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SURVIVAL AND FEEDING OF GREENLIP ABALONE (*HALIOTIS LAEVIGATA*) IN RESPONSE TO A COMMERCIALLY AVAILABLE DIETARY ADDITIVE AT HIGH WATER TEMPERATURE

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ABSTRACT Elevated mortality, triggered by increased water temperatures (>22°C) and associated factors, is a significant issue for land-based abalone farms in southern Australia. The aim of this study was to test the efficacy of the commercial animal feed product Orego-Stim (OS), containing oregano essential oil in reducing the mortality of 3-y-old greenlip abalone (*Haliotis laevigata*) exposed to high water temperature (25°C). Inclusion levels of 0.0 (commercial control diet), 0.5%, 1.0%, 2.0%, and 4.0% OS were added into a commercial feed formulation and diets were fed to 3-y-old greenlip abalone (67.98 g, 77.01 mm shell length) at two water temperatures (22°C and 25°C) for 47 days. Survival and immune parameters including phagocytic activity, total hemocyte count (THC), and activities of the enzymes superoxide dismutase (SOD) and catalase (CAT) were measured. Survival was high at 22°C with no significant differences in mortality between treatments. Irrespective of this, exposure to elevated water temperature (25°C) resulted in significantly higher mortalities for all diet treatments ($P < 0.05$), without any effect of diet. Low ferric reducing antioxidant potential values were observed for all diets. Phagocytic activity remained stable for all temperature and OS treatments (48.82% ± 1.31%). The dietary inclusion of OS and increased water temperature increased the THC compared with the commercial control diet treatment at 22°C. Superoxide dismutase was significantly elevated in greenlip abalone fed the commercial control (0.56 ± 0.08 U mL^{-1}), and CAT was significantly higher when fed the 4.0% OS diet treatment at 25°C (18.93 ± 2.25 nmol min^{-1} mL^{-1}). Despite failing to increase survival, OS significantly enhanced feed intake at both temperatures at 2.0% and 4.0% compared with the commercial control diet treatment, highlighting its ability as a feeding stimulant ($P < 0.05$).

KEY WORDS: abalone, culture, thermal stress, nutrition, anti-oxidants, survival, dietary stimulants, *Haliotis laevigata*

INTRODUCTION

In Australia, cultured greenlip abalone (*Haliotis laevigata*) account for a significant proportion of the total abalone aquaculture production (Freeman 2001). Land-based abalone culture systems are prone to large seasonal fluctuations in water temperatures ranging between 10°C and 25°C (Stone et al. 2013). When water temperatures exceed the optimal level for abalone, abalone farms may experience significant abalone mortality events (up to 50%), termed as summer mortality. This is a serious problem for the Australian abalone aquaculture industry (Vandepeer 2006, Stone et al. 2013, 2014). Summer mortality is not attributed to temperature alone; a combination of biological, nutritional, anthropogenic, and environmental factors are likely contributors (Hooper et al. 2014, Lange et al. 2014, Stone et al. 2014) with fatalities arising from complex interactions between the molluscan host, environment and surrounding pathogens (Dang et al. 2012). All these factors could contribute to oxidative stress, reduced immunity, organ damage, and potentially death, with increased biological oxygen demand at higher temperatures (Hooper et al. 2014, Lange et al. 2014, Stone et al. 2014).

Newly matured 3-y-old abalone at production size (>60 mm shell length) are particularly affected (Vandepeer 2006, Dang et al. 2011, Stone et al. 2014), indicating that maturation and spawning are likely contributors (Travers et al. 2008, 2009) as well as handling stressors (Cardinaud et al. 2014) in multiple abalone species. Heat-related mortality in greenlip abalone similar to on-farm events has been induced repeatedly in the laboratory, with survival manipulated by alternation of dietary ingredients (Lange et al. 2014, Stone et al. 2014, Duong et al. 2016). These studies provided the means to investigate dietary intervention and its responses to heat-associated stress and potential greenlip abalone deaths.

In cell-mediated immunity, all animals can produce reactive oxygen species (ROS) (Hooper et al. 2007). During this cell defense, ROS will interact with biological macromolecules and cause enzyme inactivation, lipid peroxidation, DNA damage, and cell death (Cazenave et al. 2006). Initial ROS production is controlled by the release of endogenous enzymes, creating a delicate oxidant–anti-oxidant equilibrium; excess ROS can become destructive, if there are insufficient endogenous antioxidants to neutralize them (Lushchak 2011). Increasing endogenous antioxidant concentrations with exogenous antioxidants through diets could stabilize the antioxidant–oxidant equilibrium of abalone under extreme oxidative stress such as high water temperature.

Orego-Stim (OS) is a commercial feed additive containing oregano essential oil (OEO) (Yu-Wen 2008). In the literature, OEO has been recognized for having high antioxidant, anti-inflammatory, antifungal, and antimicrobial activities (Ruberto & Baratta 2000, Santoyo et al. 2006, Viuda-Martos et al. 2007). Inclusion of OEO and its active components produced increased growth for yellowtail tetra (*Astyanax altiparanae*) (Ferreira et al. 2014) and juvenile rainbow trout (*Oncorhynchus*

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mykiss) (Ahmadifar et al. 2011). After the challenge with *Aeromonas hydrophila*, the survival, growth, and antioxidant activity of channel catfish *Ictalurus punctatus* was significantly enhanced with OS dietary inclusion at 0.05% (dose rate of 16.4 mg OS kg body weight day^{-1}) (Zheng et al. 2009). Similarly, improved survival of juvenile white leg shrimp (*Litopenaeus vannamei*) was demonstrated after exposure to *Vibrio* spp. challenge (Gracia-Valenzuela et al. 2014). Because of these beneficial properties and its commercial availability, in this study, OS was trialled as a feed additive in greenlip abalone diets. It was hypothesised that OS would maintain the antioxidant–oxidant equilibrium, act as an immunostimulant, and improve survival of greenlip abalone under heat stress. Improved survival would be of high economic benefit to the aquaculture industry and provide an insight into natural herbal immunostimulants for molluscan feed.

MATERIALS AND METHODS

Experimental Additive and Diet Preparation

Orego-Stim (Meriden Animal Health, Brassall, Queensland, Australia) was formulated into the commercial Abgrow (Eyre Peninsula Aquafeed Pty. Ltd., Lonsdale, South Australia) diet at 0.0%, 0.5%, 1.0%, 2.0%, and 4.0% (Table 1). The 2.0% inclusion of OS was designed to provide a similar dose rate to abalone to that reported to improve survival of juvenile channel catfish (16.4 mg OS kg body weight day^{-1}) (Zheng et al. 2009). This inclusion rate was adjusted, taking into account the leaching loss and feed intake rate (1.24 g kg abalone^{-1} day^{-1}) of similar-sized greenlip abalone at similar water temperatures (Lange et al. 2014).

The Abgrow diet mashes, with relevant additions of OS and water (375 mL kg^{-1} mash), were mixed using Hobart mixer (Hobart Corp., Troy, OH) and cold extruded through a TR110 pasta machine (Macchine Per Pasta SRL, Molina Di Male, Italy) to produce flat sinking diet chips (5 × 5 × 2 mm). The diet chips were dried (<10% moisture) at 50°C for 24 h and stored at –15°C until fed.

Biochemical Diet Analyses

The five formulated test diets and pure OS were assayed for antioxidant capacity using the ferric-reducing antioxidant potential (FRAP) assay, according to the methods of Xu et al. (2010) and the modifications of Cheah (2011) and Wang et al. (2015). Briefly, FRAP reagent was freshly created by mixing 300 mM acetate buffer (pH 3.6) and 10 mM of TPTZ (2,4,5-tri[2-pyridyl]-s-triazine) in 40 mM hydrochloric acid and 20 mM ferric chloride in the volume ratio 10:1:1 and held in the dark at 37°C. Diets were homogenized and dissolved in dimethyl sulfoxide (1.0 mg mL^{-1}), and 15 µL of the supernatant was assayed with 150 µL of FRAP reagent. Ferrous sulphate standards were prepared (0.0–1.0 mM) and used to generate a calibration curve. Ferric-reducing antioxidant potential assay values of diets tested are expressed as mmol Fe^{2+} g^{-1} of sample. The absorbance was read at 593 nm after 4 min in darkness on a plate reader. Vitamin C was used as a positive control (Cat. No. A5960-25g, Sigma-Aldrich, Australia).

Biochemical analyses of the moisture, ash, crude lipid, gross energy, crude protein, and amino acid composition of the commercial control diet and OS were analyzed by AsureQuality (Blockhouse Bay, Auckland, New Zealand), according to the methods of AOAC International (1995). The commercial control diet (0.0% OS) and 0.5%, 1.0%, 2.0%, and 4.0% OS diets were oven-dried for 16 h at 105°C to determine the moisture content. Crude protein (N × 6.25) was determined by the Kjeldahl method. Crude lipid was analyzed using a Soxtherm rapid extraction system (Gerhardt GmbH & Co. KG, Königswinter, Germany) with petroleum liquid (boiling

TABLE 1.

Ingredient and biochemical composition of the pure OS and experimental diets.

Item	Pure OS	OS inclusion level (%)				
		0.0*	0.5*†	1.0*†	2.0*†	4.0*†
Ingredient composition (as fed)						
Commercial diet mash (g kg^{-1})*	–	979.4	974.4	969.4	959.4	939.4
Vitamin–mineral premix (g kg^{-1})	–	2.0	2.0	2.0	2.0	2.0
Salmon oil (g kg^{-1})	–	15.0	15.0	15.0	15.0	15.0
Sodium alginate (g kg^{-1})	–	3.6	3.6	3.6	3.6	3.6
OS (g kg^{-1})	1,000.0	0.0	5.0	10.0	20.0	40.0
Biochemical composition (as fed)						
Moisture (g kg^{-1})	40.0	90.0	85.6	86.0	82.9	81.4
Crude protein (g kg^{-1})	1.0	320.0	319.9	318.2	316.1	310.1
Crude lipid (g kg^{-1})	5.0	47.0	47.0	46.8	46.5	45.7
Ash (g kg^{-1})	894.0	66.0	70.2	74.1	82.3	98.2
Carbohydrate (g kg^{-1})‡	60.0	477.0	477.3	474.6	472.2	464.6
Gross energy (MJ kg^{-1})	1.2	15.8	15.8	15.7	15.6	15.4
FRAP (mmol Fe^{2+} g^{-1})§	0.041^{ab}	0.017^{c}	0.034^{bc}	0.033^{bc}	0.031^{bc}	0.034^{bc}

Different superscripts denote significant differences (one-factor ANOVA) ($P < 0.05$).

* Diet mash was provided by Eyre Peninsula Aquafeed Pty. Ltd., Lonsdale, South Australia.

† The composition of the 0.5%, 1.0%, 2.0%, and 4.0% OS diets were calculated from the 0.0% commercial control diet and pure OS.

‡ Carbohydrate (g kg^{-1}) = 1,000 – (moisture + protein + lipid + ash).

§ FRAP = ferric reducing antioxidant potential (mmol Fe^{2+} g^{-1}), positive control vitamin C FRAP value (10.06 ± 0.48 mmol Fe^{2+} g^{-1}).

point 100°C) as the extracting solvent. Ash was determined using a muffle furnace at 550°C for 16 h. Gross energy content of the commercial control diet and OS were determined using a bomb calorimeter calibrated with benzoic acid. Carbohydrate was calculated as follows: carbohydrate (g kg^{-1}) = 1,000 – (moisture + protein + lipid + ash).

Experimental Animals

Three-year-old greenlip abalone, purchased from Pure Australian Abalone (Boston Point, Port Lincoln, South Australia), were held in a flow-through system within 180 L tanks at the South Australian Research and Development Institute Aquatic Sciences Center (West Beach, Adelaide, South Australia). Greenlip abalone were fed the 5 mm Abgrow diet (Table 1, 0.0% OS diet) *ad libitum* for 58 days.

Experimental System

The experimental system comprised two identical water temperature–controlled (22°C and 25°C) flow-through subsystems, with 15 experimental tanks per system (described in Stone et al. 2013 and Lange et al. 2014), subjected to controlled photoperiod [12 h low intensity light (3.4 Lux) and 12 h of darkness] in an air temperature–controlled laboratory (22°C ± 1°C). Water temperature was controlled using 3 kW immersion heaters (240 V, JQ20, Austin and Cridland, Carlton, NSW, Australia). Each tank was gravity fed with flow-through, 30 μm sand filtered, ultraviolet-treated fresh seawater at 300 mL min^{-1}.

Tank Stocking and Acclimation

At stocking, greenlip abalone were weighed (mean weight: 67.98 ± 16.89 g $abalone^{-1}$) and measured (mean shell length: 77.01 ± 6.48 mm) (mean ± SD, $n = 300$), and 10 individuals were randomly distributed into each of the three replicate tanks for each diet at each water temperature. The initial water temperature (15°C) was not changed during the first week poststocking. Then water temperature was increased by ~0.6°C every second day, and 22°C was reached 30 days poststocking. Thereafter, the water temperature in half of the tanks was raised by ~1°C every second day, until 25°C (35 days poststocking) was reached. The experimental temperature challenge (22°C versus 25°C) period ran for 47 days and was considered to have commenced once the water temperature had surpassed 22°C. Any mortalities were removed, weighed, measured, and replaced with similar-sized tagged greenlip abalone to maintain stocking density.

Water Quality

Water quality was monitored at 1,200 h daily. A Oxyguard Handy Polaris 2 oxygen probe and meter (Oxyguard International A/S, Birkerød, Denmark), calibrated daily in water-saturated air, was used to measure DO saturation (%) and concentration (mg L^{-1}). The pH was measured using a Eutech pH Testr30 m, after calibration in pH buffer solutions (Eutech Instruments Pte. Ltd., Singapore) and water temperature measured with a laboratory thermometer (Livingstone International, Rosebery, NSW, Australia). Salinity was measured weekly with an ISSCO UR-2 handheld refractometer, calibrated in distilled water before use (model RF20, Extech Instruments, Nashua, NH).

Feeding and Feed Intake

Once stocked into experimental tanks, greenlip abalone were fed the experimental diets to excess (0.6% body weight day^{-1}) at 1,600 h daily to account for diurnal feeding behavior (Buss et al. 2015). The following morning (0830 h), tanks were cleaned and uneaten feed collected in a 500 μm sieve, weighed wet and stored at –20°C. Feed rates were adjusted accordingly to compensate for biomass changes for each tank from mortalities and replacement of dead abalone.

The dry matter leaching loss for each diet was determined in triplicate by submerging 4 g of diet in tanks at 22°C and 25°C for 16.5 h with no greenlip abalone. After 16.5 h, the remaining diet chips were collected and oven-dried at 105°C for 16 h. Apparent feed intake was calculated by subtracting the uneaten feed (dried in the same way) and leaching loss (dry weights) from the total feed amount delivered per tank, before converting values to an as fed basis, using the initial moisture content of the diets.

Feed Calculations

Feed consumed, dry basis (g $tank^{-1}$) = feed offered – (uneaten feed + leaching loss).

Average daily feed intake, dry basis at 22°C (% biomass day^{-1}) = feed consumed/[(initial biomass + final biomass)/2]/days.

Estimated average daily feed intake, dry basis at 25°C (% biomass day^{-1}) = feed consumed/(average of running biomass average over 47 days)/days.

Sample Collection and Preparation

At the end of the experiment, three greenlip abalone per tank ($n = 90$) were sampled for hemolymph, obtained from the cephalic sinus within 60 sec of initial disturbance (Lange et al. 2014), using a 23-gauge hypodermic needle and a 1-mL syringe.

Hemolymph samples for superoxide dismutase (SOD) and catalase (CAT) assays were centrifuged (model Hettich Zentrifugen Universal 320 R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Baden-Württemberg, Germany) at 4°C and 2,000 RPM (or RCF of 236) for 5 min, pooling equal amounts of serum (three greenlip abalone per tank) into cryotubes with a P1000 micropipette and stored at –80°C.

Total hemocyte count (THC) and phagocytic activity were determined immediately. Total hemocyte count was determined in duplicate from each hemolymph sample following the methods of Dang et al. (2012) and Hooper et al. (2012), with slight modifications: 50 μL of vortexed hemolymph was pipetted into 100 μL of 6% formalin and held on ice. Then, postvortexing, a hemocytometer with cover slip was filled (9 μL) and dual replicate counts of five squares per greenlip abalone were assessed under a light microscope at 400× magnification. Total hemocyte count (cells mL^{-1}) = hemocyte count of 5 squares × 5 (to give 1 mm^2 estimate) × depth correction (10) × volume correction (1,000) × dilution factor (3).

Phagocytic activity was determined according to the methods described in Stone et al. (2014) with a slight modification to hemocyte number. In brief, phagocytic activity was determined in triplicate as the percentage of phagocytic hemocytes in 30 hemocytes per greenlip abalone: Phagocytic activity (%) = number of phagocytic hemocytes/total number of phagocytes (30) × 100.

Biochemical Serum Analyses

Serum samples were analyzed for SOD and CAT activity using a SOD activity assay kit (Item Number 706,002, Cayman Chemical Company, Ann Arbor, MI) and CAT assay kit (Item Number 707,002, Cayman Chemical Company, Ann Arbor, MI), respectively. Both kits were optimized using nonexperimental abalone serum, collected using identical techniques, with multiple dilutions, to find a dilution that best fitted the curve. Undiluted serum was used. Absorbance was read on a plate reader (CLARIOstar, BMG Labtech, Ortenberg, Germany) at 450 nm for SOD and 540 nm for CAT. All absorbance outputs were generated through MARS data analysis software.

Statistical Analyses

Survival patterns were assessed using Kaplan–Meier analyses with log-rank and Breslow tests. Homogeneity of variance among means was assessed using Levene's test and normality assessed with the Shapiro–Wilk test. To ensure normal distribution and homogeneity of variance, data were square root transformed where appropriate. One-factor analysis of variance (ANOVA) with Welch test and Games–Howell *post hoc* tests were used for FRAP and initial weight as homogeneity of variance was not reached despite transformations. Two-factor ANOVAs were used to assess the main effects (water temperature and OS addition) and interactions on feed intake, water quality parameters, THC, phagocytic activity, and serum activity (SOD and CAT) across the five formulated diets. One-factor ANOVAs and Tukey HSD multiple comparison *post hoc* tests were used to assess differences across the five diets where an interaction was observed. For feed intake and phagocytic activity when the assumption of homogeneity of variances was violated for data (despite transformations), the robustness of ANOVA was assumed in these cases. A significance level of $P < 0.05$ was used. All data are presented as mean ± SE, except water quality, presented as SD. All statistical analyses were done with IBM SPSS version 22 for Macintosh (IBM SPSS Inc., Chicago, IL).

RESULTS

Diet Antioxidant Analyses

The FRAP content did not significantly differ between any of the formulated diets used in this study ($P > 0.05$, Table 1), with an overall mean of 0.03 ± 0.00 mmol Fe^{2+} g^{-1} sample. Numerically, all diets with OS inclusion were approximately twice the FRAP value of the commercial control diet, with no statistical difference in FRAP value between OS diets and pure OS (Table 1). Pure OS had a significantly higher FRAP value than the commercial control diet treatment ($P = 0.011$, Table 1).

Survival

Greenlip abalone held at elevated water temperatures (25°C) showed a significant decrease in survival, irrespective of diet compared with greenlip abalone held at 22°C ($P < 0.05$, Fig. 1). At 25°C, there was no significant difference in greenlip abalone survival, irrespective of diet, despite the commercial control diet having higher survival numerically than OS diets ($P > 0.05$, Fig. 1). At 22°C, the survival for greenlip abalone ranged from 93.3% ± 3.3% to 100.00% ± 0.0% and similarly did not significantly differ between diets ($P > 0.05$, Fig. 1).

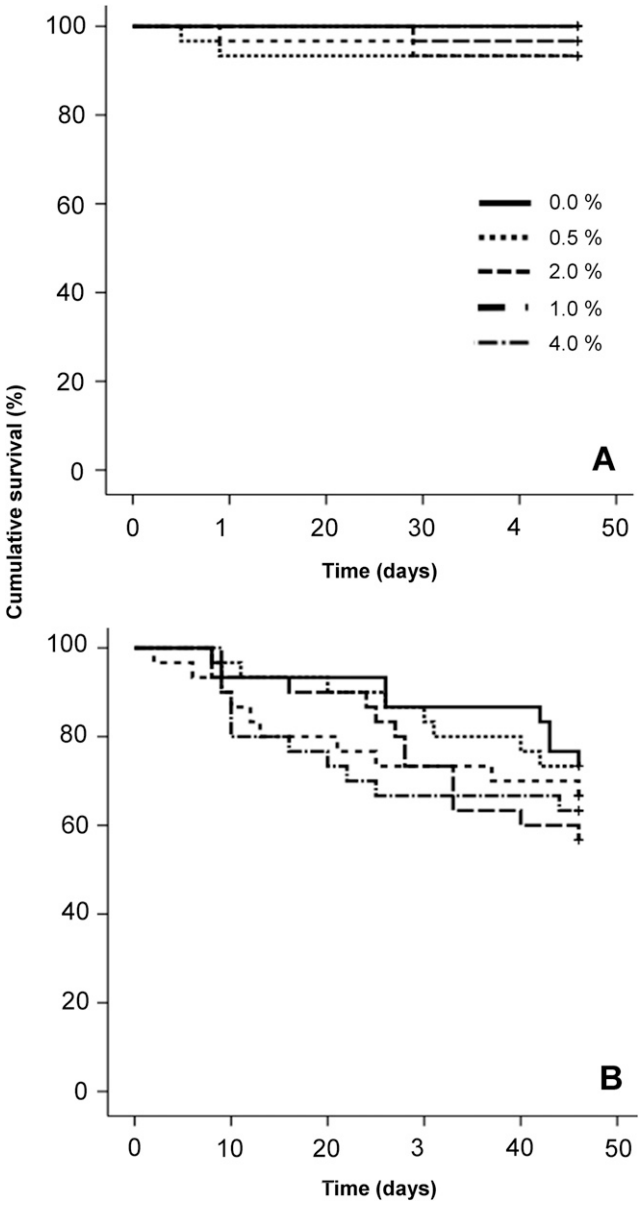

Figure 1. Kaplan–Meier survival curves for 3-y-old greenlip abalone fed diets containing OS (%) at increasing inclusion levels, including the commercial control (0.0% OS) at 22°C (A) and 25°C (B) for 47 days ($n = 30$ for each line). Survival for all formulated diets at 22°C > formulated diets at 25°C, $P < 0.05$. Survival was = between all diets at 22°C and = between all diets at 25°C, $P > 0.05$.

Feed Intake and Initial Weight

There was no significant difference in overall mean initial individual weight of greenlip abalone (67.98 ± 0.17 g, $P = 0.705$). Feed intake was significantly higher in greenlip abalone held at 22°C (0.22% ± 0.01% biomass day^{-1}) compared with 25°C (0.12% ± 0.01% biomass day^{-1}) ($P < 0.001$, Table 2), with significant differences between OS inclusion levels ($P = 0.001$, Table 2). Feed intake remained highest for 4.0% OS diet and was significantly higher than the commercial control or 1.0%

OS diets (Table 2). Greenlip abalone fed the commercial control diet had the lowest feed intake, being significantly lower than the 2.0% and 4.0% OS diets. Feed intake for greenlip abalone fed the 0.5%, 1.0%, and 2.0% OS diets did not significantly differ, and there was no significant interaction between temperature and OS inclusion level ($P = 0.416$, Table 2).

Serum SOD Activity

There was no significant effect of temperature on greenlip abalone serum SOD activity ($P = 0.138$, Table 2), nor was there any significant interaction between temperature and OS inclusion level ($P = 0.066$, Table 2). The OS inclusion level had a significant effect on serum SOD activity, with abalone-fed the commercial control having significantly higher serum SOD activity than greenlip abalone–fed the 0.5% and 1.0% OS diets ($P = 0.023$, Table 2). Greenlip abalone fed the 0.5%, 1.0%, 2.0% and 4.0% OS diets did not significantly differ in serum SOD activity ($P > 0.05$). Numerically, serum SOD activity was highest for greenlip abalone fed the commercial control diet, with lower serum SOD activity for greenlip abalone fed all diets containing OS (Table 2).

Serum CAT Activity

There was a significant interaction for serum CAT activity between OS inclusion level and temperature ($P = 0.002$, Table 2). The interaction between temperature and OS inclusion level was due to significantly higher serum CAT activity in the 25°C and 4.0% OS treatment combination than all other OS inclusion levels at both temperatures (Table 2). The OS inclusion level had a significant effect on the serum CAT activity for greenlip abalone ($P < 0.001$, Table 2). Comparatively, temperature had no significant effect on the serum CAT activity for greenlip abalone ($P = 0.083$, Table 2).

Phagocytic Activity

There was no significant effect of OS inclusion level ($P = 0.872$) or temperature ($P = 0.706$) on greenlip abalone phagocytic activity, and there was no interaction between the two factors ($P = 0.946$). Total average phagocytic activity across all treatments was 48.82% ± 1.31% (Table 2).

Total Hemocyte Count

There was a significant interaction for THC in greenlip abalone between OS inclusion level and temperature ($P = 0.048$, Table 2). The interaction was due to the effect of temperature being lost with the addition of OS; the combination of temperature and OS inclusion level resulted in no additive effect to changes in THC. There was a significant effect of temperature on greenlip abalone THC ($P = 0.002$, Table 2) but no significant effect of OS inclusion level ($P = 0.095$, Table 2). Numerically at 25°C, the 0.5% OS diet had the highest mean THC of all the OS diets, with the trend decreasing with increasing inclusion levels; however, statistically there was no difference between any OS diets at 25°C ($P > 0.05$, Table 2).

Water Quality

Water quality parameters did not significantly differ between OS inclusion level, nor were there any significant interactions ($P > 0.05$). After the commencement of the temperature challenge, significant separation of water temperatures was maintained ($P < 0.001$), with total averages being 21.97°C ± 0.00°C and 24.98°C ± 0.02°C for the two temperature treatments. The DO (mg L^{-1}) levels did not significantly differ between OS inclusion levels or temperatures ($P > 0.05$). For the duration of the experiment, the total average DO saturation was 86.12% ± 1.81% and ranged from 76% to 98% across all OS inclusion levels and temperatures. The pH was significantly higher at 25°C ($P < 0.001$). Despite this, the difference was only in the order of 0.62%, with total average pH being 8.15 ± 0.01. Salinity remained constant at 36.0 ± 0.0 ppt for the duration of the trial.

DISCUSSION

Previously, most temperature or bacterial challenges for abalone have been acute stress trials, following extended periods in normal environmental conditions (Cheng et al. 2004d, Dang et al. 2011). In this study, a chronic long-term temperature challenge and impact of dietary intervention of OS were tested comparatively, using a replicable model of on-farm summer conditions as per Lange et al. (2014) and Stone et al. (2014). When chronically exposed to elevated water temperature, the addition of OS in this study did not improve greenlip abalone survival; however, there was evidence for OS acting as a dietary stimulant.

Elevated temperature (25°C) in this study triggered significant mortality levels in 3-y-old greenlip abalone when fed commercially formulated abalone diets, 1 wk after continuous exposure (Fig. 1). This is consistent with mortality data reported for similar-sized greenlip abalone held at 26°C (Lange et al. 2014, Stone et al. 2014) and comparable with on-farm situations experienced in South Australia during periods of elevated summer water temperatures (Vandepeer 2006). Significantly lower mortality corresponded with lower water temperature treatments (22°C), which is the optimal temperature for South Australian greenlip abalone, promoting normal behavior, growth, and survival (Stone et al. 2013, Lange et al. 2014, Stone et al. 2014). Unlike previous survival success with OS addition to channel catfish diets (Zheng et al. 2009), the dietary addition of OS in this study did not improve survival, compared with abalone fed the commercial control diet at 25°C (Fig. 1). The present testing approach has already demonstrated increased survival in greenlip abalone at 25°C and 26°C when fed high antioxidant diets of dried or live sea lettuce (*Ulva lactuca*), grape seed extract, or green tea extract, in comparison with abalone fed a commercial diet at the same temperature (Lange et al. 2014, Stone et al. 2014, Duong et al. 2016). Therefore, there is little evidence for the use of this product to promote survival in this species.

By contrast, including OS in diets increased feed intake rates at either temperature, indicating that OS was a successful dietary stimulant for greenlip abalone. Overall, greenlip abalone in this study had significantly lower feed intake at 25°C compared with 22°C (Table 2). Appetite loss and reduced feed intake are common indicators of stress and onset of disease (Kaushik 1986, Wendelaar Bonga 1997). Significantly lowered feed intake at higher temperatures has also previously been described for 2 and 3-y-old greenlip abalone at 26°C (Lange et al. 2014, Stone et al. 2014). Significantly lower feed intake

TABLE 2.

Feed intake, serum SOD, serum CAT and phagocytic activities for greenlip abalone at different temperatures and OS inclusion levels.*

Temperature (°C)	22					
OS inclusion level (%)	**0.0**	**0.5**	**1.0**	**2.0**	**4.0**	**0.0**
Feed intake, dry basis (% biomass day^{-1})‡	0.19 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.08 ± 0.01
Serum SOD activity (U mL^{-1})	0.56 ± 0.08	0.23 ± 0.04	0.20 ± 0.07	0.26 ± 0.07	0.29 ± 0.10	0.23 ± 0.02
Serum CAT activity (nmol min^{-1} mL^{-1})	8.55 ± 0.69	6.69 ± 0.48	8.73 ± 1.48	9.79 ± 1.65	8.95 ± 0.49	7.73 ± 0.86
Phagocytic activity (%)	47.90 ± 3.41	50.99 ± 7.40	49.63 ± 2.57	48.64 ± 0.81	44.07 ± 8.89	51.73 ± 2.14
THC ($\times 10^6$ cells mL^{-1})	2.78 ± 0.61	5.86 ± 1.58	6.03 ± 0.41	6.94 ± 1.30	5.41 ± 0.80	7.18 ± 0.48

levels at 25°C in this study can be interpreted as indicating higher stress levels before higher mortality at this temperature (Fig. 1). In yellowtail tetra, OEO was also reported to improve feed intake at 0.5% and 1.0% inclusion levels compared with the control, with feed intake rate decreasing at higher inclusion levels (1.5%–2.5%) (Ferreira et al. 2014). This demonstrates that OS improved the appetite of greenlip abalone and acted as a suitable dietary stimulant even in elevated water temperatures. Potentially, the use of this product or other feeding stimulants previously described as attractants for black abalone (Harada 1992) may offer farmers an additional management strategy when feeding decreases.

Nutrition has previously been shown to be a limiting factor for abalone at high temperatures, particularly when diets lack antioxidants, with increased mortality repeatedly reported (Lange et al. 2014, Stone et al. 2014). The antioxidant power within a system can be measured by FRAP value, with higher values indicating greater antioxidant status (Griffin & Bhagooli 2004, Xu et al. 2010). In this study, low antioxidant status of diets is supported by the lack of significant separation in FRAP values between the commercial control and experimental OS diets (Table 1). Similar FRAP values have been reported for essential oregano oil by Teixeira et al. (2013) at 0.035 mmol Fe^{2+} g^{-1} (Table 1). This indicates higher dietary inclusions of OS may not have increased FRAP values further. This is additionally supported with Pure OS showing no significant separation to any of the OS experimental diets (Table 1). Diets containing additives with higher FRAP values (FRAP value of grape seed extract: 6.0 μmol Fe^{2+} g^{-1} sample) have previously increased greenlip abalone survival at elevated water temperature (Lange et al. 2014). If higher antioxidant diet inclusion is a solution to elevated temperature associated mortality, low FRAP values of OS diets in this study may explain the lack of survival improvement at higher temperatures, with no significant survival difference between any of the formulated diets at 25°C (Fig. 1). Additional phytochemical characterization of diets would define further clarity of these results.

The total average phagocytic activity in this study for 3-y-old greenlip abalone (48.82% ± 1.31%) is comparable with samples from greenlip abalone fed the commercial control diets seen by Stone et al. (2014) at both temperatures. Previously, when fed antioxidant rich live *Ulva* sp., significantly higher phagocytic activities were seen in abalone (Stone et al. 2014). If high antioxidant diet potential is a driver for higher phagocytic activity, then the low antioxidant diet potential seen in this study may explain levels being similar to that of the commercial control in Stone et al. (2014). Despite this, it is expected that there is more than dietary effect at play.

In this study, temperature had no significant effect on serum SOD activity with similar results seen by Lange et al. (2014). Comparatively, dietary inclusion of OS significantly increased serum SOD activity for greenlip abalone fed the commercial diet compared with 0.5% and 1.0% OS diets (Table 2). The numerical trend of higher exogenous antioxidant (FRAP) levels in the OS diets may have lowered the endogenous antioxidant enzyme SOD, as seen previously (Wan et al. 2004). Considering this, as FRAP values in this study did not significantly differ between OS inclusion levels, the effect of lowering serum SOD activity would have been small. This is further supported with no significant difference between the serum SOD activity of the commercial control and the 4.0% OS and 2.0% OS diets (Table 2). It would be expected with potentially higher antioxidant concentrations, that these results would be more pronounced.

The antioxidant enzyme CAT is only upregulated once the capacity of SOD and GPx is exceeded (Baud et al. 2004). In this study, serum CAT activity did not significantly differ and remained low for all OS inclusion levels and temperature combinations except the 4.0% OS diet at 25°C. Increased temperature (19°C) has previously triggered higher CAT activity in the gill tissue for juvenile South African abalone (*Haliotis midae*) than for abalone held at 14°C (Vosloo et al. 2013). In this study, it is possible that the combination of consuming a diet of low-antioxidant potential at increased temperature may have increased oxidative stress and ROS levels and resulted in higher serum CAT activity. Despite this, the oxidant–antioxidant equilibrium controlling oxidative stress is complex and further analysis would be required.

In previous stress studies, initial THC for abalone ranged between 2.1 and 3.5×10^6 cells mL^{-1} (Malham et al. 2003, Cheng et al. 2004a, 2004b, 2004c, 2004d, 2004e, Dang et al. 2012). These THC levels are comparable with the commercial control THC levels at 22°C in this study ($2.78 \pm 0.61 \times 10^6$ cells mL^{-1}) (Table 2). In this study, a significant interaction for THC was seen between temperature and OS inclusion level. In this case, the combination of OS inclusion level and temperature had no additive effect to THC, increasing THC only to a point. This is further supported by the commercial control, being the only diet to have significant separation of temperature, where 25°C produced significantly higher THC than the commercial control at 22°C (Table 2). Elevated THC levels have previously been seen for juvenile hybrid (*Haliotis laevigata* × *Haliotis rubra*)

TABLE 2.

continued

25				Two-factor ANOVA†						
				Temperature (*X*)	OS inclusion level (*Y*)					Interaction (*X* × *Y*)
0.5	**1.0**	**2.0**	**4.0**		**0.0**	**0.5**	**1.0**	**2.0**	**4.0**	
0.13 ± 0.01	0.11 ± 0.03	0.11 ± 0.01	0.16 ± 0.00	22 > 25	C	ABC	BC	AB	A	NS
0.17 ± 0.04	0.19 ± 0.00	0.25 ± 0.02	0.35 ± 0.07	NS	A	B	B	AB	AB	NS
6.77 ± 0.43	8.65 ± 1.01	8.58 ± 0.77	18.93 ± 2.25	NS	B	B	B	B	A§	S
51.45 ± 6.10	47.78 ± 2.52	47.53 ± 3.86	48.50 ± 3.15	NS	NS	NS	NS	NS	NS	NS
9.14 ± 1.70	8.49 ± 1.57	5.34 ± 0.35	7.92 ± 0.14	22 < 25§	NS	NS	NS	NS	NS	S

* Values were mean ± SE, $n = 3$. Significant difference when $P < 0.05$. Two-way ANOVA.

† S denotes significant interaction ($P < 0.05$). NS denotes nonsignificant ($P > 0.05$). For OS inclusion level, different letters denote significant differences (A, B, C) and common letters denote nonsignificant differences ($P > 0.05$), with letter A signifying highest and letter C signifying lowest values ($P < 0.05$). For water temperature (>) denotes that 22°C was significantly greater than 25°C and (<) denotes that 22°C was significantly less than 25°C.

‡ Feed intake measured over 47 days.

§ Denotes exceptions to significant trends (where a significant interaction was present): Significantly higher serum CAT activity for the 4.0% OS diet treatment was only true at 25°C. Significantly higher THC at 25°C was only true for the commercial control, 0.5, 1.0, and 4.0% OS diets compared with the commercial control diet at 22°C.

abalone (Hooper et al. 2014), adult blacklip abalone (*H. rubra*) (Dang et al. 2012), and Taiwan abalone (*Haliotis diversicolor*) at increased water temperature (Cheng et al. 2004e), indicating elevated THC levels may be a general cellular immune response for abalone. This is additionally supported by the significantly higher mortalities at 25°C (Fig. 1).

CONCLUSION

The addition of the commercially available dietary additive OS to a commercial abalone diet did not improve survival of greenlip abalone when exposed to high water temperature (25°C) at the levels tested in this study. Despite this, OS inclusion significantly increased the feed intake irrespective of temperature. This suggests its potential as a dietary stimulant for greenlip abalone over a range of temperatures. Given the low antioxidant activity of OS, testing a more enriched product or higher inclusion level would be warranted in future studies, to further improve feed intake and reduce summer mortality on greenlip abalone farms.

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LITERATURE CITED

Ahmadifar, E. B., B. Falahatkar & R. Akrami. 2011. Effects of dietary thymol-carvacrol on growth performance, haematological parameters and tissue composition of juvenile rainbow trout, *Oncorhynchus mykiss*. *J. Appl. Ichthyology* 27:1057–1060.

AOAC International. 1995. Official methods of analysis of AOAC International, 16th edition, vol. 2. Arlington, VA: Association of Analytical Communities.

Baud, O., A. E. Greene, J. Li, H. Wang, J. J. Volpe & P. A. Rosenberg. 2004. Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *J. Neurosci.* 24:1531–1540.

Buss, J. J., D. A. Jones, A. Lumsden, J. O. Harris, M. S. Bansemer & D. A. J. Stone. 2015. Restricting feed ration has more effect than diet type on the feeding behaviour of greenlip abalone (*Haliotis laevigata*). *Mar. Freshw. Behav. Physiol.* 48:51–70.

Cardinaud, M., C. Offret, S. Huchette, D. Moraga & C. Paillard. 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.

Cazenave, J., M. A. Bistoni, S. F. Pesce & D. A. Wunderlin. 2006. Differential de-toxification and anti-oxidant response in diverse organs of *Corydoras paleatus* experimentally exposed to microcystin RR. *Aquat. Toxicol.* 76:1–12.

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

Cheng, W., I.-S. Hsiao & J.-C. Chen. 2004a. Effect of nitrite on immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Dis. Aquat. Organ.* 60:157–164.

Cheng, W., I.-S. Hsiao & J.-C. Chen. 2004b. Effect of ammonia on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Fish Shellfish Immunol.* 17:193–202.

Cheng, W., I.-S. Hsiao & J.-C. Chen. 2004c. Effect of dissolved oxygen on the immune response of *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Aquaculture* 232:103–115.

Cheng, W., I.-S. Hsiao, C.-H. Hsu & J.-C. Chen. 2004d. Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Fish Shellfish Immunol*. 17:235–243.

Cheng, W., F.-M. Juang & J.-C. Chen. 2004e. The immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus* at different salinity levels. *Fish Shellfish Immunol*. 16:295–306.

Dang, V. T., P. Speck & K. Benkendorff. 2012. Influence of elevated temperatures on the anti-viral and anti-bacterial immune response of abalone, *Haliotis rubra*. *Fish Shellfish Immunol*. 32:732–740.

Dang, V. T., P. Speck, M. Doroudi, B. Smith & K. Benkendorff. 2011. Variations in the anti-viral and anti-bacterial activity of *Haliotis laevigata*, *H. rubra* and their hybrid in South Australia. *Aquaculture* 315:242–249.

Duong, D. N., J. G. Qin, J. O. Harris, T. H. Hoang, M. S. Bansemer, K.-L. Currie, K.-Y. Phan-Thien, A. Dowell & D. A. J. Stone. 2016. Effects of dietary grape seed extract, green tea extract, peanut extract and vitamin C supplementation on metabolism and survival of greenlip abalone (*Haliotis laevigata* Donovan) cultured at high temperature. *Aquaculture* 464:364–373.

Ferreira, P. M. F., L. S. Nascimento, D. C. Dias, D. M. V. Moreira, A. L. Salaro, M. B. D. de Freitas, A. P. S. Carneiro & J. A. S. Zuanon. 2014. Essential oregano oil as a growth promoter for the yellowtail tetra, *Astyanax altiparanae*. *J. World Aquacult. Soc*. 45:28–34.

Freeman, K. A. 2001. Aquaculture and related biological attributes of abalone species in Australia—a review. No. 28. Perth, WA: Department of Fisheries.

Gracia-Valenzuela, M. H., M. J. Vergara-Jiménez, M. E. Baez-Flores & F. Cabrera-Chavez. 2014. Anti-microbial effect of dietary oregano essential oil against *Vibrio* bacteria in shrimps. *Arch. Biol. Sci*. 66:1367–1370.

Griffin, S. P. & R. Bhagooli. 2004. Measuring anti-oxidant potential in corals using the FRAP assay. *J. Exp. Mar. Biol. Ecol*. 302:201–211.

Harada, K. 1992. Feeding attraction activity of fragrant and pungent spice extracts in black abalone (*Haliotis discus*) (studies on the feeding attractants for fishes and shellfish XVII). In: Shepherd, S. A., M. J. Tegner & S. A. Guzmán del Proó, editors. Abalone of the world: biology, fisheries and culture. Victoria, Australia: Fishing New Books. pp. 193–200.

Hooper, C., R. Day, R. Slocombe, K. Benkendorff, J. Handlinger & J. Goulias. 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.

Hooper, C., R. Day, R. Slocombe, J. Handlinger & K. Benkendorff. 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., R. Slocombe, R. Day & S. Crawford. 2012. Leucopenia associated with abalone viral ganglioneuritis. *Aust. Vet. J*. 90:24–28.

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

Lange, B., K.-L. Currie, G. Howarth & D. A. J. Stone. 2014. Grape seed extract and dried macroalgae, *Ulva lactuca* Linnaeus, improve survival of greenlip abalone, *Haliotis laevigata* Donovan, at high water temperature. *Aquaculture* 433:348–360.

Lushchak, V. I. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol*. 101:13–30.

Malham, S., A. Lacoste, F. Gelebart, A. Cueff & S. Poulet. 2003. Evidence for a direct link between stress and immunity in the mollusc *Haliotis tuberculata*. *J. Exp. Zool*. 295A:136–144.

Ruberto, G. & M. Y. Baratta. 2000. Anti-oxidant activity of selected essential oil components in two lipid model systems. *Food Chem*. 69:167–174.

Santoyo, S., S. Cavero, L. Jaime, E. Ibañez, F. J. Señoráns & G. Reglero. 2006. Supercritical carbon dioxide extraction of compounds with anti-microbial activity from *Origanum vulgare* L.: determination of optimal extraction parameters. *J. Food Prot*. 69:369–375.

Stone, D. A. J., M. S. Bansemer, B. Lange, E. N. Schaefer, G. S. Howarth & J. O. Harris. 2014. Dietary intervention improves the survival of cultured greenlip abalone (*Haliotis laevigata*) at high water temperature. *Aquaculture* 430:230–240.

Stone, D. A. J., J. O. Harris, H. Wang, G. J. Mercer, E. N. Schaefer & M. S. Bansemer. 2013. Dietary and protein level and water temperature interactions for greenlip abalone (*Haliotis laevigata*). *J. Shellfish Res*. 32:119–130.

Teixeira, B., A. Marques, C. Ramos, C. Serrano, O. Matos, N. R. Neng, J. M. F. Nogueira, J. A. Saraiva & M. L. Nunes. 2013. Chemical composition and bioactivity of different oregano (*Origanum vulgare*) extracts and essential oil. *J. Sci. Food Agric*. 93:2707–2714.

Travers, M. A., O. Basuyaux, N. Le Goïc, S. Huchette, J. L. Nicolas, M. Koken & C. Paillard. 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. Change Biol*. 15:1365–1376.

Travers, M. A., N. Le Goic, S. Huchette, M. Koken & C. Paillard. 2008. Summer immune depression associated with increased susceptibility of the European abalone, *Haliotis tuberculata* to *Vibrio harveyi* infection. *Fish Shellfish Immunol*. 25:800–808.

Vandepeer, M. 2006. Preventing summer mortality of abalone in aquaculture systems by understanding interactions between nutrition and water temperature. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Australia. 85 pp.

Viuda-Martos, M., Y. Ruíz-Navajas, J. Fernández-Lopez & J. A. Pérez-Álvarez. 2007. Chemical composition of essential oils obtained from some species widely used in Mediterranean region. *Acta Chim. Slov*. 54:921–926.

Vosloo, D., L. van Rensburg & A. Vosloo. 2013. Oxidative stress in abalone: the role in temperature, oxygen and L-proline supplementation. *Aquaculture* 416–417:265–271.

Wan, M., K.-S. Mai, H.-M. Ma, W. Xu & Z.-G. Liufu. 2004. Effects of dietary selenium and vitamin E on antioxidant enzyme activities in abalone, *Haliotis discus hannai* Ino. *Acta Hydrob. Sinica* 5:496–503.

Wang, H., S. E. P. Bastian, A. Lawrence & G. S. Howarth. 2015. Factors derived from *Escherichia coli* Nissle 1917, grown in different growth media, enhance cell death in a model of 5-fluorouracil-induced caco-2 intestinal epithelial cell damage. *Nutr. Cancer* 67:316–326.

Wendelaar Bonga, S. E. 1997. The stress response in fish. *Physiol. Rev*. 77:591–625.

Xu, X., H. Xie, Y. Wang & X. Wei. 2010. A-type proanthocyanidins from lychee seeds and their anti-oxidant and antiviral activities. *J. Agric. Food Chem*. 58:11667–11672.

Yu-Wen, J. T. 2008. Orego-Stim: the proven and researched alternative to infeed antibiotic growth promoters and coccidiostats. *Analele IBNA* 24:48–55.

Zheng, Z. L., J. Y. W. Tan, H. Y. Liu, X. H. Zhou, X. Xiang & K. Y. Wang. 2009. Evaluation of oregano essential oil (*Origanum heracleoticum* L.) on growth, anti-oxidant effect and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*). *Aquaculture* 292:214–218.