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Short-term exposure to high pCO_2 leads to decreased branchial cytochrome C oxidase activity in the presence of octopamine in a decapod

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ABSTRACT

In a recent mechanistic study, octopamine was shown to promote proton transport over the branchial epithelium in green crabs, *Carcinus maenas*. Here, we follow up on this finding by investigating the involvement of octopamine in an environmental and physiological context that challenges acid-base homeostasis, the response to short-term high pCO_2 exposure (400 Pa) in a brackish water environment. We show that hyperregulating green crabs experienced a respiratory acidosis as early as 6 h of exposure to hypercapnia, with a rise in hemolymph pCO_2 accompanied by a simultaneous drop of hemolymph pH. The slightly delayed increase in hemolymph HCO_3^- observed after 24 h helped to restore hemolymph pH to initial values by 48 h. Circulating levels of the biogenic amine octopamine were significantly higher in short-term high pCO_2 exposed crabs compared to control crabs after 48 h. Whole animal metabolic rates, intracellular levels of octopamine and cAMP, as well as branchial mitochondrial enzyme activities for complex I + III and citrate synthase were unchanged in posterior gill #7 after 48 h of hypercapnia. However, application of octopamine in gill respirometry experiments suppressed branchial metabolic rate in posterior gills of short-term high pCO_2 exposed animals. Furthermore, branchial enzyme activity of cytochrome C oxidase decreased in high pCO_2 exposed crabs after 48 h. Our results indicate that hyperregulating green crabs are capable of quickly counteracting a hypercapnia-induced respiratory acidosis. The role of octopamine in the acclimation of green crabs to short-term hypercapnia seems to entail the alteration of branchial metabolic pathways, possibly targeting mitochondrial cytochrome C in the gill. Our findings help advancing our current limited understanding of endocrine components in hypercapnia acclimation.

Summary statement: Acid-base compensation upon short-term high pCO_2 exposure in hyperregulating green crabs started after 6 h and was accomplished by 48 h with the involvement of the biogenic amine octopamine, accumulation of hemolymph HCO_3^-, and regulation of mitochondrial complex IV (cytochrome C oxidase).

1. Introduction

Acid-base regulation is one of the major physiological systems for maintaining overall systemic homeostasis and efficient biological functions in all organisms, including crustaceans (Henry and Wheatly, 1992). To ensure proper oxygenation of blood and functioning of proteins and enzymes extra- and intracellular pH must be kept in a narrow optimal range (Riggs, 1988; Somero, 1986; Truchot, 1975). Many aquatic animals live in highly variable environments that challenge internal acid-base homeostasis, including coastal and intertidal zones. These environments are characterized by fluctuations in different abiotic water parameters including carbon dioxide (CO_2). As part of the aquatic carbonate buffer system, CO_2 is in equilibrium with H^+ and HCO_3^- ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3^- \leftrightarrow H^+ + CO_3^{2-}$). The natural range of the European green crab, *Carcinus meanas*, for example, extends to areas in which pCO_2 can exceed 230 Pa (>2300 μatm), such as the Baltic Sea in late summer/early fall due to upwelling events (Thomsen et al., 2010). Fluctuations and increases of pCO_2 due to upwelling events also play a

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relevant role in marine coastal areas, where CO_2 from the depth is brought up to the surface, usually lasting 2–3 days (Bednaršek et al., 2020). These events might become more drastic with current practices of carbon storage and their potential leakage (Kim et al., 2016; Vinca et al., 2018). Furthermore, pCO_2 in intertidal rockpools can vary between 30 and 400 Pa within 12–24 h intervals accompanied by a drop in pH of up to 0.8 units (Truchot, 1986; Truchot and Duhamel-Jouve, 1980). Consequently, green crabs must have evolved specific mechanisms to cope with short-term fluctuations (i.e., hours) of high/elevated pCO_2 hypercapnia. In addition, the ongoing anthropogenic increase in atmospheric CO_2 equilibrating with the ocean surface will further mount allostatic load on the acid-base regulation in aquatic organisms (IPCC, 2023). Therefore, these conditions might become challenging even for species like green crabs that adapted to cope with current levels of environmental fluctuation, such as those found in intertidal and coastal zones (Bednaršek et al., 2020; Thomsen et al., 2010).

Similar as in sea water, CO_2, H^+ and HCO_3^- in the green crabs' hemolymph constitute to the major extracellular acid-base buffering system (Fehsenfeld and Weihrauch, 2017). Accordingly, acid-base regulation in *C. maenas* is mainly comprised of adjustments in branchial epithelial ion exchange including H^+ and HCO_3^-, CO_2 excretion, and overall ammonia regulation (i.e., NH_3/NH_4^+ as an acid-base pair) (Weihrauch et al., 2004; Fehsenfeld and Weihrauch, 2016b; Fehsenfeld and Weihrauch, 2017; Weihrauch et al., 2017).

One of the responses of *C. maenas* to hypercapnia is to buffer extra- and intracellular fluids to minimize changes in pH (Fehsenfeld and Weihrauch, 2013, 2016a). Pioneering work of Truchot in the 1970s on seawater acclimated osmo-conforming green crabs, for example, showed their complete restoration of extracellular pH due to accumulation of HCO_3^- within 115 h (Truchot, 1975; Truchot, 1979, 1986). However, our own data suggested that 48 h might not be sufficient to fully compensate for the drop in pH in response to hypercapnia (1% CO_2 = 1 kPa) in osmo-conforming green crabs of Canada's Pacific west coast (Fehsenfeld and Weihrauch, 2016a). The observed accumulation of HCO_3^- in the hemolymph could be due to branchial transport processes, dissolution of the carapace, or simple a shift in the extracellular carbonate system (Cameron, 1985; Ries et al., 2009). However, a clear mechanism and/or source for HCO_3^- accumulation has not been identified to date. Furthermore, no data for brackish water acclimated, hyperregulating green crabs on responses to short-term hypercapnia is yet available.

While minimizing changes in extracellular pH by buffering, acid equivalents still need to be removed to restore acid-base equilibrium. *C. maenas*' gills have been determined to be the major site for active acid-base regulation (Fehsenfeld and Weihrauch, 2017). H^+ and HCO_3^-, for example, directly feed branchial epithelial transporters like V-type H^+-ATPase, Na^+/H^+-exchangers (NHE), Na^+/HCO_3^--cotransporter and Cl^-/HCO_3^--exchanger (Fehsenfeld and Weihrauch, 2013, 2016a). Excreted acid-base equivalents like H^+ and CO_2 would then accumulate in the branchial chamber until being flushed out.

In some decapod species, counteracting the experienced respiratory acidosis (i.e., elevated hemolymph pCO_2 leading to a drop in hemolymph pH) upon hypercapnia comes at metabolic costs. In fact, metabolic depression was observed in the stenohaline osmo-conforming Dungeness crab, *Metacarcinus magister* (Dana, 1852) (Hans et al., 2014) and osmo-conforming *C. maenas* (Maus et al., 2018) after medium- to long-term acclimation to hypercapnia. In the latter, a decrease of metabolic rate is correlated with seawater [HCO_3^-], suggesting this as the source of elevated extracellular [HCO_3^-] (Maus et al., 2018). For short-term exposures, however, it seems more feasible for aquatic animals in a highly variable environment to employ more subtle metabolic shifts on a tissue-specific level rather than "shutting down" on a whole-body level. In case of the green crabs' gill as one of the major acid-base regulatory organs, this could entail branchial enzymatic adjustments of the respiratory transport chain as observed in fish (Michaelidis et al., 2007; Strobel et al., 2012, 2013).

As a first and fast detection system of translating environmental change into a physiological compensatory response in the body, the (decapod) endocrine system uses hormones to communicate between the "sensors" (i.e., nervous structures) and the organs that adjust performance to maintain homeostasis (Fehsenfeld, 2023). As the equivalent to norepinephrine in vertebrates the biogenic amine octopamine (OCT) has been considered as a potential stress hormone also in invertebrates (Roeder, 1999). However, its role to date has mainly been investigated within a behavioral context rather than physiological acclimation. This includes agonistic behavior in *C. maenas* (Sneddon et al., 2000) and the grapsid rainbow crab *Neohelice granulata* (former: *Chasmagnathus granulatus*) (Dana, 1851) (Pedetta et al., 2010), as well as circadian rhythms in the Madeira cockroaches *Leucophaea maderae* (Fabricius, 1781) (Schendzielorz et al., 2012) and the tobacco hornworm *Manduca sexta* (Linnaeus, 1763) (Schendzielorz et al., 2015).

Interestingly, recent data in shrimps indicates that OCT is involved in acclimation to salinity changes (Pan et al., 2019). Furthermore, Dolzer et al. (2001) indicated a potential role for OCT in acid-base regulation, as it led to decreased V-type H^+-ATPase activity in *M. sexta*.

Recently, Fehsenfeld et al. (2023) have identified OCT to be involved in branchial proton excretion in *C. maenas*, and hence suggested a role for OCT in the general maintenance of steady-state acid-base maintenance in the gill.

After the recent mechanistic study on the involvement of OCT in branchial acid-base regulation (Fehsenfeld et al., 2023), the present study aimed to investigate the involvement of OCT in an environmentally (i.e., hypercapnia) and physiologically (i.e., hyperregulation in brackish environment) relevant context. We chose a short time frame (48 h) to account for the likely quick activation of endocrine components (OCT). We hypothesized that: (i) hyperregulating green crabs quickly and effectively counteract an organismal acid-base disturbance caused by short-term hypercapnia, (ii) the biogenic amine OCT plays a role in the acid-base compensatory response in an eco-physiological context, and (iii) *C. maenas* adjust their aerobic metabolism in response to short-term hypercapnia.

To test our hypotheses, we monitored acid-base related hemolymph parameters, as well as branchial chamber water, of brackish water acclimated (i.e., hyperregulating) green crabs over the first 48 h of acclimation to hypercapnia. We measured changes in the biogenic amine/neurohormone OCT by mean of ELISA tests. As a potential target organ for OCT, we also monitored intracellular levels of OCT and cAMP in osmoregulatory active posterior gills using ELISA. We measured the whole-body as well as posterior gill 7 oxygen consumption rate using respirometry to assess changes in energy expenditure for the hypercapnia acclimation. Lastly, we measured enzyme activities for the mitochondrial electron transport chain in anterior and posterior gills.

2. Material and methods

2.1. Specimens' collection and maintenance

North Atlantic adult male intermolt European green crabs, *Carcinus maenas*, were caught in Northern Placentia Bay (NL, Canada) and transferred to the Animal Holding Facility at the University of Manitoba (Winnipeg, MB, Canada). A maximum of 75 specimens each were kept in three 1200 L tanks filled with recirculating artificial sea water (Fritz Reef Pro Mix (RPM), Fritz Aquatics, Mesquite, TX, USA) at salinity of 32 ppt, pH 8.1, pCO_2 of 44 Pa, temperature of 16 °C, and on a 12:12 h light/dark cycle for up to 8 months. Stones and PVC pipes were provided to act as shelters, enabling specimens to hide and hence lowering aggressive interactions. Green crabs were fed ad libitum with frozen bay scallops twice a week and leftovers were removed from the tanks to maintain water quality. In preparation to the experiments, 20 green crabs with a carapace width of 5–6 cm were transferred to a 200 L recirculating tank to be acclimated to a salinity of 10.6 ± 0.3, a pH of 7.85 ± 0.05, a temperature of 15.3 ± 0.2 °C and a pCO_2 of 55.3 ± 3.3 Pa for one week

as to mimic an estuarine environment (Supp. Table 1). Green crabs were fasted two to three days before acclimation to high pCO_2 experiments to reduce the effects of feeding (Quijada-Rodriguez et al., 2022). Overall, the crabs were hence not fed for four to five days which has been shown to not negatively affect their performance (Weihrauch, 1999).

All procedures followed recommendations by the University of Manitoba Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care, as well as ARRIVE guidelines (https://arriveguidelines.org).

2.2. Experimental exposure and sampling

To characterize the responses to short-term high pCO_2 of *Carcinus maenas* in an estuarine environment, two sets of six brackish water acclimated crabs were transferred to 50 L recirculating tanks and exposed to either continued control conditions, or short-term high pCO_2 conditions with a pCO_2 of 400 Pa corresponding to a pH of 7.10 for 48 h (Supp. Table 1). Hypercapnia was initiated by influx of pure CO_2 gas under the control of a regulatory computer module (IKS, Aquastar®, Karlsbad, Germany) which was set to monitor and control the pH accordingly. Water parameters were monitored daily. The water carbonate system was measured with an infra-red CO_2/ H_2O gas analyzer (LI-850, LICOR, Lincoln, NE, USA) (Allen et al., 2021) to determine total CO_2. A pH electrode (InLab Micro Combination pH Electrode, Mettler-Toledo, Greisensee, Switzerland) calibrated to NIST standards (Thermo Scientific™ Orion™ Standard All-in-One™ pH Buffer Kit, Fisher Scientific, Ottawa, Ontario, Canada) and connected to the pH-ISE meter model 225 (Denver Instruments, Gottingen, Germany) was used to measure pH_{NBS}. Subsequently, pCO_2 and HCO_3^- were calculated using the CO2SYS software (Lewis and Wallace, 1998, www. OSTI.gov) as an Excel add-in with dissociation constants according to Dickson (1990) and Mehrbach et al. (1973).

After 48 h, the crabs were rapidly euthanized by perforation of the ventral ganglion. For collection of the branchial water to measure the carbonate system parameters, crabs were held upright to keep the hemolymph in the lower part of the body, while the carapace was quickly removed. This was done by cracking it at the posterior end at the connection to the telson and immediately flipping the carapace over towards the front so that only the branchial water would collect in the "bowl" of the carapace. This remaining branchial water fluid was collected with a syringe (see Supp. Fig. 1). It was assured that only samples of branchial water were taken from intact carapaces: i.e., hypodermis still fully attached. Gills 5 and 7 were dissected, and either flash frozen in liquid nitrogen, or immediately used for respirometry. For enzymatic analyses, flash frozen gills were shipped on dry ice to the Molecular Biology Laboratory at the University of Montreal (Montréal, QC, Canada), where they were stored in a −80 °C freezer until the measurements.

Table 1
Branchial water carbonate parameters in comparison to hemolymph in control green crabs or after 48 h of high pCO_2 exposure.

Treatment	Compartment	pH	pCO_2 [Pa]	HCO_3^- [mmol L^{-1}]
Control	BW	7.53 ± 0.13*	760 ± 172*	9.1 ± 0.3 ^{A}
Control	H	7.85 ± 0.01	395 ± 46^{a}	8.9 ± 0.9^{a}
High pCO_2	BW	7.52 ± 0.06*	627 ± 120	7.5 ± 0.2^{B,*}
High pCO_2	H	7.85 ± 0.02	755 ± 21^{b}	17 ± 1.0^{b}

^{A,B}, different uppercase letters highlight significantly different between the control and the high pCO_2 condition in the branchial water (Student's *t*-test, $P < 0.05$); ^{a,b}, different lower case letters indicate significant differences between the control and the high pCO_2 condition in the hemolymph (Student's t-test, $P < 0.05$); *, indicate the presence of a significant difference in the mean trait measure between the branchial water (BW) and hemolymph (H), in the respective treatment group (Student's t-test, $P < 0.05$). All values are represented as means ± SE ($N = 6$). BW, branchial water; H, hemolymph.

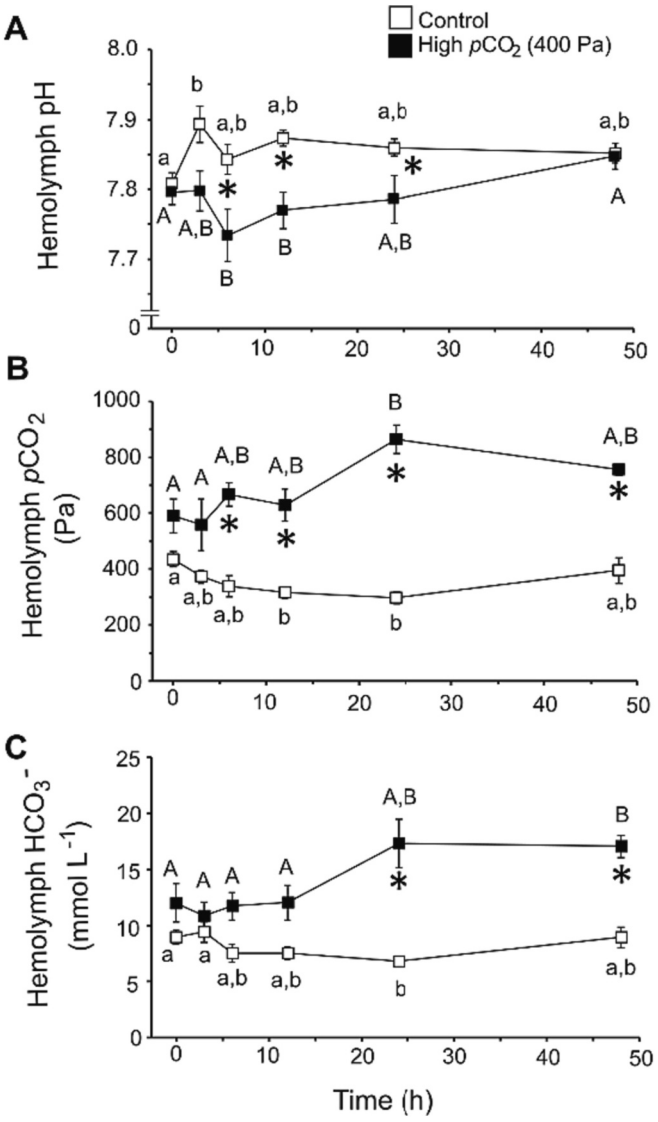

Fig. 1. The relationship between time of exposure to high pCO_2 and hemolymph carbonate parameters in the brackish water acclimated green crab, *Carcinus maenas*. Hemolymph (A) pH, (B) pCO_2 (in Pa), and (C) HCO_3^- concentration (in mmol L^{-1}). Open squares represent crabs exposed to control conditions, black squares represent specimens exposed to short-term high pCO_2 (400 Pa) as means ± SE (N = 6). Lower case letters indicate significant changes in hemolymph carbonate syste parameters over time in crabs kept under control conditions, whereas upper case letters denote significant changes in crabs acclimated to high pCO_2 conditions (REML with Tukey's post-hoc test, $P < 0.05$). Asterisks denote significant differences between green crabs acclimated to control and high pCO_2 condition at a given time point (Student's t-test or Mann-Whitney Test, $P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Hemolymph, branchial water and gill analyses – measurement of carbonate system and hormones

To assess the acid-base and hormonal status of the hemolymph, ca. 200–300 µL hemolymph were sampled at 0 h, 2 h, 6 h, 12 h 24 h, and 48 h from each specimen by inserting a needle into the arthrodial membrane at the base of a posterior walking leg. Collection of hemolymph was conducted carefully as to avoid excessive air bubbles in the syringe and exerted as rapidly as possible to avoid gas equilibration with the ambient air. Hemolymph, as well as the branchial water collected as described above, were analyzed immediately using the custom-built gas CO_2 analyzer (Lee et al., 2018) connected to the LI-850 (LICOR, Lincoln,

NE, USA) as described above for measuring water parameters. To avoid excessive formation of bubbles in the hemolymph sample due to the introduction of the gas, anti-foam reagent was added to the chamber. Hemolymph pCO_2 and HCO_3^- were subsequently calculated using the Henderson-Hasselbalch equation and respective constants as determined by Truchot (1976) (see also Fehsenfeld and Weihrauch, 2013).

OCT concentrations in hemolymph and posterior gill 7 (cytoplasm) were measured using an octopamine ELISA kit (#MBS726911, MyBioSource, San Diego, CA, USA). Gill 7 was chosen based on the recent study by Fehsenfeld et al. (2023) that showed OCT to alter proton (and ammonia) flux in this gill as components of branchial acid-base regulation.

Preparation of the samples was modified after Schendzielorz et al. (2012, 2015). In brief, either 100 μL hemolymph were mixed with 250 μL 10 mmol L^{-1} EDTA pH 7.2 and 100 μL PCA 7%, or whole gills were homogenized with metal beads for 45 s at a frequency of 30/min in 250 μL 10 mmol L^{-1} EDTA pH 7.2 and 100 μL PCA 7%. All samples were then centrifuged for 15 min at 4 °C and 1000 x g. 300 μL supernatant were mixed with 300 μL 10 mmol L^{-1} EDTA pH 7.2 and 300 μL chloroform / trioctylamine (1:1) and again centrifuged at 4 °C and 1000 xg for 5 min. 100 μL of the latter supernatants were then analyzed by ELISA following the instructions/protocol provided with the kit.

Cytoplasmatic gill cAMP levels were measured with the General Cyclic Adenosine Monophosphate (cAMP) Competitive ELISA Kit (#MBS1602789, MyBioSource, San Diego, CA, USA). Sample preparation was modified after Battelle and Kravitz (1978) and Schendzielorz et al. (2012, 2015). Briefly, whole gills were homogenized in 300 μL homogenization buffer for 30 s. The buffer contained 300 mmol L^{-1} NaCl, 50 mmol L^{-1} 3-morpholinopropane-1-sulfonic acid (MOPS), 1 mmol L^{-1} 3-isobutyl-1-methylxanthine (IBMX), 1 mmol L^{-1} 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), 1 mmol L^{-1} dithiotriol (DTT), 0.05% sodium cholate, pH 7.2. Samples were then centrifuged for 15 min at 4 °C and 1000 ×g. 50 μL of the supernatants were used in the ELISA following the instructions/protocol provided with the kit.

The performance of both ELISA kits was ensured by initial testing with dilutions series on (i) natural, unprocessed hemolymph / tissue, (ii) hemolymph / tissue "spiked" with added known concentration of either OCT or cyclic adenosine monophosphate (cAMP) (standard 1 of the respective kit), and lastly (iii) hemolymph / tissue as processed above (natural and "spiked"). OCT and cAMP levels in the gill were normalized to gill weight.

2.4. Determination of whole-organism metabolic rates

Whole-organism metabolic rate was determined by measuring oxygen uptake ($\dot{M}O2$). $\dot{M}O2$ was measured using intermittent flow respirometry (Loligo Systems, Viborg, Denmark) after 48 h of either control or high pCO_2 exposure. The respirometry setup consisted of eight acrylic chambers (587.4 ± 10.0 mL; mean ± S.D.) with tubing along the oxygen sensors (PreSens, Regensburg, Germany) to which Witrox 4 Oxygen Meter (Loligo Systems, Viborg, Denmark) were connected to measure air saturation levels of oxygen at 1 Hz. Oxygen sensors were calibrated at 0 and 100% with 2% sodium sulfite and fully oxygenated water, respectively. Prior to the experimentation, all crabs were fasted for a total of four to five days, and three crabs each were placed into individual metabolic chambers that were submerged in two 200 L tanks in a temperature controlled environmental chamber at 16 °C. One tank was kept at control conditions, whereas the second tank held high pCO_2 water (pH = 7.15). The tanks were covered with plastic panels to minimize external visual disturbances during the measurement. Based on a preliminary trial, specimens were allowed to settle down and acclimate to the chambers for 2 h. This was then followed by a subsequent 1 h period used for calculations in which $\dot{M}O2$ generally stabilized (as observed in preliminary trials; Yoon *pers. obs.*). The parameters for one intermittent measurement cycle were 300 s flush – 60 s waiting period – 300 s measurement. A fourth chamber in each tank was left empty to allow for measurement of background respiration. The average ratio between body mass and chamber volume was 14.6 ± 3.9 (mean ± S.D.) while the ratio between background respiration to metabolic rate was found to be negligible (< 1%). All slopes had $R^2 > 0.9$, and data were averaged to calculate metabolic rate following the subtraction with background respiration.

2.5. Determination of gill metabolic rates

Gill metabolic rates were measured using a high precision respirometer (O2K, Oroboros, Innsbruck, Austria). Experimental runs were performed with the O2K chamber volume adjusted to 3 mL, a stir speed of 750 rpm, and data acquisition every 0.5 s. The respirometry chambers were calibrated to fully oxygenated brackish water and brackish water depleted of O_2 by addition of sodium dithionite according to manufacturer instructions. The media used in the respirometers was brackish water at either control or high pCO_2 levels used for experimental acclimations (see above).

For respirometry trials, *C. maenas* posterior gill 7 was excised from both sides of the crab and perfused either with control *C. maenas* saline (in mmol L^{-1}: 260 NaCl, 5 $CaCl_2$, 7 $MgCl_2$, 8 KCl, 7 $NaHCO_3$, 0.1 NH_4Cl, 0.3 glucose, 0.1 glutathione, 0.5 glutamine, pH = 7.9; according to Fehsenfeld and Weihrauch, 2013), or *C. maenas* saline containing 2 ng mL^{-1} OCT. This concentration of OCT was chosen based on the literature as well as this study, to be slightly elevated as experienced by the short-term high pCO_2 exposed crabs compared to control animals with ca. 1–1.5 ng mL^{-1}, respectively (Sneddon et al., 2000). Gills were perfused for a total of 3 min with a peristaltic pump at a speed of 250 μL min^{-1}, tied off to prevent hormone leakage from the gill and allowed to incubate for 20 min before being placed into the respirometry chambers. Preliminary trials with bromophenol blue in the perfusion saline demonstrated that the above protocol was sufficient to distribute saline throughout the gill and leakage was preventable by tying off the gill. Gill oxygen consumption was measured over a time span of 10 min where the last 6 min of data were used to calculated metabolic rate as noise levels were higher in the first few minutes after introducing the gill. The gill respiration time was limited to 10 min to prevent over depletion of chamber oxygen. In our lab, isolated gill preparation of various crustaceans in the oroboros O2k respirometer have shown that metabolic rate remains stable and repeatable over at least a one-hour period (Quijada-Rodriguez; *pers. obs.*). After measurements were complete, chambers were open, gills removed, and the respiration media was allowed to oxygenate for 5 min before being sealed to determine each chamber's biological oxygen demand (BOD) in the absence of the gill (subsequent 5 min measurement). Metabolic rates were calculated according to equation below:

$$Gill\ MO2 = (Uncorrected\ MO2 - BOD) * \frac{Chamber\ volume}{Gill\ Mass} \quad (1)$$

Units: Uncorrected MO_2 = pmol mL^{-1} s^{-1}; BOD = pmol mL^{-1} s^{-1}; chamber volume = mL; Gill mass = mg.

2.6. Determination of gill enzymatic activities

To define gills' enzyme activities, gill samples were shipped to the University of Montreal on dry ice and ensured to still be frozen upon arrival. The enzymatic analyses included anterior gill 5 to identify potential differences in the effect of hypercapnia on the different gill clusters (i.e., gill 5 as respiratory gill, gill 7 as metabolically active gill). Anterior gill 5 and posterior gill 7 were homogenized in five volume/weight (μL mg^{-1}) of respective ice-cold buffers (see below) at pH 7.5 using a Polytron homogenizer (Polytron PT 1200 homogenizer, Kinetica AG, Malters, LU, Switzerland) with the 7 mm rod for 15 s, three times with a rest time on ice of 30 s between each step, then with the 5 mm rod for 15 s, three times as well with a rest time on ice of 30 s between each

step. The protocols used for the enzymatic analyzes were adapted from Bergmeyer (1983) for complexes I and III of the electron transport chain, and from Thibault et al. (1997) and Mélançon et al. (2023) for cytochrome C oxidase and citrate synthase, respectively.

The activity of complexes I and III of the electron transport chain (ETS, EC 7.1.1.2 and 7.1.1.8) was measured at 490 nm ($\varepsilon 490 = 15.9$ mmol L^{-1} cm^{-1}) by following the reduction of iodonitrotetrazolium (INT) in a reaction medium at pH 8.5 containing 100 mmol L^{-1} imidazole-HCl, 2 mmol L^{-1} INT, 0.03% (*v*/v) triton X-100, and 0.85 mmol L^{-1} NADH (omitted for the control).

The activity of cytochrome C oxidase (COX, EC 7.1.1.9) was measured at 550 nm ($\varepsilon 550 = 18.5$ mmol L^{-1} cm^{-1}) following the oxidation of reduced cytochrome *c* in a reaction medium at pH 8.0 containing 100 mmol L^{-1} potassium phosphate, 0.05% (v/v) Tween 20, and 100 μmol L^{-1} bovine cytochrome *c*. Cytochrome c was reduced with 4.5 mmol L^{-1} sodium dithionite (DTT, without excess).

Citrate synthase (CS, EC 2.3.3.1) activity was measured at 412 nm following the conversion of 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) into TNB ($\varepsilon 412 = 14.15$ mmol L^{-1} cm^{-1}). The reaction medium (pH 8.0), consisted of 100 mmol L^{-1} imidazole-HCl, 0.1 mmol L^{-1} DTNB, 0.1 mmol L^{-1} acetyl-CoA and 0.15 mmol L^{-1} oxaloacetic acid (omitted for the control reaction).

The protein concentration (mg mL^{-1}) of the samples was measured according to the bicinchoninic acid (BCA) method of Smith et al. (1985) which uses a standard curve from the absorbance values measured at 562 nm.

2.7. Statistical analyzes

All data was checked for normal distribution (Shapiro-Wilk test in case of direct comparisons, residuals/normal probability plots with Shapiro-Wilk test in case of time series), as well as the homogeneity of variances (F-test) as implemented in PAST3 software (http://palaeo-electronica.org/2001_1/past/issue1_01.htm; (Hammer et al., 2001)). Outliers were identified by Grubb's outlier test (Grubbs, 1969) and if present, eliminated from the analyses (max. One *per* data set). In case of non-normal distribution and/or heterogeneity of variances, the data were log-transformed or tested using non-parametric tests (Mann-Whitney test for comparison of the respective rank sums) if they still did not meet the criteria for parametric testing after transformation.

For the hemolymph carbonate system and circulating OCT levels a repeated measures mixed-effects model (REML) with "time" as the fixed factor was performed with GraphPad Prism 10.0.2. for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Subsequently, post-hoc Tukey's multiple comparisons were applied to identify significantly different time points with the same software. Branchial water parameters were tested with Student's *t*-test for comparison of control vs. short-term high pCO_2 data (PAST3) and compared to hemolymph values by Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparison test using GraphPad Prism 10.0.2. Intracellular levels of OCT, cAMP, whole-organism metabolic rate, gill metabolic rates and enzyme activities were analyzed with Student's t-test as implemented in PAST3. Data was considered statistically different with $\alpha < 0.05\%$ ($P < 0.05$). Graphs were merged using Inkscape 1.2.2. (https://inkscape.org/).

3. Results

3.1. Changes in body fluids and tissues after short-term exposure to high pCO_2

3.1.1. Carbonate system

Hemolymph. Hemolymph pH (pH_{hemo}) varied significantly over time in control ($F_{5,25} = 5.252$, $P < 0.05$) as well as short-term high pCO_2 exposed ($F_{5,19} = 4.732$, $P < 0.03$) crabs. Post-hoc analysis identified for control crabs to exhibit a slight transient increase in pH_{hemo} during the first 3 h after being transferred to the new control tank after which pH_{hemo} returned to initial values and remained stable for the 48 h acclimation period (Fig. 1A; $P < 0.01$). In contrast, short-term high pCO_2 exposed *C. maenas* exhibited a significant drop in pH_{hemo} (ca. 0.1 units) after 6 h ($P_{3vs6h} = 0.0495$), that lasted until 24 h of exposure and was fully restored to control levels after 48 h ($P_{6vs48h} = 0.004$; Fig. 1A). pH_{hemo} was significantly lower in short-term high pCO_2 exposed crabs compared to control crabs during 6–24 h of hypercapnia ($P < 0.040$; Fig. 1A).

Hemolymph pCO_2 ($pCO_{2\text{-hemo}}$) varied significantly over time in control ($F_{5,25} = 5.107$, $P < 0.002$) as well as short-term high pCO_2 exposed ($F_{5,19} = 4.060$; $P < 0.01$) crabs. While control specimen experienced a slight decrease of $pCO_{2\text{-hemo}}$ throughout the duration of the experiment (significant between 12 and 24 h with $P < 0.011$), short-term high pCO_2 exposed crabs experienced a pronounced increase in $pCO_{2\text{-hemo}}$ after 12 h that stayed elevated even after 48 h ($P < 0.030$; Fig. 1B). $pCO_{2\text{-hemo}}$ levels were significantly higher in short-term high pCO_2 exposed *C. maenas* compared to control specimen during 6–24 h of the exposure and remained elevated even after 48 h ($P < 0.005$; Fig. 1B).

Hemolymph $[HCO_3^-]$ ($[HCO_3^-]_{hemo}$) varied significantly over time in control ($F_{5,23} = 4.023$, $P \leq 0.009$) as well as short-term high pCO_2 exposed ($F_{5,19} = 5.288$, $P < 0.01$) crabs.

Despite control crabs experiencing a slight drop in $[HCO_3^-]_{hemo}$ at 24 h ($P = 0.018$), levels remained relatively stable over 48 h. Short-term high pCO_2 exposed *C. maenas* exhibited elevated $[HCO_3^-]_{hemo}$ after 48 h of exposure ($P = 0.042$). When compared to control specimen, $[HCO_3^-]_{hemo}$ was significantly higher already at 24 h of exposure ($P < 0.01$; Fig. 1C).

Branchial water. Generally, the fluid collected from the branchial chamber was more variable with regards to branchial chamber pH (pH_{bc}; Fig. 2A) and branchial chamber pCO_2 ($pCO_{2\text{-bc}}$; Fig. 2B) compared to the hemolymph (Figs. 1A, B), but tightly regulated for HCO_3^- (HCO_{3bc}^-). There was no observed difference in either pH_{bc} or $pCO_{2\text{-bc}}$ between control and short-term high pCO_2 exposed crabs (Figs. 2A, B). $[HCO_3^-]_{bc}$, however, was significantly lower in short-term high pCO_2 exposed crabs compared to control ones ($P = 0.006$; Fig. 2C), as was total CO_2 in general (from 9.6 ± 0.3 mmol L^{-1} to 7.9 ± 0.2 mmol L^{-1}; Student's *t*-test with $P = 0.02$).

When compared to the hemolymph (Table 1), pH_{bc} was significantly lower compared to pH_{hemo} in control crabs ($P = 0.030$), as well as after 48 h of hypercapnia acclimation ($P = 0.003$), corresponding with a higher $pCO_{2\text{-bc}}$ compared to $pCO_{2\text{-hemo}}$ only in control crabs ($P = 0.052$). Control crabs exhibited an almost 2-fold higher $pCO_{2\text{-bc}}$ compared to $pCO_{2\text{-hemo}}$ and >10-fold higher compared the ambient brackish water ($pCO_{2\text{-BW}}$). After acclimation to hypercapnia, however, $pCO_{2\text{-bc}}$ was similar to $pCO_{2\text{-hemo}}$ of short-term high pCO_2 exposed green crabs. $[HCO_3^-]_{bc}$ was similar to $[HCO_3^-]_{hemo}$ in control specimen, but exhibited the opposite adjustment in short-term high pCO_2 exposed crabs so that $[HCO_3^-]_{bc}$ was ca. 50% lower than $[HCO_3^-]_{hemo}$ ($P < 0.001$; Table 1).

3.1.2. Hemolymph and gill octopamine (OCT)

OCT concentration in the hemolymph stayed constant under control conditions ($F_{1.176,\ 7.058} = 1.126$) although there seemed to be a slight trend to decrease after 48 h (from $t_{0h} = 1.30 \pm 0.31$ ng mL^{-1} to $t_{48h} = 0.96 \pm 0.13$ ng mL^{-1}). Also in high pCO_2 exposed animals, circulating OCT levels remained fairly constant, however, contrastingly as in control crabs, there was an observable trend for increasing OCT levels after 3 h (from $t_{0h} = 1.36 \pm 0.20$ ng mL^{-1} over $t_{3h} = 1.87 \pm 0.29$ to $t_{48h} = 1.80 \pm 0.22$ ng mL^{-1}, $F_{2.097,\ 9.088} = 0.96$; Fig. 3A). Ultimately, short-term high pCO_2 exposed *C. maenas* had significantly higher levels of hemolymph OCT compared to control specimen after 48 h ($P = 0.008$; Fig. 3A).

Intracellular levels of OCT were 3-fold higher compared to hemlymph levels. Neither intracellular levels of OCT (Fig. 3B) nor second messenger cyclic adenosine monophosphate (cAMP, Fig. 3C) were significantly different in posterior gill 7 of hypercapnia-exposed vs.

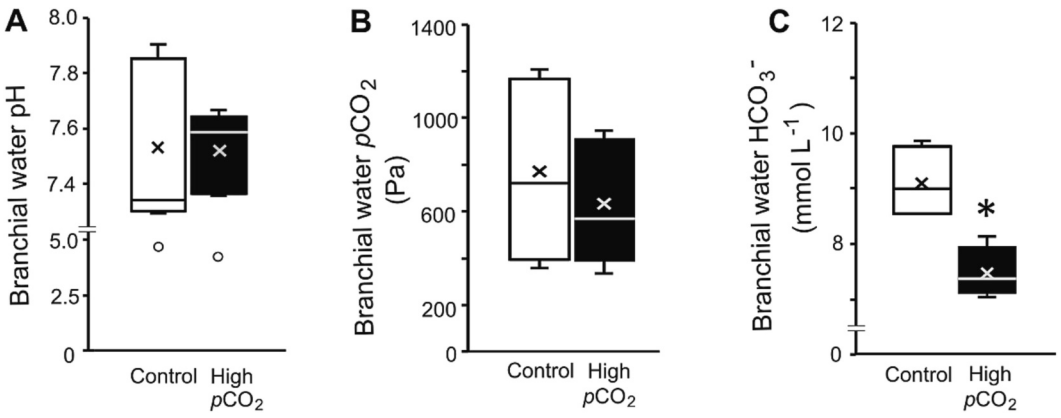

Fig. 2. The effect of short-term (48 h) exposure to high pCO_2 on the carbonate parameters of the branchial chamber fluid of *C. maenas*. Hemolymph (A) pH, (B) pCO_2 (in Pa), and (C) HCO_3^- concentration (in mmol L^{-1}). White box plots represent the control conditions, and black ones the high pCO_2 treatment. The middle line of the box represents the median, whereas the x represents the mean. The top and bottom line indicate the 1st quartile and 3rd quartile with whiskers extending to the minimum and maximum value, respectively. Asterisk denotes significant differences between mean trait measured in control and high pCO_2 acclimated green crabs (Student's t-test, $P < 0.05$, $N = 5$). Outliers that were removed from the statistical analysis are indicated as open circles in (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

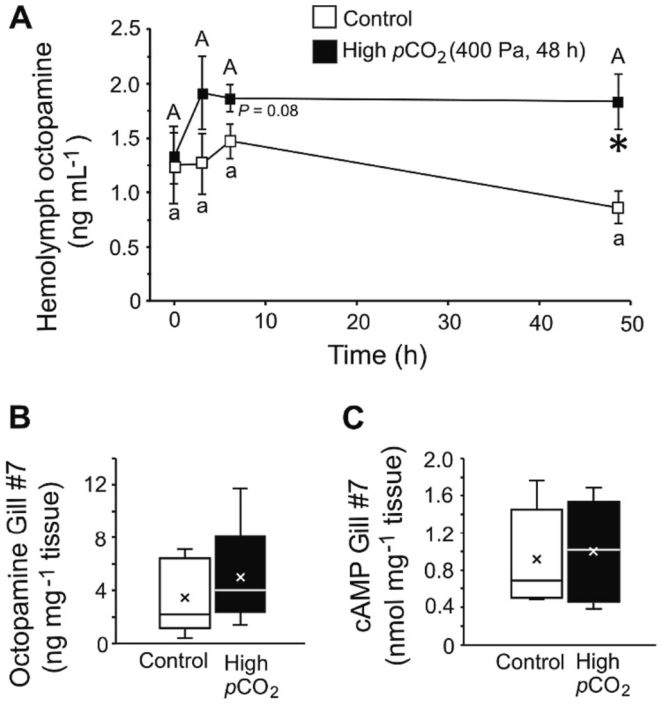

Fig. 3. Endocrine response of green crabs exposed to short-term (48 h) high pCO_2. (A) Circulating levels of octopamine. Open squares represent crabs acclimated to control conditions, black squares represent specimens exposed to high pCO_2 (400 Pa) as means ± SE (N = 6). Lower case letters indicate significant changes over time in control green crabs, whereas upper case letters denote significant changes in high pCO_2 acclimated animals (REML with Tukey's post hoc test, $P < 0.05$). Asterisks denote significant differences between control and high pCO_2 acclimated green crabs at the given time point (Student's t-test, $P < 0.05$). Branchial (B) octopamine levels in posterior gill #7 (in ng mg^{-1} tissue), and (C) cAMP levels in posterior gill #7 (in nmol mg^{-1} tissue). Box and Whisker Plots: The middle line of the box represents the median, whereas the x represents the mean. The top and bottom line indicate the 1st quartile and 3rd quartile with whiskers extending to the minimum and maximum value, respectively. $N = 6$. No significant differences were detected in branchial octopamine and cAMP levels (Student's t-test, $P > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control green crabs.

3.1.3. Metabolic rate

Neither whole-organism metabolic rates (38.9 ± 3.2 nmol g^{-1} min^{-1} vs. 46.4 ± 4.7 nmol g^{-1} min^{-1}; Fig. 4A), nor basic branchial metabolic rate of posterior gill 7 (Fig. 4B) changed after 48 h of hypercapnia (1092.9 ± 53.8 nmol g^{-1} min^{-1} vs. 1082.4 ± 65.7 nmol g^{-1} min^{-1}; Fig. 4B). A significant decrease of >30% in posterior gill metabolic rate in the presence of OCT was observed in short-term high pCO_2 exposed specimen but not in gill 7 of control crabs (Fig. 4B).

3.1.4. Mitochondrial enzymes in the gills

In control crabs, enzyme activities for complexes I + III, complex IV (cytochrome C oxidase) and citrate synthase (as representative for the Krebs cycle) were significantly higher in posterior gills 7 compared to anterior gills 5 (Supp. Fig. 2). Enzyme activities in anterior gill 5 did not change significantly after 48 h of acclimation to high pCO_2 (Fig. 5A-C). In posterior gill 7, only cytochrome C oxidase activity decreased significantly after 48 h of short-term high pCO_2 exposed *C. maenas* compared to control crabs (Fig. 5E), but not Complex I + III (Fig. 5D) or citrate synthase (Fig. 5F).

4. Discussion

4.1. Carbonate system in hemolymph and branchial water

Many decapod crustaceans have been shown to elevate hemolymph bicarbonate (HCO_3^-) levels to offset and buffer a respiratory acidosis in response to elevated environmental pCO_2 (hypercapnia) (Fehsenfeld and Weihrauch, 2013, 2016a, 2017; Hans et al., 2014; Klymasz-Schwarz et al., 2019; Rastrick et al., 2014). Hyperregulating *C. maenas*, for example have been shown to have hemolymph pH completely restored after 7 d (Fehsenfeld and Weihrauch, 2013) and maintain the new acid-base homeostasis for at least 10 weeks without showing much other impairment, i.e., changes in feeding rate and growth (Appelhans et al., 2012).

It is less clear to date at what point exactly hyperregulating decapods start to compensate for the rapidly lowered pH in their environment. Our data implies that brackish-water acclimated green crabs can tolerate the decreased extracellular pH for at least 6 h, if not 12–24 h. Only after this initial period do they start counteracting the persistent respiratory acidosis by accumulation of HCO_3^- and extracellular pH is slowly recovered to control values. Contrastingly, it has been found that fish

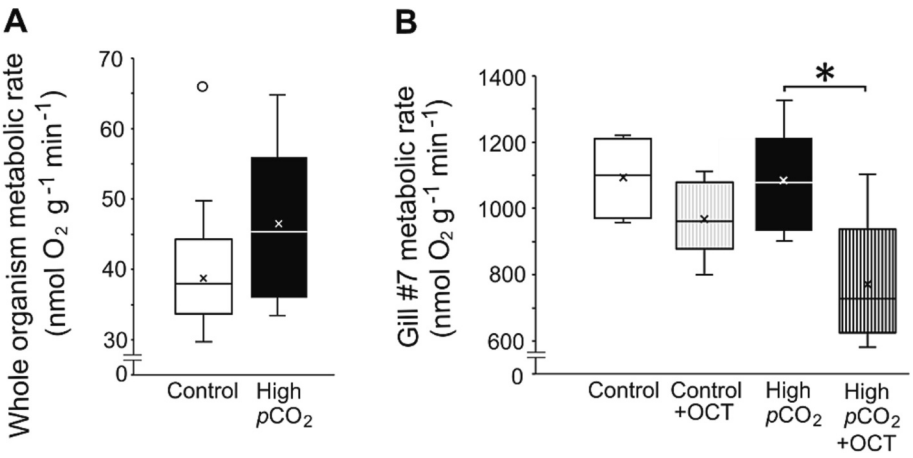

Fig. 4. Whole organism metabolic rates of *C. maenas* in response to short-term high pCO_2 acclimation (400 Pa, 48 h). (A) Whole organism metabolic rate, and (B) branchial metabolic rates of posterior gill #7 in the absence or presence of octopamine (OCT). The middle line of the box represents the median, whereas x represents the mean. The top and bottom line indicate the 1st quartile and 3rd quartile whiskers extending to the minimum and maximum value, respectively. No significant differences were detected in whole-organism metabolic rates. Asterisk indicates significant differences in branchial metabolic rates (Student's t-test, $P < 0.05$, N = 6). The open circle in (A) indicates an outlier that had been removed from the statistical analysis.

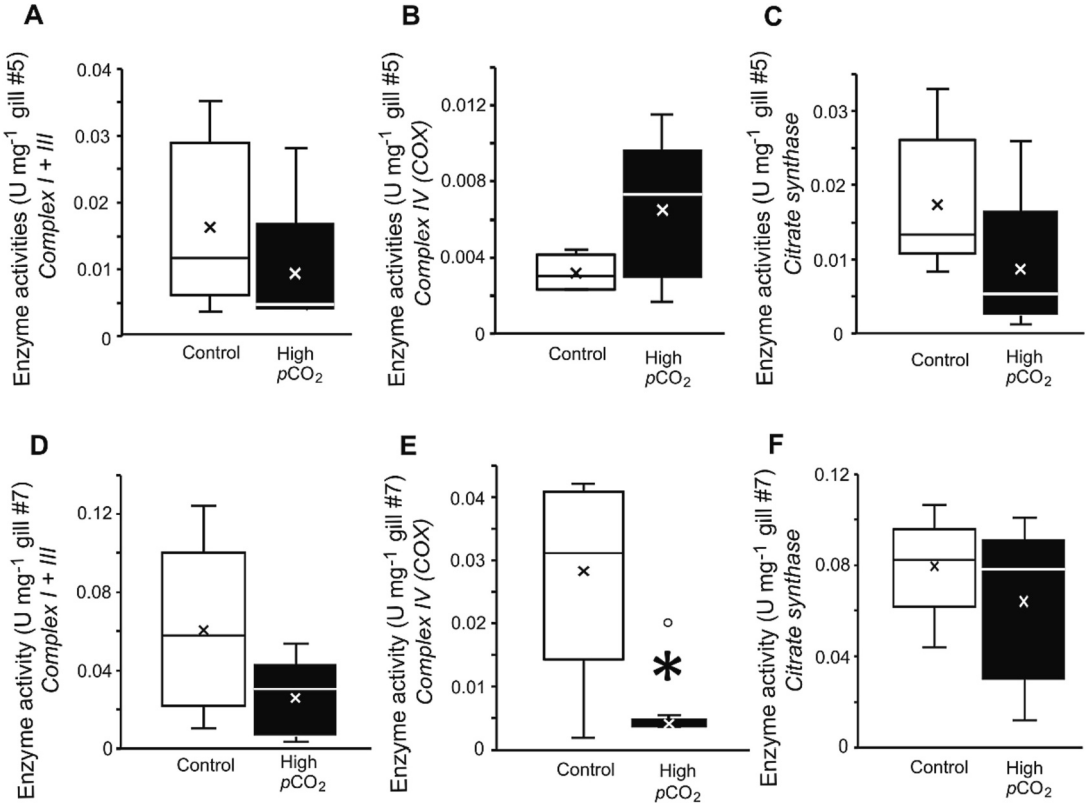

Fig. 5. Mitochondrial enzyme activities in green crab anterior gill #5 (A-C) and posterior gill #7 (D–F) of *Carcinus maenas* exposed to control or short-term high pCO_2 conditions. (A,D) Changes in the electron transport chain of complex I + III, (B,E) changes in cytochrome C oxidase (COX) activity representing complex IV, and (C,F) changes in citrate synthase activity as the first enzyme of the Krebs cycle. White boxes represent control conditions, black boxes represent short-term high pCO_2 conditions. The middle line of the box represents the median, whereas x represents the mean. The top and bottom line indicate the 1st quartile and 3rd quartile with whiskers extending to the minimum and maximum value, respectively. Asterisk denotes significant differences (Student's t-test $P < 0.05$, N = 6). Note that the scale of the y-axis differs between gill #5 and gill #7, resembling the generally lower enzyme activities in gill #5, respectively (see also Supp. Fig. 2). The open circle in (E) indicates an outlier that had been removed from the statistical analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

accumulate HCO_3^- immediately (Claiborne et al., 2002) after a pH disturbance in their blood, as do osmo-conforming green crabs (Fehsenfeld and Weihrauch, 2016a; Truchot, 1975). The observed time frame for hyperregulating green crabs likely correlates to fluctuations they would encounter daily in their environment (Thomsen et al., 2010). Only when hypercapnia persists beyond this time threshold crabs start to counteract their acidosis.

Furthermore, our study shows that branchial water in short-term

high pCO_2 exposed green crabs possesses significantly decreased levels of HCO_3^- after 48 h, while maintaining a stable pH and pCO_2. It should be noted that even these decreased levels of branchial water HCO_3^- in short-term high pCO_2 exposed crabs are still much higher than those found in ambient water (ca. 7 vs. 1.5 mmol L^{-1}). It is hence highly likely that the observed elevated $[HCO_3^-]_{hemo}$ is the consequence of selective retention of this crucial ion by the gill due to decreased ion transport. In fact, mRNA for a Cl^-/ HCO_3^- -anion exchanger (AE) from the SLC family 4 (member 1; [GenBank:CX994129.1]) was significantly down-regulated after 3 d of acclimation to hypercapnia in posterior gills of *C. maenas* (Fehsenfeld et al., 2011), supporting a role for this transporter in HCO_3^- retention. Although the exact location for AE in the green crab gill is currently unknown, it is thought that either a basolateral and/or apical presence would assist Cl^- uptake in hyper-regulating crabs. For example, an apical AE as identified in the crayfish apical hypodermis (Abehsera et al., 2021) would promote HCO_3^- transport from cytoplasm to environment, whereas basolaterally located it would aid in transporting HCO_3^- from the hemolymph /extracellular fluid into the cytoplasm. A decrease in the abundance (and subsequent activity) of this transporter would consequently assist in retaining HCO_3^- in the hemolymph and provide less excretion into the branchial water. This needs to be further investigated in future studies.

4.2. Involvement of OCT in acid-base compensation

Circulating levels of OCT significantly increased only after 48 h of hypercapnia exposure, indicating a relevant role for OCT in acid-base regulation rather than simply as a general stress hormone/factor. For a potential role as a stress hormone, OCT could have been expected to rather be elevated within the initial hours of exposure. The fact that at the same time levels of branchial OCT are not different from control crabs implies that the gill itself does not seem to be a relevant source for OCT. This would resemble findings for the antennal hearts of the locust *Schistocerca gregaria* (Antemann et al., 2017) or the antennae of *M. sexta* (Schendzielorz et al., 2015) in which OCT is rather controlled by the nervous system. However, it should be noted that we might observe branchial OCT levels that are diluted by hemolymph as we did not flush the gills. In the Chinese mitten crab, *Eriocheir sinensis*, for example, branchial OCT was 20-fold higher compared to circulating levels (Pequeux et al., 2002). Here we only measured 3-fold higher levels and hence might not have been able to observe a clear branchial response. On the other hand, OCT acts within milliseconds (Schendzielorz et al., 2015) and is then quickly degraded (Goosey and Candy, 1980a, 1982). Consequently, an accumulation of circulating OCT in the gill might simply not be observed due to the transient nature of the hormone.

Furthermore, in contrast to *M. sexta* (Schendzielorz et al., 2015), our data does not suggest an involvement of the second messenger cAMP in the branchial signalling cascade of OCT. Alternatively, the involvement of α-adrenergic OCT receptors might rather initiate an IP3-Ca^{2+} signalling cascade, as discussed below.

4.3. Metabolic components of short-term hypercapnia acclimation

In contrast to long-term acclimation of osmo-conforming green crabs to hypercapnia especially in an HCO_3^- -depleted environment (Maus et al., 2018), short-term exposure to high pCO_2 of hyperregulating *C. maenas* did not affect whole animal metabolic rates. This is not surprising, as brackish water green crabs must be accustomed to these environmental fluctuations and a whole-body response would seem rather drastic. It should be noted, however, that MO2 as measured in this study was approximately 4-fold higher than the standard metabolic rate (SMR) observed by Maus et al. (2018). This discrepancy (and variability between individuals) may indicate spontaneous activity in the metabolic chambers. While our data is sufficient to provide a relative comparison of metabolic rate between treatments, an automated longer-term measurement of metabolic rate should be considered for future studies, especially with a focus on metabolic scope.

With regards to the branchial metabolic rate, we decided to investigate posterior gills only since (i) enzyme activities remained stable in the anterior gill after exposure to hypercapnia, and (ii) posterior gills are the metabolically more active gills (Compere et al., 1989; Henry et al., 2012). Intriguingly, we observed a significant decrease in branchial metabolic rate in posterior gill 7, but only in short-term high pCO_2 exposed green crabs in the presence of OCT.

Cellular metabolism is performed by the mitochondria and here specifically by the electron transport chain present in the inner mitochondrial membrane (Campbell and Reece, 2008; Kühlbrandt, 2015). It is not surprising that all measured mitochondrial enzyme activities (complex I + III, complex IV (cytochrome C oxidase, COX) and citrate synthetase (CS)) are significantly higher in the metabolically more active posterior gills which are rich in mitochondria (Compere et al., 1989; Henry et al., 2012).

Furthermore, measuring enzyme activities in posterior gill 7 we observed a significant reduction only in COX activity in hypercapnia exposed crabs. COX mediates the final reaction on the electron transport chain, i.e., it passes electrons to oxygen, forming water and pumping H^+ out of the mitochondrial matrix into the intermembrane space. The generated proton gradient is then used by the ATP synthase to produce ATP (Campbell and Reece, 2008; Kühlbrandt, 2015). It has been shown that if COX is inhibited, cellular metabolism decreased in human muscle cells due to a higher demand for molecular oxygen to offset the inhibitory effect (Alonso et al., 2003). The decrease in branchial COX activity and metabolic rate as result of elevated circulating OCT at high pCO_2 would lead to (i) a reduction in protons in the mitochondrial intermembrane space, and/or (ii) a decrease in net mitochondrial CO_2 production, as a decreased COX activity will slow down the whole respiratory transport chain and result in negative feedback for CO_2-producing glycolysis and citric acid cycle. Less protons potentially leaking from the intermembrane space over the porous outer mitochondrial membrane (Kühlbrandt, 2015) into the cytoplasm, and/or less CO_2 entering the cytoplasm from the mitochondrion and being converted to $H^+ + HCO_3^-$, could indeed explain the decrease in H^+ excretion in posterior gills of *C. maenas,* as recently observed by Fehsenfeld et al. (2023). However, it should be noted that this differs from what is known for locust flight muscles where circulating OCT was observed to rather increase cellular metabolic rates in this tissue via glucose metabolism (Goosey and Candy, 1980b).

4.4. Proposed mode of action for OCT in posterior gills of C. maenas

To date only one branchial receptor for OCT has been identified in *C. maenas* gills (Towle and Smith, 2006), which resembles β-adrenergic-like OCT receptors of the family of G-protein receptors (Fehsenfeld; *pers. obs*). This class of receptors solely modulate cAMP levels (Farooqui, 2007) as initially identified in the Jonah crab *Cancer borealis* (*pers. obs*.; Northcutt et al., 2016). While present in both gill clusters in the green crab, anterior gills tend to have a higher mRNA abundance for this identified receptor compared to posterior gills (Fehsenfeld et al., 2023). The fact that no response in mitochondrial enzyme activities was observed in anterior gills might hence indicate the presence of β-adrenergic-like OCT receptor in only anterior gills, and a signalling cascade involving cAMP not being relevant.

While not characterized in crustaceans to date, α-adrenergic-like octopamine receptors as found in insects (Farooqui, 2007; Nakagawa et al., 2022; Sujkowski et al., 2020) have also been predicted to exist in some crab species including the swimming crab, *Portunus trituberculatus* (GenBank acc. no. XM_045283256.1/ XP_045139191.1). In contrast to β-adrenergic receptors, α-adrenergic-like OCT receptors rather activate an inositol triphosphate (IP3) and Ca^{2+} cascade as has been shown for Hawkmoths' antennae (Schendzielorz et al., 2015). The presence of an α-adrenergic-like OCT receptors only in posterior gills could hence explain their observed sensitivity towards OCT after high pCO_2 exposure

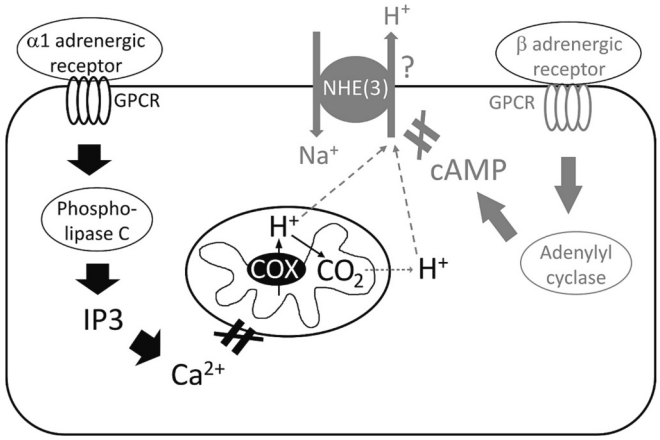

Fig. 6. Proposed model for the action of octopamine in acid-base regulation of posterior gills of *C. maenas*. In response to a respiratory acidosis, octopamine is likely to bind to a yet to be identified α1-adrenergic-like receptor and activate a signalling cascade via phospholipase C (black pathway). Phospholipase-derived IP3 then increases cellular Ca^{2+} that in turn inhibits mitochondrial COX activity, hence failing to translocate H^+ into the transcellular space and ultimately excretion at the apical cell membrane. Octopamine activating β-adrenergic-like receptor, increases in cAMP and inhibition of NHE(3) might still be relevant for anterior gills and/or other physiological processes (grey pathways). cAMP, cyclic adenosine monophosphate; COX, cytochrome C oxidase; GPCR, G-protein coupled receptor; IP3, Inositol trisphosphate; NHE(3), Na^+/H^+-exchanger (type 3).

without an elevation of cAMP. Furthermore, COX has been shown to possess a direct binding site for cations and is inhibited by Ca^{2+} (Vygodina et al., 2017). Consequently, the absence of a response of complex I + III and CS and the sole reduction of COX activity in the gill might indicate a more targeted action of OCT on the last enzyme of the mitochondrial electron transport chain. More specifically, OCT could bind to an α-adrenergic-like OCT receptor initiating the IP3-Ca^{2+} signalling cascade, with elevated Ca^{2+} levels then inhibiting COX activity, leading to reduced net CO_2/H^+ production and consequently reduced apical excretion of H^+ via NHE(3) (see Fig. 6).

5. Conclusion

Our study is the first to investigate the initial hours of hypercapnia exposure in a brackish water acclimated hyperregulating decapod, as well as accounting for endocrine aspects and investigating components of aerobic metabolism. Our work hence represents relevant and complementing results to an existing body of literature on the responses to hypercapnia in crustaceans in general, and a major invasive marine species of global concern in particular.

In response to short-term hypercapnia, *C. maenas* starts to counteract a resulting respiratory acidosis as early as 6 h of exposure. Full pH compensation in the hemolymph occurs after 48 h and it is likely accomplished by retention of HCO_3^- by the (posterior) gills. This pattern differs from seawater-acclimated green crabs that take longer to fully restore hemolymph pH even though starting to accumulate HCO_3^- earlier. Furthermore, the presence of OCT seemed to be correlated with reduced branchial COX activity as well as reduced gill tissue respiration at high pCO_2. Hence, OCT may play an important role in regulating the branchial mitochondrial function of *C. maenas* in a brackish-water environment.

Further research will be necessary to clarify the exact branchial metabolic and endocrine implications and mechanisms with regards to acid-base regulation and responses to hypercapnia. In particular, it will be of interest to better characterize presently known OCT receptors, identify alternative ones, and to elucidate their respective signalling cascades in *C. maenas* gills.

Author contributions

SF, SB, PC and DW conceived of the study. SB (enzymes) and DW (acclimation) provided the infrastructure, experimental installations, and equipment for all experiments, while SB, PC and DW provided consumables and reagents. SB and PC provided financial support to HKT during summer 2022. SF (acclimation, hormones), GRY (whole animal metabolic rate), ARQR (gill metabolic rates), and HKT (enzyme assays) conducted the respective experiments, collected the samples, and analyzed the data. SF generated the graphs and wrote the initial manuscript. All authors contributed to the final version of this MS.

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CRediT authorship contribution statement

Sandra Fehsenfeld: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Gwangseok R. Yoon:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Alex R. Quijada-Rodriguez:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Haluka Kandachi-Toujas:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Piero Calosi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Sophie Breton:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Dirk Weihrauch:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

Data availability

The data that support the findings of this study are openly available in FigShare at https://doi.org/10.6084/m9.figshare.24036591.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111603.

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