Biological Rhythm Research
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/nbrr20

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Accepted author version posted online: 06 Jan 2012.Version of record first published: 21 Feb 2012.

To cite this article: E. Azpeitia , C. Vanegas-Pérez , E. Moreno-Sáenz , M. Betancourt-Lozano & M. Miranda-Anaya (2013): Effect of chronic ammonia exposure on locomotor activity in the fiddler crab Uca princeps upon artificial tides and light cycles, Biological Rhythm Research, 44:1, 113-123

To link to this article: http://dx.doi.org/10.1080/09291016.2011.652864

Biological Rhythm Research
Vol. 44, No. 1, February 2013, 113–123

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Effect of chronic ammonia exposure on locomotor activity in the fiddler crab *Uca princeps* upon artificial tides and light cycles

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(*Received 19 October 2011; final version received 20 December 2011*)

The aim of this study was to evaluate the effect of continuous exposure to sublethal ammonia concentrations upon the characteristics of the locomotor activity rhythms in *Uca princeps*, exposed to artificial tides and light cycles. Adult male crabs were used for standardized 24 h ammonia acute toxicity tests. Sublethal ammonia concentrations were selected considering the results obtained in the acute bioassays as well as the unusual high ammonia concentrations registered in their natural habitat. Three independent groups of eight crabs each were exposed during 15–20 days to 0.125, 3.5, and 18 mg N-TA/L in artificial sea water. Locomotor activity was recorded individually by means of infrared light crossings. Artificially created tidal and light–dark cycles were used along the experiments. The 24 h-LC_{50} was 186.02 mg N-TA/L. No mortality was registered in the crabs exposed to the sublethal ammonia concentrations. In control groups, organisms showed tidal activity onset mainly in low tide (ebb) and no response to light cycles while ammonia exposed crabs shifted their onset of activity to high tide, and a general increasing activity was observed along the experiment. The obtained results indicate that ammonia affects the entraining features of the tidal activity rhythm and demonstrate that under ammonia sublethal exposure, tides may present relative coordination with light cycles in the tidal activity rhythm of the crabs.

Keywords: circadian and circatidal rhythms; locomotor activity; ammonia exposure; *Uca princeps*

Introduction

Studying the mechanisms underlying the biological timing enhances our understanding of the temporal relationship of animal behavior and geophysical changes (Marquez and Waterhouse 2004; Morgan 2004). Intertidal zone, particularly, represents an extremely challenging environment in which many organisms are found. Tides are different regarding the location of study and display temporal structure differing along coast lines over the world. Therefore, the way in which intertidal organisms interact with tides is complex, in such way that biological timing

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ISSN 0929-1016 print/ISSN 1744-4179 online

http://dx.doi.org/10.1080/09291016.2011.652864
http://www.tandfonline.com

in tidal rhythms constitutes a specific adaptation that may allow the organism to take advantage of its environment (Naylor 1997; Thurman 2004).

Different species of the fiddler crab *Uca* have been studied in order to increase the comprehension of circa tidal regulation (Honeger 1976; Lehman 1976; Thurman 1998, 2004; Thurman and Broghammer 2001). Locomotor activity in *Uca princeps* has been referred as an example of a biological model to study circatidal rhythms according to the pattern of tide that may influence its activity (Stillman and Barnwell 2004) and it is possible that such regulation would be altered by a polluted environment.

U. princeps is widely distributed in the Pacific coast, including Mexican shores dominated by mixed tides (Campos and Lopez 1998). *U. princeps* displays both daily and circatidal rhythms when exposed to light–dark cycles; particularly, organisms collected in San Blas, Nayarit (México) showed a bimodal circadian component shortly after the lights off and after lights on, and a particular delay in onset of each component is shown when the tropical tide occurs (Stillman and Barnwell 2004).

U. princeps is a common inhabitant of the estuarine environment in the NW pacific coast of México. A particular population of *U. princeps* is located in Urias estuary at Isla Soto, Mazatlán, Sinaloa, México. This population is frequently exposed to high ammonia levels due mainly to intense shrimp aquaculture and agriculture activities as well as industrial and urban wastes (Hernandez-Cornejo and Ruiz-Luna 2000; Ruiz-Luna and Berlanga-Robles 2003). In this area, both agriculture and aquaculture represent the 63.4% of the total nitrogen input to the coastal environment (Paez-Osuna et al. 1998, 1999). Shrimp aquaculture in tropical regions is associated with high concentrations of total ammonia as a result of high rates of shrimp excretion and feed loading, which has an adverse effect in productivity and results in negative impacts on coastal waters (Camargo and Alonso 2006).

In the aquatic environment, ammonia is present as an ionized ($NH_4{}^+$) and un-ionized (NH_3) molecule, where the toxic effect is mainly attributed to the un-ionized (NH_3) form; the aqueous equilibrium of the ionized and the un-ionized form is dependent on the pH, temperature, and salinity of the medium (Bower and Bidwell 1978). It has been widely reported the neurotoxic effect of ammonia enhanced by hypoxia, alkalosis, and hyperthermia (Cooper and Plum 1987). When ionized, ammonia has a similar effect than K^+ depolarizing the membrane potential (Randall and Tsui 2002). Acute intoxication with large ammonia doses leads to activation of N-methyl-D-aspartate type glutamate receptors in central nervous system (CNS), resulting in oxidative stress and disturbance of mitochondrial function (Kosenko et al. 2007; review in Ortiz-Santaliestra et al. 2010), and astrocytes represent a major target at the CNS of its toxicity. In hepatic encephalopathy, ammonia is a major factor in its pathogenesis (Albrecht and Norenberg 2006).

Among the decapod crustaceans the effects of ammonia are widely documented in prawns and shrimps, although related studies are relatively scarce in crabs. Several authors have studied the physiological effects of ammonia toxicity in crabs including survival and molting (Niel et al. 2005), ontogenetic tolerance (Niel et al. 2005; Romano and Zeng 2007a), osmoregulation (Spaargaren 1990; Rebelo et al. 1999, 2000; Romano and Zeng 2007b, 2010, 2011; Martin et al. 2011) and metabolic and immune responses (Hong et al. 2007). However to date no studies of the effect of ammonia on biological rhythms of activity in crustaceans have been published. Laboratory studies of the effect of pollutants on circadian rhythms in marine animals are scarce (Handy 2003; Gerhardt et al. 2005). This kind of studies is important in order to understand the ecological significance of how the biological clock functions and the way that its timing is affected due to environmental stressors related with human activities.

The present study was carried out in order to evaluate, under laboratory conditions, if continuous exposure to sublethal ammonia concentrations produces detectable effects upon the characteristics of the locomotor activity rhythms in *U. princeps*, exposed to artificial tides and light cycles.

Materials and methods

Capture and maintenance of organisms

Adult male crabs (2.5–3.5 cm wide of cephalothorax) were captured in the Urías estuary, Mazatlán, Sinaloa, México, during the ebb. Crabs were transported in containers to the Centro de Investigación en Alimentación y Desarrollo (CIAD, Mazatlán, México), then sent by air transportation and maintained in laboratory conditions at the Facultad de Ciencias [Universidad Nacional Autónoma de México (UNAM)], México City. Crabs were maintained during 10 days in 250 L aquaria with circulating artificial seawater and without tide stimulus; organisms were fed daily *ad libitum* with *Artemia* dry pellets. Physicochemical parameters such as dissolved oxygen (polarographic electrode Yellow Spring Instruments (YSI) ± 0.05 mL/L O_2), pH (pH meter; Walk Lab ±0.005), temperature (Brannan; ±0.05°C) and salinity (ATAGO Optical Refractometer; ±0.5 ppt) were daily monitored and maintained during acclimation and along the experiments at 25 ± 1°C, 35 ± 1 psu, 8.2 ± 0.01 pH and 5.8 ± 0.2 mg Dissolved Oxygen (DO)/L, respectively. Photoperiod was fixed at 12:12 h light:dark (LD). During acclimation and experimental control assays, total ammonia (N-TA) was kept below 0.125 mg N-TA/L (0.006 mg NH_3/L). Actual ammonia concentrations were measured using the indophenol method (Rodier 1981) adapted for microplate reading. Un-ionized ammonia concentrations were calculated by taking into account ammonia dissociation considering water temperature, salinity, and pH of each experimental aquarium (Bower and Bidwell 1978).

Experimental assays

Two experimental assays were conducted. The first one was designed to establish the median lethal concentration of ammonia (LC_{50}) to *U. princeps* in order to derive the sublethal ammonia concentrations considered for the second protocol. The second protocol was conducted to test two sublethal concentrations of ammonia (3.5 and 18 mg N-TA/L) on long-term recordings (15 days) of locomotor activity of *U. princeps* in order to evaluate its effect upon entraining to artificial tides and photoperiod. Studies on biological rhythms in crustaceans by using locomotor activity as a measure of the output of the biological clock have been performed in organisms kept in our laboratory (Miranda-Anaya et al. 2003).

Ammonia acute toxicity

Short-term static toxicity tests of 24 h without water replacement were conducted to evaluate the acute toxicity of ammonia. Crabs were not fed 12 h prior the tests or during the exposure period. Five experimental groups and a control were considered:

control (0.125), 42.5, 103.4, 197.3, 254.1, and 351.9 mg N-TA/L, corresponding to 0.006, 2.1, 5.1, 9.8, 12.6, and 17.4 mg N-NH_3/L, respectively. Eight organisms were randomly distributed in each 8 L test aquaria; bioassay was conducted in duplicate. The desired concentrations of N-TA test solutions were prepared from a concentrated ammonia stock solution of NH_4Cl (100 g/L; Merck; 95% purity).

Dead animals were recorded every hour and were immediately withdrawn from the aquaria to maintain water quality; dead organisms were detected by the lack of movement when animals were prodded gently with a glass rod. The median lethal concentration of ammonia (24 h-LC_{50}) was calculated through probit analysis using the DORES program; model significance was estimated with a χ^2 test (UNEP 1987). Once obtained the ammonia lethal concentration curve, sublethal concentrations were intrapolated.

Ammonia sublethal exposure

Sublethal ammonia concentrations (3.5 and 18 mg N-TA/L) were used for chronic exposure considering 2% and 10% of mortality in results obtained in the acute bioassays (24 h-LC_2, 24 h-LC_{10}) as well as the concentrations registered in their natural habitat in events associated with elevated N input (Paez-Osuna et al. 1999; *in situ* recordings).

Three independent groups of nine crabs each were exposed during 15–20 days to reconstituted sea water with control group (0.125 mg N-TA/L), 3.5 and 18 mg N-TA/L. For each experimental group a water closed flow system was used to control the artificially created tides, which were simulated through a water distribution system activated by a water pump (Dolphin P-2000 professional, 700 L/min) controlled with a timer. During the ebb, water was kept as a static layer of 2 cm deep, while in high tide, water was circulating and kept in a layer of 6–8 cm. Tides were daily programmed as predicted by the Center of Scientific Research and Superior Education (Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California (CICESE)) México, for the locality of Mazatlán, Sinaloa.

Crabs were placed individually in 10 L glass aquaria equipped with infrared light crossings distributed at the bottom of each aquarium (Miranda-Anaya et al. 2003). Light regime was controlled by independent timers in LD cycles 12:12 (07:00–19:00 h). LD cycles were set with white light lamps (Phillips, 200 lx) located 30 cm above the aquaria.

During the assays, water parameters were controlled (25 ± 1°C. 35 ± 1 psu, pH 8.05 ± 0.05, and DO higher than 5.2 mg/L) and 50% of the artificial marine water was replaced daily adjusting the respective water ammonia concentrations. Every other day each crab was provided with 0.1 g of *Artemia salina* pellets.

Locomotor activity recordings

Crab locomotor activity was recorded individually in each aquaria equipped with infrared light crossings and was considered as the number of beam crossings each 10 min according to Miranda-Anaya et al. (2003). Recordings were made in a controlled environmental room of the Facultad de Ciencias facilities at 25 ± 1°C. Data were captured in a PC with a data acquisition program (NAFRI S.A., México) and stored until analysis. Cyclic properties of data were analyzed using double-plotted actograms and χ^2 periodograms (Sokolove and Bushell 1978); period values were significant when its amplitude was above the confidence limit of $p = 0.05$, denoted as a diagonal line in each periodogram. Average waveforms of activity fitted to a dominant period were obtained by means of DISPAC software (IFC-UNAM).

The length of activity (α), its phase onset and relationship with tides (ψ), and the activity rest ratio relationship (α/ρ) were calculated for each cycle during, at least, 7 days of steady state rhythms. Data were plotted separately for diurnal tide (DT) and nocturnal tide (NT). Phase relationship values during different ammonia conditions were compared using Student's *t* test for independent samples (software Statistica: Jandel Scientific, San Rafael, CA, USA). Significant results were considered when $p < 0.05$.

Results

Ammonia acute toxicity

No mortalities were observed in the control groups or in those exposed to concentrations below 18 mg of N-TA/L. The ammonia median lethal concentration (24 h-LC_{50}) was calculated in 186.02 mg N-TA/L, corresponding to 176.8 mg N-NH_4^+/L and 9.2 mg N-NH_3/L, respectively.

Effect of sublethal exposure to ammonia upon locomotor activity in crabs maintained in artificial LD and tidal cycles

During 15–20 days of chronic exposure to ammonia (3.8 and 18 mg N-TA/L) no mortalities were registered. Three representative actograms of animals, control group, and crabs exposed to the ammonia sublethal concentrations (3.5 and 18 mg N-TA/L) are shown in Figure 1, as well as their correspondent periodograms. Black bars above each actogram represent the LD conditions, and gray shadows, along the right side of each actogram, represent the artificial high tide.

The actogram as an example of control group is shown in Figure 1a. The onset of locomotor activity of each main bout was present close the ebb and seems not to be affected by the LD cycle. The bout associated with the NT was notably larger than the one associated to DT. Locomotor activity was recorded along 15 days and only one animal showed fading of tidal activity by the end of the experiment (not shown). Average period values (±standard error, SE) of 24.7 ± 0.14 h and a tidal period of 12.13 ± 0.09 h were also present.

Figure 1b depicts a typical record of the locomotor activity of a crab exposed to 3.5 mg of N-TA/L during 18 days of recording. In this group the main bout of activity was present during the diurnal phase of the LD cycle, always coupled with DT. Nocturnal bout of activity was also associated to NT; nevertheless, it was often short and unstable during the days of recording. A particular observation in all the animals used in this experimental group was the fading of the activity when the high tide was present during the middle of the photophase. Each bout reappears when the high tide coincides with the lights on. All the organisms in this experimental group presented the above mentioned pattern of activity; average period values (±SE) were 23.76 ± 0.3 h and 11.86 ± 0.13 h.

Figure 1c shows a representative 20 days' actogram of a crab exposed to 18 mg N-TA/L. The length of the activity bouts along all the recording was notably increased in both nocturnal and diurnal phases. About the 10th day, a lack of stability of each component was observed regarding the tides and arrhythmic activity

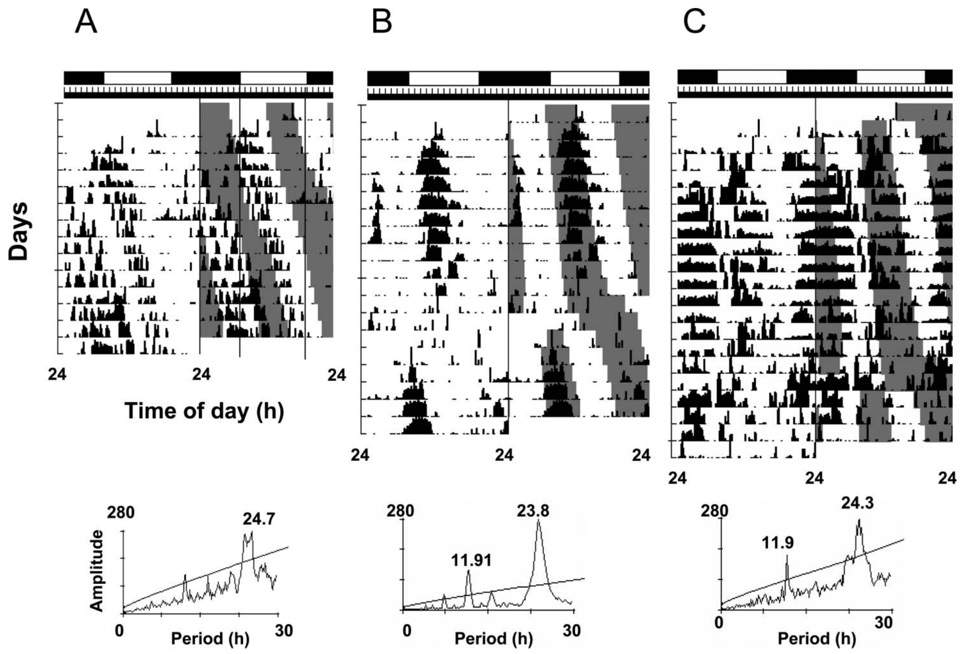

Figure 1. Actograms of locomotor activity in *Uca princeps* exposed to artificial tides (gray areas in right side of each graph) and LD cycles (upper bars). Crabs of the control group (a) displayed maintained rhythmicity entrained to ebb cycles along light and dark cycles, whereas in crabs exposed to 3.5 mg (b) and 18 mg (c) N-TA/L it was mainly maintained during the high tide. Periodograms below each actogram show main period values for each example.

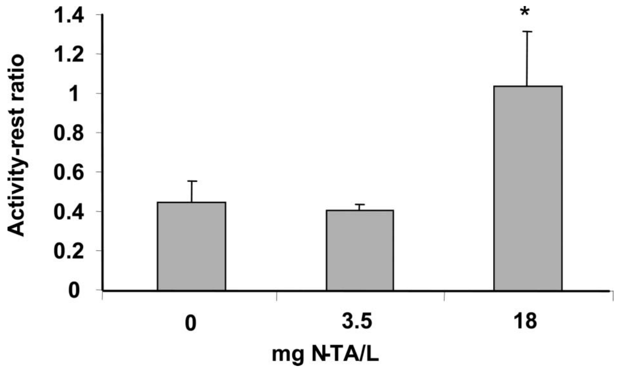

Figure 2. Activity rest ratio (α/ρ) for each tidal cycle in control groups shows no differences compared with organisms exposed to 3.5 mg of N-TA/L; α/ρ is higher in the group exposed to 18 mg N-TA/L. Statistical significance (Student's *t* test, $p < 0.05$) between groups is indicated with stars.

was noted during the last 4–6 days of the experiment in all the animals tested in this group. Period values were calculated using days of stable rhythms, usually from 3rd to 10th day. Average period of activity ($\pm$SE) in this experimental group was 23.64 ± 0.53 h and 11.55 ± 0.33 h.

Activity rest ratio (α/ρ) and phase relationship of tidal rhythm

Activity rest ratio was considered by using at least 7 days of steady state rhythms. Figure 2 presents the average (±SE) of α/ρ for both components of activity during entrainment. A tendency to decrease the length of the activity bout was noted in a group exposed to 3.5 mg, as compared with controls, and it increased almost twice when animals were exposed to 18 mg of N-TA/L (t test, $p < 0.01$), which indicates a higher level of activity related with its correspondent resting period.

Onset of activity and artificial tide regimen

Figure 3 shows the average values in hours (±SE) of the onset of activity regarding the onset of NT and DT (zero in each ordinate). Significant differences were noted among control and experimental groups ($p < 0.05$). Control group displayed activity during the ebb, in average (±SE) 6.7 ± 0.611 h after the onset of DT and 6.04 ± 0.316 h after NT, mainly at the beginning of the ebb (t test, $p < 0.05$). Crabs exposed to 3.5 mg of N-TA/L displayed different onsets of activity anticipating the onset of each tide (DT = -0.80 ± 0.20, NT = -2.37 ± 0.98 h; t test, $p < 0.05$). Crabs exposed to 18 mg of N-TA/L presented a similar tendency than the previous group (DT = 0.65 ± 0.35 h, NT = 0.77 ± 0.86 h; t test, $p < 0.001$).

Discussion

Ammonia acute toxicity

U. princeps at Estero de Urias, Sinaloa, is frequently exposed to high ammonia levels due mainly to shrimp farming and intensive agriculture activity as well as industrial and urban wastes (Paez-Osuna et al. 1998, 1999; Hernandez-Cornejo and Ruiz-Luna 2000; Ruiz-Luna and Berlanga-Robles 2003). This exposition takes about 24 h to be washed out by natural water circulation, particularly during lunar crescent or wane. However, the continuous elevated nitrogen input can exert severe impacts on the structure and function of the coastal biological systems (Paez-Osuna et al. 1999;

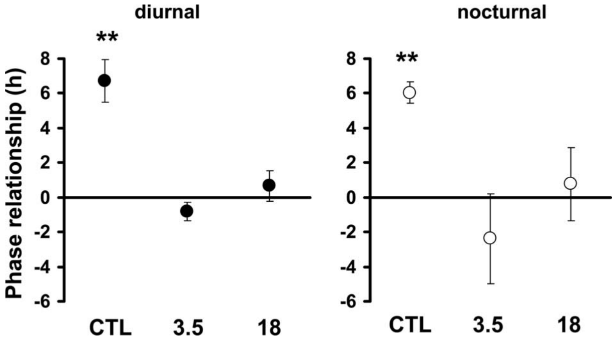

Figure 3. Average onset of activity (±SD) in *Uca princeps* of control group (CTL; 0.125 mg N-TA/L) and exposed to ammonia (3.5 and 18 mg N-TA/L). In each graph zero in ordinate indicates the onset regarding diurnal and NT. Both groups exposed to ammonia shifted their onset around the beginning of the high tide. Stars indicate statistical differences ($p < 0.001$) between control and ammonia exposed crabs.

Clavier et al. 2005; Camargo and Alonso 2006; Romano and Zeng 2007b, 2010). The 24 h-LC_{50} ammonia value obtained in *U. princeps* (186.02 mg N-TA/L) is higher than the 96 h-LC_{50} values reported for juveniles of *Scylla serrata* (95.35 mg N-ammonia/L; Romano and Zeng 2007a) and *Portunus pelagicus* (43.4–75.2 mg N-ammonia/L; Romano and Zeng 2010) but in the range of the 96 h-LC_{50} values reported for the Brazilian estuarine crab *Chasmagnatus granulata* adults of 141.50–250.07 mg N-ammonia/L (Rebelo et al. 1999). Obviously the ammonia tolerance differences are related to biotic (i.e., age and nutritional status of experimental organisms) and abiotic parameters (i.e., exposure period, salinity, temperature, pH) as well as the particular habitats of the species. However the relatively similar LC_{50} value obtained in *U. princeps* but exposed a shorter period to ammonia than *C. granulata* suggests that the former species is less tolerant to the toxic effect of ammonia than the latter.

The ammonia sublethal concentrations used in this study to evaluate the chronic exposure effects on *U. princeps* locomotor activity (3.5 and 18 mg N-TA/L), are equivalent to the 24 h-LC_2 and 24 h-LC_{10}, concentrations that are ecologically relevant, since they are frequently recorded in the distribution area of the species (*in situ* measurements) and can be associated with several toxicological and ecological effects in the coastal and estuarine environments (Clavier et al. 2005; Camargo and Alonso 2006).

Locomotor activity rhythms

The fiddler crab *U. princeps* is a suitable model to study circatidal activity coupled to artificial tides. We corroborate in our lab that circatidal endogenous rhythmicity in free running is also present in fresh field collected animals. However, they become easily arrhythmic while spending long-term period of acclimation and recording period (unpublished data). Locomotor activity in the same species collected in San Blas, México, and maintained in LD and no tides displayed a bimodal rhythm where one component is delayed when the equatorial tide should occur (Stillman and Barnwell 2004). Also in Estero de Urias, Mazatlán, tides shift between one and two peaks per day regarding the declinational cycle; therefore the structural pattern of tides and locomotor activity could be similar. The present work includes artificial tides using the tide pattern predicted by the CICESE in order to preserve the tidal activity pattern present in the field, since in this species, the tidal activity profile has an important relationship according to their habitat (Stillman and Barnwell 2004; Thurman 2004).

Different species of *Uca* have been studied in order to understand the regulation of circatidal activity (Honeger 1976; Lehman 1976). Data collected from *Uca subcylindrica* indicate that circadian or circatidal rhythms are observed regarding the light conditions and in species located far from tidal influence (Thurman 1998; Thurman and Broghammer 2001). When the crabs were subjected to variable photoperiod, they changed their activity accordingly with day length, suggesting that seasonality in locomotor activity is regulated by two clocks with circadian properties more than circatidal.

Physiological mechanisms of circatidal regulation are not clear. The nature of circatidal rhythm regulation in crabs has been discussed in the context of the presence of circadian or circatidal oscillators (Naylor 1997). The fact that some *Uca* species exposed to tides show both circadian and circatidal rhythms (Thurman and Broghammer 2001; Thurman 2004) and that the two intervals of activity sometimes show different periodicities indicates that activity in these species may be controlled by two independent oscillators. Circadian rhythms in crabs are regulated by neuroendocrine system (for review see Strauss and Dircksen 2010); however, physiological basis for circatidal control has yet to be elucidated.

It is possible to suggest that in *U. princeps*, the strength of coupling among tidal and circadian oscillators may be affected by the toxicity of ammonium. Strength of coupling among oscillators may change the onset of activity (Pittendrigh and Daan 1976). Therefore different phase relationships regarding high tide may be an indicator that ammonia might affect the coupling between circadian and circatidal oscillators in *Uca*. Considering that maybe a circadian component is also regulating the activity rhythm in *Uca*, an interesting relative correlation was observed in animals exposed to 3.5 mg N-TA/L, where strong diurnal bout of activity is faded when the high tide is present in the second half of the photophase. This behavior, however, was not clearly observed on the control group.

At 18 mg of N-TA/L, locomotor activity is intense and the daily/tidal rhythms fade in noisy bouts of activity after 10 days of chronic exposure to ammonia. This behavior is consistent with the evaluation of the activity rest ratio (Figure 3), which indicates that high sublethal concentrations of ammonia may have an opposite effect than low doses, in which ammonia might exert a narcotic effect.

Field studies are needed to confirm that the behavior changes observed in laboratory conditions are expressed in the natural environment, where the high ammonia sublethal concentrations are present in the crab habitat. Moreover, whereas ammonia directly or indirectly is affecting endogenous circadian and circatidal oscillators controlling biological rhythms and the mechanisms underlying it at the cellular and molecular levels is a matter for further studies.

Considering the biological rhythm properties as an output of the way in which the environmental changes tune biological clocks, there should be an additional indicator that can be taken into account for environmental changes produced by human activities.

Acknowledgments

This research was supported by the grants of Departments of Ecology and Natural Resources as well as Cell Biology at Faculty of Sciences of the UNAM. We would like to thank the Unidad de Análisis Ambiental and M. en C. Sebastián Zúñiga Lagunes for their technical assistance during the experiments. We would also like to thank the technical assistance of Hipólito Olague Sifuentes for his assistance (CIAD, Mazatlán). We also thank Ma. Luisa Arias for partial financial support to E. Azpeitia.

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