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TITLE PAGE

Variation in the antiviral and antibacterial activity of abalone *Haliotis laevigata*, *H. rubra* and their hybrid in South Australia

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ABSTRACT

Abalone (Haliotidae), well-known commercial gastropods, have experienced large scale disease outbreaks such as abalone viral ganglioneuritis caused by a herpesvirus and summer mortality typically caused by bacteria such as *Vibrio harveyi*. Identification of the factors that influence antimicrobial activity could assist future management of disease in the abalone industry. A proportion of abalone naturally survive these outbreaks (5-40 %) raising the possibility that some abalone are relatively resistant. Identifying such abalone could enable breeding of resistant populations. This study applied *in vitro* assays to investigate antiviral and antibacterial activity of abalone haemolymph. Comparisons were made among *Haliotis laevigata* (greenlip), *H. rubra* (blacklip) and their hybrid. Intraspecific variation was examined at the individual scale, as well as between commercial aquaculture family lines and natural populations. Abalone sourced from the wild showed higher antiviral and antibacterial activities than those from a land-based farm. We found no significant difference in antiviral activity between greenlip, blacklip and hybrid abalone ($p>0.05$). The antibacterial activity of greenlip abalone was also similar to blacklip, but significantly lower than hybrid ($p=0.001$). There was substantial individual variation among abalone (maximum range of 31-69 % for antiviral activity and 4-46 % for antibacterial activity) within the same family line or geographic location. Antiviral and antibacterial activity increased slightly with an increase in shell length, and a 2yr old family line had lower activity than 3yr old family lines. There was no significant effect of gender or reproductive activity on antiviral or antibacterial status ($p>0.05$). Further investigation is required to establish whether the individual variability in antimicrobial activity is inheritable in breeding programs and whether higher activity confers greater resistance to disease.

47 INTRODUCTION

48 Abalone are herbivorous marine molluscs and important economic species
49 worldwide (Fleming *et al.* 1996; Cook 1998; Godoy *et al.* 1998; Gordon *et al.* 2001;
50 2004). Among more than 11 species found along the Australian coast, greenlip
51 *Haliotis laevigata* and blacklip *H. rubra* are the two most commercially important
52 (Freeman 2001). A significant threat for the Australian abalone industry is the
53 emerging disease, abalone viral ganglioneuritis, reported in *H. laevigata, H. rubra*
54 and their hybrid cross (Hooper *et al.* 2007a). Similar viral infections have been
55 reported in *H. diversicolor supertexta* in Taiwan (Chang *et al.* 2005) and *H.*
56 *diversicolor reeve* in China (Wang *et al.* 2004). In Australia, abalone ganglioneuritis
57 is caused by a herpesvirus (Savin *et al.* 2010), impairing the cerebral nerves and
58 resulting in up to 95% mortality within 14 days from the onset of clinical signs (Chang
59 *et al.* 2005; Hooper *et al.* 2007a).

60 Another major threat for the abalone industry is associated with *Vibrio* bacteria,
61 some of which are common marine pathogens (Austin *et al.* 2006). *V. harveyi* and *V.*
62 *splendidus I* have been isolated from moribund abalone during disease outbreaks in
63 Tasmania, Australia (Handlinger *et al.* 2005). *V. harveyi* has been linked to summer
64 mortality in *H. tuberculata* populations in France (Travers *et al.* 2008; Travers *et al.*
65 2009) and is also thought to contribute to summer mortality on aquaculture farms in
66 South Australia (Vakalia *et al.* 2005). *V. harveyi*, (syn *V. carchariae)*, is a cause of
67 high mortality of abalone in Japan (Nishimori *et al.* 1998) and France (Nicolas *et al.*
68 2002). These Gram-negative bacteria can cause septicaemia, resulting in up to 80%
69 mortality (Nicolas *et al.* 2002; Handlinger *et al.* 2005). Typically *Vibrio* spp. are
70 opportunistic and lead to disease when immunity is suppressed as a result of
71 stressors such as high temperature, poor water quality or handling (Malham *et al.*
72 2003; Cheng *et al.* 2004a; Cheng *et al.* 2004b; Cheng *et al.* 2004c; Cheng *et al.*
73 2004d; Cheng *et al.* 2004e; Hooper *et al.* 2007b; Day *et al.* 2010).

74 Reproduction is an important stressor that can impact the immunity of molluscs
75 (Taskinen *et al.* 1999; Duchemin *et al.* 2007; Li *et al.* 2007a; Matozzo *et al.* 2010),
76 including abalone (Travers *et al.* 2009). In oysters, it has been shown that the high
77 energetic cost of reproduction diverts energy from other biological processes,
78 resulting in immune depression (Pouvreau *et al.* 2003; Soletchnik *et al.* 2006; Li *et al.*

79 2007a; Li *et al.* 2009a). Antiviral activity of oyster haemolymph against herpes
80 simplex virus type 1 (HSV-1) was found to increase from winter/spring to
81 summer/autumn, when the spawning typically occurs (Olicard *et al.* 2005a). Ripe
82 abalone (*H. tuberculata*) were found to be more susceptible to bacterial infection
83 than immature abalone and mortality increased in conjunction with reproductive
84 stress in the field (Travers *et al.* 2009). Furthermore, during gametogenesis there
85 was evidence of immune-depression, such as reduced phagocytosis and
86 phenoloxidase activity, and the overall haemolymph profile reached a minimum just
87 after spawning (Travers *et al.* 2008). Thus, reproductive status and activity both
88 appear to be factors influencing the intraspecific variability of immune defense in
89 bivalve molluscs.

90 Abalone immunity is principally based on their circulating hemocytes and secreted
91 effectors (Hooper *et al.* 2007b). Hemocytes are involved in numerous innate
92 defenses such as chemotaxis, lectin-mediated pathogen recognition, phagocytosis,
93 encapsulation and elimination of pathogens via enzymatic destruction and/or by
94 production of antimicrobial compounds (Cheng 1981; Mitta *et al.* 2000; Bachère *et al.*
95 2004; Hooper *et al.* 2007b). Antimicrobial compounds are a major component of
96 innate immunity and can be constitutively expressed and rapidly induced to provide a
97 prompt response to invading microorganisms (Tincu *et al.* 2004; Otero-Gonzalez *et*
98 *al.* 2010). The antibacterial activity of abalone haemolymph has been demonstrated
99 against marine *Vibrios* using *in vitro* assays (Vakalia *et al.* 2005; Day *et al.* 2010).
100 Recently, we have shown that plasma of abalone haemolymph also contains antiviral
101 compounds using the plaque assay for HSV-1 (Dang *et al.* 2011). In the absence of
102 mollusc cell lines for culturing abalone herpesvirus, the use of another herpesvirus
103 on a compatible cell line (Vero) facilitates antiviral screening. Genome sequence and
104 phylogenetic analysis of DNA polymerase proteins suggest that the abalone
105 herpesvirus of the family *Malacoherpesviridae* is related to other members of the
106 *Herpesviridae* including HSV-1 (Fegan *et al.* 2009; Savin *et al.* 2010). These *in vitro*
107 antibacterial and antiviral assays have been broadly applied to investigating
108 immunity of molluscs (Defer *et al.*, 2009), particularly the pacific oyster *Crassostrea*
109 *gigas* (Olicard *et al.* 2005a; Olicard *et al.* 2005b; Li *et al.* 2007a; Li *et al.* 2009c; b; Li
110 *et al.* 2009a) and thus are useful tools for screening antimicrobial defense in
111 abalone.

112 The small proportion of abalone that survive microbial disease outbreaks may
113 provide insights into their resistance mechanisms. It is possible that survivors have
114 better adapted immune mechanisms than animals that succumb. For AVG infection,
115 some healthy abalone have been found to contain viral DNA using real time
116 polymerase chain reaction (PCR) (Crane *et al.* 2009), suggesting these animals had
117 a subclinical infection. In the oyster *C. gigas*, larvae and juveniles are more sensitive
118 to herpesvirus infections than adults (Renault *et al.* 2001; Arzul *et al.* 2002a) and
119 viral DNA was found in adults of normal appearance (Arzul *et al.* 2002b). Regarding
120 *Vibrio* infection, a low level of mixed *Vibrio* species has been isolated from clinically
121 normal abalone held for experimental trials (Handlinger *et al.* 2005) and from healthy
122 abalone on aquaculture farms (Vakalia *et al.* 2005). Thus the notion that some
123 abalone possess an enhanced ability to resist infections relative to others is
124 plausible. Identification of abalone with stronger immunity could be used to develop
125 disease resistant populations. The first step towards investigating this is to examine
126 intra and inter-specific variability in abalone immunity. We used *in vitro* assays to
127 screen for antiviral activity against HSV-1, as well as antibacterial activity against *V.*
128 *harveyi* in the hemolymph of *H. laevigata, H. rubra*, and their commercial hybrid. We
129 also investigated variability between individuals, sexes, farmed family lines and wild
130 populations from different geographic locations, as well as for farmed greenlip
131 abalone before and after spawning.

132 **MATERIALS AND METHODS**

133 2.1 Abalone

134 Nine male and nine female abalone were obtained from each of four farmed family
135 lines: greenlip FL-1 and FL-2 bred from different sets of commercial parents in 2007
136 and 2006, respectively; greenlip FL-3 from wild Elliston parents in 2006; and hybrid
137 FL-4 from commercial greenlip and blacklip in 2006. These family lines were grown
138 separately in concrete raceways on a land-based aquaculture farm in Port Lincoln,
139 South Australia under the same diet and water conditions, including temperature,
140 oxygen saturation and salinity. Wild greenlip abalone were collected in July 2009
141 from South Australian coastal sites including Cowell (n = 4), Elliston (n = 11), Farm
142 Beach (n = 25) and Blackfellows Caves (n = 8). Wild blacklip abalone were collected
143 from Elliston (n = 11), O'Sullivan Beach (n = 9) and Blackfellows Caves (n = 11). All

collected abalone appeared healthy and firmly attached to the substrate. They were transported to holding facilities on the farm in Port Lincoln or Flinders University in Adelaide and acclimatised prior to testing. Abalone were tagged on arrival using 3mm spring tags (Mollusc Pty Ltd, Victoria) and fed a standard 5mm commercial abalone diet (EP Aquafeeds, South Australia).

2.2 Spawning induction

Haemolymph samples were collected from 26 abalone of the FL-2 family line (day 1). Sixteen of these abalone were then induced to spawn (spawned group, 8 males and 8 females) using standard commercial protocols, while the remaining ten (unspawned/control group, 5 males and 5 females) were kept in a commercial broodstock tank. To achieve spawning, water temperature was raised by 5 °C using portable 300w heaters (Aqua One) with ultraviolet light passing through the reproduction tanks for 3 hrs (Hahn 1989; Uki 1989). Spawning occurred between 12 and 24 hrs post-induction (day 2). Additional haemolymph samples were then taken from all abalone of spawned and control groups (day 3).

2.3 Haemolymph collection

All abalone were measured for maximum shell length with callipers (accurate to 0.1 cm) before collection of haemolymph samples. The maximum volume of haemolymph collected from each abalone was 50 µl g^{-1} (Chen *et al.* 1996). This non-lethal bleeding procedure was performed from the anterior sinus of abalone using a pre-cooled sterile syringe and 25G needle. Haemolymph samples were frozen at -80 °C until required.

2.4 Cell culture and virus

African green monkey kidney cells (Vero) were grown in EMEM (Sigma) supplemented with 10 % newborn calf serum (NCS; Sigma) and 1 % antibiotics (PCS; 10,000 IU ml^{-1} penicillin, 25,000 IU ml^{-1} colymicin, 10 mg ml^{-1} streptomycin; Sigma) at 37 °C in a humidified atmosphere of 5 % CO_2. A well-characterized strain, SC16 (Speck *et al.* 1991; Speck *et al.* 1992) of wild type HSV-1 was obtained from Dr Tony Simmons at the Institute of Medical and Veterinary Science, Adelaide. Virus

173 titer was calculated using the standard limiting dilution method (Reed and Muench
174 1938, Defer *et al.* 2009; Dang *et al.* 2011).

175 2.5 Anti-HSV assay

176 Antiviral activity of abalone extract against HSV-1 was determined by plaque
177 reduction assay, as described by Russell (1962), with minor modifications. Briefly,
178 Vero cell monolayers were infected in triplicate with about 40-50 plaque-forming
179 units (PFU), in a volume of 0.3 ml, in presence of haemolymph (6 %, v/v) for 1 h in
180 24 well-plates. Haemolymph (6%) was used throughout to compare antiviral activity
181 of abalone (EC_{50} = 6.23 %, v/v) (Dang *et al.* 2011). During incubation, plates were
182 gently shaken every 15 min. After 1 h incubation, medium containing abalone extract
183 and unabsorbed virus was removed. Cells were then washed twice with sterile PBS,
184 and overlaid with fresh medium with the same concentration of abalone haemolymph
185 and 1 % methylcellulose. Cells were incubated for 2 days at 37 °C. Monolayers were
186 fixed with 5 % formaldehyde and stained with 4 % toluidine blue in PBS, and plaques
187 were counted using a light microscope. Antiviral activity was expressed as
188 percentage reduction of plaque numbers.

189 2.6 Bacteria

190 Stock cultures of *V. harveyi* TCFB 1477 were obtained from the Fish Aquatic Health
191 Unit, Department of Primary Industries, Tasmania, and cultured directly into broth for
192 use in the antibacterial assays. The bacterial isolate was identified using MicroSys
193 V36 identification kit for the *Vibrios* (Carson *et al.* 2006). For antibacterial assays, *V.*
194 *harveyi* was cultured in sterile nutrient broth (1 g NaCl, 2 g yeast extract and 1 g
195 peptone per 100 ml distilled H_2O) overnight at 37 ºC on an orbital mixer shaker
196 (Ratek) at 200 rpm. The cultures were diluted to OD600nm = 0.1 on a
197 spectrophotometer (Metertech, UV/VIS SP8001) and returned to exponential growth
198 phase (OD600nm=0.18-0.2) prior to use in antimicrobial assays.

199 2.7 Antibacterial assay

200 Antibacterial activity in the haemolymph plasma against *V. harveyi* was measured
201 using MTS assay as described by Li et al. (2007b). The MTS assay was based on
202 reduction of MTS tetrazolium to a red formazan product by dehydrogenase enzymes

203 from live cells (Cory *et al.* 1991). 90 µl of haemolymph plasma and 10 µl of *V.*
204 *harveyi* in exponential growth culture were added into a 96-well plate in triplicate.
205 Negative controls had 90 µl haemolymph in 10 µl nutrient broth and positive controls
206 had 10 µl of *V. harveyi* in 90 µl of nutrient broth. After 30 min incubation, 20 µl of
207 CellTitre 96® Aqueous One Solution (Promega, NSW, AUS) was added to each well.
208 The plates were then incubated for a further 2 hrs or until development of the red
209 formazan product in positive controls. Absorbance was measured at 492 nm using a
210 96-well plate reader (FluoStar Omega). Antibacterial activity (%) was calculated from
211 100-((treatment absorbance-negative control absorbance)/positive control
212 absorbance*100).

213 2.8 Statistical analysis

214 All data are presented as means and standard error from at least three repeat
215 experiments. Mixed model univariate analyses were undertaken using Primer V6
216 with PERMANOVA add on. A minimum of 999 permutations of the residuals were
217 undertaken using Euclidean distance resemblance matrices from the antiviral and
218 antibacterial activity data. A three-factor PERMANOVA was performed with fixed
219 factors for source (farmed vs. wild), species (*H. laevigata*, *H. rubra* and the hybrid)
220 nested in source and population (family line or geographic location of wild
221 populations) nested in species and source. To investigate the influence of gender
222 and size on the antiviral and antibacterial properties, a three-factor ANCOVA was
223 performed on the *H. laevigata* data. Size was used as a covariate, with gender,
224 source (farm vs. wild) and populations (nested in source) used as fixed factors in the
225 analysis. Pairwise tests were undertaken when a significant difference among
226 species or populations was detected. The limit of significance was lowered to
227 α=0.025 according to Bonferroni adjustment, for repeated use of the same data in
228 the two analyses (Hochberg 1988; Abdi 2007).

229 The correlation between antiviral activity and antibacterial activity of all abalone
230 haemolymph samples was tested using Pearson's correlation coefficient
231 (PASW/SPSS statistics 18). A two-way repeated-measures ANOVA on PASW/SPSS
232 statistics version 18 was used to compare the same spawned and non-spawned
233 abalone, at day 1 (prior to spawning) and day 3 (after spawning).

234 **RESULTS**

235 Using plaque reduction assay for screening antiviral activity of abalone haemolymph
236 against HSV-1, we found a large variation (maximum range of 31-69 %) across
237 individuals within the same farmed family line or wild population (Figure 1A). The
238 coefficient of variation within populations (33.6 %) was higher than between
239 populations (12.6 %), demonstrating that most of the variation is at the individual
240 level. MTS assay also revealed a large variation in antibacterial activity of
241 haemolymph against *V. harveyi* (maximum range of 6-46 %) across individuals
242 within the same farmed family line or wild population (Figure 1B). The coefficient of
243 variation within populations (26.4 %) was higher than between populations (18.1 %).
244 There was a weak correlation in antimicrobial activity of abalone haemolymph
245 samples when tested across all individuals (n=190), with only 3% of the variation in
246 antiviral activity being explained by variation in antibacterial activity (Pearson
247 correlation, r=0.17, p=0.02)

248 Overall, haemolymph from farmed abalone was found to have lower antiviral activity
249 than that from wild-sourced abalone (Figure 2A) and the three factor PERMANOVA
250 revealed a significant difference according to source (Pseudo F=27.87, p=0.001).
251 There was no difference in antiviral activity of haemolymph of the different species
252 (Pseudo F=1.98, p=0.14), encompassing comparison of *H. laevigata* and *H. rubra*
253 from wild populations (Fig 2A), as well as *H. laevigata* family lines and the hybrid
254 cross (FL-4, Fig 1A). There was also no significant difference in antiviral activity
255 between the different geographic populations of wild abalone or the farmed greenlip
256 family lines (Pseudo F=1.18, p=0.3).

257 Similar to antiviral activity, the antibacterial activity of abalone haemolymph collected
258 from the farm was significantly lower than that of abalone from wild populations (Fig
259 2B, Pseudo F=42.7, P=0.001). A significant difference in antibacterial activity was
260 detected according to species (nested in source, Pseudo F=3.92, p=0.026). Pairwise
261 tests revealed that the *H. laevigata* x *H. rubra* hybrid (FL-4) had higher antibacterial
262 activity than the other *H. laevigata* family lines (Fig. 1B, t=3.51, p=0.001). However,
263 there was no significant difference between wild populations of *H. laevigata* and *H.*
264 *rubra* (Fig. 2B, t =0.31, p=0.75).

265 Further investigation of the factors influencing antimicrobial activity were undertaken
266 using populations of the green lip abalone *H. laevigata.* ANCOVA revealed that the
267 shell length of abalone significantly influenced their antiviral activity (Pseudo
268 F=11.12, p=0.001). Antiviral activity was found to increase slightly with an increase in
269 shell length, although shell length explains less than 9% of the total variation in
270 activity (Fig. 3A). Overall, abalone sourced from the wild populations were larger
271 (Fig. 3) and antiviral activity was again found to vary according to source (farm vs.
272 wild, Pseudo F=7.46, p=0.009). There was no significant difference in antiviral
273 activity between populations nested within source (Pseudo F=1.27, p=0.29).
274 However, farmed greenlip abalone from line FL-2 were larger (mean 9.61±0.14 cm),
275 than the other lines (Fig. 3), including two in the same age cohort (3yrs, FL-4 hybrid
276 mean 8.39±0.28; FL-3 mean 7.69±0.22 cm). There was no significant difference in
277 antiviral activity according to gender (Pseudo F=0.003, p=0.96) and no interactions
278 between gender and/or size with source or family lines (p>0.2). This implies that the
279 effect of size was consistent across all populations, whereas gender did not affect
280 the activity in any of the populations.

281 Regarding antibacterial activity, ANCOVA again revealed a significant effect of shell
282 length (Pseudo F=41.8, p=0.001). However, there was also a significant interaction
283 between the shell length and gender (Pseudo F=4.9, p=0.045), implying that the
284 nature of correlation between antibacterial activity and shell length depends on
285 gender. In both sexes, antibacterial activity increased with shell length (Fig 3B),
286 however shell length explains 38.8 % the total variation in antibacterial activity of
287 females and less than 10 % in males. Nevertheless, there was no overall significant
288 effect of gender on antibacterial activity (Pseudo F=1.8, p=0.17). When size was
289 taken into consideration, there was no significant difference in antibacterial activity
290 according to source (Pseudo F=1.83, p=0.2) and no interactions between source and
291 size (Pseudo F=1.92, p=0.19) or source and sex (Pseudo F=0.15, p=0.72). However,
292 there was a significant difference in antibacterial activity between populations nested
293 within source (Pseudo F=3.03, p=0.022). Pairwise tests on the farmed family lines
294 revealed that the 3yr hybrid FL 4 had significantly greater antimicrobial activity than
295 the 2yr greenlip family FL-1 (Fig. 1B, t=4.69, p=0.001). There was a tendency for FL-
296 1 to have lower activity than the 3yr greenlip family line FL-3, but this was not
297 significant (t=2.08, p=0.051). There was no significant different in the antibacterial

298 activity between the hybrid FL-4 and the two 3 yr greenlip family lines (FL-2 & FL-3;
299 p>0.05). There were no significant differences between the wild populations from
300 different geographical locations (p>0.27). There were also no significant interactions
301 between population and gender or size (p>0.3)

302 Reproductive status of the abalone did not appear to impact their antiviral activity
303 (two-way repeated-measures ANOVA, F=0.18, p=0.67) or antibacterial activity (two-
304 way repeated-measures ANOVA, F=0.13, p=0.72), as no significant difference was
305 found in prespawning vs. spawned animals from family line FL-2 (Figure 4A&B).
306 There was no significant interaction between day and spawning for antiviral activity
307 (F=0.19, p=0.67) or antibacterial activity (F=0.29, p=0.61). The lack of significant
308 difference between days both in the control and spawned groups illustrates that
309 repeated sampling of the haemolymph did not affect their antiviral (F=0.83, p=0.37)
310 or antibacterial (F=0.08 p=0.78) activity.

311 **1. DISCUSSION**

312 Abalone haemolymph is involved in innate defense (Cheng 1981; Mitta *et al.* 2000;
313 Bachère *et al.* 2004; Hooper *et al.* 2007b) and represents a valuable resource for
314 studying antiviral and antibacterial activity at individual, family line, population or
315 species level. Bleeding of abalone is a fast and non-lethal procedure, and offers an
316 advantage over other commercial molluscs (e.g. snails, cuttlefish, octopuses, squids,
317 clams, mussels, oysters and scallops), which typically require anesthetisation to
318 obtain the haemolymph (Gunkel *et al.* 2008) if they are not killed. Non-lethal
319 sampling with minimal handling allows for detection of immune responses from
320 relatively unstressed animals and facilitates identification of individuals with
321 enhanced immunity for future breeding and heritability studies. Our study has applied
322 *in vitro* antibacterial and antiviral assays, which have been broadly used for the
323 investigation of immune defense in molluscs (Yasin *et al.* 2000; Maier *et al.* 2001;
324 Olicard *et al.* 2005a; Olicard *et al.* 2005b; Vakalia *et al.* 2005; Li *et al.* 2007a; Li *et al.*
325 2009a; Li *et al.* 2009b; Dang *et al.* 2011) and confirms antiviral activity against HSV-
326 1 and antibacterial activity against *V. harveyi* in the haemolymph of greenlip *H.*
327 *laevigata,* blacklip *H. rubra* and their hybrid cross.

328 Antimicrobial activities have been previously reported in the haemolymph and
329 organic extracts from a range of other molluscan species (see reviews by Mitta *et al.*
330 2000, Li *et al*. 2009d, Benkendorff 2010). In particular, four classes of antibacterial
331 peptides have been isolated from bivalves (Mitta *et al*. 2000; Li *et al*. 2009d). More
332 recently, abhisin and a defensin peptide have been reported from the abalone *H.*
333 *discus discus* with antibacterial activity against marine *Vibrio*s (De Zoysa *et al.* 2009;
334 De Zoysa *et al.* 2010). In general, marine invertebrate antimicrobial peptides appear
335 to be amphiphilic and cationic to interact with bacterial membranes (Tincu *et al.*
336 2004; Li *et al*. 2009d; Otero-Gonzalez *et al.* 2010). Our preliminary studies indicate
337 that the haemolymph antibacterial compounds in *H. rubra* and *H. laevigata* could be
338 small peptides, but they are not proteins, as the activity was retained after
339 proteinase-K and heat treatment (121°C for 20min, Benkendorff unpublished data).
340 Attempts to purify the active factors resulted in antibacterial activity spreading across
341 a number of column fractions. Similar findings on molluscan antibacterial activity
342 spreading across column fraction have been reported by Defer *et al*. (2009) and
343 indicate that there may be more than one antibacterial factor may be present. In
344 addition to peptides, a wide range of lipophilic antibacterial compounds have been
345 isolated from gastropod molluscs, including polyunsaturated fatty acids and alkaloids
346 (Benkendorff *et al.* 2000, 2001, 2005; Benkendorff 2010). We have found
347 antibacterial activity in lipophilic extracts after passing abalone hemolymph through a
348 dianion resin HP20 column (Benkendorff unpublished data). Consequently, abalone
349 hemolymph may include both ubiquitous antibacterial peptides, as well as potentially
350 novel lipophylic antibacterial compounds.

351 Relatively less is known about the compounds responsible for antiviral activity in
352 marine molluscs. An anti-HIV peptide has been reported from oyster protein
353 hydrolysate (Lee *et al.* 1998) and anti-HSV-1 compounds have been reported in
354 acidic extract (40-80% acetonitrile SPE-fraction) from a range of bivalves and
355 gastropods (Defer *et al.* 2009a; b), suggestive of amphiphilic peptides. Synthetic
356 analogues of mytilin appear to have both antibacterial and antiviral activity (Roch *et*
357 *al*. 2008). However, the antiviral and antibacterial activities of abalone haemolymph
358 appear to be attributed by different compounds, since we found no correlation
359 between the level of activity detected in the antiviral and antibacterial assays across
360 all abalone samples in this study. Preliminary studies on antiviral factors from

361 abalone by Li (1960) and Li *et al* (1962) indicated they were likely to be
362 macromolecules such as glycoproteins. Subsequently, De Zoysa *et al.* (2007) report
363 the presence of a gene for myxovirus resistance (Mx) protein in the abalone *Haliotis*
364 *discus discus.* However, our studies on *H. laevigata* haemolymph indicate that the
365 anti-herpes virus compounds are unlikely to be proteins, as no significant loss of
366 activity was detected after proteinase-K, trypsin and/or high temperature treatment
367 (Dang *et al.* 2011). The antiviral activity of *H. laevigata* hemolymph was also lost by
368 lipophylic extraction on SPE columns, suggesting the active compounds are mostly
369 likely to be sugars, acids or small polar heat resistant peptides (Dang *et al.* 2011).
370 Ultimately, further research is required to elucidate the chemical structures of both
371 the antiviral and antibacterial compounds from abalone hemolymph.

372 The lack of significant difference in antiviral and antibacterial activity between wild
373 greenlip *H. laevigata* and blacklip *H. rubra* suggests that neither species has
374 inherently stronger antimicrobial defense properties than the other. This is consistent
375 with the fact that neither of the above species is more resistant to AVG or Vibriosis
376 than the other (Handlinger *et al.* 2005; Hooper *et al.* 2007a). Since our study only
377 assesses general antiviral activity against a model virus, HSV-1 in Vero cells, it does
378 not address the possibility of species-specific adaptation to endemic diseases. The
379 situation of abalone herpes-like viral infection in Taiwan provides an example, where
380 resistant abalone *H. discus* share the same habitat with susceptible *H. diversicolor*
381 *supertexta* (Chang *et al.* 2005). All abalone from both species used in this study
382 were collected from South Australia, where *V. harveyi* is endemic but viral outbreaks
383 have not been previously detected. The fact that some antiviral activity was detected
384 in all haemolymph samples tested implies that *H. laevigata* and *H. rubra* both
385 constitutively express antiviral defense factors, but it remains to be seen whether
386 these are induced by infection and/or effective against abalone herpesvirus.

387 Though interspecies hybridization between blacklip *H. rubra* and greenlip *H.*
388 *laevigata* has been applied in Australia, evidence for hybrid vigour or heterosis,
389 including immune fitness or growth rate, has not been previously reported. Previous
390 studies on marine invertebrate species indicate that interspecies hybrids can
391 sometimes show decreased vigour, for example hybrid larvae from mussels *Mytilus*
392 *edulis* and *M. galloprovincialis* grew slower than larvae of either pure species

393 (Beaumont *et al.* 2004). However, increased vigour was reported in hybrids from
394 Pacific sea urchins *Echinometra* sp. A and *Echinometra mathaei*, which showed the
395 superiority of growth traits compared to their parents (Rahman *et al.* 2005); and no
396 significant difference in growth rates was reported for *Penaeus monodon* and *P.*
397 *esculentus* hybrids (Benzie *et al.* 1995). In this study, there was no clear evidence for
398 hybrid vigour or hybrid depression in the farmed *H. laevigata* x *H. rubra* abalone. The
399 hybrid FL-4 had a similar mean shell length to the other farmed green lip (*H*
400 *laevigata*) family lines in the same age cohort. There was also no significant different
401 in antiviral activity of the hybrid compared to the farmed family lines. On average,
402 antibacterial activity of the hybrid was higher than the farmed *H. laevigata*, however,
403 when size was taken into consideration, pairwise tests revealed that the hybrid
404 differed only from 2yr old family line FL-1 and was not different to the two family lines
405 in the same 3yr age cohort. Thus, despite maintaining genetic variability by
406 outbreeding, the *H. laevigata* x *H. rubra* hybrid does not appear to benefit from
407 increased growth or immune fitness. Genetic variability at the species level does not
408 appear to contribute to variation in the constitutively expressed antiviral or
409 antibacterial activity. However, this does not rule out the possibility that variability at
410 the individual level is genetically based and thus inheritable.

411 Significant variation of *in vitro* antiviral and antibacterial activity in abalone was found
412 at the individual level (up to 69% and 46% of the variation, respectively). This
413 variability in antimicrobial defense may explain the observed differences in
414 susceptibility of individual abalone towards the herpesvirus (Hooper *et al.* 2007a;
415 Crane *et al.* 2009) and *Vibrio* bacteria infections (Handlinger *et al.* 2005; Vakalia *et*
416 *al.* 2005) within abalone farms and in natural populations. The large variation in
417 antimicrobial activity of abalone haemolymph may relate to history of exposure in
418 individual abalone, as antimicrobial compounds are expressed not only
419 constitutively, but can also be induced in response to a particular stimulus (Li *et al.*,
420 2009d). Several antimicrobial peptides such as defensins and mytilins have been
421 isolated from the haemolymph of immune-challenged mussel *M. edulis* (Charlet *et al.*
422 1996) and *M. galloprovincialis* (Mitta *et al.* 2000), clam *R. decussatus* (Gestal *et al.*
423 2007) and scallop *Argopecten irradians* (Zhao *et al.* 2007). Antimicrobial peptides in
424 marine molluscs or invertebrates have been thoroughly reviewed by Li *et al* (2009d)
425 or Otero-Gonzalez *et al.* (2010) and Tincu *et al.* (2004) and they are continuously

426 expressed and rapidly induced at different cellular levels to interact directly with
427 infectious agents or pathogenic microorganisms. A study by Li et al. (2009c)
428 demonstrates that antibacterial activity in oysters significantly dropped in the week
429 following a simulated bacterial challenge, whilst haemolymph protein significantly
430 increased. Thus the variation between abalone may result from a combination of
431 their recent infection history and their overall immune fitness.

432 The main factor found to influence antiviral and antimicrobial activity of abalone was
433 whether they were sourced from natural populations or a farm. Environmental factors
434 that impact the immune response could explain the higher antimicrobial activity in
435 wild greenlip abalone compared to farmed ones. Abalone in farms could experience
436 immunodepression from stress resulting from a change in their living environment
437 (e.g. handling, water conditions and density) (Malham *et al.* 2003; Cheng *et al.*
438 2004a; Cheng *et al.* 2004b; Cheng *et al.* 2004c; Cheng *et al.* 2004d; Cheng *et al.*
439 2004e; Hooper *et al.* 2007b). The artificial diets on farms may also result in lower
440 antiviral activity than natural populations that feed on algae, as antiviral and
441 antimicrobial activity have been widely reported from marine algae (Ohta *et al.* 1998;
442 Ponce *et al.* 2003; Lee *et al.* 2004; Smit 2004; Talarico *et al.* 2004; Puglisi *et al.*
443 2007; Salvador *et al.* 2007; Stirk *et al.* 2007; Mandal *et al.* 2008; Kamenarska *et al.*
444 2009). Overall, a combination of biotic and abiotic environmental factors that impact
445 the immune system and individual history of exposure to disease are all likely to
446 contribute to the high levels of observed variability in antiviral and antibacterial
447 activity of abalone haemolymph.

448 The size or age of the abalone may also influence their antimicrobial properties.
449 Shell length was significantly correlated to antiviral and antibacterial activity in
450 greenlip abalone *H. laevigata*, although in both cases only a weak positive
451 relationship with size was observed. For antibacterial activity, 2yr old abalone tended
452 to have lower activity than 3yr old family lines. These results suggest that abalone
453 may develop stronger antimicrobial defense compounds and/or synthesize higher
454 levels of the active compounds as they grow. Juvenile Pacific oysters *C. gigas*
455 experience much higher mortality due to Ostreid Herpesvirus 1 infection than adults
456 and adults can carry the virus without displaying clinical signs (Arzul *et al.* 2002b),
457 supporting the development of improved immunity in molluscs with age. However,

458 previous field observations on the AVG outbreak in farmed abalone indicate that high
459 mortality occurs both in juvenile abalone from one to four years old and the older
460 brood stock (Hooper *et al.* 2007a). The gender of the abalone did not influence their
461 antimicrobial activitity and surprisingly, we found no effect of spawning on antiviral
462 and antibacterial activity in abalone haemolymph. Gametogenesis and spawning has
463 been linked to decreased immune function (Travers *et al.* 2008) and increased
464 mortality due to *Vibriosis* in *H. tuberculata* (Travers *et al.* 2009). These studies were
465 conducted over a longer time frame and hence it is possible that the abalone
466 antimicrobial reserves within the haemolymph have not been exhausted within the
467 short time frame of our studies (one day post spawning). Spawning is known to
468 cause a instant reduction in the energy reserves for 5-8 days (e.g. glycogen or
469 protein) and results in reduced antibacterial activity in oysters from day 3 to 25 (Li *et*
470 *al.* 2007a; Li *et al.* 2010). Consequently, over a longer time frame, it would be
471 expected that the antibacterial reserves in abalone also become depleted in a few
472 days post-spawning and may not be replenished until their condition improves
473 (Travers *et al.* 2009).

474 In conclusion, we found considerable variability in the levels of antiviral and
475 antibacterial activity in haemolymph of blacklip *H. rubra*, greenlip *H. laevigata* and
476 their hybrid cross. This extends our previous reports of antiviral activity in greenlip
477 abalone *H. laevigata* (Dang et al. 2011). Plaque assay for antiviral activity against
478 HSV-1 represents a useful tool for comparing antiviral activity within and between
479 abalone species. Further study on antiviral activity against marine herpesvirus (i.e.
480 salmon herpesvirus type 1 and 2) is underway to confirm the variation of antiviral
481 activity against ecologically relevant pathogens. *V. harveyi* provides a useful model
482 for screening *in vitro* antibacterial activity in haemolymph. The variation in antiviral
483 and antibacterial activity of abalone within the same family line or geographic
484 location may explain why some individuals are more resistant to AVG and *Vibrio*
485 infections than others. Further research is required to investigate whether high
486 antiviral and antibacterial activity translates to reduced susceptibility towards
487 infections.

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496 **REFERENCES**

497 Abdi, H., 2007. The Bonferonni and Šidák corrections for multiple comparisons. in: Salkind, N.J. (Ed.),
498 Encyclopedia of measurement and statistics. Sage, Thousand Oaks, CA.
499 Arzul, I., Renault, T., 2002a. Herpesvirus infections in marine bivalves. Virologie. 6, 169-174.
500 Arzul, I., Renault, T., Thebault, A., Gerard, A., 2002b. Detection of oyster herpesvirus DNA and proteins in
501 asymptomatic *Crassostrea gigas* adults. Virus Research. 84, 151-160.
502 Austin, B., Zhang, X.H., 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates.
503 Letters in Applied Microbiology. 43, 119-124.
504 Bachère, E., Gueguen, Y., Gonzalez, M., De Lorgeril, J., Garnier, J., Romestand, B., 2004. Insights into the anti-
505 microbial defense of marine invertebrates: the *penaeid* shrimps and the oyster *Crassostrea gigas*.
506 Immunological Reviews. 198, 149-168.
507 Beaumont, A.R., Turner, G., Wood, A.R., Skibinski, D.O.F., 2004. Hybridisations between *Mytilus edulis* and
508 *Mytilus galloprovincialis* and performance of pure species and hybrid veliger larvae at different
509 temperatures. Journal of Experimental Marine Biology and Ecology. 302, 177-188.
510 Benkendorff, K. 2010. Molluscan biological and chemical diversity: Secondary metabolites and medicinal
511 resources produced by marine molluscs. Biological Reviews. 85, 757-775 Doi:10.111/j.1469-
512 185X.2010.00124x
513 Benzie, J.A.H., Kenway, M., Ballment, E., Frusher, S., Trott, L., 1995. Interspecific hybridization of the tiger
514 prawns *Penaeus monodon* and *Penaeus esculentus*. Aquaculture. 133, 103-111.
515 Carson, J., Higgins, M.J., Wilson, T.K., Gudkovs, N., Bryant, T.N., 2006. Aquatic animal health subprogram.
516 *Vibrios* of aquatic animals: development of a national standard diagnostic technology. Final report for
517 project 2001/628, Fisheries Research & Development Corporation, Canberra, ISBN 1 86295 368 6, pp.
518 347.
519 Chang, P.H., Kuo, S.T., Lai, S.H., Yang, H.S., Ting, Y.Y., Hsu, C.L., Chen, H.C., 2005. Herpes-like virus
520 infection causing mortality of cultured abalone *Haliotis diversicolor supertexta* in Taiwan. Diseases of
521 Aquatic Organisms. 65, 23-27.
522 Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J., Bulet, P., 1996. Innate immunity: isolation of
523 several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. The Journal of
524 Biological Chemistry. 271, 21808 –21813.
525 Chen, J.H., Yang, H.Y., Peng, S.W., Chen, Y.J., Tsai, K.Y., 1996. Characterisation of abalone (*Haliotis*
526 *diversicolor*) hemocytes *in vitro*. The Biological Bulletin. 31, 31-38.
527 Cheng, T.C., 1981. Bivalves. in: Ratcliffe, N.A., Rowley, A.F. (Eds.), Invertebrate blood cells. Academic Press,
528 London, pp. 233–299.
529 Cheng, W., Hsiao, I.S., Chen, J.-C., 2004a. Effect of ammonia on the immune response of Taiwan abalone
530 *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. Fish and Shellfish
531 Immunology. 17, 193-202.
532 Cheng, W., Hsiao, I.S., Chen, J.C., 2004b. Effect of nitrite on immune response of Taiwan abalone *Haliotis*
533 *diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. Diseases of Aquatic
534 Organisms. 60, 157-164.
535 Cheng, W., Hsiao, I.S., Hsu, C.H., Chen, J.C., 2004c. Change in water temperature on the immune response of
536 Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. Fish
537 and Shellfish Immunology. 17, 235-243.

538 Cheng, W., Juang, F.M., Chen, J.C., 2004d. The immune response of Taiwan abalone *Haliotis diversicolor*
539 *supertexta* and its susceptibility to *Vibrio parahaemolyticus* at different salinity levels. Fish and
540 Shellfish Immunology. 16, 295-306.
541 Cheng, W., Li, C.H., Chen, J.C., 2004e. Effect of dissolved oxygen on the immune response of *Haliotis*
542 *diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus* Aquaculture. 232, 103-115.
543 Cook, P., 1998. The current status of abalone farming in South Africa. Journal of Shellfish Research. 17, 601-
544 602.
545 Cory, A.H., Owen, T.C., Barltrop, J.A., Cory, J.G., 1991. Use of an aqueous soluble tetrazolium formazan assay
546 for cell-growth assays in culture. Cancer Communications. 3, 207-212.
547 Crane, M.S.J., Corbeil, S., Colling, A., Williams, L.M., Wong, F., Savin, K., Cogan, N., Fegan, M.,
548 Mohammad, I., Sunarto, A., Handlinger, J., Pyecroft, S., Douglas, M., Murdoch, B., Warner, S., 2009.
549 Development and validation of a real-time (TaqMan) PCR assay for the detection and identification of
550 abalone herpes-like virus (AbHV) 4th FRDC Aquatic Animal Health Subprogram Scientific
551 Conference, Cairns.
552 Dang, V.T., Benkendorff, K., Speck, P., 2011. *In vitro* antiviral activity against herpes simplex virus in abalone
553 *Haliotis laevigata*. Journal of General Virology. DOI:10.1099/vir.0.025247-0.
554 Day, R., Hooper, C., Benkendorff, K., Slocombe, R., Handlinger, J., 2010. Investigations on the immunology of
555 stressed abalone, Final report for FRDC project number: 2004/233. Fisheries Research and
556 Development Corporation and The University of Melbourne.
557 De Zoysa, M., Kang, H.S., Song, Y.B., Jee, Y., Lee, Y.D., Lee, J., 2007. First report of invertebrate Mx:
558 cloning, characterization and expression analysis of Mx cDNA in disk abalone (*Haliotis discus discus*).
559 Fish & Shellfish Immunology. 23, 86-96.
560 De Zoysa, M., Nikapitiya, C., Whang, I., Lee, J.-S., Lee, J., 2009. Abhisin: a potential antimicrobial peptide
561 derived from histone H2A of disk abalone (*Haliotis discus discus*). Fish & Shellfish Immunology. 27,
562 639-646.
563 De Zoysa, M., Whang, I., Lee, Y., Lee, S., Lee, J.-S., Lee, J., 2010. Defensin from disk abalone *Haliotis discus*
564 *discus*: molecular cloning, sequence characterization and immune response against bacterial infection.
565 Fish & Shellfish Immunology. 28, 261-266.
566 Defer, D., Bourgougnon, N., Fleury, Y., 2009a. Detection and partial characterisation of an antimicrobial
567 peptide (littorein) from the marine gastropod *Littorina littorea*. International Journal of Antimicrobial
568 Agents. 34, 188-190.
569 Defer, D., Bourgougnon, N., Fleury, Y., 2009b. Screening for antibacterial and antiviral activities in three
570 bivalve and two gastropod marine molluscs. Aquaculture. 293, 1-7.
571 Duchemin, M.B., Fournier, M., Auffret, M., 2007. Seasonal variation of immune parameters in diploid and
572 triploid Pacific oysters, *Crassostrea gigas* (Thunberg). Aquaculture. 264, 73–81.
573 Fegan, M., Kvalheim, N., Wong, F., Mohammad, I., Savin, K., Lancaster, M., Crane, M.S.J., Warner, M.S.,
574 2009. Development of an *in situ* hybridisation assay for the detection and identification of the abalone
575 herpes-like virus, 4th FRDC Aquatic Animal Health Subprogram Scientific Conference, Cairns.
576 Fleming, A.E., Hone, P.W., 1996. Abalone aquaculture. Aquaculture. 140, 1-4.
577 Freeman, K., 2001. Aquaculture and related biological attributes of abalone species in Australia-a review.
578 Fisheries Research Report No. 128. Fisheries Western Australia. 48.
579 Gestal, C., Costa, M., Figueras, A., Novoa, B., 2007. Analysis of differentially expressed genes in response to
580 bacterial stimulation in hemocytes of the carpet-shell clam *Ruditapes decussatus*: identification of new
581 antimicrobial peptides. Gene. 406, 134 –143.
582 Godoy, C., Jerez, G., 1998. The introduction of abalone in Chile: ten years later. Journal of Shellfish Research.
583 17, 603-605.
584 Gordon, H.R., Cook, P.A., 2001. World abalone supply, markets and pricing: historical, current and future.
585 Journal of Shellfish Research. 20, 567-570.
586 Gordon, H.R., Cook, P.A., 2004. World abalone fisheries and aquaculture update: supply and market dynamics.
587 National Shellfisheries Association, pp. 935-939.
588 Gunkel, C., Lewbart, G.A., 2008. Anesthesia and analgesia of invertebrates. in: Fish, R.E., Brown, M.J.,
589 Danneman, P.J., Karas, A.Z. (Eds.), Anesthesia and analgesia in laboratory animals. Elsevier Science
590 & Technology, pp. 535-538.
591 Hahn, K.O., 1989. Handbook of culture of abalone and other marine gastropods. CRC Press, Boca Raton,
592 Florida.
593 Handlinger, J., Carson, J., Donachie, L., Gabor, L., Taylor, D., 2005. Bacterial infection in Tasmanian farmed
594 abalone: causes, pathology, farm factors and control options. in: Walker, P., Lester, R., Bondad-
595 Reantaso, M.G. (Eds.), Proceedings of the 5th Symposium on Diseases in Asian Aquaculture, Australia,
596 pp. 289-300.
597 Hochberg, Y., 1988. A sharper Bonferroni procedure for multiple tests of significance. Biometrika. 75, 800-802.

598 Hooper, C., Day, R., Slocombe, R., Handlinger, J., Benkendorff, K., 2007b. Stress and immune responses in
599 abalone: limitations in current knowledge and investigative methods based on other models. Fish and
600 Shellfish Immunology. 22, 363-379.
601 Hooper, C., Hardy-Smith, P., Handlinger, J., 2007a. Ganglioneuritis causing high mortalities in farmed
602 Australian abalone (*Haliotis laevigata* and *Haliotis rubra*). Australian Veterinary Journal. 85, 188-193.
603 Kamenarska, Z., Serkedjieva, J., Najdenski, H., Stefanov, K., Tsvetkova, I., Dimitrova-Konaklieva, S., Popov,
604 S., 2009. Antibacterial, antiviral, and cytotoxic activities of some red and brown seaweeds from the
605 Black Sea. Botanica Marina. 52, 80-86.
606 Lee, J.B., Hayashi, K., Maeda, M., Hayashi, T., 2004. Antiherpetic activities of sulfated polysaccharides from
607 green algae. Planta Medica. 70, 813-817.
608 Lee, T.G., Maruyama, S 1998. Isolation of HIV-1 protease-inhibiting peptides from thermolysis hydrolysate of
609 osyer proteins. Biochemical and Biophysical Research Communications. 253, 604-608.
610 Li, C.P. 1960. Antimicrobial effect of abalone juice. Proceedings of the Society for Experimental Biology &
611 Medicine 103, 522-524.
612 Li, C.P., Prescott, B., Jahnes, W.G. 1962. Antiviral activity of a fraction of abalone juice. Proceedings of the
613 Society for Experimental Biology & Medicine 109, 534-538.
614 Li, Y., Qin, J.G., Abbot, C.A., Li, X., Benkendorff, K., 2007a. Synergistic impacts of heat shock and spawning
615 on the physiology and immune health of *Crassostrea gigas*: an explanation for summer mortality
616 in Pacific oysters. The American Journal of Physiology - Regulatory, Integrative and Comparative
617 Physiology 293, 2353–2362.
618 Li, Y., Qin, J.G., Abbott, C.A., Li, X.X., Benkendorff, K., 2007b. Synergistic impacts of heat shock and
619 spawning on the physiology and immune health of *Crassostrea gigas*: an explanation for summer
620 mortality in Pacific oysters. American Journal of Physiology-Regulatory Integrative and Comparative
621 Physiology. 293, R2353-R2362.
622 Li, Y., Qin, J.G., Li, X., Benkendorff, K., 2009a. Monthly variation of condition index, energy reserves and
623 antibacterial activity in Pacific oysters, *Crassostrea gigas*, in Stansbury (South Australia). Aquaculture.
624 286, 64-71.
625 Li, Y., Qin, J.G., Li, X., Benkendorff, K., 2009b. Spawning-dependent stress response to food deprivation in
626 Pacific oyster *Crassostrea gigas*. Aquaculture. 286, 309-317.
627 Li, Y., Qin, J.G., Li, X., Benkendorff, K., 2009c. Spawning-dependent stress responses in pacific oysters
628 *Crassostrea gigas*: a simulated bacterial challenge in oysters. Aquaculture. 293, 164-171.
629 Li, Y., Qin, J.G., Li, X., Benkendorff, K., 2010. Assessment of metabolic and immune changes in postspawning
630 Pacific oyster *Crassostrea gigas*: identification of a critical period of vulnerability after spawning.
631 Aquaculture Research. 41, 155-165.
632 Li, C.H., Zhao, J.M., Song L.S. 2009d. A review of advances in research on marine molluscan antimicrobial
633 peptides and their potential in aquaculture. Molluscan Research. 29, 17-26.
634 Maier, M.S., Roccatagliata, A.J., Kuriss, A., Chludil, H., Seldes, A.M., Pujol, C.A., Damonte, E.B., 2001. Two
635 new cytotoxic and virucidal trisulfated triterpene glycosides from the antarctic sea cucumber
636 *Staurocucumis liouvillei*. Journal of Natural Products. 64, 732-736.
637 Malham, S.K., Lacoste, A., Gélébart, F., Cueff, A., Poulet, S.A., 2003. Evidence for a direct link between stress
638 and immunity in the mollusc *Haliotis tuberculata*. Journal of Experimental Zoology Part A:
639 Comparative Experimental Biology. 295A, 136-144.
640 Mandal, P., Pujol, C.A., Carlucci, M.J., Chattopadhyay, K., Damonte, E.B., Ray, B., 2008. Anti-herpetic activity
641 of a sulfated xylomannan from *Scinaia hatei*. Phytochemistry. 69, 2193-2199.
642 Matozzo, V., Marin, M., 2010. First evidence of gender-related differences in immune parameters of the clam
643 *Ruditapes philippinarum* (Mollusca, Bivalvia). Marine Biology. 157, 1181-1189.
644 Mitta, G., Vandenbulcke, F., Roch, P., 2000. Original involvement of antimicrobial peptides in mussel innate
645 immunity. FEBS Letters. 486, 185–190.
646 Nicolas, J.L., Basuyaux, O., Mazurie, J., Thebault, A., 2002. *Vibrio carchariae*, a pathogen of the abalone
647 *Haliotis tuberculata*. Diseases of Aquatic Organisms. 50, 35-43.
648 Nishimori, E., Hasegawa, O., Numata, T., Wakabayashi, H., 1998. *Vibrio carchariae* causes mass mortalities in
649 Japanese abalone, *Sulculus diversicolor supratexta*. Fish Pathol. 33, 495-502.
650 Ohta, S., Ono, F., Shiomi, Y., Nakao, T., Aozasa, O., Nagate, T., Kitamura, K., Yamaguchi, S., Nishi, M.,
651 Miyata, H., 1998. Anti-Herpes Simplex Virus substances produced by the marine green alga,
652 *Dunaliella primolecta*. Journal of Applied Phycology. 10, 349-356.
653 Olicard, C., Didier, Y., Marty, C., Bourgougnon, N., Renault, T., 2005a. *In vitro* research of anti-HSV-1 activity
654 in different extracts from Pacific oysters *Crassostrea gigas*. Diseases of Aquatic Organisms. 67, 141-
655 147.
656 Olicard, C., Renault, T., Torhy, C., Benmansour, A., Bourgougnon, N., 2005b. Putative antiviral activity in
657 hemolymph from adult Pacific oysters, *Crassostrea gigas*. Antiviral Research. 66, 147-152.

658 Otero-Gonzalez, A.J., Magalhaes, B.S., Garcia-Villarino, M., Lopez-Abarrategui, C., Sousa, D.A., Dias, S.C.,
659 Franco, O.L., 2010. Antimicrobial peptides from marine invertebrates as a new frontier for microbial
660 infection control. Journal of the Federation of American Societies for Experimental Biology. 24, 1320-
661 1334.
662 Ponce, N.M.A., Pujol, C.A., Damonte, E.B., Flores, M.L., Stortz, C.A., 2003. Fucoidans from the brown
663 seaweed *Adenocystis utricularis*: extraction methods, antiviral activity and structural studies.
664 Carbohydrate Research. 338, 153-165.
665 Pouvreau, S., Enriquez-Diaz, M., Le Souchu, P., Connan, J.P., Le Roy, B., Mingant, C., Moal, J., Delaporte, M.,
666 Le Coz, J.R., Samain, J.F., 2003. Reproduction, bioenergetic and summer mortality of *Crassostrea*
667 *gigas*: experimental approach. Journal of Shellfish Research. 22, 351.
668 Puglisi, M., Engel, S., Jensen, P., Fenical, W., 2007. Antimicrobial activities of extracts from Indo-Pacific
669 marine plants against marine pathogens and saprophytes. Marine Biology. 150, 531-540.
670 Rahman, M.A., Uehara, T., Lawrence, J.M., 2005. Growth and heterosis of hybrids of two closely related
671 species of Pacific sea urchins (Genus *Echinometra*) in Okinawa. Aquaculture. 245, 121-133.
672 Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. The American Journal of
673 Hygiene. 27, 493-497.
674 Renault, T., Lipart, C., Arzul, I., 2001. A herpes-like virus infecting *Crassostrea gigas* and *Ruditapes*
675 *philippinarum* larvae in France. Journal of Fish Diseases. 24, 369-376.
676 Roch, P., Yang, Y., Toubiana, M., Aumelas, A., 2008. NMR structure of mussel mytilin, and antiviral-
677 antibacterial activities of derived synthetic peptides. Dev. Comp. Immunol. 32, 227-238.
678 Russell, W.C., 1962. A Sensitive and precise plaque assay for herpes virus. Nature. 195, 1028-1029.
679 Salvador, N., Garreta, A.G., Lavelli, L., Siguán, M.A.R., 2007. Antimicrobial activity of Iberian macroalgae.
680 Scientia Marina. 71, 101-113.
681 Savin, K., Cocks, B., Wong, F., Sawbridge, T., Cogan, N., Savage, D., Warner, S., 2010. A neurotropic
682 herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus
683 associated with the amphioxus genome. Virology Journal. 7, 308.
684 Smit, A.J., 2004. Medicinal and pharmaceutical uses of seaweed natural products: a review. Journal of Applied
685 Phycology. 16, 245-262.
686 Soletchnik, P., Faury, N., Goulletquer, P., 2006. Seasonal changes in carbohydrate metabolism and its
687 relationship with summer mortality of Pacific oyster *Crassostrea gigas* (Thunberg) in Marennes-Oléron
688 bay (France). Aquaculture. 252, 328–338.
689 Speck, P.G., Simmons, A., 1991. Divergent molecular pathways of productive and latent infection with a
690 virulent strain of herpes simplex virus type 1. Journal of Virology. 65, 4001-4005.
691 Speck, P.G., Simmons, A., 1992. Synchronous appearance of antigen-positive and latently infected neurons in
692 spinal ganglia of mice infected with a virulent strain of herpes simplex virus. Journal of General
693 Virology. 73, 1281-1285.
694 Stirk, W., Reinecke, D., van Staden, J., 2007. Seasonal variation in antifungal, antibacterial and
695 acetylcholinesterase activity in seven South African seaweeds. Journal of Applied Phycology. 19, 271-
696 276.
697 Talarico, L.B., Zibetti, R.G.M., Faria, P.C.S., Scolaro, L.A., Duarte, M.E.R., Noseda, M.D., Pujol, C.A.,
698 Damonte, E.B., 2004. Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds
699 *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*. International Journal of Biological
700 Macromolecules. 34, 63-71.
701 Taskinen, J., Saarinen, M., 1999. Increased parasite abundace associated with reproductive maturity of the clam
702 *Anodonta piscinalis*. The Journal of Parasitology. 85, 588-591.
703 Tincu, J.A., Taylor, S.W., 2004. Antimicrobial peptides from marine invertebrates. Antimicrobial Agents and
704 Chemotherapy. 48, 3645-3654.
705 Travers, M.A., Basuyaux, O., Le GoÏC, N., Huchette, S., Nicolas, J.L., Koken, M., Paillard, C., 2009. Influence
706 of temperature and spawning effort on *Haliotis tuberculata* mortalities caused by *Vibrio harveyi*: an
707 example of emerging vibriosis linked to global warming. Global Change Biology. 15, 1365-1376.
708 Travers, M.A., Le Goic, N., Huchette, S., Koken, M., Paillard, C., 2008. Summer immune depression associated
709 with increased susceptibility of the European abalone, *Haliotis tuberculata* to *Vibrio harveyi* infection.
710 Fish & Shellfish Immunology. 25, 800-808.
711 Uki, N., 1989. Abalone seedling production and its theory. International Journal Aquaculture Fisheries
712 Technology. 1, 3-15.
713 Vakalia, S., Benkendorff, K., 2005. Antimicrobial activity in the haemolymph of *Haliotis laevigata* and the
714 effects of a dietary immunostimulant. in: Fleming, A. (Ed.), The 12th Annual Abalone Aquaculture
715 Workshop. Abalone Aquaculture Subprogram, Fisheries Research and Development Corporation,
716 Canberra, Australia, McLaren Vale, South of Australia.

717 Wang, J.Y., Guo, Z.X., Feng, J., Liu, G.F., Xu, L.W., Chen, B.S., Pan, J.P., 2004. Virus infection in cultured
718 abalone, *Haliotis diversicolor reeve* in Guangdong Province, China. Journal of Shellfisheries
719 Research. 23, 1163–1168.
720 Yasin, B., Pang, M., Turner, J.S., Cho, Y., Dinh, N.N., Waring, A.J., Lehrer, R.I., Wagar, E.A., 2000.
721 Evaluation of the inactivation of infectious herpes simplex virus by host-defense peptides. European
722 Journal of Clinical Microbiology and Infectious Diseases. 19, 187-194.
723 Zhao, J., Song, L., Li, C., Ni, D., Wu, L., Zhu, L., Wang, H., Xu, W., 2007. Molecular cloning, expression of a
724 big defensin gene from bay scallop *Argopecten irradians* and the antimicrobial activity of its
725 recombinant protein. Molecular Immunology. 44, 360–368.
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729 **FIGURES**

730 Figure 1. Variation in A) antiviral activity (%) and B) antibacterial activity (%) at
731 individual, family line, population and species scale. All haemolymph plasma
732 samples were tested in triplicates at the same concentration against HSV-1 (6%, v/v)
733 using plaque reduction assay and *V. haveyi* using MTS assay. Antiviral and
734 antibacterial activity was compared for three greenlip family lines (FL-1, FL-2 and FL-
735 3) and one greenlip and blacklip hybrid (FL-4), as well as four wild populations of
736 greenlip abalone *H. laevigata* (FB-Farm Beach, EL-Elliston, CO-Cowell, BC-
737 Blackfellows Caves) and three wild populations of blacklip abalone *H. rubra* (EL, OS-
738 O'Sullivan Beach, BC).

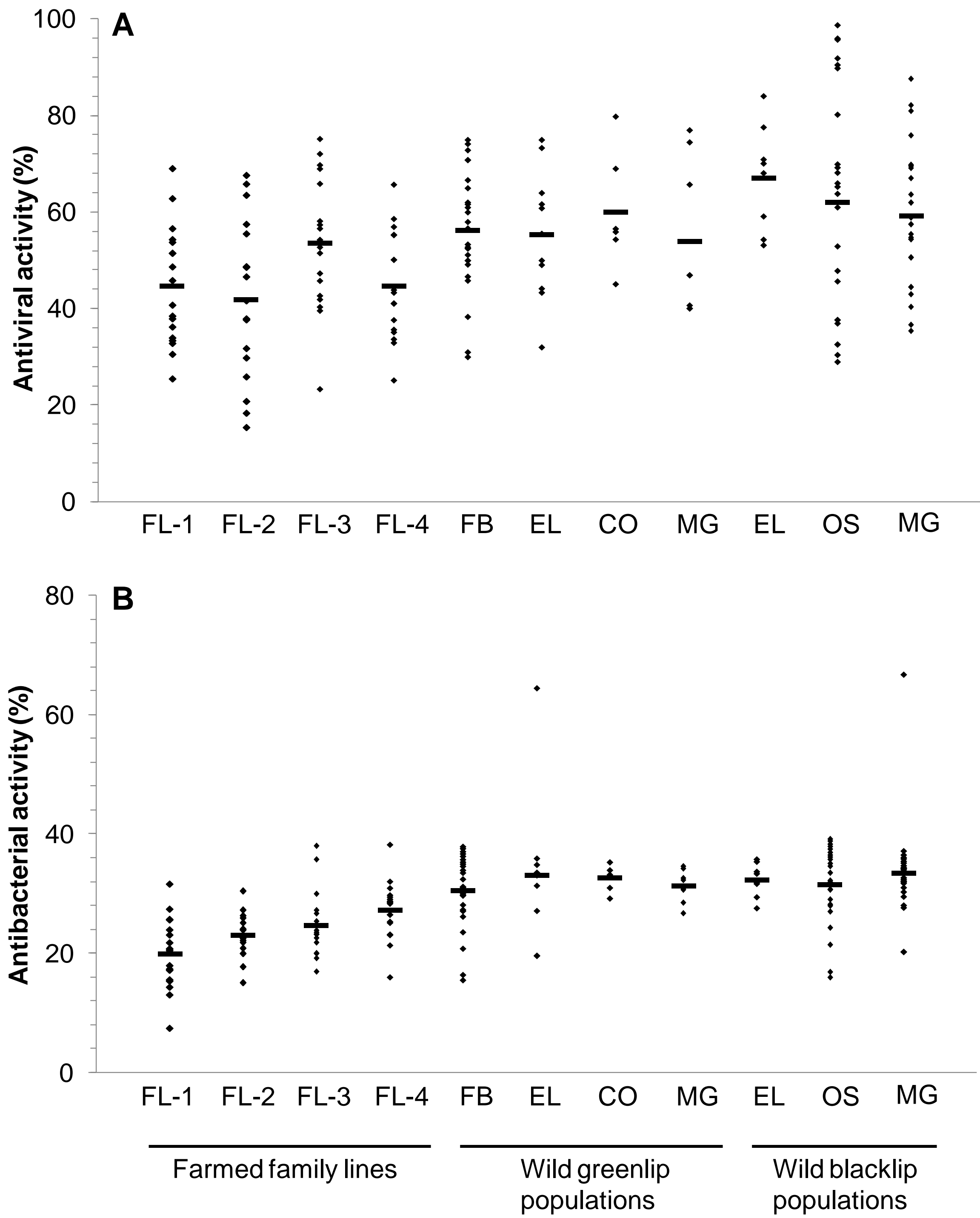
739 Figure 2. Comparison of A) antiviral activity (%) and B) antibacterial activity (%)
740 between farmed greenlip *H. laevigata* (n=54), farmed hybrid *H. laevigata* x *H. rubra*
741 (n=18), wild greenlip *H. laevigata* (n=51) and wild blacklip *H. rubra* (n=59) abalone.

742 Figure 3. Correlation between the shell length (cm) and A) antiviral activity (%) and
743 B) antibacterial activity (%). In A, data is grouped according to farmed family lines
744 (FL 1-4) and geographic location for wild populations to illustrate the differences in
745 shell length between populations and farmed family. In B, data is grouped according
746 to sex, due to a significant interaction between size and sex in the PERMANOVA for
747 antibacterial activity ($P = 0.045$).

748 Figure 4. Comparison of A) antiviral activity (%) and B) antibacterial activity (%)
749 between pre- and post-spawning. Abalone for spawning induction was from family
750 line FL-2 Control or unspawned group (n=10) was shown in straight line while
751 spawned group (n=16) was shown in dashed line.

752

753 Figure 1.



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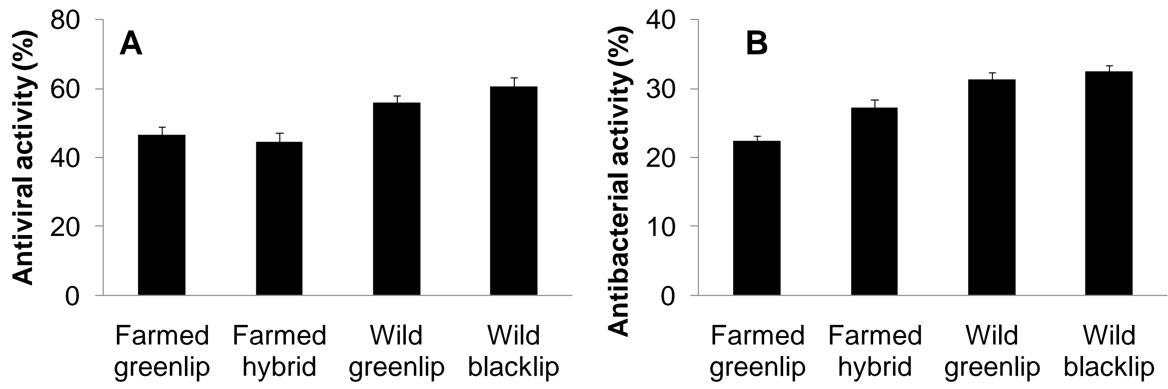
756 Figure 2



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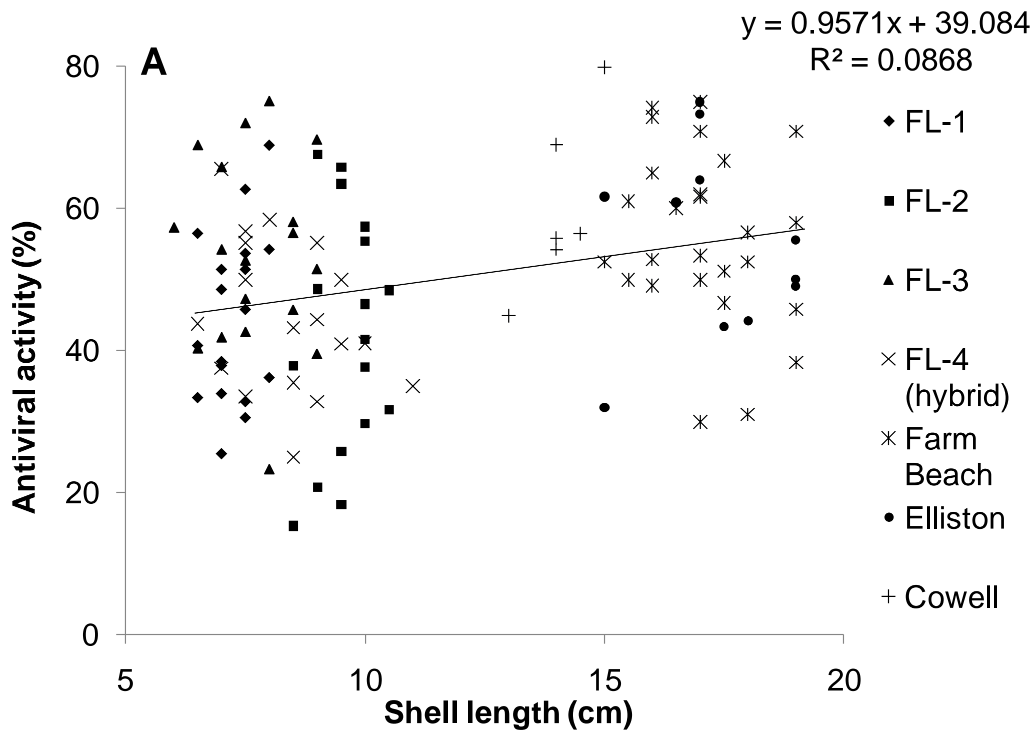
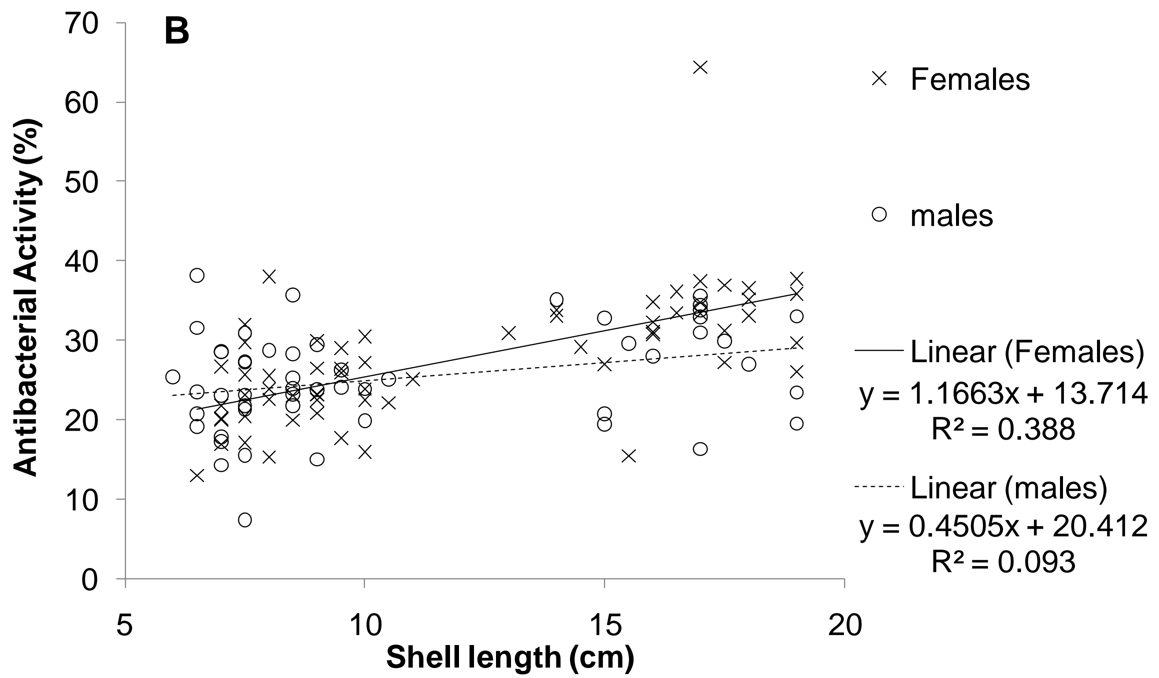
760 Figure 3





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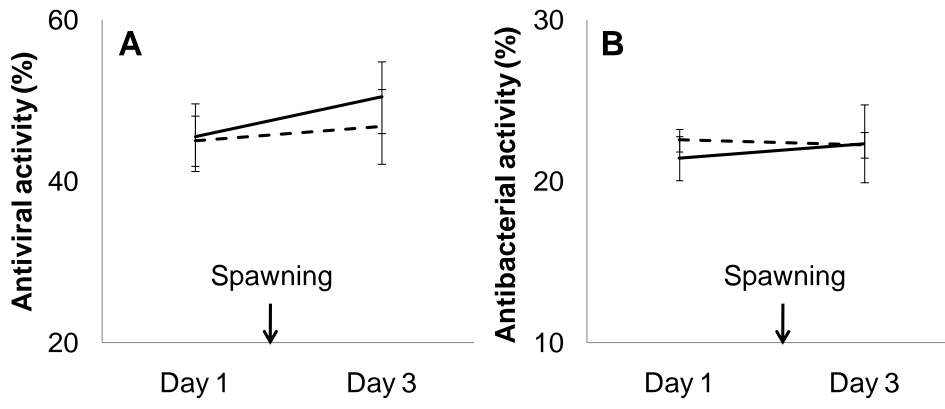
763 Figure 4



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