Aquaculture 433 (2014) 348–360

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Grape seed extract and dried macroalgae, *Ulva lactuca* Linnaeus, improve survival of greenlip abalone, *Haliotis laevigata* Donovan, at high water temperature

Brett Lange [a], Krishna-Lee Currie [b], Gordon S. Howarth [a,c], David A.J. Stone [a,b,d,e,*]

[a] School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy Campus, SA 5371, Australia
[b] School of Biological Sciences, Flinders University, Adelaide, SA 5001, Australia
[c] Centre for Paediatric and Adolescent Gastroenterology, Children, Youth and Women's Health Service, North Adelaide, SA 5006, Australia
[d] Australian Seafood CRC, Bedford Park, SA 5042, Australia
[e] South Australian Research and Development Institute, Aquatic Sciences Centre, Marine Innovation Southern Australia, PO Box 120, Henley Beach, SA 5022, Australia

ARTICLE INFO

Article history:
Received 13 May 2014
Received in revised form 24 June 2014
Accepted 24 June 2014
Available online 3 July 2014

Keywords:
Haliotis laevigata
Ulva lactuca
Grape seed extract
Temperature stress
Survival

ABSTRACT

Summer mortality (SM) in greenlip abalone (*Haliotis laevigata* Donovan) heavily affects productivity of land-based abalone farms in Southern Australia. It has been associated with conditions of high water temperature (>23 °C), low dissolved oxygen levels, and a range of other stressful factors in the culture water during summer months. This study aimed to alleviate mortality experienced by abalone at high water temperatures (26 °C), by dietary intervention using grape seed extract (GSE) and dried *Ulva lactuca* Linnaeus, two products which contain antioxidative and bioactive compounds. These products were formulated into a commercial abalone diet at levels of 5 and 30%. The diets were fed to 3-year-old greenlip abalone (26.8 g; 57.9 mm) at a water temperature of 22 or 26 °C for 38 days. No mortalities were observed at 22 °C. Compared to the unaltered commercial diet, both GSE and dried *U. lactuca* additive diets significantly increased the survival of abalone at the 26 °C water temperature ($P < 0.05$). GSE addition also significantly increased serum superoxide dismutase activity, feed intake, and meal acceptance of the abalone ($P < 0.05$). These results demonstrate the potential for GSE or dried *U. lactuca* to act as dietary additives to reduce mortality and improve productivity on abalone farms subjected to high summer water temperatures.

1. Introduction

Greenlip abalone (*Haliotis laevigata*) are cultured at seasonally fluctuating water temperatures (10-25 °C) in land-based facilities in Southern Australia (Stone et al., 2013). As a result, abalone are exposed to high water temperatures (>23 °C) for an extended period of time during summer. Abalone have a low tolerance for increasing water temperature, whether the increase be chronic or acute (Gilroy and Edwards, 1998). Exposure of greenlip abalone to water temperatures of >23 °C on South Australian land-based farms may result in the development of a condition referred to as summer mortality (SM) (Vandepeer, 2006). Summer mortality can result in stock mortality of up to 50% in land-based abalone aquaculture (Dang et al., 2011a; Stone et al., 2014; Vandepeer, 2006). However, differences in mortality rates have been reported between large and small greenlip abalone in South Australian farms. Typically, larger (>60 mm SL) 3-year-old abalone are more susceptible to mortality during these periods (Dang et al., 2011a; Stone et al., 2014; Vandepeer, 2006).

A range of stressful conditions arise from the high summer water temperatures, such as reduced dissolved oxygen (DO) in the water, and proliferation of bacteria, particularly *Vibrio* spp. (Davis and Sizemore, 1982; Miles et al., 1997). High water temperature and low DO have been known to increase metabolism and respiration levels in many aquatic animals, and cause oxidative stress by disrupting the oxidant–antioxidant equilibrium (Lushchak, 2011). High water temperatures have also been reported to reduce the antibacterial capacity of greenlip abalone, rendering them vulnerable to bacterial infection (Dang et al., 2012).

An important factor reported in SM is the vulnerability of abalone to bacterial infection from pathogenic species such as *Vibrio harveyi* (Nicolas et al., 2002; Travers et al., 2008, 2009, 2010), *Vibrio alginolyticus* and *Vibrio parahaemolyticus* (Lee et al., 2001). However, it is also believed that any opportunistic pathogens present may simply be taking advantage of the immune-suppressed abalone (Hooper et al., 2007). The ability of pathogenic species to infect abalone is further exacerbated by high water temperatures of 26 °C causing damage to the gill epithelium, which is a portal of entry for bacteria (Hooper et al., 2014). In

* Corresponding author at: South Australian Research and Development Institute, Aquatic Sciences Centre, Marine Innovation Southern Australia, PO Box 120, Henley Beach, SA 5022, Australia. Tel.: +61 882075350.
E-mail address: david.stone@sa.gov.au (D.A.J. Stone).

http://dx.doi.org/10.1016/j.aquaculture.2014.06.028
0044-8486/

addition, when elevated water temperatures were maintained at 26 °C for a further 7 days, Hooper et al. (2014) reported no significant recovery of gill epithelia. Therefore, any harmful bacteria present during periods of elevated water temperatures could be even more damaging, given the abalone are already compromised.

Feeding of antioxidants could improve the antioxidant capacity of abalone exposed to oxidative-stress-inducing culture conditions. Antioxidant enzymes are synthesised endogenously in organisms at the cellular level to maintain the oxidant–antioxidant balance, and non-enzymatic antioxidants from dietary sources may also supplement them. Many aquatic and terrestrial plants contain compounds such as vitamins, carotenoids and phenols, which are well known for their ability to protect against oxidative stress when ingested (Ahn et al., 2002). This indicates that dietary formulation or supplementation is a potential method for maintaining oxidant–antioxidant balance in abalone challenged by various stressors.

Grape seed extract (GSE) contains a range of compounds, which have antioxidative properties such as phenols (Ahn et al., 2002; Balu et al., 2005), catechins, epicatechins (Jayaprakasha et al., 2003), procyanidins and proanthocyanidins (Davies et al., 2009; Jayaprakasha et al., 2003). Positive health benefits of GSE supplementation have also been identified in other animal models such as rats and horses (Ahn et al., 2002; Balu et al., 2005; Çetin et al., 2008; Cheah, 2011; Davies et al., 2009; Farbood et al., 2009; Sarkaki et al., 2007).

Live macroalgae also contain a variety of bioactive compounds, such as polyphenols (Chojnacka et al., 2012; Dang et al., 2011b; McShane et al., 1994; Rhimou et al., 2010), sulphated polysaccharides (Chandini et al., 2008; Chojnacka et al., 2012; Dang et al., 2011b; Rhimou et al., 2010), peptides, phycobiliproteins, polyphenols, carotenoids, tocopherols, sterols (Abirami and Kowsalya, 2011; Chandini et al., 2008; Tierney et al., 2010) and a high concentration of vitamins and minerals (Chandini et al., 2008; Chojnacka et al., 2012). Wan et al. (2004) reported that abalone fed with kelp maintained a better antioxidant status than abalone fed with an artificial diet. Bansemir et al. (2006) reported that extracts of certain species of macroalgae had antimicrobial activity, and Dang et al. (2011b) reported that greenlip abalone fed live macroalgae had improved antiviral and antibacterial activity. Macroalgae extracts have been reported to exhibit antioxidative properties through high free radical scavenging abilities (Athukorala et al., 2003), and inhibition of lipid oxidation (Chandini et al., 2008).

This study aimed to test GSE and dried *Ulva lactuca* Linnaeus as feed additives to reduce mortality in abalone cultured at high water temperatures. These products were tested using the method previously developed by Stone et al. (2014), which utilised a set of diets (*U. lactuca*, positive survival control; commercial abalone diet, negative survival control) to monitor mortality patterns of greenlip abalone at high water temperatures. It was hypothesised that feeding greenlip abalone a commercial abalone feed supplemented with GSE or dried *U. lactuca* would improve their oxidant–antioxidant balance. Therefore, detrimental effects associated with high water temperature would be lessened and mortality levels would reduce.

2. Materials and methods

2.1. Experimental animals

One-year-old greenlip abalone, which had not been used in any other experiments, were purchased from SAM Abalone (Boston Point, Port Lincoln, SA, Australia) and held at the South Australian Research and Development Institute (SARDI) Aquatic Sciences Centre (ASC) (West Beach, SA, Australia). The experimental animals were held in a 5000-L tank supplied with aeration and flow-through seawater at ambient temperature and photoperiod, and fed a 5 mm commercial abalone diet (Eyre Peninsula Aquafeed Pty Ltd. (EPA), Lonsdale, SA, Australia) ad libitum daily.

2.2. Experiment 1

In order to validate haemolymph collection methods, a preliminary time course experiment was performed to investigate the effect of an acute handling stress on serum superoxide dismutase (SOD) activity and ferric-reducing antioxidant potential (FRAP) values. Forty 3-year old greenlip abalone (weight 18.06 ± 3.24 g; shell length 51.30 ± 3.04 mm) were housed at ambient water temperature (22 ± 0.5 °C) in a 250-L tank provided with flow-through aerated seawater, and fed the commercial EPA abalone diet (5 mm) ad libitum daily for 14 days prior to sampling.

Haemolymph samples were extracted from the cephalic sinus of the abalone, using a 23-gauge needle and 3 mL syringe, at four time points: 0.5, 2, 4 and 16 min after being removed from the tank. Ten abalone were sampled at each time point. Haemolymph was then centrifuged at 4 °C and 2000 ×*g* for 5 min, and the serum was pipetted into cryotubes and frozen at −80 °C, until assayed as per Section 2.3.6.

2.3. Experiment 2

2.3.1. Experimental test ingredients, diets and preparation

GSE (GSeedEX grape seed tannin, Tarac Technologies Pty Ltd., Nuriootpa, SA, Australia) was formulated into the diets to achieve a delivery dose rate of 100 mg kg bw^{-1} day^{-1} at 26 °C. This level was chosen based on research reporting that this dose rate in rodent models promoted free radical scavenging, oxidative stress reducing activity (Balu et al., 2005; Çetin et al., 2008), and other health benefits (Farbood et al., 2009; Sarkaki et al., 2007). Dietary inclusion of GSE was also calculated taking into consideration the targeted dose rate, expected abalone feed intake, decline in feed intake due to temperature, and potential loss of GSE compounds through leaching.

Live *U. lactuca* was collected from Gulf St Vincent, SA, Australia, and placed into a 4000-L tank at SARDI ASC. The live *U. lactuca* was enriched in seawater with 8 L of Guillard's F2 nutrient media (sodium nitrate component substituted with ammonium chloride, 4.75%) (Guillard, 1975; Guillard and Ryther, 1962), and re-enriched every 15 days. The enriched *U. lactuca* was maintained throughout the experimental trial, being topped up with bore water (to prevent increasing salinity) as required. To produce the dried *U. lactuca*, some of the enriched *U. lactuca* was sun-dried for 8 h, before being oven-dried at 60 °C for 48 h. The dried *U. lactuca* was blended into fine powder using a laboratory blender (model HGBTWT53, Waring Commercial, Torrington, CT, USA), and stored at −20 °C until the diets were manufactured.

The rationale for the inclusion of 30% dried *U. lactuca* in the commercial diet was the anticipation of delivering approximately the same amount of *U. lactuca* on a dry basis as would be consumed if abalone were fed the live *U. lactuca* alone at 26 °C. The inclusion level was based on delivering the reported daily feed intake of ~0.5 g of live *U. lactuca* kg bw^{-1} day^{-1} reported for 70 mm greenlip abalone at 26 °C (Stone et al., 2014).

The five experimental diets used were: 1) the commercial EPA abalone diet; 2) the commercial EPA diet + 5% GSE; 3) the commercial EPA diet + 30% dried *U. lactuca*; 4) the commercial EPA diet + 5% GSE + 30% dried *U. lactuca*; and 5) The live enriched *U. lactuca* (Table 1). GSE was included as an additive at 5%, by adding 50 g of GSE to 1 kg of existing EPA diet mash. Dried *U. lactuca* was included as an ingredient at 30%, by removing 30% of the EPA diet mash.

To manufacture the experimental formulated diets, the required amounts of dry ingredients were weighed out and mixed in a Hobart mixer (Hobart Corp., Troy, OH, USA) for 5 min. Water (30%) was then added to the EPA diet mash and mixed for a further 3 min. The diets were manufactured using a TR110 pasta machine (Macchine Per Pasta SRL, Molina Di Malo, VI, Italy) to produce a 5 mm flat sinking pellet.

Table 1
Ingredients, biochemical composition, and ferric-reducing antioxidant potentials (FRAP) of experimental diets fed to greenlip abalone.[a,b]

Diet	Commercial diet	Comm. + 5% GSE	Comm. + 30% dried *U. lactuca*	Comm. + 5% GSE + 30% dried *U. lactuca*	Live *U. lactuca*
Ingredient (as fed)					
Commercial diet mash (g kg^{-1})	983.0	936.2	683.0	650.5	na
Dried *U. lactuca* (g kg^{-1})	0.0	0.0	300.0	285.7	na
Vitamin–mineral premix (g kg^{-1})	2.0	1.9	2.0	1.9	na
Salmon oil (mL kg^{-1})	15.0	14.3	15.0	14.3	na
GSE (g kg^{-1})	0.0	47.6	0.0	47.6	na
Live *U. lactuca* (g kg^{-1})	0.0	0.0	0.0	0.0	1000.0
Sum	*1000.0*	*1000.0*	*1000.0*	*1000.0*	*1000.0*
Biochemical composition (as fed)					
Moisture (g kg^{-1})	100.0	100.0	100.0	100.0	780.0
Crude protein (g kg^{-1})	306.0	292.9	295.7	283.1	59.8
Crude lipid (g kg^{-1})	45.0	42.9	44.1	42.1	9.3
Ash (g kg^{-1})	62.0	59.2	93.4	89.1	36.7
Carbohydrate (g kg^{-1})	518.7	497.0	421.7	404.6	42.9
NFE (g kg^{-1})[c]	587.0	605.0	566.7	585.7	114.3
Gross energy (MJ kg^{-1})[d]	17.9	17.2	16.0	15.3	3.7
Antioxidant potential (as fed)					
FRAP value (μmol Fe^{2+} equivalent g^{-1})[e]	0.92	14.66	0.68	7.78	0.4
FRAP value after 6 h in water (μmol Fe^{2+} equivalent g^{-1})[f]	0.64	29.10	0.35	12.09	na

[a] na = not applicable.
[b] GSE = grape seed extract = GSeedEX grape seed tannin = (g kg^{-1} as fed): moisture, >10; crude protein, 45.0; crude lipid, 2.0; ash, 2.5; carbohydrate 791.0; NFE, 950.5; gross energy 1.48 MJ kg^{-1} (Tarac Technologies Pty Ltd., Nuriootpa, SA, Australia).
[c] NFE = nitrogen-free extract was calculated by difference, = 1000 − (crude protein + total fat + ash).
[d] Gross energy content of enriched *U. lactuca* was determined using values of 17.2, 23.6, and 39.5 MJ kg^{-1} for carbohydrate, protein and lipid, respectively (NRC, 2011).
[e] FRAP = ferric-reducing antioxidant potential was measured by homogenising the ingredient and feed sample in DMSO and assaying the supernatant. Test ingredient FRAP values (μmol Fe^{2+} equivalent g^{-1}): GSE, 6.00; live *U. lactuca*, 0.40; dried enriched *U. lactuca*, 1.72.
[f] FRAP was also measured after immersing of the feed sample in distilled water for 6 h, then collecting the solid feed on filter paper, homogenising it in DMSO and assaying the supernatant.

2.3.2. Experimental system

The experiment was conducted in a photoperiod controlled (12 h of low-intensity light (3.4 lx, 7:15 am to 7:15 pm) and 12 h of dark (7:15 pm to 7:15 am)) and air temperature controlled (20 ± 1 °C) laboratory. The experiment used two identical water temperature controlled systems (22 and 26 °C) described in Stone et al. (2013) with 30 μm sand-filtered, UV treated seawater. Each system consisted of fifteen 12.5-L blue plastic experimental tanks (Nally IH305, Viscount Plastics Pty Ltd.; length, 39.2 cm; width, 28.8 cm; depth, 11.0 cm; and bottom surface area, 1129 cm^2), with a water depth of 5 cm controlled by a standpipe, resulting in a tank water volume of 5.4 L. The experimental tanks were gravity fed flow-through aerated water from a reservoir at a flow rate of 300 mL min^{-1}. This flow rate resulted in ~80 tank water exchanges per day. Tank water flow rate and depth were checked twice a week and adjusted if needed. Water temperature was controlled using 3 kW immersion heaters (240 V, JQ20, Austin and Cridland, Carlton, NSW, Australia). The experiment lasted for 38 days.

2.3.3. Tank stocking

Abalone were weighed (26.8 ± 14.7 g) and measured (shell length, 57.9 ± 11.6 mm), and 12 abalone were systematically interspersed into 3 replicate tanks per treatment combination. The water temperature was slowly adjusted from ambient (23 °C) to the required treatment water temperatures (22 and 26 °C) over a period of seven days. Tank water temperatures were then maintained within a tolerance of ±1.5 °C until the end of the experiment.

2.3.4. Feeding

On the day after stocking, abalone in each tank were fed the appropriate experimental formulated diet to excess (1.0 to 1.2% bw day^{-1} as the experiment progressed) daily. Live enriched *U. lactuca* was also fed to excess to the appropriate tanks at a rate of 1.5% bw day^{-1} for the first week and then was increased to 2.5% bw day^{-1} for the remainder of the experiment. Feeding occurred at 4:00 pm. At 8:30 am the following day, tanks were cleaned and uneaten feed was collected in a 500 μm sieve and transferred to containers. The containers were weighed daily and stored at −20 °C. At the end of the experiment, all uneaten feed samples were oven-dried (16 h at 105 °C for experimental formulated diets; 48 h at 60 °C for the live *U. lactuca*) to obtain the dry weights. To account for feed leaching losses (or growth for the *U. lactuca* diets), the same method was used with tanks containing no abalone. Apparent feed intake was calculated by subtracting the uneaten feed (dry weight) and the amount lost due to leaching (dry weight) or gained due to growth, 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.

Dead animals were removed, weighed, measured and replaced with tagged abalone to maintain stocking density. Feed rates were adjusted accordingly to compensate for any biomass changes arising from mortalities and replacements.

2.3.5. Sample collection

At the end of the experiment, all abalone were removed for sampling. Systematically, one by one, each abalone was removed from the tank, weighed, and measured, and haemolymph was sampled. Haemolymph was obtained by inserting a 23-gauge needle with 3 mL syringe (Livingstone International, Rosebery, NSW, Australia) into the cephalic sinus and drawing out the haemolymph within 0.5 min after abalone were removed from the tank. Haemolymph was vortexed and expelled into microtubes (Eppendorf AG, Hamburg, Germany). Haemolymph samples were centrifuged at 4 °C for 5 min at 2000 ×g to separate the serum from the cell pellet. The serum was pipetted into cryotubes (Sarstedt AG and Co., Numbrecht, Germany) and kept on dry ice until storing at −80 °C.

2.3.6. Biochemical analyses

Serum samples from the abalone were analysed for SOD activity using a superoxide dismutase activity assay kit (Item Number 706002; Cayman Chemical, Ann Arbor, MI, USA). The antioxidant capacities of the GSE, dried *U. lactuca*, test diets and abalone serum samples were

determined using the FRAP methods of Xu et al. (2010), with the modifications of Cheah (2011). Equal amounts of serum from abalone from each tank were pooled for FRAP and SOD analyses. GSE, dried *U. lactuca* and test diets were homogenised in dimethyl sulfoxide (DMSO) and the supernatants were analysed for FRAP. To evaluate leaching loss, the FRAP activity of the four formulated diets was also tested after soaking in water for 6 h.

The biochemical compositions of the commercial diet, and the live and dried *U. lactuca* were analysed according to the methods of the AOAC International (1995). Crude protein (N × 6.25) was determined by the Kjeldahl method. Crude lipid was analysed with a Soxtherm rapid extraction system (Gerhardt GmbH and Co. KG, Konigswinter, Germany) with petroleum liquid (BP 100 °C) as the extracting solvent. Ash was determined using a muffle furnace at 550 °C for 16 h. Gross energy of *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 determined using the Molisch test (Lampman et al., 2010) and a glucose standard curve.

2.3.7. Growth and performance calculations

Weight gain (g $abalone^{-1}$) = final weight − initial weight

Daily growth rate (g day^{-1}) = (final weight − initial weight) / number of days

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

Feed intake (g $kg\ abalone^{-1}\ day^{-1}$ as fed basis) = (grams fed − grams uneaten) − grams leached / kg tank biomass ∗ number of days

Weekly meal acceptance (%) = (number of days part of meal was consumed by abalone / 7 days) ∗ 100

2.3.8. Water quality

Water quality parameters (DO saturation level, DO concentration, temperature, salinity and pH) were measured twice daily at 6:45 am (dark) and 3:30 pm (immediately prior to feeding). Water DO was measured in percentage (saturation) and milligrams per litre (concentration) using an Oxyguard Handy Polaris 2 oxygen probe and metre (Oxyguard International A/S, Birkerod, Denmark). Salinity was measured in grams per litre using an ISSCO UR-2 handheld refractometer (model RF20; Extech Instruments, Nashua, NH, USA). pH was measured with a Eutech pH Testr30 metre (Eutech Instruments Pte Ltd., Singapore, Singapore). Water total ammonia levels were measured weekly at 7:30 am using an API ammonium/ammonia aquarium test kit (Mars Fishcare, Chalfont, PA, USA). Dissolved CO_2 gas levels were measured at 6.45 am on day 38 of the experiment using an Oxyguard CO_2 Analyser (Oxyguard International A/S, Birkerod, Denmark).

2.3.9. Statistical analysis

In order to ensure normal distribution, data was transformed where appropriate, from percentage values to arcsine values. Levene's test for equality of variance was used to assess the homogeneity of variance among means. For Experiment 1, One-factor ANOVAs and Student–Newman–Keuls (SNK) post-hoc tests were used to assess differences in serum values across time points. For Experiment 2, One-factor ANOVAs with SNK post-hoc tests were used to assess differences in initial weights and initial shell lengths. Three-factor ANOVAs were used to assess the main affects (water temperature, GSE addition, and dried *U. lactuca* addition) and interactions, on water quality parameters, growth, performance, and health traits, across the eight formulated diet treatment combinations. Where an interaction was observed, One-factor ANOVAs with SNK post-hoc tests were used to assess differences across the eight formulated diet treatment combinations. Data from the two live *U. lactuca* treatments were omitted for all Three-factor analyses. Additionally for Experiment 2, all ten treatments were assessed using Dunnett's tests to compare the control treatment (live *U. lactuca* at the 22 °C water temperature) against the other nine treatments. Survival patterns were assessed using Kaplan–Meier analyses with Log-Rank and Breslow tests, and Cox proportional-hazards regression analyses. Feed intake and meal acceptance were assessed using a linear mixed model ANOVA, utilising diet, week, and their interaction as fixed effects, and tank number as a random effect. Statistics were computed using SPSS for Windows (version 20, IBM Corp., Armonk, NY, USA). A significance level of $P < 0.05$ was used, and unless otherwise stated, all values are presented as mean ± standard error.

3. Results

3.1. Experiment 1

The results of the haemolymph sampling validation experiment indicated a significant progressive decrease in serum SOD activity of abalone that occurred between 0.5 and 2 min ($P < 0.05$; One-factor ANOVA; Fig. 1) following removal from the tank. SOD activity remained significantly depressed at 4 min, and returned to initial levels 16 min after the handling disturbance ($P < 0.05$; Fig. 1). For this reason, haemolymph samples for Experiment 2 were collected within 0.5 min after abalone were removed from the tank. A similar, but opposite and more variable trend was observed for serum FRAP values, but was not significant ($P > 0.05$; Fig. 2).

3.2. Experiment 2

3.2.1. Survival

Survival of abalone for the 22 °C water temperature treatments was 100%, whereas at 26 °C, survival rates varied significantly among diets ($P < 0.05$, Figs. 3, 4 and 5).

There was a significant effect of both GSE and dried *U. lactuca* additions on the survival of abalone ($P = 0.001$ and $P = 0.029$, respectively; Kaplan–Meier; Log-Rank tests; Figs. 3 and 4). Abalone fed diets with no GSE were 2.8 times more likely to die than abalone fed diets with GSE ($P = 0.002$; Cox proportional-hazards regression). Similarly, abalone fed diets with no dried *U. lactuca* were 1.2 times more likely to die than abalone fed diets with dried *U. lactuca* ($P = 0.037$). The chance of surviving was not significantly affected by the interaction between GSE addition and dried *U. lactuca* addition ($P = 0.468$).

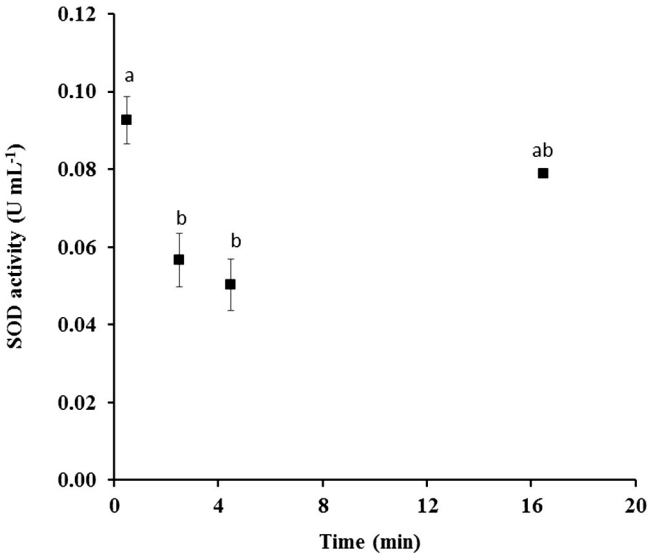

Fig. 1. Abalone serum superoxide dismutase (SOD) activities following a handling stress. Values are mean ± SE; n = 2; means with different superscripts are significantly different ($P < 0.05$; One-factor ANOVA).

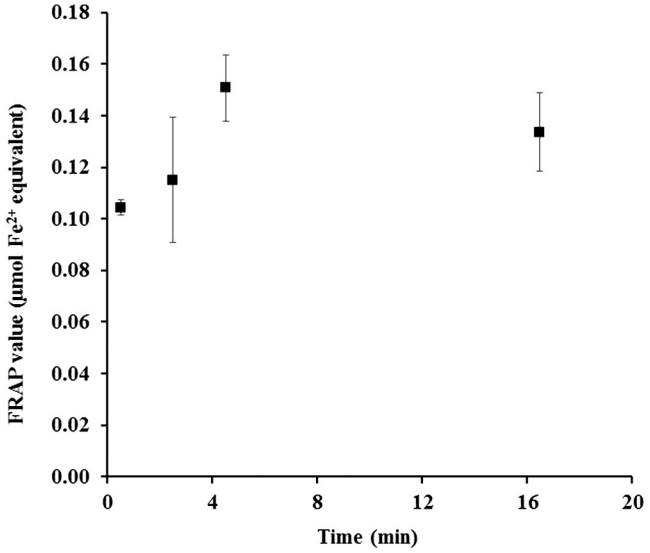

Fig. 2. Abalone serum ferric-reducing antioxidant potential (FRAP) values following a handling stress. Values are mean ± SE; n = 2; means with different superscripts are significantly different ($P < 0.05$; One-factor ANOVA).

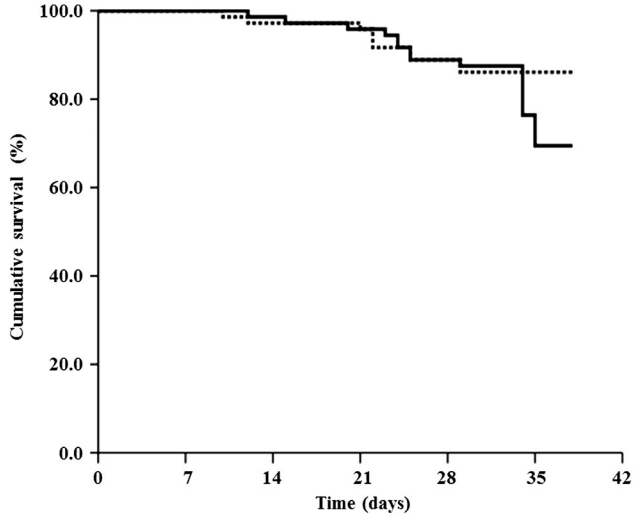

Fig. 4. Kaplan–Meier survival curves of greenlip abalone fed diets with dried *U. lactuca* (·····) and without dried *U. lactuca* (—). Survival rate of abalone fed the dried *U. lactuca* diets > no dried *U. lactuca* diets ($P = 0.029$; Log-rank and Breslow tests). Data is for the 26 °C water temperature treatments only, as no mortality was observed for the 22 °C treatments.

There was a significant difference in the survival of abalone among diet types at 26 °C ($P < 0.001$; Kaplan–Meier; Log-Rank test; Fig. 5). Abalone fed the live *U. lactuca* diet were 20.5 times more likely to survive than abalone fed the commercial diet ($P = 0.003$; Cox proportional-hazards regression). However, there was no significant difference between the survival of abalone fed the live *U. lactuca* and any of the other three diets at the 26 °C water temperature (commercial + 5% GSE $P = 0.192$; commercial + 30% dried *U. lactuca* $P = 0.052$; commercial + 5% GSE + 30% dried *U. lactuca* $P = 0.319$).

3.2.2. Feed intake and feeding behaviour

Feed intake (as fed basis) by abalone over the whole experiment was significantly affected by water temperature ($P < 0.001$; 22 > 26 °C; Table 4) and GSE addition ($P < 0.001$; GSE > no GSE), but not by dried *U. lactuca* addition ($P > 0.05$). However, feed intake was also significantly affected by the interaction between water temperature and dried *U. lactuca* addition ($P = 0.017$), and by the interaction between GSE addition and dried *U. lactuca* addition ($P \leq 0.001$, Table 4). There were no significant three-way interactions between water temperature, GSE addition and dried *U. lactuca* addition ($P > 0.05$, Table 4). The interaction between temperature and dried *U. lactuca* addition was due to the higher response in feed intake by abalone at 26 °C compared to 22 °C (Table 4). While the interaction between GSE addition and dried *U. lactuca* addition was due to the positive response in feed intake by abalone due to the addition of GSE to the diet compared to the neutral response observed when dried *U. lactuca* was added (Table 4).

Compared to the live *U. lactuca* at the 22 °C water temperature, the feed intake (as fed basis) of abalone was significantly lower ($P < 0.05$; Table 5) compared to all other treatments.

Over the course of the experiment at the 22 °C water temperature, feed intake (as fed basis) was significantly affected by time ($P < 0.001$; linear mixed model; Fig. 6a), diet type ($P < 0.001$), and the interaction between the two factors ($P < 0.001$). The interaction may be explained

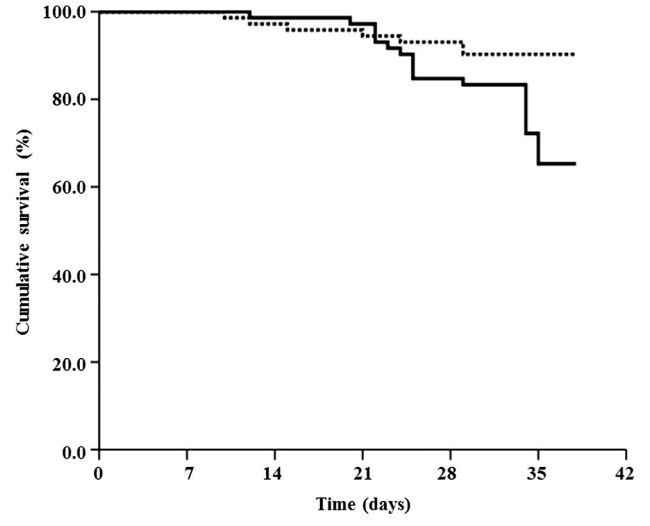

Fig. 3. Kaplan–Meier survival curves of greenlip abalone fed diets with grape seed extract (GSE) (·····) and without GSE (—). Survival rate of abalone fed the GSE diets > no GSE diets ($P = 0.001$; Log-rank and Breslow tests). Data is for the 26 °C water temperature treatments only, as no mortality was observed for the 22 °C treatments.

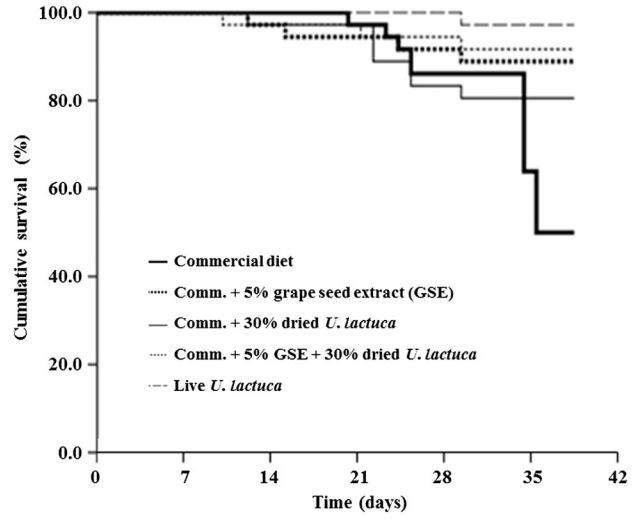

Fig. 5. Kaplan–Meier survival curves for the abalone fed different diets at the 26 °C water temperature. Survival rate of abalone fed the live *U. lactuca* diet > commercial diet ($P < 0.001$; Log-rank and Breslow tests).

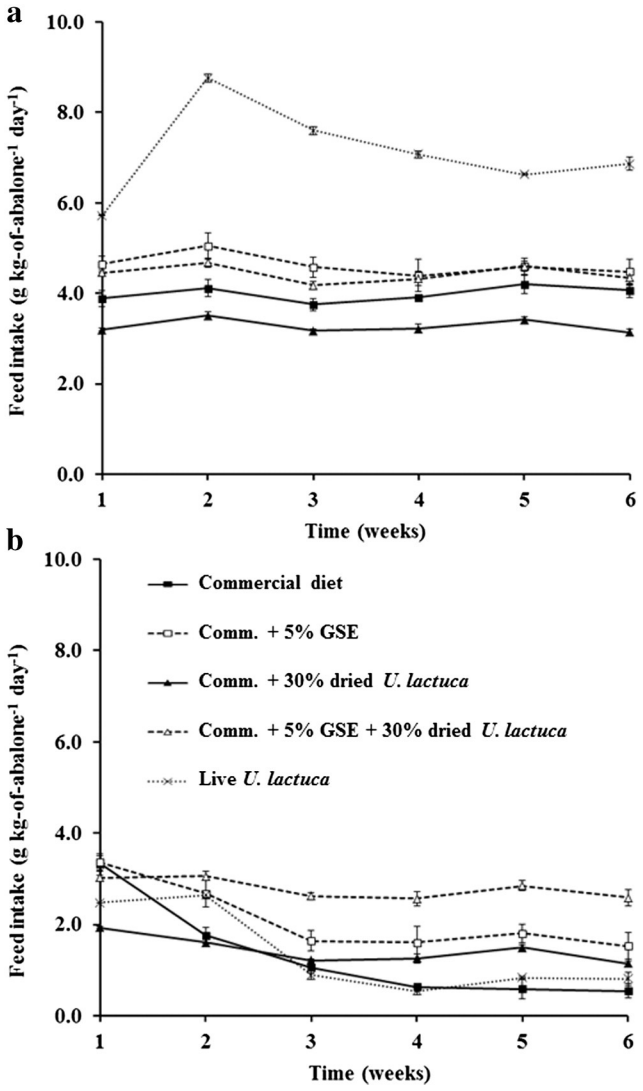

Fig. 6. Weekly average feed intake (as fed basis) of greenlip abalone fed experimental diets at the (a) 22 °C and (b) 26 °C water temperature. For each diet, each weekly value is compared back to the initial value (week 1) and assessed for significant differences (linear mixed model). Values are mean ± SE, n = 3.

by there being no significant change in feed intake by abalone over time ($P > 0.05$), for any of the formulated diets at 22 °C (Fig. 6a). Whereas, for the live *U. lactuca* at the same water temperature, feed intake by abalone increased significantly ($P < 0.001$) from week one to week two during the acclimation period, and stayed significantly higher ($P < 0.05$) for the remainder of the experiment.

Over the course of the experiment at the 26 °C water temperature, feed intake (as fed basis) by abalone was significantly affected by time ($P < 0.001$; linear mixed model; Fig. 6b), diet ($P = 0.003$), and their interaction ($P < 0.001$). The interaction between diet type and time was due to significant differences in the rate that feed intake decreased over time for different diets ($P < 0.05$). The feed intake of the commercial diet significantly decreased ($P = 0.002$) from week one to week two, and stayed significantly lower ($P < 0.001$) for the remainder of the experiment. The feed intake of the commercial + 5% GSE diet, and the live *U. lactuca* diet, did not significantly change ($P > 0.05$) from week one to week two, but the feed intake for both was significantly lower ($P = 0.001$) by week three, and stayed significantly lower ($P < 0.001$) for the remainder of the experiment. The feed intake of the commercial + 30% dried *U. lactuca* diet and the commercial + 5% GSE + 30% dried *U. lactuca* diet did not significantly change ($P > 0.05$) over the first five weeks, but had significantly decreased ($P < 0.05$) by week six.

Over the course of the experiment at the 22 °C water temperature, meal acceptance by abalone was 100% each week, for all diets. In contrast, at the 26 °C, meal acceptance was significantly affected by time ($P < 0.001$; linear mixed model; Fig. 7), diet ($P < 0.001$), and their interaction ($P < 0.001$) over the course of the same period. The interaction between time and diet type was due to significant differences in the proportion that weekly meal acceptance changed over time for different diets ($P < 0.05$). Meal acceptance did not significantly change ($P > 0.05$) over time for the commercial + 5% GSE diet, commercial + 30% dried *U. lactuca* diet, or the commercial + 5% GSE + 30% dried *U. lactuca* diet. However, for the commercial diet, meal acceptance was significantly lower ($P < 0.05$) at week four and week six. For the live *U. lactuca* diet, meal acceptance was only significantly lower ($P < 0.05$) at week four.

3.2.3. Water quality

At 6:45 am, the DO saturation levels (%) of the culture water were significantly affected by GSE addition (GSE > no GSE; $P = 0.037$; Three-factor ANOVA; Table 2), but not by water temperature ($P = 0.566$) or dried *U. lactuca* addition ($P = 0.465$), and there were no significant interactions between water temperature, GSE addition and dried *U. lactuca* addition ($P > 0.05$, Table 2). While at 3:30 pm, DO saturation levels of the culture water were not significantly affected by water temperature ($P = 0.744$), GSE addition ($P = 0.401$) or dried *U. lactuca* addition ($P = 0.418$) and there were no significant interactions between the any of the factors ($P > 0.05$, Table 2).

At 6:45 am, the DO concentrations (mg L^{-1}) were significantly affected by water temperature ($P < 0.001$; 22 > 26 °C) and GSE addition (GSE > no GSE; $P = 0.041$), but not by dried *U. lactuca* addition ($P > 0.05$; Table 2), and there were no significant interactions between any of the factors ($P > 0.05$). While at 3:30 pm, DO concentrations were significantly affected by water temperature ($P < 0.001$; 22 > 26 °C), but not by GSE addition ($P = 0.406$) or dried *U. lactuca* addition ($P = 0.328$), and there were no significant interactions between any of the factors ($P > 0.05$, Table 2).

Culture water pH levels at both 6:45 am and 3:30 pm were significantly affected by water temperature ($P < 0.001$; 26 > 22 °C), but not by GSE addition (6:45 am, $P = 0.159$; 3:30 pm, $P = 0.442$) or dried

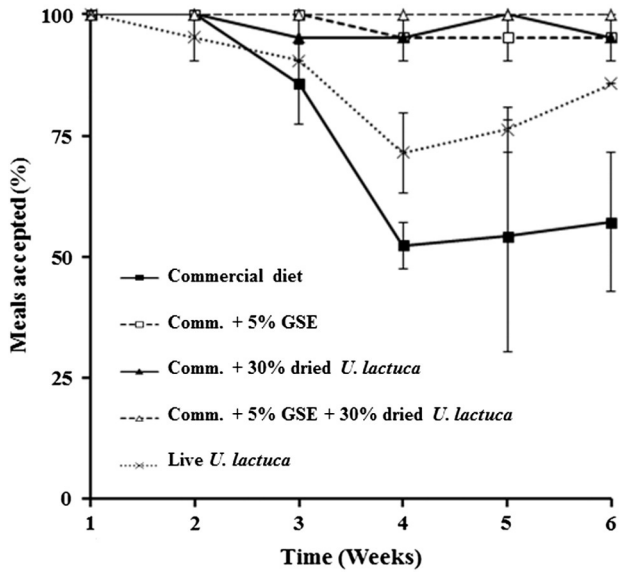

Fig. 7. Meal acceptance (percentage of meals partially consumed each week) of greenlip abalone fed experimental diets at the 26 °C water temperature. For each diet, each weekly value was compared back to the initial value (week 1) and assessed for significant differences (linear mixed model). Values are mean ± SE, n = 3.

Table 2
Three-factor ANOVA comparing the effects of water temperature, grape seed extract (GSE) addition and dried *U. lactuca* addition, and interactions on water quality parameters.[a,b,c,d]

Main effect	Water temperature		GSE addition		Dried *U. lactuca* addition		Three-factor ANOVA					
	22 °C	26 °C	With	Without	With	Without	Temp (A)	GSE add. (B)	Dried *U. lactuca* add. (C)	Temp * GSE add. (A * B)	Temp * dried *U. lactuca* add. (A * C)	GSE add. * dried *U. lactuca* add. (B * C)
DO (% sat) 6:45 am	84.24 ± 0.50	83.82 ± 0.50	84.83 ± 0.50	83.22 ± 0.50	84.29 ± 0.50	83.76 ± 0.50	0.566	0.037*	0.465	0.529	0.923	0.314
DO (% sat) 3:30 pm	88.66 ± 0.53	88.91 ± 0.53	89.11 ± 0.53	88.46 ± 0.53	89.10 ± 0.53	88.47 ± 0.53	0.744	0.401	0.418	0.577	0.519	0.245
DO (mg L^{-1}) 6:45 am	5.98 ± 0.04	5.65 ± 0.04	5.87 ± 0.04	5.76 ± 0.04	5.82 ± 0.04	5.81 ± 0.04	<0.001*	0.041*	0.765	0.392	0.562	0.427
DO (mg L^{-1}) 3:30 pm	6.28 ± 0.04	5.97 ± 0.04	6.15 ± 0.04	6.10 ± 0.04	6.15 ± 0.04	6.10 ± 0.04	<0.001*	0.406	0.328	0.406	0.659	0.144
pH 6:45 am	8.12 ± 0.01	8.16 ± 0.01	8.14 ± 0.01	8.13 ± 0.01	8.14 ± 0.01	8.14 ± 0.01	<0.001*	0.159	0.629	0.471	0.809	0.104
pH 3:30 pm	8.13 ± 0.01	8.16 ± 0.01	8.15 ± 0.01	8.14 ± 0.01	8.14 ± 0.01	8.15 ± 0.01	<0.001*	0.442	0.796	0.607	0.607	0.135

[a] Values are estimated marginal means ± SE, n = 3.
[b] Data from the seven day acclimatisation period at the start of the trial is excluded.
[c] *P*-values with * were significant ($P < 0.05$).
[d] There were no significant three-way interactions ($P > 0.05$).

U. lactuca addition (6:45 am, $P = 0.629$; 3:30 pm, $P = 0.796$), and there were no significant interactions between any of the factors ($P > 0.05$: Table 2).

Compared to the live *U. lactuca* positive control diet at the 22 °C water temperature, the 6:45 am DO saturation levels for the live *U. lactuca* diet at the 26 °C water temperature were not significantly different ($P > 0.05$; Dunnett's test; Table 3). However, DO saturation levels at 6:45 am were significantly lower ($P < 0.05$) for all formulated diets, when compared to the live *U. lactuca* at the 22 °C water temperature. At 3:30 pm, the 22 °C commercial diet, 22 °C commercial + 5% GSE diet, 26 °C commercial diet, 26 °C commercial + 30% dried *U. lactuca* diet, and the 26 °C commercial + 30% dried *U. lactuca* + 5% GSE diet treatments all had significantly lower DO saturation levels than the live *U. lactuca* at the 22 °C water temperature.

DO concentrations at 6:45 am were significantly lower ($P < 0.05$; Dunnett's test; Table 3) in all treatments when compared to the live *U. lactuca* at the 22 °C water temperature. However, at the 3:30 pm, the 22 °C commercial diet + 30% dried *U. lactuca* treatment and the 22 °C commercial diet + 30% dried *U. lactuca* + 5% GSE treatment were not significantly different ($P > 0.05$) to the live *U. lactuca* at the 22 °C water temperature; whereas all other treatments were significantly lower ($P < 0.05$).

Culture water pH levels at 6:45 am were significantly lower ($P < 0.05$; Dunnett's test; Table 3) for all 22 °C formulated diet treatments, when compared to the live *U. lactuca* at the 22 °C water temperature. However, there was no significant difference ($P > 0.05$) between the live *U. lactuca* at the 22 °C water temperature and the 26 °C water temperature treatments. At 3:30 pm, the 22 °C commercial diet had a significantly lower ($P < 0.05$) pH than the live *U. lactuca* at the 22 °C water temperature, whereas all other treatments were not significantly different ($P > 0.05$).

Dissolved CO_2 gas levels of the culture water were <1 mg L^{-1} for all formulated diet treatments, and 1 mg L^{-1} for the live *U. lactuca* treatments.

3.2.4. Growth performance

There were no significant differences ($P > 0.05$; One-factor ANOVA) in initial weight (26.8 ± 14.7 g) or initial shell length (57.9 ± 11.6 mm) of abalone allocated to each treatment combination at the commencement of the experiment.

Weight gain, daily growth rate, and shell growth rate of abalone were significantly affected by water temperature ($P < 0.001$; 22 > 26 °C; Three-factor ANOVA; Table 4), but not significantly affected by GSE addition ($P > 0.05$) or dried *U. lactuca* addition ($P > 0.05$), and there were no significant interactions ($P > 0.05$).

Compared to the abalone fed the live *U. lactuca* at the 22 °C water temperature, weight gains, daily growth rates and shell growth rates were all significantly higher ($P < 0.05$; Dunnett's test; Table 5) for the abalone held at 22 °C water temperature and fed any of the formulated diets. However, weight gains, daily growth rates, and shell growth rates of the abalone fed all formulated diets at the 26 °C water temperature were not significantly different ($P > 0.05$) to the live *U. lactuca* at the 22 °C water temperature. Shell growth rate of abalone fed the live *U. lactuca* diet at the 26 °C water temperature was significantly lower ($P < 0.05$) than for those fed the live *U. lactuca* at the 22 °C water temperature.

3.2.5. Serum SOD and FRAP

Serum SOD activity of abalone was significantly affected by GSE addition ($P = 0.038$; GSE > no GSE; Table 4), but not significantly affected by water temperature ($P = 0.439$) or dried *U. lactuca* addition ($P = 0.879$), and there were no significant interactions between any of the factors ($P > 0.05$, Table 4).

Serum SOD activity of the abalone fed the live *U. lactuca* at the 22 °C water temperature was not significantly different ($P > 0.05$; Table 5) to those of abalone from any other treatment at either water temperature.

Serum FRAP values were not significantly affected by water temperature ($P = 0.649$; Table 4), GSE addition ($P = 0.316$) or dried *U. lactuca* addition ($P = 0.774$), and there were no significant interactions between the factors ($P > 0.05$).

However, the serum FRAP value of abalone fed the live *U. lactuca* at the 22 °C water temperature was significantly higher ($P < 0.05$; Table 5) than those of the abalone fed the commercial diet at the 26 °C water temperature. Additionally, the serum FRAP value of abalone fed the live *U. lactuca* at the 22 °C water temperature was not significantly different ($P > 0.05$) to those of abalone fed any other diet at either water temperature (Table 5).

3.2.6. GSE dose rate

The addition level of GSE to the experimental diets was ~5%. Based on the daily feed intake rates presented in Table 5, the average consumption of dried GSE for the commercial + 5% GSE diet by abalone was ~238 and 113 mg kg abalone^{-1} day^{-1} for the 22 and 26 °C water temperatures, respectively. The average consumption of GSE for the commercial + 5% GSE + 30% dried *U. lactuca* diet was ~234 and 137 mg kg abalone^{-1} day^{-1} for the 22 and 26 °C water temperatures, respectively.

3.2.7. Dried U. lactuca intake

The dietary inclusion level of dried *U. lactuca* to the experimental diets was ~30%. Based on the daily feed intake rates presented in

Table 3
Water quality parameters across all treatments for comparison using Dunnett's tests.[a,b,c,f]

Water temperature (°C)		22 °C					26 °C				
Diet		Live *U. lactuca* (Dunnett's test control value)	Commercial diet	Comm. + 5% GSE	Comm. + 30% dried *U. lactuca*	Comm. + 5% GSE + 30% dried *U. lactuca*	Live *U. lactuca*	Commercial diet	Comm. + 5% GSE	Comm. + 30% dried *U. lactuca*	Comm. + 5% GSE + 30% dried *U. lactuca*
DO (% sat) 6:45 am[d]	Mean	90.7 ± 1.81	82.5 ± 0.19***	84.7 ± 0.78**	83.9 ± 1.89**	85.9 ± 1.35*	89.5 ± 1.50	83.3 ± 1.12**	84.5 ± 0.31**	83.2 ± 0.41**	84.3 ± 0.69**
	Range	80–100	70–96	69–99	68–95	72–97	74–101	68–94	69–98	64–94	69–94
DO (% sat) 3:30 pm[d]	Mean	92.7 ± 0.41	87.5 ± 0.55**	88.3 ± 1.75*	89.6 ± 0.69	89.3 ± 1.77	92.6 ± 0.56	88.3 ± 0.37*	89.8 ± 0.89	88.5 ± 0.85*	89.1 ± 0.68*
	Range	82–98	78–95	77–98	76–97	66–100	85–101	75–99	80–102	70–99	74–99
DO (mg L^{-1}) 6:45 am[d]	Mean	6.49 ± 0.12	5.87 ± 0.02***	6.04 ± 0.10**	5.94 ± 0.10**	6.09 ± 0.10**	6.04 ± 0.10**	5.60 ± 0.09***	5.72 ± 0.02***	5.62 ± 0.05***	5.64 ± 0.05***
	Range	5.7–7.1	4.9–6.8	4.9–7.1	4.9–6.6	5.2–6.8	5.1–6.9	4.6–6.4	4.7–6.8	4.3–6.4	4.0–6.4
DO (mg L^{-1}) 3:30 pm[d]	Mean	6.55 ± 0.08	6.20 ± 0.02*	6.22 ± 0.13*	6.36 ± 0.09	6.34 ± 0.11	6.20 ± 0.08*	5.92 ± 0.05***	6.04 ± 0.05**	5.92 ± 0.04***	5.98 ± 0.04***
	Range	5.9–7.1	5.5–6.8	5.1–6.8	5.4–6.9	5.1–7.1	5.4–6.9	5.1–6.8	5.5–6.9	4.8–6.8	5.0–6.8
pH 6:45 am[e]	Mean	8.18 ± 0.01	8.11 ± 0.01**	8.12 ± 0.01**	8.11 ± 0.01**	8.14 ± 0.01*	8.21 ± 0.01	8.16 ± 0.01	8.17 ± 0.01	8.15 ± 0.00	8.15 ± 0.01
	Range	8.03–8.24	7.96–8.17	8.02–8.22	7.89–8.21	7.96–8.20	8.09–8.36	8.02–8.24	8.05–8.23	8.00–8.23	8.00–8.24
pH 3:30 pm[e]	Mean	8.16 ± 0.00	8.12 ± 0.01*	8.13 ± 0.01	8.13 ± 0.01	8.14 ± 0.01	8.19 ± 0.01	8.16 ± 0.00	8.17 ± 0.00	8.15 ± 0.01	8.15 ± 0.01
	Range	8.03–8.22	7.97–8.19	8.00–8.23	7.91–8.23	7.97–8.20	8.07–8.24	8.03–8.25	8.04–8.23	8.03–8.25	8.03–8.22
Temp (°C) 6:45 am	Mean	21.9 ± 0.01	21.9 ± 0.04	21.9 ± 0.03	21.9 ± 0.04	21.9 ± 0.06	25.4 ± 0.08	25.5 ± 0.03	25.4 ± 0.11	25.5 ± 0.17	25.4 ± 0.12
	Range	21.0–22.5	21.1–22.9	21.0–22.6	20.9–22.8	21.0–22.8	24.9–26.5	25.2–26.5	25.0–26.5	25.1–26.8	25.0–26.9
Temp (°C) 3:30 pm	Mean	21.7 ± 0.01	21.7 ± 0.10	21.7 ± 0.05	21.7 ± 0.05	21.8 ± 0.09	25.5 ± 0.10	25.5 ± 0.03	25.4 ± 0.14	25.5 ± 0.23	25.4 ± 0.07
	Range	20.6–22.1	20.6–22.4	20.8–22.1	20.9–22.3	20.7–22.4	25.0–26.5	24.5–26.6	24.6–26.7	25.0–26.5	24.9–26.5

[a] Dunnett's tests use the live *U. lactuca* at the 22 °C water temperature treatment as the control value to compare against all other treatments.
[b] Data from the seven day acclimatisation period at the start of the trial is excluded.
[c] Range represents the lowest and highest recorded values. Means are ± SE, n = 3. No analyses performed for temperature.
[d] Dunnett's 1-tailed test, * denotes value is significantly different to control value (live *U. lactuca* at the 22 °C water temperature).
[e] Dunnett's 2-tailed test, * denotes value is significantly different to control value (live *U. lactuca* at the 22 °C water temperature).
[f] GSE = grape seed extract
* $P < 0.05$
** $P < 0.01$.
*** $P < 0.001$.

Table 4
Three-factor ANOVA comparing main effects of water temperature, grape seed extract (GSE) addition and dried *U. lactuca* addition, and interactions on growth, performance and health traits of greenlip abalone.[a,b,c]

Water temp (°C)	22 °C				26 °C				Three-factor ANOVA					
GSE addition	Without GSE		With GSE		Without GSE		With GSE		Water temp (A)	GSE add. (B)	Dried *U. lactuca* add. (C)	Temp * GSE add. (A * B)	Temp * dried *U. lactuca* add. (A * C)	GSE add. * dried *U. lactuca* add. (B * C)
Diet	Commercial diet	Comm. + 30% dried *U. lactuca*	Comm. + 5% GSE	Comm. + 5% GSE + 30% dried *U. lactuca*	Commercial diet	Comm. + 30% dried *U. lactuca*	Comm. + 5% GSE	Comm. + 5% GSE + 30% dried *U. lactuca*						
Weight gain (g abalone^{-1})	8.4 ± 0.9	8.0 ± 0.4	8.3 ± 0.4	7.6 ± 0.1	3.3 ± 0.9	4.9 ± 0.8	4.1 ± 1.5	4.3 ± 0.4	<0.001*	0.948	0.757	0.779	0.428	0.208
Daily growth rate (mg day^{-1})	220.6 ± 23.8	209.6 ± 10.3	219.1 ± 10.6	200.4 ± 3.5	85.6 ± 18.3	129.4 ± 21.5	109.1 ± 39.4	113.1 ± 11.7	<0.001*	0.934	0.642	0.805	0.496	0.229
Shell growth rate (μm day^{-1})	120.3 ± 13.0	126.9 ± 0.5	112.1 ± 3.7	113.5 ± 4.8	44.6 ± 7.5	45.4 ± 4.5	35.2 ± 3.7	56.2 ± 4.8	<0.001*	0.314	0.145	0.250	0.449	0.486
Feed intake (g kg abalone^{-1} day^{-1})[c,d,e]	4.00 ± 0.10[b]	3.31 ± 0.05[c]	4.76 ± 0.05[a]	4.68 ± 0.11[a]	1.24 ± 0.05[f]	1.43 ± 0.13[f]	2.25 ± 0.16[c]	2.74 ± 0.10[d]	<0.001*	<0.001*	0.828	0.399	0.017*	0.001*
Serum SOD activity (U mL^{-1})	0.090 ± 0.01	0.097 ± 0.01	0.121 ± 0.01	0.130 ± 0.03	0.098 ± 0.01^	0.085 ± 0.01	0.115 ± 0.02	0.105 ± 0.01	0.439	0.038*	0.879	0.563	0.926	0.394
Serum FRAP value (μmol Fe^{2+} equivalent)	0.120 ± 0.01	0.112 ± 0.01	0.129 ± 0.00	0.114 ± 0.00	0.096 ± 0.01^	0.126 ± 0.01	0.128 ± 0.01	0.113 ± 0.02	0.649	0.316	0.774	0.766	0.093	0.194

[a] Values are means ± SE. n = 3, except for superscript ^, where n = 2.
[b] *P*-values with * were significant ($P < 0.05$).
[c] There were no significant Three-way interactions ($P > 0.05$).
[d] Denotes One-factor ANOVA performed due to significant interactions ($P < 0.05$). Means with different superscripts are significantly different ($P < 0.05$).
[e] As fed basis.

Table 5
Growth, performance and health traits of greenlip abalone across all treatments for comparison using Dunnett's tests.[a,b,f]

Water temperature (°C)	22 °C					26 °C				
Diet	Live *U. lactuca* (Dunnett's test control value)	Commercial diet	Comm. + 5% GSE	Comm. + 30% dried *U. lactuca*	Comm. + 5% GSE + 30% dried *U. lactuca*	Live *U. lactuca*	Commercial diet	Comm. + 5% GSE	Comm. + 30% dried *U. lactuca*	Comm. + 5% GSE + 30% dried *U. lactuca*
Weight gain (g abalone^{-1}) [d]	3.5 ± 0.6	8.4 ± 0.9**	8.3 ± 0.4**	8.0 ± 0.4**	7.6 ± 0.1**	1.0 ± 0.4	3.3 ± 0.9	4.1 ± 1.5	4.9 ± 0.8	4.3 ± 0.4
Daily growth rate (mg day^{-1}) [d]	91.8 ± 15.8	220.6 ± 23.8**	219.1 ± 10.6**	209.6 ± 10.3**	200.4 ± 3.5**	26.4 ± 11.1	85.6 ± 18.3	109.1 ± 39.4	129.4 ± 21.5	113.1 ± 11.7
Shell growth rate (μm day^{-1}) [d]	49.6 ± 7.2	120.3 ± 13.0***	112.1 ± 3.7***	126.9 ± 0.5***	113.5 ± 4.8***	13.5 ± 1.2**	44.6 ± 7.5	35.2 ± 3.7	45.4 ± 4.5	56.2 ± 4.8
Feed intake (g kg abalone^{-1} day^{-1}) [c,e]	7.06 ± 0.10	4.00 ± 0.10***	4.76 ± 0.05***	3.31 ± 0.05***	4.68 ± 0.11***	1.40 ± 0.10***	1.24 ± 0.05***	2.25 ± 0.16***	1.43 ± 0.13***	2.74 ± 0.10***
Serum SOD activity (U mL^{-1}) [c]	0.132 ± 0.02	0.090 ± 0.01	0.121 ± 0.01	0.097 ± 0.01	0.130 ± 0.03	0.108 ± 0.02	0.098 ± 0.01^	0.115 ± 0.02	0.085 ± 0.01	0.105 ± 0.01
Serum FRAP value (μmol Fe^{2+} equivalent) [c]	0.136 ± 0.01	0.120 ± 0.01	0.129 ± 0.00	0.112 ± 0.01	0.114 ± 0.00	0.118 ± 0.00	0.096 ± 0.01*^	0.128 ± 0.01	0.126 ± 0.01	0.113 ± 0.02

[a] Dunnett's tests use the live *U. lactuca* at the 22 °C water temperature treatment as the control value, to compare against all other treatments.
[b] Values are mean ± SE. n = 3, except for superscript ^, where n = 2.
[c] Dunnett's 1-tailed test, *denotes value is significantly different to control value (live *U. lactuca* at the 22 °C water temperature).
[d] Dunnett's 2-tailed test, * denotes value is significantly different to control value (live *U. lactuca* at the 22 °C water temperature).
[e] As fed basis.
[f] GSE = grape seed extract.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 5, the average consumption of dried *U. lactuca* on a dry basis for the commercial + 30% dried *U. lactuca* diet was ~0.99 and 0.43 g kg abalone^{-1} day^{-1} for the 22 and 26 °C water temperatures, respectively. The average consumption of dried *U. lactuca* on a dry basis for the commercial + 5% GSE + 30% dried *U. lactuca* diet was ~1.40 and 0.82 g kg abalone^{-1} day^{-1} for the 22 and 26 °C water temperatures, respectively.

4. Discussion

In the present study, feeding the commercial diet at 26 °C, we were able to produce mortality patterns in larger 3-year-old abalone similar to those experienced on South Australian abalone farms during periods of high summer water temperatures. Using the methods outlined by Stone et al. (2014), a positive control diet, live *U. lactuca*, was also established, which resulted in high survival (>95%) at the 26 °C water temperature. The negative control commercial abalone diet performed as expected, with low (~50%) levels of survival, which was analogous to that experienced in events of SM on-farm in southern Australia (Shane McLinden, Manager, Southseas Abalone Pty Ltd., personal communication; Stone et al., 2014; Vandepeer, 2006). Mortality was not an issue at the water temperature of 22 °C, as this temperature is within the range tolerated by South Australian strains of greenlip abalone to express normal behaviour, growth and survival (Stone et al., 2013, 2014).

The primary aim of this study was to investigate the potential of two dietary additives, GSE and dried *U. lactuca*, to reduce the oxidative stress and mortality in greenlip abalone associated with chronic exposure to high water temperatures. GSE was selected due to its high level and variety of antioxidants and bioactive compounds such as phenols, catechins, epicatechins, procyanidins and proanthocyanidins (Ahn et al., 2002; Balu et al., 2005; Davies et al., 2009; Jayaprakasha et al., 2003). GSE has been found to enhance the antioxidant status and reduce oxidative-related damage in rodent models (Ahn et al., 2002; Balu et al., 2005; Çetin et al., 2008). GSE has also been reported to have antibacterial (Jayaprakasha et al., 2003) and antiviral (Su and D'Souza, 2011, 2013) activities. In the present study, significantly improved survival was demonstrated in greenlip abalone fed diets containing 5% GSE (Fig. 3). The addition of GSE also resulted in significantly higher DO levels (saturation and concentration) at 6:45 am (Table 2). Adequate DO saturation levels are essential for abalone to maintain optimal oxygen consumption rates, growth rates (81% saturation) (Harris et al., 1999) and immune functions (Cheng et al., 2004; Vosloo et al., 2013). The dissolved oxygen saturation levels (min 66%) for all treatments recorded during the present study were also always above those demonstrated to affect survival of greenlip abalone (63%) by Harris et al. (1999), using animals within the size range used in the present study.

The improved survival of the abalone fed diets containing GSE may have been partly due to the increased DO levels. GSE addition may have increased the DO levels via a number of processes. Jayaprakasha et al. (2003) demonstrated that GSE inhibited bacterial growth in vitro, therefore, it is possible that GSE leaching from the diet may have reduced the total bacterial levels in the culture tank, thus reducing bacterial oxygen demand and bacterial CO_2 production in the environment (Rivkin and Legendre, 2001). GSE may have also improved the oxidative status of the abalone, consequently reducing their respiration and oxygen consumption rates, which are known to increase with rising water temperatures (Britz et al., 1997; Lushchak, 2011).

Another benefit of GSE addition to the diets at 26 °C was the significant increase in feed intake (Tables 4 and 5; Fig. 6b) and meal acceptance (Fig. 7). Loss of appetite and reduced feed intake are common symptoms of stress and the onset of disease (Fox et al., 2002; Kaushik, 1986; Wendelaar Bonga, 1997). If an animal consumes less food, it will subsequently obtain lower levels of energy and essential nutrients, and health may be further compromised. The results of this study suggest that either GSE retains the appetite of abalone in declining health, or more likely when considering survival rates, that GSE improves the oxidative status and health profile of the abalone, resulting in retained appetite. If GSE improves the oxidative status of the abalone, then it is possible that the abalone are able to metabolise the energy needed for feeding, without producing threatening levels of free radicals in the process (Lushchak, 2011). The feed intake and meal acceptance of abalone were improved when fed the GSE diets, which would likely have caused the abalone to be in a more nutritionally nourished state, and in turn, have promoted the ability to tolerate the temperature stress and improve survival.

In Experiment 1, a validation trial was performed to determine if handling stress affected the SOD activity and FRAP values in the serum, indicating that the abalone were exhibiting a stress response similar to that exhibited by fish (Wendelaar Bonga, 1997). Following the initial disturbance, a significant decline in SOD activity and a non-significant increase in FRAP value were observed. These results formed the basis for haemolymph sampling in Experiment 2, so that sampling techniques were timely and consistent for all animals sampled.

In Experiment 2, dietary GSE addition significantly increased serum SOD activity of the abalone (Table 4). SOD is known to increase in response to a physiological stress (Monk et al., 1989), in order to combat excess free radicals formed. The increase in SOD activity which occurred in this study may indicate that the abalone fed the GSE diets were more readily able to tolerate the handling stress during sampling. This finding could imply that those abalone would also be able to tolerate other stressors which may arise in their environment. Similar findings of increased SOD activity or expression have been observed in other studies. Wu et al. (2011) reported that mRNA expression of SOD increased in *Haliotis discus hannai* when given feed supplemented with 34 and 711 mg zinc kg^{-1}, but declined when supplemented with excessive amounts of zinc (3463 mg kg^{-1}). Similarly, Wang et al. (2009) reported that 3.8 mg kg^{-1} of dietary copper increased SOD activity in *H. discus hannai* hepatopancreas and serum, but inclusion levels over 3.8 mg kg^{-1} did not have this effect. Hepatopancreas SOD and glutathione peroxidase activities were also reported to increase in *H. discus hannai*, when supplemented with the antioxidant alpha-lipoic acid (Zhang et al., 2010). When feeding *H. discus hannai* diets supplemented with vitamin E, Fu et al. (2007) reported tissue SOD activity to be unaffected, but observed an increase in activity of two other key antioxidant enzymes, catalase and glutathione peroxidase. When supplemented with menadione, *H. discus hannai* were reported to have decreased SOD activity in the viscera, but increased SOD activity in the muscle (Fu et al., 2012). These findings indicate that the effect of dietary supplementation on SOD activity is quite dependent on dose rate and sample type. Tolerance to hypoxic conditions has been associated with higher levels of antioxidant defence (Gorr et al., 2010; Vosloo et al., 2013). Therefore, the higher SOD activity observed in the serum of greenlip abalone fed GSE diets in the present study may have been due to GSE improving the antioxidant defence of the abalone, and subsequently improving their tolerance of the more hypoxic 26 °C water. This may have contributed to the improved survival observed in the GSE treatments.

Although no significant differences in serum FRAP values were found in abalone response to GSE or *U. lactuca* addition in this study at 22 or 26 °C (Table 4), there was a significant reduction in serum FRAP values of abalone fed the commercial diet at 26 °C, compared to abalone fed the live *U. lactuca* and all other diets in the present study (Table 5). Similarly, Stone et al. (2014) found abalone fed live *U. lactuca* had significantly higher serum FRAP values than those fed a commercial diet, which was suggested to indicate a higher antioxidant status of those abalone fed the live *U. lactuca* diet. Further research into the oxidative status of abalone in response to dietary intervention is warranted.

GSE has also been identified to exhibit antimicrobial activity which may have improved abalone survival in this study. Jayaprakasha et al. (2003) reported that GSE inhibited growth of particular bacteria in vitro, such as *Bacillus* spp., *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Similarly, Su and D'Souza (2011, 2013)

found that GSE was able to reduce the infectivity of viral surrogates and hepatitis A virus in vitro. Similar antibacterial and antiviral effects may have occurred in the present study, which could have reduced bacterial growth in the culture water, on the gills, or in the gut of the abalone. Bissett et al. (1998) also found that bacteria tended to proliferate on uneaten feed, therefore, the increased feed intake associated with GSE addition would have reduced the amount of uneaten feed present for bacteria to colonise. Further studies are warranted to determine the exact effects GSE has on bacterial and viral populations in the culture water, and in the abalone.

The current study also aimed to test the use of dried *U. lactuca* as a dietary additive to improve survival of greenlip abalone at the 26 °C water temperature. The selection of dried *U. lactuca* was based on the knowledge that live *U. lactuca* is a component of the natural greenlip abalone diet (Shepherd, 1973), and has been reported to significantly improve survival of greenlip abalone at water temperatures of 26 °C (Stone et al., 2014). However, it is not currently commercially viable to use as a live feed in Australian abalone aquaculture. *U. lactuca* has also been reported to contain a variety of bioactive compounds such as peptides, phycobiliproteins, polyphenols, carotenoids, tocopherols and sterols (Abirami and Kowsalya, 2011; Chandini et al., 2008; Tierney et al., 2010), which may be beneficial to the health of abalone. Feeding live *U. lactuca* and extracts from *U. lactuca* to greenlip abalone has also been reported to enhance their immune response (Dang et al., 2011b). Tendencia and de la Pena (2010) found that adding live *Gracilaria heteroclada* or *Kappaphycus striatum* into shrimp culture tanks reduced the bacterial load in the water, indicating antibacterial activity of the macroalgae. The antibacterial activity of macroalgae reported by Tendencia and de la Pena (2010) may have also occurred with the *U. lactuca* in the present study, and may have decreased bacterial levels in the culture water. In the present study, dried *U. lactuca* was included in the commercial diet at 30% to deliver an amount comparable, on a dry weight basis, to feeding exclusively live *U. lactuca* at 26 °C. At 26 °C, the survival of abalone was higher when fed the formulated diets containing dried *U. lactuca*, than those without (Fig. 4); however, the effect was smaller than that observed for GSE addition. Antibacterial properties of *U. lactuca* may be another contributing factor responsible for the high survival observed in this study.

Unlike GSE, the addition of dried *U. lactuca* did not significantly improve dissolved oxygen levels (Table 2). This may partly explain why survival was not as high for the abalone fed the dried *U. lactuca* diets, compared to those fed the GSE diets. However, like the GSE diets, the dried *U. lactuca* diets had a significant effect on feed intake and meal acceptance. Feed intake and meal acceptance at 26 °C were significantly higher, and took longer to decline for abalone fed diets containing dried *U. lactuca* compared to those without (Table 4; Figs. 6b and 7). A possible explanation for this higher survival is that dried *U. lactuca* may retain the bioactive compounds of live *U. lactuca*, which may have improved the immune function of the abalone. Another possibility is that the addition of dried *U. lactuca* diluted the ingredients in the commercial diet, therefore, also diluting any negative dietary aspects which may have exacerbated mortality (Vandepeer, 2006). Further research is required to understand and define the benefits of feeding dried *U. lactuca* to greenlip abalone.

In the current study, live *U. lactuca* was used as a positive control comparison to assess any potential benefits associated with feeding diets containing GSE or dried *U. lactuca*. Aside from the previously mentioned bioactive compounds present in macroalgae, live macroalgae can also act as a bio-filter, as it has the ability to remove CO_2 and ammonia from the water and produce oxygen. The abalone from the live *U. lactuca* treatments had very high (>95%) levels of survival (Fig. 5), and their culture water also contained significantly higher levels of DO compared to any of the formulated diets (Table 3). The higher DO levels could be attributed to photosynthetic production of oxygen by *U. lactuca* during the light period; however, this may not completely explain the occurrence. Jan et al. (1981) measured oxygen consumption of small *Haliotis diversicolour supertexta* after exposing them to *Ulva* spp. extract, and reported oxygen consumption of the abalone temporarily reduced. A similar effect may have occurred in this experiment after the introduction of the live *U. lactuca*. At 6:45 am, the pH levels of the live *U. lactuca* treatments were higher than those for the formulated diet treatments. If the live *U. lactuca* reduced bacterial levels in the culture water, then it is likely that bacterial CO_2 production would have decreased, and subsequently increased the culture water pH. Dissolved CO_2 gas readings ranged from 1 mg L^{-1} to <1 mg L^{-1} and were well below reported harmful levels (Manon and Hossain, 2013).

The abalone fed the live *U. lactuca* also had significantly decreased growth compared to abalone fed formulated diets (Table 5). Although decreased growth is not beneficial from a production viewpoint, it may have benefits which promote survival on two fronts. Slower growing organisms require less oxygen and energy inputs, therefore, the lower oxygen requirement may have led to increased oxygen levels in the culture water. Alternatively, if abalone invest less energy on growth, more energy may be available for essential immune functions and heat tolerance (Lushchak, 2011; Somero, 2002). The feed intake of abalone fed live *U. lactuca* at the 22 °C increased significantly over the first week (Fig. 6a), which was likely due to the satiation feed rate being underestimated initially, and hence was raised in the second week. Overall feed intake was higher for abalone fed the live *U. lactuca* at 22 °C compared to the other diets at the same water temperature (Table 5). The increased feed intake may have been a result of the abalone compensating for the relatively lower nutrient density of the live *U. lactuca* compared to the formulated diets (Table 1). Stone et al. (2014) reported that similar sized greenlip abalone voluntarily consumed a higher ration of live *U. lactuca* (5.9% bw day^{-1}) compared to a commercial diet (3.52% bw day^{-1}). Stone et al. (2013) also reported that similar sized greenlip abalone, grown at the same water temperature, compensated for dietary protein intake by consuming a higher ration in response to a lower dietary protein content. The meal acceptance of abalone fed live *U. lactuca* was reduced at the 26 °C water temperature, which was a similar result to that of the commercial diet. However, while abalone fed the commercial diet would often fast for periods of three to five days in a row, abalone fed the live *U. lactuca* tended to develop a daily alternation between eating and not eating in the latter weeks of the experiment. This indicates that for the 26 °C water temperature treatments, abalone fed live *U. lactuca* were accepting their meal more consistently over time, compared to those fed the commercial diet.

Summer mortality has often been associated with a number of potential stressors and it is believed that there may be a threshold level, above which, the compounded stressors in the system precipitate an outbreak of mortality as a result of a ubiquitous bacterial infection, particularly due to *Vibrio* spp. (Handlinger et al., 2005). Therefore, it is pertinent that a reduction of one stressor may benefit the entire system and avoid exceeding this outbreak threshold. Bearing this in mind, a reduction of multiple stressors may be even more beneficial. When tested within the bounds of the model developed by Stone et al. (2014), diets in the current study containing GSE and dried *U. lactuca* produced benefits in multiple areas concerning stress. These benefits may have all partly contributed to preventing the abalone from exceeding the hypothesised outbreak threshold, resulting in improved survival of the abalone at the high water temperature (26 °C) in this study.

An important stressor which was not investigated in detail in this study was microbial populations. Several reports of SM, also known as 'summer immune depression', place a high level of emphasis on microbial infections, particularly with *Vibrio* spp., being the final cause of death (Lee et al., 2001; Nicolas et al., 2002; Travers et al., 2008, 2009, 2010). This indicates that both biotic and abiotic stressors compound to cause the final result of death, and both would need to be studied further in order to gain a full understanding of SM.

Although the use of GSE has demonstrated benefits in this study, the use of some antioxidants and trace elements has been reported to exhibit reduced positive effects, or negative effects, when used at excessive

concentrations (Fu et al., 2006, 2007; Wan et al., 2004; Wu et al., 2011). We aimed to include GSE in the diets at a level which was high enough to elicit a response (dose rate of ~100 mg kg bw^{-1} day^{-1}), as determined by similar dose rates in other research which yielded positive results (Balu et al., 2005; Çetin et al., 2008; Farbood et al., 2009; Sarkaki et al., 2007). In the present study, an average dose rate of ~125 mg GSE kg bw^{-1} day^{-1} was given to the abalone fed GSE diets at the 26 °C water temperature, which was within an acceptable range of the targeted dose rate. The abalone fed GSE diets at the 22 °C water temperature received, on average, almost double the targeted dose rate (~236 mg kg bw^{-1} day^{-1}) due to the higher feed intake, but displayed identical survival to the other treatments at the 22 °C water temperature. This indicated that the higher dose rate at the 22 °C water temperature did not have any substantial disadvantages. Rather than decreasing, the FRAP value of the GSE diets increased after soaking in water (Table 1). This may have indicated that rather than the water leaching away the antioxidative compounds of GSE, it softened the feed, making those compounds more readily available for ingestion and uptake by the abalone. In the present study, positive improvements in survival were recorded from the dose rate of GSE (~125 mg kg bw^{-1} day^{-1}) consumed by abalone at 26 °C. However, it is possible that this level is beyond the optimum for promoting an ideal oxidative balance. Further dose response testing may reveal a dose rate which not only further increases survival, but also costs less to implement.

In conclusion, this study has demonstrated that addition of 5% GSE to a commercial abalone diet significantly improved the survival of greenlip abalone chronically exposed to high water temperature (26 °C) and associated stressors. It has also demonstrated the potential of dried *U. lactuca* as a dietary additive to improve survival at high water temperatures. These methods of dietary intervention can be built upon to not only investigate the effect of different dose rates, but to also test other additives which may be more effective at improving survival, or more cost-effective to implement, as a solution to reduce mortality during periods of high summer water temperature events on-farm and improve industry productivity.

Acknowledgments

The authors wish to acknowledge Dr James Harris, Elise Schaefer, Matthew Bansemer, Thanh Hoanghai and Duong Duong of Flinders University for technical and academic assistance; Dr Sasi Nayar, Raymond Cultura, Mark Gluis, Kriston Bott (SARDI Aquatic Sciences), Hanru Wang, Amy Cheah, Elizabeth Hickey, Sarah Manning and Dr Graham Webb (The University of Adelaide) for additional technical assistance; and Dr Michelle Hebart and Dr David Rutley (The University of Adelaide) for statistical support, and Dr Ron Smernik for academic writing guidance. The authors also wish to thank Joel Scanlon (Adam and Amos Abalone Foods) and Dr Tom Coote and Kym Heidenreich (Eyre Peninsula Aquafeeds) for their contributions to the supply and manufacture of the diets. Brett Lange would also like to acknowledge the financial support of the Playford Memorial Trust Inc. 2013, Adelaide, SA, Australia.

References

Abirami, R., Kowsalya, S., 2011. Nutrient and nutraceutical potentials of seaweed biomass *Ulva lactuca* and *Kappaphycus alvarezii*. J. Agric. Sci. Technol. 5, 107–115.

Ahn, H.S., Jeon, T.I., Lee, J.Y., Hwang, S.G., Lim, Y., Park, D.K., 2002. Antioxidative activity of persimmon and grape seed extract: in vitro and in vivo. Nutr. Res. 22, 1265–1273.

AOAC International, 1995. Official Methods of Analysis of AOAC International, 16th edition, vol. 2. Association of Analytical Communities, Arlington, VA, USA.

Athukorala, Y., Lee, K.W., Song, C., Ahn, C.B., Shin, T.S., Cha, Y.J., Shahidi, F., Jeon, Y.J., 2003. Potential antioxidant activity of marine red alga *Grateloupia filicina* extracts. J. Food Lipids 10, 251–265.

Balu, M., Sangeetha, P., Haripriya, D., Panneerselvam, C., 2005. Rejuvenation of antioxidant system in central nervous system of aged rats by grape seed extract. Neurosci. Lett. 383, 295–300.

Bansemir, A., Blume, M., Schröder, S., Lindequist, U., 2006. Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. Aquaculture 252, 79–84.

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.

Britz, P.J., Hecht, T., Mangold, S., 1997. Effect of temperature on growth, feed consumption and nutritional indices of *Haliotis midae* fed a formulated diet. Aquaculture 152, 191–203.

Çetin, A., Kaynar, L., Kocyigit, I., Hacioglu, S.K., Saraymen, R., Ozturk, A., Sari, I., Sagdic, O., 2008. Role of grape seed extract on methotrexate induced oxidative stress in rat liver. Am. J. Chin. Med. 36, 861–872.

Chandini, S.K., Ganesan, P., Suresh, P., Bhaskar, N., 2008. Seaweeds as a source of nutritionally beneficial compounds — a review. J. Food Sci. Technol. 45, 1–13.

Cheah, K., 2011. Grape sourced bioactives: a potential new treatment strategy for intestinal mucositis and colon cancer(Ph.D thesis) The University of Adelaide (131 pp.).

Cheng, W., Li, C.H., Chen, J.C., 2004. Effect of dissolved oxygen on the immune response of *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. Aquaculture 232, 103–115.

Chojnacka, K., Saeid, A., Witkowska, Z., Tuhy, Ł., 2012. Biologically active compounds in seaweed extracts — the prospects for the application. Open Conf. Proc. J. (Suppl. 1-M4), 20–28.

Dang, V.T., Speck, P., Doroudi, M., Smith, B., Benkendorff, K., 2011a. 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., Li, Y., Speck, P., Benkendorff, K., 2011b. 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., Benkendorff, K., 2012. Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*. Fish Shellfish Immunol. 32, 732–740.

Davies, J.A., Krebs, G.L., Barnes, A., Pant, I., McGrath, P.J., 2009. Feeding grape seed extract to horses: effects on health, intake and digestion. Animal 3, 380–384.

Davis, J.W., Sizemore, R.K., 1982. Incidence of *Vibrio* species associated with blue crabs (*Callinectes sapidus*) collected from Galveston Bay, Texas. Appl. Environ. Microbiol. 43, 1092–1097.

Farbood, Y., Sarkaki, A., Badavi, M., 2009. Preventive effect of grape seed hydroalcoholic extract on dementia type of Alzheimer's disease in aged male rats. Int. J. Pharmacol. 5, 257–262.

Fox, J.G., Anderson, L.C., Loew, F.M., Quimby, F.W., 2002. Laboratory animal medicine, 2nd edition. American College of Laboratory Animal Medicine SeriesAcademic Press, San Diego, CA, USA.

Fu, J.H., Zhang, W.B., Mai, K.S., Feng, X.N., Xu, W., Liufu, Z.G., Tan, B.P., 2006. Effects of dietary vitamin A on antioxidant responses of abalone *Haliotis discus hannai* Ino. Acta Oceanol. Sin. 25, 141–150.

Fu, J., Zhang, W., Mai, K., Feng, X., Xu, W., Liufu, Z., Ma, H., Ai, Q., 2007. Effects of vitamin E on antioxidant enzyme activities and fatty acid compositions in juvenile abalone *Haliotis discus hannai* Ino. J. Shellfish Res. 26, 809–814.

Fu, J., Xu, W., Mai, K., Zhang, W., Feng, X., Liufu, Z., 2012. Effects of dietary menadione on the activity of antioxidant enzymes in abalone, *Haliotis discus hannai* Ino. Chin. J. Oceanol. Limnol. 30, 118–123.

Gilroy, A., Edwards, S.J., 1998. Optimum temperature for growth of Australian abalone: preferred temperature and critical thermal maximum for blacklip abalone, *Haliotis rubra* (Leach), and greenlip abalone, *Haliotis laevigata* (Leach). Aquac. Res. 29, 481–485.

Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010. Hypoxia tolerance in animals: biology and application. Physiol. Biochem. Zool. 83, 733–752.

Guillard, R.R., 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., Ryther, J.H., 1962. Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. 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., Goulias, J., 2014. Effects of severe heat stress on immune function, biochemistry and histopathology in farmed Australian abalone (hybrid *Haliotis laevigata* × *Haliotis rubra*). Aquaculture 432, 26–37.

Jan, R.Q., Shao, K.T., Chang, K.H., 1981. Extract of sea lettuce *Ulva* reduces oxygen consumption of abalone (*Haliotis diversicolour supertexta* Lischke). B. I. Zool. Acad. Sin. 20, 83–85.

Jayaprakasha, G.K., Selvi, T., Sakariah, K.K., 2003. Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. Food Res. Int. 36, 117–122.

Kaushik, S.J., 1986. Environmental effects on feed utilization. Fish Physiol. Biochem. 2, 131–140.

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.).

Lee, K., Liu, P., Chen, Y., Huang, C., 2001. The implication of ambient temperature with the outbreak of vibriosis in cultured small abalone *Haliotis diversicolor supertexta* Lischke. J. Therm. Biol. 26, 585–587.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.
Manon, M.R., Hossain, M.D., 2013. Ecology of *Cyprinus carpio* var. *specularis* (Physico-chemical conditions of the habitat). J. Sci. Found. 9, 133–139.
McShane, P.E., Gorfine, H., Knuckey, I., 1994. Factors influencing food selection in the abalone *Haliotis rubra* (Mollusca: Gastropoda). J. Exp. Mar. Biol. Ecol. 176, 27–37.
Miles, D.W., Ross, T., Olley, J., McMeekin, T.A., 1997. Development and evaluation of a predictive model for the effect of temperature and water activity on the growth rate of *Vibrio parahaemolyticus*. Int. J. Food Microbiol. 38, 133–142.
Monk, L.S., Fagerstedt, K.V., Crawford, R.M., 1989. Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress. Physiol. Plant. 76, 456–459.
Nicolas, J.L., Basuyaux, O., Mazurie, J., Thebault, 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.).
Rhimou, B., Hassane, R., Jose, M., Nathalie, B., 2010. The antibacterial potential of the seaweeds (Rhodophyceae) of the Strait of Gibraltar and the Mediterranean Coast of Morocco. Afr. J. Biotechnol. 9, 6365–6372.
Rivkin, R.B., Legendre, L., 2001. Biogenic carbon cycling in the upper ocean: effects of microbial respiration. Science 291, 2398–2400.
Sarkaki, A., Farbood, Y., Badavi, M., 2007. The effect of grape seed extract (GSE) on spatial memory in aged male rats. Pak. J. Med. Sci. 23, 561–565 (July–September).
Shepherd, S., 1973. Studies on Southern Australian abalone (genus *Haliotis*). I. Ecology of five sympatric species. Aust. J. Mar. Freshwat. Res. 24, 217–258.
Somero, G.N., 2002. Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. Integr. Comp. Biol. 42, 780–789.
Stone, D.A.J., Harris, J.O., Wang, H., Mercer, G.J., Schaefer, E.N., Bansemer, M.S., 2013. Dietary protein level and water temperature interactions for greenlip abalone *Haliotis laevigata*. J. Shellfish Res. 32.
Stone, D.A.J., Bansemer, M.S., Lange, B., Schaefer, E.N., Howarth, G.S., Harris, J.O., 2014. Dietary intervention improves the survival of cultured greenlip abalone (*Haliotis laevigata* Donovan) at high water temperature. Aquaculture 430, 230–240.
Su, X.W., D'Souza, D.H., 2011. Grape seed extract for control of human enteric viruses. Appl. Environ. Microbiol. 77, 3982–3987.
Su, X.W., D'Souza, D.H., 2013. Grape seed extract for foodborne virus reduction on produce. Food Microbiol. 34, 1–6.
Tendencia, E.A., de la Pena, M.R., 2010. Potentials of *Kappaphycus striatum* (Schnitz) and *Gracilaria heteroclada* Zhang (Ad Xia) to control the growth of luminous bacteria *Vibrio harveyi*. Philipp. Agric. Sci. 93, 109–115.
Tierney, M.S., Croft, A.K., Hayes, M., 2010. A review of antihypertensive and antioxidant activities in macroalgae. Bot. Mar. 53, 387–408.
Travers, M.A., Le Goic, 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 Goic, N., Huchette, S., Nicolas, J.L., Koken, M., Paillard, C., 2009. 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., Meistertzheim, A.-L., Cardinaud, M., Friedman, C.S., Huchette, S., Moraga, D., Paillard, C., 2010. Gene expression patterns of abalone, *Haliotis tuberculata*, during successive infections by the pathogen *Vibrio harveyi*. J. Invertebr. Pathol. 105, 289–297.
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 Mol. Integr. Physiol. 164, 192–199.
Wan, M., Mai, K.-S., Ma, H.-M., Xu, W., Liufu, Z.-G., 2004. Effects of dietary selenium and vitamin E on antioxidant enzyme activities in abalone, *Haliotis discus hannai* Ino. Acta Hydrobiol. Sin. 28, 496–503.
Wang, W., Mai, K., Zhang, W., Ai, Q., Yao, C., Li, H., Liufu, Z., 2009. Effects of dietary copper on survival, growth and immune response of juvenile abalone, *Haliotis discus hannai* Ino. Aquaculture 297, 122–127.
Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.
Wu, C., Zhang, W., Mai, K., Xu, W., Zhong, X., 2011. Effects of dietary zinc on gene expression of antioxidant enzymes and heat shock proteins in hepatopancreas of abalone *Haliotis discus hannai*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 154, 1–6.
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.
Zhang, W., Chen, Q., Mai, K., Xu, W., Wang, X., Liufu, Z., 2010. Effects of dietary α-lipoic acid on the growth and antioxidative responses of juvenile abalone *Haliotis discus hannai* Ino. Aquac. Res. 41, e781–e787.