A role for octopamine and crustacean hyperglycemic hormone (CHH) in branchial acid-base regulation in the European green crab, *Carcinus maenas*

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Running title: Endocrine control of crustacean gills' acid-base regulation

Abstract

Crustaceans' endocrinology is a vastly understudied area of research. The major focus of the studies on this topic to date has been related to the molting cycle and in particular, the role of crustacean hyperglycemic hormone (CHH), as well as the role of other hormones in facilitating physiological phenotypic adjustments to salinity changes. Additionally, while many recent studies have been conducted on the acclimation and adaptation capacity of crustaceans to a changing environment, only few have investigated internal hormonal balance especially with respect to an endocrine response to environmental change. The current study hence aimed to identify and characterize endocrine components of acid-base regulation in the European green crab, *Carcinus maenas*. We show that both the biogenic amine octopamine (OCT) as well as CHH are regulatory components of branchial acid-base regulation. While OCT suppresses branchial proton excretion, CHH seemed to promote it. Both hormones were also capable of enhancing branchial ammonia excretion. Furthermore, mRNA abundance for branchial receptors (OCT-R), or G-protein receptor activated soluble guanylate cyclase (sGC1b), are affected by environmental change such as elevated pCO_2 (hypercapnia) and high environmental ammonia (HEA). Our findings support a role for both OCT and CHH in the general maintenance of steady-state acid-base maintenance in the gill, as well as regulating the acid-base response to environmental challenges that *C. maenas* encounters on a regular basis in the habitats it dwells in and more so in the future ocean.

Keywords: Endocrine control, carbonate parameters, ocean acidification, ammonia, neurotransmitter, neuropeptide, gill

Introduction

Pioneering work in the early 20th century identified the X-organ/sinus gland to be a major endocrine organ of the eyestalks (Zeleny 1905; Hanström 1935) of crustaceans. The following discovery that crustaceans' pigmentation was also under endocrine control (Koller 1927; Perkins 1928) validated crustaceans as relevant models for invertebrate endocrinology early on. Fast forward a few decades, and several important physiological processes in decapods have been looked at under an "endocrinological lens" (Fehsenfeld 2023). These include first and foremost molting as the detrimental process for growth and reproduction, and the involvement of crustacean hyperglycemic hormone (CHH) and other members of the CHH superfamily (Webster et al. 2012). CHH – arguably one of the most widely studied crustacean hormones – is also heavily involved in decapods' glucose/carbohydrate metabolism (Abramowitz et al. 1944) and water balance (Chung et al. 1999). Furthermore, more recent studies have indicated a role for CHH, as well as biogenic amines like dopamine, serotonin and norepinephrine, in (branchial) osmoregulation (Sommer and Mantel 1988; Spanings-Pierrot et al. 2000; Chung and Webster 2006; Pequeux et al. 2012; Webster et al. 2012). Our knowledge of how endocrine aspects are involved in responses to environmental change, however, is still rather limited – especially when it comes to acid-base regulation.

Many aquatic crustaceans, like the European green crab *Carcinus maenas* (Linnaeus 1758), live in estuaries and the intertidal zone, and experience highly variable environments not only in salinity, temperature, and pO_2, but also in pH and pCO_2 (Thomsen et al. 2010). Furthermore, most crustacean adults live a benthic lifestyle and (especially during molting) hide for hours at a time in burrows that can quickly accumulate waste products like ammonia (NH_3/NH_4^+) and CO_2, creating unfavorable conditions (Bellwood 2002; McGaw 2004). Consequently, physiological homeostasis in the adults of this species is constantly challenged. As a major component of physiological homeostasis, a constant pH in the blood/hemolymph, tissues and cells is indispensable in all living organisms for the proper function of proteins and enzymes (Fehsenfeld and Weihrauch 2017). On top of that, the ongoing anthropogenically induced rise in atmospheric temperature and CO_2 results in elevated ocean temperature, pCO_2 (hypercapnia), and a drop of ocean pH (ocean acidification). Consequently, possessing an efficient acid-base regulatory ability will become increasingly important for aquatic animals to respond to their changing environment, and a factor that will shape biodiversity in a future ocean (Melzner et al. 2009; Whiteley 2011; Calosi et al. 2013).

Decapod acid-base regulation involves multiple components, including ammonia excretion, ion exchange, and CO_2 transport (Fehsenfeld and Weihrauch 2017; Weihrauch et al. 2017). CO_2 itself is in equilibrium with H^+ and HCO_3- as the major physiological acid-base buffering system, the carbonate system (CO_2 + H_2O) ←→ $H^+ + HCO_3^-$). As such, H^+ and HCO_3^- on the other hand then feed (epithelial) transporters like

Na^+/H^+-exchanger, Na^+/HCO_3^--cotransporter and Cl^-/HCO_3^--exchanger and hence link acid-base with osmoregulation. As a third component, ammonia also exists in an acid-base equilibrium ($NH_3 + H^+ \leftrightarrow NH_4^+$) and can be used as an additional acid-base buffering component in the hemolymph (Fehsenfeld and Weihrauch 2013). As one of the major regulatory organs in decapods, the gills accomplish most of the osmoregulatory and acid-base related ion movements, as well as ammonia excretion. *C. maenas* for example possesses 9 paired gills that can be divided into two different clusters, the anterior (gill 1-6) and posterior gills (gills 7-9)(Compere et al. 1989). While the posterior gills are mainly responsible for osmoregulation indicated by a much higher abundance of the epithelial transporter Na^+/K^+-ATPase (Compere et al. 1989; Onken and Siebers 1992; Riestenpatt et al. 1996), acid-base regulation and ammonia excretion seem to be accomplished more universally by both anterior and posterior gills (Fehsenfeld and Weihrauch 2013, 2016a).

Our current understanding on how crabs detect and evaluate acid-base-disrupting changes in their environment, however, is still very limited. Furthermore, little is known on how external changes are communicated internally to initiate the appropriate compensatory physiological response. A respective adjustment of acid-base homeostasis likely involves the endocrine system, as has been suggested for decapods with respect to hypoxia, emersion, and changes in temperature and salinity (Fehsenfeld 2023). While numerous candidate hormones, hormone receptors, and acid-base-sensors have been identified for mammals and teleost fish in this context (*i.e.*, soluble adenylate cyclase in dogfish (Tresguerres et al. 2010)), next to nothing is known about endocrine control of acid-base balance in (aquatic) invertebrates, which represent the vast majority of animal diversity (http://www.scienceclarified.com/He-In/Invertebrates.html).

As mentioned above, the neurohormone CHH has been a major focus in crustacean endocrinology, especially with regards to the molting process (Webster et al. 2012). In fact, CHH in *C. maenas* was the first to be fully sequenced (Kegel et al. 1989). In addition to external acid-base disturbances, molting itself poses an internal acid-base challenge as it initially mobilizes and then re-incorporates HCO_3^- (a major part of the carbonate equilibrium $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$, as mentioned above) into the new exoskeleton. CHH seems therefore a likely candidate to be involved also in general acid-base regulation, despite that it was shown to be involved in many other physiological processes (Fehsenfeld 2023). However, it has not yet been investigated directly with regards to acid-base regulation.

Considered the stress hormone of invertebrates (*i.e.*, fight/flight/freeze response), the biogenic amine octopamine (OCT) has a very similar chemical structure to norepinephrine in vertebrates (Roeder 1999). Its physiological role, however, is restricted to invertebrates and includes agonistic behavior and aggression (Sneddon et al. 2000a), as well as generating circadian rhythms (Schendzielorz et al. 2012, 2015). As a neurohormone/neuromodulator/neurotransmitter, OCT is suggested to modulate almost every

peripheral organ, sense organ and numerous targets within the central nervous system (CNS) (Roeder 1999). To the author's knowledge, however, no study in decapods has so far looked at the involvement of OCT in responses to environmental change and/or disruption of internal homeostasis. Two other neurotransmitters/biogenic amines, namely dopamine and serotonin, have been identified to be involved in the response to salinity changes in decapod crustaceans (Zatta 1987; Sommer and Mantel 1988) and are thought to be involved in regulating e.g., the Na^+/K^+-ATPase activity in the Chinese mitten crab, *Eriocheir sinensis* (H. Milne Edwards,1853) (Trausch et al. 1989; Mo et al. 1998). It is therefore likely that OCT also plays a role in restoring internal homeostasis upon external acid-base challenges.

The present study aimed to elucidate the endocrine control of acid-base regulation in *C. maenas*, using the neurotransmitter/biogenic amine OCT and the neurohormone CHH. More specifically, we hypothesized that OCT, as well as CHH play a role in branchial acid-base regulation. We performed electrophysiological Ussing-chamber experiments on split gill lamellae to determine the concentration in which regular major ion transport processes (*i.e.*, NaCl uptake *via* $Na^+/K^+/2Cl^-$-cotransporter) would be unaffected by the respective hormone to single out effects on osmoregulatory-independent acid-base components only. Concentrations of both hormones that did not affect the short-circuit current were then applied in gill perfusion experiments to determine their involvement in branchial transport of acid-base equivalents (*i.e.*, H^+, pCO_2, HCO_3^-, NH_3/NH_4^+). Lastly, qPCR was performed to evaluate changes of mRNA abundance of hormone-specific receptors in response to external acid-base disturbances.

Material and methods

Specimens' collection, transport and maintenance

Adult intermolt male European green crabs, *Carcinus maenas*, were caught in Northern Placentia Bay (NF, Canada) and transported by plane on ice to the Animal Holding Facility at the University of Manitoba (Winnipeg, Manitoba, Canada). Specimens were kept in 1200 L tanks with recirculating artificial sea water (Fritz Reef Pro Mix (RPM), Fritz Aquatics, Mesquite, TX, USA) at a salinity of 32, pH 8.1, 16 °C, and a light:dark cycle of 12:12 h for up to one year. Shelter was provided by plastic structures made of cuts of a PVC pipes. Crabs were fed *ad libitum* with frozen bay scallops twice a week, and excessive food was removed to maintain water quality. For Ussing-chamber and gill perfusion experiments, green crabs were transferred to brackish water in re-circling 1200 L tanks with artificial seawater (Fritz Reef Pro Mix (RPM), Fritz Aquatics, Mesquite, TX, USA) at a salinity of 11, pH 7.9, 16 °C, and a light:dark circle of 12:12 h.

Green crabs were starved for at least 2 days before Ussing-chamber and gill perfusion experiments, as well as during the different exposures to environmental changes, to avoid effects of alternating

hemolymph ammonia levels. For all environmental exposure experiments, six crabs were transferred to individual 200 L tanks enriched with stones and shelter as mentioned above. Water conditions were monitored daily and mean of the environmental parameters are reported in Table 1.

Electrophysiological experiments – split gill

In order to investigate the effect of OCT and CHH on the branchial short circuit current I_{SC} as a measure of ion fluxes, Ussing chamber experiments were performed at room temperature with the EM-CSYS-6 Ussing chamber system (Physiologic Instruments, San Diego, CA, USA) with the automatic clamping device (VCC 600, Physiologic Instruments) and specific sliders (P2308) that provided a circular aperture with an open epithelial surface area of 8×10^{-3} cm^2. Tissue preparations were obtained by splitting single filaments of posterior gill 7 according to Schwarz and Graszynski (1989). Silicon grease was used to seal the edges of the sliders to prevent potential edge damage. Up to six preparations were investigated in parallel in one experimental run and were aerated with ambient air throughout. To account for active ion transport (or the lack thereof for singled-out acid-base components), symmetrical conditions were achieved by adding identical salines to both the apical and basolateral side of the tissue. Based on Riestenpatt et al. (1996) the saline contained (in mmol L^{-1}): 248 NaCl, 5 $CaCl_2$, 4 $MgCl_2$, 5 KCl, 2 $NaHCO_3$, 5 HEPES and 2 glucose (pH 7.9). Two pairs of Ag/AgCl electrodes for measuring trans-epithelial potential difference (PD_{te}) and short circuit current (I_{SC}) were connected to the preparations using 3% agarose bridges in 3 M KCl. Electrodes were calibrated according to the device instructions. I_{SC} was directly recorded simultaneously for every channel using Acquire Analyze software (version 2.2). The dose-response curves for OCT and CHH on the I_{SC} were obtained by stepwise increasing of the concentration of the hormone on the basolateral side after the former equilibration had levelled off (ca. 10-15 min) and stayed stable for >1 minute. To account for time-dependent decay on the I_{SC}, one split gill lamella per experimental run was kept under control conditions during the full duration of the experiment. Consequently, it was determined that the I_{SC} under control conditions decreased by <5% over a time span of 2 h.

Perfusion experiments – isolated gill

Posterior gills 7 were perfused according to the procedure described in Fehsenfeld and Weihrauch (2013), following the technique of (Siebers et al. 1985). Each perfusion consisted of an initial 30 min control step, followed by the application of the respective hormone (wash-in for 10 min, then collection for 30 min), and a third wash-out step (10 min adjustment + 30 min collection) to ensure the performance/survival of the gill tissue. Hormones were applied on the basolateral side (*i.e.*, perfusion saline) of the gill by addition

to the perfusion solution either in physiological concentrations based on the literature (OCT: 6 pmol mL^{-1} (Sneddon et al. 2000b); CHH: 0.05 pmol mL^{-1} (Chung and Webster 2005)), or singled-out acid-base concentration by Ussing chamber experiments as described above (OCT: 10^{-11} mL^{-1}, CHH: 10^{-6} mL^{-1}). The perfusion solution consisted of in mmol L^{-1} (after Fehsenfeld and Weihrauch (2013)): 260 NaCl, 8 KCl, 7 $NaHCO_3$, 7 $MgCl_2$, 5 $CaCl_2$, 0.5 Glutamine, 0.3 Glucose, 0.1 Glutathione, 0.1 NH_4Cl, pH 7.9. The bathing solution was ambient sea water from the crabs' tanks. To ensure the gills' performance over time, one gill per each round of perfusions was included to which no hormones were applied in the second step (see Supplemental Figure 1).

Perfusates were analyzed for carbonate system parameters immediately after collection starting with the determination of total CO_2, followed by measurement of the pH. Samples were then frozen at -80 °C until determination of ammonia content. To assess total CO_2, 10 µL sample was injected into a custom-made glass chamber connected to the LI-850 infra-red CO_2/ H_2O gas analyzer (LI-COR, Lincoln, NE, USA) equilibrated with $NaHCO_3^-$ standards as described by Allen et al. (2021). pH (NBS scale) was measured with the InLab Micro Combination pH electrode (Mettler-Toledo, Greisensee, Switzerland) calibrated to NIST standards (Thermofisher Orion) and connected to the pH-ISE meter model 225 (Denver Instruments, Gottingen, Germany). pCO_2 and HCO_3^- were then calculated using the Henderson-Hasselbalch equation (Fehsenfeld and Weihrauch 2013) and respective constants as determined by Truchot (1976). Total ammonia (NH_3 + NH_4^+) in the perfusates were determined colorimetrically by the sodium salicylate-hypochlorite method, as described by Verdouw Verdouw et al. (1978).

Quantitative PCR

The sequence for octopamine receptor (OCT-R) was obtained from the EST database generated by Towle and colleagues (Towle and Smith 2006; Towle et al. 2011). The sequence for soluble guanylate cyclase (sGC1b) was obtained from the study of Abuhagr et al. (2014). Primers for OCT-R and sGC1b were generated using Primer3 software (https://primer3.ut.ee/) as follows: OCT-R forward primer: 5'GAGGTGTCTGTGGGGACAAT / reverse primer: 5' GGCTAAGGACACCACGAAGT (147 bp amplicon); sCG1b forward primer: 5'GCAAATCACCATCGGCATCC / reverse primer: 5'AAGGGACGAGTCAACGTGTC (144 bp amplicon). Fragments were sequenced at The Centre for Applied Genomics (Toronto, ON, CA) and blasted against the NCBI database to ensure the right amplicon. cDNA for tissues and gills in response to high environmental pCO_2 (7 d) was used from the study by Fehsenfeld and Weihrauch (2013). cDNA for gills in response to short-term HEA was obtained from the recent study by Fehsenfeld et al. (2023). Accordingly, for the long-term HEA experiments, RNA was isolated using TRIZOL while RNA from short-term high pCO_2 was isolated using the Monarch®

Total RNA Miniprep Kit (New England Biolabs, Whitby, ON, Canada). RNA was checked for DNA contamination by regular PCR (*i.e.*, absence of signal) and DNA-free RNA was reverse transcribed using the LunaScript® RT SuperMix Kit (New England Biolabs, Ontario, Canada). Quantification of cDNA in the quantitative PCR (qPCR) was based on a 1:3 dilution series of pooled experimental cDNA (*i.e.*, 5 µL was taken out of every 20 µL final cDNA sample; range of standards = 1:1 – 1:81). qPCR was performed in a total volume of 15 µL including 7.5 µL 2x concentrated Luna® Universal qPCR Master Mix (New England Biolabs, Ontario, Canada), 2µL of cDNA (1:4 diluted relative to first standard), and 0.5 µL of each 20 µmol L^{-1} primer, and run at an annealing temperature of 58 ºC for 30 s for 40 cycles (Miniopticon, Biorad, Mississauga, ON, Canada). A subsequent melt curve was performed to ensure specificity of the gene product. Primer efficiencies were 99.8% for OCT-R and 100.5% for sGC1b, respectively.

Reference genes were identified by RefFinder (http://blooge.cn/RefFinder/) as the geometric mean of arginine kinase (forward primer: 5'- CGCTGAGTCTAAGAAGGGATT, reverse primer: 5'-CCCAGGCTAGTCTTCTTGGCC; efficiency: 102.9%; annealing temperature: 58 ºC) and ribosomal gene 3 (Rbs3; forward primer: 5'-GTCCCTTTTCACCAAGGACA / reverse primer: 5'-CAAGGCCAAACTCAACAGGTT; efficiency: 100.8%; annealing temperature: 58 ºC).

Statistical analysis

All data were tested for normal distribution (Shapiro-Wilks Test) and 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). If any, no more than one outlier was identified per data set and consequently removed from the analysis. Dose-response curves (Ussing-chamber experiments) and repeated measures ANOVAs (gill perfusion experiments) were generated using GraphPad Prism 9 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). mRNA abundance data (ANOVA for tissue expression, Student's t-test for environmental exposures) were analyzed with PAST3. All results were considered significant with $\alpha < 0.05\%$ ($P < 0.05$). Graphs were generated with Inkscape 1.2.2. (https://inkscape.org/).

Results

Effects of octopamine and CHH on branchial short-circuit current

Under pCO_2 control conditions, split posterior gill 7 lamellae of brackish water acclimated green crabs exhibited an average transepithelial potential difference of *ca*. 5.0 mV: 5.2 ± 0.3 mV in octopamine

(OCT) experiments, 4.8 ± 0.3mV in crustacean hyperglycemic hormone (CHH) experiments. When voltage clamped, this corresponded with an average short-circuit current (I_{SC}) of -312.5 mA cm^{-2} (-305.4 ± 26.4 mA cm^{-2} in OCT, -319.8 ± 30.3 mA cm^{-2} in CHH experiments).

Both the application of OCT (> 10^{-4} pmol L^{-1}) and CHH (> 10^{-1} pmol L^{-1}) resulted in a decrease of the short circuit current (I_{SC}) of 15 and 25 %, respectively (Fig. 1). Already a very low concentration of OCT was effective (EC50 = $7.1*10^{-10}$ pmol mL^{-1}; Fig. 1A), whereas the EC50 of CHH was much higher by a factor 10^6 (EC50 = $1.1*10^{-4}$ pmol mL^{-1}; Fig.1B). A concentration below the EC50 was considered to not impact NaCl (Cl^-) flux (Riestenpatt et al. 1996) but still potentially relevant for the transport of acid-base components (OCT = 10^{-11} pmol mL^{-1}, CHH = 10^{-6} pmol mL^{-1}, Fig. 3).

In both cases, physiological/circulating concentrations for the hormones as found in the literature (Sneddon et al. 2000a; Chung and Webster 2005) were toward the high end of the investigated range (indicated by brackets; Fig. 1).

Effects octopamine and CHH on branchial acid-base regulation

Posterior gills 7 in control runs without the application of hormones exhibited stable fluxes for acid-base equivalents throughout the duration of perfusion experiments (Supp. Fig. 1). The application of physiological concentrations of OCT (6 pmol mL^{-1} (Sneddon et al. 2000b)) significantly reduced the capability for proton excretion over the branchial epithelium as demonstrated by a lower ΔpH in the perfusate (*i.e.,* the perfusate had a lower decrease in pH after one gill passage compared to the perfusion solution when OCT was applied; Fig. 2A). This effect lasted throughout the experiment and was not reversed in the wash out step. Contrastingly, the application of physiological concentrations of CHH (0.05 pmol mL^{-1} (Chung and Webster 2005)) led to a reversible increase in branchial proton excretion that was reversed in the wash step (Fig. 2A'). Both hormones when applied in physiological concentrations did not affect the excretion of CO_2 or HCO_3^- (Fig. 2B,B',C,C').

Ammonia excretion was increased both with the application of physiological concentrations of OCT (Fig. 2D) as well as CHH (Fig. 2D'): this was indicated by a lower remaining ammonia concentration in the perfusate after gill passage. This effect was reversed in the wash out step in both cases (Fig. 2D,D').

Based on the findings in Ussing-chamber experiments (Fig. 2), a concentration of 10^{-11} pmol mL^{-1} (OCT) and 10^{-6} pmol mL^{-1} (CHH) were chosen to apply in whole gill perfusion experiments as a concentration that could single out effects on branchial acid-base regulation without affecting osmoregulatory NaCl movements (Riestenpatt et al. 1996) (Fig. 3). When concentrations of OCT and CHH were reduced to those low levels, OCT still resulted in a significant decrease in branchial proton excretion which this time was reversible (Fig. 3A), and a delayed increase (only in the wash step) of ammonia excretion (Fig. 2D).

CHH on the other hand resulted in a delayed decrease of proton excretion (Fig. 3A'), but still an immediate increase of ammonia excretion (Fig. 3D'). Interestingly this time, not OCT (Fig. 3B) but CHH also significantly elevated CO_2 excretion (higher negative pCO_2 in the perfusate *vs*. the perfusion solution) in the wash out step (Fig. 3B'), whereas HCO_3^- excretion remained unaltered with either hormone (Figs. 3C, C').

mRNA tissue distribution and abundance of hormone receptors

The octopamine receptor (OCT-R) as first identified by Towle and colleagues (Towle and Smith 2006; Towle et al. 2011)was present in all investigated tissues but showed highest mRNA abundance in anterior and posterior gills, followed by cerebral ganglion, heart and hypodermis. Only low mRNA abundance of OCT-R was detected in antennal gland and muscle (Fig. 4A).

The receptor for CHH in the hepatopancreas of *E. sinensis*, CHHBP (Li et al. 2016), could not be found in the *C. maenas* transcriptome (Verbruggen et al. 2015) or EST library (Towle and Smith 2006; Towle et al. 2011) applying blastn search (BLAST+ executables V2.7.1, https://www.ncbi.nlm.nih.gov/books/NBK131777/). Furthermore, using regular PCR and gene specific primers we were unable to reliably amplify either membrane guanylyl cyclase (GC) or soluble NO-insensitive GC known to be involved in MIH and/or CHH signalling in the Y-organ of *C. maenas* (Abuhagr et al. 2014) from gill tissue in efforts of this study.

Alternatively, soluble guanylate cyclase (sGC-1b) as a known cGMP-producing enzyme involved in the signaling pathway of G-protein coupled receptors like CHH (Abuhagr et al. 2014) was considered a promising candidate and hence investigated. mRNA abundance for sGC-1b was highest in the cerebral ganglion followed by the hypodermis. Moderate levels of sCG-1b mRNA were identified in heart, antennal gland and both anterior and posterior gill, while it was barely detectable in muscle (Fig. 4B).

Both OCT-R and sCG1b responded with a change in mRNA abundance when exposed to environmental change in a different pattern (Fig. 5). OCT-R mRNA abundance significantly increased in posterior gill 7 only upon exposure to long-term elevated pCO_2 (7 d of 400 Pa hypercapnia) (Fig. B'). Contrastingly, sGC-1b mRNA abundance significantly decreased by ca. 80% upon both long-term elevated pCO_2 (Fig. 5B'), but also in response to short-term exposure to high environmental ammonia (HEA, 24h 1 mmol L^{-1}) (Fig. 5A).

Discussion

To our knowledge, the current study is the first to investigate endocrine control of acid-base regulation in a decapod. Here we show that the biogenic amine octopamine can alter acid-base equivalent transport

over the gill epithelium. On the other hand, while the neuropeptide crustacean hyperglycemic hormone (CHH) has been linked to physiological responses to changes in salinity and temperature (Chung and Webster 2005, 2006)), it had not been looked at under an acid-base angle. Our gill perfusion experiments validated that CHH is also involved in branchial acid-base regulation.

Octopamine (OCT)

To date the role of OCT in invertebrates has mainly been investigated within a behavioral context (reviewed by Roeder 1999), including agonistic behavior in *C. maenas* (Sneddon et al. 2000b) and *Chasmagnathus granulatus* (Pedetta et al. 2010), and circadian rhythms in cockroaches (Schendzielorz et al. 2012) and the hawkmoth *Manduca sexta* (Schendzielorz et al. 2015)). However, Kamemoto (1991) indicated a broader physiological role for this biogenic amine based on its capability to increase branchial Na^+ flux in blue crabs *Callinectes sapidus*. Pequeux et al. (2012) also connected OCT to salinity acclimation in Chinese mitten crabs. In this study, our results indicate an additional role for OCT in crustacean acid-base regulation.

Branchial transport of acid-base components

Besides its involvement in regulating acid-base components, Ussing chamber experiments implicated a role for OCT in general ion-regulation (*i.e.*, Na^+ and/or Cl^-). Biogenic amines including OCT have been suggested to influence the activity of the Na^+/K^+-ATPase (NKA) *via* cAMP and proteinase K- mediated phosphorylation in green crabs (Sommer and Mantel 1991; Lucu and Flik 1999). While Sommer and Mantel (1991) reported an increase of branchial cAMP in brackish water acclimated *C. maenas*, Lucu and Flik (1999) discovered that branchial cAMP levels are negatively correlated to NKA-activity. NKA itself is the major transporter in *C. maenas* to generate a negative membrane potential and drives the uptake of Na^+ and Cl^- in hyperregulating green crabs *via* apical $Na^+/K^+/2Cl^-$-cotransporter and basolateral Cl^- channels as suggested by Riestenpatt et al. (1996). Our observations here showed that OCT (when applied in physiological concentrations) led to a decreased Cl^- and/or Na^+ flux, This could then be explained by the hormone induces branchial [cAMP], subsequently reducing NKA activity, resulting in less apical Cl^--uptake *via* $Na^+/K^+/2Cl^-$-cotransporter and Cl^--channels.

Interestingly, (Dolzer et al. 2001) observed in tobacco hornworm, *M. sexta*, that OCT decreased the transepithelial potential (TEP) of sensillar accessory cells – similar to what the current study showed here in Ussing chamber experiments as implied by the decrease in the short-circuit current. TEP in *M. sexta*, however, is generated by the activity of V-type H^+-ATPase and so the authors concluded that OCT influenced (*i.e.*, decreased) V-type H^+-ATPase activity, possibly also *via* the second messenger cAMP as

observed in *C. sapidus* (Kamemoto 1991) and *E. sinensis* (Onken et al. 2000). While the major transporter in *C. maenas* to generate TEP is NKA rather than V-type H^+-ATPase (cite my paper: The Journal of Experimental Biology 204, 25–37 (2001) it shows that bafilomycine has no effect on PDTe) , our gill perfusion experiments showed that OCT indeed diminished H^+-flux over the branchial epithelium. Accordingly, OCT targeting V-type H^+-ATPase also in *C. maenas* could indeed explain the here observed decrease in H^+-excretion.

Furthermore, application of OCT in the gill perfusion experiments resulted in an increased branchial ammonia excretion, yet another physiological process linked to acid-base regulation in green crabs (Fehsenfeld and Weihrauch 2013, 2016a, b, 2017). The fact that H^+ flux decreased while ammonia excretion increased implies that the proposed mechanisms of vesicular ammonia trapping including a vesicle-associated V-type H^+-ATPase ($NH_3^+ + H^+ \rightarrow NH_4^+$) (Weihrauch et al. 2001, 2004) might not be targeted by OCT. Alternatively, solely membrane-bound V-type H^+-ATPase might be affected. Interestingly, V-type H^+-ATPase was shown to relocate to the basolateral membrane in response to hypercapnia in dogfish gills (Tresguerres et al. 2007). While a basolateral V-type H^+-ATPase and/or its relocation has not been investigated in decapods, this could possibly also explain the decrease in H^+ flux. The fact that the lower concentrations of OCT resulted in a similar (though in some cases delayed) effect as the higher, physiologically relevant concentrations, strengthens the fact that OCT targets acid-base relevant processes independent from general osmoregulatory processes. This again indicates the involvement of V-type H^+-ATPase but could also include Na^+/H^+-exchanger, Na^+/HCO_3^--cotransporter and/or HCO_3^-/Cl^--exchanger which are all known to be present in posterior gills of *C. maenas* (Fehsenfeld and Weihrauch 2013). While these transporters would be directly linked to Na^+ and Cl^- transport, they likely do not much if at all contribute to generating I_{SC} as do NKA and $Na^+/K^+/2Cl^-$ cotransporter. Consequently, especially the activity of a potential basolateral Na^+-H^+-exchanger (NHE) (Fehsenfeld and Weihrauch 2013) might not be reflected in changes of the I_{SC} and could still be targeted by low concentrations of OCT. In case of an inwardly directed flux for Na^+ as suggested by Fehsenfeld and Weihrauch (2013) this would mean that OCT increases NHE activity so that H^+ is rather maintained in the hemolymph. As we did not observe changes in HCO_3^-, it is less likely for Na^+/HCO_3^--cotransporter and/or HCO_3^-/Cl^--exchanger to be affected by OCT. Further experiments would be needed to elucidate the exact mechanisms.

Branchial receptor and physiological relevance

Except for antennal gland and muscle, the mRNA for the OCT receptor was ubiquitously abundant in all investigated transcripts, indicating a broad and universal physiological role for this hormone. Towle et al. (2011) were the first to annotate OCT receptor in their databank of expressed sequence tags (EST)(Towle

and Smith 2006). When acclimated to low salinity, the receptor mRNA abundance immediately increased 2-fold in brackish water *vs*. seawater acclimated green crabs (Towle et al. 2011) supporting a role for the biogenic amine in osmoregulation as described above. After this initial response, however, the mRNA abundance stayed constant over a period of 48 h. This might explain the absence of any observable change in receptor abundance in the present study after 48 h of high pCO_2 exposure, as an adjustment might have occurred earlier. Interestingly, however, mRNA abundance is increased after 7 d of acclimation to hypercapnia, indicating a genetic/molecular adjustment rather after long-term exposure to the stressor. In general, increasing a hormone's effectiveness can be accomplished in two different ways: either altering hormones levels, or altering receptor abundance. In the case of hypercapnia-acclimation of green crabs, the specimen seemed to rather increase circulating level of OCT in the short-term (48 h, Fehsenfeld, pers. communication) so there would be no need to alter receptor abundance. Maintaining high levels of OCT, however, might not be sustainable given the universal role it seems to play. Consequently, the receptor abundance can be adjusted specifically in the target organ, here the posterior gill, to elucidate a more specific response tailored to the need in the longer run. It must be kept in mind, however, that mRNA abundance might not necessarily reflect protein abundance. For future experiments it would therefore be highly desirable to generate an antibody for the OCT receptor to validate the findings of the present study.

While OCT increased branchial ammonia in gill perfusion experiments, interestingly no change in receptor mRNA abundance was observed in the response to either short- or long-term acclimation to high environmental ammonia. As described above, branchial ammonia excretion in green crabs is linked to the actions of a (vesicular) V-H^+-ATPase and protons (Weihrauch et al. 2001, 2004, 2017). The absence of a response upon HEA acclimation on the molecular level hence strengthens the fact that OCT is rather involved in acid-base regulation. Effects on ammonia excretion as observed in the gill perfusions might consequently be only a secondary effect of the availability of protons and/or activity of V-H^+-ATPase.

Crustacean hyperglycemic hormone (CHH)

In comparison to OCT, CHH has already been the focus of multiple physiological studies in green crabs in an environmental context. For example, an increase in circulating hormone levels in this species was observed in response to hypoxia, emersion, and/or temperature (Chung and Webster 2005), as well as salinity (Chung and Webster 2006). The present study is the first, however, to suggest CHH to also be involved in (branchial) acid-base regulation.

Branchial transport of acid-base components

Like OCT, application of CHH in Using chamber experiments reduced the short-circuit current, implying that active Cl^- influx is decreased (Riestenpatt et al. 1996) and hence suggesting a role for CHH in general branchial ion regulation. Indeed, when green crabs are exposed to dilute salinity, circulating CHH levels increase together with branchial cellular cGMP and branchial glucose levels (Chung and Webster 2006). It seems counterintuitive, however, why the application of CHH in Ussing chamber experiments here led to a decrease in osmoregulatory Cl^- influx when green crabs would need to increase the uptake to hyperregulate. In fact, Spanings-Pierrot et al. 2000) showed that CHH-containing sinus gland extracts resulted in an increased transepithelial potential difference and consequently increased Na^+ influx in perfused gills of the marbled (rock) crab, *Pachygrapsus marmoratus*. However, the authors did not quantify the applied [CHH] and it is possible that a much higher concentration was applied at which CHH exhibits a bi-phasic response in the isolated gill. Furthermore, marbled rock crabs are efficient hyper-hypo-regulators and might use (slightly) different branchial mechanisms / strategies for ion uptake. Clearly, further research will be needed to fully characterize the branchial osmoregulatory response of *C. maenas* to CHH, including second messengers and signaling pathways.

With regards to acid-base balance, application of CHH in gill perfusion experiments resulted in the opposite observation as with OCT: H^+ excretion significantly increased, and in this case might be directly coupled to the observed increase in branchial ammonia excretion *via* vesicular ammonia trapping ($NH_3 + H^+ \rightarrow NH_4^+$) involving a vesicular V-H^+-ATPase as suggested for *C. maenas* (Weihrauch et al. 2001, 2004, 2017). One possible explanation could also be the direct activation of a membrane bound V-H^+-ATPase, however, to the knowledge of the authors no connection between this transporter and CHH has been made to date for crustaceans.

Furthermore, other than with the application of OCT, branchial CO_2 excretion was significantly elevated when CHH was added to the extracellular bath. This indicates an even stronger involvement for the neuropeptide in acid-base regulation compared to the biogenic amine. This would make sense as CHH is also one of the major molting hormones, and this process is irrevocably linked to acid-base regulation, *i.e.*, through the requirement of HCO_3^- for hardening the exoskeleton (Abehsera et al. 2021). HCO_3^- on the other hand is part of the carbonate buffer system and stands in direct equilibrium with CO_2 ($CO_2 + H_2O \leftarrow\rightarrow H^+ + HCO_3^-$). Furthermore. the fact that differences in CO_2 excretion were observed adds a potential (cellular) metabolic component to CHH function with regards to acid-base regulation. As CHH is well known for its involvement in systemic glucose metabolism (Webster et al. 2012) it might indeed lead also to elevated branchial glucose levels. Metabolizing this excess glucose would then result in an increased branchial CO_2 production in the mitochondria and subsequent excretion as observed in this study.

As with OCT, the fact that the lower concentrations of CHH resulted in similar (though delayed in the case of H^+ and CO_2 excretion) effects as observed with the higher, physiologically relevant concentrations, again strengthens the fact that CHH can indeed target acid-base relevant processes independent from ion-regulatory processes.

Branchial receptor and physiological relevance

While Chung and Webster (2006) verified binding sites for CHH in the green crabs' gill, no specific branchial receptor has been characterized to date. CHHBP has been identified in the hepatopancreas of *E. sinensis* (Li et al. 2016), but could not be found in the *C. maenas* transcriptome (Verbruggen et al. 2015) or EST library (Towle and Smith 2006; Towle et al. 2011) (*pers. obs.*). Furthermore, while membrane bound guanylyl cyclase has been suggested as CHH-receptor in the Y-organ of crabs (Abuhagr et al. 2014), we failed to amplify it from the green crab gills with specific primers (data not shown). Instead, we were only able to reliably amplify the soluble guanylate cyclase 1b (sGC1b) in *C. maenas'* gills, an enzyme activated by G-protein coupled receptors promoting cGMP signaling in the Y-organ during molt in response to another neurohormone closely relate to CHH, molt-inhibiting hormone MIH (Abuhagr et al. 2014). A similar molecule, soluble adenylate cyclase (sAC), had also been shown to be involved in branchial acid-base regulation in dogfish gill *via* cAMP and HCO_3^- sensing (Tresguerres et al. 2010). Consequently, sGC1b seemed a highly suitable candidate to investigate instead of the actual receptor. However, the identification of the exact membrane CHH receptor is still necessary to fully clarify the mode of action for CHH in the decapod gill.

sGC1b mRNA abundance responded to environmental acid-base challenges and was significantly decreased upon either short-term HEA or long-term hypercapnia, indicating indeed a role for this enzyme – and therefore CHH as a potential ligand of the associated G-protein coupled receptor – in physiological acid-base related processes. The involvement in both, HEA and hypercapnia rather than only one as in the case for OCT might hint towards a more universal application of CHH in acid-base regulatory processes. A downregulation of sGC1b mRNA abundance especially in response to short-term hypercapnia could indicate a lower presence of this enzyme and consequently, a decrease of branchial ammonia excretion. This would lead to an increase in hemolymph ammonia levels, which has been observed for green crabs acclimated to hypercapnia for 7 d (Fehsenfeld and Weihrauch 2013). In contrast, hemolymph ammonia in green crabs acclimated to high ammonia return to control levels after 24 h (Fehsenfeld et al. 2023) which could explain the absence of changes in mRNA abundance of sGC1b.

Conclusion

The present study demonstrates an involvement of the biogenic amine octopamine (OCT), as well as the neuropeptide crustacean hyperglycemic hormone (CHH) not only in general branchial ion regulation, but also specifically in branchial acid-base regulatory properties and in particular the excretion of H^+ and ammonia. In contrast to OCT, however, CHH also seems to include a metabolic component, as brachial CO_2 flux was affected as well. Furthermore, the regulatory properties of both hormones seem to be relevant for the acclimation of green crabs to the environment and in particular scenarios that challenge acid-base homeostasis like high environmental pCO_2 (hypercapnia) and ammonia (HEA). While the current study clearly shows an involvement of the hormones in acid-base regulation, future efforts should go toward elucidating their actual modes of actions. For example, it will be a priority to identify which brachial transporters are targeted, as well as further characterize the respective signaling cascades.

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Competing interests

The authors declare no competing interests.

Author's contributions

SF and DW conceived of the study followed by discussion with PC. DW provided the infrastructure, experimental installations and equipment for all experiments. PC and DW provided the consumables and specific reagents. SF performed the Ussing chamber and gill perfusion experiments, the acclimation of green crabs to short- and long-term hypercapnia, as well as long-term HEA, collected the respective samples and processed the respective cDNA. AQR performed the acclimation of green crabs to short-term HEA, collected the respective samples and processed the respective cDNA. SF performed all quantitative PCRs. SF analyzed the data and conducted the statistical analyses and wrote the first draft of this manuscript. All author contributed to the final version of this MS.

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Figures

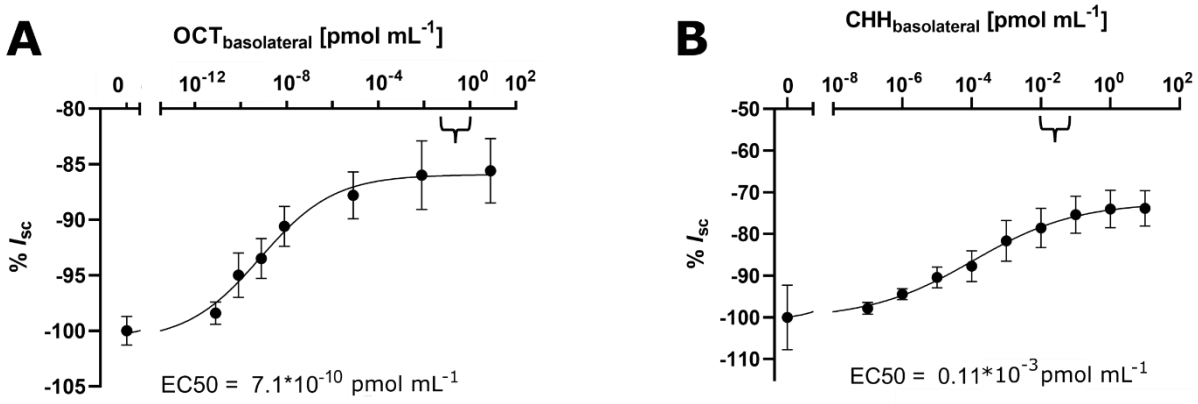


Figure 1. Dose-response curve of hormones on short-circuit current in split gill filaments (posterior gills #7) of osmoregulating the green crab *Carcinus maenas* applying Ussing-chamber experiments. Application of (A) Octopamine (OCT) or (B) Crustacean hyperglycemic hormone (CHH) led to a decrease of Cl^- flux as reflected in a more positive short-circuit current (I_{SC}). Brackets indicate physiological concentrations of hormones retrieved from the literature (Sneddon et al. 2000 for OCT (6-9 pmol mL^{-1}), Chung and Webster 2005 for CHH (0.05 pmol mL^{-1})). The average short circuit current (I_{SC}) without application of hormones accounted for (A) -305.4 ± 26.4 mA cm^{-2} and (B) -319.8 ± 30.3 cm^{-2} with a corresponding transepithelial potential difference of (A) 5.2 ± 0.3 mV and (B) 4.8 ± 0.3 mV, respectively. N = 6 for OCT, N = 5 for CHH.

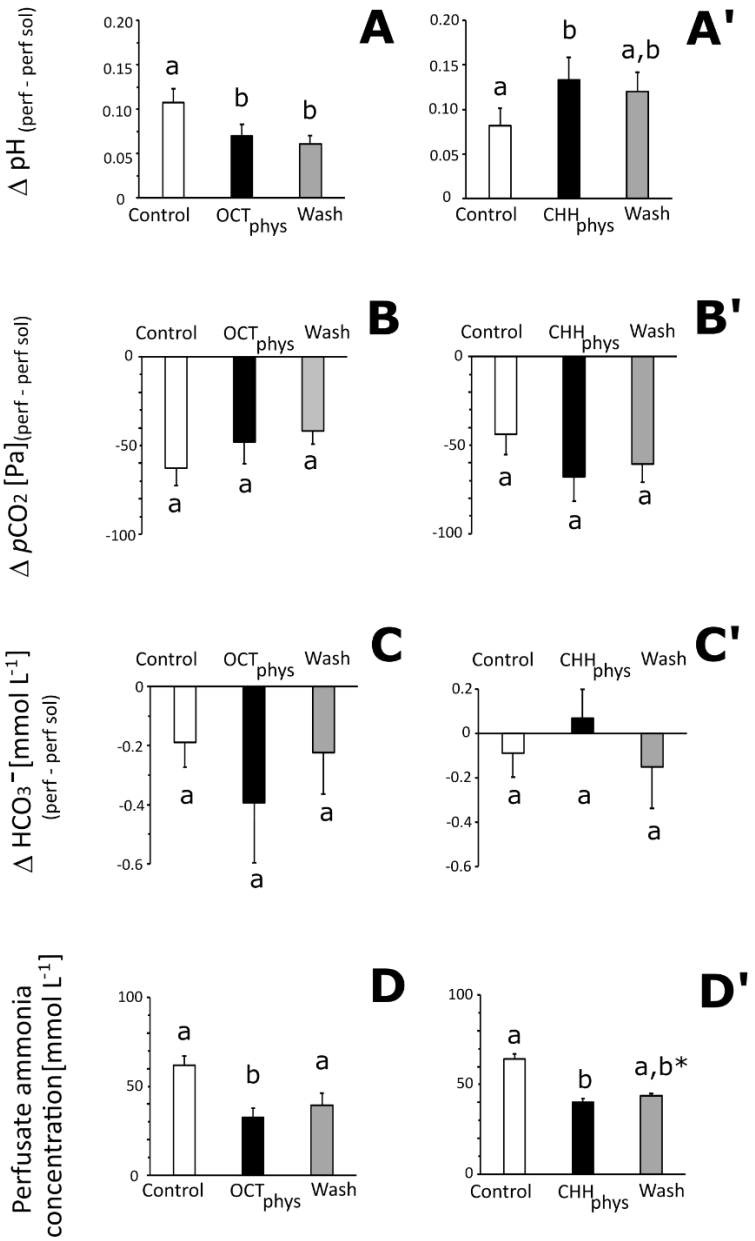


Figure 2. Effects of physiological concentrations of hormones on the acid-base regulatory capacity of posterior gill 7 of *C. maenas*. Gill perfusions were conducted in three consecutive steps: (1) control perfusion, (2) application of (A-D) 6 pmol mL^{-1} octopamine or (A'-D') 0.05 pmol mL^{-1} Crustacean hyperglyccemic hormone (CHH), and (3) wash out with control saline. (A, A'), effect on perfusate pH; (B, B') effect on branchial pCO_2 excretion; (C, C'), effect on branchial excretion of HCO_3^-, and (D,D') effect on branchial ammonia excretion. Lower case letters indicate significant differences between the different steps ($P < 0.05$, N = 5). Asterisk indicates $P = 0.07$ between the application of CHH and the wash step in panel D'.

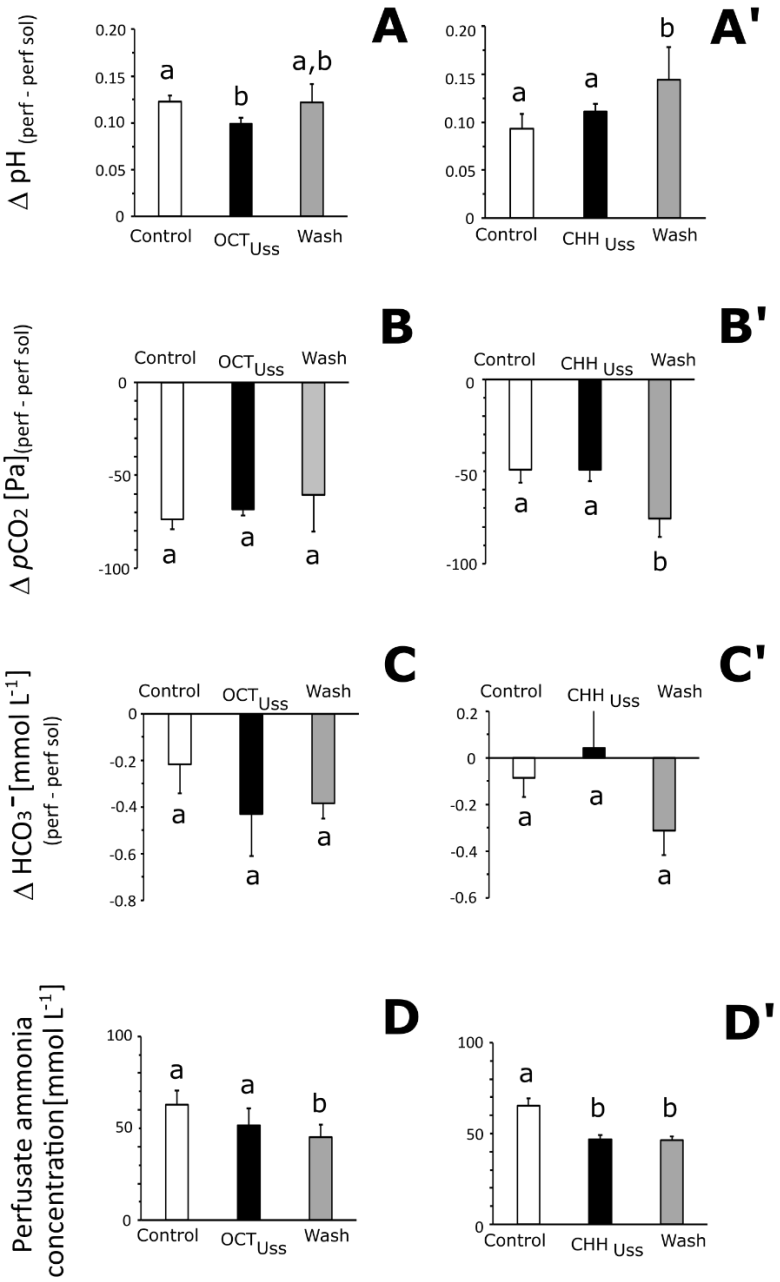


Figure 3. Effects of hormones with concentrations as determined by Ussing experiments on the acid-base regulatory capacity of posterior gill 7 of *C. maenas*. Gill perfusions were conducted in three consecutive steps: (1) control perfusion, (2) application of (A-C) 10^{-11} pmol mL^{-1} octopamine or (A'-C') 10^{-6} pmol mL^{-1} Crustacean hyperglycemic hormone (CHH), and (3) wash out with control saline. (A, A'), effect on perfusate pH; (B, B') effect on branchial pCO_2 excretion; (C, C'), effect on branchial excretion of HCO_3^-, and (D,D') effect on branchial ammonia excretion. Lower case letters indicate significant differences between the different steps ($P < 0.05$, N = 5).

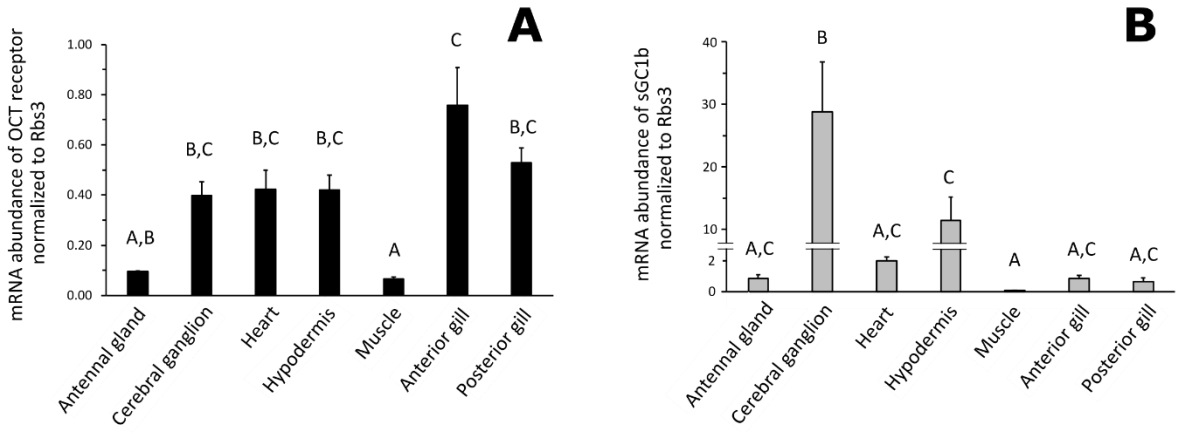


Figure 4. Tissue distribution for (A) Octopamine-receptor and (B) potential crustacean hyperglycemic hormone activated sGC1b. For CHH no membrane receptor could be identified in the gill so that soluble guanylate cyclase (sGC1b) as G-Protein activated enzyme in the signalling cascade for CHH is depicted instead. Uppercase letters denote significant differences between different tissues ($P < 0.05$, N = 4-5). mRNA abundance has been normalized to Rbs3.

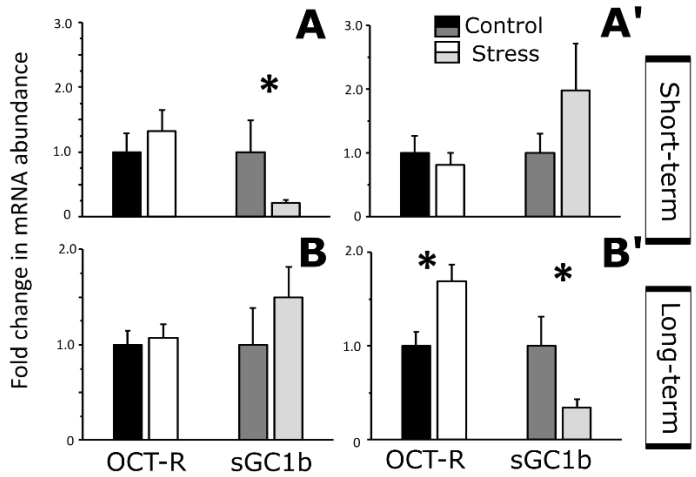
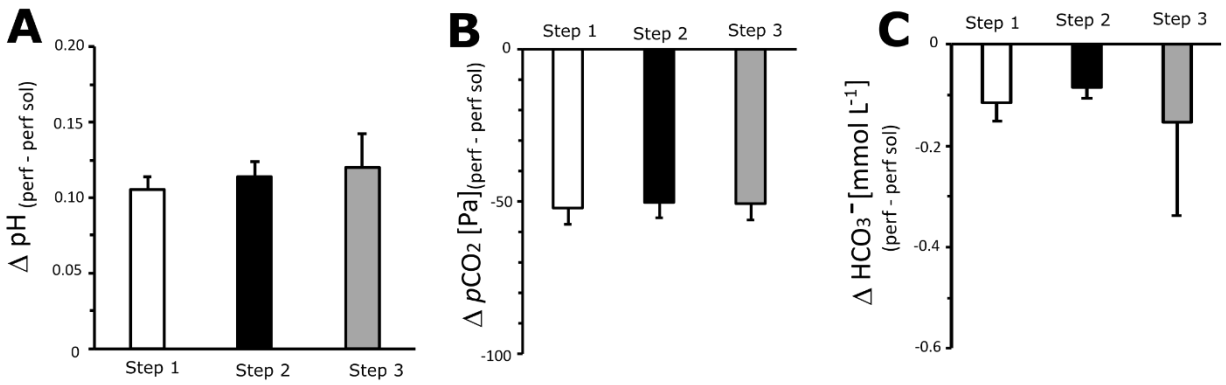


Figure 5. Fold change in mRNA abundance for Octopamine-receptor (OCT-R) and soluble guanylate cyclase (sGC1b) in response to environmental stress. Quantitative PCR was performed on posterior gill 7 in brackish water acclimated green crabs exposed to either (A) short-term high environmental ammonia (HEA, 24 h), (A') short-term high pCO_2 (48 h), (B) long-term HEA (7 d), or (B') long-term high pCO_2 (7 d). Asterisks denote significant differences ($P < 0.05$, N = 5). Data were normalized to the geometric mean of arginine kinase (ArgKin) and ribosomal gene 3 (Rbs3; as determined by RefFinder (http://blooge.cn/RefFinder/).



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722 **Supplemental Figure 1. Control perfusion to ensure the gills performance over time.** One posterior
723 gill 7 of brackish water acclimated green crabs, *Carcinus maenas*, was run in parallel which each round of
724 the perfusion experiments, but no hormone was applied in the second step. (A) effect on perfusate pH; (B)
725 effect on branchial pCO_2 excretion, and (C) effect on branchial excretion of HCO_3^-.