Effects of bilateral eyestalk ablation on gonadal maturity, moulting and biochemical changes in the hemolymph of female *Potamon persicum* crabs (Decapoda, Brachyura, Potamidae)

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Abstract: Adult female crabs (*Potamon persicum*; n=65) were collected from the Jajrood River in the east of Tehran, Iran. Both eyestalks were removed in crabs that weighed 27.6 ± 2.1 g. Changes in the levels of glucose and protein titers in the hemolymph, the number of hemocytes, gonadal and hepatic indices, body weight and carapace length were determined. The level of glucose in the hemolymph was significantly decreased at the end of the first week following eyestalk ablation and significantly increased by the end of the second and third weeks following eyestalk removal compared to the control groups with intact eyestalk ($p < 0.05$). A significant increase was observed in the total protein level of the hemolymph of destalked crabs at the end of the third week compared to those in the control group ($p < 0.05$). The fractions of total protein showed significant changes at the end of the third week compared to the control group ($p < 0.05$). The mean total hemocytes count (THC) in the hemolymph was significantly increased in ablated crabs at the end of the second and third weeks compared to the control group ($p < 0.05$). The gonadal and hepatic indices of the ablated crabs were significantly increased and decreased, respectively, at the end of the second week compared to the control groups ($p < 0.05$). The mean body weight of the ablated crabs was increased significantly at the end of the second and fourth weeks compared to the control group ($p < 0.05$). No significant change was observed in the mean carapace length of the ablated crabs at the end of the second week, but it had increased significantly by the end of the fourth week compared to the control group ($p < 0.05$). The ablated crabs moulted four weeks after the removal of their eyestalks, but no precocious moulting was observed in the control group.

Keywords: *Potamon persicum*, eyestalk ablation, hemolymph, biochemistry, gonad and hepatic indices, moulting, growth.

Introduction

Decapod crustaceans, which include shrimps, prawns, crabs, crayfish and lobsters, represent a large, diverse biological group with significant potential as an aquacultural resource. Some of the major sites of endocrine activity in decapod crustaceans are the sinus glands, which are located in the eyestalks, y-organ and mandibular organs (Van Herp and Soyes, 1997). Since the sinus glands are the sites that release gonad inhibiting hormone (GIH) and moult inhibiting hormone (MIH), the removal of the eyestalks leads to an increased rate of gonadal

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development and precocious moulting (Laufer *et al*., 1993).

On the other hand, there is only scant data available regarding the biochemistry of hemolymph in crustaceans. Many of the fundamental features of this class of crustacea are reflected in the fluctuating nature of the internal medium, such as changes in the constituents of hemolymph during different stages of the moult cycle (Barnes, 1980). Therefore, this study was designed to determine the concentrations of the major biochemical constituents of hemolymph, such as glucose, protein and hemocytes, in the hemolymph of normal and ablated *P. Persicum* crabs. This will help to a better understanding of factors involved in crustacean reproductive and growth control and their hemolymph biochemical components which are fundamental to successful aquaculture.

Materials and Methods

Adult female *P. persicum* crabs (n=65) were collected by means of baited traps from the Jajrood River in the east of Tehran in Iran. The body weight and carapace length of the crabs were 27.6 ± 2.1 g and 33.13 ± 0.7 mm, respectively. Only intermoult animals were used in this experiment. The moult stages were determined by the examination of the exoskeleton upon sacrifice of the initial control group (Skinner, 1985). Crabs were kept in a 2,000 liter glass aquarium with aerated pH 8 freshwater, at a temperature of 25 ± 1.2°C, with normal day-night illumination. They were fed with 1.5% of their body weight of gizzard per day. They were acclimatized to the laboratory conditions for one week prior to experiment.

Biochemical measurements

The first sampling was carried out at week 0 from intact female crabs (n=16). The eyestalks of eight crabs were removed bilaterally as described below and subsequent samplings from the destalked (n=8) and control (n=8) groups was performed at the end of weeks 1, 2 and 3. Measurements were taken for a glucose assay and the total hemocytes counts (THCs) in all four weeks of the experiment (weeks 0, 1, 2 and 3). Protein quantification was only performed at weeks 0 and 3.

Eyestalk ablation

The eyestalks of the crabs (n=8) that were anesthetized by cooling them in ice for 3-5 mins were cut bilaterally at their bases by cautery(Farooqui and Nagabhushanam, 1982). The crabs were released back to the rearing system immediately after ablation.

Hemolymph sampling

Samples (0.5 ml) of hemolymph were withdrawn by puncture of the perioarthrodal membrane at the base of the fourth walking leg (Mattson and Spaziani, 1985; Eddy *et al*., 2007). Sampling for the glucose assay was performed after 12 hours of starvation and was related to the cyclic physiological changes in hemolymph glucose in order to minimize any possible errors. All of the samplings were conducted between 10:00 am and 11:00 am. (Kue and Yang, 1999). For the THCs and protein assays, the hypodermic syringe contained 0.05 ml of EDTA.

Glucose assay

The determination of hemolymph glucose levels was carried out manually by the Enzymatic, Colorimetric Method (God-Pap Zistshimy Glucose Diagnostic Kits; Tietze, 1994; Adamczewska and Morris, 2001).

Protein assay

The total protein assay of the hemolymph was performed manually by the Biuret method (Tietze, 1994; Eddy *et al.*, 2007). Electrophoretic analysis of the total protein content was performed by cellulose acetate paper electrophoresis (Tietze, 1994).

Total hemocytes counts(THCs)

Each THC was performed using a Neubauer-chamber and a procedure similar to that used for red blood cell counts. Each experiment was repeated three times (Eddy *et al*., 2007; Manjula *et al*., 1997).

Morphological measurements

Forty adult female crabs were divided into three groups:

1- Initial controls (n=8), which were sacrificed at the start of the experiment (week 0) to determine the baseline gonadal and hepatic indices.
2- Ablated group (n=16), whose eyestalks were removed at the end of week 0. Half of these were sacrificed after two weeks.
3- Control group (n=16), where half of these were sacrificed after two weeks.

The carapace length, body weight, ovarian and hepatic indices of all of the crabs (initial controls, ablated and controls) were recorded. The ovarian and hepatic indices were calculated by the following formula (Radhakrishnan and Vigayakumaran, 1984).

$$GI = \frac{\text{Gonadal Wet Weight}}{\text{Weight of the crab}} \times 100$$

$$HI = \frac{\text{Hepatopancreas Wet Weight}}{\text{Weight of the crab}} \times 100$$

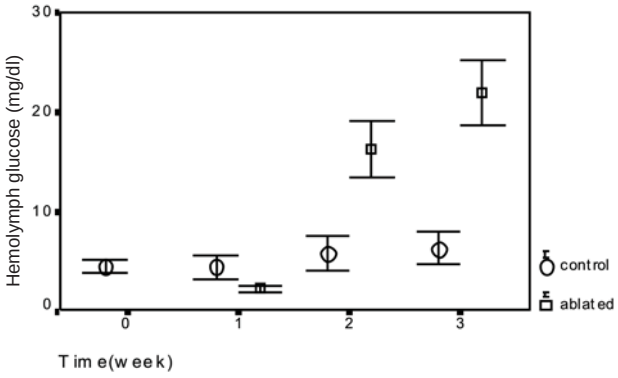
The data were analyzed using an analysis of variance (ANOVA), the Duncan multiple range test, and t-tests for independent and paired samples. All analyses were subjected to SPSS software, version 11.

Results

Hemolymph glucose

The mean glucose titer in the hemolymph was 4.8 ± 0.35 mg/dl(mean ± standard error [SE]) in the initial control crabs at week 0 and 2.4 ± 0.13 mg/dl in the ablated crabs at week 1, which was significantly lower than in control group (4.88 ± 0.35 mg/dl; $p < 0.05$; Figure 1). At the end of the second and third weeks, the mean hemolymph glucose titers of destalked crabs peaked at 16.27 ± 1.2 mg/dl and 21.96 ± 1.25 mg/dl, respectively, which were significantly higher than the peak levels in the control groups (4.9 ± 0.35 mg/dl; $p < 0.05$). The mean glucose content of the hemolymph of 18-21 mg/dl was measured in ablated moulted crabs four weeks after the removal of the eyestalks.

Figure 1: Hemolymph glucose concentrations in female *P. persicum* crabs. Values are shown as means ± SE in intact crabs at week 0 and in ablated and control groups at weeks 1, 2 and 3.

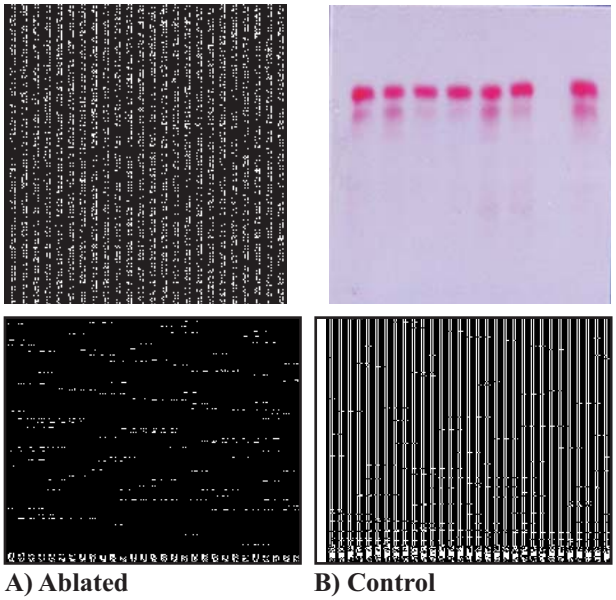


Hemolymph protein

The mean total protein level in hemolymph was 5.1 ± 0.5 g/dl at the start of experiment (week 0) in intact control crabs, and was 6.2 ± 0.5 g/dl and 5.2 ± 0.8 g/dl in ablated and control group respectively at week 3. Mean total protein was significantly increased in ablated crabs compared to the control groups at weeks 3 and 0 ($p < 0.05$).

The electrophoretic pattern of hemolymph total protein is shown in Figure 2. The electrophorograms of the ablated and control crabs showed four and three fractions, respectively, at the end of the third

Figure2: Hemolymph protein bands and electrophorograms of the female *P. persicum* crabs obtained by cellulose acetate paper electrophoresis.



A) Ablated **B) Control**

week. In the ablated crabs, the mean amount of fraction 1 was significantly decreased and the mean amounts of fractions 2 and 3 were significantly increased when compared to the control groups at week 3 ($p < 0.05$; Figure 3).

Figure 3: Fractions of hemolymph total proteins in ablated and control *P. persicum* crabs (values are shown as means ± SE)

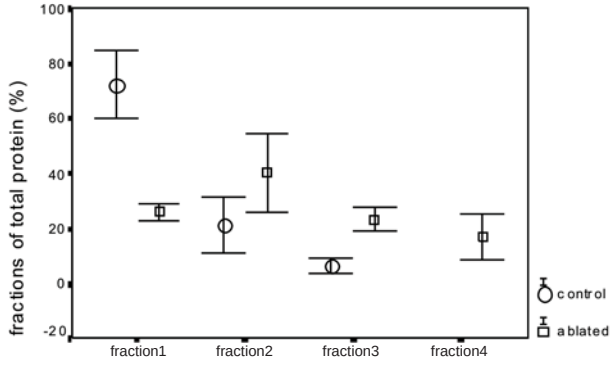
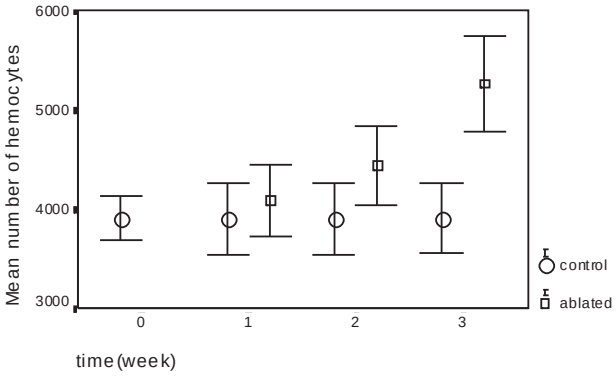


Figure 4: Total hemocyte count (THC) per mm^3 of hemolymph of the *P. persicum* crabs. Values are shown as means ± SE in intact crabs at week 0 and in ablated and control groups at weeks 1, 2 and 3.



Total hemocytes counts (THCs)

The mean THC of intact crabs at week 0 was 3,921 ± 103. No significant difference was observed between the THCs of ablated (4,096 ± 147) and control (3,916 ± 153) crabs at week 1, although this was not significant ($p > 0.05$). The THC of ablated crabs was 4,449 ± 165 at week 2 and 5,273 ± 190 at week 3, which was significantly increased when compared to the control group at week 2 (3,913 ± 152) and week 3 (3,917 ± 151; $p < 0.05$; Figure 4).

Effects on the ovaries and hepatopancreas

Ablation of the eyestalks accelerated ovarian development in adult female crabs. The gonadal indices of the initial control crabs and ablated crabs are shown in Figure 5. The gonadal indices were 0.65 ± 0.12 in the initial controls, 0.69 ± 0.16 in the controls and 1.47 ± 0.35 in destalked crabs. There was a significant increase in destalked crabs compared to the initial and control groups ($p < 0.05$).

Figure 5: Ovarian index of the *P. persicum* crabs. Values are shown as means ± SE in the ablated, control and initial control groups.

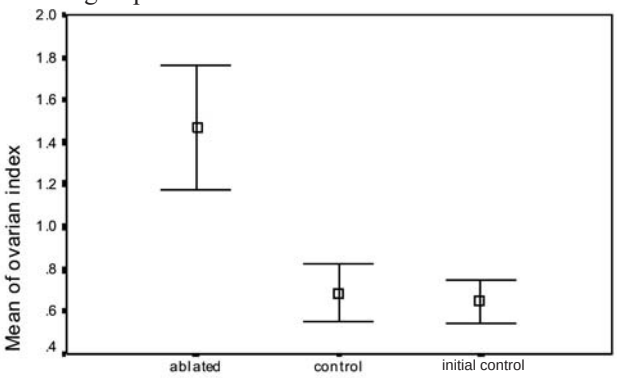
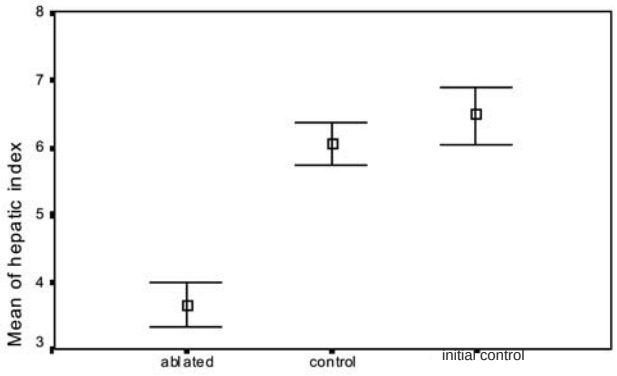


Figure 6: Hepatic index of the *P. persicum* crabs. Values are shown means ± SE in the ablated, control and initial control groups.



The hepatopancreas in ablated crabs lost the normal coloration and looked rather like flimsy bags. The hepatic indices in the initial control, control and ablated crabs were 6.5 ± 0.5, 6.05 ± 0.38 and 3.7 ± 0.4, respectively. There was a significant decrease in the hepatic index of ablated crabs compared to the initial controls and control group ($p < 0.05$; Figure 6).

Figure 7: Body weight of the *P. persicum* crabs. Values are shown as means ± SE in intact crabs at week 0 and in ablated and control groups at weeks 2 and 4.

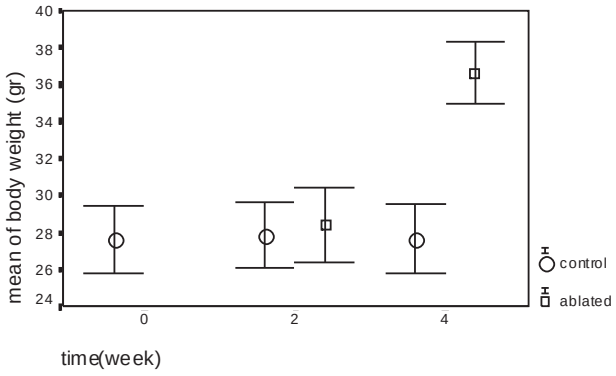
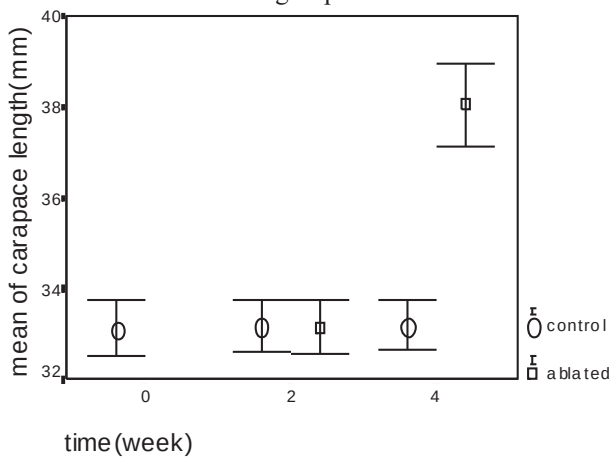


Figure 8: Carapace length of the *P. persicum* crabs. Values are shown as means ± SE in intact crabs at week 0 and in ablated and control groups at weeks 2 and 4.



Effects on moulting and growth

Destalked crabs moulted four weeks after eyestalk removal. Their body weight and carapace length were 36.6 ± 2 g and 38.4 ± 1.1 mm prior to moulting, respectively, which were significantly increased compared to the control group that had a mean body weight of 27.6 ± 2.1 g and a carapace length of 33.13 ± 0.7 mm at week 4, respectively ($p < 0.05$; Figures 7 and 8).

Discussion

The mean hemolymph glucose titers of the destalked female crabs were significantly decreased at the end of the first week after removal of the eyestalks. This was probably due directly to the removal of the eyestalk, which is a major source of crustacean hyperglycemic hormone (CHH). CHH is responsible for glucose homeostasis. It acts in carbohydrate metabolism by stimulating the glycogenolysis in muscles and the hepatopancreas, and it inhibits the synthesis of glycogen (Sedlmeier, 1982; Keller and Sedlmeier, 1988; Keller and Orth, 1990).

Marked elevation of hemolymph glucose levels was observed at both two and three weeks after the removal of the eyestalks. Two hypotheses are suggested to explain this: firstly, there are other sites distinct from the eyestalks that are sources of CHH; and secondly, factors other than CHH are involved in hyperglycemic effects. Chang *et al*. (1998) detected significant levels of CHH in *Homarus americanus* lobsters with ablated eyestalks. They suggested that other structures in the central nervous system potentially have the capacity to synthesize and secrete CHH. This hypothesis is supported by reports of CHH gene expression in the ventral nerve cord of *H. americanus* (De Kleijin *et al*., 1995; Reddy *et al*., 1997) and CHH immunoreactivity in the brain, thoracic ganglion and pericardial organs of *Carcinus maenas* (Keller *et al*., 1985; Dircksen and Heyn, 1998). These observations were also supported by evidence from enzyme-linked immunosorbent assays (ELISA) of distinct areas of the CNS from both intact and destalked animals (Chang *et al*., 1998).

The role of biogenic amines and peptidergic neuroregulators on the hyperglycemic responses of crustaceans has been discussed by some authors. Dopamine (DA) and serotonine (5-HT) induced hyperglycemic responses through the release of CHH in the eyestalks of *Penaeus monodon* (Kuo *et al*., 1995). In *Carcinus maenas,* removal of the eyestalks alone led to a considerable hyperglycemia within a few hours, which persisted at the same level for two days (Lueeschen *et al*., 1993). The possibility that biogenic amines modulate the hyperglycemic response independently of CHH can therefore be postulated. Hyperglycemic responses that are induced by injections of norepinephrine (NE) and octopamine (OA) were nearly identical between the

intact and destalked *Macrobrachium rosenbergii* prawns. It has proposed as a consequence of these findings that the hyperglycemic effects of NE and OA are directly on the target tissues and are not mediated through CHH (Kuo and Yang., 1999).

The electrophoretic pattern of the total protein of hemolymph in intact *P. persicum* crabs showed three fractions. The main proteins of hemolymph in decapod crustaceans were identified by Durliat (1983) as hemocyanin, coagologen, heteroagglutinins, vitellogenins and moult-related proteins. In sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses of the *Squilla mantis* shrimp hemolymph, three major bands were recognized as female specific proteins or vitellogenins with a molecular weight of 125 KD and one major band was identified as hemocyanin with a molecular weight of approximately 70 KD (Ferrero *et al*., 2005). In the *Orchestria gammarellus* amphipod, hemocyanin forms 60% of the total protein content of hemolymph (Chan and Weeks, 1992). A copper-bonded molecule with peroxidase activity, two subunits and molecular weight of 88 KD was reported in the hemolymph of *Gammarus pulex* by Bentley and Hurd (1993) and in the hemolymph of the *Cancer magister* crab by Terwilliger and Dumler (2001) and Terwilliger and Ryan (2001), respectively. This molecule was recognized as hemocyanin, the respiratory pigment, and was the most concentrated fraction in the electrophorogram of *G. pulex* (Bentley and Hurd, 1993).

In the electrophorogram of the hemolymph in ablated female *P. persicum* crabs, four fractions were observed. According to Ferrero *et al*., 2005, the amount of vitellogenins increased with increasing ovarian maturity. The appearance of a new fraction in the electrophorogram of ablated female *P. persicum* crabs may be due to the increase of vitellogenin and ovarian maturity as a consequence of GIH removal following eyestalk ablation. This conclusion is in accordance with a significant increase of the amount of total protein content in hemolymph in the ablated *P. persicum* crabs in comparison with the control group.

Bilateral ablation of the eyestalks induced a significant increase in the value of THC in ablated *P. persicum* crabs at the end of the second and third weeks of ablation. In crustaceans, hemopoietic activity is under hormonal control. The stimulatory effects of the x-organ sinus gland complex, which is located in the eyestalks, and the inhibitory effects of the y-organ on crustacean haematopoiesis have been discussed (Ghiretti-Magaldi *et al*., 1977; Johansson *et al*., 2000). In each nodule of hemopoietic organ, stem-cells or hemoblasts undergo regular mitosis to produce various kinds of hemocytes (Bauchau, 1981; Johansson *et al*., 2000; Jiravanichpaisal *et al*., 2006). A hormonal control of hemopoietic activity has been advocated correctly in *P. persicum* crabs, since removal of the sinus glands in the eyestalks induced a marked increase in mitosis. This is probably under the influence of the y-organ, once it has been freed from the inhibition by the sinus gland.

The eyestalk factors regulate the storage and mobilization of organic reserves that are utilized for moulting and reproduction (Liu and Laufer, 1998). Eyestalk ablation in female *P. persicum* crabs resulted in a low hepatic index and a high gonad index, which may be indicative of the utilization of these reserves in tissue synthesis. Depletion of the hepatopancreatic reserves due to eyestalk ablation was reported in *H. americanus* by Aiken (1980).

We also observed precocious moulting in destalked *P. persicum* crabs, which was probably related to the removal of MIH and consequent activation of the y-organ, which produces the moulting hormone (MH) as discussed by (Laufer *et al*., 1993). On the other hand, accelerated gonad development in female *P. persicum* due to bilateral eyestalk ablation may indicate the presence of GIH in the eyestalk and Gonad Stimulating Hormone (GSH), which is another hormone that takes part in the reproductive process. GSH is secreted from the brain and thoracic ganglion (Laufer *et al*., 1993).

As in many other decapods, reproduction and somatic growth are antagonistic in normal *P. persicum.* Since ablation accelerated the moult cycle

and rate of gonadal development simultaneously, this antagonistic relationship seems to be altered by bilateral eyestalk ablation, therefore, eyestalk ablation may be used as a tool to stimulate gonadal maturity and growth in crustacean aquaculture. However it needs further investigation.

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