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IMPACT OF VITAMIN K_1 ON TISSUE VITAMIN K LEVELS, IMMUNITY, AND SURVIVAL OF GREENLIP ABALONE, *HALIOTIS LAEVIGATA*, AT SUMMER WATER TEMPERATURES

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ABSTRACT Summer mortality impacts the productivity of greenlip abalone, *Haliotis laevigata*, on land-based farms in South Australia. It is associated with high water temperature (greater than 23°C), low dissolved oxygen levels, increased bacterial loads, and immune system suppression during summer months. This study aimed to alleviate mortality rates of greenlip abalone by dietary intervention using vitamin K_1 to support the innate immune system and oxidative status. Dietary vitamin K_1 at 0.0, 0.5, 1.0, and 5.0 mg kg^{-1} was added to a commercially formulated diet mash. An additional diet containing 0.5 mg kg^{-1} of K_3 was also used for comparison. Diets were fed to 3-y-old abalone (71.51 g; 79.91 mm) at 22 and 25°C water temperatures for 39 days. No mortalities were observed at 22°C; however, high mortalities were observed in all dietary treatments at the water temperature of 25°C. Compared with the negative control diet (0.0 mg additional inclusion of K_1 or K_3 kg^{-1}) at 25°C, the inclusion of vitamin K_1 or K_3 did not improve survival of greenlip abalone ($P > 0.05$). Vitamin K_1 inclusion level resulted in significant increases in vitamin K_1 concentration of visceral organ and muscle tissues ($P < 0.05$). Steady-state levels of vitamin K_1 were not reached. Steady-state levels of K_2-MK-4 in visceral organ and muscle were reached when analyzed levels of dietary vitamin K_1 reached 0.02 mg kg^{-1}. This was also true for K_2-MK-7, but in the visceral organ only. Vitamin K_1 inclusion level did not significantly affect total hemocyte count, phagocytic activity, or phagocytic index ($P > 0.05$). Increasing water temperature to 25°C resulted in significant increases in serum catalase activity (22 < 25°C) and vitamin K_1 concentration in muscle tissue (22 < 25°C). Comparison of vitamin K_1 or K_3 at 0.5 mg kg^{-1} resulted in significant changes to serum catalase activity ($K_1 > K_3$) and vitamin K_1 concentration in visceral organ ($K_1 > K_3$). In conclusion, vitamin K_1 at the doses tested, resulted in significant increases in vitamin K_1 concentration in visceral organ and muscle tissues, but failed to improve immune function, oxidative status, or survival of greenlip abalone at high summer water temperatures.

KEY WORDS: abalone, *Haliotis laevigata*, vitamin K_1, vitamin K_3, high water temperature, survival, tissue concentration

INTRODUCTION

Greenlip abalone, *Haliotis laevigata*, is cultured in land-based facilities in South Australia and is reliant on formulated feeds (Stone et al. 2013). Seasonal fluctuations in water temperatures expose abalone to temperatures ranging from 10 to 25°C (Stone et al. 2013). On farm, water temperatures may exceed 23°C for extended periods in the summer months. Reportedly, abalone has a low tolerance to exposure to acute or chronic water temperature increases (Gilroy & Edwards 1998, Day et al. 2010, Hooper et al. 2014). In Australia, exposure to high water temperatures may lead to a condition referred to as summer mortality which can result in mortality levels of up to 50% of larger more valuable stock (Vandepeer 2006, Dang et al. 2012, Stone et al. 2014). Older, 3-year old abalones are more susceptible to summer mortality than younger abalones (Stone et al. 2014).

The causative factors of summer mortality in abalone are suspected to be a combination of biotic and abiotic factors. High water temperatures in summer months give rise to reduced dissolved oxygen content (Lange et al. 2014). These conditions result in increased metabolism and respiration, oxidative stress (Lange et al. 2014), depressed immunity (Hooper et al. 2014), and reduced antibacterial activity, leaving abalone vulnerable to infection (Dang et al. 2012). Heat stress has also been demonstrated to damage tissue epithelium in the gills and gut (Hooper et al. 2014). It has been suggested that epithelial damage acts as a portal of entry for bacteria, including *Vibrio* species (Cheng et al. 2004). The immune status of abalone can be assessed by focusing on immune parameters including total hemocyte count, phagocytic activity, and antioxidant activity (Hooper et al. 2014).

Dietary intervention has been investigated as a possible solution for summer mortality through improvements in immune function (Lange et al. 2014, Stone et al. 2014). Dietary supplementation with the antioxidant grape seed extract at 5% improved phagocytic activity and increased survival by up to 50% (Lange et al. 2014). The impact of vitamins, other than vitamin C (Duong et al. 2016), on abalone survival in a temperature challenge trial is yet to be assessed. Vitamins have been assessed for in growth trials in optimal and fluctuating temperature conditions (Mai 1998, Tan & Mai 2001, Fu et al. 2007). Vitamin C also showed significant effect on tissue concentration (Mai 1998) and vitamin E, at inclusion levels of 50 mg kg^{-1}, increased levels of antioxidant enzymes (Fu et al. 2007).

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Identification of vitamin K–dependent proteins in the transparent sea squirt, *Ciona intestinalis*, has demonstrated the presence of a vitamin K–dependent Gla domain before the divergence of vertebrates and urochordates, suggesting new roles for vitamin K in addition to its role in blood coagulation (Kulman et al. 2006). Growth arrest–specific 6 is a vitamin K–dependent protein that has potential involvement in the innate immune system and phagocytosis (Hafizi & Dahlback 2006). These are essential to abalones for the immune response to bacteria.

Vitamin K is available in natural and synthetic forms. All K vitamers share a common 2-methyl-1, 4-naphthoquinone ring with side chains of different lengths (Krossoy et al. 2011). Phylloquinone (K_1) is a natural vitamer synthesized by plants and algae. Menaquinones (K_2), such as K_2-MK-7, are synthesized by bacteria and can have different length side chains of between 3 and 12 carbons. Menadione (K_3) is a synthetic vitamer that is converted to K_1 or K_2 in the form of K_2-MK-4 after ingestion (Grahl-Madsen & Lie 1997, Tan & Mai 2001, Krossoy et al. 2011, Fu et al. 2012). Vitamin K_3 is not biologically active until partially converted and it can be easily excreted (Krossoy et al. 2011). By contrast, vitamin K_1 has a higher retainment rate in chicken tissues than vitamin K_3 (Griminger 1984). Moreover, Griminger and Brubacher (1966) reported that a major proportion of vitamin K_1 fed to chicks was deposited in the liver.

Vitamin K as a dietary component in abalone feeds has yet to be comprehensively studied. Mai et al. (2001) reported an inclusion of 4 mg vitamin K_3 kg^{-1} diet for Pacific abalone, *Haliotis discus hannai*. Tan and Mai (2001) reported no effect of vitamin K_3 on survival in Pacific abalone maintained in seawater that fluctuated between 9.8 and 26.4°C. Fu et al. (2012) reported changes in superoxide dismutase and catalase activity with K_3 supplementation to diets. Tan and Mai (2001) recommended that 10 mg kg^{-1} of vitamin K_3 was sufficient for maintenance of steady-state tissue concentrations of K_2-MK-4; however, this was the minimum concentration included in their study.

The present study aimed to test graded levels of vitamin K_1 inclusion to abalone feeds at water temperatures of 22 and 25°C as a dietary intervention to reduce summer mortality. The effect of increasing dietary inclusion levels of vitamin K_1 on the concentration and conversion of vitamer types in visceral organ and muscle tissue was assessed. Total hemocyte count, phagocytic activity and phagocytic index, and serum antioxidant activity were determined to assess innate immune system function and oxidative stress, respectively.

MATERIALS AND METHODS

Experimental Design, Diets, and Preparation

Five experimental diets were used. A series of four diets containing nominal graded levels of vitamin K_1 (0.0, 0.5, 1.0, and 5.0 mg kg^{-1}) were chosen, taking into consideration recommended levels of vitamin K for abalone (Mai et al. 2001), fish (Krossoy et al. 2011), and poultry (Hubert Regtop personal communication, Agricure Scientific Organics, Breamar, New South Wales, Australia) (Table 1). An additional diet containing the nominal level of 0.5 mg kg^{-1} vitamin K_3 (Table 1) was included for comparison, as this is the form of vitamin K predominantly used in abalone diets (Tan & Mai 2001, Fu et al. 2012). The nutritional composition of the test diets is displayed in Table 2. Abalones were then exposed to a temperature challenge test at 22 and 25°C described by Stone et al. (2014). Briefly, water temperatures at optimal (22°C) and high (25°C) levels replicated conditions of summer mortality that abalones are subjected to on land-based farms. The control diet (0.0 mg kg^{-1} vitamin K_1) at 22 and 25°C served as positive and negative survival controls, respectively. Vitamin K_1 and K_3 were sourced from Agricure Scientific Organics (Breamar, New South Wales, Australia).

The commercial abalone Abgrow Premium diet mash, provided by Eyre Peninsula Aquafeeds Pty Ltd. (Lonsdale, South Australia, Australia) was used as the base for all test diets. To ensure that levels of vitamin K were controlled within the diets, a vitamin and mineral premix was formulated with no included vitamin K, based on previous reported dietary levels (Mai et al. 2001). Agricure Scientiic Organics (Breamer, New South Wales, Australia) manufactured the mix according to these specifications. To manufacture experimental diets, the required amounts of dry mash, vitamin premix, fish oil, and sodium alginate were weighed (Table 1) and mixed, as per manufacturer's

TABLE 1.

Composition of experimental diets.

Nominal vitamin K inclusion level (mg kg^{-1})	0.0 K_1	0.5 K_1	1.0 K_1	5.0 K_1	0.5 K_3
Ingredient (dry basis)					
Diet mash (g kg^{-1})*	955.0	955.0	955.0	954.0	955.0
Fish 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
Vitamin mineral premix (g kg^{-1})†	20.0	20.0	20.0	20.0	20.0
Carbohydrate carrier	6.4	6.4	6.4	7.4	6.4
Vitamin K_1 (mg kg^{-1})	0.0	0.5	1.0	5.0	0.0
Vitamin K_3 (mg kg^{-1})	0.0	0.0	0.0	0.0	0.5
Sum (g kg^{-1})	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0

* Commercial abalone Abgrow Premium diet mash, provided by Eyre Peninsula Aquafeeds Pty Ltd (Lonsdale, South Australia, Australia).
† Vitamin mineral premix based on Mai et al. (2001), excluding vitamin K: 100 g^{-1} contains thiamin HCI 0.6 g, riboflavin 0.5 g, folic acid 0.15 g, para-aminobenzoic acid 2.0 g, pyridoxine HCI 0.2 g, niacin 4.0 g, Ca pantothenate 1.0 g, D-biotin 60.0 mg, ascorbic acid 20.0 g, vitamin E 0.25 g, cyanocobalamin (b12) 900.0 μg, retinol 0.15 g, cholecalciferol 0.250 mg, ethoxyquin 2.0 g, inositol 20.0 g.

TABLE 2.

Biochemical composition and analyzed vitamin K_1 and K_3 levels of experimental diets fed to greenlip abalone at 22 and 25°C (dry basis).

Vitamin K inclusion level	0.0 K_1	0.5 K_1	1.0 K_1	5.0 K_1	0.5 K_3
Moisture (g kg^{-1})	104.0	98.0	95.0	98.0	91.0
Crude protein (g kg^{-1})	276.0	271.0	271.0	271.0	275.0
Crude lipid (g kg^{-1})	50.0	46.0	45.0	47.0	45.0
Ash (g kg^{-1})	62.0	61.0	61.0	62.0	62.0
NFE (g kg^{-1})*	508.0	524.0	528.0	522.0	527.0
Gross energy (MJ kg^{-1})	15.2	15.1	15.3	15.2	15.3
Vitamin K_1 (mg kg^{-1})	0.02	0.26	0.32	1.73	0.02
Vitamin K_3 (mg kg^{-1})†	ND	ND	ND	ND	0.59

* NFE, nitrogen free extract calculated by 1,000 – crude protein – crude lipid – ash – moisture.
† ND, not detectable; Vitamin K_1 and K_3 assay detectable limits were 0.5 μg kg^{-1}.

specifications, in a Hobart mixer (Hobart Corp., Troy, OH) for 5 min. A carbohydrate carrier contacting the vitamin K source at the required level, 0.0, 0.5, 1.0, and 5.0 mg kg^{-1}, was dissolved in warm water (~40°C) and added to the mash then mixed for a further 3 min. Diets were manufactured using a Tr110 pasta machine (Machine Per Pasta SRL; Molina Di Malo, Vicenza, Italy), to produce a 5 × 5 × 2 mm flat sinking chip. Diets were then dried at ~50°C for ~30 h until the moisture level was less than 10%. To reduce the impact of light on the activity of vitamin K_1, diets were transferred into black bags and stored at –20°C until fed to abalones.

Experimental Animals

Three-year-old greenlip abalones, which had not been used in any previous experiments, were purchased from SAM Abalone (Boston Point, Port Lincoln, South Australia, Australia) in September 2015. The abalones were held in 200 L tanks at the South Australian Research and Development Institute (SARDI), South Australian Aquatic Sciences Center at West Beach, South Australia, in a flow-through seawater system at ambient water temperatures (16°C–18°C). They were fed a 5 mm commercial Abgrow Premium diet *ad libitum* daily until stocking.

Experimental System

The experiment was conducted in a photoperiod-controlled (12 h of low-intensity light [7:00 AM to 7:00 PM] and 12 h of dark [7:00 PM to 7:00 AM]) and air-temperature–controlled (21.8 ± 0.7°C) laboratory. Two identical water-temperature–controlled systems (22 and 25°C) were used as described in Stone et al. (2013) with 30 μm sand-filtered, ultraviolet-treated flow-through seawater. Each system consisted of 15 12.5 L blue plastic experimental tanks (Nally IH305; length, 39.2 cm; width, 28.8 cm; depth, 11.0 cm; bottom surface area, 1,129 cm^2; Viscount Plastics Pty Ltd.), with a water depth of 6 cm controlled by a standpipe, resulting in a tank water volume of 6.8 L. Experimental tanks were gravity-fed aerated seawater from a reservoir at a flow rate of 300 mL min^{-1}. Tank water flow rates were checked and adjusted three times per week. Water temperature was controlled using 3 kW immersion heaters (240 V, AB122–1; Hotco, Williamstown, South Australia, Australia). The experiment was of 39 days duration in line with previous temperature challenge experiments (Lange et al. 2014, Stone et al. 2014)

Stocking

Experimental tanks were randomly and evenly allocated a water temperature and diet treatment, in triplicate. Greenlip abalones were weighed (71.5 ± 0.2 g) and measured (shell length 79.91 ± 0.56 mm) and 10 abalones were randomly placed into each of the 30 tanks. Water temperature was adjusted from ambient temperature (18.5°C) at stocking to the required treatment water temperatures (22 and 25°C) by a maximum increment of 1°C day^{-1}. Tank water temperatures were then maintained within ±1.0°C until the end of the experiment.

Feeding

Abalones were fed their allocated diets to excess (0.6% body weight) daily at 4:00 PM Uneaten feed was collected at 8:30 AM the following day and transferred to containers which were weighed daily and stored at –20°C. Every 7 days, uneaten feed was oven-dried at 105°C for 16.5 h to obtain dry weights. To account for feed leaching losses, a measured amount of feed was left in tanks containing no abalone over the same time period, then collected, dried, and weighed using the same methods as uneaten feed. Apparent feed intake was calculated by subtracting the uneaten feed (dry weight) and the amount lost to leaching (dry weight) from the total amount of feed delivered to each tank. Dead abalones were removed, weighed, and measured each morning. Feed rates were adjusted to compensate for biomass changes to individual tank arising from mortalities.

Feed Intake Rates Calculation

Feed intake rates and vitamin K intake rates were calculated as follows:

$$\text{Feed intake rates}\left(\text{g kg abalone}^{-1}\,\text{day}^{-1}\,\text{dry basis}\right) = \left(\text{g fed} - \text{g uneaten}\right) - \text{g leached/kg tank biomass} \times \text{number of days}$$

$$\text{Vitamin K intake rates}\left(\mu\text{g kg abalone}^{-1}\,\text{day}^{-1}\,\text{dry basis}\right) = \left[\left(\text{feed intake rate g kg abalone}^{-1}\,\text{day}^{-1}\,\text{dry basis} \times 1,000\right) \times \text{vitamin K concentration in diet } \mu\text{g kg}^{-1}\right]$$

Sample Collection and Analysis

At the conclusion of the experiment, all abalones were weighed and measured, and haemolymph was collected using 23 gauge needles and 10 mL syringes from three abalones per tank via the cephalic sinus. To avoid stress-related elevations in antioxidant activities, the time taken to procure the haemolymph after initial disturbance was recorded to ensure collection was within 0.5 min as per Lange et al. (2014). Fresh haemolymph (200 μL) was used for total hemocyte count, phagocytic activity, and phagocytic index analysis. To obtain total hemocyte count, 50 μL of haemolymph was fixed in 6% formalin in

100 µL 35 parts per thousand (ppt) saline in an eppendorf tube and kept on ice. Samples were gently vortexed and loaded into both sides of a Neubauer-improved haemocytometer counting chamber. Cells were counted in five squares on both sides using a microscope (Olympus CX40). Mean hemocyte number was calculated and converted to obtain hemocyte count per mL. Phagocytic activity was measured using the methods of Dang et al. (2011). A yeast solution for phagocytosis assay was prepared by autoclaving 2.5% baker's yeast (*Saccharomyces cerevisiae*) (Tandaco; Cerebos Foods, Seven Hills, New South Wales, Australia) in 4% Congo red (Sigma) in filtered seawater (FSW). Stained yeast cells were centrifuged at 1,500 g for 10 min, washed three times with FSW, and resuspended in FSW (0.2 µm) at 1×10^7 cells mL^{-1}. Fresh haemolymph (150 µL) was added to an Eppendorf tube at room temperature with 40 µL yeast suspension, lightly vortexed, and then rested for 10 min in the dark. Tubes were then vortexed and two drops (~50 µL) placed onto a glass slide with a coverslip. Phagocytic rate was determined in triplicate as percentage of phagocytic hemocytes in 30 hemocytes under a microscope at 400× magnification. Number of yeast cells engulfed per hemocyte was recorded to determine phagocytic index. Remaining haemolymph was centrifuged at 4°C for 5 min at 2,000 × *g* to separate serum from cell pellet. Serum was pipetted into cryotubes (Sarstedt AG & Co., Numbrecht, Germany) and kept on dry ice until storage at –80°C. Abalone serum was later assayed for catalase activity (Cayman Chemical, Ann Arbor, MI).

Two abalones per tank were shucked and frozen at –20°C for further dissection into muscle and visceral organ tissue samples. Frozen samples were thawed, and the visceral organ tissue samples were obtained by removing the organ using a sterile disposable scalpel. The sample contained the digestive tract (lower esophagus, crop, stomach, cecum, and intestine), heart, and kidneys. Special attention was used to ensure gills were removed. Abalone muscle samples were collected with a minimum size of 1.5 cm^2. All muscle samples were taken from the same location on the anterior of the abalone foot and refrozen at –20°C. Muscle and visceral organ tissue samples were washed to remove any uneaten feed and analyzed for vitamin K_1, K_2 (MK-4 and Mk-7) and K_3 by Agricure Scientific Organics using high-performance liquid chromatography methods. Vitamin K_2 was measured as K_2-MK-4 and K_2-MK-7. Vitamin K_2-MK-4 is produced by tissue-specific conversion of vitamin K_1 or K_3 whereas Vitamin K_2-MK-7 is synthesized by bacteria such as *Bacillus subtilis* spp. which have been reported in the digestive tract of a range of marine organisms (Wang et al. 2008, Walther et al. 2013). Feed samples were analyzed for proximate composition and energy (National Measurement Institute, Lindfield, New South Wales, Australia) and vitamin K_1 and K_3 concentrations (Agricure Scientific Organics, Breamar, New South Wales, Australia).

Water Quality

Water quality was measured daily at 12:00 PM. Temperature (°C) was measured using a hand-held thermometer (Livingstone glass alcohol laboratory thermometer; Rosebery, New South Wales, Australia). Dissolved oxygen saturation (%) and concentration (mg L^{-1}) were measured using an Oxyguard Handy Polaris 2 oxygen probe and meter (Oxyguard International A/S, Birkeroed, Denmark) and ranged from 80% to 92%, and from 5.5 to 6.3 mg L^{-1}, respectively. Salinity was measured (ppt) using an ISSCO UR-2 hand-held refractometer (Industrial Scientific Supply Co. Pty Ltd., Concord West, New South Wales, Australia) and ranged from 34 to 36 ppt. pH was measured with a Eutech pH testr30 m (Eutech Instruments Pty Ltd, Singapore, Singapore) and ranged from 8.17 to 8.24.

Statistical Analysis

Statistical analyses were carried out using SPSS for Windows (Version 23; IBM Corp., Armonk, NY). Data were assessed for homogeneity of variance and normality using the Levene's test for equality of variance and Shapiro-Wilk test, respectively. One-factor analysis of variance (ANOVA) was used to assess initial weights and shell lengths at stocking. Survival was assessed using Kaplan–Meier survival analysis with Log-Rank and Breslow tests. Two-factor ANOVA was used to assess the main effects of water temperature (22 or 25°C) and vitamin K_1 level (0.0, 0.5, 1.0, or 5.0 mg kg^{-1}), or vitamin K_1 and Vitamin K_3 (vitamer type) at the 0.5 mg kg^{-1} inclusion level, on treatment responses. Two-factor ANOVA was also used to assess the main effects of vitamin K_1 level (0.0, 0.5, 1.0, or 5.0 mg kg^{-1}) and time (weeks 1–6) on feed intake rates at each separate water temperature (22 or 25°C). Where significant interactions were observed, individual means were compared using one-factor ANOVA. The Student Newman Keuls post-hoc test was used to assess differences among treatment means. A significance level of $P < 0.05$ was used for all analysis and values are presented as mean ± SE of three replicate tanks unless otherwise stated.

RESULTS

Survival

There was no significant difference in mean initial weight (71.51 ± 0.2 g; $n = 30$) or shell length (shell length 79.91 ± 0.56 mm; $n = 30$) at stocking ($P > 0.563$; one-factor ANOVA).

At 22°C, 100% survival was observed at the completion of the trial for all diet treatments (Fig. 1; Kaplan–Meier; Log-Rank and Breslow test). At 25°C, survival of abalone declined to between 60% and 73%. There was no significant difference between the five dietary treatments (Fig. 1; $P > 0.363$; Kaplan–Meier; Log-Rank and Breslow test). When compared with the survival of the corresponding diets at 22°C, all abalone fed diets at 25°C showed a significant decrease in survival (Fig. 1; $P < 0.003$; Kaplan-Meier; Log-Rank, and Breslow test).

Feed Intake Rates

The average feed intake for abalone across the 39 day study ranged from 1.67 to 3.27 g kg^{-1} abalone d^{-1} (Table 3). Inclusion of vitamin K_1 at graded levels had no significant effect on average feed intake ($P = 0.116$; two-factor ANOVA). There was no significant interaction ($P = 0.068$). Feed intake was significantly lower at 25°C compared with 22°C ($P < 0.001$; two-factor ANOVA). Feed intake at 25°C was between 1.67 and 1.83 g kg^{-1} abalone d^{-1}, and at 22°C between 2.98 and 3.27 g kg^{-1} abalone d^{-1}.

Comparison of vitamers K_1 and K_3 at 0.5 dietary inclusion level also showed no significant effect of diet ($P = 0.354$; two-factor ANOVA) on feed intake with rates between 1.20 and

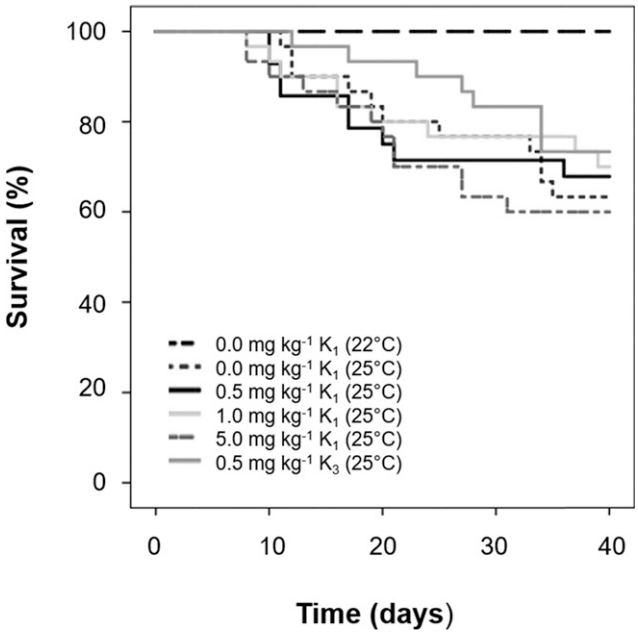

Figure 1. Kaplan–Meier survival curves of greenlip abalone fed control diets at two water temperatures (22 and 25°C) and diets with added vitamin K_1 or K_3 at 25°C. Survival rate of abalone at 22°C was significantly greater than at 25°C ($n = 30$; $P < 0.003$; Log-Rank and Breslow test).

3.30 g kg^{-1} abalone d^{-1} (Table 3). Temperature again significantly decreased feed intake at 25°C ($P < 0.001$), although there was no significant interaction ($P = 0.277$).

Vitamin K Intake

Analyzed dietary vitamin K_1 levels were between 31% and 51% of targeted levels, with diets 0.0 K_1, 0.5 K_1, 1.0 K_1 and 5.0 K_1 recording levels at 0.02, 0.26, 0.32, and 1.73 mg kg^{-1}, respectively (Table 2). Despite lower than desired levels, they were still able to deliver significantly increasing quantities of vitamin K_1 ($P < 0.001$; one-factor ANOVA; Table 3).

Visceral Organ and Muscle Tissue K Vitamer Concentrations

Vitamin K_1 was bioaccumulated in the visceral organ and muscle tissues. In the visceral organs, the increasing level of vitamin K_1 dietary inclusion had a significant positive effect, increasing tissue vitamin K_1 concentration ($P < 0.001$; two-factor ANOVA). No significant effect of temperature was observed ($P = 0.785$), and there was no significant interaction ($P = 0.961$). Visceral organ vitamin K_1 concentrations were variable and increased progressively from 1.42 μg g^{-1} in the control diet to 23.74 μg g^{-1} in the diet containing 1.0 mg kg^{-1} vitamin K_1 and then significantly increased to 95.38 μg g^{-1} in the diet containing 5.0 mg kg^{-1} vitamin K_1 (Fig. 2).

Vitamin K_1 concentrations were significantly higher at 25°C compared with 22°C water temperature in muscle tissue ($P = 0.002$; two-factor ANOVA). A significant increase in vitamin K_1 concentration was also observed with increasing vitamin K_1 dietary inclusion level ($P = 0.039$). No significant interaction was observed ($P = 0.943$). Concentrations of vitamin K_1 in muscle tissue progressively increased and ranged from 1.89 μg g^{-1} in the control diet to 5.59 μg g^{-1} in the diet containing 5.0 mg kg^{-1} vitamin K_1 (Fig. 3).

There were no detectable levels of vitamin K_3 in visceral organ or muscle tissue of abalone fed increasing levels of vitamin K_1 at 22 and 25°C.

When comparing the response of abalone fed 0.5 mg kg^{-1} of vitamins K_1 or K_3, vitamer type had a significant effect on vitamin K_1 concentrations in the visceral organ (vitamin $K_1 > K_3$) ($P = 0.004$; two-factor ANOVA; Table 4). There was no significant effect of temperature ($P = 0.500$) or the interaction between the two factors ($P = 0.514$) on vitamin K_1 concentrations in the visceral organ (Table 4). There were no detectable levels of vitamin K_3 in visceral organ or muscle tissue fed 0.5 mg kg^{-1} of vitamins K_1 or K_3 at 22 and 25°C (Table 4).

TABLE 3.

Feed and vitamin K_1 and K_3 intake rates of greenlip abalone fed diets at 22 and 25°C (dry basis).

Vitamer type	K_1	K_1	K_1	K_1	K_3
Nominal dietary inclusion (mg kg^{-1})	0.0	0.5	1.0	5.0	0.5
Feed intake rate (g kg abalone^{-1} day^{-1})					
22°C	2.99 ± 0.066	3.15 ± 0.081	3.26 ± 0.020	3.23 ± 0.128	3.10 ± 0.101
25°C	1.85 ± 0.068	1.44 ± 0.117	1.81 ± 0.082	1.65 ± 0.088	1.86 ± 0.311
Vitamin K_1 intake rate (μg kg abalone^{-1} day^{-1})					
22°C	0.05 ± 0.001	0.84 ± 0.023	1.08 ± 0.012	5.80 ± 0.234	0.07 ± 0.002
25°C	0.03 ± 0.001	0.39 ± 0.032	0.59 ± 0.033	3.21 ± 0.229	0.04 ± 0.007
Vitamin K_3 intake rate (μg kg abalone^{-1} day^{-1})*					
22°C	ND	ND	ND	ND	1.90 ± 0.061
25°C	ND	ND	ND	ND	1.12 ± 0.200

* ND, not detectable; Vitamin K_1 and K_3 assay detectable limits were 0.5 μg kg^{-1}.

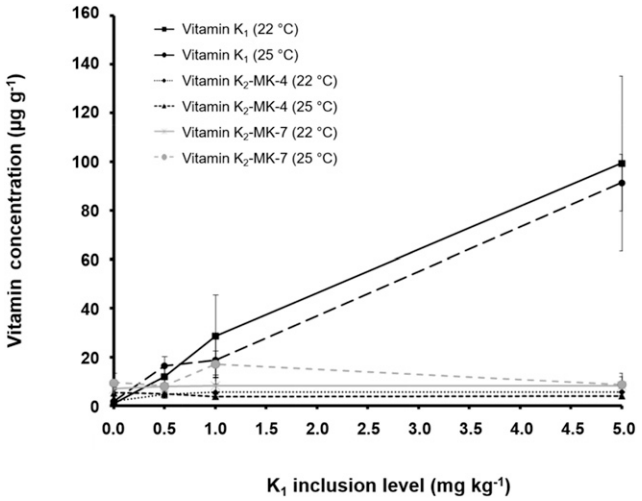

Figure 2. Concentration of K vitamers in visceral organ tissue with increasing dietary K_1 inclusion. Data presented as mean ± SE, $n = 3$.

There was no significant effect of vitamin K_1 inclusion ($P = 0.934$; $P = 0.553$), temperature ($P = 0.833$; $P = 0.277$), or the interaction between the two factors ($P = 0.342$; $P = 0.641$) on vitamin K_2-MK-4 or vitamin K_2-MK-7 visceral organ tissue concentrations, respectively (two-factor ANOVA; Fig. 3).

There were no significant effects of water temperature ($P = 0.490$; $P = 0.897$) or vitamer type (0.5 mg kg^{-1} vitamins K_1 or K_3) ($P = 0.104$; $P = 0.586$) on the vitamin K_2-MK-4 and K_2-MK-7 concentrations of visceral organ tissues (Table 4; two-factor ANOVA). There was also no significant interaction between temperature and vitamer type ($P = 0.368$; $P = 0.954$, respectively). Because of the high occurrence of nondetectable levels of vitamin K_2-MK-4 and vitamin K_2-MK-7 concentrations in muscle tissue, results were not statistically analyzed (Table 4).

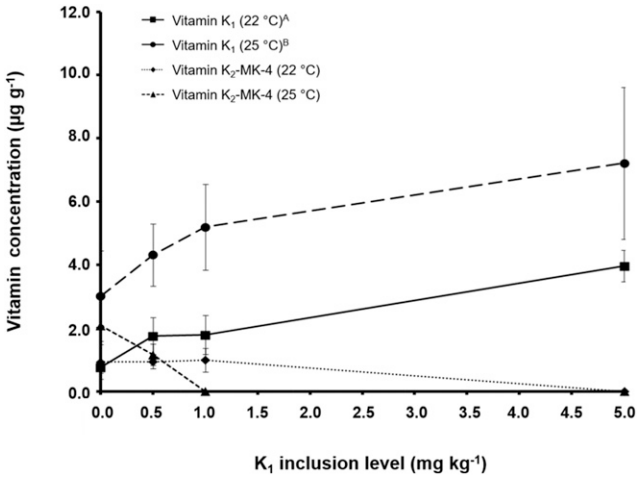

Figure 3. Concentration of K vitamers in muscle tissue with increasing dietary K_1 inclusion. Data presented as mean ± SE, $n = 3$. Vitamers in the legend with different uppercase subscripts showed a significant difference owing to water temperature ($P = 0.002$; two-factor ANOVA; Student Newman Keuls; $n = 12$). K_2-MK-7 was below detectable levels in muscle tissue.

Immune Parameters

For abalone fed graded levels of vitamin K_1, there were no significant effects of temperature, vitamin K_1 inclusion level, or interaction between the two factors for total hemocyte count (Table 5; $P = 0.677$, $P = 0.418$, and $P = 0.159$, respectively; two-factor ANOVA), phagocytic activity ($P = 0.344$, $P = 0.904$, and $P = 0.311$, respectively) and phagocytic index ($P = 0.507$, $P = 0.713$, and $P = 0.455$, respectively) (Table 5).

Comparison of vitamers K_1 and K_3 at the 0.5 mg kg^{-1} inclusion level showed no significant effect of temperature, vitamer type, or interaction on total hemocyte count ($P = 0.480$, $P = 0.737$, and $P = 0.120$; two-factor ANOVA; Table 4). A significant effect of temperature was observed on phagocytic activity ($P = 0.003$; two-factor ANOVA) with a higher phagocytic activity at 22°C compared with 25°C. No significant effect of vitamer type ($P = 0.588$) or interaction between the two factors was observed ($P = 0.431$; two-factor ANOVA). Phagocytic index analysis showed no effect of temperature ($P = 0.176$) or vitamer type ($P = 0.378$; two-factor ANOVA). A significant interaction between temperature and vitamer type was observed for phagocytic index ($P = 0.045$; two-factor ANOVA). On closer examination, it was not possible to discern the cause of the interaction as the one-factor ANOVA did not have the power to detect a significant difference between means ($P = 0.101$; Table 4).

Serum Antioxidant Activity

A significant increase in serum catalase activity was observed at 25°C compared with 22°C water temperature. ($P = 0.023$; two-factor analysis; Table 5), whereas there was no significant effect of vitamin K_1 inclusion level ($P = 0.499$) and no significant interaction between the two factors ($P = 0.164$).

When comparing vitamin K_1 and K_3 vitamers at the 0.5 mg kg^{-1} dietary inclusion level, serum catalase activity was significantly higher with vitamin K_1 inclusion compared with K_3 ($P = 0.009$; two-factor ANOVA; Table 4), and significantly higher at 25°C compared with 22°C water temperature ($P < 0.001$). There was no significant interaction between the two factors ($P = 0.700$).

DISCUSSION

In the present study, the high water temperature of 25°C generated mortality rates of 37% in the negative control abalone fed (0.0 mg kg^{-1} vitamin K_1 supplementation) which were similar to those reported in previous temperature challenge trials by Lange et al. (2014) (38 days), Stone et al. (2014) (36 days), and Duong et al. (2016) (38 days). Observed mortality rates were also comparable to rates between 15% and 50% reported for greenlip abalone on South Australian abalone farms during periods of high water temperatures during summer months (Stone et al. 2013). In addition, control-fed greenlip abalone in the present study displayed 100% survival at 22°C consistent with results reported by Lange et al. (2014) and Stone et al. (2014) in previous temperature challenge studies with greenlip abalone. In the present study, this suggested the temperature challenge method developed by Stone et al. (2014) was successful at replicating mortality rates similar to those observed on-farm for summer mortality.

Improved survival by dietary intervention with the inclusion of vitamin K_1 at the water temperature of 25°C was the main

TABLE 4.

Cellular immune parameters, antioxidant activity, and visceral organ and muscle tissue vitamin K vitamer concentrations of greenlip abalone fed vitamin K_1 and K_3 vitamer types at 22 and 25°C.*

Water temperature (°C)	22		25		Two-factor ANOVA		
Vitamin inclusion level (mg kg^{-1})	0.5 K_1	0.5 K_3	0.5 K_1	0.5 K_3	Temperature (°C) (A)†	Vitamer type (B)‡	Interaction (A × B)
Cellular parameters							
Total hemocyte count ($\times 10^6$ mL^{-1})	9.88 ± 1.34	7.49 ± 1.55	7.05 ± 8.46	8.60 ± 3.54	0.480	0.737	0.120
Phagocytic activity (%)	56.70 ± 3.21	60.25 ± 3.11	48.27 ± 2.04	47.59 ± 7.41	0.003 (22 > 25)	0.588	0.431
Phagocytic index (%)	11.36 ± 4.06	13.95 ± 3.32	12.96 ± 2.22	7.04 ± 2.43	0.176	0.378	0.045§
Antioxidants (units mL^{-1})							
Catalase	10.10 ± 0.79	7.18 ± 0.53	14.31 ± 0.72	12.00 ± 0.95	<0.001 (22 < 25)	0.009 ($K_1 > K_3$)	0.700
Visceral organ (μg g^{-1})							
K_1	11.95 ± 5.02	1.21 ± 0.33	16.40 ± 3.94	1.25 ± 0.51	0.500	0.004 ($K_1 > K_3$)	0.514
K_3	ND	ND	ND	ND	NA	NA	NA
K_2-MK-4	4.57 ± 1.45	2.86 ± 0.88	4.80 ± 0.78	2.03 ± 0.87	0.490	0.104	0.368
K_2-MK-7	7.86 ± 3.01	11.79 ± 2.45	8.10 ± 1.47	15.21 ± 3.18	0.897	0.586	0.954
Muscle (μg g^{-1})							
K_1	1.75 ± 0.59	0.61 ± 0.11	4.32 ± 0.98	2.57 ± 1.88	0.074	0.227	0.792
K_3	ND	ND	ND	ND	NA	NA	NA
K_2-MK-4	0.94 ± 0.22	0.62 ± 0.11	1.16 ± 0.34	ND	NA	NA	NA
K_2-MK-7	ND	ND	ND	ND	NA	NA	NA

NA, not statistically analyzed because of insufficient data. ND, not detected. Vitamer types at the level of less than 0.5 μg mL^{-1}.

* Data are presented as means ± SE, $n = 3$.

† Values in parentheses for water temperature indicate that 22°C is greater than or less than 25°C ($P < 0.05$; two-factor ANOVA; $n = 6$).

‡ Values in parentheses for vitamer indicate that K_1 is greater than or less than K_3 ($P < 0.05$; two-factor ANOVA; $n = 6$).

§ The interaction for phagocytic index (A × B) was close to $P = 0.05$. On closer examination, it was not possible to discern the cause of the interaction as the one-factor ANOVA did not have the power to detect a significant difference between means ($P = 0.101$).

aim of the present study, and this was not achieved. Compared with the control diet, vitamin K_1 supplementation did not lead to an improvement in abalone survival at 25°C (Fig. 1). This is the first completed temperature challenge study involving vitamin K_1 with greenlip abalone. Previous studies have used a different K vitamer and have not used temperature challenge conditions. Dietary supplementation with vitamin K_3 has previously shown no effect on survival of abalone (Tan & Mai 2001, Fu et al. 2012). Tan and Mai (2001) fed Pacific abalone with vitamin K_3–supplemented diets at fluctuating water temperatures between 9.8 and 26.4°C; well outside the reported temperature optimum of 20°C for the tested species (Cho & Kim 2012); whereas Fu et al. (2012), also using Pacific abalone, maintained a close to optimal temperature range of 17.5°C–19°C.

Vitamin K_1 is a fat-soluble vitamin reported to play an important role in the innate immune system in all vertebrates including fish, reptiles, and mammals. Vitamin K_1 has also been reported to be more bioavailable than Vitamin K_3 (Krossoy et al. 2011). The National Research Council recommends levels of vitamin K between 0.4 and 1.75 mg kg^{-1} diet to avoid deficiency signs in chickens including impaired blood coagulation and hemorrhaging (NRC 1994). In the present study, the diet series was designed to contain graded levels of vitamin K_1 to encompass the recommended levels for both chickens (Hubert Regtop, personal communication, Agricure Scientific Organics) and abalones (Mai et al. 2001). The potential negative effect of high water temperature on feed intake rate and nutrient delivery was also considered in the experimental design phase.

As anticipated, feed intake rate was reduced in the present study by approximately 50% at the water temperature of 25°C compared with 22°C (Table 3). The reduction in feed intake rate at 25°C is consistent with previous results reported for greenlip abalone (Lange et al. 2014, Stone et al. 2014, Duong et al. 2016) and demonstrates an effect of high water temperature on voluntary feed intake because of stress (Kaushik 1986).

Vitamin K_1 was present in all tested diets (Table 2). Diets formulated with added vitamin K_1 contained lower than anticipated levels of vitamin K_1, which were at least 50% below the expected values. By contrast, the level of vitamin K_3 in the diet supplemented with 0.5 mg kg^{-1} vitamin K_3 was consistent with the expected values. This suggested poor stability, or a potential form of antagonism, between vitamin K_1 and other nutrients within the diet matrix. Vitamin K_3 stability in diets has been reported to be low over prolonged storage periods, with Tavčar-Kalcher and Vengušt (2007) reporting vitamin K_3 losses of up to 80% after 12 mo of storage. Graff et al. (2010) also reported low levels of vitamin K_3 (0–46.5 mg kg^{-1}) in diets compared with targeted levels of 0–1,000 mg kg^{-1}. Vitamin K_1 produced by Agricure Scientific Organics and used in the present study has been reported to be heat stable but light sensitive (Hubert Regtop, personal communication, Agricure Scientific Organics; Kreutler & Czajka-Narins 1987). Diets in this study were produced using low heat (less than 50°C) and were also stored frozen in the dark. This suggests some other unexplained form of degradation occurred and further research is required to improve the stability of vitamin K_1 for use in abalone feeds. Greenlip abalone still consumed appreciable

TABLE 5.

Cellular immune parameters, antioxidant activity, and different K vitamer concentrations in visceral organ and muscle tissue of greenlip abalone fed graded levels of K_1 at 22 and 25°C.†

Water temperature (°C)	22				25				Two-factor ANOVA		
Vitamin K_1 inclusion level (mg kg^{-1})	0.0	0.5	1.0	5.0	0.0	0.5	1.0	5.0*	Temperature (°C) (A)‡	K_1 level (B)	Interaction (A × B)
Cellular parameters											
Total hemocyte count ($\times 10^6$ mL^{-1})	6.66 ± 1.27	9.88 ± 1.34	8.28 ± 8.54	6.23 ± 4.49	8.11 ± 9.31	7.05 ± 8.46	7.38 ± 1.24	7.28 ± 5.84	0.677	0.418	0.159
Phagocytic activity (%)	47.70 ± 4.42	56.70 ± 3.21	54.10 ± 15.5	54.70 ± 12.7	55.70 ± 9.64	48.27 ± 2.04	44.70 ± 3.96	50.70 ± 7.14	0.344	0.904	0.311
Phagocytic index (%)	12.40 ± 1.67	11.36 ± 4.06	12.80 ± 7.82	10.10 ± 1.54	11.50 ± 1.61	12.96 ± 2.22	7.78 ± 3.03	10.40 ± 2.96	0.507	0.713	0.455
Antioxidants (units mL^{-1})											
Catalase	11.33 ± 1.07	10.10 ± 0.79	9.52 ± 0.92	9.53 ± 0.99	10.26 ± 0.43	14.32 ± 0.72	11.52 ± 1.05	12.59 ± 2.30	0.023 (22 < 25)	0.499	0.164

NA, not statistically analyzed because of insufficient data.

† Data are presented as mean ± SE, $n = 3$ tanks. Except* where $n = 2$ for visceral organ and muscle at 5.0 mg kg^{-1} K^1 at 25°C.

‡ Values in parentheses for water temperature indicate that 22°C is greater than or less than 25°C ($P < 0.05$; two-factor ANOVA; $n = 12$).

levels of vitamin K_1, reflected by significantly increasing dietary inclusion levels. Increasing visceral organ and muscle tissue vitamin K_1 levels indicated that graded levels of vitamin K_1 had been delivered to abalones.

Tissue concentrations of different K vitamers [K_1, K_2 (K2-MK-4 and K2-MK-7), and K_3] were measured in visceral organ and muscle tissue of greenlip abalone. Vitamin K_3 was not detected in any tissue analyzed in the present study. This was likely because of the synthetic vitamin K_3 being interconverted to another active form or K vitamer following uptake (Krossoy et al. 2011). Vitamin K_1 concentration increased in muscle tissue at 25°C compared with 22°C (Fig. 3). Temperature had no significant effect on vitamin K_2 in muscle or any K vitamer in the visceral organ. Levels of vitamin K_1 storage differed depending on tissue type (Figs. 2 and 3). Vitamin K_1 activity has been reported to be high in the liver, pancreas, kidney, femur, and brain tissue in rats (Sato et al. 2003), and the visceral organ of Pacific abalone (Tan & Mai 2001). The vitamin K_1 concentrations in visceral organ were higher than observed in the muscle tissue and both increased with increasing dietary inclusion of vitamin K_1. Steady-state levels of vitamin K_1 were not achieved in either tissue (Figs. 2 and 3).

As with vitamin K_1, vitamin K_2-MK-4 and K_2-MK-7 concentrations were higher in visceral organ compared with muscle tissue. Similar findings were reported for K_2-MK-4 by Tan and Mai (2001) in Pacific abalone viscera and muscle tissues. The inclusion of the digestive tract in the visceral organ sample could contribute to the higher concentrations of vitamers in these samples. Vitamin K_2-MK-4 levels in viscera and muscle tissues of Pacific abalone increased with dietary inclusion of vitamin K_3 of 10 mg kg^{-1} but showed no significant increase at higher vitamin K_3 inclusion levels of up to 320 mg kg^{-1} (Tan & Mai 2001). In the present study, muscle tissue recorded its highest levels of vitamin K_2-MK-4 at low dietary inclusion levels with a decrease below detectable levels as dietary level of vitamin K_1 increased. In addition, vitamin K_2-MK-7 levels within visceral organ showed no significant changes with Vitamin K_1 inclusion level and were below detectable levels in muscle tissue. Tan and Mai (2001) did not record K_2-MK-7 as they postulated that no rich source of vitamin K–synthesizing microorganisms had been described in fish, and were unlikely to be active in abalone, and that long-chain menaquinones make minor contributions to hepatic stores in rats and chicks (Will et al. 1992). Based on the diet and tissue concentrations of vitamin K_1 and K_2 that were measured in the present study, a dietary level of 0.02 mg kg^{-1} of vitamin K_1 would be sufficient to sustain steady-state levels of vitamins K_2-MK-4 and K_2-MK-7 in abalone tissues. Further studies are required to determine the minimum dietary requirements to obtain steady-state levels for vitamin K_1.

The importance of intestinal production of the different forms of vitamin K has not been established in Pacific abalone (Tan & Mai 2001), crustaceans, or fish (Krossoy et al. 2011). Increased visceral organ tissue concentrations of this vitamer indicated a potential bacterial contribution. Analysis of the visceral organ, which included the digestive tract, showed the presence of vitamin K_2-MK-7 at levels above those of vitamin K_2-MK-4. Further investigation of the vitamin K–synthesizing bacteria in the digestive tract of abalone would assist in understanding K_2-MK-7 synthesis.

Total hemocyte count, phagocytic activity, and phagocytic index were measured to assess the immune status of greenlip abalone, as they are important components of the innate immune system. The dietary inclusion of vitamin K_1 had no effect on these immune parameters. It has been suggested that total hemocyte count and phagocytic activity are short term responses to heat stress in hybrid abalone, *Haliotis laevigata* × *Haliotis rubra* (Day et al. 2010). Stone et al. (2014), using greenlip abalone and an identical experimental setup to the present study, recorded a decrease in phagocytic activity from 53% at 22°C to 42% at 26°C after a prolonged exposure period. When comparing vitamins K_1 or K_3 fed at 0.5 mg kg^{-1}, increasing water temperature did reduce phagocytic activity (Table 4). Phagocytic index has not been widely recorded in temperature challenge experiments with abalone. Increasing salinity levels have been reported to cause a significant reduction of phagocytic index in red abalone *Haliotis rufescens* but no effect in black abalone *Haliotis cracherodii* (Martello et al. 2000). In the present study, the lack of significant improvement in the immune parameters was not sufficient evidence to confirm or discount the presence of vitamin K–dependent proteins. Further research into the presence of vitamin K–dependent proteins, such as growth arrest–specific 6, is important as they may have a role in reducing inflammation and oxidative stress in abalone (Hafizi & Dahlback 2006).

The haemolymph antioxidant activity in greenlip abalone is important as increased temperatures and excessive reactive oxygen species generation leads to oxidative stress. Dietary vitamin K_1 inclusion had no effect on serum catalase activity in greenlip abalone. By contrast, an increase in serum catalase activity, indicating a higher oxidative stress level, was observed in greenlip abalone at 25°C compared with 22°C. South African abalone, *Haliotis midae*, also showed increases in gill catalase activity at increased water temperatures of 19°C compared with 14°C (Vosloo et al. 2013). Vitamer type also caused a significant change with greenlip abalone fed vitamin K_1 showing significantly increased serum catalase activity when compared with vitamin K_3 at the 0.5 mg kg^{-1} inclusion level (Table 4). These results conflict with the findings reported by Fu et al. (2012), in which the dietary inclusion of vitamin K_3 at levels of up to 1,000 mg kg^{-1} caused an increase in muscle and viscera tissue catalase activity in Pacific abalone.

In conclusion, dietary vitamin K_1 did not influence survival, feed intake, antioxidant status, or immune parameters in greenlip abalone. The delivery of the prescribed level of vitamin K_1 proved problematic, and further research into developing a more stable form for addition into abalone feeds may be beneficial. Tissue deposition of vitamin K_2 vitamers in visceral organ and muscle tissues were enhanced by feeding vitamin K_1; however, steady-state levels of vitamin K_1 were not achieved in these tissues. By contrast, dietary levels of 0.02 mg kg^{-1} of vitamin K_1 resulted in steady-state levels of K_2-MK-4 in visceral organ and muscle tissue and K_2-MK-7 in visceral organ tissue appeared to be reached or exceeded. Further research into the synthesis of K_2 vitamers, particularly vitamin K_2-MK-7, in the digestive tract of greenlip abalone will provide insight into the importance of the digestive tract microbiome in response to vitamin K. Dietary inclusion of 0.5 mg vitamin K_3 kg^{-1} diet had a slight influence on immune status, and further research investigating inclusion levels of this vitamin may prove beneficial in enhancing the immune status and survival of greenlip abalone in response to temperature stress.

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