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Spawning-dependent stress response to food deprivation in Pacific oyster *Crassostrea gigas*

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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

1. Introduction

Oysters can encounter low food availability in the ocean due to heterogeneous distribution of seston particles at both spatial and temporal scales. Berg and Newell (1986) reported that chlorophyll *a* fluctuated eight-fold from summer to autumn in the same location. Even within a tidal cycle, both quantity and quality of seston can vary as much as across seasons (Peterson and Black, 1987). Particularly in nutrient poor areas (i.e., with low terrestrial nutrient input or upwelling), oysters are frequently subject to low food supply (Zhang and Li, 2006). Most nutrition studies in oysters have focused on the effects of dietary deficiency on growth (e.g., Whyte et al., 1990; Garcia-Esquivel et al., 2002), but there has been little consideration of summer mortality as a consequence of food deprivation concomitant with reproduction (but see Delaporte et al., 2006a).

In recent decades, mass summer mortality of Pacific oyster in aquaculture has become a widespread phenomenon in the world (Perdue et al., 1981; Cheney et al., 2000; Huvert et al., 2004; Garnier et al., 2007). It has been estimated that up to 50% of the harvestable crop can be lost in a given year, and these losses can be even higher in some areas (Renault and Cochennec, 1994; Cheney et al., 2000). Summer mortality is thought to be caused by a combination of biological and environmental stressors acting in combination with microbial pathogens (Samain et al., 2007). With respect to the energy cost for gametogenesis, reproduction is considered an important reason for mass mortality in summer (Cheney et al., 2000; Delaporte et al., 2007). In order to adapt to food deprivation, pre-spawning oysters can absorb gonad as a source of nutritional self-supply (Mathieu and Lubet, 1993; Delaporte et al., 2006b), while post-spawning oysters may

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

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

Similar to glycogen reserves, somatic protein is another important energy source during gonad maturation in bivalves, particularly as it supplies energy to maintain physiological function under extended food deprivation (Whyte et al., 1990). In Pacific oysters, excess utilization of protein is observed under enforced starvation (Garcia-Esquivel et al., 2001) and during the spawning period, especially after spawning (Mao et al., 2006). Therefore, the conflict in protein demand may affect energy metabolic resilience to food deprivation in post-spawning oysters. As reproductive output derives energy reserves from scallop body tissues under starvation (Lodeiros et al., 2001), the changes of glycogen and protein contents in various tissues may reflect the energy metabolic status of oysters after spawning when malnutrition occurs.

When oysters are under stress, any immunosuppression would compromise their defense system against opportunistic parasites and pathogens (Harvell et al., 1999). The immune system of mollusks comprises both cellular and humoral functional components (Glinski and Jarosz, 1997; Hooper et al., 2007). Cellular immunity is implicated via hemocytes, with phagocytosis providing the primary line of immunity (Tripp, 1960; Ordas et al., 2000). The humoral immunity involves many antibacterial components (Anderson and Beaven, 2001), which can be assessed by monitoring antibacterial activity in the hemolymph (Li et al., 2007). A recent study showed that hemocyte density and phenoloxidase activity decreased significantly under starvation (Butt et al., 2007). The stability of lysosomal membrane was also significantly decreased in starved oysters (Zhang and Li, 2006). Therefore, food availability can affect oysters' immunological defense (Delaporte et al., 2006a). Immunosuppression has been observed in association with spawning in various studies, suggesting a strong link between reproductive

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

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

2. Materials and Methods

2.1. Animals

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

population, while the other half received a spawning stimulation through thermal escalation at 28°C to obtain post-spawning oysters. Water became turbid after oysters expel their gametes. Soon after the seawater in the spawning tank became clear, the spawned oysters were removed to 15 ºC seawater.

2.2. Food deprivation treatment and animal sampling

In the food deprivation treatments, three replicate batches (n = 75) of both pre- and post-spawning oysters were kept in 5 μm filtered seawater. In the feeding treatments (control), the same number of pre- and post-spawning oysters were used in each replicate and the oysters were fed a mixture of three microalgal species, *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans* (2×10^9 cells per oyster per day). The water was maintained at 15°C and aerated. After 80 days of food deprivation, the starved oysters were supplied with the same diet as the controls. Oysters from all treatments were sampled on days 1, 4, 9, 16, 26, 40, 59, 80, 110 and 132. On each sampling day, six oysters were randomly selected from each replicate. An extra fifteen oysters were included in each replicate to account for unintentional mortality. Three oysters were used for assessing the condition index and hemolymph immunity. The other three were shucked for tissue sampling. Mortality was recorded on each sampling day.

2.3. Condition index measurement

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

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

2.4 Glycogen and protein analyses

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

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

The level of proteins in gill, mantle and adductor were measured according to the method of Li et al. (2000). Briefly, 100 mg of sample frozen powder was added into 8 mL of 20 mM Tris-HCI buffer containing 2% NaCl and 0.1% $NaNO_3$ (pH 8.0) and centrifuged at × 1500 g for 30 min at 4 °C. The supernatant protein portion was pippetted into an eppendorf tube on ice. Then 10 μL of protein was analysed by the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) at 590 nm on a spectrophotometer (Spectra Max 250) using the bovine serum albumin standard.

2.5. Immunity analysis

Hemolymph of oysters was withdrawn from the pericardium using a 1 mL insulin syringe with a 29-gauge needle. Hemolymph samples from each replicate were pooled and stored in a test tube on ice. A subsample (300 μL) was then mixed with an equal volume of filtered seawater (0.2 μm) (FSW) in a flow-cytometer tube (n = 3). To assess the phagocytic activity of hemocytes, fluorescent beads (Fluoresbrite® YG Microspheres, 1.75 μm) were added to each tube, as 4 μL of a stock suspension of 2.5% solids in FSW per mL. The samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, 488nm Laser) after 60-min incubation at 20ºC in darkness (Goedken and De Guise, 2004). Phagocytic activity was expressed as the percentage of cells that ingested at least three fluorescent beads.

The remaining hemolymph was frozen in liquid nitrogen until the antimicrobial activity assay, as described by Li et al. (2007). Cultures of the marine pathogen *Vibrio harveyi* were obtained from the Tasmania Department of Primary Industries Fish Health

Unit and grown in a sterile nutrient broth (1 g NaCl, 2 g yeast extract and 1 g peptone per 100 mL distilled H_2O), incubating overnight at 37 ºC on an orbital mixer shaker (Ratek) at 200 rpm. The cultures were returned to exponential growth phase prior to antimicrobial assays. The defrosted hemolymph was centrifuged at × 500 g for 5 min to pellet the hemocytes and then 90 µL aliquots of plasma were pippeted into a 96 well plate in triplicate. A 10 µL aliquot of *V. harveyi* culture was then added into each well. Negative controls consisted of 90 µL hemolymph incubated with 10 µL nutrient broth and positive controls comprised 10 µL of *V. harveyi* in 90 µL of nutrient broth. After 30 min incubation, 20 µL of CellTitre 96® Aqueous One Solution (Promega) was pipetted into each well, then the plates were returned to the incubator (37 ºC) for 2 h or until development of the red formazan product in control wells. The absorbance was measured at 492 nm using a 96 well plate reader (Spectra Max 250). The background absorbance from hemolymph broth controls was subtracted from the treatment wells and then cell viability was calculated as a percentage of the absorbance in positive control cultures. Antimicrobial activity was calculated by the equation of 100 – cell viability.

2.6. Statistic analysis

To compare the response of oysters to feeding manipulations, the experimental period was divided into the food deprivation period (day 1-80) and the refeeding period (day 80-132). Repeated-measures ANOVA were used to test the effects of food deprivation and spawning status within these two periods. The analysis of oyster mortality was conducted by two-way ANOVA. Least significant differences (LSD) procedure was used for the

post hoc comparisons when a significant treatment effect was detected. Data were transformed by logarithm or square root to meet assumptions of normality and homogeneity of variance if necessary. A significance level of 0.05 was used for all tests.

3. Results

After food deprivation for 80 days, most post-spawning oysters had a slightly translucent gonad-visceral mass and thin mantle tissue. Meanwhile, the gonad size of pre-spawning oysters was reduced, as indicated by a dwindled gonad-visceral mass and the reduction of milky coloration, especially under food deprivation. Pre-spawning oysters still possessed gonad residue after a period of 80-day food deprivation. The average mortality during 132 days was < 4% and was not different across treatments ($P > 0.05$).

3.1. Condition Index

During the 80-day food deprivation, the interactions between spawning and time, and between food deprivation and time were significant ($P < 0.001$, Table 1). Pre-spawning oysters had a higher CI value than post-spawning oysters in the first 40 days ($P \leq 0.017$, Fig. 1). The impact of food deprivation was significant in both pre-and post spawning groups between day 26 and day 80, with higher CI in the control ($P \leq 0.04$). Under food deprivation, the CI in post-spawning oysters was less than that in pre-spawning oysters. After refeeding, the interaction impact between spawning and food deprivation became significant ($P = 0.003$) and the effects of food deprivation depended on time ($P = 0.001$,

Table 1). There was no difference between pre- and post-spawning oysters in the control ($P > 0.05$, Fig. 1). But in the refed group, post-spawning oysters had lower CI than pre-spawning oysters ($P < 0.001$). The CI of refed pre- and post-spawning oysters increased over time ($P \leq 0.029$) and all reached the control level by day 132 ($P > 0.05$).

3.2 Tissue glycogen

Among the mantle, adductor and gill tissues, the glycogen content was greatest in the mantle tissue. In the first 80 days, the interaction between spawning, food deprivation and day was marginally significant in mantle glycogen ($P = 0.055$, Table 1). In the controls, mantle glycogen increased over time ($P < 0.05$), with the post-spawning oysters gaining a significantly higher mantle glycogen than pre-spawning oysters after day 59 ($P < 0.05$, Fig. 2A). In contrast, under food deprivation, pre-spawning oysters had higher mantle glycogen than post-spawning oysters before day 59 ($P \leq 0.048$). From day 80 to day 132, mantle glycogen increased over time and the interactive effect of spawning and food deprivation on mantle glycogen was significant ($P < 0.001$, Table 1). In the control, more mantle glycogen was detected in post-spawning oysters ($P < 0.001$, Fig. 2A). In the refed group, mantle glycogen in post-spawning oysters remained lower than that in pre-spawning oysters ($P < 0.05$).

For adductor glycogen, the interaction between spawning and food deprivation in the first 80 days was significant ($P = 0.001$, Table 1). There was no difference between pre- and post-spawning oysters in the control ($P > 0.05$), but post-spawning oysters contained less adductor glycogen than pre-spawning oysters in the food deprivation group ($P <$

0.05, Fig. 2B). The impact of food deprivation was not significant in pre-spawning oysters ($P > 0.05$). After feeding resumed in starved oysters, the significant interaction between spawning and food deprivation still existed ($P = 0.001$, Table 1). Adductor glycogen in refed oysters was less than the control level regardless of spawning status ($P < 0.05$, Fig. 2B), and it did not change over time ($P = 0.276$, Table 1). Glycogen in the gill was neither altered by spawning nor by food deprivation ($P > 0.05$, Table 1).

3.3 Tissue protein

Tissue protein was measured in the gill, mantle and adductor. Although adductor protein was higher than the mantle and gill proteins, it was not affected by the treatment manipulation during the experimental period ($P > 0.05$, Table 1). Under food deprivation, the mantle protein was significantly affected by the interactions between time and spawning, between time and food deprivation, and between spawning and food deprivation ($P \leq 0.001$, Table 1). Mantle protein was lower in post-spawning oysters between day 1 and day 4 and the impact of food deprivation became significant from day 16 ($P < 0.05$, Fig 3A). However, mantle protein in the starved group stabilized between day 40 and day 80 ($P > 0.05$). Regardless of time, there was no significant difference between pre- and post-spawning oysters in the control ($P > 0.05$), but under food deprivation, the mantle protein in post-spawning oysters was lower than that in pre-spawning oysters ($P < 0.05$). The impact of food deprivation on mantle protein was significant in both pre- and post-spawning oysters ($P < 0.05$). After refeeding, the mantle protein in refed oysters remained lower than that in the control ($P < 0.001$), and the

difference between pre- and post-spawning oysters became marginally significant (P = 0.052, Table 1). The change in mantle protein during the refeeding period depended on time (P = 0.017, Table 1) and significantly increased in refed oysters from day 80 to day 132 ($P < 0.05$, Fig. 3A).

From day 1 to day 80, the interactive effect between food deprivation, spawning and time was marginally significant on gill protein (P = 0.052, Table 1). In the control, there was no difference between pre- and post-spawning oysters ($P > 0.05$, Fig. 3B). However, under food deprivation, gill protein levels were reduced from day 9 in post-spawning oysters, but only reduced from day 40 in pre-spawning oysters ($P < 0.05$). Gill protein in post-spawning oysters remained low between day 9 and day 80, whereas it was stabilized at a similar level in pre-spawning oysters between day 59 and day 80 ($P > 0.05$). The impact of spawning on gill protein was significant between day 9 and day 26 in starved oysters ($P < 0.05$). After refeeding, gill protein was still affected by the interaction of the above three factors (P = 0.026, Table 1). In the refed treatment, gill protein in pre-spawning oysters was higher than that in post-spawning oysters ($P < 0.05$), and was not significantly different from the control level by day 132 ($P > 0.05$, Fig. 3B).

3.4 Immunocompetence

During the period of food deprivation, hemocyte phagocytosis increased in the control and decreased in the food deprivation treatment (Fig. 4A). The hemocyte phagocytic activity was affected by an interaction between spawning, food deprivation and day (P = 0.021, Table 1). In the control, although phagocytosis in post-spawning

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

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

significantly increased over time ($P < 0.05$) and the food deprived pre-spawning oysters reached the control level by day 132 ($P > 0.05$, Fig. 4B). After refeeding, pre-spawning oysters showed higher antimicrobial activity than post-spawning oysters ($P < 0.001$).

4. Discussion

Food deprivation and spawning have a rapid and dramatic effect on oyster condition. During a period of 80-day food deprivation, the mantle tissue thinned out and the body became slightly translucent in post-spawning oysters. In a pioneer study, however, similar symptoms were observed in oysters after starvation for 147 days (Whyte et al., 1990). This delay in response was likely due to the fact that the experimental oysters used by Whyte et al. (1990) had not spawned. Although the 80-day food deprivation did not cause significant mortality, this study revealed that food deprivation could significantly change the physiological and immunological status of oysters, especially in post-spawning oysters. To our best knowledge, this study is the first to examine the synergistic effects of spawning and food deprivation on the biological condition and stress responses of oysters, and our experimental evidence has demonstrated the crucial role of food supply for the recovery of oysters after spawning.

In this study, both spawning and food deprivation significantly reduced condition index, which is consistent with previous studies (Gabbott and Stephenson, 1974; Wright and Hetzel, 1985; Whyte et al., 1990). Although the oysters had reduced condition index (CI) after spawning, further reductions of CI occurred under food deprivation, indicating gonad reabsorption and additional depletion of stored metabolic resources. Noticeably, CI

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

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

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

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

In the present study, the gill protein levels in pre- and post-spawning oysters all reduced to a stable low level under food deprivation, concomitant with the low protein levels in the mantle by day 80. With a reduction of the metabolic activity of starved oysters (Rodhouse and Gaffney, 1984), it is conceivable that a stable low level of protein is required to sustain the basic physiological functions, e.g., respiration in the gills. Similarly, Whyte et al. (1990) also found that a minimal protein threshold existed in starved oysters to maintain a sustainable nutritional condition. This study revealed that this threshold level of protein in post-spawning oysters was reached earlier than that in pre-spawning oysters. As a result, protein recovery in post-spawning oysters was slower than in pre-spawning oysters upon refeeding. Nevertheless, post-spawning oysters still appear able to allocate bioenergetic resources between body tissues as a physiological response to nutritional stress under food deprivation.

As reported in brackish water clams *Rangia cuneuta* (Lane, 1986), the Pacific oyster was able to derive energy from glycogen and protein reserves to withstand food deprivation. However, the sequence of biochemical catabolism varies among species. For instance, protein was catabolized in mussels and clams only after carbohydrate reserves were almost depleted during food deprivation (Gabbott and Bayne, 1973; Beninger and Lucas, 1984). In contrast, oysters in this study mobilized protein even when glycogen was still relatively abundant. This is possibly because most amino acids can be converted into gluconeogenic precursors and enter the energy cycle at the same time as glucose enters the Krebs cycle in *C. gigas* (Gabbott, 1976; Whyte et al., 1990). Although Whyte et al. (1990) did not find that spawning altered energy budget, we observed that mantle glycogen and protein were reduced during spawning. After 80-day food deprivation, the

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

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

In contrast to hemocyte phagocytosis, spawning status did not affect the hemolymph antimicrobial activity, as long as sufficient food was available. This is consistent with Li et al. (2007) and manifests that antimicrobial activity can counteract the deficiency of cellular immunity in post-spawning oysters during the recovery phase. However, in the absence of food, the reduction of hemolymph antimicrobial activity was significantly greater in post-spawning oysters compared to pre-spawning individuals. Butt et al. (2007) commented that oysters may have some starvation-relevant immune components, which could be reduced with increased stress, but otherwise maintained during times of restricted feeding. Our results support the notion of the presence of spawning-relevant immune components in oyster hemolymph. As the antimicrobial activity is associated with plasma peptides (Montes et al., 1995; Montes et al., 1997; Anderson and Beaven, 2001), the ability to synthesis antimicrobial peptides may be reduced by both food deprivation and spawning activity. In the present study, food resumption enhanced the antimicrobial activity, but pre-spawning oysters showed a faster recovery than post-spawning oysters, similar to the pattern observed for hemocyte phagocytosis. As oyster immunity is the primary defense against pathogenic microorganisms (Anderson and Beaven, 2001), the spawning-suppressed immune response to food deprivation could easily make oysters susceptible to pathogens and parasites. In a worse case scenario, the synergistic effect of spawning and poor nutritional condition could lead to mass mortality, especially in summer under high temperature stress when pathogens prevail.

Overall, this study demonstrates that spawning can suppress oysters' metabolic and immune responses and their recovery from food deprivation. Pre-spawning oysters can mitigate the stress of food deprivation by gonad absorption, resulting in a better

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

Acknowledgment

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Table 1. Summary of the probability values of difference after the analysis of repeated measure ANOVA on various immune and metabolic parameters in Pacific oysters. The treatments involved spawning status (pre-spawning and post-spawning; SP) and food supply (with and without feeding). Each variable was separately analyzed during food deprivation (FD, first row) and refeeding (Refeed, second row). The time effect refers to either the period of food deprivation (days 1-80, first row) or the refeeding period (days 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

Figure legends

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

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

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

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

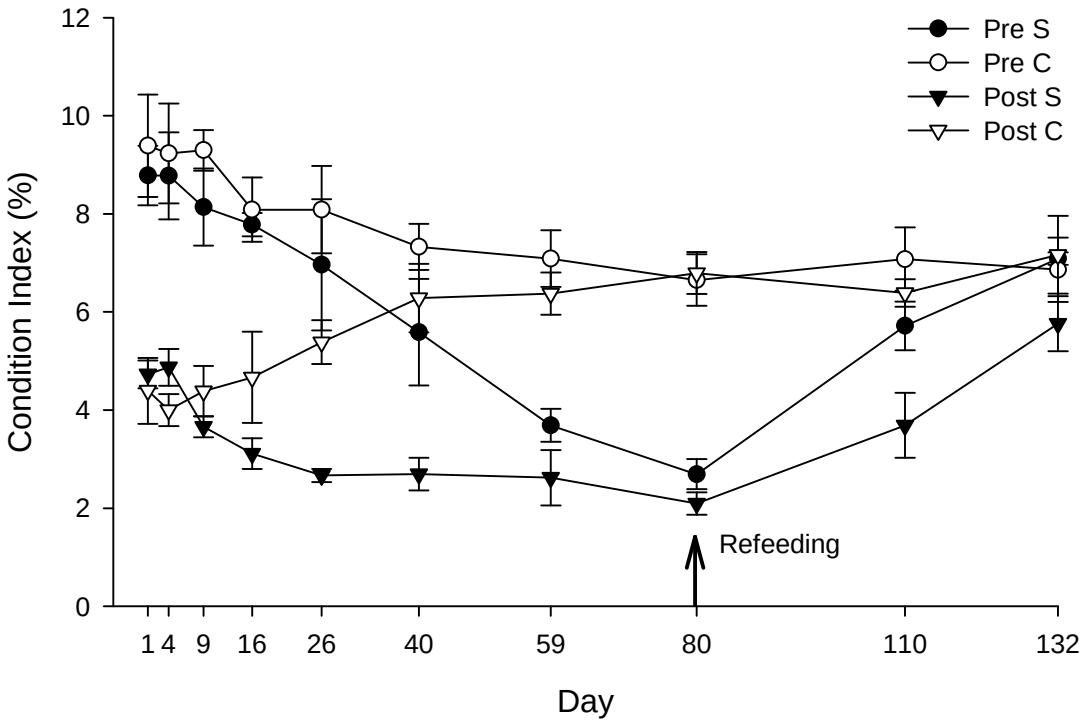

Pre S
Pre C
Post S
Post C
Condition Index (%)
12
10
8
6
4
2
0
Refeeding
1 4 9 16 26 40 59 80 110 132
Day

Figure 1.

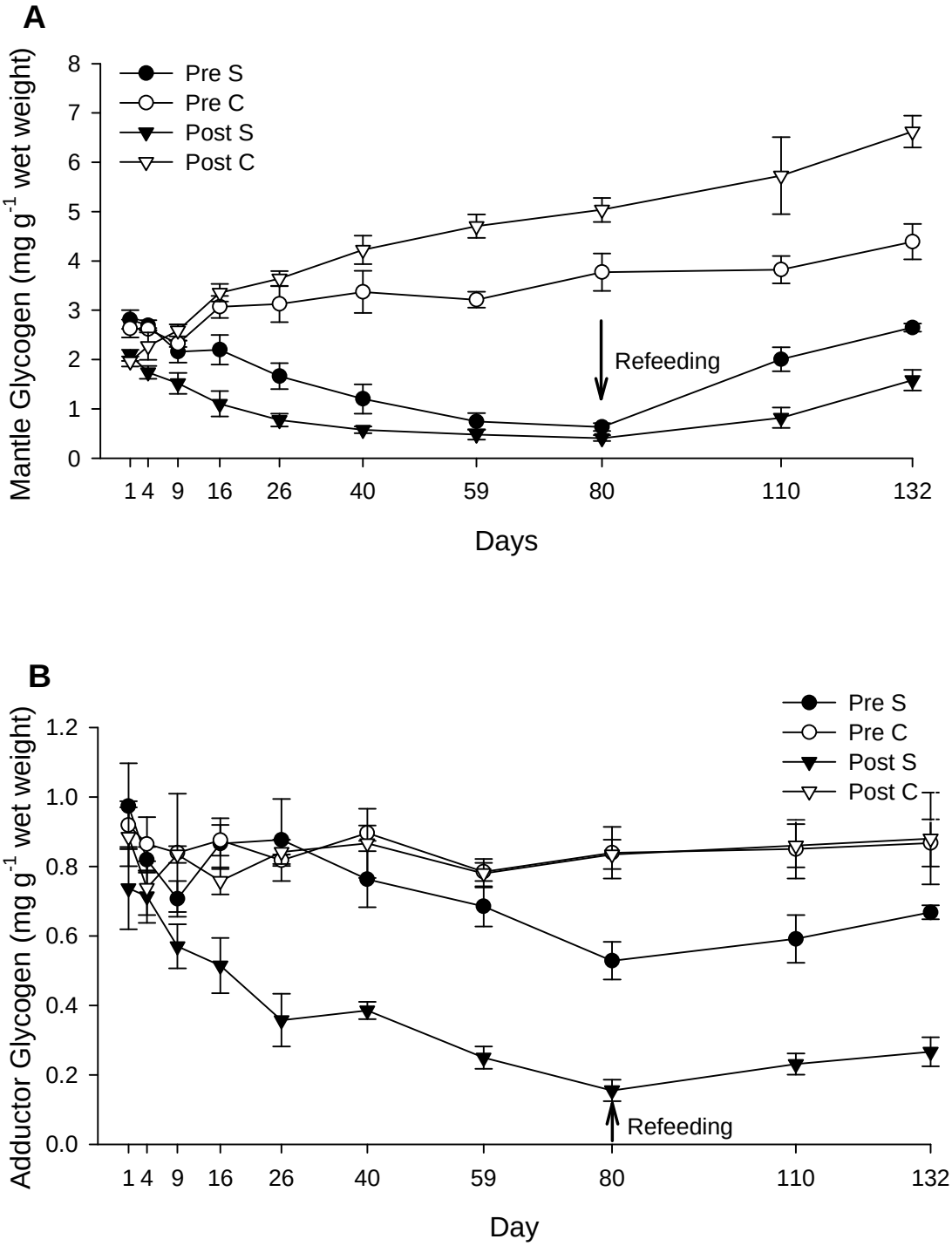
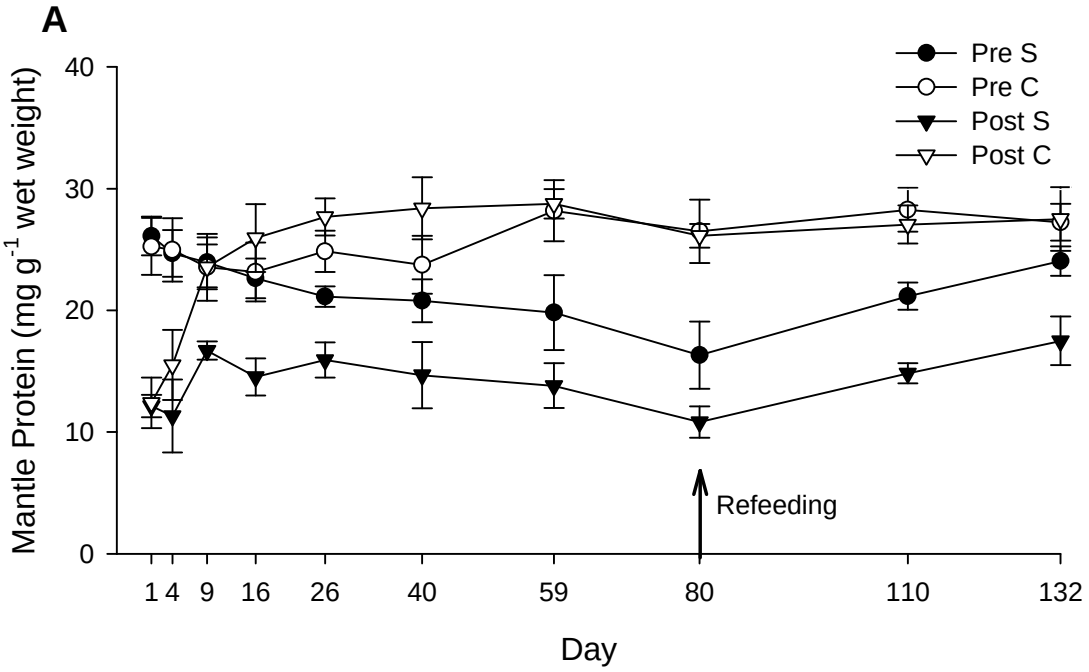

A
Mantle Glycogen (mg g-1 wet weight)
Pre S
Pre C
Post S
Post C
Refeeding
Days
B
Adductor Glycogen (mg g-1 wet weight)
Pre S
Pre C
Post S
Post C
Refeeding
Day

Figure 2.


A
Pre S
Pre C
Post S
Post C
Mantle Protein (mg g-1 wet weight)
40
30
20
10
0
Refeeding
1 4 9 16 26 40 59 80 110 132
Day

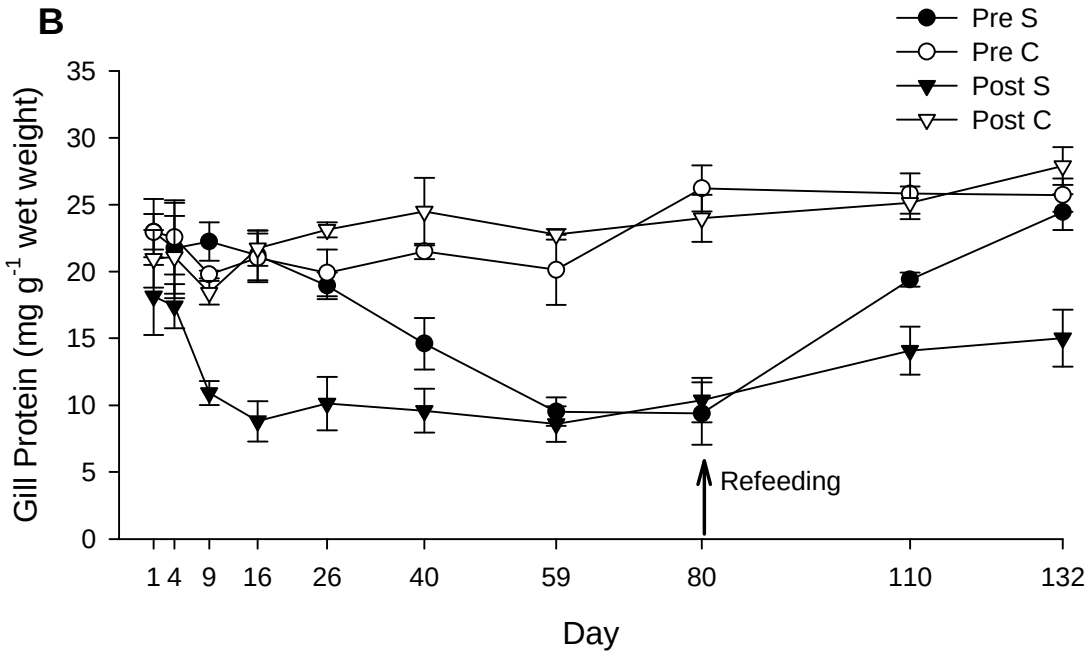
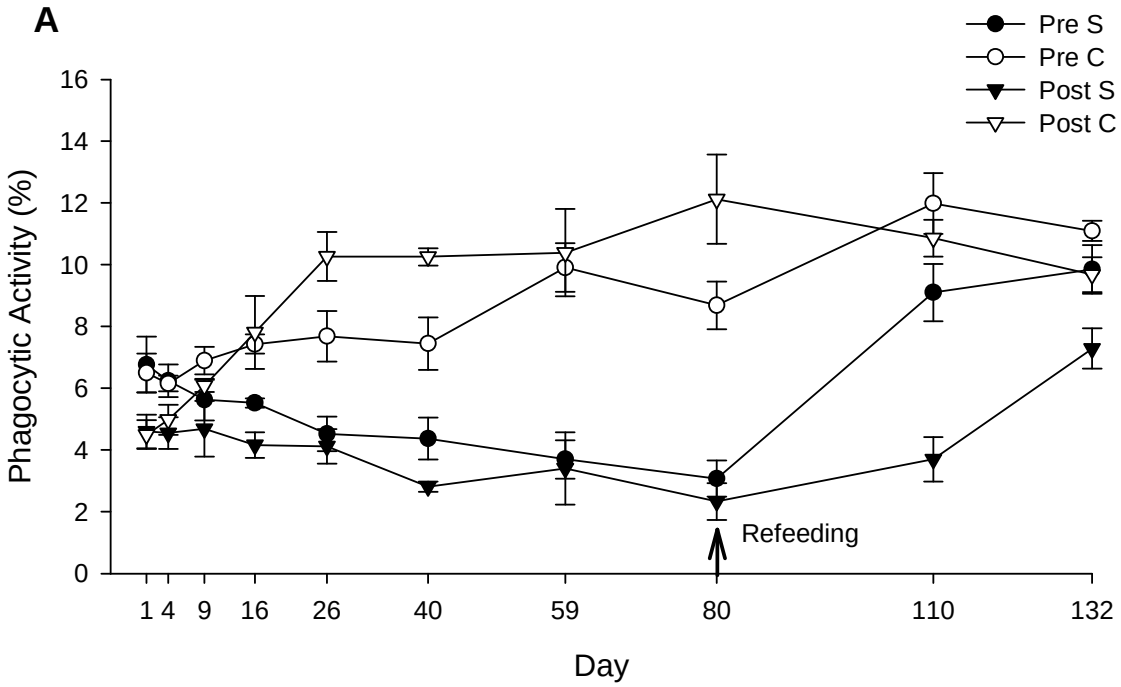

B
Pre S
Pre C
Post S
Post C
Gill Protein (mg g-1 wet weight)
35
30
25
20
15
10
5
0
Refeeding
1 4 9 16 26 40 59 80 110 132
Day

Figure 3.


A
Pre S
Pre C
Post S
Post C
Phagocytic Activity (%)
16
14
12
10
8
6
4
2
0
Refeeding
1 4 9 16 26 40 59 80 110 132
Day

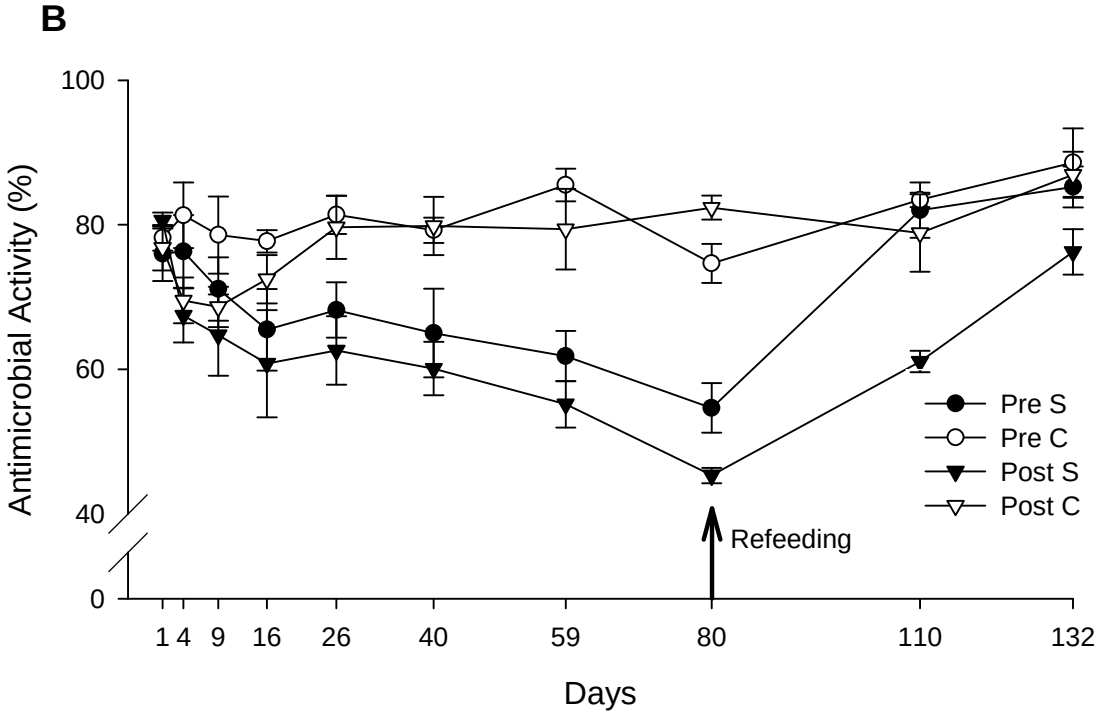

B
Antimicrobial Activity (%)
100
80
60
40
0
Pre S
Pre C
Post S
Post C
Refeeding
1 4 9 16 26 40 59 80 110 132
Days

Figure 4.