# Investigating the Role of *Vibrio aestuarianus* in Summer Mortality of Farmed *Crassostrea gigas* in Baynes Sound, British Columbia

by

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BA, University of California Berkeley, 2009

Post Degree Diploma, Vancouver Island University 2019

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We acknowledge and respect the lək$^{w}$əŋən peoples on whose traditional territory the university stands and the Songhees, Esquimalt and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day

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**ABSTRACT**

Marine aquaculture is already vital to global food security and will continue to become more important in the coming years. *Crassostrea gigas* (Pacific oysters) is the primary oyster species cultivated worldwide. The FAO and IPCC predict that climate change will create uncertainty and challenges for marine aquaculture. Baynes Sound, British Columbia, is a productive region for aquaculture, producing >50% of British Columbia's total annual bivalve production by live weight and value. Major summer mortality events have been documented in farmed *Crassostrea gigas* globally since the 1950's. These events are believed to be caused by a multiplicity of factors including changes induced by anthropogenic climate change. One of the major contributors to summer mortality is the proliferation of *Vibrio* bacteria, specifically *Vibrio aestuarianus*, which has been shown to increase in abundance and virulence when seawater temperatures rise. Despite this connection and the economic importance of oyster farming in the region, little is known about the presence of *V. aestuarianus* in Baynes Sound. Our 17-month study sampled 7 sites in Baynes Sound on 33 occasions from May 2019 to September 2020. We found a positive correlation between seawater temperature and total *Vibrio* detected in water samples in Baynes Sound, an association that was stronger when the overall temperature regime was warmer. We found no significant correlation between any of the bacterial assays tested and salinity, pH, or $\Omega_{arag}$ saturation. We also did not identify a geographic pattern to bacterial abundance or virulence amongst test *C. gigas* in the field. Understanding that flagellates are the predominant type of microalgae present in Baynes Sound when summer mortality events occur, in lab trials, we found that incorporating *V. aestuarianus* into marine aggregates with flagellate microalgae caused higher mortality

than aggregates with diatoms or planktonic *V. aestuarianus*. These results were not statistically significant but led us to look at how exposure to husbandry stress pre and post inoculation with *V. aestuarianus* incorporated into marine aggregates affects mortality. We found that stress was a significant driver of mortality, particularly when administered 24h post inoculation, suggesting that farmers should avoid sorting or tumbling their oysters in the summer, and particularly immediately after a marine heatwave. Oysters lack adaptive immune systems and are grown in an open ocean environment where it is not possible to eliminate their exposure to pathogens. These factors make it impossible to use vaccines or antibacterial disinfectants to combat diseases. Therefore, breeding genetic resistance to *V. aestuarianus* may be the most effective way to fight summer mortality. Creating a repeatable and accurate protocol for inoculating oysters with marine bacteria is key to accurate heritability measurements and the estimation of breeding values of different families. Key factors include controlling for dose per animal, laboratory efficiency, and inoculation via a mechanism that mimics real-world infection and does not bypass the animal's immune defenses. We designed a protocol which controls for these factors, separating each animal into individual containers and adding a controlled dose of planktonic bacteria to each. Previously used methods of injection or using an infected "donor" animal in a group tank do not control for dose or bypass parts of the oysters' natural immune system, potentially creating inaccuracies in survival data generated with these methods. After designing this protocol, we tested 32 full-sib families and estimated the heritability of survival to *V. aestuarianus* on the observed and underlying liability scales to be 0.095 (SE = 0.043), and 0.15 (SE = 0.068) respectively. We also found a strong negative correlation between oyster

size and survival, with a gram of additional weight creating a 73% increase in the risk of death. Fast growth and large size are two traits which have been explicitly bred into *C. gigas* stock. Our work suggests that breeding a slower-growing, smaller animal may reduce summer mortality.

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# 1. CHAPTER 1: INTRDUCTION TO SUMMER MORTALITY AND PACIFIC OYSTER AQUACULTRE IN BAYNES SOUND

Global population is rising at unprecedented rates, trending towards 9.7 billion people by 2050. Along with this population rise, demand for protein and nutrient-dense food is also rising. The cost of terrestrial protein production is ever-increasing due to the limited availability of arable land (Flachowsky et al., 2017) and production of food from wild fisheries has stagnated due to overfishing (FAO, 2020). Per capita fish consumption rose from 9.0 kg in 1961 to 20.5 kg in 2018 (FAO, 2020). According to the FAO's 2020 report "The State of World Fisheries and Aquaculture 2020", world aquaculture production continues to grow and now provides half of all fish for human consumption. From 1990 to 2018, there was a 122% rise in total food fish consumption, only a 14% rise in global capture fisheries production, and a 527% rise in global aquaculture production, with a total farmgate sale value of USD 263.6 billion (FAO, 2020). Despite this economic growth, the FAO and IPCC predict that climate change will create uncertainty and challenges for aquaculture, including temperature and sea-level rise, increased occurrence of disease, shifts in precipitation, freshening from glacier melt, changing ocean productivity and circulation patterns, increasing occurrence of extreme climatic events, eutrophication, and ocean acidification (FAO, 2018b; IPCC, 2019).

Debate about the sustainability of fin-fish aquaculture centers around the use of wild-caught fish in feed. The rate of protein conversion from fishmeal to farmed fish has gotten significantly lower, and therefore more sustainable, in the last decade, but is still a major source of concern (Ghamkhar and Hicks, 2020, Ahmed et al., 2019, Troell et al. 2014). Small pelagic fish which are used for aquaculture fish-feed also play a key role

in in supporting species across the marine food web including larger fish, birds, mammals, and crustaceans (Smith et al. 2010). By contrast, responsible aquaculture production of bivalves such as oysters is considered to be environmentally sustainable, and even to have a positive environmental impact (Shumway et al., 2003; FAO, 2020). Marine bivalves, filter-feeding organisms that extract organic matter from water for growth are grown extensively in aquaculture. In 2018 17.7 million tonnes of bivalves were produced through aquaculture, accounting for 21.5% of fish production (FAO, 2020). Bivalves provide diverse ecosystem services such as improving water quality, increased spatial complexity, regulating concentrations of plankton and microorganisms, and remove waste materials from the water column thereby lowering nutrient load (FAO, 2020; Shumway et al., 2003, reviewed by Lemasson et al, 2017, Coen et al., 2007). When farmed in the same area with fed species like finfish, bivalves benefit the environment by removing waste, thus lowering the impact of fed aquaculture on surrounding environments (FAO, 2020).

The Pacific oyster, *Crassostrea gigas* has been introduced to every inhabited continent, forming the backbone of a global shellfish industry worth USD 3.5 billion (FAO, 2020). *C. gigas* is a resilient species that grows rapidly and can succeed in a wide range of environments, making it one of the most widely cultured bivalve species in the world (Quayle, 1988; Gillespie et al., 2012; FAO, 2020). *C. gigas* is the main oyster species cultivated worldwide, an industry worth USD 1.4 billion (FAO 2020, Azéma et al, 2017). Canadian oyster production is valued at CAD 54 million (DFO, 2019) with British

Columbia accounting for CAD 15M of that total. The Baynes Sound region of British Columbia produces roughly 50% of the oysters grown in BC annually.

While *C. gigas* can tolerate a wide range of physical conditions, they are sedentary when mature and are thus unable to escape when their environment fluctuates beyond tolerable levels. Additionally, their temperature and salinity tolerance vary widely in genetically distinct populations distributed across the globe, so individual populations may not tolerate extreme environmental conditions that other populations could survive. Major summer mortality events have been documented in farmed *C. gigas* since the 1950's in Japan (Imai et al., 1965), the USA (Chaney and Gracey, 2011), Europe (Soletchnik et al., 2007), Australia (Li et al., 2009b) and Canada (Cassis et al., 2011). These summer mortality events are likely caused by a confluence of many factors including temperature fluctuations which induce stress and possibly allow pathogens to proliferate (Garnier et al., 2007). Summer mortality events typically occur when seawater temperature exceeds a threshold of 19$^{o}$C (Soletchnik et al., 2007, Soletchnik et al., 2003). In general, rising seawater temperatures have been linked to increased disease incidence in marine ecosystems (reviewed by Burge et al., 2014). Marine heatwaves and warming seawater temperatures have been shown to significantly increase death in *C. gigas* due to a proliferation of bacteria (from 4.3% to 77.4%), notably an increase in the proportion of *Vibrio* bacteria when seawater is rapidly warmed (Green et al., 2019; Wendling et al, 2013).

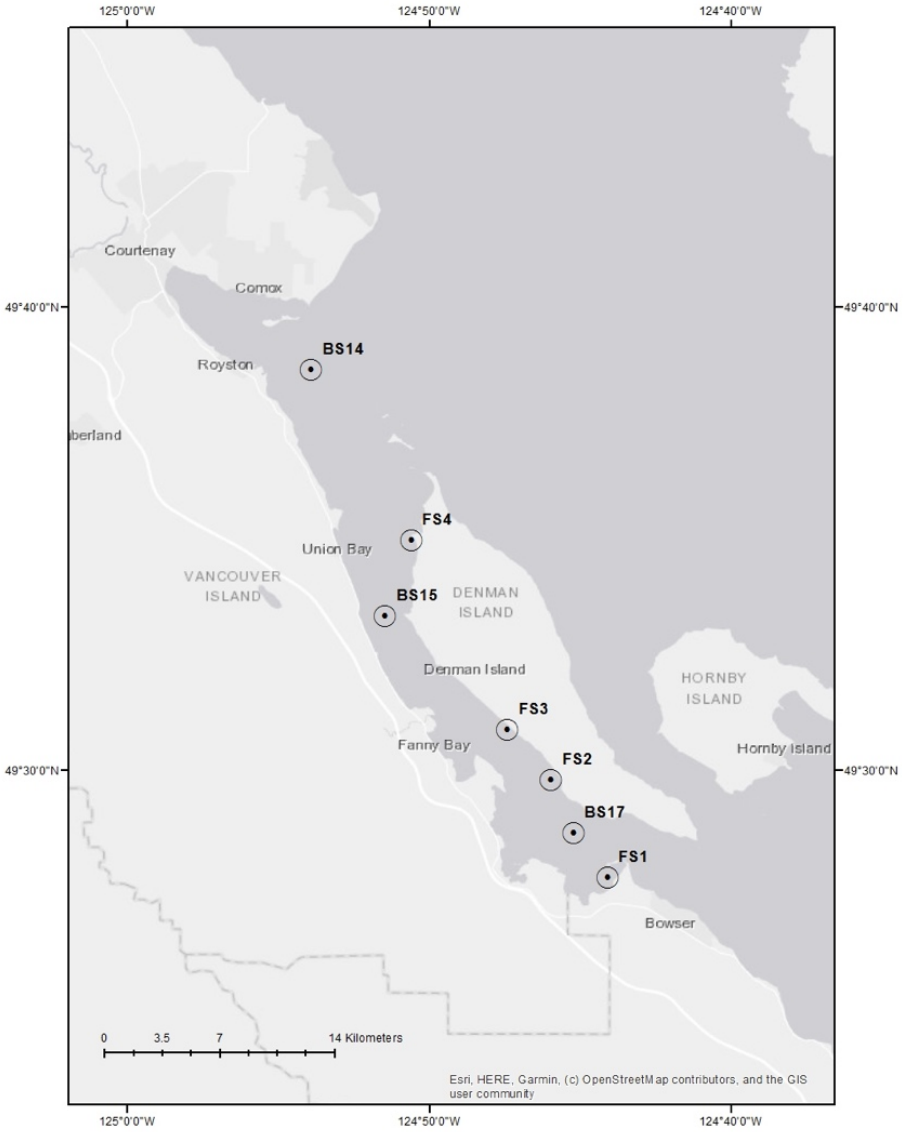



**Figure 1.1**: Baynes Sound is located within the Strait of Georgia and is part of the Salish Sea region. Sampling stations are shown. FS1, FS2, FS3, and FS4 are farm sites. Samples were collected at 3m below sea surface at farm sites. At BS17, samples were collected at 5m, 30m, and 70m below sea surface. At BS14 and BS15 samples were collected from 5m and 30m below sea surface.

Baynes Sound British Columbia is an important oyster growing region and is the geographic area of focus for this thesis. Baynes Sounds is a roughly 40km by 4km stretch of the Strait of Georgia between Vancouver Island and Denman Island (Figure

1.1). The area supports both wild and farmed harvests of many bivalve species including Manila clams (*Venerupis philippinarum*), geoduck clams (*Panopea generosa*), mussels (*Mytillus trossulu*s and *M. galloprovincialis*) and oysters (*Crassostrea gigas*). Climate change is affecting seawater temperatures in the region. The frequency of marine heatwaves in Baynes Sound is documented to be rising, and local oyster farms have reported significant summer mortality events of up to 90% losses since 2015 (Okey et al., 2014; anecdotal BSCGA). Most recently, in late June and early July 2021, temperatures reach significantly higher than ever before, reportedly causing die offs of up to 70% of cultivated oysters, along with other shellfish (anecdotal BCSGA).

While there have been studies in other parts of the world, there has not been a comprehensive study of Summer Mortality Syndrome in Baynes Sound. Research suggests temperature (Bai et al, 2015; Malham et al, 2009; Samain et al, 2008), reproductive state (Bodoy et al, 1988; Cotter et al, 2010; Samain et al, 2008), and pathogens (Bai et al, 2015; Garnier et al, 2007; Engelsma et al, 2008; Travers et al, 2019) are primary contributors to Summer Mortality Syndrome (Figure 2). Secondary factors such as food supply (Samain et al, 2008), salinity (Bodoy et al, 1988; Samain et al, 2008), harmful algal blooms (Cannuel et al, 2007; Cassis et al, 2006; Samain et al, 2007) and host genetic susceptibility (Samain et al, 2007; Rosa et al, 2012), have been associated with Summer Mortality Syndrome in some contexts.

Higher seawater temperatures could induce increased mortality in *C. gigas* through a variety of mechanisms, including increased abundance and virulence of pathogens

(Kimes et al., 2012), and effects on host physiology (Li et al, 2007 and 2009b, Welding et al., 2013)). Changing temperatures affect the metabolic rate of organisms (Green et al, 2018). Metabolic stress could result in higher susceptibility to disease as well as increasing the population density of pathogenic bacteria such as *Vibrio* and changing the community composition in ways that increase virulence (Green et al, 2018). Previous work suggests that temperature stress also can effect the microbial dynamics and composition of communities in the hemolymph of *C. gigias (*Lokmer et al, 2015). It has been shown that microbiota are vital to the survival and development of host organisms (McFall-Ngai et al., 2013). In stressed or compromised hosts such as those in unfavorable environmental conditions these symbionts can act as pathogens (Garnier et al., 2007; Cerf-Bensussan and Gaboriau-Routhiau, 2010; Lokmer et al., 2015; Olson et al., 2014). Temperatures above 20–25 °C have been shown to have negative impacts on *C. gigas* feeding activity (filtration rate), and temperatures above 20 °C increase respiration exponentially (Bougrier et al., 1995; Le Gall et al.; 1998, Ren et al., 2000). Temperatures above 21 °C are likely to cause physiologically stress in *C. gigas* due to reduced aerobic scope and a mismatch between energy acquisition and expenditure (Bougrier 1995, Le Gall et al.; 1998). This may cause physiological trade-offs that divert energy from processes such as immunity towards basic maintenance (Lanning et al., 2006).

Over the last decade, microbiological analysis of Pacific oysters during summer mortality events in Europe has routinely detected *Vibrio* spp. (Garnier et al, 2007; Samain et al, 2008). *Vibrio* bacteria are Gram-negative rod-shaped bacteria that are widespread in

marine environments. *Vibrio* infections have been shown to cause more mortality in *C. gigas* as water temperature rises (Green et al., 2019). Specifically, *Vibrio aestuarianus* subspecies *aestuarianus* is associated with mortality events in adult farmed oysters (Gay et al., 2004; Lemire et al., 2015; Travers et al., 2017). A controlled laboratory experiment studying comparative virulence of *Vibrio tubiashii, Vibrio anguillarum, Vibrio alginolyticus* and *Vibrio aestuarianus* found that at 5 days post injection, the cumulative mortality rates were 36%, 40%, 40% and 76%, respectively, indicating that *V. aestuarianus* induced the highest death rate (Meng et al., 2015). Of the species identified, *V. aestuarianus* is the most well-studied in Summer Mortality Syndrome (Garnier et al, 2007).

While *Ostreid herpesvirus* 1 (OsHV-1) is a cause of summer mortality in some geographies (Burge et al., 2006, 2007; Renault et al., 1994, 1998, 2000a,b), it has not yet been detected in Baynes Sound, so cannot be the primary pathogenic driver of mortality in the region. Additionally, laboratory challenge demonstrated that *V. aestuarianus* causes higher mortality in adult oysters, whereas OsHv-1 causes higher mortality in juveniles (Azema et al., 2016; Green et al., 2016). Concentrations as low as 5 x $10^6$ *V. aestuarianus* per oyster can cause >90% mortality (Gay et al, 2004; Travers et al, 2017). Finally, a comparative laboratory challenge study examining reproductive investment, thermal stress and *Vibrio* infection concluded that these factors contribute cumulatively to oyster mortalities, and that pathogenic *Vibrio* spp infection was of the highest importance (Wendling et al, 2013). For this reason, this thesis focuses on examining the

link between seawater temperature and abundance of *Vibrio* bacteria on the mortality of adult *C. gigas* in Baynes Sound.

Studies have demonstrated that resistance to infection by *V. aestuarianus* and other *Vibrio*s may be heritable. One study using the controlled infectious challenges showed that resistance was heritable in adult *C. gigas* in 1 of the 2 genetic stocks tested, with realized heritability estimated at the first generation of mass selection for the 1 stock which could breed resistance ranging from 0.05 to 0.30 (Degremont et al., 2020). They also found that the realized heritability for resistance was higher for oysters selected for dual resistance to both OsHV-1 and *V. aestuarianus*, ranging from 0.47 to 0.80, than those only selected for *V. aestuarianus* (Degremont et al., 2020). This result suggests that selection for dual resistance in *C. gigas* could limit the impact of both OsHV-1 and *V. aestuarianus* on summer mortality (Degremont et al., 2020). It is also possible that these dual-resistant animals are generally heartier with stronger immune systems, suggesting that there may be another quality about these animals which could be more effectively bred for than resistance to individual bacterial species or viruses. Another study focused on heritability of resistance to *V. alginolyticus* tested 52 families and found high levels of phenotypic variation in survival (0% to 56.25%) but low to moderate heritability, ranging from 0.133 to 0.257 (Zhai et al., 2021). The same study found that the genetic and phenotypic correlation between resistance to *V. alginolyticus* and growth traits were low, suggesting the feasibility of simultaneous genetic improvement of both growth, which is a desirable trait for farmers, and resistance traits. This correlation has not been examined for resistance to *V. aestuarianus* and growth traits.

Genetic selection for oyster resistant to summer mortality syndrome is considered an important strategy to safeguard the shellfish industry in a warming climate. To develop a genetic selection program, knowledge of the genetic control of the trait of selection is required to formulate a breeding strategy and a process to test the survival of individuals in the breeding population against summer mortality syndrome needs to be developed. Summer mortality has been shown to occur based on complex unpredictable spatial and temporal dynamics *in situ,* which precludes selection of broodstock solely upon the basis of field trials (Lang et al., 2009). To accurately calculate heritability of summer mortality resistance, controlled studies must be performed in the lab. Historically there have been two widely used mechanisms for infecting *C. gigas* with pathogens for lab trials which each present problems. [1] Direct injection of all animals: researchers inject adult oysters with the pathogen, directly in the adductor muscle. This method is labor intensive, limiting the practicality of conducting large lab trials with many subjects. It also requires the subjects to be large enough to be injected, so precludes the study of spat and younger *C. gigas*. It requires that the shell be ground down to create a hole in the shell, introducing an element of physical stress. Most importantly, direct injection does not mimic real-world disease transmission and bypasses several immune mechanisms (De Decker & Saulnier, 2011). [2] Cohabitation: One or more "donor" *C. gigas* is injected with the pathogen and is placed in a shared tank with other oysters, shedding bacteria and infecting the healthy oysters. Bacterial shedding from the infected donor oyster will not be consistent across all donors, creating a potentially significant difference in dose per replicate tank (De Decker & Saulnier, 2011; Azéma et al., 2015).

A goal of this thesis is to develop essential knowledge to increase the survival and commercial viability of *C. gigas* farmed in Baynes Sound. As part of this effort, we seek to determine the degree to which resistance to *V. aestuarianus* is heritable in the locally found population, as well as to identify specific resistant individuals which could be bred into the Vancouver Island University breeding program. We will also seek to examine the phenotypic and genetic correlation between animal size and resistance to *V. aestuarianus* infection. Our work also describes a repeatable experimental framework for bacterial infection which controls both for dose, as direct injection does, but also mimics real-world infection pathways, like cohabitation does.

# 2. CHAPTER 2: UNDERSTANDING HOW OYSTER FARMING, TEMPERATURE, AND MARINE AGGREGATES INFLUENCE THE DYNAMICS OF PATHOGENIC *VIBRIO AESTUARIANUS* IN BAYNES SOUND

## 2.1 ABSTRACT

The FAO and IPCC predict that climate change will create uncertainty and challenges for marine aquaculture. Baynes Sound, British Columbia, is a productive region for aquaculture, producing >50% of British Columbia's total annual bivalve production by live weight and value. *Vibrio aestuarianus* has been associated with recurrent mass mortalities of adult *Crassostrea gigas,* threatening oyster farming worldwide. *V. aestuarianus* has been shown to increase in abundance and virulence when seawater temperatures rise. However, knowledge of the ecology of *V. aestuarianus* in marine environments which are used for oyster farming remains scarce. A better understanding of this would increase our capacity to predict and mitigate disease occurrence. This study is the first attempt to understand the spatiotemporal factors which contribute to *V. aestuarianus* abundance in Bayes Sound. Our 17-month study sampled 7 sites in Baynes Sound on 33 occasions from May 2019 to September 2020. We found is a positive correlation between seawater temperature and total *Vibrio* detected in water samples in Baynes Sound, an association that was stronger when the overall temperature regime was warmer. We found no significant correlation between any of the bacterial assays tested and salinity, pH, or $\Omega_{arag}$ saturation. We also did not identify a geographic pattern to bacterial abundance or virulence amongst test *C. gigas* in the field. Understanding that flagellates are the predominant type of microalgae present in Baynes Sound when summer mortality events occur, in lab trials, we found that incorporating *V. aestuarianus* into marine aggregates with flagellate microalgae caused higher mortality than aggregates

with diatoms or planktonic *V. aestuarianus*. These results were not statistically significant but led us to look at how exposure to husbandry stress pre and post inoculation with *V. aestuarianus* incorporated into marine aggregates effects mortality. We found that stress was a significant driver of mortality, particularly when administered 24h post inoculation, suggesting that farmers should avoid sorting or tumbling their oysters in the summer, and particularly immediately after a marine heatwave.

## 2.2 INTRODUCTION

Warming sea surface temperatures will have detrimental effects on the health of marine bivalve populations (reviewed by Burge et al., 2014). Warming seawater increases the frequency of disease outbreaks in bivalve populations, and stresses their immune systems, impairing immune response (Green et al., 2014, Gagnaire et al., 2006, Malham et al., 2009, Petton et al., 2013). In a small but growing body of literature, marine heatwaves, which are becoming more frequent and more intense (Frolicher et al., 2018), are documented to trigger outbreaks of infectious disease in farmed bivalves (Green et al., 2019, Scanes et al., 2020).

*Vibrio* bacteria are Gram-negative rod-shaped bacteria which are globally distributed marine *Gammaproteobacteria* (Destoumieux-Garzon et al., 2020). *Vibrio* species cause epizootics, zoonoses, and epidemics in a diverse group of organisms, including serious illness such as cholera (*Vibrio cholerae*) in humans (Austin, 2010; Le Roux et al., 2015; Lopez-Joven et al., 2018). It has been suggested that strains of *Vibrio* may be a cause of summer mass mortality events in farmed *C. gigas* populations (Austen, 2010, Wendling

et al, 2013). Specifically, strains of *Vibrio aestuarianus* are associated with mortality events in adult farmed oysters (Gay et al., 2004; Lemire et al., 2015; Travers et al., 2017), and seawater temperature influences the growth and abundance of *Vibrio* pathogens in the marine environment (Vezzulli et al., 2012). Concentrations as low as 5 x $10^6$ of *V. aestuarianus* per oyster can cause >90% mortality (Gay et al, 2004; Travers et al, 2017).

Strains of *Vibrio aestuarianus* and those of the Splendidus clade have been associated with summer mortality among farmed *C. gigas,* a phenomenon reported globally since the 1950's (Soletchnik et al., 1999; Gay et al., 2004; Lemire et al., 2015, Imai et al., 1965; Chaney and Gracey, 2011; Soletchnik et al., 2007; (Li et al., 2009; Cassis et al., 2011).). Oyster farming is a globally important industry and also regionally important to Canada, with Canadian production valued at CAD 54 million (DFO, 2019) with British Columbia accounting for CAD 15M of that total. *V. aestuarianus* is known as a major pathogen to adult oysters (Travers et al., 2017) while strains of the Splendidus clade (*Vibrio tasmaniensis* and *Vibrio crassostreae*) are associated with a multifactorial disease affecting spats and juveniles (Lopez-Joven et al., 2018; Gay et al., 2004; Lemire et al., 2015; Bruto et al., 2017; de Lorgeril et al., 2018), which is triggered by herpes virus OsHV-1 µVar (Segarra et al., 2010; Martenot et al., 2011). Summer mortality events typically occur when seawater temperature exceeds a threshold of 19ºC (Soletchnik et al., 2007, Soletchnik et al., 2003; Pernet et al., 2012, 2014).

Strains of *V. aestuarianus* isolated during adult oyster mortalities are mostly pathogenic and form phylogenetically coherent virulent lineages (Lopez-Joven et al., 2018). As a

consequence of the species' high degree of clonality, *dnaJ* detection is considered a reliable way of identifying the presence of *V. aestuarianus* (Saulnier et al., 2009) in the environment (Lopez-Joven et al., 2018).

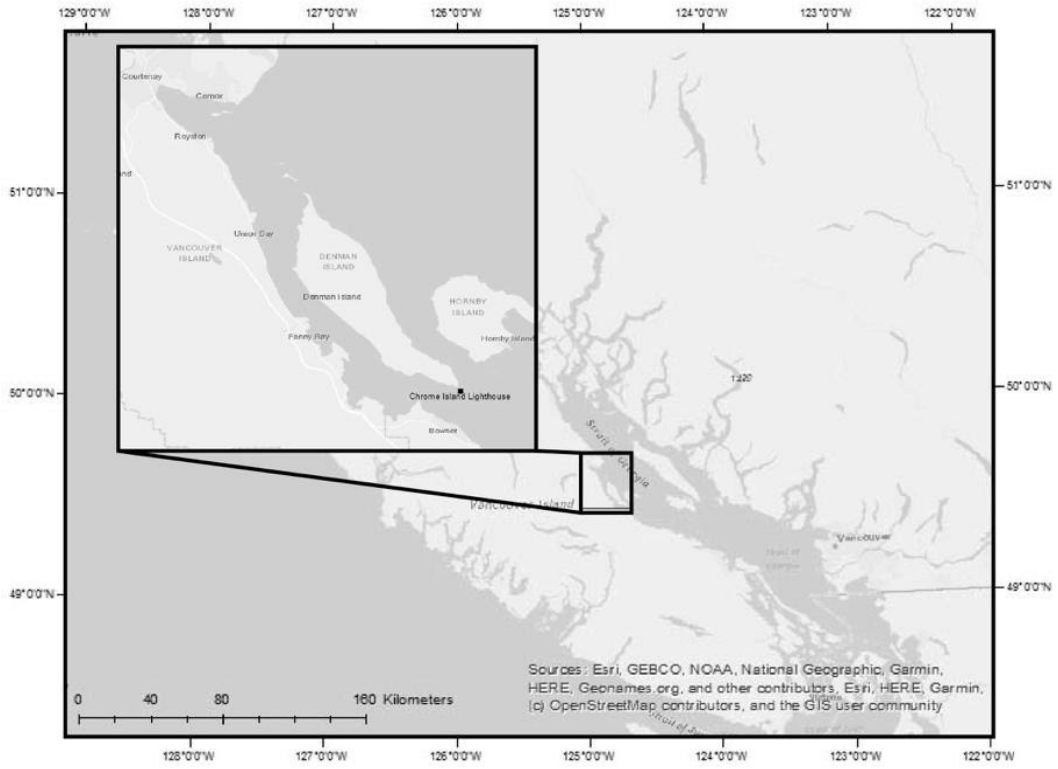



**Figure 2.1:** Baynes Sound study area in British Columbia, Canada.

Baynes Sound, BC is the focus region for this study due to its importance to Canadian oyster farming. Baynes Sound is a narrow stretch of water with moderate tidal flow, which is approximately 8700 ha. It is the stretch of water bordered by the east coast of Vancouver Island and partially bordered by west coast of Denman Island, British Columbia, Canada (Figure 2.1). Baynes Sound is a highly productive habitat for shellfish, with wild and farmed populations of various species including Manila clams (*Venerupis philippinarum*), geoduck clams (*Panopea generosa*), mussels (*Mytillus trossulus* and *M. galloprovincialis*) and oysters (*Crassostrea gigas*). *C. gigas* is dominant species farmed

in the Sound, comprising >50% of British Columbia's total annual bivalve production by live weight and value (Dumbauld et al., 2009, Carswell et al., 2006). *C. gigas* are grown both on intertidal beaches and in deep-water culture suspended from rafts. Climate change may have an outsized impact on shellfish aquaculture in Baynes sound because of its geographic proximity to the Gulf of Alaska, which is predicted to have significant temperature changes driven by anthropogenic climate change (Oakey et al., 2014, Hobday & Pecl, 2014).

While mortalities of adult farmed oysters have been documented repeatedly in recent years causing dramatic losses to British Columbia oyster production, environmental drivers of *V. aestuarianus*' recurrence have been poorly investigated. To our knowledge, there has been no previous long-term study of *Vibrio* in the Baynes Sound region. In other regions environmental surveys exploring the spatial and temporal distribution of *V. aestuarianus* have been conducted (Vezzulli et al., 2015, Lopez-Joven et al., 2018). These studies found *V. aestuarianus* in summer and at very low levels in the sediment in winter. To predict and mitigate disease occurrence, it is paramount to understand the recurrence of summer mortality syndrome in regions used for oyster farming. This requires gaining more insight on the environmental reservoirs of *V. aestuarianus* and environmental factors driving their dynamics and transmission to oysters. To this end, our study was a 17-month effort to attempt to understand the link between proliferation of *V. aestuarianus* in Baynes Sound and its relationship to geography, seawater temperature, salinity, and pH.

In addition to our effort to understand the spatial and temporal distribution of *V. aestuarianus* and the seawater conditions which cause it to proliferate locally, we are interested in the potential role of marine aggregates as an environmental factor which may increase the virulence of *V. aestuarianus.* Marine aggregates, often called marine snow, are a naturally occurring part of marine aquatic systems. These particles can consist of microalgae, fecal pellets, larvacean houses, microbes, or inorganics (Froelich et al., 2013). Aggregate particles are brought together by exocellular polymers and physical/chemical forces (Alldredge et al., 1988) The aggregation of these suspended particles is an important process for the ecosystem because it increases the downward vertical transport of material through the water column, sinking them to the ocean floor (Crocker & Passow, 1995; Kiørboe et al., 1990; Passow & Wassmann, 1994).

Many bacterial pathogens are found within marine aggregates including *V. cholerae, V. parahaemolyticus, V. vulnificus, V. alginolyticus, Aeromonas hydrophila, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacteria* sp. (Lyons et al., 2007; Venkateswaran et al., 1990). It is understood that *Vibrio*s proliferate more abundantly in environments rich with organic matter (Tinta et al., 2012). Additionally, it has been shown that *Vibrio*s are often found living associated with different substances as a biofilm (Shikuma et al., 2010), phytoplankton and zooplankton, and are common symbionts with a number of metazoans (Thompson et al., 2004). There is a strong association between *Vibrio*s and marine aggregates. In fact, field studies have shown *Vibrio*s in high abundances in marine snow, and absent or in low abundances in ambient

seawater (Vojvoda et al., 2014). One 2015 study showed that *V. aestuarianus* readily attached to chitin particles and copepods, and formed biofilms (Vezzulli el at., 2015).

As suspension feeders, bivalves such a *C. gigas* process large volumes of seawater. As part of this process, oysters uptake a variety of microorganisms and particulate matter (Riisgård, 1988). These microorganisms are captured with varying efficiency, based on their size. The gills act as a multi-layered sieve. Particles of optimum size are caught and moved into the digestive tract via the labial palps. Particles that are too large are stopped and passed from the oyster as pseudofeces. Particles that are smaller than optimum are captured with an efficiency that decreases asymptotically with decreasing particle size (Riisgård, 1988), and are passed through the gills uncaptured (Froelich et al., 2013). Most bivalve species capture particles smaller than 1 μm at an efficiency <20% (Kach & Ward, 2008; Møhlenberg & Riisgård, 1978; Riisgård, 1988; Ward & Shumway, 2004).

The incorporation of bacteria into larger marine snow aggregates has been demonstrated to increase the rate at which oysters ingest bacteria, presumably because it is not filtered out as effectively when part of a larger mass. The rate at which *E. coli* and *V. vulnificus* are ingested by shellfish increases significantly when the bacterial cells are incorporated into aggregates (Froelich et al., 2013; Kach & Ward, 2008). Typically, the microalgae blooms in the Strait of Georgia follow a régime of predominance of diatoms in the spring, and flagellates in the summer, with overall volume of microalgae increasing dramatically in the summer (Costalago et al., 2020; Sukhanova et al., 2009; Albright & McCrae 1987). Summer mortality events in the region tend to occur during the flagellate-

dominant later summer months. In a laboratory challenge, we ask if marine aggregate formed with diatoms or flagellates is more pathogenic to *C. gigas*. We hypothesize that marine aggregates combining *V. aestuarianus* and a flagellate microalga will cause higher mortality than aggregates containing *V. aestuarianus* and a diatom.

Our first trial testing marine aggregates with *V. aestuarianus* combined with either diatoms or flagellates did not achieve significant mortality. This result made us include another factor which farmed oysters experience: husbandry stress. Our aim is to find results which can help aquaculture farmers in Baynes Sound avoid summer mortality events, so our experimental design aimed to mimic husbandry practices. In real-world aquaculture*, C. gigas* are often grown in stacks of trays suspended from rafts (Quayle, 1988). These stacks are occasionally removed from the water, thereby exposing the oysters to temperature shock of ambient air temperatures. As part of typical oyster husbandry practice oysters are tumbled to remove sharp shell frill, grade the oysters by size, achieve a deeper cup-shaped shell which is more marketable, and clean them. This exposes oysters to physical stress. Understanding that oyster farmers need to make decisions about when to handle their animals, we designed a second and third laboratory challenge trial with the purpose of determining if these husbandry techniques contribute to summer mortality in *C. gigas*, especially when combined with infection of *V. aestuarianus* incorporated into marine snow. Trial 2 was designed to test if stress combined with *V. aestuarianus* inoculation increased mortality when tested against animals exposed to just stress, and animals exposed to *Vibrio aestuarianus* but no stress. Understanding that husbandry stress is inevitable, Trial 3 was designed to test stress 24 h

pre-inoculation had a different result than stress 24 h post-inoculation with *Vibrio aestuarianus*. This experiment was designed to see if we could help farmers make decisions about when to handle their oysters before or after a marine heatwave.

## 2.3 METHODS

### 2.3.1 Baynes Sound Survey

#### 2.3.1a Study Location

The study was carried out at 7 sites distributed throughout Baynes Sound which is a roughly 40km by 4km stretch of the Strait of Georgia between Vancouver Island and Denman Island in British Columbia, Canada (49.5362° N, 124.8393° W) (Figure 1.1). Sampling stations were chosen inside and outside oyster farms to determine the potential impact of shellfish farming on pathogen dynamics. Four sample sites were at commercial oyster farms, with samples being taken off oyster rafts at 3m depth, a depth similar to many suspended oyster culture stacks. Three sites outside of farms were chosen based on their distribution throughout the sound, BS17 being the southernmost site and located at the deepest point in the sound (Figure 1.1).

#### 2.3.1b Environmental Drivers of *Vibrio* abundance

A total of 198 seawater samples were collected on 33 occasions from May 2019 to September 2020 at sites shown in Figure 1.1. Samples were collected from depths of 3, 5, 30, or 70 m with Niskin bottles (General Oceanics, United States) deployed on a line and tripped with messenger weights. Niskin bottle target depth was measured using an

A.G.O Environmental Ltd EWC-6 Electronic Wire Counter Module. Water samples for DNA extraction were collected in duplicate and were transferred from Niskin bottles to sterile 1 L amber soda-lime glass bottles. Sample bottles were rinsed three times with sample, filled, and stored in a cooler and transferred immediately to the lab. Duplicate 500 ml of seawater samples were vacuum filtered twice from each bottle using Analytical Test Filter Funnels (Nalgene; Nunc, United States) fitted with 0.22-µm pore-size hydrophilic polyvinylidene fluoride (PVDF) filters. Filters were removed from funnels and placed in sterile 15 ml screwcap tubes and immediately frozen at −80°C until DNA extraction. Filters were stored for a maximum of 3 months before processing. Additional seawater samples were collected from each Niskin bottle, using 350 mL amber soda-lime glass bottles that were filled from the bottom with care not to introduce bubbles to minimize gas exchange. Sample bottles were rinsed three times with sample, filled, fixed with 200 µl of a solution of saturated mercuric chloride, and crimp-sealed using polyurethane-lined metal caps. NIST traceable thermometers (VWR PN 23609-176) to record *in situ* temperature with 0.2°C factory reported accuracy. *In situ* temperature and salinity were recorded by a conductivity-temperature-depth (CTD) profiler (Seabird SBE 19plus V2 SeaCAT Profiler CTD) used immediately prior to Niskin bottle collection for samples. These 350 mL fixed samples were sent to Hakai Institute for further processing, using methods described in Evans et al. 2019. Briefly, Hakai Institute measured seawater sample salinity, pH, $TCO_2$, $pCO_2$, $pCO_2$, $pH_T$, TA, and $\Omega_{arag}$ (Evans et al, 2019).

**2.3.1c Spatial and Temporal Patterns of Oyster Mortality in Baynes Sound**

Three trays of 80 oysters were deployed at a depth of 3m at FS1, FS2, FS3, and FS4 (Figure 1.1). Black plastic oyster aquaculture trays were used, and each was deployed on an individual line. Oysters were donated by Mac's Oysters Ltd., were all of the same genetic stock originally sourced from a hatchery in Chile, and same age (~2 years old). Oysters were sampled on 6 dates throughout the summer, 2 weeks apart. Mortality was recorded, and dead oysters were discarded. 3 live oysters were removed from each tray, weighed, and gill tissue was sampled from each. Gill tissue samples were immediately frozen at −80°C and remained frozen until DNA extraction.

### 2.3.1d *Vibrio* Abundance in Samples

The abundance of bacteria in samples was estimated by targeting the 16S rRNA gene using quantitative PCR. Firstly, genomic DNA was extracted from 396 water filters using the Qiagen DNeasy Powersoil Extraction Kit (Qiagen, Valencia, CA). The filters were rolled up, cut into 2 mm strips, and placed into bead tubes before DNA purified according to the manufacturer's instructions. DNA from oyster gill tissue samples was extracted using Qiagen Blood and Tissue extraction kit, according to the manufacturer's instructions (Qiagen, Valencia, CA). Immediately after extraction, DNA was stored at -80°C. DNA yield and purity were determined by spectrophotometry.

### 2.3.1e DNA Amplification and Quantification

Quantitative polymerase chain reaction (qPCR) was performed to measure abundance of 16s, total *Vibrio*, and *Vibrio aestuarianus.*

Isolates of *Vibrio spp*. were obtained by Dr. Timothy Green of Vancouver Island University from oyster homogenate of sampled oysters collected during mortality events in Baynes Sound, BC. These were identified as *Vibrio aestuarianus* based on comparisons with confirmed 16S rRNA, pyrH and recA nucleotide gene sequence. These *V. aestuarianus* sequences were used to design species-specific recA primers and probes for use in quantitative polymerase chain reaction 58 (qPCR).

Water sample DNA was investigated for qPCR inhibition using serial dilutions. Briefly, samples were diluted in two series, 5-fold (1:5, 1:25) and 10-fold (1:10, 1:100), to find the minimal dilution needed to dilute out inhibitors but not lower the target concentration below the detection limit. A 1:25 dilution was found to be optimal and was used for all water samples. Sampled oyster DNA were diluted to 25 ng µl-1 to normalize the concentration of DNA per sample.

Three assays were run for each sample: detection of *V. aestuarianus,* detection of total *Vibrio spp.,* and detection of total bacteria using the 16s marker. Contents of qPCR reactions are listed in Table 2.1. All samples were run with qPCR duplicates in a 384-well hard-shell PCR plate (Bio-Rad) on a CFX384 Real-Time System using the Bio-Rad CFX Maestro version 1.1 software (Bio-Rad). Standard curves were created by amplifying a dilution series from DNA extracted from a known concentration cell culture. The resulting concentration of cells per ul was converted to cells per ng of DNA using the concentration of DNA per qPCR reaction.

**Table 2.1:** Details of contents of qPCR reaction wells for all assays.

| *V. aestuarianus* | Total *Vibrio* | Total Bacteria 16s |
|---|---|---|
| 6.5 µl SSO Advanced Universal Probes Supermix (Bio-Rad, Hercules, CA, USA | 6.5 µl Syber Probe Supermix (Bio-Rad, Hercules, CA, USA) | 6.5 µl SSO Advanced Universal Probes Supermix (Bio-Rad, Hercules, CA, USA |
| 0.25 µl of forward (5′ AGGTTCGATCATGCG CCTAG3') primer | 0.25 µl of forward (5′GGCGTAAAGC GCATGCAGGT 3') primer | 0.25 µl of forward (5′ ACTCCTACGGGAGGCA G 3') primer |
| 0.25 µl of reverse (5′ CTGACGATTCCGGGCCATA G 3′) primer | 0.25 µl of reverse (5′ GAAATTCTACCC CCCTACAG 3′) primer | 0.25 µl of reverse (5′ GACTACCAGGGTATCT AATCC 3′) primer |
| 0.1 µl probe (5′[HEX] TACGATGGATGTTGAAACC ATCTCTACTG[BHQ1]3′) | | 0.1 µl probe (5′ [6FAM]TGCCAGCAGCC GCGGTAATAC[TAM] 3′) |
| 0.65 µl of dsH2O | 0.75 µl of dsH2O | 0.65 µl of dsH2O |
| 5 µl of extracted DNA from each sample | 5 µl of extracted DNA from each sample | 5 µl of extracted DNA from each sample |

### 2.3.1f Statistical Analysis

Linear regression, two-way ANOVA, row statistics analysis, and graphing were performed using GraphPad Prism version 9.0.0 for MacOS (GraphPad Software, San Diego, California USA, www.graphpad.com).

## 2.3.2 Marine Snow Lab Challenges

### 2.3.2a Bacteria Growth

*Vibrio aestuarianus* (strain 2018-BS-032, see Table 3.1) was grown for 24 hours in tryptone soy broth containing 2% NaCl at 20°C under constant agitation at 200 rpm. The culture was purified by centrifuged (1000×*g*, 20 min), and cell pellet was rinsed and resuspended in sterile seawater. This purification process was repeated twice to remove

extracellular products and culture media. The titre of *Vibrio* inoculum was estimated by serial dilution and plating on tryptone soy agar + 2% NaCl. Colonies were enumerated after incubation at 20°C for 36 h. The *V. aestuarianus* (strain 2018-BS-032) used in this disease challenge is a pathogenic strain isolated from a mass mortality event of juvenile and adult *C. gigas* in Baynes Sound, Canada in August 2018 (Table 3.1).

### 2.3.2b *C. gigas* Origins and Rearing

All *C. gigas* used in the described laboratory trials were 1-year-old and originated from spawning group, bred and raised at Vancouver Island University's Deep Bay Marine Field Station.

### 2.3.2c Marine Aggregate Production

Marine aggregates (marine snow) were generated using methods similar to those described by Shanks and Edmondson in 1986, with the following modifications. Briefly, algal and *V. aestuarianus* cells were added to seawater from Baynes Sound filtered to 1 micron in 250-ml glass bottles, 10 ug /liter $^{-1}$ hyaluronic acid was added, and the bottles were constantly rotated at 200rpm at 20°C for 24 h. Algal species used were the flagellate *Tisochrysis lutea* (Tiso) and diatom *Thallasiosira pseudonanna* (3H). Each treatment was a total of 200mL. The titre of *V. aestuarianus* added to marine snow treatments was found to have a concentration of 4.4 x $10^7$ CFU/mL, and 5mL of this titre were added to the treatments which included bacteria. Details of the treatments for Trial 1 are listed in Table 2.2, Trial 2 are listed in Table 2.3, and Trial 3 are listed in Table 2.4.

**Table 2.2:** Marine snow treatment details for Trial 1: Diatom vs. Flagellate.

| Treatment | Temp (°C) | *Vibrio* | Algae | Hyaluronic Acid | Tanks | N |
|---|---|---|---|---|---|---|
| A | 20 | No | N/A | 10ugL^-1 | 3 | 20 |
| B | 20 | 5mL | N/A | 10ugL^-1 | 3 | 20 |
| C | 20 | 5mL | Diatom | 10ugL^-1 | 3 | 20 |
| D | 20 | No | Diatom | 10ugL^-1 | 3 | 20 |
| E | 20 | 5mL | Flagellate | 10ugL^-1 | 3 | 20 |
| F | 20 | No | Flagellate | 10ugL^-1 | 3 | 20 |

**Table 2.3:** Treatments for Trial 2: Marine Snow + Stress. This trial was designed to test if stress combined with *Vibrio aestuarianus* inoculation increased mortality when tested against animals exposed to just stress, and animals exposed to *Vibrio aestuarianus* but no stress.

| Treatment | Temp (°C) | *Vibrio* | Algae | Hyaluronic Acid | Tanks | N | Stress |
|---|---|---|---|---|---|---|---|
| A | 20 | No | Flagellate | 10ugL^-1 | 3 | 10 | No |
| B | 20 | No | Flagellate | 10ugL^-1 | 3 | 10 | 24 h post-inoculation |
| C | 20 | Yes | Flagellate | 10ugL^-1 | 3 | 10 | No |
| D | 20 | Yes | Flagellate | 10ugL^-1 | 3 | 10 | 24 h post-inoculation |
| Control | 20 | No | N/A | 10ugL^-1 | 1 | 10 | No |

**Table 2.4:** Treatments for Trial 3: Marine Snow + Stress Pre and Post Inoculation. This trial was designed to test stress 24 h pre-inoculation had a different result than stress 24 h post-inoculation with *Vibrio aestuarianus*.

| Treatment | Temp (°C) | *Vibrio* | Algae | Hyaluronic Acid | Tanks | N | Stress |
|---|---|---|---|---|---|---|---|
| A | 20 | No | N/A | 10ugL^-1 | 3 | 18 | N/A |
| B | 20 | Yes | Flagellate | 10ugL^-1 | 3 | 18 | 24 h pre-inoculation |
| C | 20 | No | Flagellate | 10ugL^-1 | 3 | 18 | 24 h pre-inoculation |
| D | 20 | Yes | Flagellate | 10ugL^-1 | 3 | 18 | 24 h post-inoculation |
| E | 20 | No | Flagellate | 10ugL^-1 | 3 | 18 | 24 h post-inoculation |

### 2.3.2d Trial 1 Experiment Setup and Sampling

20 animals were placed in each sterilized 15-gallon fish tank, which was filled halfway with seawater filtered to 1 micron. They were held in the tanks for 24 h prior to inoculation. The temperature was maintained at 20°C for the duration of the experiment. Treatment bottles were added to tanks. Experimenters examined each tank every 24 hours for 168 hours post inoculation, removing any dead animals each day. Gill tissue was taken from each dead oyster and stored at -80°C for further analysis.

### 2.3.2e Trial 2 Experiment Setup and Sampling

10 animals were placed in each sterilized 15-gallon fish tank, which was filled halfway with seawater filtered to 1 micron. Stress was defined as the combination of temperature shock, desiccation, and tumbling. Oysters which were exposed to stress were removed from their tanks and placed into glass beakers which were sealed with parafilm to prevent cross-contamination between tanks. They were exposed to 28°C air for 4 h, then shaken for 10 min at 350 RPM. This trial was designed to test if stress combined with *V. aestuarianus* inoculation increased mortality when tested against animals exposed to just stress, and animals exposed to *V. aestuarianus* but no stress. Treatments for this trail are outlined in Table 2.3. Experimenters examined each tank every 24 hours for up to 120 hours post inoculation, removing any dead animals each day. Gill tissue was taken from each dead oyster and stored at -80°C for further analysis.

### 2.3.2f Trial 3 Experiment Setup and Sampling

18 animals were placed in each sterilized 15-gallon fish tank, which was filled halfway with seawater filtered to 1 micron. Stress was defined as the combination of temperature shock, desiccation, and tumbling. Oysters which were exposed to stress were removed from their tanks and placed into glass beakers which were sealed with parafilm to prevent cross-contamination between tanks. They were exposed to 28°C air for 4 h, then shaken for 10 min at 350 RPM. This trial was designed to test if stress 24 h pre-inoculation had a different result than stress 24 h post-inoculation with *Vibrio aestuarianus*. Treatments for this trail are outlined in Table 2.4. Experimenters examined each tank every 24 hours for up to 168 hours post inoculation, removing any dead animals each day. Gill tissue was taken from each dead oyster and stored at -80°C for further analysis.

#### 2.3.2g Statistical Analysis

Linear regression, two-way ANOVA, row statistics analysis, and graphing were performed using GraphPad Prism version 9.0.0 for MacOS (GraphPad Software, San Diego, California USA, www.graphpad.com).

## 2.4 RESULTS

### 2.4.1 Baynes Sound Survey

#### 2.4.1a Spatiotemporal Analysis of *Vibrio aestuarianus* and Water Quality

On September 6, 2019, seawater temperatures at Farm Sites 1 and 2 reached above 19 ºC (Figure 2.2), a temperature previously shown to increase risk of mortality in oysters. An increase in mortality rate was measured at the farm sites on September 10, 2019, a few days after the temperature spike, indicating that there could be a link between

temperature increase and increased oyster mortality (Figure 2.11). The quantity of *V. aestuarianus* DNA found in samples did not correlate to seawater temperature in 2019, however samples taken in 2020 show levels of *V. aestuarianus* increasing and decreasing with sea temperature fluctuations (Figure 2.2).

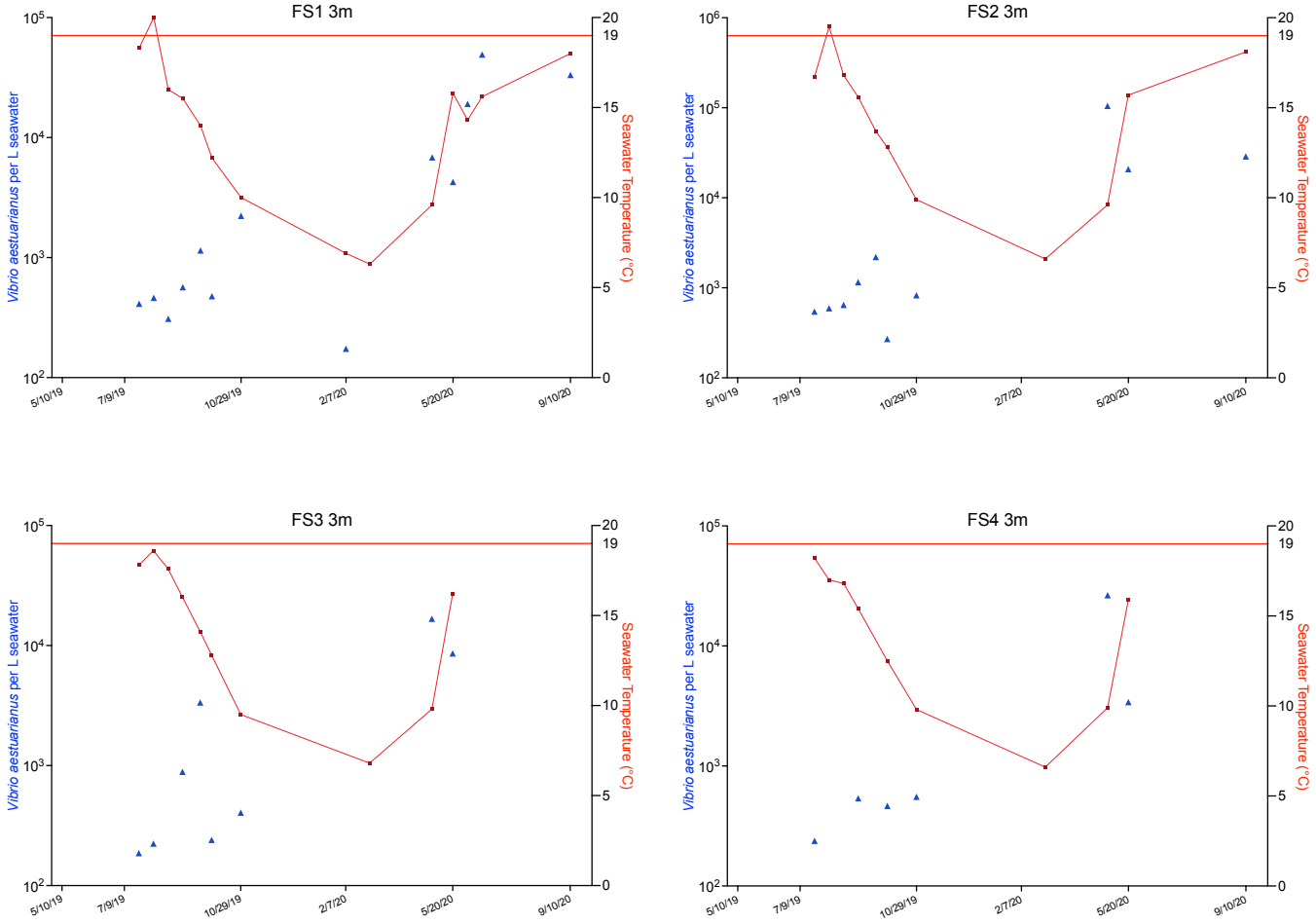



**Figure 2.2:** Scatter plot of the number of copies of *Vibrio aestuarianus* per L of seawater, on the left axis. Seawater temperature (ºC) measured in the Niskin bottle at the time of collection is plotted in red on the right axis, with a line at 19 ºC, the limit defined for marine heatwaves in Baynes Sound.

*V. aestuarianus* is most abundant at site BS17, at 5m depth (Figure 2.3, Figure 2.4). Temperatures at BS17 were more often near 19 ºC than they were at BS14 or BS15 in

2019 (Figure 2.3, Figure 2.4). Abundance of *V. aestuarianus* is higher at the shallowest depth measured, 5m, for all sites (Figure 2.3 and Figure 2.4).

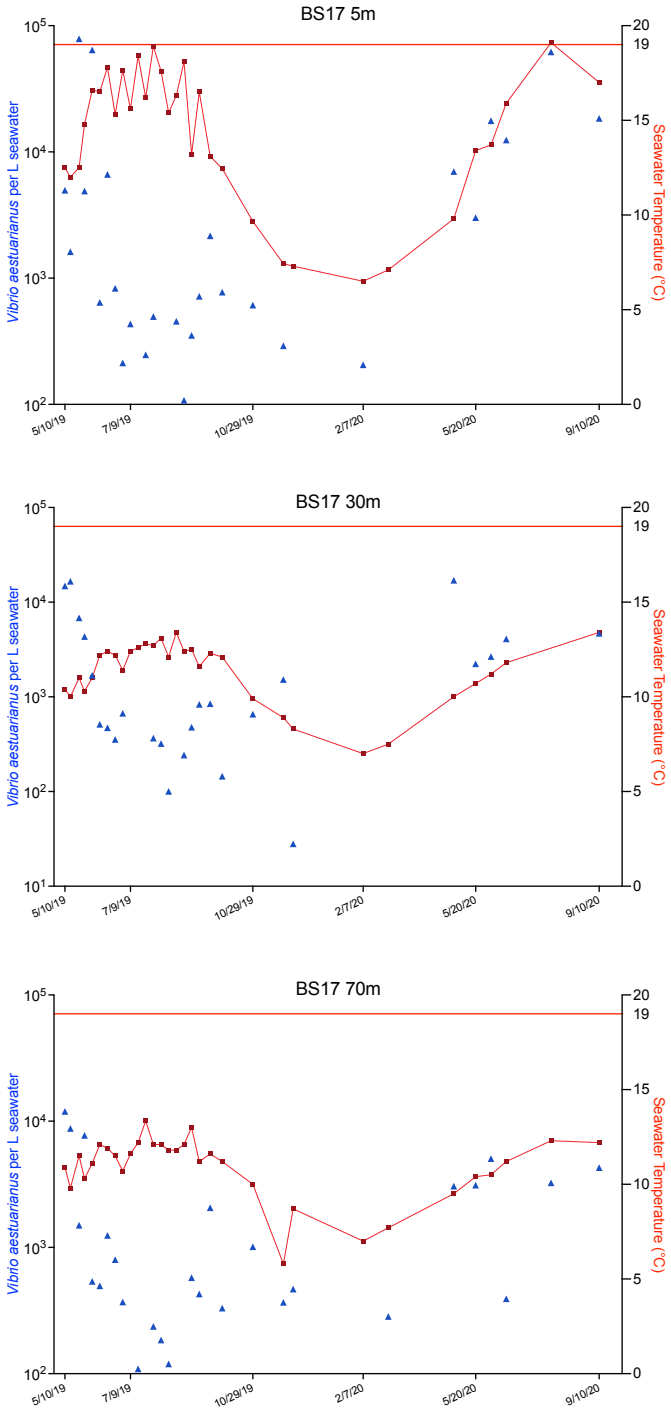



**Figure 2.3:** Abundance of *V. aestuarianus* at site BS17 (Figure 1.1) and seawater temperature (ºC) measured in the Niskin bottle sample, horizontal line at 19 ºC. Measurements shown at 5m, 30m, and 70m depth below sea surface.

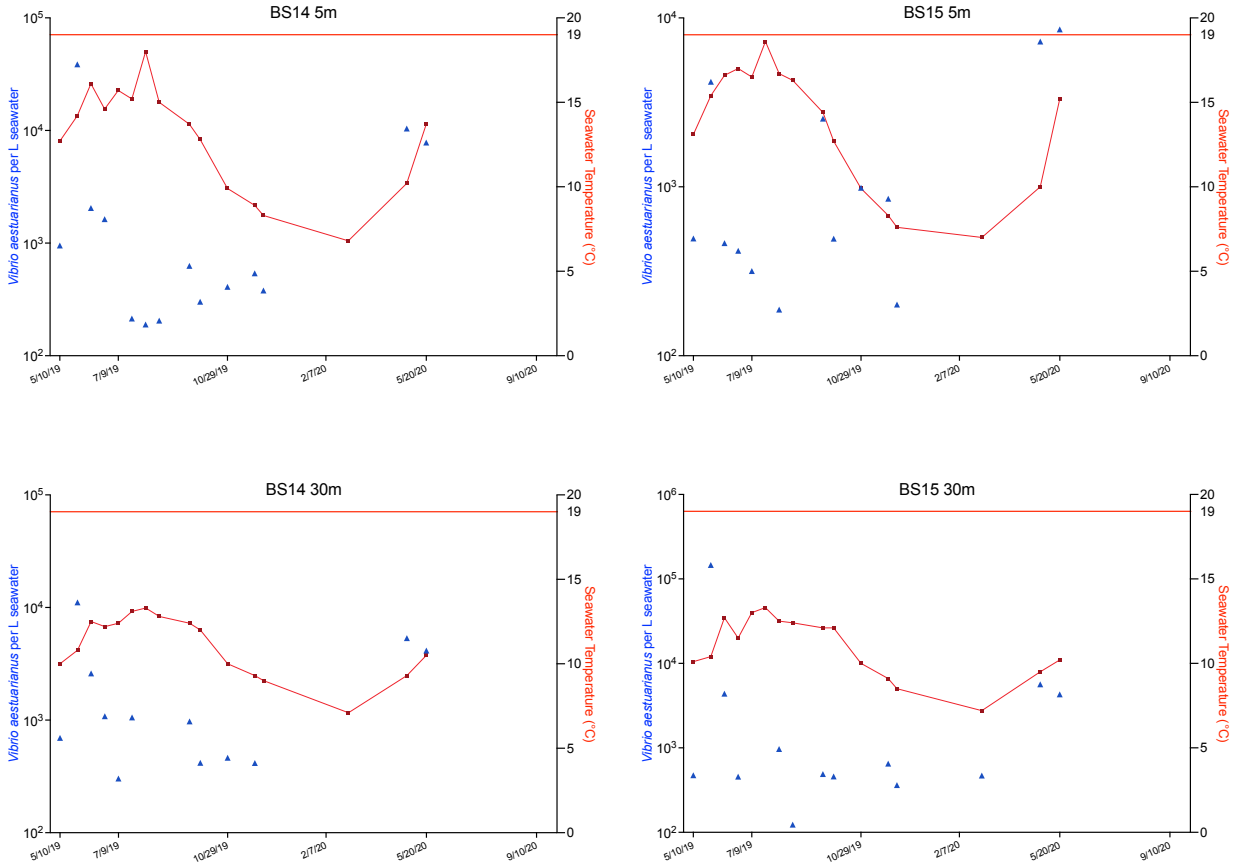



**Figure 2.4:** Abundance of *V. aestuarianus* at sites BS14 and BS15 (Figure 1.1) and seawater temperature (ºC) measured in the Niskin bottle sample, horizontal line at 19 ºC. Measurements shown at 5m and 30m depth below sea surface.

Sea surface temperature data collected daily at Chrome Point Lighthouse (Lat 49.472, Long -124.6845) located on an island at the South end of Baynes Sound is shown in Figure 2.5. Summer sea surface temperatures were lower with fewer heatwave events reaching over 19 ºC than the two previous years.

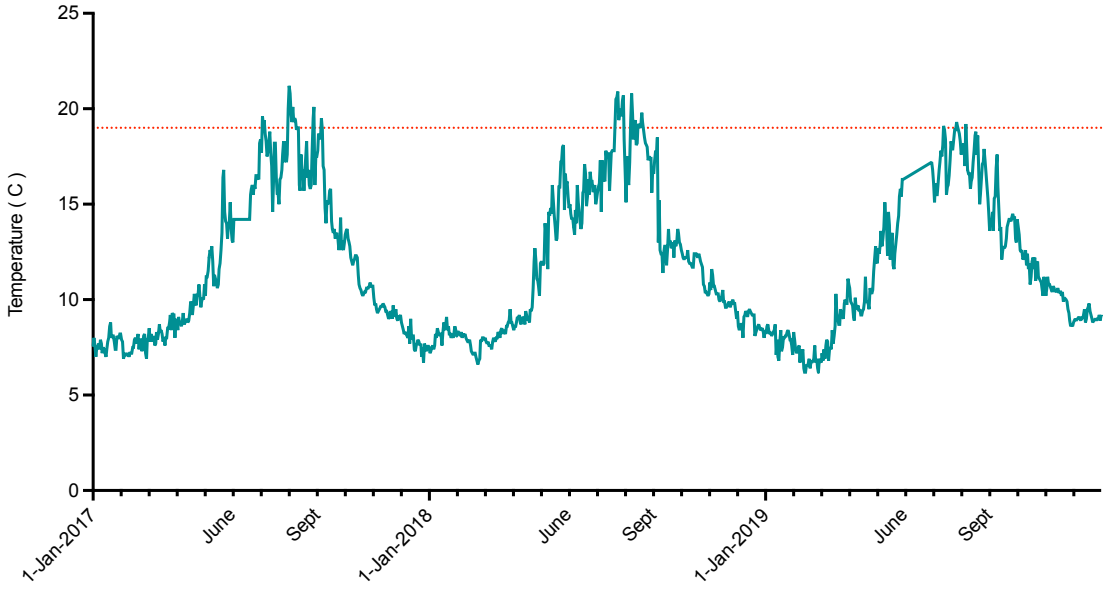



**Figure 2.5:** Daily sea surface temperature measured by the Canadian Department of Fisheries and Oceans at Chrome Point Lighthouse (Lat 49.472, Long -124.6845), with line at 19 ºC.

In a linear regression, seawater temperature was shown to have a significant positive relationship to the total bacteria found in a sample ($p = 0.0023$), and the total amount of *Vibrio spp.* ($p = <0.0001$) in each liter of seawater measured (Figure 2.6 and Table 2.5). However, corresponding R squared values were lower than the acceptable threshold (0.2), R squared for total bacteria was 0.0234, and for total *Vibrio* was 0.103. *Vibrio aestuarianus* was not found to have a significant linear relationship to temperature (Figure 2.6 and Table 2.5). Interestingly, variance in the amount of total bacteria 16s around the regression line increased as temperature increased (Figure 2.6).

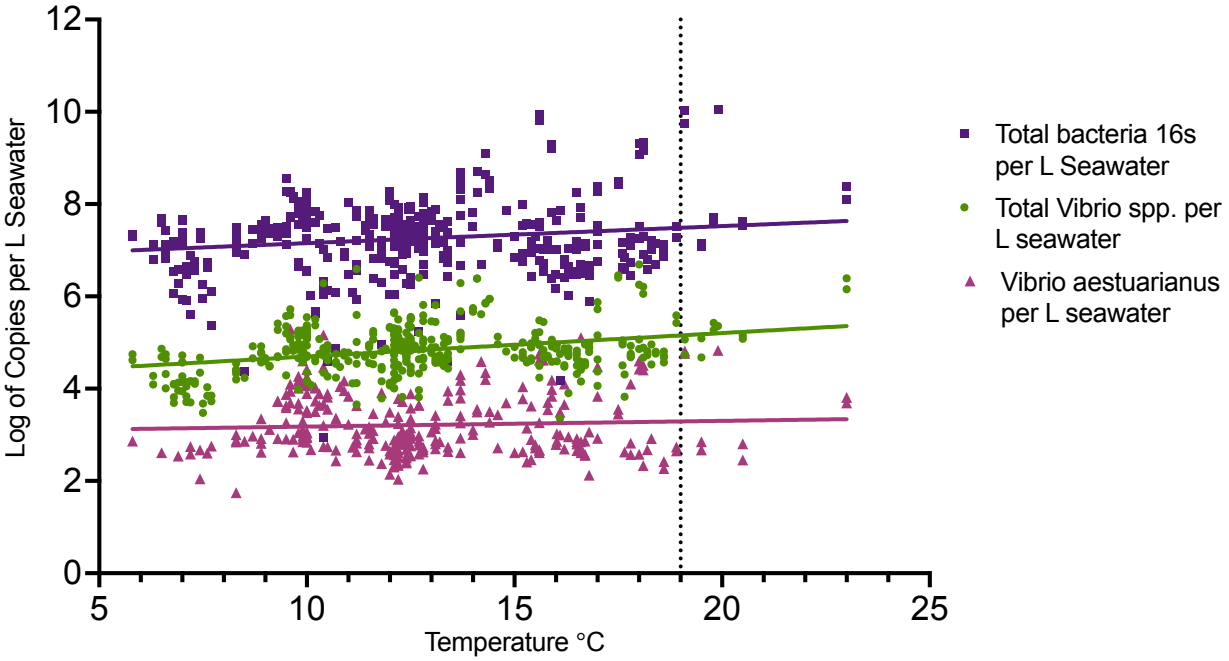



**Figure 2.6:** All data, 2019-2021. Scatter plot of the log of the average number of copies of each assay at a particular temperature, measured in the Niskin bottle at time of collection. Vertical line at 19 ºC. Linear regression lines shown.

**Table 2.5:** All data, 2019-2021. Linear regression of the log of the average number of copies of each assay at a particular temperature.

| | Total *Vibrio* | Total Bacteria 16s | *Vibrio aestuarianus* |
|---|---|---|---|
| 95% Confidence Intervals: | | | |
| Slope | 0.03589 to 0.06569 | 0.01325 to 0.06068 | -0.01331 to 0.03808 |
| Y-intercept | 3.994 to 4.384 | 6.473 to 7.095 | 2.714 to 3.395 |
| X-intercept | -122.0 to -60.88 | -534.5 to -106.8 | -infinity to -71.48 |
| | | | |
| Goodness of Fit | | | |
| R squared | 0.103 | 0.0234 | 0.00335 |
| Sy.x | 0.5117 | 0.8159 | 0.6708 |
| | | | |
| Is slope significantly non-zero? | | | |
| F | 44.91 | 9.393 | 0.9008 |
| DFn, DFd | 1, 391 | 1, 392 | 1, 268 |
| P value | <0.0001 | 0.0023 | 0.3434 |
| Deviation from zero? | Significant | Significant | Not Significant |
| | | | |
| Equation | Y = 0.05079*X + 4.189 | Y = 0.03697*X + 6.784 | Y = 0.01239*X + 3.055 |

Data from 2020 was analyzed separate from 2019 to show possible effect of the lower sea surface water temperatures which occurred in 2019. In 2020, total bacteria (P = <0.0001, R squared = 0.384) total *Vibrio (P* = <0.0001, R squared = 0.409) and *Vibrio aestuarianus* (P = 0.0013, R squared = 0.367) were all found to have significant linear relationships to temperature, meaning that as temperature increased, the amount of bacteria in the samples increased (Figure 2.7 and Table 2.6). There is not a significant linear relationship between the log of total bacteria (16s) per L of seawater and salinity (p = 0.9745, R squared = 4.533e-005), or between the log of total bacteria (16s) per L of seawater and $\Omega_{arag}$ (p = 0.1049, R squared = 0.1151).

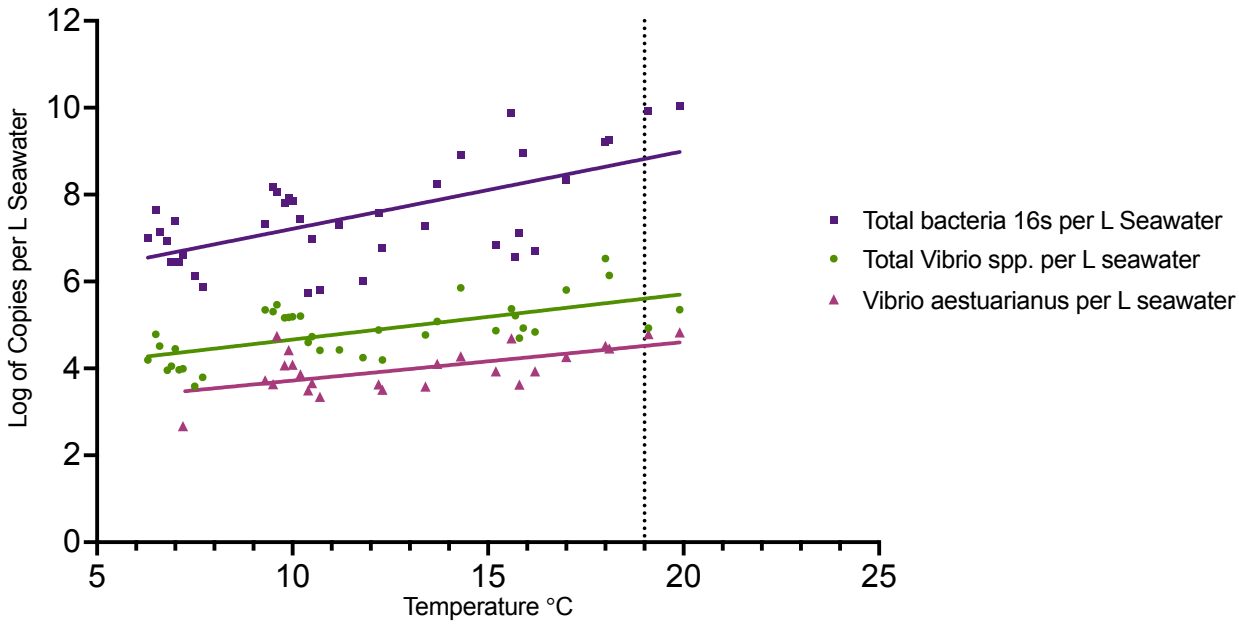



**Figure 2.7:** Data from 2020 only. Scatter plot of the log of the average number of copies of each assay at a particular temperature, measured in the Niskin bottle at time of collection. Vertical line at 19 ºC. Linear regression lines shown.

**Table 2.6:** Data from 2020 only. Linear regression of the log of the average number of copies of each assay at a particular temperature.

| | Total *Vibrio* | Total Bacteria 16s | *Vibrio aestuarianus* |
|---|---|---|---|
| 95% Confidence Intervals | | | |
| Slope | 0.06202 to 0.1469 | 0.1022 to 0.2552 | 0.03844 to 0.1389 |
| Y-intercept | 3.095 to 4.145 | 4.480 to 6.372 | 2.151 to 3.516 |
| X-intercept | -66.45 to -21.19 | -61.93 to -17.67 | -90.93 to -15.58 |
| | | | |
| Goodness of Fit | | | |
| R squared | 0.409 | 0.384 | 0.367 |
| Sy.x | 0.5131 | 0.9249 | 0.428 |
| | | | |
| Is slope significantly non-zero? | | | |
| F | 24.91 | 22.44 | 13.34 |
| DFn, DFd | 1, 36 | 1, 36 | 1, 23 |
| P value | <0.0001 | <0.0001 | 0.0013 |
| Deviation from zero? | Significant | Significant | Significant |
| | | | |
| Equation | Y = 0.1045*X + 3.620 | Y = 0.1787*X + 5.426 | Y = 0.08866*X + 2.833 |

Temperature is the only water quality measure collected in this study that has a significant relationship to abundance of bacteria in Baynes Sound. pH and salinity remained relatively consistent and did not correlate to bacterial abundance (Figure 2.8). More data would be required to determine if there is a correlation between aragonite saturation and total bacteria.

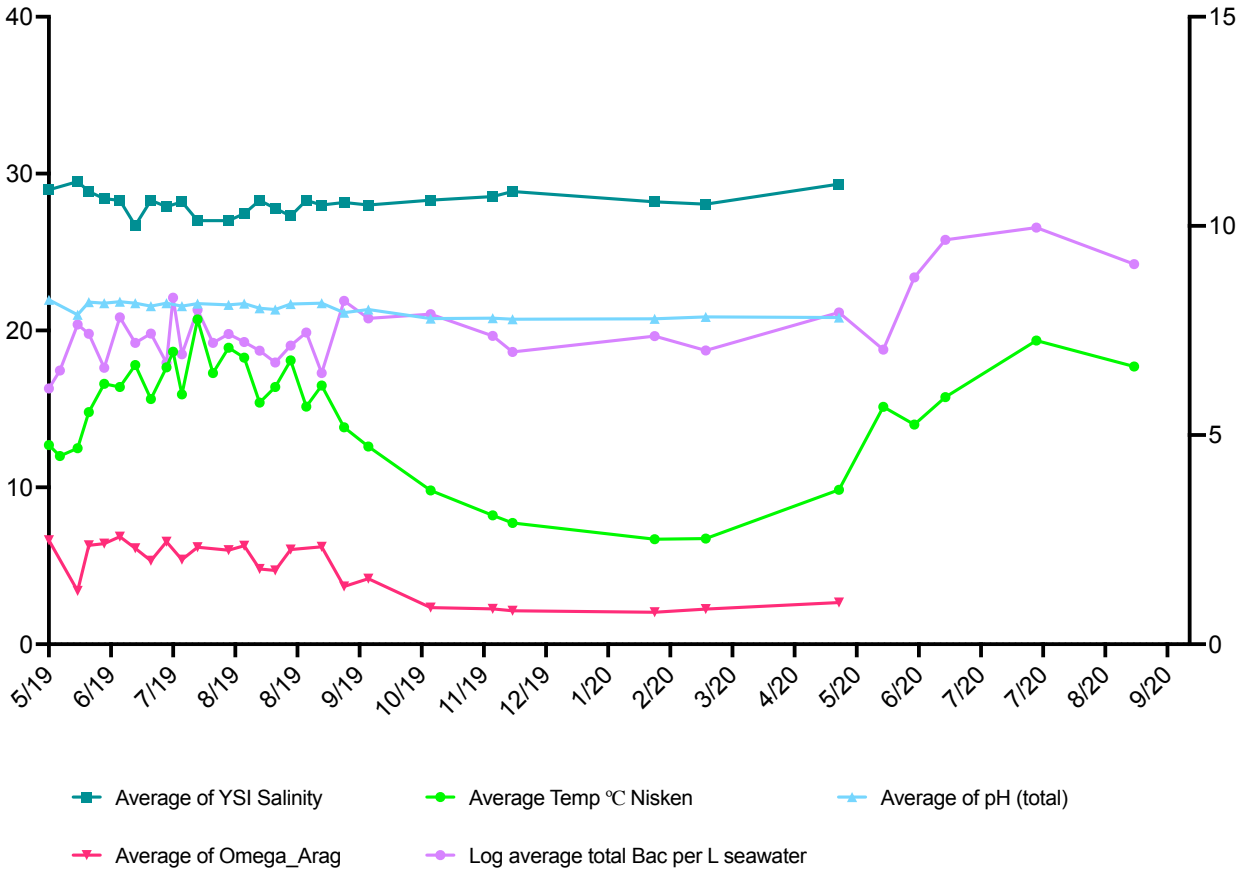



**Figure 2.8:** Average water quality measures for 0m, 3m, and 5m observations, averaged by date.

### 2.4.1b Farm Site Oyster Survival and Growth

Oyster wet weight was measured over time at each farm site (Figure 2.9). Although the averages were different between sites, they were not significantly different over the course of the summer or between any consecutive weeks. No farm site was shown to have a significant advantage to others in promoting oyster growth over the course of the summer.

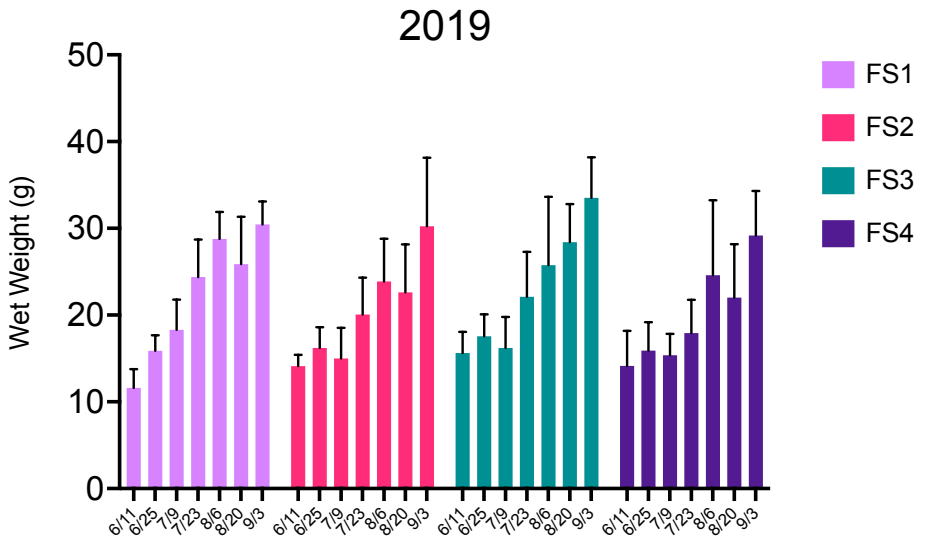



**Figure 2.9:** Average wet weight (N=9 per site per day) measured in grams at 4 farm sites measured over time between 6/11/19 and 9/3/19.

No significant difference shown between survival at the various sites (Figure 2.10). There was a significant difference between average survival at all sites at time points 1 and 2 with a P value of 0.0297, and between 8 and 9 with a P value of 0.0377 (Figure 2.11). The decline in survival between time points 8 and 9 is significant, but the reduction in survival rate of ~2% is not large enough to constitute a true summer mortality event. This same timepoint, measured on 9/10/2019 also showed an increase in growth rate at all sites, although not a statistically significant one, which could correlate to the slight decrease in survival measured at that same time period (Figure 2.11).

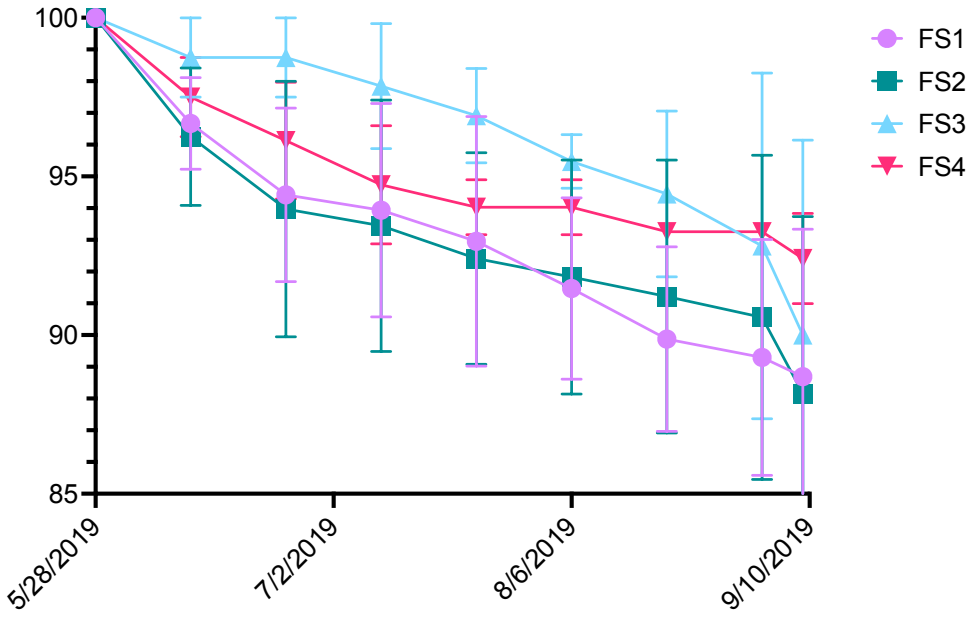



**Figure 2.10:** Average percent survival by farm site. Average of 3 trays per site, with 80 oysters in each tray.

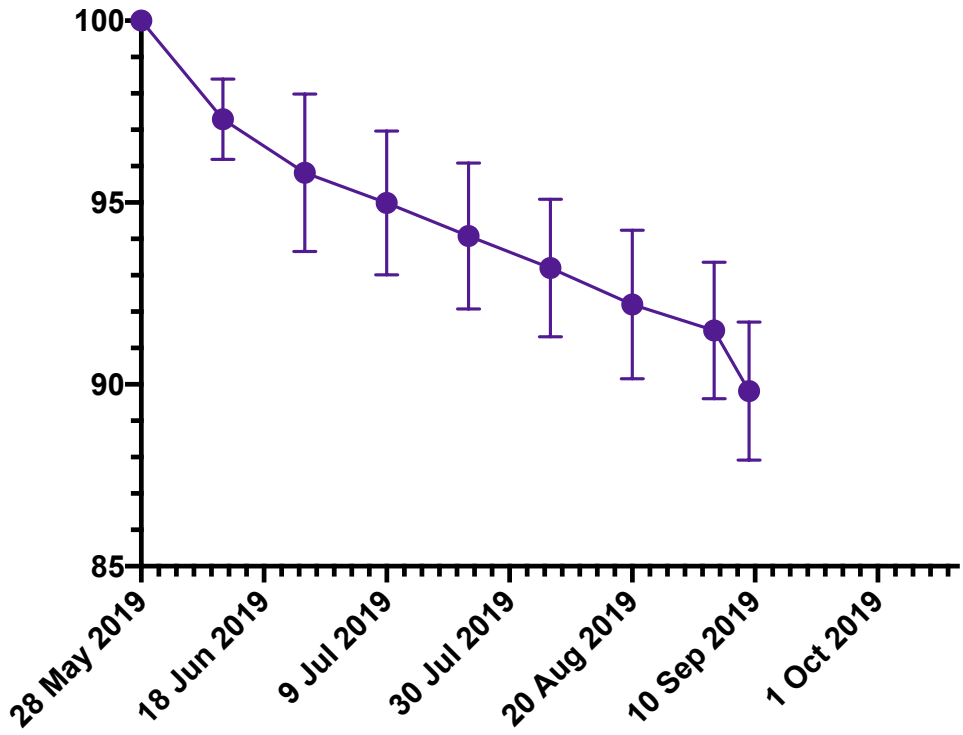



**Figure 2.11:** Average oyster survival from all 3 traps of 80 oysters at all 4 farm sites.

### 2.4.2 Marine Snow Lab Challenges

#### 2.4.2a Trial 1: Marine Snow Flagellate vs. Diatom

No significant difference was found between treatments, but there was mortality in the treatments which included the flagellate *T. lutea*, while no mortality was found in treatments with planktonic *V. aestuarianus* or the diatom *T. pseudonanna* (Figure 2.12).

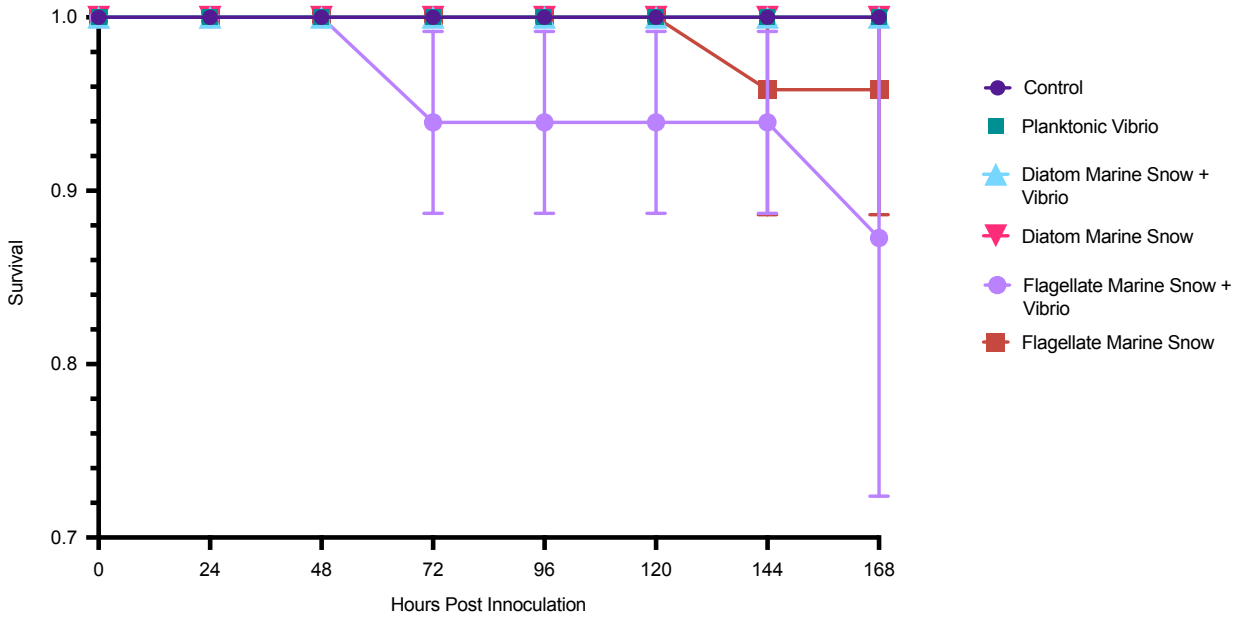
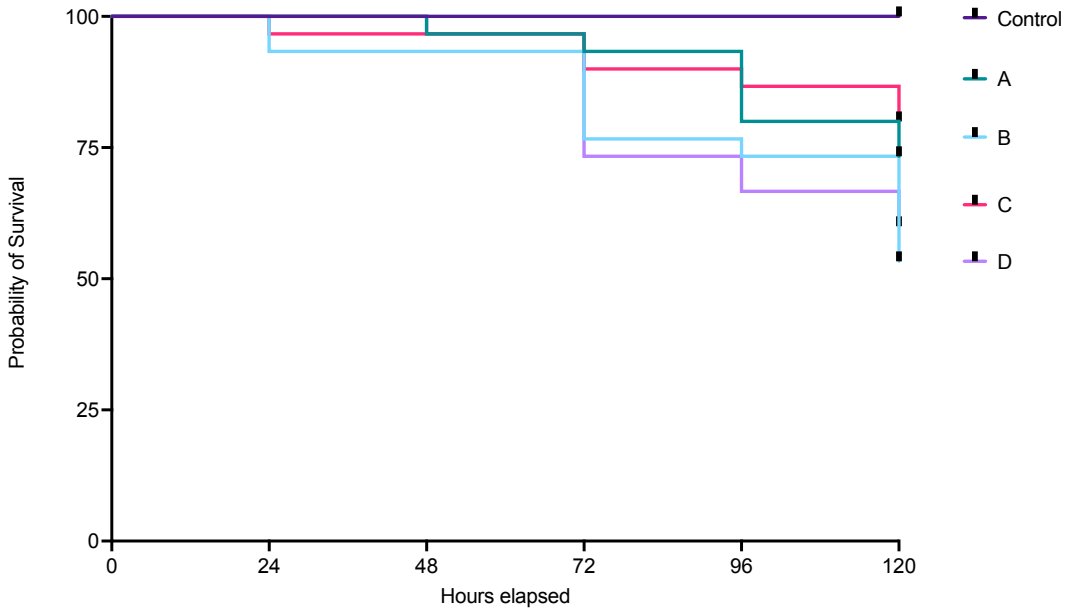



**Figure 2.12:** Survival rate for all treatments in Marine Snow Trial 1. N = 20, 3 tanks per treatment. Details of treatments in Table 2.2.

#### 2.4.2b Trial 2: Marine Snow + Stress

Trial 1 failed to achieve significant mortality for any treatment protocol. Because bacterial infection alone was not enough to induce a mortality event, for Trial 2 we introduced stress which mimicked typical oyster husbandry techniques. Treatments B and D showed the lowest probability of survival in the Marine Snow + Stress Challenge, Trial 1 (Figure 2.13). These two treatments both included stress as defined in the methods section of this paper. Treatment B showed the lowest survival proportion (55.3) despite the fact that this treatment did not include *Vibrio aestuarianus*, indicating that

husbandry stress may be a large factor in summer mortality (Figure 2.13). P values were calculated for comparisons between all treatment combinations, with the comparison between B and C being the only significant result (Table 2.7). The difference between C and D is nearly significant with a P value of 0.0911. Treatment C was inoculated with *Vibrio aestuarianus*, but was not placed under stress, while treatment D was both inoculated with *V. aestuarianus* and placed under stress (Table 2.3). This could indicate that the combination of stress and *V. aestuarianus* infection is more deadly than infection alone.




| | A | B | C | D | Control |
|---|---|---|---|---|---|
| Survival proportion at 120 h: | 73.33 | 53.33 | 80 | 60 | 100 |

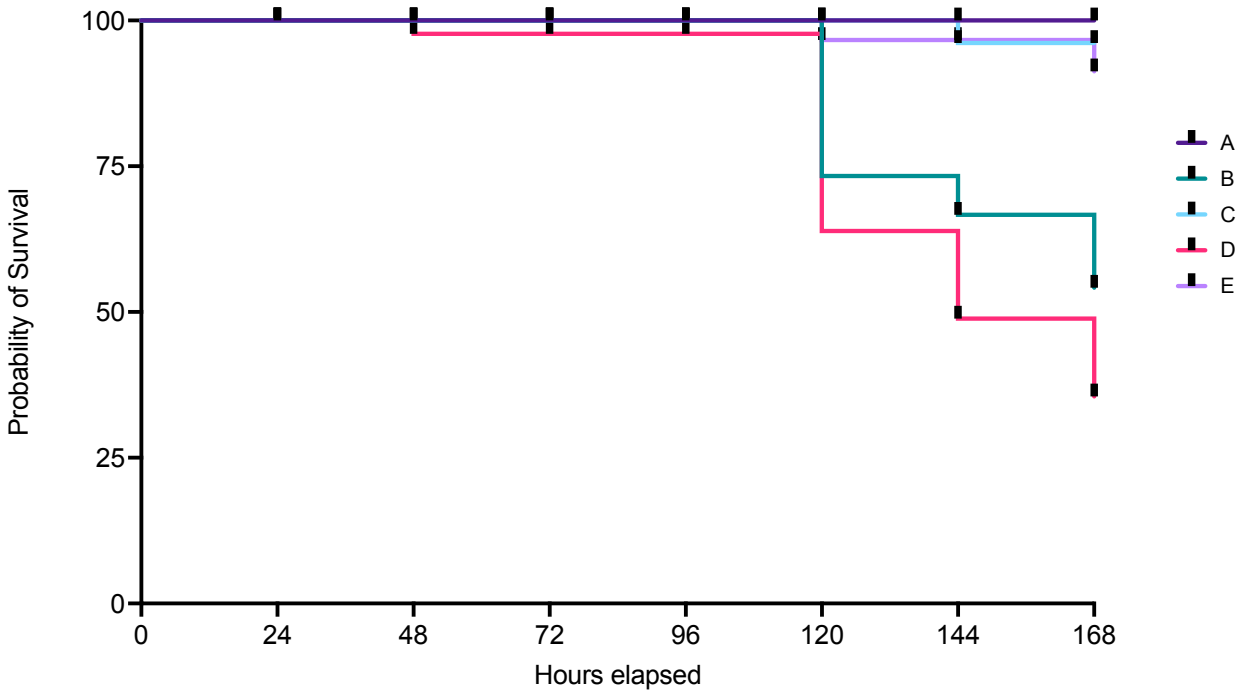
**Figure 2.13**: Probability of survival for Trial 2: Marine Snow + Stress. Details of treatments in Table 2.3

**Table 2.7:** P values of individual treatments compared using the adaptive method of Benjamini, Krieger and Yekutieli, with Q value set at 30% (Benjamini et al., 2006).

| Treatments compared | P Value | Q value |
|---|---|---|
| A+B | 0.1162 | 0.2518 |
| A+C | 0.5732 | 0.7452 |
| A+D | 0.2306 | 0.3747 |
| B+C | 0.0342 | 0.2223 |
| B+D | 0.7021 | 0.7606 |
| C+D | 0.0911 | 0.2518 |

### 2.4.2c Trial 3: Marine Snow + Stress Pre and Post Inoculation

Treatments where animals were both stressed and inoculated with *V. aestuarianus* (B and D) show the lowest survival proportion (Figure 2.14). Animals in treatment group D were stressed 24h post-inoculation, animals in group B were stressed 24h prior to inoculation. Between these two treatment groups, there is not significantly difference (p value =0.1349 see Table 2.8). That said, treatment group D showed nearly a 20% lower probability of survival than treatment group B, so it is possible that husbandry stress after infection with *V. aestuarianus* could cause more mortality than stress prior to infection. In our results, treatments with *V. aestuarianus* and stress are significantly different than treatments with just stress without *V. aestuarianus* (Table 2.8). Although treatments with stress without *V. aestuarianus* (C and E) showed more mortality than the control (Figure 2.14), the difference is not statistically significant (A + E have p value of 0.1484, A + C have p value of 0.3268 see Table 2.8).




| | A (control) | B | C | D | E |
|---|---|---|---|---|---|
| Survival proportion at 168 h: | 100 | 54.166 | 96.15 | 35.53 | 91.29 |

**Figure 2.14:** Probability of survival for Marine Snow + Stress Trial 2. Details of treatments in Table 2.4

**Table 2.8:** P values of individual treatments compared using the adaptive method of Benjamini, Krieger and Yekutieli, with Q value set at 5% (Benjamini et al., 2006).

| Treatments compared | P Value | Q value |
|---|---|---|
| B+C | 0.0003 | 0.00021 |
| B+D | 0.1349 | 0.05666 |
| B+E | 0.0019 | 0.001 |
| C+D | 0.00001 | 0.00001 |
| C+E | 0.5157 | 0.1805 |
| D+E | 0.00001 | 0.00001 |

## 2.5 DISCUSSION

The main findings of this study indicate that there is a correlation between seawater temperature and abundance of bacteria, *Vibrio*, and specifically *V. aestuarianus* in Baynes Sound, BC. This is consistent with Le Roux et al.'s 2016 finding that *Vibrio* infections of shellfish are expected to become more frequent as coastal waters

warm, supporting larger *Vibrio* populations (Le Roux et al., 2016). This relationship is more significant in data collected in 2020 than in 2019 observations. This finding is consistent with the fact that there were fewer summer mortality events at oyster farms in Baynes sound in 2019, when compared with 2018 or 2020 (anecdotal, BC Shellfish Growers Association). For this reason, we believe that our data could reflect an accurate indication of the harmful bacteria in the sound in 2019. While our data did not continue through the summer of 2020 (complication of field sampling due to Covid-19), sea surface temperate is monitored daily at the mid-day high tide from Chrome Point Lighthouse at the southern end of Baynes Sound (Lat 49.472, Long -124.6845). We obtained this dataset from the Canadian Department of Fisheries and Oceans (https://open.canada.ca/data/en/dataset/719955f2-bf8e-44f7-bc26-6bd623e82884) and found that the sea surface temperatures in the summers of 2017 and 2018 reached higher high temperatures, and did so more frequently for longer duration than in the summer of 2018 (Figure 2.5). This could indicate a connection between the 2019 lower occurrences of major summer mortality in the region and lower seawater temperatures. Further study is warranted to determine if *Vibrio* is connected to these events. Additionally, data about seawater quality including pH, salinity, and $\Omega_{arag}$ were not processed for samples collected after May 2020, which does not allow us to draw strong conclusions about their correlation to *Vibrio* abundance.

We did not detect a significant difference between oyster growth rates or survival at four sites distributed throughout the region, indicating that all parts of the sound studied are similarly useful for farmers. The farm site oysters survival experiment showed that

mortality increased just as growth rates spiked (Figure 2.9 + Figure 2.11), which could indicate a correlation between increased oyster growth and increasing mortality. While we did not record a massive summer mortality event in our experimental oysters, about 10% of oysters died over the course of the experiment (Figure 2.11) which is significant summer mortality and could have a significant economic impact on the bottom line for a farmer. The decline in survival between time points 1 and 2 could be explained due to the shock of putting the oysters into the trays on the farm and therefore is not likely a summer mortality event (Figure 2.11). While we did not find a significant difference in survival between sites, real-world summer mortality events occur in a localized manner, effecting individual farms, even localizing to individual stacks of oysters. This suggests that infection is transmissible between oysters, and if a dense population has individuals with higher susceptibility to infection, they may increase the bacterial load in the water, causing oysters which wouldn't succumb to a lower bacterial load to become sick and die. Additionally, summer mortality does seem to effect older oysters more severely than younger ones (Chaney and Gracey, 2011), suggesting that size or maturity could be a contributing factor to survival of summer mortality.

Although our oysters all came from the same hatchery stock, parental genetics were not controlled. An area for further study would be to investigate if size contributes to mortality within genetic families of the same age. Additional questions arise about the potential for genetic breeding of resistance to extreme temperature events, as well as the potential for early-life spat conditioning, or exploring the limits of *C. gigas'* epigenetic plasticity to increase tolerance to climate change conditions. The majority of *C. gigas*

cultivated in Baynes Sound originate from aquaculture hatcheries, where seawater temperature is tightly controlled. This suggests a possible link between conditions experienced in early-life, and the limits of thermal tolerance for farmed oysters. We will explore these questions further in Chapter 3.

Our first marine snow lab trial found that there was mortality in the treatments which included the flagellate *T. lutea*, while no mortality was found in treatments with planktonic *V. aestuarianus* or aggregated which included the diatom *T. pseudonanna.* While this difference was not significant statistically, it could indicate that there is a mechanism of interaction between *V. aestuarianus* and flagellate microalgae which causes the bacteria to become more virulent to *C. gigas*. Another hypothesis is that perhaps ingesting *T. lutea* makes *C. gigas* more susceptible to infection. The results of this challenge could indicate that that *V. aestuarianus* infects and kills oysters more readily when incorporated into a marine snow aggregate than when planktonic. *C. gigas* are suspension feeders which uptake particles selectively, based on size (Froelich et al., 2013). Therefore, planktonic bacterial cells are taken up at a lower rate than those associated with a marine aggregate (Froelich et al., 2013). *C. gigias* survival to *V. aestuarianus* infection is dose dependent, so it is possible that the incorporation of *V. aestuarianus* into marine snow could increase the effective dose enough to induce mortality. That said, it is likely that this trial did not achieve statistical significance due to an insufficiently high dose of bacteria in any of the treatments. More study would be required to determine if this result is meaningful.

Typically, the microalgae blooms in Baynes Sound follow a régime of diatoms in the spring, and flagellates in the summer. Our study does not definitively confirm the hypothesis that *V. aestuarianus* in aggregate with flagellate microalgae is more deadly to *C. gigas* than *V. aestuarianus* in aggregate with a diatom. More study would be needed to prove or disprove this hypothesis. Additionally, if true, more study would be warranted to determine the cause.

Our lab trials which combined physical stress with bacterial infection showed that physical stress in the form of desiccation, increased temperature, and shaking was a significant factor in causing mortality, particularly if combined with *V. aestuarianus* infection. This is consistent with the generally held consensus that summer mortalities in *C. gigas* seems to be complex and multicausal, occurring because of interactions between the physiological status of the animal, the environment, and pathogens such as bacteria of the genus *Vibrio* (Samain and McCombie, 2008; Sauvage et al., 2009). Additionally, we found that stressing oysters with husbandry practices after infection had 20% more mortality than the group stressed prior to infection, although the result was not statistically significant. Understanding that oysters in cultivation must periodically be checked throughout the summer, these results indicate that minimizing handling and desiccation during summer months could result in less mortality. Monitoring seawater temperature and delaying husbandry techniques which cause stress until periods of cold or normal temperature could also minimize risk of mortality. If nothing else, oysters should not be handled immediately after a marine heatwave. More study would be

needed to determine how long after a heatwave farmers should wait before handling their oysters.

Injecting oysters with bacteria such as *V. aestuarianus* is standard practice for survival experiments in lab settings. This does not mimic real-world infection mechanisms and requires extensive time and resources for trials with large numbers of subjects. An alternative to injection is to add free-floating bacteria to seawater, however this approach is limited by the low uptake levels for planktonic bacterial cells. Incorporating bacteria into a marine aggregate appears to be a way to increase uptake of bacteria by bivalves in laboratory settings.

Baynes Sound, BC is an important region for Canadian oyster farming. Our study is the first long-term spatiotemporal study of the pathogen *V. aestuarianus* in the region. Our results indicate that as global warming continues to increase temperatures in the region, summer mortality in C. gigas induced by *V. aestuarianus* will become more common. To mitigate this, farmers can avoid stressing their oysters after heatwaves, when animals may have been exposed to *V. aestuarianus.*

# 3. CHAPTER 3: DESIGNING A PROTOCOL TO ESTIMATE GENETIC PARAMATERS OF RESISTANCE TO *VIBRIO AESTUARIANUS* IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* AND UNCOVERING THE ROLE OF OYSTER SIZE ON SURVIVAL

## 3.1 ABSTRACT

The Pacific oyster (*Crassostrea gigas*) is one of the most important aquaculture species in the world. However, this industry has been economically hampered by mass summer mortality caused by pathogenic factors including *Vibrio* bacteria, specifically *Vibrio aestuarianus*. Because *C. gigas* lack adaptive immune systems, selective breeding of oyster with high resistance to *V. aestuarianus* bacteria would be an effective and sustainable approach to reducing summer mortality. Estimation of genetic parameters for disease resistance is a critical step toward selective breeding. A preliminary investigation found that inoculation dose is a significant factor in mortality. In order to accurately calculate heritability of resistance to *V. aestuarianus*, a lab protocol needed to be designed which allows dose to be controlled and does not bypass the oyster's immune system's first lines of defense. Currently popular modes of inoculation do not allow researchers to both control dose per oyster and allow the oyster to take in the pathogen via a pathway that mimics real-world infection. In this study, we tested 32 full-sib families using *C. gigas* with diverse genetic backgrounds and performed an artificial infection experiment to assess disease resistance among families and estimate genetic parameters for resistance to *V. aestuarianus* infection. To do this effectively, we designed a repeatable protocol which isolates oysters into individual dishes and uses a titre of planktonic vibrio for inoculum. The survival rate of the 32 full-sib families ranged from 8.3% to 83.3%, suggesting high levels of phenotypic variation in resistance to *V.*

*aestuarianus* infection. Genetic parameters for resistance to *V. aestuarianus* infection revealed low to moderate heritability. The heritability of survival on the observed scale was estimated to be 0.095 (SE = 0.043), and the heritability on the underlying liability scale was estimated to be 0.15 (SE = 0.068). We also found a strong negative correlation between oyster size and survival, with a gram of additional weigh creating a 73% increase in the risk of death. Fast growth and large size are two traits which have been explicitly bred into *C. gigas* stock. Our work suggests that breeding a slower-growing, smaller animal may reduce summer mortality.

## 3.2 INTRODUCTION

The Pacific oyster, *Crassostrea gigas* has been introduced to every inhabited continent, forming the basis of a global shellfish industry worth $US 3.5 billion (FAO 2020). The natural distribution of *C. gigas* is the intertidal zone and estuaries of Japan and costal Asia (Cognie, 2006; Quayle, 1988; Orensanz et al., 2002). *C. gigas* is the main oyster species cultivated worldwide, accounting for 98% of the global oyster production, an industry worth $US 1.4 billion (FAO 2018, Azéma et al, 2017). Canadian oyster production is valued at $CAD 54 million (DFO, 2019) with British Columbia accounting for $CAD 15M of that total. The Baynes Sound region of British Columbia produces roughly 50% of the oysters grown in BC annually. Climate change is affecting seawater temperatures in the region. The history of rising seawater temperatures and marine heatwaves in the region is well documented (reviewed by Okey et al., 2014). Most recently, in late June and early July 2021, temperatures reach significantly higher than

ever before, reportedly causing die offs of up to 70% of cultivated oysters, along with other shellfish (anecdotal BCSGA).

Major summer mortality events have been documented in farmed *C. gigas* since the 1950's in Japan (Imai et al., 1965), USA (Chaney and Gracey, 2011), Europe (Soletchnik et al., 2007), Australia (Li et al., 2009) and Canada (Cassis et al., 2011). As discussed in Chapter 2, warming seawater temperature seems to be a primary driver in the increased abundance of *Vibrio* sequences in Baynes Sound. In general, rising seawater temperatures have been linked to increased disease incidence in marine ecosystems (reviewed by Burge et al., 2014). Marine heatwaves and warming seawater temperatures have been shown to significantly increase death in *C. gigas* due to a proliferation of bacteria (from 4.3% to 77.4%), notably an increase in the proportion of *Vibrio* bacteria when seawater is rapidly warmed (Green et al., 2019; Wendling et al, 2013).

Over the last decade, bacterial analysis of Pacific oysters during summer mortality events in Europe has routinely detected *Vibrio* spp. (Garnier et al, 2007; Samain et al, 2008). *Vibrio* bacteria are Gram-negative rod-shaped bacteria that are widespread in marine environments. Strains of *Vibrio aestuarianus* are associated with mortality events in adult farmed oysters in France and other European countries (Gay et al., 2004; Lemire et al., 2015; Travers et al., 2017). Of the species identified, *V. aestuarianus* and members of the *V. splendidus* clade are the most well-studied in Summer Mortality Syndrome (Garnier et al, 2007). Concentrations as low as $5 \times 10^6$ *V. aestuarianus* per oyster can cause >90% mortality (Gay et al, 2004; Travers et al, 2017). Finally, a comparative laboratory

challenge study examining reproductive investment, thermal stress and *Vibrio* infection concluded that these factors contribute cumulatively to oyster mortalities, and that pathogenic *Vibrio spp* infection was of the highest importance (Wendling et al, 2013).

The shellfish industry in British Columbia has identified genetic selection as a priority to reduce summer mortality of farmed *C. gigas*. Common methods used to fight disease in traditional agriculture are vaccination and chemotherapeautics. Oysters lack adaptive immune systems so cannot be vaccinated and are grown in an open ocean environment where it is not possible to eliminate their exposure to pathogens or treat with chemotherapeautics such as antibacterial disinfectants (Prado-Alvarez et al., 2016; Alfaro et al., 2019). Therefore, genetic improvement of disease resistance of oyster may be the only currently viable method to solve the disease problem in farmed *C. gigas* (Dégremont, 2013; Stear et al., 2001; Yáñez et al., 2013). Selective breeding is only an effective technique if the heritability of the desired trait is sufficiently high, therefore it is critical to estimate the genetic parameters for the target trait (Taylor et al., 2009; Liang et al., 2017; Sun et al., 2015; Wang and Ma, 2019). To date, most selective breeding of *C. gigas* has focused on traits that increase marketability and profitability including increasing growth traits, shell color, and meat composition (Li et al., 2011; Wang et al., 2012; Zhang et al., 2019a, Zhang et al., 2019b; Wan et al., 2017; Xu et al., 2019b; Han and Li, 2020; Wan et al., 2020). For disease resistance breeding, the genetic improvement of resistance to *Ostreid herpesvirus 1* (OsHV-1) has been well studied (Dégremont et al., 2015; Azéma et al., 2017; Camara et al., 2017; Azéma et al., 2017a; Azéma et al., 2017b; Dégremont et al., 2019). *Vibrio aestuarianus* has received less attention, but

preliminary studies indicate that resistance to infection by *V. aestuarianus* and other *Vibrios* may be heritable at high enough levels to make breeding for this trait worthwhile. One study using the controlled infectious challenges showed that resistance was heritable in adult *C. gigas* in 1 of the 2 genetic stocks tested, with realized heritability estimated at the first generation of mass selection for the 1 stock which could breed resistance ranging between 0.05 to 0.30 (Degremont et al., 2020). They also found that the realized heritability for resistance was higher for oysters selected for dual resistance to both OsHV-1 and *V. aestuarianus*, ranging from 0.47 to 0.80, than those only selected for *V. aestuarianus* (Degremont et al., 2020). This result suggests that selection for dual resistance in *C. gigas* could limit the impact of both OsHV-1 and *V. aestuarianus* on summer mortality (Degremont et al., 2020). Another study focused on heritability of resistance to *V. alginolyticus* tested 52 families and found high levels of phenotypic variation in survival (0% to 56.25%) but low to moderate heritability, ranging from 0.133 to 0.257 (Zhai et al., 2021). The same study found that the genetic and phenotypic correlation between resistance to *V. alginolyticus* and growth traits were low, suggesting the feasibility of simultaneous genetic improvement of both growth, which is a desirable trait for farmers, and resistance traits. To my knowledge, the correlation between resistance to *V. aestuarianus* and growth has not been examined.

As discussed above, accurately calculating heritability of disease resistance is key to helping the economically important aquaculture industry survive summer mortality in our changing climate. However, summer mortality has been shown to occur based on complex unpredictable spatial and temporal dynamics *in situ*, which precludes selection

of broodstock solely upon the basis of field trials (Lang et al., 2009). To accurately calculate heritability of summer mortality resistance, controlled studies must be performed in the lab. Historically there have been two widely used mechanisms for infecting *C. gigas* with pathogens for lab trials which each introduce uncontrolled elements to the experimental design. [1] Direct injection of all animals: researchers inject adult oysters with the pathogen, directly in the adductor muscle. This method is highly labor intensive, limiting the practicality of conducting large lab trials with many subjects. It also requires the subjects to be large enough to be injected, so precludes the study of spat and younger *C. gigas*. It requires that the shell be ground down to create a hole in the shell, introducing an element of physical stress. Most importantly, direct injection does not mimic real-world disease transmission and bypasses several immune mechanisms (De Decker & Saulnier, 2011). [2] Cohabitation: One or more "donor" *C. gigas* is injected with the pathogen and is placed in a shared tank with other oysters, shedding bacteria and infecting the healthy oysters. Bacterial shedding from the infected donor oyster will not be consistent across all donors, creating a potentially significant difference in dose per replicate tank (De Decker & Saulnier, 2011; Azéma et al., 2015).

Beyond testing the specific genetic resistance of families for a Baynes Sound *C. gigas* breeding program, this study describes a repeatable experimental framework which controls both for dose, as direct injection does, but also mimics real-world infection pathways, like cohabitation does. We conducted a series of lab trials to address this question with controlled laboratory challenge trials which are described below.

The goal of Challenge 1 was to determine which isolate of *V. aestuarianus* was most pathogenic by measuring the lowest dose to cause 50% mortality (LD50) and the time to reach 50% mortality. It is documented that different isolates of the same *Vibrio* species can have significantly different pathogenicity to marine life (Zhang et al., 2000, Liuxy et al., 1996). Seven *Vibrio spp*. were isolated from moribund oysters in Baynes Sound during the summer of 2018. The purpose of our first challenge trial is to determine which of these 7 *V. aestuarianus* and *V. harveyi* isolates have a higher pathogenicity to *C. gigas,* and if there is a dose response. Different concentrations were given to determine LD50 for each isolate, and to determine the pathogenicity of the isolates at different doses.

The purpose of the second challenge (Challenge 2) was to determine the heritability of resistance to *V. aestuarianus* and the estimated breeding values (EBVs) for *C. gigas* in Vancouver Island University's selective breeding program. Selective breeding programs have been used to enhance disease resistance in oysters. This is one key strategy used to create oyster stock that is optimized for localized conditions and resistant to increased instances of disease (reviewed by Dégremont et al. in 2015). Limited trials have been conducted to estimate the ability to selectively breed resistance to *V. aestuarianus* infection in *C. gigas*. Recently, one trial has been conducted by Dégremont et all in 2020, which found moderate to low realized heritability to increase resistance to *V. aestuarianus* infection after one generation of mass selection in *C. gigas*. The purpose of this challenge is to estimate the heritability of survival of infection of *V. aestuarianus* by infecting 32 full-sib families with this pathogen.

In Challenge 2, we discovered a highly significant positive correlation between oyster size and increased mortality. Challenge 1 showed that mortality is highly dose dependent. We suspected that it was possible that larger oysters were receiving a higher dose of planktonic *V. aestuarianus* when in shared seawater with many oysters. The purpose of Challenge 3 was to determine if we could develop a better lab methodology to test the hypothesis that when *C. gigas* are together in a common tank and that tank is inoculated with planktonic bacteria, larger animals will filter more water quickly and thereby receive a higher bacterial dose. We are interested to see if larger animals in shared tanks have higher mortality because they receive a larger dose, or if there is another physiological mechanism at work which makes them more susceptible to infection. This challenge was designed to [1] find any correlation between clearance rate and survival, and [2] remove dose variability between individuals. To achieve this, oysters from 32 full-sib families were isolated so that their individual clearance rate could be determined. After clearance rate was tested, each oyster received an identical dose of planktonic *V. aestuarianus* bacteria, and survival was monitored. A second trial was carried out with similar methods, testing fewer genetic families with higher N per family to validate results.

## 3.3 METHODS

### 3.3.1 Bacterial strains and growth conditions

For all challenges, *Vibrio spp.* were grown for 24 hours in tryptone soy broth containing 2% NaCl at 24°C (unless otherwise noted) under constant agitation at 200 rpm. The culture was purified by centrifuged (1000×*g*, 20 min), and cell pellet was rinsed and

resuspended in sterile seawater. This purification process was repeated twice. The titre of *Vibrio* inoculum was estimated by serial dilution and plating on tryptone soy agar + 2% NaCl. Colonies were enumerated after incubation at 24°C for 36 h.

**Table 3.1**: Bacterial isolates of *Vibrio spp*. known to be pathogenic to Pacific oysters were isolated from sick and dead oysters in Baynes Sound by Dr. Timothy Green of Vancouver Island University.

| | Species | Identifier | Collection Date | Collection Location Notes |
|---|---|---|---|---|
| S1 | *Vibrio harveyi / owensii* | BS-2018-005 | 7/26/2018 | KR Farm |
| S2 | *Vibrio aestuarianus* | BS-2018-006 | 7/26/2018 | KR Farm |
| S3 | *Vibrio aestuarianus* | BS-2018-028 | 7/26/2018 | Isolated from juvenile, KR farm |
| S4 | *Vibrio aestuarianus* | BS-2018-032 | 8/3/2018 | KR Farm |
| S5 | *Vibrio aestuarianus* | BS-2018-035 | 8/3/2018 | KR Farm |
| S6 | *Vibrio harveyi* | BS-2018-042 | 8/3/2018 | Big Rock |
| S7 | *Vibrio aestuarianus* | BS-2018-043 | 8/3/2018 | Big Rock |

### 3.3.2 Challenge 1: *Vibrio aestuarianus* Isolate Pathogenicity

Six-month-old *C. gigas* spat were obtained from Vancouver Island University's Deep Bay Marine Field Station. Ten spat were added to each 100mm petri dish, along with 12 mL filtered seawater. Spat were allowed to acclimate to 24°C for 24 h prior to inoculation, and the temperature was maintained for the duration of the experiment. Each inoculation was performed in triplicate, with a total of N = 30 spat per bacterial treatment. Three trays of spat were also held as control, with 1 mL sterile seawater added in place of bacterial titre. Each day, the spat were fed 300µL of *Tisochrysis lutea* (Tiso) per petri dish, which was kept at 4°C for the duration of the experiment to arrest growth

and prevent colony crash. The *Tisochrysis* had a starting concentration of 8 million cells / mL and was grown under axenic conditions.

In 2018, Seven isolates of *Vibrio spp*. known to be pathogenic to Pacific oysters were isolated from sick and dead oysters in Baynes Sound by Dr. Timothy Green of Vancouver Island University (Table 3.1). These strains have been stored at -80° C since their isolation and purification. These 7 strains were grown according to procedure outlined above. Each strain was diluted into 5 concentrations (Table 3.2) which were measured for optical density. These 5 concentrations of our 7 bacterial isolates (S1-S7) were the treatments added to dishes of *C. gigas* (Table 3.2). 1 mL of bacterial treatment was added to each petri dish containing 10 spat, with triplicate dishes for each treatment. Each dilution was tested in 3 replicates of 10 spat. 6 control dishes of 10 spat received a treatment of 1 mL of autoclaved seawater. A serial dilution was plated on to tryptone soy agar with 2% NaCl and allowed to grow for 24 hours at 24° C before being counted to calculate colony forming units.

Experimenters examined each petri dish every 24 hours for 264 hours post-inoculation, removing any dead spat each day. Dead spat were stored at -80°C for further study. Slope and intercepts were calculated using a simple linear regression performed using GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com.

**Table 3.2:** Bacterial colony forming units for dilutions of bacterial isolates from dead and dying oysters in Baynes Sound collected by Dr. Timothy Green in 2018.

| Colony Forming Units / mL seawater | | | | | |
|---|---|---|---|---|---|
| Concentration: | 1/1 | 1/2 | 1/4 | 1/8 | 1/16 |
| S1 | 835478 | 456711 | 216304 | 113943 | 57598 |
| S2 | 858642 | 419461 | 215678 | 109248 | 54467 |
| S3 | 796975 | 609470 | 206287 | 109248 | 56345 |
| S4 | 708388 | 357168 | 179366 | 96100 | 51337 |
| S5 | 620113 | 303639 | 148689 | 82014 | 38816 |
| S6 | 1076199 | 564081 | 315222 | 157767 | 87962 |
| S7 | 1073068 | 560637 | 292370 | 145872 | 79823 |

### 3.3.3 Challenge 2: *Vibrio aestuarianus* Survival Heritability

*C. gigas* were spawned and reared at Deep Bay Marine Field Station. Briefly, a total of 32 full-sib families were produced in two 2020 spawnings (FR1 and FR2), using 12 dams and 11 sires with diverse genetic backgrounds, in a hatchery farm located in Deep Bay (British Columbia, Canada). The detailed information of the family crosses and broodstock pedigree are provided in Appendix 1. Oysters were conditioned artificially through the manipulation of water temperature to induce development and maturation of gonads and were strip-spawned. After rearing to spat in the hatchery, families were transported to ocean for culture on suspended longlines on a raft in at FS1 in Baynes Sound (Figure 1.1) which is one of the main areas for shellfish farming of *C. gigas* in Canada. At the time of the trials, oysters from FR1 were 11 months old and from FR2 were 10 months old.

12 spat from each family (FR1 + FR2) were placed into 100mm plates with 30mL seawater filtered to 1 micron. A control plate of 12 spat was also produced for each

family, except family 2.9 due to a lack of spat available in that family. The spat were kept at 24°C for the duration of the experiment.

1.5 mL of suspended *V. aestuarianus* (strain 2018-BS-032) culture with an optical density of 7.696 was added to each treatment plate. No bacteria were added to the controls. 600µL of *Tisochrysis lutea* (Tiso) was fed to both the treated and control plates daily. The Tiso culture was grown at Deep Bay Marine Field Station in seawater filtered to 1 micron and had a cell density of 8 x $10^6$ cells / mL at time zero. The algae was kept at 4°C for the duration of the experiment to arrest growth and prevent crash. 24 hours post-inoculation, an additional 30mL of filtered seawater was added to each plate. Live/dead counts were performed daily. Dead spat were recognized by their gaping/open shells, and inability to close their adductor when prodded with sterilized tweezers. Dead oysters were removed and measured for length using calipers from the hinge to the furthest lateral edge of the frill. The dead animal was then removed from inside its shell and frozen at -80°C. 120 hours post-inoculation, 5 mortalities were found in control dishes, and the experiment was ended. Remaining oysters were measured and frozen.

**Table 3.3.** Summary of Challenge 2 bacterial challenge with *V. aestuarianus* in *C. gigas*

| Items | Number |
|---|---|
| Families | 32 |
| Oysters | 770 |
| Age FR1 (months) | 11 |
| Age FR2 (months) | 10 |
| Sires | 11 |
| Dams | 12 |
| Final Mortality (%) | 58.3 |
| Challenge test time (days) | 5 |
| Average shell length (mm) | 14.378 |

### 3.3.4 Challenge 3: Clearance Rate + Survival Heritability

*C. gigas* rearing and breeding are described in Challenge 2. Pedigree details for all families available in Appendix 1. Two trials were conducted as part of this challenge. [1] Trial 1 tested all families with an N of 12 per family, and [2] Trial 2 tested 6 families from FR2 with a higher N per family (See Table 3.4)

*V. aestuarianus* (strain 2018-BS-032) was grown according to the procedure outlined at the top of this section. Dose varied by trial, see Table 3.4.

**Table 3.4:** Optical density, dose volume, and colony forming units of *V. aestuarianus* added to Challenge 3 Trials 1 and 2.

| | OD 600 | Dose per oyster | CFU / mL |
|---|---|---|---|
| Trial 1 | 0.772 | 500ul | 59,355,000 |
| Trial 2 | 0.197 | 1mL | 12,100,000 |

Individual *C. gigas* were weighed, measured, and placed alone into individual wells of 6-well plates. 10 mL seawater filtered to 1 micron was added to each well, and animals acclimated to 24°C overnight before the trial began. Algae used for the challenge was *Chaetoceros calcitrans*, which was raised in sterile hatchery conditions at Vancouver Island University's Deep Bay Marine Field Station. Details of setup are in Table 3.4.

It has been shown that there is a linear dependence between light absorbance and algal cell count (Havlik et al., 2013). The optimal absorbance wavelength to measure *Chaetoceros calcitrans* is known to be 688nm (Santos-Ballardo et al., 2015). In Trial 1, clearance rate was determined for each oyster using the following procedure. Cell density was counted using a sedgewick rafter counting chamber and was found to have a cell

count of 7.4*10^6 cells / mL with an OD 688 of 0.112. Seawater was emptied from oyster trays, along with any accumulated waste, and oyster wells were refilled with the seawater *C. calcitrans* mixture (OD 688 = 0.112). After 15 min, 500ul of liquid was taken from each oyster's tray and measured for OD 688. This was repeated 4 times.

Clearance rate is described as:

CR = (Algal density (To) – Algal density (Tx))/time/weight

24h after clearance rate was measured, each oyster was given an identical volume of *V. aestuarianus* (Table 3.4). Live/dead counts were performed daily. Dead spat were recognized by their gaping/open shells, and inability to close their adductor when prodded with sterilized tweezers. Dead oysters were removed and frozen at -80°C.

**Table 3.5:** Summary of Challenge 3 bacterial + clearance rate challenge with *V. aestuarianus* in *C. gigas*.

| Trial 1: | | Trial 2: | |
|---|---|---|---|
| Items | Number | Items | Number |
| Families | 32 | Families | 6 |
| Oysters | 396 | Oysters | 293 |
| Age FR1 (months) | 11 | Age FR2 (months) | 10 |
| Age FR2 (months) | 10 | Sires | 4 |
| Sires | 11 | Dams | 4 |
| Dams | 12 | Final Mortality (%) | 21.8% |
| Final Mortality (%) | 57.5% | Challenge test time (hours) | 168 |
| Challenge test time (hours) | 96 | N family 2.2 | 84 |
| Average shell length (mm) | 28.12 | Average weight (g) | 1.47 |
| Average weight (g) | 2.6 | N family 2.10 | 42 |
| N per family | 12 | N family 2.16 | 30 |
| | | N family 2.20 | 29 |
| | | N family 2.21 | 42 |
| | | N family 2.24 | 48 |
| | | N control | 18 |

### 3.3.5 Statistical Analysis

The Kaplan-Meier estimate of the survival function (Kaplan et al, 1958) was used to plot survival curves of families using GraphPad Prism software (version 8). The survival distribution function is:

$$\widehat{S}(t) = \Pi_{t_i < t} \left(1 - \frac{d_i}{n_i}\right)$$

Where $t_i$ is death time at day i, $d_i$ is the number of oysters that die at $t_i$ and $n_i$ is the number of surviving oysters before $t_i$.

To classify families into resistant or susceptible to *V. aestuarianus*, a Cox proportional hazard regression analysis (Cox, 1972) was used to measure the survival time of each family, performed using the Survival package in RStudio (Therneau, 2020).
The risk of death for families was compared based on a hazard ratio HR = hi (t)/hr (t), where hi (t) denotes the mortality risk in family i, hr (t) denotes the mortality risk in the reference family, and the survival rate in the reference family is 0%. Families with hazard ratio value <1 were classified as resistant to *V. aestuarianus*, while families with hazard ratio > 1 were classified as susceptible to *V. aestuarianus*. Put briefly, the hazard is the instantaneous event probability at a given time, or the probability that an individual under observation experiences the event in a period centered around that point in time (Bradburn et al, 2003). The statistical significance was set as $P < 0.05$.

Phenotypic and genetic data were analyzed using techniques similar to Divilov et al, in their 2021 publication “Genetic improvement of survival in Pacific oysters to the

Tomales Bay strain of OsHV-1 over two cycles of selection". Briefly, a linear mixed model $\mathbf{y} = \mathbf{Xb} + \mathbf{Zu} + \mathbf{e}$ was used, in the *sommer* package (Covarrubias-Pazaran, 2016) in R (R Core Team 2021). In this model, **y** was a vector of survival phenotypes (dead = 0, alive = 1). **X** was a design matrix for the fixed effects, and **b** was a vector of fixed year effects. **Z** was an incidence matrix for the random effects, and $\mathbf{u} \sim \mathbf{N}(0, \mathbf{A}\sigma_a^2)$ was a vector of random additive genetic effects where **A** was the additive relationship matrix calculated from a pedigree linking the oysters to the founders of the Vancouver Island University breeding program (de Melo et al., 2016) and $\sigma_a^2$ was the additive genetic variance. Lastly, $\mathbf{e} \sim \mathbf{N}(\mathbf{0}, \mathbf{I}\sigma_e^2)$ was a vector holding the error where **I** was an identity matrix and $\sigma_e^2$ was the error variance. Length was included as a fixed effect.

The heritability on the observed scale was calculated as $h_o^2 = \sigma_a^2/(\sigma_a^2 + \sigma_e^2)$. The heritability on the underlying liability scale, which is a more accurate estimate of heritability with binomial data (Divilov et al., 2021), was calculated as $h_u^2 = h_o^2 p(1 - p)/z^2$, where p was the proportion of survivors and *z* was the standard normal probability density function at a threshold value, which was the standard normal quantile function at $1 - p$ (Dempster and Lerner, 1950).

The genetic trend of survival was obtained by calculating the mean survival EBV of families within each cohort ($\overline{EBV}$) and converting the proportions to percentages.

## 3.4 RESULTS

### 3.4.1 Challenge 1: *Vibrio aestuarianus* Isolate Pathogenicity

In Figure 3.1 we can see that at 168 h post-inoculation, survival rates were under 50% for the highest dose concentrations of all strains, excepting S2. Because of the large differences in dose concentration between strains (Table 3.2), it's difficult to rely on graphic representation of survival to show pathogenicity. In fact, in figure 3.2 we can see that dose, regardless of strain, has a linear relationship with time to 50% mortality (R squared = 0.6058, P = <0.0001). Dose may be a more important factor in mortality than other differences between isolates.

Oysters treated with S4, an isolate of *V. aestuarianus*, reached 50% mortality faster and at a lower dose than the other isolates (Figure 3.1). S3, which was isolated from juvenile oysters at the same site as S4, shows a similar LD50 at a slightly higher dose. 2 of the 3 trays exposed to S4 had 1 individual animal out of 10 die at 96 hours post-inoculation (total of 2/30 at that dose), 48 hours before any mortality was observed in S3 (Figure 3.1). It is possible that S4 would therefore be more deadly in-situ than lab results reflect because dead oysters were removed from the trial, and therefore could no longer shed additional bacteria into the environment. S5 is an isolate of *Vibrio aestuarianus* collected from the same site on the same day as S4, which appears to show similar pathogenicity at its highest dose, but at lower doses takes longer to reach LD50, indicating a less pathogenic isolate (Figure 3.1). Survival and mortality rate is dose-dependent (Figure 3.2). No mortality was observed in controls or with doses lower than 148689.4445 CFU/ml.

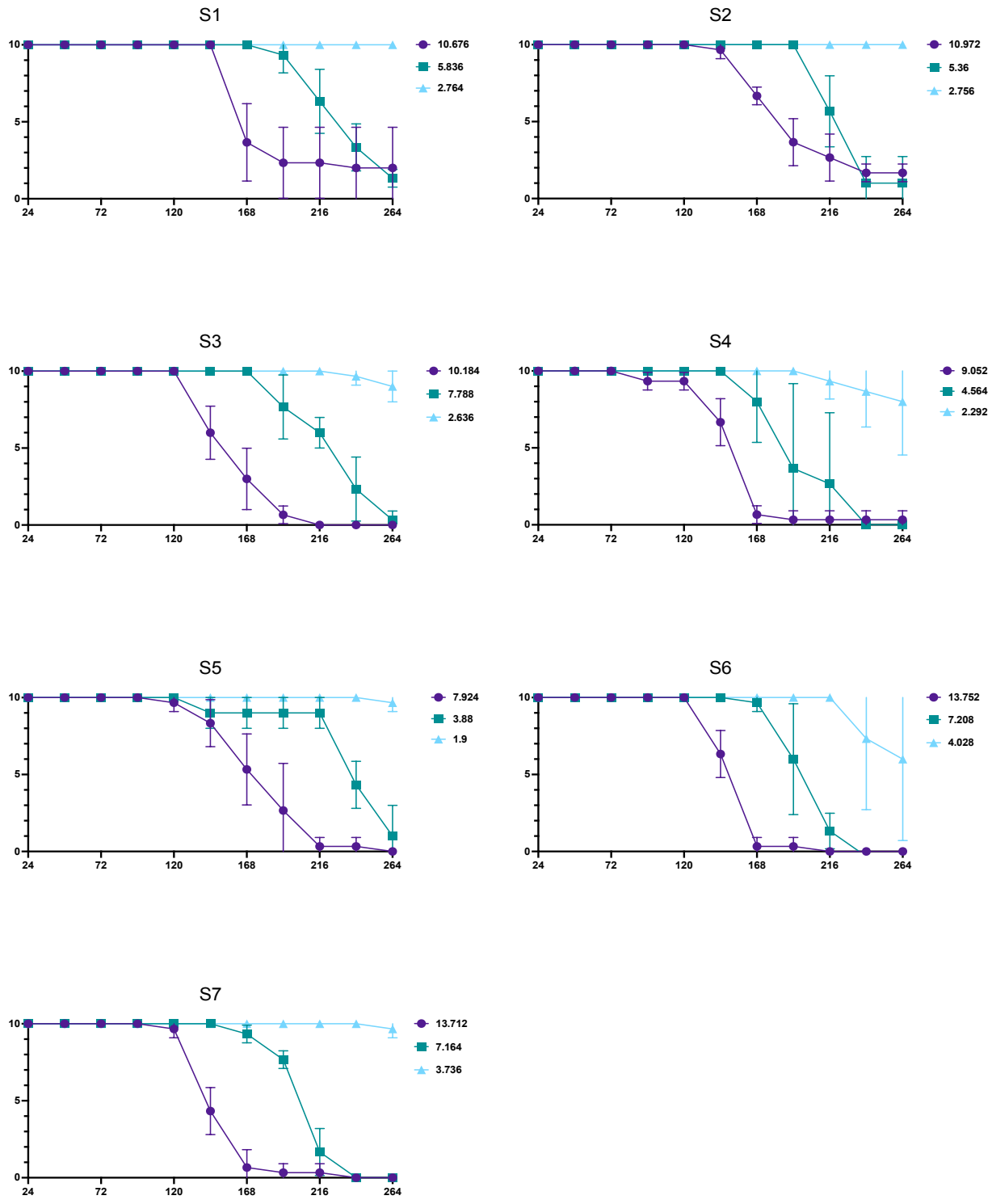



**Figure 3.1:** Survival curves for all strains tested (Table 3.1), time is measured I hours on the x-axis. Concentrations 1/1, ½, and ¼ are shown, as they were the only dose concentrations that induced mortality (Table 3.2). Concentrations are identified by OD 600 values.

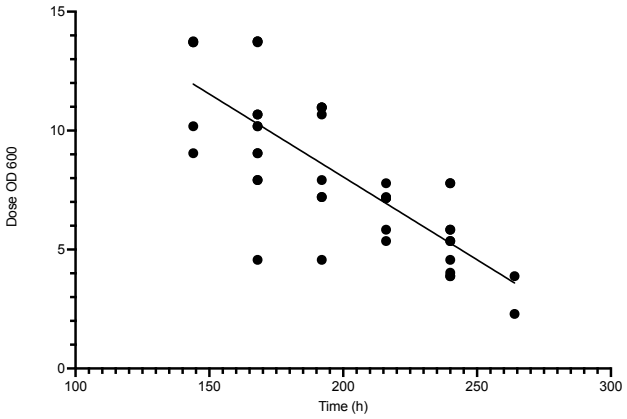



**Figure 3.2:** Time to reach 50% mortality, compared to dose measured in OD 600. All doses of all bacterial isolates shown (Table 3.2).

### 3.4.2 Challenge 2: *Vibrio aestuarianus* Survival Heritability

#### 3.4.2a Comparison of survival rate among families

The artificial infection experiment of *V. aestuarianus* was carried out with 32 families for 5 days (Table 3.3). The aggregate daily mortality and survival curve for all 32 families during 5 days of infection with *V. aestuarianus* were shown in Figure 3.3. It's apparent that daily mortality first increased, was highest 3 days post-inoculation with *V. aestuarianus*, then decreased. The challenge test was terminated when there were 2 mortalities in the control trays, at 120 hours. There was a cumulative mortality rate of 58.3% for all families. Survival rate varied widely among families, suggesting a significant phenotypic variation associated with resistance to *V. aestuarianus* (Figure 3.4). The survival rate was 0% in 2 families (2.13 and 2.20), and the family with the highest survival rate was family 2.21 (70%) (Table 3.6).

Hazard ratio values of each family based on Cox proportional regression analysis were shown in Figure 3.5. In the Cox proportional hazard model, length was included as a covariate in the multivariate analysis. Length was included because the lengths of the oysters selected from each family were not representative of the lengths of the entire family. In the Cox analysis the p-value for length is highly significant (2e-16), with a hazard ratio HR = exp(coef) = 1.1848, indicating a strong relationship between the oysters' length and increased risk of death (Figure 3.5). The hazard ratios of covariates are interpretable as multiplicative effects on the hazard. For example, holding the other covariates constant, having more length increases the hazard by a factor of 1.18. The hazard ratio (1.18) means that for every 1 mm increase in length the risk of death goes up by 18%, regardless of the family. The p-value for all three overall tests (likelihood, Wald, and score) are significant, indicating that the model is significant. These tests evaluate the omnibus null hypothesis that all of the betas (β) are 0. In the above example, the test statistics are in close agreement, and the omnibus null hypothesis is soundly rejected. Three families were shown to have significant increase in hazard ratio values (1.8, 1.18, 2.13). We can conclude from this that length is an important variable in determining hazard ration, as families 1.8 and 1.18 had middle-of-the-pack survival proportions (Table 3.6). With this in mind, we examined the linear relationship between survival time and length, shown in Figure 3.6. We analyzed FR1 and FR2 separately, because they were different ages and we wanted to isolate size as a variable (Figure 3.6). For both FR1 and FR2 there was a significant linear relationship between smaller size and longer survival time (FR1: p value = <0.00001, R squared = 0.3355; FR2: p value = <0.00001, R squared = 0.3652). The slope of the linear regression of FR1 was -2.346, and

the slope of linear regression of FR2 was -1.975 (Figure 3.6), suggesting a slightly stronger correlation between size and survival in FR1 than FR2.

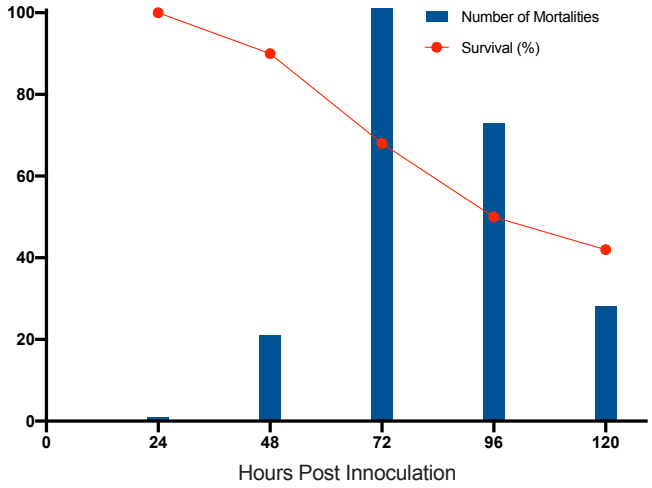



**Figure 3.3:** The daily mortality and survival curve for all 32 families (n = 770) during 5 days of challenge with *V. aestuarianus*.

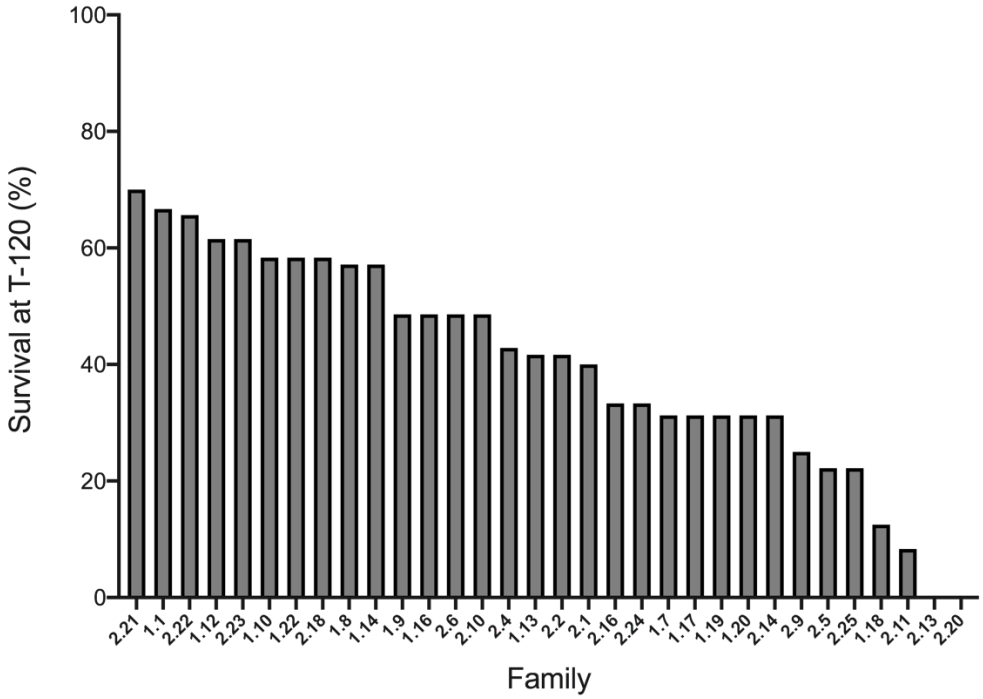



**Figure 3.4:** The survival rate of 32 families at day 5 after challenge 2 with *V. aestuarianus*.

**Table 3.6:** Survival proportions of all 32 families at 96 h post-inoculation and 120 h post inoculation.

| Family: | 2.13 | 2.20 | 2.11 | 1.18 | 2.5 | 2.25 | 2.9 | 1.7 | 1.17 |
|---|---|---|---|---|---|---|---|---|---|
| Survival proportion at 96 h | 16.7 | 41.7 | 25.0 | 25.0 | 33.3 | 33.3 | 37.5 | 41.7 | 41.7 |
| Survival proportion at 120 h | 0.0 | 0.0 | 8.3 | 12.5 | 22.2 | 22.2 | 25.0 | 31.3 | 31.3 |

| 1.19 | 1.20 | 2.14 | 2.16 | 2.24 | 2.1 | 1.13 | 2.2 | 2.4 | 1.9 | 1.16 | 2.6 | 2.10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 41.7 | 41.7 | 41.7 | 58.3 | 50.0 | 50.0 | 50.0 | 58.3 | 57.1 | 58.3 | 58.3 | 58.3 | 58.3 |
| 31.3 | 31.3 | 31.3 | 33.3 | 33.3 | 40.0 | 41.7 | 41.7 | 42.9 | 48.6 | 48.6 | 48.6 | 48.6 |

| 1.8 | 1.14 | 1.10 | 1.22 | 2.18 | 1.12 | 2.23 | 2.22 | 1.1 | 2.21 |
|---|---|---|---|---|---|---|---|---|---|
| 66.7 | 64.3 | 66.7 | 75.0 | 66.7 | 69.2 | 76.9 | 75.0 | 66.7 | 90.0 |
| 57.1 | 57.1 | 58.3 | 58.3 | 58.3 | 61.5 | 61.5 | 65.6 | 66.7 | 70.0 |

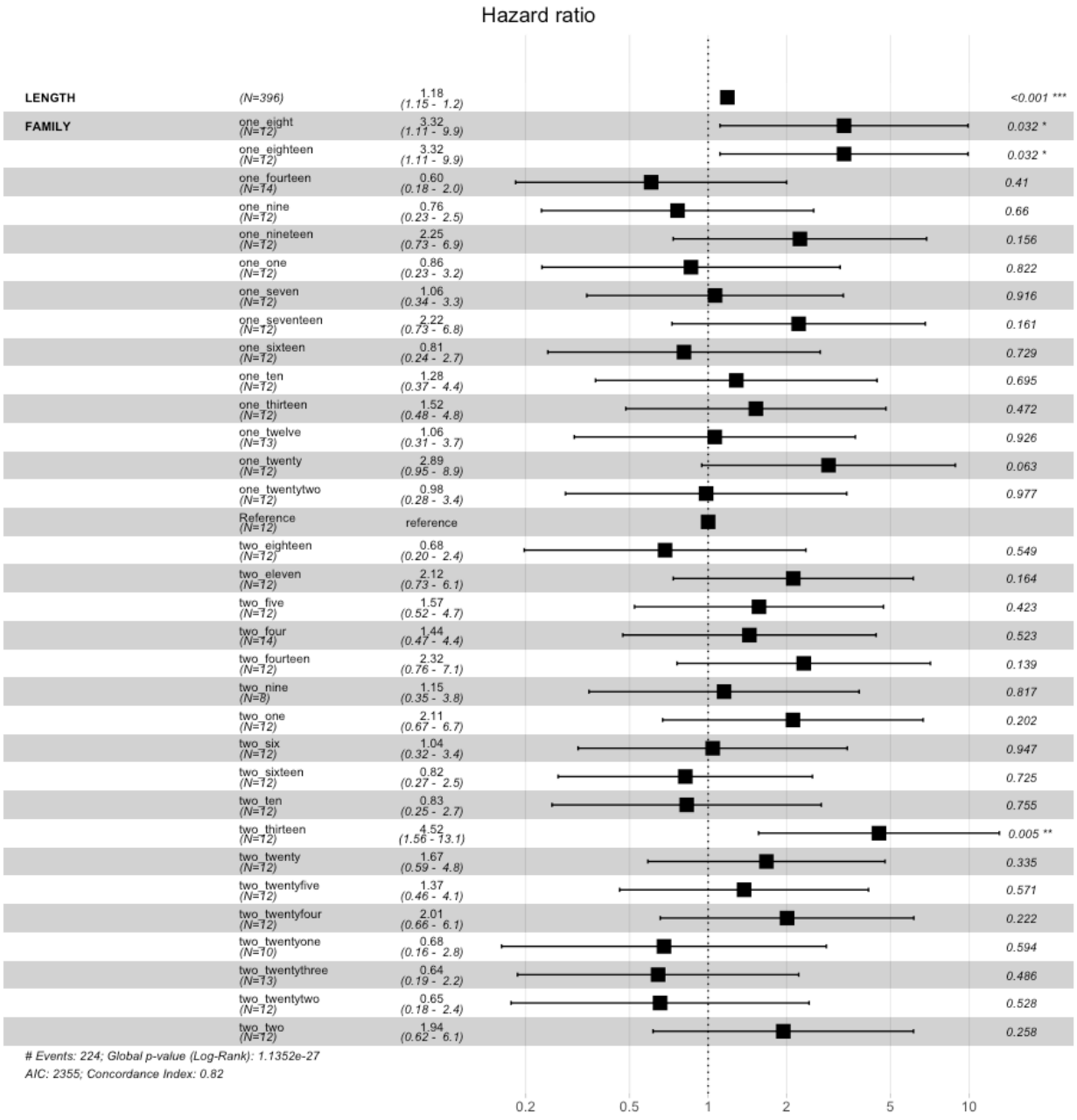



**Figure 3.5:** Hazard ratio plot based on Cox regression analysis for resistance to *V. aestuarianus* in *C. gigas*. Families with hazard ratio values <1 (P < 0.05) were classified as resistant, and families with hazard ratio values >1 (P < 0.05) were classified as susceptible. HR = 1: No effect, HR < 1: Reduction in the hazard, HR > 1: Increase in Hazard 1.8, 1.18, 2.13

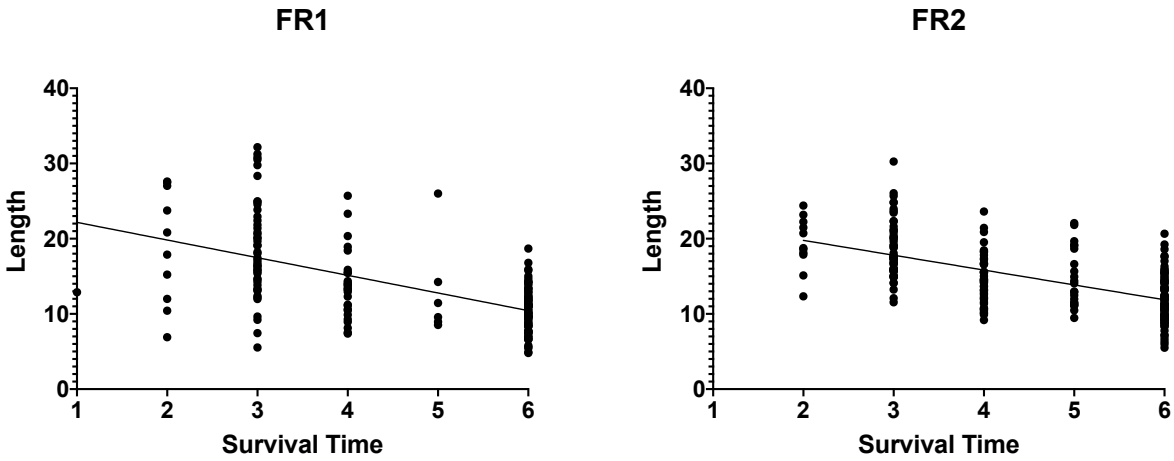



**Figure 3.6**: Survival time post-inoculation with *V. aestuarianus*, compared with shell length. Day 6 represents oysters that survived past the end of the experiment, which was at 120 h (5 days). Slope of linear regression of FR1 is -2.346, slope of linear regression of FR2 is -1.975.

### 3.4.2b Heritability

Length was included as a fixed effect in this model. The heritability of survival on the observed scale was estimated to be 0.034 (SE = 0.029), and the heritability on the underlying liability scale was estimated to be 0.054 (SE = 0.047). This result indicates that phenotypic survival to *V. aestuarianus* infection is not highly heritable. The dam MAF had the highest EBV of the breeding parents included in the pedigree (Table 3.7).

**Table 3.7:** EBVs for all families and parents. Five lowest highlighted in red, five highest highlighted in yellow.

| 2.11 | 2.20 | UKF | 1.17 | 2.13 | 1.18 | 2.14 |
|---|---|---|---|---|---|---|
| -0.08051 | -0.07941 | -0.07209 | -0.06331 | -0.05845 | -0.06 | -0.057 |
| 1.19 | PLF2 | 1.20 | PLM | PLF3 | PSM3 | PSM4 |
| -0.05643 | -0.04507 | -0.03841 | -0.0322 | -0.03125 | -0.03 | -0.028 |
| PLF | 2.16 | 2.1 | 2.4 | TAM | MAM | 2.9 |
| -0.02413 | -0.02331 | -0.02319 | -0.02162 | -0.02134 | -0.02 | -0.018 |
| 2.2 | TAF | 2.5 | 2.24 | 2.6 | PSM1 | 1.22 |
| -0.01752 | -0.01365 | -0.00925 | -0.00734 | -0.00154 | 0.004 | 0.006 |
| 1.13 | PSF2 | PSM2 | CHM | CHF | 1.10 | 2.10 |
| 0.00683 | 0.00856 | 0.00926 | 0.01305 | 0.01443 | 0.015 | 0.019 |
| PSF4 | PSF3 | UKM | 1.12 | 2.25 | 1.16 | PSF1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.01871 | 0.01966 | 0.02131 | 0.0226 | 0.02378 | 0.026 | 0.03 |
| 2.18 | 2.21 | PLM1 | 1.7 | PLF1 | 1.14 | PLM4 |
| 0.032 | 0.0339 | 0.03477 | 0.03838 | 0.03911 | 0.041 | 0.051 |
| 1.8 | MAF | 2.23 | 1.9 | 1.1 | 2.22 | |
| 0.05205 | 0.05549 | 0.05936 | 0.06076 | 0.06274 | 0.065 | |

### 3.4.3 Challenge 3: Clearance Rate + Survival Heritability

#### 3.4.3a Trial 1 Comparison of Survival Rates Among Families

A simple linear regression between the percent survival per family at 120 h post-inoculation and average weight per family was significant, showing that higher weight is negatively correlated to survival ($p = 0.0013$, r squared = 0.2939). This result is consistent with earlier findings in Challenge 2, but due to the isolation of the animals and the standardization of dose, we can postulate that the earlier result was not simply due to larger oysters ingesting a larger amount of bacteria than their smaller counterparts. Families showed significant phenotypic differences in survival rate (8.33%-83.3%), with families 1.14, 1.18, 2.11, 2.16, and 2.18 demonstrating the lowest survival and 2.1, 2.21, and 2.22 demonstrating the highest survival (Figure 3.7 and Table 3.8).

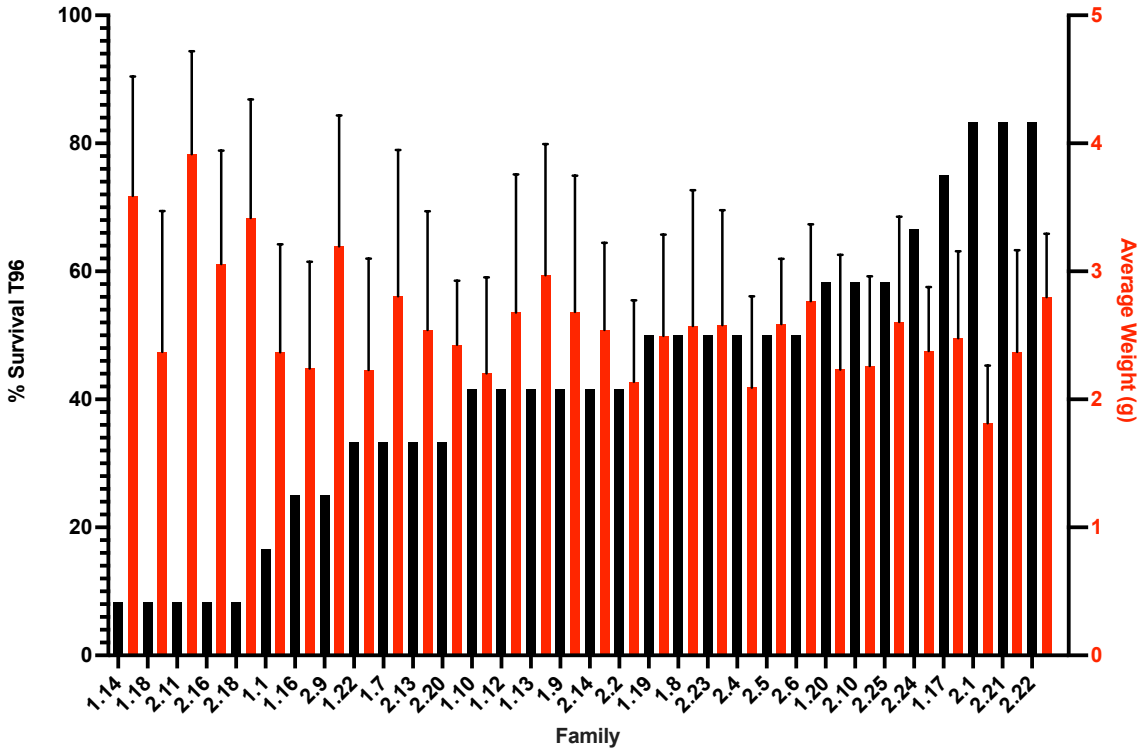



**Figure 3.7:** Shows the percent survival by family at 120 hours post-inoculation in black, and the average weight (g) in red. N = 12 animals per family.

**Table 3.8:** Survival proportions of each family 120 hours post-inoculation.

| Family: | 1.18 | 2.11 | 2.16 | 2.18 | 1.14 | 1.1 | 1.16 | 2.9 | 1.7 |
|---|---|---|---|---|---|---|---|---|---|
| Survival proportion at 120 h | 8.33 | 8.33 | 8.33 | 8.33 | 9.09 | 16.7 | 25 | 25 | 33.3 |

| 1.22 | 2.13 | 2.20 | 1.9 | 1.10 | 2.2 | 2.14 | 1.12 | 1.13 | 1.8 | 1.19 | 2.4 | 2.5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 33.3 | 33.3 | 33.3 | 41.7 | 41.7 | 41.7 | 41.7 | 45.5 | 45.5 | 50 | 50 | 50 | 50 |

| 2.6 | 2.23 | 1.20 | 2.10 | 2.25 | 2.24 | 1.17 | 2.1 | 2.21 | 2.22 |
|---|---|---|---|---|---|---|---|---|---|
| 50 | 50 | 58.3 | 58.3 | 58.3 | 66.7 | 75 | 83.3 | 83.3 | 83.3 |

There is a clear positive correlation between *C. gigas* weight and clearance rate (Figure 3.8). The linear relationship is significant (p value = <0.0001, R squared = 0.2559).

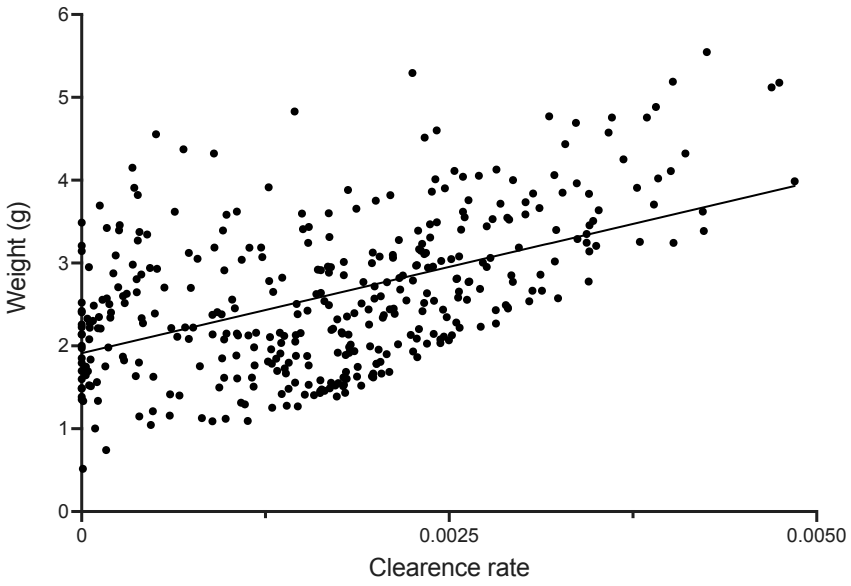



**Figure 3.8:** Simple linear regression of clearance rate and weight (g). Clearance rate is calculated as CR = (Algal density (To) – Algal density (Tx))/time/weight. Algal density was measured in optical density with 688 nm wavelength.

The average clearance rate per family is shown to have a negative linear relationship with the average family percent survival (Figure 3.9). The relationship is significant with a p value of 0.0247 although the R squared is slightly low, at 0.1572. There is more variance in clearance rate within the group of families with low survival. When the clearance rate of individual oysters is related to time to death (number of days survived), the linear relationship is weakly significant, with a p value of 0.0134, an R squared value of 0.1595, and a nearly flat slope (-0.0001178). This indicates that clearance rate is not a good predictor of individual survival, but average clearance rate per family is a weak predictor of family average survival. Because the mean has a stronger correlation than the data of the individuals, perhaps a trial with more animals would reveal a stronger correlation.

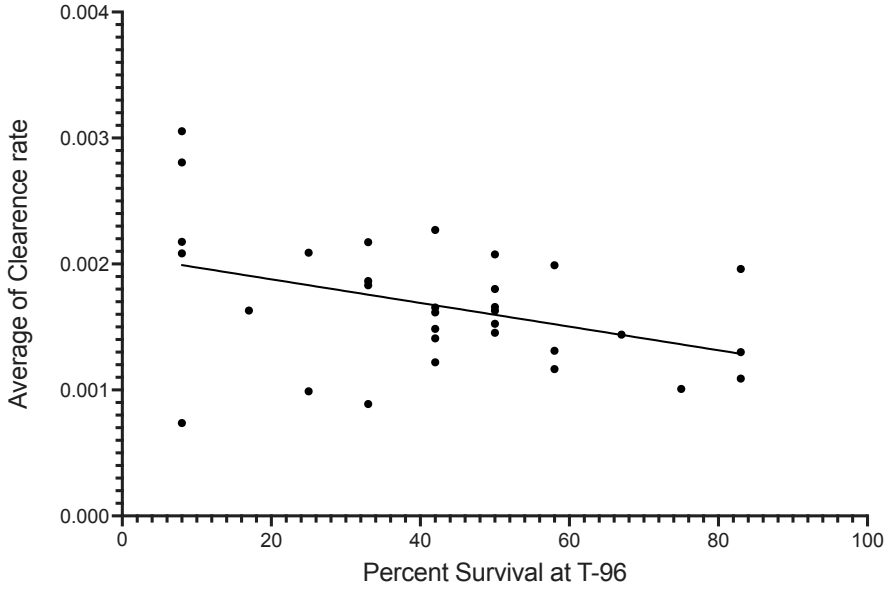



**Figure 3.9:** Simple linear regression of average clearance rate and the average percent survival at 96 h post-inoculation by family. Clearance rate is calculated as CR = (Algal density (To) – Algal density (Tx))/time/weight. Algal density was measured in optical density with 688 nm wavelength.

Stated more specifically, we calculated the Cox Hazard Ratio (HR) for weight (g) and the log of clearance rate (called "log" in Figure 3.10). Weight has a significant HR of 1.73 (Figure 3.10), indicating a strong relationship between the oysters' weight and increased risk of death. The hazard ratios of covariates are interpretable as multiplicative effects on the hazard. For example, holding the other covariates constant, having more weight increases the hazard by a factor of 1.73. The hazard ratio (1.73) means that for every 1g increase in weight the risk of death goes up by 73%. The HR for the log of clearance rate is not significant, but shows a slight negative correlation to survival, which mimics the results we found in our linear regression shown in Figure 3.9. The distribution of the regression was not found to be normal.

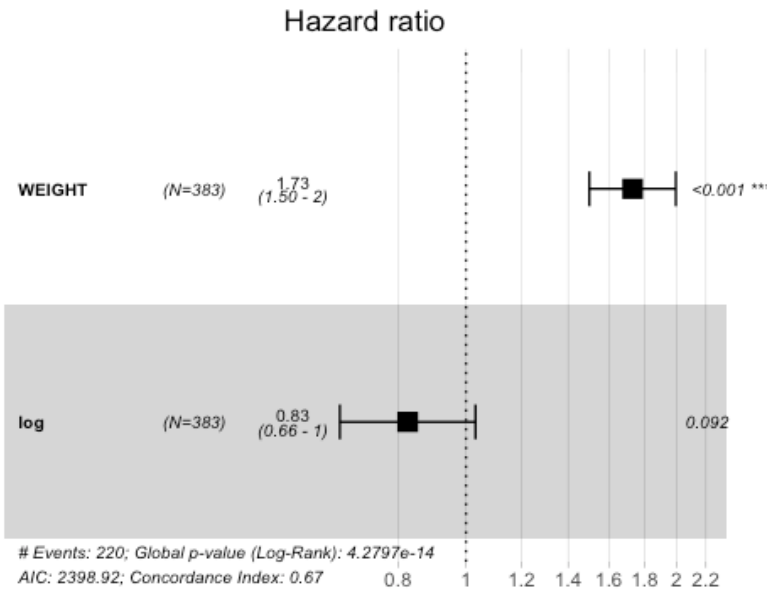



**Figure 3.10**: Hazard ratio plot based on Cox regression analysis for survival of *V. aestuarianus* in *C. gigas*. We examine the effect of weight (g) and the log of clearance rate on survival. Factors with hazard ratio values <1 ($P < 0.05$) were classified as resistant, and families with hazard ratio values >1 ($P < 0.05$) were classified as susceptible.

### 3.4.3b Trial 1 Heritability

Because clearance rate is not a significant factor in survival (Figure 3.10), we did not include it as a factor in our heritability model. We included weight as a fixed effect. The heritability of survival on the observed scale was estimated to be 0.095 (SE = 0.043), and the heritability on the underlying liability scale was estimated to be 0.15 (SE = 0.068). Survival EBV¯s of all families, sires, and dams visible in Table 3.8. The 5 highest EBV's (highlighted in yellow in Table 3.9) align with the highest survival proportions shown in Table 3.7. The dam MAF which has a high EBV was mother to families 2.21, 2.22, 2.23, 2.24, and 2.25 (Appendix 1) indicating that she may have passed resilience to *V. aestuarianus* to her offspring. Dam PLF, which has the lowest

EBV of any parent, was mother to families 2.16, 2.18, and 2.20 (Appendix 1), all of which have negative EBVs (Table 3.9).

**Table 3.9:** Estimated breeding values for all families and parents. Five highest EBV's highlighted in yellow, five lowest highlighted in red.

| 1.1 | 1.12 | 1.13 | 1.14 | 1.16 |
|---|---|---|---|---|
| -0.16846 | -0.02148 | 0.07372 | -0.08289 | -0.13556 |
| 1.17 | 1.18 | 1.19 | 1.22 | 1.7 |
| 0.13363 | -0.19299 | 0.07101 | -0.11702 | -0.02032 |
| 1.8 | 1.9 | 1.10 | 1.20 | 2.1 |
| 0.0331 | 0.02192 | -0.02946 | 0.02418 | 0.13897 |
| 2.11 | 2.13 | 2.14 | 2.16 | 2.18 |
| -0.02958 | -0.06874 | -0.01949 | -0.1182 | -0.13111 |
| 2.2 | 2.21 | 2.22 | 2.23 | 2.24 |
| -0.00188 | 0.24086 | 0.26896 | 0.08789 | 0.15515 |
| 2.25 | 2.4 | 2.5 | 2.6 | 2.9 |
| 0.14911 | 0.0079 | 0.05335 | 0.07396 | -0.01414 |
| 2.10 | 2.20 | CHF | CHM | MAF |
| 0.05882 | -0.07744 | 0.02635 | 0.06372 | 0.24136 |
| MAM | PLF | PLF1 | PLF2 | PLF3 |
| 0.0338 | -0.14071 | -0.08298 | 0.10778 | -0.08984 |
| PLM | PLM1 | PLM4 | PSF1 | PSF2 |
| 0.00062 | -0.10058 | 0.00799 | -0.07878 | -0.01621 |
| PSF3 | PSF4 | PSM1 | PSM2 | PSM3 |
| 0.00107 | 0.04457 | -0.00499 | 0.00502 | -0.06496 |
| PSM4 | TAF | TAM | UKF | UKM |
| 0.04311 | 0.03791 | 0.07108 | -0.0505 | -0.05481 |

### 3.4.3c Trial 2 Comparison of Survival Rates Among Families

This challenge was conducted to determine if previous results linking size of animal to more risk of mortality were repeatable with a higher N per family. A simple linear regression between the percent survival per family at 168 h post-inoculation and average weight per family was significant, showing that higher weight is negatively correlated to survival ($p =<0.0001$, r squared = 0.7847) (Figure 3.11).

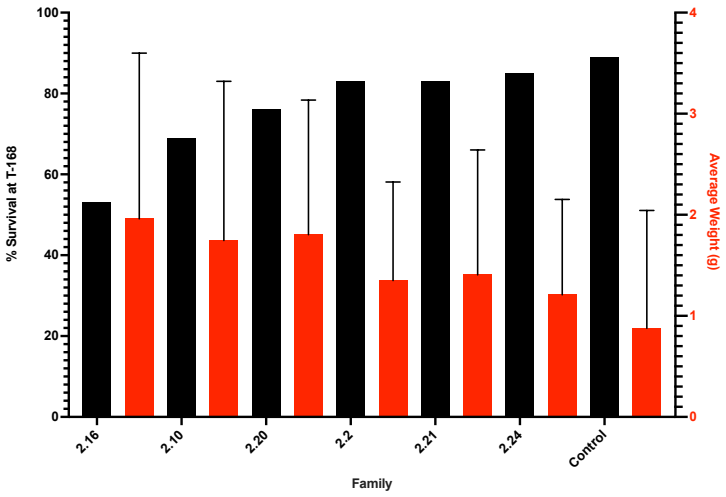



**Figure 3.11:** Percent survival by family at 168 hours post inoculation in black, and the average weight (g) in red. For N per family, see Table 3.11.

**Table 3.10:** Survival proportions of each family 168 hours post-inoculation.

| Family: | 2.16 | 2.10 | 2.20 | 2.2 | 2.21 | 2.24 | Control |
|---|---|---|---|---|---|---|---|
| Survival proportion at 168 h | 53% | 69% | 76% | 83% | 83% | 85% | 89% |

**Table 3.11:** Number of *C. gigas* tested per family for Trial 2.

| Family | N |
|---|---|
| 2.10 | 42 |
| 2.16 | 30 |
| 2.2 | 84 |
| 2.20 | 29 |
| 2.21 | 42 |
| 2.24 | 48 |
| Control | 18 |
| Total | 293 |

Of the families measured in both Trial 1 and 2, family 2.16 consistently had the lowest survival proportion, and the highest average weight (Figure 3.7 and Figure 3.11). Families 2.24 and 2.21 consistently had the highest survival proportions. When compared with Challenge 2 some of the results are inconsistent (Table 3.12) which is explainable due to the differences in methodologies used. Family 2.21 is consistently a top performer

in terms of survival proportion. The inconsistency in performance could be due to differences in dose, which we know from Challenge 1 can greatly effect pathogenicity (Figure 3.1).

**Table 3.12:** Survival proportions for the families tested in all 3 *V. aestuarianus* challenge trials.

Challenge 2

| Family: | 2.10 | 2.16 | 2.20 | 2.2 | 2.21 | 2.24 |
|---|---|---|---|---|---|---|
| Survival proportion at 96 h | 58.3 | 58.3 | 41.7 | 58.3 | 90.0 | 50.0 |
| Survival proportion at 120 h | 48.6 | 33.3 | 0.0 | 41.7 | 70.0 | 33.3 |

Challenge 3, Trial 1

| Family: | 2.10 | 2.16 | 2.20 | 2.2 | 2.21 | 2.24 |
|---|---|---|---|---|---|---|
| Survival proportion at 120 h | 58.33 | 8.333 | 33.33 | 41.67 | 83.33 | 66.67 |

Challenge 3, Trial 2

| Family: | 2.10 | 2.16 | 2.20 | 2.2 | 2.21 | 2.24 |
|---|---|---|---|---|---|---|
| Survival proportion at 168 h | 69.05 | 53.34 | 75.86 | 83.34 | 83.34 | 85.42 |

Percent survival per family was not consistent between Challenge 2, where 10 animals from each family were pooled into containers together, and Challenge 3, where each oyster was in its own individual container (Figure 3.12). Planktonic *Vibrio* was added in both challenges.

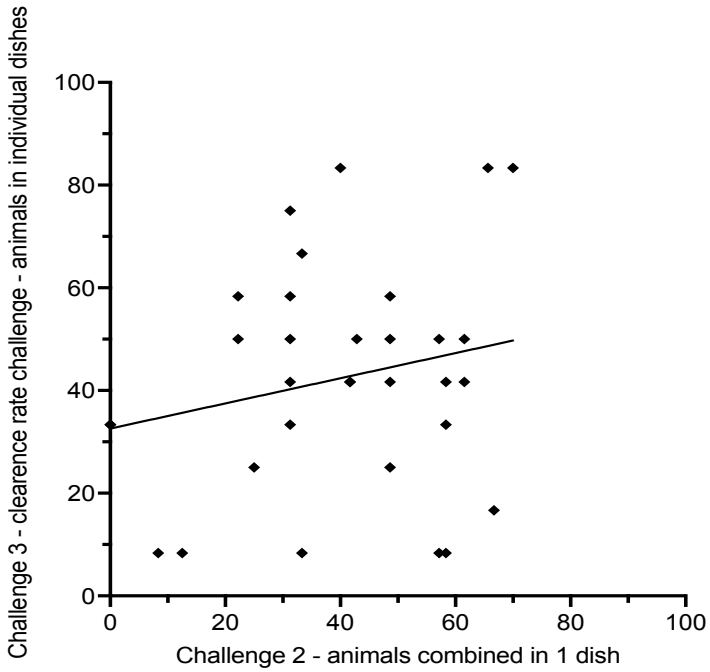



**Figure 3.12:** Percent survival for each family in Challenge 2, where animals were pooled into 1 container, and Challenge 3 Trial 1, where animals were isolated in individual containers. Linear regression shown is not significant.

## 3.5 DISCUSSION

### 3.5.1 Challenge 1: *Vibrio aestuarianus* Isolate Pathogenicity

*Vibrio spp.* isolates were found to have significantly different LD50s, with dose being a significant factor in pathogenicity. The tested isolates were all taken from dead and dying *C. gigas* Baynes Sound in the summer of 2018, indicating that virulence of *Vibrio aestuarianus* can be changed by highly localized and temporal factors. That said, our results indicate that dose is more important than specific isolate strain. The method of inoculating oysters for lab trials using one "donor" oyster infected through injection and then placed in a tank of healthy animals does not control dose, as each infected donor oyster will shed different amounts of bacteria at different speeds. Understanding that dose is a critical factor which effects LD50, we can say that this method of inoculation is

not effective at controlling dose. Currently, the typical aquaculture practice for *C. gigas* is to grow oysters in high density environments, whether it be a stacking tray system suspended from a raft, or baskets floating at the surface (Quayle, 1988). This high-density environment may provide the perfect breeding ground for a killingly high dose of *V. aestuarianus* to infect nearby animals quickly, as shown through lab cohabitation studies which showed secondary infections in as little as 2 hours after an infected oyster was placed in a tank with healthy oysters (De Decker & Saulnier, 2011; Azéma et al., 2015). If one more susceptible animal gets infected and dies, it sheds lots of bacteria, potentially infecting the other animals around it who may have otherwise survived a low-dose infection from the natural environment with a higher, killing dose. Study into the effect of reducing animal density in farm environments could be warranted. Further study of the sequence of these bacterial isolates as well as their genetic upregulation could also be warranted. Strain 4 (BS-2018-032) *Vibrio aestuarianus* was found to have the lowest LD50 and was thus used in all subsequent lab challenge trials.

### 3.5.2 Challenges 2 + 3: *Vibrio aestuarianus* Survival Heritability + Clearance Rate

Results from the three trials in these two challenges showed that there are significant phenotypic differences between survival to *V. aestuarianus* infection in our 32 full-sib families. There is some consistency across all the trials as to best and lowest performing families, notably the offspring of dam MAF performed well, and the offspring of dam PLF performed poorly. Genetic heritability on the underlying liability scale was estimated to be 0.15 (SE = 0.068). This confirms the findings of Degremont et al.'s 2020 study, as it is within the range of the heritability calculated by that lab. While this only a

moderate heritability, it may still be worth breeding for resistance to *V. aestuarianus* infection, as it can cause significant mortality in cultured *C. gigas*, and an increase in survival could have a large economic impact for famers.

Maternal immune priming has been shown to have an effect on the survival of bivalve larvae (Yue et al., 2013; Robinson & Green, 2020). Maternal immune priming is the transfer of immunity from mother to offspring, which may reduce the offspring's risk of disease from a pathogen that previously infected its mother (Robinson & Green, 2020). Because the parents of our oyster families are from wild stock and animals who lived on farms exposed to bacterial infection, it is possible that the observed phenotypic variation in survival is a result of maternal immune priming, rather than genetics. This possibility is supported by the strength of one dam's offspring in our results, dam MAF, which was collected from wild stock. This result is significant for oyster farmers in Baynes sound, as it will be incorporated in a breeding program to create heartier oysters which are more resistant to local strains of *V. aestuarianus.*

Our first heritability trial showed a very strong link between survival and size, with each 1 mm increase in length increasing the risk of death by 18%, regardless of the genetic family. This result led us to wonder whether larger animals were perhaps getting a higher dose of bacteria due to increased speed of water filtration. We controlled dose by isolating oysters and giving them the same volume of bacteria titrate. While we did not find a strong link between clearance rate and survival, it was correlated to both size and survival, leading us to believe that size is the more important variable. We again found a highly significant correlation between animal size and survival, even with infection dose

controlled. This finding is perhaps more significant than the genetic heritability of resistance to *V. aestuarianus* because a multitude of environmental factors play a role in determining oyster size beyond the genetic. This finding calls into question any heritability calculations which do not include animal size as a factor in the model. Additionally, fast growth rate is one of the physical traits that currently existing breeding programs for *C. gigas* focus most closely on, because it decreases time to market and therefor profitability for farmers (Li et al., 2011; Wang et al., 2012; Zhang et al., 2019a). We suspect that global oyster breeding programs may be selecting animals for both larger size and faster growth rates, and we suggest that further study is needed to determine if this selection is creating genetic stock which may be more susceptible to infection by *Vibrio aestuarianus*. We ask: what is the economic value of a fast-growing large oyster vs. the value of a disease resistant, slower growing oyster? Another area for further study could be to determine if slower growing families which are more resistant to infection with *Vibrio aestuarianus* become more susceptible as they reach market size, even if they do so more slowly. This will be of particular concern as disease becomes more common in our warming oceans.

We found that the clearance rate of individual animals varied significantly, even within genetic full-sib families. Because of this, if an experiment requires that dose be a controlled variable, we expect that inoculating oysters by adding planktonic bacteria or an infected donor oyster to a tank containing multiple healthy animals would not be a good experimental design. Dose is a significant factor in mortality rate for *C. gigas* exposed to *Vibrios*, so any experiment measuring mortality rates requires more precise

dose control, particularly when calculating heritability. Isolating oysters into smaller containers allows the experimenter to add the same dose of bacteria to each animals' environment, while still mimicking real-world infection mechanisms. An additional benefit of this method is that it removes the possibility of a tank effect.

Challenges 2 and 3 showed different mortality rates per family. This could have been caused by the differences in experimental setup, with Challenge 2 pooling multiple animals into one shared tank and thereby not controlling the inoculum dose per oyster. It also could be an effect of individual oyster size being a more predictive factor of mortality than genetic family. It was imposable to have oysters of the exact same size for each of the challenges, so mortality rate for families would be impacted by the size of the individuals used in the challenges.

# 4. CHAPTER 4: CONCLUSION

This thesis is an examination of the role of *Vibrio aestuarianus* in Baynes Sound, BC.

The questions initially posed were:

1) What environmental factors appear to contribute to the incidence of summer mortality in Baynes Sound?

   There is a positive correlation between seawater temperature and total *Vibrio* detected in water samples in Baynes Sound (Figure 2.6, Figure 2.7, Table 2.5, Table 2.6). This correlation was stronger and more significant in 2020 than in 2019. There was also a significant positive correlation between abundance of *V. aestuarianus* and seawater temperature in 2020, but not in 2019. This discrepancy between years corresponds with lower regional seawater temperatures in 2019 (Figure 2.5). There is no significant correlation between any of the bacterial assays tested and salinity, pH, or $\Omega_{arag}$ saturation. We found *V. aestuarianus* to be distributed evenly throughout Baynes Sound, with no distinct pattern of geographic distribution. We found there to be more abundant *Vibrio* above the thermocline, in warmer waters than in the deeper cooler water sampled, although it was still present there as well.

2) Is resistance to *V. aestuarianus* heritable?

There are significant phenotypic differences between families survival rates to infection with *V. aestuarianus.* Oyster size is significantly negatively correlated to survival to

infection from *V. aestuarianus*. The Cox proportional hazard ratio calculation showed that for every 1g increase in weight the risk of death goes up by 73%, regardless of the genetic family. Analyzing survival data from 396 oysters from 32 full-sib families, we estimated the heritability of survival on the observed and underlying liability scales to be 0.095 (SE = 0.043), and 0.15 (SE = 0.068) respectively. The heritability on the underlying liability scale is a more accurate estimate of heritability with binomial data. We were able to identify the breeding values for the families in Vancouver Island University's *C. gigas* breeding program, which will be used to breed resistance to *V. aestuarianus* infection into future generations of seed stock for the region.

To date, most selective breeding efforts for *C. gigas* have focused on traits which enhance marketability and speed of production such as increasing growth rates. Some effort has been made to select for resistance to disease, particularly *Ostreid herpesvirus*, but efforts to breed resistance to *Vibrio* infection are just beginning. Our results indicate that larger, faster growing oysters may be more susceptible to *V. aestuarianus*, even within the same genetic family. Current selection efforts for increased size and growth rate may be creating a population which is more susceptible to summer mortality, thereby negating the economic advantage of faster growing oysters which reach marketability earlier.

3) Can we design a better protocol for laboratory bacterial infection of *C. gigas* and other bivalves?

   The two primary methods to study bacterial infection of oysters are currently injection and cohabitation. In order to test for heritability of resistance against

disease it is important to both control the dose administered to each animal, and to mimic real-world infection pathways that do not bypass the oysters' natural immune response. Our study describes a repeatable experimental framework which controls both for dose, as direct injection does, but also mimics real-world infection pathways, like cohabitation does.

4) What can oyster farmers in the region do to minimize mortality on their farms?

Our study did not locate a geographic reservoir for *Vibrio*, instead suggesting that it occurs throughout the water column in the entirety of Baynes Sound but becomes more prolific when seawater temperatures go up. Our study found that husbandry stress may be a contributing factor in summer mortality, especially when that stress comes after oysters have been infected with *Vibrio*. Because our findings also indicate that temperature predicts *Vibrio* abundance, our study suggests that farmers avoid stressing their oysters immediately after a marine heatwave and during hotter seawater temperatures.

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**APPENDIX 1:** Detailed pedigree for *Crassostrea gigas* families bred at Vancouver Island University's Deep Bay Marine Field Station. FR1 was produced on May 23, 2020, FR2 was produced on June 26, 2020.

| Abbreviation | Origin |
|---|---|
| PS | Pipestem Inlet, BC |
| PL | Pipers Lagoon, BC |
| UK | Guernsey, UK |
| CH | Chile |
| MA | Marina Island, BC |
| TA | Taylor's shellfish |

| Family | Dam | Sire |
|---|---|---|
| 1.1 | PSF1 | PLM1 |
| 1.7 | PSF2 | PLM4 |
| 1.8 | PSF3 | PLM4 |
| 1.9 | PSF4 | PLM4 |
| 1.10 | PSF3 | PSM1 |
| 1.12 | PLF1 | PSM1 |
| 1.13 | PSF4 | PSM2 |
| 1.14 | PLF1 | PSM2 |
| 1.16 | PLF1 | PSM3 |
| 1.17 | PLF2 | PSM3 |
| 1.18 | PLF3 | PSM3 |
| 1.19 | PLF2 | PSM4 |
| 1.20 | PLF3 | PSM4 |
| 1.22 | PLF3 | PLM1 |
| 2.1 | TAF | TAM |
| 2.2 | TAF | CHM |
| 2.4 | TAF | PLM |
| 2.5 | TAF | MAM |
| 2.6 | CHF | TAM |
| 2.9 | CHF | PLM |
| 2.10 | CHF | MAM |
| 2.11 | UKF | TAM |
| 2.13 | UKF | UKM |
| 2.14 | UKF | PLM |
| 2.16 | PLF | TAM |

| | | |
|---|---|---|
| 2.18 | PLF | UKM |
| 2.20 | PLF | MAM |
| 2.21 | MAF | TAM |
| 2.22 | MAF | CHM |
| 2.23 | MAF | UKM |
| 2.24 | MAF | PLM |
| 2.25 | MAF | MAM |