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Exercise and emersion in air and recovery in seawater in the green crab (*Carcinus maenas*): Effects on nitrogenous wastes and branchial chamber fluid chemistry

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Abstract

At low tide, the green crab, which is capable of breathing air, may leave the water and walk on the foreshore, carrying branchial chamber fluid (BCF). N-waste metabolism was examined in crabs at rest in seawater (32 ppt, 13°C), and during 18-h recovery in seawater after 1 h of exhaustive exercise (0.25 BL s^{-1}) on a treadmill in air (20°C–23°C), or 1 h of quiet emersion in air. Measurements were made in parallel to O_2 consumption ($\dot{M}O_2$), acid-base, cardio-respiratory, and ion data reported previously. At rest, the ammonia-N excretion rate ($\dot{M}_{Amm}$ = 44 µmol-N kg^{-1} h^{-1}) and ammonia quotient (AQ; $\dot{M}_{Amm}/\dot{M}O_2$ = 0.088) were low for a carnivore. Immediately after exercise and return to seawater, $\dot{M}_{Amm}$ increased by 65-fold above control rates. After emersion alone and return to seawater, $\dot{M}_{Amm}$ increased by 17-fold. These ammonia-N bursts were greater, but transient relative to longer-lasting elevations in $\dot{M}O_2$, resulting in temporal disturbances of AQ. Intermittent excretion of urea-N and urate-N at rest and during recovery indicated the metabolic importance of these N-wastes. Hemolymph glutamate, glutamine, and PNH_3 did not change. Hemolymph ammonia-N, urea-N, and urate-N concentrations increased after exercise and more moderately after emersion, with urate-N exhibiting the largest absolute increments, and urea-N the longest-lasting elevations. All three N-wastes were present in the BCF, with ammonia-N and PNH_3 far above hemolymph levels even at rest. BCF volume declined by 34% postemersion and 77% postexercise, with little change in osmolality but large increases in ammonia-N concentrations. Neither rapid flushing of stored BCF nor clearance of hemolymph ammonia-N could explain the surges in $\dot{M}_{Amm}$ after return to seawater.

KEYWORDS
ammonia-N, ammonia quotient, excess postexercise ammonia-N excretion (EPANE), urate-N, urea-N

Abbreviations: ANOVA, analysis of variance; AQ, ammonia quotient; BCF, branchial chamber fluid; BL, body lengths; BMSC, Bamfield Marine Sciences Centre; ECFV, extracellular fluid volume; EPANE, excess postexercise or postemersion ammonia-N excretion; EPOC, excess postexercise oxygen consumption; $\dot{M}_{Amm}$, rate of total ammonia-N excretion; $\dot{M}_N$, rate of nitrogenous waste excretion; $\dot{M}O_2$, rate of oxygen consumption; NH_3, ammonia gas; NH_4^+, ammonium ion; PCO_2, partial pressure of carbon dioxide; pK, dissociation constant; PNH_3, partial pressure of ammonia gas; T_{Amm}, concentration of total ammonia = $NH_3 + NH_4^+$; TCO_2, total carbon dioxide concentration = $CO_2 + HCO_3^- + CO_3^{2-}$; αNH_3, ammonia gas solubility coefficient.

1 | INTRODUCTION

The European green crab (*Carcinus maenas*), now invasive throughout much of the world (Behrens-Yamada, 2001; Klassen & Locke, 2007), has become a model organism in decapod physiology (Leignel et al., 2014). It is capable of breathing air, emersing itself at low tide or from unfavorable conditions in seawater rockpools. Its physiology during and after air-exposure has been studied extensively (reviewed by Simonik & Henry, 2014), but its ability to actually exercise while emersed in air has received no attention until recently. Wood & Po (2022) presented the first information on its exercise capacity during air-exposure, the physiological changes which occur, and the nature of its postexercise recovery in seawater. Principal findings were an ability to walk continuously on a treadmill in air at low speed (0.25 body lengths s^{-1}) for up to 60 min, accompanied by marked changes in heart and scaphognathite rates. The postexercise period was characterized by a severe acidosis of both respiratory origin (greatly elevated hemolymph PCO_2) and metabolic origin (greatly elevated hemolymph [lactate] and reduced [HCO_3^-]), and a large excess postexercise oxygen consumption (EPOC). However, there was a rapid recovery of hemolymph pH due to respiratory compensation (decreased PCO_2) despite a profound and long-lasting metabolic acidosis. The metabolic acid load in the hemolymph persisted for at least 8 h postexercise despite evidence of the mobilization of $CaCO_3$ and $MgCO_3$ from the carapace as a source of buffer base. In general, all these responses were much greater in animals than had been exercised in air relative to those of crabs that had been quietly emersed in air for the same time period.

The present paper investigates the nitrogenous waste metabolism of the crabs during these same experiments. It is well documented that all three major nitrogenous wastes (ammonia, urea, and urate; Wright, 1995) occur in the hemolymph and tissues of the green crab (Binns, 1969; Durand & Regnault, 1998; Durand et al., 1999; Spaargaren, 1982). With respect to excretion, most focus has been on ammonia-N (Durand & Regnault, 1998; Fehsenfeld & Weihrauch, 2016; Henry et al., 2012; Simonik & Henry, 2014; Spaargaren, 1982; Weihrauch et al., 1998, 1999, 2002, 2004, 2017), though Durand & Regnault (1998) and Weihrauch et al. (1999) reported that urea-N excretion to the water amounted to only 13% −21% of ammonia-N excretion under resting conditions in green crabs. There appear to no reports on urate-N excretion rates, and no reports on N-waste excretion after exercise in this species. Exercise is of particular relevance to nitrogen metabolism because of the potential liberation of N-wastes from increased rates of amino acid oxidation and adenylate breakdown (Hochachka, 1985). In the blue crab (*Callinectes sapidus*), ammonia-N excretion increased greatly during enforced swimming activity in seawater (Booth et al., 1984; Milligan et al., 1989). The present study on *C. maenas* therefore focuses on responses in ammonia-N, urea-N, and urate-N concentrations in the hemolymph as well as their excretion rates induced by 60 min of treadmill exercise in air, and the changes which occur during the subsequent 18 h of recovery in seawater. Patterns are compared with those seen after 60 min of quiet emersion in air.

Many species of air-breathing crabs, including *C. maenas* (Wheatly & Taylor, 1979) are known to carry water from their aquatic environment with them in the gill chambers when they emerse into air (Burnett, 1988), and there are reports of high concentrations of ammonia-N building up in this branchial chamber fluid (BCF) (Burnett & McMahon, 1987; Regnault, 1994). In the present study, the finding of very high rates of ammonia-N excretion occurring when crabs were returned to seawater after exercise or emersion prompted a quantitative investigation of the chemistry of the BCF, and its possible contribution to rates of N-waste excretion.

Specific hypotheses were that exercise would differentially elevate the hemolymph concentrations and excretion rates of all three N-wastes during recovery, that these changes would be greater than after simple quiet emersion, and that ammonia-N would predominate, reflecting its much lower metabolic cost for manufacture (Wright, 1995). An additional hypothesis was that that the flushing of BCF after return to water, when scaphognathite rates are exceptionally high (Wood & Po, 2022), would make a substantial contribution to the observed surge in N-excretion rate after exercise and emersion.

2 | METHODS

2.1 | Animal collection, maintenance, and handling

With the exception of the BCF collections (*Series 4*), these experiments were performed on the same male green crabs (40–90 g) as reported in Wood & Po (2022), so the origin and husbandry of the crabs was identical. They were collected by traps baited with fish fillets in areas near Seddall Island, Ecoole, Regional District of Alberni-Clayoquot, British Columbia, Canada (49°05′00.0″ N 125°10′21.6″W) under Fisheries and Oceans Canada permits XR-180-2020 and XR-136-2021. The crabs in *Series 4* were collected the following year, from the same sites, at the same time, and held identically at Bamfield Marine Sciences Centre. The mean weights, carapace widths (widest point), and body lengths of all the crabs used in the present study were 56.18 ± 1.23 g, 6.07 ± 0.04 cm, and 8.19 ± 0.06 cm (means ± SEM, $N = 155$). As crabs generally run sideways, body length was taken as the distance between the first hinges of the longest left and right walking legs when the crab was standing upright. Experimental temperature was 12°C–13°C, and salinity was 32 ppt. The animals were fasted for 3 days before experiments. They were then placed in individual shielded 500-ml glass jars for respirometry. The jars were served with flowing seawater and aeration, and the crabs were allowed to settle for 24 h before data collection commenced, so the total fasting period was 4 days. Experiments were approved by the BMSC Animal Care Committee.

The two experimental treatments were either exercise on a treadmill in air (20°C–23°C) at 0.25 BL s^{-1} for 1 h, or simple emersion in air for 1 h in dry chambers, performed as described by Wood & Po

(2022). The crabs were then returned to their 500-ml glass jars, and followed for 18 h during recovery in seawater.

2.1.1 | Series 1—weight loss during exercise and emersion

This series was performed as described by Wood &Po (2022), and yielded the weight change data at the end of 1 h of exercise in air, or at the end of 1 h of emersion, values that were used in calculations of BCF volume changes for comparison to the results of *Series 4*.

2.1.2 | Series 2—N-waste excretion

Seawater samples for the measurement of ammonia-N excretion ($\dot{M}_{Amm}$), urea-N excretion, and urate N-excretion were collected simultaneously with the $\dot{M}O_2$ measurements reported in *Series 2* of Wood & Po (2022). The flushing and sampling cycles have been described in that paper. Supporting Information: Figure S1 lays out the time-line for the flux measurements. Flux measurements were made in crabs ($N = 6$) under control resting conditions in seawater (2 × 30-min periods) before exercise. The crabs were then exercised in air at 0.25 BL s^{-1} for 1 h, following which the crabs were returned to seawater. Flux measurements were made continuously during the first 2 h (8 × 15-min periods) after return to seawater, and then at 5, 8, and 18 h (2 × 30-min periods each, bracketing the times). An identical protocol was used before and after 1 h of emersion in air ($N = 7$). The jars were sealed during the flux measurements. Additionally, to measure N -excretion into the emersion containers during the 1-h ($N = 7$) and 10–12-h periods ($N = 6$) of air exposure (the latter during overnight $\dot{M}O_2$ measurements in air as described by Wood & Po, 2022), the chambers were thoroughly rinsed at the end with 50 ml of fresh seawater, which was collected. All seawater samples were immediately frozen at −20°C for the later measurement of ammonia-N, urea-N, and urate-N concentrations.

2.1.3 | Series 3—hemolymph N-metabolites

Hemolymph samples for measurement of N-metabolites were aliquoted separately as part of the 0.5-ml samples taken in *Series 3* of Wood & Po (2022). Samples were taken from the arthrodial membrane of the posterior walking legs of crabs (i.e., venous hemolymph) while they remained under water. Sampling times in the exercise series were resting control in seawater (pre-exercise) and at 0, 0.5, 1, 2, 5, 8, and 18 h postexercise during recovery in seawater, with $N = 6–7$ samples at each time. Sampling times in the emersion series were control (pre-emersion) and at 0, 2, 5, 8, and 18 h postemersion during recovery in seawater, with $N = 6–7$ samples at each time. Samples were immediately frozen in liquid nitrogen, then stored at −80°C for later analyses.

2.1.4 | Series 4—BCF volume and composition

Various methods were tried to collect BCF, both by implantation of chronic cannulae into the chambers through holes drilled at the lateral borders of the dorsal carapace (Burnett & McMahon, 1987), and by inserting cannulae acutely through the inhalant pathways—the Milne-Edwards openings under the chelipeds and through smaller openings at the bases of the walking legs, the latter as described by Regnault (1992). However, most yielded only small or no volumes in emersed crabs. As it was important to obtain measurements of total BCF volumes for our calculations, a more robust method was used, based on the technique used by Depledge (1984) to deplete branchial chamber water. The crab was removed from its experimental treatment, and dried in a standardized fashion without tilting; the same procedure as used in Series 1. This involved holding the crab upright, and then drying it with pieces of cotton towel. The crab would usual grab the first piece of towel with its chelipeds, so subsequent pieces could be used to remove external moisture. The crab was then briefly placed on a dry surface, when it would invariably drop the towel, so that the animal could be picked up again and placed into a tared plastic beaker and weighed. The crab was then then held over a large piece of parafilm. The animal was gently shaken by hand over the parafilm for a 3-min period, with continuous changes in the angle at which the crab was held. Initial trials showed that negligible additional fluid was released after 3 min. All droplets deposited on the parafilm were then collected using a 250-µl gas-tight syringe (Hamilton) and transferred to a tared micro-centrifuge tube, which was then weighed to determine volume. Total CO_2 and pH in BCF were measured as for hemolymph samples in *Series 3* (Wood & Po, 2022) and the remaining sample was then frozen in liquid nitrogen and stored at −80°C for later analyses of N-wastes and osmolality. Crabs were sampled when resting, fully immersed in flowing seawater (control, $N = 8$), at the end of 1 h of standard emersion ($N = 8$), and at the end of 1 h of treadmill exercise at 0.25 Bl s^{-1} ($N = 8$). Additionally, flowing seawater samples ($N = 8$) were taken from the same source at the time when these experiments were performed and subjected to the same analyses.

2.2 | Analytical techniques and calculations

The concentrations of ammonia-N and urea-N (2N per urea molecule) in seawater and BCF were determined colorimetrically by the methods of Verdouw et al. (1978) and Rahmatullah & Boyde (1980) respectively, with seawater used as a blank, and standards made up in seawater. Concentrations of urate-N (4N per urate molecule) were assayed with a DIUA-250 QuantiChrom™ uric acid assay kit (BioAssay Systems), with seawater used as a blank, and standards made up in seawater. Excretion rates ($\dot{M}_N$, µmol-N kg^{-1} h^{-1}) of ammonia-N, urea-N, and urate-N were calculated as:

$$\dot{M}_N = [([N]_{final} - [N]_{initial}) \times V]/(W \times T), \quad (1)$$

where [N] was measured in μmol-N L^{-1}, *V* (L) was the volume of the respirometer, *W* (kg) was the body mass (kg), and *T* was time (h). Tests with empty chambers demonstrated that there was no detectable blank production of any of the three N-wastes. For tests where N-excretion to the dry emersion chambers was measured, *V* was 0.05 L, representing the 50 ml of seawater used to rinse the chambers at the end. The detection limits for submerged crabs in the 500-ml respirometry jars were about 2 μmol-N kg^{-1} h^{-1} for both ammonia-N excretion and urea-N excretion, and about 15 μmol-N kg^{-1} h^{-1} for urate-N excretion. For the 50-ml rinses from the initially dry chambers used for emersion of crabs, the detection limits were about 10-fold lower.

The ammonia quotients (AQs, ratio of $\dot{M}_{Amm}$ to $\dot{M}O_2$) were calculated based on simultaneous measurements of $\dot{M}_{Amm}$ (reported in Figure 1a,b) and $\dot{M}O_2$ (reported in Wood & Po, 2022) in these same crabs. The excess postexercise or postemersion ammonia-N excretion (EPANE) was calculating by integrating the area under the individual $\dot{M}_{Amm}$ versus time plots, relative to the pretreatment resting control values in seawater.

Concentrations of total ammonia-N (T_{Amm}) in hemolymph were measured enzymatically (L-glutamate dehydrogenase) on freshly thawed samples using an AA0100 ammonia assay kit (Sigma-Aldrich) which had been cross-validated with the colorimetric water ammonia assay using common standards. Hemolymph concentrations of urea-N and urate-N were measured as in seawater, again using common standards. In the urate-N assay, correction was made for the blank absorbance due to hemolymph (Lallier et al., 1987). The samples for urate-N analyses were freshly thawed, whereas the urea-N analyses were performed on the second thaw. Tests showed that hemolymph urea-N concentration was not affected by several freeze-thaw cycles. Glutamine and glutamate concentrations in hemolymph were determined with an EGLN-100 EnzyChrom™ assay kit (BioAssay Systems).

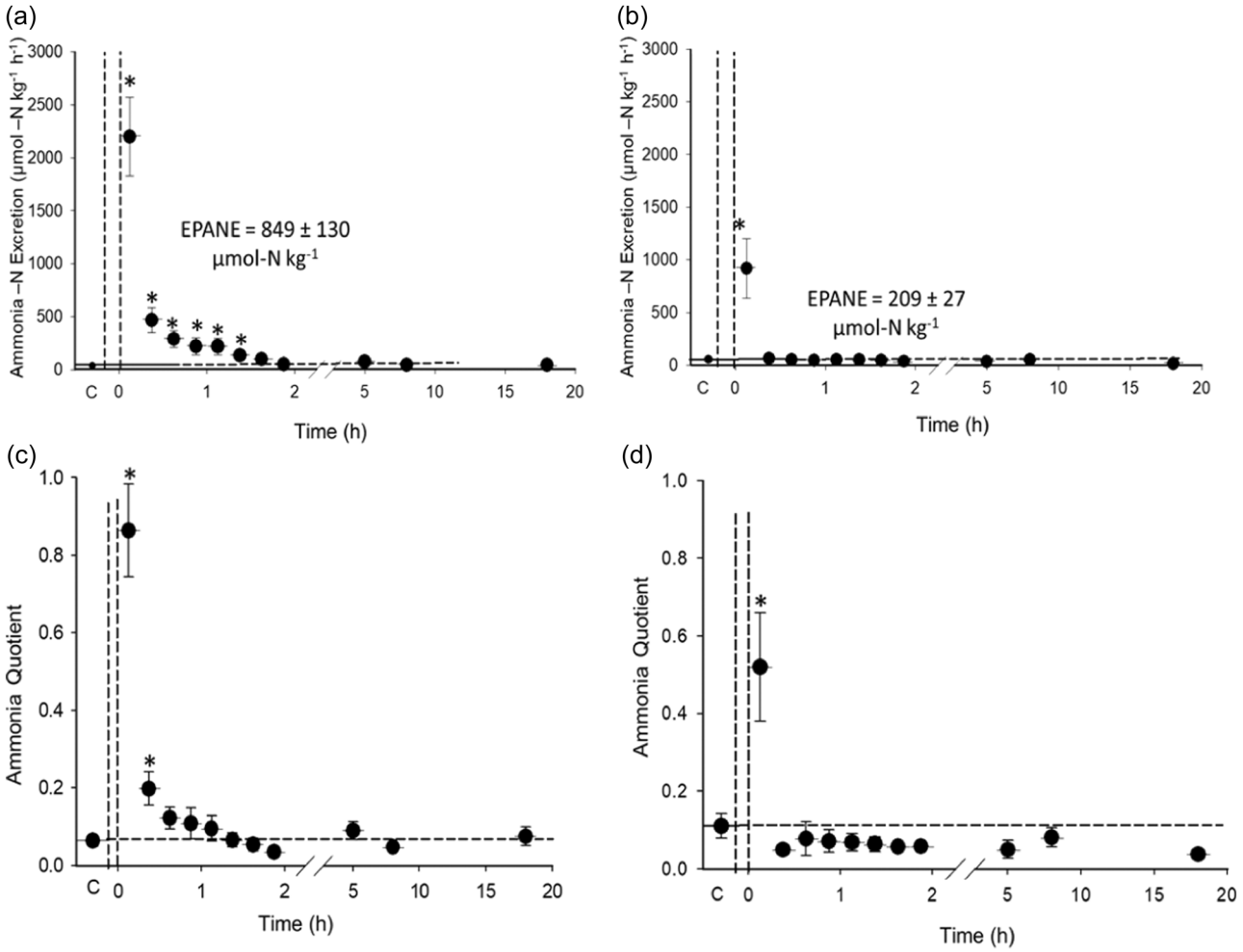

FIGURE 1 (a) and (b) Ammonia-N excretion rates ($\dot{M}_{Amm}$) and (c) and (d) the corresponding ammonia quotients (AQs), the ratio of simultaneously measured $\dot{M}_{Amm}$ to $\dot{M}O_2$ (from Wood & Po, 2022) of *Carcinus maenas* in *Series 2* in response to 1 h of exercise in air (left-hand panels, *N* = 6), or 1 h of simple emersion in air (right-hand panels, *N* = 7), followed by 18-h recovery in seawater. Calculated excess postexercise or postemersion ammonia-N excretions (EPANE) are also shown. The air-exposure period is indicated by dashed vertical lines, and resting control rates in seawater by dashed horizontal lines. Means ± 1 SEM. Two-way repeated measures ANOVA during recovery for $\dot{M}_{Amm}$: treatment ($p < 0.001$), time ($p < 0.015$), and interaction ($p < 0.001$). For AQ: treatment (p = n.s.), time ($p < 0.001$), and interaction (p = n.s.). Asterisks indicate significant differences ($p \leq 0.05$) from control values.

Constants for pK and ammonia solubility at the appropriate temperature and ionic strength from Cameron and Heisler (1983) were used to calculate the fractionation of ammonia in hemolymph, BCF, and seawater using the following equations:

$$[NH_3] = \frac{Plasma T_{Amm} \times \text{antilog}\,(pH - pK)}{1 + \text{antilog}\,(pH\text{-}pK)}, \quad (2)$$

$$[NH_4^+] = T_{Amm} - [NH_3], \quad (3)$$

$$PNH_3 = [NH_3]/\alpha NH_3, \quad (4)$$

where T_{Amm} was measured in μmol L^{-1} and αN_{H3} was the solubility coefficient of NH_3 (μmol L^{-1} μTorr^{-1}). Hemolymph pH values were taken from measurements on these same samples reported by Wood & Po (2022).

Total CO_2 (TCO_2, mmol L^{-1}) in BCF and seawater was measured using a Corning 965 analyser (Corning Instruments) calibrated with $NaHCO_3$ standards. On these same samples, pH values were measured anaerobically in a temperature-controlled water bath, using an MI-4156 Micro-Combination pH probe (Microelectrodes Inc.) and Accumet pH meter (Fisher Scientific), calibrated with Radiometer precision buffers (Radiometer-Copenhagen). PCO_2 in seawater was calculated using the CO_2Sys EXCEL macro of Lewis & Wallace (1998). Osmolality in BCF and seawater was determined with a 5100C vapor pressure osmometer and Opti-Mole™ standards (Wescor).

2.3 | Statistical analyses

Statistical analyses paralleled those in Wood & Po (2022), and a significance level of $p \leq 0.05$ was used throughout. Data have been expressed as means ± 1 SEM (N) where N = number of crabs. Most analyses were parametric. All data were first checked for normality of distribution and homogeneity of variance, and if necessary, standard transformations (log, square route, inverse) were applied. If data still failed, nonparametric alternatives were used. Student's two-tailed t-test (or the Mann–Whitney U test for nonparametric comparisons) were used to compare the resting control measurements in seawater between the exercise series and the emersion series for all parameters. These tests were also used for comparing some calculated physiological parameters between or within the two treatment groups. To detect significant overall effects of treatment, recovery time, and their interactions, two-way ANOVAs were performed on the 0–18 h recovery data for the exercise and emersion treatments. In *Series 2* where the same animals were followed over time, these were repeated measures two-way ANOVAs. One-way ANOVAs (regular or repeated measures as appropriate) followed by Dunnett's post hoc test were then used within each treatment to identify significant differences from resting control measurements in seawater. One-way ANOVAs followed by Tukey's post hoc tests were used to identify differences amongst treatments for the BCF samples of *Series 4*. The Kruskall Wallis ANOVA on ranks, followed by Dunn's post hoc test, was performed for nonparametric comparisons.

3 | RESULTS

3.1 | N-waste excretion

Rates of ammonia-N excretion ($\dot{M}_{Amm}$) for resting crabs in seawater under control conditions did not differ between the two experimental treatments, averaging 44 ± 11 (N = 13) μmol-N kg^{-1} h^{-1} (Figure 1; Table 1). $\dot{M}_{Amm}$ was significantly elevated after 1 h in air, with a much larger 65-fold increase after exercise (Figure 1a) relative to the 17-fold elevation after emersion (Figure 1b), measured during the first 15 min after return to seawater. $\dot{M}_{Amm}$ also declined much more quickly in the emersed crabs, returning to control levels by 0.25–0.50 h (Figure 1b), whereas the elevation stayed significant through 1.25–1.50 h in the exercised crabs (Figure 1a). Two-way ANOVA identified significant overall effects of treatment ($p < 0.001$), time ($p = 0.015$), and their interaction ($p < 0.001$), with $\dot{M}_{Amm}$ being consistently higher in the exercised crabs than in the emersed crabs throughout recovery. The EPANEs were 849 ± 130 (N = 6) μmol-N kg^{-1} after exercise, and 209 ± 27 (N = 7) μmol-N kg^{-1} after emersion, a significant difference.

The AQs ($AQ = \dot{M}_{Amm}/\dot{M}O_2$) were the same in the two groups under control conditions, averaging 0.088 ± 0.019 (N = 13) (Figure 1c,d). During recovery, there were significant overall effects of only time ($p < 0.001$); treatment and interaction effects were not significant. $\dot{M}_{Amm}$ increased to a much greater extent than $\dot{M}O_2$ immediately after return to water in both treatments, so the AQ initially rose significantly to about 0.86 at 0–0.25 h postexercise (Figure 1c), and to 0.52 (Figure 1d) at 0–0.25 h postemersion. Thereafter AQ values quickly declined, returning to control levels by 0.75–1.0 h in the exercised treatment and 0.25–0.50 h in the emersion treatment. The AQ for EPANE relative to the EPOC was

TABLE 1 N-waste excretion rates measured by washing the containers after crabs had been emersed in air for 1 or 10–12 h compared with those measured under resting control conditions in seawater

	Resting control in seawater	1 h emersion in air	10–12 h emersion in air
Ammonia-N excretion rate (μmol-N kg^{-1} h^{-1})	44.4 ± 11.2 (13)^{A} 13/13$^{\#}$	7.6 ± 1.7 (23)^{B} 23/23$^{\#}$	2.6 ± 0.8 (6)^{C} 6/6$^{\#}$
Urea-N excretion rate (μmol-N kg^{-1} h^{-1})	16.9 ± 4.7 (13)^{A} 9/13$^{\#}$	8.7 ± 1.4 (23)^{A} 23/23$^{\#}$	1.4 ± 0.5 (6)^{B} 6/6$^{\#}$
Urate-N excretion rate (μmol-N kg^{-1} h^{-1})	210.3 ± 81.2 (13)^{A} 5/13$^{\#}$	22.5 ± 11.9 (23)^{A} 8/23$^{\#}$	2.7 ± 2.5 (6)^{A} 2/6$^{\#}$

Note: For each treatment and N-waste, the fraction of crabs exhibiting detectable excretion is given, indicated by #. Means ± 1 SEM, where all undetectable values were included as zero. Means sharing the same letter within individual rows are not significantly different ($p > 0.05$).

0.112 ± 0.016 (N = 6) in the exercised crabs, not significantly different from 0.079 ± 0.010 (N = 7) in the emersed crabs. Both values were also similar to the pretreatment control values.

Rates of urea-N excretion were highly variable both within and among crabs, and were sometimes undetectable. For this reason, the individual rates have been plotted in Figure 2 on a logarithmic scale, with the number of "zero" values (i.e., below the detection limit of about 2 µmol urea-N kg^{-1} h^{-1}) tabulated just above the X-axis. Most, but not all crabs (five of six in the exercise control, four of seven in the emersion control) excreted detectable amounts of urea-N during the resting periods in seawater (Figure 2). The two control rates (including the "zero" values), were not significantly different, averaging 17 ± 5 (N = 13) µmol-N kg^{-1} h^{-1} (Figure 2a,b; Table 1). After both exercise (Figure 2a) and emersion (Figure 2b, rates tended to increase, but there were no significant differences by either two-way or one-way ANOVA, and it was not practical to calculate an excess postexercise or postemersion urea-N excretion. Nevertheless, during the first 2 h postexercise or postemersion, the time-averaged mean rates (including all "zero" values) were 55 ± 19 (N = 6) µmol-N kg^{-1} h^{-1} in the exercise treatment and 31 ± 2 µmol-N kg^{-1} h^{-1} (N = 7) in the emersion treatment. Only the latter was significantly greater than its respective resting control value.

Even greater variability and similar intermittency occurred in the rates of urate-N excretion (Figure 3), so the data have been

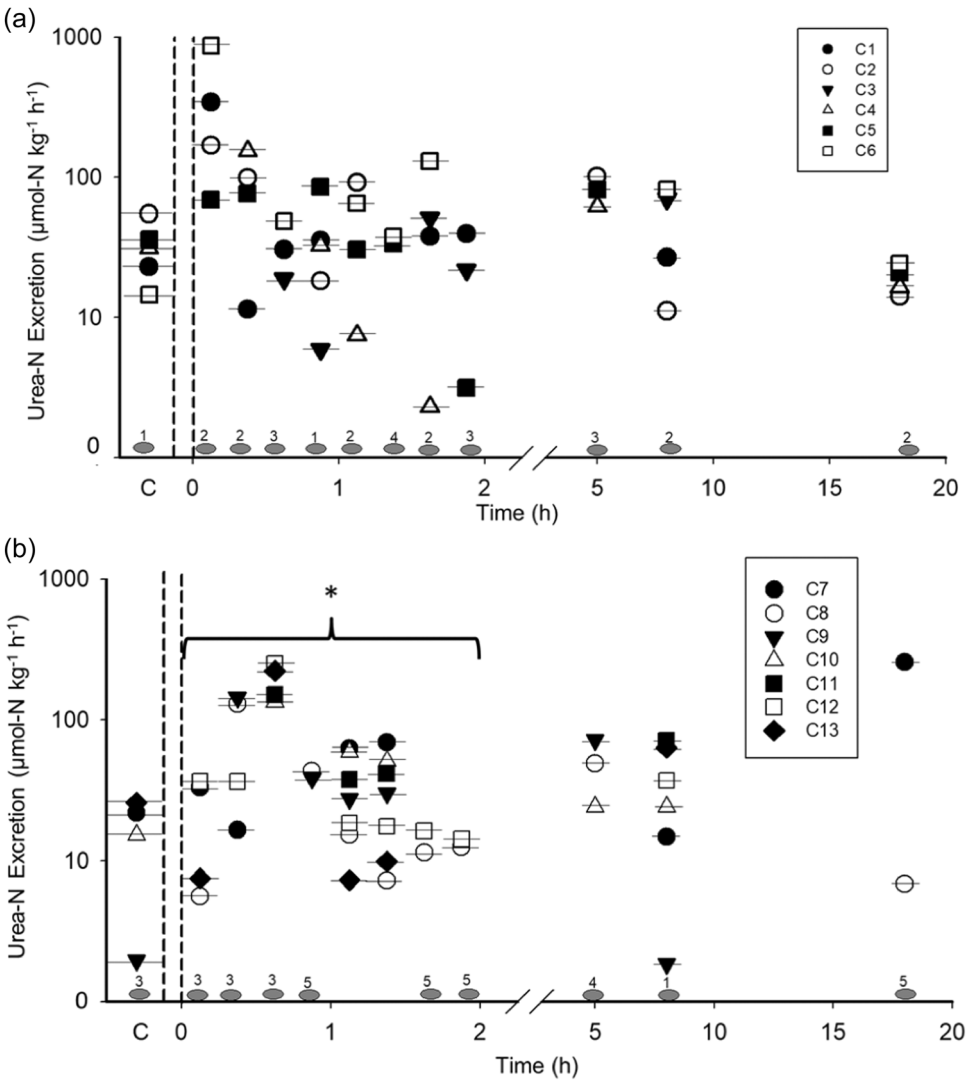

FIGURE 2 Urea-N excretion rates of *Carcinus maenas* in *Series 2* in response to (a) 1 h of exercise in air (N = 6), or (b) 1 h of simple emersion in air (N = 7), followed by 18-h recovery in seawater. The air-exposure period is indicated by the dashed vertical lines. In light of the high variability, individual rates for each crab over each measurement period are shown, and the Y-axis has a logarithmic scale to capture this variability. The number of "zero" values (i.e., below the detection limit of about 2 µmol urea-N kg^{-1} h^{-1}) have been plotted just above the X-axis in each time period. Two-way repeated measures ANOVA during recovery: treatment (p = n.s.), time (p = n.s.), and interaction (p = n.s). The bracket and asterisk indicate that the time-averaged urea-N excretion (including all "zero values") over the first 2 h postemersion was significantly elevated ($p < 0.05$) relative to the control rate (Mann–Whitney U test). This was not the case for time-averaged urea-N excretion over the first 2 h after exercise

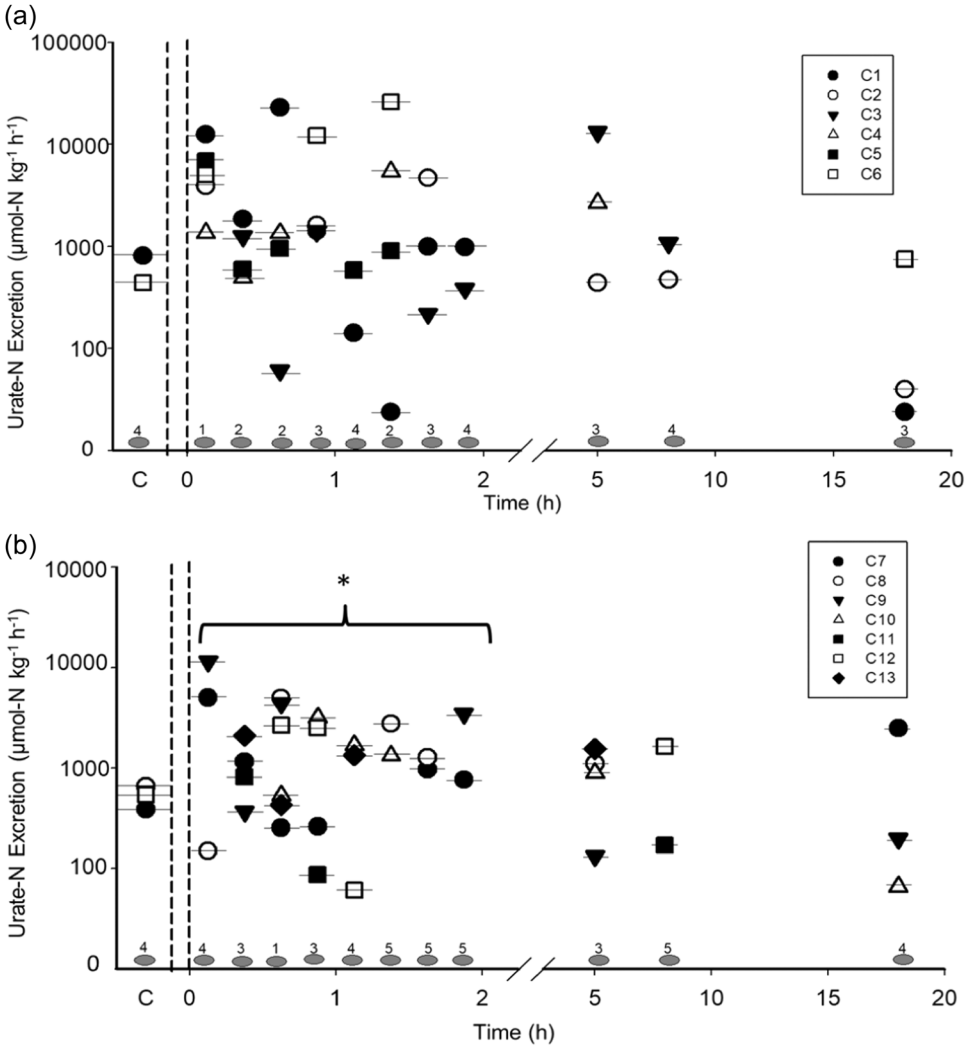

FIGURE 3 Urate-N excretion rates of *Carcinus maenas* in *Series 2* in response to (a) 1 h of exercise in air ($N = 6$), or (b) 1 h of simple emersion in air ($N = 7$), followed by 18-h recovery in seawater. The air-exposure period is indicated by the dashed vertical lines. In light of the high variability, individual rates for each crab over each measurement period are shown, and the *Y*-axis has a logarithmic scale to capture this variability. The number of "zero" values (i.e., below the detection limit of about 15 µmol urea-N kg^{-1} h^{-1}) have been plotted just above the *X*-axis in each time period. Two-way repeated measures ANOVA during recovery: treatment (p = n.s.), time (p = n.s.), and interaction (p = n.s). The bracket and asterisk indicate that the time-averaged urate-N excretion (including all "zero values") over the first 2 h postemersion was significantly elevated ($p < 0.05$) relative to the control rate (Mann–Whitney *U* test). This was not the case for time-averaged urate-N excretion over the first 2 h after exercise.

plotted as for urea-N excretion. In the exercise and emersion treatments, only two of six crabs and three of seven crabs respectively excreted detectable amounts of urate-N during the control periods in seawater (Figure 3). Including the "zero "values (i.e., below the detection limit of about 15 µmol urate-N kg^{-1} h^{-1}), the two control rates were not significantly different, averaging 210 ± 81 ($N = 13$) µmol-N kg^{-1} h^{-1}. After both exercise (Figure 3a) and emersion (Figure 3b), urate-N excretion rates tended to increase. However, as with urea-N excretion, there were no significant differences by either two-way or one-way ANOVA, and it was not practical to calculate an excess postexercise or postemersion urate-N excretion. During the first 2 h postexercise or postemersion, the time-averaged mean urate-N excretion rates (including all "zero" values) were 2424 ± 901 ($N = 6$) µmol-N kg^{-1} h^{-1} in the exercise treatment and 983 ± 314 µmol-N kg^{-1} h^{-1} ($N = 7$) in the emersion treatment. Again, only the latter was significantly greater than its respective control value.

A comparison of N-waste excretion rates measured by washing the containers after crabs had been emersed in air for 1 h or 10–12 h with those measured under resting control conditions in seawater revealed that detectable ammonia-N and urea-N excretion continued in air in all crabs, albeit at much lower rates (Table 1). Ammonia-N excretion rates were significantly reduced by about 83% over 1 h, and 94% over 10–12 h, whereas only the 93% reduction in urea-N excretion rate over 10–12 h was significant. The 89% and 99% reductions in absolute urate-N excretion rates over 1 h and 10–12 h

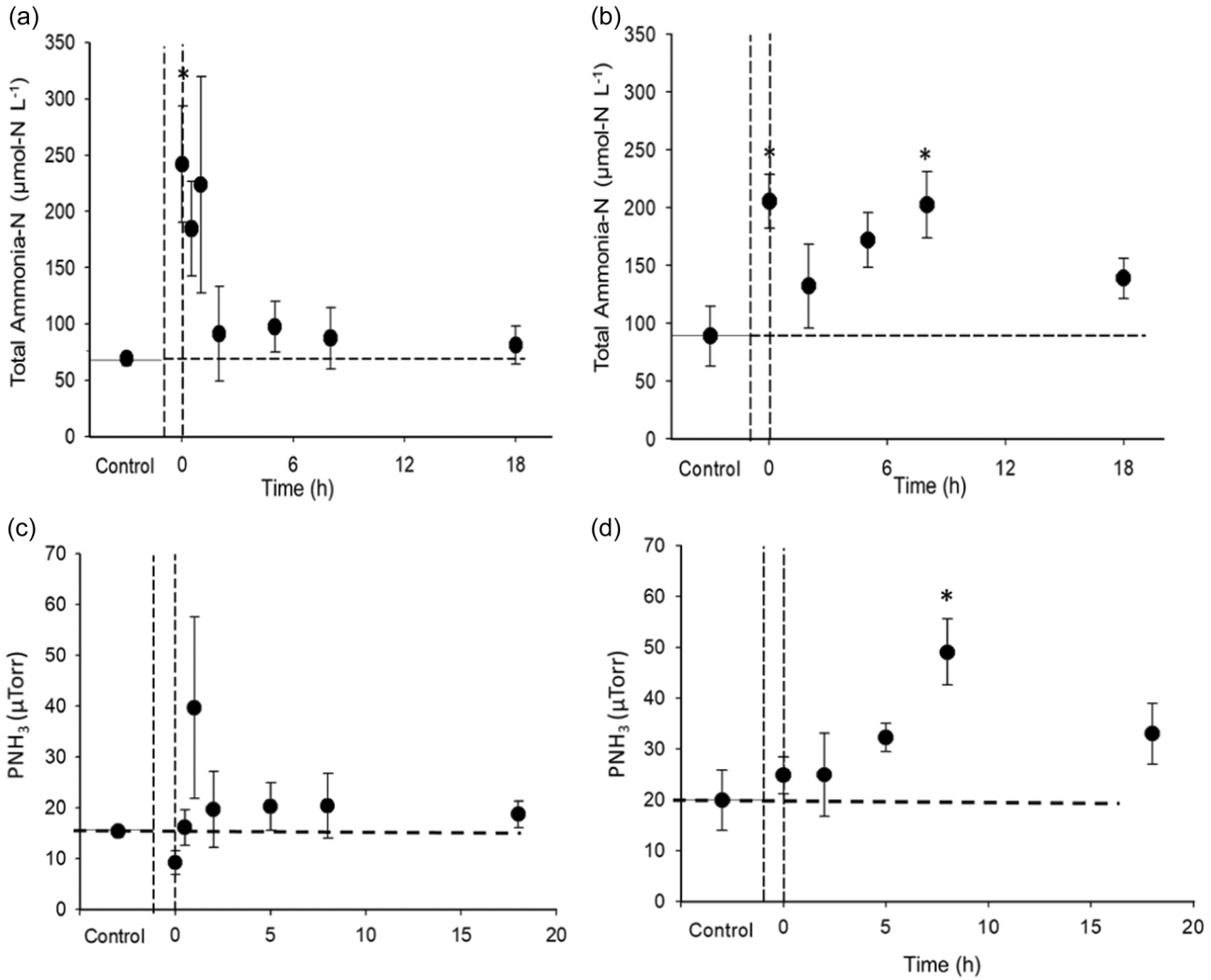

FIGURE 4 (a) and (b) Total ammonia-N (T_{Amm}) and (c) and (d) PNH_3 responses in venous hemolymph of *Carcinus maenas* in *Series 3* in response to 1 h of treadmill exercise in air (left-hand panels, $N = 6–7$), or 1 h of simple emersion in air (right panels, $N = 6–7$) followed by 18-h recovery in seawater. The air-exposure period is indicated by the dashed vertical lines, and the control resting values in seawater by the dashed horizontal lines. Means ± 1 SEM. Two-way ANOVA during recovery for T_{Amm}: treatment ($p < 0.001$), time ($p < 0.001$), and interaction ($p < 0.001$). For PNH_3: treatment ($p = 0.004$), time ($p = 0.04$), and interaction (p = n.s.). Asterisks indicate significant differences ($p \leq 0.05$) from control values.

respectively were not significant, reflecting great variability and many "zero" values in the data.

3.2 | Hemolymph N-Metabolites

Hemolymph total ammonia-N (T_{Amm}) and PNH_3 levels under resting control conditions in seawater were not significantly different among the two groups, averaging 79 ± 13 ($N = 14$) µmol-N L^{-1} (Figure 4a,b) and 18 ± 3 ($N = 14$) µTorr (Figure 4c,d) respectively. Immediately after both exercise (Figure 4a) and emersion (Figure 4b), T_{Amm} concentrations increased significantly by about three-fold, but declined quickly thereafter, though there was a secondary significant elevation at 8 h in the emersion treatment. Two-way ANOVA revealed significant overall effects of treatment ($p = 0.003$) and time ($p = 0.01$), but no significant interaction (Figure 4a,b). In contrast to T_{Amm}, PNH_3 levels did not change significantly after exercise because of the substantial hemolymph acidosis at the times of T_{Amm} elevation (see Wood & Po, 2022). After emersion, there was a significant increase in PNH_3 only at 8 h (Figure 4c,d). By two-way ANOVA, there were significant effects of treatment ($p = 0.004$) and time ($p = 0.04$), but no significant interaction.

Hemolymph urea-N concentrations were similar to T_{Amm}, levels in resting crabs, and did not differ significantly between the two control groups, averaging 120 ± 12 ($N = 14$) µmol-N L^{-1} (Figure 5a,b). Responses during recovery in the two treatments were similar, so there were no significant treatment or interaction effects by two-way ANOVA, though the effect of time was significant ($p < 0.001$). After both exercise (Figure 5a) and air-emersion (Figure 5b), hemolymph urea-N approximately doubled and remained significantly greater than control levels at most times through 8 h, but had returned to resting levels by 18 h.

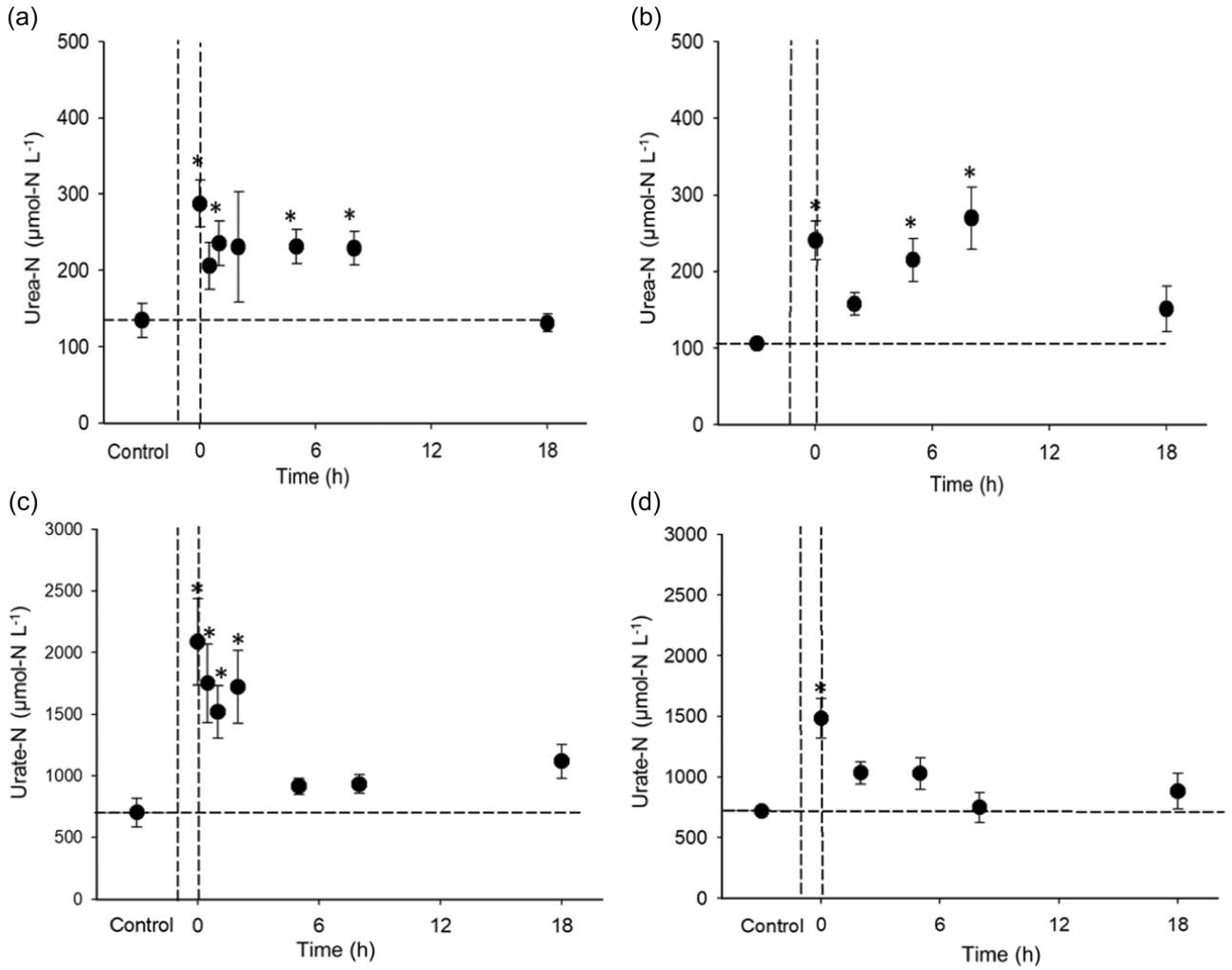

FIGURE 5 (a) and (b) Hemolymph urea-N and (c) and (d) urate-N responses of *Carcinus maenas* in *Series 3* in response to 1 h of treadmill exercise in air (left-hand panels, $N = 6–7$), or 1 h of simple emersion in air (right-hand panels, $N = 6–7$), followed by 18-h recovery in seawater. The air-exposure period is indicated by the dashed vertical lines, and the control resting concentrations in seawater before treatment by the dashed horizontal lines. Means ± 1 SEM. Two-way ANOVA during recovery for urea-N: treatment (p = n.s), time ($p < 0.001$), and interaction (p = n.s.). For urate-N: treatment (p = n.s.), time ($p < 0.001$), and interaction (p = n.s). Asterisks indicate significant differences ($p \leq 0.05$) from control values.

Under resting control conditions in seawater, hemolymph urate-N concentrations were much higher than either T_{amm}-N or urea-N levels, and were virtually identical in the two treatments, averaging 710 ± 60 ($N = 14$) µmol-N L^{-1} (Figure 5c,d). Urate-N concentrations increased by three-fold immediately after exercise, and by two-fold immediately after emersion, and then returned to control levels by 5 h in the exercised crabs and 2 h in the emersed crabs. Two-way ANOVA confirmed that response patterns were similar in the two treatments, so while the overall influence of time was significant ($p < 0.001$), this was not the case for treatment or for interaction.

On a molar basis, the concentrations of glutamate (521 ± 56 µmol L^{-1}, $N = 14$; Figure 6a,b) and glutamine (537 ± 42 µmol L^{-1}, $N = 14$; Figure 6c,d) in the hemolymph were about equal, and did not differ between the two treatment groups under control resting conditions in seawater. However, it is important to note that there is only 1N in glutamate and 2N in glutamine, so the actual concentration of N in hemolymph glutamine was two-fold higher. There were no significant treatment, time, or interactive effects by two-way ANOVA, and no significant differences from control values in either treatment group by one-way ANOVA despite the almost 50% fall in glutamine that was greatest at 2 h after exercise (Figure 6c).

3.3 | BCF volume and composition

The volume of BCF that could be collected dropped significantly from about 10.4 ml kg^{-1} in control resting crabs when they were initially lifted out of seawater to about 2.4 ml kg^{-1} after 1 h of exercise in air (Figure 7a). The latter was also significantly different from 6.9 ml kg^{-1} after 1 h of emersion in air. These values may be compared with whole body weight losses of 26.6 ± 1.3 ($N = 27$) g kg^{-1} in identically

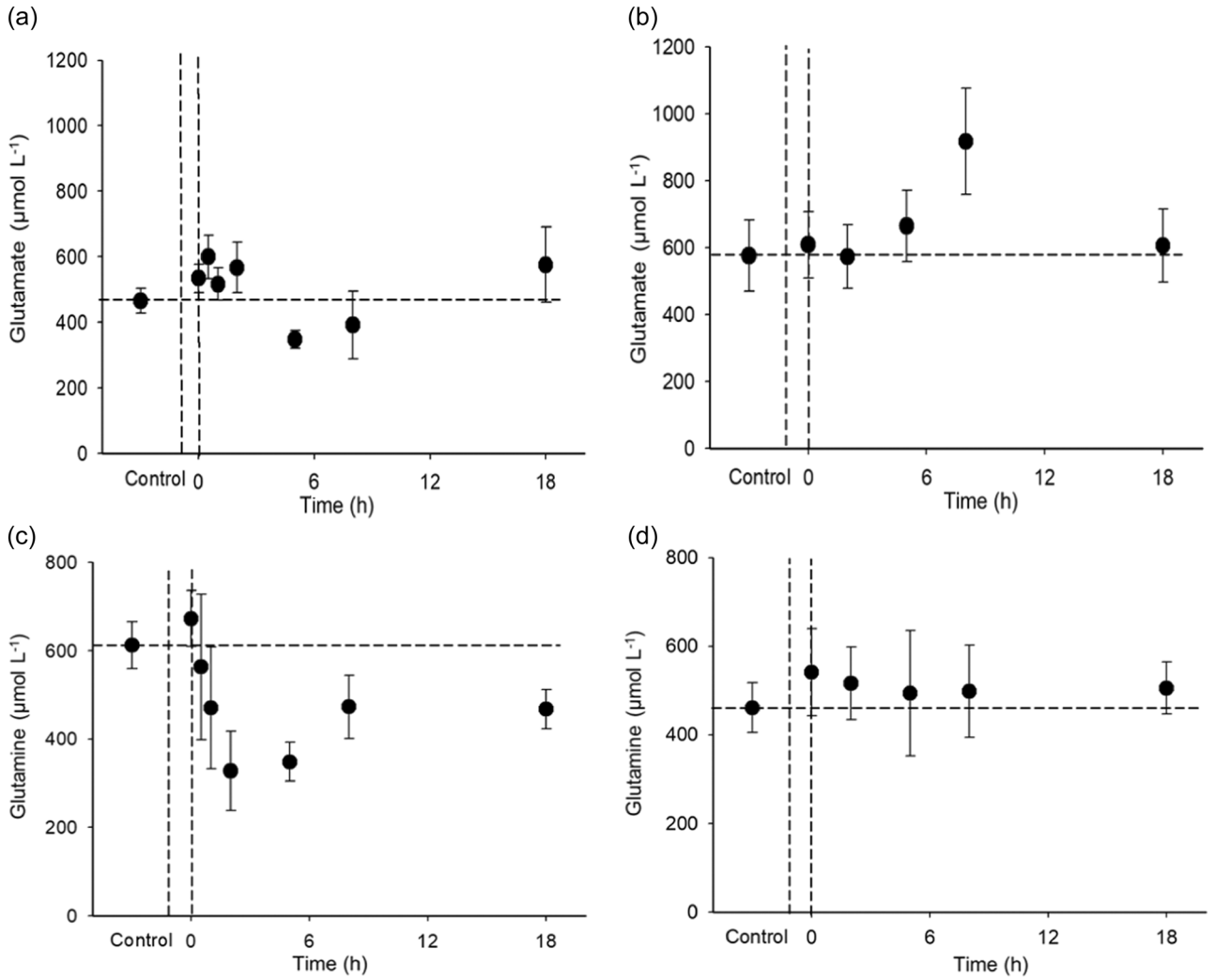

FIGURE 6 (a) and (b) Hemolymph glutamate and (c) and (d) glutamine responses of *Carcinus maenas* in *Series 3* in response to 1 h of treadmill exercise in air (left-hand panels, $N = 6–7$), or 1 h of simple emersion in air (right-hand panels, $N = 6–7$), followed by 18-h recovery in seawater. The air-exposure period is indicated by the dashed vertical lines, and the control resting concentrations in seawater before treatment by the dashed horizontal lines. Means ± 1 SEM. Two-way ANOVAs during recovery revealed no significant effects of treatment, time, or their interaction for either glutamate or glutamine, and there were no significant differences from control values.

exercised crabs and 10.1 ± 0.8 ($N = 23$) g kg^{-1} in identically emersed crabs of *Series 1* as reported in Wood & Po (2022). Thus, the measured decrements of BCF could account for 30% of the mass loss of exercised crabs, and 35% of the mass loss of emersed crabs.

In contrast, changes in osmolality were minimal. In all treatments, BCF osmolality was slightly but significantly higher than in seawater, but there were no significant differences among treatments (Figure 7b). Osmolality in BCF of exercised crabs was only 6% higher than in control crabs, in contrast to the 77% decrease in BCF volume, ruling out evaporation as a major cause of chamber fluid loss. BCF osmolalities remained close to those of hemolymph (Figure 7b) reported for these treatments by Wood & Po (2022).

The pH of the BCF did not vary significantly from that of the seawater in the various treatments, though it became more variable in the exercised crabs (Figure 8a). In control crabs, the BCF had the same total CO_2 (TCO_2, Figure 8b) and PCO_2 (Figure 8c) as the surrounding seawater. However, exercise and emersion in air tended to raise these values with significant 1.9-fold increases in TCO_2 after emersion (Figure 8b) and 2.3-fold increases in PCO_2 after exercise. Smaller increases in TCO_2 after exercise and in PCO_2 after emersion were not significant. In comparison to venous hemolymph measurements reported for these treatments in Wood & Po (2022), BCF pH values were comparable under control conditions but higher after emersion and exercise (Figure 8a), while BCF TCO_2 concentrations (Figure 8b) and PCO_2 tensions (Figure 8c) were considerably lower in all treatments, favouring CO_2 movements from hemolymph to BCF.

Background concentrations of total ammonia-N (T_{Amm}), urea-N, and urate-N were undetectable in seawater. However, in the BCF of control crabs resting in seawater, T_{Amm} was surprisingly high (about 1550 µmol-N L^{-1}), and increased significantly to about 3200 µmol-N L^{-1} after emersion, and 6800 µmol-N L^{-1} after exercise (Figure 9a).

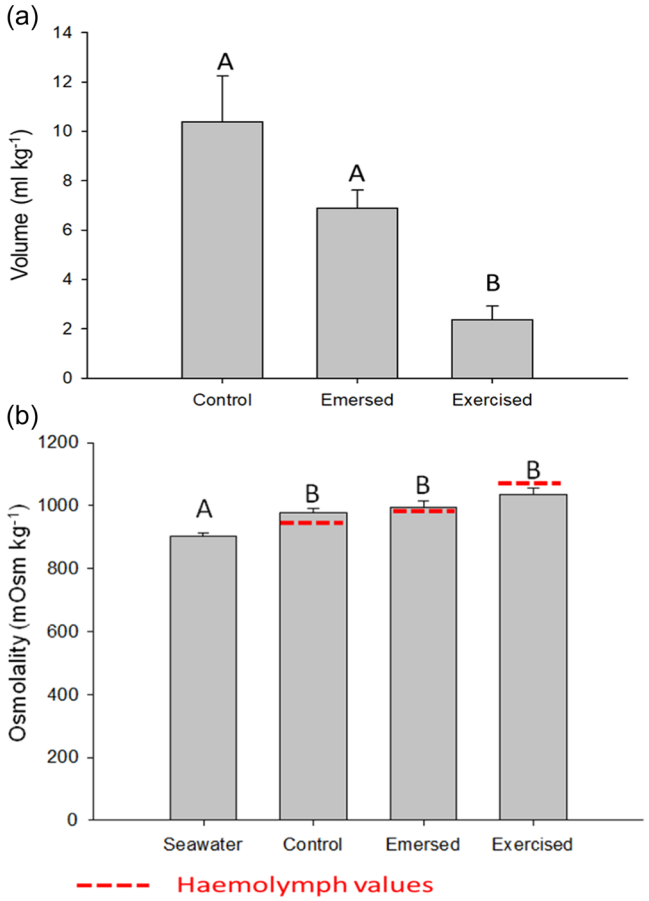

FIGURE 7 (a) Branchial chamber total fluid volumes, and (b) osmolalities collected from *Carcinus maenas* in *Series 4* sampled when resting, immediately after removal from flowing seawater (control, $N = 8$), immediately after 1 h of simple emersion in air ($N = 8$) or exercise in air ($N = 8$). Means ± 1 SEM. In (b), the osmolality of flowing seawater ($N = 8$) sampled at the same time is also shown and the dashed horizontal red lines represent the mean osmolalities in venous hemolymph in these treatments reported by Wood & Po (2022). Bars not sharing the same letters are significantly different ($p \leq 0.05$) from one another.

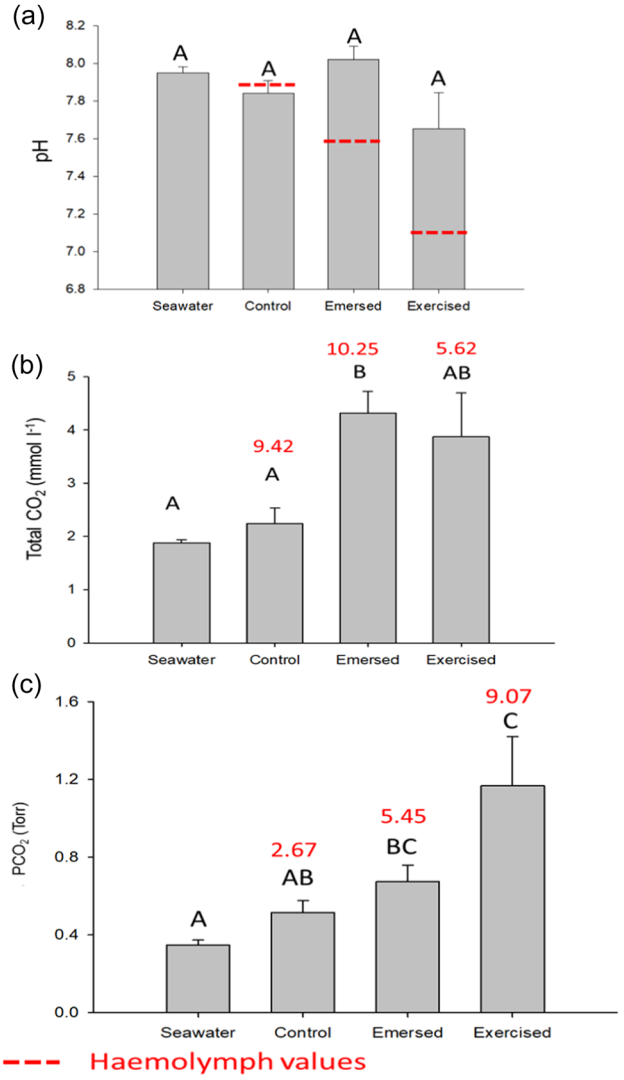

FIGURE 8 (a) pH, (b) total CO_2 concentrations (TCO_2), and (c) PCO_2 of branchial chamber fluid collected from *Carcinus maenas* in *Series 4* sampled when resting, immediately after removal from flowing seawater (control, $N = 8$), immediately after 1 h of simple emersion in air ($N = 8$) or exercise in air ($N = 8$). Comparable values for flowing seawater ($N = 8$) sampled at the same time are also shown. Means ± 1 SEM. The dashed horizontal lines or values in red indicate the mean values in venous hemolymph in these treatments reported by Wood & Po (2022). Bars not sharing the same letters are significantly different ($p \leq 0.05$) from one another.

In contrast, mean hemolymph T_{Amm} concentrations were only 79 (control), 206 (postemersion), and 242 μmol-N L^{-1} (postexercise) in these same treatments (cf. Figure 4a,b). Similarly, PNH_3 levels in BCF were comparably high, increasing significantly from about 320 μTorr in control crabs to 920 μTorr in emersed crabs and 1250 μTorr in exercised crabs (Figure 9b). These may be compared with mean hemolymph PNH_3 values of only 18 (control), 9 (postemersion), and 20 μTorr (postexercise) in these treatments respectively (cf. Figure 4a,b). Clearly diffusion gradients would not favor ammonia movements from hemolymph to BCF.

Urea-N (60–200 μmol-N L^{-1}, Figure 9a) and urate-N (75–125 μmol-N L^{-1}, Figure 9b) were detected in the BCF of all crabs but did not increase significantly with emersion or exercise. These remained slightly below hemolymph urea-N concentrations (120–290 μmol-N L^{-1}; Figure 5a,b) and greatly below hemolymph urate-N concentrations (700–2100 μmol-N L^{-1}; Figure 5c,d) in these treatments, suggesting that diffusion gradients could favor movement of these N-wastes from hemolymph to BCF.

4 | DISCUSSION

4.1 | Overview

The hypotheses that exercise in air would differentially elevate the hemolymph concentrations of all three N-wastes, and that these changes would be greater than after a comparable period of quiet

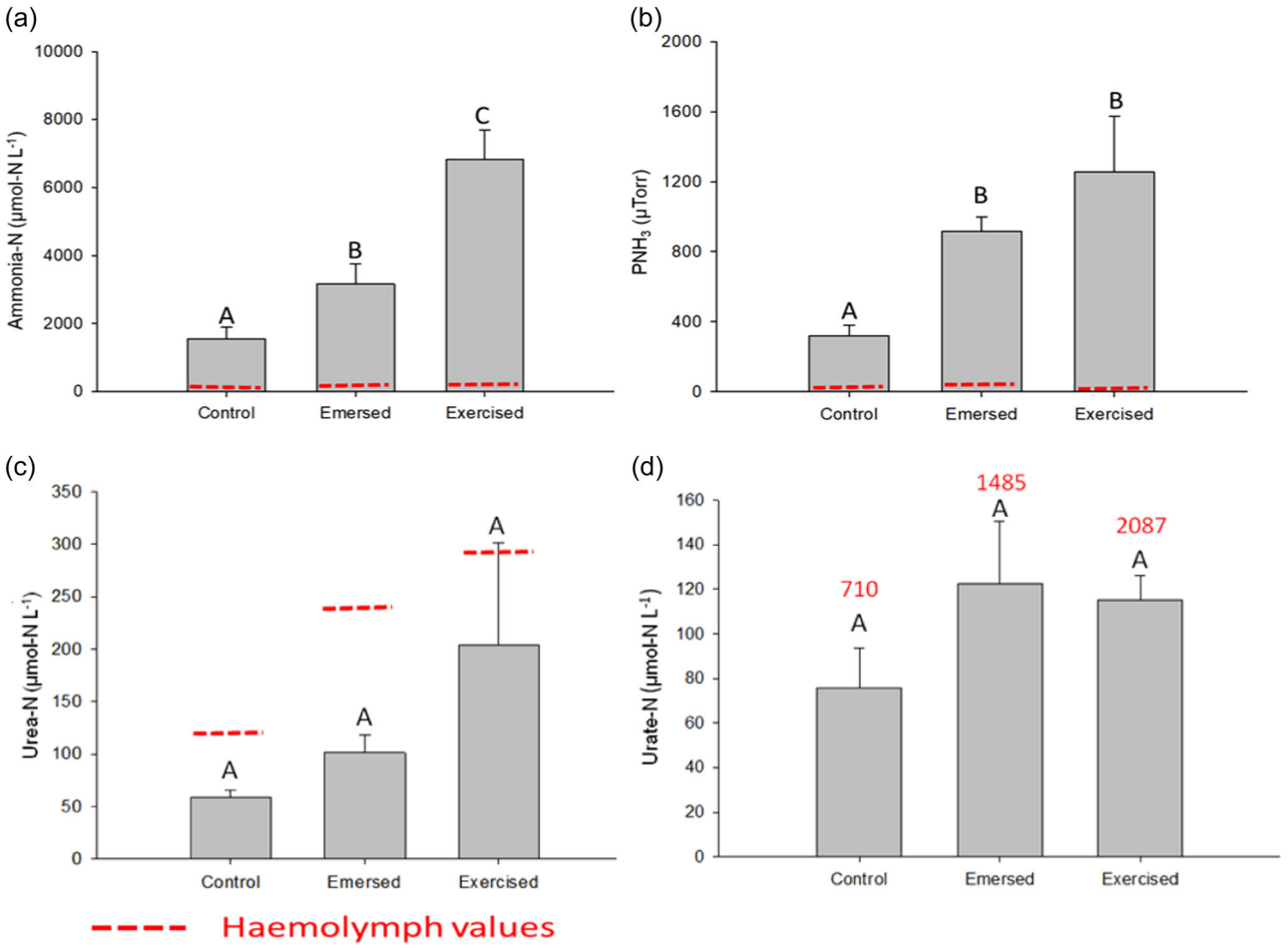

FIGURE 9 (a) Total ammonia-N concentrations (T_{Amm}), (b) PNH_3, (c) urea-N concentrations, and (d) urate-N concentrations of branchial chamber fluid collected from *Carcinus maenas* in *Series 4* sampled when resting, immediately after removal from flowing seawater (control, $N = 8$), immediately after 1 h of simple emersion in air ($N = 8$) or exercise in air ($N = 8$). Means ± 1 SEM. The dashed horizontal lines or values in red indicate the mean values in venous hemolymph in these treatments reported by Wood & Po (2022). Bars not sharing the same letters are significantly different ($p \leq 0.05$) from one another.

emersion in air, were both confirmed. For both treatments, the largest relative increases in hemolymph concentrations were for ammonia-N, but surprisingly, the longest-lasting increases were for urea-N, and the largest absolute increases by far were for urate-N, reflecting its content of 4N per molecule. This may play an important role in protecting hemolymph O_2 transport against the negative effects of acidosis during and after emersion and exercise in air. The hypothesis that the excretion rates of all three N-wastes would be elevated during recovery was generally supported, with particularly dramatic increases in ammonia excretion. Additionally, we report for the first time that substantial amounts of urate-N, as well as lesser amounts of urea-N, are excreted intermittently by *C. maenas*. While the sites and mechanisms are unknown, this finding may explain why the AQ seems unusually low in this carnivorous species, where high protein utilization in oxidative metabolism is expected. However, based on measurements of BCF chemistry at the end of exercise and emersion in air, the hypothesis that the flushing of stored BCF would make a substantial contribution to elevated rates of N-excretion after the return to water was not supported.

4.2 | Resting N-metabolism

Comparisons with previous studies reporting resting concentrations of these three N-wastes in the hemolymph of immersed *C. maenas* are confounded by variability in the literature and possible confusion as to units. However, our measurements of resting ammonia-N (79 μmol-N L^{-1}) appear to be at the lower end of the range (81–940 μmol-N L^{-1}) in previous reports, while our measurements of resting urea-N (120 μmol-N L^{-1}) are also close to the lower end of the range (48–1760 μmol-N L^{-1}), yet our resting urate-N measurements (710 μmol-N L^{-1}) are at the upper end of the range (100–560 μmol-N L^{-1}) (e.g., Binns, 1969; Durand & Regnault, 1998; Lallier et al., 1987; Quijada-Rodriguez et al., 2022; Simonik & Henry, 2014; Spaargaren, 1982; Weihrauch et al., 1999, 2017). At least in part, these differences may reflect the 4-day fasting and the nonstressful control conditions employed in our study, as signaled by very low resting $\dot{M}O_2$ and hemolymph lactate concentrations in these crabs (Wood & Po, 2022), as well as very low rates of ammonia-N excretion. Control $\dot{M}_{Amm}$ values (44 μmol-N kg^{-1} h^{-1}; Figure 1a,

Table 1) contrast with values ranging from 71 to 250 μmol-N kg^{-1} h^{-1} in other studies (Quijada-Rodriguez et al., 2022; Simonik & Henry, 2014; Spaargaren, 1982; Weihrauch et al., 1999). Interestingly, our measurements of ammonia in the BCF indicate that even in resting, submerged crabs, this ammonia excretion is occurring against both concentration and PNH_3 gradients (Figure 9a,b), in accord with abundant evidence that ammonia excretion can occur by active transport mechanisms in the gills of *C. maenas* (Weihrauch et al., 1998, 1999, 2002, 2004, 2017).

$\dot{M}O_2$ (Wood & Po, 2022) and $\dot{M}_{Amm}$ (Figure 1a) were measured simultaneously, so we were able to calculate the AQ which averaged 0.088 under resting control conditions in seawater after 4 days of fasting (Figure 1b). Interestingly, an even lower resting AQ (0.043) can be calculated from $\dot{M}_{Amm}$ and $\dot{M}O_2$ data reported by Simonik and Henry (2014) for 2-day fasted *C. maenas* at similar temperature, even though both absolute rates were much higher in that study. Theoretically, if all aerobic metabolism were fueled by protein (amino acid) oxidation, the AQ would be 0.27 (Lauff & Wood, 1996; Wang et al., 2021a), so these AQ values would suggest that only 33% (present study) or 16% (Simonik & Henry, 2014) of resting metabolism is based on protein oxidation in the green crab. These values seem low relative to the few other measurements on moderately fasted carnivorous decapods, where AQs >0.135 indicated that protein was the major metabolic fuel (reviewed by Wang et al., 2021a, 2021b). For example, AQ was 0.20 in both the shrimp *Crangon crangon* (Regnault, 1981) and the spiny lobster *Sagmariasus verreauxi* (Wang et al., 2021b).

The low AQ in *C. maenas* probably occurs because some of the N-waste from protein metabolism is incorporated into urea-N and/or urate-N, and therefore AQ underestimates protein oxidation in this species. According to Durand & Regnault (1998) and Weihrauch et al. (1999), urea-N excretion amounted to 13%–21% of ammonia-N excretion, whereas the present data (16.9 as urea-N vs. 44 μmol-N kg^{-1} h^{-1} as ammonia-N) indicate a somewhat higher proportion (38%; Table 1). Recalculation of control data in Quijada-Rodriguez et al. (2022) also suggests a 38% figure. However, interpretation is difficult because urea-N excretion was intermittent, detectable in only 9 of 13 crabs under control conditions in the present study, and was clearly intermittent after experimental treatments (Figure 2a,b). Notably Quijada-Rodriguez et al. (2022) found no increase in urea-N excretion after feeding despite a seven-fold increase in hemolymph urea-N concentration in the green crab. There appear to be no previous measurements of urate-N excretion rates in *C. maenas*. Surprisingly, urate-N excretion was by far the largest component on an absolute basis in the present study, but was detected in only 5 of 13 crabs under control conditions (Table 1) and again was clearly intermittent after experimental treatments (Figure 2c,d).

The pathways of intermediary N-metabolism are complex and incompletely characterized in decapods (reviewed by Linton et al., 2017; Regnault, 1987; Weihrauch et al., 2004, 2017). Urate may originate from *de novo* synthesis by the uricotelic pathway directly from dietary protein, or indirectly from the degradative metabolism of purines, while urea may result from the degradation of urate or arginine. Urea synthesis by the ornithine-urea cycle is thought to be absent. Nevertheless, Quijada-Rodriguez et al. (2022) proposed that urea may serve as a temporary storage product for excess N after feeding so as to avoid ammonia toxicity. In terrestrial decapods, urate-N is generally recognized as a "storage-excretion" product that can be eliminated in the molted carapace or perhaps as granules in the feces (Linton et al., 2017). Both urea-N and urate-N increased in tissues of the swimming crab *Portunus trituberculatus* exposed to high environmental ammonia (Pan et al., 2022). Our experiments did not reveal the sites of excretion of either urea-N or urate-N in *C. maenas*. We cannot eliminate the gills and branchial chamber epithelia, which are universally recognized as the major route of ammonia excretion in crabs (e,g. Henry et al., 2012; Regnault, 1987; Weihrauch et al., 2017). Certainly, the present BCF measurements (Figure 9c,d) indicate that both substances are present in the BCF, and that positive hemolymph-to-BCF diffusion gradients exist for both urea-N and urate-N, even in animals that have been emersed and exercised in air. However, given the intermittency and completely different patterns of urea-N and urate-N from ammonia-N excretion (Figure 2a–d vs. Figure 1a,b), routes other than the gills and branchial chamber epithelia may make an important contribution. These could include urinary, fecal, and emetic routes, and especially in the case of urate-N, the release of small bits of carapace by fragmentation. Given the apparent importance of both urea-N and urate-N excretion to N-balance in the green crab, this is an important subject for future investigation.

4.3 | N-metabolism during and after exercise and emersion in air

All control and recovery measurements were made in crabs submerged in seawater at the acclimation temperature of 12°C–13°C, but the exercise and emersion occurred in 20°C–23°C air. While this protocal may complicate interpretation, it is environmentally realistic for temperatures measured during the day on the local foreshore during August, when these experiments were performed, as discussed in greater detail by Wood & Po (2022).

The absolute increases in hemolymph urate-N concentrations immediately after exercise (Figure 5c), and emersion (Figure 5d), were respectively about eight-fold and six-fold greater than the absolute increases in ammonia-N (Figure 4a,b) or urea-N levels (Figure 5a,b). However, increases in urea-N persisted longer than for the other two N-metabolites. Again, the present experiments provide no evidence on the biochemical pathways by which urate-N and urea-N were generated, but they do indicate the quantitative importance of these N-wastes which has generally been overlooked in previous studies. Exceptions include reports in *C. maenas* of greatly elevated hemolymph urea-N concentrations after feeding (Quijada-Rodriguez et al., 2022) and urate-N concentrations after exposure to hypoxia or high temperature (Lallier et al., 1987) and prolonged emersion (Durand & Regnault, 1998). Urate-N also increased after prolonged

emersion in the edible crab, *Cancer pagurus* (Regnault, 1992, 1994). One obvious benefit of elevated hemolymph urate-N is that it will increase the O_2 affinity of hemocyanin (Lallier et al., 1987; Morris et al., 1985). Therefore, in combination with the elevations in hemolymph Ca^{2+}, Mg^{2+} (Truchot, 1975), and lactate concentrations (Truchot, 1980) that were also observed in the present crabs (Wood & Po, 2022), it will help to protect hemolymph O_2 transport against the negative effects of acidosis during and after emersion and exercise in air. Interestingly, neither urate-N (Lallier & Walsh, 1990) nor Ca^{2+} and Mg^{2+} (Booth et al., 1984) were elevated during or after aquatic exercise in the blue crab (*C. sapidus*), though there were substantial elevations in T_{Amm} and ammonia excretion rates (Booth et al., 1984; Milligan et al., 1989). Hemolymph glutamate and glutamine were also measured as these amino acids are often involved in N-shuttling mechanisms, as discussed by Regnault (1992) and Allen et al. (2021). However, in accord with the findings of Allen et al. (2021) on emersed *Helice formosensis* (the semi-terrestrial thick crab), we found no significant changes in either glutamate or glutamine (Figure 6a–d).

The remarkable increases in ammonia-N excretion ($\dot{M}_{Amm}$) during the first 15 min of return to water after exhaustive exercise (65-fold; Figure 1a) and emersion (17-fold; Figure 1b) were temporally disconnected from the much smaller simultaneous increases (4.4-fold and 3.9-fold respectively) in $\dot{M}O_2$ reported by Wood & Po (2022). While elevated $\dot{M}_{Amm}$ lasted only 0.25 h to 1.5 h, elevated $\dot{M}O_2$ lasted 2 h (postemersion) to 8 h (postexercise). This explains why the AQs were initially greatly elevated immediately after return to water for both treatments and then tended to decrease nonsignificantly below control values during the remainder of the recovery period (Figure 1c,d). Interestingly, when AQ values were calculated from the ratio of the short-lasting EPANE to long-lasting EPOC values, they remained the same as the original control values in submersed crabs. This may suggest that the relative usage of protein/amino acids as oxidative fuels did not change during emersion, exercise in air, and recovery thereafter. However, interpretation is confounded by the significant postemersion increases, and non-significant postexercise increases in urea-N and urate-N excretion that occurred over the first 2 h after return to water (Figures 2 and 3). It is also confounded by the report of Simonik & Henry (2014) that green crabs maintained $\dot{M}_{Amm}$ unchanged during emersion by volatilization into the air. These workers detected no increase in $\dot{M}_{Amm}$ upon return to seawater and no changes in hemolymph T_{Amm} during emersion or recovery, both of which directly contrast with the present results (Figures 1b and 4b) and a previous report (Durand & Regnault, 1998). There appear to be no other reports of ammonia volatilization in *C. maenas*. As our experiments did not address the possibility of aerial ammonia excretion, and as our control rates of $\dot{M}O_2$ and $\dot{M}_{Amm}$ were much lower than those of Simonik & Henry (2014), it is difficult to explain these differences.

The present crabs did continue to excrete ammonia-N, urea-N, and urate-N into their dry containers during emersion, although urate-N excretion was detected in only one-third of the air-exposed crabs (Table 1). The large reductions in the absolute rates relative to control values in seawater were not due to depressed metabolism, as $\dot{M}O_2$ more than doubled during emersion, and there was a substantial EPOC during recovery (Wood & Po, 2022). The EPANE during recovery (Figure 1b) was more than sufficient to match the elevated $\dot{M}O_2$ at unchanged AQ. Small droplets of fluid were often seen in the containers at the end of the emersion period, which were presumably BCF that had leaked out. Assuming that the mean concentrations in the BCF of each N-product during this 1-h period were the averages of the measured control and 1-h emersion concentrations (Figure 9a,c,d), then the measured ammonia-N excretion to the dry chamber (Table 1) could be explained by the "spillage" of 3.2 ml kg^{-1} of BCF. This seems very reasonable, inasmuch as the measured BCF volume decreased from 10.4 to 6.9 ml kg^{-1} over this period (Figure 7a). However, analogous calculations yielded values of 109 ml kg^{-1} for the BCF loss needed to explain measured urea-N excretion, and 223 ml kg^{-1} needed to account for measured urate-N excretion., both of which are clearly impossible. Thus BCF "spillage" could account for the observed ammonia excretion during emersion in the air-exposed crabs. However, as the rates were only 17% of the control rates in water (Table 1), dumping of BCF, at the expense of further desiccation, does not appear to be a viable strategy for excreting ammonia during air exposure, and is more likely accidental. Furthermore, it could not explain the observed excretion rates of the other two N-wastes. This again raises questions as to the routes of urea-N and urate-N excretion. For all three N-wastes, the rates of excretion measured over 10–12 h of emersion were only a small fraction of the rates measured over 1 h (Table 1), indicating that rates declined greatly over time.

There are no previous N-waste data on green crabs exercised in either water or in air, and in future, a comparative study to look at how N-waste patterns may differ during and after exercise in these two different media would be valuable. However, in the present study, in the first 0.25 h after re-immersion in seawater, exercised crabs excreted ammonia-N at 65-fold the control rate (Figure 1a) and emersed crabs at 17-fold the control rate (Figure 1b). There is certainly precedent for very high rates of ammonia-N excretion immediately after return to water in air-exposed crabs. In *C. pagurus* emersed for 18 h (Regnault, 1994), and *C. maenas* emersed for 72 h (Durand & Regnault, 1998), $\dot{M}_{Amm}$ values were 60-fold the control rate in the first 5 min after re-immersion in seawater and three to five-fold the control rate in the subsequent 0.5–1.0 h. Simple calculations based on hemolymph T_{Amm} concentrations (Figure 4) and estimates of extracellular fluid volume (ECFV = 330 ml kg^{-1} in *C. maenas*, Harris & Andrews, 1982) suggest that most of this rapid excretion observed in the present study did not originate from the hemolymph. Complete clearance of all the ammonia-N stored in the ECFV could account for only 15% (postexercise) to 29% (postemersion) of the observed excretion in the first 0.25 h. Regnault (1994) reached a similar conclusion in *C. pagurus* based on comparable calculations. Our attention therefore turned to the BCF as the possible source of this burst of ammonia-N excretion in *C. maenas*.

4.4 | The role of BCF

The initial volume of BCF (10.4 ml kg^{-1}) carried into air when the crab was removed from seawater represents about 25% of the total branchial chamber volume if the anatomy of *C. maenas* is similar to that of *C. pagurus* (Regnault, 1994). Using a radiolabel washout technique, Burnett & McMahon (1987) measured a similar BCF volume at the start of emersion (13 ml kg^{-1}) in the striped shore crab (*Pachygrapsus crassipes*) that has a similar intertidal lifestyle as *C. maenas*. The BCF volume of the present crabs decreased to 6.9 ml kg^{-1} after 1 h of emersion, and 2.4 ml kg^{-1} after 1 h of treadmill exercise (Figure 7a). These measured decrements of BCF volume could account for 30% of the whole-body mass loss of exercised crabs (26.6 g kg^{-1}), and 35% of the mass loss of emersed crabs (10.1 g kg^{-1}). Presumably the other 65-70% of the whole-body mass losses were due mainly to evaporation. Note that that osmolality in the BCF of emersed and exercised crabs was only 2% and 6% higher respectively than in control crabs (Figure 7b), in contrast to the 34% and 77% decreases in BCF volume (Figure 7a). This observation rules out evaporation as a major cause of BCF volume losses. More likely, the BCF losses were due to spillages mentioned above, which would be greater during exercise (and were sometimes observed as droplets on the treadmill belt), and to equilibration of the BCF into the ECFV so as to mitigate whole-animal evaporative losses.

It is recognized that the technique used for collection of BCF involved considerable air-exposure of the sample, and that the sample would likely include some urine with the branchial chamber seawater. However, urine is known to comprise part of the BCF in emersed crabs (Harris & Santos, 1993), and at the measured pHs, loss of ammonia would have been negligible. In contrast, there was likely some loss of CO_2, so pH values of BCF samples (Figure 8a) may have been over-estimated, while TCO_2 (Figure 8b) and PCO_2 values (Figure 8c) may have been under-estimated. Regardless, these data show the acid-base status of the BCF is intermediate between those of hemolymph and seawater, and responds to treatments that affect the former. It is also possible that BCF volumes were underestimated by our technique, if a film of water remained on the gills and/or branchial chamber walls after the shaking procedure. However, at most, this would be no more than 20% of the collected volumes, and would not substantially impact the preceding conclusions, or the calculations below about the role of the BCF in N-waste excretion.

It is intriguing that even in resting crabs lifted directly out of seawater (controls), BCF T_{Amm} concentrations (Figure 9a) were considerably greater than in the ambient seawater. This likely reflects the fact that in resting *C. maenas*, ventilation of the branchial chambers was often unilateral and intermittent (Wood & Po, 2022), such that excreted ammonia built up in one or both of them. Regnault (1994) suggested the same explanation for similarly high BCF T_{Amm} in submerged *Cancer pagurus*. The marked increases in both T_{Amm} and PNH_3 after emersion and especially after exhaustive exercise in air raised the BCF-to-hemolymph concentration and partial pressure gradients opposing diffusion to as much as 100-fold (Figure 9a,b). This reinforces the conclusion that ammonia-N excretion must occur by some sort of active process. This was not the case for the much lower BCF levels of urea-N and urate-N, which both remained lower than in hemolymph, and did not increase greatly after emersion or exercise (Figure 9c,d).

At first glance, the elevated BCF T_{Amm} levels would seem to support our hypothesis that the flushing of stored BCF would make a substantial contribution to elevated rates of ammonia-N excretion after both emersion and exercise. However, simple calculations using the BCF volumes of Figure 7 and N-waste concentrations of Figure 9 convincingly falsify the hypothesis. Assuming that all of the N-wastes in the BCF were flushed out in the first 0.25 h postemersion or postexercise, this mechanism could account for only 2.9% (postexercise) and 9.5% (postemersion) of the observed net ammonia-N "bursts" after return to seawater (Figure 1a,b). The % values for urea-N excretion and urate-N excretion are even lower. This agrees with conclusions of both Regnault (1994) and Durand & Regnault (1998), and points to the need to identify other origins, especially for the rapidly mobilized ammonia-N. As pointed out earlier, ammonia-N clearance from the hemolymph does not appear to be the explanation.

4.5 | Conclusions

This study has shown that in addition to continuous ammonia-N excretion, intermittent urea-N and urate-N excretion are important components of the nitrogen metabolism of the green crab when in seawater. Excretion of all three continues during air exposure, but at reduced rates. After both exercise in air or quiet emersion in air, crabs exhibit elevated hemolymph ammonia-N, urea-N, and especially urate-N. BCF volume is reduced, but its osmolality barely changes; its ammonia-N levels increase above those in the hemolymph, while urea-N and and urea-N do not. Upon return to seawater, massive, short-lived increases in ammonia-N excretion occur, but these do not appear to result from either flushing of BCF or clearance from the hemolymph. Their origin deserves future investigation.

AUTHOR CONTRIBUTIONS

Chris M. Wood designed the study, obtained funding, did the experiments, and wrote the manuscript.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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