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The effects of seeding density on production traits and stress biomarkers of the Greenshell^{TM} mussel, *Perna canaliculus*

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Conservation Biology at Massey University, Albany, New Zealand

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2021

General Abstract

The Greenshell^{TM} mussel, *Perna canaliculus,* is New Zealand's most important aquaculture species and holds great economic significance. Losses of juvenile mussels (spat) are common and can severely reduce the potential yield of *P. canaliculus*, however the exact drivers of these losses are unknown. Environmental variation inevitably contributes to mussel performance, however, environmental factors are difficult to control in a water-based aquaculture system, such as *P. canaliculus* farms. Therefore, it is important to understand how controllable factors, such as commercial procedures, impact mussel performance so modifications can be implemented. In this study, I assessed the impacts of seeding density (257, 515, and 1030 mussels 10cm^{-1} of culture rope) on *P. canaliculus* performance on commercial mussel farms in the Marlborough Sounds. In a spatially replicated field experiment during the austral summer in the initial stages of culture when spat loss is common, I explored the effects of seeding density on production traits: *P. canaliculus* abundance and size, as well as accumulation of biofouling on culture ropes and stress biomarkers in *P. canaliculus* by assessing a stress effect: total antioxidant capacity (TAC), and stress responses: oxidative stress, and viability (valve closure during freshwater immersion). At the end of my experiment, *P. canaliculus* abundance remained greatest at the highest seeding density, however losses were also greatest at the highest seeding density, and *P. canaliculus* growth was greater at shallow depths. The abundance of a problematic biofouling species, *Mytilus galloprovincialis*, was greatest at low seeding densities and shallow depths, and varied between sites. *Mytilus galloprovincialis* were also larger at shallow depths. Shallower depths and Site 2 attracted a greater biomass of non-mussel biofouling. Although, TAC in *P. canaliculus* varied among seeding density treatments, the impacts of seeding density were inconsistent among depths, sites, and time. Nonetheless, while mussel viability remained high in all treatments oxidative stress appeared greater near the end of summer. Ameliorating challenging conditions increases the capacity of mussels to respond to additional stressors, including uncontrollable environmental factors and future climate change. However, my results indicate seeding density influences whole-organism and stress biomarkers differently and underlies trade-offs between favourable mussel production traits for farmers to consider.

Acknowledgements

Firstly, a big thanks to Rodney Roberts from SpatNZ for coordinating all initial meetings and guiding me every step of the way with all my questions and uncertainty, you have been a great support through my Masters experience! Thanks to Sanford Ltd for funding the field work for this study, Dave Hebert for sharing your expertise and Wayne Hollis for skippering our boat trips, providing much needed coffee for the early starts and chasing dolphins along the way. Massive thanks to Andy Day for organising all field work, spat supply, and leading the field trips even when I couldn't fly down in time, you made everything happen so thank you. Additional thanks to Norman Ragg, Steve Webb, and Jess Ericson from Cawthron Institute, and Sarah Cummings from SpatNZ for your contribution in the initial planning stages of this project, thanks for being so accommodating and sharing your ideas. Thanks to Danielle Kok, Erin Moffet and Mena Welford for assistance in the labs. Thank you so much to my co-supervisors Natali Delorme and Paul South from Cawthron Institute for your constant support and valued feedback, helping on the field trips and in the lab and overall taking me under your wing, it has been awesome to work with you both.

Secondly, I'd like to thank my family; Jose, John, Lissa, Megan and Georgia, you guys are my absolute rocks. Your support and positivity throughout my Masters has encouraged me to stay motivated even when things felt hard. Thanks to my close friends especially Lana and Lucie for your support and study breaks and fellow students and best mates Georgia, Emily, Julia, Jenny-Ann, Alex, and Katie for the laughs and important snack runs.

Last but not least, a great big thanks to my primary supervisor, Dave Aguirre, for your endless encouragement and guidance in this journey. Your positivity and reassurance has kept me on track and focused, your laid back attitude made nothing seem too scary. The hours you invested into my work, restructuring, editing and fixing many errors, it is all hugely appreciated! Thanks for the constant support, even when we were stuck at home for weeks and Zoom was our best friend (thanks Covid). Huge appreciation for all you have done for me, thanks Dave.

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Chapter 1: General introduction

1.1 Rationale for study

With an expanding human population, the demand for aquatic food resources is growing. Concerning seafood, there are two main options; wild-caught seafood, or cultured seafood (Klinger et al., 2013). Global production of seafood including wild-caught and cultured seafood has more than quadrupled between 1960 and 2015 (Anderson et al., 2017; Ritchie & Roser, 2019). The growing demand for seafood has seen overexploitation of wild populations (Burgess et al., 2013; Mansfield, 2010). Consequently, wild-caught seafood production has reached maximum productivity and remained stagnant since 1980, and alternatively aquaculture production has consistently increased since 1970 (Anderson et al., 2017; Ritchie & Roser, 2019). Now, aquaculture provides over 50% of global seafood supply, and there is hope that continued development of this sector will alleviate overfishing of wild stocks (Anderson et al., 2017; Ritchie & Roser, 2019).

Global aquaculture production comprises mainly fish, aquatic plants and molluscs (Garlock et al., 2020; Naylor et al., 2021; Tacon, 2020). Depending on the species and location, fish are typically cultured in freshwater or coastal ponds, tanks or cages (Bostock et al., 2010), aquatic plants/seaweeds are grown in coastal floating or suspended culture, or in ponds or tanks (Kim et al., 2017), and molluscs are grown on the seafloor, freshwater and coastal cages as well as raceways, ponds, hatcheries, coastal longline systems or in raft systems (Tidwell, 2012). The environment and aquaculture system in which seafood is cultured can influence the efficiency of production.

Molluscs, particularly shelled molluscs (hereafter referred to as ‘shellfish’ unless otherwise specified), are essential to global aquaculture production, making up 12.2% of global aquaculture value, and 15.5% of global aquaculture production weight (Tacon, 2020). Despite the mass production of molluscs, there are also many limiting factors in shellfish cultivation. Inefficiency in shellfish production generally stems from losses due to mortality

(Soletchnik et al., 2005), secondary settlement and migration (South et al., 2020), or reduced retention in longline farming systems (Alfaro, 2006). Therefore, high retention, survival, and growth are favourable production traits in shellfish cultivation. Shellfish aquaculture typically occurs in the natural aquatic environment where changes in abiotic factors such as temperature (Lachance et al., 2008), carbon dioxide (CO_2) levels (Talmage & Gobler, 2011) and water movement (Alfaro, 2006; Hayden & Woods, 2011) can influence production traits. For example, shell growth in the hard clam, *Mercenaria mercenaria,* was significantly lower at 28°C compared with 24°C, and survival in the bay scallop, *Argopecten irradians,* decreased with greater CO_2 levels (Talmage & Gobler, 2011). Biotic factors including predation (Šegvić-Bubić et al., 2011), and competition for resources due to overcrowding (Bordignon et al., 2021; Liu et al., 2019) can also influence shellfish production traits such as increasing migration which impacts abundance, as well as reducing growth and survival. Additional to biotic and abiotic factors, commercial seafood species are also exposed to commercially induced pressures including transportation and handling during farming, which are known to negatively impact cultured species such as trout (López-Patiño et al., 2014; Tacchi et al., 2015), though these effects are less understood in shellfish.

While whole-organism responses such as changes in growth or survival are immediately recognisable by farmers and are critically important for shellfish production, stress biomarkers are also impacted by a range of abiotic and biotic factors. Importantly, stress biomarkers often precede whole-organism responses in both terrestrial and marine organisms, making them possible indicators of an organism's response to the environment. For example, increased temperature and food availability can alter stress biomarkers and subsequently growth (Hébert & Dunlop, 2020), as well as reproductive behaviour (Lynn et al., 2010). In the Catarina scallop, *Argopecten ventricosus,* oxidative damage in the swimming muscle increased in response to predator-exposure, and as scallops neared spawning, oxidative damage also increased in the gills and mantle compared with predator-free scallops (Guerra et al., 2013). Interestingly, after four months, the muscle weight in predator-exposed scallops was greater than predator-free scallops (Guerra et al., 2013). Commercial procedures can also impact stress biomarkers and whole-organism responses in shellfish. For example, high stocking density in the noble scallop, *Chlamys nobilis*, increased the bacterial load and reactive oxygen species levels, indicative of higher stress, resulting in decreased survival and growth compared with low stocking densities (Liu et al., 2019). Stress biomarkers and whole-organism responses in shellfish to external pressures are dependent on the species and growing environment, so it is important to assess how certain pressures impact valuable aquaculture species to allow beneficial procedural modifications to be implemented.

The Greenshell^{TM} mussel, *Perna canaliculus,* is the primary aquaculture species in New Zealand, with approximately 97,000t harvested each year, creating revenue of NZD$381 million annum^{-1} (Stenton-Dozey et al., 2020). New Zealand's coastline is well suited for shellfish aquaculture due to its abundance of sheltered bays, inlets and islands, and high primary productivity (Stenton-Dozey et al., 2020). The major locations for *P. canaliculus* farming in New Zealand are the Marlborough Sounds, Hauraki Gulf, Golden Bay and Stewart Island (Jeffs et al., 1999). *Perna canaliculus* are farmed using a longline system, where a double backbone is suspended from buoys, and culture ropes holding mussels are looped over the backbone and extend approximately 10m deep (Jeffs et al., 1999). A mussel farm of 3ha includes up to 10 longlines (Jeffs et al., 1999; Stenton-Dozey et al., 2020). Juvenile mussels (spat) are seeded onto the culture ropes using mechanical seeding equipment on a purposely designed vessel, where they are held against the polypropylene culture ropes inside a polyester mesh stocking (Jeffs et al., 1999; South et al., 2021). Over time, the stocking and remaining beach-cast material disintegrate, allowing the spat to re-attach to the culture rope or migrate elsewhere (Skelton & Jeffs, 2020). The nursery stage continues until spat reach 10-20mm, then mussels are stripped from the culture rope, most biofouling removed, and then re-seeded at a lower density, called inter-seeding (Jeffs et al., 1999; South et al., 2020). Typically, this process is repeated once more, before a final grow-out period when mussels are harvested and sold after a total growth period between 18 months and five years (Jeffs et al., 1999; South et al., 2020; Woods et al., 2012).

New Zealand's mussel industry is heavily reliant on wild-caught spat, with approximately 80% of spat sourced from beach-cast material arriving on Ninety Mile Beach in the far north of New Zealand (Alfaro et al., 2010; Jeffs et al., 1999). Approximately 200t of beach-cast material is collected each year and transported in refrigerated vehicles to the main mussel farming locations (Alfaro et al., 2010; Jeffs et al., 1999; Jeffs et al., 2018). The arrival, abundance and quality of spat on Ninety Mile beach is highly dependent on sea and wind conditions, and can be unpredictable (Alfaro et al., 2010). A small amount of spat is also caught on artificial ropes at sites neighbouring the main mussel farming locations (Jeffs et al., 1999). Additionally, hatchery technology has been created to promote selective breeding of *P. canaliculus,* which provides a highly successful source of spat for the industry to reduce the heavy reliance on beach-cast material (Ragg et al., 2010). Nonetheless, with such a high reliance of wild-caught spat, making the most of this resource is crucial.

Unfortunately, up to 99% of *P. canaliculus* spat can be lost in the early stages of aquaculture (Skelton & Jeffs, 2021b; South et al., 2020). Mortality and secondary settlement (migration) are the two main drivers of loss in *P. canaliculus,* often while spat are still attached to beach-cast material inside the stocking; however, there is still great uncertainty on factors influencing mortality and migration (Carton et al., 2007; Hayden & Woods, 2011; Skelton & Jeffs, 2020; South et al., 2020). Overcrowding of shellfish is a factor that can impact biofouling and conspecific settlement (Cubillo et al., 2015; Fletcher et al., 2013) and intraspecific and interspecific competition for nutrients and space (Bordignon et al., 2021), which can consequently impact shellfish growth, survival, abundance, as well as migration (Cubillo et al., 2015; McGrorty & Goss-Custard, 1995). For example, a high initial seeding density had negative impacts on growth and survival in the Manila clam, *Ruditapes philippinarum* (Bordignon et al., 2021). Seeding density also had negative impacts on performance of the mussel, *Mytilus galloprovincialis,* where growth was reduced when density of mussels increased (Cubillo et al., 2012b). As a procedure which tends to impact shellfish performance and can be controlled by mussel farmers, it is a priority to investigate *P. canaliculus* performance at different seeding densities so farming protocols can be modified, ultimately reducing stress and loss, and enhancing mussel production.

Mussel farms often accumulate an abundance of biofouling, consisting of macroalgae, suspension-feeding organisms such as ascidians and other mussel species, and mobile invertebrates including amphipods and crustaceans (South et al., 2019; Woods et al., 2012). Biofouling can negatively impact mussel performance through direct competition for space (South et al., 2019; Woods et al., 2012) or by decreasing water flow and nutrient delivery to mussels (Sievers et al., 2013), thus indirectly reducing growth and retention of mussels. Blue mussels, *M. galloprovincialis*, are the most problematic biofouling species on *P. canaliculus* farms due to the intense resource competition for food and space (Atalah et al., 2016; Forrest & Atalah, 2017; Woods et al., 2012). The composition and biomass of biofouling accumulating on mussel farms can vary with depth (Woods et al., 2012), spat type (Forrest & Atalah, 2017), location (Sievers et al., 2014), the density of mussels on the rope and time of year (South et al., 2017). Although most biofouling in *P. canaliculus* cultivation is removed during inter-seeding and final seeding (Woods et al., 2012) to allow *P. canaliculus* to dominate space on the culture rope, biofouling species are often fast growing and fast reproducing, thus can persist and remain problematic on mussel farms (Fletcher et al., 2013). Therefore, understanding the factors underlying variation in biofouling accumulation, and how seeding density impacts biofouling accumulation on *P. canaliculus* farms across a spatial scale to account for abiotic variation, is essential to mitigate overcrowding and competition.

1.2 Overview of thesis

This thesis contributes to the understanding of how seeding density impacts *P. canaliculus* performance in New Zealand. This thesis comprises two empirical data chapters (Chapters two and three), and a general discussion chapter (Chapter four). Chapters two and three are written in manuscript format as I expect to submit these for peer-review as co-authored manuscripts shortly after receiving the examiner's comments, so I use "we" (first-person plural) to acknowledge this. Nonetheless, this thesis is my own work, completed under the guidance of my supervisors. Given that these chapters are intended as independent publishable units, there will also be some repetition of background information and methodologies throughout the thesis.

Chapter two analyses the effects of seeding density on *P. canaliculus* performance by measuring production traits: abundance and growth of wild-sourced *P. canaliculus* spat, as well as biofouling accumulation on commercial mussel farms in the Marlborough Sounds. I predict that *P. canaliculus* abundance will be greatest at high seeding density, but that less mussels will be lost, and growth will be greatest at low seeding density. I also predict that biofouling accumulation will be greatest at the low seeding density. Additionally, the intensity of the impacts of seeding density on *P. canaliculus* performance and biofouling accumulation will likely vary across space and time due to variation in marine environmental factors. These data will indicate whether seeding density consistently impacts *P. canaliculus* production traits and biofouling accumulation despite spatial and temporal variation.

Chapter three investigates the impacts of seeding density on stress biomarkers of *P. canaliculus* from commercial mussel farms in the Marlborough Sounds by measuring a stress effect (total antioxidant capacity), and two stress responses (oxidative stress and viability [valve closure during freshwater immersion]). I predict that oxidative stress will be greatest at high seeding density, and that viability will be greatest at low seeding density, implying that low seeding densities are less stressful for *P. canaliculus.* These data will examine if seeding density consistently impacts stress biomarkers and whole-organism responses, and whether stress biomarkers could indicate changes in production traits such as abundance and growth.

Initially, I had planned to conduct another sampling trip in March 2020, to analyse mussel performance for an extended period during the initial months of mussel cultivation. However, due to the lockdowns and restricted travel during the COVID-19 pandemic, this plan was unable to advance. Nonetheless, I feel I progressed an ambitious research plan and have produced two data chapters of publication quality and look forward to the examiner's suggestions for improvement.

Chapter 2: Impacts of seeding density on the abundance and size of the Greenshell^{TM} mussel, *Perna canaliculus*

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In preparation for publication

2.1 Abstract

The Greenshell^{TM} mussel, *Perna canaliculus*, is economically significant and extensively farmed in New Zealand. Significant losses in commercial populations of *P. canaliculus* in the Marlborough Sounds, particularly in juvenile mussels (spat), severely limit potential harvest, though the drivers of these losses are largely unknown. Here, we investigated how seeding density impacts the abundance and size of *P. canaliculus* spat at four depths from five mussel farms at two sites in the Marlborough Sounds. We also recorded the abundance and size of blue mussels, *Mytilus galloprovincialis*, which are a problematic biofouling species, as well as the biomass and composition of the biofouling community accumulating on mussel culture ropes under the same experimental treatments. Although abundance of *P. canaliculus* remained greatest at the highest seeding density, the highest seeding density also experienced the greatest losses. *Perna canaliculus* size on the other hand, was greater at shallower depths and at Site 2. *Mytilus galloprovincialis* was most abundant at low seeding densities, shallow depths and at Site 1, while *M. galloprovincialis* were larger at shallower depths. Shallow depths and Site 2 accumulated greater biomass than deeper depths and Site 1. Overall, it is important for mussel farmers to prioritise cultivation in the most favourable conditions; however, given the results we present here it appears there will be impactful trade-offs in production traits (e.g., abundance and growth) for farmers to consider.

2.2 Introduction

Aquaculture is essential for global food supply and is primarily comprised of fish, aquatic plants and molluscs (Garlock et al., 2020; Naylor et al., 2021; Tacon, 2020). Since 1997, global aquaculture production has tripled in live weight volume, and the number of cultured species has increased by 40% (Metian et al., 2020; Naylor et al., 2021). The demand for aquatic cultured food is predicted to grow, however there are many limiting factors including environmental fluctuations and anthropogenic interactions which can cause aquaculture production to become inefficient (Gephart et al., 2020; Nguyen & Alfaro, 2020). Shellfish aquaculture is particularly susceptible to environmental fluctuations, due to cultivation typically occurring in the natural aquatic environment (Broekhuizen et al., 2021; Chang et al., 2003), and the sessile nature of most shellfish species which restricts movement away from challenging conditions. Losses of cultured shellfish are generally driven by increased mortality, migration, and reduced retention, which can be impacted by elevated temperature (Lachance et al., 2008), water movement (Alfaro, 2006; Hayden & Woods, 2011), as well as commercial procedures including handling and overstocking (Bordignon et al., 2021; Harding et al., 2004). Globally, shellfish contribute 12.2% to global aquaculture value, and 15.5% of global aquaculture production weight (Tacon, 2020). Due to the high global demand for commercial shellfish, understanding drivers of large losses is essential to ensure efficient shellfish production.

The Greenshell^{TM} mussel, *Perna canaliculus,* is New Zealand's most important aquaculture species with approximately 97,000t harvested each year, creating revenue of NZD$381 million annum^{-1} (Stenton-Dozey et al., 2020). Juvenile mussels (spat) for *P. canaliculus* farming in New Zealand are primarily wild-caught, with approximately 80% being collected from beach-cast material at Ninety Mile Beach in the far north of New Zealand (Alfaro et al., 2010; Jeffs et al., 1999). The density of spat can range from 200 to 200M mussels kg^{-1} of beach-cast material, and the shell length ranges between 250μm and 10mm (Hickman, 1976; Jeffs et al., 2018). Approximately 200t of beach-cast material is collected each year and transported in refrigerated vehicles to the main mussel farming locations up to 1600km away: Hauraki Gulf, Marlborough Sounds, Golden Bay and Stewart Island (Alfaro et al., 2010; Jeffs et al., 1999; Jeffs et al., 2018). Aside from beach-cast material at Ninety Mile Beach, a small amount of spat is caught in the wild on artificial ropes at sites neighbouring the major mussel farming locations (Jeffs et al., 1999). Additionally, hatchery technology has been created to promote selective breeding of *P. canaliculus,* which provides a highly reliable and successful source of spat for the industry (Ragg et al., 2010). However, with much of the spat source for the mussel industry dependent on the environment delivering beach-cast material at Ninety Mile Beach, ensuring efficient use of the available source is essential.

Perna canaliculus farms are made up of longline systems which consist of a double backbone supported by buoys, and culture ropes seeded with mussels are continuously looped over the backbone and extend 5-10m deep (Jeffs et al., 1999). Each mussel farm is approximately 3ha and includes up to 10 longlines (Jeffs et al., 1999; Stenton-Dozey et al., 2020). Spat are initially seeded onto the polypropylene culture ropes inside a polyester mesh stocking, and over time the stocking and remaining beach-cast material disintegrate which allows the spat to re-attach to the culture rope or migrate elsewhere (Skelton & Jeffs, 2020). Once spat that attach to the culture rope reach 10-20mm in length, the nursery stage, all mussels are stripped from the culture rope, cleared of most biofouling, and re-seeded at a lower density, a process called inter-seeding (Jeffs et al., 1999; South et al., 2020, 2021). Later, mussels are usually stripped and re-seeded at a lower density once more for a final grow-out period, called final seeding, then they are harvested and sold after a total growth period between 18 months and five years (Jeffs et al., 1999; South et al., 2020; Woods et al., 2012).

Losses of cultured *P. canaliculus* are common, with up to 99% of *P. canaliculus* spat being lost as the stocking disappears from the culture rope during the nursery stage of cultivation (Skelton & Jeffs, 2021b; South et al., 2020). Mortality and secondary settlement (migration) are likely to contribute to the loss of seeded *P. canaliculus*, though there is still uncertainty on the exact drivers of excessive mortality and migration in *P. canaliculus* (Carton et al., 2007; Hayden & Woods, 2011; South et al., 2020). However, abiotic factors such as water temperature (Lachance et al., 2008), turbulence (Alfaro, 2006) and nutrient availability, and biotic factors including competition for resources (Bordignon et al., 2021) and predation (Šegvić-Bubić et al., 2011) can impact growth, retention and survival of commercial mussels and other cultivated shellfish. The abundance of *P. canaliculus* spat remaining on culture ropes at inter-seeding is therefore a renowned bottleneck for the commercial population and often determines the overall yield of adult *P. canaliculus.*

Seeding density is the density in which cultured mussels are spaced on the ropes at initial seeding, and can impact intraspecific competition between cultured mussels, biofouling accumulation and therefore interspecific competition between cultured mussels and biofouling species with demand for similar resources (Cubillo et al., 2015). For example, high seeding density decreased growth and survival in the Manila clam, *Ruditapes philippinarum*, likely due to intraspecific competition for food and space (Bordignon et al., 2021). Similarly, growth in the mussel, *Mytilus galloprovincialis,* decreased at higher seeding densities due to stronger intraspecific competition at high compared with low seeding density (Cubillo et al., 2012b). The level of intraspecific competition between cultured mussels and biofouling accumulation as a result of seeding density can impact self-thinning of cultured mussels, therefore directly contribute to overall losses (Guiñez, 2005; Lauzon-Guay et al., 2005b). Seeding density is therefore a proposed method to manage losses of *P. canaliculus* spat in the nursery stage of aquaculture as it is a commercial procedure which mussel farmers can control. Accordingly, it is important to understand how seeding density impacts *P. canaliculus* performance so beneficial changes to commercial procedures can be made.

Mussel farms accumulate large quantities of biofouling, including macroalgae, suspension-feeding species including other mussels and ascidians, and mobile invertebrates such as amphipods and other crustacea (South et al., 2019; Woods et al., 2012). Blue mussels, *M. galloprovincialis*, are the most problematic biofouling species on *P. canaliculus* farms due to their demand for similar resources (Atalah et al., 2016; Woods et al., 2012). The density of mussels can influence the accumulation and composition of biofouling on culture ropes. For example, higher density of adult *P. canaliculus* increased the accumulation of *M. galloprovincialis* as well as some amphipods (South et al., 2017). Also, density of small *P. canaliculus* (20-40mm shell length at seeding) had a significant negative relationship with the biomass of an invasive ascidian species, *Didemnum vexillum* (Fletcher et al., 2013). Although there are systems in place to remove biofouling during inter-seeding and final seeding, excessive biofouling can persist due to the fast growth and reproduction of many biofouling species (Fletcher et al., 2013). Therefore, understanding how seeding density impacts biofouling accumulation, and how this relates to *P. canaliculus* performance will allow farmers to practically manage biofouling accumulation in the future.

Here, we examined the effects of seeding density on the performance of wild-sourced *P. canaliculus* spat in a field experiment in the Marlborough Sounds. Specifically, we measured production traits: *P. canaliculus* spat abundance and growth, as well as *M. galloprovincialis* abundance and growth and non-mussel biofouling accumulation. The field experiment tested three seeding densities at four depths at five farms within two main mussel farming sites in the Marlborough Sounds to account for variation in the abiotic environment. This experiment was conducted during the nursery stage of mussel cultivation to capture the period where spat losses are common.

2.3 Materials and methods

2.3.1 Study sites

This study assessed the performance of *P. canaliculus* spat from culture ropes held in conventional farming systems during the nursery stage of the aquaculture production cycle. The field experiment was carried out at five commercial mussel farms at two sites in the outer Marlborough Sounds, New Zealand.

2.3.2 Source of *Perna canaliculus* and initial seeding

Beach-cast material including seaweed, hydroids and *P. canaliculus* spat was harvested from Ninety Mile Beach, New Zealand (34°56'54.4"S 173°07'14.0"E) on 14th September 2019. Three 10kg bags of beach-cast material were collected and transported to Havelock (41°16'54.5"S 173°46'01.9"E) in a refrigerated truck (6-7°C), where it was amalgamated to produce 30kg of spat material for the field experiment. The experimental culture ropes were prepared on a purpose-designed mussel seeding boat. Seeding the mussels onto the culture ropes involved sprinkling spat material onto the culture rope by hand as it was mechanically pulled past a seeding hopper before being secured in a polyester stocking and deployed in the water. The rate at which the spat material was added to the culture rope was adjusted to achieve the different seeding densities (Table 1). All experimental culture ropes were initially deployed at Site 1 on 16th September due to logistical constraints and then half of the culture ropes were moved to the five farms at Site 2 on the 20th September 2019. For the study, five farms at both Site 1 and Site 2 each held a 16m loop of culture rope of each seeding density spaced 0.5m apart.

Table 1. The weight of spat material required to achieve the experimental seeding densities for each 16m loop of culture rope.

Seeding density target (m of culture rope $10kg^{-1}$ bag of spat material)	Equivalent grams of spat material m^{-1} of culture rope	Equivalent grams of mussels m^{-1} of culture rope	Number of mussels m^{-1} of culture rope	Number of mussels $10cm^{-1}$ of culture rope
150m	66g	15.9g	10,309	1,030
300m	33g	7.96g	5,154	515
600m	16.5g	3.98g	2,577	257

To measure initial spat abundance, we haphazardly collected and froze (-20°C) five samples (37-54g) of the spat material for later analysis. We measured wet weight (g) of each sample, then washed each sample over a series of mesh sieves to separate the contents into three size classes based on mussel shell width (62μm – 250μm, 250μm – 1000μm, and >1000μm). Contents >1000μm were separated, and mussels were counted. The shell length (SL) of 30 individual mussels (>1000μm shell width) was determined by imaging under a dissecting microscope and using ImageJ software (Schneider et al., 2012). The contents of the two smaller size classes (62μm – 250μm and 250μm – 1000μm) were subdivided into eighths using a plankton splitter. One subsample of each size class was photographed under a dissecting microscope to count all individuals and measure the SL of 30 randomly selected mussels. Overall, the mean (± standard error, SE) SL of *P. canaliculus* spat in the spat material was 1.132mm ± 0.005 mm, and the mean (±SE) number of mussels g^{-1} of spat material was 156 ± 15 (Table 1).

2.3.3 Experimental design

We measured *P. canaliculus* performance at three levels of seeding density 257, 515 and 1030 mussels $10cm^{-1}$ of culture rope (Table 1). The effects of these three seeding densities were examined at four depth strata: 0-1m, 2-3m, 4-5m, and 6-7m, represented as 0.5m, 2.5m, 4.5m and 6.5m respectively. Samples were taken from both sides of each loop of culture rope, at two sites in the outer Marlborough Sounds: Site 1 and Site 2, and five replicate farms within each site. Sampling occurred in December 2019 and February 2020. Some deeper replicates at Site 2 were lost due to rough weather between the December

2019 and February 2020 sampling occasions, giving a total of 240 samples for the first sampling occasion on 9th December 2019, and 192 samples for the second sampling occasion on 13th February 2020.

2.3.4 Sample collection

Collecting samples required raising each experimental culture rope out of the water using a davit and winch mounted on a purpose-designed vessel (Figure 1a). At each sampling occasion (December 2019 and February 2020), a 10cm section from each culture rope was sampled using a stainless-steel measuring tool (Figure 1b). The culture rope was cut on either side of the measuring tool (Figure 1c), the sample then placed in a zip-lock bag and immediately placed into a chiller bin with an ice pack. The remaining culture rope was then reconnected with two large cable ties before returning to the water. Samples were transported to the laboratory where they were frozen for analysis.

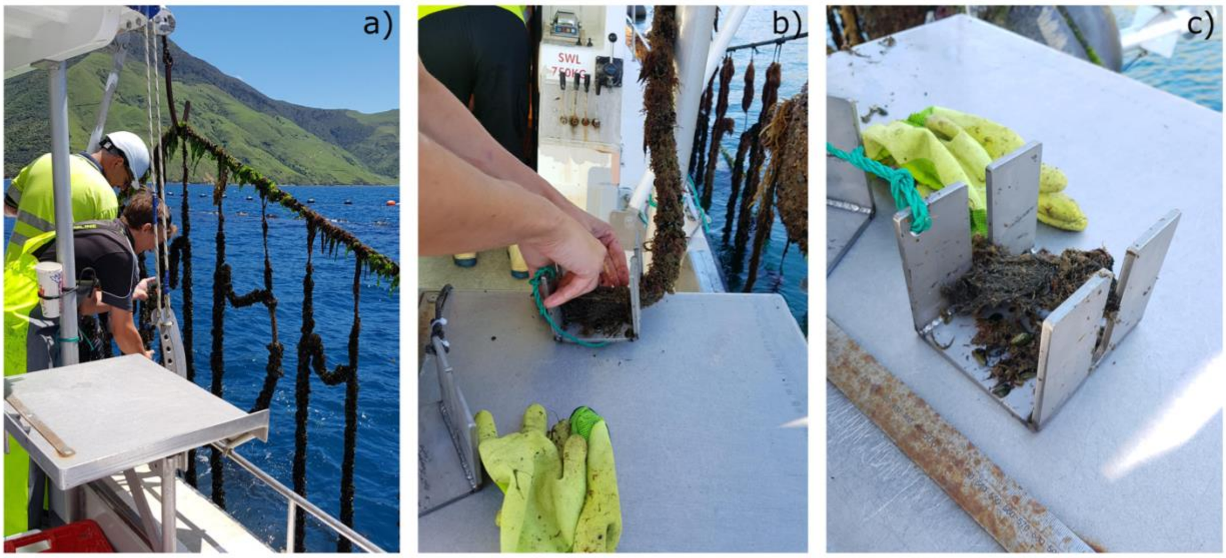

Figure 1. The procedure to collect samples from the experimental culture ropes in December 2019 and February 2020. This required raising each experimental culture rope out of the water with a davit and winch on a purposely-designed vessel (a), and at the selected depths, the culture rope was slotted into the 10cm stainless-steel measuring tool (b), and then cut on either side of the measuring tool (c) to produce a 10cm sample of culture rope.

2.3.5 Mussel abundance and size as well as biofouling composition and biomass

The contents from each sample were separated from the 10cm section of culture rope and rinsed through a 1000μm sieve. In each sample, *P. canaliculus* retained in the 1000μm sieve (>1000μm shell width) were counted to measure abundance (number of mussels 10cm^{-1} of culture rope). The percentage of *P. canaliculus* lost was estimated from the mussel abundance estimates at initial seeding (1030, 515 and 257 mussels 10cm^{-1} of culture rope), and the abundance estimates in December 2019 and February 2020. To assess *P. canaliculus* size, thirty mussels (>1000μm shell width) from each sample were randomly selected, photographed, and shell length (SL) measured using ImageJ software (Schneider et al., 2012). To analyse biofouling composition and biomass, the contents of the 1000μm size class were separated into six categories: *P. canaliculus, M. galloprovincialis*, mobile invertebrates, sessile invertebrates, algae and fish. *Mytilus galloprovincialis* in each sample were counted, and 30 randomly selected individuals were photographed, and SL measured using ImageJ software (Schneider et al., 2012). The four non-mussel biofouling categories were dried separately at 60°C for 48h, and the dry weight was quantified to the nearest 0.0001g for biofouling biomass quantification.

2.3.6 Statistical analysis

To examine the impacts of the fixed effects and interactions on mussel abundance and growth, as well as biofouling accumulation we used linear and generalised linear mixed models fit using R package lme4 (Bates et al., 2018) in RStudio version 1.4.1106 (RStudio Team, 2020). Our analyses included four fixed effects; seeding density with three levels: 257, 515 and 1030 mussels 10cm^{-1} of culture rope, depth with four levels: 0.5m, 2.5m, 4.5m and 6.5m, site with two levels: Site 1 and Site 2, time with two levels: December 2019 and February 2020, as well as the interactions between all combinations of fixed effects. To account for the variation among farms and culture ropes, there were two random effects; farm nested within site with 10 levels (five farms at each site), and rope nested within farm with 30 levels (three loops of culture rope at each farm) to account for the repeated measures on each culture rope (two samples from each depth, both sides of every culture loop at each sampling occasions).

The response variable for mussel abundance was the number of mussels (*P. canaliculus* and *M. galloprovincialis*) 10cm^{-1} of culture rope which was treated as a Poisson distributed variable. For mussel size analyses the response variable was the mean SL of mussels (*P. canaliculus* and *M. galloprovincialis*) 10cm^{-1} of culture rope. To assess non-mussel biofouling biomass the response variable was the total dry weight (g) of all biofouling categories. For mussel abundance and size as well as biofouling biomass analyses, we used a backward model selection by single term deletions using the drop1 function in the R package lmerTest (Kuznetsova et al., 2015) to test for significant effects (i.e., P-value<0.05). Tests were only produced if a fixed effect was not involved in a significant higher-order interaction. To examine variation in biofouling community composition data we used redundancy analysis (RDA) implemented in the R package vegan (Oksanen et al., 2013) with 9999 permutations of residuals to test the significance of the fixed effects and interactions. The response variable for biofouling community composition was a distance matrix containing the Euclidean distances between samples based on the dry weights (g) of each biofouling category. Last, to assess the relationship between the raw observed abundance of *P. canaliculus* and the raw observed abundance of *M. galloprovincialis,* as well as the biomass of non-mussel biofouling we fit a simple log-log linear regression without any additional fixed or random effects.

Table 2. Backward model selection for mussel abundance and size, as well as biomass and composition of biofouling communities. Abbreviations: SD (seeding density of *Perna canaliculus*), S (site), DP (depth), T (time), F (farm) and R (rope). For mussel abundance analyses the response (Y) was the total number of *Perna canaliculus* or *Mytilus galloprovincialis* mussels 10cm^{-1} culture rope, for mussel size analyses the response variable (Y) was shell length (cm) of *Perna canaliculus* or *Mytilus galloprovincialis*, for biofouling community composition the response (Y) was a Euclidean distance matrix for the biomass of biofouling taxa in each category, and for the overall biofouling biomass the response (Y) was the total biomass of non-mussel biofouling (g). The notation (1|F) or (1|R) was used to denote that the model assumes random intercepts for each level of the random effect indicated after the bar.

Best supported model for:	Model terms
Perna canaliculus abundance	Y = S + SD + DP + T + S×SD + S×DP + S×T + SD×DP + SD×T + DP×T + S×SD×DP + S×SD×T + S×DP×T + SD×DP×T + S×SD×DP×T + (1|F) + (1|R)
Perna canaliculus size	Y = S + SD + DP + T + S×SD + S×DP + S×T + SD×DP + SD×T + DP×T + S×SD×DP + S×DP×T + (1|F) + (1|R)
Mytilus galloprovincialis abundance	Y = S + SD + DP + T + S×SD + S×DP + S×T + SD×DP + SD×T + DP×T + S×SD×DP + S×SD×T + S×DP×T + SD×DP×T + S×SD×DP×T + (1|F) + (1|R)
Mytilus galloprovincialis size	Y = S + SD + DP + T + S×SD + S×T + SD×DP + SD×T + DP×T + S×SD×T + SD×DP×T + (1|F) + (1|R)
Non-mussel biofouling community composition	Y = S + SD + DP + T + S×SD + S×DP + S×T + SD×DP + SD×T + (1|F) + (1|R)
Non-mussel biofouling biomass	Y = S + SD + DP + T + S×SD + S×DP + SD×DP + SD×T + S×SD×DP + (1|F) + (1|R)

2.4 Results

2.4.1 Effects of seeding density on *Perna canaliculus* abundance and size

The impacts of seeding density on *P. canaliculus* abundance varied with depth, site and time (Figure 2a-d; Table 3: Site×Density×Depth×Time). In December 2019, at Site 1 there was a clear positive relationship between seeding density and *P. canaliculus* abundance, and at the intermediate and high seeding density abundance decreased with increasing depth where abundance was 35-55% greater at 0.5m compared with 6.5m (Figure 2a). However, at Site 2 in December 2019, *P. canaliculus* abundance was only impacted by depth at the lowest seeding density where abundance was greater at shallow depths (Figure 2b). At Site 2 in December 2019, *P. canaliculus* abundance was approximately 50% greater at the high seeding density compared with intermediate or low seeding density in which abundances of *P. canaliculus* did not significantly differ (Figure 2b). At Site 1 in February 2020*, P. canaliculus* abundance remained greatest at the high seeding density, and lowest at the low seeding density though the difference in abundance between seeding densities was smaller in February 2020 than in December 2019 (Figure 2c). Depth only impacted mussels at the low seeding density at Site 1 in February 2020 in which *P. canaliculus* abundance was approximately 60% greater at 6.5m compared with 0.5m (Figure 2c). At Site 2 in February 2020, *P. canaliculus* abundance significantly increased with higher seeding density, however at the intermediate seeding density, abundance increased with increasing depth, so at the deeper depths (4.5m and 6.5m) the intermediate and high seeding density did not significantly differ in abundance (Figure 2d).

Across all depths and sites, the percent loss of *P. canaliculus* between initial seeding and the first sampling occasion in December 2019 was 77.6%, 74.5% and 68% for the seeding densities 1030, 515 and 257 mussels 10cm^{-1} of culture rope respectively. By the February 2020 sampling occasion, the percent loss since initial seeding was 87.9%, 80.6% and 80.8% for seeding densities 1030, 515, and 257 mussels 10cm^{-1} of culture rope respectively. Losses were high across all seeding densities, and although losses of *P.*

canaliculus were greatest at the highest seeding density, the abundance of *P. canaliculus* in February 2020 generally remained greatest at the highest seeding density.

Table 3. Summary statistics for the backward model selection procedure for *Perna canaliculus* abundance and size. All models retained the same random effects (farm and rope). Significant results are bolded, and tests were not produced for fixed effects if higher-order interactions involving those fixed effects were significant. Abbreviations: SD (seeding density of *Perna canaliculus*), S (site), DP (depth), T (time), F (farm) and R (rope).

	Abundance			Size			
Fixed effect	df	LRT	Pr(Chi)	NumDF	DenDF	F	Pr(>F)
S	-	-	-	-	-	-	-
SD	-	-	-	-	-	-	-
DP	-	-	-	-	-	-	-
T	-	-	-	-	-	-	-
S×SD	-	-	-	-	-	-	-
S×DP	-	-	-	-	-	-	-
S×T	-	-	-	-	-	-	-
SD×DP	-	-	-	-	-	-	-
SD×T	-	-	-	2	11812	45.496	**<0.001**
DP×T	-	-	-	-	-	-	-
S×SD×DP	-	-	-	2	11846	5.463	**0.004**
S×SD×T	-	-	-	2	11710	1.215	0.297
S×DP×T	-	-	-	1	11832	16.837	**<0.001**
SD×DP×T	-	-	-	2	11811	1.932	0.145
S×SD×DP×T	2	24.936	**<0.001**	2	11746	0.538	0.584

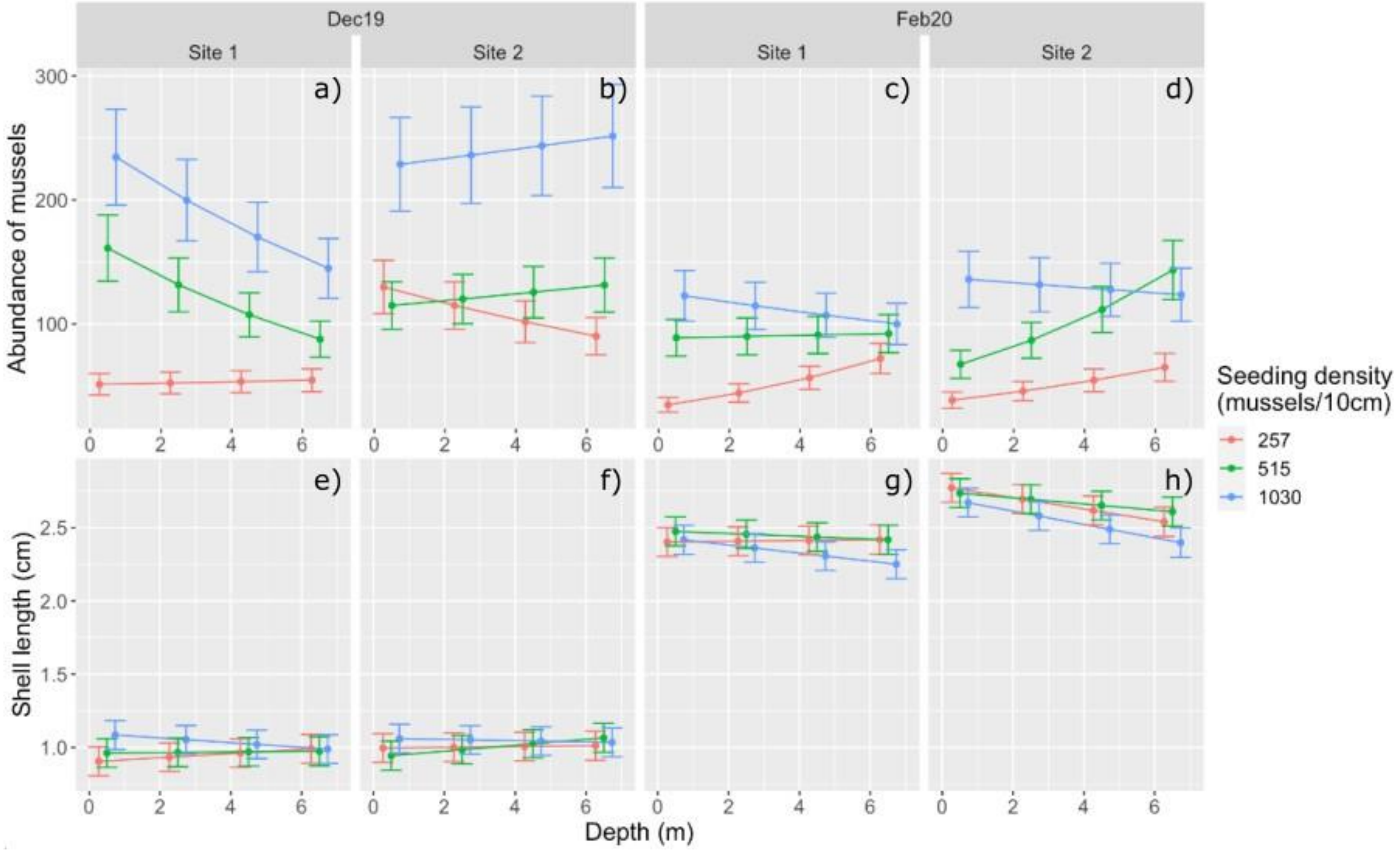

Figure 2. Mean (±SE) abundance (number of mussels; a, b, c and d) and size (shell length (cm); e, f, g and h) of *Perna canaliculus* 10cm^{-1} of culture rope at three seeding densities, four depths, at Site 1 and Site 2 in the Marlborough Sounds in December 2019 and February 2020.

Growth of *P. canaliculus* was greatest at the shallowest depths at Site 2 (Figure 2f, 2h; Table 3: Site×Depth×Time). At Site 1, the impacts of seeding density and depth on *P. canaliculus* size were insignificant (Figure 2e, 2g), whereas at Site 2 size generally decreased as depth increased, with minor variation in size among seeding densities (Figure 2f, 2h; Table 3: Site×Density×Depth). In December 2019, there was no difference in *P. canaliculus* size among seeding densities, whereas in February 2020, size was slightly lower at the highest seeding density (mean shell length (SL): 2.43cm) compared with intermediate (mean SL: 2.56cm) or low seeding density (mean SL: 2.53cm) (Figure 2e, 2f, 2g, 2h; Table 3: Density×Time).

2.4.2 Effects of seeding density on *Mytilus galloprovincialis* abundance and size

The impacts of seeding density on the abundance of *M. galloprovincialis* varied with depth, site and time (Figure 3a-f; Table 4: Site×Density×Depth×Time). At Site 1 in December 2019 the abundance of *M. galloprovincialis* was not significantly different among seeding densities or depth (Figure 3a). However, at Site 2 in December 2019, *M. galloprovincialis* abundance was significantly lower at the highest seeding density compared with low or intermediate seeding density at 0.5m and 2.5m (Figure 3b). At Site 2 in December 2019, *M. galloprovincialis* abundance was greater at shallower compared with deeper depths (abundance at 0.5m 80-90% greater than abundance at 6.5m), with very few *M. galloprovincialis* accumulated at 4.5m and 6.5m (<15 mussels $10cm^{-1}$ of culture rope, Figure 3b). At Site 1 in February 2020, *M. galloprovincialis* abundance was greatest at the lowest seeding density and decreased as seeding density increased and was also greater at shallower depths where abundance was 40-60% greater at 0.5m than 6.5m (Figure 3c). At Site 2 in February 2020, *M. galloprovincialis* abundance was significantly lower at the highest seeding density compared with low or intermediate seeding density at 0.5m, but as depth increased, *M. galloprovincialis* abundance decreased across all seeding densities (Figure 3d). Overall, across all fixed effects, *M. galloprovincialis* abundance was approximately 58% greater in February 2020 compared with December 2019, and approximately 68% greater at Site 1 than Site 2.

Table 4. Summary statistics of the backward model selection procedure for *Mytilus galloprovincialis* abundance and size. All models retained the same random effects (farm and rope). Significant results are bolded, and tests were not produced for fixed effects if higher-order interactions involving those fixed effects were significant. Abbreviations: SD (seeding density of *Perna canaliculus*), S (site), DP (depth), T (time), F (farm) and R (rope).

	Abundance			Size			
Fixed effect	df	LRT	Pr(Chi)	NumDF	DenDF	F	Pr(>F)
S	-	-	-	-	-	-	-
SD	-	-	-	-	-	-	-
DP	-	-	-	-	-	-	-
T	-	-	-	-	-	-	-
S×SD	-	-	-	-	-	-	-
S×DP	-	-	-	-	-	-	-
S×T	-	-	-	-	-	-	-
S×DP	-	-	-	1	7713.9	0.162	0.687
S×T	-	-	-	-	-	-	-
SD×DP	-	-	-	-	-	-	-
SD×T	-	-	-	-	-	-	-
DP×T	-	-	-	-	-	-	-
S×SD×DP	-	-	-	2	7709.9	0.066	0.936
S×SD×T	-	-	-	2	7161.0	3.182	**0.042**
S×DP×T	-	-	-	1	7714.4	0.122	0.727
SD×DP×T	-	-	-	2	7708.3	4.787	**0.008**
S×SD×DP×T	2	6.783	**0.034**	2	7440.0	0.643	0.526

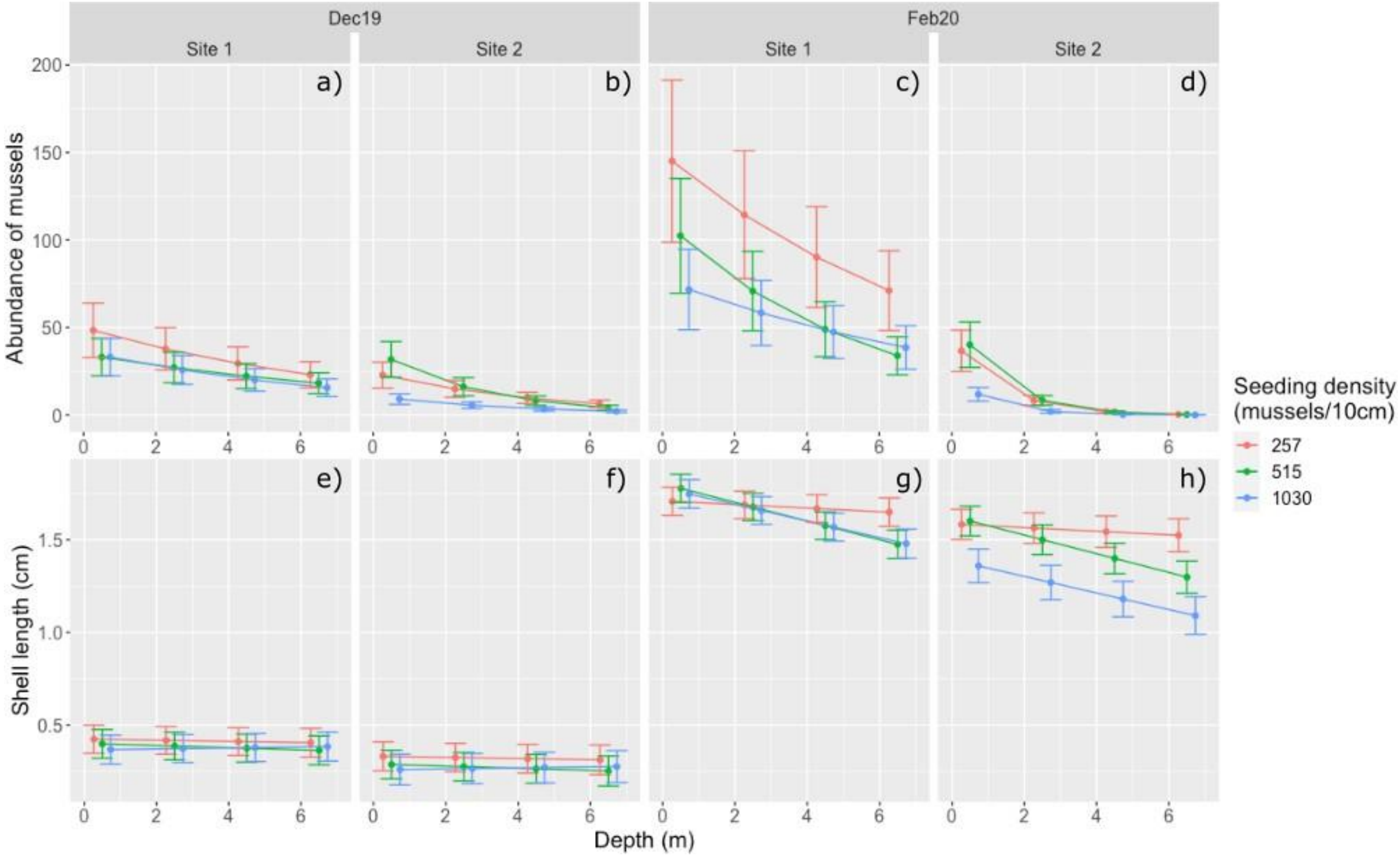

Figure 3. Mean (±SE) abundance (number of mussels; a, b, c and d) and size (shell length (cm); e, f, g and h) of *Mytilus galloprovincialis* 10cm^{-1} of culture rope at three seeding densities, four depths, at Site 1 and Site 2 in the Marlborough Sounds in December 2019 and February 2020.

In December 2019, *M. galloprovincialis* size did not differ among seeding densities at either site (Figure 3e, 3f), however at Site 2 in February 2020, size was greater at the lower seeding densities (Figure 3h; Table 4: Site×Density×Time). *Mytilus galloprovincialis* were typically larger at Site 1 than at Site 2, though site differences were most apparent in February 2020 where mussels were up to 25% larger at Site 1 than at Site 2. In December 2019, we detected no difference in *M. galloprovincialis* size among depth and seeding density; however, in February 2020, the effects of depth were most apparent at intermediate and high seeding densities, where *M. galloprovincialis* were 16-19% larger at 0.5m compared with 6.5m (Figure 3e-h; Table 4: Density×Depth×Time).

2.4.3 Biofouling community composition and biomass

The first (RDA1) and second (RDA2) axes of our redundancy analysis explained 61% of the and 29% of the variance respectively and indicated significant changes in non-mussel biofouling community composition in a model considering all fixed and random effects (RDA1: F=33.59, P(perm)=0.001; RDA2: F=11.22, P(perm)=0.001). RDA1 was characterised by a gradient in community composition from samples dominated by algae and mobile invertebrates to samples dominated by sessile invertebrates. RDA2 was characterised by a gradient in community composition from samples dominated by mobile invertebrates to samples dominated by algae and sessile invertebrates.

In December 2019, low and intermediate seeding densities favoured mobile and sessile invertebrates, and high seeding density favoured mainly algae and mobile invertebrates, as well as sessile invertebrates, whereas in February 2020, all seeding densities favoured mainly algae and sessile invertebrates (Figure 4a-d; Table 5: Density×Time). With increasing depth, culture ropes with low and intermediate seeding density favoured mainly mobile invertebrates, but also algae and sometimes sessile invertebrates, whereas culture ropes with high seeding density favoured mainly algae and mobile invertebrates, and occasionally sessile invertebrates with increasing depth (Figure 4a-d; Table 5: Density×Depth). In December 2019 most of the non-mussel biofouling on the culture ropes consisted of algae and mobile invertebrates, whereas in February 2020 the non-mussel biofouling consisted of mostly algae and sessile invertebrates (Figure 4a-d; Table

5: Site×Time). At Site 1, increasing depth favoured mainly algae and sessile invertebrates, whereas at Site 2, increasing depth favoured mostly algae and mobile invertebrates (Figure 4a-d; Table 5: Site×Depth). At Site 1, the three seeding densities favoured mainly sessile invertebrates and algae, whereas at Site 2, the low seeding density favoured mobile invertebrates, as well as algae, the intermediate seeding density favoured mainly mobile invertebrates and algae, as well as sessile invertebrates, and the high seeding density favoured mostly algae and mobile invertebrates (Figure 4a-d; Table 5: Site×Density).

Table 5. Summary statistics of the backward model selection procedure for non-mussel biofouling community composition and mean non-mussel biofouling biomass. All models retained the same random effects (farm and rope). Significant results are bolded, and tests were not produced for fixed effects if higher-order interactions involving those fixed effects were significant. Abbreviations: SD (seeding density of *Perna canaliculus*), S (site), DP (depth), T (time), F (farm) and R (rope).

	Non-mussel biofouling community composition			Total non-mussel biofouling biomass			
Fixed effect	df	F	P(perm)	NumDF	DenDF	F	Pr(>F)
S	-	-	-	-	-	-	-
SD	-	-	-	-	-	-	-
DP	-	-	-	-	-	-	-
T	-	-	-	-	-	-	-
S×SD	1	4.134	**0.017**	-	-	-	-
S×DP	1	16.847	**0.001**	-	-	-	-
S×T	2	2.678	**0.017**	1	405.64	0.116	0.734
SD×DP	2	4.093	**0.001**	-	-	-	-
SD×T	1	6.494	**0.002**	2	405.44	4.384	**0.013**
DP×T	0	0.000	0.000	1	399.59	3.601	0.058
S×SD×DP	2	1.896	0.087	2	397.60	4.192	**0.016**
S×SD×T	1	2.282	0.051	2	403.59	1.697	0.185
S×DP×T	2	1.169	0.323	1	397.73	1.444	0.230
SD×DP×T	0	0.000	0.000	2	398.65	1.218	0.297
S×SD×DP×T	0	0.000	0.000	2	398.77	1.292	0.276

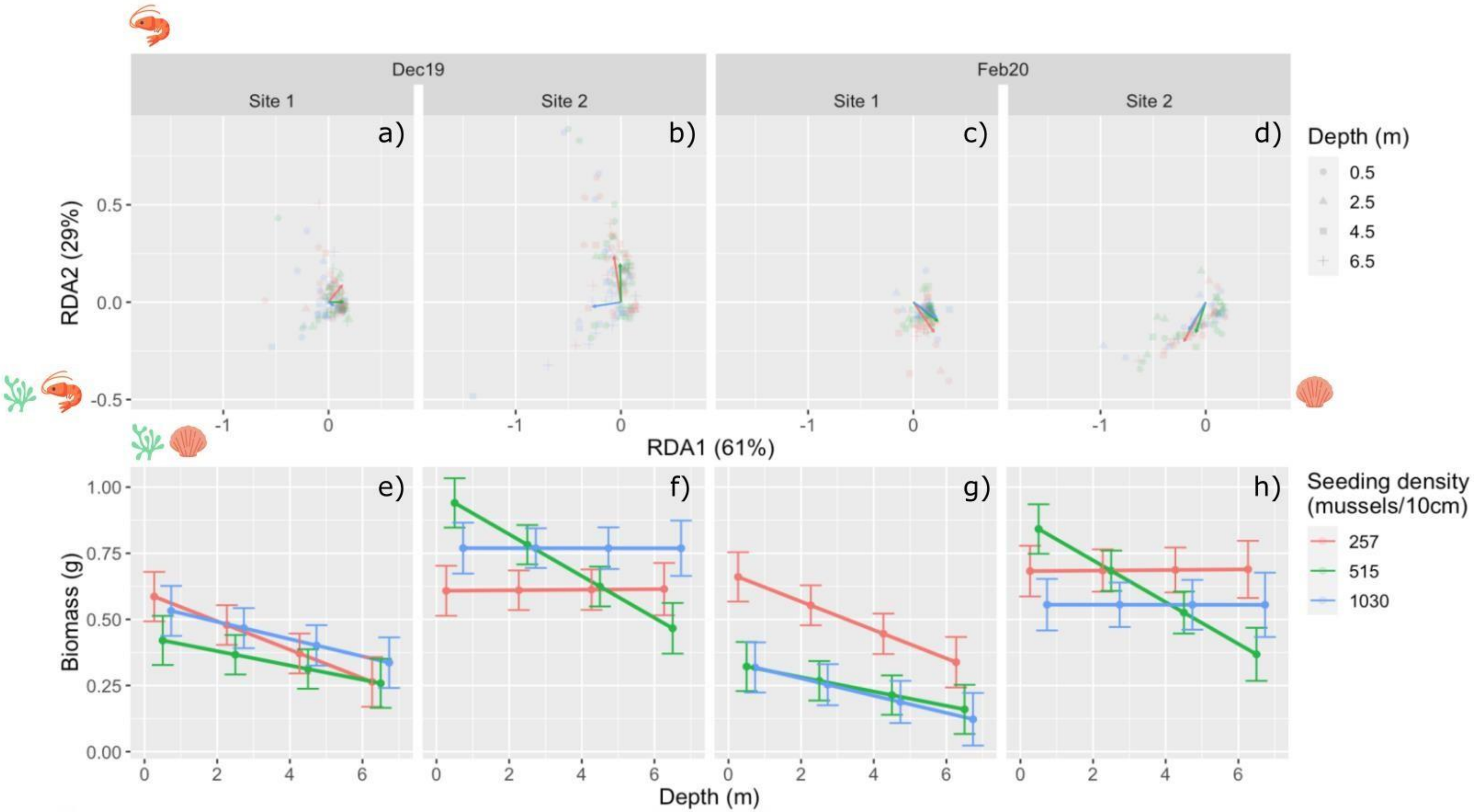

Figure 4. Redundancy analysis (RDA) of variability in community composition for non-mussel biofouling 10cm^{-1} of culture rope (a, b, c and d), as well as the mean (±SE) biomass (dry weight in grams) of total non-mussel biofouling (e, f, g and h) at three seeding densities, four depths, at five farms at Site 1 and Site 2 in the Marlborough Sounds in December 2019 and February 2020. The arrows in panels a, b, c and d represent the effects of increasing depth on community composition, and the icons represent the biofouling categories with the greatest contributions to RDA1 (positive values favour sessile invertebrates and negative values favour algae and mobile invertebrates) and RDA2 (positive values favour mobile invertebrates, and negative values favour algae and sessile invertebrates).

At Site 1, the mean biomass of non-mussel biofouling decreased 45-60% with increasing depth (from 0.5m to 6.5m) across all seeding densities, with a slightly greater biomass at the lowest seeding density, whereas at Site 2, the biomass of non-mussel biofouling decreased by about 50% as depth increased from 0.5m to 6.5m at the intermediate seeding density, and was not impacted by depth at low and high seeding densities (Figure 4e-h; Table 5: Site×Density×Depth). Overall, the biomass of non-mussel biofouling was approximately 46% greater at Site 2 compared with Site 1. In December 2019, without accounting for depth, the mean biomass of non-mussel biofouling had a positive relationship with seeding density, whereas in February 2020, the mean biomass of non-mussel biofouling had negative relationship with seeding density (Figure 4e-h; Table 5: Density×Time).

More biofouling accumulated at the shallower depths (Figure 4e-h), driven primarily by sessile invertebrates and algae (Figure 4a-d). The greater biofouling accumulation at Site 2 compared with Site 1 (Figure 4e-h) was driven by algae and mobile invertebrates (Figure 4a-d). The biomass of non-mussel biofouling was slightly greater at higher seeding densities in December 2019 (Figure 4e, 4f) and was mainly driven by greater algae and mobile invertebrate accumulation (Figure 4a, 4b). Last, the greater biomass of non-mussel biofouling at lower seeding densities in February 2020 (Figure 4g, 4h) was driven by a combination of mobile invertebrates, algae and sessile invertebrates (Figure 4c, 4d).

If we ignore variation accounted for by the different fixed and random effects in our experimental design and examine relationships between raw values of *P. canaliculus* abundance and *M. galloprovincialis* abundance as well as biomass of non-mussel biofouling we observed an overall significant negative association between *P. canaliculus* abundance and *M. galloprovincialis* abundance (Figure 5a; ANOVA, $F_{1, 430}$= 45.332, p<0.001, correlation=-0.309), but not between *P. canaliculus* abundance and biomass of non-mussel biofouling (Figure 5b; ANOVA, $F_{1, 430}$= 0.0566, p=0.8121, correlation=-0.011).

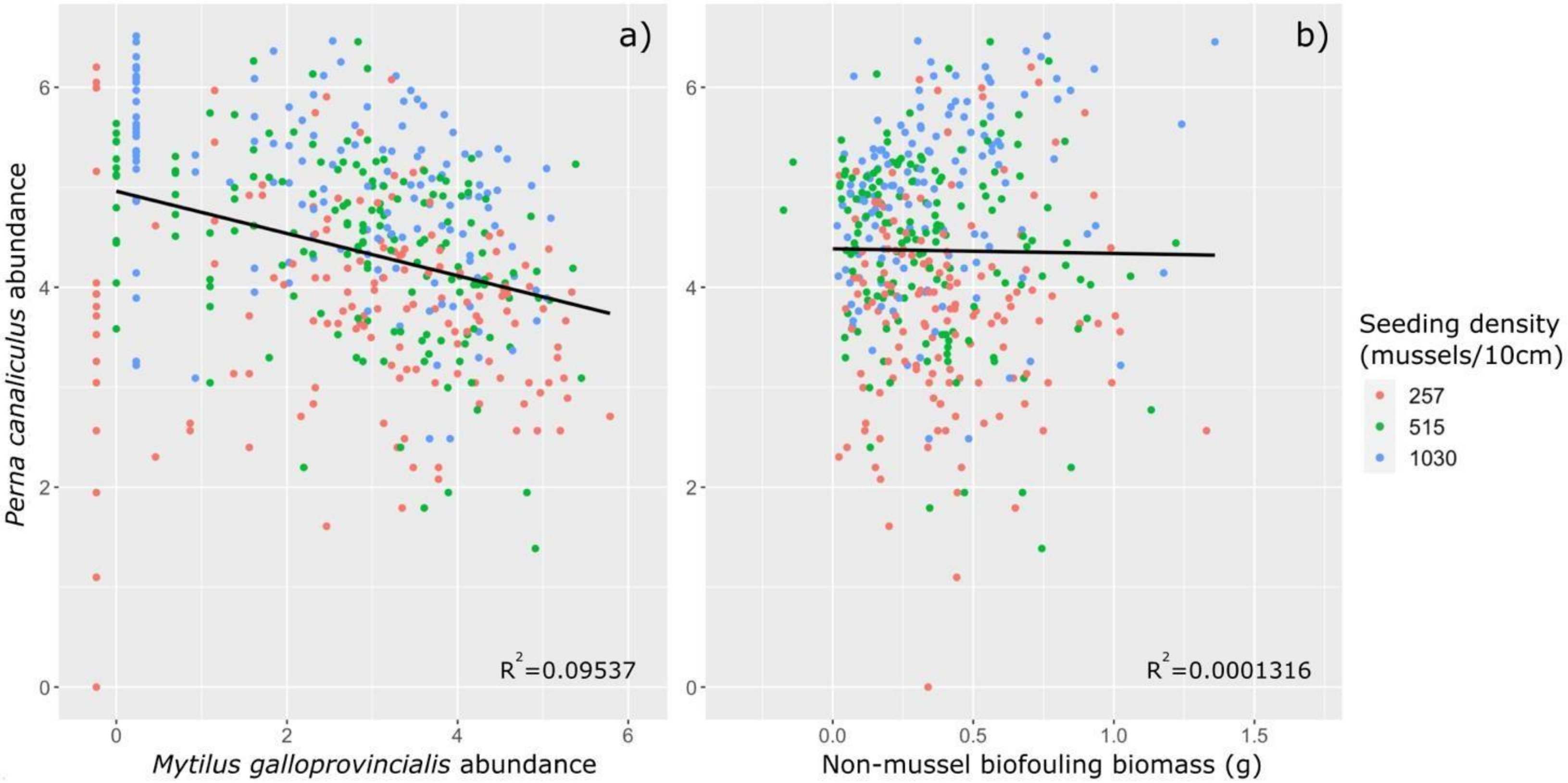

Figure 5. Relationship between *Perna canaliculus* abundance (number of mussels 10cm^{-1} of culture rope) and *Mytilus galloprovincialis* abundance (number of mussels 10cm^{-1} culture rope; a) and *Perna canaliculus* abundance and the biomass (g) of non-mussel biofouling (b). Values on both the x- and y-axes were log-transformed, and the three levels of the seeding density treatment are indicated for visual purposes.

2.5 Discussion

Seeding density is an important factor in *P. canaliculus* farming that can impact mussel production (Rosland et al., 2011), though the impacts of seeding density on our results were inconsistent and demonstrate important trade-offs in production traits. Lower seeding density can reduce intraspecific competition for food and space, which can minimise self-thinning and increase growth (Cubillo et al., 2012b; Lauzon-Guay et al., 2005b; Xavier et al., 2007). Like the results presented here, losses in *Mytilus edulis* throughout a three-year growth period were greatest at higher seeding density due to greater intraspecific competition compared with lower seeding densities (Capelle et al., 2016b). Growth was greater for *P. canaliculus* at shallow depths in our study. The abundance of *M. galloprovincialis* was generally greater at lower seeding density, at Site 1, and *M. galloprovincialis* were also more abundant and larger at shallow depths. The impact of seeding density on non-mussel biofouling biomass varied spatially and temporally. High seeding density can also be favourable as it reduces available space for biofouling accumulation, as *M. galloprovincialis* accumulation was generally greater at low seeding density and allows more cultured mussels to be seeded in a certain farm area (Kamermams & Capelle, 2019). These trade-offs highlight how important it is for mussel farmers to consider which production traits they wish to prioritise; greater abundance or size of *P. canaliculus,* lower abundance or size of *M. galloprovincialis*, or minimal accumulation of non-mussel biofouling, and focus farming on practices that enhance the most favourable outcomes.

The impacts of seeding density on mussels are typically dependent on species, age, and the surrounding abiotic and biotic environment. For instance, Cubillo et al. (2012b) examined the impacts of stocking density on the growth of cultured *M. galloprovincialis* >40mm shell length (SL) from 1-6m. *Mytilus galloprovincialis* growth was greater at the lower densities, where the maximum estimated SL was 21% and 26.4% larger for individuals at the lowest densities (220 and 370 individuals m^{-1} respectively), than the highest density (1150 individuals m^{-1}), most likely due to greater intraspecific competition at higher density (Cubillo et al., 2012b). The consistent impacts of stocking density on *M. galloprovincialis* growth suggest that these larger mussels may be less affected by a fluctuating environment than *P. canaliculus* spat in our study, or that environmental variation was greater in our study area than in Cubillo et al. (2012b), and thus the effects of stocking density in our study were less apparent. Lauzon-Guay et al. (2005a) assessed the impacts of initial density on the growth and survival of various sized *M. edulis*: small spat (13.62mm mean SL), medium spat (23.12mm mean SL), and large spat (28.53mm mean SL). From two sites investigated, initial density only impacted large spat at St Peter's Bay, Prince Edward Island, and small spat at New London Bay, in which SL was greater at higher density compared with the same sized spat at lower density (Lauzon-Guay et al., 2005a). *Mytilus edulis* survival did not differ between sites, and small spat at the high density had lower survival than all other mussels at other densities early in the experiment, but the impacts of density on mussel survival disappeared as the experiment developed (Lauzon-Guay et al., 2005a). The results of Lauzon-Guay et al. (2005a) suggest different aged mussels respond differently to challenging conditions, and younger spat tend to be most vulnerable to density effects and environmental differences among sites. This is reflected in our study where losses of *P. canaliculus* were greatest at the highest seeding density and seeding density inconsistently impacted mussel size between sites and sampling occasions, indicating the environment played an important role in mediating *P. canaliculus* performance in our study.

Over settlement by *M. galloprovincialis* can reduce the yield of crop-sized *P. canaliculus* (Forrest & Atalah, 2017). In our study, losses of *P. canaliculus* were greater at high seeding density compared with low seeding density, whereas *M. galloprovincialis* accumulation was greater at low seeding density. It is therefore unlikely that *M. galloprovincialis* were a dominant cause of *P. canaliculus* loss among seeding densities, but more likely that resource availability including nutrients and space contributed to retention and therefore loss of *P. canaliculus* (Supono et al., 2021). Intraspecific competition for nutrients and space was likely greater at high seeding density, and would have intensified as mussels grew, causing more mussels to die or migrate at high compared with low seeding density (Cubillo et al., 2012b). The accumulation of *M. galloprovincialis* was consistently greater at shallow depths, which has also been reported by Woods et al. (2012), where accumulation of *M. galloprovincialis* on *P. canaliculus* farms in the Marlborough Sounds was greater at 2m deep compared with 8m. Thus, shallow depths in the Marlborough Sounds appear most favourable for *M. galloprovincialis* recruitment to *P. canaliculus* culture ropes.

Accordingly, the greater accumulation of *M. galloprovincialis* at shallow depths may have contributed to a lower abundance of *P. canaliculus* at shallow depths particularly at the intermediate and low seeding density in February 2020. Based on 40 years of data, *M. galloprovincialis* abundance also varies among sites in the Marlborough Sounds (Atalah et al., 2017), and we observed greater abundance at Site 1 compared with Site 2. However, *P. canaliculus* abundance was not greatly impacted by site, suggesting that the greater abundance of *M. galloprovincialis* at Site 1 did not directly increase loss of *P. canaliculus,* but instead may have increased resource competition, and thus contributed to reduced size of *P. canaliculus* at Site 1. Controlled experiments to analyse how *M. galloprovincialis* abundance can influence nutrient availability on culture ropes would provide better understanding of the relationship between *M. galloprovincialis* accumulation and *P. canaliculus* spat performance.

Non-mussel biofouling biomass varied among combinations of experimental treatments; however non-mussel biofouling did not have a significant impact on *P. canaliculus* abundance. Some biofouling species such as the decorator crab *Notomithrax minor* (Van de Ven, 2007), and ascidian, *D. vexillum* (Fletcher et al., 2013) can negatively influence the abundance of *P. canaliculus* spat. However, South et al. (2019) found that the accumulation of mobile invertebrates, algae, and sessile invertebrates on *P. canaliculus* farms did not impact spat abundance. While the accumulation of certain biofouling species may have contributed to variation in *P. canaliculus* abundance and size in some experimental treatments, future experiments are needed to test the direct impacts of biofouling species on *P. canaliculus* performance to determine which biofouling species have neutral, negative or positive effects on spat performance.

The impacts of seeding density on mussel performance varied spatially and temporally. Factors such as water temperature (Lachance et al., 2008) and turbulence (Alfaro, 2006; Hayden & Woods, 2011) often vary spatially and temporally in the marine environment, and can impact mussel attachment, and therefore mussel abundance. For example, water temperature and turbulence were two major contributors to the retention of cultured *M. edulis* in the Gulf of St. Lawrence, Canada, where attachment strength was greater in temperatures between 10°C and 15°C compared with temperatures >15°C, and attachment strength was greater in higher turbulence (Lachance et al., 2008). Also, Alfaro (2006) tested the effects of different water velocities on the retention of *P. canaliculus* spat and found that retention was greater at 10cm s^{-1} compared with 5cm s^{-1} or 1cm s^{-1}. Higher water flow allowed quicker nutrient replenishment around the culture ropes, allowing the mussels to direct more energy toward byssus growth and less toward feeding (Alfaro, 2005, 2006). Many studies have shown that mussel growth can also vary with factors such as food provisioning (Karayücel & Karayücel, 2000; Page & Hubbard, 1987), competition (Azpeitia et al., 2018) and temperature (Fuentes et al., 2000), all of which are expected to vary with depth, site, and time. For example, in the Santa Barbara Channel, California, *M. edulis* grew fastest at 9m compared with 2m or 18m which was correlated with elevated chlorophyll *a* and particulate organic carbon (Page & Hubbard, 1987). Additionally, Fuentes et al. (2000) showed that *M. galloprovincialis* in the Mediterranean, grew faster at warmer, shallower depths (2.5m deep), than mussels grown deeper (7.5m depth). The water temperature in the Marlborough Sounds is typically highest in February and March, where it can be ~1°C warmer than December (Broekhuizen et al., 2021; Hickman et al., 1991). Chlorophyll *a,* particulate carbon and organic matter, and salinity also vary temporally in the Marlborough Sounds (Hickman et al., 1991). Therefore, environmental variation in the Marlborough Sounds through space and time was undoubtedly an important determinant of *P. canaliculus* performance in our experiment.

Greater abundance and size of mussels as well as minimal biofouling are desirable traits for successful *P. canaliculus* farming. We found that these traits varied among seeding densities and in space and time. Importantly, lower seeding density reduced losses of spat but increased the presence of a problematic biofouling species which implies trade-offs in critical production traits are likely for the mussel farming industry. Recording environmental data such as temperature, water velocity, chlorophyll *a* and particulate organic carbon at different seeding densities and across a spatial and temporal scale, will help to describe the environmental conditions mussels are experiencing at different seeding densities and perhaps explain variability in spat performance. Additionally, it is important to assess the nutrient and spatial requirements of specific biofouling species that persist on *P. canaliculus* farms, particularly *M. galloprovincialis,* and thus how biofouling accumulation impacts resource availability at different seeding densities to better understand the role biofouling plays on *P. canaliculus* performance. Last, more detailed examination of stress biomarkers, such as oxidative stress, in *P. canaliculus* in response to different seeding densities will strengthen the understanding of how different farming conditions impact *P. canaliculus* as the most valuable aquaculture species in New Zealand.

Chapter 3: Effects of seeding density on total antioxidant capacity, oxidative stress, and viability in the Greenshell^{TM} mussel, *Perna canaliculus*

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In preparation for publication

3.1 Abstract

The Greenshell^{TM} mussel, *Perna canaliculus*, is New Zealand's most important aquaculture species. Unfortunately, losses of juvenile mussels (spat) are common and can drastically reduce the yield of adult *P. canaliculus*. While environmental fluctuations are inevitable in the marine environment, modifying seeding density is a potential method to manage loss of spat by reducing biofouling and intraspecific competition. In challenging conditions, stress biomarkers that display meaningful change in an organism's physiology can precede more severe whole-organism responses such as reduced growth and survival and can therefore serve as a warning system for impending losses. Here, we analysed the impact of seeding density (257, 515, and 1030 mussels 10cm^{-1} of culture rope) on a stress response total antioxidant capacity (TAC), and stress effects: lipid peroxidation (LP) as a marker of oxidative stress, and viability (valve closure during freshwater immersion) to determine the physiological condition of spat at the time of sampling. The spatially structured field experiment in the Marlborough Sounds lasted for five months during summer 2019/2020 to capture temporal variation in stress biomarkers of mussels when temperature induced stress is expected to be greatest. The effect of seeding density on TAC was variable among sampled depths and times. In contrast, seeding density had no impact on LP. TAC was lower, and LP highest in February 2020 than December 2019, indicating oxidative stress was greater when mussels were older/larger and water temperatures higher. Viability remained high in all treatments. Our results suggest that the relatively mild summer conditions and the seeding densities we examined were within the tolerance of normal stress capacities for cultured *P. canaliculus* spat.

3.2 Introduction

The natural marine environment is largely uncontrollable, and stress biomarkers in marine organisms to environmental stochasticity often contribute to determining whole-organism responses such as survival and growth, and overall population growth and resilience (Fuller et al., 2010; Seebacher et al., 2015). For instance, the colonial ascidian, *Didemnum vexillum,* exhibited significant changes in DNA methylation at 27°C compared with 19 and 25°C which correlated with an overall decrease in colony growth (Hawes et al., 2018). Additionally, Leung et al. (2020) found the physiological performance and energy budget of the marine gastropod, *Austrocochlea concamerata,* reduced its capacity for shell building in warmer and more acidic conditions (24°C compared with 21°C and $p\text{CO}_2$ levels of 940ppm compared with 400ppm). If stress biomarkers pre-empt whole-organism responses and the two responses can be associated together, the predictability of future whole-organism responses is possible by examining organism stress biomarkers.

A variety of abiotic and biotic stressors can influence stress biomarkers in marine invertebrates (Gazeau et al., 2014; Marčeta et al., 2020; Marigomez et al., 2017). For example, stress biomarkers including hemocyte concentration, reactive oxygen species (ROS) production, and total antioxidant capacity (TAC) in hemolymph and gill tissue in the Greenshell$^{\text{TM}}$ mussel, *Perna canaliculus,* exposed to heat shock (29°C and 30°C) indicated greater stress compared with the control group (7.3°C) (Delorme et al., 2021). Durán et al. (2018) found that activity of the antioxidants superoxide dismutase and glutathione peroxidase increased, indicating greater stress, at a pH of 7.5 compared with a pH of 8.0 in octocorals, ascidians, crabs, prawns, bivalves and sea cucumbers. Additionally, the change of temperature from between 24-28°C, to extremes of 16-36°C increased antioxidant activities more at a pH of 7.5, compared with 8.0, showing that abiotic factors can cumulatively increase stress (Durán et al., 2018). Biotic factors including predator exposure (Sheriff et al., 2009) and competition for resources (Chase et al., 2016; Comendant et al., 2003) can also influence stress biomarkers. For instance, two species of amphipod crustaceans, *Dikerogammarus villosus* and *Gammarus jazdzewskii*, exhibited increased respiration rates after short (35 min) and chronic (1 or 7 days) exposures to predation risk (Eurasian perch), and *G. jazdzewskii* had increased catalase activity, heat shock protein concentration and oxidative damage, which are stress biomarkers, after the short exposure to predation risk (Jermacz et al., 2020).

Aquaculture is essential to global food production (Garlock et al., 2020; Naylor et al., 2021; Tacon, 2020); and many commercial aquatic species are cultured in water-based systems where they are exposed to natural environmental fluctuations (Bannister et al., 2014; Buschmann et al., 2012; Jeffs et al., 2018; Matozzo et al., 2013). Commercial aquatic species also have the additional challenge of overcoming direct anthropogenic effects which can induce stress such as handling (López-Patiño et al., 2014), transportation (Tacchi et al., 2015), farming procedures, and other challenges that can stem from cultivation such as overcrowding due to high densities (El-Khaldi, 2010). Due to the limited ability to control biotic and abiotic factors in water-based aquaculture systems and the high demand for an abundant and quality product, it is critical to understand the effect of farming induced stressors which can result in poor performance or even death.

One of the most common measures of stress in animals is oxidative stress, which is the imbalance of reactive oxygen species (ROS) and antioxidants (Halliwell & Gutteridge, 2015). ROS are produced during cell metabolism and are responsible for many important physiological processes, such as regulating gene expression and initiating important immune responses (Alfadda & Sallam, 2012; Hancock et al., 2001; Lesser, 2006; Lushchak, 2014). Under benign conditions, ROS are non-destructive due to antioxidant molecules and enzymes which are responsible for eliminating free radicals (Livingstone, 2001; Soldatov et al., 2007). However, under stressful conditions (e.g., foreign substances, changes in temperature, pH, predation), the production of ROS exceeds the eliminating abilities of antioxidants, causing ROS to become toxic and damage cellular structures including DNA, lipids, proteins and membranes, and can lead to cell death (Bergamini et al., 2004; Gillis et al., 2014; Hermes-Lima & Zenteno-Savín, 2002; Matozzo et al., 2013; Storey, 2004). As a stress effect that is responsive under challenging conditions, oxidative stress is a great indicator of an organism’s physiological state prior to more conspicuous whole-organism responses.

The Greenshell^{TM} mussel, *P. canaliculus,* is New Zealand's most important aquaculture species with approximately 97,000t harvested each year, creating revenue of NZD$381 million annum^{-1} (Stenton-Dozey et al., 2020). The major sites for *P. canaliculus* farming in New Zealand include the Marlborough Sounds, Hauraki Gulf, Golden Bay and Stewart Island (Jeffs et al., 1999). A commercial mussel farm is typically 3ha and consists of approximately 10 longlines made up of a double backbone system supported by buoys, with culture ropes seeded with mussels continuously looped over the backbones extending 5-10m deep (Jeffs et al., 1999; Stenton-Dozey et al., 2020). Juvenile mussels (250µm-10mm) are typically referred to as "spat" and initially seeded onto the polypropylene culture ropes inside a mesh stocking (Jeffs et al., 1999). The stocking and remaining beach-cast material disintegrates over time, allowing mussels the option to re-attach to the culture rope or to re-settle elsewhere (Skelton & Jeffs, 2020). Unfortunately, losses of cultured *P. canaliculus* spat are common during the initial growth period after seeding (the nursery stage) during the disappearance of the stocking, when up to 99% of *P. canaliculus* spat can be lost (Skelton & Jeffs, 2021b; South et al., 2020). Mortality and secondary settlement (migration) both contribute to *P. canaliculus* spat loss; however, the drivers of high migration and mortality are largely unknown (Carton et al., 2007; Hayden & Woods, 2011; South et al., 2020). Once mussels have reached 10-20mm in shell length, they are stripped from the culture rope and re-seeded at a lower density, a process called inter-seeding (Jeffs et al., 1999; South et al., 2020). Because spat loss is greatest in the nursery stage of cultivation, the proportion of spat remaining at inter-seeding is a major determinant of the overall harvesting yield of adult *P. canaliculus*.

Modifying seeding density is one possible method to manage spat losses in *P. canaliculus* cultivation as it can alter biofouling accumulation and mitigate overcrowding on the culture ropes (Bordignon et al., 2021; Cubillo et al., 2015). High seeding density is known to negatively impact the survival of *Mytilus edulis* spat in Canada (Lauzon-Guay et al., 2005a), and decrease growth and increase losses in *Mytilus galloprovincialis* spat in the Black Sea (Karayücel et al., 2015). Similarly, a greater density decreased the length/height and length/width ratio of cultivated *M. galloprovincialis* in Spain, likely as a morphological adaptation to reduce the effects of intraspecific competition (Cubillo et al., 2012a). A high seeding density which increases competition for resources can result in self-thinning of mussels which can increase losses (Guiñez, 2005; Lauzon-Guay et al., 2005b). Therefore, investigating the effects of seeding density on stress biomarkers of *P. canaliculus* could provide a method to calibrate the internal response of seeded spat to farming procedures, and how stress might relate to more obvious, whole-organism responses. Understanding how seeding density impacts stress biomarkers in *P. canaliculus* will also help to implement beneficial modifications to seeding procedures to minimize mussel stress and mitigate stress-on-stress situations (Delorme et al., 2020). In this study, we assessed the effect of seeding density on *P. canaliculus* physiology by measuring a stress response, total antioxidant capacity (TAC), and two stress effects, lipid peroxidation (LP) as a marker of oxidative stress, as well as mussel viability by conducting a freshwater immersion test to determine the physiological condition of mussels at the time of sampling. The field experiment was spatially and temporally replicated in the Marlborough Sounds over five months during the nursery stage of cultivation to capture the stage when spat loss is common.

3.3 Materials and methods

3.3.1 Study sites

This study assessed stress biomarkers including total antioxidant capacity, oxidative stress and viability of *P. canaliculus* spat from five conventional longline mussel farms at two sites in the outer Marlborough Sounds, New Zealand. Our study occurred during the nursery stage of the aquaculture production.

3.3.2 Source of *Perna canaliculus* and initial seeding

Three 10kg bags of beach-cast material including seaweed, hydroids and *P. canaliculus* spat were harvested from Ninety Mile Beach, New Zealand (34°56'54.4"S 173°07'14.0"E) on 14th September 2019 and transported in a refrigerated truck (6-7°C) to Havelock (41°16'54.5"S 173°46'01.9"E). Here, the three bags were amalgamated to produce 30kg of spat material for the field experiment. A purpose-designed vessel was used to seed the mussels onto the culture ropes, which involved sprinkling the spat material onto the culture rope by hand as it was mechanically pulled past a seeding hopper. A mesh stocking was placed around the culture rope and spat material before deployment in the water. To achieve the three seeding densities (Table 1), the rate at which the spat material was added to the culture rope was adjusted. All experimental culture ropes were initially deployed at Site 1 on 16th September due to logistical constraints and then half of the culture ropes were moved to five farms at Site 2 on the 20th September 2019. For the study, five farms at both Site 1 and Site 2 each held a 16m loop of culture rope of each seeding density spaced 0.5m apart.

Table 1. The weight of spat material required to achieve the experimental seeding densities for each 16m loop of culture rope.

Seeding density target (m of culture rope 10kg^{-1} bag of spat material)	Equivalent grams of spat material m^{-1} of culture rope	Equivalent grams of mussels m^{-1} of culture rope	Number of mussels m^{-1} of culture rope	Number of mussels 10cm^{-1} of culture rope
150m	66g	15.9g	10,309	1,030
300m	33g	7.96g	5,154	515
600m	16.5g	3.98g	2,577	257

At the time of initial seeding, five samples of the spat material (37-54g) were haphazardly removed and frozen at -20°C to determine the initial spat abundance for each seeding density treatment (Table 1). Each of the five samples were weighed (wet weight) and washed over a series of mesh sieves to separate the sample into three size classes to simplify counting, based on mussel shell width (62μm-250μm, 250μm-1000μm, and >1000μm). Mussels in the >1000μm size class (>1000μm shell width) were counted on a white tray, whereas the two smaller size classes of each sample were subdivided into eighths using a plankton splitter, with one subsample (one eighth of the original sample) of each size class photographed under a dissecting microscope and counted. The counts of mussels in each size class were pooled and divided by the wet weight of each initial sample to provide an estimate of spat density g^{-1} of spat material. The mean (± standard error, SE) mussel density was 156 ± 15 mussels g^{-1} of spat material (Table 1).

3.3.3 Experimental design

To assess how *P. canaliculus* stress varied among seeding densities, we measured the total antioxidant capacity (TAC), lipid peroxidation (LP) and viability of *P. canaliculus* at three seeding densities: 257, 515 and 1030 mussels 10cm^{-1} of culture rope (Table 1). For TAC and LP analyses, at each seeding density, samples were taken from one side of each loop of culture rope at two depth strata: 0-1m and 6-7m represented as 0.5m and 6.5m respectively, at three replicate farms from each site. For mussel viability analyses, at each seeding density, samples were taken from both sides of each loop of culture rope from four depth strata: 0-1m, 2-3m, 4-5m, and 6-7m, represented as 0.5m, 2.5m, 4.5m and 6.5m respectively, at five replicate farms at each site. Some deeper samples at Site 2 were lost due to rough weather between the December 2019 and February 2020 sampling dates, so for TAC and LP analyses, supplementary samples were taken from a fourth farm at Site 2 in February 2020. This resulted in a total of 36 samples for both TAC and LP from each sampling occasion which occurred on 9th December 2019 and 13th February 2020, and 240 samples in December 2019, and 192 samples in February 2020 for mussel viability.

3.3.4 Sample collection and preparation

For sample collection in December 2019 and February 2010, a davit and winch on a purpose-designed vessel was used to raise the experimental culture ropes out of the water (Figure 1a). At the required depths, the culture rope was placed against a 10cm stainless steel measuring tool and cut on either side to provide a 10cm section of culture rope containing mussels and biofouling (Figure 1b, 1c). The remaining culture rope was reconnected with two large cable ties and returned to the water once all samples from each culture rope were collected. Samples for TAC and LP analyses required removing 2-6 mussels from the samples collected at 0.5m and 6.5m depth. These mussels were opened using a scalpel blade and the tissue excised and placed into 3.6ml cryogenic tubes then snap-frozen in liquid nitrogen and stored at -70°C. The remaining samples were placed in a zip-lock bag and immediately placed into a chiller bin with an ice pack for viability tests. For TAC and LP analyses, the frozen mussel tissues were individually ground and homogenised into a frozen powder using a mortar and pestle (previously chilled in liquid nitrogen) and weighed (to the nearest 0.1mg).

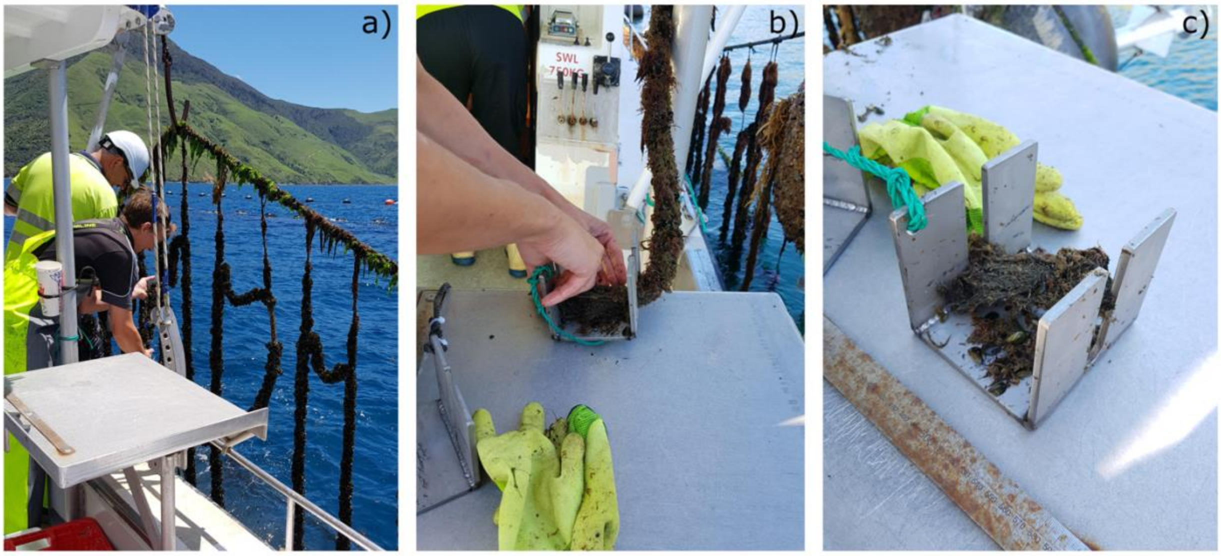

Figure 1. The procedure to collect samples from the experimental culture ropes in December 2019 and February 2020. This required raising each experimental culture rope out of the water with a davit and winch on a purposely-designed vessel (a), and at the selected depths, the culture rope was slotted into the 10cm stainless-steel measuring tool (b), and then cut on either side of the measuring tool (c) to produce a 10cm sample of culture rope.

3.3.5 Total antioxidant capacity assay

The total antioxidant capacity (TAC) can be used to quantify a mussel's ability to eliminate the accumulation of free radicals, including ROS, thus indicating a mussel's preparedness to deal with oxidative stress (Birben et al., 2012; Delorme et al., 2021). For the TAC assay, an aliquot of 100mg ± 10mg was collected from the homogenised tissue powder for each sample. We measured TAC with the Antioxidant Assay Kit (Sigma-Aldrich®, catalogue number: CS0790) following manufacturer's instructions. Measuring TAC using this assay involves a process where a ferryl myoglobin radical and hydrogen peroxide oxidizes 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS) to produce a radical cation, $ABTS^{\cdot+}$, which is a green soluble chromogen that can be measured spectrophotometrically. The antioxidants in the sample prevent the production of $ABTS^{\cdot+}$, thus the colour intensity detected using a spectrophotometer is negatively correlated with $ABTS^{\cdot+}$ concentration. For the assay, the powdered samples (100mg ± 10mg) were homogenised in assay buffer solution, using 1.5ml Eppendorf tubes and glass beads (Qiagen 5mm) in a Qiagen TissueLyser II for 5 minutes. The homogenate was then removed from the tube, placed into 2ml Eppendorf tubes and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant from each tube was removed, and frozen in aliquots at -70°C. A clear 96-well plate was used for the assay, with 10µL of supernatant added to each well in duplicate, together with the reagents specified in the protocol. Where required, samples were diluted 5-fold in assay buffer solution to standardise the range of possible absorbance values. The TAC values were then obtained from reading the absorbance at 405nm using a BioTek Synergy 2 plate reader and Gen5 3.05 software. The TAC values are the concentration (mM) of (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylix acid (Trolox) solution, thus a greater Trolox concentration indicates greater TAC. A standard curve of the Trolox standard concentrations 0.00 – 0.42mM was generated for the assay to calculate the TAC in the test samples (with a mean (±SE) R^2 of 0.712 ± 0.059 across all 96-well plates).

3.3.6 Lipid peroxidation assay

Lipid Peroxidation (LP) is the degradation of lipids as a result of oxidative stress and can be measured by analysing malondialdehyde (MDA), an end product of the reaction of an oxidative attack by ROS. For the LP assay, an aliquot of 20mg ± 3mg was collected from the homogenised tissue powder for each sample. Here, we measured LP using the Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich®, catalogue number: MAK085), following the manufacturer's instructions and adjusting the volume of reagents according to the tissue mass in each sample. In this assay, LP is determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric product, proportional to the MDA present. Each sample (20mg ± 3mg powdered mussel tissue) was homogenised for 5 minutes in 1.5ml Eppendorf tubes with beads (Qiagen 5mm) using a Qiagen TissueLyser II in 600µL MDA Lysis buffer containing 6µL of butylated hydroxytoluene (100×). The samples were then centrifuged at 13,000 × *g* for 10 minutes at ambient temperature. Then, 200µL of the supernatant from each sample was transferred to a microcentrifuge tube with 600µL of TBA and incubated at 95°C for 60 min. Once cooled to room temperature, 200µL of the supernatant and TBA mixture was added into 96-well plates in triplicates, and the absorbance was read at 532nm using a BioTek Synergy 2 plate reader and Gen5 3.05 software. A standard curve of the MDA standard concentrations 0 – 20nmol was prepared for the assay to calculate the MDA concentration in the test samples (with a mean (±SE) R^2 of

0.991 ± 0.028 across all 96-well plates).

3.3.7 Viability test

On the day after collection from the field, mussels were exposed to a freshwater immersion test to examine stress and viability. During freshwater immersion mussels experience an osmotic shock and mussels that are in a good physiological condition (low stress) react to this osmotic shock by closing their valves to isolate themselves from the hostile external environment. However, when mussels are dead or in a poor physiological condition (high stress), they are not able to quickly respond to this osmotic shock and their valves remain open. Thus, during a freshwater immersion test, mussels can appear either 'viable' (closed valves, alive and low stress), or 'non-viable' (open valves and highly stressed, or empty and dead at the time of collection). The freshwater immersion test consisted of removing all contents from the 10cm culture rope samples and rinsing the contents of each sample through a 1000μm sieve. All contents retained in the 1000μm sieve were immersed in freshwater and the number of open and empty mussels were counted after 30 seconds immersion. Samples were then placed at -20°C and stored until the total abundance of mussels in each sample was quantified. This allowed for the calculation of the proportion of mussels that were closed, open or empty at the time of collection.

3.3.8 Statistical analysis

To examine the impacts of the fixed effects and interactions on TAC, LP and viability we used linear and generalised linear mixed models fit using R package lme4 (Bates et al., 2018) in RStudio version 1.4.1106 (RStudio Team, 2020). Our analyses included four fixed effects: seeding density with three levels: 257, 515 and 1030 mussels 10cm^{-1} of culture rope, depth with two levels for TAC and LP: 0.5m and 6.5, and four levels of depth for viability: 0.5m, 2.5m, 4.5m and 6.5m, site with two levels: Site 1 and Site 2, and time with two levels: December 2019 and February 2020, as well as the interactions between all combinations of fixed effects. There were three random effects in TAC and LP statistical analyses: farm, rope, and sample ID, to account for the variation among farms, culture ropes and sample replicates (i.e., the duplicates or triplicates of each sample), while viability analysis had farm and rope as random effects.

For the TAC assay the response variable was the mean total antioxidant concentration (mM Trolox equivalent), and for the LP assay the response variable was the mean MDA concentration (nmol). For viability analysis we assumed a binomial error distribution where the response variable was the number of viable (closed valves) and non-viable mussels (open valves, or empty mussels) in each sample. For *P. canaliculus* viability, the time between exiting the ocean and the freshwater immersion test were recorded as 'aerial exposure' (10-36 hours) for each sample and added as an offset in viability statistical analysis given the strong relationship between aerial exposure and viability (F_1=82.72, p<0.001). For TAC, LP and viability analyses we used a backward model selection by single term deletions using the drop1 function in the R package lmerTest (Kuznetsova et al., 2015) to determine significant effects (i.e., P-value < 0.05). Tests were only produced if a fixed effect was not involved in a significant higher-order interaction.

Table 2. Backward model selection for total antioxidant capacity, lipid peroxidation and viability in *Perna canaliculus*. Abbreviations: SD (seeding density of *Perna canaliculus*), S (site), DP (depth), T (time), F (farm), R (rope) and ID (identification of each sample with duplicates and triplicates). For the total antioxidant capacity assay the response (Y) was the observed total antioxidant concentration (mM Trolox equivalent) in *Perna canaliculus* from each sample, and for the lipid peroxidation assay the response variable (Y) was the observed MDA concentration (nmol) in *Perna canaliculus* from each sample. For the viability test, the response (Y) was the probability of closing valves during freshwater immersion. The notation (1|F), (1|R) or (1|ID) was used to denote that the model assumes random intercepts for each level of the random effect indicated after the bar.

Best supported model for:	Model terms:
Total antioxidant capacity	Y = S + SD + DP + T + S×SD + S×DP + S×T + SD×DP + SD×T + DP×T + S×SD×DP + S×SD×T + S×DP×T + SD×DP×T + S×SD×DP×T + (1|F) + (1|R) + (1|ID)
Lipid peroxidation	Y = T + (1|F) + (1|R) + (1|ID)
Viability	Y = S + SD + DP + T + S×SD + S×DP + S×T + SD×DP + SD×T + DP×T + S×SD×DP + S×DP×T + (1|F) + (1|R)

3.4 Results

Seeding density had inconsistent effects on total antioxidant capacity (TAC) across depths, sites and times (Figure 2a-d; Table 3: Site×Density×Depth×Time). In December 2019, at the lowest seeding density TAC decreased with increasing depth by approximately 16% at Site 1 (Figure 2a) and approximately 52% at Site 2 (Figure 2b). At intermediate and high seeding densities in December 2019, the only change in TAC with depth occurred at the highest seeding density at Site 2, where TAC increased by approximately 28% with increasing depth (Figure 2b). In February 2020, TAC was impacted by seeding density at Site 2 only, where TAC increased by approximately 77% with increasing depth at the lowest seeding density (Figure 2d). However, on average TAC was 71% greater in December 2019 compared with February 2020 (Figure 2a-d). Lipid peroxidation (LP) was only significantly impacted by time, where LP was, on average, 25% greater in February 2020 compared with December 2019 (Figure 2e-h; Table 3: Time).

Table 3. Summary statistics for the backward model selection procedure for the total antioxidant capacity, lipid peroxidation and viability in *Perna canaliculus*. All models retained the same random effects (farm and rope). Significant results are bolded, and tests were not produced for fixed effects if higher-order interactions involving those fixed effects were significant. Abbreviations: S (site), SD (seeding density), DP (depth), T (time), F (farm) and R (rope).

	Total antioxidant capacity				Lipid peroxidation				Viability		
Fixed effect	NumDF	DenDF	F	Pr(>F)	NumDF	DenDF	F	Pr(>F)	df	LRT	Pr(Chi)
S	-	-	-	-	1	210	0.343	0.559	-	-	-
SD	-	-	-	-	2	210	0.565	0.569	-	-	-
DP	-	-	-	-	1	210	0.084	0.773	-	-	-
T	-	-	-	-	1	210	5.646	**0.018**	-	-	-
S×SD	-	-	-	-	2	201	0.144	0.866	-	-	-
S×DP	-	-	-	-	1	201	0.695	0.405	-	-	-
S×T	-	-	-	-	1	201	1.619	0.205	-	-	-
SD×DP	-	-	-	-	2	201	0.292	0.747	-	-	-
SD×T	-	-	-	-	2	201	0.162	0.851	2	82.754	**<0.001**
DP×T	-	-	-	-	1	201	0.136	0.713	-	-	-
S×SD×DP	-	-	-	-	2	194	0.002	0.998	2	33.902	**<0.001**
S×SD×T	-	-	-	-	2	194	0.554	0.576	2	0.662	0.718
S×DP×T	-	-	-	-	1	194	1.373	0.243	1	32.836	**<0.001**
SD×DP×T	-	-	-	-	2	194	0.546	0.580	2	4.334	0.115
S×SD×DP×T	2	93.392	19.441	**<0.001**	2	192	0.440	0.645	2	1.798	0.407

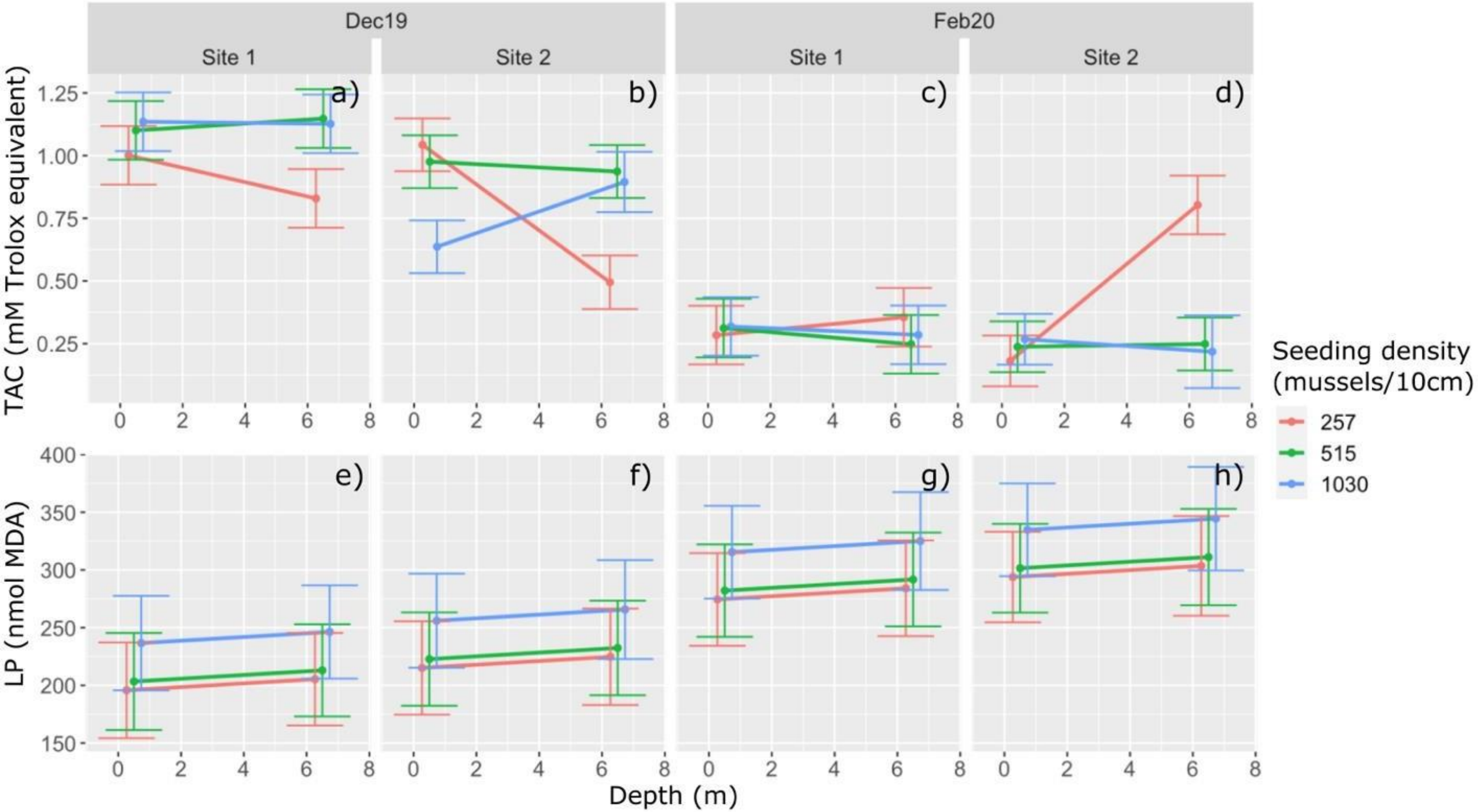

Figure 2. Mean (±SE) total antioxidant capacity (TAC, mM Trolox equivalent; a, b, c and d) and lipid peroxidation (LP; e, f, g and h) represented by mean (±SE) MDA concentration (nmol) in *P. canaliculus* at three seeding densities, two depths, at Site 1 and Site 2 in the Marlborough Sounds in December 2019 and February 2020.

Accounting for the effect of aerial exposure, viability varied among sites, seeding densities, and depths (Figure 3a-d; Table 3: Site×Density×Depth). At intermediate and high seeding densities at Site 1, viability was up to 3% greater at 6.5m compared with 0.5m (Figure 3a, 3c). At Site 1, viability was slightly greater (approximately 1%) at intermediate and high seeding density compared with low seeding density (Figures 3a, 3c). However, at Site 2, seeding density and depth had lesser impact on viability (Figure 3b, 3d). In December 2019, *P. canaliculus* viability had a positive relationship with seeding density; however, in February 2020, there was a slight negative relationship between seeding density viability (Figure 3a-d; Table 3: Density×Time). Additionally, the effects of depth on *P. canaliculus* viability were more apparent in December 2019, where viability was typically greater at deeper compared with shallower depths at Site 1, but lower at deeper depths at Site 2 (Figure 3a, 3b; Table 3: Site×Depth×Time). Differences in the effects of depth on *P. canaliculus* viability between sites were reduced in February 2020 (Figure 3c, 3d). Despite small differences in viability among the experimental treatments, *P. canaliculus* viability remained high across all treatments (>0.9 probability of resisting freshwater immersion; Figure 3).

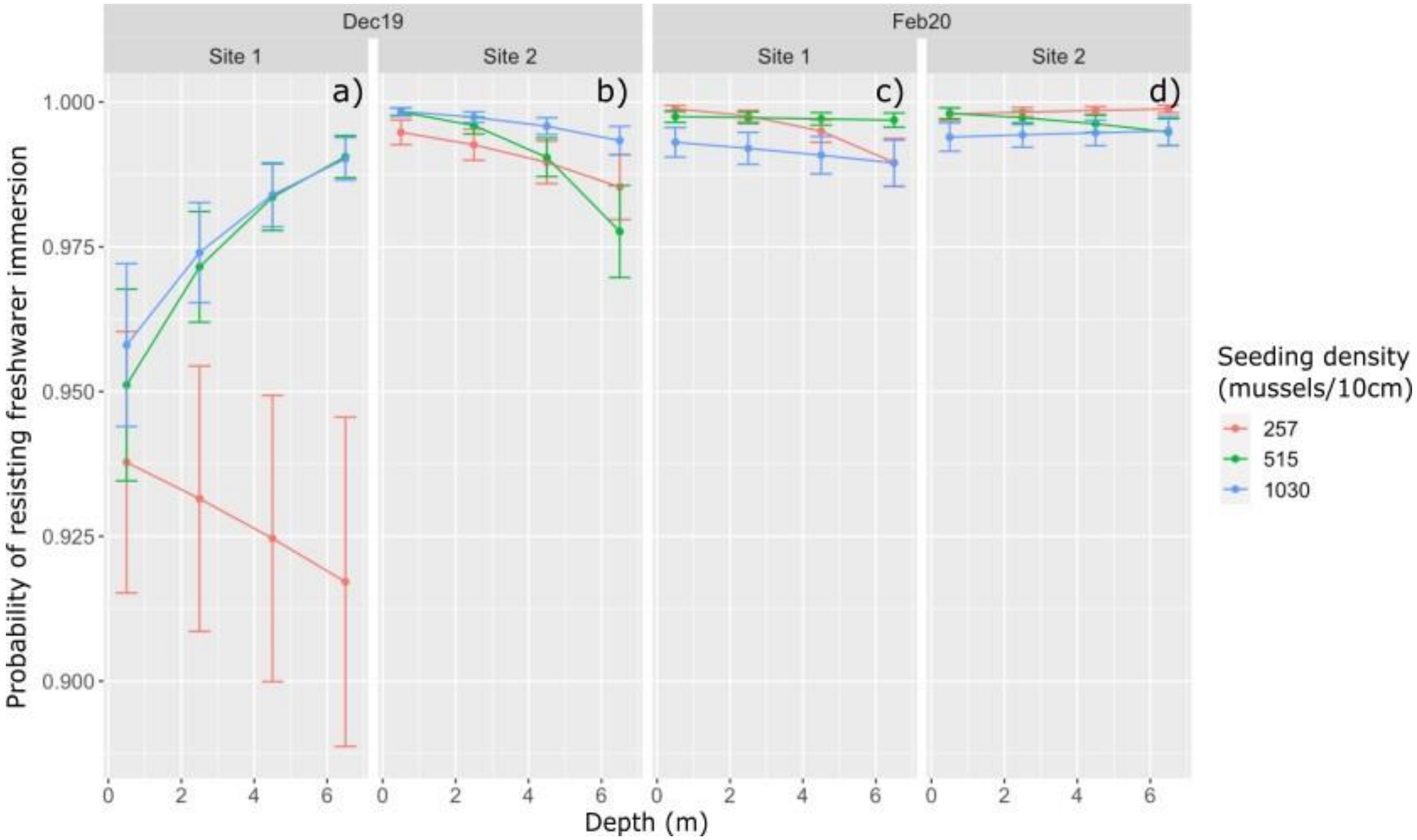

Figure 3. Mean (±SE) viability (probability of resisting freshwater immersion by closing valves) of *P. canaliculus* at three seeding densities, four depths, at Site 1 and Site 2 in the Marlborough Sounds in December 2019 and February 2020.

3.5 Discussion

Sessile or sedentary species that have limited ability to escape stressful conditions must either tolerate environmental challenges or perish. Tolerating challenging conditions requires a response, either a physiological or whole-organism response. Lower seeding density can reduce overcrowding and intraspecific competition and maximise shellfish survival and growth (Cubillo et al., 2012b; Griffiths & Hockey, 1987; Xavier et al., 2007), but requires greater farm space to seed a certain abundance of mussels while allowing more space for biofouling to accumulate. On the other hand, higher seeding density reduces space for biofouling, and allows more mussels to be seeded in a smaller farm area but can increase overcrowding and intraspecific competition (Kamermams & Capelle, 2019). Thus, understanding the impacts of seeding density on mussel stress biomarkers, and how these compares to whole-organism responses is important to determine a seeding density that balances stress and efficient mussel farming.

Variation in total antioxidant capacity (TAC) and viability were likely influenced by differences in oxygen and nutrient availability among the three seeding densities. For instance, antioxidant activities in the brown mussel, *Perna perna*, increased with prolonged air exposure (hypoxia), suggesting reduced resistance to oxidative stress (Nogueira et al., 2017). Hyperoxia has also been shown to induce oxidative stress in the Antarctic scallop, *Adamussium colbecki,* and Mediterranean scallop, *Pecten jacobaeus* (Viarengo et al., 1995). Similarly, Delorme et al. (2020) showed that increased fasting duration increased markers of oxidative stress and a reduced ability to enhance antioxidant activity in *P. canaliculus*. Delorme et al. (2020) also found protein and carbohydrate content, energy reserves and tolerance to additional stress decreased with greater fasting, indicating that prolonged periods of low food availability can have detrimental effects on mussel performance. While we did not measure or control abiotic factors, it is likely that oxygen and nutrient availability contributed to variation in TAC and viability in *P. canaliculus* among seeding densities. It seems plausible that abiotic factors not associated with differences in seeding density influenced the oxidative metabolism of mussels and obscured the effects of seeding density if they occurred in our study.

Overall, mussels appeared under greater oxidative stress near the end of our experiment during the peak of the austral summer. Water temperature in the Marlborough Sounds is historically highest in the months February and March, where it can be ~1°C warmer than in December (Broekhuizen et al., 2021; Hickman et al., 1991). During this experiment, the water temperature at 5m depth in Waitata Bay, Marlborough Sounds (~9km from Site 1, ~25km from Site 2) was 15.5-16.7°C in December 2019, and 16.9-17.7°C in February 2020 (New Zealand King Salmon, 2020), supporting the findings of Broekhuizen et al. (2021). Water temperature also typically varies with depth and location in the marine environment (Broekhuizen et al., 2021; Fuentes et al., 2000). Elevated water temperature can enhance oxygen consumption and alter metabolism which can increase the production of reactive oxygen species (ROS), thus inducing oxidative stress in aquatic animals (Bocchetti et al., 2008; Lushchak, 2011; Verlecar et al., 2007). Therefore, the elevated water temperature in February 2020 likely increased oxidative stress in *P. canaliculus* compared with December 2019. While TAC was impacted inconsistently by seeding density, depth, site and time; seeding density and depth did not impact LP, suggesting that these factors are not negatively impacting the prooxidant-antioxidant balance in *P. canaliculus* at the levels tested in this study.

Oxidative damage in mussels is sometimes linked to more severe, whole-organism responses such as reduced survival (Nguyen & Alfaro, 2020; Wang et al., 2018), which can also be influenced by increased water temperature (McFarland et al., 2015; Yuan et al., 2016). The viability test carried out here is a whole-organism response of mussels to osmotic shock but also an indicator of the mussel's physiological state. The water temperature in the Pelorus Sound during summer 2019/20 was consistently cooler than the two previous summers (New Zealand King Salmon, 2020). Mussel viability remained high across all treatments (>90% probability of valve closure during freshwater immersion), which suggests the temperatures during the summer 2019/20 were within the stress tolerance range of *P. canaliculus* at the time of sampling. However, we would still expect to see a decrease in viability between December 2019 and February 2020 due to increased water temperature in February, but instead there was a slight increase in viability which may be influenced by mussel age. Therefore, mussel viability was not impacted by temporal variation the same way as oxidative stress and is likely to be impacted by factors additional to temperature.

Additional to temperature; chlorophyll *a,* particulate carbon and organic matter, and salinity also vary temporally in the Marlborough Sounds (Hickman et al., 1991). Typically, chlorophyll *a,* particulate carbon and particulate organic matter are slightly lower, and salinity greater in the month of February compared with December (Hickman et al., 1991), with spatial differences also expected. Because fluctuations in nutrient availability can impact mussel stress (Delorme et al., 2020), it is possible that differences in nutrient availability between December 2019 and February 2020, caused temporal variation in oxidative stress and viability. Although both our sites were exposed to oceanic salinities, salinity change can cause a variety of stress effects and responses in marine organisms, many of which can increase ROS production and cause oxidative stress (Liu et al., 2007; Lushchak, 2011). For example, *Perna viridis* exposed to three salinities: 15%, 22%, and 30%, had significantly lower ROS production at the low salinity (Wang et al., 2012). Salinity fluctuations can also increase mussel mortality (McFarland et al., 2015; Yuan et al., 2010). Furthermore, it is also possible that spat growth during this experiment reduced available space on the culture ropes by February 2020, increasing intraspecific competition and possibly contributing to greater stress. Therefore, while further examination is required, a combination of temperature and nutrient availability, as well as intraspecific competition likely contributed to greater oxidative stress in *P. canaliculus* in February 2020, as well as impacting mussel viability differently throughout time.

Although the effects of seeding density on TAC and viability varied spatially and temporally, and LP varied only temporally; the overall differences in *P. canaliculus* stress among seeding density treatments were minimal. The seeding density treatments in our experiment were low, for example the seeding density of 150m $10kg^{-1}$ spat material typically seeds 20,000 – 50,000 spat m^{-1} of culture rope (Skelton & Jeffs, 2021a), whereas in our study the same treatment seeded only 10,309 spat m^{-1} of culture rope. As the quality and quantity of spat within beach-cast material from Ninety-Mile Beach is highly variable and uncontrollable (Alfaro et al., 2010), my results may have displayed greater impacts of seeding density on stress biomarkers in *P. canaliculus* if the typical density of spat (20,000 – 50,000 spat m^{-1} of culture rope) for the highest seeding density treatment was achieved. In future, recording environmental data such as temperature, water velocity, chlorophyll *a* and particulate organic carbon (POC) on the mussel farms at different seeding densities will better describe how seeding density changes the growing environment for *P. canaliculus* spat. Additionally, laboratory experiments, where multiple environmental factors can be manipulated to mimic growing conditions at different seeding densities, with oxidative stress, viability, and whole-organism responses such as abundance and size of *P. canaliculus* measured under these manipulated conditions will increase the understanding of how seeding density impacts *P. canaliculus* performance*.* Finally, measuring oxidative stress and viability in both cultured spat and adult mussels among seeding densities and manipulated environmental conditions will show how the tolerance of *P. canaliculus* to environmental and anthropogenic stressors changes throughout ontogeny. The more we understand the impacts of seeding density on *P. canaliculus* performance, the greater opportunity there is to alter farming procedures to mitigate large losses of *P. canaliculus* and enhance production.

Chapter 4: General discussion

This thesis describes the effects of seeding density on *Perna canaliculus* performance and biofouling accumulation on commercial mussel farms in the Marlborough Sounds. In mussel farming, greater abundance, size and viability, and lower oxidative stress of the cultured species are favourable for production. However, I found the impacts of seeding density on *P. canaliculus* performance and biofouling accumulation typically varied spatially and temporally, and there was an indication of trade-offs between favourable production traits and stress biomarkers. Although the highest seeding density I examined had the greatest mussel abundance at the end of the experiment, the highest seeding density also experienced the greatest loss throughout this experiment. My experiments focused only on the nursery stage of mussel cultivation, and it would be important to examine if these losses carry-over in subsequent stages of cultivation. Seeding density had inconsistent impacts on both whole-organism responses including abundance and size, and stress biomarkers including oxidative stress indicators and viability in *P. canaliculus*. This suggests that whole-organism responses and stress biomarkers are not consistently impacted by external pressures, and that stress biomarkers were not necessarily a pre-cursor for changes in abundance and size in *P. canaliculus*. Seeding density generally negatively impacted *Mytilus galloprovincialis* abundance and had inconsistent impacts on *M. galloprovincialis* size as well as non-mussel biofouling composition and biomass. The overall inconsistency of these results suggest that *P. canaliculus* performance and biofouling accumulation were heavily impacted by factors not considered in our study.

Inter- and intraspecific interactions can affect mussel performance. *Mytilus galloprovincialis* is a problematic biofouling species on *P. canaliculus* farms due to its niche similarity and therefore similar demand for resources (Atalah et al., 2016; Woods et al., 2012). The greater biomass of non-mussel biofouling at shallow depths and Site 2, and the greater abundance of *M. galloprovincialis* at shallow depths and in February 2020 may have increased interspecific competition, and thus contributed to greater oxidative stress in *P. canaliculus* in some cases. Similarly, the greater size of both *P. canaliculus* and *M. galloprovincialis* in February 2020 likely minimised available space on the culture ropes, thus increasing competition and oxidative stress for seeded *P. canaliculus*. Where variation in *P.*

canaliculus viability occurred between treatments, this did not correlate with a difference in *M. galloprovincialis* abundance, suggesting that *M. galloprovincialis* accumulation was not strongly associated with *P. canaliculus* viability. Some non-mussel biofouling species such as the decorator crab, *N. minor* (Van de Ven, 2007), and an ascidian, *Didemnum vexillum* (Fletcher et al., 2013) can also decrease the abundance of *P. canaliculus* spat on culture ropes. While biofouling taxa identification was not conducted, it is possible that these biofouling species were present and *N. minor* predated on *P. canaliculus* and *D. vexillum* elevated spatial competition, thus impacting *P. canaliculus* performance. However, biofouling accumulation can have non-significant impacts on *P. canaliculus* spat retention and abundance on commercial mussel farms (South et al., 2019). For instance, the accumulation of amphipods, macroalgae, mussels and sessile invertebrates had minor, inconsistent impacts on the abundance of *P. canaliculus* spat, suggesting a weak influence of biofouling accumulation on spat retention (South et al., 2019). The inconsistent impacts of biofouling species on *P. canaliculus* performance highlights the importance to explore their effects further.

The influence of environmental variation on mussel performance is unavoidable in a water-based cultivation system. Temperature, food availability and salinity are three common abiotic factors influencing mussel performance and fluctuate monthly in the Marlborough Sounds (Broekhuizen et al., 2021; Hickman et al., 1991; Ren et al., 2019). Water temperature inconsistently impacts mussel production traits. For example, *Mytilus edulis* retention was greatest in temperatures 10-15°C compared with >15°C (Lachance et al., 2008), whereas growth in *M. galloprovincialis* was greater at shallow depths which were a maximum 3°C warmer than deeper depths (Fuentes et al., 2000). Water temperature also impacted oxidative stress in *Perna viridis*, where heat stress (32°C) increased the activities of antioxidants compared with 20°C, implying an increase in the production of reactive oxygen species (ROS), activating antioxidants to reduce ROS accumulation (Verlecar et al., 2007). Increased water temperature typically reduces mussel survival (McFarland et al., 2015; Yuan et al., 2016), as seen in *P. canaliculus*, where 35.4% of mussels at the shallower part of the mussel bed at Banks Peninsula were dead and gaping, whereas 0% of mussels died at the deeper edge of the mussel bed which was consistently >2°C cooler (Petes et al., 2007). Food availability is another factor that can influence mussel performance, and it appears that increased food availability has consistent positive impacts on mussels. For example, greater food availability can increase shell growth (Melzner et al., 2011), attachment strength (Babarro et al., 2008), tolerability to other environmental stressors (Schneider et al., 2010) and antioxidant activity thus reducing oxidative stress (Delorme et al., 2020) in various species of mussels. Salinity is another factor that can influence mussel performance in the marine environment, where fluctuations and extreme salinities can influence mussel growth (Line & Pleissner, 2012), increase mussel mortality (McFarland et al., 2015; Yuan et al., 2010), and increase ROS production and initiate oxidative stress (Liu et al., 2007). Multiple other abiotic factors such as oxygen availability and water flow can also influence mussel performance, by impacting attachment (Alfaro, 2006), oxidative stress biomarkers (Giannetto et al., 2017), and growth (Hayden & Woods, 2011).

During this experiment, the environmental conditions that *P. canaliculus* was exposed to on the mussel farms were unmeasured and uncontrolled, limiting our understanding of the drivers underlying inconsistent impacts of seeding density on *P. canaliculus* performance. During the sampling trip in December 2019, eight Onset HOBO temperature loggers were deployed on two farms at both sites to capture the temperature on the culture ropes at 0.5m and 6.5m depth. However, due to rough weather between December 2019 and February 2020, most of the temperature loggers were lost from the culture ropes, and others were unable to be retrieved. Some sections of the culture ropes were also lost, particularly at the deeper depths bringing the samples at 6.5m down to two replicates at some farms at Site 2 in February 2020. Therefore, the overall number of samples totalled 240 in December 2019 and 192 in February 2020. This experiment was undertaken during five months in summer 2019/2020 at two main sites in the Marlborough Sounds, however examining these effects at more sites and for longer with more frequent sampling would help to elucidate if the variability in the impacts of seeding density I found are generalisable at greater spatial and temporal scales. The seeding densities in my experiment were low, for example the 150m 10kg^{-1} spat material treatment typically seeds 20,000 – 50,000 spat m^{-1} of culture rope (Skelton & Jeffs, 2021a), whereas in my study, the same procedure seeded only 10,309 spat m^{-1} of culture rope. Thus, the overall low stress of *P. canaliculus* in this study could reflect the lower general seeding density across all treatments in my study. While the quality and quantity of spat within beach-cast material from Ninety-Mile Beach is highly variable and uncontrollable (Alfaro et al., 2010), my results may have displayed greater impacts of seeding density on mussel performance if the typical density of spat (20,000 – 50,000 spat m^{-1} of culture rope) for the highest seeding density treatment was achieved. While this experiment provided important insight to *P. canaliculus* performance and biofouling accumulation in the nursery stage of mussel cultivation, research assessing the abiotic environment on mussel farms, and direct impacts of seeding density and biofouling taxa on *P. canaliculus* performance is important.

The nursery stage is the foundation for mussel aquaculture but is also the stage where the greatest losses occur, significantly impacting potential yield (South et al., 2020). Spat in the nursery stage are vulnerable to many stressors, including factors such as predation (Capelle et al., 2016a; Morrisey et al., 2006) and stress from initial seeding and transportation (Calderwood et al., 2014), and are also at a stage where secondary settlement/migratory behaviour naturally occurs (Carton et al., 2007; South et al., 2020). Losses of *P. canaliculus* are often heightened during summer, a phenomenon called 'summer mortality', although, like mortality and secondary settlement behaviours increasing loss in *P. canaliculus,* the exact drivers of summer mortality are unknown (Li et al., 2020). However, it is suspected that during summer pathogen loads increase and elevated water temperature can cause thermal stress, therefore elevating mortality and loss during summer (Alfaro et al., 2019; Dunphy et al., 2015). With future climate change, sea temperatures and levels are expected to rise (Fan et al., 2014; Walsh et al., 2012), ocean acidification will increase (Dupont & Pörtner, 2013), weather events are predicted to intensify (Fleischhut et al., 2020; Kendon et al., 2014), and chemical and biological composition of the water column is expected to change (Sarmiento et al., 2004). These future predicted changes for the marine environment cause major concern for future loss and summer mortality events, particularly for valuable aquaculture species such as *P. canaliculus*.

While *P. canaliculus* production in New Zealand is a highly successful industry, harvestable product is largely limited by low spat retention on ropes (approximately 5% between seeding and harvest) and by available spat supply (Aquaculture New Zealand, 2020). To achieve the industry's goal to increase retention to over 5% by 2035 (Aquaculture New Zealand, 2020), stressors which increase spat losses need to be mitigated, and a consistent and high-quality spat supply is crucial. Ultimately, methods such as removing *M. galloprovincialis* from mussel ropes would greatly benefit *P. canaliculus* production; however, this requires colour sorters which cost approximately $1M and can't be implemented until mussels are 30-50mm. Hatchery grown spat currently produce up to 20% of the industry's spat supply from a single hatchery. To increase hatchery supply it would be most beneficial to develop additional hatcheries in other regions to mitigate the risk of biosecurity issues and natural disasters (Aquaculture New Zealand, 2020). While extensive plans are in place to optimise spat retention and supply by 2035, simple methods such as altering seeding density and farming procedures to increase mussel abundance and performance are therefore great methods to mitigate loss in the interim.

Future research should involve environmental monitoring including temperature, chlorophyll *a,* particulate organic matter, salinity, oxygen and water flow on commercial *P. canaliculus* farms at various seeding densities, depths and sites. This will show how the abiotic environment varies among seeding density and spatio-temporal variation on commercial mussel farms. Then, manipulative laboratory experiments could assess the impacts of seeding density on *P. canaliculus* retention, size, oxidative stress and viability in carefully controlled abiotic environments. Manipulating seeding density in a constant environment will identify direct impacts on *P. canaliculus* performance without confounding abiotic factors. Additionally, manipulative experiments can be conducted on *M. galloprovincialis* to understand how different seeding densities of *P. canaliculus* on commercial farms and abiotic factors affect the accumulation and size of *M. galloprovincialis*. Also, measuring total antioxidant capacity, oxidative stress and viability in *M. galloprovincialis* which settle on *P. canaliculus* farms would allow comparison of the stress biomarkers of both species to different seeding densities. Last, understanding the relationship between *P. canaliculus* and certain biofouling species which accumulate on the culture ropes can be explored through controlled experiments, measuring *P. canaliculus* performance with varying abundances of biofouling species.

Aquaculture species contribute more than 50% of global seafood (Anderson et al., 2017; Ritchie & Roser, 2019), reinforcing the importance to mitigate losses in shellfish production in the face of climate change. The significance of *P. canaliculus* to New Zealand aquaculture highlights the need to understand drivers behind major losses, and how commercial farming procedures can mitigate stress. Reducing stressors during the aquaculture process for *P. canaliculus* will reduce stress-on-stress responses and the ability for the stress coping mechanism to respond to additional challenges such as future climate change is increased (Delorme et al., 2020). Therefore, further research into the impacts of seeding density and abiotic and biotic factors on *P. canaliculus* performance will aid mussel farmers in making correct procedural modifications, and ultimately mitigate devastating losses in the future.

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