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Hemolymph Agglutination and Coagulation Inside the Body of *Penaeus monodon* (Crustacea, Decapod) and Effect of Sudden Changes of Salinity on Oxygen Consumption and Metabolic Rate

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Abstract: *Penaeus monodon* shrimps were collected by cast nets, put into large buckets and immediately brought to the laboratory, and kept in aquaria. The shrimps were fed with artificial and formulated feed and kept at a salinity of 25 ppt at room temperature (27-28°C). The shrimps were acclimatized for about 7 days. Experiments were conducted, when the shrimp were kept in 1 ppt to 45 ppt. The shrimp were transferred from the aquaria to the respiratory chamber, which was kept in the B.O.D. incubator at ambient temperature (28°C). The shrimp were kept in higher saline waters, especially at 40 ppt and 45 ppt, the antennal scales, the branchiostegite region of the carapace, and the uropods got swollen. A cross section of this swollen region has shown hemolymph agglutination and coagulation inside the body. This is certainly due to an osmotic imbalance, which has an impact on the sudden changes of salinities in the shrimps. Pearson's correlation coefficient compares the strength and direction of an association between body weight and consumed oxygen at various salinities to the R value strong +ve (0.98-0.99) and <0.00001 significant level at p <0.05. One Way ANOVA test, the difference between the averages oxygen consumption of some groups are big enough to be statistically significant.

Keywords: Dissolved oxygen, Salinity, Branchiostegite, Hemolymph, BOD, Pearson's correlation, ANOVA

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Introduction

P. monodon commonly called as Tiger shrimp, is a key coastal aquaculture species in South-East Asia, contributing considerably to the economies of several nations (Duan *et al.*, 2020). *P. monodon* inhabits brackish water and tolerates a wide range of salinities, from pure freshwater to pure sea water, and salinities higher than pure sea water. Salinity is a significant abiotic element that influences the distribution and general functioning of many aquatic animals, including penaeid shrimps. According to Pearse and Gunte (Pearse and Gunter, 1957). *P. monodon* juveniles were

adaptable to extremely low salinity, with a 64% survival rate at 0 ppt. Juveniles, on the other hand, exhibited the greatest tolerance, with a survival rate of 100% at 20 ppt. at 52 ppt, there was mortality. According to these observations, older stages are more tolerant of higher salinities (Motoh, 1981). *P. monodon*, as a euryhaline species, can withstand a broad range of salinities, from slightly brackish (1-2 ppt) to hypersaline (40 ppt). Numerous studies have been undertaken to investigate the effects of short-term salinity change on metabolism as represented in respiration rates, owing in part to the widespread use of penaeid shrimp in aquaculture (Rosas *et al.*, 1997).

Salinity is an important abiotic factor in marine and estuarine biota survival, growth, maturity, spawning, and migration. The combination of salinity and temperature is one of the most important physical characteristics, and the biological effects of these elements are complex and diverse. Variations in osmotic concentration, solute proportion, dissolved gas saturation, and other factors can all be influenced by salinity (Varghese, 1999). However, few researches investigated the long-term effects of salinity and body weight on metabolic rate in prawns acclimated to test conditions prior to experimental testing. To understand the metabolic rate of animals, oxygen consumption must be measured. There are a few practical applications, such as maintaining oxygen levels during growth or transportation (Roy *et al.*, 2007).

The main challenge for acclimation and survival in low-salinity environments is to maintain ionic regulation (osmoregulation) between body fluid (hemolymph in crustaceans) and the surrounding medium (McNamara *et al.*, 2015; Rahi *et al.*, 2020). A large-scale change in ambient salinity can cause significant osmotic stress in organisms, resulting in decreased development and increased disease susceptibility and mortality. Crustaceans are well recognized in this regard for their capacity to swiftly control these internal biological processes in response to salinity changes (Vogt, 2013). The present study was conducted to investigate the physiological O_2 consumption, metaboloic rate and, haemolymph coagulation of *P. monodon* at nine different experimental salinity levels (1‰, 5‰, 10‰, 15‰, 20‰, 25‰, 30‰, 35‰, and 40‰) during the study period.

Materials and Methods

Shrimps were collected by cast nets, put into large buckets of oxygenated sea water, and immediately brought to the laboratory, and kept in aquaria. The shrimps were fed with artificial and formulated feed and kept at a salinity of 25 ppt at room temperature (27-28°C). Before beginning the study's execution, the shrimps were acclimatized for about 7 days. The shrimps were transported from the aquaria to respiratory chambers based on the experimental salinities from 1 ppt to 45 ppt. The intermoult shrimps for oxygen consumption were chosen with consideration (Drach, 1939). Pure sea water was mixed with fresh water to make low-saline waters. Crystallised salt from the salt fields was dissolved in sea water to make high-saline water > 35 ppt, and salinity values were done by using a salinity refractometer following calibration with distilled water.

Initially, the respiratory chambers were kept in the B.O.D. incubator, and shrimp were placed into the chambers when the water temperature reached the ambient temperature (28°C). Air bubbles were carefully avoided accumulating between the lid and the water column of the respiration chamber. The shrimp were allowed in the respiratory chamber for one hour, and the oxygen consumed by the shrimp was measured. The method was a modification of Winkler's method for determining dissolved oxygen in seawater (Montgomery *et al.*, 1964; Caritt and Carpenter, 1966). The dissolved oxygen values, which are tabulated by individual body weight for the Pearson's correlation coefficient measures the strength and direction of the relationship between two variables are compared to the values of R and significant level (p).

Hemolymph samples were taken from eight experimental individuals of *P. monodon* for microscopic examination. A 500 µl (hypodermic 23-gauge needle) syringe pre-filled with 100 µl of anticoagulant (0.1% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.0) was used to collect 100 µl of hemolymph. The needle was inserted into the inter-segmental membrane between the cephalothorax region and the antennal scale to collect hemolymph (Sang and Fotedar, 2004).

Swollen portions were fixed with 10% formaldehyde, Susa, Bouin's, and formol calcium fixatives and processed for histological examinations (Pearse, 1968; Culling *et al*., 1985). The components were dehydrated in a graded series of alcohols before being cleaned in xylene and embedded in paraffin wax following infiltration at 58-60°C. Sections were cut for histological and histochemical examinations. Mallory's triple and Heidenhain's Azan connective tissue stains were used for histological differentiation of cells and tissues (Humason, 1965; Culling *et al*., 1985). The periodic acid/Schiff (PAS) was used for carbohydrates and associated components, while the Alcian blue procedure at pH 1.0 and pH 2.5 was used for mucopolysaccharide investigations, mercuric bromophenol blue was utilized to demonstrate basic proteins (Pearse, 1968; Mazia *et al*., 1953). Sudan black B method was utilised for histochemical demonstration of lipids in cells and tissues (Chifflle and Putt, 1951).

Results and Discussion

Experiments were conducted to study the influence of salinity on the oxygen consumption of shrimp in different age groups (Tables 1, 2). The present experiments were conducted in order to reveal the probable effect of sudden variations in salinities on the oxygen demand and metabolic rate of these shrimps in aquaculture practices. It has been observed that the change in salinity certainly has an influence on oxygen consumption, and the demand for oxygen changes with an increase or decrease in salinity, especially when the shrimp were exposed to salinities of 30 ppt and above (Table 2, Fig. 1). At room temperature (27°C–30°C), the shrimp are very active without much change in their oxygen consumption, even after being exposed to various salinities. The increase in metabolic rate with increasing salinity is not much and is very much dependent upon the ambient temperature and age of the shrimp (Tables 1, 2). The similar results were observed by Rama Rao and Babu (2022) who have reported the oxygen consumption and metabolic rates at varying salinities from 1 ppt to 45 ppt to effect on 15- 35°C temperatures in *P. monodon*. The results reveled that the shrimps were inactive and the metabolic rate was minimal at 15°C. With an increase in temperature the activity of the shrimp changed, resulting in more oxygen consumption. The shrimps were active at temperatures ranging from 25-30°C. Scott *et al*. (2009) studied ecophysiological responses in *Litopenaeus vannamei* as a function of ambient salinity and animal size. *L. vannamei*'s growth rate, routine metabolic rate, limiting oxygen concentration, and marginal metabolic scope were all determined after it was acclimated to and tested at salinities of 2, 10, and 28 ppt at 28 °C. RMR, measured as the oxygen consumption rate per unit body weight for fasting, habitually active shrimp, was not affected by salinity but declined as shrimp weight increased.

In the present study, the shrimp's oxygen consumption percentage was observed at 10 ppt intervals from 10 to 45 ppt. The results indicated t 8.05% increase from 15–25‰ and 4.75% decrease from 35–45‰ in 5.3 g body weight. In 7.23 g body weight, 13.7% increase from 15–25‰, and 3.50% decrease from 35–45‰. In 11.12 g body weight, 13.51% increase from 15–25‰, and 2.86% decrease from 35–45‰ (Fig. 2). It is very clearly indicated that with the increasing salinity up to 35 ppt, the shrimp are normal. As the salinity increased to 45 ppt, the percentage of consumed oxygen decreased.

In the sub-adults, the percentage increase in metabolic rate further decreased with increasing salinities. Experiments on the oxygen

Table 1: *Penaeus monodon* oxygen consumption and metabolic rate in various salinities at 28 °C

			1ppt Salinity		5ppt Salinity		10 ppt Salinity		15 ppt Salinity		20 ppt Salinity	
S. No.	Length cm	Weight g	O_2 ml/l	MR	O_2 ml/l	MR	O_2 ml/l	MR	O_2 ml/l	MR	O_2 ml/l	MR
1	7.8	4.65	0.72	0.16	0.73	0.16	0.82	0.18	0.83	0.18	0.83	0.18
2	8.5	5.3	0.78	0.15	0.78	0.15	0.88	0.17	0.89	0.17	0.88	0.17
3	9	5.85	0.83	0.14	0.82	0.14	0.91	0.16	0.92	0.16	0.94	0.16
4	9.8	6.1	0.89	0.14	0.87	0.14	0.98	0.16	0.98	0.16	0.99	0.16
5	10	7.23	0.91	0.3	0.91	0.13	1.11	0.15	1.11	0.15	1.11	0.15
6	10.2	7.72	0.95	0.12	0.98	0.12	1.18	0.15	1.18	0.15	1.19	0.15
7	10.6	9.84	1.2	0.12	1.22	0.12	1.33	0.14	1.37	0.14	1.39	0.14
8	11.4	11.12	1.27	0.11	1.27	0.11	1.45	0.13	1.48	0.13	1.46	0.13

Table 2: *Penaeus monodon* oxygen consumption and metabolic rate in various salinities at 28 °C

			25 ppt Salinity		30 ppt Salinity		35 ppt Salinity		40 ppt Salinity		45 ppt Salinity	
S. No	Length cm	Weight g	O_2 ml/l	MR	O_2 ml/l	MR	O_2 ml/l	MR	O_2 ml/l	MR	O_2 ml/l	MR
1	7.8	4.65	0.85	0.19	0.89	0.15	0.96	0.21	0.97	0.21	1.01	0.22
2	8.5	5.3	0.93	0.18	0.95	0.18	0.99	0.19	1.04	0.2	1.11	0.21
3	9	5.85	0.97	0.17	0.99	0.17	1.04	0.18	1.09	0.19	1.19	0.2
4	9.8	6.1	1.03	0.17	1.04	0.17	1.12	0.18	1.14	0.19	1.2	0.2
5	10	7.23	1.16	0.16	1.13	0.16	1.21	0.17	1.27	0.18	1.31	0.18
6	10.2	7.72	1.19	0.15	1.16	0.15	1.26	0.16	1.34	0.17	1.34	0.17
7	10.6	9.84	1.41	0.14	1.37	0.14	1.44	0.15	1.53	0.16	1.62	0.16
8	11.4	11.12	1.49	0.13	1.53	0.14	1.59	0.14	1.63	0.15	1.75	0.16

consumption of adult shrimp of 15 g size were not possible due to limitations in the size of the respiratory chambers. The similar results were observed by Rama Rao (2023) who reported the effect of salinity on the oxygen consumption and metabolic rate of *Penaeus monodon* of varying body mass. The shrimps were exposed to salinities from 0 to 45 ppt. The adults were not very comfortable with sudden increasing salinities at all the temperatures studied. The percentage increase in the metabolic rate of juvenile shrimp is about 37% at 25°C and 12% at 30°C with increasing salinity from 1 to 45 ppt. Cawthorne *et al*. (1983) reported that the changes extended up to three days and entailed the immediate transfer of juvenile *Penaeus monodon*. Within 4 h of being exposed to fresh water, all animals died. Young shrimps (40 days post-metamorphosis) survived

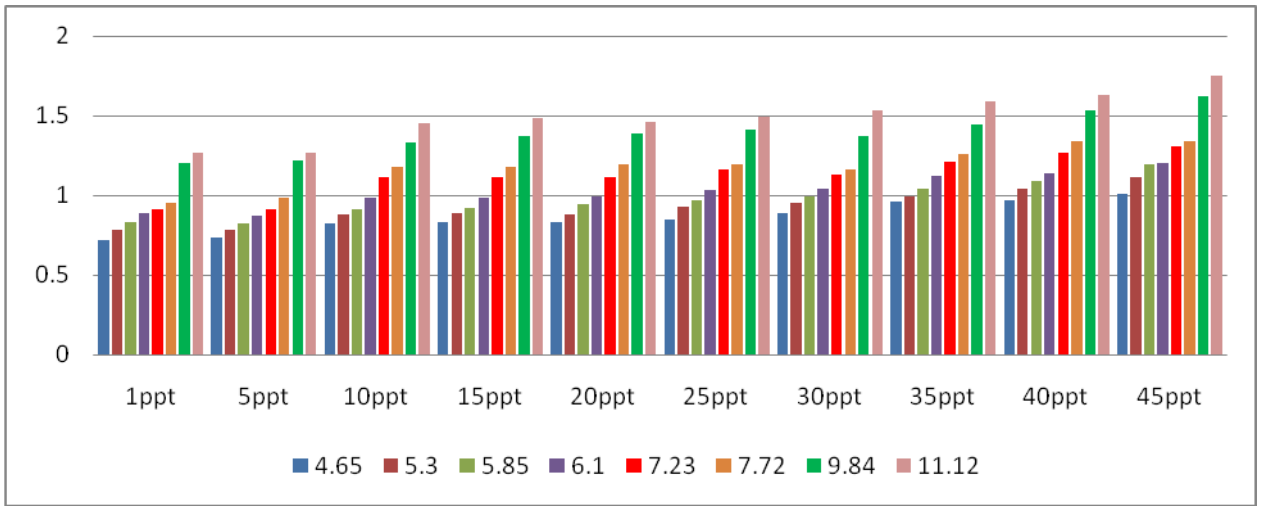

Fig. 1: Oxygen consumption at 28°C in *Penaeus monodon* at varied salinities.

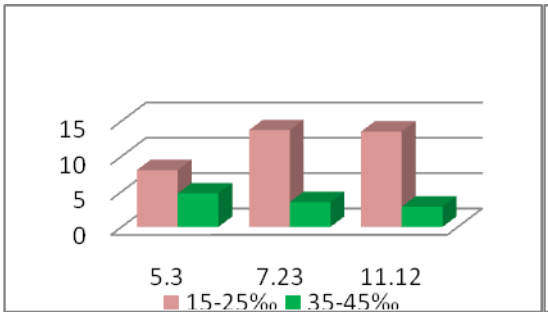

Fig. 2: oxygen consumption percentage at every 10 ppt intervals.

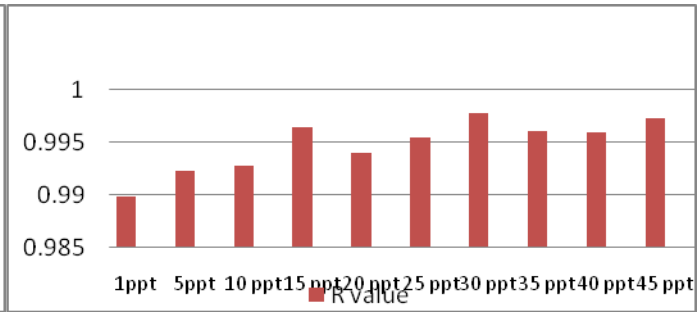

Fig 3. R value of Dissolved Oxygen at various salinities.

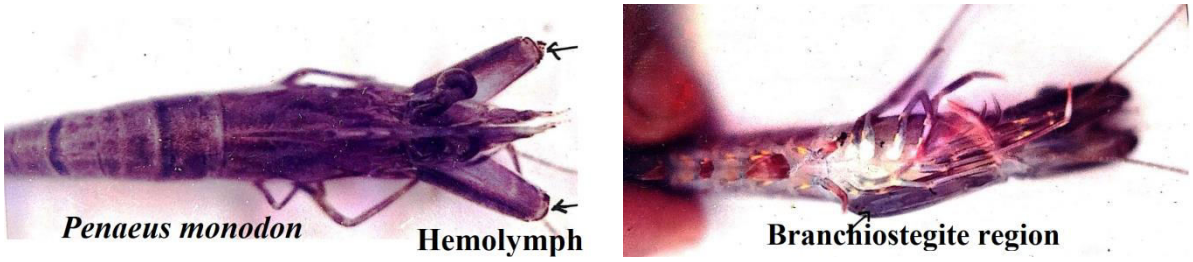

Fig. 4: *Penaeus monodon* swollen parts.

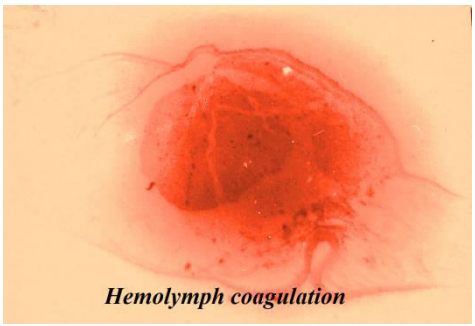

Fig. 5: Hemolymph clottable protein.

for 6 to 3 days longer than older animals and flourished in low-salinity water. The necessity for a complex medium containing more than one salt was proven by shrimps subjected to dilute artificial media. A laboratory-reared shrimp osmoregulation curve that was 150 days old was also produced.

When the shrimp were kept in higher saline waters, especially at 40 ppt and 45 ppt, the antennal scales, the branchiostegite region of the carapace, and the uropods got swollen (Fig. 4). A cross-section of this swollen region has shown hemolymph agglutination and coagulation inside the body (Fig. 5). This is certainly due to an osmotic imbalance, which has an impact on the sudden changes in salinities in the shrimp. In general, when the shrimp were kept in higher saline waters, the movement of the shrimp was erratic, resulting in an increase in oxygen consumption. Juvenile shrimps were more comfortable in different salinities without much change in their oxygen consumption. Maw-Sheng *et al*. (2007) reported the clottable protein (CP) which is involved in the hemolymph coagulation of *Penaeus monodon*. Furthermore, the concentrations and regulating of plasma CP under normal and intentionally stressful settings were investigated. Coagulation of hemolymph is an important defensive mechanism in crustaceans with an open circulatory system. It inhibits both fluid loss and the spread of opportunistic infections (Tait, 1911). The quantity of penaeid shrimp clottable protein was more in the summer than in the winter and fluctuated with the moulting cycle; its plasma levels increased 2-fold before moulting and dropped to the normal level after moulting (Chen *et al.*, 1993; Maw-Sheng *et al.*, 1998).

In the present study Pearson's correlation coefficient compares the strength and direction of an association between body weight and consumed oxygen at various salinities to the R value Strong +ve (0.98-0.99) and <0.00001 significant level at $p < 0.05$ (Fig. 3). One Way ANOVA test, the difference between the averages oxygen consumption of some groups are big enough to be statistically significant. P-value equals 0.032, it means the smaller the p-value the stronger it support of the experimental results. The test statistic F equals 2.202, which is not in the 95% region of acceptance. The $\eta 2$ equals 0.22. It means that the group explains 22.1% of the variance from the average (similar to R2 in the linear regression). The similar observations were recorded by Allan Geoff and Greg (1992)) who determined that the least permissible pH, defined as the pH that lowered growth by 5% over a period of 23 days, was 5.9 at a salinity of 30 ppt. Long-term (23 days) administration to low pH (4.9) at 30 ppt salinity substantially lowered prawn dry matter content ($P < 0.001$) and increased moulting frequency ($P < 0.05$) compared to a pH of 7.8.

Conclusion

Even after being exposed to varied salinities, the shrimps are quite active at room temperature (27°C–30°C) with no change in oxygen consumption. The increase in metabolic rate with increased salinity is minimal and highly dependent on the ambient temperature and shrimp age. The results indicated 8.05% increase from 15–25‰ and 4.75% decrease from 35–45‰ in 5.3 g body weight. In 7.23 g body weight, 13.7% increase from 15–25‰, and 3.50% decrease from 35–45‰. In 11.12 g body weight, 13.51% increase from 15–25‰, and 2.86% decrease from 35–45‰. The antennal scales, the branchiostegite area of the carapace, and the uropods swelled when the prawns in higher saline conditions, particularly at 40 and 45 ppt. Inside the body, a cross-section of this enlarged area revealed hemolymph agglutination and coagulation. The clottable protein was observed in *Penaeus monodon* culture ponds for sudden changes of salinity before moulting and dropped to the normal level after moulting

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