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Acute exposure to high environmental ammonia (HEA) triggers the emersion response in the green shore crab

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ABSTRACT

The physiological effects of high environmental ammonia (HEA) exposure have been well documented in many aquatic species. In particular, it has recently been demonstrated that exposure to ammonia in fish leads to a similar hyperventilatory response as observed during exposure to hypoxia. In littoral crabs, such as the green crab (*Carcinus maenas*), exposure to severe hypoxia triggers an emersion response whereby crabs escape hypoxia to breathe air. We hypothesized that exposure to HEA in green crabs would lead to a similar behavioural response which is specific to ammonia. Using an experimental arena containing a rock bed onto which crabs could emerse, we established that exposure to HEA (4 mmol/l NH_4HCO_3) for 15 min triggers emersion in crabs. In experiments utilizing $NaHCO_3$ controls and NH_4HCO_3 injections, we further determined that emersion was triggered specifically by external ammonia and was independent of secondary acid-base or respiratory disturbances caused by HEA. We then hypothesized that emersion from HEA provides a physiological benefit, similar to emersion from hypoxia. Exposure to 15 min of HEA without emersion (no rock bed present) caused significant increases in arterial haemolymph total ammonia (T_{amm}), pH, and [HCO_3^-]. When emersion was allowed, arterial haemolymph T_{amm} and [HCO_3^-] increased, but no alkalosis developed. Moreover, emersion decreased haemolymph partial pressure of NH_3 relative to crabs which could not emerse. Overall, we demonstrate a novel behavioural response to HEA exposure in crabs which we propose may share similar mechanistic pathways with the emersion response triggered by hypoxia.

1. Introduction

Ammonia is the primary nitrogenous waste produced by amino acid and protein metabolism and must be eliminated from the body due to its toxic effects at elevated levels. In solution, ammonia is present in equilibrium as ionic NH_4^+ and gaseous NH_3 with a reaction pK between 9.0 and 9.5; therefore, the majority of ammonia exists as NH_4^+ at physiological pH. In most aquatic organisms, ammonia gas is simply diffused into the surrounding environment and as such, NH_3 is considered to be the third respiratory gas in ammonotelic fish species (Cameron and Heisler, 1983; Randall and Ip, 2006). In aquatic crabs, ammonia is excreted across the gills primarily via the combined actions of a basolateral $Na^+/K^+(NH_4^+)$ pump or a NH_4^+/K^+ channel coupled to the apical release of H^+-ATPase-acidified vesicles that trap NH_3 as NH_4^+ (see Weihrauch et al., 2004, 2009 for review). Notably, ammonia can also travel passively across membranes as NH_3 but its diffusion may be facilitated by ammonia-transporting Rhesus (Rh) proteins that have been identified in many species of aquatic animals including the green crab *Carcinus maenas* (RhCM; see Weihrauch et al., 2004).

Ammonia has recently received attention for its role in the control of breathing in fish. Initial evidence of the ventilatory effects of ammonia in an ammonotelic teleost, the rainbow trout, was presented by McKenzie et al. (1993), and later studies further demonstrated a direct role of internal ammonia in stimulating both ventilatory frequency and amplitude in this species (Zhang and Wood, 2009; Zhang et al., 2011). This has now also been observed in a ureotelic elasmobranch, the dogfish shark (De Boeck and Wood, 2015). Exposure to external ammonia (high environmental ammonia; HEA) also stimulates ventilation, though most evidence to date supports the notion that this is largely an effect of the eventual rise in plasma T_{amm} rather than an effect of the external presence of ammonia (reviewed by Zhang et al., 2015). These ventilatory effects appear to be mediated, at least in part, by branchial neuroepithelial cells (NECs) (Zhang et al., 2011; Zhang et al., 2015), the same cells responsible for effecting ventilatory changes in response to alterations in internal and external oxygen tensions (see Zachar and Jonz, 2012 for review).

Abbreviations: HEA, high external ammonia; PCO_2, partial pressure of CO_2; PO_2, partial pressure of O_2; P_{NH3}, partial pressure of NH_3; T_{amm}, total ammonia.

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In littoral crabs such as the green crab (*Carcinus maenas*), as well as in fish, exposure to hypoxia leads to increases in ventilation (Taylor and Butler, 1973; Taylor et al., 1973; Taylor, 1976; Taylor et al., 1977). Unique to crabs, however, is the reversal of the irrigation direction of the branchial chamber in response to hypoxic exposure (Taylor and Butler, 1973; Taylor et al., 1973). This latter response is particularly important because at sufficiently low oxygen tensions, these amphibious crabs straighten their walking legs and partially emerse themselves such that the exhalant openings of the branchial chamber become aerially exposed (Robertson et al., 2002; Taylor and Butler, 1973; Taylor et al., 1973; Taylor et al., 1977). The reversal of the irrigation direction serves to pull air through the exhalant openings, aerating the water which bathes the gills within the branchial chamber (Taylor and Butler, 1973; Taylor et al., 1973; Taylor et al., 1977). Complete whole-body emersion in response to hypoxia has also been observed when crabs are provided with access to aerially exposed platforms (Davenport and Wong, 1987; de Lima et al., 2015) and in severe hypoxia, access to air can prevent hypoxia-induced mortality (de Lima et al., 2015). While helping O_2 supply under these circumstances, aerial exposure can also lead to the accumulation of CO_2 and subsequent systemic acidosis (Burnett and McMahon, 1987; DeFur et al., 1988; Depledge, 1984; Simonik and Henry, 2014; Truchot, 1975), demonstrating a trade-off in this behavioural response.

The overall aim of the present study was to characterize the behavioural response to high environmental ammonia (HEA) exposure in *Carcinus maenas*. We hypothesized that, similar to hypoxia, HEA would trigger an emersion response in crabs held in experimental arenas containing rock beds onto which they could emerse. We further hypothesized, based on the ventilatory response described in fish (Zhang et al., 2015), that this emersion response would be attributed to ammonia specifically, rather than to any secondary acid-base or respiratory disturbances caused by ammonia exposure. As such, we simultaneously observed the behavioural response to HEA exposure and collected arterial haemolymph samples for the determination of acid-base (pH, HCO_3^- concentration) and respiratory parameters (partial pressures of O_2 (PO_2) and CO_2 (PCO_2)). Moreover, we predicted that emersion from HEA would provide a physiological benefit relative to crabs which were not allowed to emerse, similar to that observed in hypoxia where crabs emerse from unfavourable oxygen gradients to breathe air. These experiments represent the first attempt at understanding the behavioural response to ammonia exposure in crabs.

2. Material and methods

2.1. Crabs

Male green crabs (*Carcinus maenas*; 50–65 g) were collected by trapping in the intertidal zone of Effingham Inlet in British Columbia, Canada (49.05766, −125.11825) under collecting permit XR2772015 (Dept. of Fisheries and Oceans Canada). Crabs were transferred to outdoor holding tanks at the Bamfield Marine Sciences Centre (BMSC; Bamfield, BC, Canada) supplied with flow-through BMSC sea water [12 °C; salinity = 30 ppt; pH ~ 8; PO_2 = 140–150 Torr; total ammonia concentration (T_{amm}) 10 μmol/l]. Crabs were fed 3 times per week with a satiating meal of dead hake (*Merluccius productus*) and were held at the station for a minimum of 3 weeks prior to any experimentation. Crabs were fasted for 48 h prior to individual experiments.

2.2. Surgery

In Series 2–5, each crab was fitted with an arterial haemolymph sampling site which was covered with a double layer of dental dam. To do so, crabs were placed on ice in order to anaesthetize them and the carapace of the crab was then thoroughly dried, and a small, shallow hole was drilled through it at the anterior margin of the pericardium using a Dremel tool (Dremel, Mount Prospect, IL, USA) fitted with a dentistry drill bit. A small dental dam patch was then glued over this hole using cyanoacrylate glue (Krazy Glue, Westerville, OH, USA). A few minutes later, a second layer of dental dam was glued over the first, leading to a tight seal through which haemolymph could be collected using a syringe. A dot was marked with a ballpoint pen to visually mark the sampling portal. Crabs were allowed to recover for 24 h after the surgical procedure and showed no apparent changes in behaviour as a result of the surgery. We used this arterial sampling site rather than venous sampling from the arthrodial membranes as it would reflect the oxygen tension and acid-base balance of the haemolymph following passage through the gills. Furthermore, it facilitated sampling from submerged crabs; we did not want to unnecessarily expose animals to air when drawing haemolymph samples as many of the factors we were examining (pH, PCO_2, PO_2) are sensitive to air exposure. These crabs were then used in 4 different experimental series (Series 2-5) which assessed the emersion response to HEA, the parameters which trigger this behavioural response, and the potential beneficial effects of emersion from HEA.

2.2.1. Series 1 – emersion behaviour in response to 10 mmol/l HEA

Fasted crabs were randomly selected from holding tanks and placed individually into plastic experimental arenas containing 4 l of aerated sea water. These arenas, depicted in Fig. 1 (width = 50 cm; length = 80 cm, height = 50 cm), contained a rock bed occupying half of the total arena such that crabs had access to aerial exposure by emersing onto the rock bed. Importantly, the water level within the arena was high enough such that crabs could not emerse themselves by straightening their walking legs. Crabs were allowed to adjust to the experimental arena for 10 min. Following this period, stock concentrations of NH_4HCO_3 (HEA) or $NaHCO_3$ (HCO_3^- control; both adjusted to pH 8) were added to the sea water in the arena to a final concentration of 10 mmol/l, marking the onset of the 15-min exposure period. The total time spent completely emersed from the water (i.e., on the rock bed) was recorded for each crab during this period. After the 15-min exposure, crabs were removed from their respective treatment and placed into the opposite treatment and emersion time was again recorded over a 15-min period. In this manner, each crab was exposed to both HEA and the HCO_3^- control, with 50% of crabs tested in each sequence.

2.2.2. Series 2 – physiological status at the point of emersion from 4 mmol/l HEA

In light of the positive results from Series 1, a concentration of 4 mmol/l NH_4HCO_3, the lowest concentration triggering emersion in a pilot experiment, was chosen for Series 2. Crabs fitted with arterial sampling sites were randomly selected and placed individually into the experimental arenas described in Series 1. Following a 10-min adjustment period, NH_4HCO_3 was added to the sea water within the arena to a final concentration of 4 mmol/l, marking the onset of the experimental period. Crabs were observed throughout the exposure. At the point at which each crab started to emerse (began to climb the rock bed), it was

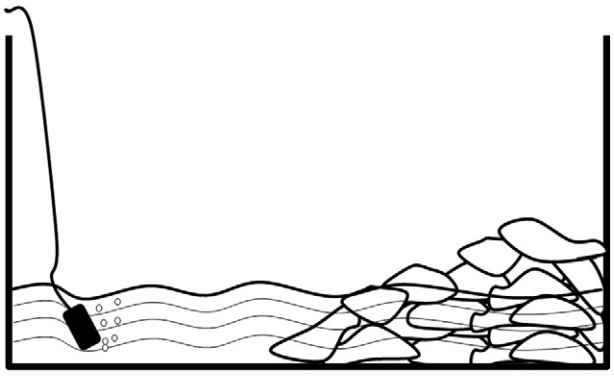

Fig. 1. Schematic representation of the experimental arenas used in experimental Series 1–5.

stopped and held under water, not allowing exposure to air. Then an 800-μl arterial haemolymph sample was collected immediately using a 1-ml "insulin" syringe with a 28.5 gauge needle (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA) which was inserted to a depth of approximately 1 cm through the dental dam seal. Arterial haemolymph pH and PO_2 were measured immediately (see Analytical techniques), after which the haemolymph sample was snap frozen in liquid nitrogen and stored at −80 °C until subsequent total ammonia (T_{amm}) and total CO_2 (TCO_2) analyses. In this series, an additional group of control crabs were run through the exact same protocol in parallel except that NH_4HCO_3 was not added and crabs were simply exposed to the experimental arena for approximately the same amount of time as the HEA-exposed crabs prior to haemolymph sampling. These crabs never emersed from control seawater. The purpose of this treatment was to serve as a control for any stress effects associated with handling and restraint of crabs which were trying to emerse. Following each treatment, crabs were weighed and water samples were collected for determination of pH, T_{amm}, and TCO_2. Seawater pH was determined immediately (see Analytical techniques) and the water sample was then stored at −20 °C until subsequent analyses.

2.2.3. Series 3 – emersion behaviour in response to ammonia injection

In Series 3, crabs fitted with arterial haemolymph sampling sites were weighed, placed into experimental arenas containing control sea water, and allowed to adjust to the setup for 10 min. Thereafter, crabs were injected with a volume of saline equal to 3% of haemolymph volume, assuming a haemolymph volume of 30% wet crab weight (Robertson, 1960) [saline in mmol/l: 260 NaCl, 5 $CaCl_2$, 7 $MgCl_2$, 8 KCl, 7 $NaHCO_3$, 0.3 glucose; pH 7.9; adapted from Fehsenfeld and Weihrauch, 2013]. "Saline control" crabs were injected with regular crab saline, while "150 mmol/l NH_4HCO_3 saline" crabs were injected with the same saline containing 150 mmol/l NH_4HCO_3, with pH corrected to 7.9. Crabs were observed for 15 min in order to determine if elevated haemolymph T_{amm} elicited a behavioural response similar to that seen in response to HEA. Following 15 min, 600-μl haemolymph samples were removed following the protocol described in Series 2, pH was measured immediately (see Analytical techniques), and the sample was then snap frozen and stored at −80 °C.

2.2.4. Series 4 – role of haemolymph O_2 in triggering emersion during HEA exposure

In this series, the arterial haemolymph PO_2 (P_aO_2) and emersion behaviour data recorded under normoxia in Series 2 were compared to those of crabs tested under hyperoxia. In these experiments, crabs were exposed to the same level of HEA as in Series 2 except that the water of the experimental arenas (for both control and HEA exposures) was made hyperoxic (180–200% of air O_2 saturation) by bubbling pure O_2 into the water and monitoring with an Accumet AP84 combined pH and dissolved oxygen (DO) meter (Cole-Parmer, Vernon Hills, IL, USA). Haemolymph sampling and P_aO_2 analysis were conducted in the same manner as in the normoxic experiments of Series 2. The goal of these additional hyperoxia experiments was to determine if saturating the haemolymph with O_2 would attenuate the emersion response to HEA.

2.2.5. Series 5 – physiological responses to HEA exposure with and without emersion

In this series, crabs fitted with haemolymph sampling sites were placed individually into experimental arenas and following a 10-min adjustment period, initial 400-μl haemolymph samples were collected, analyzed, and handled in the same manner as in Series 2 and 3. Haemolymph P_aO_2 was not assessed in this series. Crabs were allowed to recover from sampling for an additional 10 min and thereafter either NH_4HCO_3 (HEA) or $NaHCO_3$ (HCO_3^- control) was added to a final concentration of 4 mmol/l, marking the onset of the experimental period. Crabs were monitored for a total of 15 min and the time spent emersed (on the rock bed) was recorded for each individual crab. After 15 min, a second haemolymph sample was collected from each crab. All the HCO_3^- control crabs were sampled in sea water, as they never emersed. However in the HEA experiments, where crabs would periodically emerse from the experimental water, crabs were sampled in the media (water or air) in which they were found at the 15 min time point. Sufficient experiments were conducted such that we collected an equal sample size for crabs sampled in water as in air. In addition, a third set of crabs was exposed individually to 4 mmol/l NH_4HCO_3 for 15 min in a plastic container containing no rocks so that they were not able to emerse from HEA. All sampling and handling in this third set of experiments followed the same protocol described above. All haemolymph samples were collected, analyzed, and stored in the same manner as in Series 2 and 3.

2.3. Analytical techniques

In both pH and PO_2 analyses, arterial haemolymph samples were thermostatted to 12 °C. Haemolymph (600-μl samples) and water (1-ml samples) pH were measured with a pH microelectrode (PerpHecT ROSS pH microelectode, Thermo Scientific, Waltham, MA, USA) connected to a handheld H160 pH meter (Hach, Mississauga, ON, Canada). PO_2 in 600-μl arterial haemolymph samples was measured using a polarographic blood oxygen electrode (Radiometer, Copenhagen, Denmark) connected to a polarographic amplifier (model 1900, A-M Systems, Everett, WA, USA). TCO_2 concentrations were measured with a 965 Total CO_2 analyzer (Corning, Corning, NY, USA). Previously snap-frozen haemolymph samples (stored at −80 °C) and water samples (stored at −20 °C) were thawed and analyzed for TCO_2 immediately. T_{amm} in these water and haemolymph samples was then measured using the indophenol salicylate method described by Verdouw et al. (1978). Haemolymph samples were first deproteinized by adding 1 vol of 8% perchloric acid and incubating for 5 min; samples were then neutralized by adding 0.5 vols of 3 mol/l KOH followed by centrifugation. Deproteinization of haemolymph samples was necessary as the presence of protein inhibits the assay reaction.

2.4. Calculations

Arterial partial pressure of CO_2 (P_aCO_2; Torr) in haemolymph samples was calculated using a modified Henderson-Hasselbalch equation and pK′ and CO_2 solubility coefficient (αCO_2; mmol/l/Torr) values obtained from Truchot (1976):

$$P_aCO_2 = TCO_2/\left[\left(1 + \text{antilog}\left(pH - pK'\right)\right) \times \alpha CO_2\right] \quad (1)$$

From this, arterial $[HCO_3^-]$ ($[HCO_3^-]_a$; mmol/l) was determined using the following equation:

$$[HCO_3^-] = TCO_2 - (P_aCO_2 \times \alpha CO_2) \quad (2)$$

Arterial $[NH_4^+]$ and partial pressure of NH_3 (P_{NH3}) were also calculated using the Henderson-Hasselbalch equation and pK′ and NH_3 solubility coefficient (αNH_3; μmol/l/μTorr) values adjusted to seawater ionic strength, tabulated by Cameron and Heisler (1983). This approach has been used previously in crustacean studies (Weihrauch et al., 1998). $[NH_4^+]$ (μmol/l) was calculated using the following equation:

$$\left[NH_4^+\right] = T_{amm}/\left[1 + \text{antilog}\left(pH - pK'\right)\right] \quad (3)$$

where T_{amm} is total ammonia (μmol/l). P_{NH3} (μTorr) was then calculated using the following equations:

$$[NH_3] = T_{amm} - \left[NH_4^+\right] \quad (4)$$

$$P_{NH3} = [NH_3]/\alpha NH_3 \quad (5)$$

2.5. Statistical analyses

All data are presented as means ± SEM (n = number of crabs) and statistical significance was accepted at the $P < 0.05$ level. Comparisons between two unpaired treatment groups (e.g., as in Series 2 and 3) were made using an unpaired two-tailed Student's *t*-test. Comparisons made between more than two unpaired treatment groups (Series 4) were made using a one-way ANOVA followed by a Holm-Sidak post-hoc test. In Series 5, paired two-tailed Student's *t*-tests were used to determine significant differences between initial and final haemolymph parameters within each experimental group while a one-way ANOVA followed by a Holm-Sidak post-hoc test was used to determine significant differences among final haemolymph parameters among experimental groups. Data were checked to ensure normality of distributions (Shapiro-Wilk test) and homogeneity of variances prior to performing parametric tests. All statistical analyses were conducted using SigmaStat v3.5 (Systat Software, Inc.). Linear regressions in Series 5 and corresponding r (Pearson's correlation coefficient) and P values were obtained using SigmaPlot v11.0 software integrated with SigmaStat v3.5 (Systat Software, Inc.).

3. Results

3.1. Series 1: emersion response to 10 mmol/l HEA

Following a 10-min adjustment period without additions, crabs exposed to 10 mmol/l HEA as NH_4HCO_3 spent an average of 421 s, 46.8% of the entire 15-min period, emersed on the rock bed of the experimental arena (Fig. 2A). When these crabs were then transferred to 10 mmol/l $NaHCO_3$ (HCO_3^- control treatment) only 0.2% of the total experimental period was spent emersed (Fig. 2A). Crabs initially exposed to 10 mmol/l $NaHCO_3$ spent <1% of the 15 min period emersed, but when transferred to HEA, these same crabs spent over 25% of the experimental period emersed (Fig. 2A). The average time to first emersion in response to HEA was 321 ± 23 s in the group exposed to HEA first (i.e. before the HCO_3^- control treatment), and was 373 ± 31 s in crabs which were exposed to HEA second (i.e. after the HCO_3^- control treatment) (Fig. 2B). There were no statistically significant differences in either total time spent emersed or time to first emersion between the two experimental groups in response to HEA.

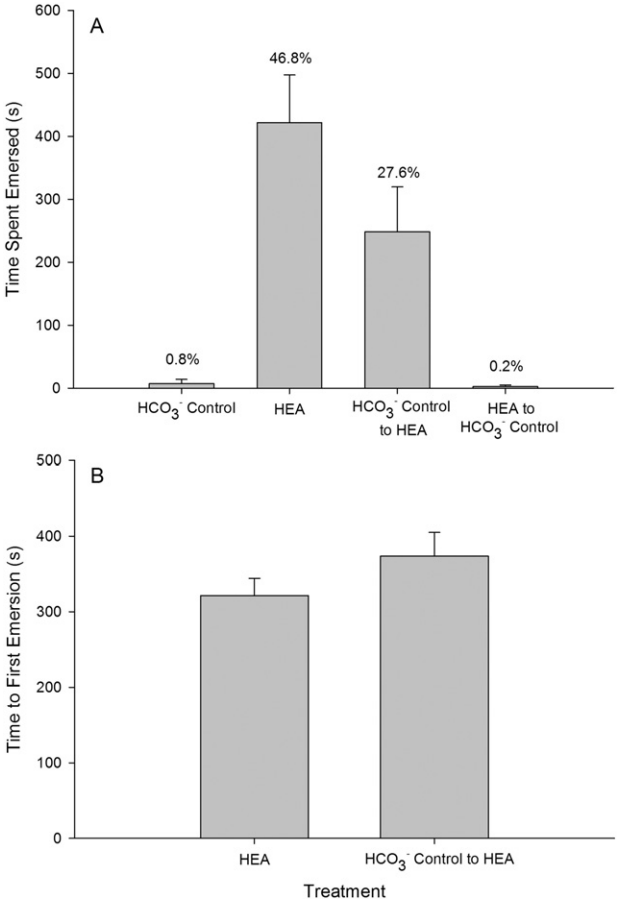

Fig. 2. Time spent emersed (A) and time to first emersion (B) in crabs exposed to 10 mmol/l $NaHCO_3$ (HCO_3^- Control), 10 mmol/l NH_4HCO_3 (HEA), 10 mmol/l NH_4HCO_3 with previous exposure to 10 mmol/l $NaHCO_3$ (HCO_3^- Control to HEA), and 10 mmol/l $NaHCO_3$ with previous exposure to 10 mmol/l NH_4HCO_3 (HEA to HCO_3^- Control). Time spent emersed expressed as a percent of total exposure time is included above each mean in panel A. Means ± 1 SEM (n = 5 for all treatments).

3.2. Series 2: haemolymph parameters at the point of emersion from 4 mmol/l HEA

A pilot series of experiments (data not shown) revealed that 4 mmol/l NH_4HCO_3 was the lowest concentration that elicited the emersion response in crabs exposed to HEA for 15 min. This exposure concentration was thus chosen for subsequent experiments. The average time to first emersion in Series 2 was 546 ± 136 s (Fig. 5B). Crabs in this series were not allowed to fully emerse from the water; instead, haemolymph samples were collected in order to determine physiological status at the point of emersion. Overall, crabs experienced a mild metabolic alkalosis and a dramatic increase in $[NH_4^+]$ and P_{NH3} at this time. Arterial haemolymph T_{amm} in control crabs was 347.2 ± 14.9 μmol/l and increased approximately 5-fold to 1798 ± 82 μmol/l in HEA-exposed crabs (Fig. 3A). Haemolymph pH_a increased significantly from 7.87 ± 0.03 in control crabs to 8.00 ± 0.04 in HEA-exposed crabs (Fig. 3B). P_aCO_2 remained unchanged (Fig. 3C). The rise in $[HCO_3^-]_a$ (Fig. 3D) was not significant but was nearly equal to the rise in arterial $[NH_4^+]$ (Fig. 3E; both increased by approximately 1.5 mmol/l). Note that arterial $[NH_4^+]$ increased by approximately 5-fold (Fig. 3E) while arterial P_{NH3} increased by 7-fold (Fig. 3F), reflecting the concomitant increase in pH_a.

3.3. Series 3: responses to NH_4HCO_3 injections

The large elevation in haemolymph T_{amm} measured at the point of emersion in Series 2 suggested that this might be the signal triggering the emersion response. Therefore, crabs in Series 3 were injected with either control saline or control saline containing 150 mmol/l NH_4HCO_3. Based on an average injection load of 1.18 ± 0.02 mmol/kg and a haemolymph volume of 300 ml/kg (Robertson, 1960), an approximate 4 mmol/l increase in T_{amm} and $[HCO_3^-]$ was expected. However, this treatment not only did not lead to an emersion response, but 15 min post-injection, haemolymph T_{amm} was only 625 ± 34 μmol/l (Fig. 4A). Haemolymph pH_a, P_aCO_2 and $[HCO_3^-]_a$ were not statistically different between the two experimental groups although an increase in $[HCO_3^-]_a$, likely caused by the direct injection of a bicarbonate salt, was accompanied by an increase in P_aCO_2 which maintained pH_a (Fig. 4B,C,D). Interestingly, 15 min following the injection, mean $[HCO_3^-]_a$ had risen by 1.61 mmol/l in the NH_4HCO_3 injected crabs relative to the control saline group, which was approximately 2.5 mmol/l lower than the expected level; $[NH_4^+]$ had increased by only 0.36 mmol/l, >3.5 mmol/l below the expected level (Fig. 4).

3.4. Series 4: P_aO_2 at the point of emersion from 4 mmol/l HEA and effects of hyperoxia

P_aO_2 measured at the point of emersion from HEA under normoxia in the crabs of Series 2 was 35.0 ± 8.5 Torr and in control normoxic crabs was 32.0 ± 8.5 Torr (Fig. 5A). In order to provide further evidence for the rejection of the hypothesis that HEA alters haemolymph oxygen content, thereby triggering emersion, crabs were exposed to either HEA or control conditions in hyperoxic sea water (180–200% air O_2 saturation). Haemolymph P_aO_2 was significantly higher in hyperoxic crabs,

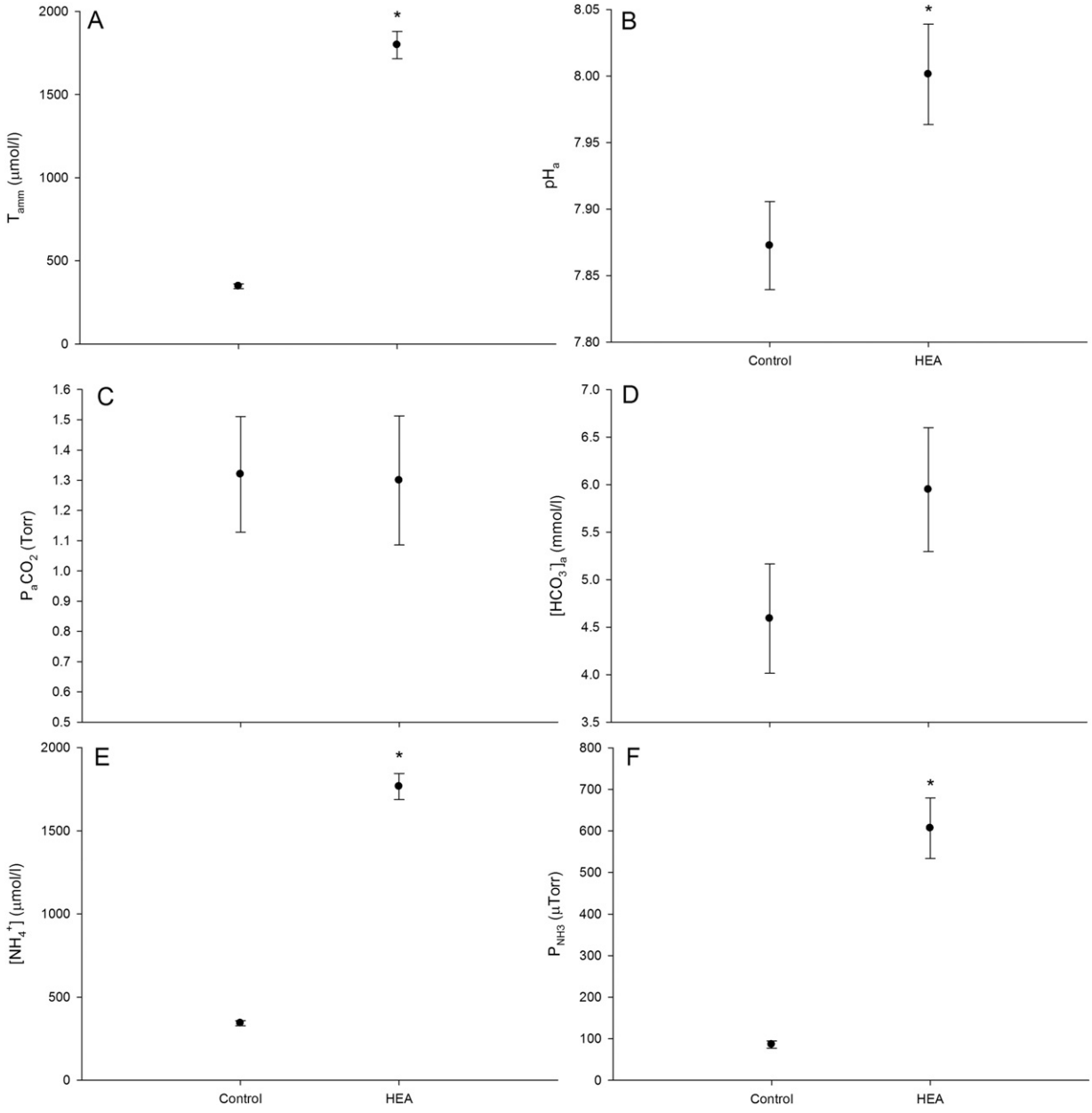

Fig. 3. Total ammonia (T_{amm}; A), pH_a (B), partial pressure of carbon dioxide (P_aCO_2; C), $[HCO_3^-]_a$ (D), $[NH_4^+]$ (E), and partial pressure of NH_3 (P_{NH3}; F) of arterial haemolymph collected from control crabs and at the point of emersion in crabs exposed to HEA. Asterisks denote significant differences within a given parameter between control and HEA means as determined by an unpaired two-tailed Student's *t*-test. Means ± 1 SEM (n = 8 for all parameters).

reaching values of 90.5 ± 5.1 Torr and 99.9 ± 12.9 Torr in control and HEA-exposed crabs, respectively (Fig. 5A). However, this did not alter the time to emersion from HEA which was 546 ± 136 s in normoxia and 395 ± 53 s in hyperoxia (Fig. 5B); hyperoxia alone did not cause crabs to emerse onto the rock bed.

3.5. Series 5: effects of 15 min exposures to 4 mmol/l HEA with and without emersion

In this series, the aim was to understand whether being able to emerse from HEA provided a physiological benefit to crabs. Thus, the series consisted of three experimental treatments: 4 mmol/l $NaHCO_3$ (HCO_3^- control) in which crabs could emerse but did not, 4 mmol/l NH_4HCO_3 with a rock bed present to allow emersion (HEA + E), and 4 mmol/l NH_4HCO_3 with no rock bed present so that the crabs could not emerse (HEA). There were no significant differences in seawater pH or PCO_2 between any of the treatments (Table 1). Arterial haemolymph T_{amm} increased approximately 5-fold in both the HEA and HEA + E groups and final T_{amm} was not significantly different between the two groups at the end of the 15 min period (Fig. 6A). In the HCO_3^- control treatment, crabs demonstrated a significant haemolymph alkalosis, as did crabs in the HEA treatment (Fig. 6B). However, in the HEA + E treatment, pH_a was unchanged by the end of the 15 min exposure (Fig. 6B). A significant increase in P_aCO_2 was observed when crabs were allowed to emerse from HEA, whereas P_aCO_2 was not altered in crabs which were not allowed to emerse from HEA (Fig. 6C). In the HCO_3^- control treatment, P_aCO_2, fell significantly (Fig. 6C) but $[HCO_3^-]_a$ was unchanged (Fig. 6D). Both HEA groups experienced significant, approximate 1.5-mmol/l increases in $[HCO_3^-]_a$ which were roughly equimolar to the increases in $[NH_4^+]$ observed in each group (Fig. 6D,E). Interestingly, while post-exposure T_{amm} and $[NH_4^+]$ did not differ between HEA groups, haemolymph P_{NH3} was significantly lower in HEA + E crabs (Fig. 6F). In this series, half of HEA + E crabs were sampled in air for post-exposure values, while the other half were sampled in water, depending on where in the

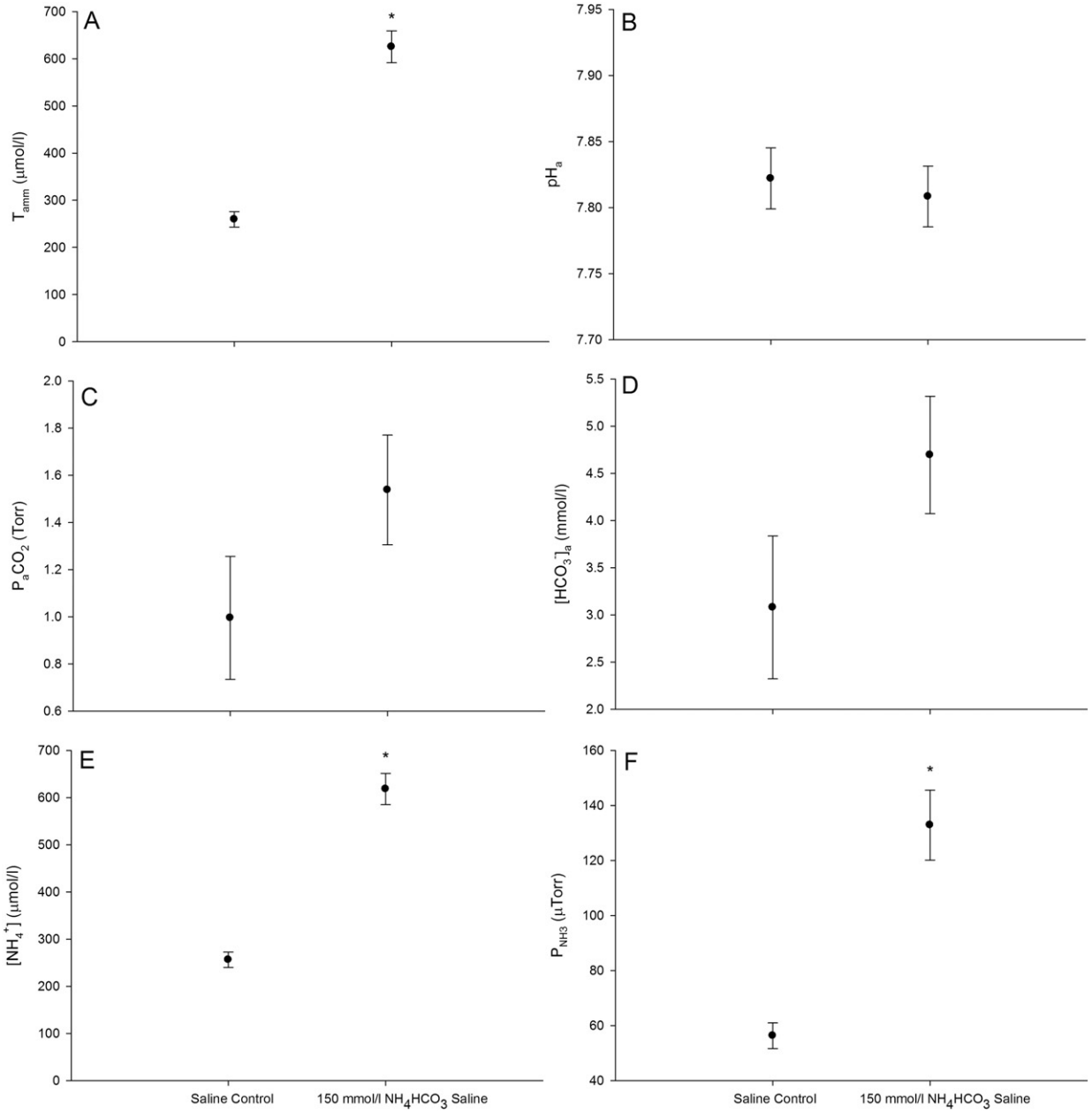

Fig. 4. Arterial haemolymph total ammonia (T_{amm}; A), pH_a (B), partial pressure of carbon dioxide (P_aCO_2; C), $[HCO_3^-]_a$ (D), $[NH_4^+]$ (E), and partial pressure of NH_3 (P_{NH3}; F) collected 15 min following injection in crabs injected with control saline or saline containing 150 mmol/l NH_4HCO_3. Asterisks denote significant differences within a given parameter between control saline and 150 mmol/l NH_4HCO_3 saline means as determined by an unpaired two-tailed Student's *t*-test. Means ± 1 SEM (n = 5 for saline control; n = 6 for 150 mmol/l NH_4HCO_3 saline).

experimental arena the crab was situated at the end of the exposure. The medium in which crabs were sampled did not have any statistically significant effects on any of the measured parameters (Table 2).

The total amount of time spent emersed by a given crab in the HEA + E treatment varied substantially from 33 to 778 s (Fig. 7), allowing for the overall effect of emersion to be assessed. As crabs spent more time emersed, T_{amm} and $[NH_4^+]$ both decreased, likely as a function of an overall shorter exposure to HEA (Fig. 7A,E). Similarly, pH decreased linearly with time spent emersed (Fig. 7B) but this was not the case for P_aCO_2 (Fig. 7C) or $[HCO_3^-]_a$ (Fig. 7D) which demonstrated little to no dependence on time spent emersed. Arterial haemolymph P_{NH3} also decreased with increasing time spent emersed and this correlation ($r = -0.854$) was much stronger than that of T_{amm} ($r = -0.671$) or $[NH_4^+]$ ($r = -0.665$) (Fig. 7). It is noteworthy that the intercept (i.e., 0 s) of the fitted linear relationships was remarkably similar to the values measured in HEA crabs which spent no time emersed from HEA. The intercept values and measured HEA values respectively were 2115 versus 2066 ± 106 μmol/l for T_{amm}, 7.92 versus 7.95 ± 0.04 for pH_a, 2086 versus 2029 ± 85 μmol/l for $[NH_4^+]$, and 556 μTorr versus 574.97 ± 66.44 μTorr for P_{NH3}.

4. Discussion

4.1. High environmental ammonia triggers the emersion response in crabs

In the present study, exposure to HEA at either 4 or 10 mmol/l NH_4HCO_3 triggered the emersion response in green crabs (Figs. 2 and 3), similar to the response to hypoxia (Davenport and Wong, 1987; de Lima et al., 2015; Robertson et al., 2002; Taylor and Butler, 1973; Taylor et al., 1973; Taylor et al., 1977). In fish, exposure to HEA or infusion of ammonia increases ventilation and it is now clear that ammonia alone, in the absence of any secondary changes in blood pH, plasma

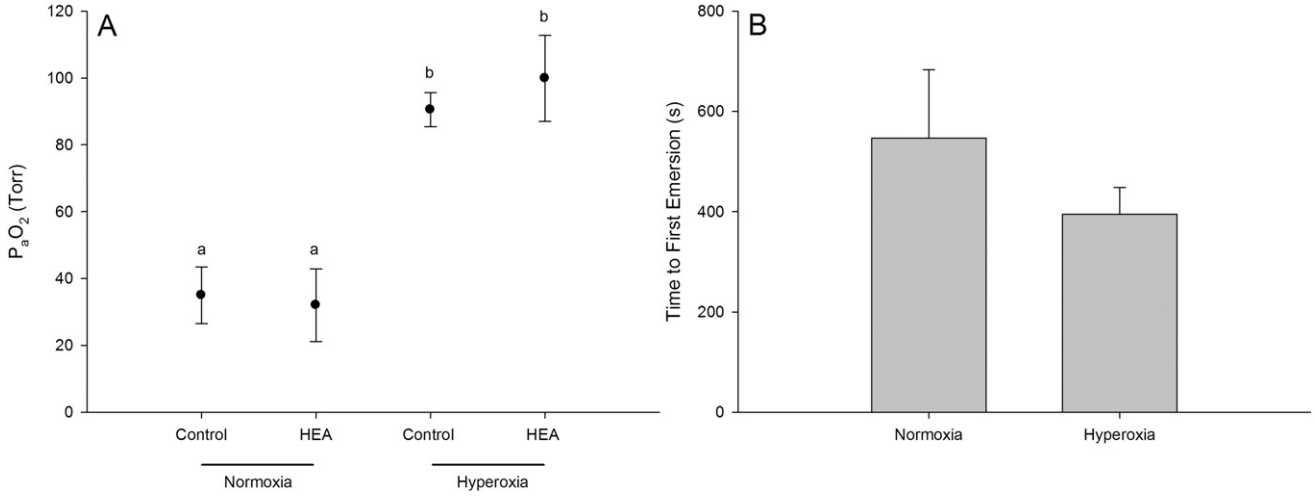

Fig. 5. P_aO_2 (A) of haemolymph collected from control crabs and at the point of emersion in crabs exposed to HEA under normoxic and hyperoxic conditions. Time to first emersion (B) in HEA under normoxic and hyperoxic conditions. Means not sharing the same letter are significantly different from one another as determined by a one-way ANOVA followed by a Holm-Sidak post-hoc test. Means ± 1 SEM (n = 8 for normoxic exposures; n = 4 for hyperoxic exposures).

PCO_2, or plasma $[HCO_3^-]$ can elicit this response, effectively demonstrating that ammonia directly drives ventilatory changes similar to those observed in response to hypoxia (see Zhang et al., 2015 for review).

In Series 2, arterial haemolymph samples were collected to determine respiratory and acid-base status at the point of emersion. Exposure to 4 mmol/l NH_4HCO_3 led to a significant increase in T_{amm} (and $[NH_4^+]_a$ and P_{NH3}) and a significant metabolic alkalosis; the increase in $[HCO_3^-]_a$ was nearly equimolar to the increase in $[NH_4^+]_a$ (Fig. 3). Alkalosis in response to HEA exposure also occurs in fish (Cameron and Heisler, 1983; Wilson et al., 1994) and is thought to result from the fact that NH_3 crosses the gill epithelium more readily than NH_4^+. After entering the blood or haemolymph, NH_3 increases pH by reacting with H^+, producing NH_4^+ and resulting in a metabolic alkalosis and an equimolar increase in $[HCO_3^-]$. It is therefore possible that emersion was triggered by haemolymph alkalosis. In Series 3, crabs were directly injected with NH_4HCO_3 resulting in the addition of NH_4^+ to the haemolymph without a consequent alkalosis. These crabs did not emerse, further suggesting that systemic alkalosis may trigger emersion, although this idea is discounted below. This could in fact represent an adaptive response to systemic alkalosis given that air exposure tends to increase haemolymph PCO_2 and decrease pH (Burnett and McMahon, 1987; DeFur et al., 1988; Depledge, 1984; Simonik and Henry, 2014; Truchot, 1975). Moreover, Taylor (1982) suggested that changes in internal pH and/or $[HCO_3^-]$ may drive some ventilatory responses in crustaceans. However, crabs exposed to 4 mmol/l $NaHCO_3$ in Series 5 also experienced a significant increase in pH_a (Fig. 6B) but these crabs never emersed. Similarly, crabs injected with NH_4HCO_3 in Series 3 displayed a significant increase in $[HCO_3^-]_a$ (Fig. 4D) but this also did not result in emersion. It is also important to note that in both HEA and HCO_3^- control treatments in Series 5, water pH and PCO_2 were equal (Table 1), suggesting that changes in external pH or PCO_2 also did not account for the emersion response observed in HEA. In addition, exposure to HEA had no effect on haemolymph P_aO_2 (Fig. 5A), which is also the case in response to ammonia infusion in rainbow trout (McKenzie et al., 1993; Zhang and Wood, 2009). Zhang and Wood (2009) could not, however, discount the possibility that ammonia alters haemoglobin O_2 binding, leading to a potential decrease in blood O_2 content. The hyperoxia experiments in Series 4, designed to maximize haemocyanin O_2 saturation, more than doubled P_aO_2 (Fig. 5A) but had no effect on the timing of first emersion in crabs. This discounted the possibility that haemolymph oxygen status is altered by ammonia and subsequently triggers emersion from HEA. Overall, these data demonstrate that ammonia itself is responsible for triggering emersion in crabs, although future studies assessing the behavioural responses to ammonia-independent alkalosis may help further demonstrate the specific role of ammonia, rather than HEA-induced alkalosis, in triggering the emersion response.

Table 1
Total ammonia (T_{amm}), pH, and PCO_2 in water samples collected in Series 5 experiments.

	$NaHCO_3$	NH_4HCO_3 (+Emersion)	NH_4HCO_3 (No emersion)
T_{amm} (μmol/l)	11.61 ± 1.62[a]	4261.29 ± 71.16[b]	4067.67 ± 151.66[b]
pH	8.12 ± 0.03	8.03 ± 0.03	8.07 ± 0.04
PCO_2 (Torr)	0.52 ± 0.05	0.61 ± 0.03	0.55 ± 0.04

Means not sharing the same letters within a given measured parameter are significantly different from one another as determined by a one-way ANOVA with a Holm-Sidak post-hoc test. Means ± 1 SEM (n = 5–6).

4.2. Potential mechanisms for ammonia sensing

In fish, neuroepithelial cells (NECs), the cells responsible for sensing respiratory gases, are now considered to be tri-modal, responsive to O_2, CO_2, and NH_3 (Perry and Tzaneva, 2016). While these cells are believed to sense both internal and external levels of O_2 (Zachar and Jonz, 2012), NECs are likely only moderately sensitive to external ammonia in trout (Zhang et al., 2011, 2015) and not at all sensitive to external ammonia in dogfish sharks (De Boeck and Wood, 2015). While ventilation was not assessed in the present study, it is possible that gas sensors in crustaceans are responsible for driving the emersion response during hypoxic and high ammonia exposures. Two different types of gas sensors have been described in crustaceans. The first are internal sensors, comprising of branchial nerves which terminate in the walls of the branchiocardiac veins, and increase in firing rate in response to hypoxia (Ishii et al., 1989). The second are external sensors housed within small, rigid gill spines that have sockets exposed to the surrounding water, but these have not been functionally characterized (Laverack and Saier, 1993).

The presence of both internal and external sensors is in accordance with the notion that crabs are sensitive to both internal and external oxygen tensions (Taylor, 1982). In contrast, the emersion response in crabs appears to be sensitive predominantly to external ammonia. Crabs injected with NH_4HCO_3 did not emerse from control seawater. In Series 3, the injected ammonia load was expected to be approximately 4 mmol/l in the haemolymph (see Results). While NH_4CO_3 injection led to an increase of 1.6 mmol/l in $[HCO_3^-]_a$ by 15 min following injection, $[NH_4^+]$ increased by only approximately 0.4 mmol/l (Fig. 4), suggesting that crabs are able to rapidly clear ammonia from the haemolymph. *C. maenas* has been demonstrated to possess a branchial mechanism for active NH_4^+ excretion mediated by basolateral $Na^+/K^+(NH_4^+)$-ATPase

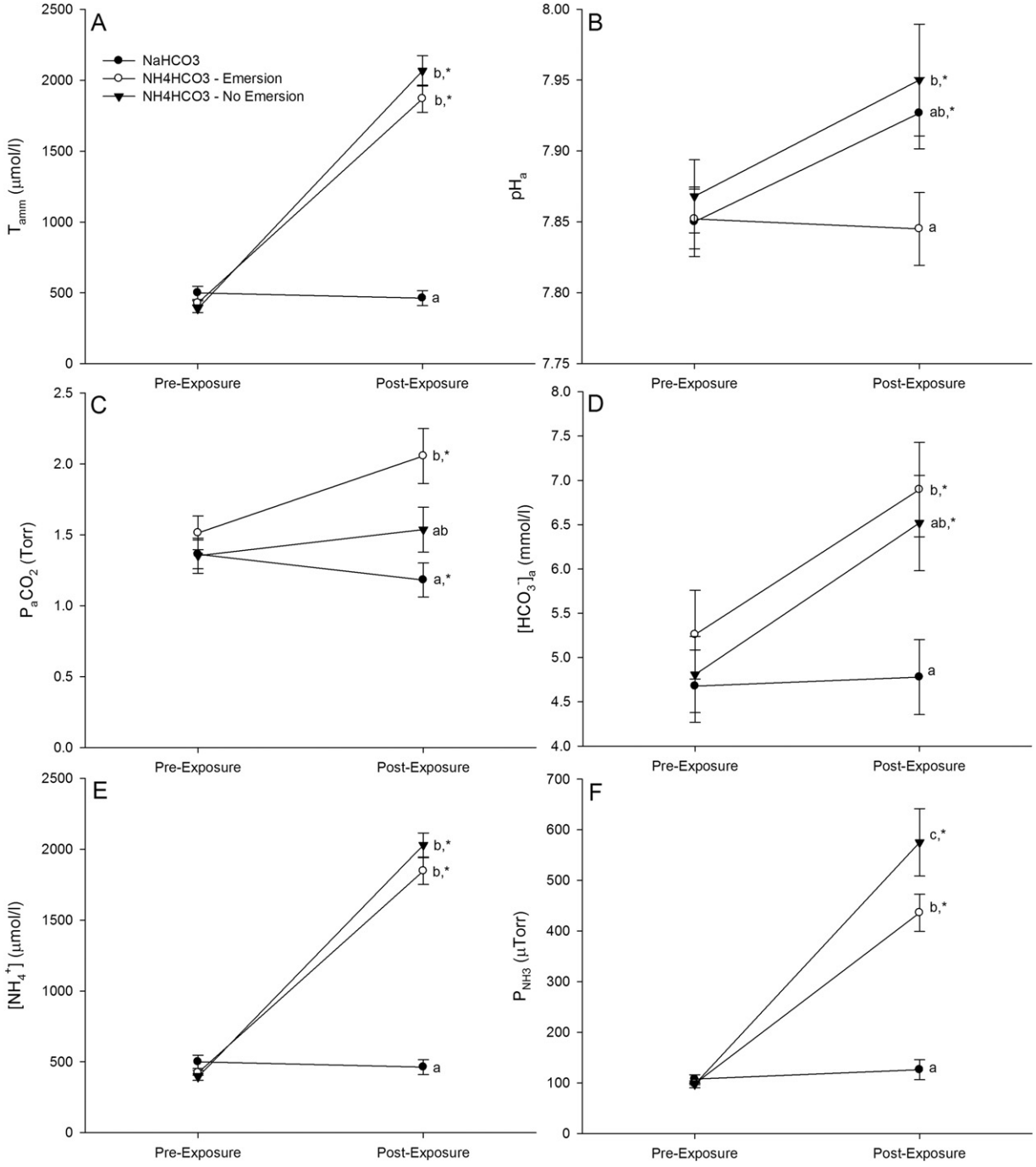

Fig. 6. Total ammonia (T_{amm}; A), pH_a (B), partial pressure of carbon dioxide (P_aCO_2; C), $[HCO_3^-]_a$ (D), $[NH_4^+]$ (E), and partial pressure of NH_3 (P_{NH3}; F) of arterial haemolymph collected from crabs before and after 15 min exposures to 4 mmol/l $NaHCO_3$, 4 mmol/l NH_4HCO_3 with emersion allowed, and 4 mmol/l NH_4HCO_3 with no emersion allowed. Asterisks represent a significant difference between pre- and post-exposure means within a given treatment as determined by a paired two-tailed Student's *t*-test. Post-exposure means of different treatments not sharing the same letter are significantly different from one another as determined by a one-way ANOVA followed by a Holm-Sidak post-hoc test. Means ± 1 SEM (n = 6 for $NaHCO_3$; n = 8 for NH_4HCO_3-Emersion; n = 6 NH_4HCO_3-No Emersion).

and vesicular H^+-ATPase (Weihrauch et al., 2002), which likely coordinated this rapid clearance of ammonia following the injection. The notion that emersion is triggered specifically by external ammonia is also supported by the emersion response displayed in Series 1. Crabs exposed to 10 mmol/l NH_4HCO_3 for 15 min (Fig. 2A; HEA) must have accumulated significant levels of ammonia, based on the results from those exposed to 4 mmol/l NH_4HCO_3 (Fig. 6A,E,F), yet when these crabs were subsequently transferred to the HCO_3^- control, emersion did not occur (Fig.2; HEA to HCO_3^- control). Thus, it is unlikely that internal ammonia levels are responsible for triggering the emersion response. Indeed, it would probably be maladaptive for emersion to be triggered by internal ammonia levels, which likely increase naturally in response to feeding or exercise, given that exposure to air in *C. maenas* tends to reduce ammonia excretion and increase haemolymph T_{amm} (Durand and Regnault, 1998). It is important to note, however, that at least one study has demonstrated the capacity for ammonia volatilization during air exposure in this species (Simonik and Henry, 2014). In future studies, it will be interesting to examine the role of the proposed gas sensors present in the

Table 2
Post-exposure arterial haemolymph total ammonia (T_{amm}), pH_a, carbon dioxide partial pressure (P_aCO_2), $[HCO_3^-]_a$, $[NH_4^+]$, and partial pressure of NH_3 (P_{NH3}) of crabs exposed to 4 mmol/l NH_4HCO_3 sampled in air or water in Series 5.

	Sampled in air	Sampled in water	P-value
T_{amm} (μmol/l)	1697.05 ± 102.60	2040.96 ± 126.74	0.068
pH_a	7.81 ± 0.04	7.88 ± 0.03	0.249
$PaCO_2$ (Torr)	2.33 ± 0.32	1.79 ± 0.17	0.177
$[HCO_3^-]_a$ (mmol/l)	7.34 ± 0.93	6.45 ± 0.56	0.441
$[NH_4^+]$ (μmol/l)	1677.57 ± 100.06	2014.87 ± 125.48	0.069
P_{NH3} (μTorr)	372.65 ± 52.26	499.13 ± 36.40	0.082

No significant differences were observed between crabs sampled in air or water in any parameter. P values determined via a two-tailed unpaired Student's *t*-test are listed. Means ± 1 SEM (n = 5).

branchiocardiac vessels (Ishii et al., 1989) and gill spines (Laverack and Saier, 1993) of crustaceans in sensing hypoxia and HEA, and their potential involvement in triggering the emersion response in crabs.

4.3. Emersion from HEA is physiologically beneficial

Crabs exposed to HEA which were unable to emerse presented significant increases in haemolymph T_{amm}, pH_a, and $[HCO_3]_a$ (Fig. 6). When allowed access to aerial exposure, haemolymph T_{amm} and $[HCO_3^-]_a$ increased but no alkalosis developed (Fig. 6). Therefore, emersion from HEA may indirectly serve to maintain acid-base balance even though pH_a is not directly responsible for mediating this behavioural response. In previous work, exposure to air in crabs resulted in a decrease

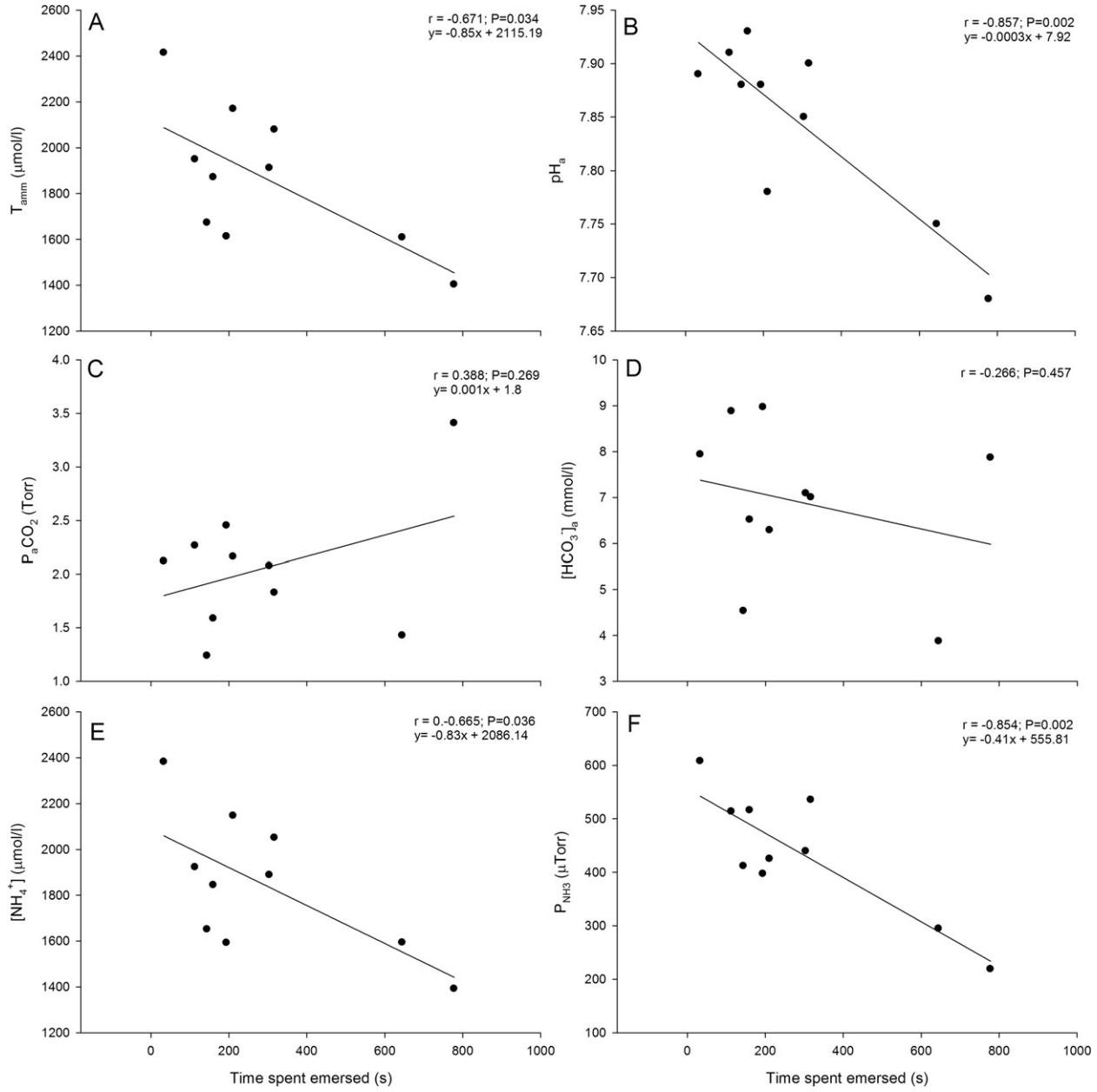

Fig. 7. Arterial haemolymph total ammonia (T_{amm}; A), pH_a (B), partial pressure of carbon dioxide (P_aCO_2; C), $[HCO_3^-]_a$ (D), $[NH_4^+]$ (E), and partial pressure of NH_3 (P_{NH3}; F) of crabs exposed to 4 mmol/l NH_4HCO_3 for 15 min as a function of time spent emersed. The Pearson's correlation coefficient and its statistical significance are given for each parameter, together with the equation of the regression relationship. (n = 10 for all parameters).

in haemolymph pH typically attributed to a rise in PCO_2 (Burnett and McMahon, 1987; DeFur et al., 1988; Truchot, 1975). Indeed, P_aCO_2 increased significantly in HEA + E crabs in the present study (Fig. 6) and pH_a decreased as a function of time spent emersed ($r = -0.857$; Fig. 7A). However, P_aCO_2 only showed a very weak, non-significant correlation with time spent emersed ($r = 0.387$; Fig. 7C), suggesting that the maintenance of pH_a is not a function of increased CO_2 retention during emersion. Notably, most crabs that emersed from HEA would periodically re-immerse in water. Upon re-immersion, any CO_2 accumulated during emersion would rapidly diffuse into the surrounding water due to its high solubility, causing a subsequent increase in pH_a (Truchot, 1975). In other words, if CO_2 retention was the only mechanism by which emersion reduced pH_a, this would likely be only transient, returning to pre-emersion levels when crabs return to water. Alternatively, pH_a could also be reduced through the volatilization of NH_3. While not directly assessed in the present study, NH_3 volatilization has been described in some terrestrial crab species (see Weihrauch et al., 2004) and has been observed in at least one instance in *C. maenas* (Simonik and Henry, 2014). Air exposure in green crabs has also been demonstrated to result in only a minor increase in haemolymph T_{amm} which remained constant over a 72 h period and was not associated with sequestration of ammonia into muscle (Durand and Regnault, 1998). Though ammonia volatilization was not assessed in the latter study, it could potentially account for the relative maintenance of T_{amm} observed in their study (Durand and Regnault, 1998) and the maintenance of pH_a observed in HEA + E crabs in our study (Fig. 6B). Therefore, while emersion does not significantly reduce the total ammonia load (Fig. 6A,E), it does appear to eliminate HEA-induced alkalosis and attenuate the increase in P_{NH3}, which is generally considered to be the more toxic form of ammonia (Randall and Tsui, 2002). These results demonstrate that emersion from HEA provides a physiological benefit to crabs similar to the case of emersion from severe hypoxia which allows crabs to breathe air and attenuate the decrease in haemolymph PO_2 associated with exposure to progressive hypoxia (Taylor et al., 1973).

4.4. Perspectives and future directions

The many functions of ammonia in various physiological processes are an emerging topic in the field of comparative physiology (Wright and Wood, 2012). Ammonia clearly has the capacity to directly elicit changes in behaviour in crabs. The emersion response described for hypoxia in crabs, which occurs at a given threshold oxygen tension in response to progressive hypoxia, is preceded by cardiorespiratory alterations at a higher critical oxygen tension (Taylor et al., 1973). Therefore, one hypothesis might be that at lower ammonia concentrations, HEA exposure elicits a hyperventilatory response driven by internal ammonia levels. Such a response would normally serve to clear elevated internal ammonia of endogenous origin from the haemolymph, similar to the mechanism described in fish (De Boeck and Wood, 2015; Zhang and Wood, 2009; Zhang et al., 2011, 2015). However, at higher concentrations external ammonia would drive the emersion response described in our study. This could be analogous to the changes in ventilation which precede emersion in response to progressive hypoxia exposure in *C. maenas* (Taylor et al., 1977). No study to date, however, has addressed the ventilatory effects of ammonia in a crab species.

One question which remains is why does this behavioural response to HEA exist? In natural settings, littoral crabs are likely routinely exposed to levels of ammonia only in the low micromolar range, much lower than the threshold of 4 mmol/l observed in the present study. Indeed, levels up to 2–5 mmol/l are observed only in specific circumstances such as in tide pools surrounded by dense avian populations (Loder et al., 1996), and it is possible that crabs are not regularly exposed to such environments. Weihrauch et al. (1999) proposed that the high ammonia environment (1–2 mmol/l) of sediments which can surround some crab burrows may have driven the evolution of active ammonia excretion mechanisms in crabs. Is it also possible that this same selective pressure similarly drove the evolution of a sensory mechanism which is specific to external ammonia? An ecological approach to determine the degree to which crabs are exposed to elevated external ammonia in the wild may be the next step in addressing this question. It is clear from our work that ammonia specifically triggers the emersion response in crabs and that this appears to provide a physiological benefit. It is now necessary to understand the potential role of this response as a behavioural adaptation to life in habitats which may be periodically exposed to high levels of ammonia.

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