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ORIGINAL RESEARCH

Tolerance response to ammonia and nitrite in hatchlings paralarvae of *Octopus vulgaris* and its toxic effects on prey consumption rate and chromatophores activity

Paloma Feyjoo · Rodrigo Riera · Beatriz C. Felipe · Alí Skalli · Eduardo Almansa

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Abstract Ammonia and nitrite are among the most important water quality parameters. The aim of this study was to determine the acute toxicity of unionized ammonia and nitrite on newly hatched *Octopus vulgaris* paralarvae. Concretely, survival, feeding and chromatophore activity were examined under different NH_3 and NO_2^- concentrations. The median lethal concentration (LC 50) determined for 24 h was 10.7 ppm for NH_3 and 19.9 ppm for NO_2^-. Based on these results, a concentration range was chosen to test the effect of this environmental contaminants in feeding and chromatophore activity (range between 2.5 and 20 ppm for feeding and 10 and 30 ppm for chromatophore activity), resulting in modifications in both parameters. In fact, a significant decrease ($P < 0.05$) in *Artemia* nauplii consumption by the paralarvae was observed with an increase in NH_3 and NO_2^-. Similarly, the chromatophore activity was also affected by concentration of these contaminants, with decreasing response ($P < 0.05$) when submitted to stressful situations, as the ammonia and nitrite concentration was increased.

Keywords Ammonia · Chromatophore activity · Nitrites · Nitrogen · *Octopus vulgaris* · Paralarvae · Predation

P. Feyjoo
Department of Marine Biology, School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth, Devon PL4 8AA, UK

R. Riera
Centro de Investigaciones Medioambientales del Atlántico (CIMA SL), Arzobispo Elías Yanes, 44, La Laguna, 38206 Santa Cruz de Tenerife, Canary Islands, Spain

B. C. Felipe · E. Almansa (✉)
Instituto Español de Oceanografía, Centro Oceanográfico de Canarias, Avenida 3 de Mayo 73, Apdo. 1373, 38005 Santa Cruz de Tenerife, Canary Islands, Spain
e-mail: eduardo.almansa@ca.ieo.es

A. Skalli
Unidad de Cultivos Experimentales, Centro de San Carlos, Instituto de Investigación y Tecnología Agroalimentarias (IRTA), Crta. Poble Nou km. 6. San Carlos de la Rápita, 43540 Tarragona, Spain

Introduction

Environmental changes may impact upon various biological processes of an organism (Pierce et al. 2006). Slight changes in water quality for instance, are likely to influence mortality rate, especially during the early stages of development (Parra and Yúfera 1999). Some substances excreted by living organisms, such as those produced by metabolism, might accumulate up to the point of resulting poisonous for them. Ammonia is the major end product in the breakdown of proteins and is excreted by aquatic invertebrates, among other groups. Total ammonia nitrogen (TAN) in seawater is present in two forms: unionized ammonia (NH_3) and ionized ammonia (NH_4^+), the former being more toxic than the latter (Meade 1985). The ratio of these two forms is a function of pH, temperature and salinity (Bower and Bidwell 1978; Chen and Chin 1989; Durborow et al. 1997). Ammonia induces the start of the nitrogen cycle. This takes place through oxidation by bacteria and results in the formation of nitrite (NO_2^-). Thus, whenever ammonia levels are elevated, a rise of nitrites will soon follow. Although, unlike ammonia, nitrite is not a severe toxicant, its concentration increases in cases of failure of water treatment in closed systems (Parra and Yúfera 1999; Menasveta et al. 2001), and it may also be followed by the organism's poisoning (Brownell 1980; Rodrigues et al. 2007).

As short-lived species, cephalopods are highly vulnerable to environmental changes (Pierce et al. 2006). Specifically, octopuses are particularly affected by ammonia excretion, since its production has been found to be much higher within this family (Vaz-Pires et al. 2004). For instance, cephalopods excrete between two and three times the amount of ammonia per kilogram of body weight compared with fish (Lee 1994). Boletzky and Hanlon (1983) have previously reviewed tolerance levels of nitrogenous waste in cephalopods. These authors determined 15 ppm of nitrite as the 96-h medium tolerance limit in *Octopus joubini* hatchlings.

This study focuses on the effects of unionized ammonia and nitrites on survival, feeding and chromatophore control. A decline in prey consumption (Artemia) was expected as a result of an increase in the pollutant concentration. Regarding the capacity of octopus of change colour at will, because it is under direct neural control (Wells 1978), any abnormality in their activity might be attributed to an alteration in the nervous system. Ammonia has both excitant and depressant effects on the nervous system (Jack 1982). Considering the effects of ammonia and nitrites on other species' nervous response (e.g. Foss et al. 2003), an alteration in skin colour display was expected in individuals exposed to the aforementioned nitrogenous pollutants.

Materials and methods

Octopus vulgaris eggs

This study was carried out at the culture facilities of the Spanish Oceanographic Institute in Tenerife (Canary Islands) during July and August 2008. *Octopus vulgaris* eggs were obtained by natural spawning from three different captive adult females, each of which was kept in a 1-m^3 tank with constant water flux of 120% per hour and air supply. Temperature was maintained at 23°C, and pH ranged between 7.9 and 8.2. Clay bowls inside the water tanks served as shelter for the animals.

From the first hatching (July, 9), paralarvae were collected by siphoning and transferred into a 1 × 0.5 × 0.5-m rectangular white plastic bucket with air supply, from which the

desired number of specimens for experimentation was collected. After every collection, the tanks containing the adult animals were drained of remaining paralarvae, in order to ensure that the following collection would aim on 0-day-old paralarvae. The process was repeated once per day. Dry weights of paralarvae from the three females were determined along the spawning season to assure a similar size of experimental paralarvae, obtaining an average weight of 0.26, 0.26 and 0.30 mg.

All adults and paralarvae were handled according to welfare considerations (see Moltschaniwskyj et al. 2007). Animals were sacrificed by means of hypothermic shock (0°C water) to minimize their pain.

Preparation of the solution and previous assays:

The solutions used for NH_3 and NO_2^- were ammonium chloride and sodium nitrite, respectively. The calculations were made as follows:

- $NH_4Cl = 53.49$ g/mol, so $NH_3 = 18$ g. So forth, in order to prepare a solution of 10 g NH_3, 29.7 g NH_4Cl were added to a litre of distilled water. The desired concentration of NH_4Cl for each different analysis was taken from this initial stock solution and diluted in one litre of filtered seawater per treatment (100 g/l SW is 1 mg/ml = 1 ppm).
- $NaNO_2 = 69$ g/mol, so $NO_2^- = 15$ g. Thus, in this case, 15 g of $NaNO_2$ were added to a litre of distilled water to prepare a 10 g NO_2^- solution. Likewise as for ammonia, the desired concentrations of NO_2^- were taken from this initial stock solution and diluted into one litre of filtered sea water.

Evaluation of tolerance to ammonia and nitrite

The limits of *Octopus vulgaris* paralarvae's tolerance to the effects of non-ionized ammonia and nitrite were determined by a series of preliminary exposure experiments. We selected freshly hatched (0-day old) paralarvae in order to obtain a homogeneous pool of specimens with similar condition. Given that hatchling paralarvae could survive at least 2–3 days without feeding (Quintana 2010; Villanueva and Norman 2008), we did not supply any prey during experimental period. A short exposure period (24 h) was selected to assure minimum changes on paralarval condition non-related with experimental treatments. Twelve different concentrations of NH_3 (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 55, 70, 75 ppm) were used. Experiments were made in 2 l plastic bottles with air supply and filled up to 1 l with filtered sea water, plus the determined concentration for each. Each treatment consisted of three replicates, each of which contained 25 larvae during a 24-h exposure period. Before the addition of the pollutant, the organisms test were left for 1 h to allow them acclimatize. Temperature ranged between 22 and 25°C, pH ranged between 7.9 and 8.2 (Meter–Hanna pHep Plus), and salinity was constant at 35 PSU. Light intensity was averaged 230 lux throughout the day. Larvae condition was determined at the end of the experiment according to their swimming ability. Swimming larvae were considered alive, while those at the bottom, even if moving, dead. Anyway, should be taking into account that several specimens that were considered live after 24 h could be died after 48 h due to experimental treatments. However, this effect could not be determined in this experiment and should be tested in further experiments. The exact same experiment was carried for 12 concentrations of NO_2^- (0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ppm). The experiments were repeated daily as follows: three different concentrations plus the control were

prepared per day, each with its correspondent replicates. After 24 h, the paralarvae found alive were counted and the mortality rate determined. Then, the next experiments were set up for the next three concentrations. The median lethal concentration (LC 50) was determined from a linear regression of the probit of mortality percentage against logarithmic concentrations (Abel and Axiak 1991). The LC 50 was then taken into consideration for the choice of concentrations used in the rest of experiments.

First-feeding

Before the start of this experiment, *Artemia* nauplii tolerance to ammonia and nitrite was tested by exposing 20 specimens to the highest concentrations planned to be used upon the feeding assays (50 and 30 ppm for NH_3 and NO_2^-, respectively). The total exposure period was of 24 h, after which death rate was measured by direct counting. Previous studies (Iglesias et al. 2006) stated that *O. vulgaris* paralarvae start capturing prey from the first day of their life, increasing their captures at 2-day old. In the present experiment, hatchlings (0-day old) paralarvae were selected according to the reasons exposed in the previous section although they were previously tested to assure a significant capture rate.

In order to study food consumption by paralarvae of *O. vulgaris*, methodology described by Márquez et al. (2007) was used with slight modifications. Each paralarvae was deposited in a volume of 100 ml, without renovation, with 0.1 *Artemia*/ml (on-grown *Artemia*, 1.5–2 mm) and lower concentrations for NH_3 (2.5, 6, 12, 20 ppm) and NO_2^- (15, 20, 30). After 24 h, the number of non-consumed *Artemia* was counted, and the consumption percentage was calculated. Artificial illumination was maintained overnight, because octopuses are visual predators (Guerra 1978) and hence the number of prey captures was lower in the dark (Márquez et al. 2007). Anyway, other sensory system components have been described in *Octopus* paralarvae (Villanueva and Norman 2008), and their role in prey capture should not be discarded. With this method, some paralarvae were also dead after the exposure period. Every test where the larva was found dead or moribund was repeated.

Chromatophoric activity

In order to examine the differences in skin colour in relation to the background tone, a previous minor experiment was carried out. A total of 400 specimens were used for this test. The paralarvae were separated in two groups and placed in two different buckets, one of them white and the other black, both of $1 \times 0.5 \times 0.5$ m and filled up with filtered sea water to a depth of 0.25 m. After 1 h for acclimatization, the proportion of pale and dark paralarvae was determined.

The chromatophore activity was measured by direct observation according to changes in colour. A total of 200 paralarvae were exposed to each concentration of ammonia (3, 6, 12 and 20 ppm) and nitrite (10, 20, 30 ppm) in 800-ml glass beakers for 24 h. The time taken to recover the initial state of transparency after being absorbed up with a pipette (which makes the paralarvae become dark brown) and released again after 2 s into the same beaker was recorded by direct observation. Each paralarvae was removed from the beaker after this procedure.

Statistical analysis

The 24-h LC 50 values were calculated from the regression probit module (Abel and Axiak 1991). Kolmogorov-Smirnoff test was achieved to confirm normal distribution. Levene's

test for the homogeneity of variances was applied to confirm the possibility of assumption for this equality. Data, presented as mean ± SD, were analysed for statistical differences among groups by one-way ANOVA or by Kruskal–Wallis one-way ANOVA on ranks if data complied or not with normality and homogeneity of variance, respectively. In the first case, ANOVA was followed by Student–Newman–Keuls (SNK) while in the second by Dunn's multiple-comparison procedures, both with a significance level (P) of 0.05. To all data expressed as percentage, arcsin transformation was applied directly (Fowler et al. 2002). All statistical analysis were done with $\alpha = 0.05$ using SPSS v. 16.0.

Results

Tolerance response and mortality

Unionized ammonia and nitrite had a clearly negative effect on paralarvae survival. Tolerance response to unionized ammonia and nitrite is illustrated in Fig. 1a and b, respectively. Mortality showed a tendency to increase with increasing exposure concentrations.

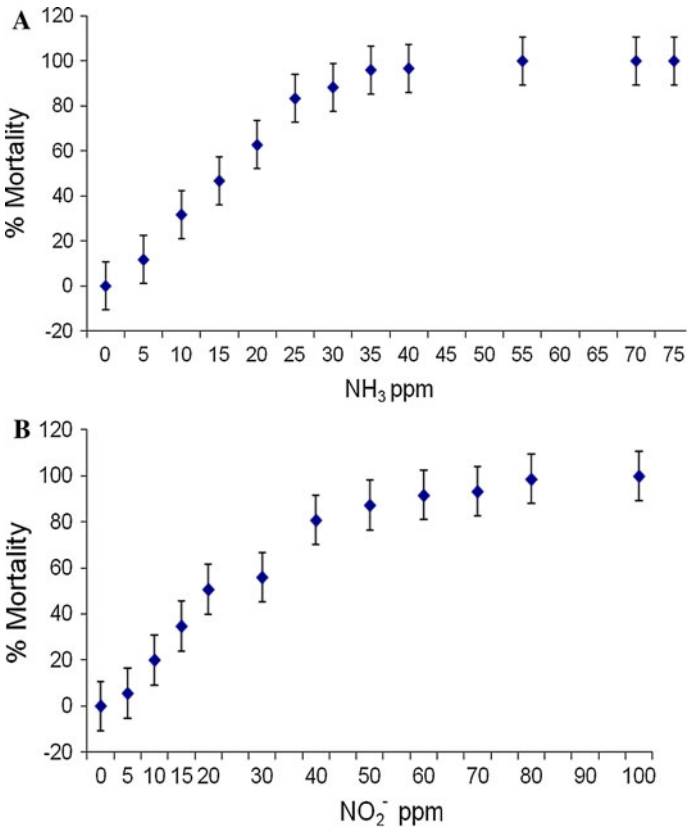

Fig. 1 Mortality percentage ± SD of *Octopus vulgaris* paralarvae after 24-h exposures to different concentrations of non-ionized ammonia (**a**) and nitrite (**b**)

Total mortality was reached at 45 ppm NH_3, whereas a concentration of almost double that amount was required for NO_2^-.

The 24-h LC 50 values calculated from the estimates of the probit test were 10.7 ppm NH_3 and 19.9 ppm NO_2^-. Probit plot against log-10 transformation of concentration is shown in Fig. 2. (NH_3) and b. (NO_2^-). Pearson goodness-of-chi-square test from the probit analysis revealed that the significant level was less than 0.150 for NH_3 data, so a heterogeneity factor was used in the calculation of confident limits. In the case of NO_2^-, the chi-square was non-significant ($P > 0.150$), hence a heterogeneity factor was not used, and model fit is accepted as adequate. Confidential limits were calculated using a value of 1.96.

Effect of NH_3 and NO_2^- on feeding

A decrease in *Artemia* consumption by the paralarvae was observed with an increase in NH_3 and NO_2^- concentration, falling from an average of 51.7% (±17.22) of *Artemia* nauplii consumed on the control, to 7% (±21.91) at 20 ppm of NH_3 (Fig. 1a) and from 60 (±12.65) to 10% (±10) at 30 ppm NO_2^- (Fig. 1b). Levene's test revealed the existence of homogeneity of variances within the data ($P > 0.05$) on all analysis, thus this assumption has been made accordingly. Levene test statistic is 2.656 for NH_3 and 0.650 for NO_2^- (Figs. 3 and 4).

One-way ANOVA conducted on the data for unionized ammonia provided the evidence of a statistically significant difference between the means of prey consumption percentages of octopus paralarvae ($P < 0.05$; Fig. 3). However, multiple comparisons with Tukey Post-Hoc results indicated that the only significantly different outcome was significant between the control and 20 ppm.

In the test for consumption under nitrite exposure, One-way ANOVA provided overwhelming evidence of statistical difference of prey consumption of octopus paralarvae ($P = 0.003$; Fig. 4). Further multiple comparisons (Tukey HSD) resulted in significantly different outcomes between control means and each treatment, all of which were highly significant.

Chromatophore activity

The background chosen as the basis of the preliminary skin colour assays was clear, as under these conditions, homogeneous transparent skin coloration was exhibited among paralarvae. This was taken as the reference normal colour before inducing the stimulus.

The time for the chromatophores to return to the relaxed transparent state after the darkening display (resulted from the stimulus), clearly decreased with increasing ammonia concentration. Figure 5 illustrates this negative trend in chromatophore activity (time) against the increase in NH_3 concentration. Levene test statistic obtained was 0.656, and the assumption of variances' homogeneity ($P > 0.05$) was done. One-way ANOVA analysis revealed significant statistical difference in mean time taken to paralarvae to return to the relaxed state under the different treatments. Moreover, differences appeared to be significant in all pairings (control treatments and among treatments), except when comparing 12 and 20 ppm (Fig. 5).

The same pattern was observed from the error bar graph for nitrite (Fig. 6). Levene test was non-significant ($P > 0.05$), so the assumption of equal variances was accordingly met. One-way ANOVA revealed strong evidence of significant differences ($P < 0.05$) among all comparisons except one: 20–30 ppm ($P > 0.05$).

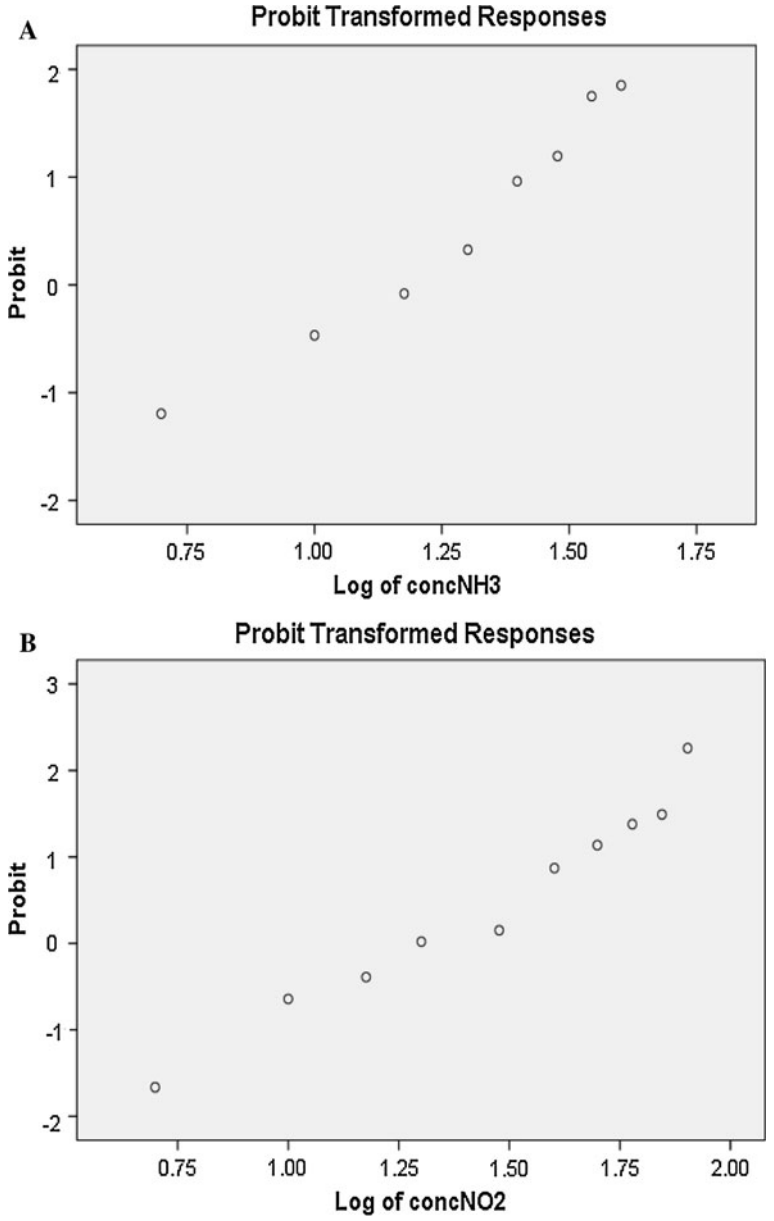

Fig. 2 Regression plot obtained by log-10 transformation of non-ionized ammonia (**a**) and nitrite (**b**) concentrations against probit transformation of mortality percentage

Discussion

The toxicity of environmental nitrogen has been demonstrated for fish (Brown and McLeay 1975; Meade 1985; Durborow et al. 1997) and other marine animals such as molluscs and crustaceans (Chen and Chin 1989; Chen et al. 1990). In all cases, nitrogenous compounds

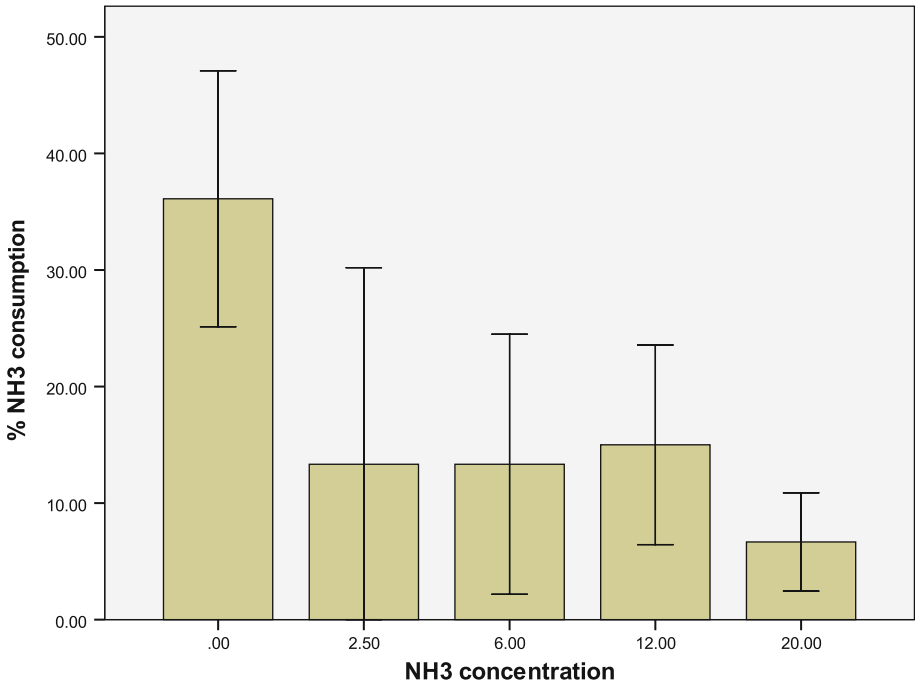

Fig. 3 Mean *Artemia* consumption percentage ± SD of 0-day-old *O. vulgaris* paralarvae at different concentrations of non-ionized ammonia

have been reported to be toxic when certain levels are exceeded. Pollutants accumulate in the tissues of the organism up to an extent of exerting toxic effects (Abel and Axiak 1991). This point when the compound becomes toxic is dependant of the concentration. The reduction in *O. vulgaris* consumption of prey with an increase in NH_3 and NO_2^- levels can be translated as a loss of appetite due to the environmental toxicity to the extent of stopping feeding.

Larval response to ammonia (NH_3) and nitrite (NO_2^-) was analysed in 24-h tests. Octopus paralarvae appear to be highly sensitive to ammonia and in a lesser extent, to nitrite. The 24-h LC 50 value for NH_3 (10.7 ppm) in octopus paralarvae observed in this study was higher than those recorded for *Sparus aurata* larvae (0.28 ppm; Parra and Yúfera 1999) and juvenile sea bass (0.54 ppm; Weirich and Riche 2006).

The results obtained for NO_2^- in the present study indicate that octopus paralarvae are very sensitive to nitrites concentration, showing a 24-h LC 50 value of 19.9 ppm. This values are lower than those described for *Penaeus chinensis* juveniles (339 ppm 24-h LC 50 and 37 ppm 96-h LC 50 according to Chen et al. 1990) and has strong differences with values described by Brownell (1980) and Parra and Yúfera (1999) in different marine fish larvae species with 24-h LC 50 values ranged between 1.000 and 2.700 ppm. Nitrite toxics effects are related with depression effects of oxygen affinities in respiratory pigments such as haemoglobin and haemocyanin, which have been extensively described in *Octopus vulgaris* haemocyanin by Salvato et al. (1989). Fish larvae do not show respiratory pigments until several days after hatching (Rombough 1988); however, in cephalopods, haemocyanin presence has been detected from embryonic stage in *Sepia officinalis* (Decleir et al. 1971) and has been suggested in *O. vulgaris* hatchlings (Villanueva and Bustamante 2006). Therefore, these data suggest that the high sensitivity of *O. vulgaris*

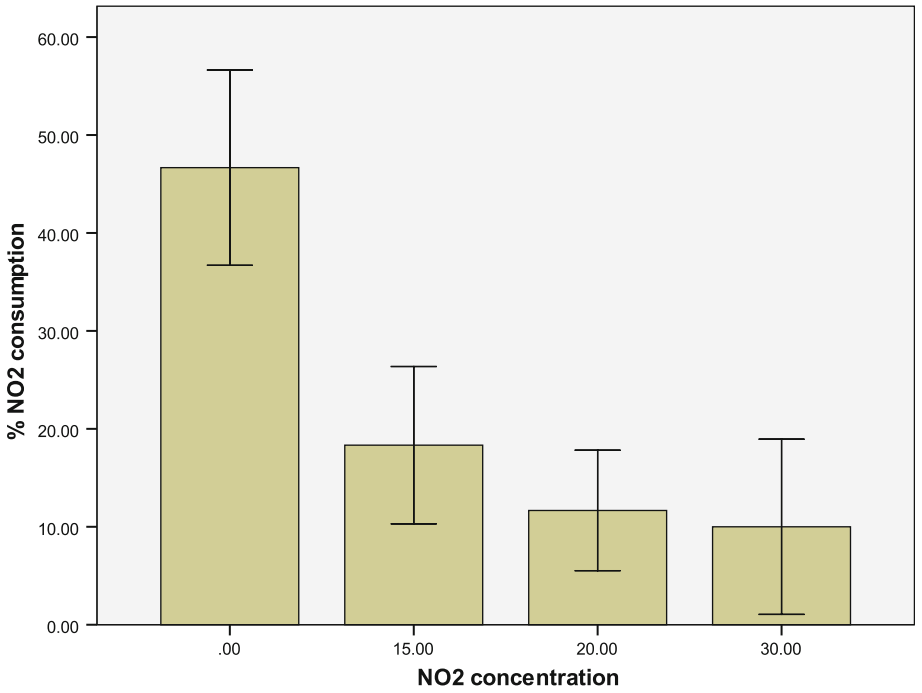

Fig. 4 Mean *Artemia* consumption percentage ± SD of 0-day-old *O. vulgaris* paralarvae at different concentrations of nitrite

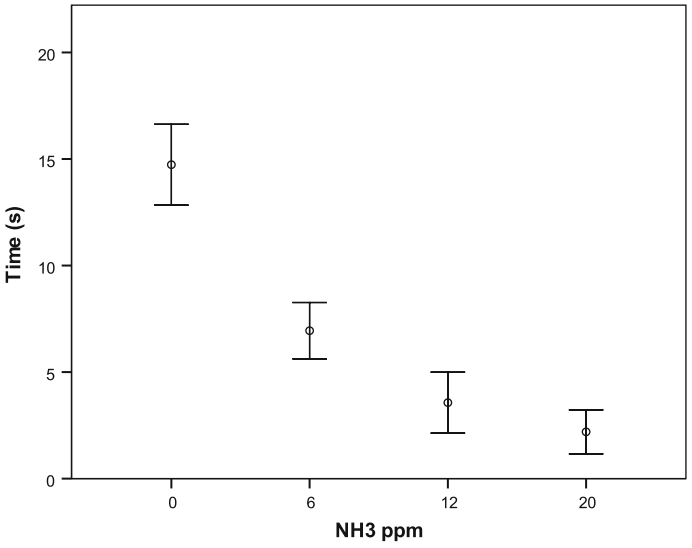

Fig. 5 Mean times (s) ± SD taken in 0-day-old *O. vulgaris* paralarvae chromatophores to relax after stimuli for the different concentrations of non-ionized ammonia

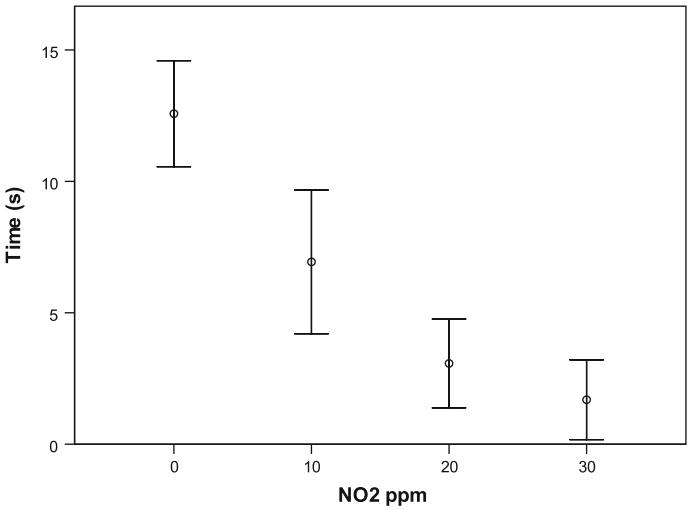

Fig. 6 Mean times (s) ± SD taken in 0-day-old *O. vulgaris* paralarvae chromatophores to relax after stimuli for the different concentrations of nitrite

paralarvae to nitrites concentration could be related to its effect on oxygen transport, which could be critical in a high metabolic species like *O. vulgaris.* Anyway, other toxic effects of nitrite related with different oxidation processes should not be discarded (Colt and Armstrong 1981). Finally, is remarkable the lower toxicity of nitrite *vs*. non-ionized ammonia (Bianchini et al. 1995), which agrees with the results obtained in this study.

There is a need to further analyse the effects of paralarvae predation. In addition, although the trends found look fairly clear, it would be suitable to complete gaps by testing concentrations not included in the analysed ranges. For instance, there is a large decline in prey consumption from the control to the lower concentration (2.5 ppm NH_3 and 15 ppm NO_2^-). This needs to be investigated to a bigger extent, testing for larger ranges of concentrations. Moreover, it would be appropriate to use exposure periods longer than 24 h, as this might be undermining the results of the concentrations.

One of the objectives of the present study was to determine the acute toxicity of NH_3 and NO_2^- in chromatophore activity of octopus paralarvae. Each chromatophore consists of an elastic bag full of pigment granules, surrounded by muscular filaments that are enervated by nervous system. Hence, colour change is entirely under neuromuscular control, directly from the brain (Wells 1978). The chromatophore activity was clearly reduced under exposure to NH_3 and NO_2^-. This suggests that these compounds affect the animal's nervous system and their available energy, resulting in a drop on their ability to maintain the respond to stimuli, becoming generally less responsive.

Solutions with ammonia and ammonium chloride in body fluids are crucial for control buoyancy in cephalopods according to Pörtner and Zielinski (1998). Ammonium chloride is stored within fluid-filled cavities, which are found in the muscles and thus can be determined the mechanisms of failure of chromatophore activity as a response to exposure to high concentrations of ammonia. Although it is known that pH within these ammonium-containing cavities is very low and hence great part of the NH_3 is converted into NH_4^+ (Voight et al. 1994), however, it is unknown whether this is related to tolerance to NH_3 in the chromatophore activity.

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