Aquatic Animal Health Subprogram: Determination of susceptibility of various abalone species and populations to the various known AbHV genotypes

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Abbreviations

AAHL	Australian Animal Health Laboratory
AbHV	Abalone herpesvirus
AVG	Abalone viral ganglioneuritis
CSIRO	Commonwealth Scientific and Industrial Research Organisation
df	Degrees of freedom
Dil	Dilution
GSW	Great Southern Waters
L	Litre
mg	Milligram
mm	Millimetre
neg ctrl	Negative control
OIE	World Organisation for Animal Health (Office International des Épizooties)
pos ctrl	Positive control
SA	South Australia
Std Dev:	Standard Deviation
Tas	Tasmania
Vic	Victoria
WA	Western Australia

Executive Summary

Background

Abalone viral ganglioneuritis (AVG) caused by infection with abalone herpesvirus (AbHV) was first detected in Victorian abalone in late 2005/early 2006 and contributed to a reduced wild-catch fishery from 1614 tonnes in 2004/05 to a low of 827 tonnes in 2010/11 in Victoria. Subsequently, there has been a recovery in volume of production to 1196 tonnes in 2012/13. Interestingly, abalone herpesvirus (AbHV) was also detected in Tasmania from 2008 but there was no disease in wild stocks and volume of production in Tasmania has remained relatively steady at 2500-3000 tonnes since 1998/99. The need to understand the epidemiology of the various virus subtypes and their effects on abalone stocks and species and requirement for biosecurity incited us to undertake this study. Information acquired will allow authorities to improve management plans, including avoiding possible translocation of the disease where susceptible abalone species are present. Previous research on AbHV was successful in developing an experimental infection model at CSIRO Australian Animal Health Laboratory high-biosecure aquarium facility for studying pathogenesis in target species. This model was used to determine whether or not *Haliotis rubra* (blacklip), *H. laevigata* (greenlip) and *H. conicopora* (brownlip), originating from various States in Australia, are susceptible to infection by the five known AbHV subtypes and/or abalone viral ganglioneuritis (AVG). This two-year study was undertaken using a well-studied positive control hybrid Jade Tiger abalone (*H. laevigata X H. rubra*) obtained from a commercial farm (Great Southern Waters Pty Ltd (GSW; now known as Craig Mostyn Group Jade Tiger abalone Pty Ltd)). In addition, the genomes of AbHV subtypes Tas3, Tas4 and Tas5 were fully sequenced. Analysis and alignment with previously sequenced AbHV subtypes (Vic1, Tas1 and Tas2) were performed.

Aims/Objectives

1 Determine the susceptibility of greenlip, blacklip, hybrid and other readily available abalone species to abalone herpesvirus (AbHV) genotypes.

2 Determine the complete genome sequences for AbHV Tas3 and Tas4 to gain insights into how and over what timeframe they have arisen, whether genetic recombination is contributing to this variation and which genome regions might affect virulence, as well as instructing on how diagnostic methods for their detection and differentiation can be refined.

Methodology

Objective 1: The susceptibility of greenlip, blacklip and brownlip abalone species to known AbHV genotypes was determined in bioassays using the biosecure aquarium facilities at CSIRO AAHL. Abalone (1-2 years old), particularly species of high commercial value, were obtained from various States in Australia with the assistance of private aquaculture companies and Government agencies (e.g. Tasmania, Victoria, South Australia and Western Australia). These abalone were sourced from regions with no history of disease and transported to AAHL. Experimental groups of 8 abalone were exposed by immersion to a low, medium or high dose of AbHV inoculum using standardised bioassay procedures developed in FRDC Project 2009/032. Uninfected controls were housed in a separate room of the biosecure aquarium facility. Known susceptible abalone (1-2 year old hybrids sourced from Craig Mostyn Group Jade Tiger abalone Pty Ltd, previously known as Great Southern Waters Pty Ltd (GSW)) were used as positive controls. Abalone were monitored daily for gross

disease signs for 9 days post-exposure. Tissue samples were collected from abalone exhibiting disease signs or that had died since they were last observed, as well as any that survived the 9-day bioassay period, and processed subsequently to assess AbHV infection severity by histology and/or PCR. The use of 3 dilutions of each inoculum prepared to the AbHV Vic1, Tas1, Tas2, Tas3 and Tas4 subtypes allowed evaluation of their pathogenicity in different abalone species from different geographical regions using the susceptible GSW hybrid abalone as a benchmark positive control. Quantitative PCR (qPCR) was used to evaluate the viral loads in each sample tissue.

Objective 2: The complete genome sequences of the most recently identified AbHV Tas3 and Tas4 subtypes were determined from DNA amplified from preparations of AbHV semi-purified from infected abalone using procedures developed in FRDC Project 2009/032 (the whole genome sequences for Tas1 and Tas2 have been obtained previously). Because the Tas-5 sub-type became available during the course of the project we also performed its full sequencing and alignment. Briefly, ganglia in AbHV-infected and diseased abalone were harvested in such a way as to minimise the amount of muscle tissue collected. The harvested ganglia were homogenised and the homogenate subjected to various rounds of gradient ultra-centrifugation. Virus-containing fractions (monitored by qPCR) were pooled to obtain a semi-purified viral preparation. DNA from this semi-purified viral fraction was extracted and amplified using a multiple-displacement method prior to preparation for whole genome sequencing using next gen (454 and/or Illumina) technology. Sequences obtained were used to construct the whole viral genome for Tas3, Tas4 and Tas5 which were compared to each other and to AbHV Vic1, to Tas1 and to Tas2 genotype sequences obtained in FRDC Project 2009/032. Such comparisons helped identify genome regions that are most conserved or most consistently divergent in an attempt to associate specific sequences with differences in infectivity and/or pathogenicity of these subtypes as determined in the bioassays, as well as provide information on any need to refine PCR tests used for diagnosis and epidemiology.

Results

Results demonstrated that all abalone tested are susceptible to infection by the five AbHV subtypes used (Vic1, Tas1, Tas2, Tas3, Tas4). Due to limited availability of healthy greenlip abalone from WA, challenge trials using AbHV Tas2, Tas3 and Tas4 could not be performed. Histopathological analysis performed on moribund abalone did not show differences in AVG lesions between virus subtypes or abalone species.

Sequence analysis of AbHV Tas3, Tas4 and Tas5 revealed high identity/homology with the previously sequenced AbHV subtypes (Vic1, Tas1 and Tas2).

Implications for relevant stakeholders

AVG remains a serious concern to all State jurisdictions with significant commercial investments in abalone fisheries and aquaculture. This study emphasised the potential risk of all known subtypes of AbHV to infect the main Australian abalone species of commercial interest.

Recommendations

All abalone species in Australia, including those that have not been tested in this project, should *a priori* be considered likely to be susceptible to infection with AbHV and the disease AVG since so far no species in Australia has demonstrated significant resistance to infection to AbHV subtypes by experimental infection.

Keywords

Abalone viral ganglioneuritis (AVG); abalone herpesvirus (AbHV); experimental challenge; susceptibility to AVG; abalone; *Haliotis laevigata*; *Haliotis rubra; Haliotis conicopora*.

Introduction

Abalone viral ganglioneuritis (AVG), caused by infection with abalone herpes virus (AbHV), was first reported in late 2005 from abalone farms in Victoria (Hooper *et al*., 2007). Since then it has been recorded in wild abalone populations along a restricted length of the Victorian coastline. Interestingly, while AVG has not been reported to have ever occurred in wild abalone in Tasmania, disease outbreaks have occurred in Tasmanian processing plants during 2008 to 2012 and surveys have shown positive PCR results from a small number of abalone in the wild. Of further interest, AbHV subtypes causing these disease occurrences have exhibited genetic diversity requiring refinement to existing diagnostic tests to provide robust detection and identification (see FRDC Projects 2007/006 and 2009/032). The existence of genetic variants (subtypes) of AbHV in wild Tasmanian abalone populations that do not cause disease under normal circumstances raised a number of issues of concern to both industry and regulators including (i) the ability of available tests to detect sub-clinical infections (see FRDC Project 2009/032), (ii) the geographical range of AbHV as opposed to where disease has occurred, (iii) the biosecurity risks of sourcing wild broodstock for aquaculture purposes (see FRDC Project 2011/046) and (iv) the pathogenic potential of AbHV variants existing in abalone populations inhabiting distinct geographical regions within Australia. These issues have been discussed with various stakeholders including Great Southern Waters Pty Ltd (now known as Craig Mostyn Group Jade Tiger Abalone Pty Ltd); Australian Abalone Growers Association; Department of Primary Industries, Parks, Water & Environment, Tasmania; Department of Fisheries WA; FRDC Aquatic Animal Health Subprogram, and this research project emanated from these discussions. Specifically, this project aims to extend previous research to obtain a better understanding of the pathogenicity of known AbHV subtypes and on the susceptibility of three abalone species of major commercial importance in Australia. In addition, the project aims at gaining knowledge on viral genomic factors important in determining pathogenicity.

Need

With the emergence of AVG in Victorian abalone in 2005/6, the subsequent discovery of several genetic variants of the causative agent, AbHV, associated with disease outbreaks in Tasmanian processing plants, and apparent variations in AbHV pathogenicity observed in different abalone species, there is the need to understand what factors precipitate disease in infected hosts. This project aims to address this knowledge gap and is relevant to all jurisdictions with abalone fisheries/aquaculture. Access to biosecure aquarium facilities, major abalone species and all known AbHV subtypes provides CSIRO-AAHL with a unique capability to investigate which factors influence disease outcomes following infection, including the nature of the AbHV genotype as well as abalone species or geographic origins from locations spanning the primary wild fisheries in Victoria, Tasmania, Western Australia and South Australia. An improved understanding of the susceptibility of abalone of different species, and origins, to infection by the various AbHV subtypes, and whether or not different subtypes vary in their potential for transition from sub-clinical infection to acute disease is important for informing fisheries and aquaculture policies and strategies to avoid infection and disease. This project addresses specific priorities detailed in the current FRDC Aquatic Animal Health Subprogram R&D Plan (viz. 6.2.1 Nature of disease and host-pathogen interaction), priorities identified in the National Abalone Health Work Plan developed by a committee of national representatives following the second Abalone Virus Scientific and Management Forum held in Melbourne in 2007, and FRDC's 2013 Annual Competitive Round Call for Expressions Of Interest (viz. Determining risk factors for AVG).

Objectives

1. Determine the susceptibility of greenlip, blacklip, brownlip to abalone herpesvirus (AbHV) genotypes.

2. Determine the complete genome sequences for AbHV Tas3, Tas4 and Tas-5 to gain insights into how and over what timeframe they have arisen, whether genetic recombination is contributing to this variation and which genome regions might affect virulence, as well as instructing on how diagnostic methods for their detection and differentiation can be refined.

Methodology

Abalone herpesvirus subtypes

The subtypes used for this study originated from diagnostic submissions to AAHL during the period from 2006 to 2012. Following completion of the diagnostic testing, tissues were used as the inoculum to expand each of the subtypes in Jade Tiger hybrid abalone for storage in liquid nitrogen and future use. The origin of each of the AbHV subtypes is shown in Table 1.

Table 1. Origin of AbHV subtypes used in this study

AbHV subtype	Date isolated	Location and abalone species of origin
Vic1	Jan 2006	Victoria/Greenlip
Tas1	Aug 2008	Tasmania/n.a.
Tas2	Aug2009/Nov 2011	Tasmania/NSW/Blacklip/Greenlip
Tas3	Dec 2010/Jan 2011	Tasmania/Blacklip
Tas4	Feb 2011	Tasmania/ Blacklip
Tas5	Nov 2011	Tasmania/NSW/Greenlip

n.a.: info not available

Preparation of inocula

Jade Tiger hybrid abalone were obtained from GSW, Indented Head, relocated to AAHL and allocated to each of the experimental groups (to be inoculated with either AbHV subtype Vic1, Tas1, Tas2, Tas3 or Tas4) for production of infectious water to be used in the challenge experiments.

To prepare the inocula for the challenge experiments a standard dose (determined previously: 100 µL of an AbHV stock containing ~10^4 gene copies) of stock virus was inoculated intra-muscularly into each of 15 GSW blacklip x greenlip hybrid abalone (~70mm). Virus-inoculated abalone were placed in a single tank and maintained for 4-5 days for the collection of shed virus. 100% water exchange was carried out daily. At day 4 (for Vic1 and Tas1, Tas3, Tas4 subtypes) and day 5 (for Tas2 subtype) post-injection 20 litres of the infectious water (containing shed AbHV) were collected and used to make three serial dilutions (1:2, 1:10 and 1:100) to be used for the challenge experiments. [NB. The use of three viral doses ensures that morbidity/mortality is induced within an appropriate range for at least one dose in susceptible abalone species. Dilution 1:2 gave the most meaningful results and was used for mortality curves graphs in the result section].

Challenge experiments

For the susceptibility trials, experimental abalone were obtained from the following farms:

- South Australian greenlip (grown in Victoria): 68 Snapper Point Rd, Allestree, VIC 3305
- Western Australian greenlip and brownlip: 888 Abalone Pty Ltd, Bremer Bay WA 6338
- Victorian greenlip and blacklip: Ocean Wave Seafoods Pty Ltd, Victoria 3212
- Tasmanian greenlip and blacklip Abtas Marketing Pty Ltd, Beaconsfield, Tasmania 7270

Each group of experimental abalone and positive control hybrid abalone were placed in their experimental groups and allowed to acclimate in the AAHL aquarium. Following acclimation, experimental abalone were bathed in their respective treatment water in individual buckets - AbHV infectious water (3 dilutions) in individual tanks overnight: Abalone were placed in 2-litre tanks (one abalone/tank) and challenged individually for 20 hours before the infectious water was replaced by fresh seawater in each tank. From then on abalone were monitored daily and water changed every other day for 9 to 10 days.

Positive controls included 70-mm GSW hybrid abalone (known to be susceptible to the virus) exposed to the 1:10 dilution to confirm pathogenicity of the virus. Greenlip abalone were also maintained in a virus-free room as negative controls.

Animals were monitored daily and experiments were terminated on day 9 or 10 post-immersion. Samples were taken from any moribund and dead abalone during the daily monitoring and processed for laboratory analysis.

Laboratory analyses

Samples were collected for qPCR analysis using the ORF-66 primers and probe (OIE, 2015) and/or histological examination to determine presence of AbHV and tissue lesions typical of AVG (Hooper et al., 2007; Corbeil et al., 2012). Log-rank (Mantel-Cox) test was run on mortality curves to determine any statistical difference.

Whole genome sequence analysis

AbHV Tas3, Tas4 and Tas5 subtypes were determined from DNA amplified from preparations of AbHV semi-purified from infected abalone using procedures developed in FRDC Project 2009/032 (the whole genome sequences for Tas1 and Tas2 have been obtained previously). Briefly, ganglia in AbHV-infected and diseased abalone were harvested in such a way as to minimise the amount of muscle tissue in the harvested tissue. The harvested ganglia were homogenised and the homogenate subjected to various rounds of gradient ultra-centrifugation. Virus-containing fractions (monitored by qPCR) from the centrifugations were pooled to obtain a semi-purified viral preparation. DNA from this semi-purified viral fraction was extracted and amplified using a multiple-displacement method prior to preparation for whole genome sequencing using next gen (454 and/or Illumina) technology (Tan *et al*., 2008). Sequences obtained were used to construct the whole viral genomes for Tas3, Tas4 and Tas5 (PhylosSift V1.01 software) which were compared to each other and to AbHV Vic1 and to Tas1 and Tas2 subtype sequences obtained in FRDC Project 2009/032. Such comparisons helped to identify genome regions that are most conserved or most consistently divergent in an attempt to associate specific sequences with differences in infectivity and/or pathogenicity of these subtypes as determined in the bioassays, as well as provide information on any need to refine PCR tests used for diagnosis and epidemiology.

Results, Discussion and Conclusion

Objective 1: Determine the susceptibility of greenlip, blacklip, brownlip to abalone herpesvirus (AbHV) genotypes

Cumulative Mortality

Low to high levels of morbidity and mortality occurred in groups of challenged abalone over the project trials (Figures 1a-e). Histopathology typical of AVG was observed in samples taken from moribund abalone (not shown). No mortality occurred and no abnormal histology was observed among the negative control abalone. Based on observations in the negative control group of brownlip abalone, the white matter of the nerve cords is much more cellular than most greenlip, blacklip and hybrid abalone that have been examined at AAHL.

Mortality curves of abalone species challenged with AbHV subtypes dilution 1:2

Figure 1a shows that all abalone species from all four States were susceptible to AbHV Vic1 subtype. Morbidity began within three days post-challenge as typically seen with this subtype in previous studies.

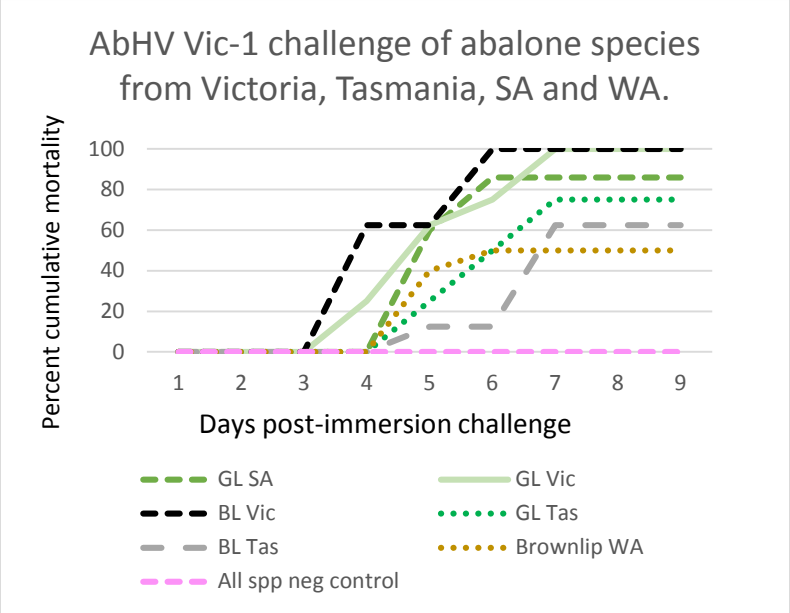


Figure 1a. Cumulative mortality curves for immersion trial of Victorian greenlip and blacklip, Tasmanian greenlip and blacklip, SA greenlip and WA brownlip abalone, challenged with AbHV Vic1. The difference between final cumulative mortality and 100% represents percentage survival. SA= South Australia, WA= Western Australia, GL=greenlip, BL=blacklip. Note that curves from abalone originating from different States cannot be compared with each other as they were not challenged on the same day and therefore not with the same batch of infectious water.

Figure 1b shows that all abalone species from all four States were also susceptible to AbHV Tas1 subtype. Morbidity began between three to five days post-challenge.

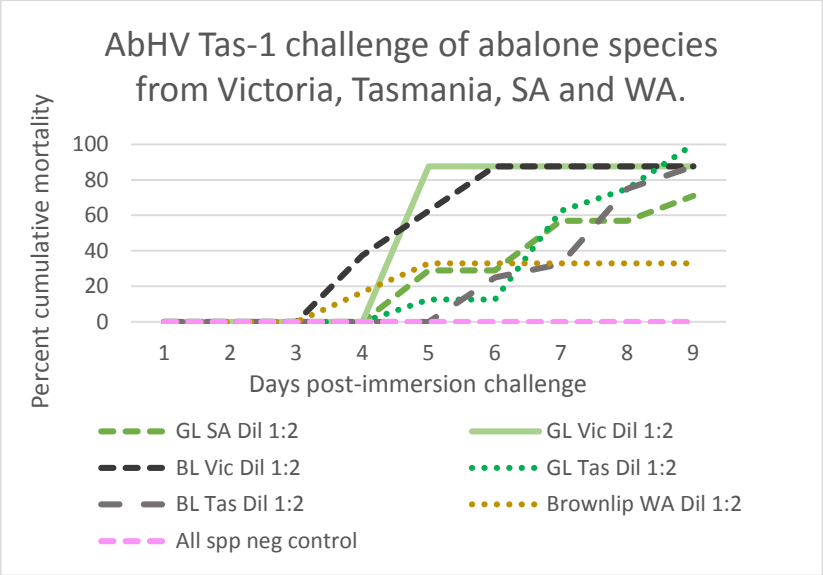


Figure 1b. Cumulative mortality curves for immersion trial of Victorian greenlip and blacklip, Tasmanian greenlip and blacklip, SA greenlip and WA brownlip abalone, challenged with AbHV Tas1. The difference between final cumulative mortality and 100% represents percentage survival. SA= South Australia, WA= Western Australia, GL=greenlip, BL=blacklip. Note that curves from abalone originating from different States cannot be compared with each other as they were not challenged on the same day and therefore not with the same batch of infectious water.

Figure 1c shows that all abalone species from all four States were also susceptible to AbHV Tas2 subtype. Morbidity began between two to four days post-challenge.

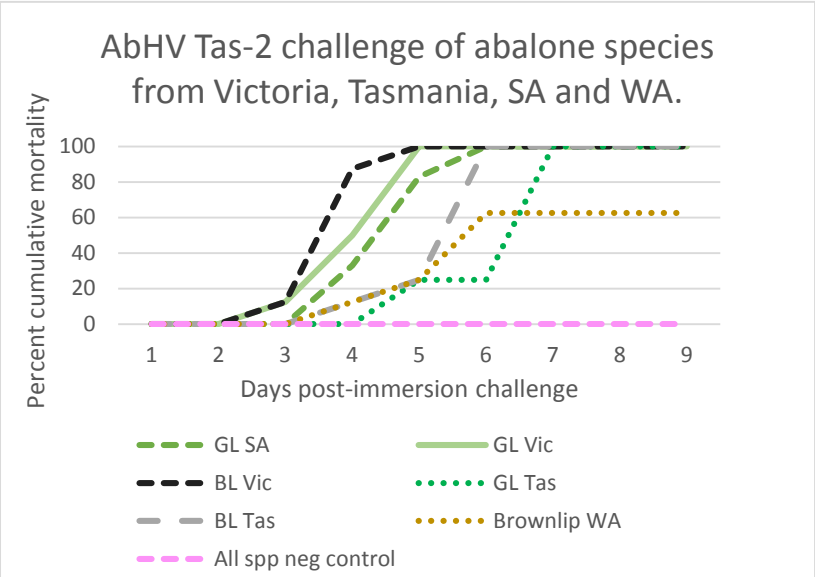


Figure 1c. Cumulative mortality curves for immersion trial of Victorian greenlip and blacklip, Tasmanian greenlip and blacklip, SA greenlip and WA brownlip abalone, challenged with AbHV Tas2. The difference between final cumulative mortality and 100% represents percentage survival. SA= South Australia, WA= Western Australia, GL=greenlip, BL=blacklip. Note that curves from abalone originating from different States cannot be compared with each other as they were not challenged on the same day and therefore not with the same batch of infectious water.

Figure 1d shows that all abalone species from all four States were also susceptible to AbHV Tas3 subtype. Morbidity began between three to four days post-challenge.

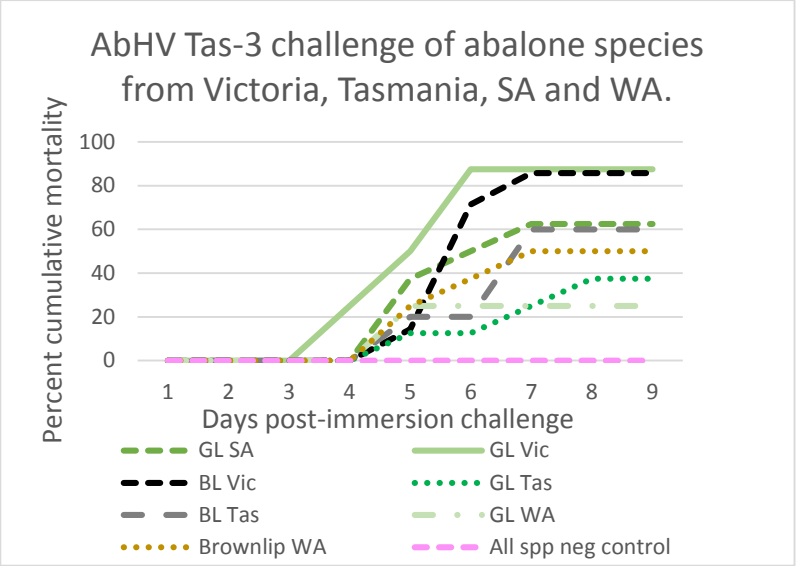


Figure 1d. Cumulative mortality curves for immersion trial of Victorian greenlip and blacklip, Tasmanian greenlip and blacklip, SA greenlip and WA brownlip and greenlip abalone, challenged with AbHV Tas3. The difference between final cumulative mortality and 100% represents percentage survival. SA= South Australia, WA= Western Australia, GL=greenlip, BL=blacklip. Note that curves from abalone originating from different States cannot be compared with each other as they were not challenged on the same day and therefore not with the same batch of infectious water.

Figure 1e shows that all abalone species from all four States were also susceptible to AbHV Tas4 subtype. Morbidity began at day three up to day 6 post-challenge.

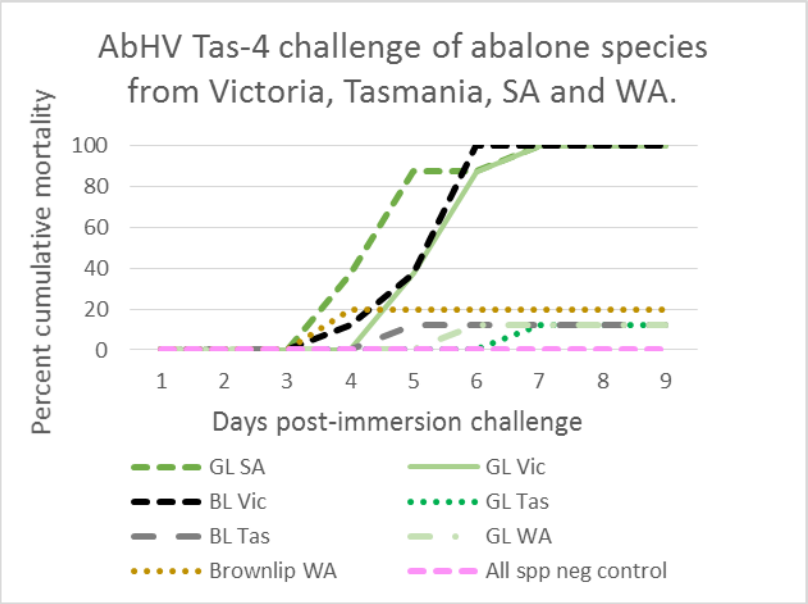


Figure 1e. Cumulative mortality curves for immersion trial of Victorian greenlip and blacklip, Tasmanian greenlip and blacklip, SA greenlip and WA brownlip and greenlip abalone, challenged with AbHV Tas4. The difference between final cumulative mortality and 100% represents percentage survival. SA= South Australia, WA= Western Australia, GL=greenlip, BL=blacklip. Note that curves from abalone originating from different States cannot be compared with each other as they were not challenged on the same day and therefore not with the same batch of infectious water.

Comparative susceptibility of Victorian greenlip and blacklip abalone to AbHV Vic1, Tas1, Tas2, Tas3 and Tas4.

Log-rank (Mantel-Cox) test (GraphPad Prism) demonstrated no significant difference in mortality between Victorian greenlip and blacklip abalone challenged with all AbHV subtypes at all dilutions used (results not shown), with the exception of subtype Tas-2 at dilution 1:10 (Table 2).

Table 2. Log-rank (Mantel-Cox) test on Victorian greenlip and blacklip abalone challenged with AbHV Tas4

Victorian greenlip and blacklip	AbHV Tas2 Dilution 1:10
Chi square	9.0
df	1
P value	0.002
Curves significantly different (p< 0.05)	Yes

Comparative susceptibility of Tasmanian greenlip and blacklip abalone to AbHV Vic1, Tas1, Tas2, Tas3 and Tas4

Log-rank (Mantel-Cox) test demonstrated no significant difference in mortality between Tasmanian greenlip and blacklip abalone challenged with all AbHV subtypes at all dilutions used (results not shown), with the exception of subtypes Tas2 at dilution 1:2 and Tas3 at dilution 1:10 (Table 3).

Table 3. Log-rank (Mantel-Cox) test on Tasmanian greenlip and blacklip abalone

Victorian greenlip and blacklip	AbHV Tas2 Dilution 1:2	AbHV Tas3 Dilution 1:10
Chi square	5.6	5.04
df	1	1
P value	0.01	0.02
Curves significantly different (p< 0.05)	Yes	Yes

Comparative susceptibility of Western Australian greenlip and blacklip abalone to AbHV Tas3 and Tas4.

Log-rank (Mantel-Cox) test demonstrated no significant difference in mortality between Western Australian greenlip and blacklip abalone challenged with all AbHV subtypes at all dilutions used (results not shown).

Summary tables of viral load in tissues greenlip, blacklip and brownlip abalone.

Table 4a: Viral load/mg tissue in Victorian blacklip abalone

Dilution\subtype	Vic1	Tas1	Tas2	Tas3	Tas4
1:2 Mean	6.72E+08	4.56E+07	1.20E+08	1.66E+07	2.29E+07
Std Dev	8.05E+08	3.47E+07	1.13E+08	1.04E+07	1.74E+07
1:10 Mean	1.75E+08	3.77E+07	1.75E+08	1.41E+07	1.97E+07
Std Dev	1.82E+08	1.78E+07	1.14E+08	1.70E+07	1.89E+07
1:100 Mean	2.86E+08	2.09E+07	2.18E+08	5.25E+06	1.01E+07
Std Dev	4.59E+08	1.33E+06	3.77E+08	6.05E+06	1.39E+07

Table 4b: Viral load/mg tissue in Victorian greenlip abalone

Dilution\subtype	Vic1	Tas1	Tas2	Tas3	Tas4
1:2 Mean	1.60E+08	9.97E+06	1.04E+08	9.52E+05	2.22E+06
Std Dev	1.10E+08	1.21E+07	7.83E+07	1.47E+06	1.87E+06
1:10 Mean	3.35E+07	1.29E+07	6.62E+07	3.47E+04	7.60E+06
Std Dev	5.32E+07	2.58E+07	6.31E+07	6.02E+04	1.87E+06
1:100 Mean	1.01E+08	1.05E+06	n/a	1.67E+00	4.88E+05
Std Dev	1.42E+08	7.38E+05	n/a	1.54E+00	7.38E+05

Table 4c: Viral load/mg tissue in Tasmanian blacklip abalone

Dilution\subtype	Vic1	Tas1	Tas2	Tas3	Tas4
1:2 Mean	1.86E+07	1.91E+06	8.10E+07	2.05E+06	2.26E+06
Std Dev	2.67E+07	2.14E+06	5.36E+07	2.85E+06	1.53E+13
1:10 Mean	6.13E+06	9.20E+06	8.63E+06	6.93E+00	n/a
Std Dev	8.66E+06	1.48E+07	1.75E+07	1.76E+00	n/a
1:100 Mean	n/a	1.91E+06	3.66E+02	n/a	n/a
Std Dev	n/a	4.09E+06	5.60E+02	n/a	n/a

Table 4d: Viral load/mg tissue in Tasmanian greenlip abalone

Dilution\subtype	Vic1	Tas1	Tas2	Tas3	Tas4
1:2 Mean	4.10E+07	4.56E+06	1.51E+07	4.28E+06	2.06E+01
Std Dev	3.37E+07	7.18E+06	1.26E+07	5.43E+06	2.11E+01
1:10 Mean	9.93E+05	2.60E+06	1.62E+07	3.65E+06	n/a
Std Dev	1.72E+06	3.83E+06	3.18E+07	3.54E+06	n/a
1:100 Mean	00E+00	4.19E+04	1.44E+05	2.21E+01	n/a
Std Dev	00E+00	9.36E+04	3.15E+05	2.55E+01	n/a

Table 4e: Viral load/mg tissue in South Australian greenlip abalone

Dose\Subtype		Vic1	Tas1	Tas2	Tas3	Tas4
1:2	Mean:	2.82E+07	4.31E+06	2.21E+07	2.40E+07	2.03E+07
	Std Dev:	4.44E+07	3.63E+06	1.75E+07	1.75E+07	2.92E+07
1:10	Mean:	4.77E+07	n/a	1.07E+07	2.31E-05	3.97E+07
	Std Dev:	6.74E+07	n/a	9.15E+06	2.80E-05	5.08E+07
1:100	Mean:	3.49E+06	3.95E+06	4.11E+07	3.18E-04	1.54E+07
	Std Dev:	4.93E+06	3.78E+06	4.21E+07	5.90E-04	2.65E+07

Table 4f: Viral load/mg tissue in Western Australian greenlip abalone

Dose\Subtype		Vic1	Tas1	Tas2	Tas3	Tas4
1:2	Mean:	n/a	n/a	n/a	2.56E00	3.83E+05
	Std Dev:				2.79E00	6.63E+05
1:10	Mean:	n/a	n/a	n/a	4.86E00	2.84E00
	Std Dev:				2.26E00	1.18E00
1:100	Mean:	n/a	n/a	n/a	1.60E+05	4.42E+02
	Std Dev:				2.70E+05	6.16E+02

n/a: not available

Table 4g: Viral load/mg tissue in Western Australian brownlip abalone

Dose\Subtype		Vic1	Tas1	Tas2	Tas3	Tas4
1:2	Mean:	5.49E+04	1.05E+05	1.42E+04	1.72E+07	1.50E+05
	Std Dev:	1.70E+05	1.98E+05	2.46E+04	2.65E+07	2.35E+05
1:10	Mean:	n/a	n/a	0.00E+00	n/a	n/a
	Std Dev:	n/a	n/a	0.00E+00	n/a	n/a
1:100	Mean:	n/a	8.42E-01	0.00E+00	n/a	n/a
	Std Dev:	n/a	1.27E+00	0.00E+00	n/a	n/a

n/a: not available

Histological analysis

Results showed histological lesions typical of AVG in neural tissues from all abalone challenged with AbHV. No histopathological differences were seen between abalone species or virus subtype involved. Additionally all abalone showing AVG lesions were also positive by qPCR. All negative control mock challenged abalone were negative by histological analysis and qPCR. Only one difference was seen within the white matter of the nerve cords in uninfected brownlip abalone which are much more cellular than uninfected greenlip, blacklip and hybrid abalone.

Discussion

Mortality curve interpretation

Amongst the three virus dilutions (doses) used to perform the challenges, the 1:2 dilution induced in all trials (with the exception of two groups of abalone) the highest levels of mortality. It is of interest that a gradual loss in pathogenicity amongst virus subtypes was noticed in the last 12 months of the project. This was likely due to long-term storage of the virus stocks in liquid nitrogen. Repeated trials with freshly grown virus gave marginally higher percent mortality. Overall, the challenge methodology was sufficiently reproducible to evaluate the susceptibility of abalone species to various viral subtypes. With regards to the production of infectious water for the immersion challenges, we noticed highly variable molecular viral titers between experiments during the course of this project. This variation made it impossible to compare mortality rates between abalone species originating from different states. Indeed, because titration of AbHV in cell culture has not yet been successful and molecular titration measures viral DNA present and is not a measure of virus infectivity, precise comparative pathogenicity is not yet achievable. Also, although this study tested abalone of similar age and size, a previous study showed that both juvenile and mature stages were susceptible to infection and disease (Crane et al., 2013).

Comparative susceptibility between abalone species (within a given trial)

For most trials we did not observe significant differences between greenlip and blacklip or brownlip. However, in three cases (Table 2 and Table 3) a statistical difference was shown. It is likely that variation in the size/age between abalone species tested influenced apparent susceptibility rather than a species-specific host factor. A characterization of the host gene expression upon viral infection of each abalone species could shed some light on possible difference, however, only a clear difference in mortality between species would justify such undertaking. It is interesting to note that abalone of Tasmanian origin were susceptible to infection and disease following experimental exposure to AbHV subtypes of Tasmanian origin. These observations suggest that the under the right conditions (involving environmental factors and viral dose, for example) infection and disease in Tasmanian wild abalone could occur similar to the disease outbreaks in live holding facilities and farms.

Viral titers in tissues

Viral titers in abalone tissues varied considerably in all dilution groups as shown by high standard deviations suggesting that there was no consistency between animal with regards to virus replication in their tissues (Tables 4a to 4f). During trials, abalone surviving the challenge would often show presence of virus but at lower titers than moribund/dead abalone. These observations suggest that exposure of some abalone resulted in a lower infection rate during the

immersion challenges and the infection process was consequently slower in these survivors than in abalone that showed morbidity signs or died within 9 days. Alternatively, some level of resistance in specific abalone may also lead to delayed viral replication. This phenomenon was observed in a previous study conducted in our laboratory (FRDC Project No. 2011/003). However, it was not possible to determine the specific cause of this delay in the infection process within the current project.

Due to logistic issues and the long distance between the Western Australian farm and AAHL, the WA greenlip abalone did not always arrive in a healthy state at Geelong and it was not possible to undertake studies with AbHV subtypes Vic1, Tas1 and Tas2. However, based on the results obtained with other abalone, it is most likely that the WA greenlip would also be susceptible to these subtypes.

Conclusion

Results obtained in this study show that AbHV subtypes Vic1, Tas1, Tas2, Tas3 and Tas4 are able to infect abalone via natural routes and can cause disease and mortality in greenlip, blacklip and brownlip species originating from Victoria, Tasmania and Western Australia. No histopathological differences were seen between abalone species and AbHV subtypes.

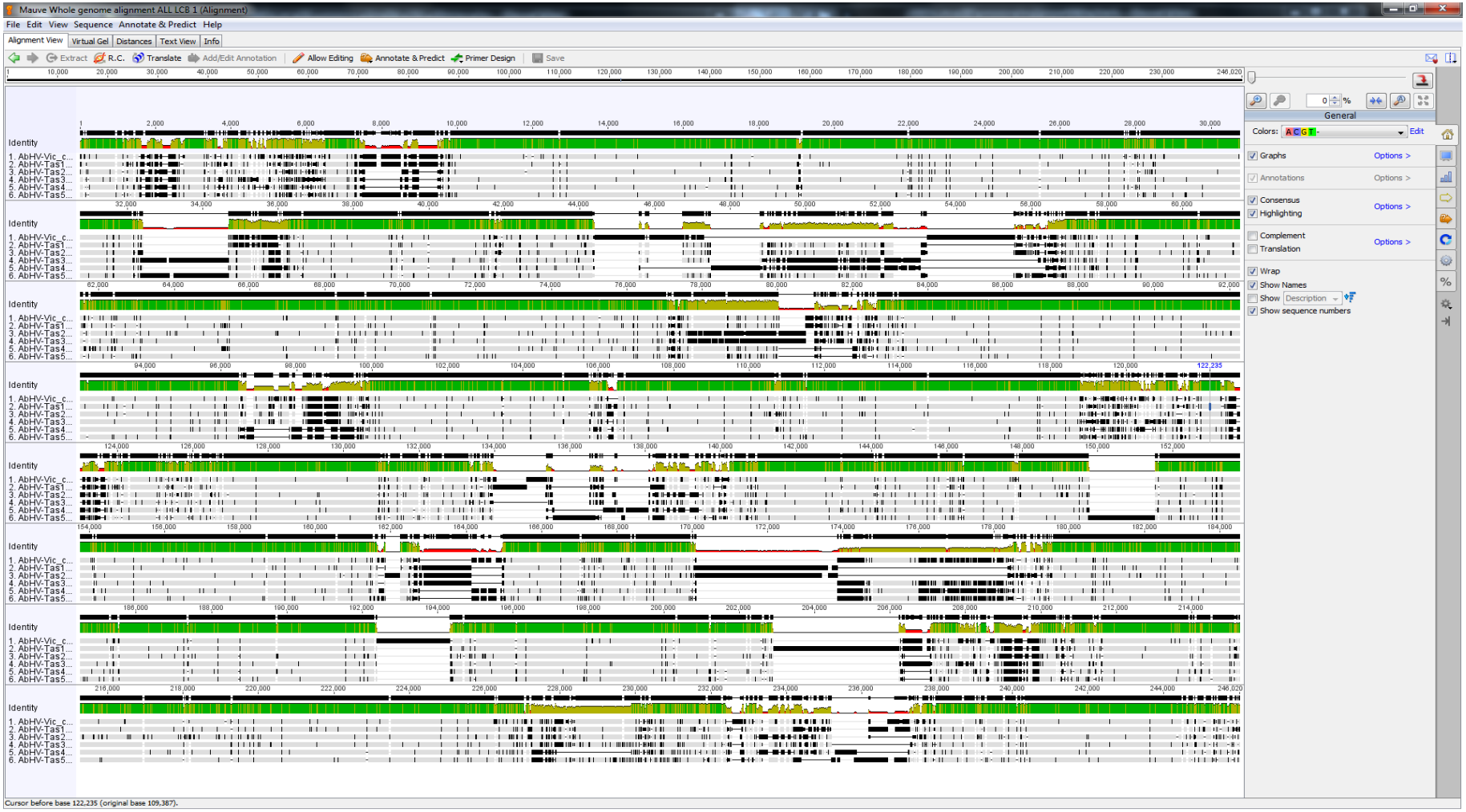
Results, Discussion and Conclusion

Objective 2: Determine the complete genome sequences for AbHV Tas3, Tas4 and Tas5 to gain insights into how and over what timeframe they have arisen, whether genetic recombination is contributing to this variation and which genome regions might affect virulence, as well as instructing on how diagnostic methods for their detection and differentiation can be refined.

Abalone herpesvirus sequence analysis

Genome sequencing of AbHV subtypes Tas3, Tas4 and Tas5 has been completed. Genome sequence alignment of AbHV subtypes Vic1 (2007), Tas1 (2008), Tas2 (2009), Tas3 (2010), Tas4 (2011) and Tas5 (2011) is shown in Figure 2 and demonstrates that the genomes of the different subtypes are quite different in many regions. Due to the large volume of data only a summary is presented here.

Figure 2. Genome sequence alignment of AbHV subtypes Vic1, Tas1, Tas2, Tas3, Tas4 and Tas5



Positions of variable sequences are highlighted in black

AbHV genome sequence differences

Due to substantial sequence deletions/insertions, particularly outside of protein-coding regions, the complete AbHV genome sequence assemblies varied in length from 207,874 bp for the Tas4 subtype to 216,273 bp for the Tas1 subtype (Table 5a). Variations in the numbers of repeated DNA elements accounted for much of the length and sequence variation. In comparison, nucleotide sequence variation was relatively low in discrete genes encoding putative proteins of either predicted or unknown function. Overall levels of nucleotide sequence identity among the AbHV subtypes determined from a Mauve alignment (Darling et al., 2004) are shown in Table 6b.

Table 5a. Variations in lengths predicted for the complete genome sequence and discreet genome components of AbHV subtypes examined from Victoria and Tasmania

AbHV subtype	Genome components (lengths = bp)						
	TRL	UL	IRL	IRS	US	TRS	Total
Vic1	5,877	101,253	5,877	12,105	78,638	12,082	215,832
Tas1	5,871	100,361	5,871	12,100	79,970	12,100	216,273
Tas2	5,968	99,549	5,968	11,338	76,592	11,338	210,753
Tas3	5,995	101,709	5,995	11,352	77,521	11,352	213,842
Tas4	5,945	97,777	5,945	11,924	74,359	11,924	207,874
Tas5	5,978	101,058	5,978	12,082	77,870	12,082	215,048

TRL = Terminal repeat long; IRL = Internal repeat long; UL = Unique long region; IRS = Internal repeat short; TRS = Terminal repeat short; US = Unique short region; bp = base pairs

Table 5b. Nucleotide sequence identity levels (%) based on complete genome sequence comparisons of the AbHV subtypes identified to date among abalone from Victoria and Tasmania

	Vic1	Tas1	Tas2	Tas3	Tas4
Tas1	86.14				
Tas2	85.00	91.68			
Tas3	88.36	87.53	89.81		
Tas4	85.36	83.44	86.81	89.59	
Tas5	86.69	86.79	86.61	90.11	89.55

Alignment of the complete genome sequences using the Mauve Multiple Genome Alignment software (Darling et al., 2004) showed nucleotide identity levels between AbHV subtypes to range from 85.0% (AbHV-Vic1 compared with AbHV-Tas2) to 91.7% (AbHV-Tas1 compared with AbHV-Tas2). However, there were no obvious trends of any one strain being particularly more divergent from all other subtypes.

For more refined examination of strain relatedness, the PhylosSift V1.01 software (Darling et al., 2014) was used to interrogate each AbHV genome sequence for reference marker genes included in its viral species database based on their known phylogenetic usefulness. The 3 marker genes identified in each AbHV genome were ribonucleotide-diphosphate reductase subunit alpha (AbHV-Vic1; CDS 33313-35790), ribonucleotide-diphosphate reductase subunit beta (AbHV-Vic1; CDS 52648-50720) and ATP-dependent DNA ligase (AbHV-Vic1; CDS 134376-132487). Concatenations of these 3 gene sequences were then aligned and used to generate a maximum-likelihood phylogenetic tree (Figure 3).

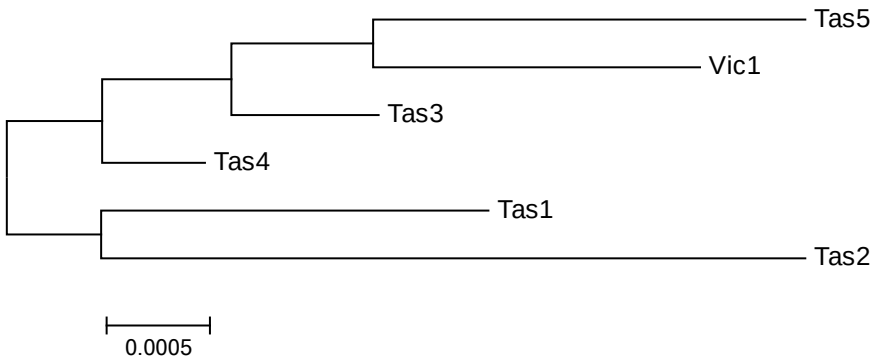


Figure 3. Maximum-likelihood phylogenetic tree showing the relatedness of Vic1, Tas1, Tas2, Tas3, Tas4 and Tas5 AbHV subtypes based on an alignment of the concatenated nucleotide sequences of the ribonucleotide-diphosphate reductase alpha and beta subunit and the ATP-dependent DNA ligase genes.

While closely related, differences in the nucleotide sequences of the 3 genes examined clearly separated each of the 5 AbHV subtypes to lesser or greater extents. Such distinctions were not unexpected based on the subtypes being identified at different geographical locations and at different times between 2007 and 2011. Interestingly, the AbHV-Vic1 subtype responsible for mortality in farmed and wild abalone in Victoria beginning in 2005-06 was not clearly demarcated from Tasmanian subtypes associated with disease episodes at different wild abalone processing premises between 2008 and 2011. However, whether or not the AbHV-Vic1 subtype should be expected to be unique from the Tasmanian subtypes cannot be inferred based on these few gene sequences, their unknown rate of genetic drift over time, and the lack of detailed information on the level of AbHV genome variation in abalone populations inhabiting disparate regions of Tasmania.

Implications

In this current study all abalone species (greenlip, blacklip, brownlip and hybrid (greenlip x blacklip)) from different geographical origins were shown to be susceptible to the five known AbHV subtypes. This information should be taken into consideration by authorities and industry regarding management and translocation of abalone within regions and inter-state. Where movements of live abalone require test regimes, fit – for –purpose diagnostic tests should be used (Corbeil et al., 2014). Furthermore, complete genome sequences determined previously for the Victorian AbHV subtype and 2 Tasmanian subtypes (Tas1, Tas2) found associated with processing plant outbreaks of AVG, as well as here for 3 additional Tasmanian subtypes (Tas3, Tas4, Tas5) associated with similar AVG outbreaks, highlights the likelihood of these different subtypes having evolved in discrete populations of abalone. If this is the case, there is potential for translocating AbHV subtypes across wild abalone populations. Risks highlighted by these data have contributed to the implementation of restrictions in wild abalone movements across fishing zone boundaries specified around the Tasmanian coastline, and in the requirements for receiving processing plants to have biosecurity measures in place to decontaminate water prior to its release back into the environment.

Recommendations

The outputs from this project are:

- Information on the susceptibility of greenlip, blacklip, brownlip, originating from Tasmania, Victoria, South Australia and Western Australia, to infection with AbHV.
- Information on the pathogenicity of 5 subtypes of AbHV (Vic1, Tas1, Tas2, Tas3 and Tas4).
- Information on the onset of AVG and on viral loads of challenged abalone.

This new knowledge will contribute to better control and management of AbHV and associated disease (AVG) and should be made available to the abalone industry sector and government regulators.

The new information will also be published in a peer-reviewed scientific journal for broader distribution.

Further development

While the results of this project demonstrate that all abalone species tested are susceptible to all known AbHV subtypes, further research is required to address the following:

- Refine and better understand the challenge system to improve the precision and repeatability of results (e.g. develop molluscan cell lines that are susceptible to infection by AbHV thus permitting a more accurate titration and indicator of infectious virus dose).
- Determination of the stress factors (e.g. duration of transportation, change in temperature before challenge, water quality) that influence the onset of disease in AbHV-infected abalone.
- Better understand the stability of the virus in liquid nitrogen storage and the effect of passaging the virus in live abalone with regards to loss of pathogenicity.
- Continue to examine the epidemiology of AbHV subtypes associated with AVG outbreaks.

Extension and Adoption

The purpose of this project was to increase our knowledge about abalone herpesvirus and its effect on Australian abalone species of commercial importance so that industry sectors and regulators are able to make better-informed decisions concerning management of this fishery in the presence of this virus and of its various subtypes. The non-technical summary will be provided in the next issue of the *Health Highlights* Newsletter and the final report will be available on the FRDC website.

Results were reported in person at scientific and industry conferences and through peer-reviewed scientific publication.

Copies of the final report will be forwarded to state authorities, AAGA and ACA.

Presentations made by project staff at scientific and industry meetings/conferences

Corbeil S, Williams LM, Cowley JA, Moody NJG, McColl KA, Mohr PG, Crane MSJ. 2015. Determination of susceptibility of various abalone species and populations to the various known abalone herpesvirus genotypes. 3rd FRDC Australasian Aquatic Animal Health, Cairns, 6-10 July 2015.

Corbeil S, Williams LM, Cowley JA, Moody NJG, McColl KA, Mohr PG, Crane MSJ. 2015. Determination of susceptibility of various abalone species and populations to the various known abalone herpesvirus genotypes. Craig Mostyn Group Jade Tiger abalone Pty Ltd, Indented Head, Victoria, 27 July 2015.

Crane M. 2015. Abalone herpesvirus (AbHV): Research Up-date. 2015 AAGA Annual Workshop, South Australian Aquatic Sciences Centre, West Beach South Australia, 13-14 August 2015.

Publications in peer-reviewed scientific journals

Corbeil Serge, Lynette M Williams, Kenneth A McColl, Mark St.J. Crane. 2016. Determination of susceptibility of Australian abalone species (*Haliotis laevigata*, *Haliotis rubra and Haliotis conicopora*) to infection from various abalone herpesvirus genotypes**.** Diseases of Aquatic Organisms. In press.

Project materials developed

Corbeil Serge, Lynette M Williams, Kenneth A McColl, Mark St.J. Crane. 2016. Determination of susceptibility of Australian abalone species (*Haliotis laevigata*, *Haliotis rubra* and *Haliotis conicopora*) to infection from various abalone herpesvirus genotypes**.** *Diseases of Aquatic Organisms* **119:** 1010-106.

Appendices

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References

Corbeil S, McColl KA, Williams LM, Mohammad I, Hyatt AD, Crameri SG, Fegan M, Crane MStJ. 2012. Abalone viral ganglioneuritis: Establishment and use of an experimental immersion challenge system for the study of abalone Herpes virus infections in Australian abalone. *Virus Res* **165:** 207-213.

Corbeil S, Williams LM, Kube P, King H, Elliott N, Crane MStJ. 2013. Aquatic Animal Health Subprogram: Investigation into the genetic basis of resistance to infection by abalone herpes-like virus. Fisheries Research and Development Corporation Project No. 2011/003.

Corbeil S, Williams LM, Warner S, Fegan M, Moody NJG, Mohammad I, Ellard K, Caraguel C, Deveney M, Cowley J, Crane MStJ. 2014. Aquatic Animal Health Subprogram: Characterisation of abalone herpes-like virus infection in abalone. Fisheries Research and Development Corporation Project No. 2009/032.

Crane MStJ, Corbeil S, Fegan M, Warner S. 2009. Aquatic Animal Health Subprogram: Development of molecular diagnostic procedures for the detection and identification of herpes-like virus of abalone (*Haliotis* spp.). Fisheries Research and Development Corporation Project No. 2007/006.

Crane MStJ, Corbeil S, Williams LM, McColl KA, Gannon V. 2013. Evaluation of abalone viral ganglioneuritis resistance amongst wild abalone populations along the Victorian coast of Australia. *J Shellfish Res* **32:** 67-72.

Darling ACE, Mau B, Blatter FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Research 14, 1394-1403.

Darling AE, Jospin G, Lowe E, Matsen FA IV, Bik HM, Eisen JA. 2014. PhyloSift: phylogenetic analysis of genomes and metagenomes. PeerJ 2:e243 http://dx.doi.org/10.7717/peerj.243

Hooper C, Hardy-Smith P, Handlinger J. 2007. Ganglioneuritis causing high mortalities in farmed Australian abalone (*Haliotis laevigata* and *Haliotis rubra*). *Aust. Vet. J.* **85:** 188-193.

OIE. 2015. Infection with abalone herpesvirus. Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.4.1.
http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.4.01_INF_ABALONE.pdf

Tan J, Lancaster M, Hyatt A, van Driel R, Wong F, Warner, S. 2008. Purification of a herpes-like virus from abalone (*Haliotis* spp.) with ganglioneuritis and detection by transmission electron microscopy. *J. Virol. Meth.* **149:** 338-341.