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Effect of unilateral and bilateral eyestalk ablation in *Litopenaeus vannamei* male and female on several metabolic and immunologic variables

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Abstract:

Eyestalk ablation is the most common procedure to induce gonadic maturation in commercial hatcheries of penaeid shrimp. In addition to reproduction, other physiological and metabolic processes are affected by removal of the X-organ sinus gland complex located in the eyestalk. In this study, the effect of unilateral and bilateral eyestalk ablation on the concentration of several hemolymph metabolites and phenoloxidase system in female and male shrimp was investigated. As a consequence of reducing or suppressing molt-inhibiting hormone (MIH) production, the duration of the molting cycle was significantly shorter in eyestalk-ablated shrimp: bilaterally (10 days), unilaterally (17 days), and shrimp that were not ablated (24 days). Mortality was significantly higher in unilaterally (35%) and bilaterally (68%) ablated shrimp than in untreated shrimp (2%), probably caused by impairment of several physiological functions mediated by hormones from the eyestalk and direct injury of the nervous system. Males and females were affected differently by eyestalk ablation in terms of concentrations of glucose, triglycerides, and protein in hemolymph. Glucose and lactate levels were lower in bilaterally ablated shrimp, as expected by the role of crustacean hyperglycemic hormone in glucose metabolism. Cholesterol and hemocyte count were not significantly different among the three treatments. Prophenoloxidase and phenoloxidase activities were significantly lower in bilaterally, but not in unilaterally ablated shrimp. This could suggest an endocrine control of this mechanism of the effector immune response or reflect the level of physiological trauma caused by bilateral eyestalk ablation in this species.

Keywords: Hemolymph metabolites; Phenoloxidase; Shrimp; Sinus gland

1. Introduction

Eyestalk ablation (hereafter called ablation) has been used since 1970 to improve the aquaculture production of *Penaeus* spp. larvae (Bray and Lawrence, 1992). Besides improving reproductive performance, there is evidence of other metabolic consequences that are not fully understood. The X-organ sinus gland complex, located in the eyestalks, is the principal neuroendocrine gland in crustaceans (Beltz, 1988; Chang, 1992). In this gland, hormones are synthesized, stored, and secreted to the hemolymph to regulate several metabolic processes (Chang, 1992). The most studied processes are vitellogenesis (Fingerman, 1995; Palacios et al., 1999), food intake, digestion, and nutrient transport (Rosas et al., 1995), molting (Chang and O'Connor, 1988), metabolism of lipids (Teshima et al., 1988; Santos et al., 1997), regulation of glucose and proteins in hemolymph (Santos and Keller, 1993a,b; Teshima et al., 1988; Chen and Cheng, 1995), hydromineral balance, regeneration and pigment production (Keller and Sedlmeier, 1988).

Despite the numerous studies of the prophenoloxidase (proPO) activating system (for review, see Söderhäll and Smith, 1986; Sritunyalucksana and Söderhäll, 2000), little information exists about its endocrine control. Perazzolo et al. (2002) observed a decrease in total phenoloxidase (PO) activity seven days after ablation of shrimp, but explained the decrease because of stress, instead of endocrine control. In insects, it is known that ecdysone modulates the expression of proPO-activating enzyme at the mRNA level (Ahmed et al., 1999; Zou et al., 2005). In crustaceans, ecdysone from the Y-organ is under the control of the sinus gland (Chang and O'Connor, 1988) and could have a similar role.

Most of the studies related to the removal of eyestalks in penaeid shrimp have focused on reproduction (for reviews, see Bray and Lawrence, 1992; Racotta et al., 2003). Only a few studies analyzed the metabolic or immunologic consequences (Rosas et al., 1993; Palacios et al., 1999; Perazzolo et al., 2002; Maggioni et al., 2004). Rosas et al. (1993) found differences between the sexes in energy balance after ablation, although these differences were related to the different reproductive efforts of males and females. In this study, the effect of unilateral and bilateral ablation on biochemical composition of the hemolymph and related immune system variables was analyzed in non-reproductive *Litopenaeus vannamei* females and males.

2. Materials and methods

2.1. Experimental conditions

A total of 100 female and male whiteleg shrimp *L. vannamei* (15.5 ± 1.5 g) were transferred to circular tanks (1.5 m diameter × 0.8 m high) at a density of 16 shrimp per tank, in a closed circulating system at 24 °C and salinity of 34 with 400% daily water exchange and a 12 h:12 h photoperiod. Shrimp were fed every morning with a commercial pellet diet containing 40% protein, 7% lipids, 10% moisture, and 7% ash (Piasa, La Paz, México) and before darkness with fresh squid. Shrimp were individually marked by different cutting of the uropods, which allows 16 different combinations. After molting, these marks are still present in the exuviae, allowing the identification of molted individuals (Racotta and Hernández-Herrera, 2000).

2.2. Eyestalk ablation and sampling in relation to the molt cycle

Two days after ecdysis, shrimp were ablated unilaterally (left eyestalk only) or bilaterally (both eyestalks) by cutting the eyestalks under water at the base of the peduncle and applying pressure to the wound for 15 s to minimize fluid loss and help coagulation. Control animals (not ablated) were manipulated in a similar way two days after ecdysis. Overnight fasting shrimp were sampled between 08:00 and 09:00 h, hemolymph was withdrawn from the ventral sinus with a 1.0 ml syringe containing a shrimp salt solution with EDTA as the anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM hepes, and 10 mM EDTA-Na_2 at pH 7.3) (Vargas-Albores et al., 1993). Hemolymph was kept on ice for all measurements. Only intermolt shrimps were sampled, based on the individual's last molting and observation of uropods (Chang et al., 1988).

2.3. Biochemical analyses

Glucose, lactate, triglycerides, and cholesterol were measured with commercial kits from Merck and Sigma. Total proteins were determined by the technique described by Bradford (1976). These protocols were standardized at 450 mM salinity in a microplate reader, using appropriate calibration curves for each variable (Palacios et al., 1999). For each variable, 10 to 50 µl of a sample, depending on the particular analysis, were mixed with 200 µl reagent solution and incubated at 24 °C for 10 to 30 min, depending on maximum reaction and stability of each analysis. Absorbance was read at 492 nm for glucose, triglycerides, and cholesterol, at 560 nm for lactate, and 595 nm for proteins.

2.4. Total hemocyte count

Hemolymph was diluted 1:10 with sterile shrimp salt solution. From this dilution, hemocytes were counted in triplicate with a Neubauer chamber under a light microscope and total hemocyte count was reported as the number of hemocytes ml^{-1} of hemolymph.

2.5. Determination of proPO content and PO activity

Hemocytes were separated from plasma by centrifugation at 3000 *g* for 3 min. Hemocytes were suspended in 450 µl cacodylate buffer (10 mM sodium cacodilate at pH 7). PO activity was determined by recording the formation of dopachrome from L-dihydroxiphenylalanine, a reaction catalyzed by PO (Hernández-López et al., 1996). Fifty µl cacodilate buffer were added to 50 µl plasma and then 50 µl L-dopa (3 mg ml^{-1} dH_2O). The solution was incubated 10 min at 25 °C, then 800 µl cacodylate buffer was added and the absorbance was measured. Cacodylate buffer was used as a control. Total activity was expressed as the change in absorbance at 492 nm min^{-1} ml^{-1} of hemolymph sampled.

To determine whole PO activity (activated proPO + PO), the proPO sample was first activated with trypsin (0.1 mg ml^{-1} in distilled H_2O). Then, proPO content was calculated as the absorbance obtained for whole PO activity from samples incubated with trypsin minus the absorbance obtained for PO activity from samples incubated without trypsin. Originally, proPO and PO were analyzed separately in the plasma and the cellular pellet; however, data obtained for both fractions were summed to correct for accidental degranulation or rupture of hemocytes.

2.6. Statistics

Normal distribution and homoscedasticity were examined for each group of data. The effect of ablation treatment and sex were analyzed by two-way ANOVA. When significant differences were found by ANOVA, data were analyzed with an *a posteriori* Tukey test for different sample size. Only when a significant interaction between ablation treatment and sex was obtained, individual means (each ablation treatment-sex combination) were compared; otherwise only global means (i.e. pooled means for ablation treatment or sex) were compared.

3. Results

Duration of the molt cycle significantly decreased with ablation: bilateral took 10 days and unilateral took 17 days, while the control group took 24 days ($P < 0.01$; Fig. 1) and with no significant differences between sexes. Mortality was 2% for the control group, 33% in the unilaterally ablated group, and 68% in the bilaterally ablated group, again, without significant differences between sexes (not shown).

A significant interaction between sex and ablation ($P < 0.05$) was obtained for the concentration of glucose (Fig. 2a). Compared to controls, glucose levels increased in unilaterally ablated males and decreased in unilaterally ablated females. In general, bilaterally ablated shrimp had lower levels of glucose, compared to control group (females) or unilaterally ablated group (males). Concentration of lactate was significantly higher in females than in males (main effect of sex, $P < 0.001$; Males 3.5 ± 0.43 mg dl^{-1}, females 6.5 ± 0.7 mg dl^{-1}). Lactate concentrations were lower in the bilaterally ablated group compared to the unilateral ablated group, with intermediate levels in the control group (Fig. 2b; main effect of ablation treatment, $P < 0.001$).

For concentration of triglycerides, ablation affected females and males differently, as shown by a significant interaction ($P < 0.05$; Fig 3a). Within the control group, females had significantly higher levels than males. Triglycerides were lower in unilaterally ablated females, compared to the control group, but no effect was observed in ablated males. No significant effects were observed in cholesterol concentration (Fig. 3b).

A significant interaction ($P < 0.05$) was also detected for the concentration of protein (Fig. 4). In the control group, protein was significantly lower in females than in males, whereas the opposite effect occurred in unilaterally ablated shrimp. In females, unilateral ablation decreased protein levels, while in males, protein levels were increased by unilateral ablation.

Content of proPO and activity of PO were significantly lower in bilaterally ablated shrimp compared to the control group or to unilaterally ablated shrimp (main effect of ablation, $P < 0.05$; Figs. 5a and 5b). Sex did not affect proPO or PO; interactions were not significant. Total hemocyte count was not significantly affected by ablation or sex, although a trend toward a decrease with degree of ablation was observed (Fig. 5c).

4. Discussion

As in other reports, the duration of the molt cycle decreased in both sexes of whiteleg shrimp as an effect of ablation. Similar results were obtained by Chan et al. (1990) with shrimp of the same size but maintained at 22 °C, rather than 24 °C, where the molt cycle duration for intact, unilaterally and bilaterally ablated shrimp was 23.4, 15.9, and 9.1 days, respectively. However, in two related species (blue shrimp *Litopenaeus stylirostris* and white shrimp *L. setiferus*) maintained at higher temperatures (27–29 °C),

the effect of ablation was less pronounced: the molt cycle was 13.6 days in intact shrimp and 11.5 days in unilaterally ablated shrimp (Robertson et al., 1987). The decrease in molt cycle duration following ablation is mainly attributed to the lower concentration of molt-inhibiting hormone caused by ablation. This hormone exerts an inhibitory action on ecdysteroids biosynthesis (Chang and O'Connor, 1988; Lachaise et al., 1993). In ablated shrimp, 20-hydroecdysone (20E) is synthesized and secreted at a higher rate. Studies with *L. stylirostris* (Gendrop-Funes and Valenzuela-Espinosa, 1995) and other crustaceans (Carlisle, 1953) failed to obtain a decrease in molt cycle duration after ablation. Chan et al. (1990) suggested that this was a consequence of the molt stage or reproductive stage of the shrimp at the time of ablation. According to our results, the duration of the molt cycle after ablation is the same in females and males. Sexual dimorphism is apparent in shrimp >20 g (Otoshi et al., 2003) or when sexual maturity is attained at ~30 g (Racotta et al., 2003). Therefore, possible differences in duration of the molt cycle between females and males would occur in shrimp larger than those used in our study.

Mortality was directly related to the degree of ablation. This was expected, considering the strong physiological stress caused by partial or total removal of the main endocrine gland, the X-organ sinus gland complex. Ablation not only removes this organ complex, it produces severe trauma, destroys a mayor portion of the nervous system, and renders the animal blind (Chang and O'Connor, 1988; Chang, 1989).

Function of the humoral and cellular defense system has been widely investigated in crustaceans and insects (Söderhäll and Smith, 1986; Olafsen, 1988; Johanson and Söderhäll, 1989; Vargas-Albores, 1995; Hernández-López et al., 1996; Moullac et al., 1997). Recent studies in insects indicate that several neuroendocrine systems modulate

198 the humoral and cellular defense system. In unilaterally ablated female *Farfantepenaeus*
199 *paulensis*, a decrease in total hemocyte count was observed (Perazzolo et al., 2002). In *L.*
200 *vannamei*, the decline was not significant (Maggioni et al., 2004); in our study, only a
201 non-significant trend, related to the degree of ablation was observed for males. In
202 *Drosophila melanogaster*, Sorentino et al. (2002) found that the lack of ecdysteroids
203 compromised the cellular immune responses reducing hemocytes proliferation and
204 encapsulation.

205 Beside the evidence that 20E affect cellular activity, Ahmed et al. (1999)
206 demonstrated that 20E up-regulates the expression of proPO gene in *Anopheles gambiae*.
207 In our study, and as suggested by duration of the molt cycle, ablated shrimp probably
208 have higher levels of 20E that should, in turn, increase the level of proPO. However, we
209 observed a decline in proPO in bilaterally ablated shrimp. Ahmed et al. (1999) and
210 Müller et al. (1999) found different proPO genes in insects and demonstrated that 20E
211 can stimulate, inhibit, or not affect the expression of the different proPO genes. The
212 decline in proPO in ablated shrimp in our study could be an inhibitory action of 20E on
213 the expression of proPO gene(s) in hemocytes. Alternatively, a decline in one or several
214 particular hormones from the eyestalk with a putative positive effect on a proPO gene
215 could also be involved and remains to be investigated.

216 The non-significant decrease of hemocytes in bilaterally ablated shrimp,
217 particularly in males, could also contribute to the reduced proPO activity produced by
218 semi-granular and granular hemocytes (Sritunyalucksana and Söderhäll, 2000).
219 Moreover, reduced PO activity in bilaterally ablated shrimp could be a direct
220 consequence of lower levels of proPO or decreased activity of a proPO-activating

enzyme, a serine proteinase that converts proPO into PO (Sritunyalucksana and Söderhäll, 2000). According to Zou et al. (2005), 20E reduced the mRNA levels of the proPO-activating proteinase in *Manduca sexta*. Perazzolo et al. (2002) found reduced whole PO (proPO + PO) in unilaterally ablated *Farfantepenaeus paulensis*, but Maggioni et al. (2004) found no effect in *L. vannamei*. In our study, this effect occurred only in bilaterally ablated shrimp.

Beside the participation of PO in the internal defense system, the enzyme participates in the process of cuticular melanization in crustaceans (Benjakul et al., 2005) and insects (Hiruma and Riddiford, 1993). The acceleration of the molting process caused by ablation presumably produces increased melanin production through the PO system. However, it is not known if PO in hemocytes participates in melanin incorporated in the exoskeleton. In insects, the PO responsible for cuticular melanization is produced in the epidermis (Hiruma and Riddiford, 1993).

It is well known that crustacean hyperglycemic hormone (CHH) secreted from the sinus gland located in the eyestalk stimulates hyperglycemia (Santos and Keller, 1993a), but it is not clear if CHH controls the baseline levels of circulating glucose because ablation does not necessarily modify these levels. In lobsters *Panulirus argus*, only bilaterally, but not unilaterally ablated specimens had lower levels of hemolymph glucose (Diaz-Iglesias et al., 1987). This suggests that some CHH is needed to maintain a certain level of glucose. Controversial results have been reported for the glucose concentration in the crab *Chasmagnathus granulate*: no effect was reported by Santos and Colares (1986), whereas Santos et al. (1988) reported a decreased in glucose concentration 24 hours after bilateral ablation. The last authors attributed the discrepancy to the method used for

glucose analysis, since the reduced levels occurred only when a specific enzymatic procedure was used. However, utilizing the same method used in our study, no effect of bilateral ablation was observed in *Carcinus maenas* (Lüschen et al., 1993; Santos and Keller, 1993c) and a slight decrease occurred in *Orconectes limosus* (Santos and Keller, 1993c) and kuruma shrimp *Marsupenaeus japonicus* (Kuo et al., 1995). In our study, lower glucose concentrations occurred in unilaterally and bilaterally ablated females and higher concentrations occurred in unilaterally ablated males. Absence of a clear relation between glucose levels and degree of ablation suggests that CHH is not directly involved in maintaining glucose levels, as originally suggested by Santos and Colares (1986). In addition to CHH, monoamines, such as catecholamines and serotonin produces clear hyperglycemic responses in ablated *Carcinus maenas* (Lüschen et al., 1993), suggesting that others neuroendocrine mechanisms are involved in glucose regulation and metabolism without participation of CHH. In contrast to glucose, a low lactate concentration in bilaterally ablated shrimps in our study could result from suppression of the principal source of CHH, which apparently stimulates anaerobic glycolysis with lactate production (Santos and Keller, 1993b).

Hormones from the sinus gland are also involved in lipid metabolism, as clearly indicated by the use of ablation to induce gonad development (for reviews, see Bray and Lawrence, 1992; Racotta et al., 2003), a process involving accumulation of lipids in gonads (Teshima et al., 1988; Palacios et al., 1999). The general effects of ablation on reproduction, including mobilization of lipids from the hepatopancreas to the gonad, are mainly attributed to the concomitant reduction in the levels of gonad inhibiting hormone produced by the sinus gland. However, it was also suggested that CHH stimulates lipid

mobilization from the hepatopancreas with a concomitant increase in several lipid classes in the hemolymph (Santos et al., 1997). In *Carcinus maenas* males, ablation produces a significant decrease of total lipids, but not of triglycerides in the hemolymph (Santos et al., 1997). In our study, unilateral, but not bilateral, ablation significantly decreased triglycerides levels only in hemolymph of females. Lack of a relationship between triglyceride levels and the degree of ablation does not allow a clear interpretation of our results that addresses possible antagonic effects of CHH and GIH on metabolism of lipids.

In previous studies, contradictory results were obtained for the effect of ablation on protein levels in hemolymph of penaeid shrimp. Perazzolo et al. (2002) reported that protein levels decreased by 50% in unilaterally ablated *Farfantepenaeus paulensis* females. In contrast, Maggioni et al. (2004) reported a non-significant increase in protein of 27% in unilaterally ablated *L. vannamei* females. In our study, unilaterally ablated males and females produced opposite effects, and thus should be discussed together with sex-dependent effects.

Several effects of ablation were related to sex and differences between sexes were observed in the control group. Few studies have systematically compared metabolic responses between males and females. Chen and Cheng (1993) did not find differences in protein and hemocyanin levels between females and males in southern pink shrimp *Marsupenaeus japonicus*. Rosas et al. (1993) observed that ablation in *Farfantepenaeus notialis* increased ingestion of food and assimilation rates to a greater extent in females than males. Moreover, a more efficient use of energy in ablated females than in ablated males was apparently related to different energy requirements to support ablation-induced

gonad development (Rosas et al., 1993). Although, shrimp in our study had not reached sexual maturity, it seems that metabolic differences can occur before maturity.

Higher levels of lactate were observed in females in all treatments. Females could be more susceptible to stress during the sampling procedure, since increased lactate is a typical stress response in penaeid shrimp (Racotta and Palacios, 1998). Lower glucose levels in unilaterally ablated females, in contrast to males, could be related to higher glucose use in anaerobic glycolysis, as reflected by the marked increase in lactate levels in unilaterally ablated females. For the control group, triglycerides levels were lower, whereas protein was higher in males than in females. Both components represent circulating reserves that could satisfy increased tissue metabolism induced by ablation as suggested by oxygen consumption (Nan et al., 1995). If so, the major circulating reserve for each sex should be preferentially used, although it would not explain the increase in protein in unilaterally ablated females.

5. Conclusions

The duration of the molting cycle and survival decreased in relation to the degree of eyestalk ablation. The capacity of immune response inferred from proPO content and PO activity decreased only in bilaterally ablated shrimp, suggesting higher susceptibility to pathogens. Differences between males and females in biochemical composition of hemolymph in the control group, as well as the sex-related effect of ablation, suggest that metabolic differences between sexes appear before individuals reached reproductive age and weight.

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Figures captions

Fig. 1. Effect of eyestalk ablation (EA) on the duration of the molt cycle in *Litopenaeus vannamei*. (C) control shrimps, (U) unilaterally ablated and (B) bilaterally ablated females and males. Data are presented as mean ± SD. Following two-way ANOVA (see text for statistical significances), different capital letters on the bars indicate significant difference ($P < 0.05$) between global means (pooled for sex) of the different ablation treatment groups.

Fig. 2. Effect of eyestalk ablation (EA) on hemolymph levels of glucose (a) and lactate (b) in *Litopenaeus vannamei*. (C) control shrimps, (U) unilaterally ablated and (B) bilaterally ablated females and males. Data are presented as mean ± SD. Following two-way ANOVA (see text for statistical significances). Only when a significant interaction between EA and sex was obtained (Fig. 2a), individual means (each EA-sex combination) were compared with *a posteriori* test of Tukey analysis for different sample size. α= 0.05. Otherwise (Fig. 2b) only global means (i.e. pooled means for EA) were compared and significant differences are indicated with capital letters.

Fig. 3. Effect of eyestalk ablation (EA) on hemolymph levels of triglycerides (a) and cholesterol (b) in *Litopenaeus vannamei*. (C) control shrimps, (U) unilaterally ablated and (B) bilaterally ablated females and males. Data are presented as mean ± SD. See figure 2 for statistical details.

Fig. 4. Effect of eyestalk ablation (EA) on hemolymph levels of protein in *Litopenaeus vannamei*. (C) control shrimps, (U) unilaterally ablated and (B) bilaterally ablated females and males. Data are presented as mean ± SD. See figure 2 for statistical details.

Fig. 5. Effect of eyestalk ablation (EA) on hemolymph prophenoloxidase content (a), phenoloxidase activity (b) and total haemocytes count (THC) (c) in *Litopenaeus vannamei*. (C) control shrimps, (U) unilaterally ablated and (B) bilaterally ablated females and males. Data are presented as mean ± SD. See Fig. 2 for statistical details.

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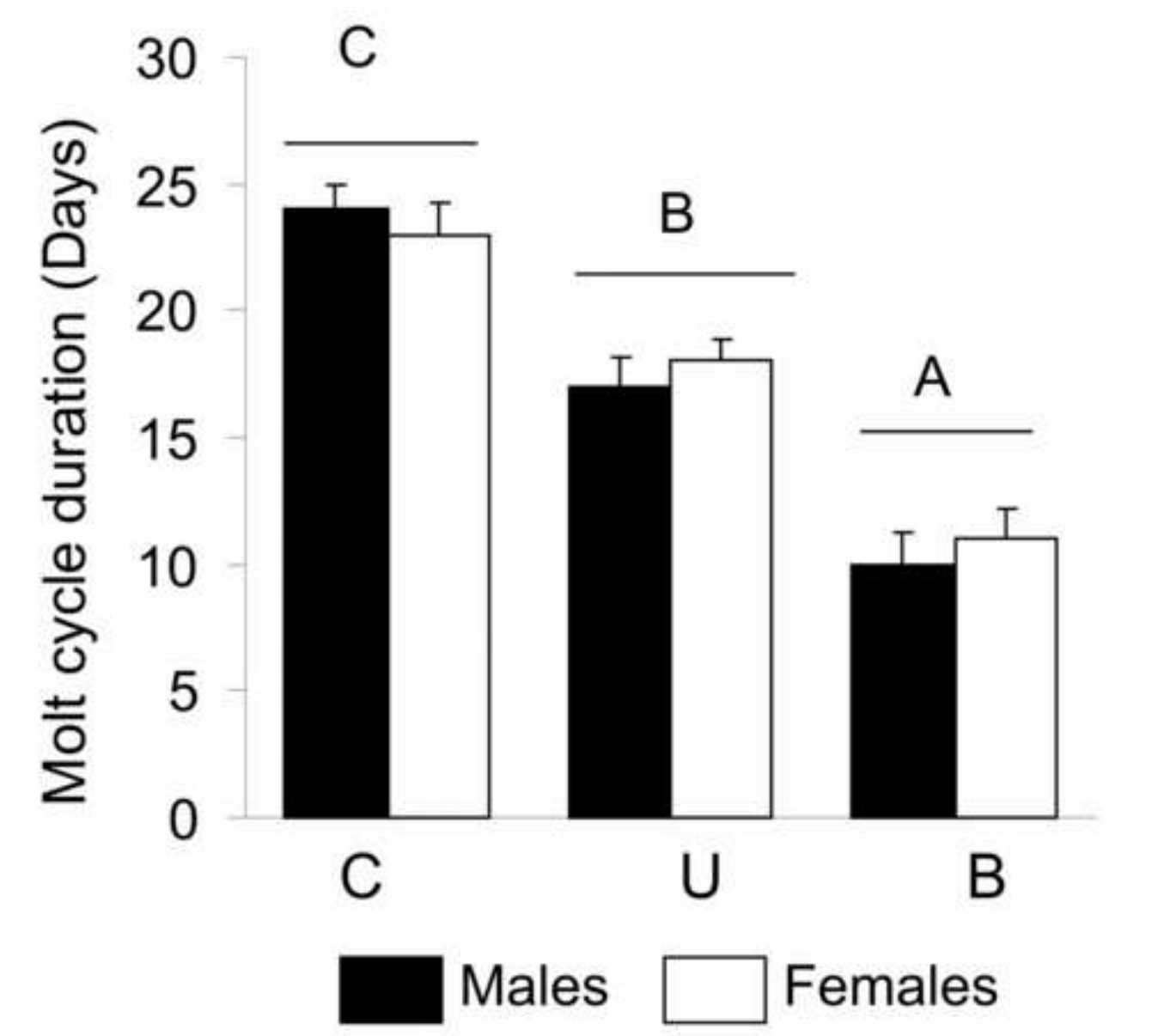

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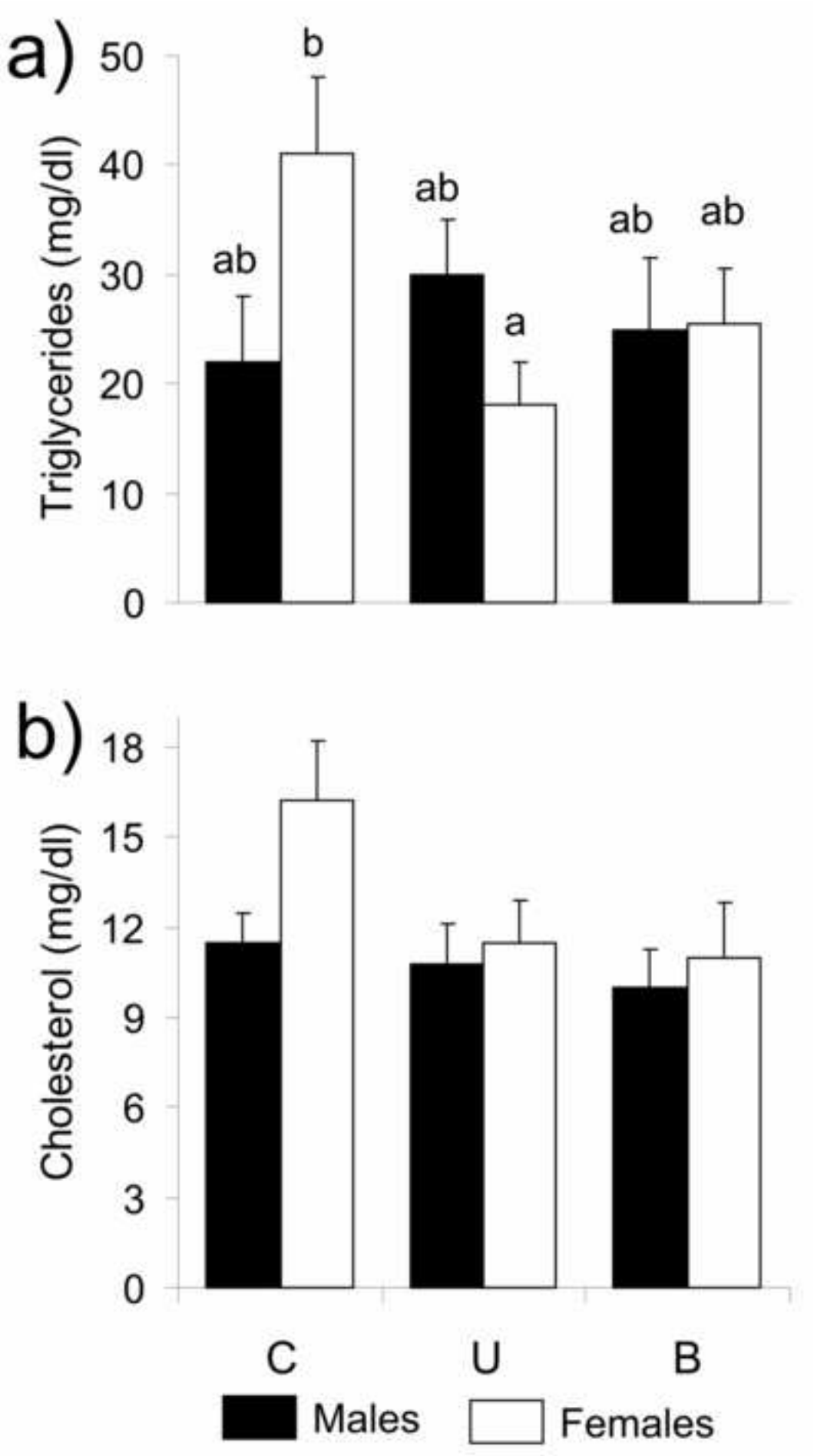

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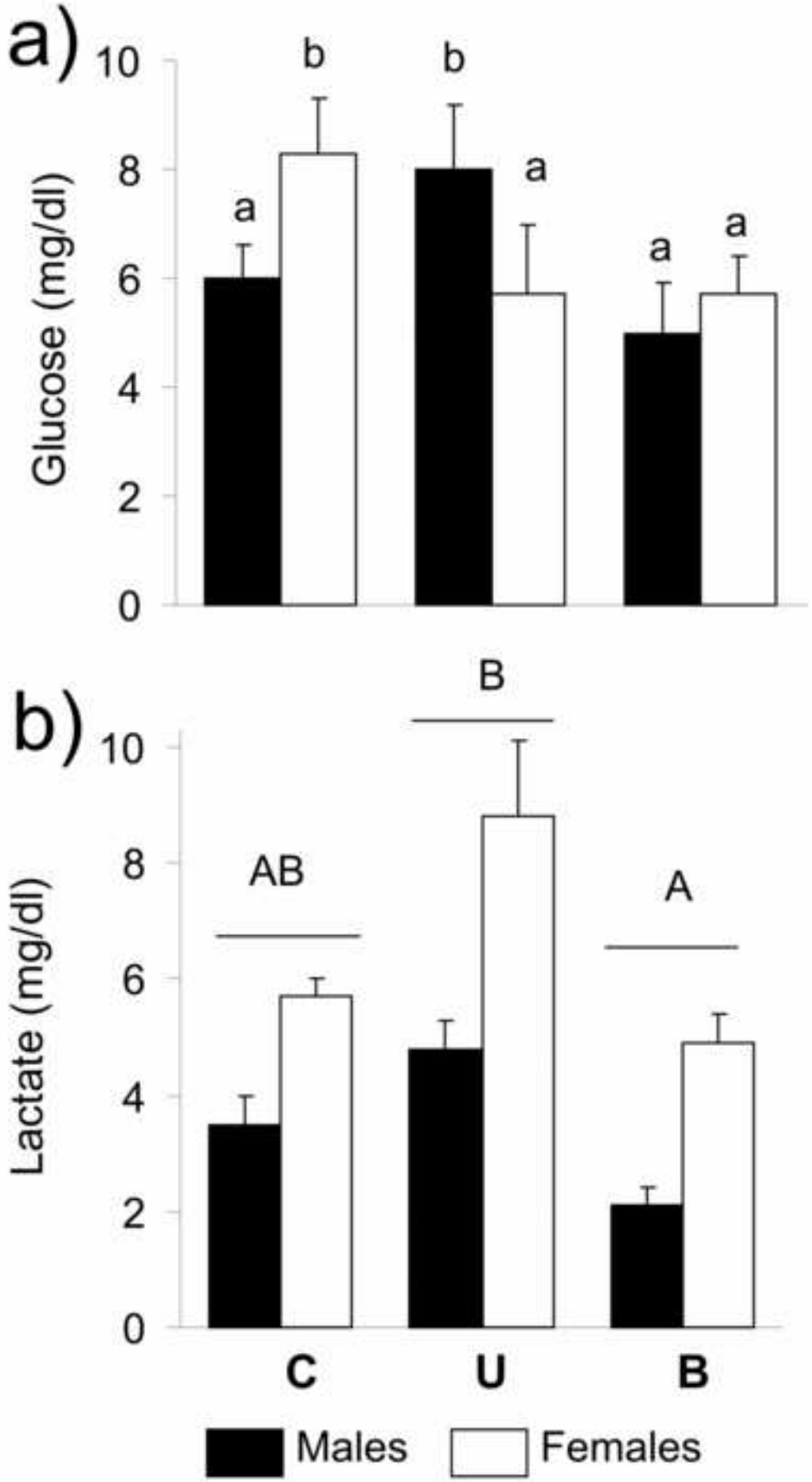

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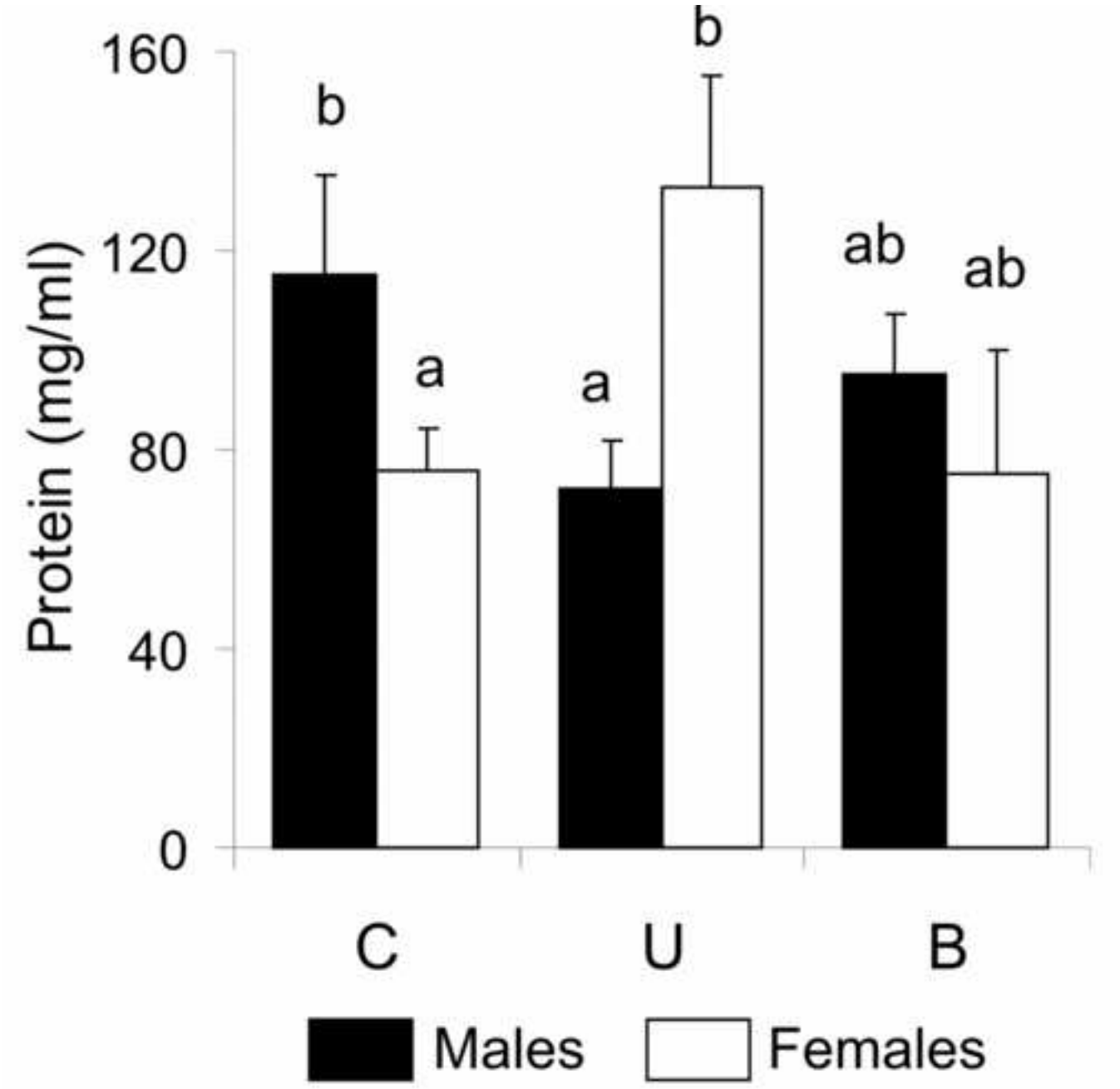

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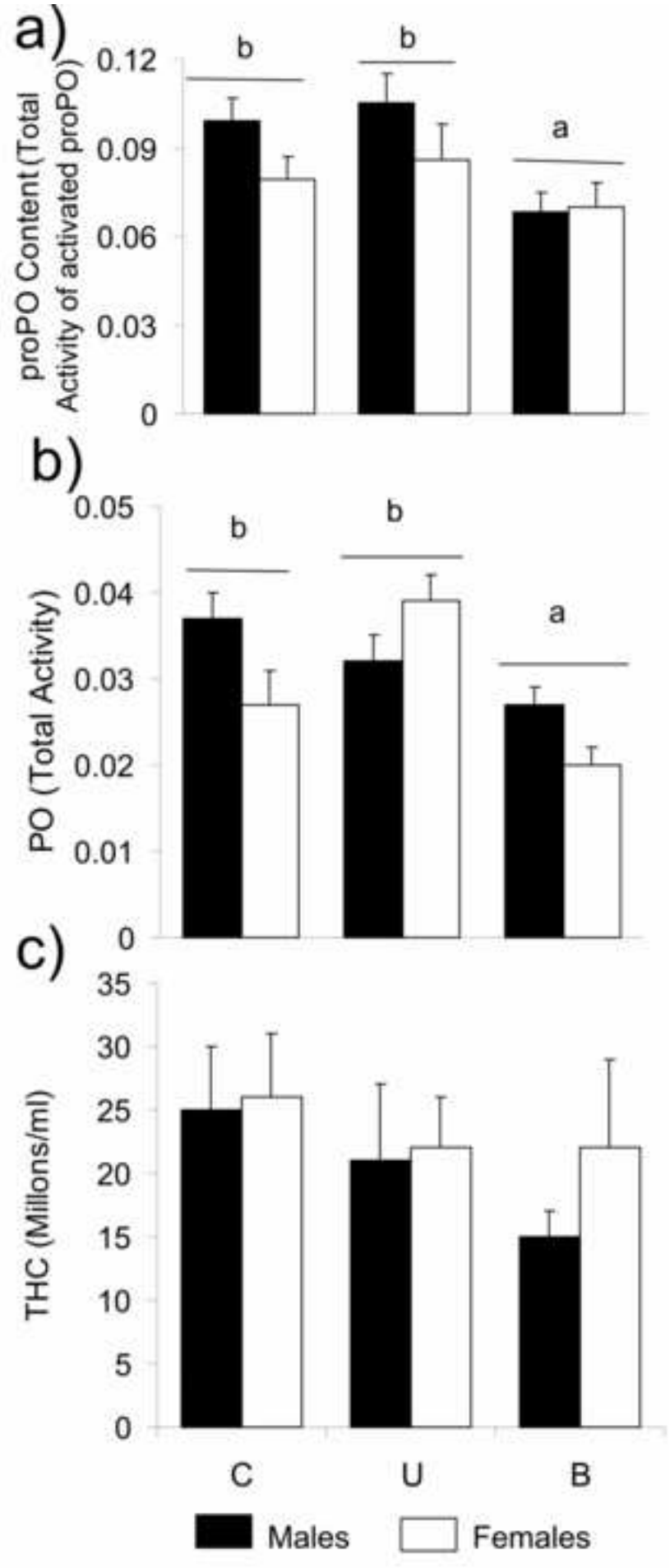

*** Response to Reviewers**

Dear Dr. Donaldson

I am sending you back the revised version of the manuscript AQUA- D-07-00020), "Effect of unilateral and bilateral eyestalk ablation in *Litopenaeus vannamei* males and females on several metabolic and immunologic variables" by J.C. Sainz-Hernández, I.S. Racotta, S. Dumas and J. Hernández-López submitted to Aquaculture.

Some specific corrections are highlighted with yellow in the revised manuscript. However complete sections were rewritten as indicated. All suggestions of referees were addressed as indicated below:

Reviewer No. 1

English language was revised by our institutional editor whose first language is English

A) Methods and Results sections

- Time of hemolymph sampling was now indicated (line 91).
- We used a calibration curve and it is now indicated (line 103).
- We did not evaluate the different groups in different times; the first sentence of the results section was rephrased for clarity to state that the duration of the molt cycle was different between groups (lines 146-147).
- Conventional signs ($P<0.05$) were used to indicate significant differences throughout the whole text (also suggested by Reviewer No. 2).
- All figures were re-drawn as suggested. Results were re-written according to the new order and style was changed for clarity.

B) Discussion

In general discussion was shortened and rewritten with a more integrative perspective and less speculation.

- The first two paragraphs were deleted as suggested
- It is now specified that there is a decrease in the duration of the molt cycle (line 178 and line 317)
- The discussion on the immune condition was in general improved and a consideration about the participation of PO in the tanning process during the molt cycle was now considered (lines 237-243).
- Causes of mortality were reconsidered and severe physiological disruptions including nervous system damage are now included (201-203).
- Mistakes in the literature were corrected

Reviewer No. 2

- One sentence was deleted from the abstract as suggested
- The section of hormonal control of proPO system in the Introduction was rewritten (lines 52-60)
- Register of molting occurrence was confusing in the previous version of the MS; a more detailed description of shrimp and exuviae marking was now included in the corrected version (see lines 80 to 83)
- All style corrections were done, particularly for the Results and Discussion sections that were practically rewritten.