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SHORT COMMUNICATION

Effect of moulting, eyestalk ablation, starvation and transportation on the immune response of the Indian spiny lobster, *Panulirus homarus*

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The Indian spiny lobster, *Panulirus homarus* (Linnaeus 1758), is of great commercial importance and has a high demand in the international market. However, due to the lack of larval rearing technology, traders depend heavily on the wild-caught undersized lobsters that are normally maintained in impoundments for fattening. Overstocking and poor management of these holding and fattening systems can result in stress, which can eventually lead to disease outbreak. Crustaceans respond to these stress factors with their innate immune system, consisting of both cellular and humoral factors.

The cellular immune response in crustaceans is modulated by phagocytosis, encapsulation, nodule formation and phenoloxidase (PO) activity, whereas the humoral response is mediated by agglutination and bactericidal activity (Söderhäll & Cerenius 1992). Haemocytes play a crucial role in the immune response by actively taking part in phagocytosis, encapsulation, nodule formation and cytotoxic mediation (Söderhäll & Cerenius 1992). Immune response parameters such as total haemocyte counts (THC) (Evans, Fan, Finn, Dawson, Siva & Lee 1992; Sequiera, Tavares & Aralachaves 1996; Jussila, Jago, Tsvetnenko, Dunstan & Evans 1997; Jussila, Paganini, Mansfield & Evans 1999), bactericidal activity (Söderhäll & Cerenius 1992), haemolymph clotting (Jussila, Mc Bride, Jago & Evans 2001), PO activity (Le Moullac, Soyez, Saulnier, Ansquer, Avarre & Levy 1998; Cheng & Chen 2000) and superoxide anion production (Sung, Chang, Her, Chang & Song 1998) have all been used as stress indicators in crustaceans.

Despite its commercial importance, the effects of stress factors on *P. homarus* are poorly understood. Herein, we report on the immune response of the Indian spiny lobster to the stress factors that result from moulting, eyestalk ablation, starvation and transportation by measuring different immune parameters (e.g. PO activity and THC). This study will have important implications in the management of fattening and farming operations of spiny lobsters.

Lobsters weighing 100–120 g were collected from Vizhinjam (8.41 °N, 77.0 °E) on the southwest coast of India and were transported to the Research Centre of Central Marine Fisheries Research Institute (CMFRI) at Calicut, 300 km north of Vizhinjam, in thermocol boxes containing cool saw dust. The lobsters were maintained in individual fibreglass-reinforced plastic tanks containing 100 L filtered seawater and were acclimatized for 2 weeks (temperature: 25 ± 0.3 °C, salinity: 35‰, pH: 7.8 ± 0.2 and dissolved oxygen: 5 ± 1.4 mL L^{-1}). The lobsters were fed with green mussel (*Perna viridis*) tissue, equal to 10% of their bodyweight, once daily. Except for the moulting studies, all experimental lobsters were in the inter-moult stage.

For moulting studies, the moult stages were determined using the standard procedure described by Berry (1971) and Radhakrishman (1989). The lobsters were observed at three different stages: the pre-moult (stage D), the inter-moult (stage C) and the post-moult (stages A and B). THC and PO activities were measured during each moulting stage (pre-, inter- and post-moult). The moulting studies ranged from 45 to 50 days. Lobsters

in the inter-moult stage were subjected to bilateral eyestalk ablation using an electro-cauterizer to minimize bleeding as well as to reduce the rate of infection. Intermoult lobsters were starved for 4 weeks to study the effect of starvation on the immune system.

Transportation of live lobsters for long distances using moist sawdust is one of the common methods practiced in India (Vijayakumaran & Radhakrishnan 1997). One of the objectives of the present study is to assess the stress caused by transportation. A total of 15 lobsters were collected from the fattening pond and transported to the research centre to investigate the effect of transportation on stress factors. The haemolymph was collected twice: once before transportation (control) and again after the lobsters arrived at the research centre. To avoid stress caused by excessive handling, haemolymph was drawn only once from each individual lobster. To lower their metabolic rate, lobsters were kept at a water temperature of 16 °C for 1 h before dry packing. Cold seawater was circulated to maintain the water temperature at 16 °C. The lobsters were also given a slow dip in this water and draped with several layers of damp paper towels. Wood shavings were laid over the thermocol box floor and bottles of seawater ice were used to maintain a temperature of 16 °C inside the box. The lobsters were laid in rows to minimize congestion and to increase their survival rate. Boxes were then sealed tightly to maintain the temperature and transported to the hatchery within 15 h of containment. As soon as the boxes reached the hatchery, the haemolyph samples were drawn and the lobsters were released into fresh seawater.

Haemolymph (0.1 mL) was drawn from the ventral sinus of each lobster using a 2 mL syringe containing 0.9 mL of anticoagulant (0.114 M sodium citrate, 0.10 M sodium chloride, pH 7.5). A drop of the haemolymph in the anticoagulant was placed in a Neubauer haemocytometer (Hausser Scientific Company, Horsham, PA, USA), and the THC was counted using a phase-contrast microscope (Perazzolo & Barracco 1997; Verghese, Radhakrishnan & Padhi 2007).

In the spiny lobster (genus: *Panulirus*), unlike other crustaceans, most of the PO activity has been detected in cell-free plasma (Hernández-López, Gollas-Galván, Gómez-Jiménez, Portillo-Clark & Vargas-Albores 2003). In addition, Perazzolo and Barracco (1997) reported a significant level of PO activity in the serum of *Penaeus paulensis*, suggesting that serum be used for PO activity assays. Considering this evidence, we used serum for PO assay measurements in the present study. PO activity was measured spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine as described by Verghese *et al.* (2007).

Each assay (THC and PO) was repeated at least five times with at least 10 individuals for each data point, and each lobster was bled only once. One-way ANOVA, followed by *post hoc* multiple comparisons was carried out using the statistical program SPSS (ver. 14.0), with significance levels for all analyses set at $P < 0.05$.

Our results demonstrated that the mean THC was significantly higher during the inter-moult period. No significant differences were observed in the mean THC between pre-moult (stages D_0, D_1, D_2, D_4) and post-moult (stages A and B) stages (Fig. 1). An inverse relationship was observed between PO activity and THC (Fig. 1).

Although the THC appeared to increase with respect to duration, there were no significant differences among the estimates (Fig. 2). In contrast, the PO activity showed significant variation, with the highest and lowest PO activities occurring after 2 h and 1 week following ablation respectively (Fig. 2).

Our results showed significant decreases in both PO activity and THC as the duration of starvation increased (Fig. 3). Like starvation, transportation also had a significant effect on the THC and PO activities of the lobsters. Our results clearly showed a significant decrease in THC and PO activities after the lobsters were transported for long distances (Fig. 4).

The present study investigated the effect of moulting, eyestalk ablation, starvation and transportation on the immune response of the Indian spiny lobster, *P. homarus*. Our study revealed that the significant variation ($P < 0.05$) in the PO and THC is directly associated with the moult cycle, eyestalk ablation, starvation and transportation, thus indicating that these

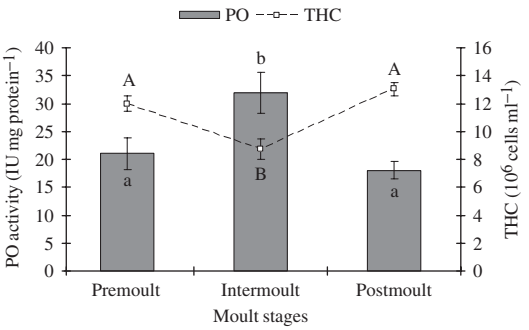

Figure 1 Mean (± SE) phenoloxidase (PO) activity and total haemocyte count (THC) of *Panulirus homarus* during different moult stages. Data with different letters are significantly different ($P < 0.05$).

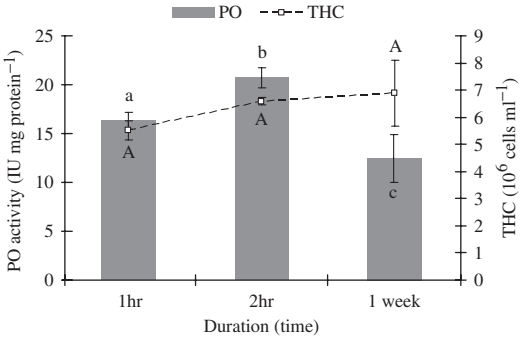

Figure 2 Effect of eyestalk ablation on the phenoloxidase (PO) activity and the total haemocyte count (THC) of *Panulirus homarus*. Data with different letters are significantly different ($P < 0.05$).

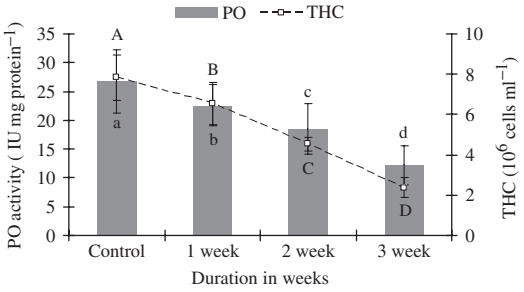

Figure 3 Effect of starvation on the phenoloxidase (PO) activity and the total haemocyte count (THC) of *Panulirus homarus*. Data with different letters are significantly different ($P < 0.05$).

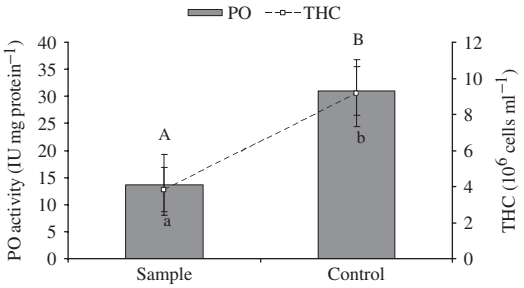

Figure 4 Effect of transportation on the phenoloxidase (PO) activity and the total haemocyte count (THC) of *Panulirus homarus*. Data with different letters are significantly different ($P < 0.05$).

two immune parameters could be used as stress indicators.

Our results showed a significant variation in THC during different moulting stages of *P. homarus*, thus suggesting that *P. homarus* has a differential stress response during moult cycles. The significant reduction in THC observed during the inter-moult stage of *P. homarus* (Fig. 1) is consistent with previous studies of several other crustacean species (e.g. *Sicyonia ingentis*: Tsing, Arcier & Bréhélin 1989; *Penaeus japonicus*: Chen & Cheng 1993; and *Penaeus stylirostris*: Le Moullac, Le Groumellec, Ansquer, Froissard, Levy & Aquacop 1997). Neuroendocrine hormones might play a crucial role in the variation in THC, as they regulate moulting, colour variation and glycaemia (Truscott & White 1990). The enhanced THC during the pre- and post-moult stages could either be due to the compensation of immunodeficiency caused during moulting (Chen & Cheng 1993) or due to the detachment of the old cuticle, which would put the lobster at a higher risk of bacterial attack. Therefore, an increased supply of haemocytes to the epidermis of the new cuticle at the pre-moult stage could confer sufficient protection against pathogen invasion (Le Moullac *et al.* 1997).

The present study has also revealed significant differences in PO activity during the different moult cycles. The PO activity is shown to be significantly higher during inter-moult, which is consistent with the results observed in other crustacean species (*P. stylirostris*: Le Moullac *et al.* 1997; *Litopenaeus vannamei*: Liu, Yeh, Cheng & Chen 2004). This increase in PO activity could be due to the active participation of granulocytes releasing enzymes needed for tanning of the cuticle during the pre- and post-moult stages (LeMoullac *et al.* 1997). The decrease in PO activity during the pre- and post-moult stages may be due to the increased amount of inhibitors of the PO system (Johansson & Söderhäll 1989). Nevertheless, these observed, significant fluctuations in PO activity during the different moult stages clearly indicate that this physiological process keeps the lobster under stress.

Although bilateral eyestalk ablation has been shown to have a stimulating effect on the moulting frequency and weight gain in *P. homarus* (Radhakrishnan & Vijayakumaran 1984; Juinio-Menez & Ruinata 1996), the present findings revealed that bilateral eyestalk ablation may also have adverse effects on the growth and survival of *P. homarus* resulting from a significant stress response. The THC activity in the bilaterally ablated lobsters showed a sudden increase after 2 h of ablation. This result is consistent with the earlier studies on injury in *Panulirus cygnus* (Jussila *et al.* 1997). When injury occurs, haemocytes are mobilized towards the site of injury, as haemocyte activation is the primary response against invasion and wound healing (Le Moullac

et al. 1998). The increase in THC during ablation may be due to the release of haemocytes into circulation from storage sites such as haematopoietic tissue, which is the store house for mature haemocytes (Jussila *et al*. 1997). The increase in PO activity during ablation could be a defensive reaction to repair the damage caused by injury. In arthropods, melanin synthesis is involved in the process of sclerotization, wound healing of the cuticle and in defence reactions against invading microorganisms (Söderhäll 1982; Ratcliffe, Rowley, Fitzgerald & Rodes 1985; Sugumaran 1996).

Our results showed a significant reduction in THC within 3 weeks of starvation of *P. homarus*, indicating a direct relationship between haemocyte numbers and the nutritional status of the lobster. The association between starvation and the significant reduction in THC may affect the storage of carbohydrate in the haemocytes (Johnston, Elder & Davies 1973) and haemocyanin (Mosco, Riggo & Savoini 1989). Consistent with the present findings, previous studies have also reported a reduction of 41.11% in THC during starvation in *P. homarus* (Manjula, Rahman & Abraham 1997). This significant decrease in THC during starvation could be due to the decrease in circulating haemocytes (e.g. Stewart, Cornick & Dingle 1967; Cheng & Chen 2001). The serum PO activity of lobsters in this study was also found to decrease, indicating that poor nutrition may reduce the immune status, which in turn can make the lobster more vulnerable to infection.

In this study, the significant reduction in THC and PO activities after a 15-h transportation period implied that transportation causes a significant stress response in lobsters. Similar observations have been reported with other crustacean studies (e.g. Jussila *et al*. 1997; Jussila *et al*. 1999; Gomez-Jimenez, Uglow & Gollas-Galvan 2000; Fotedar, Evans & Jones 2006). Although the mode of transportation is one of the contributing factors of stress, the intensity of the stress response in the transported lobsters might be better associated with the maintenance of the lobsters (e.g. high stock density) before transportation, handling of the lobsters, minimal air exposure and drastic reduction in temperature during the transportation (Vijayakumaran & Radhakrishnan 1997).

In summary, the significant variation in THC and PO activities due to moulting, eyestalk ablation, starvation and transportation indicates that these factors are important when considering how to minimize the stress of captured lobsters. Accounting for these factors may significantly affect the survival and growth of the lobsters and the lobster farming industry as well. Thus, routine monitoring of health status is of great importance with regard to successful aquaculture practices.

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References

Berry P.F. (1971) The biology of the spiny lobster *Panulirus homarus* (Linnaeus) off the east coast of Southern Africa. *Investigational Report Oceanographic Research Institute, South Africa* **28**, 1–75.

Chen J.C. & Cheng S.Y. (1993) Studies on haemocyanin and haemolymph proteins levels of *Penaeus japonicus* based on sex, size and moulting cycle. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **106**, 293–296.

Cheng W. & Chen J.C. (2000) Effects of pH, temperature and salinity on immune parameters of the freshwater prawn, *Macrobrachium rosenbergii*. *Fish and Shellfish Immunology* **10**, 387–391.

Cheng W. & Chen J.C. (2001) Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*. *Fish and Shellfish Immunology* **11**, 53–63.

Evans L.H., Fan A., Finn S.P., Dawson S.A., Siva C.J. & Lee I.R. (1992) Nutritional status assessment studies in the freshwater crayfish, *Cherax tenuimanus*. In: *Proceedings of the Aquaculture Nutrition Workshop, Salamander Bay* (ed. by G.L. Allan & W. Dall), pp. 87–91. NSW Fisheries, Brackish Water Fish Research Station, Salamander Bay, Australia.

Fotedar S., Evans L. & Jones B. (2006) Effect of holding duration on the immunue system of western rock lobster, *Panulirus cygnus*. *Comparative Biochemistry and Physiology Part A: Molecular Integrative Physiology* **143**, 479–487.

Gomez-Jimenez S., Uglow R.F. & Gollas-Galvan T. (2000) The effects of cooling and emersion on total haemocyte count and phenoloxidase activity of the spiny lobster *Panulirus interruptus*. *Fish and Shellfish Immunology* **10**, 631–635.

Hernández-López J., Gollas-Galván T., Gómez-Jiménez S., Portillo-Clark G. & Vargas-Albores F. (2003) In the spiny lobster *Panulirus interruptus* the prophenoloxidase is located in plasma not in haemocytes. *Fish and Shellfish Immunology* **14**, 105–114.

Johansson M.W. & Söderhäll K. (1989) Cellular immunity in crustacean and the proPO system. *Parasitology Today* **5**, 171–176.

Johnston M.A., Elder H.Y. & Davies P.S. (1973) Cytology of *Carcinus* haemocytes and their function in carbohydrate metabolism. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **46**, 569–581.

Juinio-Menez M.A. & Ruinata J. (1996) Survival, growth and food conversion efficiency of *Panulirus ornatus* following eyestalk ablation. *Aquaculture* **146**, 225–235.

Jussila J., Jago J., Tsvetnenko E., Dunstan B. & Evans H. (1997) Total and differential haemocytes count in western rock lobsters (*Panulirus cygnus* George) under post harvest stress. *Marine and Freshwater Research* **48**, 863–867.

Jussila J., Paganini M., Mansfield S. & Evans L.H. (1999) On physiological responses, haemolymph glucose, total hemocyte count and dehydration, of marron (*Cherax tenuimanus*) to handling and transportation under simulated conditions. *Freshwater Crayfish* **12**, 154–167.

Jussila J., Mc Bride S., Jago J. & Evans L.H. (2001) Haemolymph clotting time as an indicator of stress in western lobsters (*Panulirus cygnus* George). *Aquaculture* **199**, 185–193.

Le Moullac G., Le Groumellec M., Ansquer D., Froissard S., Levy P. & Aquacop (1997) Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against vibriosis. *Fish and Shellfish Immunology* **7**, 227–234.

Le Moullac G., Soyez C., Saulnier D., Ansquer D., Avarre J.C. & Levy P. (1998) Effect of hypoxia stress on the immune response and the resistance to vibriosis of the shrimp *Penaeus stylirostris*. *Fish and Shellfish Immunology* **8**, 621–629.

Liu C.H., Yeh S.T., Cheng S.Y. & Chen J.C. (2004) The immune response of the white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio* infection in relation with the moult cycle. *Fish and Shellfish Immunology* **16**, 151–161.

Manjula P.L., Rahman K.M.D. & Abraham T.J. (1997) Haemocytes classification and differential counts in the Indian spiny lobster *Panulirus homarus* (Linnaeus). *Journal of Aquaculture in Tropics* **12**, 113–121.

Mosco A., Riggo D. & Savoini A. (1989) Molecular characteristics, site of synthesis and tissue distribution of *Squilla mantis* haemocyanin. In: *Biology of Stomatopods: Selected Symposia and Monographs, U.Z.I., 3* (ed. by E.A. Ferrero), pp. 147–152. Mucchi, Modena, Italy.

Perazzolo L. & Barracco M. (1997) The phenoloxidase activating system of shrimp *Penaeus paulensis* and associated factors. *Developmental and Comparative Immunology* **21**, 385–395.

Radhakrishnan E.V. (1989) *Physiological and Biochemical Studies on the Spiny Lobster, Panulirus Homarus*. University of Madras, India.

Radhakrishnan E.V. & Vijayakumaran M. (1984) Effect of eyestalk ablation in spiny lobster *Panulirus homarus* (Linnaeus) on moulting and growth. *Indian Journal of Fisheries* **31**, 130–147.

Ratcliffe N.A., Rowley A.F., Fitzgerald S.W. & Rodes C.P. (1985) Invertebrate immunity: basic concepts and recent advances. *International Review of Cytology* **97**, 183–350.

Sequiera T., Tavares D. & Aralachaves M. (1996) Evidence for circulation hemocyte proliferation in the shrimp *Penaeus japonicus*. *Developmental and Comparative Immunology* **20**, 97–104.

Söderhäll K. (1982) Prophenoloxidase activating system and mealnisation – a recognition mechanism of arthropods? A review. *Developmental and Comparative Immunology* **6**, 601–611.

Söderhäll K. & Cerenius L. (1992) Crustacean immunity. *Annual Review of Fish Diseases* **2**, 3–23.

Stewart J.E., Cornick J.W. & Dingle J.R. (1967) An electronic method for counting lobster, *Homarus americanus* Milne Edwards. Hemocytes and the influence of diet on hemocyte numbers and hemolymph proteins. *Canadian Journal of Zoology* **45**, 291–304.

Sugumaran M. (1996) Roles of insect cuticle in host defense reactions. In: *New Directions in Invertebrate Immunity* (ed. by K. Söderhäll, S. Iwanaga & G.R. Vasta), pp. 355–374. SOS Publications, Fair Haven, NJ, USA.

Sung H.H., Chang H.J., Her C.H., Chang J.C. & Song Y.L. (1998) Phenoloxidase activity of haemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. *Journal of Invertebrate Pathology* **71**, 26–33.

Truscott R. & White K.N. (1990) The influence of metal and temperature stress on the immune system of crabs. *Functional Ecology* **4**, 455–461.

Tsing A., Arcier J.M. & Bréhélin M. (1989) Haemocytes of penaeids and palaemonid shrimps: morphology, cytochemistry and hemograms. *Journal of Invertebrate Pathology* **53**, 64–77.

Verghese B., Radhakrishnan E.V. & Padhi A. (2007) Effect of environmental parameters on immunue response of the Indian spiny lobster, *Panilurus homarus* (Linnaeus, 1758). *Fish and Shellfish Immunology* **23**, 928–936.

Vijayakumaran M. & Radhakrishnan E.V. (1997) Live transport and marketing of spiny lobster in India. *Marine and Freshwater Research* **48**, 823–828.

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