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Risk factors and management strategies associated with summer mortality in Australian abalone

Matthew Bansemer, Jessica Buss, Georgia Macaulay, Shane Roberts, Tracey Bradley, Graeme Knowles, Cecile Dang, James Harris, Kathryn Wiltshire and Nick Savva

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Researcher Contact Details
Name: Dr Matthew Bansemer
Address: PIRSA Fisheries and Aquaculture
2 Hamra Avenue
West Beach, 5024, South Australia.
Phone: 08 8429 2100
Fax: N/A
Email: matthew.bansemer@sa.gov.au

FRDC Contact Details
Address: 25 Geils Court
Deakin ACT 2600
Phone: 02 6122 2100
Email: frdc@frdc.com.au
Web: www.frdc.com.au

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Abbreviations

AAGA	Australian Abalone Growers Association
CF	Condition
DO	Dissolved oxygen
FRDC	Fisheries Research and Development Corporation
OIE	World Organisation for Animal Health
OTC	Oxytetracycline
PIRSA	Primary Industries and Regions South Australia
SGR	Specific growth rate
SL	Shell length

Introduction and Executive Summary

In 2018, the Australian farmed and wild-fished abalone industry was valued at ~$220 million, which is forecasted to expand (Mobsby, 2020). In Australian abalone aquaculture, higher-than-normal mortality rates have been reported to coincide with warm water temperatures. Abalone mortality during warm water temperatures has been termed summer mortality and is a research priority for the Australian abalone industry (AAGA, 2020).

Summer mortality is often used as an umbrella term and lacks a true definition for what constitutes a summer mortality event and their associated risk factors. Mortalities attributed to summer mortality can accumulate into large stock losses. Mortality rates of up to 50% reported farmed in 3-year-old Greenlip Abalone (*Haliotis laevigata*) have been attributed to summer mortality (Vandepeer, 2006). Since 2010, eight of the ten warmest years on record were documented for Australia's ocean surface temperatures (CSIRO, 2018). As the frequency of marine heatwaves is forecast to increase (Roberts et al., 2019), it is important to determine what factors increase the risk of summer mortality in abalone farms and identify any causative agents. Prior to this project, there was no case definition for summer mortality. Identifying what management practices could reduce stock loss and prevent summer mortality was therefore difficult.

In this project, we reviewed the scientific literature and collaborated with Australian abalone growers to develop a case definition for summer mortality. The case definition developed for summer mortality is as follows:

i. Chronic mortality of unknown cause (if in doubt take this to mean >0.15% mortality of individuals in a tank per day ($tank^{-1}$ day^{-1}) for at least one week) above the farm's winter baseline mortality rate in >1 year old abalone, and

ii. occurs in at least two culture units, and

iii. occurs between January and April, but,

iv. excludes those diagnosed with an OIE notifiable disease as the primary cause of mortality.

Using this case definition we conducted a case-control study. Case-control studies allow frequency of exposure of case/controls to potential risk factors to be determined retrospectively. We used farm mortality data (2015-2021) to identify tanks which fit the case definition (cases) and tanks which did not (controls). We compared exposure of cases and controls to a variety of risk factors (e.g. water quality, husbandry, climate, biology).

Results from the case-control study indicated that warm water temperatures and an increase in mortality two weeks after last handling or grading were associated with increased risk of summer mortality. Abalone that were in tanks that experienced summer mortality the year prior were also more prone to subsequent summer mortality. Several factors had an interactive effect on risk of summer mortality, including:

- previous year summer mortality and abalone age.
- previous year summer mortality and size at nursery removal.
- size at nursery removal and abalone age.
- post-grading mortality and feed rate.
- feed rate and abalone age.

In this project we also investigated summer mortality events during the life of the project to rule out primary pathogens and infectious agents. *Vibrio* spp. (including *Vibrio harveyi*) were detected in both case and control abalone, with a higher prevalence in case animals. Bacterial presence did not always translate to clinical disease. In some animals from case tanks, *V. harveyi* and *Vibrio spp*. caused disease as there were gross findings consistent with vibriosis. However, mixed bacterial growth, and not one uniform pathogen, was detected in both case and control abalone and there was no evidence that *V. harveyi* and *Vibrio spp.* were consistently the primary cause of mortality. In conclusion, the bacterial pathogens detected were secondary (opportunistic) to some primary stressor(s).

This project has provided the industry with new information about summer mortality, by identifying several factors that increase the risk of summer mortality and confirming that it is a non-infectious process, which is particularly important for trade and market access. Consequently, the key factors attributed to summer mortality will help direct future research avenues and refinement of husbandry practices.

Keywords

Greenlip Abalone (*Haliotis laevigata*), Blacklip Abalone (*Haliotis rubra*), hybrid abalone (*H. laevigata x H. rubra*), summer mortality, case control study, disease investigation.

Objectives

In this project, our objectives were to:

1. Develop a case definition for summer mortality (Objective 1);
2. Summarise summer mortality research, retrospective mortality investigations and laboratory submissions for Australian abalone (Objective 2); and
3. Investigate summer mortality events during the life of the project to comprehensively rule out primary pathogens and infectious agents (Objective 3).

Chapter 1: Review of summer mortality in Australian abalone aquaculture

1. Introduction

When water temperatures exceed the thermal tolerance of molluscs, mortality events can occur. Molluscs affected by warm water mortality events include oysters (Goulletquer et al., 1998), mussels (Myrand et al., 2000), scallops (Xiao et al., 2005), clams (Fiori et al., 2004) and abalone (Vandepeer, 2006; Roberts et al., 2019). In Australian abalone aquaculture, this phenomenon has been termed summer mortality, but the causal factors have been unclear. Summer mortality is a research priority for the Australian abalone industry (AAGA, 2020).

In Australia, Greenlip Abalone (*H. laevigata*), Blacklip Abalone (*H. rubra*), and a hybrid of these species (*H. laevigata x H. rubra*) are cultured (Lleonart et al., 2003; Schaefer et al., 2013). Summer mortality has been reported in all cultured species in Australia (Vandepeer, 2006). Summer mortality on Australian abalone farms is often reported as continuous and low (<2% mortality per day) over the summer period (Vandepeer, 2006, pers. comm. Australian Abalone Industry 2020). Vandepeer (2006) reported high cumulative mortality (up to 50%) associated with summer mortality in cultured Greenlip Abalone.

Summer mortality likely involves complex interactions between biological, environmental, anthropogenic, and nutritional stressors (Hooper et al., 2014; Romalde et al., 2014). These stressors lead to states of physical and immunological compromise in abalone, increasing susceptibility to opportunistic bacterial infection, and therefore, death (Hooper et al., 2007). While increased presence of bacteria (predominately *Vibrio* spp.) have been described from abalone mortality events (Vandepeer, 2006; Hooper et al., 2014; Romalde et al., 2014), no pathogen has been identified as a causative agent for summer mortality.

In this Chapter, we reviewed the literature on summer mortality in Australian abalone aquaculture and summarise potential risk factors that may contribute to summer mortality. Chapter 1 partly fulfills Objective 2 of this project by summarising summer mortality research. We scoped the *Google Scholar* database for publications (English language publications from all years) on abalone health and summer mortality using the following search terms: (aquaculture OR farm* OR culture*) AND (mollusc* OR abalone OR gastropod OR bivalve OR shellfish OR mussel OR oyster OR scallop) AND (summer mortality OR mortality syndrome OR non infectious disease OR unknown aetiology OR unknown cause OR syndrome OR winter mortality OR disorder OR undiagnosed OR husbandry). Papers were manually checked by title and abstract and eliminated if the subject matter was clearly unrelated to the search terms, or otherwise after reading the full text. Additional publications missed by our initial search were discovered by examining reference lists of appropriate articles and additional investigative searches of *Google Scholar* up until 16 September 2021. Supplementary papers missed by these searches and grey literature known by co-authors to be relevant were also reviewed.

2. Potential risk factors for summer mortality

2. 1. Water quality

2.1.1. Water temperature

Abalone are marine ectotherms, meaning their body temperature is regulated by external environmental temperature (Vandepeer, 2006). Water temperature underpins key physiological processes in abalone (Morash and Alter, 2016). Abalone that are exposed to water temperatures outside of their optimal thermal range can exhibit poor growth (Onitsuka et al., 2008; Byrne et al., 2011), altered development (Grubert and Ritar, 2004; Rogers-Bennett et al. 2010), increased vulnerability to disease (Raimondi et al., 2002; Rosenblum et al., 2005; Vilchis et al., 2005), and mortalities (Searle et al., 2006). Water temperature can therefore influence population ecology of abalone in the wild (Morash and Alter, 2016) and on-farm. Water temperature during grow-out affects almost every aspect of production in abalone aquaculture, including feed intake and growth rates (Britz et al., 1997). In Australia, abalone aquaculture typically occurs in land-based tank systems, where water temperatures range from below 10 °C in Tasmania during winter, to above 24 °C in South Australia during summer (Stone et al., 2013; Stone et al., 2014a). Optimal water temperature for best growth of 1- and 2-year-old *H. laevigata* is reported to be 22˚C (Stone et al., 2013).

The 50% critical thermal maxima is defined as the point in which 50% of a group of abalone held at elevated temperature are unable to hold onto substrate and is considered equivalent to mortality (Gilroy and Edwards, 1998). For Tasmanian sourced *H. laevigata* stock (82 mm shell length [SL]), the 50% critical thermal maxima is 27.5˚C (Gilroy and Edwards, 1998), whereas it is 29.5˚C for South Australian sourced *H. laevigata* (Madigan et al., 2000). Differences in critical thermal maxima indicates a genetic difference in stock from different climates. A heat shock protein 70 (HSP70) associated with thermal stress, and genes associated with abalone displaying resistance to heat exposure, have been identified in *H. laevigata* (Shiel et al., 2015, Shiel et al., 2017; Sandoval-Castillo et al., 2018).

During extreme temperature exposure, temporary abalone survival is sustained by anaerobic metabolism, heat shock proteins and antioxidative defence (Pörtner, 2002; Morash and Alter, 2016). Under laboratory conditions, high water temperatures (>25˚C) can lead to mortalities in cultured abalone, particularly in larger (>60 mm shell length, SL) 3-year-old abalone (Lange et al., 2014; Stone et al., 2014a; Buss et al., 2017). High mortality in older abalone on-farm can result in substantial stock loss for growers, as 3-year-old abalone are market size and most valuable (Vandepeer, 2006).

When water temperatures exceed the thermal preference of abalone, this increases the metabolic rate (Duong et al., 2016). Increased metabolism due to warm water are energetically demanding and may decrease the ability of abalone to cope with additional stressors (Duong et al., 2016). Indirect effects of rising water temperatures on-farm can result in additional stressors for abalone, including increased biofouling, increased bacterial growth in water, higher bacterial virulence, and low dissolved oxygen (DO) (Hooper et al., 2014). High water temperatures coupled with low DO levels can result in oxidative stress (Lushchak, 2011).

2.1.2. Dissolved oxygen

Abalone have paired bipectinate gills (ctenidia) and at rest rely on their right gill for oxygen intake, but when stressed can direct more hemolymph to the left gill to enhance oxygen uptake (Ragg and Taylor, 2006). At full capacity, left gill perfusion rates will increase 30-fold and match the right gill's oxygen uptake (Ragg and Taylor, 2006). An animal's fitness is dependent on oxygen supply to their body and there is a delicate relationship between

oxygen and temperature (Pörtner, 2010). If water temperatures increase beyond the optimal temperature limit for a species, aerobic scope will decrease due the difference between oxygen availability and demand.

Decreased aerobic scope can lead to onset of functional insufficiency, tissue hypoxia and tissue failure (Pörtner, 2002; Vosloo et al., 2013). Dissolved oxygen is a crucial parameter to measure on farm. Below 63% DO saturation, *H. laevigata* can experience significant reductions in food consumption, growth and survival (Harris et al., 1999a) and exposure to >120% DO saturation caused mortality in Red Abalone *Haliotis rufescens* (Leitman, 1989). The delivery of oxygen into land-based farms is dependent on the DO levels of the water supply, while the DO levels of the water supply and the flow rate into tanks dictates the amount of oxygen the farmed abalone receive (Vandepeer, 2006). DO levels may be increased with mechanical aeration such as pumps or through air/oxygen diffusion (Kepenyes and Váradi, 1984).

2.1.3. Nitrogenous products

Nitrogenous products are introduced into the aquaculture system through the break-down of uneaten feed or excreted waste (Harris et al., 1997; Lazzari and Baldisserotto, 2008; Tomasso, 1994). Ammonia is the primary source of excreted nitrogen in aquatic animals. Ammonia undergoes nitrification, a process where bacteria oxidise ammonia waste to nitrite and then nitrate (Tomasso, 1994). Nitrate is the end product of nitrification and is least harmful to juvenile aquatic species (not held in recirculating aquaculture systems (RAS), where nitrate can become problematic), whereas ammonia and nitrite are toxic at low concentrations to aquatic species (Tomasso, 1994; Harris et al., 1997). Ammonia is either ionised or un-ionised and the final form is pH and temperature dependent, with un-ionised forms considered more toxic (Harris et al., 1998). Greenlip abalone are sensitive to both nitrite and ammonia levels (Harris et al., 1997; Harris et al., 1998). Nitrite levels >0.56 milligrams per litre (mg L^{-1}) significantly decrease growth (length and weight) and nitrite levels >4.29 mg L^{-1} decrease oxygen consumption in *H. laevigata* (Harris et al., 1997). Free Ammonia-Nitrogen (FAN) is the unionised and more toxic form of inorganic nitrogen. For *H. laevigata*, FAN exposure >0.110 mg FAN^{-1} decreases feed intake, >0.054 FAN^{-1} decreases growth rate, >0.073 mg FAN^{-1} increases oxygen consumption and energy expenditure and >0.188 mg FAN^{-1} induces mortality (Harris et al., 1998). Nitrate levels >250 mg $N\text{-}NO_3$ 1^{-1} decreased growth rate in the European abalone *Haliotis tuberculata* (Basuyaux and Mathieu, 1999). Nitrogenous products are important to manage in abalone farms. Decreasing nitrogenous wastes in an aquaculture system may be achieved by optimising dietary protein, feed rates and waste management (Harris et al., 1997).

2.1.4. pH

pH is a measure of hydrogen ion concentration in water. pH fluctuates naturally during the day due to variations in carbon dioxide (CO_2) concentration from changes in respiration or photosynthesis of aquatic life and atmospheric gas exchange (Boyd et al., 2011). Outside of their optimal pH niche, abalone shell growth, shell quality, and body growth are compromised (Cummings et al., 2019; Auzoux-Bordenave et al., 2020). Burke et al. (2001) compared growth rates and survival of Greenlip and Blacklip Abalone. Greenlip Abalone grow best in pH range of 7.78-8.77, while Blacklip Abalone growth was fastest in the pH range of 7.93-8.46. Significant mortalities were recorded for both species at pH less than 7.16 or higher than 9.01 (Burke et al., 2001). A 50% reduction in growth rates were reported at pH 7.39 for Greenlip Abalone and 7.37 and 9.02 for Blacklip Abalone (Harris et al., 1999b). As pH affects several functions in abalone, managing appropriate pH levels on-farm is important.

2.1.5. Salinity

In abalone aquaculture salinity is largely dependent on the salinity of incoming water supply which varies with the climate and physical characteristics of the coastline the farm is located. For example, warm temperatures can increase evaporation and salinity in inverse estuaries (Wolanski, 1986), while high freshwater inputs in bays can lower salinity (Richmond et al., 2019). Abalone have a large muscular foot they use for locomotion and substrate attachment. The muscular foot is a large and permeable area (not protected by shell) of abalone (Burton, 1983). Changing salinity directly impacts ionic concentrations in the hemolymph and sudden variations in salinity will cause the hemolymph to equilibrate with the external water. Abalone that are saline stressed seal themselves to the substrate to reduce equilibrating with the external environment. This behavioural strategy of firm attachment to the substrate forms a small water layer between the foot and the substrate that shields against external sub-optimal-saline water from encountering the abalones' permeable surface (Burton, 1983). Abalone that have been previously stressed however, may have a dampened response to fluctuating salinity, leaving them more vulnerable to sub-optimal salinity (Burke et al., 2001). Exposure to low salinity before Greenlip and Blacklip abalone could fully attach to a substrate significantly increased probability of mortality. Salinity within the range of 25-40 g L^{-1} is tolerable for Greenlip and Blacklip Abalone, and 2 g L^{-1} and variation either side of this range induced mortality (Burke et al., 2001).

2. 2. Husbandry and farm environment

Abalone life-history stage, environmental or anthropogenic (handling or air exposure) stressors may be associated with summer mortality. Maturation and spawning stress are associated with higher mortality rates in mature (4-year-old) and spawning Ass's-ear Abalone *Haliotis asinina* compared to immature (1.5-year-old) *H. asinina* when exposed to elevated water temperature (>17˚C) (Travers et al., 2009). When abalone are stressed, energy is diverted to support essential metabolic functions, feeding ceases and growth rates decline (Tomanek, 2010; Stone et al., 2014a; Morash and Alter, 2016).

Routine husbandry practices in abalone aquaculture often involve physical handling of abalone. Grading and manual handling abalone for stock assessment (growth, health checks and harvest) or translocation, have the potential to induce stress in abalone, and may decrease feeding rates and growth, and increase mortality rates (Morash and Alter, 2016). Handling or air exposure immunocompromised *H. asinina* (immature or mature), and exposure to high water temperatures (>19˚C) exacerbated this effect (Cardinaud et al., 2014).

Common handling methods in abalone aquaculture include chipping (manual removal of abalone from substrate with a blunt spatula) (Hooper et al., 2011; Robinson et al., 2013) or muscle relaxants (Chacón et al., 2003). Accidental incisions during abalone chipping can result in hemolymph loss and death if tissue damage is severe and/or energy loss through excessive mucus production (Chacón et al., 2003). A favoured method, therefore, involves chemical muscle relaxants, which prevent muscle contraction (Ross and Ross, 2008) and allow for abalone removal from substrate without force (Aquilina and Roberts, 2000). Properties of a good relaxant include rapid induction, minimal stress, fast recovery, unaltered behaviour post treatment and no mortality (Ross and Ross, 2008). Multiple chemicals have been trialled in different abalone species (see Chacón et al., 2003). Magnesium sulphate (PER86963), 2-phenoxyethanol (PER83233), magnesium chloride (PER83238) and benzocaine (PER14638) are chemical relaxants approved by the Australian Pesticides and Veterinary Medicines Authority (APVMA) for use in Australian abalone culture. Of these, only

magnesium chloride and magnesium sulphate are suitable for harvest due to 0-day withholding periods.

Either intentionally, through selective breeding, or unintentionally, abalone in aquaculture are undergoing domestication. Lachambre et al. (2017) compared the behavioural and immunological responses to stress of hatchery-reared and wild abalone finding farmed abalone had a greater capacity to recover after laboratory-based stress tests. Individuals that can better recover from farm related stressors are more fit for commercial abalone aquaculture (Robinson et al., 2013). Selecting for stress-tolerant abalone may be another option to further improve the coping capacity of aquaculture stock to on-farm husbandry.

Stocking density can also induce stress in farmed abalone. High stocking densities result in increased feed per culture unit and therefore higher faecal production and potential waste feed. If there is inadequate waste management this can lead to poor water quality (Vandepeer, 2006). There are different cleaning methods used on farm in Australia including scrubbing, high pressure cleaners, spray bars or tippers. The type of method used typically depends on the tank type and shape at each farm. Different cleaning equipment/methods can impact abalone health. For example, water jets from high pressure hoses have potential to cause injury to abalone. Cleaning frequency can also induce stress in abalone; frequent cleaning can cause stress, but infrequent cleaning can lead to nitrogen build-up from uneaten feed, poor water quality and environmental stress (Vandepeer, 2006). During on-farm experimental trials, Stone et al. (2014b) recommended that tanks are cleaned twice weekly in winter and three times per week in summer.

In the wild, abalone are nocturnal feeders. In darkness, locomotion increases in *H. iris*, feeding activity maximises in *H. asinina* (Tahil and Juinio-Menez, 1999; Allen et al., 2006) and feed intake, growth rates and feed conversion efficiencies all increase for *H. rufescens* (Ebert and Houk, 1984). In aquaculture, decreasing feed ration can cause a shift from nocturnal to diurnal feeding in *H. laevigata* (Buss et al., 2015). Continual low light intensity is therefore a favoured environment for rearing abalone. On farm, light intensity can be minimised with shade cloth over the raceways or tanks and abalone can be provided with additional day time shelters or hides (Heasman and Savva, 2007). Inadequate provision of shade or shelter may cause stress in cultured abalone. Abalone may also experience increased heat stress in warmer months, as shallow water exposed to full sun will heat faster than water that is partially shaded.

2. 3. Feed

In the wild, juvenile abalone primarily consume diatoms, while adult abalone shift to a predominately consuming macroalgae (Tutschulte and Connell, 1988; Stepto and Cook, 1993; Naidoo et al., 2006). Formulated feeds are typically used on Australian land-based abalone farms (Fleming et al., 1996; Stone et al., 2013; Bansemer et al., 2016b). In Australia, there has been significant research effort on optimising formulated feed for farmed abalone to improve growth, health, and survival. Dietary inclusions of macroalgae, antioxidants and vitamins have been trialled in formulated diets to improve abalone survival when exposed to warm water temperatures (>25˚C) (Lange et al., 2014; Duong et al., 2016; Buss et al., 2017; Thomson et al., 2018; Table 1).

Table 1. Dietary intervention studies aimed to improve the survival of abalone.

Abalone species	Abalone age	Diet type	Temperature	Result	Reference
H. laevigata	**Experiment 1:** 2-year-old 3-year-old **Experiment 2:** 3-year-old	**Experiment 1:** -Live *Ulva* sp. Non-enriched -Commercial diet (Comm.) **Experiment 2:** -Live *Ulva* sp. Nitrogen enriched -Comm.	**Experiment 1:** 18°C, 22°C and 26°C **Experiment 2:** 22°C and 26°C	**Experiment 1:** <u>2-year-old:</u> -26°C: No significant difference in survival between diets: -Feed intake significantly higher in abalone fed Comm. than those fed *Ulva* sp. Non-enriched. <u>3-year-old:</u> -26°C: Significantly higher survival for abalone fed *Ulva* sp. Non-enriched than those fed the Comm. diet. -18°C and 22°C: No significant difference in abalone survival between diets for either age. -Feed intake significantly higher for Comm. diet than those fed *Ulva* sp. Non-enriched **Experiment 2:** <u>3-year-old:</u> -26°C: Significantly higher survival for abalone fed *Ulva* sp. Enriched than those fed the Comm. diet. -22°C: No significant difference in abalone survival between diets. -Significantly higher feed intake at 22°C than at 26°C.	Stone et al. (2014a)
H. laevigata	3-year-old	-Comm. -Comm. + 5% Grape Seed Extract (GSE) -Comm. + 30% dried *Ulva* sp. -Comm. + 5% GSE + 30% dried *Ulva* sp. -Live *Ulva* sp.	22°C and 26°C	-Feed intake was significantly higher at 22°C than at 26°C. -22°C: Significantly higher daily growth rate for abalone fed any formulated feed than those fed live *Ulva* sp. -26°C: No significant difference in abalone daily growth rate between diets. -22°C: Abalone survival was 100% across all diets. -26°C: Significantly higher abalone survival when fed GSE, dried *Ulva* sp. Diets and live *Ulva* sp. Inclusions than those fed the Comm. diet.	Lange et al. (2014)

H. laevigata	3-year-old	-Comm. (at 22°C and 25°C) -Peanut skin extract (PE) (0.5%, 1%, 2.5% or 5%) -Green tea extract (GTE) (0.5%, 1%, 2.5% or 5%) -Vitamin (Vit) C (1%, 1% Vit C + 1%GTE, 1% Vit C + 1% PE) -GSE 5%	22°C and 25°C	-Feed intake significantly decreased for all diets at 25°C compared to 22°C -Significantly lower survival for abalone fed Comm. at 25°C than abalone held at 22°C -No significant difference in survival between 5% GSE at 25°C and Comm. diet at 22°C -At 25°C: Significantly higher survival for abalone fed 0.5% GTE or 2.5% GTE than abalone fed the Comm. diet. - PE or Vit C had no beneficial effects on abalone survival.	Duong et al. (2016)
H. laevigata	3-year-old	-Comm. -Vit K_1 (0.5%, 1%, 1.5%) -Vit K_3 (0.5%)	22°C and 25°C	-At 25°C: No significant improvement in abalone survival Vit K_1 or K_3. -Vit K_1 had no effect on immune parameters (total hemocyte count, phagocytic activity or index) -Significantly lower catalase activity in abalone fed Vit K_3 than those fed Vit K_1 -Significantly lower catalase concentration in visceral organ (Vit K_3<Vit K_1) -Significantly lower catalase activity in abalone at 22°C than those at 25°C.	Thomson et al. (2018)
H. laevigata	3-year-old	-Comm. -Orego-Stim (OS) (0.5%, 1%, 2%, 4%)	22°C and 25°C	-Significantly higher abalone survival and feed intake at 22°C than at 25°C. -Significantly higher feed intake with higher OS diet inclusion: 4% OS than at 1.0% OS and the Comm. diet.	Buss et al. (2017)

3. Abalone immune system and laboratory diagnosis of summer mortality

3.1. Abalone immune system

Aquaculture environments limit the mobility of abalone, therefore farmed abalone have a restricted ability to physically remove themselves from stressful or temporarily unfavourable conditions. When abalone experience adverse environmental conditions in aquaculture, including high water temperature, they therefore rely mainly on their innate immune system for defence.

Molluscs, including abalone, have a simple immune system that, when compromised, can leave them vulnerable to stress and opportunistic pathogens. Molluscan external barriers including shell, mucus, and epithelia provide the first line of defence against pathogens (Sokolova, 2009). Abalone lack an adaptive immune system and rely on their innate (or non-specific) immune system for protection, which comprises humoral, and cell mediated immunity (Hughs et al., 2010). Abalone humoral immune responses include lectins, lysosomal enzymes and anti-microbial peptides, which detect and mark pathogens for further destruction (Sokolova, 2009). Cell mediated immunity is the central defence system for molluscs, whereby circulating hemocytes destroy pathogens via phagocytosis or oxidative burst (Sokolova, 2009; Hughs et al., 2010). Phagocytosis is a complex cellular process that recognises, adheres, ingests, encapsulates, and destroys foreign cells. Oxidative burst eliminates pathogens through rapid formation of reactive oxygen species (ROS) (Sokolova, 2009), which can cause enzyme inactivation, lipid peroxidation, DNA damage and cell death (Winston and Di Giulio, 1991; Roch, 1999). Common ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), alkyl peroxides, singlet oxygen ($^1O_2$), and hydroxyl radicals (OH.) (De Zoysa et al., 2009). To protect against excessive oxidative damage, the effects of ROS are controlled by the production of endogenous antioxidants including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), thiol peroxidase (TPx) and thioredoxin (TRx) (Winston and Di Giulio, 1991; Cazenave et al., 2006; De Zoysa et al., 2009). In turn, there is a delicate oxidant-antioxidant balance in molluscan cell mediated innate immunity (De Zoysa et al., 2009).

3.2. Pathology of summer mortality

Common gross observations for abalone experiencing summer mortality (bloating, excess mucus production, foot atrophy or abscesses) (Vandepeer, 2006) can be analogous to gross observations for other abalone diseases notifiable to the World Organisation for Animal Health (OIE). Histopathological lesions observed during summer mortality events include hemocyte infiltration into abalone digestive gland, loss of gill epithelium and gill necrosis (Hooper et al., 2014). Gill damage from exposure to high water temperatures has been demonstrated in abalone (Pedler et al., in press) and other aquatic species including Japanese flounder (*Paralichthys olivaceus*) (Yifan et al., 2015) and sea bream (*Sparus aurata*) (Madeira et al., 2014). Damage to the gills may provide an entryway for bacteria (Pichon et al., 2013).

No single causative pathogenic agent for summer mortality has been identified. In some summer mortality cases, *Vibrio* spp. are present, but are likely secondary and opportunistic infections in immune-compromised abalone (Vandepeer, 2006; Hooper et al., 2007; Dang et al., 2011).

Vibrio species, including *V. alginolyticus, V. parahaemolyticus*, *V. splendidus* and *V. harveyi,* are associated with mortality in abalone*. Vibrio alginolyticus* is associated with mortality in red abalone (*H. rufescens*), and abscesses and ulcers in small abalone (*H. diversicolor supertexta*) larvae (Buller, 2004; Handlinger et al., 2006). *Vibrio parahaemolyticus* is associated with mass mortality in small abalone (Buller, 2004; Handlinger et al., 2006). In Australian *Haliotis* spp., there have been no mortality events due to *V. alginolyticus* or *V. parahaemolyticus* (Handlinger et al., 2006). Handlinger et al. (2006) reported *V. splendidus* infections in Tasmania and New South Wales, but these were described as incidental infections, which do not cause abscesses. Clinical disease and mortality events are associated with *V. harveyi* on abalone farms in Victoria, Tasmania and South Australia, and infections in Western Australia, but not in wild abalone populations (Handlinger et al., 2006).

Increased stress, including elevated temperature, can lead to an immunocompromised state in animals (Sun et al., 2019). Opportunistic pathogens are then able to infect immunocompromised animals (Hooper et al., 2007). *Vibrio* spp. are opportunistic bacteria associated with summer mortality events in immunocompromised cultured abalone (Vandepeer, 2006; Dang et al., 2011; Dang et al., 2012) and wild abalone mortality events (Roberts et al. 2019). *Vibrio* spp. are Gram-negative bacteria with curve-shaped rod structures that can cause vibriosis (a haemorrhagic septicaemic disease) in fish and molluscs (Bøgwald and Dalmo, 2014). *Vibrio* spp. can enter abalone farms through water sources or abalone broodstock (Theil et al., 2004). Clinical vibriosis in abalone is characterised by blister-like lesions in the foot and high mortality. After initial infection in the foot, *Vibrio* spp. infection spreads to other organs, which lose natural function and abalone die due to starvation and exhaustion (Li et al., 1998). Clinical vibriosis in abalone has been suggested to be related to high stocking density, poor water quality (high silt and organic matter loads), inadequate water flow rates and tanks designs (Jones and Stephens, 2006; Vandepeer, 2006). Elston (1990) referred to vibriosis as a management disease, and suggested *Vibrio* spp. occurrences can be mitigated with effective hygiene practices through improved water quality or reduced stocking densities.

4. Conclusion

When water temperatures exceed the thermal tolerance of abalone, their health may be impacted which may result in mortality. In Australian abalone aquaculture, this has been termed summer mortality and it remains an issue for the industry. Abalone are marine ectotherms, subject to water temperature fluctuations. For defence they rely on physical barriers and on innate (or non-specific) immune system. Nutritional research indicates farmed abalone can boost their immune defence against environmental stress through dietary inclusion of macroalgae or antioxidants.

Warm water does not always result in summer mortality. Summer mortality is likely driven by a combination of abiotic and biotic factors. Abalone are sensitive to environmental change (e.g. fluctuating or deteriorating water quality) and may become stressed by anthropogenic interference (e.g. grading and handling). Moreover, the sensitivity of abalone to various stressors is likely influenced by life-stage, genetics, and the number of prior stressful events they have experienced. No sole pathogen is described as the causative agent for summer mortality and gross lesions described are consistent with many other diseases. Pathogenic bacteria have been associated with summer mortality in some studies, but without robust evidence (Vandepeer, 2006; Dang et al., 2011; Dang et al., 2012). Globally, *Vibrio* spp. have been associated with high mortality in abalone during warm water temperatures (Nicolas et

al., 2002; Pichon et al., 2013; Cardinaud et al., 2015). These opportunistic pathogens and may be present due to the immune-compromised nature of moribund abalone stressed by warming waters. A detailed investigation into the primary drivers of summer mortality and determining if causal pathogens are involved will aid the industry in developing appropriate mitigation strategies in a warming climate.

Chapter 2: A case-control study for summer mortality in Australian abalone aquaculture

1. Introduction

Greenlip and hybrid abalone are predominantly cultured in land-based systems throughout southern Australia (Stone et al., 2013). During their grow-out, abalone are exposed to water temperatures that range from 10˚C to 25˚C. When water temperatures exceed abalone's preferred optimum during the summer period, Australian abalone farmers have reported higher-than-normal mortalities, which have been termed summer mortality by industry and in the literature. Summer mortality is therefore a high research priority for the Australian abalone industry (AAGA, 2020).

Summer mortality likely involves complex interactions between biological, environmental, anthropogenic, and nutritional stressors (Hooper et al., 2014; Romalde et al., 2014). Abalone genetics (Shiel et al., 2015; Shiel et al., 2017), age (Travers et al., 2009), diet (Stone et al., 2014a), water quality (Harris et al., 1998; Harris et al., 1999a) and previous exposure to stress (Morash and Alter, 2016) can all influence how abalone respond to stress. Such factors may therefore play a role in how susceptible abalone are to the warm water temperatures that coincide with summer mortality events. Previous research on summer mortality has largely been conducted using heat stress model trials in small tanks to investigate dietary intervention (e.g. Vandepeer, 2006; Stone et al., 2014a). These trials have identified useful dietary ingredients to reduce mortality (e.g. grape seed extract). The conditions of these trials differ to the realities experienced by abalone in commercial farming systems. The short term and controlled nature of laboratory trials (feed rates, handling, water temperature etc.) are often not reflective of the commercial on-farm environment.

Case-control studies are a well-established epidemiological method for determining if exposure to risk factor(s) is associated with a defined outcome (presence or absence of a disease) (Lewallen and Courtwright, 1998). Frequency of exposure of case/controls to each risk factor can be determined retrospectively and this method permits examination of multiple risk factors at a time. A case-control study for summer mortality would allow the investigation of multiple potential risk factors for summer mortality (e.g., water temperature, age, husbandry, and the presence/absence of a pathogen).

Summer mortality in abalone lacks a clear case definition and a description of pathology in the literature. While farmers have indicated the mortality pattern of summer mortality is slow and cumulative (AAGA, pers. comm.), which may slowly accumulate up to 50% over a summer period (Vandepeer, 2006; Stone et al., 2014a). Based on the current published literature, it was difficult to develop a case definition for summer mortality. In this Chapter, we consulted with industry (survey and workshop) and abalone health experts, used mortality data (2015-2021) and analysed literature to develop a case definition for summer mortality which encapsulates the commercial summer mortality experience. Our aim was to identify risk factors associated with summer mortality through examining retrospective summer mortality data from 2015 – 2021 using a case-control study. This satisfies Objectives 1 and 2 of this project.

2. Methods

2. 1. Developing a case definition for summer mortality

After reviewing the scientific literature in Chapter 1, a survey was developed and distributed to Australian abalone growers in August 2020 (Appendix 1). This survey allowed farms to confidentially submit their experience of summer mortality and explain how they define summer mortality. Survey results (Appendix 2) were used to refine the preliminary case definition.

An industry-government summer mortality workshop was then held on 3 March 2021. This workshop was attended by abalone farm managers, industry representatives, and state government aquatic animal health representatives (South Australia, Western Australia, Victoria, and Tasmania). In this workshop, we discussed the summer mortality survey results and preliminary case definition, the risk factors that were explored in the case-control study for summer mortality (for meeting agenda, presentation and meeting minutes see Appendices 3 – 5). In the workshop, additional suggestions to the case definition were provided (see Appendix 3). The preliminary case definition was subsequently amended.

This case definition was then reviewed against retrospective mortality data provided by three farmers. This process confirmed that the definition was comprehensive and captured elevated and chronic mortality above the farm's winter baseline without being overly inclusive.

2. 2. Identifying case, matched control and background control tanks

A retrospective case-control analysis was conducted using each tank as one epidemiological unit. Summer mortality case and control tanks were identified from 2015 to 2021. Six farms provided data, three farms indicated that they did not have tanks that fit the case definition, and two other farms did not participate. Individual farms were de-identified during analysis for confidentiality reasons.

Case tanks were identified using the above case definition (Section 2.1). Tanks that met the case definition during a summer period were excluded from being a case tank again for that year and were also excluded from being matched or background controls for the summer period.

Matched control tanks were selected using a random number generator (Microsoft Excel, 2022). The start date of a matched control tank was systematically aligned with the start date of a corresponding case tank. Once matched control tanks were identified, tanks were excluded from being used as background control within the same summer period.

Background control dates were systematically selected so that there were no tanks on the farm that fit the case definition for summer mortality. Once background control dates were selected, background control tanks were then selected using a random number generator (Microsoft Excel, 2022). For each farm, at least three background control tanks were identified from January to April. Once background controls were selected, the corresponding tank was excluded from being a background control for that year.

We identified case (n =264 tanks), matched control (n = 217 tanks) and 59 background control (n = 59 tanks). In the analysis, matched and background control tanks were analysed together as a control group (n = 276). After case tanks, matched controls and background controls were identified, their exposure to a range of risk factors for summer mortality was assessed (full list in Appendix 7).

2. 3. Risk factors

We used data from the literature review (Chapter 1), industry survey and industry workshop to identify 93 potential risk factors (e.g. related to temperature, husbandry, animals, feed, water quality, farm characteristics and practices, laboratory diagnosis; see Appendix 3-5). Given the breadth of risk factors explored, data for all risk factors were not always available for case or control tanks (61 factors were tested in final models). See Appendix 7 for final risk factors provided to farmers and Appendix 10 for risk factors excluded due to lack of data availability.

2. 4. Climate data

Climate data (2009-2021) including total daily precipitation (millimetres, mm), daily maximum air temperature (degrees Celsius, °C) and daily maximum wind gust speed (kilometres per hour, km/h), for the nearest active weather station to each farm were sourced from the Bureau of Meteorology (BOM) (website: http://www.bom.gov.au/climate/data-services/data-requests.shtml).

2. 5. Sea Surface Temperatures

Sea surface temperature (SST) anomaly maps of Australia showing satellite data (SA Integrated Marine Observing System) were obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) website: http://www.cmar.csiro.au/remotesensing/oceancurrent/sst_anom/. SST anomaly data compares a 6-day average SST to the historical average SST at the same time of year. We used these SST data to determine if SSTs were more than 3˚C above the historical SST average one week prior to case or control at the location of each farm.

2. 6. Data exploration and analysis

The data set contained many potential predictors of summer mortality, although data was not obtained for all potential predictors. Several variables were invariant across cases and controls and hence were excluded from further consideration (see Appendix 10 for list of excluded variables), along with variables for which data was not available, providing 61 potential predictor variables. Many of these predictor variables were expected to be collinear (Table 2), especially because several variables were collected that related to the same parameter, for example minimum and maximum water temperature, or that were related biologically (e.g. age, size and mass). To explore associations between other potential predictors (not expected to be collinear), we determined correlations between continuous variables using Kendall's Tau (τ). Associations between different factors and between factors and continuous variables were investigated using Fisher's exact test and Kruskal-Wallis tests respectively. Initial data exploration and subsequent analyses were all carried out using R statistical software (R Core Team 2021).

Models should aim to use predictors that are biologically relevant and most likely to be directly related to the outcome of interest, because this results in the most plausible models and aids interpretability (Dormann et al., 2013). Indirect variables with good predictive power can also be useful to include, especially where drivers of an outcome are not well understood (Dormann et al., 2013). We determined the predictive power of each potential variable based on the Watanabe-Akaike Information Criterion (WAIC) from model fit using the same method as for final models (see below) but using each variable as the sole predictor. All continuous variables were standardised before use in models. Where WAIC was similar (difference < |10|) between two or more of the models using related variables,

we selected the variable most likely to be directly related to the outcome. From this process we obtained a set of uncorrelated variables for use in initial models.

Models were built using step-wise forward selection and WAIC (see Table 4). At the first stage of model building, we added the predictors that were most likely to be biologically relevant, being those selected from the correlated sets representing temperature, age, and potential stressors. WAIC was determined for models using each combination of two of these biologically relevant factors, and the model with lowest WAIC selected. We then added further uncorrelated variables, assessed as those having $\tau \leq 0.4$ or no significant Fisher or Kruskal-Wallis test with variables already in the model, one at a time based on their WAIC in single-variable models. Variables were retained in the model where their inclusion provided a reduction of WAIC of more than 2. Once no further improvement in WAIC resulted, we then explored the addition of all other potential variables. For this stage, we built models using the selected model from the previous stage and examining WAIC for all potential models with one additional predictor. Where a model with one additional predictor showed WAIC reduction of 2 or more, that model was selected, and the process repeated. When no further reduction in WAIC was achieved, we explored the addition of interactions, using only pairs of variables already included in the models. At the final stage of model selection, we examined the effect of removing each parameter or interaction. Where removal of an interaction increased WAIC by more than 2, that interaction, and main effects of parameters in the interaction, were retained in the model. For parameters not included in retained interactions, parameters were retained where their removal resulted in an increase in WAIC of more than 2.

We applied generalised linear mixed models (GLMM) using the binomial family and logistic link. Models were fitted with a Bayesian hierarchical modelling approach with integrated nested Laplace approximations (Rue et al., 2009) run with R-INLA package (Martins et al., 2013; Lindgren and Rue, 2015; Rue et al., 2017). Farm and tank were included in all models as random effects. We used penalised complexity priors (Fuglstad et al., 2019) for the precision of random effects, with probability of standard deviation >1 set to 0.05. and default R-INLA priors for fixed effects.

Table 2. Risk factors (predictor variables) that were determined to be the most biologically relevant and interpretable with associated collinear proxies. Proxies were added in a stepwise fashion to determine if they improved the fit of the model compared to the selected biologically relevant risk factors. Note risk factors 5-10 did not have collinear proxies.

Risk factors	Collinear proxies
Water temperature 1. (Q25) Maximum water temperature in one week prior to the Case/Control event? (during that week)	1. a. (Q21) Maximum water temperature, 1 week prior to case/control date? (°C) (on the day, 1 week prior). 1. b. (Q22) Maximum water temperature, 2 weeks prior to Case/Control date? (°C) (on the day, 2 weeks prior) 1. c. (Q24) Minimum water temperature in one week prior to the Case/Control event? (during that week) 1. d. (Q87) Maximum air temperature since abalone were last graded (°C) (in between grading date and case date)
Abalone biology 2. (Q66) Age of abalone in this tank? (in MONTHS)	2. a. (Q60) Average weight (g) of abalone/tank, 1 grading BEFORE Case/Control. 2. b. (Q57) Average weight (g) of abalone/tank, 2 gradings BEFORE Case/Control. 2. c. (Q98) Average abalone growth rate between grading BEFORE Case/Control and grading AFTER Case/Control 2. d. (Q99) Average abalone growth rate between 2 gradings BEFORE Case/Control 2. e. (Q70) Average feed rate for one month before case/control? (% body weight/day) 2. f. (Q58) Total weight (kg) of abalone/tank, 2 gradings BEFORE Case/Control. 2. g. (Q45) Have abalone spawned in past 3 months, in this tank? (Yes/No)
Stress 3. (Q51) Average abalone mortality rate for this tank, 2 weeks after last grading or handling (%)	3. a. (Q46) Is this a partial harvest tank? 3. b. (Q41) Were animals in this tank mixed during grading? 3. c. (Q88) Max air temp (°C) of on day of last grading 3. d. (Q53) Grading date, 1 grading BEFORE Case/Control 3. e. (Q52) Grading date, 2 gradings BEFORE Case/Control
4. (Q44) Month and year abalone were removed from nursery plates/weaning tank.	4. a. (Q43) Abalone size when removed from nursery plates/weaning tanks (shell length, mm).
5. (Q47) Did abalone in this tank experience summer mortality last summer?	
6. (Q35) Tank cleaning method	
7. (Q36) Tank coverage description	
8. (Q38) Is there algal growth in this tank?	
9. (Q55) Size/volume of Case/Control tank (m^2)	
10. (Q1) Date of Case/Control	

2. 7. Odds ratios

An odds ratio is the measure of association for a case-control study. Odds ratios quantify the association between exposure to a risk factor (e.g. water temperature) and the occurrence of summer mortality. An odds ratio of 1 (or close to 1) indicates exposure does not increase or decreases the risk of summer mortality. An odds ratio greater than 1 indicates that the odds of exposure to a risk factor of interest are greater among case tanks than control tanks, indicating that exposure increase the likelihood of summer mortality. An odds ratio less than 1 indicates the odds of exposure among case tanks are lower than odds of exposure among controls, the exposure could be a protective factor against summer mortality.

To illustrate effects, we extracted odds ratios for main effects at selected levels and selected levels of the other interacting parameter. Odds ratios were calculated by exponentiating logistic predictions generated from the final selected model using the inla.make.lincombs function. 95% highest density intervals (HDI) were calculated for model parameters and odds ratios using the inla.hpdmarginal function.

3. Results

3.1. Case definition

Review of current literature resolved there was no clear case definition for summer mortality in abalone aquaculture. To ensure our case definition reflected experience on farm, we consulted with industry to develop a case definition for summer mortality in Australian abalone aquaculture.

A working case definition was developed and based on discussions held at the Australian Abalone Growers Association (AAGA) workshop in 2019:

> A >1% increase (absolute) in daily tank mortality rate from the farms' baseline rate from December to April, excluding those diagnosed with an OIE notifiable disease as the primary cause of mortality.

In August 2020, a survey was distributed to industry and in December 2020, the case definition was revised to include all industry survey responses.

i. Chronic mortality in >1 year old abalone above the farms' baseline mortality rate (if in doubt take this to mean > 0.15% $tank^{-1}$ day^{-1}), and:

ii. occurs in at least two culture units, and

iii. occurs between January and April, but

iv. excludes those diagnosed with an OIE notifiable disease as the primary cause of mortality.

An industry-government summer mortality workshop was held on 3 March 2021. Within the workshop, two suggestions were made by industry to improve the Case Definition:

- the farms' baseline mortality should be calculated from winter months only, as some farms had baseline mortalities during summer close to the mortality value specified in the case definition (0.15% $tank^{-1}$ day^{-1}).
- the case definition should exclude mortalities of known causes (e.g. power failure or no water in tank).

Incorporating feedback from this workshop, the summer mortality case definition was revised and the final working case definition for summer mortality in farmed abalone aquaculture is:

i. Chronic mortality of unknown cause (if in doubt take this to mean >0.15% tank^{-1} day^{-1} for at least one week) above the farms' winter baseline mortality rate in >1 year old abalone, and

ii. occurs in at least two culture units, and

iii. occurs between January and April, but,

iv. excludes those diagnosed with an OIE notifiable disease as the primary cause of mortality.

3.2. Case-control study

The final selected model contained six mean effects and five two-way interactions (Table 4). The inclusion of any higher-order interactions involving parameters selected in this model did not provide any further improvement to model fit. Except for maximum water temperature in the week prior to the case/control date, all parameters selected in the final model were involved in one or more interactions. Interactions were included between age and each of previous year summer mortality, weaning size and feed rate. There were also interactive effects of previous year summer mortality and weaning size, and of feed rate and post-grading mortality (Table 4). Main effects of each selected parameter were positive, i.e. associated with an increased risk of summer mortality, while interactions were negative (Table 5). For a list of variable definitions, refer to Table 3.

Table 3. Names and description of predictor variables tested in the final model.

Predictor variables	Description
Maximum water temperature	Maximum water temperature (°C) in the week prior to case/control date.
Size at weaning	Abalone size when removed from nursery plates/weaning tanks (shell length, mm)
Previous year summer mortality	Did abalone in this tank experience summer mortality last summer (yes/no)
Post-grading mortality	Average abalone mortality rate for this tank, 2 weeks after last grading or handling (%)
Age	Abalone age (in months) at time of case/control date
Feed rate	Average feed rate (% body weight/day) for one month before case/control

Table 4. Watanabe-Akaike information criterion (WAIC) of selected model at each stage of the model selection process. For the forward selection of main terms, the selected model (lowest WAIC) is shown in bold, and the lowest WAIC of all other alternative models listed (X indicates any other parameter). For the selection of interaction terms, the selected interactions are shown in bold and the lowest WAIC of alternative models listed (XX = any other interaction). For backward selection, the deselected parameters are shown. Removal of any other term resulted in an increase to WAIC.

Parameters in model	**Lowest WAIC**
Intercept (Int) only	749.8
Forward selection of main terms	
Int + [1] Prev SM	**647.6**
Int + any other parameter	652.6
Int + Prev SM + [2] Grading mort	**580.0**
Int + Prev SM + X	632.5
Int + Prev SM + Grading mort + [3] Max T	**569.2**
Int + Prev SM + Grading mort + X	582.0
Int + Prev SM + Grading mort + Max T + Age	**569.2**
Int + Prev SM + Grading mort + Max T + X	571.3
Int + Prev SM + Grading mort + Max T + Age + [4] Feed	**543.2**
Int + Prev SM + Grading mort + Max T + Age + X	564.6
Int + Prev SM + Grading mort + Max T + Age + Feed + [5] Wean size	**535.2**
Int + Prev SM + Grading mort + Max T + Age + Feed + X	538.2
Int + Prev SM + Grading mort + Max T + Age + Feed + Wean size + [6] Shade	**531.5**
Int + Prev SM + Grading mort + Max T + Age + Feed + Wean size + X	532.6
Int + Prev SM + Grading mort + Max T + Age + Feed + Wean size + Shade + [7] Volume	**529.2**
Int + Prev SM + Grading mort + Max T + Age + Feed + Wean size + Shade + X	531.7
Int + Prev SM + Grading mort + Max T + Age + Feed + Wean size + Shade + Volume + X	529.5
Forward selection of interactions	
+ Prev SM x Wean size	**502.3**
+ XX	515.5
+ Prev SM x Wean size + Grading mort x Feed	**491.1**
+ Prev SM x Wean size + XX	493.3
+ Prev SM x Wean size + Grading mort x Feed + Age x Feed	**485.3**
+ Prev SM x Wean size + Grading mort x Feed + XX	486.2
+ Prev SM x Wean size + Grading mort x Feed + Age x Feed + Prev SM x Age	**475.9**
+ Prev SM x Wean size + Grading mort x Feed + Age x Feed + XX	482.5
+ Prev SM x Wean size + Grading mort x Feed + Age x Feed + Prev SM x Age + Wean size x Age	**465.8**
+ Prev SM x Wean size + Grading mort x Feed + Age x Feed + Prev SM x Age + XX	467.5
+ Prev SM x Wean size + Grading mort x Feed + Age x Feed + Prev SM x Age + Wean size x Age + XX	467.7
Backward selection	
− Volume	464.3
− Volume – shade	461.5
Final selected model	
Int + Prev SM + Grading mort + Max T + Age + Feed + Wean size + Prev SM x Wean size + Grading mort x Feed + Age x Feed + Prev SM x Age + Wean size x Age	**461.5**

[1] Previous summer mortality in the year prior (yes or no),
[2] Average abalone mortality rate for this tank, 2 weeks after last grading or handling (%),
[3] Maximum water temperature (°C) in the week prior to case/control date,
[4] Average feed rate (% body weight/day) for one month before case/control,
[5] Abalone size when removed from nursery plates/weaning tanks (shell length, mm),
[6] Tank shade level
[7] Tank volume (metres cubed, m^3).

Table 5. Coefficients of the selected model. Coefficients are on the log-odds (logit) scale, with the exponentiated coefficient demonstrating the multiplicative change in odds for that parameter.

Parameter	**Mean coefficient**	**95% highest density interval (HDI)**	
		0.025 HDI	***0.975 HDI***
Intercept	-1.54	-2.12	-0.97
Max water temperature in week prior	0.60	0.30	0.90
Mortality after last grading/handling (Grading mort)	3.08	1.70	4.53
Age	0.92	0.55	1.28
Previous year summer mortality (Prev SM)	3.29	2.67	3.91
Size at weaning/nursery plate removal (Wean size)	2.05	1.31	2.83
Feed rate	0.30	-0.31	0.90
Prev SM x Wean size	-1.83	-2.78	-0.90
Grading mort x Feed rate	-3.07	-5.10	-1.07
Age x Feed rate	-0.75	-1.12	-0.38
Age x Prev SM	-1.38	-1.95	-0.81
Age x Wean size	-0.90	-1.40	-0.43

Previous year summer mortality and age interactive effect

There was an interactive effect of abalone age and previous summer mortality (Table 4). For abalone that had no previous summer mortality experience, the risk of subsequent summer mortality increased with age; summer mortality was two times more likely for every 6-month increase in age. For abalone in tanks that did experience summer mortality the previous year, odds of summer mortality decreased with age (Table 5). For abalone in tanks that experienced summer mortality the year prior, animals were more likely to experience summer mortality at all ages, but the size of this effect decreases in older abalone.

Table 6. Odds ratios for exposure to factors included in the final model. * Signifies where the odds ratio is different from one (in bold).

Exposure to factors	Odds ratio (95% HDI)
Water temperature	
2 °C increase in max weekly water temperature	**2.02 (1.42 – 2.86)***
Effect of age with and without previous SM	
6 month increase in age – no previous SM	**2.07 (1.55 – 2.78)***
6 month increase in age – previous SM	**0.69 (0.50 – 0.96)***
Effect of previous SM at selected ages	
Previous year summer mortality – age 24 months	**38.95 (19.53 – 77.58)***
Previous year summer mortality – age 30 months	**13.00 (7.23 – 23.32)***
Previous year summer mortality – age 36 months	**4.34 (1.96 – 9.54)***
Effect of weaning size with and without previous SM	
5 mm increase in weaning size – no previous year SM	**3.40 (2.16 – 5.36)***
5 mm increase in weaning size – previous year SM	1.14 (0.78 – 1.66)
Effect of previous SM at selected weaning sizes	
Previous year summer mortality – 15 mm weaning size	**54.59 (25.30 – 117.69)***
Previous year summer mortality – 20 mm weaning size	**18.32 (9.86 – 33.98)***
Previous year summer mortality – 25 mm weaning size	**6.15 (2.50 – 15.08)***
Effect of weaning size at selected ages	
5 mm increase in weaning size – age 18 months	**6.06 (3.41 – 10.74)***
5 mm increase in weaning size – age 24 months	**3.95 (2.47 – 6.30)***
5 mm increase in weaning size – age 30 months	**2.57 (1.61 – 4.09)***
Effect of age at selected weaning sizes	
6 month increase in age – 15 mm weaning size	**2.75 (1.93 – 3.91)***
6 month increase in age – 20 mm weaning size	**1.79 (1.34 – 2.39)***
6 month increase in age – 25 mm weaning size	1.16 (0.79 – 1.71)
Effect of post grading mortality at selected feed rates	
0.2% increase in grading mortality – 0.25% feed	**22.42 (6.13 – 81.95)***
0.2% increase in grading mortality – 0.50% feed	**14.40 (4.96 – 41.80)***
0.2% increase in grading mortality – 1.00% feed	**5.94 (2.82 – 12.51)***
Effect of feed rate at selected levels of post-grading mortality	
0.5% increase in feed rate – 0% post grading mortality	**2.04 (1.53 – 2.72)***
0.5% increase in feed rate – 0.2% post grading mortality	0.84 (0.50 – 1.42)
0.5% increase in feed rate – 0.4% post grading mortality	0.35 (0.12 – 1.01)
Effect of feed rate at selected ages	
0.5% increase in feed rate – age 18 months	**2.96 (1.67 – 5.23)***
0.5% increase in feed rate – age 24 months	**1.50 (1.04 – 2.16)***
0.5% increase in feed rate – age 30 months	0.76 (0.50 – 1.14)
Effect of age at selected feed rates	
6 month increase in age – 0.25% feed	**3.77 (2.38 – 5.97)***
6 month increase in age – 0.50% feed	**3.18 (2.14 – 4.73)***
6 month increase in age – 1.00% feed	**2.27 (1.67 – 3.07)***
6 month increase in age – 1.50% feed	**1.61 (1.21 – 2.16)***

Previous year summer mortality and size at weaning interactive effect

Previous year summer mortality also had an interactive effect with abalone size at nursery removal (Table 4). For abalone that did not experience summer mortality the previous year, the risk of summer mortality increased if abalone had been a larger size at nursery removal (3.4 times more likely with every 5 mm increase in weaning size; Table 6). For abalone held in tanks that did experience previous year summer mortality, the odds of summer mortality did not change significantly with weaning size (odds ratio ~ 1; Table 6).

Abalone that experienced summer mortality the year prior were more likely to experience subsequent summer mortality across all nursery removal sizes, but this effect becomes less severe for abalone removed from the nursery at larger sizes (Fig. 2). Abalone with previous year summer mortality were 55 times more likely to experience subsequent summer mortality if they had been removed from the nursery at 15 mm, compared to six times more likely if they had been removed from nurseries at 25 mm (Table 6).

Size at weaning and age interactive effect

There was an interactive effect of abalone size at nursery removal and age (Table 4). The effect of size at nursery removal on summer mortality risk decreases as abalone age (Fig. 1). For 18-month-old abalone, a 5 mm increase in nursery removal size means the risk of summer mortality was six times more likely. For 30-month-old abalone, however, a 5 mm increase in nursery removal size means the risk of summer mortality was 2.5 times more likely (Table 6).

The effect of age on risk of summer mortality decreases as size at nursery removal increases. For abalone that were removed from the nursery at 15 or 20 mm, risk of summer mortality increased with age (Table 6). For a weaning size of 25 mm there is no change in summer mortality risk with age (odds ratio ~1) (Table 6).

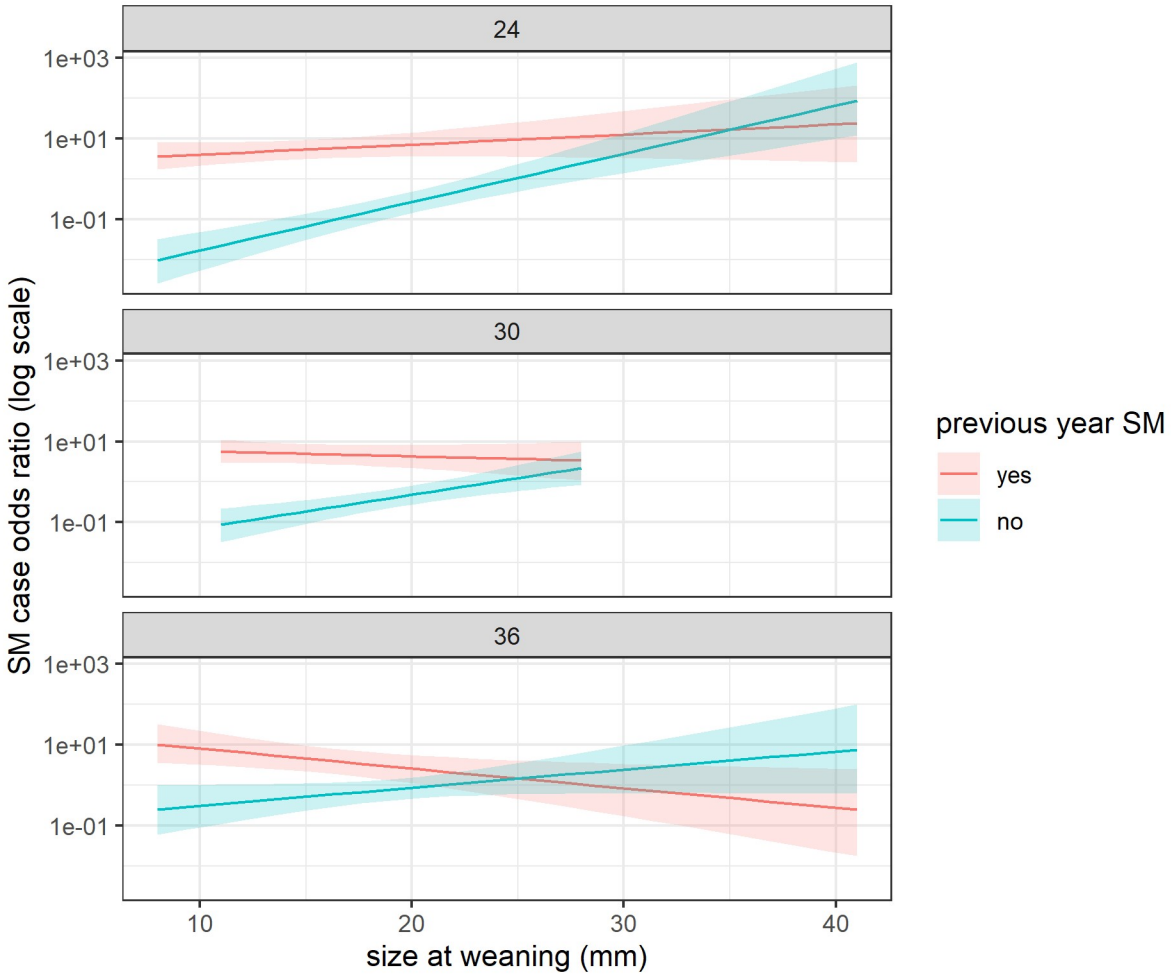

Figure 1. Effect of previous year summer mortality and size at weaning (mm) on odds of summer mortality for selected abalone ages (24, 30 and 36 months old). The effect is shown for the range of weaning sizes in the data set for abalone of the same age. Odds ratios are presented on a logarithmic scale for ease of interpretation.

Feed rate and post-grading mortality interactive effect

There was an interactive effect of feed rate and post-grading mortality on risk of summer mortality in abalone. For abalone that were in tanks with no post-grading mortality, the risk of summer mortality increased with increasing feed rates, but there was no effect of feed rate (odds ratio ~ 1) in tanks experiencing 0.2 or 0.4% post-grading mortality. Examining the raw data showed that 100% of tanks which had post-grading mortality rates over 0.4% experienced summer mortality. Comparatively, 45% of tanks that had post-grading mortality less than 0.4% experienced summer mortality. Any occurrence of post-grading or handling mortality was associated with a greatly increased risk of summer mortality in the model; the size of this effect decreased at higher feed rates (Fig. 2). Feed rate data, however, was missing for many of the cases that experienced post-grading mortality, meaning this interaction should be interpreted with caution.

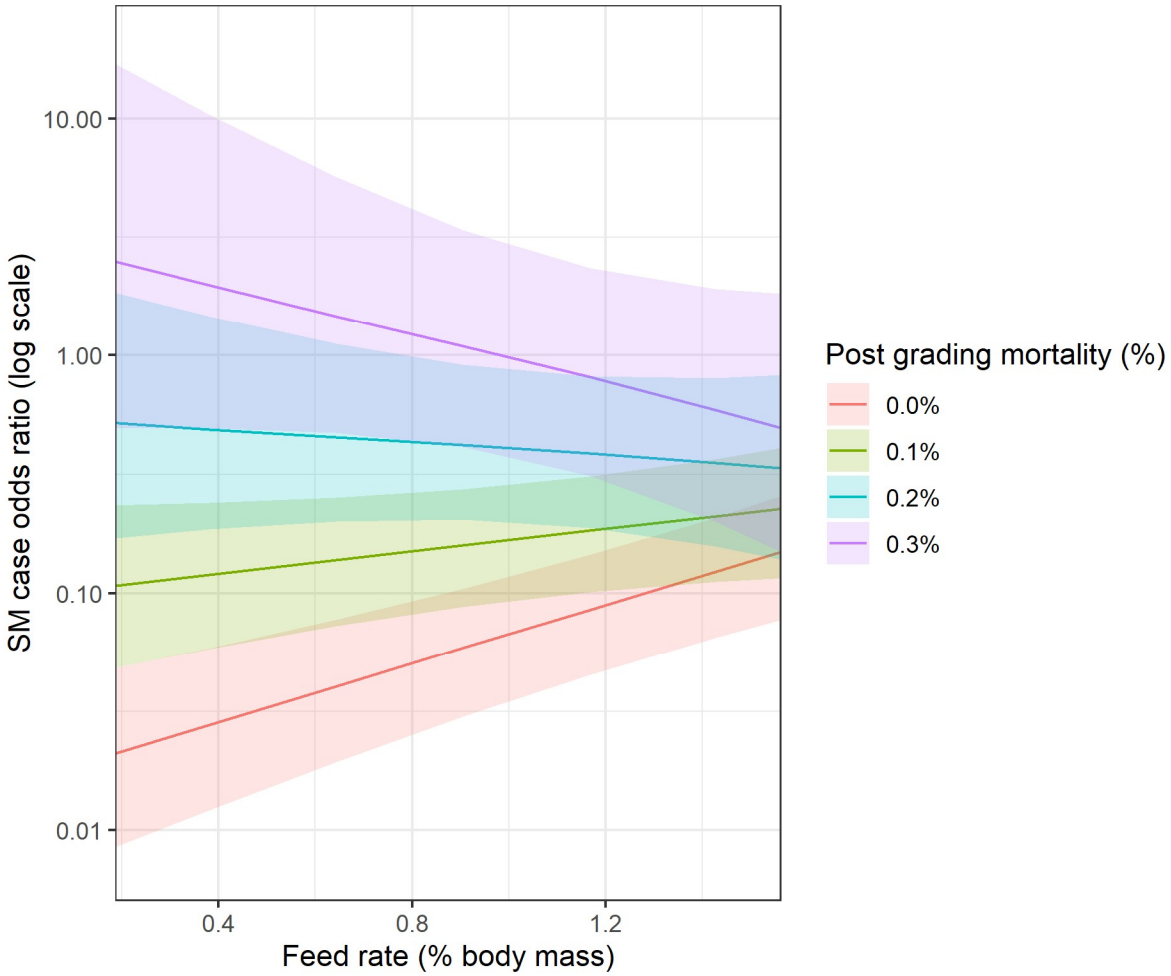

Figure 2. Effect of feed rate (percentage of abalone body mass) on odds ratio of summer mortality (log scale) for tanks with selected degrees of post-grading mortality (0.0-0.3%). Odds ratios are presented on a logarithmic scale for ease of visualisation.

Feed rate and age interactive effect

There was an interactive effect of feed rate and age on risk of summer mortality in abalone (Table 4). The effect of feed rate on summer mortality risk decreases with increasing abalone age. In 18-and-24-month-old abalone, an increase in feed rate was associated with an increased risk of summer mortality, while for 30-month-olds an increase in feed rate had no effect on summer mortality risk (odds ratio ~1) (Table 6). Older abalone typically had lower feed rates, which likely contributed to this interaction. Risk of summer mortality increased with abalone age, but the size of this effect was less at higher feed rates (Fig. 3).

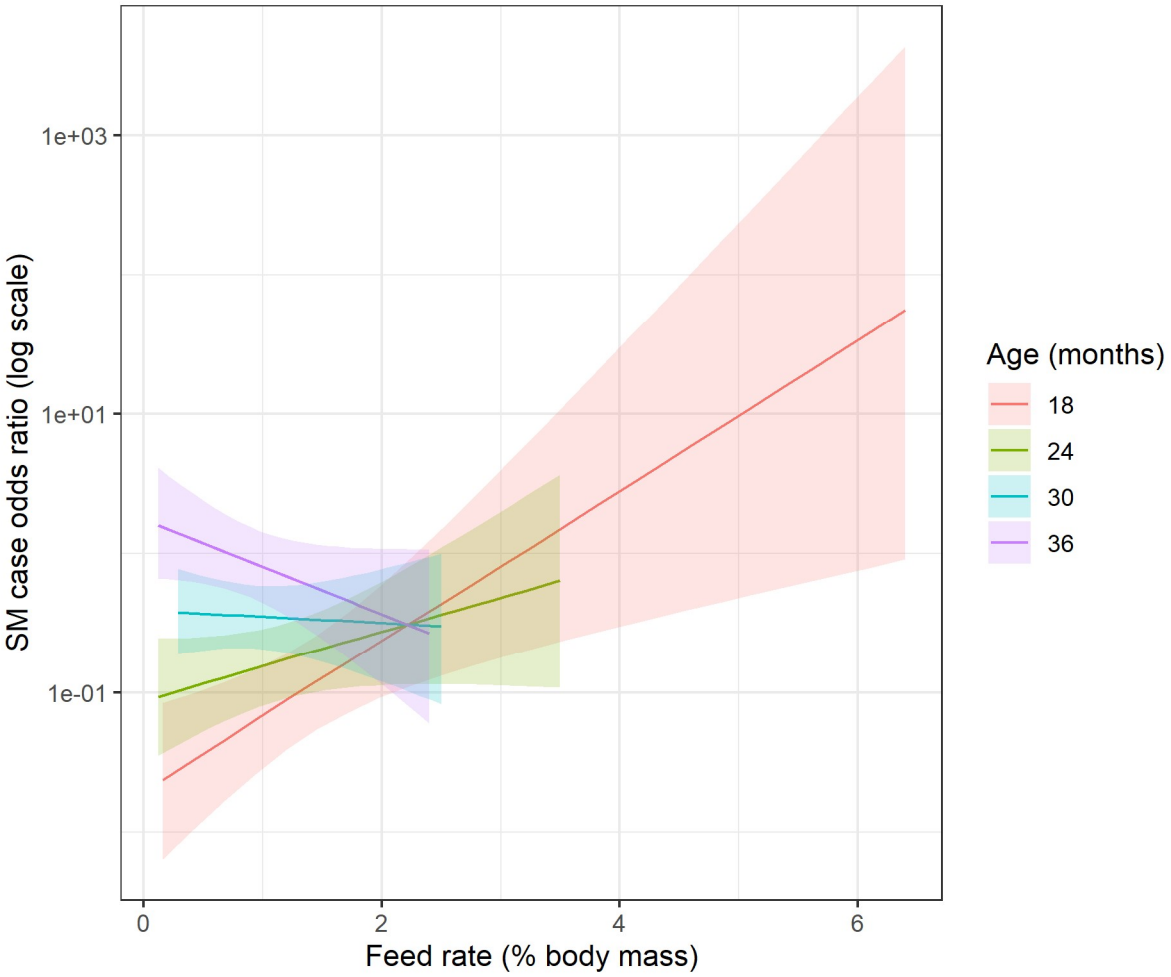

Figure 3. Effect of feed rate on odds of summer mortality for selected abalone ages (18, 24, 30 and 36 months old). The effect is shown for the range of feed rates in the data set for abalone of the same age ± 3 months. Odds ratios are presented on a logarithmic scale for ease of visualisation.

Maximum water temperature effect

Higher water temperature in the week leading up to the case date is associated with an increased risk of summer mortality (Fig. 4). The odds of summer mortality are two times greater for every 2 °C increase in maximum weekly water temperature (Table 6) (data range = 16.0-27.2 °C).

Abalone in tanks that experienced summer mortality the previous year were at a higher risk of summer mortality compared to abalone that did not experience summer mortality the previous year. The effect of previous summer mortality was consistent across temperatures (Fig. 4).

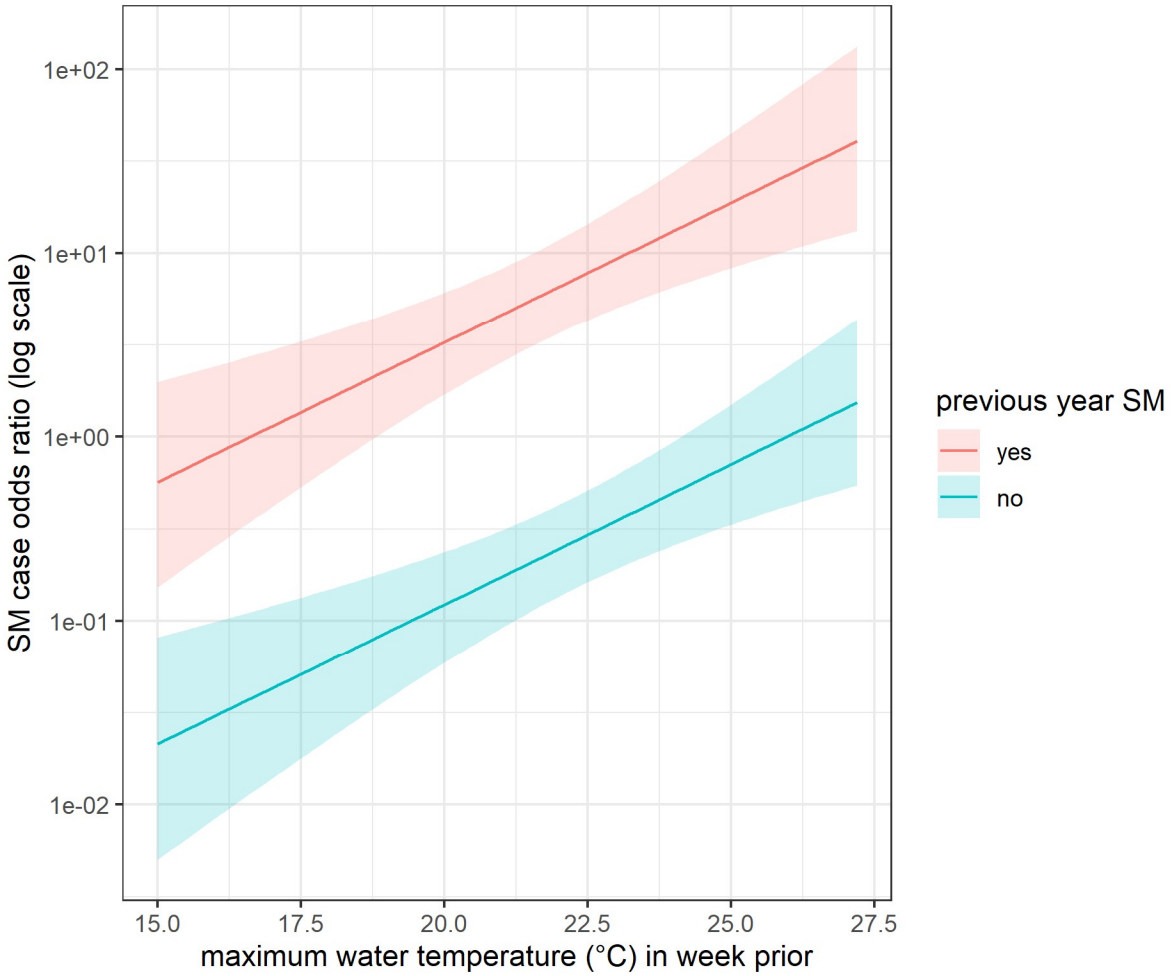

Figure 4. Effect of water temperature (˚C) and previous year summer mortality on odds of summer mortality. Odds ratios are presented on a logarithmic scale for ease of visualisation.

4. Discussion

In this project, we conducted a case-control study to determine associations between summer mortality case tanks and a range of putative risk factors. Retrospective data (from 2015-2021) provided by participating AAGA farms was used in this study. Below, we discuss identified factors that increase the likelihood of summer mortality in Australian abalone aquaculture.

Maximum water temperature

We found the risk of summer mortality was two times more likely for every 2 °C increase in maximum weekly water temperature experienced in the week prior (Maximum water temperatures ranged from 16.0-27.2 °C). Water temperature governs molluscan body temperature and influences important physiological processes in abalone including survival, stress, growth and metabolism (Bansemer et al., 2016c; Morash and Alter, 2016). At water temperatures above their optima the abalone immune system becomes inhibited and increases vulnerability to infections (Hooper et al., 2007; Wendling and Wegner, 2013). High water temperatures at abalone farms may also increase pipe biofouling, decrease water quality, and increase bacterial growth (Hooper et al., 2014). These factors can act as additive stressors for thermally stressed abalone. Results from this project are consistent with previous research (Travers et al., 2009; Stone et al., 2014a).

Farmers are currently unable to economically control incoming water temperature. Holding abalone at lower water temperatures would require sophisticated water temperature regulating technology, such as that in recirculating aquaculture systems (RAS) (Ahmed and Turchini, 2021). In Australia, abalone are predominately cultured in flow-through outdoor tank or slab systems. Adapting these systems into RAS or other systems capable of water temperature regulation would require high capital investment and complex engineering (Badiola et al., 2012) and is unlikely to be commercially feasible. As RAS technology continues to evolve, the technology will likely become cheaper in the future. Aside from high costs, additional challenges with rearing abalone in RAS include ensuring adequate waste removal. For example, Harris et al. (1997) demonstrated that extremely low levels of nitrite in the water reduces abalone growth. Initial site selection of farms is an important factor to consider to ensure the water temperature is optimal for survival and growth. There are some farm design and management strategies that may reduce water temperature on farm. Extending intake pipes to access colder water, tank design, increased flow rate and shade level may reduce water temperatures (Wassnig et al., 2010).

Feed rate and post-grading mortality

Abalone growth is reported to increase when cultured with the same-size conspecifics (Mgaya and Mercer, 1995). As abalone grow, they are moved and handled to adjust stocking densities or are sorted by size (Mgaya and Mercer, 1995). Grading or moving abalone on farm typically involves applying anaesthesia to a tank followed by scooping up the abalone into large trays. Abalone are then distributed into other tanks. There may also be a grading process where the abalone are passed through a sieve with differing hole sizes to group similar sized abalone stocked together (Hooper et al., 2011). This process involves multiple events known to cause stress in farmed abalone (Morash and Alter, 2016), including removal from substrate via anaesthetic or chipping, extended time outside of water, exposure to variable air temperatures and manual or mechanical handling (Hooper et al., 2011).

In the current study, post-grading mortality was associated with an increased risk of summer mortality, but the size of the effect decreased at higher feed rates. When post-grading mortality increased (> 0%), the risk of summer mortality decreased as feed rate increased. In

general, any tanks with post-grading mortality rates > 0% were at a much greater risk of summer mortality. In the current study, all tanks with post-grading mortality rates over 0.4% experienced summer mortality. It should be noted that feed rate data was missing for many of the cases that experienced post-grading mortality, meaning this interaction should be interpreted with caution. For example, there was no single tank that had a feed rate greater than 1.5% and a post-grading mortality rate greater than 0.5 %. Model predictions in this range are therefore extrapolations, and likely contributed to the appeared effect where higher feed rates decreased summer mortality risk in abalone with higher rates of post-grading mortality. When there was no post-grading mortality (e.g. 0%), the risk of summer mortality increased as feed rate increased.

We also found older abalone were more susceptible to higher levels of post-grading mortality, which may be explained by a longer history of exposure to on-farm stressors (Travers et al., 2009). Hooper et al. (2011) previously suggested that abalone are sensitive to stock movement practices. The authors reported elevated haemocyte counts and depressed phagocytic rates, neutral red retention times and antibacterial activity for abalone that had been anaesthetised with benzocaine and moved (Hooper et al. 2011). While the authors reported abalone recovered back to baseline levels within one day, this contrasts with results from the current study that indicate abalone may have extended recovery times after additive stressors on-farm. In the current study, post-grading mortality increases summer mortality risk, which suggests surviving abalone from tanks with elevated post-grading mortality may not have fully recovered before experiencing further stress.

Handling is necessary in abalone aquaculture to complete a production cycle, and although procedures are in place to protect abalone from stress during these events, abalone may still become stressed during handling. Where possible, sufficient time in between handling events should be planned to promote recovery. Avoiding handling on extreme weather days will also prevent exposing abalone to elevated air temperatures (Hooper et al., 2011). Taking extra care when handling older abalone is also encouraged, as they were more prone to post-grading mortality. Keeping daily stock counts and recording number of mortalities in each tank may allow farmers to identify tanks that have high mortality rates after a handling event and as a result an increased risk of summer mortality.

Previous year summer mortality

Generally, abalone that were in tanks that experienced summer mortality the previous year were at a higher risk of subsequent summer mortality. This may be due to extended recovery times from stress for abalone. Multiple stressful events likely delay full recovery, and recovery times from additive stressors are thought to be much longer than recovery from a single stress in abalone (Morash and Alter, 2016). The effect of multiple stressors on abalone are often delayed (Lachambre et al., 2017). When abalone are stressed, they rapidly use up their energy reserves and become less able to protect themselves from adverse conditions (Vandepeer, 2006), but may be experiencing sub-clinical physiological and/or secondary pathological effects. During this time, they may appear and behave normally (Vandepeer, 2006). Visible signs of stress or low level mortalities may be difficult to detect, as they can occur weeks after a stress event. Delayed mortality after stress in abalone has previously been reported in experimental studies. For example, Bansemer et al. (2016) and Stone et al. (2013) reported mortalities typically occurred two to three weeks after experimental abalone were handled. Recording what tanks experience summer mortality is a useful method for farmers to identify and track abalone that have increased risk to summer mortality the subsequent summer season. A greater insight into abalone recovery rates post-stress in aquaculture would aid in optimising husbandry in abalone aquaculture to promote health and this should be considered an avenue for future research.

Previous year summer mortality and age

For abalone that did not experience summer mortality the year prior, the risk of subsequent summer mortality increased with age. Laboratory summer mortality models have demonstrated 3-year-old abalone are more susceptible to heat stress 2-year-old abalone (Lange et al., 2014; Stone et al. 2014a). Body size affects a range of physiological functions in animals, including respiration which generally increases with body mass as larger animals require more energy and oxygen for cellular respiration (Glazier, 2005). As water temperatures increase, DO levels decrease (Danladi Bello et al., 2017), which likely places larger abalone at a higher risk of hypoxia in summer. Older abalone also allocate energy to maturation, which may leave them less able to cope with other stressors (Travers et al., 2009). Pacific oysters have also been reported to be more susceptible to opportunistic *Vibrio* sp. infection and mortality during summer after spawning due to depressed immunity (Wendling and Wegner, 2013). In addition to differences in size and life-stage, younger abalone have had less exposure to other stressors compared to older abalone.

Older abalone were less likely to experience summer mortality again if they had experienced summer mortality the year prior compared to younger abalone. Larger abalone (>30-month-old) are approaching harvest age, so they are therefore, less likely to be in the farm environment for another summer period to experience subsequent summer mortality. With older abalone being generally more susceptible to summer mortality and post-grading mortality, reducing number of summers abalone spend in production may be a strategy to reduce the impact of summer mortality on production. This may be achieved through increasing growth rate to shorten the production cycle or alternatively by developing market demand for smaller two-year-old abalone (Stone et al., 2014c).

Size at weaning

As discussed above, abalone that experienced summer mortality the year prior were more likely to experience subsequent summer mortality, however, the risk was less for abalone removed from the nursery plates/weaning tanks at larger sizes. For abalone that did not experience summer mortality the previous year, the risk of summer mortality increased if abalone were a larger size at nursery removal (larger than 20 mm). The risk of summer mortality was also affected by the interaction between size at nursery removal and abalone age. In general, the effect of size at nursery removal decreased with increasing abalone age; the more time abalone had to recover from a disturbance (e.g., nursery removal), the less that disturbance affected them. The effect of abalone age on risk of summer mortality decreased as size at nursery removal increased. This may indicate early stressors experienced in the production cycle may affect abalone throughout their production. A previous survey undertaken by Vandepeer (2006) reported that one farm managed and reduced summer mortality and bloat in abalone by removing animals from nursery plates at a larger size. Weaning abalone on to formulated feeds too early can result in reduced growth rates and higher mortality rates (Dyck et al., 2010). As abalone growth is fast in the nursery environment, it can be difficult to maintain adequate supply of algal food to keep up with grazing pressure (Dyck et al., 2010). Delaying weaning for too long can result in a lack of diatom food supply and mortality events (Heasman et al., 2004). Further investigation into the optimal size for nursery removal to reduce the risk of summer mortality may be an opportunity for future research.

Feed rate and age

The risk of summer mortality increased with age, but the size of the effect was less at higher feed rates. The effect of age on summer mortality has previously been reported by Stone et al. (2014a). The authors demonstrated that 26 °C water caused mortalities in larger three-

year-old abalone but did not raise mortality rates in smaller two-year-old abalone. In the current study, the risk of summer mortality in younger abalone increased with higher feed rates, while increasing feed rates for 30-month abalone had no effect on the risk of summer mortality (odds ratio ~1). Previous research has reported the age (and size) dependent response of feed intake, growth, survival and digestion physiology of greenlip abalone (Stone et al., 2013; Bansemer et al., 2015a; 2015b; 2016a; 2016d). Due to their nocturnal and slow feeding behaviour, farmed abalone are typically fed to excess and provided with feed throughout the night (Bansemer et al., 2015b). Feed rates are adjusted seasonally and are dependent on abalone age (Stone et al., 2016). Feed intake (on a %body weight basis) is higher for younger abalone, however the total feed volumes (on a kg basis) are considerably less than those fed to larger abalone (Stone et al., 2014c). As a result, it may be easier to overfeed younger abalone than older animals and may be a possible explanation for the interaction observed in the current study. Excess feed in abalone aquaculture has been linked to increased waste, a reduction in water quality (high ammonia and nitrites and lowered DO) and an increase in bacterial and or algal growth in the tank (Stone et al., 2013). An opportunity identified in AAGA STRATEGIC PLAN 2020-2025 was to embrace technology, including monitoring and management of water quality and feeding. Developing predictive tools for feed management in younger abalone during the summer period may be an avenue for future research to reduce summer mortality in this cohort of animals.

Other factors

We found that some factors including maximum stocking density, cleaning method and cleaning frequency were not associated with summer mortality. While these factors were not associated with summer mortality, maintaining optimal stocking densities and an appropriate cleaning schedule are important farm management practices that promote abalone health (Wassnig et al., 2010). High stocking densities and infrequent cleaning can lead to poor water quality and impact abalone production (Jones and Stephens, 2006; Vandepeer, 2006). A likely explanation for this finding in the current project is abalone were held at a stocking density and in tanks that were adequately cleaned to not be associated with summer mortality.

Data was not available for multiple water quality parameters identified as potential risk factors. These parameters could therefore not be analysed in this study (see Appendix 10 for list of variables where no data was available). The role of other water quality parameters on abalone health and biology have been well-documented in the literature (see Chapter 1). In this study, water temperature may have acted as a proxy for other water quality parameters, as DO, pH, ammonia, and salinity for example. In addition, there were other factors identified as potential risk factors where no data was available for analyses, include spawning and feed type. These factors have been demonstrated to be important biological factors that influence abalone health (Travers et al., 2009; Stone et al., 2014a; Morash and Alter, 2016).

There is another FRDC project running concurrently to this project: Benchmarking for health and productivity in aquaculture project (FRDC: 2018-180, principal investigator Tracey Bradley). This project is working with various Australian aquaculture industries, including the abalone industry, to standardise data collection. In Australian aquaculture, data collection on farms ranges from basic, to high level data collection where data is recorded across multiple sites and can be used for remedial action. By benchmarking mortality and growth rates, health and environmental data, individual farms will be able to assess and compare and may be able to reduce the tanks exposure to risk factors associated with summer mortality. Another FRDC project: Application of a machine learning approach for effective stock management of farmed abalone (FRDC: 2019-151, principal investigator Jan Strugnell) is developing artificial intelligence software that can be used to measure, count and identify

abalone from an image to predicts abalone weight and stocking density. In the future, this technology may reduce manual handling for stock assessment. Handling less frequently may also reduce handling stress and consequently summer mortality.

Conclusion

This study has developed a case definition for summer mortality in Australian abalone aquaculture and identified associated risk factors. Warming water temperatures and elevated levels of mortality after grading or handling increased the risk of summer mortality. Generally, abalone that were in tanks that experienced summer mortality the previous year and survived had increased risk of summer mortality the following year. There were several interactive effects that influenced the likelihood of summer mortality. Some risk factors identified in the current study, such as water temperature could be managed through infrastructure changes (e.g. extend intake pipe to deeper cooler water, water chillers or RAS systems). The benefits of these options should be assessed against benefits on a farm-by-farm basis. Other risk factors, such as post-grading mortality, may be managed through improved husbandry and handling practices. Future technological advancements in stock assessment may facilitate a reduction in the frequency abalone are handled. Risk factors identified in the current study suggest that abalone have extended recovery times after stress events. Good record keeping would allow farmers to identify abalone from tanks that are at increased risk of summer mortality and aim to reduce exposure of identified vulnerable abalone to other risk factors associated with summer mortality.

Chapter 3: Surveillance of farmed abalone affected by summer mortality

1. Introduction

Notifiable diseases negatively impact abalone production globally. Withering Syndrome (infection with *Xenohaliotis californiensis*) is exotic to Australia but has devastated wild abalone populations in the United States (Crosson et al., 2014) and Abalone Viral Ganglioneuritis (AVG) has caused significant losses in both wild and farmed abalone populations in Asia and Australia (Corbeil, 2020). Disease poses a threat to ongoing globalised trade and market access (Jennings et al., 2016), especially for aquaculture production industries that rely on international export markets. The majority (>90%) of farmed and wild-caught Australian abalone are sold overseas to Japan, Hong Kong, China, Singapore, Taiwan, USA, Canada, and the EU (AAGA, 2020). Adequate knowledge of disease status of farms supports trade and market access for industry.

In Australia, fish farmers are required to report sudden high or unexplained mortality events, and suspect or confirmed disease, on their farm to relevant state authorities, so investigations can occur to rule out infectious disease or for emergency responses be coordinated if disease is detected (DAWE, 2022). Emergency response plans for aquatic diseases in aquaculture typically have a mortality trigger value that when detected on-farm will instigate a disease investigation. Summer mortality in abalone aquaculture is characterised by low-level, elevated (~0.15% above winter baseline) mortality over the summer period (Jan-April), in at least two culture units (Chapter 2). The persistent chronic nature of the summer mortality means stock losses accumulate slowly, especially when compared to sudden high mortality caused by notifiable diseases such as AVG or Withering Syndrome. Summer mortality would therefore not typically hit the trigger values for a disease rule-out investigation (sudden high (acute) mortality event). The pathology of summer mortality is currently underexamined and pathogenic bacteria have been associated with summer mortality in some studies, but without robust evidence (Vandepeer, 2006; Dang et al., 2011; Dang et al., 2012). Globally, *Vibrio* spp. have been associated with mortality event in abalone at warm water temperatures (Nicolas et al., 2002; Pichon et al., 2013; Cardinaud et al., 2015). Vibriosis in abalone resulting in mortality have been related to sudden rises in water temperature during summer (Handlinger et al., 2005).

No pathogen is described as the causative agent for summer mortality in any mollusc species (Go et al., 2017; King et al., 2019). As discussed in Chapter 1, summer mortality in abalone aquaculture is most likely a non-infectious syndrome caused by a combination of adverse environmental and husbandry factors that likely leave abalone more vulnerable to opportunistic pathogens. Consistent and coordinated data collection during summer mortality events would provide a comprehensive assessment to rule out primary pathogens.

In this chapter, our aim was to summarise laboratory submissions from 2010 to 2020 for wild and farmed abalone across southern Australia to determine whether the presence of any pathogen was attributable to mortality. We also aimed to rule out infectious disease in abalone from tanks that fit the summer mortality case definition.

2. Methods

2.1. Review of ten years of laboratory submission data

State representatives from Tas, Vic, SA and WA compiled 10 years of data on abalone investigations submitted to state laboratories between 2010 and 2020. For example, in South Australia, the Primary Industries Information Management System (PIIMS) database was searched for records of all abalone mortality investigations between 2010-2020. Data examined for all laboratory submissions included: event date, state, farm or wild abalone, submission number, abalone species, reason for submission, number of animals submitted, type of samples submitted, days between sample collection and laboratory processing, number and type of testing, laboratory findings - molecular biology, laboratory findings – microbiology, laboratory findings – histopathology, diagnoses, and availability of stored samples. For all states, detailed case information on notifiable diseases were reviewed but excluded from the results, as summer mortality has no associated notifiable diseases, and all notifiable pathogens have their own definition and are separately investigated and reported (e.g. Quarterly Aquatic Animal Disease Report (enaca.org)).

2.2. Investigating summer mortality events during the life of the project to comprehensively rule out primary pathogens and infectious agents

Draft state specific guidelines for investigating summer mortality events during the life of the project were developed and presented to industry during the industry government workshop. These guidelines detailed how industry can collect and submit abalone from affected tanks that fit the summer mortality case definition (case tanks) and non-affected tanks that do not fit the summer mortality case definition (control tanks). Suggestions from industry were made regarding targeting similar size abalone for both case and control animals during sampling (see Appendix 5 for workshop minutes).

Objective 3 was achieved by using guidelines developed during industry consultation (Component 1) to comprehensively rule out primary pathogens and infectious agents during any summer mortality events throughout the life of the project. This involved affected farmers submitting both affected (case) and non-affected (control) abalone across two summer summers (Jan-April 2021 and 2022) and four states. Industry guidelines were adapted to include relevant contact information and legislative protocol specific for each state, but the collection of animals and sampling methods did not change. As an example of state specific guidelines, see Appendix 8 for South Australian guidelines.

Farmers submitted 30 affected abalone (cases) from tanks that met our case definition (for definition see Chapter 2) and 10 non-affected abalone from tanks that did not meet our case definition (controls) during a mortality event as per the OIE guidelines for disease detection (OIE, 2000; OIE, 2019). As the case definition describes a summer mortality event occurring over at least two culture units, at least two case tank replicates (or more) existed per summer mortality event. The number of animals sampled per tank was determined by the number of tanks meeting the case or control definition (for example, if 5 tanks met the case definition, 6 animals were sampled per tank to provide the 30 case samples).

Sampling of affected animals used a risk-based approach biased towards moribund animals so that if a pathogen was present, then there was a higher chance of detecting it. Mortalities were avoided due to their limited diagnostic value. Risk-based surveillance increases surveillance sensitivity with a smaller number of animals, compared to random, representative surveillance. Control animal selection was randomised, using a collection method described in Stone et al. (2014b). In short, a transect line was drawn across a culture tank at a point 1/3 and 2/3 of the distance along three non-affected (control) tanks, abalone were randomly sampled from each along each transect. The number of control abalone selected per tank was divided by number of control tanks sampled (n = 10 control abalone per event). Selected case and control abalone were placed into individual and labelled

sealed plastic bags, with selection and packaging for control animals occurring prior to case animals to avoid any potential disease transmission risk. All sample bags were placed indirectly on ice and sealed in a foam container. Samples were couriered to relevant state laboratories, arriving within 24 hours from collection, and analysed using histopathology and microbiology techniques to comprehensively rule out primary pathogens and infectious agents. Polymerase chain reaction (PCR) is used for notifiable disease rule out during a mortality investigation. Any notifiable disease rule-out investigations using PCR techniques were investigated through the respective state authority and their allocated budget, noting that each state investigates mortality events differently.

To ensure uniform sample collection for all State laboratories, a Standard Operating Procedure (SOP) was also developed to ensure consistency in analyses of abalone submitted for this project component (Appendix 9). The SOP was developed with input from each state laboratory and state representative to ensure accurate representation of up-to-date techniques and protocol.

There were funds to investigate four mortality events per year for two years (summer of 2021 and summer of 2022) using histopathology and microbiology. To ensure each state was represented, initially we limited each state (WA, SA, Vic and Tas) to one mortality investigation between January and March. If by April, there were less than four investigations, a state that already had a summer mortality investigation was able to submit abalone for investigation, on a first-in basis.

3. Results

3.1. Review of 10 years of lab submissions

All States combined

- Mortality, health and/or disease investigations occurred in all seasons but were more common during warmer seasons (austral summer, autumn and spring).
- Mortality and health/disease investigations were not restricted to one abalone species, nor one population type (wild or farmed); mortality or health/disease investigations occurred across farmed and wild populations of Greenlip, Blacklip or hybrid abalone. The abalone species affected was dependent on both source (farmed or wild) and state.

Bacteria

- Between 2010 and 2020, Australia-wide there were 63 abalone submissions with bacterial presence.
- Bacterial species identified include *Shewnella* sp., *Pseudoalteromonas* sp., *Staphylococococcus* sp., *Vibrio* spp., *V. splendidus* and *V. harveyi-I, V. harveyi-II, V. lentus, V. jasicida. V. gigantis, V. pomeroyi, V. rotiferianus, V. fortis, V. cyclitrophicus, V. alginolyticus* and *V. mediterranei*.

Industry survey results: laboratory submission summary

Based on the industry survey (see Chapter 2), six farms (out of nine respondents) submitted abalone to their respective state laboratory between 2010 and 2020 from what they characterised as a summer mortality event. Three farms did not have any abalone mortality events between 2010 and 2020. In total there were 18 laboratory submissions between 2010 and 2020 relating to summer mortality across Australia. Laboratory submissions had either

bacterial myositis, abscesses, or non-conclusive diagnoses. Of bacteria identified in summer mortality submissions, only *Vibrio* spp. were listed.

3.2. Investigating summer mortality events during the life of the project

We conducted a risk-based surveillance to increase surveillance sensitivity with a smaller number of animals, compared to random, representative surveillance. Farmers voluntarily submitted animals when they had a summer mortality event that met the case definition developed in Chapter 2. This approach was more suitable than a managed surveillance program because the disease investigation for summer mortality is unlike a typical disease investigation (low-level elevated mortality over months). During the life of the project, 120 case and 40 control abalone were submitted from four farms over two summer periods (three farms over January to April 2021 and one farm in January to April 2022). Due to our approach of aiming to include submissions from each state, three reports of summer mortality were not investigated (two in 2021 and one in 2022) as states had already submitted samples in the relative summer period.

Laboratory submissions were diagnosed with either vibriosis, bacteraemia including bacterial myositis, micro-abscesses, or non-conclusive diagnoses. Of bacteria identified in summer mortality submissions, only *Vibrio* species were listed, and these were detected in both case and control abalone with variable growth rates (heavy, moderate, light, and occasional growth identified in both groups). Mixed bacterial growth, and not one uniform pathogen, was detected in both case and control abalone and there was no evidence that *V. harveyi* and *Vibrio* spp. were the primary cause of mortality. The bacterial pathogens detected were described as secondary (opportunistic) to some primary stressor(s).

Microbiology

- Both case and control abalone had bacterial growth (of varying colony size) detected in their hemolymph culture
    - Bacterial growth was identified in 98% of case animals and 75% control animals.
- Bacterial species identified in hemolymph culture include *Vibrio* spp*., and V. harveyi*
    - 68% of case animals and 28% of controls had *Vibrio* species detected.
    - 35% of case animals and 5% of controls had *V. harveyi* detected.

Histopathology

- In submission 1 the 30 case (affected) abalone had bacterial myositis with culture of *Vibrio harveyi* biotype II or mixed *Vibrio* spp. all 30 animals consistent with vibriosis. Additional findings from these animals included increased numbers of haemocytes surrounding the oesophagus and digestive glands, with intralesional bacteria. Control animals did not have bacterial myositis but *Vibrio* spp. were isolated from their hemolymph.
- In submission 2, case (affected) animals had hemocyte infiltration of the foot (18 of 30 case animals). 3 of 30 case animals had hemocytes in the digestive gland, 4 of 30 case animals had hemocytosis in the gills and 1 of 30 had hemocytosis in the kidney. Hemocytosis and tissue rarefaction of the oesophagus was present in 12 of the 30 case animals. 3 of 30 case abalone had heamocytosis in intestinal connective tissue. 6 case abalone had bacteria present in the hemocytes. 1 of 10 control abalone had

heamocytosis in intestinal connective tissue with bacteria. All other control abalone showed no significant changes.

- In submission 3, 19 of 30 case (affected) abalone had bacterial myositis with culture of *Vibrio* spp. or mixed Vibrio spp. 3 of 30 animals had increased numbers of haemocytes surrounding the oesophagus with intralesional bacteria. These findings were consistent with Vibriosis. No control abalone has bacterial myositis but some had *Vibrio* sp. cultured from their hemolymph.
- In submission 4, the 30 case (affected) abalone had bacterial myositis with culture of *Vibrio harveyi* or mixed *Vibrio* sp. from 27 of 30 animals, consistent with vibriosis. Additional findings from these animals included increased numbers of haemocytes surrounding the oesophagus and digestive glands and stomach, with intralesional bacteria. Control animals did not have bacterial myositis, but *Vibrio* sp. were isolated from their haemolymph.

4. Discussion

In the current study, our aim was to summarise laboratory submissions from 2010 to 2020 for wild and farmed abalone across southern Australia to detect the presence of any pathogen. We also aimed to rule out infectious disease in abalone from tanks that fit the summer mortality case definition that were investigated during the life of the project.

We found no single infectious pathogen consistently detected in laboratory submissions of abalone. Bacterial species identified from past submissions included a range of species, however, only *V. harveyi* was associated with abscesses and mortality in abalone*. Vibrio splendidus* is associated with incidental infections in abalone following environmental or husbandry stress, but is not associated with abscesses (Buller, 2004). *V. alginolyticus* and *V. mediterranei* have been associated with abalone mortality and/or lesions (Buller, 2004). Both *V. splendidus* and *V. alginolyticus* have also been associated with mortality events in Pacific oysters (*Magallana gigas*) (Go et al., 2017).

Vibrio spp. were associated with 49 of the 63 past (2010-2020) submissions that reported the isolation of bacteria (mortality, disease, or surveillance investigations), from both wild and farmed abalone populations. Bacterial presence did not always translate to clinical disease; of the 63 bacteria-associated submissions Australia-wide, there were only 19 reports of vibriosis; all were associated with myositis and one with abscesses. Abscesses were reported in an additional five abalone submissions (four associated with *Vibrio* species), but final diagnoses did not confirm vibriosis. *Vibrio* spp. were detected in all months of the year but were most common in summer. *Vibrio* spp. are abundant in aquatic environments, and pathogenic *Vibrio* species grow well in warm and low saline coastal water (Baker-Austin et al., 2017). *Vibrio* bacteria are abundant in the abalone culture environment and sources include broodstock, feed, substrates used by abalone, and incoming seawater (Lizárraga-Partida et al., 1998). The lack of a consistent single infectious pathogen in 10 years of disease investigations indicates summer mortality in Australian abalone cannot be attributed to one infectious pathogen. National data consolidated from this project indicates summer mortality events are attributed more commonly, but not always to a multitude of different opportunistic bacteria (mainly *Vibrio* species) present in the environment.

This component of the project compiled both case and control abalone submitted by commercial abalone growers during confirmed summer mortality events to rule-out infectious disease. We found no single infectious pathogen associated with abalone affected by summer mortality. Microbiology (hemolymph cultures) revealed the presence of *Vibrio* species in both case and control abalone, which are common opportunistic pathogens that can cause secondary bacterial infections after a stress event. *Vibrio harveyi* is a known

pathogen of abalone and other molluscs and was detected in 35% of case abalone and 5% of control abalone in the current study. Case abalone had a higher proportion of *Vibrio* species detections compared to control abalone. A higher proportion of *Vibrio* detections in case abalone is likely due to active targeting during sampling of moribund individuals that are susceptible to opportunistic infections, and the larger sample size of case (n = 120) versus control abalone (n = 40). A higher sample number for case abalone was incorporated into experimental design to improve chances of pathogen detection in affected abalone. *Vibrio* species are often secondary opportunistic pathogens and cause clinical disease in animals that are already stressed or immunocompromised, rather than causing disease in healthy animals (Handlinger et al., 2005). For example, sick fish that are continuously exposed to sub-lethal levels of harmful algae (an additional stressor) are vulnerable to developing vibriosis that eventually causes mortality (Albright et al., 1993; Roberts et al., 2019). Recently, Pedler et al. (in press) reported gill damage in greenlip abalone after exposure to high water temperatures. This damage may facilitate bacterial infiltration (Pichon et al. 2013). In this project, 4 out of 120 case abalone had bacteria identified in their gills and there was not a consistent diagnosis of the gill tissue among case abalone. Vibriosis in abalone can occur after handling or other stressful events that make abalone susceptible to infection. Limiting exposure to these stresses may decrease the likelihood of vibriosis (Elston and Lockwood, 1983).

There are several factors which can directly affect the pathogenicity of *V. harveyi* and other *Vibrio* spp. in molluscs. High-water temperature, grading or handling trauma, exposure to anaesthetics or increasing salinity occurred prior to disease caused by *Vibrio* in Tasmanian abalone aquaculture (Handlinger et al., 2002). Furthermore, prolonged recovery from bacterial infections may occur if abalone are in sub-optimal environmental conditions (Morash and Alter, 2016), including high water temperatures experienced in summer. Isolated, low levels of mixed *Vibrio* species have been observed in clinically healthy abalone, indicating bacterial carriage of *Vibrio* in abalone is normal and infections are likely secondary or opportunistic, when the hosts defences are decreased (Handlinger et al., 2002). This is in line with pathologists' comments in the laboratory submissions for this project.

Previous attempts to establish a link between *Vibrio* levels and abalone mortality have failed due to highly variable *Vibrio* levels within tanks (Vandepeer, 2006). Non-pathogenic bacteria are bacteria that do not cause disease, whereas pathogenic bacteria cause disease (Pigłowski, 2019). As many species of *Vibrio* are non-pathogenic and coexist with abalone, monitoring *Vibrio* levels within tanks may not a viable strategy to predict mortality events (Vandepeer, 2006). *Vibrio crassostreae*, *V. splendidus* biovar II, *V. harveyi* and *V. alginolyticus* were all detected in Pacific oysters after a mass mortality event in NSW (Go et al., 2017). The authors reported they could not be sure whether high *Vibrio* spp. levels caused mortality or levels increased after oysters died (Go et al., 2017).

Previous studies have tested the efficacy of various antibiotics (oxytetracycline [OTC], amoxicillin, oxolinic acid, and a trimethoprim / sulphadiazine mixture) in treating *V. harveyi* infections in abalone aquaculture (Handlinger et al., 2005). OTC was effective as an in-feed treatment against *V. harveyi* infections (Handlinger et al., 2005). Post treatment, however, there were high OTC tissue residues, which would result in long withholding periods, and this therefore limits the suitability of OTC for commercial use (Handlinger et al., 2005). Other antibiotics tested were not effective in reducing natural *V. harveyi* infections (Handlinger et al., 2005). A crucial part of disease control is understanding and managing stress. The association between *Vibrio* outbreaks and stress demonstrate prevention via stress control is preferable to antimicrobial treatment. This can be achieved by minimising exposure to known stressors and to risk factors of summer mortality identified in Chapter 2. Because of the

lasting effect of summer mortality on surviving abalone, prevention is key. Disease surveillance can either prove disease freedom from an area or facilitate control strategies including containment (Peeler and Taylor, 2011). Disease surveillance used in this study may not be sensitive enough to detect all pathogens potentially present in abalone affected by summer mortality. For example, Handlinger et al. (2006) conducted a national health survey of wild abalone populations with the aim to detect serious disease and define any detected pathogens, using gross histological examinations recommended by the OIE. In 2006, an outbreak of AVG caused 80 % of the wild population in the Victorian Western Zone Abalone Fishery to die (FRDC, 2022). Handlinger et al. (2006) was unable to detect AVG during disease surveillance that occurred months before the detection of AVG in Victoria. The ability of a test to detect a pathogen is dependent on test diagnostic sensitivity (DSe) and specificity (DSp) (Weinstein et al., 2005). DSe and DSp will change for a test depending on disease target, if the test is used singularly or in combination with another test, and how the results of combined tests are considered. For example, histopathology was the best singular test for detecting *Bonamia exitiosa* infection in *Ostrea angasi* oysters, with higher DSe than qPCR and equal DSp (Buss et al., 2019). When using two tests in combination, however, and defining a positive from either test as an infected case, the combination of histopathology and qPCR further improved DSe and DSp than the singular histopathology test alone (Buss et al., 2019).

We are aware different tests may have been more sensitive at detecting different pathogens. It is noteworthy that summer mortality is unlike a typical disease investigation (low, chronic mortality) and features no known singular pathogen. Microbiology and histopathology, being visual tests were therefore chosen as most suitable for this surveillance, as they are not limited to detecting a singular pathogen, they can assess pathogen intensity, confirm pathogen viability (microbiology) and provide different information on whole organism health. This project also relied on state departments undertaking a disease investigation if a notifiable pathogen was suspected, which may have included using other sensitive, but specific, tests (including qPCR).

In summary, exploratory disease investigations of both case and control abalone during summer mortality events did not find one uniform pathogen among animals that fit the summer mortality case definition. *Vibrio* spp. (including *V. harveyi*) were detected in both case and control abalone, with a higher prevalence in case animals, but bacterial presence did not always translate to clinical disease. *Vibrio harveyi* and *Vibrio* spp. caused secondary infection and disease in some case animals with gross findings consistent with vibriosis. Mixed bacterial growth was detected in both case and control abalone and there was no evidence that *V. harveyi* and *Vibrio* spp. were the primary cause of mortality. The bacterial pathogens detected were secondary (opportunistic) to some primary stressor(s). Australia's strong aquatic animal health management systems provide numerous opportunities to meet importing country sanitary requirements (DAWE, 2021). The requirements of trading partners may change over time and new disease challenges may emerge. Aquatic animal health management must therefore continue to adapt to sustain and expand future market access (DAWE, 2021).

Conclusion

The main aim of this project, in collaboration with AAGA farmers, was to determine risk factors for summer mortality and to rule-out infectious agents as the cause of summer mortality. These aims were achieved.

This project has provided the industry with new information about summer mortality, by determining risk factors that increase the risk of summer mortality. We recognise that controlling summer mortality once it has begun, using methods such as regulating temperature, is generally not commercially feasible in Australian abalone aquaculture facilities. Marine heat waves are increasing in frequency and higher instances of *Vibrio* outbreaks are occurring (Baker-Austin et al., 2017; King et al., 2019). Using oceanography to forecast temperature and adjust husbandry strategies (e.g. grading, harvest) may be one management option to reduce summer mortality in abalone aquaculture. Additional long-term strategies such as selective breeding programs for heat-tolerant and stress-resistant families may also be of benefit to industry.

Currently, minimising exposure to risk factors that are within the farmer's control may be more beneficial, such as reducing stress when handled. Further research into the key factors identified in this study, including improving handling methods to reduce stress and promote abalone health, may be advantageous. Careful planning and good record keeping may enable farmers to identify abalone tanks that are at higher risk of summer mortality. This may allow farmers to adapt husbandry for these animals.

Results from this project suggest that summer mortality is a non-infectious process and may be used by industry and government to support and maintain trade and market access for Australian abalone. Abalone become stressed by environmental, biological and husbandry factors, leaving them immunocompromised and susceptible to delayed mortality or opportunistic bacteria, like *Vibrio* spp.

Implications

Summer mortality was identified as a research priority outlined in AAGA's strategic plan for 2020-2025 (AAGA, 2020). Since 2010, eight of the ten warmest years on record were documented for Australia's ocean surface temperatures (CSIRO, 2018). As climate change is forecast to increase the duration and frequency of marine heat waves (Roberts et al., 2019), it is important to determine strategies to effectively manage summer mortality in Australian abalone aquaculture. This project has identified risk factors that farmers may manage to decrease the risk of summer mortality.

This project has demonstrated that summer mortality is a non-infectious process. This finding may be used by industry and government to support and maintain trade and market access for Australian abalone.

Recommendations and Further Development

Several risk factors were identified to be associated with summer mortality, and many potential risk factors were unable to be examined due to lack of data. Industry is encouraged to use more water quality monitoring technology to collect data on additional potential factors (e.g. DO levels, pH, salinity and ammonia) which influence abalone health.

Keeping good stock number and mortality records for each tank or culture unit is crucial for calculating baseline mortality rates and building knowledge of what is considered 'normal' mortality for each farm. Good knowledge of baseline mortality is essential for detecting elevated or abnormal mortality levels. Monitoring and recording mortalities associated with routine husbandry practices, such as grading and handling, is also important. This will aid farmers in identifying abalone that have a increased risk of summer mortality. Reducing stress during grading (e.g. not grading or handling on extreme weather days) is also recommended.

Results generated from this project have been conveyed to industry and could form the basis for subsequent research projects that build upon the results reported here. In the current project, there were several limitations and areas of interest that were not able to be explored, which require further investigation. We recommend future research on summer mortality management should focus on:

- Optimising commercial handling or grading methods and timing to minimise stress and mortality.
- Investigating recovery times in abalone held in commercial environments, with a focus on recovery times after one and/or multiple stressors.
- Investigating the role of other water quality parameters unable to be investigated in the current study due to lack of data (e.g. DO and ammonia).
- Future mortality events should utilise the summer mortality case definition and continue to utilise different diagnostic methods when required to effectively rule out infectious and notifiable pathogens.
- Market development for early harvest.

Extension and Adoption

The application was developed in direct collaboration with members of the AAGA and State government representatives. This project comprised an array of collaborators which included members of AAGA and participants from abalone farms, universities (Flinders University; University of Adelaide) and State government departments (Western Australia, South Australia, Victoria and Tasmania).

Strong collaborative networks were forged with abalone farm managers. Their participation throughout the project was vital in generating data and ultimately the success of the project.

Co-investigators communicated by e-mail, telephone, and team meetings and workshops, on an ad-hoc basis. The primary purposes of these communications were to engage industry in the development and design of research, provide updates on the progress of the research. These discussions are ongoing.

This project also conducted complimentary activities aligned with another FRDC/AAGA project (FRDC Project Number: 2019-156) entitled “Does the dietary inclusion of *Ulva* meal improve the survival of abalone during summer on two Australian abalone farms?” (Primary Investigator: Professor David Stone (SARDI). These projects allowed the Principal Investigator of both projects to conduct complementary activities between project whilst on farm visits.

Apart from this final report, to date no publications have arisen from this project.

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Appendices

Appendix 1 – FRDC 2019-147: SURVEY

Background

High mortality during warm summer water temperatures has been termed summer mortality by Australian abalone growers and researchers. However, summer mortality is not a well-defined condition, which impedes analyses to determine likely causes and subsequent management options. Based on discussions held at the AAGA workshop in 2019, our preliminary case definition for summer mortality in Australian abalone is:

- A >1% increase (absolute) in daily tank mortality rate from the farms baseline from December to April, excluding those diagnosed with an OIE notifiable disease as the primary cause of mortality

Below is a survey that is part of the FRDC project 2019-147. This is an opportunity for each farm to submit their experience of summer mortality in a confidential manner. For the purpose of the survey, summer mortality is what you consider it to be. Information from this survey will be used to refine our preliminary case definition for summer mortality.

We will use the case definition to examine the association between summer mortality cases and a range of putative risk factors (eg. water temperature, husbandry, animals, feed, water quality, farm, presence/absence of a pathogen). This will involve farm visits, farm and laboratory data analyses, workshop, and may involve another survey in the future. Please see below a list of factors we are proposing to examine. We also welcome any further input and suggestions to this list.

You can fill the survey and provide input to the proposed risk factors confidentially. Results for farms that participate in the survey will be de-identified. The unit of interest (epidemiological unit) on the farm will be the tank / pen / race and the unit of interest (epidemiological unit) by state will be farm. The summary of results will not identify farms and sizes of farms (only proportion of affected tanks / races / pens per farm).

The primary outcome of this project is to improve the current understanding and management of risk factors associated with summer mortality in abalone.

Risk factors and management strategies associated with summer mortality in Australian abalone (FRDC project: 2019-147) - Survey

Where is your farm located? This will be reported as State A, B, C or D.

- ☐ Tasmania
- ☐ Victoria
- ☐ South Australia
- ☐ Western Australia

What is the ancestry of your broodstock? (ie. Number of generations from wild broodstock and were the original wild brood stock from local waters, interstate waters or mixed)

What species of abalone do you produce in your grow-out?

- ☐ Greenlip abalone (*Haliotis laevigata*)
- ☐ Blacklip abalone (*Haliotis rubra*)
- ☐ Hybrid abalone (*H. laevigata* × *H. rubra*)

What water quality parameters do you measure on farm? If you tick 'Yes' to a water quality parameter, how often do you record these?

Water quality parameter	**Yes**	**No**	**How often are they recorded (per week)**
Water temperatures (˚C)			
Dissolved oxygen (%sat or mg/L)			
pH			
Salinity (ppt)			
Ammonia (mg/L)			
Nitrite (mg/L)			
Nitrate (mg/L)			

Have you experienced summer mortality on your farm?

- ☐ Yes — If you ticked 'Yes', please continue with the survey below.
- ☐ No — If you ticked 'No', please continue to the risk factors to consider section on page 6. Please return both the survey and risk factor suggestions.

In this survey, summer mortality is what you consider summer mortality to be.

What is the typical water temperature range (minimum and maximum) when summer mortality occurs?

Which abalone species experience summer mortality events?

- ☐ Greenlip abalone (*Haliotis laevigata*)
- ☐ Blacklip abalone (*Haliotis rubra*)
- ☐ Hybrid abalone (*H. laevigata* × *H. rubra*)

What areas of the farm do you experience summer mortality?

- ☐ Broodstock tanks
- ☐ Nursery plates
- ☐ Grow-out tanks

If you ticked grow-out tanks above, what year class experience summer mortality?

- ☐ Soon after weaning (~6 months)
- ☐ 1 year old
- ☐ 2 year old
- ☐ Harvest size animals (≥3 year old)

On your farm, what units of production typically experience summer mortality?

- ☐ A single tank
- ☐ A number of tanks
- ☐ Blocks of connected tanks on the farm
- ☐ Entire farm

During a typical summer mortality event, how many tanks on your farm experience summer mortality?

How many tanks does your farm have and what is the typical biomass of a tank. This will be used standardise the level of mortality across all farms, and will not be reported.

Is there a common factor across tanks affected by summer mortality (e.g. in the same shed or tanks are supplied by same pipe)?

What level of mortality do you consider a baseline for your farm (ie. % tank/day)?

What level of mortality do you consider a summer mortality event (ie % tank/day)?

What month do you typically start to experience summer mortality?

What month do you typically stop experiencing summer mortality?

Do you engage a veterinarian and/or submit samples to a veterinary diagnostic laboratory when you experience summer mortality?

☐ Yes ☐ No

If you ticked 'Yes' to the above question, please complete the table below for each year you have experienced summer mortality. Specifically,

- How many laboratory submissions did you make relating to summer mortality?
- How many of these submissions had a conclusive diagnosis?

Year	**Number of laboratory submissions made relating to summer mortality**	**Number of submissions that had a conclusive diagnosis**
2010		
2011		
2012		
2013		
2014		
2015		
2016		
2017		
2018		
2019		
2020		

Any further comments:

Risk factors to consider

		Relevance rating			Comments recommendations
	Units	**Relevant**	**Not relevant**	**Unsure**	**(eg. type of priming events, subjective scoring, if this data is not collected or is confidential)**
Temperature					
1. Historical average (five year) water temperature during the month of the case/control	*X* ˚C				
2. Maximum water temperature one week prior to case/control	*X* ˚C				
3. Temperature difference between historical average (five year) water temperature during the month of the case/control and maximum water temperature one week prior to case/control	*X* ˚C				
4. >3 ˚C water increase in water temperature one week prior to case/control	Yes/No				
5. 3-5 day period with water temperatures >3˚C above historical average one week prior to case/control	Yes/No				
6. Average historical (five year) air temperature during the month of the case/control	*X* ˚C				
7. Maximum air temperature during the week prior to case/control	*X* ˚C				
8. Difference in average historical (five year) air temperature during the month of the case/control and maximum air temperature during the week prior to case/control	*X* ˚C				
Husbandry					
9. Average tank cleaning frequency for one month prior to case/control date	*X* per week				
10. Cleaning of the case/control tank	Categorical				e.g. tippers, broom, emptying and flushing or gurney)
11. Light intensity for the case/control tank	Subjective score based on tank location or percentage shade cloth				
12. Water depth of the case/control tank	*X* cm				
13. Algal growth in the case/control tank	Yes/No				
14. Is the tank a combination of other tanks from grading	Yes/No				
Animals					
15. Abalone species in case/control tank	Categorical (greenlip/blacklip/hybrid)				
16. Size animals in case/control tank were removed from nursery plates	*X* mm				

17. Month animals in case/control tank were removed from nursery plates					
18. Did abalone in case/control tank spawn in the past three months	Yes/No				
19. Is the case/control tank a partial harvest tank	Yes/No				
20. Did animals in the case/control tank experience summer mortality last summer	Yes/No				
21. Has the case/control tank experienced summer mortality in the past five years	Yes/No				
22. Days between grading (or handling) and case/control date	Days				
23. Maximum air temperature when abalone in the case/control tank were last graded	*X* ˚C				
24. Maximum water temperature when abalone in the case/control tank were last graded	*X* ˚C				
25. Maximum stocking density before last grading in case/control tank	kg/m^2, kg/m^3 or % tank surface area				
26. Two week mortality rate of abalone in case/control tank after last grading (or handling)	%				
27. Maximum stocking density before case/control date	kg/m^2, kg/m^3 or % tank surface area				
28. Abalone growth rate in case/control tank between the last two grading's	Specific growth rate				
29. Abalone growth rate in case/control tank between grading date and case/control date					
30. Average abalone weight in case/control tank	grams				
31. Average abalone length in case/control tank	mm				
32. Abalone condition index in case/control tank					
33. Size classification of abalone in case/control tank after grading	Small, medium or large				
34. Were the abalone pre-stressed or "primed" before the summer mortality event	Yes/No				
Feed					
35. Feed type abalone were fed in case/control tank	Categorical				
36. How many days did abalone receive a health promoting feed (e.g. inclusions of an antioxidants or algae) in the case/control tank	Days (including 0 if abalone didn't receive a health promoting feed)				
37. Average feed rate for one month before case/control date	%BW/day				
38. Feeding frequency for one month before case/control date	1 to 7 days/week				

39. Feed rate of abalone in case-control tank between the last two grading's	%BW/day				
Water quality					
40. Average dissolved oxygen level in case/control tank	mg/L or % saturation				
41. Average total gas pressure (TGP) in case/control tank	% saturation				
42. Silt present in case/control tank	Yes/No				
43. Water supply issue (e.g. pump failure, algae build up) in case/control tank	Yes/No				
44. Flow rate in case/control tank	% tank turn over/day				
45. Average ammonia concentration in case/control tank	mg/l				
46. Average nitrite concentration in case/control tank	mg/l				
47. Average nitrate concentration in case/control tank	mg/l				
48. Average pH					
49. -Average salinity in case/control tank	ppt				
Farm					
50. Case/control tank system	Categorical (e.g. Slab, Raceway, Pipe, Maze)				
51. Water re-use in case/control tank (partial re-circulation)	Yes/No				
Laboratory Diagnosis (if available)					
52. Primary pathogen in abalone from the case/control tank	Yes/No/NA				
53. Haemocyte infiltration in digestive gland of abalone from the case/control tank	Yes/No/NA				
54. Loss of gill epithelium lining and/or gill necrosis in abalone from the case/control tank	Yes/No/NA				
55. Did animal exhibit signs of bloat (swollen abdomen or floating)	Yes/No/NA				
Any suggestions or comments for risk factors to consider:					

Appendix 2 – Summary of survey results

Water quality
Nine farms returned the survey. Results were provided in confidence. All survey participants measured water temperature (from continuously to daily). Eight of nine survey participants measured dissolved oxygen (from continuously to as required). Five of nine participants measured pH (from daily to quarterly). Four of nine participants measured salinity (from every 30 min to quarterly). Six of nine participants measured ammonia, nitrite and nitrate concentrations (from once per week to quarterly).

Summer Mortality
Eight of nine survey respondents noted that they have experienced summer mortality on their farm. The below sections refer to these eight respondents.

Temperature
Survey participants reported water temperatures associated with summer mortality were variable and state dependent, and ranged between 19°C and 23.5°C.

Species
Survey results indicated that greenlip abalone (*Haliotis laevigata*), blacklip abalone (*Haliotis rubra*) and hybrid abalone (*H. laevigata* x *H. rubra*) are all susceptible to summer mortality.

Abalone age
For abalone ages susceptible to summer mortality, all respondents noted that 2-year-old and 3-year-old abalone are susceptible to summer mortality. Two respondents noted that 1-year-old abalone were also susceptible to summer mortality. The case definition was refined to reflect this (**>1 year old abalone** are included in the summer mortality case definition).

Grow-out areas
All eight survey participants reported that summer mortality only affected abalone in grow-out tanks (1-year-old to 3-year-old).

Baseline mortality and summer mortality rate
Normal baseline mortality during summer was variable between survey participants, and ranged from 0.1% to 1% per day. Mortality rates during a summer mortality event were also variable (0.03% to 0.3% per day). One farm reported an excessively high average daily mortality rate during summer (<1.5% mortality per day). This mortality rate was excluded from the data set, as it was not a defined number (reported as a maximum) and the farm had minimal production history. Incorporating summer mortality rates for the remainder of survey participants (excluding 1.5%), the average daily mortality rate for summer mortality was **~0.15%/tank/day**.
Given the variability in base line mortality rates and mortality rates associated with summer mortality, incorporating specific mortality rate in the case definition would not be representative for all farms. The chronic nature of mortalities associated with summer mortality has been incorporated into our revised case definition to reflect the survey results (**chronic mortality, e.g. 0.15%/tank/day above the farms' baseline mortality rate**).

Starting month for summer mortality

Six of eight survey participants noted that summer mortality began in January. One survey participant noted mortalities beginning in October, but that real summer mortality events occur in February. The starting month in the revised case definition was therefore updated from December to **January**, to reflect the major of the survey results.

Ending month for summer mortality
Three survey participants agreed summer mortality events end in early or late March, one participant detailed end of March/early April, three others detailed April, and one detailed April to early May. The revised case definition maintained the **same ending month of April**.

OIE notifiable disease
Six of eight participants notified a veterinarian and submitted samples during a summer mortality event. From laboratory submissions, no notifiable diseases were detected. The revised case definition continues to **exclude those diagnosed with an OIE notifiable disease as the primary cause of mortality**. Gross observations and lesions were not included from the revised case definition as molluscs show non-specific signs for a number of diseases.

Number of tanks
Only one survey participant noted summer mortality occurring in a single tank. All other participants reported that summer mortality occurs over a number of tanks and/or the entire farm. The updated case definition includes culture units (**at least two culture units**) to reflect the majority of survey results.

Common factors attributed to summer mortality
Warm water temperature, high stocking density/tank biomass and inadequate tank cleaning were factors identified by participants as common during summer mortality events.

Summary of risk factors provided to Australian abalone growers
A total of 55 risk factors were presented to Australian abalone farmers. These risk factors covered a range of factors including temperature, husbandry, animals, feed, water quality, farm and laboratory diagnosis. Four of the nine farms completed this section. The following summarises risk factors deemed most relevant by the four respondents.
All four respondents deemed the following relevant to summer mortality:

- Temperature (particularly maximum water temperature one week prior to a summer mortality event).
- Husbandry (including tank cleaning frequency and water depth).
- Water quality (average dissolved oxygen, total gas pressure, silt presence, inadequate water supply, and water flow rate.

Three respondents also deemed the following relevant to summer mortality:

- Maximum stocking density prior to the summer mortality event and before last grading.
- Spawning three months prior to a summer mortality event.
- Partial harvest tanks.
- Air or water temperature while handling or grading.
- Abalone feed type.

Appendix 3 – Industry workshop agenda and information sheet

Risk factors and management strategies associated with summer mortality in Australian abalone

- FRDC project

2019-147.

Information sheet

3 March 2021

Government of South Australia
Primary Industries and Regions SA

AGRICULTURE VICTORIA

Tasmanian Government
Department of Primary Industries, Parks, Water and Environment

Department of Primary Industries and Regional Development
GOVERNMENT OF WESTERN AUSTRALIA

FRDC

Background

In southern Australia, greenlip (*Haliotis laevigata*), blacklip (*H. rubra*) and hybrid (*H. laevigata x H. rubra*)abalone are commercially farmed. When water temperatures exceed the thermal tolerance of species, abalone populations can experience mortality events. High mortality during warm summer water temperatures has been termed summer mortality by Australian abalone growers and researchers.

A review of literature and an industry survey helped refine a case definition for summer mortality. Survey results will be summarised in the workshop. The revised case definition will be used to examinethe association between summer mortality cases and a range of putative risk factors (e.g. water quality, water temperature, husbandry, animals, feed, farm, presence/absence of a pathogen). This will provide information on risk factors associated with summer mortality. This may facilitate farm management practices to mitigate future summer mortality events.

Good evidence of disease status of Australian farms is crucial for trade and market access. Mortality events in aquaculture require comprehensive investigation to establish aetiology. Industry guidelines have been provided to abalone growers that detail submission protocol to investigate summer mortalityevents during the life of the project to comprehensively rule out primary pathogens and infectious agents, in both control and impacted abalone populations.

This workshop forms part of FRDC project: “Risk factors and management strategies associated withsummer mortality in Australian abalone –2019-147”.

Objectives – provide an update and discuss:

- Summer mortality survey results and revised case definition.
- Risk factors that will be explored in the case/control study for summer mortality
- Discuss industry guidelines for submitting abalone to rule out primary pathogens and infectiousagents for summer mortality events in 2021 and 2022.
- Discuss Australian abalone industry’s experience of summer mortality.

The primary objective of this project is to improve the current understanding and management of riskfactors associated with summer mortality in abalone.

What to bring

Please bring the following items with you to the workshop

- Computer, tablet or phone.
- Notebook and pen.
- A copy of your State’s guidelines. *Sent to you via email.*
- A copy of the risk assessment form (for case/control experiment). *Sent to you via email.*

Code of Conduct for the Workshop

1. We start on time and finish on time.
2. We all participate and contribute - everyone is given opportunity to voice their opinions.
3. We actively listen to what others have to say, seeking first to understand, then to be understood.
4. We participate in open and honest feedback in a constructive manner.
5. We strive to continually improve our workshop process.

Date and Time

Wednesday, 3 March 2021, 12:30 – 2:30 pm (AEDT).

WA: Start time: 9:30 am SA: Start time: 12:00 pm Tas: Start time: 12:30 pmVic: Start time: 12:30 pm

Venue

The workshop will be held online via a Teams link.

- Please create a Teams account prior.
    - Free Teams download links for computer desktop or mobile phone can be found at:https://www.microsoft.com/en-au/microsoft-365/microsoft-teams/download- app#desktopAppDownloadregion

Agenda

Time	Topic	Who
12:30 (AEDT)	Welcome and Introduction	Matthew Bansemer and Jessica Buss
12:40	Project background, survey results and case definition	Matthew Bansemer and Jessica Buss
1:00	Case/control study (risk factors) -Purpose -Method -Outcome	Matthew Bansemer and Jessica Buss
1:20	Industry guidelines: Purpose and summary	Matthew Bansemer and Jessica Buss
1:40	Comments, questions and discussion	All
2:30	***Finish***	

Appendix 4 – PowerPoint presentation at industry workshop

Revised case definition

- A revised summer mortality case definition integrates summarised results from the 'summer mortality farm survey' and review of the literature:
  - **Chronic mortality (if in doubt take this to mean >0.15%/tank/day for at least one week) in >1 year old abalone above the farms' baseline mortality rate, and**
  - **occurs in at least two culture units, and**
  - **occurs between January and April, but,**
  - **excludes those diagnosed with an OIE notifiable disease as the primary cause of mortality.**

Please note: this case definition may be refined as further information becomes available on summer mortality in abalone during the life of the project.

Revised case definition

The revised case definition will be used:

1. **Case/Control study**
   - Examine association between summer mortality cases and multiple putative risk factors
     - Water quality
     - Husbandry
     - Animals
     - Feed
     - Farm
     - Presence/absence of a pathogen
2. **Industry guidelines** – sent via email
   - Rule out primary pathogens and infectious diseases

Case/Control study (risk factors)

Case/Control study

Purpose

- Risk factors were sent out with survey for comment
  - 55 risk factors were presented to survey participants
- Used to determine **which risk factors** are **associated with summer mortality**
  - Understanding of risk factors may inform management decisions

Case/Control study

Risk factors

- **Water Quality**
  - Maximum water temperature one week prior to case/control
  - Maximum air temperature since abalone were last graded
  - Maximum water temperature of this tank since abalone were last graded
  - Average Dissolved Oxygen level / Average Total Gas Pressure
  - Flow rate
  - Average ammonia/ Nitrite concentration
  - Average pH
  - Average salinity

Case/Control study

Risk factors

- **Husbandry**
  - Last months' tank cleaning frequency per week? (i.e. 1-7 days per week)
  - Tank cleaning method (Tipper, broom, emptying + flushing or gurney)
  - Light intensity (subjective percentage score)
  - Water depth
  - Algae growth
  - Silt presence
  - Water supply issue
  - Were animals in this tank mixed during grading? (i.e. are abalone originally from multiple tanks?)

Case/Control study

Risk factors

- **Animals**
  - Abalone species
  - Month abalone were removed from nursery plates
  - Have abalone spawned in past 3 months
  - Is this a partial harvest tank
  - Did abalone in this tank experience summer mortality last summer
  - Have abalone in this tank experienced a summer mortality event in the past 5 years
  - Days since this tank has been graded / animals handled
  - Stocking density for this tank
    - Maximum before/after last grading
    - Current stocking density

Case/Control study

Risk factors

- **Animals**
  - Mortality rate: daily/ weekly/ 2 weeks after last grading/handling
  - Average abalone weight, length and condition factor
  - Average abalone growth rate between last grading and today
  - Days between last grading and mortality event
  - Size classification of abalone in this tank after grading
  - Year class
  - Were abalone in this tank pre-stressed or primed before this summer mortality event? (only applicable for Case tanks)

Case/Control study

Risk factors

- **Feed**
  - Feed type (formulated feed/algae/combination)
  - Were abalone from this tank fed a health promoting feed (antioxidant or algae)
    - How many days were they fed this before the case/control day
  - Average feed rate for one month before today
  - Feeding frequency one month before today
  - Feed rate between the last two gradings
- **Farm**
  - Tank system
  - Is water re-used (e.g. partial re-circulation)

Case/Control study

Risk factors

- **Laboratory diagnosis (if available)**
  - Was a primary pathogen identified from the veterinary necropsy report?
  - What was the primary pathogen (if applicable)?
  - Was hemocyte infiltration in abalone digestive gland described in the veterinary necropsy report?
  - Was loss of gill epithelium lining or gill necrosis described in the veterinary necropsy report?
  - Did abalone in this tank exhibit signs of bloat (swollen abdomen/floating)?

Case/Control study

Risk factors (sent out with survey)

- 4 /10 farms completed the Risk Factor section of the survey
- **Risk factors deemed most relevant by four respondents:**
  1. Temperature (particularly maximum water temperature one week prior to a summer mortality event).
  2. Husbandry (including tank cleaning frequency and water depth).
  3. Water quality (average dissolved oxygen, total gas pressure, silt presence, inadequate water supply, and water flow rate).

Case/Control study

Risk factors (sent out with survey)

- **Risk factors deemed most relevant by three respondents:**
  1. Maximum stocking density prior to the summer mortality event and before last grading
  2. Spawning three months prior to a summer mortality event
  3. Partial harvest tanks
  4. Air or water temperature while handling or grading
  5. Abalone feed type

Case/Control study

What is a case/control study?

Individuals which have an outcome of interest (case)	v	Individuals which DO NOT have an outcome of interest (control)

- Views data retrospectively to compare frequency of exposure to risk factors in each group (case/control).
- Frequency of exposure determines relationship between the risk factor and outcome of interest (i.e. Summer Mortality).
  - Uses data you already have.

Cigarette smoke inhalation and risk of lung cancer: a case–control study in a large Japanese population

Epidemiology and risk factors for typhoid fever in Central Division, Fiji, 2014–2017: A case-control study

RESEARCH ARTICLE

Lung cancer risk in never-smokers: a population-based case-control study of epidemiologic risk factors

Abstract

and lung cancer: effects of study type and continent

RESEARCH

Epidemiology and etiology of Traveler's diarrhea in Bangkok, Thailand, a case-control study

Case/Control study

Risk factors associated with mortalities attributed to infectious salmon anaemia virus in New Brunswick, Canada

K L Hammell and I R Dohoo

Risk Factors Associated With Outbreaks of Infectious Salmon Anemia (ISA) With Unknown Source of Infection in Norway

FRDC

Case-Control Study of Epidemic Mortality and *Cardicola forsteri*–Associated Disease in Farmed Southern Bluefin Tuna (*Thunnus maccoyii*) of South Australia

FINAL REPORT

Investigating emerging causes of disease in a commercial Australian prawn farm

A prospective trial and epidemiological data review

Spatio-temporal risk factors for viral haemorrhagic septicaemia (VHS) in Danish aquaculture

Case/Control study

Methods

- **Use case definition to examine association between summer mortality case tanks and a range of risk factors**
  - Compares Cases (summer mortality) with Controls
  - Epidemiological unit: 1 x Tank / raceway
  - Not sampling individual abalone, getting data on entire tank
- **Retrospectively determine from each group:**
  1. Frequency of exposure to each risk factor
  2. Risk factor relationship to Case/Control
- **Can simultaneously look at multiple risk factors**
- **Facilitates management of summer mortality**

Case/Control study

Definitions

Case	Tanks that meet case definition for summer mortality
Matched Control	Tanks that <u>DO NOT</u> meet case definition but fall on <u>SAME DAY</u> as a Case.
Background Control	Tanks that <u>DO NOT</u> meet case definition and fall on a <u>DIFFERENT DAY</u> to a Case.

Case/Control study

Methods

DATA SUMMARY TABLE

Please list dates and number of cases/tanks between January and April, where a Case and Matched Control occur (if possible). If there are no Cases or Matched Controls for that year, please write N/A.

Year	Number of Cases/tanks	Date of Case/Matched Control	Number of Matched Controls/tanks (same day as case)
2010	• • • etc.	• • • etc.	• • • etc.
2011	• • • etc.	• • • etc.	• • • etc.
2012	• • • etc.	• • • etc.	• • • etc.
2013	• • • etc.	• • • etc.	• • • etc.
2014	• • • etc.	• • • etc.	• • • etc.
2015	• • • etc.	• • • etc.	• • • etc.
2016	• • • etc.	• • • etc.	• • • etc.

CASE TANKS/MATCHED CONTROL

- In Case/Control document file (sent post workshop):
  - DATA SUMMARY TABLE
  - BACKGROUND CONTROL SAMPLING PLAN
  - RISK FACTOR FORM
- Individual Teams meetings to collate farm data
  - – start ~April 2021
- **For Teams meeting please bring:**
  - ✓ DATA SUMMARY TABLE (filled out)
  - ✓ List of tank names
  - ✓ Relevant data files (use RISK FACTOR FORM as guide)

Case/Control study

Methods

DATA SUMMARY TABLE

Please list dates and number of cases/tanks between January and April, where a Case and Matched Control occur (if possible). If there are no Cases or Matched Controls for that year, please write N/A.

Year	Number of Cases/tanks	Date of Case/Matched Control	Number of Matched Controls/tanks (same day as case)
2010	• • • etc.	• • • etc.	• • • etc.
2011	• • • etc.	• • • etc.	• • • etc.
2012	• • • etc.	• • • etc.	• • • etc.
2013	• • • etc.	• • • etc.	• • • etc.
2014	• • • etc.	• • • etc.	• • • etc.
2015	• • • etc.	• • • etc.	• • • etc.
2016	• • • etc.	• • • etc.	• • • etc.

CASE TANKS/MATCHED CONTROL TANKS

Assess farm records (2010 to 2021)

- Participant to fill out:
  - Number of Case Tanks/Matched Control Tanks
  - Dates of Cases/Matched Controls
  - Please bring filled out DATA SUMMARY TABLE to individual Teams meeting
- Please match number of Matched Control Tanks to (if possible)
  - If Matched Controls/Case Tanks not available, please write N/A
- DATA SUMMARY TABLE = Checklist
- Together: fill out RISK FACTOR FORMS

Case/Control study

Methods

BACKGROUND-CONTROL SAMPLING PLAN

Matt/Jess will use your provided list of tank names to create a randomised Background-Control (different day to Case) sampling plan. Please aim to sample three allocated tanks on each allocated date. If a proposed allocated date falls on a Case day, please select a new date (closest date where there are no Cases).

Year	Target date (If Case falls on this date, choose nearest Background-Control date possible)	Target Tank Name	New date (if cannot do target date – please select closest date where there are no cases)
2010		1) 2) 3)	
2011		1) 2) 3)	
2012		1) 2) 3)	
2013		1) 2) 3)	
2014		1) 2) 3)	
2015		1) 2) 3)	
2016		1) 2) 3)	
2017		1) 2) 3)	

BACKGROUND CONTROL

- Please bring list of tank names (Word or Excel) to Teams meeting
- We will use this list to provide a randomised plan (Target Background Control Tanks on Target Dates)
  - 3 x Background Control Tanks per year
- RISK FACTOR FORMS for Background Controls to be filled out <u>after the meeting</u>.
- Important - background data for the statistical model (logistic regression)
- Can have data for each year

Case/Control study

Methods

- **RISK FACTOR FORM**
  - Primary data input for this project
  - 1 x RISK FACTOR FORM **per tank**
    - Case tank
    - Matched control tank → Teams meeting (together)
    - Background control tank → After Teams meeting
  - Aim for at least 3 tanks for each Case/Control
  - Series of specific questions to be filled out **together** in a separate individual Teams meeting
    - Some data repetition on same day, likely.
  - View RISK FACTOR FORM prior to meeting to see kind of data to have on hand

Case/Control study

Outcome

- Analyse data on water quality, husbandry, animals, feed, farm and laboratory diagnosis
- Confidentiality of farms will be maintained:
  - States/farm names will de-identified and not disclosed
- Statistical modelling: examine association between summer mortality and putative risk factors (sourced from nation wide data pool)
- Identifying key risk factors can facilitate management of summer mortality

Information from this project would be considered as affecting the business/commercial affairs of the farms concerned, such information would be considered exempt from release, pursuant to Clause 7(1) of Schedule 1 of the Freedom of Information Act 1991.

Industry guidelines

Industry guidelines

Purpose

- State specific guidelines were sent to farms in December 2020.
- These guidelines provide a procedure to rule out primary pathogens and infectious diseases in summer mortality case and control populations.
  - Facilitate trade and market access

Aim

- Investigate summer mortality cases during the life of the project to comprehensively rule out primary pathogens and infectious agents.

Industry guidelines

Methods

- When a case definition for summer mortality is met:
  - Contact Jess/Matt → State lab / State representative
  - Sample abalone within 24 h
  - Submit samples to state lab for histopathology and microbiological analyses to rule out primary pathogens and infectious diseases.
- Funds to investigate **4x mortality events per year** for **2 years** (summer of 2021 and summer of 2022) using **histopathology** and **microbiology**.
- To ensure each state is represented:
  - Limit each state to 1x mortality investigation between Jan - Mar.
  - If by April, there has been <4x investigations, a state that has already had a summer mortality investigation will be able to submit abalone for investigation, on a first-in basis.

Industry guidelines

Methods

- Fill out and send with packaging:
- 1) Summer mortality sampling Form (take photo and send copy to Matt/Jess)
- 2) State specific lab form (Matt/Jess will help)
- Sample 40 abalone total:
  - 10 x abalone from Matched Control Tanks
    - Please aim for at least 3 control tanks:
      - Tank 1: 3 animals - Tank 2: 3 animals - Tank 3: 4 animals
  - 30 x abalone from Case Tanks (target moribund, but not dead abalone)
    - Split number of Case animals by number of Case tanks (e.g. if 3 Case tanks, 10 animals/tank)
- Sample sizes consistent with OIE guidelines (OIE, 2000; OIE, 2019).

Industry guidelines

Methods

- Sample Control abalone FIRST, then Case tanks
- Submit Control and Case animals in **separately labelled bags**, indirectly on ice to State labs within 24h
- If a notifiable disease is suspected or confirmed, any state legislation will take preference

Where to from here?

Where to from here?

- **Industry guidelines (collecting abalone):**
  - Collect samples for any summer mortality events over the next two summers (2021-2022) to rule out primary pathogens and infectious diseases
  - Facilitate trade and market access
- **Case/Control study (using data you already have):**
  - Assess prior farm records and note days that meet a case definition
  - Collate data for Case/Control tanks (with assistance from Jess/Matt)
  - Case/Control study will identify risk factors correlated with summer mortality cases
  - Facilitate management strategies

Questions and discussion

- **Further input and suggestions welcome**
- **Survey results and summer mortality case definition**
  - Chronic mortality (if in doubt take this to mean >0.15%/tank/day for at least one week) in >1 year old abalone above the farms' baseline mortality rate, and
  - occurs in at least two culture units, and
  - occurs between January and April.
- **Risk factors**
  - Other risk factors?
- **Case control study**
- **Industry guidelines and submitting samples**
- **Any concerns?**

Appendix 5 – Industry workshop minutes

Minutes

Summer Mortality Industry workshop

Risk factors and management strategies associated with summer mortality in Australian abalone – FRDC project 2019147

3 March 2021, held via Teams

Objectives – provide an update and discuss

- Summer mortality survey results and revised case definition.
- Risk factors that will be explored in the case/control study for summer mortality.
- Discuss industry guidelines for submitting abalone to rule out primary pathogens and infectiousagents for summer mortality events in 2021 and 2022.
- Discuss Australian abalone industry's experience of summer mortality.

The primary objective of this project is to improve the current understanding and management of riskfactors associated with summer mortality in abalone.

Discussion

Case definition

- Industry members within workshop highlighted that the revised case definition does not include circumstances where mortality is clearly due to a known event (i.e. no water in a tank or grading).
    - Workshop participants discussed that to capture this situation within the case definition,the following point should be included: “unknown cause of mortality”.
- Clarity regarding baseline mortality definition within case definition was discussed.
- Some farms indicated that they had baseline mortalities close to the mortality value within the revised case definition (0.15%/tank/day). To resolve this, workshop participants concluded that the case definition should be updated to specify baseline mortality is during winter (June-August).
- The case definition was amended to reflect this suggestion.

Case/Control study (risk factors): purpose, method, outcome

- Industry within workshop requested additional clarification on definitions for case, matched control and background control. PIRSA will revise definitions to improve readability.
- Industry suggested additional Risk Factor items to add to Case/Control project including weather patterns/storms and additional water temperature risk factors. PIRSA will include additional risk factor recommendations.

Industry guidelines

- Industry and project co-investigators requested that the industry guidelines include extra criteria about matched control selection. In particular, to include a clause that states matched control animals are similar in terms of size and age (if possible).
- A meeting of representatives from all participating state laboratories was recommended to confirm uniformity in sample collection and report formats. This was to ensure all reports provide similar formats for data / results which can then be analysed statistically by the investigators at the end of April 2022 when all testing will be completed.

Other business

- It was suggested that tissue samples submitted for industry guidelines, could have a portion of tissue stored in RNAlater for future studies involving eDNA or gut microbiome.
- An AAGA representative informed the group that future genetic studies, breeding program and nutritional health were of interest to AAGA.
- Workshop participants agreed additional samples could be stored in RNAlater and potentiallyused in future studies if:
    - Confidentiality of farms was maintained beyond this project.
    - Consent from the farm whereby the tissue originates from is first obtained.
- Tracey Bradley (DJPR) informed workshop participants that she has a benchmarking project,which will also involve collection of Nation-wide abalone data and there could be substantial cross over between the two projects.
    - PIRSA and DJPR will meet externally to assess if data would be comparable and if project timelines align (to determine viability of collaboration).
- PIRSA to send updated Case/Control study document and industry guidelines to all participantswhen finalised.

Appendix 6 – Calculations used in case-control study

Daily mortality rate

To calculate the daily mortality rate (%), we used the following formula:

$$daily\ mortality\ rate = \frac{number\ of\ mortalities}{total\ stock - (sum\ of\ previous\ mortalities)}$$

Where number of mortalities is the total number of abalone that died on a given day,
And total stock is the total number of abalone in each tank at the beginning of the month,
And sum of previous mortalities is the total of the number of abalone that had died over the course of the month in that tank.
When dead abalone were only recorded every second/third day, we accounted for this by dividing the number of mortalities by the number of days mortality data was not recorded (plus one), using the following formula:

$$daily\ mortality\ rate = \frac{no.\ of\ mortalities/days}{total\ stock - (sum\ of\ previous\ mortalities)}$$

Where days is the number of days mortality data was not recorded plus the last day mortality was recorded.

Winter baseline

To calculate a farm's winter baseline mortality, we calculated the daily mortality rate (%) (as above) for winter (June, July, and August) of the previous year and took the average daily mortality rate (%) of these winter months.

Summer mortality trigger value

In line with the case definition, once the winter baseline mortality level was calculated, we added 0.15% to this value to set the trigger value for summer mortality for the following summer. For example, the winter baseline value plus 0.15% for 2020 was used to set the summer mortality trigger for the summer of 2021.
Where data was unavailable to calculate a farm's winter baseline mortality rate per day, we used the industry survey results where we asked farmers: "What level of mortality do you consider a baseline for your farm (ie. % tank/day)?". We then added 0.15% to the reported winter baseline by the farmer to set the trigger value for summer mortality.

Stocking density

$$stocking\ density = \frac{tank\ biomass\ (kg)}{tank\ surface\ area\ (m^2)}$$

Condition factor

$$condition\ factor = 5575 \times \frac{weight\ (g)}{length\ (mm)\ 2.99}$$

Specific growth rate (SGR) (%/day)

$$SGR = \frac{(\ln(final\ weight\ (g)) - \ln(initial\ weight\ (g)) \times 100}{days}$$

Appendix 7 – Risk factors investigated in the case-control study

Question number	Factor
1	**Date of Case/Control** (as the case definition defines summer mortality as occurring over at least one week, take this to mean the date at the end of the week). Start from 2021 to 2010.
2	**Farm location (Vic, SA, Tas, WA) (this will be kept confidential)**
3	**Farm trading name (this will be kept confidential)**
4	**Tank type (Case or Matched/Background Control)**
5	**Tank identification/name**
6	**Replicate**
	Water Quality (Collect for single Case/Control tank)
7	Maximum **water temperature,** 1 week prior to case/control date? (°C) (on the day, 1 week prior)
8	Maximum **water temperature**, 2 weeks prior to Case/Control date? (°C) (on the day, 2 weeks prior)
9	**Difference** between **minimum and maximum water temperatures** on **day** of Case/Control? (°C)
10	**Minimum water temperature** in one **week prior** to the **Case/Control event?** (during that week)
11	**Maximum water temperature** in one **week prior** to the **Case/Control event?** (during that week)
12	Was there a **>3°C water increase** in water temperature one week prior to case/control? (Yes/No) (during that week)
13	Maximum **water** temperature of this tank since abalone were **last graded** (°C) - PM Temp
14	Average **dissolved oxygen** level for this tank (1 week prior to case/control) (mg/L OR % saturation) (during that week)
15	Average **Total Gas Pressure** (TGP) for this tank (% saturation) (one week prior to case/control) (during that week)
16	**Flow rate** on case/Control date? (tank turnover/day) (Tank turn over per day = 1440 (as there are 1440 min in 1 day) / (tank volume [L] / flow rate [L min-1]))
17	Average **ammonia** concentration for this tank? (one week prior to case/control) (mg/L) (during that week)
18	Average **nitrite** concentration for this tank? (one week prior to case/control) (mg/L) (during that week)
19	Average **pH** for this tank? (one week prior to case control) (during that week)
20	Average **salinity** for this tank? (one week prior to case control) (ppt) (during that week)
	Water Quality Data for INCOMING WATER ONLY (whole farm) - please write SAME VALUE for EACH replicate on SAME DATE.
21	Maximum **water temperature,** 1 week prior to case/control date? (°C) (on the day, 1 week prior)
22	Maximum water temperature, 2 weeks prior to Case/Control date? (°C) (on the day, 2 weeks prior)
23	**Difference** between **minimum and maximum water temperatures** on **day** of Case/Control? (°C)
24	**Minimum water temperature** in one **week prior** to the **Case/Control event?** (during that week)

25	**Maximum water temperature in one week prior to the Case/Control event?** (during that week)
26	Was there a **>3°C water increase** in water temperature one week prior to case/control? (Yes/No)
27	Maximum **water** temperature since abalone were **last graded** (°C)
28	Average **dissolved oxygen** level (1 week prior to case/control) (mg/L OR % saturation) (during that week) - AM Inlet
29	Average **Total Gas Pressure** (TGP) (% saturation) (one week prior to case/control) (during that week)
30	Average **ammonia** concentration? (one week prior to case/control) (mg/L) (during that week)
31	Average **nitrite** concentration? (one week prior to case/control) (mg/L) (during that week)
32	Average **pH**? (one week prior to case control) (during that week)
33	Average **salinity**? (one week prior to case control) (ppt) (during that week)
	Husbandry (per tank)
34	Last months' **tank cleaning frequency** per week? (prior to case/control) (i.e. 1-7 days per week)
35	Tank **cleaning method**? (Tipper, broom, emptying + flushing or gurney)
36	**Tank coverage description** (in terms on sunlight exposure/light intensity)
37	**Water depth** for this tank? (cm) (shallowest part)
38	Is there **algal growth** in this tank? Yes/No
39	Is **silt present** in this tank? Yes/no/N/A
40	Is there a **water supply issue** in this tank? (e.g. pump failure, algae build-up) (Yes/No)
41	**Were animals** in this tank **mixed** during grading? (i.e. do abalone originate from multiple tank sources?) (Yes/No)
	Animals (per tank)
42	**Abalone species** in this tank (greenlip, blacklip, hybrid or mixed)
43	**Abalone size** when removed from nursery plates/weaning tanks (shell length, mm).
44	**Month and year abalone** were **removed** from nursery plates/weaning tank.
45	Have abalone **spawned in past 3 months**, in this tank?(Yes/No)
46	Is this a **partial harvest** tank? (Yes/No)
47	Did **abalone** in this tank **experience summer mortality last summer**? (Yes/No)
48	Have abalone **in this tank experienced a summer mortality** event in the **past 5 years**? (Yes/No)
49	**Daily abalone mortality** for this tank (%) (day of Case/Control)
50	**Average abalone mortality** for this tank (%) (in **week** of case/control)
51	**Average abalone mortality** rate for this tank, **2 weeks after last grading** or **handling** (%)
52	**Grading date, 2 gradings BEFORE** Case/Control (day/month/year)
53	**Grading date, 1 grading BEFORE** Case/Control (day/month/year)
54	**Grading date, 1 grading AFTER** Case/Control (day/month/year)
55	**Size/volume** of Case/Control **tank (m2)**
56	**Average shell length (mm)** of abalone/tank, **2 gradings BEFORE** Case/Control
57	**Average weight (g)** of abalone/tank, **2 gradings BEFORE** Case/Control.
58	**Total weight (kg)** of abalone/tank, **2 gradings BEFORE** Case/Control

59	**Average shell length (mm)** of abalone/tank, 1 **grading BEFORE** Case/Control
60	**Average weight (g)** of abalone/tank, **1 grading BEFORE** Case/Control.
61	**Total weight (kg)** of abalone/tank, **1 grading BEFORE** Case/Control
62	**Average shell length (mm)** of abalone/tank, **1 grading AFTER** Case/Control
63	**Average weight (g)** of abalone/tank, **1 grading AFTER** Case/Control.
64	**Total weight (kg)** of abalone/tank, **1 grading AFTER** Case/Control
65	**Size** classification of abalone in this tank **after grading**? (small, medium, large)
66	**Age** of abalone in this tank? (in MONTHS)
	Feed (per tank)
67	**Feed type** abalone were fed in this tank? Formulated feed/algae/combination
68	Were abalone from this tank **fed a health promoting feed** (with anti-oxidant or algae inclusion)? (Yes/No)
69	If abalone were fed health promoting feed, **how many days** were they fed this before day of case/control?
70	**Average feed rate** for **one month** before case/control? (% body weight/day)
71	**Average Feed rate** between last grading and case/control?(% body weight/day)
72	**Feeding frequency one month** before case/control? (1 to 7 days/week)
	Farm (per tank)
73	**Tank system** of Case/ Control (Slab, Raceway, Pipe, Maze or round tank)
74	Is **water re-used** in this tank? (partial re-circulation) Yes/No
	Laboratory Diagnosis (if available)
75	**Did you submit abalone from this tank for histopathology/microbiology? (Yes/No).** **If NO, do not answer questions below.**
76	Was a **primary pathogen identified** from the veterinary necropsy report? (Yes/ No/ n/a)
77	**What was the primary pathogen** (if applicable)?
78	Was **hemocyte infiltration** in abalone digestive gland described in the veterinary necropsy report? (Yes/no/n/a)
79	Was **loss of gill epit**helium lining or gill necrosis described in the veterinary necropsy report? (Yes/NO/N/A)
80	Did **abalone in this tank exhibit signs of bloat** (swollen abdomen/floating)? (Yes/no/n/a)
81	Were **bacteria identified?** (Yes/No)
82	Which **bacteria species** (including type if possible) were identified? (If applicable)
	Extra temperature questions: Jess/Matt/Georgia to fill out
83	**Total rainfall** in week prior to Case/Control (mm)
84	**Average MAXIMUM wind speed** in **week prior** to Case/Control (km/h)
85	**Maximum wind speed**, in **week prior** to Case/Control
86	**Number of days** with wind speeds **>17 knots (31.5 km/h)**, **in one week prior**. (17 knots are classed as 'fresh winds' on Beaufort wind scale, BOM)
87	Maximum **air** temperature since abalone were last graded (°C), before case control event (inbetween grading date and case date)
88	Max air temp (°C) of **on day** of last grading before case/control
89	Historical average (5 year) **water temperature** during month of case/control.

90	**Temperature difference** between historical average (five year) **water temperature** during the month of the case/control and maximum water temperature one week prior to case/control
91	Were **sea surface temperatures >3˚C** above historical average **one week prior** to case/control (yes/no).
92	Average (of MIN and MAX daily temps) historical (over the five years prior to case/control) **air temperature** during the month of the case/control
93	Maximum **air temperature** during the week prior to case/control
94	Difference in average historical (five year) **air temperature** during the month of the case/control and maximum air temperature during the week prior to case/control
	Extra stock grading questions: Jess/Matt to fill out
95	**Days** since this tank has been **graded** / animals handled prior to Case/Control? (see Q1 and Q53, number day before last grading including last grading date - but excluding case date)
96	**Maximum stocking density** for this tank, **1 grading BEFORE Case/Control** (kg/m2, kg/m3 or % tank surface area)
97	**Maximum stocking density** for this tank, **1 grading AFTER Case/Control**? (kg/m2, kg/m3 or % tank surface area)
98	**Average abalone growth rate** between **grading BEFORE** Case/Control and **grading AFTER** Case/Control(Specific growth rate) (% / day) Specific growth rate (SGR) equals (ln average final weight - ln average initial weight) x 100 / days
99	**Average abalone growth rate** between **2 gradings BEFORE** Case/Control (Specific growth rate) (% / day) Specific growth rate (SGR) equals (ln average final weight - ln average initial weight) x 100 / days
100	Average **abalone condition at 1 grading BEFORE Case/Control**: Condition factor (CF) equals 5575 x (weight [g] / length [mm] 2.99)
101	Number of days between the **2 gradings BEFORE Case/Control**
102	Number of days between **ONE grading BEFORE** Case/Control and **ONE grading AFTER** Case/Control

Appendix 8 – Industry guidelines (for SA) provided to farmers for reporting summer mortality and submitting animals to their respective state laboratory.

Risk factors and management strategies associated with summer mortality in Australian abalone (FRDC project: 2019-147): INDUSTRY GUIDELINES FOR REPORTING SUMMER MORTALITY

Overview

High mortality during warm summer water temperatures has been termed summer mortality by Australian abalone growers and researchers. Based on the survey that was sent to all AAGA farms and the excellent information provided by participants, our working case definition for summer mortality as of December 2021:

- ***Chronic mortality of unknown cause (if in doubt take this to mean > 0.15%/tank/day for at least one week) above the farms' winter baseline mortality rate in >1 year old abalone, and***
- ***occurs in at least two culture units, and***
- ***occurs between January and April, but,***
- ***excludes those diagnosed with an OIE notifiable disease as the primary cause of mortality.***

For a detailed justification for the modified case definition, please see appendix 1.

Purpose

- These guidelines provide a procedure for the Australian abalone industry to rule out primary pathogens and infectious diseases in abalone populations impacted by summer mortality and control populations.
- These guidelines can be used by industry to sample abalone from summer mortality case tanks.

Background

- Investigate summer mortality events during the life of the project to comprehensively rule out primary pathogens and infectious agents.
  When a case definition for summer mortality is met, abalone should be sampled within 24 h and submitted to state laboratories for histopathology and microbiological analyses to rule out primary pathogens and infectious diseases.

Funds available for notifiable disease rule-out

There are funds to investigate four mortality events per year for two years (summer of 2021 and summer of 2022) using histopathology and microbiology. To ensure each state is represented, initially we will limit each state (WA, SA, Vic and Tas) to one mortality investigation between January and March. If by April, there has been less than four investigations, a state that has already had a summer mortality investigation will be able to submit abalone for investigation, on a first-in basis.

Notifiable disease rule-out from polymerase chain reaction (PCR) is not funded in this project.

Methods - Sampling procedure

Please follow these sampling methods when the case definition for summer mortality is met. Sampling numbers for case and control are consistent with OIE guidelines (OIE, 2000; OIE, 2019[1,2]). If a notifiable disease is suspected, any state legislation will take preference.

For South Australia: If notifiable disease is suspected, it should be immediately reported to PIRSA on 24-hour Fishwatch hotline **1800 065 522** (24 hours per day) or contact PIRSA directly.

When you have a tank that meets the case definition:

1) Notify Georgia/Matt to discuss event (georgia.macaulay@sa.gov.au)/ (Matthew.Bansemer@sa.gov.au) and confirm further sampling.
2) When sampling confirmed, print out Summer Mortality Sample form (page 6) and Gribbles Veterinary request form (page 7).
3) Collect from farm (within 24 h of case definition event):
    a. Control tanks (case definition for summer mortality is NOT met)

[1] Office International des Epizooties (OIE), 2019. Chapter 2.4. Diseases of Molluscs. In: Manual of Diagnostic Tests for Aquatic Animals, Paris, France.
[2] Office International des Epizooties (OIE), 2000. Chapter 1.1. General Information. In: Diagnostic Manual for Aquatic Animal Diseases, Paris, France.

i. **Collect whole abalone from control tanks first**. Control tank = tank that does not meet the case definition for summer mortality (see page 1 for case definition).
ii. Use a **random sampling approach** described in Figure 1: Mark 2 transect lines 1/3 and 2/3 along the length of the tank, and sample abalone that fall on each transect line.
iii. **Sample 10 abalone** (maximum) from at least **3 control tanks**. Note, if 3 tanks are not available, sample from 1 or 2 tanks and split sample number per tank accordingly.
iv. If possible, sampling should **target control animals** that are of **similar size** and **same cohort** to **case** animals

b. Case tanks (case definition for summer mortality is met)
i. **Collect whole abalone from case tanks** after abalone from control tanks have been collected.
ii. Sample **30 abalone** (maximum) from at least **3 case tanks** (e.g. 10 abalone per tank). Note, if 3 tanks are not available, sample over 1 or 2 tanks and split sample number per tank accordingly.
iii. If possible, sampling should **target moribund (sick and dying) abalone.** Dead abalone should not be collected as they have limited diagnostic relevance.

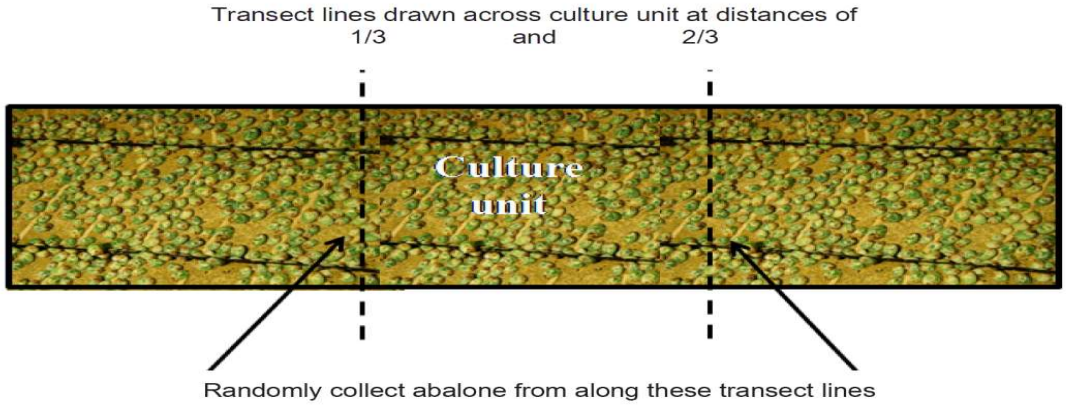

Figure 1. Random selection sampling protocol for control abalone tanks. Image is from Stone et al. (2014).

4) Place whole abalone from the same tank into the same sealed plastic bag.
a. **Label separated bags** clearly if they are **Case** or **Control** and which **tank (replicate number)** they were collected from with a permanent marker on the bag (e.g. Case tank 1, Case tank 2, Case tank 3 / Control tank 1, Control tank 2, Control tank 3).

b. **Place the 3 replicate bags** (Case or Control) into a **larger sealed plastic bag** and **label** as **Case** or **Control**.
c. Wrap the larger bags (labelled Case or Control) separately in newspaper for insulation (if possible).
d. Store larger, newspaper wrapped bags on ice within a foam container.
e. Print and fill out Summer Mortality Sample Form (see page 6), take a photo of the completed form and place within a plastic sleeve/bag (to keep dry).
f. Print and fill out Gribbles Veterinary request form (page 7), take a photo of the completed form and place within a plastic sleeve/bag.
g. Place the filled out Summer Mortality Sample Form and Gribbles Veterinary request form (protected in a plastic bag) within foam box (on top of samples).

5) **When a case definition is met** and samples are being collected, **please email:**
South Australian representatives: Georgia Macaulay (georgia.macaulay@sa.gov.au) and Matthew Bansemer (Matthew.Bansemer@sa.gov.au)
AND your state laboratory (where you will send samples to)
Glenside.Enquiries@clinicallabs.com.au
a) The purpose of your email is to notify the state reps and state laboratory that the case definition for summer mortality has been met on your farm and samples are being sent. **Please include in your email, a photo or photocopy of your filled out Summer Mortality Sample Form** and **Gribbles Veterinary request form.**
6) Please post foam containers (use express courier) to relevant state laboratory within 24h of sampling:

Vetlab, Gribbles Pathology,
Flemington Street,
Glenside, South Australia, 5065
Contact number: 08 8202 3300
Email: Glenside.Enquiries@clinicallabs.com.au

Alternatively, samples can be driven to the laboratory.

SUMMER MORTALITY SAMPLING

Summer Mortality SAMPLE FORM SOUTH AUSTRALIA	
Sampling date	…./…./….
Contact name	
Phone #	
Farm name	
Licence #	
Time samples were packaged	
Control: Number of replicate tanks abalone sampled from	
Control: Total number of abalone sampled	
Case: Number of replicate tanks abalone sampled from	
Case: Total number abalone sampled	

Please complete and include this form with your samples!

SAMPLE CHECKLIST: please ensure your samples meet these criteria:

☐ Control and Case samples are in **clearly labelled separate bags**.

☐ Abalone samples are indirectly **on ice within a Styrofoam box** (labelled bags are ideally wrapped in newspaper for extra insulation).

☐ **Summer Mortality Sample Form (THIS FORM) is filled out** and placed in a plastic bag/sleeve on top of samples within box.

☐ **Gribbles Veterinary request form is filled out** and placed in a plastic bag/sleeve on top of samples within box.

☐ Email Georgia Macaulay, Matthew Bansemer, and State laboratory that samples are being sent and provide a photocopy or photograph of this filled out sample form in the email.

Any questions? Please call PIRSA's Aquatic Animal Health Officers Georgia Macaulay 08 8429 4412 or Matthew Bansemer 08 8429 2100.

Appendix 9 – Standard Operating Procedure for State Laboratories for disease rule-out

FRDC project - 2019-147 - Risk factors and management strategies associated with summer mortality in Australian abalone:

Project background:

High mortality during warm summer water temperatures has been termed summer mortality by Australian abalone growers and researchers.

Good evidence of disease status of Australian farms is crucial for trade and market access. Mortality events in aquaculture require comprehensive investigation to establish aetiology. Industry guidelines have been provided to participating abalone growers (land farms) that detail submission protocol to investigate summer mortality events during the life of the project to comprehensively investigate for disease mechanisms or infectious agents.

When a case definition for summer mortality for abalone is met, abalone will be sampled within 24 h from tanks that fit our case definition for summer mortality and control tanks. Abalone will be submitted to state laboratories for histopathology and bacteriological analyses to rule out primary pathogens and infectious diseases.

Funding details:

There are funds to investigate four mortality events per year for two years (summer of 2021 and summer of 2022) using histopathology and bacteriology. To ensure each state is represented, initially we will limit each state (WA, SA, Vic and Tas) to one mortality investigation between January and March. If by April, there have been fewer than four investigations, a farm from a state that has already had a summer mortality investigation will be able to submit abalone for investigation, on a first-in basis.

Notifiable disease rule-out from polymerase chain reaction (PCR) is not funded in this project. Total funds available per investigation are $7800.

Standard Operating Procedure:

1) Farmers submitting samples will receive sampling instruction by Jessica Buss and Matthew Bansemer.

2) Jessica and Matthew will notify relevant State laboratory and State co-investigator of any incoming submissions.

3) Abalone growers will be instructed to submit 40 x whole abalone, indirectly on ice to State lab, comprising:

a. 10 x Control abalone

b. 30 x Case abalone

4) Case and Control abalone will be provided in separately labelled plastic bags.

a. Case: describes abalone collected from tanks which meet the case definition for summer mortality.

b. Control: describes abalone collected from control tanks that do not meet the case definition for summer mortality.

5) Please sample all 40 abalone for bacteriology and histopathology to comprehensively investigate for disease mechanisms or infectious agents.

6) For histopathology, please:

a. Use H&E stain + Grocott Methenamine Silver (only if fungi suspected)

b. Ensure your slides incorporate the following organs (irrespective of the number of sections): Buccal and pedal ganglia, cross section of oral cavity and adjacent connective, oesophagus, stomach, digestive gland, intestines, rectum, gill, mantle, pericardial sac and heart, left and right kidney, gonad (if suitable age), epipodium and muscular foot

7) For bacteriology please:

a. Use agar plates: TCBS (thiosulfate citrate bile salts sucrose agar) and ZMA (Zobell's formulation, marine agar 2216).

b. At least one replicate per abalone. i. Note: per abalone = per animal. I.e. for each animal please inoculate hemolymph onto TCBS and ZMA plates and prepare 1 smear of hemolymph on a glass slide for Gram staining.

c. Identify bacteria to species level (if possible)

8) Please see Table 1 for an example of what information to include. Results do not have to follow a table format, but please ensure results include the following information:

a. 1 animal = 1 data point for this project, therefore, please include individual animal IDs for each submission.

b. For each animal ID, please clearly identify if that particular ID is a Case or Control and list the associated tank number that animal came from. I.e. Animal 1– Case – Tank F2.

c. For each animal, please provide histopathology necropsy description, including presence/absence of any pathogen/s.

d. For each animal, please list absence/presence of pathogen/bacteria and if possible, which species.

Necropsy reports:

Please keep necropsy reports inclusive and look for any suspect pathogen or evidence of disease. If notifiable is suspected, then this falls outside the case definition for summer mortality and State requirements for notifiable disease would take preference.

An example of general terms to include for histopathology necropsy:

Were any of the following identified: Helminths (Cestodes, Trematodes, Nematodes), Protists (ciliates, Apicomplexan-like parasite, intracellular parasites), rickettsia-like bacterium, Vibrio spp. or viral-like inclusions. Does evidence of pathogen presence explain health status of that particular animal?

Table 1: Bacteriology and Histopathology results. If a pathogen is detected from either bacteriology or histopathology – please report to a species level if possible. If no pathogen detected, please write N/A.

Animal ID	Tank ID	Tank type (Case/Control)	TCBS necropsy report.	ZMA necropsy report	Histopathology: necropsy report
1	Tank E1	Control			
2	Tank E1	Control			
3	Tank E1	Control			
11	Tank F3	Case			
12	Tank F3	Case			
(etc. up to 40)	Tank B7	Case			

Appendix 10 – Variable not included as risk factors in the case-control study

Table of variables (questions presented to farmers in the risk factor form [Appendix 8]) that were not included as fixed factors in the final analysis for the case-control study because: there were no data available, data was the same for case and controls, or variables could not be causative factors (recorded after case/control or solely for use in specific growth or condition factor calculations). The response variable [was Question 4: Tank type (Case or Matched/Background Control)] and two variables were treated as random effects: Question 3: Farming trading name (kept confidential), Question 5: Tank identification/name.

Question	No data available:
7	Collect for single Case/Control tank - Maximum water temperature, 1 week prior to case/control date? (°C) (on the day, 1 week prior)
8	Collect for single Case/Control tank - Maximum water temperature, 2 weeks prior to Case/Control date? (°C) (on the day, 2 weeks prior)
9	Collect for single Case/Control tank - Difference between minimum and maximum water temperatures on day of Case/Control? (°C)
10	Collect for single Case/Control tank - Minimum water temperature in one week prior to the Case/Control event? (during that week)
11	Collect for single Case/Control tank - Maximum water temperature in one week prior to the Case/Control event? (during that week)
12	Collect for single Case/Control tank - Was there a >3°C water increase in water temperature one week prior to case/control? (Yes/No) (during that week)
13	Collect for single Case/Control tank - Maximum water temperature of this tank since abalone were last graded (°C)
14	Collect for single Case/Control tank - Average dissolved oxygen level for this tank (1 week prior to case/control) (mg/L OR % saturation) (during that week)
15	Collect for single Case/Control tank - Average Total Gas Pressure (TGP) for this tank (% saturation) (one week prior to case/control) (during that week)
16	Collect for single Case/Control tank - Flow rate on case/Control date? (tank turnover/day) (Tank turn over per day = 1440 (as there are 1440 min in 1 day) / (tank volume [L] / flow rate [L min-1]))
17	Collect for single Case/Control tank - Average ammonia concentration for this tank? (one week prior to case/control) (mg/L) (during that week)
18	Collect for single Case/Control tank - Average nitrite concentration for this tank? (one week prior to case/control) (mg/L) (during that week)
19	Collect for single Case/Control tank - Average pH for this tank? (one week prior to case control) (during that week)
20	Collect for single Case/Control tank - Average salinity for this tank? (one week prior to case control) (ppt) (during that week)
69	If abalone were fed health promoting feed, how many days were they fed this before day of case/control?
76	Was a primary pathogen identified from the veterinary necropsy report? (Yes/ No/ n/a)
77	What was the primary pathogen (if applicable)?
78	Was hemocyte infiltration in abalone digestive gland described in the veterinary necropsy report? (Yes/no/n/a)
79	Was loss of gill epithelium lining or gill necrosis described in the veterinary necropsy report? (Yes/NO/N/A)

80	Did abalone in this tank exhibit signs of bloat (swollen abdomen/floating)? (Yes/no/n/a)
81	Were bacteria identified? (Yes/No)
82	Which bacteria species (including type if possible) were identified? (If applicable)
89	Historical average (5 year) water temperature during month of case/control.
90	Temperature difference between historical average (five year) water temperature during the month of the case/control and maximum water temperature one week prior to case/control
100	Average abalone condition at 1 grading BEFORE Case/Control: Condition factor (CF) equals 5575 x (weight [g] / length [mm] 2.99)
	No difference between case/control:
40	Is there a water supply issue in this tank? (e.g. pump failure, algae build-up) (Yes/No). ****This was answered 'No' for all non-missing responses***.
67	Feed type abalone were fed in this tank? Formulated feed/algae/combination. ****This was answered as formulated feed for all non-missing results***.
68	Were abalone from this tank fed a health promoting feed (with anti-oxidant or algae inclusion)? (Yes/No). ****This was answered 'No' for all non-missing responses***
74	Is water re-used in this tank? (partial re-circulation) Yes/No. ****This was answered 'No' for all non-missing responses***.
75	Did you submit abalone from this tank for histopathology/microbiology? (Yes/No). If NO, do not answer questions below. ****This was answered 'No' for all non-missing responses***
	Non causative factors (recorded after case/control date):
50	Average abalone mortality for this tank (%) (in week of case/control)
54	Grading date, 1 grading AFTER Case/Control (day/month/year)
62	Average shell length (mm) of abalone/tank, 1 grading AFTER Case/Control
63	Average weight (g) of abalone/tank, 1 grading AFTER Case/Control.
64	Total weight (kg) of abalone/tank, 1 grading AFTER Case/Control
97	Maximum stocking density for this tank, 1 grading AFTER Case/Control? (kg/m2, kg/m3 or % tank surface area)
101	Number of days between the 2 gradings BEFORE Case/Control
102	Number of days between ONE grading BEFORE Case/Control and ONE grading AFTER Case/Control
	Response variable:
4	Tank type (Case or Matched/Background Control)
	Random effects:
3	Farm trading name (this will be kept confidential)
5	Tank identification/name