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New qPCR (quantitative polymerase chain reaction) and haematological sampling methods for monitoring *Bonamia exitiosa* infections and oyster (*Ostrea chilensis*) mortality in the Foveaux Strait fishery (OYU 5).

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EXECUTIVE SUMMARY

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A new qPCR method has been successfully developed to detect and quantify *Bonamia exitiosa* in *Ostrea chilensis* from Foveaux Strait. This method relies on two key innovations: a duplex qPCR assay and shortened bench top procedures. The characteristics of the qPCR assay include the co-amplification of the Bonamia target (in the ITS region of the ribosomal genes) and the use of *Ostrea chilensis* β-actin gene as an internal control. In addition, the assay uses a new master mix containing a robust taq polymerase mix that is able to cope with inhibitors often found in crude extracts and extracts from environmental samples. A novel system is also employed to delay the amplification of the internal control to prevent a low level Bonamia ITS amplification being outcompeted by the stronger internal control (β-actin) reaction.

This method has also successfully incorporated a shortened bench top procedure to minimise handling and was transferred to a 96 well plate format to allow the simultaneous screening of up to four Bonamia stations per hour compared to 3−4 stations per day using heart imprint methods.

An initial development trial was undertaken with samples from the 2012 Bonamia survey. A trial of repeatability of Cq values showed that amplification of the respective gene was mostly within 1 Cq value. There were relatively few samples that had no qPCR reaction: 4.2% of heart tissue samples and 5.3% of gill tissue samples had no amplification of the Bonamia target and β-actin genes. The percentages of samples of heart and gill tissue with no detectable infection from heart imprints (score = 0) that amplified the control gene β-actin were both high and similar, 90.5% for heart tissues and 93.6% for gill tissues. Gill tissues had significantly higher sensitivity for detecting Bonamia than heart tissues. To further refine this method, a variation to OYS2011/01 was proposed that included investigating:

- the effects of different sizes of tissue samples, and different types of oyster tissues (e.g. heart and gill tissue) on detection and quantification of Bonamia ITS using qPCR,
- the effect of taking imprints with heart tissues before qPCR sampling,
- validation of the primers,
- appropriate Cq cut-off values and protocols,
- differences in Bonamia ITS and β−actin amplification between gill and heart tissues.

The initial qPCR method was refined and a standard method and sampling design established. These were used to test oysters sampled during the 2013 Bonamia survey. All samples were run on 96 well plates and with negative controls and positive controls for Bonamia ITS and the β-actin gene. Levels of repeatability for Cq values for the Bonamia ITS (+ve) and negative controls were very high, Cq values for the β-actin gene in controls were more variable. The new method greatly increased the reliability of Bonamia ITS and the β-actin gene amplification. Criteria were established to determine when to repeat whole plates and individual samples, and when to reject data for analysis. Differences between heart and gill tissues are potentially attributed to structural differences between the tissues, and/or the contamination of gill tissue by water borne *Bonamia exitiosa* infective particles. Replicate qPCR samples of gill tissues were provided to Ministry for Primary Industries Biosecurity to test for concurrent infections.

A digestion time of 45 minutes was found to be the most effective and efficient for heart tissues, with slightly longer times for optimal gill digestion. There was a correlation between the size of heart

tissue digested and the DNA released (using PicoGreen), but no correlation for gills. There may be a slight benefit in shaking the extractions. An experiment designed to test the effects of taking heart imprints before qPCR analysis could not be completed as the oyster sample didn't contain sufficient oysters with Bonamia infection. The standard primers and probe used for sampling in 2012 and 2013 were found to be species specific to *B. exitiosa*, and did not amplify *B. ostreae* or other closely related genera. However, the *B. ostreae* specific probe did not amplify *B. ostreae* DNA. Establishing appropriate Cq cut-off values was problematic; the Chatham Islands oyster population may not be Bonamia free as some samples amplified very early and late in the cycling (indicating the possibility of some Bonamia DNA in the samples).

Gill tissues generally amplified Bonamia ITS earlier in the cycle than heart tissues, but the Cq values were not significantly different as a group i.e, between all gill samples combined and all heart samples combined. Heart imprints had a performance coefficient of 0.73 when compared to qPCR heart samples and 0.61 compared to gill samples. Flat-liners (no qPCR reaction for both the Bonamia ITS and the β-actin gene) were lower in 2013, 3.2 % for heart tissue samples and 2.1% for gill samples, compared to 4.2 % for heart tissue samples and 5.3% of gill tissue samples in 2012.

The quantification of Bonamia cannot be directly compared between qPCR and heart imprints as the qPCR Cq values estimate numbers of Bonamia ITS region gene copies and heart imprints scores categorise the average numbers of Bonamia cells in oyster haemocytes. Of the heart imprint samples that were positive for Bonamia infection, all but one of the Cq values from matched qPCR heart and gill samples were below the 35 Cq cut-off. Cq values for both heart and gill tissues showed a decreasing trend with increasing intensity of Bonamia infection estimated from heart imprints.

There has been an incremental improvement in the qPCR method, and while there are some outstanding issues to be addressed, the qPCR method will greatly enhance our ability to monitor Bonamia infection in Foveaux Strait oysters.

1 INTRODUCTION

Bonamiosis is a disease of dredge oysters caused by protistan parasites of the genus Bonamia. Several species of Bonamia have been recognised and the taxonomic relationships among the regional species are under review (Carnegie Virginia Institute of Marine Sciences, pers. comm.). In the Foveaux Strait oyster (*Ostrea chilensis*) fishery (OYU 5) the parasite *Bonamia exitiosa* (hereafter referred to as "Bonamia") has severely reduced oyster densities during recent epizootics, 1986−1992 (Cranfield 2005; Doonan 1994), and 1999−2012 (Michael 2013). *Bonamia exitiosa* is thought to be an endemic disease of Foveaux Strait oysters and it is likely that periodic events of disease mortality (epizootics) have been a recurrent feature of the oyster population. Declines in localised oyster populations have been reported since 1906, but *B. exitiosa* was only identified in 1964. Bonamia epizootics in the Foveaux Strait oyster population have been followed by long periods when Bonamia could not be detected (and when oyster populations have rebuilt). High oyster densities have persisted for long periods in the apparent absence of disease. The two Bonamia epizootics have shown that Bonamia mortality is a recurrent feature of the oyster population.

The size of the oyster population in Foveaux Strait is primarily driven by disease mortality and to a lesser extent recruitment (Fu & Dunn 2009). At the low exploitation rates in the fishery 1996−2012, fishing is considered unlikely to have a significant effect on future oyster population size, and mortality from Bonamia is the primary determinant of future stock status. Annual, fishery-scale Bonamia surveys provide information for management and to guide fishing strategies. The status of Bonamia infection in oysters is determined from the distribution of prevalence and intensity of infection, and the numbers of non-fatal (category 1 and 2 infections, see Diggles et al 2003) and fatally infected (category 3 and higher) oysters scaled to the size of the oyster population. The population sizes of new clocks (see Michael et al. 2013 for a definition) provide estimates of pre-survey mortality and the numbers of fatally infected oysters in the population represent the expected short-term mortality post survey. These two estimates measure mortality differently, however, when combined they provide an indicative estimate of summer mortality from Bonamia. The levels of disease mortality, expressed as percentages of the recruit-sized oyster populations, provide a reference point for stock assessment projections (see figures 41 and 42 in Fu & Dunn 2009). If summer mortality remains around or below 10%, and recruitment near the long-term average, the oyster population will continue to rebuild. It is therefore critical that there is continued annual monitoring of disease status in Foveaux Strait oysters. The development of a qPCR method for the detection and quantification of Bonamia in oyster tissue will allow a standardised sampling design and methods for annual fishery-scale monitoring of this disease to be established.

Bonamia research has been rated the highest priority by stakeholders and the Ministry for Primary Industries (MPI) (Michael 2010a, Michael 2010b, Ministry of Fisheries 2009), and is the main focus of a five year (2011–2015) strategic research plan. A key objective of this plan is to minimise losses of oysters from Bonamia infection. Research that may provide some insight into the temporal course of infection and allow future Bonamia mortality to be predicted is of primary importance to the oyster industry. The ability to predict mortality within a time frame that allows oyster density in infected areas to be fished down by targeted fishing is critical to minimising losses to the fishery from Bonamia mortality. To develop this capability requires a better ability to determine low level infections in the fishery. To advance our knowledge of Bonamia and its interaction with the fishery, a cost-effective, real-time, sensitive qPCR method to detect low level Bonamia infections is essential.

Until recently, heart imprint methods were the most cost effective method for fishery-scale monitoring of Bonamia. Surveys were used to determine *B exitiosa* infection in the oyster fishery and estimate prevalence and intensity of infection (Michael et al. 2013). Heart imprints are made by removing and lightly dabbing the heart on a slide to deposit a small amount of oyster blood (haemolymph). Slides are air dried and stained to highlight Bonamia cells, and examined under a microscope. This method lacks sensitivity, the ability to reliably detect low level infections, and

specificity, the ability to distinguish between closely related pathogens or pathogens with similar cellular structures and organelles. Correlation studies with *in-situ* hybridisation have shown that the prevalence of Bonamia estimated from heart imprints can underestimate the true infection rate by about 30% (Diggles et al. 2003). Heart imprint methods are known to consistently report the highest percentages of false negatives when compared to histology (stained sectioned tissue) and PCR methods (Balseiro et al. 2006, Bearham 2008, Carrasco et al. 2012), with up to 65% of samples returning false negatives in some trials (Balseiro et al. 2006). PCR is the most sensitive method for the detection of haplosporidian diseases (Balseiro et al. 2006, Bearham 2008, Carnegie et al. 2000, Carrasco et al. 2012, Cochennec et al. 2000, Corbeil et al. 2000, Corbeil et al. 2006, Diggles et al. 2003, Abollo et al. 2008). Although heart imprint methods require specialist skills and samples are more time consuming to process, until recently this has been the most cost effective, fishery-scale sampling method for the oyster fishery. While heart imprint methods lack specificity, combination methods of histology and PCR provide the highest specificity (Balseiro et al. 2006) for routine screening and histology, real-time PCR, and sequencing for positive identification (Bearham 2008, Carrasco et al. 2012). The World Organization for Animal Health Office International des Epizooties (OIE) manual of diagnostic tests for aquatic animals 2012 lists both heart imprints and PCR as accepted methods for screening for *B exitiosa* infection, with a caveat that both methods have their limitations.

Recent developments in molecular methods have provided the opportunity to develop a quantitative PCR (qPCR) method that is cost-effective, provides results in real-time, and has high sensitivity to detect low level *Bonamia exitiosa* infections, and other Bonamia species and haplosporid infections. A high sensitivity and specificity tool will allow us to better determine infection rates, especially in individual oysters with low and currently undetectable infections. This capability is critical if we are to investigate the temporal course of infection and drivers of epizootics.

Molecular methods have been used to detect Bonamia infections in dredge oysters and have been shown to be more sensitive and less ambiguous than standard histological and heart imprint techniques (Balseiro et al. 2006, Carnegie et al. 2000, Carnegie et al. 2003, Marty et al. 2006). Preliminary work on DNA detection of *B. exitiosa* in *O. chilensis* in New Zealand amplified a small region of the 18S rRNA gene and identification was based on the size of a relatively short (300 base pair) gene fragment (Diggles et al. 2003). Subsequently, a larger region (700 bp) of the 18S rRNA gene was amplified and sequenced following methods developed by Carnegie et al. (2000). Confirmation of the identity of the amplified product, by either sequencing or other molecular methods is essential to distinguish the amplified product from other haplosporidians (Burreson et al. 2004, Carnegie et al. 2000). However, sequencing a large number (more than 100) of individual oysters is laborious, time-consuming and expensive, and would not be practical for the Bonamia screening application envisaged. A qPCR method for fishery-scale monitoring should provide high specificity (amplify *B. exitiosa*, while other closely related species give negative results) and high sensitivity (minimise the numbers of false negatives where infection is not detected), and allow for the screening of much higher numbers of oysters, typically up to 2000 oyster tissue samples. The key objective of this study is to develop a quantitative PCR (qPCR) method in collaboration with an experienced qPCR assay developer as a sub-contractor who has a track record of developing diagnostic tools for industry (John Mackay, dnature Ltd).

The qPCR method for Bonamia should be more cost effective per sample than the heart imprint methods. The performance of both qPCR and heart imprint methods are compared using samples from the 2013 Bonamia survey of Foveaux Strait oysters. The intensity of infection (mean level of infection in infected oysters only, based on a categorical scale of 1 – 5, see Diggles et al. 2003) from heart imprints has traditionally been used to estimate the proportion of the oyster population with non-fatal infections (categories 1 and 2), and those with fatal (category 3 and greater) infections. Should qPCR methods prove to be more cost effective, cycles of quantification (Cq) from qPCR results will need to be correlated to categorise 1 to 5 infections from heart imprints. This is important as it will enable the historical time series of Bonamia data from heart imprints to be compared with data from new qPCR methods. It will allow the qPCR method to estimate fatal infections as well as presence/absence of

Bonamia. This study aims to evaluate the sensitivity and precision of Bonamia detection, speed of processing, and relative cost per sample to assess the overall performance of qPCR and heart imprint methods. In the short-term, all heart imprints will be scored for intensity of infection from oyster samples that return positive qPCR results, as well as a selection of randomly selected qPCR negative samples, and all qPCR samples with anomalous results to maintain the current Bonamia time series.

The MPI research programme OYS2011/01 aimed to develop a new qPCR method for monitoring the prevalence and distribution of Bonamia infections in Foveaux Strait oysters (objective 1). Initial trials were very promising and there was a high likelihood that a molecular tool for fishery-scale monitoring could be developed. Both the traditional heart imprint and qPCR methods were used to determine the presence/absence of infection from samples collected during the 2012 Foveaux Strait oyster Bonamia survey and the results compared (objective 2). Based on these results a standard qPCR sampling design for the annual monitoring of Bonamia was established. The initial development highlighted the need for further testing to ensure the robustness of the methods. A peer review of the initial development of the methods by a qPCR working group convened by MPI agreed on further testing through a variation to OYS2013/01

- to ensure that different sized tissue samples and different types of tissues and taking imprints with heart tissues before qPCR sampling didn't affect qPCR detection and quantification,
- to identify any issues that may be associated with the duplex assay,
- to investigate any inconsistencies in detection between qPCR and heart imprints, and
- to investigate improvements in the bench top method such as higher throughput qPCR regimes.

This report documents the development a qPCR method and high throughput system for fishery-scale monitoring of Bonamia in Foveaux Strait oysters, and includes further testing undertaken as a variation to the Ministry for Primary Industries project OYS2011/01. A standard method comprising qPCR and heart imprint methods for fishery-scale sampling and research to investigate the epidemiology of Bonamia is proposed for consideration by the Shellfish Working Group.

OBJECTIVES

1. Develop a quantitative PCR (qPCR) method for monitoring intensity[1] and extent of Bonamia [infection] in Foveaux Strait Oyster fishery

Intensity of infection [1]: Published term describing the extent of infection in individual, infected oysters using a categorical scale of 1−5, based on the numbers of Bonamia particles in oyster haemocytes.

SPECIFIC OBJECTIVES:

1. Develop and establish a qPCR method to detect *Bonamia exitiosa* that has high sensitivity, high specificity; and is high throughput and is cost effective.
2. Investigate the effects of different sizes of tissue samples, and different types of oyster tissues (e.g. heart and gill tissue) on detection and quantification of Bonamia using qPCR.
3. Investigate the effect of taking imprints with heart tissues before qPCR sampling.
4. Validate primers.
5. Establish appropriate Cq cut-off values and protocols.
6. Investigate differences in Bonamia ITS and β−actin amplification between gill and heart tissues.
7. Compare the new qPCR method to the traditional heart imprint microscopic detection method.
8. Establish a standard sampling design and method for the annual monitoring of disease.
9. Investigate concurrent infections.

2 METHODS

The development of a high throughput qPCR method to detect and quantify *Bonamia exitiosa* in *Ostrea chilensis* relies on two key innovations: a duplex qPCR assay and a shortened bench top DNA extraction methodology. Modern molecular diagnostic assays require the use of an internal control assay that confirms the presence of suitable DNA templates for amplification. Any reaction without a target or internal control amplification signal may be flagged as an invalid result. The assay needs to work well with tissue samples digested with a simple DNA extraction method, as normal DNA extraction methods are often time consuming and expensive. The innovative characteristics of the qPCR assay include the co-amplification of the Bonamia target (in the ITS region of the ribosomal genes) and β-actin gene as internal control. In addition, the assay uses a new master mix (PerfeCTa™ qPCR ToughMix™) containing a robust taq polymerase mix that is able to cope with inhibitors often found in crude extracts and extracts from environmental samples. A novel system is also employed to delay the amplification of the internal control to prevent a low level Bonamia amplification being outcompeted by the stronger internal control reaction. This method has been successfully incorporated into a shortened bench top method to minimise handling and was transferred to a 96 well plate format to allow the simultaneous screening of up to four Bonamia stations per hour compared to 3−4 stations per day using histological methods.

Develop and establish a qPCR method to detect *Bonamia exitiosa* that has high sensitivity, high specificity; and is high throughput and is cost effective.

Previous work at NIWA had identified a range of primers that could be used to successfully amplify the ITS region of *Bonamia* spp. However qPCR reactions typically require new primers due to the incorporation of a probe sequence that works in tandem with the primers. A published primer and probe set were therefore evaluated alongside a new primer/probe design and modified primer sequences for the published assay. The PCR conditions for this primer set were optimised for the PerfeCTa™ qPCR ToughMix™ taq polymerase master mix. The oyster β-actin gene was used as an internal control and a primer/probe set was designed for this gene target. A proprietary IC block was also designed and was used as part of the internal control probe design to prevent low level Bonamia amplifications being outcompeted for reagents (and thus preventing amplification) by the typically higher internal control levels (β-actin). The PCR cycling conditions were tested for the internal control, so that both the Bonamia ITS region and β-actin gene could be amplified in a single PCR reaction with equivalent sensitivity as singleplex Bonamia qPCR. The ITS probe was labelled with the FAM (6-carboxyfluorescein) and the β-actin gene with Texas-red (sulforhodamine 101 acid chloride) fluorophores to enable differentiation of the Bonamia and internal control PCR products formed, due to their different emission spectra.

A cost effective, fast and high throughput (96 well plate format) DNA extraction method was developed and optimised for obtaining amplifiable DNA from the gill and heart tissues of oysters, based on a NIWA in-house method. The extracted tissue samples were analysed using the primers, probes and taq polymerase master mix described above on a BioRad CFX96 with the following PCR cycling conditions; denaturing at 95°C for 3 minutes followed by 43 cycles of denaturing at 96°C for 5 seconds and annealing at 60°C for 20 seconds, this allowed for a run of approximately 40 minutes per plate. Included in each plate was a negative reagent control and a positive control for both *B. exitiosa* and β-actin.

The qPCR primers and probes, and the specificity and sensitivity of the method, were evaluated using infected oyster tissues sampled during the 2012 and 2013 Bonamia surveys. The need for replication of tissues was also evaluated. We investigated the variance in qPCR product formation (from replicate samples of individual oyster tissues), to determine if and at what level we would need to replicate samples from individual oyster tissues to give reliable estimates of the intensity of Bonamia infection. Samples for further testing of the initial method (variation to OYS2013/01) were taken from sites where there was the greatest chance of high Bonamia prevalence in the sample i.e., from sites where

there were signs of heightened disease mortality (increased proportions of shells of recently killed oysters known as new clocks) and the moribund oysters known as gapers.

The effects of different sizes of tissue samples, and different types of oyster tissues (e.g. heart and gill tissue) on detection and quantification of Bonamia using qPCR.

The effects of different sized tissue samples, and different types of oyster tissues (e.g. heart and gill tissue) on detection and quantification of Bonamia using qPCR were investigated. This objective included comparisons of the effects of tissue size (volume) and tissue type (heart, gill, and heart and gill together), and the effects of proteinase K digestion duration (e.g. 45 minutes to 5 hours). The amount of DNA released was measured using Quant-iT™ *PicoGreen*®. The proportion of tissue to proteinase K = buffer at about 25% w/v (weight per volume) of tissue to buffer ratio has been found to have no adverse effect on DNA extraction. We used a 96 well plate shaker inside an incubator for incubating the tissue digestions.

Experiment 1:
During the 2013 Bonamia survey, 100 oysters, about 20 in each of five size groups representing the size range of oysters from 20–90 mm were sampled for this objective. The hearts and gills were removed from 42 oysters and placed separately into pre weighed, labelled tubes. The hearts and gills were removed from a further 42 oysters which were placed together in a single pre-weighed tube for each individual. All samples were stored at -80°C until processing.

Thirty-two of the samples, either gill, hearts or heart and gill combined, were processed on consecutive days using the standard Bonamia extraction method, except that they were incubated at 52°C with shaking (300 rpm) and sub-samples (2µl) were taken from each extraction after 45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 5 hours. The 2 µl sub-sample was used for DNA quantified using Quant-iT™ *PicoGreen*® which detects double strand (ds) DNA down to 25 pg/ml in the presence of ssDNA and RNA. The standard extraction method incubated samples at 56°C in a thermocycler with no shaking.

Experiment 2:
A second set of 20 oysters (see Appendix 1, Table A4) was used to assess the effect of shaking on the DNA extraction procedure. One set of 10 oysters was incubated at 52°C with shaking at 300 rpm, and a second set (10 oysters) was incubated at 56°C in a thermocycler (no shaking, standard extraction method). The shaker could only be incubated at 52°C, however both temperatures are well within the range for effective proteinase K digestion. The amount of DNA released was measured as above after 45 minutes and 2 hours. In addition, the standard qPCR procedure was performed (Appendix 2) on the DNA extracts and the Bonamia ITS and β-actin genes were quantified.

The effect of taking imprints with heart tissues before qPCR sampling.

The effect of taking imprints with heart tissues before the hearts are used for qPCR sampling was investigated. The Cq values for half hearts, half with no treatment and the other half blotted on filter paper and used to make 30 imprints on glass slides were compared. Paired samples of gill and heart tissue, with two treatments for hearts (with and without imprinting and blotting) were also compared to provide information to help interpret differences between heart and gill tissue Cq values from the 2012 samples.

Thirty oysters were selected from the 2013 survey, from sites that showed potentially high prevalence of Bonamia. The heart was removed from 20 large oysters and cut in half. One half was blotted to provide 30 heart imprints and then placed in the qPCR plate and the other half was immediately placed in the qPCR plate. Concurrently the gills were removed and placed in qPCR plates. All samples were frozen at -80°C. The standard qPCR procedure for Bonamia was followed (Appendix 2) and Cq values between hearts, blotted hearts and gills were compared.

Primer validations.

Primer validations were undertaken. Primers were checked *in silico* against sequences (for all Bonamia species and closely related protozoan genera). These primers were trialled with DNA from closely related organisms to ensure that no other genera and/or Bonamia species would amplify, and that the primers were species and genera specific. Validations were undertaken using reference DNA at the Animal Health Laboratory at the Ministry for Primary Industries (MPI) using the same laboratory system. This included re-running some of the 2012 samples with the *Bonamia exitiosa* probe to investigate differences in probe performance and Cq values.

In silico analysis of the primers and probes was undertaken at the design stage for the initial generic Bonamia qPCR assay. *B. exitiosa* and *B. ostreae* specific probes, and two alternative primer sets had been designed, but had not been tested in the assay. The specific primers and alternative probes, and the generic set of primers and probes were tested against pure DNA of *Perkinsus olseni* ("Dermo"), *P. marinus, B. ostreae*, *B. exitiosa*, *Haplosporidium nelsoni* (MSX), and the Bonamia 2012 positive control at the Animal Health Laboratory of MPI. The Bonamia 2012 positive (Bon+ve) was made from pooled positive 2012 survey samples which were column purified using the DNA Easy Tissue kit (Qiagen). All these were tested using the standard qPCR protocol, the primers of the standard protocol and the two alternative probes using the PerfeCTa™ qPCR ToughMix (Dnature). The different primer sets were tested against the Bon+ve, and the *B. ostreae* and *B. exitiosa* DNA using SsoFast™ EvaGreen® qPCR mix (BioRad).

Appropriate Cq cut-off values and protocols.

We attempted to validate appropriate Cq cut-off values and protocols using samples of oysters (*O. chilensis*) from the Chatham Islands as negative controls, and an extract of Bonamia from infected oyster from Foveaux Strait as positive controls. During the 2013 Bonamia survey, 100 oysters of mixed sizes were sampled from the Chatham Islands for this objective. The hearts and gills were removed and analysed by the standard Bonamia qPCR protocol.

Cq cut-off levels differentiating between positive and negative infections were validated using a standard curve where the Bonamia target (ITS region of the ribosomal genes) was diluted to extinction. This work was undertaken in collaboration with Suzanne Keeling (MPI) and John Mackay (dnature).

Differences in Bonamia ITS and β−actin amplification between gill and heart tissues.

Differences in Bonamia ITS and *Ostrea chilensis* β−actin amplification between gill and heart tissues were investigated to determine why β−actin does not consistently amplify in samples with very little or no Bonamia. Replicate single assays for Bonamia ITS and β−actin were run separately for individual oyster tissues to investigate potential interactions. Further, samples that did not amplify β−actin with low Bonamia Cq values were run as single assays to further investigate the role of the IC block.

Investigate concurrent infections.

Concurrent infections of other potential pathogens were investigated with the assistance of NCBID-Wallaceville (Brendan Gould and Brian Jones). Replicate samples of gill tissues were collected from the 2013 Bonamia survey for Brian Jones of MPI biosecurity to screen for other pathogens.

Compare the new qPCR method to the traditional heart imprint method.

A comparison of presence/absence scores from qPCR heart and gill data separately and heart imprints was undertaken by sampling individual oysters from the 2013 Bonamia survey. Duplicate samples of tissues of heart and gill were taken for qPCR analysis from oysters sampled for heart imprints. The same hearts were used for both heart imprints and qPCR. The qPCR samples were taken from the first 24 oysters from the samples of 25 oysters processed for heart imprints, and from all 70 sites sampled in February 2012 and 57 sites sampled in February 2013.

Heart imprint samples were processed using standard methods as described in Michael et al. (2013). Station data and morphometric data were taken for each oyster. Each oyster was assigned a size category using oyster size rings, and also measured for length and height with callipers. Data were recorded on a standard Bonamia survey form (see Appendix 3). Heart imprints were made by removing the heart with fine forceps, draining excess water and fluid on filter paper, and lightly dabbing the heart on a slide to deposit a small amount of hemolymph. Three rows of eight to ten imprints were made on labelled slides. Slides were placed in slide racks to air dry for at least five minutes. The slides were stained with Hemacolor© and oven dried at 60°C.

Oyster heart imprints were examined under a microscope using a times 50 objective lens under oil and scored for intensity of infection using the criteria shown in Table 1 from Diggles et al. (2003). Three good heart imprints containing oyster haemocytes were located and examined on each slide, and the number of Bonamia cells counted for each. If no Bonamia cells were found, further imprints were examined to confirm the absence of Bonamia.

Table 1: Criteria used to stage intensity of Bonamia infection in oysters from Diggles et al. (2003).

0	No Bonamia observed
1	One Bonamia cell observed after examining an imprint
2	More than 1, but fewer than 10, Bonamia cells observed after examining an imprint
3	More than 10 Bonamia present in the imprint, but few in each haemocyte
4	Bonamia present in many haemocytes of each imprint and many in each haemocyte
5	Bonamia present in nearly all haemocytes of each imprint and many in each haemocyte, and extracellularly

Category 0 oysters have no detectable infection. Previous studies (Diggles et al. 2003) suggested that stage 1 and 2 level Bonamia infections are relatively light and do not appear to adversely affect the host. Stage 3 infections are much more elevated and systemic, and are associated with minor tissue damage throughout the host. It is likely that stage 3 infections will almost always progress to stage 4 (Diggles et al. 2003). Stage 4 infections are systemic, and all tissues are congested with infected haemocytes; death appears inevitable. Stage 5 infections differ from those of stage 4 in that tissue damage is extreme throughout the animal, tissues have lost their integrity, and the oyster is near death.

The qPCR data was analysed using BioRad CFX Manager™ software (Version 3.0). The quantification cycle (Cq), the fractional cycle number where fluorescence increases above the threshold (also referred to as Ct (threshold cycle) or Cp (quantification cycle)) was determined by the regression method as implemented in the option using the BioRad CFX Manager™ software (Version 3.0).

The qPCR data from oyster heart and gill samples were assessed based on the information for each plate contained within the sample sheets, plots of RFU against Cq values, and Cq values for the positive (Bonamia ITS and β-actin gene) and the negative internal control reactions. Rules are proposed for repeating qPCR reactions for each sample or plate, and the rejection of data from analysis, and are given in the results section.

All matching heart imprint slides for those samples that tested positive for Bonamia infection in either heart or gill samples were examined. At least three samples that were qPCR negative were randomly

selected from the remaining samples from each site, all samples for the 25th slide from each site (for which there is no qPCR data), and in 2013, all anomalous results such as flatliners (no reaction for both Bonamia ITS region and β-actin) and early ampers (samples that amplified in the first 10 cycles) were also examined.

The sensitivity of Cq values in replicate qPCR runs, specifically the handling process (pipetting reagents), was investigated by repeating station 10 on plate 40 twice in the same run. The 96 well plate contained duplicate wells for each of the 24 heart and gill samples. These samples were run as a single qPCR run and were analysed as described previously. Data from this trial were tabulated and summarised to investigate difference in Cq values for paired cells.

Differences in Cq values between heart and gill tissues for the ITS region and β-actin gene, and comparisons by histological score were compared graphically and with tests for statistical significance. Individual heart tissue samples that showed a difference between qPCR and heart imprint scores were read so as to score intensity of infection. In all, 197 oyster samples were reread including 119 that were scored as having no detectable infection (0); and some of the zero category samples were repeats while others were random samples to check reader consistency. Box plots were used to display heart and gill tissue Cqs against histological score.

Paired Cq values for heart and gill tissues from each oyster, grouped by histological score, were tested for significant difference in SigmaPlot 12. We used T-Tests for normally distributed data passing the Shapiro-Wilk normality test. Where data failed the test for normality, pairwise comparisons were undertaken using the Mann-Whitney Rank Sum Test, and for multiple pairwise comparisons using Dunn's method and the Holm-Sidak method for the Kruskal-Wallis test. Lower mean/median Cq values indicated greater sensitivity.

We also estimated a coefficient of similarity (S, after Balseiro et al. 2006), which compares qPCR and heart imprint performance using both positive and negative detection efficiencies:

$$S = (a+d)/p$$

where a is the total number of positives from heart imprints, d is the total number of negatives from heart imprints and p is the total number of samples combined that were examined based on qPCR positive and negative results. The qPCR is assumed to have a coefficient of 1.00 for this comparison. Data on the numbers of sites testing positive for both heart and gill samples, heart samples alone, and gill samples alone are given to highlight differences between qPCR tissues.

Establish a standard sampling design and method for the annual monitoring of disease.

Information from objectives 1 and 2 were used to develop a qPCR sampling method for the 2013 Bonamia survey. The refined standard qPCR method is given in Appendix 2. Sampling designs and protocols for Bonamia surveys are documented here. A survey design, and optimal numbers of sites and sample sizes will be developed when revised objectives for future Bonamia surveys are decided.

3 RESULTS

Initial qPCR assay development

Published primers and probes for Bonamia (Corbeil et al. 2006), novel *Bonamia* spp. designs as well as modified primers for the published assay were tested against positive Bonamia samples, using the NIWA DNA extraction method. Modified primers were tested in *in-silico* analysis and suggested that they would provide more sensitive and/or more specific detection. Each reaction mix (containing different primers and/or probes) was compared using serial dilution of the Bonamia DNA in a negative shellfish DNA matrix (mimicking increasingly low Bonamia infections). The primer/probe mix that was chosen on the basis of this optimisation was largely the assay as published by Corbeil et al. (2006) although a modified forward primer was employed for better amplification. While the newly designed assay provided comparable performance, the published assay had been tested on an international range of isolates. Several novel primer/probe combinations were designed and tested for the oyster internal control reaction and then combined with the optimal Bonamia assay. The duplex assay was then tested with a range of block concentrations in order to reduce the internal control reaction to a level that, while still providing robust amplification, allowed equivalent low level detection of Bonamia in the duplex reaction as in the singleplex Bonamia assay. The optimised reaction mix was tested with standard qPCR mastermixes (Quanta PerfeCTa FastMix II) and the newer mastermix designed for environmental inhibitors (Quanta PerfeCTa ToughMix). At higher levels of Bonamia infection, the Cq levels were equivalent (indicating similar performance) however low level detection and reproducibility of Bonamia were better with the ToughMix mastermix.

Development of protocol for 96 well plate

The 96 well plates containing the heart and gill tissue samples were frozen to -80˚C, without any preservative. Sample plates were thawed at room temperature and the tissue was digested *in-situ* in the plates. Then the 'crude' extracts were diluted and dispensed into 96-well qPCR plates for qPCR, this whole process takes approximately 1 hour. Therefore while the qPCR protocol is running another plate can be extracted and is ready as soon as the qPCR protocol finishes, this allows the analysis of approximately 6 plates containing a total of 288 oysters to be analysed for *Bonamia exitiosa* with both the heart and gill tissue per day. For single tissue samples (either heart or gill), the qPCR throughput can equate to 20−24 Bonamia sample stations per day compared with 3−4 station for heart imprints when the intensity of infection is mainly low. Screening only qPCR positive and a random selection of qPCR negative samples reduces the numbers of heart imprints that initially need to be screened to maintain the Bonamia survey time series.

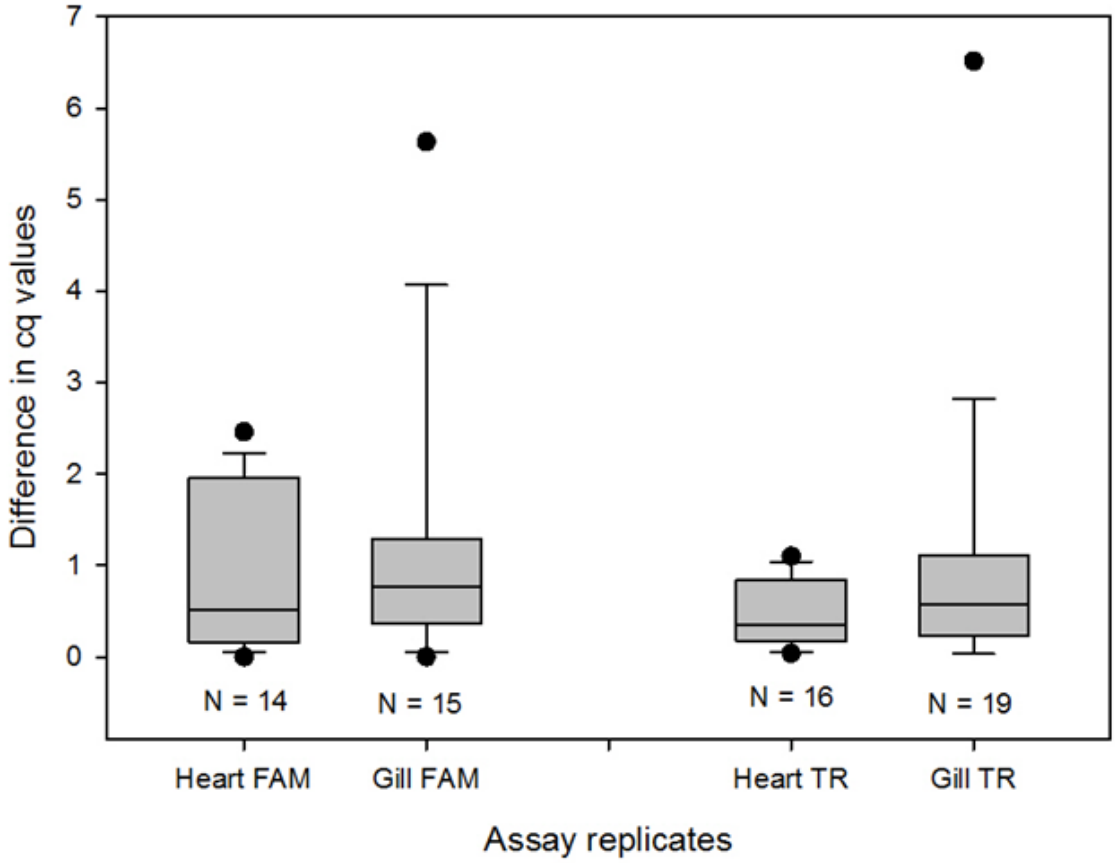
Effectiveness of method

Repeatability

The sensitivity of Cq values to replicate sampling was initially investigated in 2012 by repeating station 10 on plate number 40 twice in the same qPCR run. Most of the 24 samples in each of the 4 assays amplified (Appendix 4), but there was some apparent blocking of the β-actin gene amplification when ITS regions Cqs values were generally lower than 30 (Table 2). Seven of the 48 samples failed to amplify in either of the replicates. The replicate analysis showed that amplification of the respective gene was mostly within 1 Cq value (Figure 1), and therefore no replication was needed from individual oyster tissues.

Table 2: Differences in Cq values for two replicate samples of heart and gill tissues for the Bonamia ITS region and oyster β-actin gene from station 10.

Reactions	Heart ITS region	Heart β-actin gene	Gill ITS region	Gill β-actin gene
N	15	14	16	19
Min difference in Cq	0.00	0.04	0.00	0.03
Max difference in Cq	2.46	1.10	5.63	2.82
Median difference in Cq	0.52	0.36	0.76	0.57
Mean difference in Cq	0.84	0.46	1.16	1.04



The repeatability of Cq values was good for the purified DNA used in the positive controls and for water used as the negative control (Figures 2 and 3). Positive controls showed very similar Cq values for Bonamia and greater variability for the β-actin gene (Figures 2 and 3). One control sample did not amplify the β-actin gene (shown as zero in Figure 2), and there was some difference between two sets of reagents (Figure 3). Negative controls performed as expected (Figure 2) with the exception of one sample indicating some contamination (Figure 2). High levels of repeatability are expected from the controls because they lack inhibitors in the purified DNA.

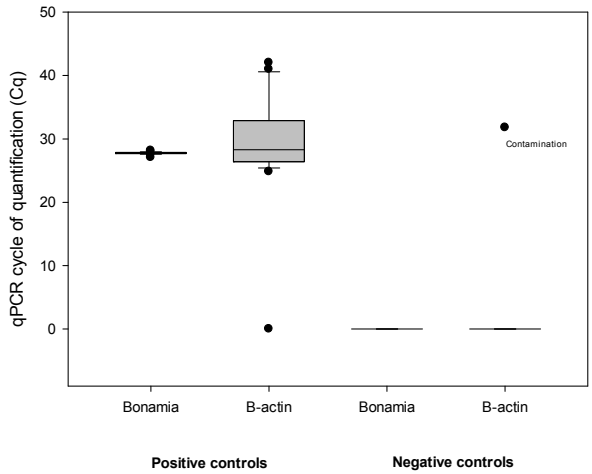


Figure 2: Boxplots of qPCR Cq values from positive and negative controls for Bonamia ITS and β-actin.

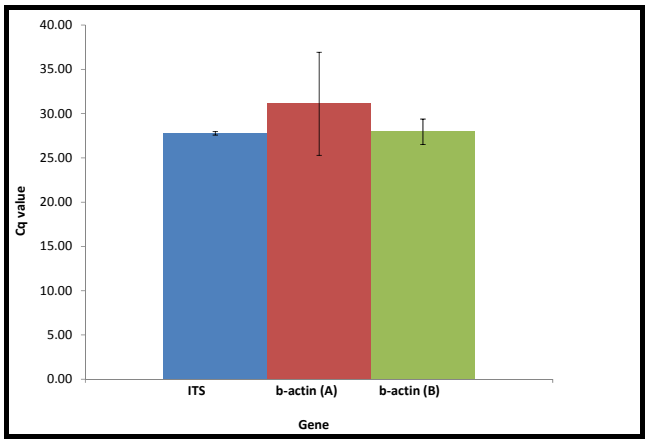


Figure 3: Boxplots of qPCR Cq values from positive controls for Bonamia ITS and β-actin using two different sets of reagents (A) and (B).

Amplification of gene sequences

Oyster gill tissue samples were mostly larger in size and volume than oyster heart tissues. A characteristic of the qPCR method is that sample volume is standardised by using a standard aliquot of extraction buffer resulting in a consistent amount of tissue digested. Therefore, differences in sample size should not unduly affect Cq values (Figure 4). Gill tissues were sampled directly from oysters and heart tissues were used for heart imprints before being placed in the qPCR plates. It is not known whether or to what extent the loss of blood from heart tissues during the heart imprint process may affect the quantification of Bonamia ITS region or the oyster β-actin gene using qPCR. Considering that the volume of tissue digested is standardised, gill tissues may contain more copies of the β-actin gene (muscle) than heart tissues, and may therefore produce lower Cq values (Figure 4). Blocking of the internal control may occur more regularly in gill tissue than in heart tissue due to the higher number of β-actin genes.

qPCR reactions for both heart and gill tissue ranged in responses from no amplification of either gene; ITS region amplification only; β-actin gene amplification only and no Bonamia ITS region, and amplification of both genes (Figure 5). There was no clear pattern of difference between heart and gill Bonamia ITS region Cq values.

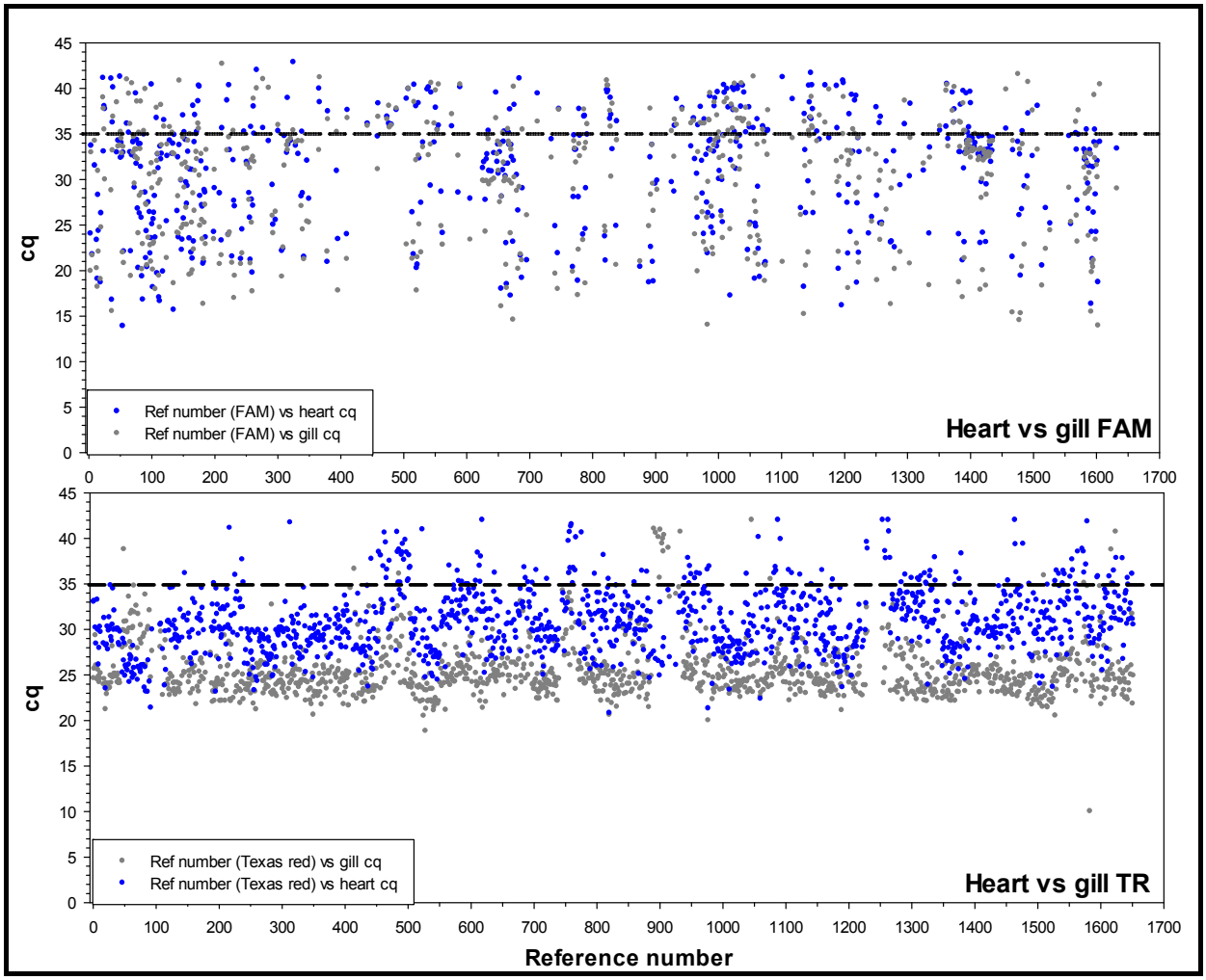


Figure 4: Cq values for paired heart and gill tissue samples that amplified using Bonamia ITS region (FAM) (top) and oyster internal control β-actin gene (Texas Red (bottom) by oyster reference number.

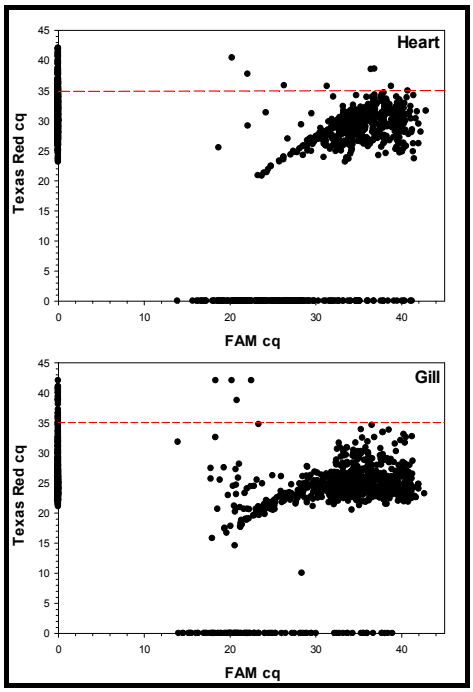


Figure 5: Scatter plots of paired Cq values for ITS region (FAM) (top) and β-actin gene (Texas Red) (bottom), for heart (top) and gill (bottom) tissue samples.

There were relatively few samples that had no qPCR reaction, i.e., no fluorescence detected above the baseline (using the regression method) for both the Bonamia ITS region (FAM) and *Ostrea chilensis* β-actin (TR): 4.2% of heart tissue samples and 5.3% of gill tissue samples. The percentages of tissues that amplified the ITS region from those samples identified as positively infected by histology (scores 1−5) were 40.1% for heart tissues and 58.4% for gill tissues (Table 3 and Figure 6), however the amplification of non-infected samples (histological score 0) was expectedly low. Overall, 41.4% of all heart samples and 51.4 % of all gill samples had amplification of the ITS region (Figure 6, Table 3). The percentages of samples of heart and gill tissue with no detectable infection using histology (score = 0) that amplified the control gene β-actin were both high and similar, 90.5% for heart tissues and 93.6% for gill tissues (Figure 6, and Table 3). There was evidence of decreasing amplification of β-actin with increasing infection (histological categories 1−5) and where ITS region Cq values were generally low. Heart tissues appeared to be more affected by this than gill tissues (Figure 6, and Table 3).

Table 3: The numbers of samples from oyster heart and gill tissues that produced cycle of quantification (Cq) values from Bonamia ITS region (FAM) and *Ostrea chilensis* β-actin (TR), by histological score of infection.

Histology	Heart					Gill				
Bonamia infection	Sample (N)	FAM Cq	% amp	TR Cq	% amp	Sample (N)	FAM Cq	% amp	TR Cq	% amp
0	1460	505	34.6	1321	90.5	1432	705	49.2	1340	93.6
1	17	15	88.2	7	41.2	17	16	94.1	16	94.1
2	39	38	97.4	9	23.1	38	37	97.4	28	73.7
3	46	44	95.7	8	17.4	46	44	95.7	30	65.2
4	60	55	91.7	9	15.0	59	53	89.8	27	45.8
5	25	25	100.0	5	20.0	25	19	76.0	7	28.0
All samples (Total)	1647	682	41.4	1359	82.5	1617	874	54.1	1448	89.5
Infected oysters (1–5)	187	177	94.7	38	40.1	185	169	91.4	108	58.4

• *The last well on the qPCR 96 well plate was used for control samples, resulting in fewer oyster gill tissue samples than heart tissue samples.*

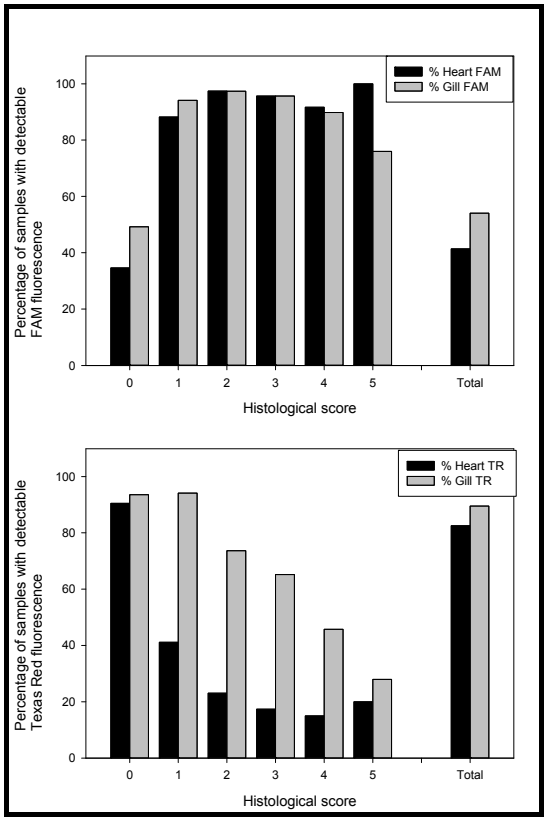


Figure 6: Percentages of heart and gill tissue samples that amplified using ITS region (FAM) and β-actin (Texas Red) by histological score.

The effects of different sizes of tissue samples, and different types of oyster tissues on detection and quantification of Bonamia using qPCR.

Experiment 1

The details of the oysters used in objective 1 are given in Appendix 1 Table A1. The oysters had an average length of 67 mm (± SD 11.6 mm) and height of 77 mm (± SD 12.8 mm). A range of different sizes and categories were used to examine the effect of oyster size. The average weight of the heart samples was 2.45 mg (± SD 1.37 mg) with the largest sample being 5.4 mg and the smallest 0.40 mg. The gill samples ranged from 1.80 mg to 18.40 mg with an average of 7.26 mg (± SD 3.21 mg). The details of oysters used for the combined heart and gill extraction are given in Appendix 1 Table A2. The average length of these oysters was 66 mm (± SD 11.6 mm) and height of 76 mm (± SD 12.2 mm). The weight of tissue used in these DNA extractions ranged from 4.90 mg to 14.00 mg, with an average of 9.26 mg (± SD 2.65 mg). None of the tissue samples used for DNA extraction exceeded a tissue to buffer ratio of 25% w / v (Table A1 and A2, Appendix 1).

The amount of DNA released after 45 minutes, 1 hour, 2, 3, 4, 5 and 24 hours by tissue type ranged from 3.73 to 288.25 ng / µl for heart tissue, 32.04 to 694.54 ng / µl for gill tissue and 11.67 to 225.20 ng / µl for the combined heart and gill tissue samples (Figure 7). There was no significant difference in the amount of DNA released from the heart tissue between 45 minutes, 1 hour and 2 hours (ANOVA, Tukey's HSD analysis), significantly less DNA was measured after 3, 4, 5 and 24 hours compare to 45 minutes ($p<0.05$) for these comparisons (ANOVA, Tukey's HSD analysis), indicating that after 2 hours degradation of the released DNA occurred either due to DNases released from the oyster tissue or from organisms in the oyster tissue. There was a significant difference between the amounts of DNA released from the gill tissue, with the most released after 2 hours, this was significantly different from all the other incubation times ($p<0.05$ for these comparisons (ANOVA, Tukey's HSD analysis). There was no difference in the amount of DNA released after 45 minutes and 1 hour (Figure 7). The amount of DNA released from the combined heart and gill samples was not significantly different between 45 minutes and 2 hours, but was significantly less at 1 hour compared to 45 minutes and 2 hours.

This experiment indicated that after 45 minutes of incubation, maximum DNA would be recovered from the heart and the heart and gill combined samples, but that a longer incubation time may be needed for gill samples.

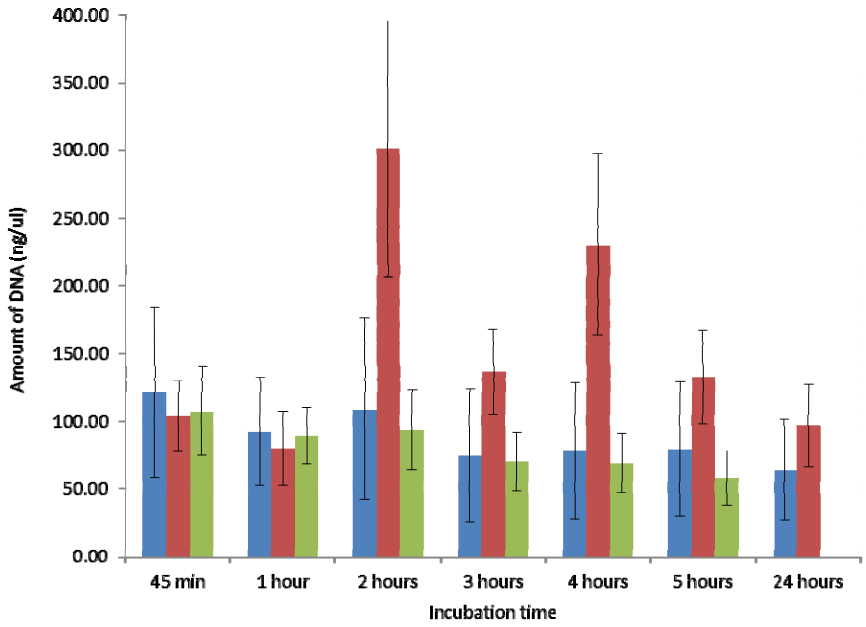


Figure 7: The average concentration of DNA (ng / µl) released after 45 minutes, 1, 2, 3, 4, 5 and 24 hours of incubation at 52°C with shaking for the different tissues heart (blue), gill (red) and the combined heart and gill (green) tissue samples. Error bars are equal to one standard deviation.

The amount of DNA released per milligram of tissue was calculated to ascertain whether the weight of the tissue had an influence on the amount of DNA released (Figure 8).

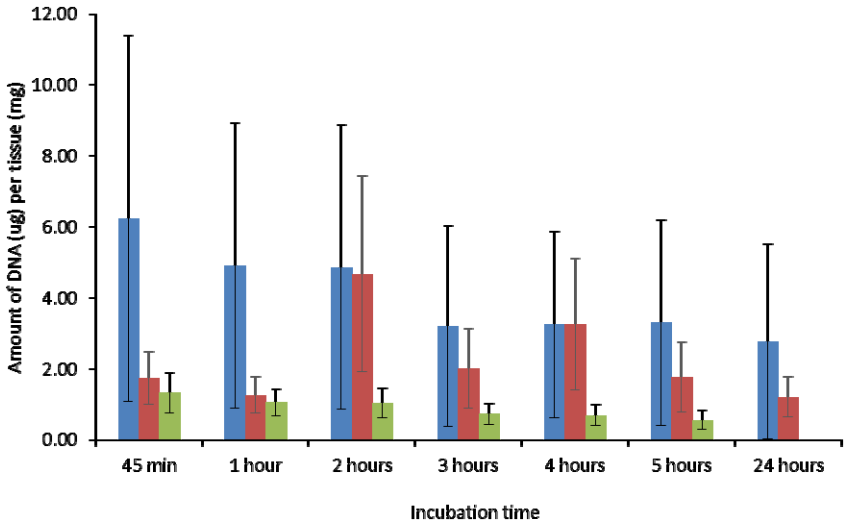


Figure 8: The average amount of DNA (µg) released per milligram of tissue after 45 minutes, 1, 2, 3, 4, 5 and 24 hours of incubation at 52°C with shaking for the different tissues heart (blue), gill (red) and the combined heart and gill (green) tissue samples. Error bars are equal to one standard deviation.

There was a correlation between the amount of tissue used in the extraction and the amount of DNA released for heart samples only at all incubations times except 24 hours (Table 4, Figure 9). Therefore the amount of tissue used in the DNA extraction does effect the amount of DNA that can be released. This may be due to heart samples being small and that the maximum amount of DNA is released after the first 45 minutes of incubation. There was no correlation between the amount of tissue used and the amount of DNA released for the other tissue.

Table 4: Correlation coefficients (r) between weight and the amount of DNA released after 45 minutes, 1 hour, 2, 3, 4, 5 and 24 hours incubation, for correlation coefficients in italics P is <0.05, bolded P is <0.01 and for bold and italics P is <0.001. Non-significant correlations coefficients are not shown.

			Incubation time			
Tissue weight	45 minutes	1 hour	2 hours	3 hours	4 hours	5 hours
Heart	***P* = *0.00099*** r = 0.55	*P* = *0.01833* r = 0.41	***P* = *0.00005*** r = 0.66	***P* = *0.00011*** r = 0.63	**P = 0.00115** r = 0.55	**P = 0.00795** r = 0.46
Gill						
Heart and Gill						

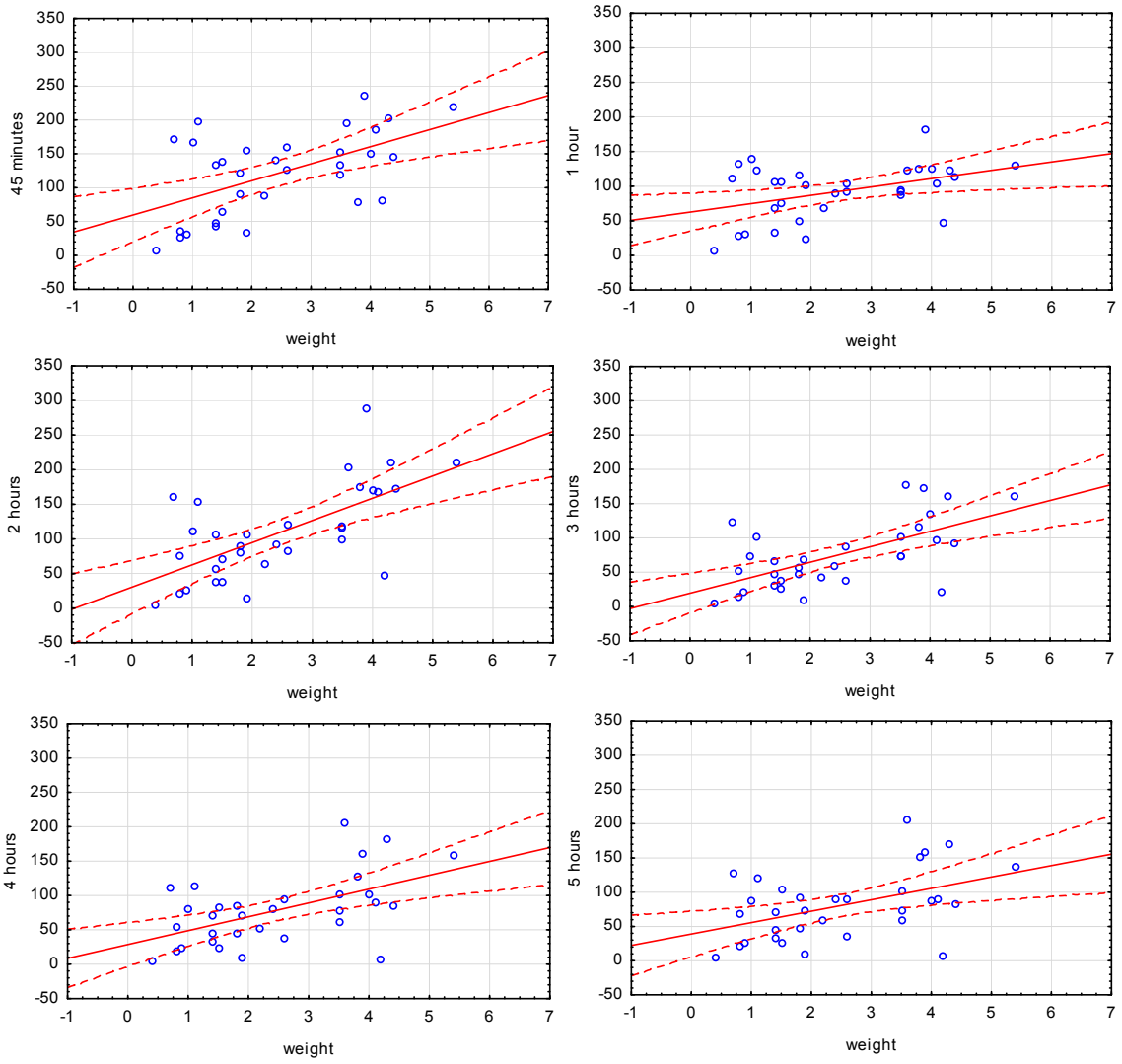


Figure 9: Scatterplots of tissue weight (mg) and amount of DNA released (ng / ul) after 45 minutes, 1 hour, 2, 3, 4 and 5 hours for heart tissue only. Dotted lines are equal to a 95% confidence interval.

Experiment 2

The effect of shaking the DNA extraction was investigated using an additional set of oysters (10 for shaking and 10 non-shaking extractions). Oyster details are given in Appendix 1 Table A3. The oysters had an average length of 68 mm (± SD 10.6 mm) and height of 77 mm (± SD 11.2 mm). The average weight of the heart samples was 1.68 mg (± SD 0.73 mg) with the largest sample being 3.20 mg and the smallest 0.60 mg. The gill samples ranged from 3.50 mg to 8.40 mg with an average of 6.30 mg (± SD 1.40 mg). The weight of combined samples ranged from 9.0 mg to 17.0 mg, with an average of 11.85 mg (± SD 2.32 mg). None of the tissue samples used for DNA extraction exceeded a tissue to buffer ratio of 25% w / v (Table A3, Appendix 1).

There was no significant difference between the amount of DNA released with and without shaking for the heart samples or for the combined heart and gill samples after 45 minutes or 2 hours of incubation or for the gill samples after 2 hours of incubation (Figure 10). There was a difference for the gill samples after 45 minutes of incubation - more DNA was released with shaking (Figure 10).

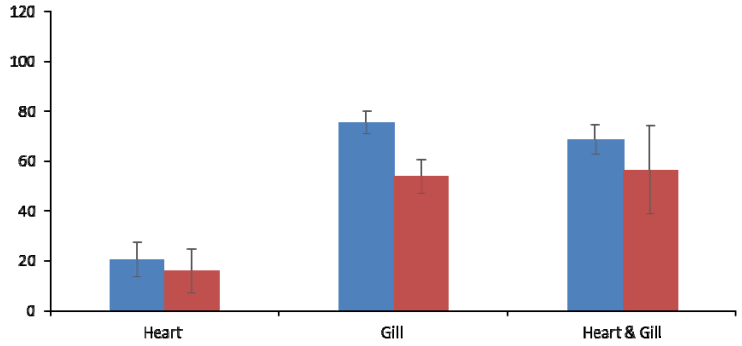


Figure 10: The amount of DNA (ng / µL) released from the heart, gill and combined heart and gill tissues with shaking (blue) and without shaking (red) after 45 minutes. Error bars are equal to one standard deviation.

The standard qPCR protocol was performed on the extracts obtained in experiment 2 to ascertain if shaking would affect the quality of the DNA, by shearing, and potentially the amplification of the β-actin gene and its quantification. There was very little difference in Cq values between the DNA extractions with and without shaking (Figure 11), indicating that shaking the extractions did not shear the DNA and did not adversely affect the quantification of the actin gene.

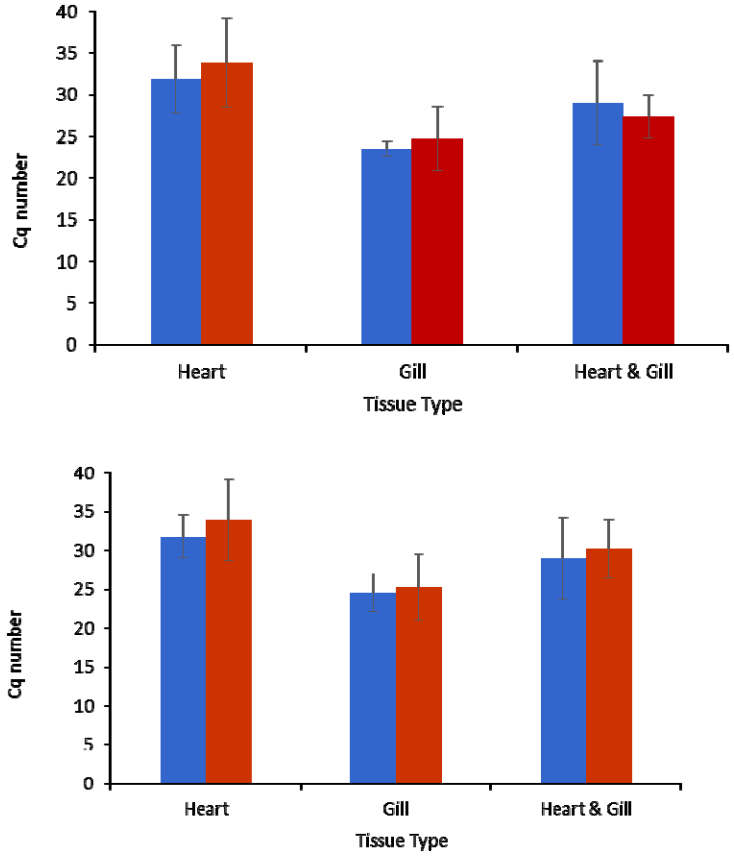


Figure 11: Cq values for the amplification of the β-actin gene for the heart, gill and combined heart and gill tissues with shaking (blue) and without shaking (red) after 45 minutes (top panel) and 2 hours (bottom panel). Error bars are equal to one standard deviation.

The two experiments conducted in objective 1 were carried out to validate the current standard extraction protocol and to ascertain if this could be improved by either lengthening the incubation time and/or incubating the extractions with shaking, and also to check if the 25% w/v ratio was exceeded in processing survey samples. Considering that the heart tissue is the focal point for Bonamia infections, the DNA protocol should be skewed and optimised for heart tissue. Therefore, on balance the current protocol of 45 minute incubation without shaking provides a good compromise between speed and optimum DNA extraction for heart tissue.

Variation objective 1.2: The effect of taking imprints with heart tissues before qPCR sampling.

Samples of oysters for this experiment came from site 10, where survey data suggested that there would be the best chance of sampling oysters with relatively high prevalence of infection. The details of the oysters used for this objective are given in Table A5 Appendix 1. The oysters had an average length of 83 mm (± SD 7.4 mm) and height of 96 mm (± SD 8.7 mm), and were slightly larger than the ones used for objective 1. This was done specifically as the heart had to be dissected into two so that one half could be blotted and the other half could be left untouched (control). The Cq values for the β-actin and Bonamia ITS gene were calculated for the unblotted heart, blotted heart and the gill tissue of each oyster (Figure 12, Table 5).

All samples that were Bonamia negative amplified β-actin, validating the qPCR assay. There were too few oysters that tested qPCR positive for Bonamia in the sample, and no analysis of the effects of blotting on heart Cq values could be tested. Only two unblotted and four blotted hearts tested positive and there were no samples with low level (Cq 35.1 and greater) amplifications. Eight gills amplified the Bonamia ITS gene, but only four were positive, and the other four low level amplifications may be due to external contamination by Bonamia.

There was no significant difference in β-actin Cq values used between the blotted and unblotted heart tissues. Cq values for gill tissues were different to hearts (Figure 12), possibly due to differences in digestion of the two types of tissues. The individual oysters that were Bonamia positive (208 and 218), unblotted hearts had Cq values about 1–1.5 lower than blotted (Table 5).

Because we could not investigate the effects of potential blood loss through blotting, and therefore reduced numbers of copies of Bonamia DNA (changes in Cq values) in the sample, the effects of blotting on qPCR sensitivity could not be addressed by these data. Blotting the oyster heart does not affect the tissue or the ability to amplify the β - actin gene.

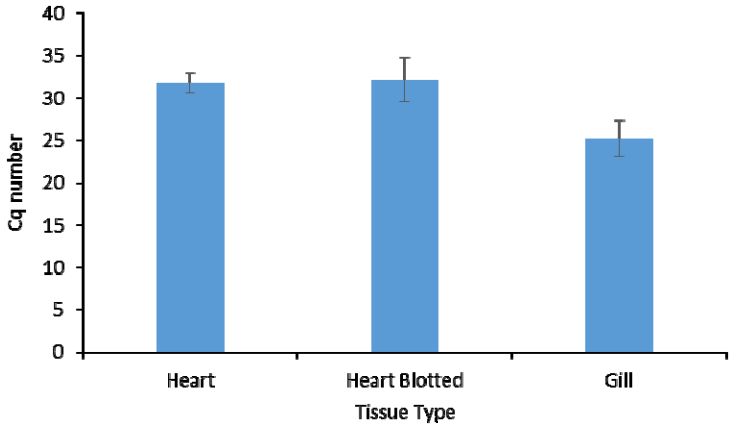


Figure 12: Cq values for the amplification of the β-actin gene for the unblotted heart, blotted heart and gill tissue. Error bars are equal to one standard deviation.

Table 5 Individual oyster qPCR results, Cq values for each tissue for each gene assayed. Oysters in italics have positive Bonamia ITS reaction in all tissue and bolded oysters have two or one tissues positive for Bonamia ITS gene, using standard cut of value of Cq <35 for positives. NA = no amplification of the gene.

	Heart		Heart Blotted		Gill	
Sample	β - actin	Bonamia	β - actin	Bonamia	β - actin	Bonamia
201	28.32	NA	34.93	NA	25.24	40.65
202	38.28	NA	NA	NA	27.95	NA
203	32.00	NA	30.81	NA	23.38	NA
204	31.69	NA	30.85	NA	28.00	NA
205	28.99	NA	30.12	NA	22.68	**34.83**
206	31.88	NA	33.38	NA	23.78	NA
207	32.30	NA	**29.65**	**32.28**	**25.17**	**33.42**
208	*NA*	*19.88*	*NA*	*20.98*	*NA*	*17.38*
209	27.34	NA	31.59	NA	25.32	NA
210	36.08	NA	38.74	NA	26.16	NA
211	28.60	NA	**31.78**	**34.69**	**24.41**	**35.76**
212	30.40	NA	32.07	NA	25.71	NA
213	32.91	NA	32.85	NA	26.11	NA
214	30.03	NA	32.24	NA	24.67	37.95
215	33.26	NA	32.17	NA	24.71	NA
216	32.73	NA	35.00	NA	27.28	NA
217	33.61	NA	26.85	NA	23.16	NA
218	*NA*	*19.77*	*NA*	*22.21*	*NA*	*17.63*
219	30.74	NA	31.31	NA	26.98	NA
220	31.91	NA	31.06	NA	23.99	38.51

Primer validations.

Previous *in silico* work had shown that the standard probe had a 100% similarity with *B. ostreae* and *B. exitiosa*. None of the other non-Bonamia organisms, *Perkinsus olseni*, *P. marinus* and *Haplosporidium nelsoni* showed any amplification with the standard Bonamia qPCR protocol, or the two new *B. exitiosa* and *B. ostreae* specific probes. The standard probe and primer sets only showed amplification with the *B. exitiosa* and Bon+ve DNA purified from Foveaux Strait oysters, and not *B. ostreae*. The new *B. exitiosa* and *B. ostreae* probes failed to show any amplification with the *B. exitiosa*, *B. ostreae* and Bon+ve DNA (Table 6). This result was unexpected as *in silico* the standard probe matches *B. ostreae* with a 100% similarity. The Bon+ve DNA was tested by dnature using their equipment and low levels of amplification were seen using the new *B. ostreae* specific probe, and the new *B. exitiosa* probe amplified the same as the standard probe. This needs further investigation. Lack of access to different stocks of *B. exitiosa* and *B. ostreae* DNA is hindering progressing this issue further.

Table 6: Predicted and observed results with the standard Bonamia probe, *B. exitiosa* and *B. ostreae* specific probes. + = amplification, - = no amplification and ? = unknown reaction.

	Predicted			Observed		
	Standard probe	*B. exitiosa* specific probe	*B. ostreae* specific probe	Standard probe	*B. exitiosa* specific probe	*B. ostreae* specific probe
Bon +ve	+	+	?	+	-	-
B. exitiosa	+	+	-	+	-	-
B. ostreae	+	-	+	-	-	-

During the design stage of the standard Bonamia qPCR two more sets of primers were designed, primer 408 - 521 and primer 427 - 521. These were tested against the Bon+ve, and the *B. ostreae* and *B. exitiosa* DNA using SsoFast™ EvaGreen® qPCR mix. All three DNA's amplified with these primers.

The current standard Bonamia qPCR assay is specific for *B. exitiosa* on the NIWA qPCR equipment and does not cross react with *B. ostreae* as the *in silico* analysis had shown. Further work will need to be conducted using different samples of *B. ostreae* DNA to confirm this result.

Appropriate Cq cut-off values and protocols.

The cycle of quantification (Cq) cut-off to determine positives from false positives was set at Cq 35based on serial dilution of Bon+ve samples to extinction. Given the large geographical isolation, the Chatham Island oyster population could potentially be Bonamia free. Samples of Chatham Island oysters could therefore provide Bonamia negative samples to validate the negative cut off value. The full results for each Chatham Island oyster tested are given in Table A6 in Appendix 1. Cq values were calculated using the standard procedure, and low and no amplification were scored using the standard procedure. Examples of positive, negative samples, low amplification and no amplification curves are given in Figure A1 in Appendix 5.

Seven oysters showed weak reactions or 'abnormal curves' with the Bonamia assay. Oysters 55, 59, 60, 72 and 78 showed very early amplification probably due to matrix autofluorescence in the sample, and an example of this type of curve is given in Figure 1 in Appendix 5. Two oysters, oyster 45 for heart (Cq = 36.13) and 89 for gill tissue (Cq = 41.49) showed high Cq values which would have been classed as Bonamia negative (Figure 13 and Table A6 in Appendix 1).

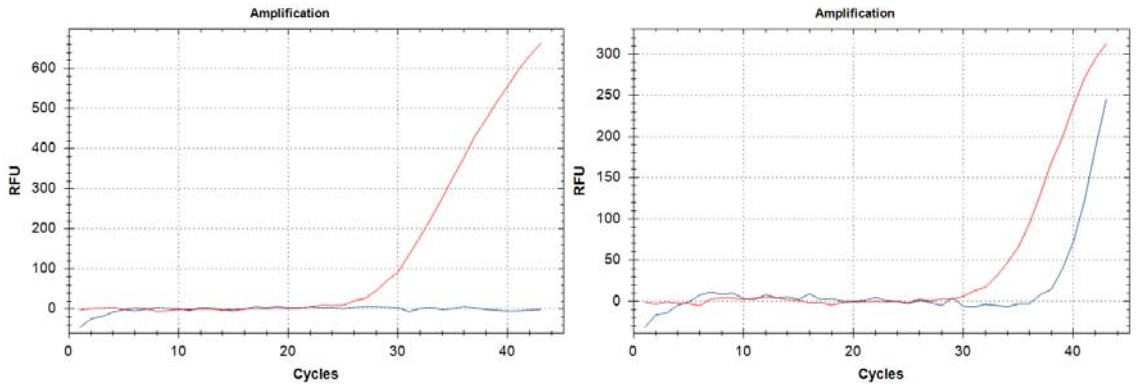


Figure 13: Oyster 45 qPCR amplification curves showing gill (left) and heart (right) amplification curves, β-actin (red) and Bonamia ITS (blue).

The unexpected results for oysters 45 and 89 need to be confirmed by dilution and cloning and sequencing the product to make sure that it is Bonamia. The primers and probes have been shown to be very specific so it is unlikely that the reaction is not due to some low level of Bonamia in these oysters. This highlights the need to know numbers of gene copies at Cq value, so that estimates of the numbers of Bonamia cells could be made.

Differences in Bonamia ITS and β−actin amplification between gill and heart tissues.

Improvements to the method before the survey was conducted reduced the concern we had about the β-actin not amplifying consistently in samples. The dilution was changed from a 1:10 dilution to a 1:20 and the volume added to the qPCR was increased to 4 µl to compensate for this. The increase in dilution helped reduce the amount of inhibitors in the crude extracts. A positive control of pooled Bonamia positive samples was made and this was added to every plate. The performance of this positive control was used to check the performance of the qPCR and plates were rejected that showed little or no amplification with the positive control (see Figures 2 and 3). qPCR is very sensitive to even minor changes in the concentration of reagents. As new reagents were purchased, all reagents were cross checked with previous batches to ensure consistent performance of the assays.

Objective 2. Comparison of the new qPCR method and the traditional heart imprint method.

Evaluation of qPCR data for inclusion in analysis

Only the first 24 of 25 oyster heart and gill tissue samples from each site were analysed with qPCR. All samples were run on 96 well plates with negative controls and positive controls for Bonamia and the β-actin gene. Output from the BioRad CFX96 were tabulated in Excel templates. Wells H6 and H12 were used for positive and negative control samples respectively. The data from these wells were separated from the sample data, and control data checked to verify performance of the assay. Sample data were examined to identify anomalies and potential amplification problems, and graphical output from the BioRad CFX96 examined (Figure 14) to identify flat-liners and unusual amplification curves (see Appendix 5 for examples).

Rules to repeat assays

qPCR assays repeated the entire 96 well plates when there was a problem with any of the controls, or when large numbers of individual sample wells showed anomalies in either the qPCR data exported to Excel files or on the BioRad CFX96 graphics of relative fluorescence units (RFU) curves against Cq values. These anomalies included:

- The sample wells on the plates were dry or missing tissue.
- Out of range Cq values for the Bonamia positive (about Cq of 28) and negative control wells.
- The Bonamia ITS and internal control (β- actin) Cq values were NAs (there were no values, also known as flat-liners).
- Either the Bonamia ITS or internal control (β- actin) amplified very early in the cycles ($Cq \leq 10$, see Figure A1, Appendix 5).
- The internal control Cqs were late ($Cq \geq 40$) together with no Bonamia amplification.
- The internal control (β-actin gene) amplified early and well before the Bonamia ITS.

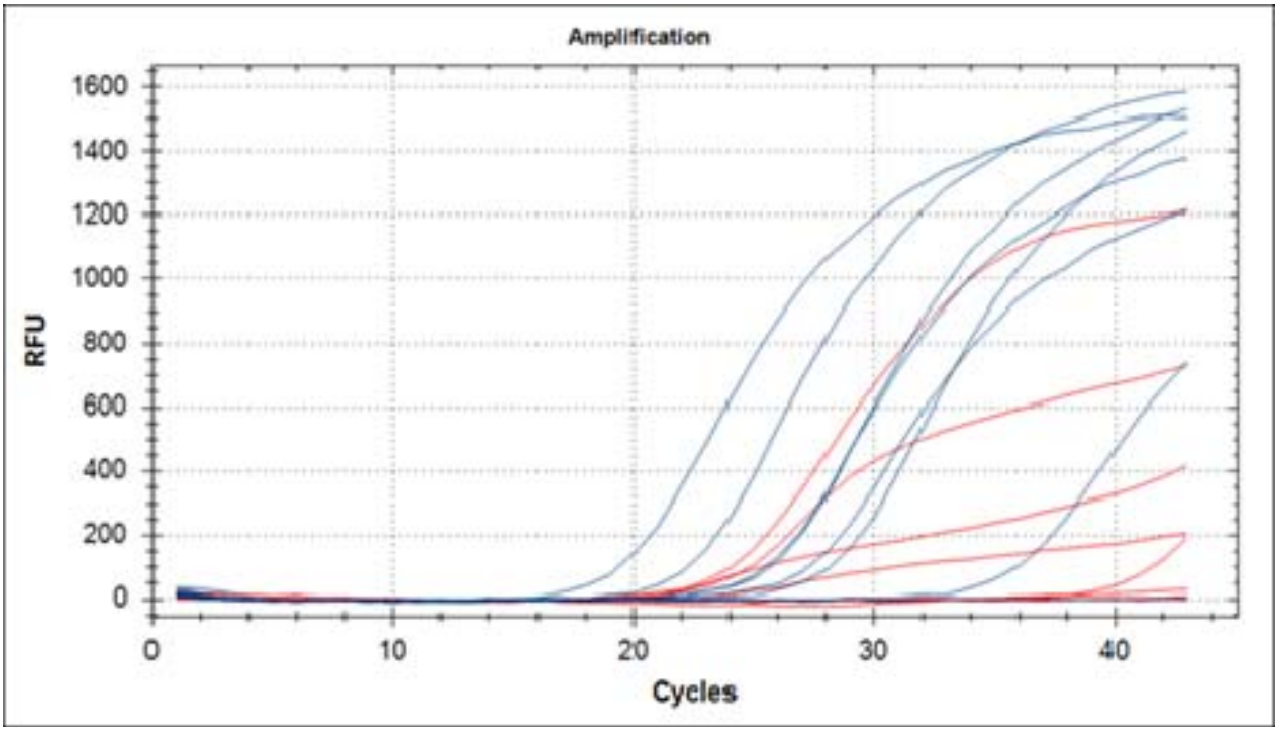


Figure 14: Plots of relative fluorescence units (RFU) against cycle for 8 wells of a 96 well qPCR plate. In seven of the eight samples, the ITS region amplified indicating the presence of Bonamia (7 blue curves). In two of these 7 samples amplifying FAM around Cqs of 20, amplification of the β-actin gene (red curves) was blocked. For one of the eight samples neither gene (FAM or TR) were amplified.

Rules to omit qPCR data from analysis

Even after assays were repeated, there were still samples that produced anomalous results. Data from samples were omitted when:

- There were out of range Cq values for the Bonamia positive (should be around Cq about 28) and negative control wells.
- The Bonamia ITS and internal control Cq values were both NAs (there were no values, known as flat-liners).
- Either the Bonamia ITS or internal control (β- actin) amplified very early in the cycles (Cq <10, see Figure A1, Appendix 5).
- The internal control (β- actin) Cq values were late (Cq values ≥ 40) together with no Bonamia ITS amplification.

Comparison of results for qPCR and histology from the 2012 survey

Initial analysis of the qPCR data identified a number of discrepancies between qPCR and histology. Fourteen plates were rerun (plates: 3, 4, 11, 12, 16, 18, 19, 22, 23, 26, 30, 35, 39, and 40) and 200 slides of heart imprints scored again. Because of the categorical scoring method, we expected some variation in scores within groups for no detectable infection (0), light infections (1−2), and heavy fatal infections (3−5). The repeated histology scores differed by 2 or more category scores in 3% of the repeated slides examined (N = 194).

The qPCR method using the generic Bonamia ITS region showed high sensitivity in the detection of Bonamia. At a cut-off of 35 Cq, 50% of the samples were positive, at 33 Cq 28% were positive, and at 30 Cq 13% were positive for Bonamia. Only 11.4% of the paired histology samples were positive for Bonamia in 2012 (Figure 15).

The quantification of Bonamia cannot be directly compared between qPCR and histology as the qPCR Cq values estimate numbers of Bonamia ITS region copies while the histology scores categorise the average numbers of Bonamia cells in oyster haemocytes. There was however a trend of decreasing Cq values with increasing histological score (infection), Figure 15. There were a number of outliers in the Cq values for infected samples (histological score 1−5), for both gill and heart tissues that may represent either some inhibition of the qPCR

reaction, very small tissue samples sizes, incomplete digestion of tissue, or the significant loss of blood during the heart imprint process (Figure 15). Overall, the qPCR method was shown to be effective in detecting low level infections of Bonamia.

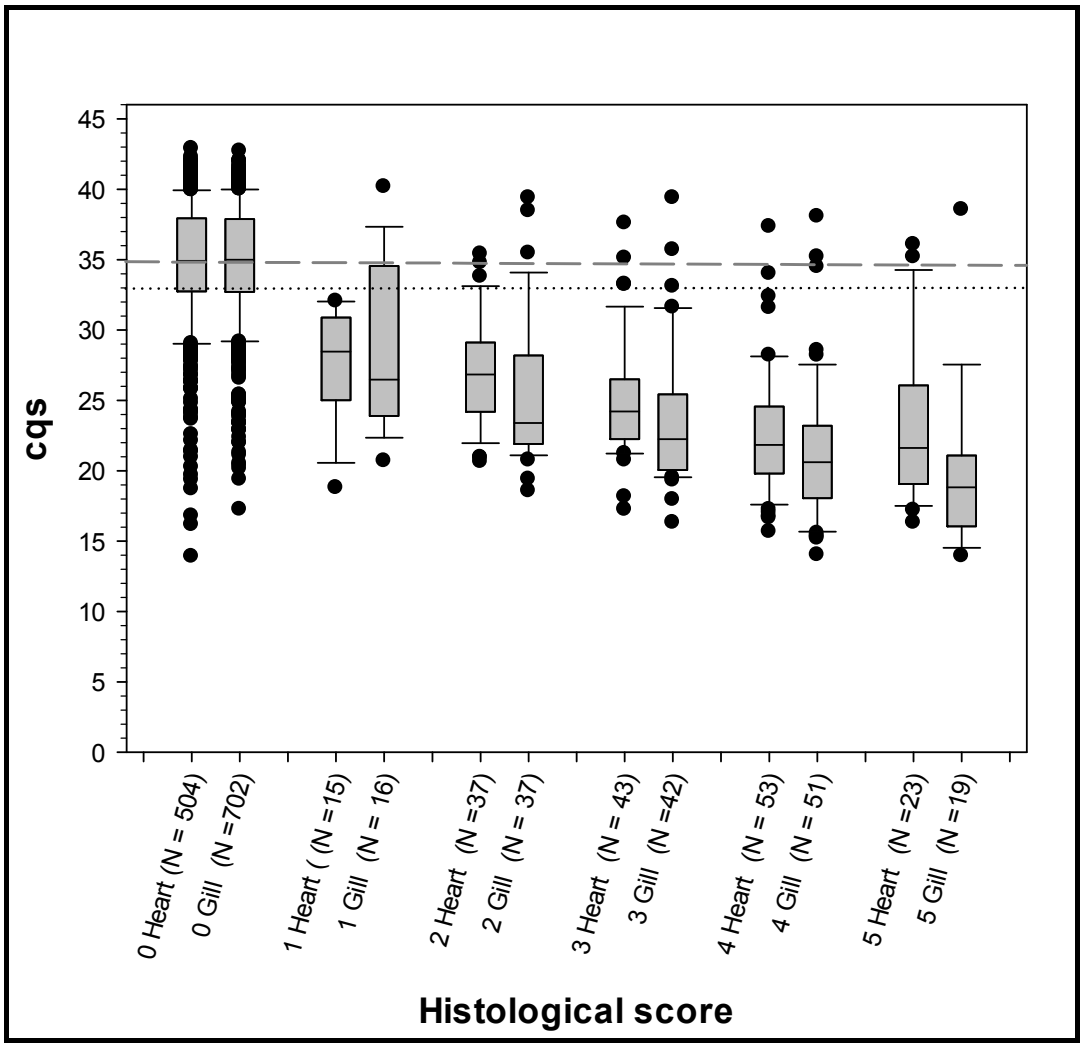


Figure 15: Boxplots of Cq values for paired samples of heart and gill tissues for ITS region (FAM) by histological score. Cut-off levels set at 35 Cq (grey dashed line) and 33 Cq (black dotted line). Box plots show medians (solid lines), boxes 25 and 75 percentiles, whiskers at 95 percentiles, and outliers shown as black circles above and below whiskers.

The results show significantly higher sensitivity for detecting Bonamia in gill tissue than in heart tissue in all oysters that had both heart and gill paired Cq values and that had histological scores 0, 2, 3, or 5 (Table 7). There was no significant difference between heart and gill tissue for histological categories 1 and 4. Sample size was small for histological category 1, but good for histological category 4.

Table 7: Tests of significance between paired heart and gill tissues from the same oyster, for oysters with ITS region Cq values for both heart and gill, grouped by histological scores. The sample size is shown as numbers of tissues (No. tissues). Test include T Tests (TT) for normally distributed data passing the Shapiro-Wilk normality test, else Mann-Whitney Rank Sum test (MWRS), P values and significance shown as not significant (NS) or significant (S). The more sensitive of the two tissues (lower mean/median Cq) is shown for each histological score.

Histological score	No. tissues	Test	P value	Significance	More sensitive
0	375	MWRS	0.003	S	Gill
1	15	TT	0.351	NS	-
2	36	MWRS	0.012	S	Gill
3	42	MWRS	0.012	S	Gill
4	52	MWRS	0.078	NS	-
5	19	MWRS	0.029	S	Gill
All	539	MWRS	0.012	S	Gill

2013 survey data summary

Heart imprints, heart and gill tissue samples were taken from each of 1422 oysters, and from the 57 sites within the Foveaux Strait oyster fishery area. A subsample of 652 slides were initially scored for Bonamia infection from the 1442 slides taken. These comprised qPCR 431 samples that were qPCR positive, 165 randomly selected samples that were qPCR negative (a minimum of 3 selected from each site), and a further 56 samples from the last oyster (25th oyster in each sample, qPCR analysis sampled oysters 1–24) in each sample. One site sampled less than 25 oysters. Nineteen of these samples were re-examined because there were differences between the histological scores and qPCR results.

Initial analysis of the 2013 qPCR data identified a number of anomalies, possibly relating to reagents used for the assays. Eight whole plates were rerun and 49 individual samples repeated (Table 8). Data from the original assays were replaced with data from the repeated assays, and any outstanding data anomalies based on the data removal criteria deleted from the final dataset.

Table 8: The plate numbers (Plate.num) of whole plates repeated, numbers of individual wells repeated for both heart and gill tissues from each station (Well1 and Well2); and the numbers of individual samples (No.samp1 and No.samp2) repeated from each plate.

Plate.num	Well1	Well2	Total
13P04	46	36	82
13P03	40	26	66
13P11	37	23	60
13P09	37	10	47
13P06	19	13	32
13P05	20	10	30
13P12	24	5	29
13P10	18	5	23
13P14	15	3	18
13P18	15		15
Total			402

Plate.num	No.samp1	No.samp2	Total
13P29	7		7
13P16	6		6
13P17	6		6
13P26	6		6
13P15	4	1	5
13P24	4		4
13P19	3		3
13P25	3		3
13P20	2		2
13P22	2		2
13P34	2		2
13P01	1		1
13P21	1		1
13P27	1		1
			49

Generally Bonamia ITS Cq values for heart and gill tissues were similar (Figure 16, top left) with the exception of two samples where the heart tissues tested positive with low Cq values and the gill tissues were negative. β-actin gene amplification was generally higher in gill tissues compared to heart tissues (Figure 16, top right). β-actin amplified later in heart tissues at Cq values of 25–35 than in gill tissues (Cq values of 20–28) (Figure 16, bottom left and bottom right respectively). β-actin dropped away earlier in gill tissues with decreasing Cq values for the Bonamia ITS than in heart tissues. These differences suggest that gill and heart tissues amplify and interact with the block slightly differently.

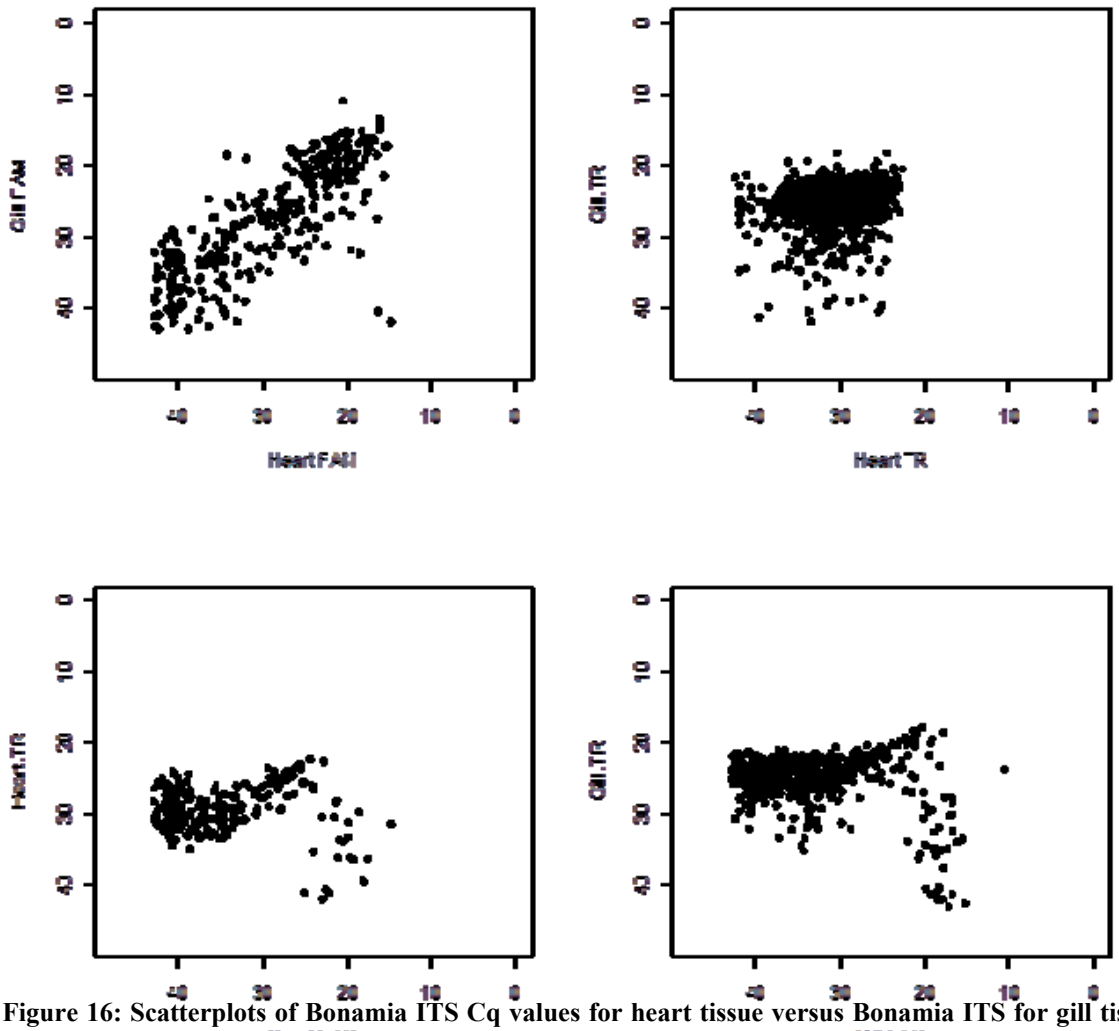


Figure 16: Scatterplots of Bonamia ITS Cq values for heart tissue versus Bonamia ITS for gill tissue (Top left), β- actin Cq values for heart tissue versus β- actin for gill tissue (Top right), Bonamia ITS Cq values for heart tissue versus β- actin for heart tissue (Bottom left), Bonamia ITS Cq values for gill tissue versus β- actin for gill tissue (Bottom right). Axes are reversed as smaller values show earlier fluorescence corresponding to larger numbers of gene copies.

The qPCR method showed high sensitivity in the detection of Bonamia. At a cut-off of 35 Cq, 50% of the samples were positive in 2012 and 11.4% of the paired heart imprint samples were positive. There were relatively few samples that had no qPCR reaction (i.e., no fluorescence detected above the baseline) in 2012: 4.2% of heart tissue samples and 5.3% of gill tissue samples had no amplification of the Bonamia ITS target and β-actin genes.

The proportion of samples that had no qPCR reaction in 2013 was lower than in 2012 (3.2 % of hearts and 2.1 % of gills) (Table 9). Excluding flatliners, 19.5% of the heart qPCR samples and 29.0 % of gill qPCR samples tested positive for *B. exitiosa* DNA; and 31.6% of qPCR samples representing the pooled hearts and gill results tested positive.

Table 9: Summary of samples screened for bonamia using qPCR (heart and gill tissues) in 2013. The total numbers of heart and gill samples tested (Sample (N)), those that tested positive (Positive (<35Cq)) and those where no bonamia DNA was detected (Negative (>35 Cq)), the numbers and percentages of flatliners and early ampers.

qPCR samples							
Bonamia infection	Sample (N)	Positive <35Cq)	Negative (>35 Cq)	Flatliners	% Flatliners	Early ampers (Cq<10)	% Early ampers
Heart	1365	258	1051	43	3.2	5	0.04
Gill	1366	388	882	29	2.1	30	2.19
Both H and G		215					
Heart only +ve		43					
Gill only +ve		173					

In all, 1422 heart imprint slides were taken, twenty five from each of all but one of the 57 sites. The number of heart imprint slides screened (N=596, Table10) included qPCR positives and randomly selected negatives. Of those samples testing positive by qPCR, 67.8% of heart imprints were positive from qPCR positive hearts and 45.1% of positive gill qPCR samples (Table 10). The performance of heart imprints against heart and gill tissue qPCR assays was estimated as coefficients of similarity, 0.73 and 0.61 for heart tissues and gill tissues respectively (Table 11).

Table 10: The summary Bonamia infection from paired qPCR samples and heart imprint slides to give heart imprint performance. qPCR assumed to be 100% reliable. The total numbers qPCR, heart tissues, and gill tissues that tested positive, and the numbers of randomly selected qPCR negative samples (Sample (N)). The numbers of corresponding heart imprint samples that scored positive for bonamia infection (Histo+ve) the percentage of false negative (failure to detect infection, % False-ve), and those that where negative (Histo-ve and % False+ve).

Sensitivity	Sample (N)	Histo+ve	% False-ve	Histo-ve	% False+ve
qPCR +ve	431	175	59.4		
Heart qPCR +ve	258	175	32.2		
Gill qPCR +ve	388	175	54.9		
qPCR -ve	165			161	2.4
All qPCR	596			NA	NA

Table 11: The performance of heart imprints against paired qPCR positives for heart tissues and gill tissues as estimated by the coefficient of similarity (S) after Balseiro et al. (2006).

Heart tissue qPCR	(175+161)/463	= 0.73
Gill tissue qPCR	(175+161)/553	= 0.61

Gill tissues generally produced lower Cq values than heart tissues (Figure 17), which may either mean they are more sensitive (provide for better amplification) and or potentially that the method is amplifying external contamination of gill tissue by water-borne Bonamia particles. Heart tissues may provide better estimates of oyster infection and gill tissues better estimates of pathogen presence in the environment.

Of the matched qPCR samples (heart and gill tissues) with a Bonamia score of 0 from heart imprints, there were more gill tissues that tested qPCR positive than heart tissues (Figure 17), but a high proportion of these gill tissue Cq values were clustered around the 35 Cq cut-off. The heart tissue Cq values for ranged from 14 to 43 while the Cq values from gill tissues ranged from 10 to 43 (Figure 18), suggesting that for a large number of qPCR positive samples (Figure 17) the infection was not detectable using heart imprints. Half of the qPCR heart tissue samples with a Bonamia score of 0 from heart imprints were positive (Figure 18).

The quantification of Bonamia cannot be directly compared between qPCR and heart imprints as the qPCR Cq values estimate numbers of Bonamia ITS region copies while heart imprint scores categorise the average numbers of Bonamia cells in oyster haemocytes. Of the heart imprint samples that were positive for Bonamia infection, all but one of the qPCR heart and gill samples were positive (values below 35 Cq). The boxplots of Cq values for both heart and gill tissues showed a decreasing trend with increasing intensity of Bonamia infection estimated from heart imprints i.e., Bonamia scores increasing from 1 to 5 (Figure 17). Gill tissues generally had lower Cq values than heart tissues.

There were a number of outliers in the boxplots (Figure 17), and these outliers (both gill and heart tissues) may represent either some inhibition of the qPCR reaction, possibly small tissue sample sizes (from small oysters), incomplete digestion of tissue, or the significant loss of blood during the heart imprint process (Figure 17).

Bonamia ITS Cq values for all gill tissues compared to all heart tissues that amplified were not significantly different (Mann-Whitney rank sum test, $P = 0.079$). Cq values for both heart and gill qPCR tissues that had matching Bonamia scores of 0 (tested negative using heart imprints) were both significantly different to their respective qPCR tissues with matching Bonamia scores of 1–5 (Kruskal-Wallis one way analysis of variance on ranks, $P = <0.001$).

Differences among Cq values grouped by Bonamia scores (1–5) for heart tissues were significant ($P = <0.001$, $\alpha = 0.050$: 1.000) with high power to detect differences. An all pairwise multiple comparison (Holm-Sidak method at a significance level = 0.05) of heart tissue Cq values by Bonamia score is shown in Table 12: all Cq groups for Bonamia scores 1–4 were significantly different, but there was no significant difference between groups of Cqs for Bonamia scores 4 and 5.

Table 12: An all pairwise multiple comparison (Holm-Sidak method at a significance level = 0.05) of heart tissue Cq values by Bonamia score, S denotes significant difference and NS no difference.

	H1	H2	H3	H4	H5
H1	-	-	-	-	-
H2	S	-	-	-	-
H3	S	S	-	-	-
H4	S	S	S	-	-
H5	S	S	S	NS	-

Differences among Cq values grouped by Bonamia scores (1–5) for gill tissues were significant ($P = <0.001$). An all pairwise multiple comparison (Dunn's method, at a significance level = 0.05) of gill tissue Bonamia ITS Cq values by Bonamia scores is shown in Table 13: Cq value groups for Bonamia scores were not as distinct with three of the ten comparisons not significantly different.

Table 13: An all pairwise multiple comparison (Dunn's method, at a significance level = 0.05) of gill tissue Cq values by Bonamia score (H1–5), S denotes significant difference and NS no difference.

	G1	G2	G3	G4	G5
H1	-	-	-	-	-
H2	NS	-	-	-	-
H3	S	S	-	-	-
H4	S	S	NS	-	-
H5	S	S	S	NS	-

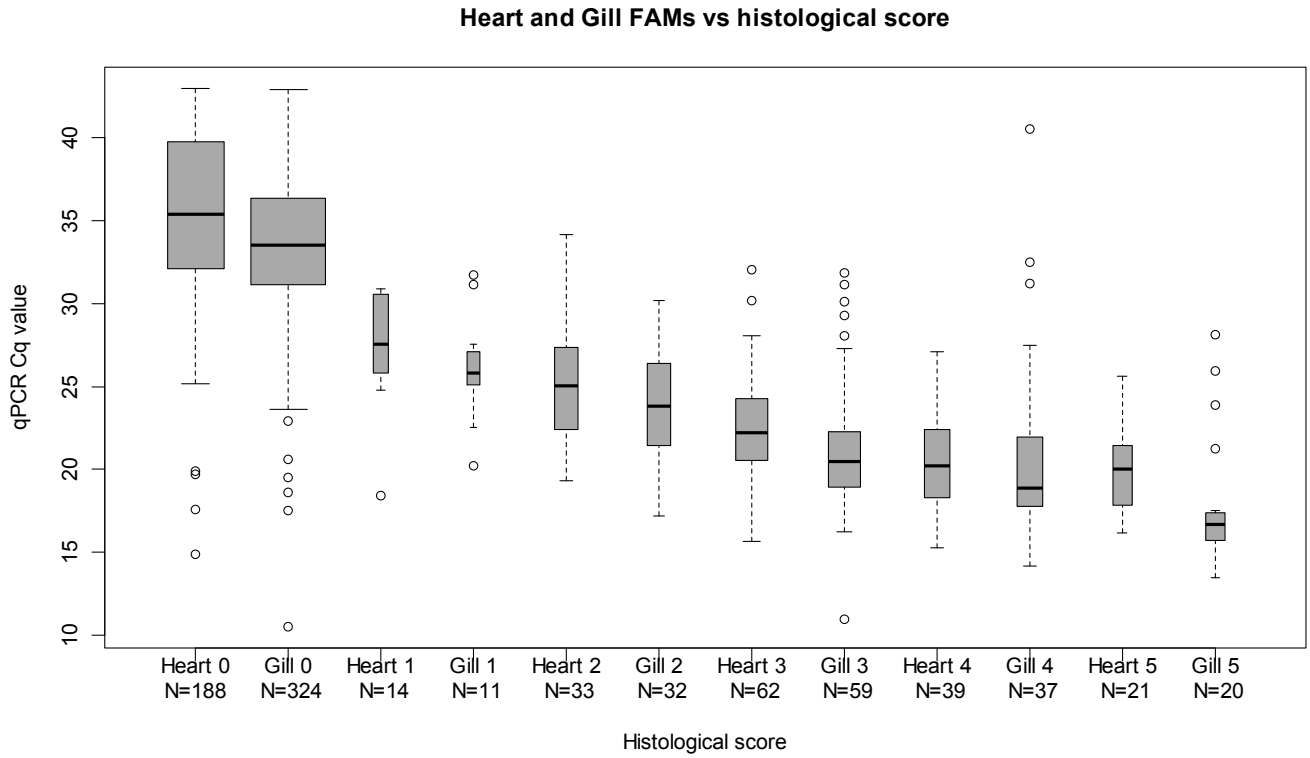


Figure 17: Boxplots of Cq values for paired samples of heart and gill tissues for ITS region (FAM) by histological score. Cut-off levels set at 35 Cq. Box plots show medians (solid lines), boxes 25 and 75 percentiles, whiskers at 95 percentiles, and outliers shown as black circles above and below whiskers.

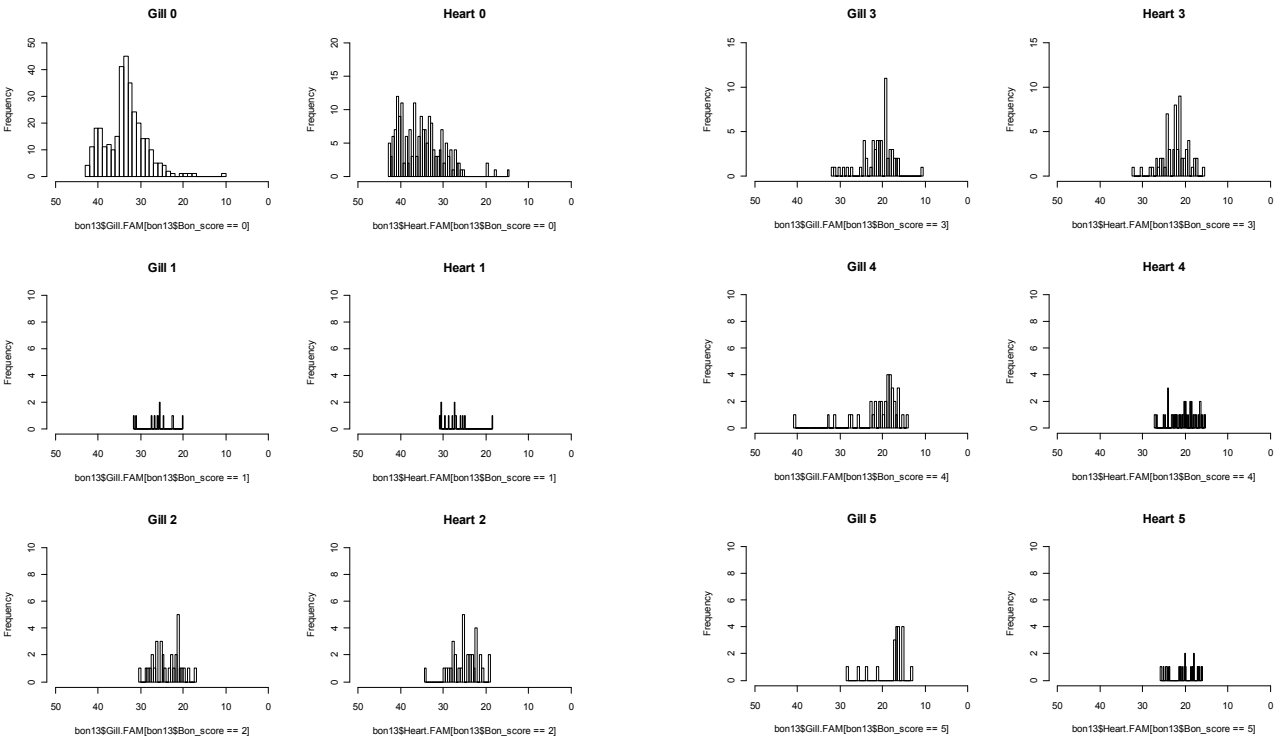


Figure 18: The distribution of Bonamia ITS Cq values for heart and gill tissue by histological Bonamia score.

Objective 3: Establish a standard sampling design and method for the annual monitoring of disease.

The Ministry for Primary Industries is reviewing the objectives of Foveaux Strait oyster Bonamia surveys and the type of information they require, and based on any changes, the Bluff Oyster Management Company may also change the information they require from these surveys. Until then, it is not possible to describe an appropriate survey design.

The main consideration is for future sampling to maintain consistency with the historical time series of survey and Bonamia data. It is envisaged that the standard survey dredge tow will remain the sampling unit. It is proposed that laboratory sampling will take histological (heart imprints and histology) samples, and qPCR samples for both heart and gill tissue from all oysters. We will retain the sample size of 25 oysters per station. All qPCR samples will be run first. A subset of histological (heart imprint slides) representing all qPCR positive samples and a random sample of at least three qPCR negative samples per site will also be examined. We will compare the qPCR and histological results and repeat any sampling required to address data anomalies between paired qPCR and histological samples. These results will be evaluated and more or all the histological slides scored if needed to maintain the time series of infection data.

4 CONCLUSIONS

The initial qPCR method for the detection and quantification of *Bonamia exitiosa* in Foveaux strait oysters *Ostrea chilensis* has been further developed with incremental gains in performance from the introduction of positive and negative controls, and an improvement in bench top procedures. Both heart and gill tissues provide greater sensitivity and specificity using this qPCR method than the heart imprint method. Heart imprints had a performance coefficient of 0.73 when compared to qPCR heart samples and 0.61 compared to gill samples (assuming qPCR is 100% efficient at detecting Bonamia infection) i.e., qPCR detects greater numbers of mostly low level infections than heart imprints and therefore has greater sensitivity. There was a decreasing trend in Cq values of samples with matching heart imprint samples with increasing Bonamia scores.

The standard method amplified *Bonamia exitiosa* genetic material (ITS region of the ribosomal genes) and purified DNA from a pooled number of samples of infected oysters from Foveaux Strait, however, it does not amplify *B. ostrea* DNA or DNA from closely related genera providing high specificity. Gill tissues generally amplified Bonamia ITS earlier in the cycle than heart tissues, i.e., generally produced lower Cq values than heart tissues, which may either mean they are more sensitive and or are potentially amplifying external contamination by water-borne Bonamia particles on gill tissue. Heart tissues may provide better estimates of oyster infection and gill tissues better estimates of pathogen presence in the environment.

There are a number of issues relating the objectives addressed in the variation to OYS201101 that may require further investigation. A meeting of a Ministry for Primary Industries Technical Group may provide some direction on the issues that should be further pursed, and determine the issues that will not affect the robustness of this qPCR method. These include:

1. The percentages of qPCR samples which had no reaction for both the Bonamia ITS and the β-actin gene (Flat-liners) were lower than in 2012, 3.2 % for heart samples and 2.1 % for gill samples in 2013. There is scope to easily further reduce the occurrence of flat-liners, but data loss is minimal at this level.
2. Standard dilution curves have validated the Cq cut-off values. A Cq cut-off value one cycle more or less could make significant differences to estimates of prevalence of infection, especially for gill tissue samples, many of which are clustered close to the 35 Cq cut-off value. Work to establish the number of Bonamia gene copies at cycle of quantification could assist to better set Cq cut-off values, and more importantly establish a qPCR protocol for intensity of infection.
3. Further work on probes and primer validation is required to investigate difference between qPCR systems, and to allow research on likely concurrent infections.
4. Further work is required on the effects of different sizes of tissue samples, and different types of oyster tissues (e.g. heart and gill tissue) on detection and quantification of Bonamia ITS using qPCR. The inability to determine whether individual oysters are infected with Bonamia before they are sampled results in “hit and miss” sampling of infected oysters. This is also a problem when investigating the effects of taking imprints with heart tissues before qPCR sampling.
5. Seasonal sampling and sampling seawater close to the seabed at survey sites could help explain differences between heart and gill tissues.

Given the well-known limitations and higher costs in using the heart imprint method alone to monitor Bonamia in the Foveaux Strait oyster fishery, the refined standard qPCR method will provide greater sensitivity and specificity to identify low level Bonamia infection oysters, it is faster and more cost effective than heart imprints alone.

5 ACKNOWLEDGMENTS

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Appendix 1: Data tables for qPCR samples

Table A1: Individual oysters used in objective 1, length (anterior–posterior axis) and height (dorsal–ventral axis), oyster category: R (recruit sized unable to pass through a 58 mm internal diameter ring), P (pre-recruits able to pass through a 58 mm internal diameter ring, but unable to pass through a 50 mm ring), and O (small oysters able to pass through a 50 mm internal diameter ring and down to 10 mm in length); and tissue size (mg) and weight per volume in the DNA extraction reaction.

Oyster	Length (mm)	Height (mm)	Category	Heart (mg)	Heart (w/v %)	Gill (mg)	Gill (w/v %)
1	77	92	R	3.6	3.27	1.8	1.64
2	66	84	R	2.2	2.00	18.4	16.73
3	70	90	R	5.4	4.91	13.1	11.91
4	71	78	R	2.4	2.18	4.9	4.45
5	67	76	R	1.8	1.64	8.9	8.09
6	65	81	R	1.5	1.36	6.8	6.18
7	84	88	R	4.0	3.64	5.9	5.36
8	64	66	R	0.8	0.73	6.0	5.45
9	64	73	R	1.5	1.36	4.8	4.36
10	52	67	P	1.9	1.73	4.8	4.36
11	44	55	O	1.4	1.27	7.3	6.64
12	76	86	R	3.8	3.45	5.4	4.91
13	54	57	P	1.4	1.27	4.9	4.45
14	52	56	P	1.1	1.00	6.5	5.91
15	57	72	R	1.4	1.27	4.8	4.36
16	64	76	R	1.9	1.73	5.8	5.27
17	66	90	R	4.3	3.91	7.4	6.73
18	74	88	R	3.5	3.18	4.8	4.36
19	79	86	R	4.2	3.82	9.4	8.55
20	52	56	O	0.9	0.82	4.9	4.45
21	80	92	R	3.5	3.18	4.7	4.27
22	77	88	R	2.6	2.36	5.6	5.09
23	72	91	R	4.1	3.73	6.9	6.27
24	81	91	R	1.8	1.64	9.8	8.91
25	76	86	R	4.4	4.00	10.9	9.91
26	92	86	R	3.5	3.18	7.9	7.18
27	81	87	R	2.6	2.36	13.8	12.55
28	68	84	R	3.9	3.55	7.2	6.55
29	61	66	R	0.8	0.73	7.1	6.45
30	55	65	P	1.0	0.91	6.6	6.00
36	47	50	O	0.4	0.36	7.7	7.00
37	67	75	R	0.7	0.64	7.4	6.73

Table A2: Individual oysters used in objective 1, Length (anterior–posterior axis) and height (dorsal–ventral axis), oyster category: R (recruit sized unable to pass through a 58 mm internal diameter ring), P (pre-recruits able to pass through a 58 mm internal diameter ring, but unable to pass through a 50 mm ring), and O (small oysters able to pass through a 50 mm internal diameter ring and down to 10 mm in length); and tissue size of the heart and gill combined (mg) and weight per volume in the DNA extraction reaction.

Oyster	Length (mm)	Height (mm)	Category	Tissue (mg)	Tissue (w/v %)
101	54	68	P	5.2	4.73
102	53	62	P	4.9	4.45
103	57	71	R	6.5	5.91
104	60	73	P	9.8	8.91
105	57	64	P	5.1	4.64
106	65	71	R	6	5.45
107	48	63	O	8.2	7.45
108	74	79	R	6.7	6.09
109	66	74	R	7.8	7.09
110	69	67	R	10.5	9.55
111	69	89	R	8.3	7.55
112	66	73	R	10.3	9.36
113	60	63	R	7.1	6.45
114	59	64	P	7.2	6.55
115	72	77	R	7.8	7.09
116	71	89	R	9.5	8.64
117	57	64	P	10.5	9.55
118	68	85	R	5.9	5.36
119	48	68	O	11	10.00
120	48	59	O	11.7	10.64
121	70	86	R	12	10.91
122	64	83	R	11.3	10.27
123	75	78	R	13.2	12.00
124	89	100	R	10.2	9.27
125	80	83	R	11.5	10.45
126	70	85	R	10.8	9.82
127	70	101	R	12.4	11.27
128	81	84	R	14	12.73
129	74	91	R	9.7	8.82
130	85	91	R	6.1	5.55
131	43	54	O	11.9	10.82
132	84	88	R	13.1	11.91

Table A3: Individual oysters used in objective 1, Length (anterior–posterior axis) and height (dorsal–ventral axis), oyster category: R (recruit sized unable to pass through a 58 mm internal diameter ring), P (pre-recruits able to pass through a 58 mm internal diameter ring, but unable to pass through a 50 mm ring), and O (small oysters able to pass through a 50 mm internal diameter ring and down to 10 mm in length);and tissue size of the heart and gill combined (mg) and weight per volume in the DNA extraction reaction. (A) were used with shaking and (B) were used with no shaking during the incubations.

(A)

Oyster #	Length (mm)	Height (mm)	Category	Heart (mg)	Heart (w/v %)	Gill (mg)	Gill (w/v %)	Heart and Gill (mg)	Heart and Gill (w/v %)
31	75	86	R	2.1	1.91	8.4	7.64		
32	63	77	R	3.2	2.91	6.4	5.82		
33	52	64	O	1.2	1.09	7.4	6.73		
34	50	55	O	1.4	1.27	7	6.36		
35	74	82	R	1.8	1.64	6	5.45		
133	73	90	R					11.2	10.18
134	76	90	R					10.2	9.27
135	67	79	R					17	15.45
136	73	79	R					9.4	8.55
137	69	89	R					14.4	13.09

(B)

Oyster #	Length (mm)	Height (mm)	Category	Heart (mg)	Heart (w/v %)	Gill (mg)	Gill (w/v %)	Heart and Gill (mg)	Heart and Gill (w/v %)
38	49	58	O	0.6	0.55	3.5	3.18		
39	59	75	P	1.7	1.55	7.4	6.73		
40	66	74	R	2.2	2.00	6.3	5.73		
41	69	68	R	1.7	1.55	5.7	5.18		
42	54	65	P	0.9	0.82	4.9	4.45		
138	72	74	R					9	8.18
139	75	77	R					13.9	12.64
140	71	71	R					10.9	9.91
141	82	96	R					10.1	9.18
142	88	88	R					12.4	11.27

Table A4: Individual oysters used in objective 2, Length (anterior–posterior axis) and height (dorsal–ventral axis), oyster category: R (recruit sized unable to pass through a 58 mm internal diameter ring), P (pre-recruits able to pass through a 58 mm internal diameter ring, but unable to pass through a 50 mm ring), and O (small oysters able to pass through a 50 mm internal diameter ring and down to 10 mm in length).

Oyster #	Length (mm)	Height (mm)	Category
201	81	81	R
202	104	118	R
203	83	97	R
204	88	99	R
205	88	106	R
206	85	104	R
207	82	87	R
208	90	98	R
209	78	92	R
210	79	85	R
211	83	96	R
212	72	83	R
213	81	93	R
214	82	96	R
215	88	102	R
216	82	97	R
217	77	89	R
218	79	92	R
219	69	104	R
220	90	98	R

Table A5: Details of oysters sampled to investigate the effect of taking imprints with heart tissues before qPCR sampling. All oysters were of recruit size. Their hearts excised and cut in half. One half was lightly blotted then 30 imprints made on a slide, and then sampled for qPCR. The other half was sampled directly for qPCR. Gill samples were also taken from each individual oyster. The oyster sample number is show with corresponding length and height.

Oyster number	Half sample blotted	Half sample Not blotted	Gill sample	Date qpcr	length (mm)	Height (mm)
201	yes	yes	yes	6/03/2013	81	81
202	yes	yes	yes	6/03/2013	104	118
203	yes	yes	yes	6/03/2013	83	97
204	yes	yes	yes	6/03/2013	88	99
205	yes	yes	yes	6/03/2013	88	106
206	yes	yes	yes	6/03/2013	85	104
207	yes	yes	yes	6/03/2013	82	87
208	yes	yes	yes	6/03/2013	90	98
209	yes	yes	yes	6/03/2013	78	92
210	yes	yes	yes	6/03/2013	79	85
211	yes	yes	yes	6/03/2013	83	96
212	yes	yes	yes	6/03/2013	72	83
213	yes	yes	yes	6/03/2013	81	93
214	yes	yes	yes	6/03/2013	82	96
215	yes	yes	yes	6/03/2013	88	102
216	yes	yes	yes	6/03/2013	82	97
217	yes	yes	yes	6/03/2013	77	89
218	yes	yes	yes	6/03/2013	79	92
219	yes	yes	yes	6/03/2013	69	104
220	yes	yes	yes	6/03/2013	90	98

Table A6: Chatham Island oyster Cq values for Bonamia OTS and β –actin gene from heart and gill tissues.

	Heart		Gill	
Oyster #	Bonamia	β -actin	Bonamia	β -actin
1		low amp		40.54
2		42.00		29.50
3		34.43		No amplification
4		No amplification		36.24
5		31.85		28.30
6		No amplification		No amplification
7		28.98		low amp
8		33.05		No amplification
9		26.50		No amplification
10		No amplification		38.94
11		42.00		No amplification
12		35.48		No amplification
13		29.49		30.47
13		26.78		Low amplification
14		No amplification		32.95
15		No amplification		No amplification
16		42.00		Low amplification
17		28.93		25.57
18		32.04		No amplification
20		32.06		Low amplification
21		28.99		28.86
22		30.40		No amplification
23		28.23		Low amplification
24		27.49	Not tested	
25		29.65		27.09
26		Low amplification		No amplification
27		34.10		Low amplification
28		Low amplification		26.12
29		Low amplification		25.51
30		28.18		27.90
31		24.53		No amplification
32		35.78		35.26
33		34.51		24.22
34		31.17		29.44
35		28.47		28.33
36		28.28		No amplification
37		30.81		25.20
38		31.18		37.58
39		28.92		Low amplification
40		30.47		No amplification

Table A6: Continued.

	Heart		Gill	
Oyster #	Bonamia	β -actin	Bonamia	β -actin
41		Low amplification		26.19
42		42.00		No amplification
43		31.50		34.46
44		32.49		26.55
45	36.13	31.96		27.65
46		29.24		25.45
47		30.51		36.38
48		33.02	Not tested	
49		34.75		36.44
50		Low amplification		32.39
51		33.71		Low amplification
52		30.09		27.40
53		36.13		30.25
54		34.65		37.60
55	1.49	No amplification		31.42
56		Low amplification		31.28
57		29.97		28.47
58		32.45		26.06
59	1.78	30.22		25.21
60		36.95	1.92	26.59
61		No amplification		25.35
62		33.35		33.00
63		Low amplification		37.99
64		Low amplification		No amplification
65		32.16		Low amplification
66		39.54		29.58
67		31.65		34.18
68		No amplification		No amplification
69		Low amplification		31.34
70		31.69		No amplification
71		No amplification		No amplification
72		No amplification	1.58	No amplification
73		31.59		33.56
74		Low amplification	not tested	
75		39.50		29.97
76		No amplification		No amplification
77		40.57		Low amplification
78		33.43	3.08	Low amplification
79		34.87		25.87

Table A6: Continued.

	Heart		Gill	
Oyster #	Bonamia	β -actin	Bonamia	β -actin
80		No amplification		No amplification
81		29.48		Low amplification
82		32.38		Low amplification
83		35.91		No amplification
84		36.16		Low amplification
85		35.39		No amplification
86		30.17		No amplification
87		30.68		23.71
88		31.64		25.37
89		30.05	41.49	29.89
90		26.02		27.46
91		17.19		24.96
92		32.18		26.90
93		32.52		26.15
94		31.44		25.27
95		32.27		24.58
96		40.70		24.66
97		35.00		24.86
98		27.14	not tested	
99		38.10		24.48
100		34.01		31.39

Appendix 2: Description of standard qPCR method for the detection and quantification of *Bonamia exitiosa* in Foveaux Strait oysters *Ostrea chilensis*.

Bonamia qPCR Summary for 2013 survey Method

Carry out work in Marine Ecology DNA Lab (i.e. sample working area)

Digestion of samples

1. **10ml buffer + 1ml proteinase K (10mg / ml)** in a sterile trough. Mix well.
2. Remove strip caps and add 110ul of working buffer solution into each well.
3. Cover with PCR plate with PCR grade sealing tape.
4. Centrifuge 96 well plate for 3000 RCF for 2 minutes @ 5°C.
5. **User: Els Programme: Bon digest, set volume for 100ul. 9600 Applied Biosystems thermoccyler file#137**
6. Centrifuge plate for 3000 RCF for 2 minutes @ 5°C.

Dilution to 1:20 of samples

1. Add PCR grade water to a sterile trough.
2. Dispense 76 µl of PCR grade water into a 96well plate.
3. Transfer 4 µl of sample from digest 96 well plate. Use a 5-100µl 12 place multichannel pipette and long filter tips. Check 2µl volume. Mix dilution by sucking up and down with tip.
4. Cover with PCR plate tape.
5. Centrifuge plate for 3000 RCF for 2 minutes @ 5°C.
6. Cover plate with PCR sealing plate tape.

qPCR set up (in Assay Lab)

1. To a defrosted pre-measured primer and probe master mix add 525 µl of Quanta qPCR tough mix.
2. Mix master mix by vortexing briefly and pulse centrifuge.
3. In biological safety cabinet, pour mastermix into a sterile trough.
4. Aliquot 6µl per well into a qPCR 96 well plate, seal plate and take to DNA lab.

Add sample (in DNA Extraction lab)

1. Transfer 4 µl of sample 1:20 dilution into each well containing the master mix.
2. Cover plate with BioRad qPCR grade sealing film.
1. Centrifuge plate for 3000 RCF for 2 minutes @ 5°C.
2. Load onto qPCR.

qPCR plate analysis (in Shared Marien Ecology Lab)

1. On desk top click in BIORAD-CFX manager, don't type in any pass word. Press OK.
2. Go to "create new experiment" . OK
3. In experiment set up , click into "select existing ".
4. Go to Bonamia and open up.
5. Go to "plate ", select existing , then click on "Quick plate 96 wells –Bonamia"
6. Go to lid and press open. Add in QPCR plate. Close lid.
7. Press "start run" bottom. Then start run again.
8. Save plate in Bonamia 2013 file. date, qpcr plate#, survey plate#

Appendix 3: Bonamia data record.

FOVEAUX STRAIT OYSTER BONAMIA DATA RECORD

Page ___ of ___

Date

Station no. | Day | Month | Year | Time NZST

Recorder

Comments

Oyster no	Length (mm)	Height (mm)	Size category [4]	Heat imprint score	Histology sample

1) Start a new form for each new station
2) Measure oysters to the nearest mm down
3) Check oysters for size; recruit (R), pre-recruit (P), and small recruit (O) size with 'oyster rings'.

Appendix 4: Differences in Cq values for FAM and Texas Red between runs from repeats of station 10, plate 26. Shaded cells denote dry wells.

Plate	Well_ Heart	Sample	Station	Oys	HF_R1	HF_R2	Diff_ HFAM	HT_R1	HT_R2	Diff_ HTR	Well_Gill	GF_R1	GF_R2	Diff_ GFAM	GTR_R1	GTR_R2	Diff_ GTR
26	A01	OH01	10	1	NA	NA	-	NA	40.12	-	B01	NA	34.49	-	30.25	23.74	6.51
26	C01	OH02	10	2	19.22	19.07	0.15	NA	NA	-	D01	25.86	27.54	1.68	NA	NA	-
26	E01	OH03	10	3	25.81	25.12	0.69	NA	NA	-	F01	22.53	21.84	0.69	19.72	18.98	0.74
26	G01	OH04	10	4	25.87	24.79	1.08	23.35	22.37	0.98	H01	26.77	26.39	0.38	22.9	22.6	0.3
26	A02	OH05	10	5	NA	NA	-	34.31	33.96	0.35	B02	35.59	35.22	0.37	23.13	23.03	0.1
26	C02	OH06	10	6	NA	36.58	-	33.15	32.26	0.89	D02	32.96	33.8	0.84	24.38	24.41	0.03
26	E02	OH07	10	7	28.9	29.16	0.26	NA	NA	-	F02	24.87	23.78	1.09	21.59	20.48	1.11
26	G02	OH08	10	8	22.86	22.34	0.52	NA	NA	-	H02	20.79	20.61	0.18	NA	20.18	-
26	A03	OH09	10	9	NA	39.27	-	30.43	30.19	0.24	B03	38.34	NA	-	23.07	23.3	0.23
26	C03	OH010	10	10	19.18	19.27	0.09	NA	NA	-	D03	22.76	23.21	0.45	20.01	20.58	0.57
26	E03	OH011	10	11	37.86	NA	-	26.38	26.23	0.15	F03	36.01	39.41	3.4	24.16	24.13	0.03
26	G03	OH012	10	12	31.95	31.79	0.16	NA	NA	-	H03	NA	NA	-	33.93	36.32	2.39
26	A04	OH013	10	13	NA	NA	-	33.98	34.25	0.27	B04	35	40.63	5.63	24.19	24.54	0.35
26	C04	OH014	10	14	NA	NA	-	27.38	27.74	0.36	D04	34.71	NA	-	24.37	23.94	0.43
26	E04	OH015	10	15	NA	35.81	-	30.94	30.98	0.04	F04	34.82	35.43	0.61	25.29	24.68	0.61
26	G04	OH016	10	16	38.06	38.06	0	32.01	32.38	0.37	H04	36.16	NA	-	24.53	25.03	0.5
26	A05	OH017	10	17	37.51	35.49	2.02	29.83	29.89	0.06	B05	34.92	34.09	0.83	23.06	23.63	0.57
26	C05	OH018	10	18	20.14	20.67	0.53	NA	NA	-	D05	18.83	18.83	0	NA	25.49	-
26	E05	OH019	10	19	21.09	20.87	0.22	NA	NA	-	F05	19.18	20.53	1.35	NA	NA	-
26	G05	OH020	10	20	35.26	37.72	2.46	28.9	29.08	0.18	H05	NA	NA	-	38.33	35.51	2.82
26	A06	OH021	10	21	NA	NA	-	29.77	29.18	0.59	B06	NA	38.01	-	24.52	25.79	1.27
26	C06	OH022	10	22	34.87	35.29	0.42	28.44	27.34	1.1	D06	32.75	32.67	0.08	23.66	23.83	0.17
26	E06	OH023	10	23	33.42	35.38	1.96	27.18	28	0.82	F06	38.61	37.57	1.04	24.05	25.12	1.07
26	G06	OH024	10	24	33.63	31.56	2.07	NA	NA	-	H06	19.24	NA	-	20.23	NA	-

Appendix 5: Graphs of relative fluorescence units (RFU) against cycle of quantification (Cq values) showing curves of different amplification patterns for Bonamia ITS and the β-actin gene.

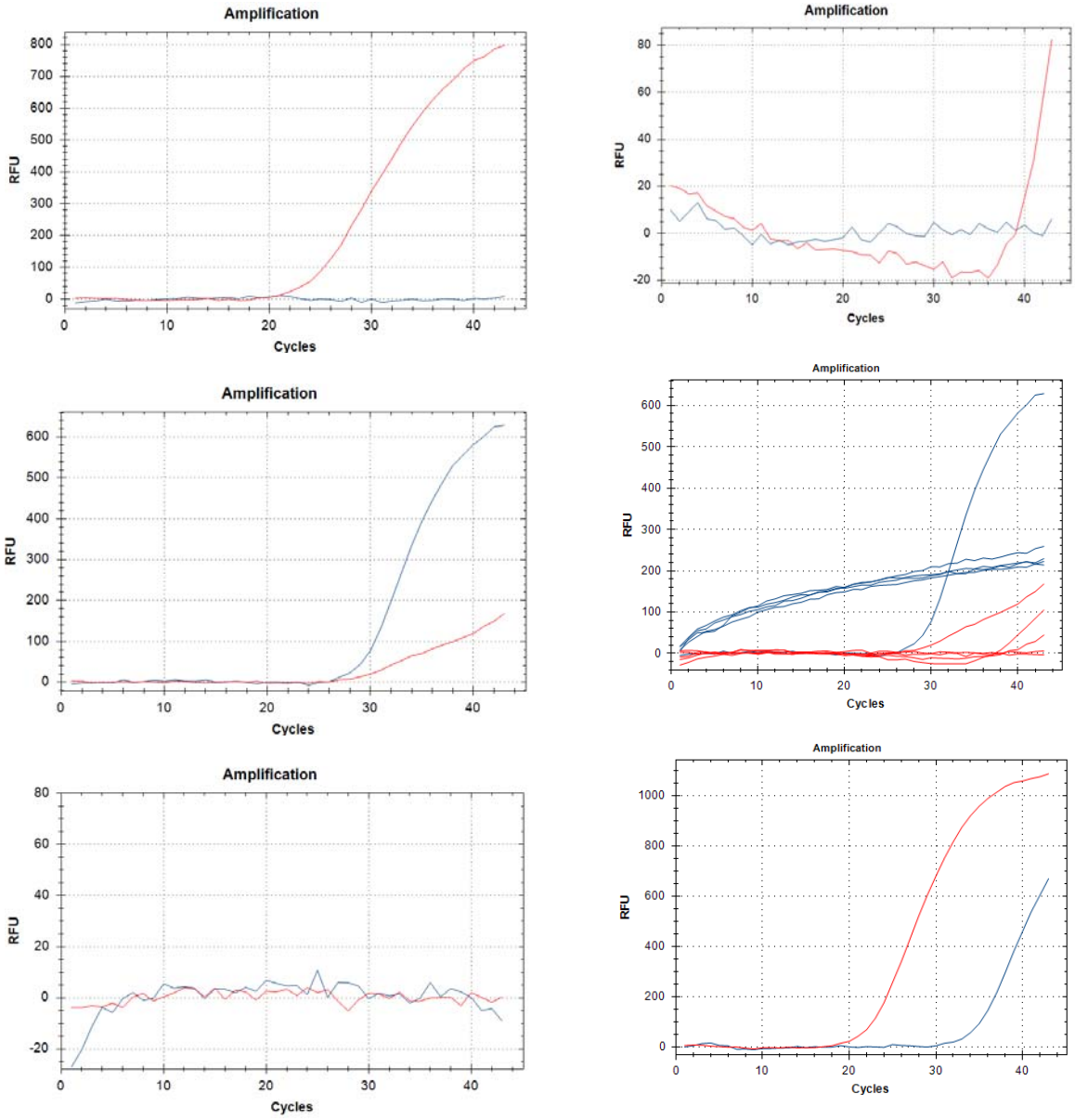

Figure A5.1: Graphs of relative fluorescence units (RFU) against cycle of quantification (Cq values) showing curves of different amplification patterns for Bonamia ITS and the β-actin gene: Top left going anticlockwise β-actin gene only, Bonamia ITS and β-actin gene, no reaction with either (flat liners), late and weak β-actin amplification, very early Bonamia amplification, and β-actin gene amplifying well before Bonamia. Blue lines denote Bonamia ITS gene amplification and red lines β-actin gene amplification.