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Original Article

The effect of prolonged starvation on blood chemistry of horseshoe crab, *Carcinoscorpius rotundicauda* (Chelicerata: Xiphosura)

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Abstract

This study investigated the effects of prolonged starvation on oxygen consumption, ammonia-N excretion, and blood chemistry of the horseshoe crab, *Carcinoscorpius rotundicauda*. Starvation over a period of 7 weeks showed no significant difference of body weight between the starved and fed groups. The oxygen consumption rate decreased during weeks 1-4 and then significantly increased after week 6 of starvation. Starvation also resulted in a significant increase in ammonia-N rate from week 3 to 7. The O:N ratios were significantly reduced in the starved group from week 3 to 7. Starving induced the reduction of hemolymph osmolality from week 5. Hemolymph Na^+ and Cl^- of the starved group decreased from week 4 for Na^+ and from week 3 to 7 for Cl^-, while hemolymph K^+ increased from week 4 to 7. Hemolymph K^+ of both groups was hyperionic during the experiment. Thus, horseshoe crab can survive starvation for more than 7 weeks.

Keywords: horseshoe crab, starvation, metabolism, osmoregulation

1. Introduction

Horseshoe crabs are a primitive group of marine chelicerate arthropods. The oldest fossils date back to the Palaeozoic Devonian period between 350 and 450 million years ago (Damen & Saridaki, 2002). Two horseshoe crab species, namely *Tachypleus gigas* and *Carcinoscorpius routundicauda*, are commonly found in the Gulf of Thailand and in the Andaman Sea. These species have long been known to co-exist also in Pattani Bay, Thailand. Recently though, only one of these species, *Carcinoscorpius rotundicauda*, was found in Pattani Bay. This could be due to excessive fishing, water pollution, starvation or habitat destruction. Horseshoe crab blood is important for medical and pharmaceutical applications as a sensitive indicator to screen against bacterial contamination. The estimated number of horseshoe crabs in Thailand has decreased tremendously, and the mid-2016 retail price per individual was about 100 THB.

Physiological changes caused by starvation have been intensively studied in many vertebrate animals, including birds (Lamsova *et al.*, 2004; Lee *et al.*, 2002), reptiles (McCue, 2008), amphibians (Hervant *et al.*, 2001), and fishes (Boetius & Boetius, 1985; Frick *et al.*, 2008; Zen *et al.*, 2012; Zhu *et al.*, 2003). Some studies have also focused on invertebrate animals, such as crab (Pellegrino *et al.*, 2013), prawn (Mahavidlaya & Maharashtra, 2015), and mollusks (Zhoa *et al.*, 2011). Many arthropods have been similarly studied, for example insects (Bansode *et al.*, 2015; Jensen *et al.*, 2010). The ability to tolerate starvation varies by species. The abundance of marine species in a locality is highly dependent on environmental factors such as water temperature, salinity, and other chemical factors, as well as co-existing interacting species. One estimate of seawater temperature rise is 4 °C by the year 2113 (Whiteley *et al.*, 2011), and the pH is predicted to drop by 0.03.

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Horseshoe crabs are omnivorous, feeding on a wide variety of benthic invertebrates, including bivalves, polychaetes, crustaceans, and gastropods. Starvation is generally considered among the most important causes of mortality, resulting in compromised population fitness (Thorson, 1950). Starvation affects metabolic rate (Zhao *et al.*, 2011) and critical swimming speed (Hu *et al.*, 2010). Animals tend to accumulate an energy reserve when nutritional conditions are favorable to survive and proliferate even in periods of low food availability (Brockington and Clark, 2001). Hu *et al.* (2010) reported that the body weights of horseshoe crab *Tachypleus tridentatus* and *C. rotundicauda* remained unchanged during prolonged starvation for seven weeks. Plasma glucose decreased continuously while lipase activity increased in these animals during their starvation. These studies have shown that metabolism during starvation could be further clarified by investigating oxygen consumption, osmoregulation, ammonia excretion, and the O:N atomic ratios.

Oxygen consumption and ammonia excretion have long been used to evaluate metabolic rates. Ammonia is an end product from amino acid and nucleic acid metabolism and is highly toxic for most animals. It is naturally excreted by most aquatic animals and arises from the degradation of both natural and anthropogenic organic matter entering the aquatic system. When ammonia production exceeds the capacity of absorption, recycling, and purification in the aquatic system, its accumulation has observable effects on the biota. The O:N ratio is an index of protein utilization in energy metabolism. O:N ratios are useful to assess the relative contribution of protein to total catabolism (Bayne & Winddows, 1978). Osmoregulation is an important and high energy consuming regulatory function in aquatic invertebrates. Thus, we hypothesize that starvation may impair osmoregulation. The aim of this present study was to investigate physiological responses to starvation of the Asian horseshoe crab, *C. rotundicaudal*, by observing oxygen consumption, ammonia-nitrogen excretion rate, and osmoregulation.

2. Materials and Methods

2.1 Collection and maintenance of animals

The study was conducted at the Coastal Aquaculture Research Station of Skom, Tapha, Songkhla, Thailand. This station is located adjacent to the Gulf of Thailand on the coast covering a length of about 100 m along the coastline. The transport time from the site of collection to the laboratory was around 1 h. Horseshoe crabs, *C. rotundicauda*, were obtained from Ban Datoa villages in Pattani Province, Thailand (Longitude 101° 20′ 24″, Latitude 6° 54′ 00″) (Figure 1). A total of 40 adult horseshoe crabs of both male and female sex, ranging in mass from 150 to 260 g (234±8.2), were transferred to the laboratory and held in a continuous 1.5 l min^{-1} flow of seawater (salinity 33-34 ppt, dissolved oxygen 6-8 mg l^{-1}) that provided oxygen and removed excess nitrogenous wastes. The water temperature was 28±1°C, pH was around 8, and the photoperiod was 12:12 (light: dark) throughout the study. Seawater was pumped from the coast adjacent to the laboratory and passed through a filter tank filled with zeolites and activated carbon. Fecal matter was removed daily by water suction after feeding. During a two-week acclimation period before the actual experiment, the *C. rotundicauda* were

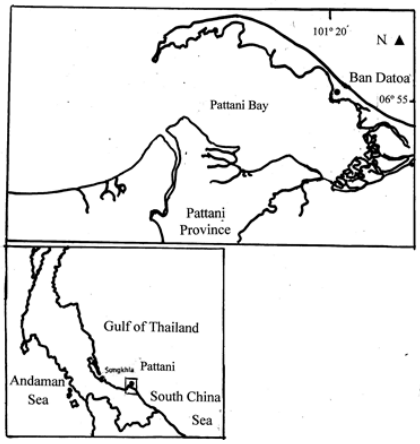

Figure 1. Map of sample collection site. A solid circle on each map represents the area of sample collection (Royal Thai Navy, 1995).

fed shellfish (*Mytilus* sp.) once daily between 16:00 and 17:00 h to satiety by visual subjective observation.

2.2 Experiment I: Effects on oxygen consumption and ammonia excretion

Starvation effects were examined over a 7–week experimental period. The subjects were divided into two experimental treatment groups, namely starved and fed animals. After one week of acclimation, the horseshoe crabs were randomly assigned to the treatment groups, weighed, and placed into randomly assigned individual tanks. The ten *C. rotundicauda* assigned to the fed treatment group had a mean±SE weight of 236.12±8.35 g and continued to receive the same food once daily between 16:00 and 17:00 h. The ten *C. rotundicauda* assigned to the starved treatment group had a mean±SE weight of 233.11±7.80 g and were individually placed in a tank. Each tank of both treatment groups measured 60 (length) x 45 (width) x 27 (height) cm. Each tank had a water volume of 60 liters and they were connected to a filter tank and aeration was applied 24 h day^{-1}. The oxygen consumption rates (MO_2) of each treatment group were determined weekly by the water bottle method. A single horseshoe crab was placed inside a sealed container (20 liters) for 1 h with continuous seawater circulation for at least 90 min to attenuate the handling stress. The water supply was suspended and the container was closed, so that the horseshoe crab could consume the oxygen in the known water volume for a period of 1 h. The difference between the dissolved oxygen (DO) in each container was measured at the start and after 1 h by the Winkler method described by Chinni *et al.* (2000), and a container without any horseshoe crab was used as a control. Briefly stated, these were the steps taken to determine the concentration of dissolved oxygen. A water sample was carefully filled into a 300 ml biological oxygen demand glass-stoppered bottle. Immediately 1 ml of 400 g L^{-1} of manganese sulfate was added to the collection bottle by inserting the calibrated pipette just below the surface of the water. Alkali-iodide-azide reagent (1 ml) was added in the same manner. The bottle was stoppered with care to assure no air was introduced. The sample was mixed several times by

inverting. If oxygen was present, a brownish-orange cloud of precipitate would appear. When this flocculence had settled to the bottom, the sample was mixed by turning it upside down several times and then letting it settle again. Concentrated sulfuric acid (1 ml) was added above the surface of the sample. The bottle was carefully stoppered and inverted several times to dissolve the flocculence. A sample of 201 ml was titrated with 0.025 mol l^{-1} sodium thiosulfate to a pale straw color. Starch solution (2-3 drops) was added to form a blue color. Titration continued until the sample turned clear. The concentration of dissolved oxygen in the sample was equivalent to the number of milliliters of titrant used. Three out of ten in a group were sampled for the weekly determination. Each treatment and control had three replicates. The oxygen consumption rate (MO_2) (Das *et al.*, 2005) was calculated as follows.

$$MO_2 = \frac{\left[(DO_0 - DO_t)V\right]}{Wt} mg.g^{-1}.h^{-1}$$

where DO_0 is the DO of the water at the start of the experiment (mg l^{-1}), DO_t is the DO of the water at the end of the experiment (mg l^{-1}), V is the volume of the container (l), W is the live wet weigh of the horseshoe crab (g), and t is the experiment time (h). The ammonia production for each treatment group was also measured weekly. An individual horseshoe crab was placed inside a sealed container (20 liters) for 1 h with seawater and the amount of ammonia produced (T_{ANE}) in 1 h was determined using the phenol-hypochlorite method described by Solorzano (1969).

$$T_{ANE} = \frac{\left[(N_t - N_0)V\right]}{Wt} \mu g^{-1}h^{-1}$$

where N_0 is the NH_4-N concentration of the water at the start of the experiment (µg l^{-1}), N_t is the NH_4-N concentration of the water at the end of the experiment (µg l^{-1}), V is the volume of the container (l), W is the live wet weight of horseshoe crab (g), and t is the experiment time (h). In addition, the oxygen: nitrogen (O:N) ratios were also calculated as the ratio of atoms of oxygen consumed to atoms of nitrogen excreted at the sampling times.

2.3 Experiment II: Effects on blood chemistry

Hemolymph (1 ml) was sampled from the cardiac sinus by means of a hypodermic needle and syringe and transferred to an iced centrifuge tube. Serum was separated by centrifugation at 10,000 g for 10 min at 4 °C, and Na^+ and K^+ were determined by flame photometry. Cl^- concentration was determined by the thiocyanate method with a commercial kit (Labtest, Brazil) and a microplate reader (MRX-HD, Dynex Technologies Inc., USA) operated at 490 nm.

2.4 Statistical analysis

All results were expressed as mean±S.E. All data were statistically analyzed by two-way analysis of variance (ANOVA) to determine the effects of starvation on the physiological responses. Mean values were compared by Student's t-test and P-values <0.05 were considered statistically significant.

3. Results and Discussion

3.1 Results

3.1.1 Body weight of horseshoe crabs

Starvation treatment had no significant effect on the body weight of the horseshoe crabs throughout the experiment. However, the body weight tended to decrease with starvation time, but this apparent trend was not statistically significant. The loss of body mass over 7 weeks of starvation was about 7% (Figure 2).

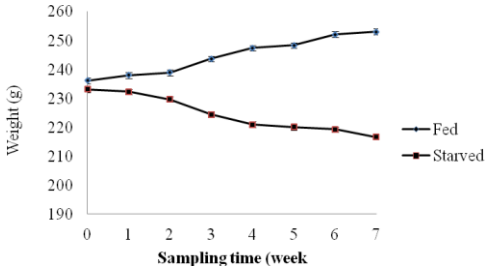

Figure 2. Effects of feeding and starvation (fed, ▲; starved, ■) on body weight in horseshoe crab, *C. rotundicauda*. Data are expressed as mean±SE (n=10). No significant differences were detected in either group throughout the sampling period (P>0.05).

3.1.2 Oxygen consumption rate

The oxygen consumption rate of *C. rotundicauda* was significantly affected by starvation after the first week which ranged from 23.48 to 26.21 µg O_2 g^{-1} h^{-1}. The oxygen consumption rate of the starved groups from week 1 to week 4 was significantly lower than that of the fed groups. However, this reversed from week 5 to week 7 which signified that the starved groups had significantly higher oxygen consumption than the fed group (Figure 3).

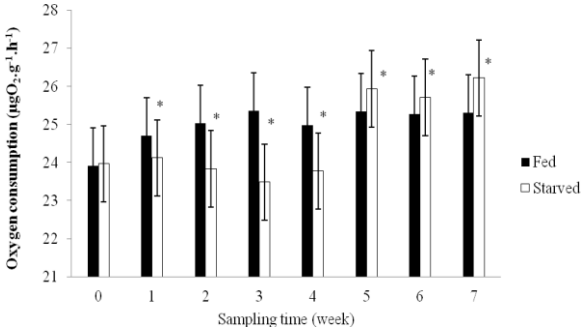

Figure 3. Effects of feeding and starvation (fed; ■ starved; □) on oxygen consumption rate in horseshoe crab, *C. rotundicauda*. Data are expressed as mean±SE (n=10). * indicates significant differences in both groups (P<0.05).

3.1.3 Ammonia-N excretion rate

Ammonia-N excretion rates of the starved horseshoe crabs increased significantly from week 3 (2.2 µg g^{-1} h^{-1}) to week 7 (2.5 µg g^{-1} h^{-1}). These excretion rates remained constant with

a mean of 1.9 µg g^{-1} h^{-1} in the fed treatment groups (Figure 4). The starved groups had significantly higher excretion rates than the fed groups excluding the initial period from week 0 to week 3.

3.1.4 O:N ratios

During week 3 to week 8 the starved groups had a significantly lower O:N ratio (19-21%) than the fed groups. In the initial period from week 0 to week 2, the O:N ratio of the fed group was slightly lower and showed no significant difference (Figure 5).

3.1.5 Osmoregulation

1) Osmolality

The fed group of *C. rotundicauda* contained an osmoconformer which maintained hemolymph osmolality. The hemolymph osmolality of the fed group (988-1035 mOsm kg^{-1}) was higher than the seawater. The fed groups had significantly ($P<0.05$) higher hemolymph osmolality than the starved groups, except week 1 to week 3. Hemolymph osmolality of the starved groups varied from 756 mOsm kg^{-1} (week 7) to 1044 mOsm Kg^{-1} (week 1) (Figure 6). After week 3, osmoregulation of the starved groups decreased and attained its lowest value at the end of the experiment (week 7).

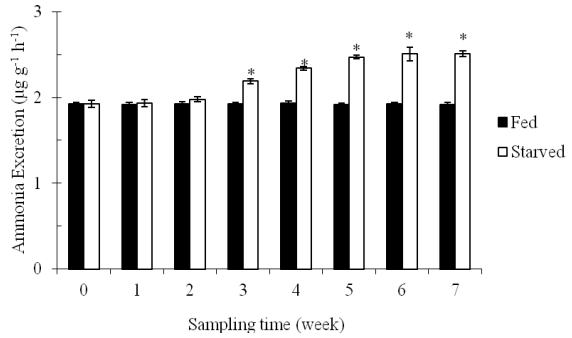

Figure 4. Effects of feeding and starvation (fed; ■; starved; □) on ammonia-N excretion rate in horseshoe crab, *C. rotundicauda*. Data are expressed as mean±SE (n=10). * represents significant differences in both groups ($P<0.05$).

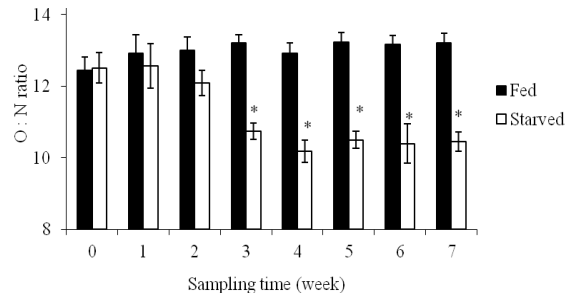

Figure 5. Effects of feeding and starvation (fed, ■ ; starved, □) on O:N ratio in horseshoe crab, *C. rotundicauda*. Data is expressed as mean±SE (n=10). * indicates significant differences in both groups ($P<0.05$).

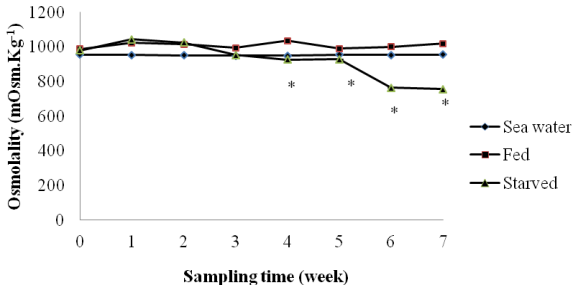

Figure 6. Effects of feeding and starvation compared with seawater as the control (fed, ■; starved, ▲; seawater, ●) on hemolymph osmolality in horseshoe crab, *C. rotundicauda*. Data are expressed as mean±SE (n=10). * indicates significance in both groups ($P<0.05$).

2) Hemolymph Na^+

From week 1 to week 7 the fed *C. rotundicauda* contained Na^+ hyperionic with mean concentrations of hemolymph Na^+ from 458 mmol l^{-1} (week 0) to 479 mmol l^{-1} (week 5). From week 4 to week 7, the starved groups had significantly lower hemolymph Na^+ than the fed group, but this was not found in the initial period from week 0 to week 3. The mean concentrations of hemolymph Na^+ for the starved *C. rotundicauda* ranged from 350 mmol l^{-1} (week 7) to -454 mmol l^{-1} (week 0) (Figure 7).

3) Hemolymph Cl^-

Hemolymph Cl^- was significantly lower in the starved group than in the fed groups, except from week 0 to week 2. Hemolymph Cl^- of the fed *C. rotundicauda* showed no significant difference compared with seawater. The values range from 491 mmol l^{-1} to 496 mmol l^{-1}. Hemolymph Cl^- ranged from 491 mmol l^{-1} to 496 mmol l^{-1} for the fed groups and from 140 mmol l^{-1} (week 7) to 496 mmol l^{-1} (week 0) for the starved groups (Figure 8).

4) Hemolymph K^+

Both the fed and the starved groups were hyperionic for K^+ throughout the experimental period. Hemolymph K^+ of

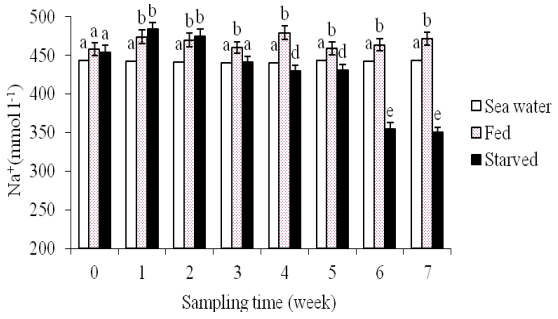

Figure 7. Effects of feeding and starvation compared with seawater as the control (seawater, □; fed, ▒; starved, ■) on hemolymph Na^+ in horseshoe crab, *C. rotundicauda*. Different letters indicate significant differences between the fed and starved groups at the same sampling time ($P<0.05$).

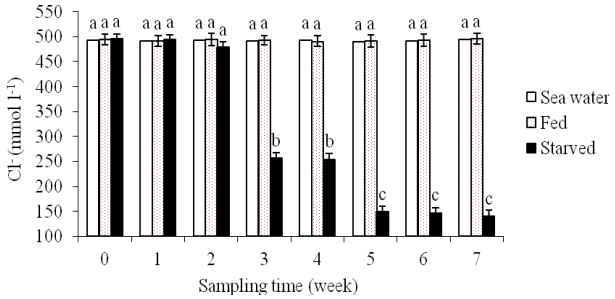

Figure 8. Effects of feeding and starvation compared with seawater as the control (seawater, □; fed, ▒ starved, ■) on hemolymph Cl^- in horseshoe crab, *C. rotundicauda*. Different letters indicate significant differences between the fed and starved groups at the same sampling time ($P<0.05$).

the starved group was significantly higher than the fed groups from week 5 to week 8. Hemolymph K^+ of the starved group ranged within 12 mmol l^{-1} to 13 mmol l^{-1}, whereas Hemolymph K+ of the fed group ranged from 11 mmol l^{-1} to 12 mmol l^{-1} (Figure 9).

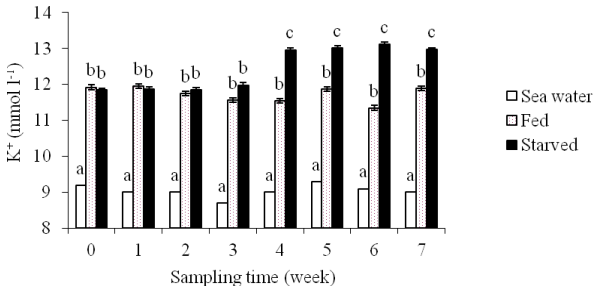

Figure 9. Effects of feeding and starvation compared with seawater as the control (seawater, □; fed, ▒ starved, ■) on hemolymph K^{+-} in horseshoe crab, *C. rotundicauda*. Different letters indicate significant differences between the fed and starved groups at the same sampling time ($P<0.05$).

3.2 Discussion

The reduction of body mass is possibly the most obvious and most frequently documented response to starvation. The present study found that both fed and starved *C. rotundicaudal* survived in good health throughout the 7-week experimental period. This suggested that *C. rotundicauda* tolerated starvation well for at least 7 weeks, despite the observed 7% reduction in body mass. Our data agreed with the findings by Hu *et al.* (2010).

The body mass of several vertebrate and invertebrate did not change during starvation, including *Litopenaeus vannamei* (Comoglio, 2004) and isopod (Hervant & Renault, 2002). It is possible that these species effectively replaced organic mass lost from tissue with water; therefore, there was virtually no net change in body mass during prolonged starvation. Increased osmotic pressure in the tissue caused by increased metabolite levels possibly contributed to this balance.

The oxygen consumption of an entire organism is commonly used as an indirect indicator for measurement of the metabolic rate (Randall *et al.*, 2002). Compared to the fed group, the oxygen consumption rate of the starved group was significantly lower from week 1 to week 4 and significantly higher from week 5 to week 7. This suggested that the reduced metabolic rate of *C. rotundicaudal* from week 1 to week 4 was supported by glucose reduction observed by Hu *et al.* (2010). It is known that the horseshoe crab stores fat, glycogen, and protein, in the yellow connective tissue of its hepatopancreas, and this may be the dominant energy storage. Hu *et al.* (2010) found increased plasma lipase activity in starved *C. rotundicauda* after week 4. It is possible that the metabolic rate of starved *C. rotundicauda* increased after week 3 with lipid and protein metabolism used as the energy source. The high oxygen consumption in the starved group from week 5 to 7 in the present study confirmed this observation. Starvation associated reduction in oxygen consumption rate was also found in other invertebrates, such as *Penaeus esculentus* (Dall & Smith, 1986), *Ostrea edulis* (Beiras *et al.*, 1994), intertidal mussels (Helson & Gardner, 2007), and gastropod, *Nassarius conoidalis* (Zhao, 2011). In addition, Hu *et al.* (2011) found a consistent drop in the oxygen consumption rate in horseshoe crab *C. rotundicauda* from week 1 to week 7, whereas we observed an increase from week 5 to week 7. The discrepancy of this observation compared with our results may be due to energy consumption during the osmoregulation process.

In decapod crustaceans, ammonia, amino acids, and urea are the 3 main end products of nitrogenous metabolism (Regnault, 1987). Salinity has a strong effect on ammonia excretion in crustaceans with increased excretion even at low salinity (*Farfantepenaeus paulensis*, Lemos *et al.*, 2001; *Penaeus chinensis*, Chen & Lin, 1994; *Scylla serrata*, Chen & Chia, 1996; *Portunus pelagicus*, Romano & Zeng, 2010). Zhao *et al.* (2011) reported that starvation reduced the ammonia excretion rate in the gastropod, *Nassarius conoidalis*, and the decrease continued over the duration of starvation. This agreed with the results of horseshoe crab by Hu *et al.* (2011). The discrepancy with our results might be caused by the reduction of hemolymph osmolality in starved *C. rotundicaudal* and by increased protein catabolism from week 5 to week 7.

The O:N atomic ratio is a direct measurement that reflects changes in the energy requirements due to either internal or external variables. Several factors are known to affect O:N, including pollution (Chen *et al.*, 2009), temperature (Wu & Sun, 2006), salinity (Wu & Sun, 2006), and starvation (Comoglio *et al.*, 2005; Wu *et al.*, 2011). In this study, the O:N atomic ratio reduced significantly to a range of 10.4-10.7 in starved *C. rotundicauda* from week 3 to week 7 and could indicate protein catabolism. However, high O:N values in the range of 12.3-13.2 were obtained in the fed group. Hu *et al.* (2011) reported mean O:N ratios of *C. rotundicauda* that ranged from 16.2 to 19.9 and increased with time of starvation. Different ranges may be due to environmental conditions, size, age or maturity of the subject animals. Mayzaud and Conover (1988) reported that organisms should have an O:N ratio of 24 where equal proportions of lipids and proteins are catabolized. The mean O:N values obtained in this study were lower than 24 which indicated that *C. rotundicauda* favors protein over glucose or lipids. The significant reduction in the O:N ratio of starved *C. rotundicauda* from week 4 to week 8 suggested that starved horseshoe crabs have to get a comparatively larger fraction of

their energy from protein than the fed animals. This seems natural. The fed animals get fat on starch or glucose in their feed, while the starved animals must consume their own protein after they have used other reserves. In some species, the hematocrit decreased during starvation (Rios *et al.,* 2002) while in others it increased (Shoemaker *et al.,* 2003). This suggested that starvation-induced changes in the hematocrit are related to changes in total body water content. Our study found that the hemolymph osmolality of the starved *C. rotundicauda* was 9% lower than the fed group. The decrease in hemolymph osmolality in starved *C. rotundicauda* from week 5 to week 7 of our study agreed with McCue (2010).

Hemolymph Na^+ of fed *C. rotundicauda* was significantly higher than in seawater, except in week 1. From week 4 to week 7, hemolymph Na^+ and Cl^- of starved *C. rotundicauda* decreased significantly to around 4-25% and 48-72%, respectively. This was associated with a reduction in the hemolymph osmolality in the starved group from 926 mOsm kg^{-1} to 757 mOsm kg^{-1}.

4. Conclusions

The present study demonstrated that prolonged 7-week starvation of horseshoe crab *C. rotundicauda* did not affect its body mass, but significantly decreased oxygen consumption from the initial period, and then significantly increased after week 5 of starvation. The observations suggest that the species has different types of energy reserves that are consumed sequentially leading to step-wise changes in metabolism during starvation. The ammonia-N excretion rate also increased after week 3, while it remained constant from week 0 to week 2. The O:N ratio reduced significantly in the starved horseshoe crab from week 3 to week 7. Starving induced a reduction of hemolymph osmolality from week 3 to week 7. Hemolymph Na^+ and Cl^- decreased during starvation in contrast to hemolymph K^+ which significantly increased from week 4 to week 7. The activities of Na^+/K^+-ATPase and Cl^-/HCO_3^- exchanger in the gills of this species need to be determined in further studies on the starvation effects of this species.

Acknowledgements

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