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RESEARCH ARTICLE

Emersion and recovery alter oxygen consumption, ammonia and urea excretion, and oxidative stress parameters, but not diffusive water exchange or transepithelial potential in the green crab (*Carcinus maenas*)

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ABSTRACT

The green crab (*Carcinus maenas*) is an inshore species affected by intertidal zonation patterns, facing periods of emersion during low tide and submersion during high tide. During these periods of air and subsequent water exposure, these species can face physiological challenges. We examined changes in O_2 consumption rate ($\dot{M}_{O_2}$), and ammonia and urea excretion rates over sequential 14 h periods in seawater (32 ppt, control), in air and during recovery in seawater after air exposure (13°C throughout). At the end of each exposure, the anterior (5th) and posterior (8th) gills and the hepatopancreas were removed for measurements of oxidative stress parameters (TBARs and catalase in the gills and hepatopancreas, and protein carbonyls in the gills). $\dot{M}_{O_2}$ remained unchanged during air exposure, but increased greatly (3.4-fold above control levels) during the recovery period. Ammonia and urea net fluxes were reduced by 98% during air exposure, but rebounded during recovery to >2-fold the control rates. Exchangeable water pools, rate constants of diffusive water exchange, unidirectional diffusive water flux rates (using tritiated water) and transepithelial potential were also measured during control and recovery treatments, but exhibited no significant changes. Damage to proteins was not observed in either gill. However, lipid damage occurred in the anterior (respiratory) gill after the air exposure but not in the posterior (ionoregulatory) gill or hepatopancreas. Catalase activity also decreased significantly in recovery relative to levels during air exposure in both the anterior gill and hepatopancreas, but not in the posterior gill. The crabs did not modify water metabolism or permeability. We conclude that $\dot{M}_{O_2}$ was maintained but not enhanced during air exposure, while ammonia and urea-N excretion were impaired. As a result, all of these parameters increase greatly during re-immersion recovery, and oxidative stress also occurs. Clearly, emersion is not without physiological costs.

KEY WORDS: Exotic species, Air exposure, Tide cycles, Desiccation stress, ROS

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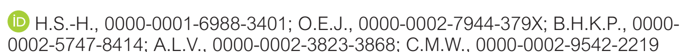
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INTRODUCTION

The transition area between marine and terrestrial ecosystems (intertidal zone) is periodically exposed to the inundations of the tides. Such an environment supports extremely productive and biodiverse habitats (such as tidal mudflats, sandy beaches, fringing coral reefs and steep rocky cliffs) (Banks et al., 2005; Luijendijk et al., 2018). In addition, intertidal environments shelter organisms that originate exclusively from the marine environment. Owing to the influence of tide cycles, these organisms are adapted to extremely dynamic conditions, displaying daily behavioral, biochemical and physiological responses to changes in temperature, salinity, dissolved oxygen and periods of emersion (Young and Elliott, 2020).

During the tidal cycle, one of the most stress-inducing situations is during emersion. The desiccation stress associated with air exposure can decrease the efficiency of respiration and lead to the eventual collapse of gas exchange and aerobic metabolism (Burnett and McMahon, 1987). Nevertheless, intertidal organisms can present adaptations that can facilitate aerobic respiration, such as specialized structures for air breathing (Stillman and Somero, 1996) and physiological adjustments that enhance oxygen delivery to the tissues (Morris and Butler, 1996; Stenseng et al., 2005; Stillman and Somero, 1996). In fact, intertidal organisms are an important group for studying genetic, biochemical, physiological and behavioral responses to environmental changes (i.e. temperature, salinity, dissolved oxygen and periods of emersion) (Somero, 2002). As common inhabitants of the intertidal zone, crustaceans such as the green crab (*Carcinus maenas*), also known as the 'shore crab', have frequently been used for such purposes.

The green crab is a very proficient invader owing to its phenotypic plasticity and omnivorous diet. Its original range included the Atlantic coast of Europe as far north as Norway, across the British Isles and Iceland, and along the northern coast of Africa to Mauritania (Darling et al., 2008). However, its ability to alter many aspects of its biology to fit particular environments has allowed *C. maenas* to become a globally invasive species (Young and Elliott, 2020), and it is now also found on the Pacific coast of Canada. Adult *C. maenas* are very tolerant to environmental alterations, e.g. low oxygen levels as well as temperature (thermotolerance) and salinity variations (euryhalinity), so can remain in moist burrows above the neap high tide line for up to 10 days until water returns with the next spring high tide (Yamada, 2002). In the present study, we employed a 14 h control period of immersion in seawater, followed by 14 h of air exposure (emersion) followed by a 14 h period of re-immersion in seawater (recovery) to examine various potential effects that might occur during a daily tidal exposure.

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List of symbols and abbreviations

^3H_2O	tritiated water
AgCl	silver chloride
αO_2	appropriate O_2 solubility coefficient
BMSC	Bamfield Marine Sciences Centre
CAT	catalase
DNPH	2,4-dinitrophenylhydrazine
EDTA	ethylenediaminetetraacetic acid
EPOC	excess post-emersion O_2 consumption
H_2O_2	hydrogen peroxide
$J_{Amm,net}$	net flux rate of ammonia
J_{H_2O}	unidirectional diffusive water flux rates
J_{net}	net flux rate
$J_{Urea,net}$	net flux rate of urea
k	diffusive water exchange
KH_2PO_4	potassium phosphate monobasic
MDA	malonaldehyde
$\dot{M}_{O_2}$	O_2 consumption rate
NH_3	non-ionized ammonia
NH_4^+	ionized ammonia
P_{O_2}	oxygen partial pressure
P_{O_2f}	final oxygen partial pressure
P_{O_2i}	initial oxygen partial pressure
POS	preparing for oxidative stress
R	total ^3H_2O radioactivity (in cpm)
ROS	reactive oxygen species
SA_{H_2O}	specific activity (cpm ml^{-1}) of the external water
T	measurement period (h)
Tamm	total ammonia
TBA	thiobarbituric acid
TBARs	thiobarbituric acid reactive substances
TEP	transepithelial potential
TEP equivalents	tetraethoxypropane equivalents
V	volume
V_{H_2O}	exchangeable water pool
X_f	final concentration of total ammonia or urea
X_i	initial concentration of total ammonia or urea

During the emersion, the water-breathing animals can experience a reduction of the capacity to regulate respiratory gas exchange and nitrogenous waste excretion. The gills are potentially impaired by the water availability, which is limited in such environments. There have been many studies comparing the O_2 consumption rate ($\dot{M}_{O_2}$) of *C. maenas* in air versus water (reviewed by Simonik and Henry, 2014 and Wood and Po, 2022). Most have reported that $\dot{M}_{O_2}$ falls by up to 50% in air (e.g. Wallace, 1972; Newell et al., 1972; Simonik and Henry, 2014; Nancollas and McGaw, 2021), though others have reported no change (Taylor and Butler, 1978) or increases during emersion (Wood and Po, 2022). This species is known to excrete waste nitrogen largely as ammonia, together with lesser amounts of urea (Dal Pont et al., 2022; Durand and Regnault, 1998; Weihrauch et al., 2004; Wood, 2023), but there is limited and conflicting information on N-waste excretion during emersion. Ammonia-N excretion has been reported to either increase greatly upon re-immersion, suggesting that it is inhibited during the period of air exposure (Durand and Regnault, 1998; Wood, 2023), or not change during emersion and re-immersion (Simonik and Henry, 2014). Clearly, there is discord in the literature on these issues, some of which may have resulted from differences in methods, especially the degree of handling disturbance of the animals (Taylor and Butler, 1978; Houlihan et al., 1984; Wilson et al., 2021) and differences in air temperature versus seawater temperature during the treatments. Therefore, the first objective of the present study was to re-examine these issues while holding temperature unchanged. We predicted that under these conditions, O_2 consumption, ammonia N-excretion and urea-N excretion would all decrease during emersion, and then increase above control levels during the re-immersion recovery period.

Our second focus was on potential oxidative stress during emersion and recovery. There is evidence that the 20- to 30-fold higher concentration of O_2 in air versus water may result in oxidative stress in air-breathing organs of bimodal breathing animals when they breathe air (reviewed by Pelster and Wood, 2018). Furthermore, regardless of whether $\dot{M}_{O_2}$ changes during air exposure, there are consistent reports of reduced heart rate during air exposure in *C. maenas* followed by increased heart rate upon re-immersion (Depledge, 1984; Simonik and Henry, 2014; Wood and Po, 2022). This would likely result in initial ischemia during the emersion (air exposure), and subsequent hyper-perfusion of key tissues during the re-immersion. Reduced oxygen availability associated with compromised performance of either the respiratory or circulatory systems during air exposure may limit aerobic pathways for energy production and increase the generation of reactive oxygen species (ROS) in a time-dependent manner (Bickler and Buck, 2007). The electron carriers of the mitochondrial respiratory chain may be reduced during ischemia, whereas the immediate re-oxidation of these carriers may occur after the re-initiation of perfusion, leading to oxyradical overproduction and oxidative damage to lipids and proteins (Rivera-Ingraham and Lignot, 2017). Also, a key physiological process underlying the adaptive strategies of intertidal organisms relies on the biological plasticity of the redox metabolism. In other words, to avoid ROS damage to biomolecules during the low oxygen stress, the organisms could increase the levels of endogenous antioxidants (Hermes-Lima et al., 2015; Moreira et al., 2017, 2021). Specifically, we hypothesized that redox metabolism would be impaired by the emersion/re-immersion events, resulting in oxidative damage. To evaluate these possibilities, we measured indices of oxidative damage to lipids (peroxidation) and proteins (carbonylation), as well a key enzyme (catalase) involved in the removal of ROS, at the end of emersion and again at the end of the re-immersion recovery period.

Our third focus was on water metabolism and permeability. Recently, Dal Pont et al. (2022) have shown, using tritiated water (^3H_2O), that the internal exchangeable water pools, rate constants of diffusive water exchange and unidirectional diffusive water flux rates in *C. maenas* were all affected by acclimation salinity. These authors also detected changes in transepithelial potential (TEP) associated with differences in salinity. All these observations suggest that gill permeability is subject to regulatory control. Therefore, we hypothesized that 14 h of air emersion would also influence these parameters so as to achieve water conservation by reduction of gill permeability. Reduced gill permeability would likely be evidenced by reductions in the rate of diffusive water exchange and reduced TEP across the gill. Specifically, we predicted that these adaptations would manifest in minimal change in the internal exchangeable water pool, but a reduction in the rate constant of diffusive water exchange, a reduction in the unidirectional diffusive water flux rate, and an attenuation of the TEP, when measured immediately after return to water. We also predicted that these changes would be partially or completely reversed by the end of the 14 h recovery period in seawater.

In summary, our study evaluated whether 14 h of air emersion followed by 14 h of re-immersion recovery would influence the rates of O_2 consumption, ammonia-N excretion and urea-N

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excretion, indices of oxidative stress, and water conservation and gill permeability parameters in green crabs facing this emersion period.

MATERIALS AND METHODS

Male green crabs [*Carcinus maenas* (Linnaeus 1758)] (mass: 63.2±2.7 g, mean±s.e.m.; *N*=60) were collected via traps in the intertidal zone of Effingham Inlet in British Columbia, Canada (collecting permit: XR2772015), and transferred to Bamfield Marine Sciences Centre (BMSC), where experiments and analyses were performed, under an approved BMSC Invertebrate AUP. Crabs were placed in constantly aerated 200-liter tanks receiving flow-through seawater (∼32 ppt, 13°C) and exposed to a natural daylight cycle (14 h:10 h light:dark). The crabs were fed on salmon heads but were fasted for 3 days prior to the start of experiments.

Measurements of $\dot{M}_{O_2}$ and sampling for oxidative stress

Crabs were individually distributed into glass respirometry chambers (volume=1000 ml) in three treatments (*N*=6–12): control immersion (seawater exposure for 14 h), emersion (air exposure for 14 h) and recovery (re-immersion recovery for 14 h after emersion for 14 h). For convenience, in the remainder of the paper, these are labelled as 'water', 'air' and 'recovery' treatments, respectively. In each case, the crabs were allowed to settle in the respirometers overnight with flow-through seawater and aeration prior to the start of the measurements to minimize any influence of handling disturbance. The glass respiratory chambers were shielded with aluminum foil to minimize disturbance and submerged in a water bath to maintain temperature at 13°C. In the water and recovery treatments, the respirometers were continuously aerated and flushed with fresh seawater, but were closed and sealed for 20-min periods four times (at approximately 4 h intervals) during the 14 h periods. Water samples (4 ml) were drawn anaerobically at the start and end of each 20 min closed period for measurement of initial P_{O_2} (P_{O_2i}) and final P_{O_2} (P_{O_2f}), respectively, then flow-through and aeration were re-established until the next closure period. The four determinations were averaged. In the air treatment, the respirometry chambers were drained and dried with minimal disturbance to the crabs, after which they were allowed to settle for 30 min. The air-filled respirometer was then sealed for 14 h. Air samples (4 ml into gas-tight syringes) were taken for P_{O_2} measurements at the start (P_{O_2i}) and end (P_{O_2f}) of the 14 h period of closure.

P_{O_2} in water and air samples was measured using a polarographic oxygen micro-electrode (Radiometer, Copenhagen, Denmark) thermostated to 13°C, and coupled to an amplifier (model 1900, A-M Systems, Everett, WA, USA). $\dot{M}_{O_2}$ (µmol kg^{-1} h^{-1}) was calculated as:

$$M_{O_2} = [(P_{O_2i} - P_{O_2f})\alpha_{O_2}V]/(MT), \quad (1)$$

where α_{O_2} is the appropriate O_2 solubility coefficient (µmol l^{-1} Torr^{-1}) at 13°C in either 32 ppt seawater (Boutilier et al., 1984) or air (Dejours, 1978), *M* is the mass (kg) of the crab, *T* is the measurement period (h) and *V* is the volume (l) of the respirometer, corrected for the mass of the crab.

At the end of each experimental treatment, crabs were weighed, placed on ice for anaesthesia, and the anterior (5th) and posterior (8th) gills, the hepatopancreas, and muscle from the pereopods were removed for measurements of the oxidative stress parameters. These tissues were used for determination of thiobarbituric acid reactive substances (TBARs) for lipid peroxidation, catalase activity in the gills, hepatopancreas and muscle, and protein carbonyls in the gill. Samples were frozen immediately in liquid nitrogen, and then stored at −80°C until analysis.

Ammonia and urea-N excretion

In a separate series, crabs were subjected to the same protocol as used in the previous section for the $\dot{M}_{O_2}$ and oxidative stress measurements. A single set of crabs (*N*=7) were followed through all three treatments (water, air and recovery). In the water and recovery treatments, 20 ml of water was collected at the start and end of the 14 h exposures. For the air treatment, the crab was briefly removed, and the chamber was thoroughly washed with distilled water and dried at the beginning, while at the end, the crab was again briefly removed, and the chamber was rinsed with 20 ml of distilled water, which was collected. Water samples were frozen at −20°C for later analyses.

In the water and recovery treatments, the net flux rates (J_{net}) in µmol-N kg^{-1} h^{-1} of total ammonia (TAmm=NH_3+NH_4^+) and urea-N (note: 2 N per urea molecule) were calculated as:

$$J_{net} = (X_i - X_f)V(TM)^{-1}, \quad (2)$$

where X_i and X_f are, respectively, the initial and final concentrations of total ammonia or urea-N (µmol l^{-1}) in the water during the flux period, *V* is the volume (l) of the water in the chamber, *T* is the duration of the flux period (h) and *M* is the crab mass (kg). In the air treatment, *V* was 0.02 liters and X_i was 0 µmol l^{-1}.

Total ammonia and urea-N concentrations in water, without dilution, were measured by colorimetric methods described by Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. All measurements were made in triplicate using standards made up in the appropriate media and appropriate blanks.

Oxidative stress

For measurement of oxidative stress, the frozen gills (5th and 8th gills), hepatopancreas and muscle samples were weighed, then homogenized on ice in 2.5 volumes of ice-cold phosphate buffer (100 mmol l^{-1} KH_2PO_4, 5 mmol l^{-1} disodium EDTA, pH 7.5 at 25°C) using a motorized homogenizer. Homogenates were centrifuged at 9000 ***g*** for 15 min at 4°C and the supernatant was used for the TBARS, catalase, protein carbonyl and total protein assays.

TBARS

Concentrations of TBARS in gills, hepatopancreas and muscle were determined as described by Satoh (1978). This indicator of lipid peroxidation was measured through the reaction of malonaldehyde (MDA) and thiobarbituric acid (TBA) with the MDA–TBA adduct formation under high temperature (100°C) and acidic conditions. The MDA–TBA adduct formation was measured colorimetrically at 530 nm. The concentrations of TBARS were expressed as µmol tetraethoxypropane (TEP) equivalents µg^{-1} protein.

Catalase (CAT)

Gills, hepatopancreas and muscle CAT activities were determined as described by Beutler (1975). The rate of enzymatic decomposition of H_2O_2 was measured in a spectrophotometer at 240 nm. Enzyme activity was expressed in µmol H_2O_2 min^{-1} mg^{-1} protein.

Protein carbonyls

The concentrations of protein carbonyls in gills were measured through a colorimetric assay kit (Protein Carbonyl assay kit, item

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no. 10005020, Cayman Chemical). The assay uses the 2,4-dinitrophenylhltdrazine (DNPH) reaction to measure the protein carbonyls in tissue homogenates. The amount of protein-hydrozone produced was spectrophotometrically measured in 96-well plates at 360 nm. The concentrations of protein carbonyls were expressed as nmol carbonyls mg^{-1} protein.

Total protein determination

Gills, hepatopancreas and muscle supernatants were used to measure the total protein content according to Bradford (1976). The amount of protein was spectrophotometrically measured in 96-well plates at 595 nm. The protein content was expressed as mg protein g^{-1} fresh tissue using bovine albumin serum as a standard.

Transepithelial potential measurements

Crabs (n=7) were cold-anesthetized on ice for approximately 5 min, then weighed and transferred to a wooden surgery table with fixtures to stabilize the crab. Using a Dremel tool fitted with a small dentistry drill bit (Dremel, Mount Prospect, IL, USA), a small hole was drilled on the top of the carapace close to the anterior margin of the pericardium of each of the crabs. Dental dam was then glued (Krazy Glue, Westerville, OH, USA) in double layers on top of the hole to create an auto-sealing access to the hemolymph of the crab. Crabs were returned to seawater for at least 24 h prior to experimentation. The same surgery was used by Zimmer and Wood (2017) and Dal Pont et al. (2022), and no changes in behavior of the crabs were observed after the surgery.

TEP measurements were made using 3 mol l^{-1} KCl–agar bridges connected via Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL, USA) to a Radiometer pHM 82 pH meter (Radiometer, Copenhagen, Denmark), which served as a high impedance voltmeter. The measurement bridge was placed into the crab's hemolymph through the hole on the carapace, and the reference bridge was placed in the external seawater. The TEP (mV) was expressed as the potential in the extracellular fluid of the crab relative to the outside seawater reference as 0 mV. TEP measurements were made in quadruplicate with correction for the junction potential, which was the voltage when the measurement bridge is placed in seawater before insertion into the crab.

Initial control TEP measurements were made while each crab was in a chamber containing 400 ml of seawater. This facilitated confinement of the crab so that the measurement bridges could be correctly placed. At the beginning of emersion, the seven crabs were placed into a 50 liter dry plastic holding tank with a lid that allowed air flow. The tank was held in a flowing seawater bath to maintain the air temperature at 13°C. After 14 h, each crab was re-immersed in 400 ml of fresh seawater in the measurement chamber for the first recording of TEP at 0 h of recovery. Thereafter, the crabs remained submerged in an aerated seawater tank for repeated TEP measurements at 1, 2, 6, 12 and 18 h of recovery after emersion, in each case facilitated by transfer to the 400 ml measurement chamber.

^3H_2O measurements of water metabolism

The rate constants of diffusive water exchange, the internal exchangeable water pools and the unidirectional diffusive water flux rates were measured using tritiated water (^3H_2O) in three treatment groups: control (N=8, immersed in seawater); post-emersion (N=6, immediately upon return to seawater after 14 h of standard air exposure; and recovery (N=6, after 20 h recovery in seawater following 14 h of standard air exposure). For initial loading with tritiated water, crabs were placed for 12 h in individual plastic containers filled with 0.5 liter of seawater to which 10 µCi of ^3H_2O (Perkin Elmer, Woodbridge, ON, Canada) had been added. Each container was equipped with an air-stone for aeration, shielded to minimize disturbance, and partially submerged in a water bath so as to maintain 13°C. Duplicate 4 ml water samples were taken at the end of the equilibration period. This duration was more than sufficient to achieve diffusive equilibration with the internal water pool of the crab, as demonstrated by preliminary experiments. The crabs were then rinsed in radioisotope-free seawater water for 1 min, before transfer to the respective experimental treatment. Note that this 12 h ^3H_2O loading period was either immediately before the control measurements (control treatment), immediately before 14 h of air exposure (post-emersion treatment), or started 8 h after return to water following 14 h of emersion (recovery treatment). In the post-emersion treatment, any water at the bottom of the emersion jar at the end of 14 h of emersion was collected and counted, to check for ^3H_2O losses during the period of air exposure (see Results).

The experimental containers were the standard respirometer jars filled with exactly 1 liter (minus crab mass) of radioisotope-free seawater at 13°C and fitted with air-stones for aeration and mixing. Four milliliters of seawater was sampled immediately at the point where the ^3H_2O-loaded crab was added (time 0) and at 5, 10, 15, 20, 25, 30, 40, 50, 60, 75 and 90 min, with final duplicate 4 ml samples taken at approximately 12 h when diffusive equilibration (washout) was complete. Eight milliliters of Ultima Gold AB fluor (Perkin-Elmer, Wellesley, MA, USA) was added to each 4 ml water sample (2:1 ratio). The concentration of ^3H_2O in the samples was determined using a liquid scintillation counter (LS6500, Beckman Coulter, Fullerton, CA, USA), after the samples had been allowed to rest in the dark for 12 h to minimize chemiluminescence. Tests showed that quenching was constant, so no corrections were made.

Calculations paralleled those of Giacomin et al. (2019) and were based on the total amount of radioactivity (cpm) taken up by each individual crab during the ^3H_2O loading period (R_{Total}). R_{Total} was therefore equal to the total radioactivity present in the crab at the start of the washout period. R_{Total} was calculated from the total amount radioactivity in the experimental container (crab+water) at the end of the washout period, plus all radioactivity removed during water sampling. At each time during the washout period, the radioactivity remaining in the crab (R_{Time}) could be back-calculated, because the cumulative radioactivity appearance in the water at every sampling time was measured. R_{Time} data were natural-log transformed and regressed against time (on a linear scale) to yield the rate constant of diffusive water exchange (k, in h^{-1}) as the slope of the line (Evans, 1967) as follows:

$$k = (\ln R_{Time1} - \ln R_{Time2})/(T_1 - T_2), \quad (3)$$

where R_{Time1} and R_{Time2} are total ^3H_2O radioactivity (in cpm) in the crab at times T_1 and T_2 (in h). Although regressions were performed over both the first 30 min and the first 90 min of washout, the 30 min regressions had marginally higher r^2 values and were used in all calculations.

The exchangeable water pool (V_{H_2O}, in ml kg^{-1}) was calculated as:

$$V_{H_2O} = R_{Total}/SA_{H_2O} \times M, \quad (4)$$

where SA_{H_2O} is the specific activity (cpm ml^{-1}) of the external water at the end of the 12 h ^3H_2O loading period and M is the crab mass (kg). In the emersion treatment, R_{Total} was corrected for any losses during the emersion period.

The unidirectional diffusive water flux rate (J_{H_2O}, ml kg h^{-1}) was calculated from the k and V_{H_2O} data:

$$J_{H_2O} = -kV_{H_2O}. \quad (5)$$

Statistical analyses

All data are reported as means±s.e.m. (N). N ranged from 6 to 12 in different experiments. Statistical significance was accepted at P<0.05. All data were tested for normality of distribution and similarity of variance; when the data did not meet these assumptions, a non-parametric test was applied. Significant differences among treatments in $\dot{M}_{O_2}$, oxidative stress parameters (TBARs, CAT and carbonyl proteins) and ^3H_2O measurements of water metabolism (exchangeable internal water pool, rate constant of diffusive water exchange, unidirectional diffusive water flux rate) were determined through one-way ANOVA. Repeated-measures ANOVAs were used for the ammonia and urea-N net fluxes, and TEP measurements. The *a posteriori* Holm–Šidák (parametric) or Dunn (non-parametric) methods were applied to assess differences among the treatments, depending on the normality of data distribution and similarity of variance. All statistical analyses and graphics employed Sigma Stat (version 3.5) and Sigma Plot (version 11.0) software (Jandel Scientific, San Jose, CA, USA).

RESULTS

$\dot{M}_{O_2}$

When crabs were air-exposed for 14 h, $\dot{M}_{O_2}$ did not change significantly from the control level of approximately 550 µmol kg^{-1} h^{-1} recorded during immersion in seawater. However, when crabs were re-immersed after 14 h of emersion, crabs had an $\dot{M}_{O_2}$ 3.4 and 2.4 times higher than the crabs from the immersion and emersion treatments, respectively (Fig. 1).

Ammonia and urea-N excretion

The control levels of $J_{Amm,net}$ and $J_{Urea\text{-}N,net}$ during immersion in seawater were approximately –65 and −10 µmol-N kg^{-1} h^{-1}, respectively. During 14 h of emersion, these rates became barely detectable, decreasing significantly to approximately 2% of the control rates. However, during 14 h of recovery after re-immersion in seawater, both $J_{Amm,net}$ and $J_{Urea\text{-}N,net}$ rebounded, increasing significantly to more than 2-fold the original control rates (Fig. 2).

Oxidative stress

At the end of air exposure, lipid damage (TBARs) was approximately 2-fold higher, a significant difference, in relation to control and recovery values, but only in the anterior gills (5th gill) of crabs air-exposed for 14 h (Fig. 3A). However, no differences were observed for posterior gills (8th gill, Fig. 4A), hepatopancreas (Fig. 5A) or muscle (Table 1). In contrast, small increases in CAT activity during emersion were not significant relative to control values in either the 5th gill (Fig. 3C) or hepatopancreas (Fig. 5B), but in both tissues, CAT activity decreased markedly after recovery, resulting in significant 60–70% declines relative to the values after air exposure. There were no changes in CAT in the 8th gill (Fig. 4C) nor in the muscle (Table 1). Protein carbonyls exhibited no changes in either anterior or posterior gills (Figs 3B and 4B).

Transepithelial potential

The TEP under control conditions (−1.5 mV) tended to become more negative at the end of the 14 h emersion period (−2.4 mV), and then gradually recovered to control levels during 18 h of re-immersion (Table 2). However, there was considerable inter-individual variability, and none of the changes were significant.

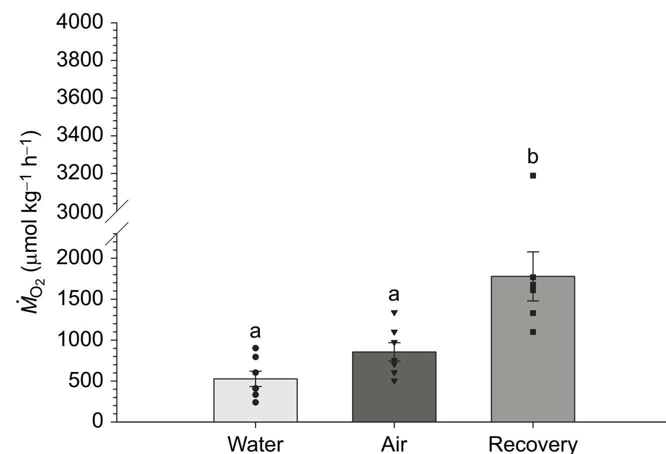

Fig. 1. Rate of oxygen consumption ($\dot{M}_{O_2}$) of *Carcinus maenas* during 14 h of water (immersion) or air exposure and during the recovery period (14 h of re-immersion after 14 h of air exposure). Values are means±s.e.m. (N=6–12). Different letters indicate significant differences among the groups (P<0.05).

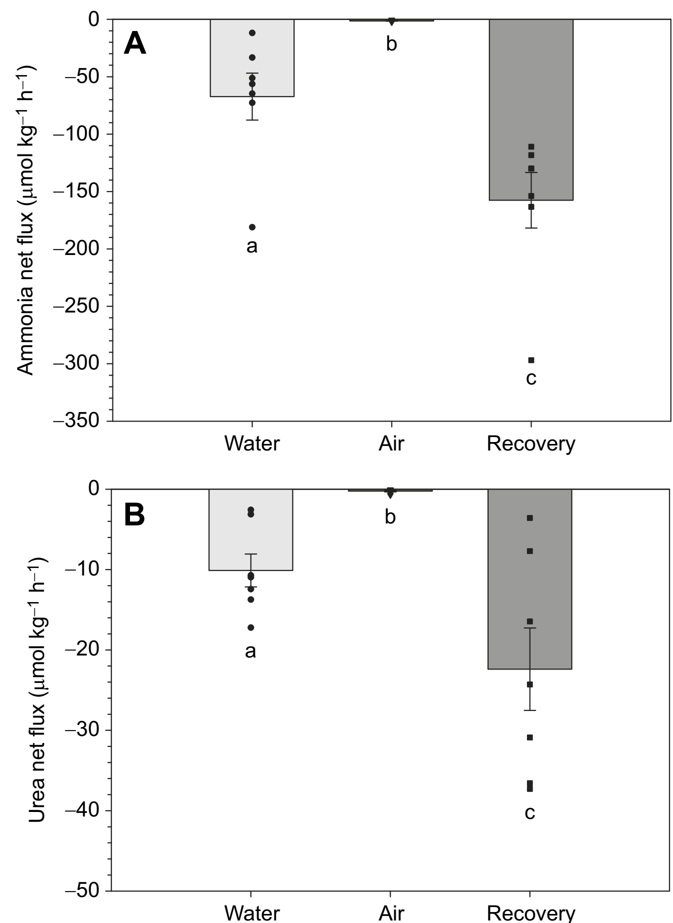

Fig. 2. Ammonia and urea net flux of *C. maenas* during 14 h of water (immersion) or air exposure and during the recovery period (14 h of re-immersion after 14 h of air exposure). (A) Ammonia net flux ($J_{Amm,net}$); (B) urea net flux ($J_{Urea\text{-}N,net}$). Values are means±s.e.m. (N=7). Different letters indicate significant differences among the groups (P<0.05).

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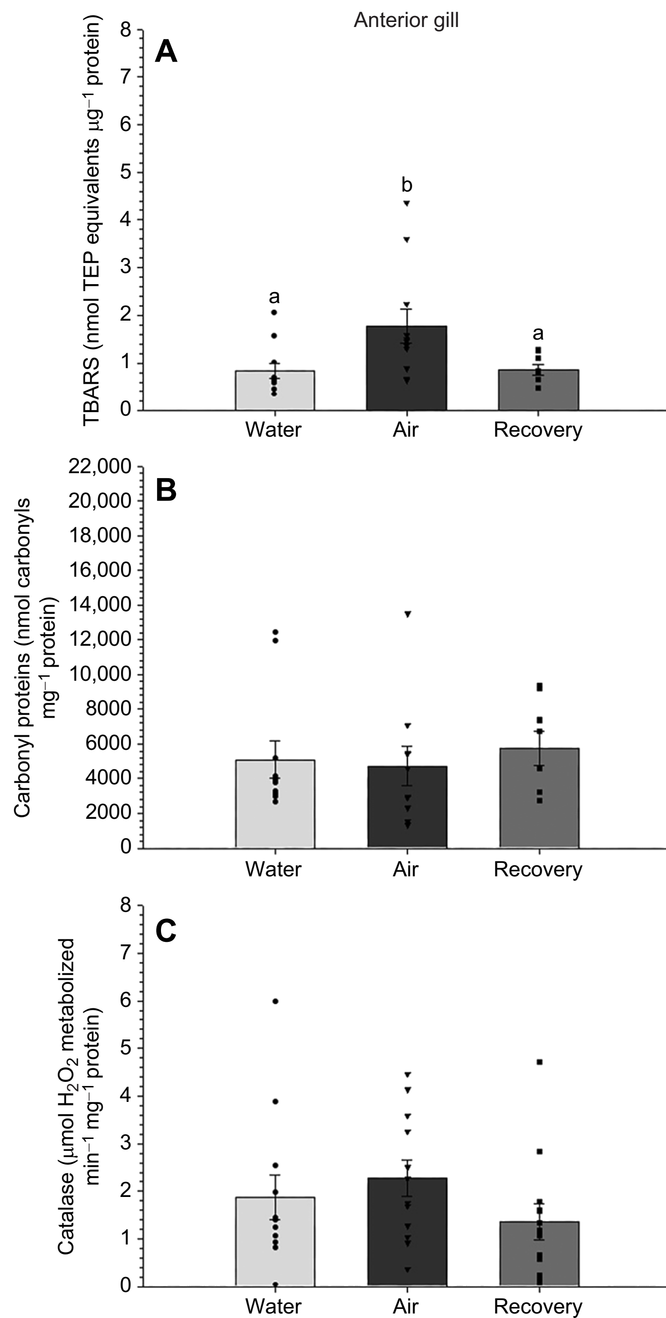

Fig. 3. Lipid and protein damage and catalase activity in the anterior gill (5th gill) of *C. maenas* after 14 h of water (immersion), 14 h of immersion plus 14 h of air exposure, or after 14 h of recovery (14 h of re-immersion after 14 h of air exposure). (A) Lipid damage (TBARS); (B) protein damage (carbonyl proteins); and (C) catalase activity. Values are means±s.e.m. (*N*=6–12). Different letters indicate significant differences among the groups (*P*<0.05).

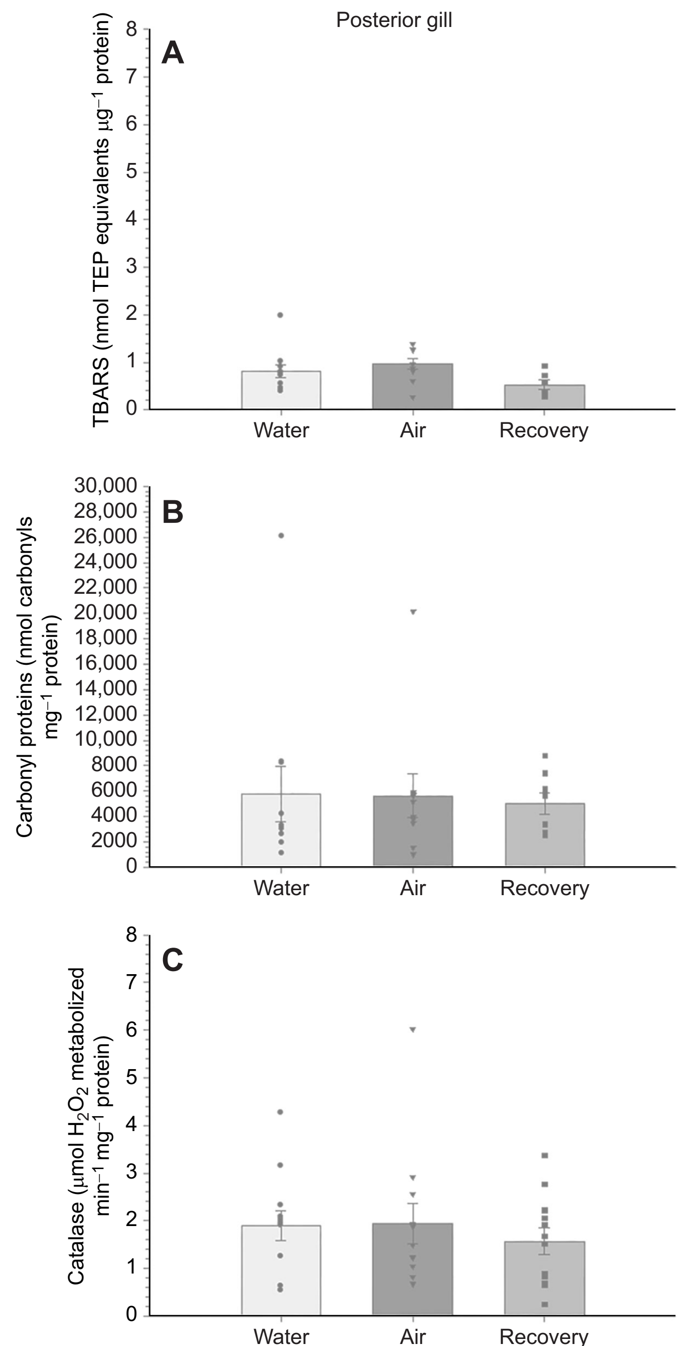

Fig. 4. Lipid and protein damage and catalase activity in the posterior gill (8th gill) of *C. maenas* after 14 h of water (immersion), 14 h of immersion plus 14 h of air exposure, or after 14 h of recovery (14 h of re-immersion after 14 h of air exposure). (A) Lipid damage (TBARS); (B) protein damage (carbonyl proteins); and (C) catalase activity. Values are means±s.e.m. (*N*=6–12). Different letters indicate significant differences among the groups (*P*<0.05).

^3H_2O measurements of water metabolism

There were no significant changes in the indices of water metabolism (Table 3). Non-significantly lower exchangeable water pools (V_{H_2O} ~640 versus ~700 ml kg^{-1}) and non-significantly higher rate constants of diffusive water exchange (k ~0.79 versus ~0.71 h^{-1}) in the post-emersion and recovery treatments resulted in almost identical unidirectional diffusive water flux rates as in the control treatment (J_{H_2O} ~500 ml kg^{-1} h^{-1}). Note that in the post-emersion treatment, these calculations take into consideration the very small losses of ^3H_2O, amounting to 1.18±0.40% (*N*=6) of the total body ^3H_2O load that was detected in the 0.60±0.15 ml kg^{-1} (*N*=6) of water collected from the originally dry emersion chambers at the end of 14 h of air exposure. These likely reflected losses of branchial chamber fluid and/or urine and had negligible impact on the calculated indices.

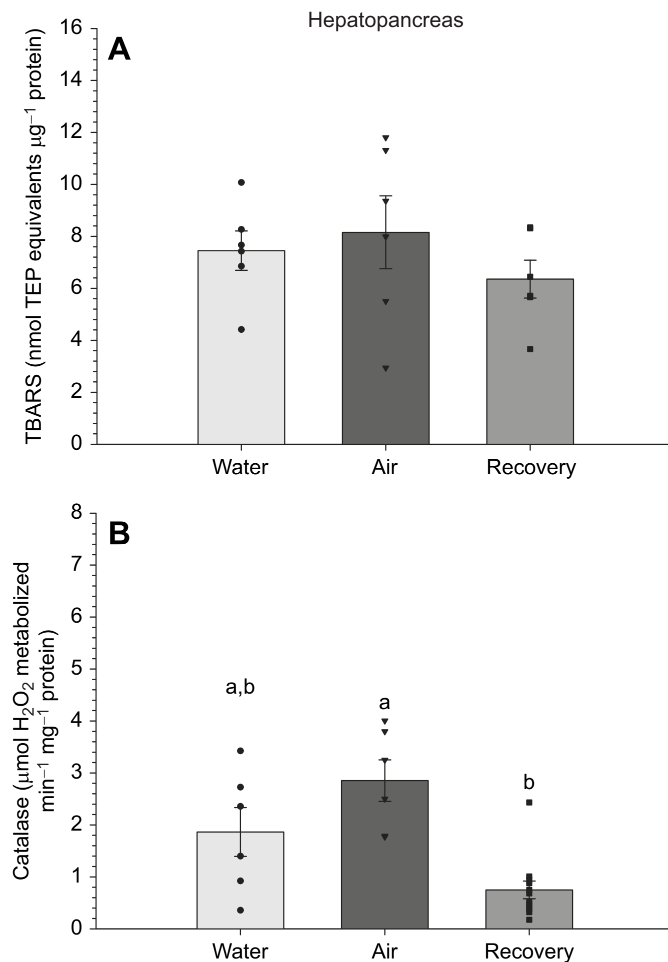

Fig. 5. Lipid and protein damage and catalase activity in the hepatopancreas of *C. maenas* after 14 h of water (immersion), 14 h of immersion plus 14 h of air exposure, or after 14 h of recovery (14 h of re-immersion after 14 h of air exposure). (A) Lipid damage (TBARS); (B) protein damage (carbonyl proteins); and (C) catalase activity. Values are means±s.e.m. (*N*=6–12). Different letters indicate significant differences among the groups (*P*<0.05).

DISCUSSION

In the present work, our prediction that the O_2 consumption would decrease during emersion and increase during recovery was partially supported. There was a marked 3.4-fold increase in $\dot{M}_{O_2}$ above control levels during the post-emersion recovery, validating the second point. However, in contrast to both our original prediction and most previous studies (see Introduction), *C. maenas* did not decrease their $\dot{M}_{O_2}$ during the emersion, but instead maintained it at a level slightly, but not significantly, elevated relative to the control (Fig. 1). We believe that the explanation lies in the fact that we took pains to minimize experimental disturbance, which is known to elevate $\dot{M}_{O_2}$ in green crabs (Taylor and Butler, 1978; Houlihan et al., 1984; Wilson et al., 2021). This resulted in very low control values of $\dot{M}_{O_2}$ in our submersed crabs, around 550 µmol kg^{-1} h^{-1}. The only other study to report such a low rate in submersed *C. maenas* under control conditions was by Wood and Po (2022), who used different respirometers but a similar settling protocol. As reviewed by Simonik and Henry (2014) and Wood and Po (2022), resting control rates in all other studies at comparable temperature ranged from approximately 850 to 4200 µmol kg^{-1} h^{-1}. Thus, the $\dot{M}_{O_2}$ that we recorded during 14 h of emersion, approximately 800 µmol kg^{-1} h^{-1}, was also below the control submersed rates reported by other workers, but was comparable to (Johnson and Uglow, 1985; Nancollas and McGaw, 2021) or below the emersed rates (Newell et al., 1972; Taylor and Butler, 1978; Simonik and Henry, 2014; Wood and Po, 2022) in many studies. In green crabs, the scaphognathites can pump air through the branchial chambers, and the gills have specialized modifications for air breathing such as widely spaced lamellae supported by chitin (Taylor and Butler, 1978) and cross-bridges (Johnson and Uglow, 1985), which prevent collapse in air. Indeed, crabs in hypoxic water are known to voluntarily emerge to breathe air when aquatic oxygen tensions fall below a critical value, a response that increases their arterial O_2 content (Taylor et al., 1973; Wheatly and Taylor, 1979).

Thus, green crabs, despite being classified as only a T1 species, the lowest rank for terrestriality among the air-breathing crabs

Table 1. Lipid damage (TBARS) and catalase activity (CAT) in muscle of *Carcinus maenas* immediately after 14 h of immersion, 14 h of air exposure and 14 h of recovery (14 h of re-immersion after 14 h of air exposure)

Treatment	TBARS (nmol TEP equivalents µg^{-1} protein)	CAT (µmol H_2O_2 min^{-1} mg^{-1} protein)
Immersion	0.879±0.298	0.259±0.046
Air	0.886±0.330	0.209±0.042
Recovery	0.804±0.184	0.175±0.037

Values are means±s.e.m. (*N*=6–12). None of the emersion/re-immersion values were significantly different from the control values (*P*>0.05).

Table 2. Transepithelial potential (TEP) of crabs (*N*=7) under control conditions (i.e. during immersion in seawater) and after 14 h of emersion followed by re-immersion into fresh seawater before TEP measurements at 0 h (actually 2 min re-immersion), 1, 2, 6, 12 and 18 h of re-immersion

Hours after emersion	TEP (mV)
Control (immersed)	−1.5±0.3
0	−2.4±0.5
1	−2.3±0.5
2	−2.0±0.5
6	−2.0±0.5
12	−1.9±0.6
18	−1.5±0.4

None of the emersion/re-immersion values were significantly different from the control value (*P*>0.05). Data are means±s.e.m.

Table 3. Exchangeable water pool (V_{H_2O}), rate constant of diffusive water exchange (*k*) and unidirectional diffusive water flux rate (J_{H_2O}) in control (immersed), post-emersion (0–0.5 h after return to seawater following 14 h of air exposure) and recovery treatments (20–20.5 h after return to seawater)

Treatment	V_{H_2O} (ml kg^{-1})	*k* (h^{-1})	J_{H_2O} (ml kg^{-1} h^{-1})
Control	701.0±42.7 (8)	0.709±0.071 (8)	499.6±59.2 (8)
Immediately post-emersion (0–0.5 h)	648.7±14.5 (8)	0.783±0.033 (6)	506.9±19.7 (6)
Recovery (20–20.5 h)	633.2±18.9 (8)	0.794±0.077 (6)	503.7±52.9 (6)

There were no significant differences among treatments in any of the parameters (*P*>0.05). Data are means±s.e.m. (control *N*=8, immediately post-emersion *N*=6, recovery *N*=6).

(Hartnoll, 1988), can maintain at least a low rate of O_2 consumption during an extended period of air exposure, equivalent to that when submersed at rest. However, the 3.4-fold elevation of $\dot{M}_{O_2}$ during recovery (Fig. 1) suggests that the rate of O_2 consumption recorded during emersion was not sufficient to support the increased metabolic demands of air exposure. Thus, this excess post-emersion O_2 consumption (EPOC) represented the cost of paying off metabolic processes that had been fuelled anaerobically or deferred during the period of air exposure. Principal amongst these would be the costs of supporting the crab's body against the effects of gravity in air, restoring acid–base, ionic and nitrogenous waste homeostasis, repairing oxidative damage after contact with the external seawater was re-established, and any activity that occurred. This finding is consistent with several previous studies that have observed an EPOC and/or lactate accumulation in *C. maenas* following emersion (Johnson and Uglow, 1985; Simonik and Henry, 2014; Wood and Po, 2022), though others have shown no such disturbances (Newell et al., 1972; Taylor and Butler, 1978; Nancollas and McGaw, 2021). EPOC and lactate accumulation were much greater when air exposure was combined with exercise (Wood and Po, 2022), so the differences among studies may reflect differences in the degree of activity of the emersed crabs in the respirometers.

In accord with the low control rate of O_2 consumption in our submersed crabs, rates of ammonia (-66 μmol-N kg^{-1} h^{-1}) and urea-N excretion (-10 μmol-N kg^{-1} h^{-1}) were similarly low (25–80%) relative to resting rates reported in most other studies on *C. maenas* (Spaargaren, 1982; Simonik and Henry, 2014; Durand and Regnault, 1998; Quijada-Rodriguez et al., 2022). However, they were comparable to rates reported for well-rested green crabs by Wood (2023) and Dal Pont et al. (2022). Supporting our original hypothesis, both ammonia and urea-N excretion rates declined to very low levels during the emersion period, and then rebounded to levels more than 2-fold greater than the control rates during post-emersion recovery (Fig. 2A,B). These findings agree well with those of Durand and Regnault (1998) and Wood (2023) on *C. maenas*, and of Regnault (1994) on *Cancer pagurus*. In these studies, the excretion of ammonia was negligible, and it built up in the hemolymph during emersion, but ammonia excretion was massively increased early in recovery. When averaged over 14 h recovery as in the present study, the elevations were comparable. The very low rates of ammonia and urea-N excretion to the dry respirometers during the 14 h of emersion were probably explained by discharge of urine and spillage of branchial chamber fluid, with which the urine mixes (Harris and Santos, 1993). However, the studies of Regnault (1994) and Wood (2023) established that the greatly elevated excretion rates during post-emersion recovery could not be explained by clearance of the small amounts of ammonia or urea-N that had accumulated in the branchial chamber fluid.

Little is known about the mechanisms of urea-N excretion, but several pathways of ammonia excretion, some of them active, have been identified in the gills of crabs. For example, Rh proteins appear to be involved in NH_3 efflux (Weihrauch et al., 2009, 2017), and experiments with intact *C. pagurus* revealed a permeability of the gills for ionic NH_4^+ (Weihrauch et al., 2004). Also, NH_4^+ ions can substitute for K^+ ions in activation of the ouabain-sensitive Na^+/K^+-ATPase (Weihrauch et al., 2004). A study performed by Weihrauch et al. (2002) indicated a basolateral membrane transport of NH_4^+ via the Na^+/K^+-ATPase and K^+ channels and a secondarily active apically located, Na^+-independent transport mechanism for NH_4^+. Pressley et al. (1981), in the crab *Callinectes sapidus*, and Lucu et al. (1989), in *C. maenas*, found the presence in the apical membrane of an amiloride-sensitive Na^+/NH_4^+ antiporter translocating NH_4^+ from the epithelial cell to the ambient medium in exchange for Na^+. Some or all of these mechanisms may have contributed to the elevated ammonia excretion seen during recovery from emersion in the present study.

Our ammonia excretion data, as well as those of Regnault (1994), Durand and Regnault (1998) and Wood (2023), are in stark contrast to those of Simonik and Henry (2014), who reported no build-up of ammonia in the hemolymph, no inhibition of ammonia excretion during 6 h of emersion, and no surge in ammonia excretion during post-emersion recovery in *C. maenas*. However, in that study, ammonia excretion was maintained by volatilization to the atmosphere. This route was not assessed in the present study or those of Regnault (1994), Durand and Regnault (1998) and Wood (2023). We are aware of no other studies showing ammonia volatilization in the green crab. This is clearly a topic worthy of future investigation.

Our second focus was on potential oxidative stress during emersion and recovery. Specifically, we confirmed our hypothesis, based on studies cited in the Introduction, that emersion and/or re-immersion events would result in oxidative damage. This was evidenced by lipid peroxidation during emersion, but only in the 5th gill (Fig. 3A) and not in the 8th gill (Fig. 4A), the hepatopancreas (Fig. 5A) or the muscle (Table 1), and there were no responses in protein carbonyls in the gills (not measured in the hepatopancreas or muscle). However, at the end of the normoxic recovery period, catalase activity was depressed relative to the air exposure levels in both the 5th gill (Fig. 3C) and the hepatopancreas (Fig. 5B).

For crabs, the posterior gills are involved with the osmoregulatory functions, whereas the anterior gills are mainly respiratory (Freire et al., 2008). In the congeneric *Carcinus aestuarii*, salinity challenge resulted in oxidative stress mainly in the posterior gills (Rivera-Ingraham and Lignot, 2017). Therefore, it is interesting that in the present work, air-exposed crabs presented lipid damage, measured through the TBARs assay, only in the anterior gill (5th gill). Oxygen availability can stimulate ROS production, where ROS production increases proportionally as O_2 concentration increases (Pelster and Wood, 2018). The O_2 content of air is 20 to 30 times greater than that of seawater. During emersion, the 5th gill is probably more in contact with the air, reflecting its respiratory function, so this could be a reasonable explanation for the higher damage in this tissue. To avoid ROS damage to biomolecules, the organisms could increase the levels of endogenous antioxidants during the air exposure. Relative to air-exposed levels, the activity of the catalase decreased in anterior gill (5th) and hepatopancreas of crabs during recovery, but did not change in the posterior gill (8th). These decreases could be due to the degradation of the enzyme during ROS neutralization generated during the air exposure because no lipid damage (TBARS) was observed during the recovery period. Notably, in a study on two species of swimming crabs, Freire et al. (2011) reported a significant decrease in catalase activity in the anterior gill but not in the posterior gill during recovery from air exposure in *Callinectes danae*. Those authors observed no significant changes in *Callinectes ornatus*. As in the present study, carbonyl proteins did not change in either anterior or posterior gills of either species, but in contrast to our findings, there were no significant changes in lipid peroxidation in either gill in both species. When facing challenging environmental situations (i.e. hypoxia, freezing, starvation or other), tolerant species are indeed capable of 'preparing for oxidative stress' (POS) (Hermes-Lima et al., 2015). This requires increases in their antioxidant defenses before triggering metabolic imbalance to counteract the burst of ROS generated upon the reperfusion (for review, see Moreira et al., 2017).

The fact that the lipid damage, measured through the TBARS assay, only occurs in the anterior gill (5th) is probably due to its direct contact with the O_2 in the air. The posterior gills sit more in the branchial chamber fluid and thus receive less direct exposure to air. The lack of direct air exposure may also be a reasonable explanation for why no alterations were found for muscle. The lack of damage in these tissues suggest that the transfer of oxygen is tightly regulated in the anterior 'respiratory' gills; in future studies, measurement of hemolymph oxyhemocyanin levels would be informative in this regard. The hepatopancreas is intermediate with no direct damage but with a 'potential' increase in catalase during emersion and a significant decline in catalase during recovery. The hepatopancreas and gill responses may be linked. Johannsson et al. (2018) have shown correlations between gill and liver antioxidant enzyme responses to hypoxia, re-oxygenation in *Cyphocharax ambroides*, a characid fish.

The neutralization of the ROS generated during the air and recovery exposures was sufficiently efficient to prevent damage to the other organs. This is not always the case. Lima et al. (2015) found increases in lipid damage in muscle of the crab *Neohelice granulata* only during the reoxygenation period (30 min), after 4 h of air exposure. Also, according to Geihs et al. (2014), the recovery period could be crucial for the normalization of ROS generation and tissue damage. These authors have shown that there was no lipid damage in the locomotor muscle of *N. granulata* during 1, 4 and 10 h of hypoxia, but after 30 min of reoxygenation in normoxic water, there was an increase in lipid damage levels that only returned to normal after 2 h of reoxygenation. In the present work, changes over such short time scales would not have been detected, because our measurements were made at 14 h intervals. Moreover, other enzymatic and non-enzymatic factors may participate in ROS neutralization (e.g. glutathione peroxidase, superoxide dismutase, glutathione tripeptide, ascorbic acid) (Lushchak, 2007). These were not measured in the present study. The actions of these factors are species- and tissue-specific (Lushchak, 2007). Thus, we have only a partial picture of the antioxidant response of the crabs to the emersion–recovery protocol, but enough to show lipid peroxidation in the respiratory gill and not in other tissues.

Our third focus was on permeability and water metabolism. For crustaceans, the gills are the main organs for exchanges between the body and its environment. The TEP is an indirect indicator of the membrane permeability through the movements of ions across the branchial epithelium (Potts, 1984). In *C. maenas*, the TEP can have both diffusive and electrogenic components; Na^+ and Cl^- are the two major ions in seawater and in hemolymph, and are the major contributors both to the diffusion component and to the active component of the potential (Siebers et al., 1985, 1989). In the present work, the TEP for *C. maenas* submerged in 100% seawater was -1.5 ± 0.3 mV. This value is in accordance with several previous measurements on intact green crabs in 100% seawater (Greenaway, 1976; Zanders, 1980; Winkler, 1986; Dal Pont et al., 2022). The lack of significant change at the end of emersion and throughout subsequent recovery suggests that disturbances in gill permeability and transport functions during air exposure were minimal.

The internal exchangeable water pool (~700 ml kg^{-1}; Table 3) was virtually identical to both a recent measurement of this parameter in *C. maenas* (Dal Pont et al., 2022), as well as to the total body water content measured in the same species acclimated to 100% seawater (Zatta, 1987). In support of our original hypothesis, there was only a small, non-significant reduction (~9%) in this internal exchangeable water pool after 14 h of air exposure. However, in contrast to our predictions, we could detect no significant changes in the rate constant (*k*) or absolute rates of diffusive water exchange either immediately or 20 h after air exposure (Table 3). This fits with the lack of significant change in TEP (Table 2), suggesting that absolute permeability is not reduced. Therefore, resistance to desiccation may rely more on behavioral changes (reduced activity) to minimize evaporation, as well as the use of branchial chamber fluid to replenish internal water reserves (Burnett and McMahon, 1987; Wood, 2023).

Conclusions

The ability of *C. maenas* to survive prolonged periods of air exposure is probably an important contributor to its success as an exotic invader (Yamada, 2002; Young and Elliott, 2020). The present study has shown that it can tolerate 14 h of emersion with minimal disturbance of its permeability and water metabolism, and can maintain its $\dot{M}_{O_2}$ at a low resting level during this period. However, metabolic costs appear to be increased during air exposure, so a substantial EPOC occurs during re-immersion recovery to reimburse those expenditures. Additionally, ammonia and urea-N excretion are both almost eliminated during air exposure, and are subsequently greatly elevated during recovery. Oxidative stress manifested as lipid damage occurs in the anterior gills that are specialized for respiratory gas exchange, and this leads to subsequent decreases in catalase activity during recovery. However, these responses do not occur in posterior gills that are specialized for osmoregulation.

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Competing interests
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Author contributions
Conceptualization: H.S.-H., C.M.W.; Methodology: H.S.-H., B.H.K.P., C.M.W.; Validation: H.S.-H., O.E.J., C.M.W.; Formal analysis: H.S.-H., O.E.J., B.H.K.P.; Investigation: H.S.-H., B.H.K.P.; Resources: C.M.W.; Writing - original draft: H.S.-H., C.M.W.; Writing - review & editing: H.S.-H., O.E.J., B.H.K.P., A.L.V., C.M.W.; Visualization: H.S.-H., A.L.V., C.M.W.; Supervision: A.L.V., C.M.W.; Funding acquisition: A.L.V., C.M.W.

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