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Manuscript

1 # Spawning-dependent stress response to food deprivation in

2 # Pacific oyster *Crassostrea gigas*

3

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11

Abstract

This study investigated the impact of spawning on metabolic and immunological responses in Pacific oysters *Crassostrea gigas* under food deprivation. Both pre- and post-spawning oysters were deprived of food for 80 days and then refed for 52 days. Overall mortality was less than 4%, but the condition index was significantly reduced by food deprivation and spawning. Mantle glycogen of post-spawning oysters was less than pre-spawning oysters and was further depleted with reduction of adductor glycogen during food deprivation. Under food deprivation, mantle and gill proteins in pre- and post-spawning oysters were also reduced and maintained at a low level until feeding recommenced. Pre-spawning oysters exhibited strong metabolic resilience to food deprivation as indicated by faster glycogen and protein recovery than post-spawning oysters upon refeeding. The results of hemocyte phagocytosis and hemolymph antimicrobial activity also demonstrated that post-spawning oysters had low immune resilience under food deprivation. This study indicates that food deprivation impedes metabolic and immunological activities in oysters, resulting in a prolonged post-spawning recovery and low metabolic and immune capacity. This finding contributes to our understanding of the factors contributing to oyster mortality in areas with low food supply.

Keywords: Oyster spawning; Food deprivation; Glycogen; Protein; Phagocytosis; Antimicrobial activity

34 **1. Introduction**

35

36 Oysters can encounter low food availability in the ocean due to heterogeneous
37 distribution of seston particles at both spatial and temporal scales. Berg and Newell
38 (1986) reported that chlorophyll *a* fluctuated eight-fold from summer to autumn in the
39 same location. Even within a tidal cycle, both quantity and quality of seston can vary as
40 much as across seasons (Peterson and Black, 1987). Particularly in nutrient poor areas
41 (i.e., with low terrestrial nutrient input or upwelling), oysters are frequently subject to low
42 food supply (Zhang and Li, 2006). Most nutrition studies in oysters have focused on the
43 effects of dietary deficiency on growth (e.g., Whyte et al., 1990; Garcia-Esquivel et al.,
44 2002), but there has been little consideration of summer mortality as a consequence of
45 food deprivation concomitant with reproduction (but see Delaporte et al., 2006a).
46 In recent decades, mass summer mortality of Pacific oyster in aquaculture has become
47 a widespread phenomenon in the world (Perdue et al., 1981; Cheney et al., 2000; Huvert
48 et al., 2004; Garnier et al., 2007). It has been estimated that up to 50% of the harvestable
49 crop can be lost in a given year, and these losses can be even higher in some areas
50 (Renault and Cochennec, 1994; Cheney et al., 2000). Summer mortality is thought to be
51 caused by a combination of biological and environmental stressors acting in combination
52 with microbial pathogens (Samain et al., 2007). With respect to the energy cost for
53 gametogenesis, reproduction is considered an important reason for mass mortality in
54 summer (Cheney et al., 2000; Delaporte et al., 2007). In order to adapt to food
55 deprivation, pre-spawning oysters can absorb gonad as a source of nutritional self-supply
56 (Mathieu and Lubet, 1993; Delaporte et al., 2006b), while post-spawning oysters may

57 postpone or prolong the recovery process (Bayne, 1973). There is however no evidence
58 suggesting that the resistance to food deprivation depends on the spawning status in
59 oysters. As spawned oysters are considered in a fragile condition (Berthelin et al., 2000a,
60 b; Cho and Jeong, 2005; Li et al., 2007), this study aimed to assess the spawning-
61 dependent stress response to food deprivation to better understand the biological reasons
62 for summer mortality.

63 The Pacific oyster, *Crassostrea gigas*, has shown high plasticity in metabolic
64 adaptation to food deprivation (Garcia-Esquivel et al., 2002). In fed oysters, materials
65 from the digestive gland are used to maintain energy reserves in other tissues (Thomason,
66 1972). These reserves can be used for basal metabolism under starvation (Bayne, 1973).
67 Being basic bio-energy fuels, carbohydrates are stored mainly as glycogen in the bivalves
68 (Li et al., 2000). Glycogen plays a central role in providing energy during gametogenesis
69 (Ruiz et al., 1992; Kang et al., 2003; Bacca et al., 2005) and its content reaches lowest
70 after spawning (Perdue et al., 1981; Berthelin et al., 2000a; Li et al., 2007). In addition,
71 glycogen phosphorylase and synthase in oysters are suppressed by spawning (Bacca et
72 al., 2005). When food deprivation occurs, reduction of glucose intake enhances utilization
73 of tissue glycogen (Zaba and Davies, 1980; Zaba, 1981; Gabbott, 1983). Any further
74 glycogen depletion could be detrimental to oysters, as reported in clams and mussels
75 (Naimo et al., 1998; 2000). Previous studies have shown positive correlations between
76 glycogen content and oyster survival (Perdue et al., 1981; Li et al., 2007). Hence, it is
77 important to examine the dynamic glycogen reserves in post-spawning oysters upon food
78 deprivation to understand mortality events relevant to spawning.

79 Similar to glycogen reserves, somatic protein is another important energy source
80 during gonad maturation in bivalves, particularly as it supplies energy to maintain
81 physiological function under extended food deprivation (Whyte et al., 1990). In Pacific
82 oysters, excess utilization of protein is observed under enforced starvation (Garcia-
83 Esquivel et al., 2001) and during the spawning period, especially after spawning (Mao et
84 al., 2006). Therefore, the conflict in protein demand may affect energy metabolic
85 resilience to food deprivation in post-spawning oysters. As reproductive output derives
86 energy reserves from scallop body tissues under starvation (Lodeiros et al., 2001), the
87 changes of glycogen and protein contents in various tissues may reflect the energy
88 metabolic status of oysters after spawning when malnutrition occurs.
89 When oysters are under stress, any immunosuppression would compromise their
90 defense system against opportunistic parasites and pathogens (Harvell et al., 1999). The
91 immune system of mollusks comprises both cellular and humoral functional components
92 (Glinski and Jarosz, 1997; Hooper et al., 2007). Cellular immunity is implicated via
93 hemocytes, with phagocytosis providing the primary line of immunity (Tripp, 1960;
94 Ordas et al., 2000). The humoral immunity involves many antibacterial components
95 (Anderson and Beaven, 2001), which can be assessed by monitoring antibacterial activity
96 in the hemolymph (Li et al., 2007). A recent study showed that hemocyte density and
97 phenoloxidase activity decreased significantly under starvation (Butt et al., 2007). The
98 stability of lysosomal membrane was also significantly decreased in starved oysters
99 (Zhang and Li, 2006). Therefore, food availability can affect oysters' immunological
100 defense (Delaporte et al., 2006a). Immunosuppression has been observed in association
101 with spawning in various studies, suggesting a strong link between reproductive

102 physiology and the immune system in oysters (e.g. Duchemin et al., 2007; Li et al.,
103 2007). Thus, we, hypothesize that the impact of food deprivation on immunity can be
104 further impaired by the spawning event in oysters.

105 In southern Australia, oyster farming areas have a lower chlorophyll *a* level (0.1-5 μg
106 L^{-1}) (Baghurst, 2002) than most oyster growing regions in northern hemisphere (1-20 μg
107 L^{-1}) (Berg and Newell, 1986; Field et al., 1998; Ngo et al., 2006). In addition, food
108 deprivation is a common stressor encountered by oysters farmed in intensive systems
109 (Zhang and Li, 2006). Therefore, this study was designed to investigate the resistance to
110 food deprivation in pre- and post-spawning Pacific oysters from South Australia. The
111 synergistic effects of spawning and food deprivation were evaluated by measuring
112 metabolic and immune responses through experimental manipulations. This research
113 endeavor reflects the likely consequences of spawning under food deprivation and
114 contributes to our understanding of the underlying reason for mass oyster mortality
115 during the post-spawning period.

116

117 2. Materials and Methods

118

119 *2.1. Animals*

120

121 Ripe oysters (shell length 9~10 cm) were obtained from an oyster farm in Ceduna,
122 South Australia in December 2005. The oysters were cleaned to remove epibionts, and
123 were acclimatized and fed with microalgae at 15 ºC at the South Australian Aquatic
124 Sciences Centre for one week. Half of the oysters were kept at 15 ^{o}C as the pre-spawning

125 population, while the other half received a spawning stimulation through thermal
126 escalation at 28°C to obtain post-spawning oysters. Water became turbid after oysters
127 expel their gametes. Soon after the seawater in the spawning tank became clear, the
128 spawned oysters were removed to 15 ºC seawater.
129
130 *2.2. Food deprivation treatment and animal sampling*
131
132 In the food deprivation treatments, three replicate batches (n = 75) of both pre- and
133 post-spawning oysters were kept in 5 µm filtered seawater. In the feeding treatments
134 (control), the same number of pre- and post-spawning oysters were used in each replicate
135 and the oysters were fed a mixture of three microalgal species, *Isochrysis* sp., *Pavlova*
136 *lutheri* and *Chaetoceros calcitrans* (2×10^9 cells per oyster per day). The water was
137 maintained at 15°C and aerated. After 80 days of food deprivation, the starved oysters
138 were supplied with the same diet as the controls. Oysters from all treatments were
139 sampled on days 1, 4, 9, 16, 26, 40, 59, 80, 110 and 132. On each sampling day, six
140 oysters were randomly selected from each replicate. An extra fifteen oysters were
141 included in each replicate to account for unintentional mortality. Three oysters were used
142 for assessing the condition index and hemolymph immunity. The other three were
143 shucked for tissue sampling. Mortality was recorded on each sampling day.
144
145 *2.3. Condition index measurement*
146

147 After drying the shells with blotting paper, the whole weight was measured on an
148 analytical scale before opening the shells. Whole flesh was taken, dried at 60°C for 48 h
149 and then weighed to the nearest 0.01 g. The shell weight was also recorded. The
150 condition index (CI) of the oyster was determined according to Crosby and Gale (1990)
151 by the formula:

152 $$CI = \frac{Drying\ Weight \times 100}{Total\ Weight - Shell\ Weight}$$

153

154 *2.4 Glycogen and protein analyses*

155

156 The gill, mantle and adductor tissues were removed separately and snap frozen in
157 liquid nitrogen. Before analysis, these tissues were ground into a fine powder using
158 frozen mortar and pestle on dry ice. Each replicate sample consisted of three pooled
159 animals. The results of glycogen and protein measurements were expressed in mg per
160 gram of tissue weight.

161 Glycogen contents of gill, mantle and adductor were determined on a Unicam UV-
162 visible spectrometer at 460 nm according to the method of Kristman (1962). A 200 mg
163 sample of frozen powder was extracted with 1 mL of perchloride acid (PCA). A 0.2 mL
164 aliquot of PCA extraction was pipetted into a microcuvette and added 1.3 mL I_2KI
165 solution (dissolved 0.26 g of iodine and 2.6 g of KI in 10mL distilled water, then 1.92 mL
166 I_2KI to 500 mL saturated $CaCl_2$ solution). The samples were then incubated at 25 ºC for
167 20 min before the analysis. Purified oyster glycogen was used as the standard at 0, 0.1,
168 0.4 and 0.7 mg mL^{-1}.

169 The level of proteins in gill, mantle and adductor were measured according to the
170 method of Li et al. (2000). Briefly, 100 mg of sample frozen powder was added into 8
171 mL of 20 mM Tris-HCI buffer containing 2% NaCl and 0.1% $NaNO_3$ (pH 8.0) and
172 centrifuged at × 1500 g for 30 min at 4 °C. The supernatant protein portion was pippetted
173 into an eppendorf tube on ice. Then 10 μL of protein was analysed by the Bio-Rad
174 Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) at 590 nm on a
175 spectrophotometer (Spectra Max 250) using the bovine serum albumin standard.
176
177 *2.5. Immunity analysis*
178
179 Hemolymph of oysters was withdrawn from the pericardium using a 1 mL insulin
180 syringe with a 29-gauge needle. Hemolymph samples from each replicate were pooled
181 and stored in a test tube on ice. A subsample (300 μL) was then mixed with an equal
182 volume of filtered seawater (0.2 μm) (FSW) in a flow-cytometer tube (n = 3). To assess
183 the phagocytic activity of hemocytes, fluorescent beads (Fluoresbrite® YG Microspheres,
184 1.75 μm) were added to each tube, as 4 μL of a stock suspension of 2.5% solids in FSW
185 per mL. The samples were analyzed on a FACScan flow cytometer (Becton Dickinson,
186 San Jose, 488nm Laser) after 60-min incubation at 20ºC in darkness (Goedken and De
187 Guise, 2004). Phagocytic activity was expressed as the percentage of cells that ingested at
188 least three fluorescent beads.
189 The remaining hemolymph was frozen in liquid nitrogen until the antimicrobial
190 activity assay, as described by Li et al. (2007). Cultures of the marine pathogen *Vibrio*
191 *harveyi* were obtained from the Tasmania Department of Primary Industries Fish Health

192 Unit and grown in a sterile nutrient broth (1 g NaCl, 2 g yeast extract and 1 g peptone per
193 100 mL distilled H_2O), incubating overnight at 37 ºC on an orbital mixer shaker (Ratek)
194 at 200 rpm. The cultures were returned to exponential growth phase prior to antimicrobial
195 assays. The defrosted hemolymph was centrifuged at × 500 g for 5 min to pellet the
196 hemocytes and then 90 µL aliquots of plasma were pippeted into a 96 well plate in
197 triplicate. A 10 µL aliquot of *V. harveyi* culture was then added into each well. Negative
198 controls consisted of 90 µL hemolymph incubated with 10 µL nutrient broth and positive
199 controls comprised 10 µL of *V. harveyi* in 90 µL of nutrient broth. After 30 min
200 incubation, 20 µL of CellTitre 96® Aqueous One Solution (Promega) was pipetted into
201 each well, then the plates were returned to the incubator (37 ºC) for 2 h or until
202 development of the red formazan product in control wells. The absorbance was measured
203 at 492 nm using a 96 well plate reader (Spectra Max 250). The background absorbance
204 from hemolymph broth controls was subtracted from the treatment wells and then cell
205 viability was calculated as a percentage of the absorbance in positive control cultures.
206 Antimicrobial activity was calculated by the equation of 100 – cell viability.
207
208 *2.6. Statistic analysis*
209
210 To compare the response of oysters to feeding manipulations, the experimental period
211 was divided into the food deprivation period (day 1-80) and the refeeding period (day 80-
212 132). Repeated-measures ANOVA were used to test the effects of food deprivation and
213 spawning status within these two periods. The analysis of oyster mortality was conducted
214 by two-way ANOVA. Least significant differences (LSD) procedure was used for the

215 post hoc comparisons when a significant treatment effect was detected. Data were
216 transformed by logarithm or square root to meet assumptions of normality and
217 homogeneity of variance if necessary. A significance level of 0.05 was used for all tests.
218
219 **3. Results**
220
221 After food deprivation for 80 days, most post-spawning oysters had a slightly
222 translucent gonad-visceral mass and thin mantle tissue. Meanwhile, the gonad size of pre-
223 spawning oysters was reduced, as indicated by a dwindled gonad-visceral mass and the
224 reduction of milky coloration, especially under food deprivation. Pre-spawning oysters
225 still possessed gonad residue after a period of 80-day food deprivation. The average
226 mortality during 132 days was < 4% and was not different across treatments ($P > 0.05$).
227
228 *3.1. Condition Index*
229
230 During the 80-day food deprivation, the interactions between spawning and time, and
231 between food deprivation and time were significant ($P < 0.001$, Table 1). Pre-spawning
232 oysters had a higher CI value than post-spawning oysters in the first 40 days ($P \leq 0.017$,
233 Fig. 1). The impact of food deprivation was significant in both pre-and post spawning
234 groups between day 26 and day 80, with higher CI in the control ($P \leq 0.04$). Under food
235 deprivation, the CI in post-spawning oysters was less than that in pre-spawning oysters.
236 After refeeding, the interaction impact between spawning and food deprivation became
237 significant ($P = 0.003$) and the effects of food deprivation depended on time ($P = 0.001$,

238 Table 1). There was no difference between pre- and post-spawning oysters in the control
239 ($P > 0.05$, Fig. 1). But in the refed group, post-spawning oysters had lower CI than pre-
240 spawning oysters ($P < 0.001$). The CI of refed pre- and post-spawning oysters increased
241 over time ($P \leq 0.029$) and all reached the control level by day 132 ($P > 0.05$).
242
243 *3.2 Tissue glycogen*
244
245 Among the mantle, adductor and gill tissues, the glycogen content was greatest in the
246 mantle tissue. In the first 80 days, the interaction between spawning, food deprivation and
247 day was marginally significant in mantle glycogen ($P = 0.055$, Table 1). In the controls,
248 mantle glycogen increased over time ($P < 0.05$), with the post-spawning oysters gaining a
249 significantly higher mantle glycogen than pre-spawning oysters after day 59 ($P < 0.05$,
250 Fig. 2A). In contrast, under food deprivation, pre-spawning oysters had higher mantle
251 glycogen than post-spawning oysters before day 59 ($P \leq 0.048$). From day 80 to day 132,
252 mantle glycogen increased over time and the interactive effect of spawning and food
253 deprivation on mantle glycogen was significant ($P < 0.001$, Table 1). In the control, more
254 mantle glycogen was detected in post-spawning oysters ($P < 0.001$, Fig. 2A). In the refed
255 group, mantle glycogen in post-spawning oysters remained lower than that in pre-
256 spawning oysters ($P < 0.05$).
257 For adductor glycogen, the interaction between spawning and food deprivation in the
258 first 80 days was significant ($P = 0.001$, Table 1). There was no difference between pre-
259 and post-spawning oysters in the control ($P > 0.05$), but post-spawning oysters contained
260 less adductor glycogen than pre-spawning oysters in the food deprivation group ($P <$

261 0.05, Fig. 2B). The impact of food deprivation was not significant in pre-spawning
262 oysters ($P > 0.05$). After feeding resumed in starved oysters, the significant interaction
263 between spawning and food deprivation still existed ($P = 0.001$, Table 1). Adductor
264 glycogen in refed oysters was less than the control level regardless of spawning status (P
265 < 0.05, Fig. 2B), and it did not change over time ($P = 0.276$, Table 1). Glycogen in the
266 gill was neither altered by spawning nor by food deprivation ($P > 0.05$, Table 1).
267
268 *3.3 Tissue protein*
269
270 Tissue protein was measured in the gill, mantle and adductor. Although adductor
271 protein was higher than the mantle and gill proteins, it was not affected by the treatment
272 manipulation during the experimental period ($P > 0.05$, Table 1). Under food deprivation,
273 the mantle protein was significantly affected by the interactions between time and
274 spawning, between time and food deprivation, and between spawning and food
275 deprivation ($P \leq 0.001$, Table 1). Mantle protein was lower in post-spawning oysters
276 between day 1 and day 4 and the impact of food deprivation became significant from day
277 16 ($P < 0.05$, Fig 3A). However, mantle protein in the starved group stabilized between
278 day 40 and day 80 ($P > 0.05$). Regardless of time, there was no significant difference
279 between pre- and post-spawning oysters in the control ($P > 0.05$), but under food
280 deprivation, the mantle protein in post-spawning oysters was lower than that in pre-
281 spawning oysters ($P < 0.05$). The impact of food deprivation on mantle protein was
282 significant in both pre- and post-spawning oysters ($P < 0.05$). After refeeding, the mantle
283 protein in refed oysters remained lower than that in the control ($P < 0.001$), and the

284 difference between pre- and post-spawning oysters became marginally significant (P =
285 0.052, Table 1). The change in mantle protein during the refeeding period depended on
286 time ($P = 0.017$, Table 1) and significantly increased in refed oysters from day 80 to day
287 132 ($P < 0.05$, Fig. 3A).
288 From day 1 to day 80, the interactive effect between food deprivation, spawning and
289 time was marginally significant on gill protein ($P = 0.052$, Table 1). In the control, there
290 was no difference between pre- and post-spawning oysters ($P > 0.05$, Fig. 3B). However,
291 under food deprivation, gill protein levels were reduced from day 9 in post-spawning
292 oysters, but only reduced from day 40 in pre-spawning oysters ($P < 0.05$). Gill protein in
293 post-spawning oysters remained low between day 9 and day 80, whereas it was stabilized
294 at a similar level in pre-spawning oysters between day 59 and day 80 ($P > 0.05$). The
295 impact of spawning on gill protein was significant between day 9 and day 26 in starved
296 oysters ($P < 0.05$). After refeeding, gill protein was still affected by the interaction of the
297 above three factors ($P = 0.026$, Table 1). In the refed treatment, gill protein in pre-
298 spawning oysters was higher than that in post-spawning oysters ($P < 0.05$), and was not
299 significantly different from the control level by day 132 ($P > 0.05$, Fig. 3B).
300
301 *3.4 Immunocompetence*
302
303 During the period of food deprivation, hemocyte phagocytosis increased in the
304 control and decreased in the food deprivation treatment (Fig. 4A). The hemocyte
305 phagocytic activity was affected by an interaction between spawning, food deprivation
306 and day ($P = 0.021$, Table 1). In the control, although phagocytosis in post-spawning

307 oysters was initially lower than that in pre-spawning oysters ($P < 0.05$), it reached the
308 level of pre-spawning oysters on day 4 and exceeded this level on day 16 and beyond (P
309 < 0.05, except day 59, Fig. 4A). Under food deprivation, phagocytosis rates between pre-
310 and post-spawning oysters showed no difference from day 9 to day 80 ($P > 0.05$). After
311 refeeding, the interaction of spawning and food deprivation was significant ($P = 0.002$,
312 Table 1). LSD test showed that the hemocyte phagocytic activity was similar between
313 pre- and post-spawning oysters in the control ($P > 0.05$), whereas the level in pre-
314 spawning oysters was higher than that in post-spawning oysters in the refed group ($P <$
315 0.001, Fig. 4A). In addition, the impacts of spawning and food deprivation were still
316 dependent on time after refeeding ($P < 0.001$ and $P = 0.006$, Table 1). Phagocytosis
317 significantly increased in refed oysters from day 80 to day 132 and it was higher than that
318 in post-spawning oysters ($P < 0.05$, Fig. 4A). The phagocytosis rate in refed pre-
319 spawning oysters became similar to the control level by day 110 ($P > 0.05$).

320 In the first 80 days, the antimicrobial activity in hemolymph was influenced by
321 spawning and food deprivation ($P = 0.015$, Table 1). There was no difference between
322 pre- and post-spawning oysters in the control ($P > 0.05$), whereas the post-spawning
323 oysters had lower antimicrobial activity than pre-spawning oysters under food
324 deprivation ($P < 0.05$, Fig. 4B). In addition, the interaction between food deprivation and
325 day was significant ($P < 0.001$, Table 1). Food deprivation did not affect antimicrobial
326 activity until day 16 ($P < 0.05$, Fig. 4B). After 80 days when feeding was recommenced,
327 the interactions between food deprivation and spawning, and between food deprivation
328 and time were still significant ($P = 0.001$ and $P < 0.001$, respectively. Table 1). Although
329 the antimicrobial activity in refed oysters was lower than that in the control, it

330 significantly increased over time ($P < 0.05$) and the food deprived pre-spawning oysters
331 reached the control level by day 132 ($P > 0.05$, Fig. 4B). After refeeding, pre-spawning
332 oysters showed higher antimicrobial activity than post-spawning oysters ($P < 0.001$).
333
334 **4. Discussion**
335
336 Food deprivation and spawning have a rapid and dramatic effect on oyster condition.
337 During a period of 80-day food deprivation, the mantle tissue thinned out and the body
338 became slightly translucent in post-spawning oysters. In a pioneer study, however,
339 similar symptoms were observed in oysters after starvation for 147 days (Whyte et al.,
340 1990). This delay in response was likely due to the fact that the experimental oysters used
341 by Whyte et al. (1990) had not spawned. Although the 80-day food deprivation did not
342 cause significant mortality, this study revealed that food deprivation could significantly
343 change the physiological and immunological status of oysters, especially in post-
344 spawning oysters. To our best knowledge, this study is the first to examine the synergistic
345 effects of spawning and food deprivation on the biological condition and stress responses
346 of oysters, and our experimental evidence has demonstrated the crucial role of food
347 supply for the recovery of oysters after spawning.
348 In this study, both spawning and food deprivation significantly reduced condition
349 index, which is consistent with previous studies (Gabbott and Stephenson, 1974; Wright
350 and Hetzel, 1985; Whyte et al., 1990). Although the oysters had reduced condition index
351 (CI) after spawning, further reductions of CI occurred under food deprivation, indicating
352 gonad reabsorption and additional depletion of stored metabolic resources. Noticeably, CI

353 reduction in post-spawning oysters was smaller than that in pre-spawning oysters,
354 coincident with their spawning status and the noticeable gonad absorption in pre-
355 spawning oysters. As gonad degradation is a process of absorption of endogenous energy
356 in pre-spawning oysters (Mathieu and Lubet, 1993; Delaporte et al., 2006b), the energy
357 reserve in gonads can mitigate the stress from low food supply. However, under food
358 deprivation, post-spawning oysters need to catabolize energy stored in other tissues, due
359 to a lack of available gonad mass for absorption and the thin mantle tissue. Despite
360 reaching a similar low level of condition index after 80-day food deprivation, the
361 recovery process of pre-spawning oysters was much quicker than that of post-spawning
362 oyster upon refeeding, suggesting that pre-spawning oysters are more resilient to food
363 deprivation than post-spawning individuals.

364 The mantle was found to be an important tissue for glycogen storage and metabolism
365 in oysters, as it was more sensitive to both spawning and food availability than adductor
366 muscle and gills in glycogen content. During feeding, mantle glycogen levels consistently
367 increased, whereas they decreased after spawning and during food deprivation. This is
368 consistent with the study of Ruiz et al. (1992) who suggested that glycogen in the mantle
369 tissue is a sensitive indicator for energy reserves in oysters. However, under food
370 deprivation, a reduction in adductor glycogen also occurred in post-spawning oysters,
371 which was not detected in pre-spawning oysters. This implies that post-spawning oysters
372 have developed adaptations to use adductor glycogen as a source of spare energy to
373 maintain their metabolism and to cope with food deprivation. In food deprived oysters,
374 the increase of tissue glycogen in post-spawning oysters was slower than that in pre-
375 spawning oysters after feeding resumed, indicating that the spawning activity retards

376 energy recovery after experiencing a period of food shortage. Berthelin et al. (2000a)
377 reported that the amount of glycogen storage could regulate metabolic activities in
378 oysters. Interestingly, in the controls, post-spawning oysters expedited glycogen recovery
379 and quickly exceeded the level of pre-spawning oysters, implying that spawning may
380 trigger the process of metabolic recovery by glycogen accumulation. The change of
381 energy storage and metabolism associated with the spawning activity may be due to the
382 energy reallocation from germinal development to somatic growth after spawning,
383 coincident with the increase of glycogen synthase enzyme in post-spawning oysters
384 (Bacca et al., 2005).

385 Variation of metabolic activity can result in the change of protein content in the
386 somatic tissues, because the reduction of metabolism under food deprivation usually
387 leads to low protein synthesis in mollusks (Takuji et al., 2002). In contrast, fluctuations of
388 protein were found in mantle and gills, but not in the adductor. This is possibly due to
389 different physiological functions among tissues, as mantle and gills are more involved
390 with metabolic activities in oysters (Evseev et al., 1996), whereas the adductor protein is
391 more likely to be structural. Mao et al. (2006) reported that protein was utilized during
392 oyster spawning as a energy source. Our data indicate that the mantle tissue is the main
393 source for protein catabolism during spawning, compared with gills and adductor
394 muscles. On the other hand, glycogen depletion was only observed in mantle and
395 adductor, but not in gills. The selective energy catabolism among various tissues in the
396 Pacific oysters has also been reported by Garcia-Esquivel et al. (2002) under the
397 condition of enforced starvation.

398 In the present study, the gill protein levels in pre- and post-spawning oysters all
399 reduced to a stable low level under food deprivation, concomitant with the low protein
400 levels in the mantle by day 80. With a reduction of the metabolic activity of starved
401 oysters (Rodhouse and Gaffney, 1984), it is conceivable that a stable low level of protein
402 is required to sustain the basic physiological functions, e.g., respiration in the gills.
403 Similarly, Whyte et al. (1990) also found that a minimal protein threshold existed in
404 starved oysters to maintain a sustainable nutritional condition. This study revealed that
405 this threshold level of protein in post-spawning oysters was reached earlier than that in
406 pre-spawning oysters. As a result, protein recovery in post-spawning oysters was slower
407 than in pre-spawning oysters upon refeeding. Nevertheless, post-spawning oysters still
408 appear able to allocate bioenergetic resources between body tissues as a physiological
409 response to nutritional stress under food deprivation.
410 As reported in brackish water clams *Rangia cuneuta* (Lane, 1986), the Pacific oyster
411 was able to derive energy from glycogen and protein reserves to withstand food
412 deprivation. However, the sequence of biochemical catabolism varies among species. For
413 instance, protein was catabolized in mussels and clams only after carbohydrate reserves
414 were almost depleted during food deprivation (Gabbott and Bayne, 1973; Beninger and
415 Lucas, 1984). In contrast, oysters in this study mobilized protein even when glycogen
416 was still relatively abundant. This is possibly because most amino acids can be converted
417 into gluconeogenic precursors and enter the energy cycle at the same time as glucose
418 enters the Krebs cycle in *C. gigas* (Gabbott, 1976; Whyte et al., 1990). Although Whyte
419 et al. (1990) did not find that spawning altered energy budget, we observed that mantle
420 glycogen and protein were reduced during spawning. After 80-day food deprivation, the

421 slower energy restoration in post-spawning oysters demonstrates that spawning could
422 suppress the metabolic response to food deprivation in oysters.

423 Under stress, the reduction of energy reserves could necessitate a down regulation of
424 immune functions (Malham et al., 2003), as the oyster immune defense also requires
425 energy supply (Delaporte et al., 2006a). Our study demonstrated that spawning and food
426 deprivation can bring changes in hemocyte phagocytic activity as well as in energy
427 reserves. The reduction of phagocytosis after spawning has been reported in other studies
428 (e.g., Delaporte et al., 2006a; Duchemin et al., 2007). As reported by Delaporte et al.
429 (2006a), there would be less energetic supply for hemocyte phagocytosis due to the cost
430 of spawning. Similarly, the low energy intake during food deprivation can also decrease
431 hemocyte phagocytosis, as found in the present study and others (Funakoshi, 2000;
432 Hégaret et al., 2004). Interestingly, the presence of food significantly enhanced
433 phagocytic activity to a greater degree in post-spawning oysters than pre-spawning
434 oysters. This enhancement of phagocytic activity was coupled by the quick storage of
435 tissue glycogen and protein. However, the decrease of phagocytic activity in pre-
436 spawning oysters was associated with gonad resorption during food deprivation. It seems
437 that hemocyte phagocytosis is positively related to energy anabolism but negatively
438 related to catabolism. Under food deprivation, the phagocytosis level became similar
439 between pre- and post-spawning oysters by day 9. When feeding was resumed,
440 phagocytosis in pre-spawning oysters increased much quicker than that in post-spawning
441 oysters, suggesting the oyster cellular immune resilience to food deprivation is spawning
442 dependent.

443 In contrast to hemocyte phagocytosis, spawning status did not affect the hemolymph
444 antimicrobial activity, as long as sufficient food was available. This is consistent with Li
445 et al. (2007) and manifests that antimicrobial activity can counteract the deficiency of
446 cellular immunity in post-spawning oysters during the recovery phase. However, in the
447 absence of food, the reduction of hemolymph antimicrobial activity was significantly
448 greater in post-spawning oysters compared to pre-spawning individuals. Butt et al. (2007)
449 commented that oysters may have some starvation-relevant immune components, which
450 could be reduced with increased stress, but otherwise maintained during times of
451 restricted feeding. Our results support the notion of the presence of spawning-relevant
452 immune components in oyster hemolymph. As the antimicrobial activity is associated
453 with plasma peptides (Montes et al., 1995; Montes et al., 1997; Anderson and Beaven,
454 2001), the ability to synthesis antimicrobial peptides may be reduced by both food
455 deprivation and spawning activity. In the present study, food resumption enhanced the
456 antimicrobial activity, but pre-spawning oysters showed a faster recovery than post-
457 spawning oysters, similar to the pattern observed for hemocyte phagocytosis. As oyster
458 immunity is the primary defense against pathogenic microorganisms (Anderson and
459 Beaven, 2001), the spawning-suppressed immune response to food deprivation could
460 easily make oysters susceptible to pathogens and parasites. In a worse case scenario, the
461 synergistic effect of spawning and poor nutritional condition could lead to mass
462 mortality, especially in summer under high temperature stress when pathogens prevail.
463 Overall, this study demonstrates that spawning can suppress oysters' metabolic and
464 immune responses and their recovery from food deprivation. Pre-spawning oysters can
465 mitigate the stress of food deprivation by gonad absorption, resulting in a better

466 metabolic and immune resilience to food deprivation than post-spawning oysters. Post-
467 spawning oysters are able to further deplete energy reserves and reduce metabolic activity
468 for physiological adaptation under food deprivation, but this results in a prolonged
469 suppression of metabolic and immune responses. Although a synergetic effect of food
470 deprivation and spawning does not directly cause mortality, the concurrent energetic and
471 immunological dysfunctions may lead to mass mortality in post-spawning oysters (Li et
472 al., 2007). In the natural environment, the starved post-spawning oysters are likely to be
473 more sensitive to other potentially lethal stresses, e.g., bacterial challenge and heat shock.
474 Therefore, to investigate histological change and relevant pathological response will be
475 worthwhile in future study.
476
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478
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670

671 Table 1. Summary of the probability values of difference after the analysis of repeated
672 measure ANOVA on various immune and metabolic parameters in Pacific oysters. The
673 treatments involved spawning status (pre-spawning and post-spawning; SP) and food
674 supply (with and without feeding). Each variable was separately analyzed during food
675 deprivation (FD, first row) and refeeding (Refeed, second row). The time effect refers to
676 either the period of food deprivation (days 1-80, first row) or the refeeding period (days
677 80-132, second row). The significant level was set up at $P = 0.05$.

Variables	Food	**Between-Subjects Effects**			**Within-Subjects Effects**			
		SP	Food	SP * Food	Time	Time * SP	Time * Food	Time * SP * Food
Condition index	FD	<0.001	<0.001	0.222	<0.001	<0.001	<0.001	0.589
	Refeed	0.001	<0.001	0.003	<0.001	0.391	0.001	0.867
Mantle glycogen	FD	0.191	<0.001	<0.001	0.593	<0.001	<0.001	0.055
	Refeed	0.035	<0.001	<0.001	<0.001	0.883	0.454	0.127
Adductor glycogen	FD	<0.001	<0.001	0.001	<0.001	0.621	<0.001	0.168
	Refeed	0.001	<0.001	0.001	0.276	0.980	0.666	0.964
Gill glycogen	FD	0.345	0.194	0.395	0.011	0.673	0.471	0.990
	Refeed	0.892	0.964	0.507	0.001	0.825	0.327	0.207
Adductor protein	FD	0.243	0.459	0.180	0.186	0.860	0.531	0.975
	Refeed	0.544	0.320	0.879	0.841	0.981	0.269	0.987
Mantle protein	FD	<0.001	<0.001	0.001	0.084	<0.001	<0.001	0.750
	Refeed	0.052	<0.001	0.084	0.001	0.896	0.017	0.874
Gill protein	FD	0.002	<0.001	<0.001	0.001	0.121	<0.001	0.052
	Refeed	0.016	<0.001	0.026	0.001	0.451	0.010	0.026
Phagocytosis	FD	<0.001	0.092	<0.001	0.004	<0.001	0.008	0.021
	Refeed	<0.001	0.007	0.002	<0.001	<0.001	0.006	0.259
Antimicrobial activity	FD	0.456	<0.001	0.015	<0.001	0.280	<0.001	0.519
	Refeed	0.001	<0.001	0.001	<0.001	0.335	<0.001	0.156

678 **Figure legends**

679

680 Figure 1. The condition index of oysters: “pre S” and “post S” represent the starved pre-
681 and post-spawning oysters; “pre C” and “post C” represent the fed pre- and post-
682 spawning oysters as controls (n = 3). Feeding recommenced on day 80 in the starved
683 groups.

684

685 Figure 2. The glycogen content in mantle tissue (A) and adductor (B) of oysters: “pre S”
686 and “post S” represent the starved pre- and post-spawning oysters; “pre C” and “post C”
687 represent the fed pre- and post-spawning oysters as controls (n = 3). Feeding
688 recommenced on day 80 in the starved groups.

689

690 Figure 3. The protein level in the mantle tissue (A) and gill tissue (B) of oysters: “pre S”
691 and “post S” represent the starved pre- and post-spawning oysters; “pre C” and “post C”
692 represent the fed pre- and post-spawning oysters as controls (n = 3). Feeding
693 recommenced on day 80 in the starved groups.

694

695 Figure 4. The hemocyte phagocytic activity (A) and antimicrobial activity in hemolymph
696 (B) of oysters: “pre S” and “post S” represent the starved pre- and post-spawning oysters;
697 “pre C” and “post C” represent the fed pre- and post-spawning oysters as controls (n = 3).
698 Feeding recommenced on day 80 in the starved groups.

699

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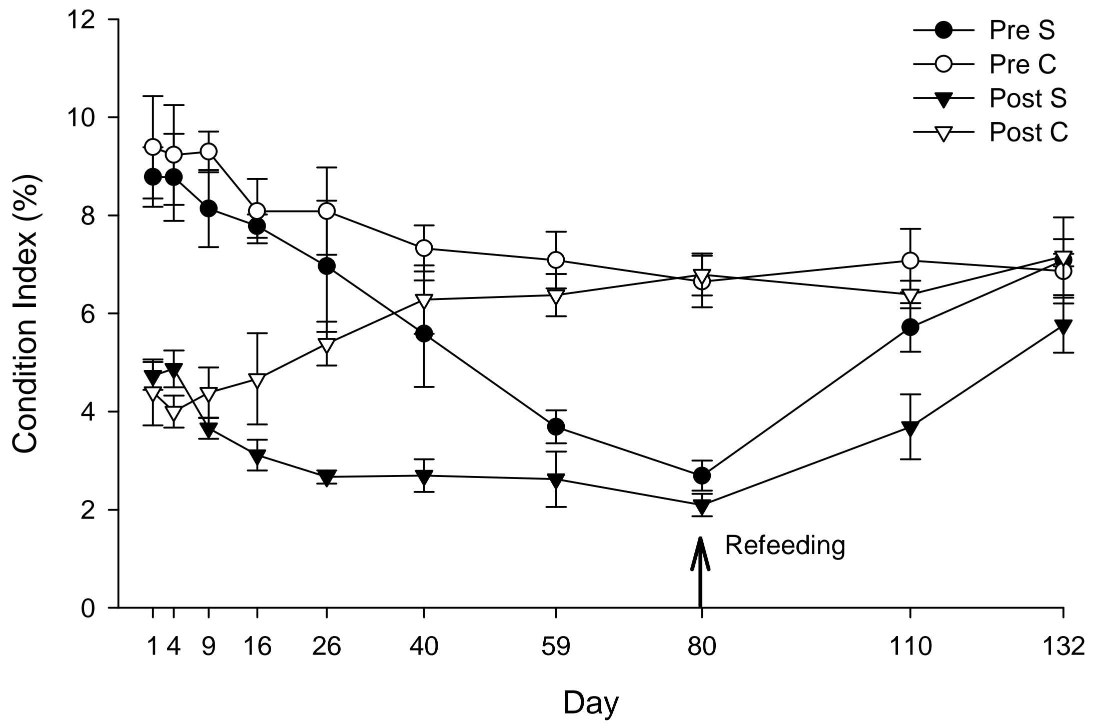

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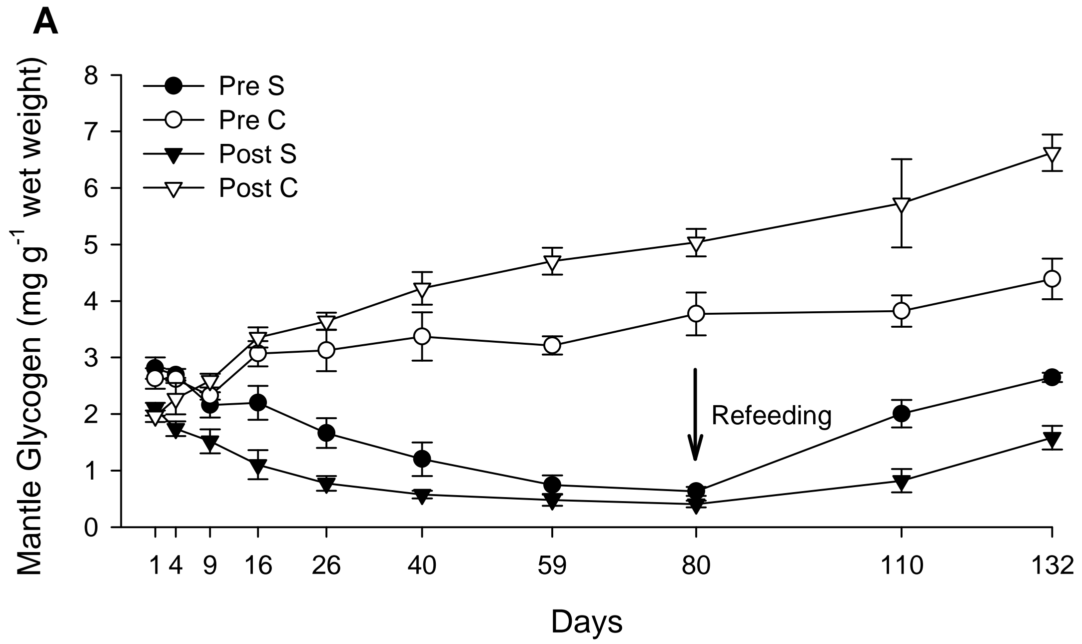
703 Figure 1.



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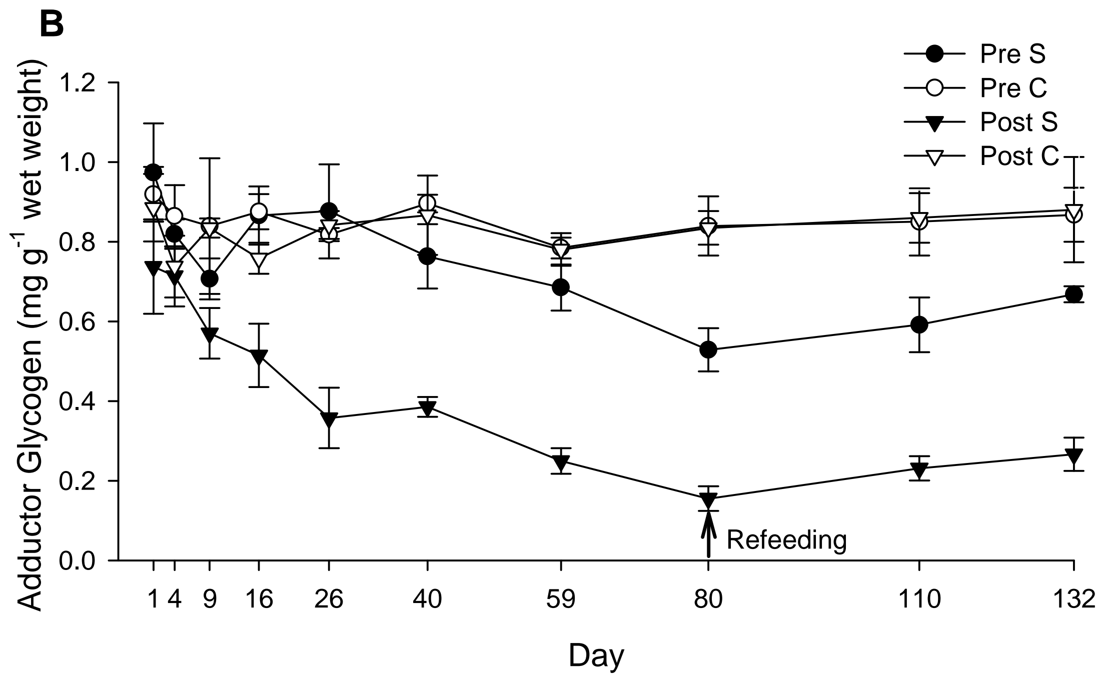

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707 Figure 2.

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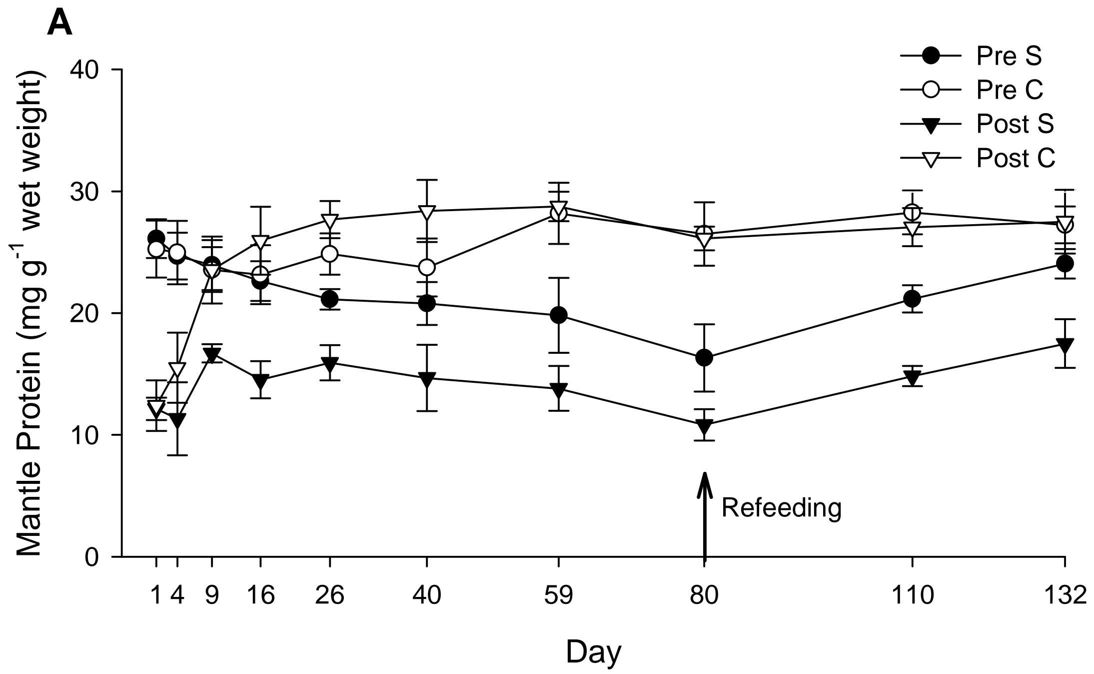

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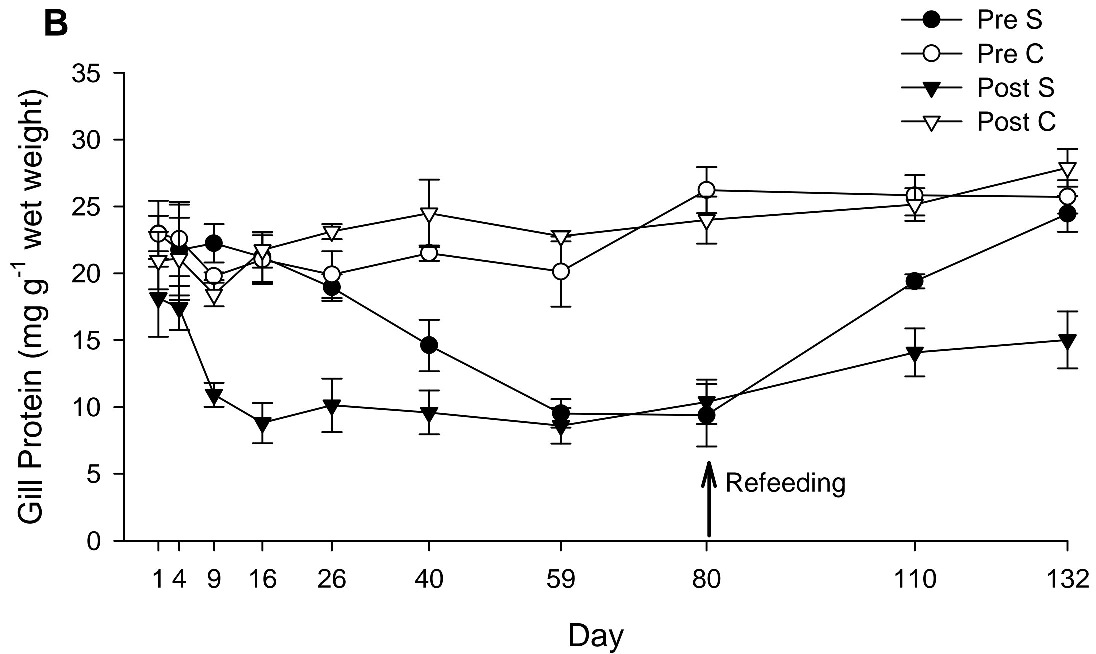

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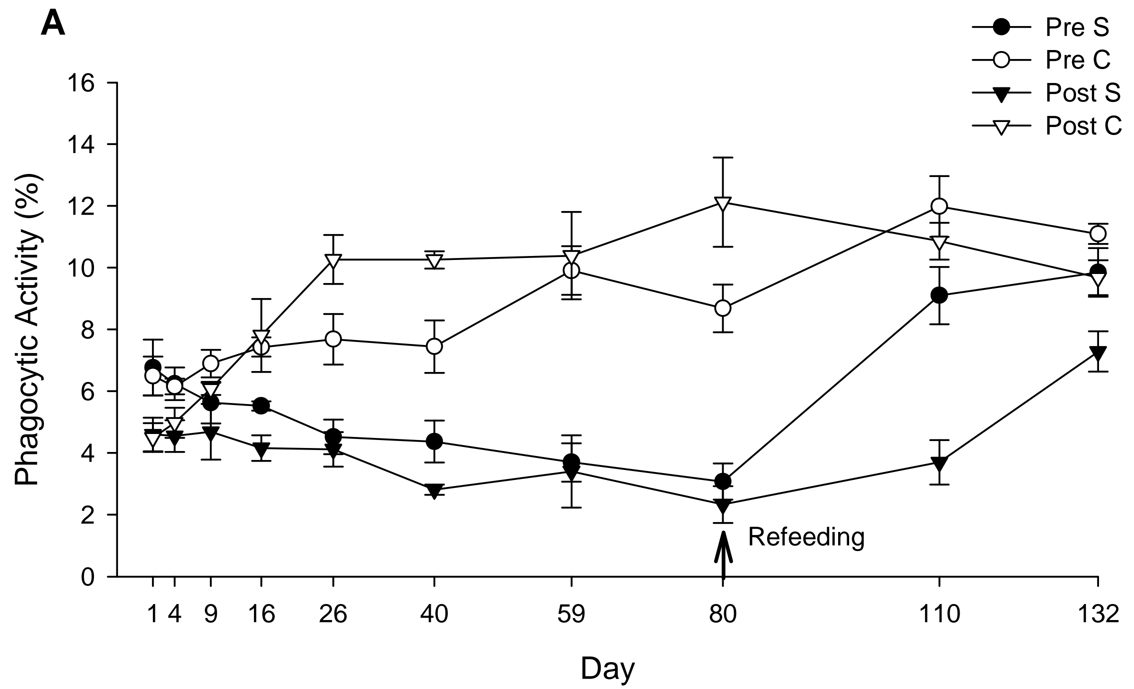
713 Figure 3.



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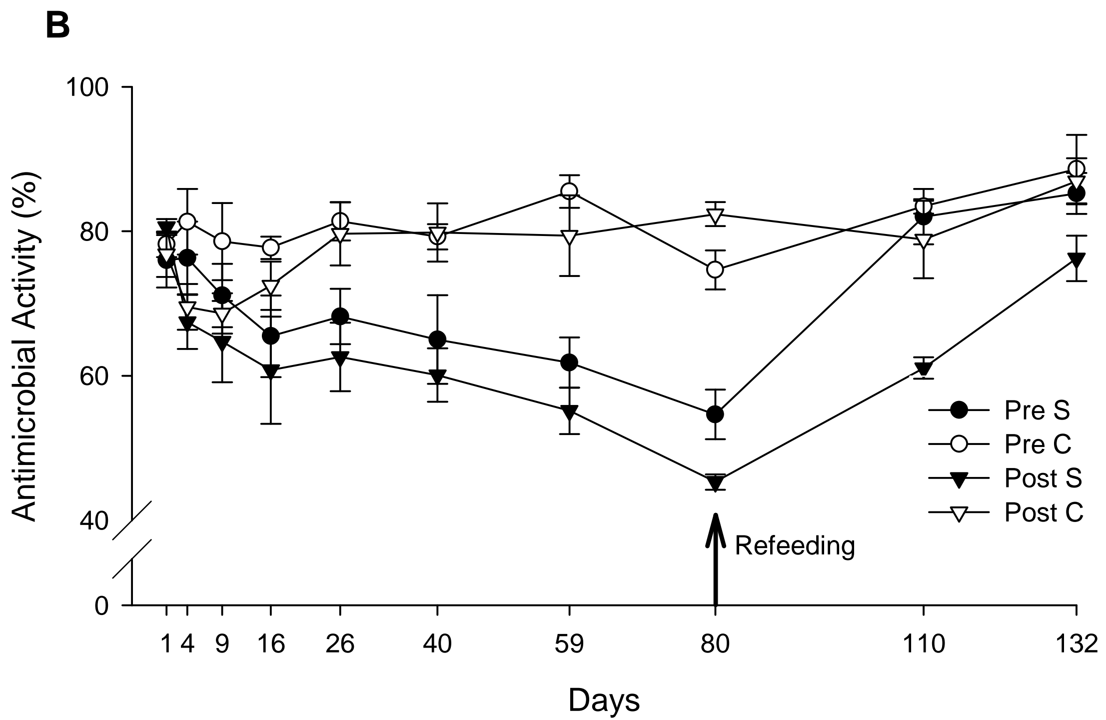

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718 Figure 4.