NZMSS STUDENT RESEARCH GRANT

Matthew Bennion

University of Waikato

Disease in a threatened New Zealand surf clam

PhD student: Matthew Bennion

Supervisors: Dr Phil Ross (University of Waikato)

Dr Henry Lane (NIWA)

Prof Ian McDonald (University of Waikato)

Firstly, I would like to extend my sincere thanks to the New Zealand Marine Science Society for awarding me the Student Research Grant in 2019. The award has contributed significantly to my research and allowed me to pursue goals only possible because of this extra funding.

Background

Toheroa are a large beach clam endemic to New Zealand. They are the largest of the *Paphies* family, and iconic kaimoana. Māori in Northern New Zealand have harvested toheroa for hundreds of years, evident from kōrero and the presence of toheroa shells within archaeological shell middens (Taikato and Ross, in prep). During the 20th century, intensive recreational and commercial harvesting of toheroa decimated populations (Williams et al., 2013, Ross et al., 2018a). Today toheroa are protected and managed under a customary permit system (Miskelly, 2016). Yet, despite protections being in place for three to four decades now, toheroa populations have not recovered. Toheroa remain within the national psyche of many New Zealanders. A taonga species, they are harvested for customary purposes still. However, many pākehā too attest to having fond memories of harvesting this highly-prized kaimoana (Stace, 1991). Their lack of recovery is of increasing concern to many communities, particularly where populations are considerably low (e.g. Kāpiti-Horowhenua). Although reasons for their limited recovery are increasingly receiving attention (Gadomski and Lamare, 2015, Ross et al., 2018a), the role disease might be playing remains a mystery (Ross et al., 2018a). A collaborative note published in 2017 highlighted the need for investigation in this area (Ross et al., 2018b), following the discovery of gas bubble disease and bacterial pathogens from toheroa at Ripiro Beach. Simultaneously, since 2012, >20 unexplained shellfish mass-mortality events have occurred in New Zealand, some raising concern among the public (https://tinyurl.com/yfrub62q). To discover what role disease might be having on the recovery of toheroa and the New Zealand shellfish mortality landscape, I began this project with the goal of developing health baselines and characterising bacterial infections within this highly-prized and mysterious surf clam.

Histopathology and co-infection in toheroa

The NZMSS Student Research Grant foremost facilitated the preparation and staining of toheroa tissue sections by hematoxylin and eosin (H&E). Traditional histopathology remains a vital investigatory tool for initial exploration of host health. Compared to tailored molecular methods the unbiased viewing of various tissues and structures can reveal unknown aetiologies and give an overarching snapshot of host health (Carella and Sirri, 2017). As toheroa are no longer commercially or recreationally fished, interest in researching their health, compared to other important kaimoana, such as flat oysters (*Ostrea chilensis*) (e.g. Lane et al., 2016) and green-lipped mussels (*Perna canaliculus)* (Trottier et al., 2012) has been

potentially limited. This reduced research capacity has meant that toheroa health is poorly understood. The aim of this initial part of my research was to construct a toheroa health 'baseline' against which to assess their health in the future. The prepared histology slides of toheroa tissue sections have been assessed for the presence of parasites and pathogens. At the same time, immune responses and reproductive health have been assessed and recorded.

A shortcoming of histopathology is that data is often discrete and/or ordinal, and inferences from data can be challenging. For example, here the presence of intracellular microcolonies (IMCs) has been graded, 0 = None, 1 = Low, 2 = Medium, 3 = High and 4 = Severe. Efforts are being made to aid the assessment of histology tissue sections using image analysis software (e.g. QuPath) (Bankhead et al., 2017) however, these tools were deemed unsuitable here due to the exploratory nature of the work being carried out. To maximise the usefulness of the histopathology data, Bayesian ordinal logistic regression models are being constructed in R using Rstan (RStan, 2016), rstanarm (Gabry and Goodrich, 2016) and bayesplot (Gabry and Mahr, 2017). These models will be used to interpret the vast array of histopathology data being gathered based on gametogenesis, intracellular microcolonies, mucus hyperplasia, lipofuscin pigmented cells, gill ciliates and a several environmental variables (e.g. SST and Chl-*a*). Below are some examples of histology images and gross pathology as well as some initial outputs from the co-infection model (fitted with preliminary data).

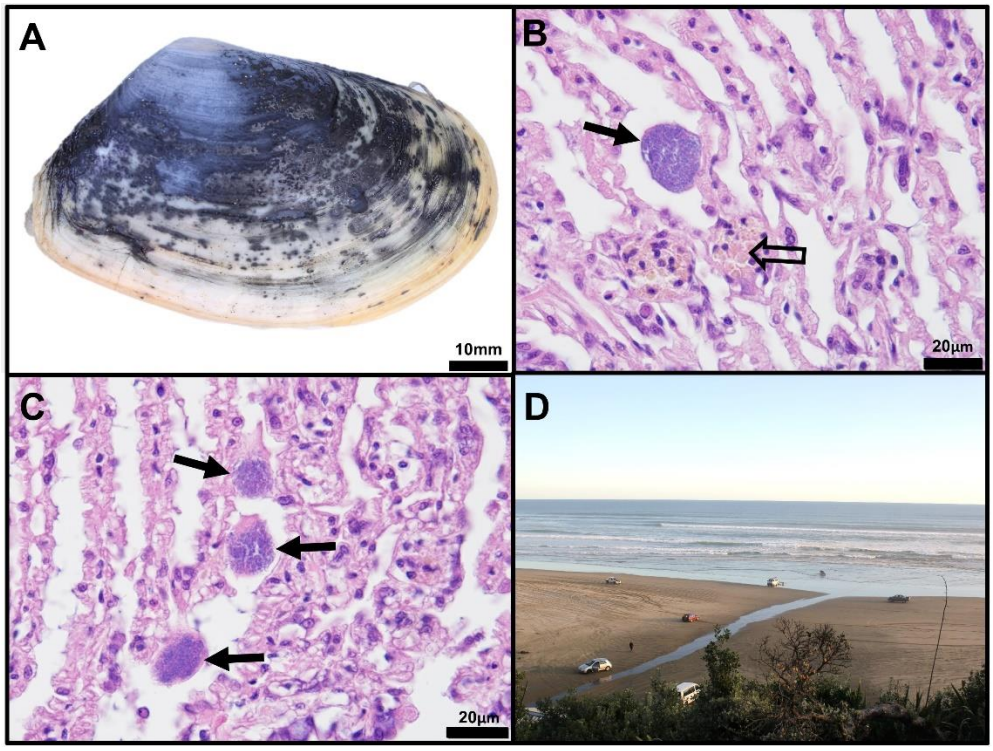

Fig. 1. A) *P. ventricosa* (toheroa) a large endemic surf clam to New Zealand, with a unique longitudinal distribution (Ross et al., 2019). B & C) Histological tissue sections (H&E stained) of toheroa gills (Olympus BX51 at x100 magnification, under oil). Filled arrows indicate cells infected with intracellular microcolonies, hollow arrow denotes lipofuscin pigmented cells. D) A freshwater stream at Ripiro Beach, preferred habitat of toheroa. Toheroa beds typically concentrate either side of freshwater streams on wide, high-energy surf beaches (Williams et al., 2013; Ross et al., 2018).

The preliminary model used here (Fig. 2) indicates that none of the variables used to construct the model describe the level of infection with IMCs on their own. That is, the level of brown cells or lipofuscin pigmented cells, gill ciliates; month, sex, condition (based on a condition index), site and digestive gland atrophy do not significantly explain to the levels of infection with IMCs in toheroa tissues. It should be noted that this model is in the development stage and does not yet contain all the data that will be obtained via continued seasonal sampling effort. This has been included to show two things, i) how it is possible to maximise the effectiveness of traditional histopathology when combined with Bayesian modelling techniques and ii) the pitfalls of histology i.e. sensitivity. For example, the model shows 'month' having a limited effect on the outcome of IMC infection level, yet more sensitive molecular techniques (qPCR) have been applied to the same samples and indicate strong seasonal variation in the infection intensity of these intracellular bacteria. With refinement and the inclusion of new data, it is hoped this model will perform better and provide a more accurate explanation of the data at hand.

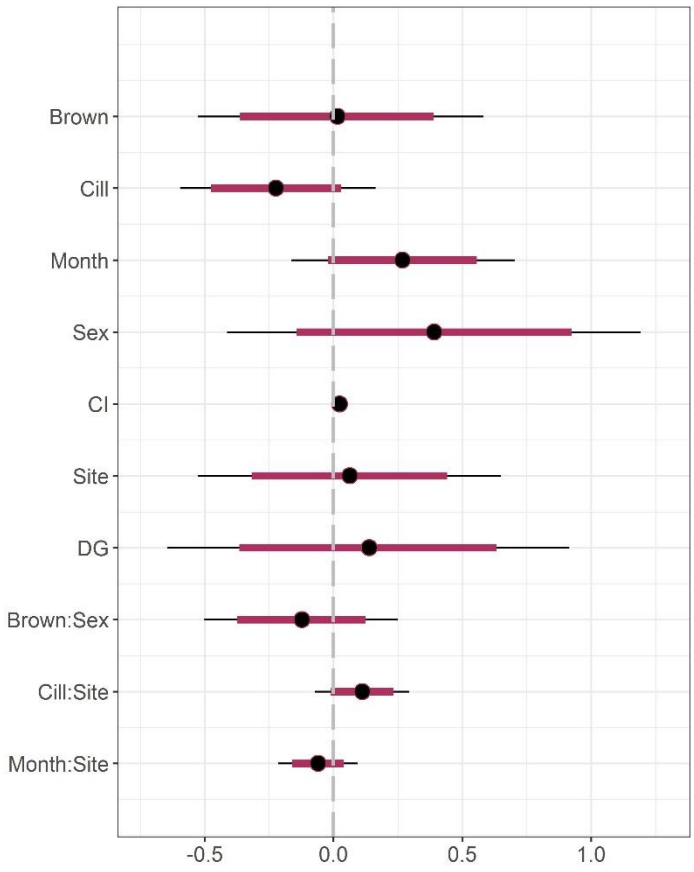

Fig. 2. Parameter estimates of population-level effects for the full model. Black dot: median, maroon line = 80% probability interval, thin black line = 95% probability interval.

The presence of intracellular microcolonies in the gills and digestive gland were a significant and reoccurring observation made in toheroa tissues. This was expected due to a first report issued in 2018 in the *Journal of Fish Diseases* (Ross et al., 2018b). The authors attributed the inclusions to *Rickettsia*-like organisms or RLOs. Often the term RLO is used to describe and unidentified intracellular bacteria (Fournier and Raoult, 2009). The term 'RLO' has been described as misleading recently in an article, which discovered that intracellular bacteria in *Pecten maximus* from Lyme Bay, UK were instead attributed to bacteria of the genus *Endozoicomonas* (Cano et al., 2018).

Following extensive histology, the next step was to characterise the bacteria present in toheroa tissues that are causing these inclusions. This work is ongoing but so far, inclusions have been attributed to *Endozoicomonas* spp., a bacterium often associated with the coral symbiome (Neave et al., 2016). This echoes the findings of Cano et al. (2018), highlighting further the potential confusion created by the widespread use of the term '*Rickettsia*-like organism".

Intracellular microcolonies: *Endozoicomonas*

To confirm the agent responsible for infected cells visible via histology (Fig.1), two typical steps are taken. The first, *in-situ* hybridization (ISH) allows for visual confirmation of the bacterium in question by staining a targeted genetic material. The second, PCR and gene sequencing. Again, to complete this part of my research, services at SVS Laboratories Ltd. were used. To carry out ISH, tissue sections need to be prepared on positively charged/adhesive slides. Slides were prepared for ISH at SVS Laboratories Ltd. The hybridization process was carried out at the Animal Health Laboratory (AHL) in Wallaceville.

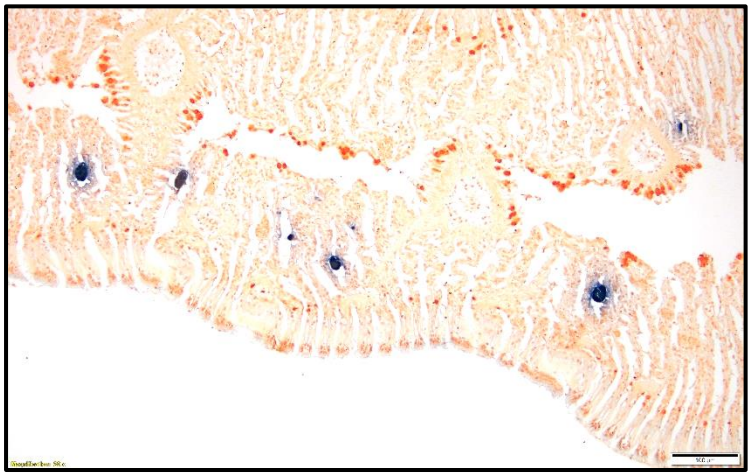

Fig. 3. *In-situ* hybridization of 16S rRNA *Endozoicomonas* gene (Mendoza et al., 2013) in toheroa gills. Dark blue staining indicates the presence of *Endozoicomonas* genetic material. Magnification x20.

The above photomicrograph of an ISH prepared slide shows the presence of *Endozoicomonas* infected cells (stained blue) in toheroa gills. Following this confirmation, two follow-up procedures were used to both i) characterise the bacterium and ii) assess infection levels. Gene sequencing of purified PCR products is underway to assess whether spatially separated populations of toheroa are infected by the same bacterium or whether multiple different *Endozoicomonas* spp. are responsible for infections seen via histology. To assess whether seasonal infection patterns exist, qPCR is being used to examine the spatial and temporal variation in gene copy numbers of *Endozoicomonas* spp. in toheroa and thus infer whether an infection gradient exists.

Since 2012 MPI have investigated more than 20 shellfish mass mortality events around New Zealand. Up to 16 of these cases have been associated with intracellular microcolonies and the majority of these mortality events have occurred in the summer months. Establishing whether a seasonal infection pattern exists could help to explain the seasonality of these mortality events and their association with these intracellular bacteria. Results from qPCR carried out thus far indicate a seasonal infection gradient does exist (Fig. 4). For example, at Ripiro Beach, the median number of *Endozoicomonas* spp. gene copies in March 2019 is almost two logs higher than that of November 2019.

Sequencing of PCR products was carried out at the Waikato DNA Sequencing Facility. Purified PCR products were sent from Tauranga to Hamilton for sequencing. Some of the funding procured from NZMSS was also used to pay for these services (Table 1).

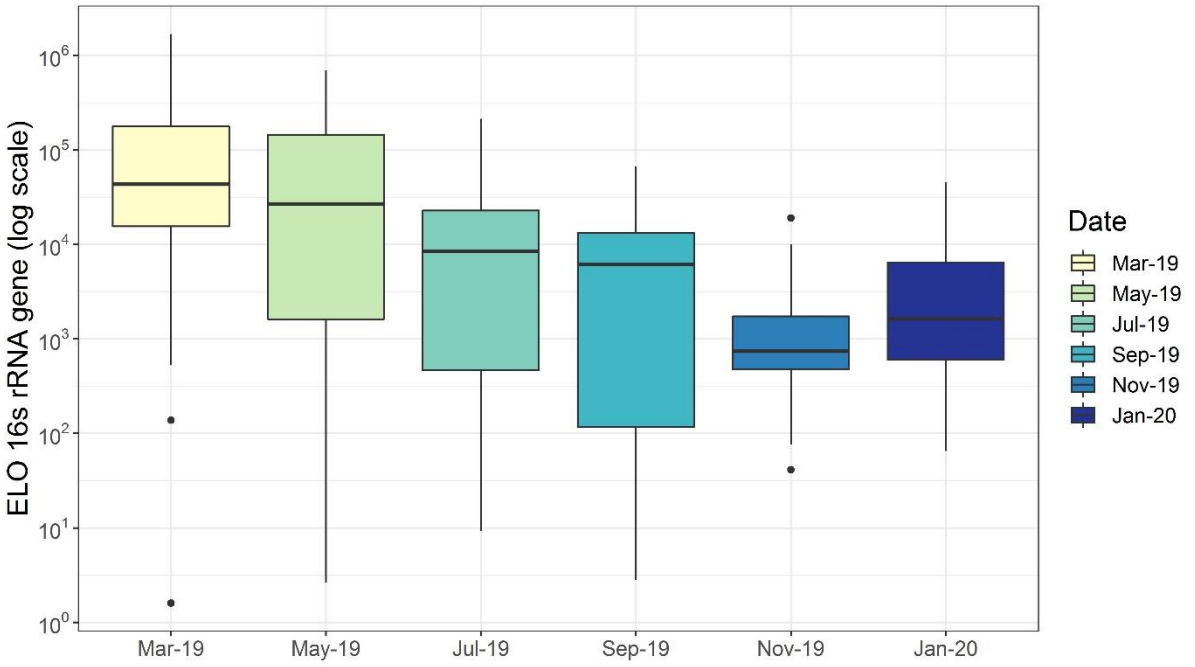

Fig. 4. Boxplot showing the seasonal variability of *Endozoicomonas* in toheroa from Ripiro Beach. Boxplot indicates the number of copies of the *Endozoicomonas* gene (log10) between March 2019 and January 2020. Line = median, box = interquartile range, thin line = min/max.

Closing remarks

This report has included some of the progress I have made since early 2019 which can be attributed to the support I received from the NZMSS student research grant. Using funds to obtain histological sections has allowed me to assess the spatial and temporal patterns of the parasites and pathogens in toheroa. Furthermore, the time that has been saved on the processing of tissue sections by general H&E has given me the chance to pursue other facets of this project at an earlier stage. Histopathology of toheroa tissue has given me insight into their reproductive cycle, following methods described by Gadomski and Lamare (2015). At the same time, I have been able to carry out a baseline health assessment. Sampling 'healthy' specimens in this case has been important because it will provide a benchmark against which to compare 'sick' individuals in the future.

Toheroa remain threatened, despite ~30 years of protection. This research is striving to provide insight into the health status of toheroa to aid their conservation and restoration. Thus far, my research suggests that disease on its own is unlikely to be the main driver of the decline of the toheroa. However, it is possible that disease may be one of several stressors combining to effect on the population dynamics of toheroa. To investigate further the role *Endozoicomonas* spp. might be having on host health, DNA metabarcoding is going to be carried out to assess what affect (if any) *Endozoicomonas* spp. have on the microbial community structure within toheroa.

Where the student grant has been spent

As indicated on the proposal submitted for this award, the bulk of this funding has been used to pay for the preparation of histological slides. Histology samples were fixed and trimmed into cassettes at Sulphur Point, Tauranga after which tissue samples in cassettes were sent to Hamilton for embedding; sectioning and staining at SVS Laboratories Ltd. (see Table 1). This process could not have been effectively achieved at our laboratory and undoubtedly not to the high standard achieved by SVS. Using this service has propelled the project forward allowing me to spend my time on other aspects within the larger research project. Furthermore, the high quality of the sections has contributed greatly to other aspects of this research such as *in-situ* hybridization carried out at the Animal Health Laboratory in Wallaceville (Fig. 3).

Table 1. A brief overview of where the NZMSS Student Research Grant was spent.

Where	Amount
Jaycar, Warehouse and Mitre 10	$146.89
Bunnings Tauranga	$120.82
Payless plastics	$21.78
Steve's Marine Supplies	$72.20
Waikato DNA Sequencing Facility & CourierPost	$137.10
SVS Laboratories Ltd. Hamilton	$2,494.36
Total	**$2,993.15**

As well as histology slide preparation, some of the funds were used to construct a bespoke 'light box' (Fig. 5) for photographing toheroa specimens on the beach. The purpose of this was to capture a snapshot of gross pathology *in-situ* (e.g. Fig. 1). Principally, we were striving to capture high-resolution images of the shells, specifically those featuring gas bubble lesions to determine whether gas bubbles were a seasonal/site-specific phenomenon. To construct this box, LEDs were purchased from Jaycar and Mitre10 and various consumables were purchased at Bunnings Tauranga.

Once again, I extend my gratitude to NZMSS for awarding me the Student Research Grant in 2019. The funds have allowed me to scale up my project as a whole by saving me funds elsewhere and awarding me *time*. Furthermore, the award has placed emphasis on my research and given me a chance to highlight the status of toheroa and the knowledge gaps that I am hoping my PhD research will be able to fill.

Signed,

Matthew Bennion, PhD Student

Date: 03/03/2020

Phil Ross, PhD Supervisor

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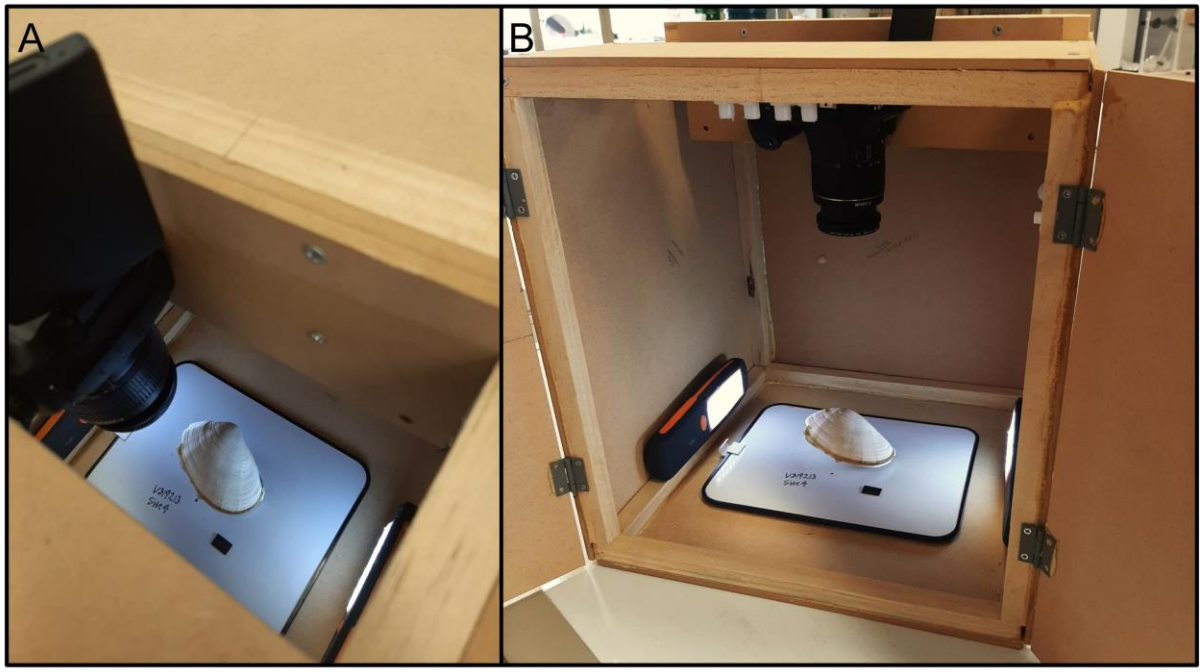

Fig. 5. A) & B) show the completed bespoke ‘gas bubble disease light box’ for photographing gross pathology of specimens *in-situ*. LEDs light the box from the top and sides, controlling the light balance and allowing for cleaner images for assessment of gas bubble intensity and extent on the shells of toheroa.