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HAEMOLYMPH AND URINE CONSTITUENTS OF A LABORATORY-HELD CRAB SPECIES, *CANCER PAGURUS* (L)

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ABSTRACT

The pattern of normal ammonia efflux during a 24-h period has been assessed for the brown crab, *Cancer pagurus* acclimatized to laboratory holding conditions. Simultaneous measurements of haemolymph and urine dissolved ammonia and pH of haemolymph glucose and lactate levels were also made.

An approximate tripling of daytime ammonia efflux occurred during darkness. The nocturnal haemolymph and urine dissolved ammonia values were significantly lower than daytime values. Haemolymph pH rose slightly but not significantly during darkness but nocturnal urine pH was significantly higher than the daytime value. Haemolymph glucose rose significantly from the daytime low of 0.4 - 0.5 mM to a nocturnal high of 0.74 ± 0.14 mM. Lactate levels showed no apparent relationship with the time of sampling.

The data are considered in relation to the behavior of the animals and the implications for their live marketing.

Keywords : Haemolymph, ammonia efflux, urine constituents, *Cancer pagurus*.

INTRODUCTION

The natural day-night cycle of light intensity (photoperiod) is an important environmental cue that co-ordinates many biological rhythms (Lees, 1971; Webb, 1976; Naylor, 1988). The daily metabolic rhythms of aquarium-maintained animals are determined primarily from the prevailing photoperiod regime and, in their natural environment, benthic species such as *C. pagurus*, would depend on the penetration of light in the water column. Light is scattered and absorbed in water and measurements at temperate latitudes indicate that the depth for

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1% surface light to be retained is 16 m (Clarke, 1954). Normally, *C. pagurus* is found at depths of 20-300 m and would seldom experience natural photoperiod. The rhythms in question are in the nature of metabolic and physiological activities and are reflected by changes to the animals' oxygen consumption, nitrogen metabolism, and levels of various haemolymph constituents. Thus, daily rhythms shown to occur in crustaceans include those of oxygen consumption (Arudpragasam and Naylor, 1964; Ansell, 1973), cardioventilatory activities (Arudpragasam and Naylor, 1964; Ansell, 1973; Pollard and Larimer, 1977), quantitative changes to various haemolymph constituents, e.g., glucose, (Dean and Vernberg, 1965; Hamann, 1974; Poolsanguan and Uglow, 1974), neuronal activity (Arechiga et al., 1974), color change (Brown et al., 1953; Fingerman, 1955), and whole animal activity (Hagerman, 1971; Rebach, 1985).

Data on changes to ammonia efflux of marine crustaceans are scant, but a tidal rhythm of ammonia efflux in *Crangon crangon* has been described (Regnault, 1983). More recently, Marangos et al. (1990) measured day/night variations of ammonia efflux in *Penaeus japonicus* and showed two nocturnal maxima of ammonia excretion, each of which was almost double the daily efflux.

The measurement of physiological parameters in 'normal' crabs is valuable in the context of identifying changes associated with stress or poor survival under commercial conditions. The live shellfish industry requires methods of assessing reliably the health of its products with 'health' in such context relating to prospects of the animals surviving post-harvest treatments. Such survival is an important attribute in the context of improving the selection of animals for holding and subsequent sale and selection on the basis of haemolymph protein levels is already being practiced by some commercial enterprises which deal, store, and distribute live lobsters.

These studies of the daily patterns of nitrogen efflux in *C. pagurus* were made in order to assess the legitimacy of using fixed times of the working day to make measurements of physsiological variables in this species and of conclusions drawn on the basis of such data.

Physiological data of this type were vbeing collected routinely for this and several other species in our laboratory, and were in danger of beinng inteerpreted without adequate appreciation of the exstent of possible rhythms of nitrogen metaabolism.

MATERIALS AND METHODS

Animal maintenance

Live crabs (265.1 - 941.6 g, mean = 505.4 ± 6.9 g) were purchased and maintained in large tanks in a temperature-controlled room at a constant 12 ° ± 1 °C, at a salinity of 35 with artificial light and photoperiod of 12:12 h. Salinity was measured daily with a hand-held refractometer (Atago), and total ammonia (TA) levels in the aquaria were monitored daily and not allowed to rise above 100 mol TA/L.

Experimental animals were maintained under such conditions and fed mussels (*Mytilus edulis libitum)* for two weeks before experimentation. This time was allowed to ensure temperature acclimation, adjustment to the light regime, and although they are subtidal, the loss of any tidal rhythms of activity. A level which has been shown to alter the ammonia efflux rate of *Cancer pagurus* minimally.

Total ammonia efflux

Groups of adult, intermoult crabs (n = 7 in each case) unselected for gender were chosen to be of similar size and each individual placed into a chamber (volume = 4.025 L). Chambers had opaque circular walls (for minimum visual disturbance), and an acrylic lid that could be sealed, airtight. Each chamber was fitted with an inflow and outflow so that aerated seawater (12° ± 1°C, 35 salinity) was constantly re-circulating through the chambers via a biofilter.

The crabs were allowed to acclimatize in these chambers for 48 h prior to experimentation, and were not fed 24 h before the experiment began. Any feces produced were removed from the chambers prior to the initial measurements being made.

Total ammonia (TA) efflux was measured by stopping the flow of water through the chamber and measuring the increase in the water TA over 1 h. Water samples (1 ml) were withdrawn with a syringe through a Suba-seal from chambers at t_0 and at t_1 1 h later. Such samples were then taken every 3 h for 24 h. Seawater samples were stored in microcentrifuge tubes and analyzed immediately for TA. The volume of water in the chamber was measured at the end of the experiment after the careful removal of the crab. A level which has been shown to alter te ammonia efflux rate of *Cancer pagurus* minimally (Hosssie, 1993).

Haemolymph sampling

Haemolymph samples were collected from groups of n = 7 individuals at 3 h intervals over 24 h by inserting a hypodermic needle through the arthrodial membrane at the base of the 4th or 5th pereiopod, into the blood sinus. A different group of crabs was used at each sampling time and no crab was sampled more than once. Each sample was transferred to a microcentrifuge tube and measured immediately for pH. Haemolymph (1 ml) was then diluted with distilled water (0.5 ml), shaken rapidly to prevent clotting, and frozen immediately (-20°C).

Haemolymph sampling took approximately 30 sec per animal. When thawed, haemolymph samples were centrifuged (2 min at 8,000 rpm) to remove any cellular debris. Haemolymph samples were analyzed for TA (Hunter and Uglow, 1993), glucose (Sigma Kit No. 510A), and lactate (Sigma Kit No.735-10).

Urine sampling

Urine samples were collected at the same time as the haemolymph. Samples were obtained by first deflecting the nephropore operculum on each antennule with a metal hook and then inserting the tip of a fire-drawn glass capillary just inside the nephropore. Urine flow usually began immediately upon operculum deflection. If this did not occur, gentle suction was applied via a plastic pipette attached to the capillary. Samples were collected until no more could be removed (ca 30 sec for each bladder). To minimize air exposure of the sample and possible pH change, the pH was immediately measured with a microelectrode. This procedure was carried out for both urinary openings, and the urine collected from each bladder was analyzed separately. The volume of urine collected was also recorded for each bladder and the remaining urine was frozen (-20°C) until analyzed for TA.

Statistical treatments

All data were examined for normality using Lillifor's Test and homogeneity of variance using Levene's Test. Comparisons between day and night levels of ammonia efflux were made using a paired t-test. Blood and urine constituent data were analyzed using one-way Analysis of Variance (ANOVA) if homogeneous and normal or the non-parametric Mann-Whitney U test.

RESULTS

Total ammonia efflux

Data relating to the mean weight-specific TA effluxes of *C. pagurus* under conditions of 12:12 lighting are shown in Figure 1 and reveal a clear pattern of low/high, daytime/nighttime TA efflux. The maximum TA efflux of 0.3-1.2 mol TA/g/h occurred between 21:00 and 00:00 h, and the minimum of 0.01-0.17 mol TA/g/h occurred at midday. These timings coincided with a distinct increase in locomotory activity by the crabs at around 21:00 h and an almost complete cessation of movement in the middle of the day. The mean daytime TA efflux of 0.17 ± 0.13 mol TA/g/h was approximately one third of the mean nighttime rate of 0.51 ± 0.06 mol TA/g/h ($P = < 0.05$), and the overall mean diel TA efflux was 0.35 ± 0.04 mol TA/g/h. No significant gender-related difference in the pattern or the magnitude of TA efflux was found.

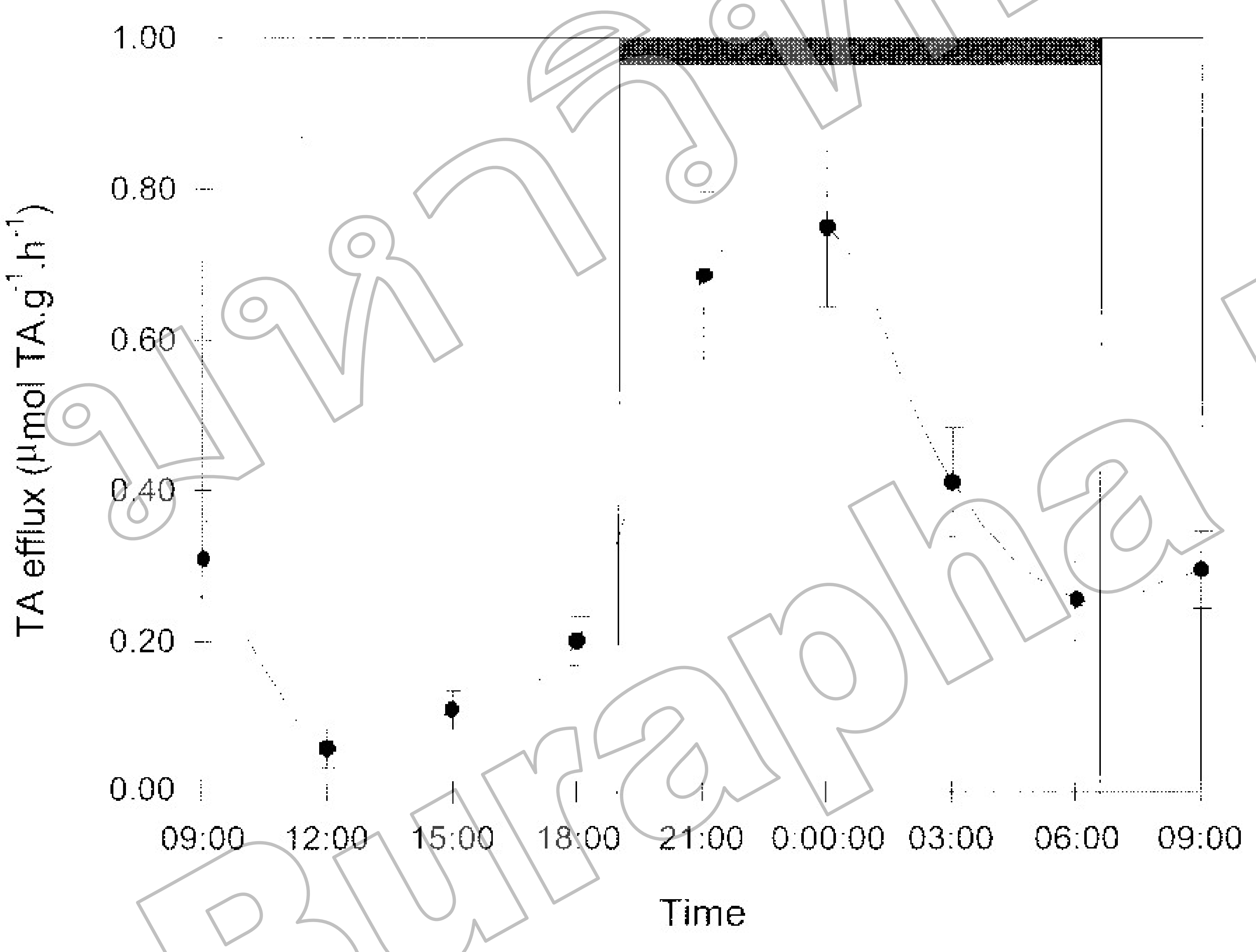

Figure 1. Total ammonia efflux of laboratory-acclimatized *Cancer pagurus* measured over a 24-h period. Each point is the mean of n = 7 with SEM. The filled area indicates the duration of the dark period.

Haemolymph and urine constituents

Urine sampling was particularly effective using the method described and overall mean volumes of 1.8 ± 0.34 ml urine/bladder and 3.44 ± 0.88 ml urine/crab were collected. Associated variability was large with maximum and minimum bladder volumes of 10.25 ml and 0.07 ml collected at a single sampling time. There were no apparent relationships between times of collection and the volumes of urine collected or between the constituents of either left or right bladder ($P = > 0.05$ in all cases), and values given here are the averages from both bladders.

The lowest TA levels in urine and haemolymph occurred during darkness, between 21:00 and 06:00 h (Figure 2). The mean nighttime urine TA (43.6 ± 4.2 mol TA/L) was significantly lower ($P = < 0.01$) than the daytime value (101.31 ± 7.56 mol TA/L). Haemolymph TA levels were more variable but followed a similar trend which changed from a low nighttime mean value of 180.27 ± 14.59 mol TA/L to a day-time mean value of 280.81 ± 29.84 mol TA/L ($P = < 0.01$). The mean daily TA concentrations for haemolymph and urine TA were 227.19 ± 17.47 and 71.15 ± 6.06 mol TA/L, respectively.

Haemolymph pH increased during darkness (Figure 3), but not significantly and reached a maximum value of 7.72 ± 0.01 at 06:00 h and decreased to a minimum value of 7.61 ± 0.01 at midday - a slight daytime acidosis. The urine pH values showed a similar trend with a maximum value of 8:01 ± 0.04 at 21:00 h and a minimum of 7.64 ± 0.08 at midday. A significant ($P = < 0.05$) increase in the urine pH occurred during the dark period. The mean daily haemolymph and urine pH were 7.68 ± 0.01 and 7.85 ± 0.02 respectively.

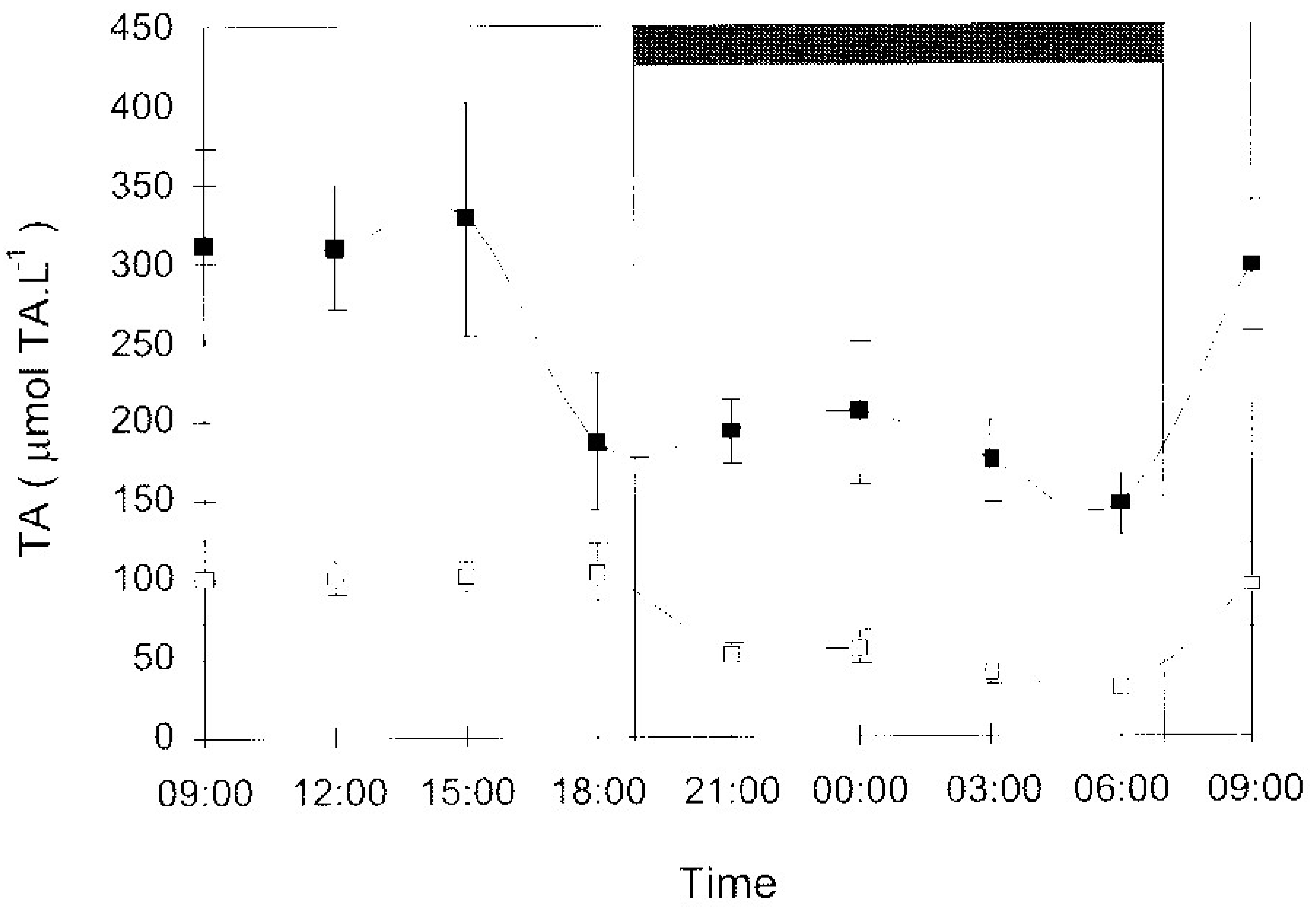

Figure 2. Changes to haemolymph (■) and urine (□) TA concentrations of laboratory-acclimatized *Cancer pagurus* measured over a 24-h period. Values are the mean of n = 7 with SEM. The filled area indicated the period of darkness.

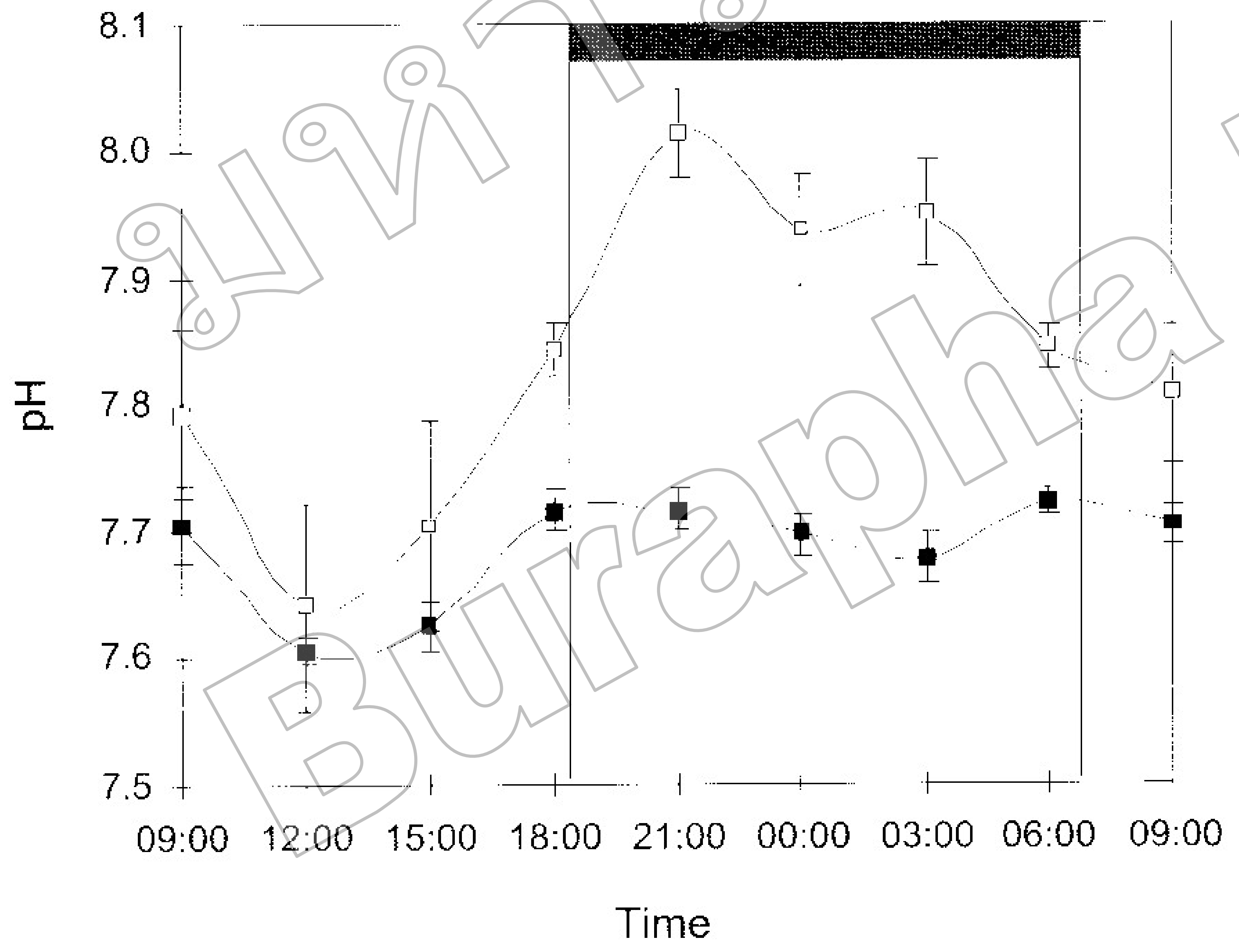

Figure 3. Changes to haemolymph (■) and urine (□) pH values in laboratory-acclimatized *Cancer pagurus* measured over a 24-h period. Values are the mean of n = 7 with SEM. The filled area indicates the period of darkness.

During darkness, haemolymph glucose concentrations increased significantly (P = <0.05) from a resting day-time level of between 0.4-0.5 mmol/L to as high as 0.74 ± 0.14 mmol/L at midnight (Figure 4). The haemolymph glucose concentration in two crabs was 5-7 times higher than those in the other crabs. There appeared to be no relationship between the time of day and the occurrence of these high concentrations and, although mentioned here, they were discounted because they were possibly the result of an undesigned hyperglycaemic response to handling (Telford, 1968, 1974). The mean daily haemolymph glucose concentration was 0.6 ± 0.06 mmol/L.

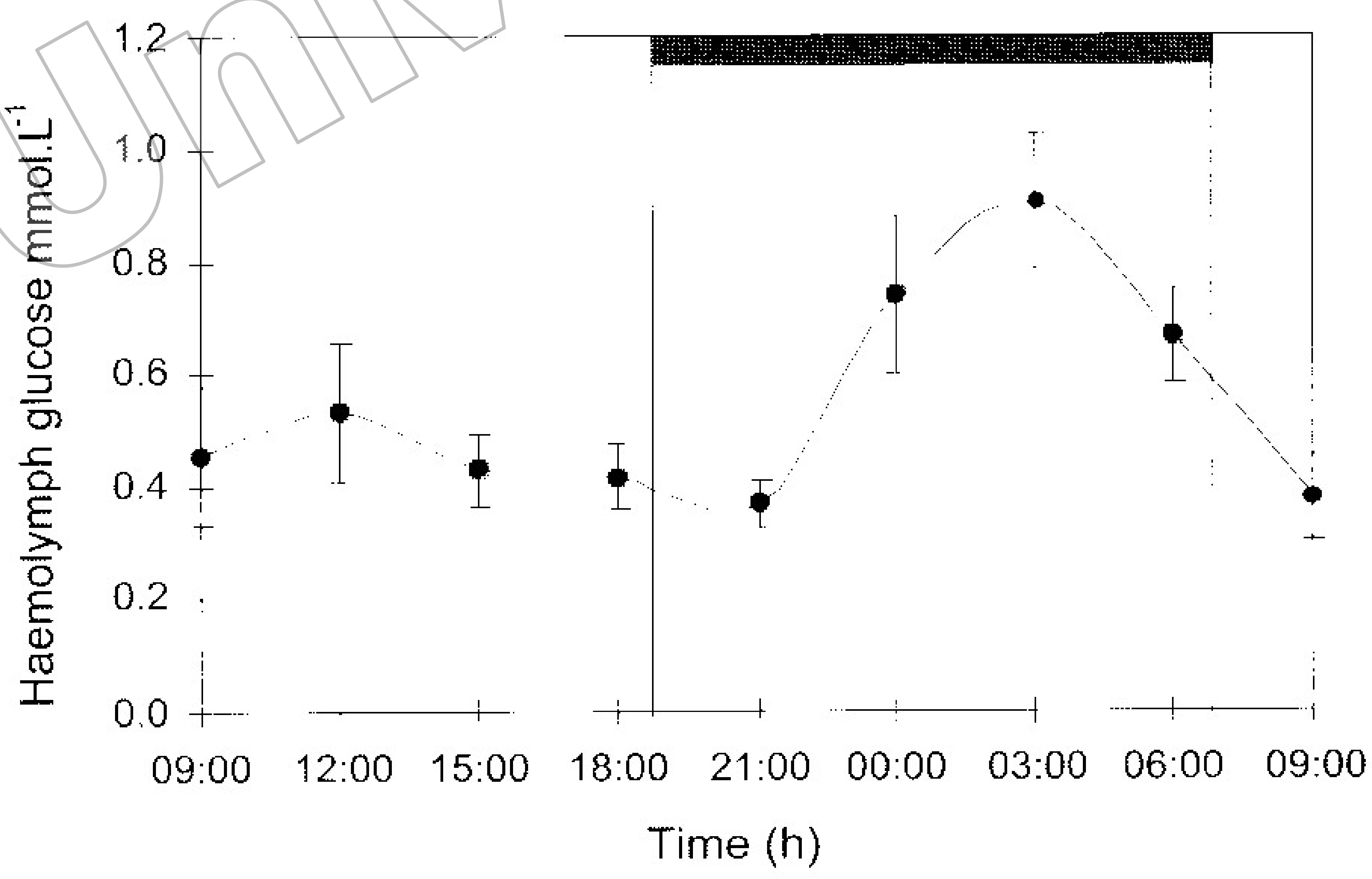

Figure 4. Haemolymph glucose concentrations of laboratory-acclimatized *Cancer pagurus* measured over a 24-h period. Values are the mean of n = 7 with SEM. The filled area indicates the period of darkness.

No significant changes to haemolymph lactate, attributable to the time of sampling, occurred during the experiment (Figure 5), and the overall daily mean haemolymph lactate concentration was 1.49 ± 0.12 mmol/L. The two crabs with high haemolymph glucose levels also had high lactate concentrations and have been discounted. One crab, however, had high haemolymph lactate but not a corresponding high glucose value, and this individual is responsible for the high mean concentration and associated standard errors measured at 15:00 h.

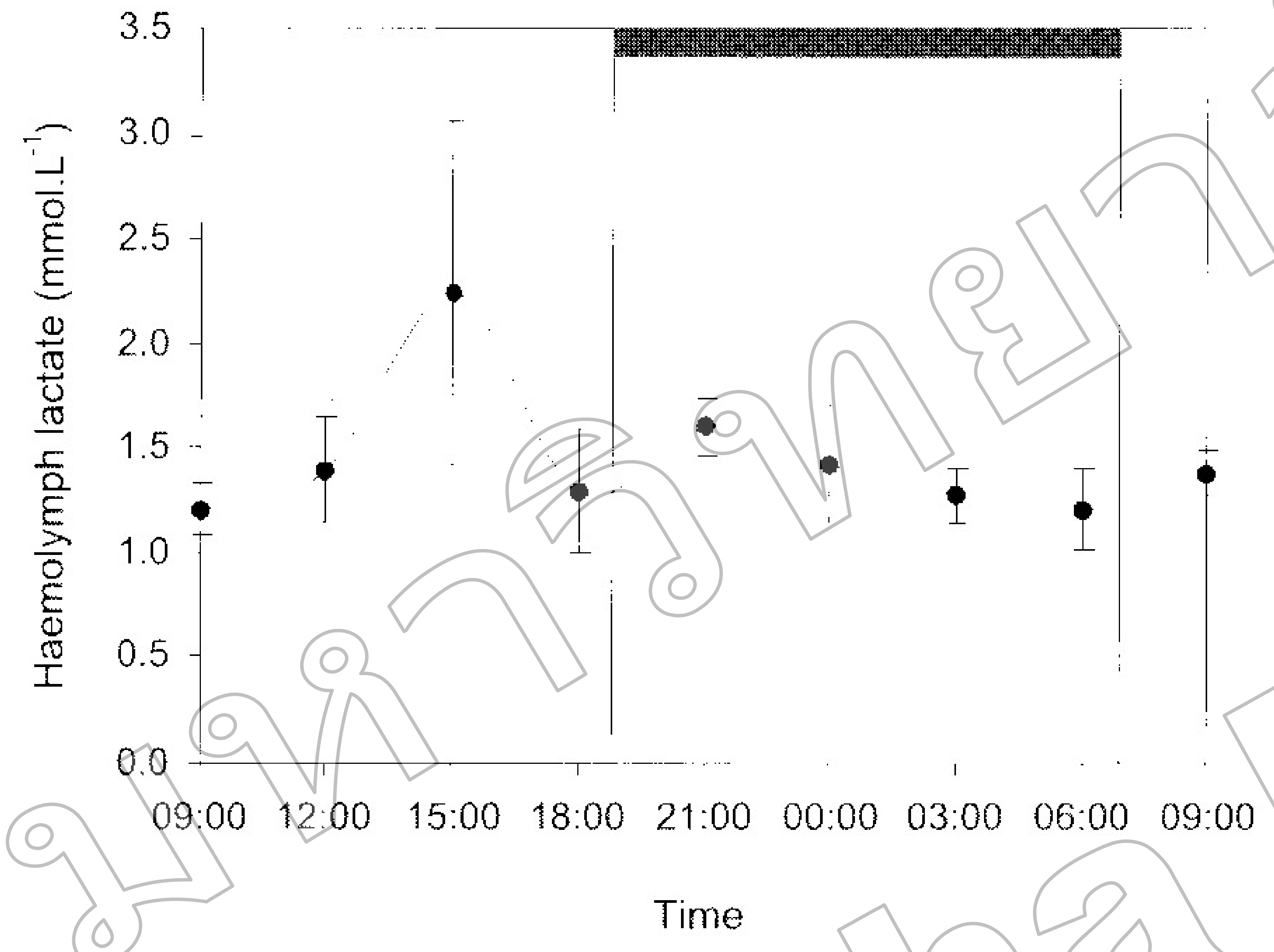

Figure 5. Haemolymph lactate concentrations of laboratory-acclimatized *Cancer pagurus* measured over a 24-h period. Values are the mean of n = 7 with SEM. The filled area indicates the period of darkness.

DISCUSSION

Rhythms of physiological events that can be correlated with activity patterns set by environmental changes such as photoperiod, occur in many crustacean species (De Coursey, 1983; Naylor, 1988). *Cancer pagurus* is a night-active species and other laboratory-acclimatized crustaceans have been shown to be nocturnal (Bolt and Naylor, 1985). The gradual changes in light intensity at dawn and dusk do not occur usually in the laboratory and laboratory lighting has a constant intensity level as well as a different spectral composition to natural daylight.

Nevertheless, laboratory acclimatized *C. pagurus* showed a clear nocturnal activity coinciding with high TA efflux, and thus accord well with the findings for *Penaeus japonicus* (Marangos et al., 1990) and with the data on oxygen consumption of *C. pagurus* (Ansell, 1973). His study showed that the nocturnal oxygen consumption rate of *C. pagurus* was up to 5 times that of the day-time rate, and this he ascribed to increase a locomotory activity and a greater alertness and responsiveness of crabs at night.

The mean daily TA efflux of *C. pagurus* at 12 °C was 0.35 ± 0.04 μmol TA/g/h. This value is similar to that of the TA excretion of other decapods found in the literature, e.g., Regnault (1994) measured the mean TA efflux of resting *C. pagurus* under normoxia (16-18 °C) to be 0.171 ± 0.016 μmol TA/g/h.

Starvation (Ansell, 1973) and feeding (Aldrich, 1975) affect normal rhythms of oxygen consumption in *C. pagurus*, and ammonia efflux is also dependent upon nutritional status (Nelson et al., 1979; Nelson and Kropp, 1985). Fed *Crangon franciscorum* may excrete as much as 7 times more ammonia than starved ones (Nelson et al., 1979). Here, crabs were deliberately acclimatized to laboratory conditions and not fed 24 h before data were collected, and it is likely that the nocturnally increased TA effluxes were a direct result of increased activity levels. The data show that nocturnal ammonia effluxes are approximately triple those that could be predicted on the basis of daytime values. The overall daily mean efflux rate was almost exactly double that of the daytime value. It was clear that the laboratory-held animals became nocturnal and that the increased ammonia efflux was associated with the nocturnal activity rhythm. Such information is of importance in the context of successful husbandry of the animals in the laboratory or commercial environment and also hints at the care that needs to be applied when making.

Circulating haemolymph TA levels decreased significantly and rapidly with the onset of darkness and had already begun an anticipatory decrease before darkness. Similarly, the onset of darkness has been found to affect activity levels in *Crangon crangon* (Hagerman, 1971). Although haemolymph TA levels are generally considered to be regulated, they showed a variability of approximately 55% (= 100 µmol TA/L) between the nocturnal low and daytime high values. Ammonia is excreted *via* the gills and, presumably, increased activity results in higher ventilation rates and higher TA effluxes. This may result in a lowered mean haemolymph TA concentration despite a higher TA production. Ventilation rates were not measured in this experiment but significant cyclical changes of gill ventilation and ventilation volume patterns occur in *Carcinus maenas* with high ventilation volumes related closely to an increased oxygen consumption (Arudpragasam and Naylor, 1964). In this study, haemolymph TA levels ranged between 69.83 and 628.00 µmol TA/L. The normal mean values of 280.81 ± 29.83 µmol TA/L (day) and 180.27 ± 14.59 µmol TA/L (night) are comparable with those in the literature for this and other decapods.

The pattern of urine TA changes were found to mirror those of the haemolymph TA, with a decrease of 50 µmol TA/L at night which suggests there may be a direct relationship between the two. Urine production rates have been estimated for several decapods and comprise 2-14% body weight/day in marine species (Parry, 1960). Urine production rates of species comparable to *C. pagurus* include those of *Cancer magister* (Holliday, 1977), and *Homarus americanus* (Breithaupt et al., 1999) are both *ca.* 2% body weight/day. A value of between 3-10% body weight/day has been quoted for *C. pagurus* (Robertson, 1939, cited in Parry, 1960). Applying this value to calculate the approximate TA excreted *via* the urine of an average sized crab (440g), yields a total of 0.04-0.14 µmol TA/day and corresponds to only 0.003-0.01% of the TA excreted daily. This contrast with estimates of urinary ammonia contributions of 1-2% of the daily ammonia excreted by freshwater-acclimated *Callinectes* (Cameron and Batterton, 1978) and < 2% in *Jasus edwardsii* (Binns and Peterson, 1969) but agrees with the negligible contribution in *Carcinus maenas* (Harris and Andrews, 1985).

Haemolymph pH is a reflection of the acid-base functioning in the animal, and haemolymph pH here varied between 7.7-7.6. This low haemolymph pH value could be caused by an increased ambient CO_2 concentration that prevents adequate removal of CO_2 at the gills and which leads to an accumulation in the haemolymph. The lowest haemolymph pH levels were measured at midday, but values remained close to 7.7 during the night.

The nighttime mean urine pH level was significantly different than the daytime value and made the urine at this time some 0.3 pH units more alkaline than the haemolymph value. This difference suggests that the antennal glands may play a role in the acid-base balance of the animal. Previous experiments have shown that the antennal gland is an important avenue for the loss and reabsorption of electrolyte and bicarbonate ions, and especially divalent ions (Wheatly, 1985).

Cancer pagurus haemolymph glucose levels increased significantly during darkness and a similar pattern of diel rhythmicity occurs also in the crayfish, *Orconectes limosus* (Hamann, 1974). Poolsanguan and Uglow (1974) found a circatidal pattern of change in haemolymph total reducing sugar levels in *Crangon crangon*, with highest levels at high tide periods and photoperiod effects not significant at the 5% level.

Circulating glucose is used frequently as an indicator of immediate stress in crustaceans (Telford, 1974), but it is essential to recognize also that the normal daily cycle produces changes to glucose concentrations.

Haemolymph lactate levels did not change over the 24-h period, and the mean level was 1.49 ± 0.12 mmol/L. Haemolymph lactate levels are accepted as an indicator of hypoxic stress to decapods (Truchot, 1980; Booth et al., 1982; Morris et al., 1986a), and it appears that no such stress occurred in these studies.

Although not designed to be a comprehensive, detailed investigation of rhythmicity, these studies indicate rhythmic changes in metabolic activity occur in *C. pagurus*. *Cancer pagurus* is a valuable species that is marketed alive in Europe and a recognition that potentially toxic ammonia is excreted in higher quantities during darkness carries implications for the optimization of holding/transporting systems for this and related species. Consignments of live shellfish are normally held under constant darkness, and it is unknown whether this causes an overall increase in ammonia production or even if the rhythm persist in prolonged darkness. Any means of reducing ammonia production would be beneficial when animals are transported over long distances in fixed volumes of water or when transported dry.

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