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Immunological changes in response to herpesvirus infection in abalone *Haliotis laevigata* and *Haliotis rubra* hybrids

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ABSTRACT

Australian abalone production has been affected by outbreaks of abalone viral ganglioneuritis (AVG) caused by a herpesvirus (AbHV). In this study, we undertook experimental transmission trials by immersion to study the abalone immune response to infection with AbHV. Representative cellular and humoral immune parameters of abalone, including total haemocyte count (THC), superoxide anion (SO) and antiviral activity against herpes simplex virus type 1 (HSV-1), were examined in apparently healthy (sub-clinical) and moribund abalone after challenge. In the early infection, sub-clinical stage (days 1-3), THC was found to increase significantly in infected abalone. TaqMan qPCR confirmed 20.5% higher viral load in moribund abalone compared to apparently healthy abalone, indicating that the abundance of AbHV within abalone is linked to their clinical signs. At the clinical stage of infection, THC was significantly lower in moribund abalone, but increased in AbHV-exposed but apparently healthy abalone, in comparison to non-infected controls. SO was reduced in all abalone that were PCR-positive for AbHV. THC and SO level were found to be negatively correlated with the presence of AbHV in abalone, but no effect of AbVH exposure was observed on the haemolymph antiviral activity. These results suggest that abalone mount an initial cellular immune response to AbHV infection, but this response cannot be sustained under high viral loads, leading to mortality.

INTRODUCTION

Abalone viral ganglioneuritis (AVG) threatens the sustainability of the abalone industries in south-eastern Australia [1]. AVG is caused by infection with a herpesvirus [2], damaging neural tissues and resulting in up to 90% mortality of farmed abalone within 7-14 days from the onset of clinical signs [1]. Phylogenetic analysis of the DNA polymerase protein indicates that the abalone herpes virus (AbHV) belongs to the family *Malacoherpesviridae* and is distantly related to other members of the *Herpesviridae* [2].

Abalone defense against viral infection relies on their innate immune system, which consists of cellular and humoral components [3]. Cellular immunity in molluscs, including abalone, is centered on the activity of haemocytes, including the elimination of infectious agents involving release of superoxide anion, phagocytosis of microbial pathogens and the recognition and elimination of infected cells [3, 4]. Antimicrobial compounds acting as humoral effectors of molluscan immunity can be constitutively expressed and rapidly induced to provide an immediate response to invading microorganisms [5, 6].

Of the AVG outbreaks reported by Hooper *et al*. [1], a small proportion of abalone survived (<10%). It is possible that survivors possessed an enhanced immunity. In this study, we performed *in vivo* infection trials (using abalone sourced from a farm with no previous history of AVG), by direct immersion in water in which AbHV was suspended [7, 8], to examine abalone immune responses during the first week after exposure to AbHV. Total haemocyte count, superoxide anion production in haemocytes and antiviral activity in the haemolymph plasma were used as representative cellular and humoral immune parameters of abalone to investigate

differences in immune responses between apparently healthy and moribund abalone after challenge with AbHV. After immersion of abalone in AbHV-infectious water, their immunity was assessed at the early subclinical stage, and later, at the onset of disease.

MATERIALS AND METHODS

Virus stock was obtained from abalone previously infected with the Victorian isolate of AbHV (designated Vic-1) by homogenizing neural tissue in Eagle's Minimal Essential Medium containing 20% foetal bovine serum, with a Dounce homogeniser, followed by filtration (0.22μm) and the filtrate stored in liquid nitrogen, until use. AbHV-infectious water used for immersion challenge was produced by injecting six abalone intramuscularly in the foot with 100 uL of this stock virus (~1 x 10^5 viral gene copies (v.g.c.)/100uL). At day 4 post-inoculation, the water was harvested, titrated using the AbHV TaqMan assay [7] and diluted 1:5 to generate the challenge AbHV-infectious water. The amount of AbHV in this water was determined to be 15.6 x 10^6 v.g.c. ml^{-1}.

H. laevigata x *H. rubra* hybrid abalone (n=150, 7-8 cm in shell length), obtained from Great Southern Waters Pty Ltd, Victoria, were placed in experimental aquaria and acclimated at 15-16 °C for a week in seawater with continuous aeration and daily 100% water exchange. Abalone (n=72) were challenged by immersion for 20hr, in triplicate 40L tanks in the infection room. Abalone (n=72) as negative controls were maintained in fresh seawater in triplicate tanks in a separate non-infection room. Haemolymph and ganglia were sampled from nine abalone from the infected and non-infected (negative control) groups at days 1, 2 and 3.

Moribund abalone, demonstrating loose attachment to the substrate, were collected on days 5, 6 and 7 for sampling their haemolymph and ganglion. On the same days, haemolymph and ganglion were also sampled from apparently healthy abalone in the infected room and negative control abalone from the non-infected room (n=9 per day, 3 from each tank).

Ganglion sections, including ganglion tissues and surrounding muscle from infected and uninfected abalone, were weighed. Nucleic acid was extracted using QIAamp DNA mini kit (QIAGEN) and stored at -80°C until required. ORF-49 qPCR [9] was carried out on DNA extracts from abalone tissues according to established protocols [7, 9]. All abalone samples were tested by real-time TaqMan PCR (7500 Fast Real-time PCR system, Applied Biosystems) in duplicate to obtain C_T values. AbHV-negative samples were identified as having C_T values >35.8 and AbHV-positive samples had C_T values <35. To quantify the number of AbHV gene copies (v.g.c. μl^{-1}) in abalone samples from the C_T value, a plasmid DNA standard curve was prepared for plasmid Topo-ORF49 using serial 10-fold dilutions [7, 9]. The qPCR assay for plasmid Topo-ORF49 produced a linear correlation between recombinant plasmid level and C_T value over 4 $\log_{10}$ dilutions (coefficient R^2=0.99).

Fresh haemolymph (100 µl) was sampled from the anterior sinus using a sterile syringe and needle (5ml, 25G), then immediately fixed in 200 µl formalin (10%), in phosphate buffered saline (PBS), in pre-cooled Eppendorff tubes and kept on ice. Total haemocyte count for each haemolymph sample was obtained using an improved Neubauer hemocytometer. Superoxide anion (SO) production by haemocytes was quantified using reduction of nitroblue tetrazolium (NBT) to formazan, as described in previous studies [10-12] and measured at 620 nm on a

105 microplate reader (Multiskan Ascent, Thermo Electron Corporation). PBS was used as negative
106 control to measure the background breakdown of NBT. Intracellular SO was expressed as
107 haemolymph sample (150 µl) absorbance - negative control absorbance.
108
109 The remaining haemolymph were centrifuged (1500 x *g*, 5 min) to obtain haemolymph plasma
110 for antiviral assays. The plaque reduction assay was used to measure antiviral activity of abalone
111 haemolymph against HSV-1, as previously described [13, 14]. Haemolymph plasma (6 %; v/v)
112 was used throughout to compare antiviral activity (EC_{50} = 6.23 %, v/v, i.e. the concentration
113 required to inhibit HSV-1 plaque formation by 50%) [13].
114
115 At the sub-clinical stage of infection (days 1-3), each abalone immune parameter (THC, SO
116 level, antiviral activity) was compared between AbHV-exposed and non-infected abalone using
117 two-factor permutational analysis of variance in Primer V6 with PERMANOVA add-on [15].
118 Post-hoc planned comparisons were undertaken between controls and treatments on each day
119 using pair-wise tests. At the clinical stage of infection (days 5-7), four infected and non-infected
120 abalone groups (1: negative control, 2: AbHV-exposed, PCR-negative and apparently healthy, 3:
121 infected PCR-positive and apparently healthy, 4: PCR-positive and moribund) were compared
122 for each immune parameter using one-factor univariate PERMANOVA. The correlation between
123 v.g.c (log_{10}) in ganglion tissues and each immune parameter from AbHV-exposed, PCR-positive
124 abalone in the immersion trial was tested using Pearson's correlation coefficient (PASW/SPSS
125 statistics 18).
126
127 **RESULTS AND DISCUSSION**

There was no mortality, or clinical signs in abalone, during the first three days after exposure to virus. All negative control abalone were PCR-negative for AbHV (C_T values > 35.8). Challenged abalone were all PCR-negative at days 1 and 2, and two out of the nine abalone were PCR-positive at day 3 (C_T values < 35.8). This confirms that qPCR can detect sub-clinical AbHV infections [7]. Viral DNA was detected in abalone ganglion tissues but not in their haemolymph.

During the subclinical phase of infection, the level of SO and antiviral activity against HSV-1 were not significantly different between AbHV-exposed and control groups (Table 1). However, the mean THC in AbHV-exposed abalone increased over time and pair-wise analysis detected significant difference in THC between AbHV-exposed and control abalone on day 3 (Table 1). An increase in circulating haemocytes (THC) is a common stress response observed in abalone in response to a wide range of abiotic (e.g. handling, water temperature, salinity) and biotic stressors (e.g. *Vibrio* sp. infections) [12, 16, 17]. Here we demonstrate that in the early stages of exposure to virus, abalone will also mount an initial immune response by increasing the number of circulating haemocytes. Circulating haemocytes play prominent roles in defense against pathogenic microbes, by mechanisms such as phagocytosis and the release of cytotoxic compounds [18].

Abalone started showing clinical signs of ganglioneuritis (e.g. loss of pedal adhesion to tank surface, loss of righting reflex) on day 5 after exposure to virus. In total, 18 moribund were sampled and all were PCR-positive for AbHV (Supplementary Fig. 1). In addition, 29 healthy-looking but virus challenged abalone were sampled from days 5-7, and of these 23 were PCR-positive, but with a greater range of AbHV viral copies than moribund abalone (Supplementary

Fig. 1). Viral DNA was not detected in a small proportion of apparently healthy abalone (n=6) that were also exposed to AbHV by immersion. Longer-term experiments would be required to confirm whether these healthy-looking AbHV- exposed abalone would ultimately succumb to the disease. Indeed, 100% cumulative mortality of abalone has been observed in previous AbHV infection trials after 10 days [7].

At the clinical infection stage (days 5-7), THC was still elevated in apparently healthy abalone that were PCR-negative (42.6%) and PCR-positive (13.6%) for AbHV, but THC was significantly lower in moribund abalone (38.8%) in comparison to the negative controls (Fig. 1a, $p<0.05$). For PCR-positive abalone exposed to AbHV- infectious water, THC was negatively correlated with number of v.g.c. (log_{10}) from abalone tissues (Pearson's correlation coefficient $r=-0.33$, $p=0.03$). This result is consistent with previous reports of leucopenia in AbHV-infected abalone, where significantly lower THC was observed in farmed abalone with microscopic lesions, in comparison to healthy abalone with no lesions indicative of AVG [19]. The decrease in circulating haemocytes in moribund abalone is most likely due to the sequestration of haemocytes to the infected neural tissue [19]. A similar effect of reduced THC has been reported for Pacific white shrimp *Litopenaeus vannamei* infected with white spot syndrome virus [20] and Taura syndrome virus [21], thus emphasizing the central role of maintaining circulating haemocytes for immunity against viruses in marine invertebrates.

A significant change in SO level was observed when abalone started showing clinical signs of disease (from day 5 post-infection), with higher levels found in apparently healthy abalone that were exposed to the virus and a significant decrease in moribund abalone, compared to negative

controls (Fig. 1b, $p<0.05$). No significant difference in SO was found between AbHV- exposed healthy and moribund abalone, which were confirmed to be PCR-positive ($p = 0.1$), or between healthy PCR-positive abalone and negative control abalone ($p=0.63$). For all abalone that were exposed to AbHV, the level of SO was positively correlated to THC ($r=0.48$, $p<0.01$) and negatively correlated to the number of v.g.c. (log10) in abalone tissues ($r=-0.53$, $p<0.01$). Since only the volume of haemolymph, and not the number of haemocytes, was standardized between samples in the SO assay, the relationship between SO and viral load is mostly likely an indirect effect contributed by the reduced THC in clinically infected abalone.

Consistent with our previous studies [11-14], a high level of baseline antiviral activity against HSV-1 was observed in all treated and control abalone. Antiviral activity against HSV-1 was not significantly higher in apparently healthy infected abalone compared with moribund abalone (Fig. 1c, $p>0.05$). Furthermore, antiviral activity did not increase significantly in abalone exposed to AbHV, in comparison to the negative controls. Consequently, there appears to be no ramping up of the constitutive levels of antiviral activity present in the unchallenged animal on exposure to AbHV. This suggests that there may not be an inducible component to the synthesis of humoral antiviral (anti-HSV-1) compounds in response to AbHV infection in abalone, which is in contrast to the inducible synthesis of antibacterial factors in bivalves [22], some of which also appear to have antiviral activity [23-25].

In summary, changes to the abalone cellular immune response, but not the humoral antiviral activity, can be observed in to the early stages of infection with AbHV. Abalone with low viral prevalence have a high number of circulating haemocytes and associated intracellular SO.

Further research is required on the heritability of the ability to mount a cellular immune response to pathogen infection and specific antiviral factors in abalone that may help confer resistance to AbHV.

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277 TABLE

278 Table 1. Effect of AbHV infection and time post-infection on A) total haemocyte count (THC,
279 cells x 10^4 per ml), B) superoxide anion (SO, OD 620nm), C) antiviral activity (%) against HSV-
280 1. Each immune parameter was measured from nine replicate abalone. The asterisk (*) indicates
281 significant differences ($p<0.05$) between virus-exposed and negative control groups.

Time post-challenge	Immune parameter	PERMANOVA		
		Non-infected control	AbHV-exposed	P value (pair-wise test)
Day 1	THC (cells x 10^4 ml^{-1})	281.33±28.1	289.33±21.07	0.84
	Intracellular SO (OD 620nm)	0.15±0.03	0.15±0.03	0.94
	Antiviral activity (%)	48.24±5.8	54.93±2.98	0.31
Day 2	THC (cells x 10^4 ml^{-1})	275.83±41.46	347.17±47.01	0.28
	Intracellular SO (OD 620nm)	0.14±0.02	0.15±0.03	0.59
	Antiviral activity (%)	45.11±4.37	51.1±6.19	0.43
Day 3	THC (cells x 10^4 ml^{-1})	276±22.63	381.5±39.08	0.04*
	Intracellular SO (OD 620nm)	0.11±0.02	0.15±0.01	0.11
	Antiviral activity (%)	47.12±3.76	49.86±3.46	0.57

282

FIGURE

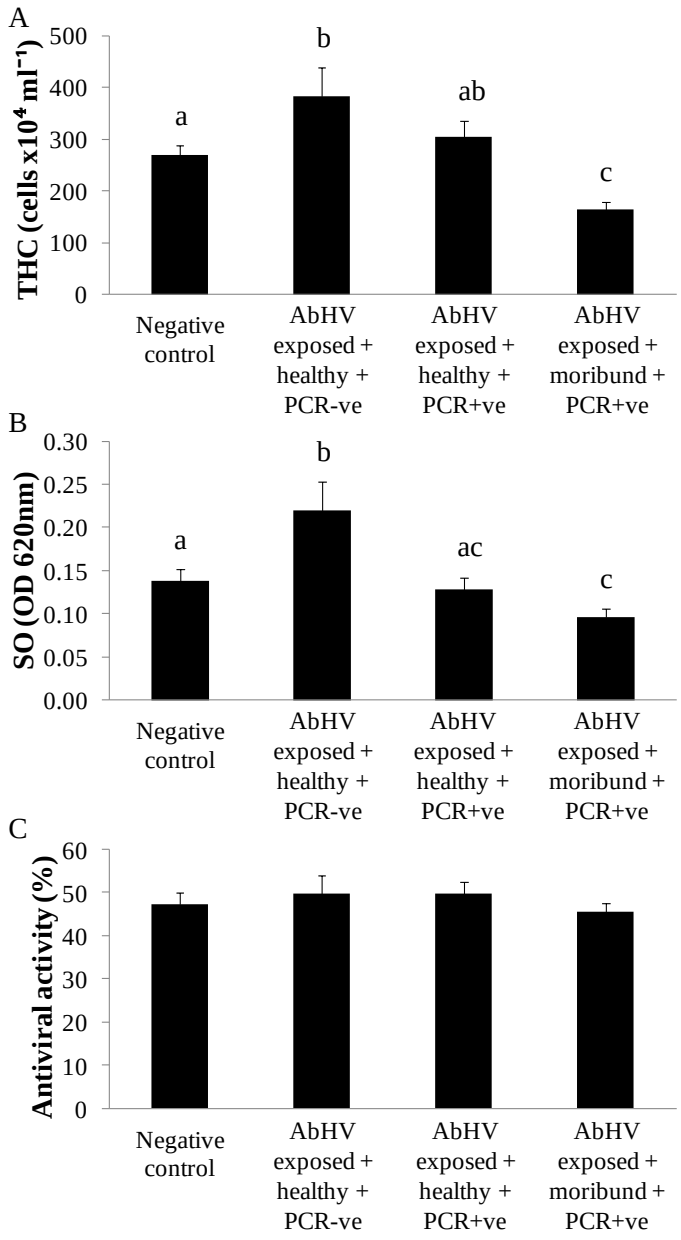
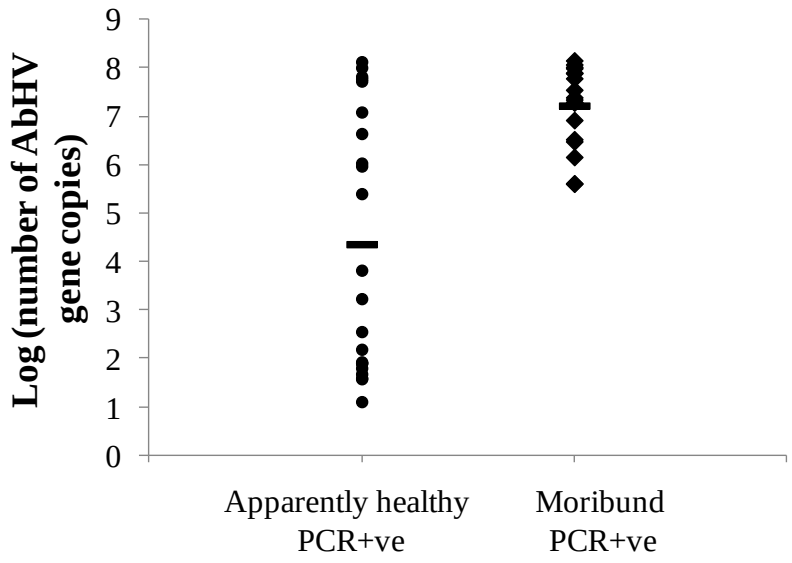


Fig. 1. Effect of AbHV infection status at the clinical stage on abalone immune responses A) total haemocyte count (THC, cells x 10^4 per ml), B) superoxide anion (SO, OD 620nm), C) antiviral activity (%) against HSV-1. Comparison was made between non-infected negative control (n=27), AbHV exposed but apparently healthy and PCR-negative (n=6), apparently healthy and PCR-positive for AbHV (n=23), and moribund and PCR-positive (n=18) abalone. Different small letters indicate significant differences ($p<0.05$) between groups.



293

294 Supplementary Figure 1. Quantification of AbHV gene copies (log_{10}) based on ORF49 qPCR Ct
295 values, in apparently healthy and moribund abalone from day 5 post-infection.

296