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MECHANISMS OF ACID-BASE REGULATION IN SEAWATER-ACCLIMATED GREEN CRABS, *CARCINUS* *MAENAS*

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Mechanisms of acid-base regulation in seawater-acclimated green crabs, *Carcinus maenas* (S. Fehsenfeld and D. Weihrauch)

Abstract

The present study investigated acid-base regulatory mechanisms in seawater-acclimated green crabs (*Carcinus maenas* (L., 1758)). In full-strength seawater, green crabs are osmoconformers so that the majority of the observed responses were attributed to ion-fluxes based on acid-base compensatory responses alone. Similar to observations in brackish-water acclimated *C. maenas*, seawater-acclimated green crabs exposed to hypercapnia rapidly accumulated HCO_3^- in their hemolymph, compensating for the respiratory acidosis caused by excess hemolymph $p\text{CO}_2$. A full recovery from the decreased hemolymph pH after 48 hours, however, was not observed. Gill perfusion experiments on anterior gill #5 indicated the involvement of all investigated genes (i.e. bicarbonate transporters, $\text{V}-(\text{H}^+)$ -ATPase, Na^+/K^+ -ATPase, K^+ -channels, Na^+/H^+ -exchanger and carbonic anhydrase) in the excretion of acid-base equivalents. The most significant effects were observed when targeting a potentially basolateral and/or cytoplasmic localized $\text{V}-(\text{H}^+)$ -ATPase, as well as potentially basolateral localized bicarbonate transporter (likely a $\text{Na}^+/\text{HCO}_3^-$ -cotransporter). In both cases, H^+ accumulated in the hemolymph and CO_2 excretion across the gill epithelium was significantly reduced or even reversed when blocking bicarbonate transporters. Based on the findings in this study, a working model for acid-base regulatory mechanisms and their link to ammonia excretion in the gill epithelium of *C. maenas* has been developed.

Keywords: green shore crab, *Carcinus maenas*, crustacea, CO_2 , ammonia, gill perfusion, inhibitors

Introduction

Over the last few decades, green crabs (*Carcinus maenas* (L., 1758)) have become one of the most successful marine invaders on the planet (Lowe et al. 2000). Originating from northern European waters, they now can be found in both the Canadian Atlantic and Pacific, where they threaten the existing natural ecosystem communities as well as commercial fisheries (Jamieson et al. 1998; Cameron and Metaxas 2005; Miron et al. 2005). The success of *C. maenas* as an invasive species can be attributed to their high capability for short-term acclimation as well as their potential for long-term adaptation to a variety of environmental challenges such as changes in salinity and temperature, high ammonia and low pO_2 / high pCO_2 (Truchot and Duhamel-Jouve 1980; Weihrauch et al., 1999a; Bellwood 2002; Thomsen et al. 2010) .

In decapod crustaceans like the green crab, this acclimation/adaptation potential is based on the capacity to maintain homeostasis of bodily fluids despite the changing environment, which is mainly achieved *via* ion exchange processes in the gill epithelium. Based on their morphological differences and lower activity of Na^+/K^+ -ATPase compared to the most posterior localized 2-3 pairs of gills, the anterior gills (4-6 pairs of gills) of euryhaline decapod crustaceans are thought to be mainly involved in respiratory and excretory processes rather than NaCl uptake (Compere et al. 1989; Pequeux 1995; Freire et al. 2008; Henry et al. 2012). While extensive work has been conducted to investigate osmoregulatory and ammonia excretory patterns in gills of decapod crustaceans (Freire et al. 2008; Henry et al. 2012; Larsen et al. 2014), hardly anything is known concerning acid-base regulation.

Resulting from tracer flux and voltage-clamp studies on split gill lamella in Ussing chamber experiments, the gill epithelium of *C. maenas* has been characterized as a (moderate) leaky epithelium, exhibiting a relatively high trans-epithelial conductance and high ion transport rates (Riestenpatt et al. 1996). Applying inhibitors in gill perfusion experiments and on split gill lamellae helped to identify basolateral Na^+/K^+ -ATPase and Cl^- -channels, as well as an apical $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -cotransporter supported by apical and basolateral K^+ -channels, to be the key-players in trans-branchial active NaCl transport in moderate hyper-osmoregulators such as *C. maenas* and *Neohelice (Chasmagnathus) granulata* (Dana, 1851) (Lucu and Siebers 1987; Riestenpatt et al. 1996; Onken et al. 2003). Briefly, these authors compared the NaCl uptake mechanism in the gill epithelium of these decapod crustaceans to the mechanism proposed for the thick ascending limb (TAL) of the Henle's loop in the mammalian kidney. Basolateral Na^+/K^+ -ATPase and Cs^+ -sensitive K^+ -channels have also been shown to be involved in ammonia excretion through the gills of *C. maenas*, as well as *via* a cytoplasmic $\text{V}-(\text{H}^+)$ -ATPase and a functional microtubule network (Weihrauch et al. 1998, 2004b).

Unfortunately, our knowledge of branchial acid-base regulation in decapod crustaceans is sparse. For example, by application of pharmacological agents and measuring the trans-epithelial potential difference (PDte) in gill perfusion experiments in brackish-water acclimated green crabs, Siebers et al. (1994) identified oxidative metabolism and carbonic anhydrase (CA), a key-player in acid-base balance by promoting the hydration of CO_2 into H_2CO_3 and the subsequent dissociation into H^+ and HCO_3^- , to play the central roles in acid-base regulation by the gill epithelium. Additionally, inhibition of branchial CA led to a dose-dependent decrease in hemolymph osmolarity and Na^+ and Cl^-

concentrations in low salinity acclimated striped shore crabs (*Pachygrapsus crassipes* (Randall, 1980), Burnett et al. 1981)), the blue crab (*Callinectes sapidus* (Rathburn, 1896), Henry and Cameron, 1983) and *C. maenas* (Henry et al., 2003). These results indicated that H^+ as well as HCO_3^- provided by CA could be directly feeding Na^+/H^+ -exchangers or Cl^- or Na^+/HCO_3^- -transporters in the gill epithelium.

In respect to the whole animal status, acid-base homeostasis of the extracellular fluid seems to be dependent on the strong ion difference (and the resulting anion gap): Besides the actual ion exchange processes at the gill as mentioned before, changes in hemolymph Na^+ and Cl^- concentrations (i.e. due to dilute salinity acclimation) are partly balanced by a shift in the weaker ions H^+ , OH^- and HCO_3^- independent of an external source, in order to maintain the electrical neutrality of the body fluids (Stewart 1978).

A direct link between acid-base regulation and whole animal ammonia status in regard to salinity acclimation in decapod crustaceans has also been supported by a few studies. When acclimated to dilute salinities, *C. sapidus* elevated hemolymph ammonia levels and ammonia excretion rates, while at the same time hemolymph pH and HCO_3^- increased at constant pCO_2 (Mangum et al. 1976; Henry and Cameron 1982). Mangum et al. (1976) speculated that this is caused by the increased deamination of intracellular free amino acids used in cell volume regulation. Based on these links of ammonia, ion and acid-base regulation in decapod crustaceans, it can be hypothesized that gill epithelial transporters known to be involved in salinity acclimation and ammonia excretion will also play an important role in acid-base regulation in decapod crustaceans.

While most studies on osmoregulation in decapod crustaceans have been conducted in brackish-water acclimated specimens, the present study concentrates on seawater-

acclimated green crabs. At 32 ppt, *C. maenas* is osmoconforming and keeps its hemolymph osmolality isotonic to the surrounding seawater. As a result, NaCl movements are believed to be mainly passive (Zanders 1980), as also supported by the high trans-epithelial conductance measured in gills of the marine osmoconforming Red rock crab (*Cancer pagurus* (L., 1759), Weihrauch et al. 1999a)). This allows for the investigation of the most basic underlying principles of acid-base regulation in these osmoconforming animals, uncoupled from a salinity-mediated response. In a first set of experiments, acid-base homeostasis in the seawater-acclimated green crabs was challenged by exposure to high environmental $p\text{CO}_2$ (hypercapnia) in order to observe the whole animal response and the capability of osmoconforming green crabs to counteract this disturbance without activated ion regulatory mechanisms in place. Additionally, pharmaceuticals for the inhibition of distinct transporters potentially involved in acid-base regulation were applied in isolated gill perfusion experiments of seawater-acclimated *C. maenas* to identify key epithelial proteins participating in this process. Based on these results, an updated, more detailed working model for acid-base regulatory mechanisms and a link to ammonia excretion is postulated for the gill epithelium of seawater-acclimated green crabs.

Material and Methods

Animals

Male green crabs were collected in Barkley Sound at the opening of the Pipestem Inlet (Vancouver Island, BC, Canada) in the summer of 2012 and 2013 under the Department

of Fisheries and Oceans collection permits XR 207 2012 and XR 235 2013, respectively. Only male animals with an approximate carapace width of 5-7 cm and a weight of 60-90 g were chosen for experimentation. Approximately 50 green crabs were kept in aerated ~500 L flow-through outdoor tanks directly connected to water from the Barkley Sound (salinity = 32 ppt) under natural light conditions (10h (dark):14h (light)) at the Bamfield Marine Sciences Centre (Bamfield, BC, Canada). Animals were fed *ad libitum* once a week with fish carcasses and fasted for 2-3 days prior to experimentation.

Acclimation to high environmental $p\text{CO}_2$ (hypercapnia)

For acclimation to hypercapnic conditions (1% CO_2 = 1013.25 Pa), two aerated flow-through plastic containers (68 L) were set up in the laboratory space and six green crabs transferred into each. A header tank for each container was established, supplying containers with either fresh seawater (controls) or seawater pre-equilibrated to 1% CO_2 (high $p\text{CO}_2$). The flow rate from the header tanks to the containers holding the animals was adjusted to 50 ml / min.

To easily draw hemolymph from the animals, a hole (diameter ~2mm) was drilled into the dorsal carapace using a Dremel[®] and sealed with a piece of dental dam. A sterile syringe with a 21.5 gauge needle was used to obtain ~200 μl hemolymph samples at 0, 6, 12, 24 and 48 hours, respectively. Hemolymph was immediately assessed for pH and total carbon (C_T) as described below. Samples were then frozen at -20°C until analyzed for ammonia content (see below).

To determine whole animal ammonia excretion rates, green crabs were transferred into small aerated containers holding 2 L of seawater, after being acclimated to either control or high $p\text{CO}_2$ seawater in the 68 L tanks for 48 h, as described above. 10 ml water

samples were taken after 10 and 40 minutes and frozen at -20°C until further analysis for ammonia (see below).

Gill perfusion with application of inhibitors

Isolated anterior gill #5 of control seawater-acclimated animals were perfused following the protocol of Siebers et al. (1985) with a flow rate of $128\ \mu\text{l min}^{-1}$, using a peristaltic pump (Sci 323 Watson–Marlow Bredel Pump, Falmouth Cornwall, England). Gills were placed in 50 ml glass beakers containing 30 ml bathing solution (seawater directly taken from Barkley Sound). The perfusion solution contained (in mmol L^{-1}): 470 NaCl, 12 CaCl_2 , 12 MgCl_2 , 11 KCl, 9 NaHCO_3 , 0.3 glucose, 0.1 glutathione, 0.5 glutamine, based on results from ion chromatography performed on hemolymph of full strength seawater-acclimated green crabs (Fehsenfeld and Weihrauch 2013). $100\ \mu\text{mol L}^{-1}\ \text{NH}_4\text{Cl}$ was only added to the perfusion solution, not the bathing solution, to account for *in vivo* conditions (Weihrauch et al., 1998). The pH of the perfusions solution was adjusted to 7.9 with $0.1\ \text{mol L}^{-1}\ \text{NaOH}$ immediately before each respective perfusion step to ensure low variability in this parameter.

The perfusion protocol consisted of three consecutive steps: following a 40 min control phase, gills were perfused with perfusion solution containing the respective inhibitor (see below) for 40 min. A third 40 min period applying perfusion solution as in the control step (step 1) was implemented to ensure that the gills were still active (returning to control levels). The gills were allowed a 10 minute equilibration period after which the perfusate was collected for 30 min.

The inhibitors and their concentrations were chosen as follows, according to the available literature: $100\ \mu\text{mol L}^{-1}$ tenidap (bicarbonate transporters, i.e. $\text{Na}^+/\text{HCO}_3^-$ -cotransporter,

NBC; Ducoudret et al. 2001), 20 $\mu\text{mol L}^{-1}$ KM91104 (V-(H⁺)-ATPase; Kartner et al. 2010), 5 mmol L^{-1} ouabain (Na⁺/K⁺-ATPase, NKA; Weihrauch et al. 1998), 100 $\mu\text{mol L}^{-1}$ amiloride (Na⁺/H⁺-exchanger, NHE; Siebers et al. 1982), 12 mmol L^{-1} BaCl₂ (K⁺-channels; Schirmanns and Zeiske 1994) and 1 mmol L^{-1} acetazolamide (Weihrauch et al. 1998). All inhibitors with the exception of ouabain were diluted from 100x concentrated stock solutions in DMSO resulting in a final DMSO concentration in the perfusion solution of 1 vol%. Ouabain was directly dissolved in the perfusion solution. Perfusion experiments with 1 vol% DMSO alone in the inhibitor step were performed and found to neither have an effect on ammonia excretion, nor acid-base equivalents ($n = 3$, data not shown).

Analysis of hemolymph and perfusate samples

All hemolymph and perfusate samples were immediately measured for the acid-base equivalents pH and C_T and temperature. pH was measured either with the InLab Micro Combination pH electrode (hemolymph – small volumes; Mettler-Toledo) or the pH/ATC electrode 300729.1 (perfusates – large volumes; Denver Instruments, Göttingen, Germany), connected to a pH-ISE meter model 225 (Denver Instruments). Total CO₂ (C_T) was measured using a Corning 965 carbon dioxide analyzer (Olympic Analytical Service, UK). $p\text{CO}_2$ and HCO₃⁻ were then calculated applying the appropriate factors and equations as generated by Truchot (1976).

Hemolymph ammonia, perfusate ammonia and ammonia contents of the seawater samples from the whole animal excretion experiment were measured using a gas-sensitive NH₃ electrode (Orion 9512 from Thermo Scientific, Cambridgeshire, England) connected to a digital mV/pH meter, following the procedure established by Weihrauch et

al. (1998). All samples were diluted (1:3 in case of the perfusates and water samples, 1:7 in case of hemolymph samples due to low volumes) as high salt has been found to interact with the electrode (User guide for Orion 9512 from Thermo Scientific; personal experience). Standard curves were diluted accordingly.

Changes in perfusate $[H^+]$ were calculated based on the measured pH differences in the perfusate compare to the initial perfusion solution as follows:

$$\Delta[H^+] = 10^{(-pH_{\text{perfusion solution}})} - 10^{(-pH_{\text{perfusate}})}$$

Accordingly, gill excretion rates for CO_2 and ammonia were assessed based on the loss of the respective component in the perfusate (= perfusion solution after one passage through the gill) compared to the initial perfusion solution for each step.

Phylogenetic analysis of Na^+/H^+ -exchanger

The 24 protein sequences for the Na^+/H^+ -exchanger as identified in the NCBI protein database (table 3.1) were aligned with the default MUSCLE algorithm (Edgar 2004) as provided by MEGA 6.06 (Tamura et al. 2011). The test function as implemented in MEGA 6.06 was applied to find the best protein model. A gene tree based on the resulting 514 amino acid alignment was constructed with the method of maximum likelihood. Accordingly, the consensus tree was constructed using the gamma-distributed LG model (Le and Gascuel 2008) based on a matrix of pairwise distances estimated using a JTT model (Jones et al. 1992) with invariant sites and two discrete gamma categories, as well as the default settings (heuristic method of Nearest-Neighbor-Interchange (NNI), automatic initial tree with NJ/BioNJ). Bootstrap values were obtained for 1000 replicates.

Statistics

Statistical analyses were performed using the software Past3 (Hammer et al. 2001) and JMP[®], Version 10 (SAS Institute Inc., Cary, NC, 1989-2007). All data sets were tested for normal distribution (Shapiro–Wilk test) and homogeneity of variances (F-test or Levene's test) prior to testing. In cases where normal distribution and/or homogeneity of variances were not fulfilled, the data sets were log-transformed. Student's t-test or paired t-test was applied comparing two means. For the time series on hemolymph parameter during high $p\text{CO}_2$ acclimation, a two-way ANOVA (independent variables: acclimation, time) was performed on pH, $p\text{CO}_2$ and HCO_3^- to identify between-group differences. Subsequently, a repeated-measures one-way ANOVA was performed on either control or high $p\text{CO}_2$ acclimated animals for the respective factors to identify within-group differences. All results with $p < 0.05$ were considered significant.

Results

Whole organism response of *C. maenas* to high $p\text{CO}_2$ exposure

The transfer of green crabs into the experimental units (inside, smaller tanks) caused a shift of hemolymph pH (not significant), $p\text{CO}_2$ and $[\text{HCO}_3^-]$ (both significant, repeated-measures ANOVA with Tukey's pairwise comparisons, $p < 0.05$, $n = 5$) in control animals in the initial 6 hours of acclimation. While the control pH was restored to pre-transfer values after 12 hours, $p\text{CO}_2$ and HCO_3^- levels remained slightly lower than observed in control animals before the transfer but remained constant over time (figure 1).

The two-way ANOVA identified time and acclimation as well as their interaction to have a significant impact on each of the investigated hemolymph parameter. Green crabs exposed to high $p\text{CO}_2$ (1% CO_2) rapidly accumulated CO_2 and HCO_3^- in their hemolymph (figure 1). A significant increase in both hemolymph parameters was observed after only 6 hours and seemed to level off (i.e. no significant differences between 24 and 48 hours) at a 4-fold increased level for $p\text{CO}_2$ (688 ± 40 Pa), and a 3-fold increased level for HCO_3^- (19 ± 2 mmol L^{-1}) after 48 hours exposure. Interestingly, the increase in HCO_3^- did not seem to be sufficient to completely counteract/buffer the respiratory acidosis and pH values decreased slightly by 0.11 units from control values of 7.87 ± 0.02 to 7.76 ± 0.03 in high $p\text{CO}_2$ crabs after 48 hours.

Hemolymph ammonia increased significantly from 93 ± 0.8 $\mu\text{mol L}^{-1}$ in control animals to 406 ± 45 $\mu\text{mol L}^{-1}$ in high $p\text{CO}_2$ crabs, as did the whole animal ammonia excretion rate (46 ± 10 versus 175 ± 34 nmol $\text{g}^{-1} \text{h}^{-1}$).

Effects of inhibitors in gill perfusion experiments of seawater-acclimated green crabs

Under control conditions, gill #5 excreted ammonia, H^+ and CO_2 (lower concentration in perfusate compared to the initial perfusion solution, figure 2). Compared to green crabs acclimated to brackish-water (10 ppt, data from Fehsenfeld and Weihrauch 2013), *C. maenas* acclimated to full-strength seawater (32 ppt) generally excreted less H^+ and CO_2 , while excretion rates for ammonia and HCO_3^- were the same as for brackish-water acclimated animals.

By far the most drastic effects on H^+ and CO_2 excretion rates by isolated gill #5 were observed with basolateral application of tenidap (bicarbonate transporters) and KM91104

(V-(H⁺)-ATPase), respectively (figure 3). Blocking potentially basolateral situated bicarbonate transporters resulted in an accumulation of protons and CO₂ in the perfusate (perfusate [H⁺]/pCO₂ exceeded [H⁺]/pCO₂ in the perfusion solution resulting in negative fold-change values; figure 3A, B). Inhibiting V-(H⁺)-ATPase basolaterally led to a similar level of accumulation of protons in the hemolymph as blocking bicarbonate transporters with tenidap (figure 3A). In contrast to tenidap, however, CO₂ excretion still took place when KM91104 was applied but was drastically reduced (*ca.* 70%, figure 3B). Basolateral bicarbonate transporters seemed not to be involved in ammonia excretion in anterior gill #5, whereas ammonia excretion was significantly reduced applying basolateral KM91104 (V-(H⁺)-ATPase; figure 3B). It should be noted that basolaterally applied KM91104 might not only affect basolateral V-(H⁺)-ATPase, but by diffusion over the basolateral membrane may also affect intracellular V-(H⁺)-ATPase.

While the apical application of tenidap (bicarbonate transporters) and KM91104 (V-(H⁺)-ATPase) led to a small but significant increase in perfusate [H⁺] (equivalent to a decrease in $\Delta H^+_{(\text{perfusion solution} - \text{perfusate})}$ implying a reduced net-excretion; figure 3A), apical application of KM91104 additionally resulted in a significant decrease of CO₂ excretion (figure 3B).

Also all of the other basolaterally applied inhibitors significantly decreased ΔH^+ (figure 3A). While blocking the Na⁺/K⁺-ATPase by ouabain and K⁺-channels with BaCl₂ resulted in *ca.* 20% increase in [H⁺], blocking, Na⁺/H⁺-exchanger (and potentially Na⁺-channels) with amiloride and carbonic anhydrase with acetazolamide resulted in approximately 50-60% increase in perfusate [H⁺] in comparison to the initial perfusion solution.

With the exception of the Na^+/H^+ -exchanger and/or potential Na^+ -channels, all investigated transporters contributed significantly to the excretion of CO_2 (figure 3B). Besides the above mentioned pronounced effects on CO_2 excretion when blocking $\text{Na}^+/\text{HCO}_3^-$ -cotransporter and $\text{V}-(\text{H}^+)$ -ATPase, inhibition of the Na^+/K^+ -ATPase by ouabain led to the lowest reduction of CO_2 excretion (*ca.* 20%), while blocking K^+ -channels with BaCl_2 and carbonic anhydrase with acetazolamide resulted in a decrease of CO_2 excretion by *ca.* 50%.

In addition to the reduced ammonia excretion observed when blocking basolateral $\text{V}-(\text{H}^+)$ -ATPase as mentioned above, blocking Na^+/K^+ -ATPase, K^+ -channels and Na^+/H^+ -exchanger/potential Na^+ -channels significantly reduced ammonia excretion across the gill epithelia to a similar extent of *ca.* 50% (figure 3C).

Maximum likelihood analysis of Na^+/H^+ -exchanger (NHE)

The Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 1.7531)). The rate variation model ([+I]) allowed for 4.7% of the sites to be evolutionarily invariable. All positions with less than 95% site coverage were eliminated resulting in a total of 228 positions in the final dataset.

As can be seen in figure 4, the 24 sequences for NHEs (*Caenorhabditis elegans* (Maupas, 1900) NHX1-9, *Homo sapiens* (L., 1758) NHE1-9, *Aedes aegypti* (Linnaeus in Hasselquist, 1762) NHE-3,8,7/9, *Hyas araneus* (L., 1758) NHE type 1,2 and *C. maenas* NHE) divided into two monophyletic groups. While the first group includes *H. sapiens* NHE-1 through NHE-5 and most of the *C. elegans* isoforms, the second group contains NHE-6 through NHE-9 from *H. sapiens* and *A. aegypti*. The NHE of *C. maenas* falls into the first group and is most similar to the basolateral NHE-3 of *A. aegypti*. The two

different types of NHE identified in the transcriptome of another crustacean, the spider crab *H. areneus*, are most similar to the potentially apical NHE-7/9 of *A. aegypti* (type 1) and NHE-8 of *H. sapiens* (apical) and *A. aegypti* (intracellular and/or subapical; type 2) in the second group.

Discussion

Systemic response of seawater-acclimated *C. maenas* upon disturbance of acid-base homoeostasis

Even though the transfer of green crabs into the experimental units caused a slight shift in the hemolymph acid-base status, values remained stable over the course of the experiment (6-48 hours). The observed alterations in hemolymph parameters during the initial 6 hours are likely due to slight differences in the external medium caused by the differing husbandry conditions (pre-transfer: big outside tanks with *ca.* 50 animals *vs.* post-transfer: small tanks with 6 animals). Seawater-acclimated green crabs *C. maenas* exhibited a rapid respiratory acidosis in response to elevated environmental $p\text{CO}_2$ (48h, 1% $\text{CO}_2 = 1 \text{ kPa} = 7.5 \text{ mmHg}$) with significantly increased hemolymph $p\text{CO}_2$ (levelling off just below environmental values) and HCO_3^- and a significant drop in pH. These general changes in acid-base status and the observed 4-fold increase in hemolymph ammonia as well as ammonia excretion rates (2.5-fold) after 48 hours were similar to what has been observed in two recent studies on brackish-water acclimated green crabs (0.4% $\text{CO}_2 = 0.4 \text{ kPa} = 3 \text{ mmHg}$; Appelhans et al. 2012 (10 weeks exposure); Fehsenfeld and Weihrauch 2013 (7 days exposure) and the marine Dungeness crab (*Metacarcinus magister* (Dana, 1852), Hans et al. 2014 (7-10 days exposure)).

Interestingly, however, the 2-fold increase in hemolymph HCO_3^- levels as observed in the current study were not able to fully compensate the pH drop resulting from the elevated hemolymph $p\text{CO}_2$. Similarly, freshwater- and seawater-acclimated *C. sapidus* were not able to fully restore blood pH in this time frame in response to 1% CO_2 exposure (Cameron 1978; Henry et al. 1981). In contrast, brackish-water acclimated green crabs restored their hemolymph pH completely after 7 days when exposed to *ca.* 0.4% CO_2 (0.4 kPa), however, no data is available for the initial phase of hypercapnia-acclimation (Fehsenfeld and Weihrauch 2013). It has to be noted that a CO_2 level of 1% is higher than what green crabs might encounter in the wild. Additionally, osmoregulating *C. maenas* are likely better equipped of counteracting the acid-base disturbance due to the activated ionoregulatory machinery at their gills. It therefore is not surprising that the osmoconforming green crabs were not able to fully compensate the acid-base disturbance. While a comparable increase in blood $p\text{CO}_2$ and HCO_3^- was observed in several seawater-acclimated fish in response to acclimation to 1% CO_2 (Brauner and Baker 2009), their capability of restoring blood pH varied strongly (Hayashi et al. 2004). For example, after a drop of blood pH by 0.1-0.3 units, pH returned to control levels after only 1-3 hours in the yellowtail (*Seriola quinqueradiata* (Temminck and Schlegel, 1845)), and the Japanese flounder (*Paralichthys olivaceus* (Temminck and Schlegel, 1845)). In contrast, blood pH in star-spotted dogfish (*Mustelus manazo* (Bleeker, 1854)) required 72 hours to recover to control levels (Hayashi et al. 2004). While the observed pH drop might not yet be harmful to the green crabs, it may help to increase $[\text{NH}_4^+]$ in the blood while reducing the amount of NH_3 , allowing for a tighter control of ammonia levels *via* active transport by the Na^+/K^+ -ATPase (Weihrauch et al. 1998). In the present study,

changes in hemolymph acid-base parameters were only monitored for 48 hours. Even though the changes in hemolymph pH, $p\text{CO}_2$ and $[\text{HCO}_3^-]$ seem to level off after this time (i.e. no significant difference between values for 24 and 48 hours), a later re-adjustment cannot be excluded and complete restoration of hemolymph pH could possibly have occurred within a longer acclimation time.

Hemolymph ammonia levels in seawater-acclimated control green crabs were initially lower than in brackish-water acclimated control animals, but then rose to twice as high in osmoconforming/hypercapnia-acclimated animals (this study) compared to osmoregulating/ hypercapnia-acclimated crabs (Fehsenfeld and Weihrauch 2013). These generally lower ammonia contents in seawater-acclimated crabs are likely due to the fact that seawater-acclimated green crabs are not osmoregulating and therefore exhibit a lower metabolic rate in contrast to the hyper-regulating brackish-water acclimated animals, a phenomenon that has been observed in other marine crustaceans like the prawn *Metapenaeus monoceros* (Fabricius, 1798) (Rao 1958). The increase in hemolymph ammonia in seawater-acclimated green crabs in response to the acid-base disturbance may be explained by an increase in the resting metabolic rate to counteract the respiratory acidosis, a response also observed in the blue mussel (*Mytilus edulis* (L., 1758)) when acclimated to 0.24 and 0.4 kPa $p\text{CO}_2$ (Thomsen and Melzner 2010). This potential increase in metabolic rate in osmoconforming/hypercapnia-acclimated green crabs stands in contrast to the response of Dungeness crabs acclimated to a comparably high $p\text{CO}_2$ (0.33 kPa), which exhibited a metabolic depression correlated with both decreased hemolymph ammonia concentration and whole animal ammonia excretion rates (Hans et al. 2014).

Interestingly, ammonia excretion rates in both seawater and brackish-water acclimated *C. maenas* increased to the same extent (2.5-fold) following high $p\text{CO}_2$ acclimation, independent of the initial hemolymph ammonia concentration (this study, Fehsenfeld and Weihrauch 2013) and the level of external $p\text{CO}_2$ (~0.4 kPa in osmoregulating vs. ~1 kPa in osmoconforming crabs). Besides the possibility that the observed values resemble the general maximum ammonia excretion that can be accomplished by the animals, it is also possible that in response to hypercapnia, hemolymph ammonia levels are highly regulated and elevated to a distinct level in accordance with the crabs' physiological state (i.e. osmoconforming vs. hyper-osmoregulating). In osmoconforming/hypercapnia-acclimated green crabs hemolymph ammonia (NH_3) seems to become increasingly important as a (proton) buffer indicated by the higher absolute level of extracellular ammonia. In contrast, the activated ion-regulatory machinery in hyper-osmoregulating/hypercapnia-acclimated crabs might already increase the crabs' capacity to counteract acid-base disturbance using a potentially more efficient transporter inventory on the gill level and therefore requiring less absolute extracellular ammonia as extracellular buffer. Accordingly, whole animal ammonia excretion rates in osmoconforming/hypercapnia-acclimated green crabs do not increase in comparison to osmoregulating/hypercapnia-acclimated *C. maenas* in order to maintain higher buffering capacity of the extracellular space.

Excretory patterns of isolated gills of seawater and brackish-water acclimated *C. maenas*

In contrast to posterior gills, anterior gills do not undergo major structural changes due to acclimation to dilute salinity (Compere et al. 1989). Interestingly, however, when

acclimated to brackish water, anterior gill epithelia were more efficient in excreting protons and CO₂ than the anterior gill epithelia of osmoconforming green crabs, but not in respect to ammonia excretion (figure 2B, C). This indicates that the adjustment of the excretion of acid-base equivalents seems to play an important role also in osmoregulatory processes during dilute salinity acclimation. Additionally, the fact that anterior gills are capable of increasing proton and CO₂ excretion without being restructured shows that the adjustment of acid-base regulatory mechanisms in anterior gills are likely based on changes in gene expression levels of already present transporters or their activity, rather than the epithelia being “re-invented” as suggested for posterior gills of *C. maenas* (Compere et al. 1989) and *N. granulata* (Luquet et al. 2002).

Interestingly, ammonia excretion rates of anterior gill #5 were independent of environmental salinity. This is surprising because whole animal excretion rates were observed to be significantly (3-fold) higher in osmoconforming green crabs (this study) compared to osmoregulating green crabs (Fehsenfeld and Weihrauch 2013). It seems, however, that full-strength seawater acclimated osmoconforming green crabs also excreted more ammonia in the urine than did osmoregulating specimens acclimated to 10 ppt (Weihrauch et al. 1999b). This finding might indicate a decreased role for gills in ammonia excretion of osmoconforming crabs, and an increased role for alternative ammonia-excretory structures like the antennal glands. In osmoconforming *M. magister*, expression of the Rhesus-like protein, known to be involved in ammonia excretion in this decapod crustacean, significantly increased in antennal glands but not in the gills when animals were exposed to high environmental ammonia (Martin et al. 2011). Clearly,

further research is required to examine the role of antennal glands in ammonia regulation and excretion in *C. maenas*.

Identified transporters to be involved in acid-base and ammonia regulatory capacities of isolated anterior gills

Opening remarks

The major aim of the present study was to increase our limited understanding and fragmentary knowledge about mechanisms involved in branchial acid-base regulation in weak osmoregulating crustaceans. Prior to discussing the results of this study, however, it has to be noted that the applied pharmaceuticals have specific limitations and the results presented and discussed in this study can only be hypothetical. The reader is therefore invited to critically evaluate the implications of the obtained data.

Unfortunately, hardly any functional studies on isolated gill epithelial transporters of decapod crustaceans are available. Accordingly, the concentration and specificity of commonly used inhibitors might differ in comparison to observations made in other model organisms (i.e. *Xenopus laevis* (Daudin, 1802) oocytes expressing a specific ion transporter). In contrast to these functional studies on individual transporters, the present study used the intact, isolated gill in gill perfusion experiments. Therefore, it can neither be excluded that indirect effects on other ion fluxes occur when one transporter is blocked (potentially leading to a simultaneous effect on the observed parameters), nor that multiple transporters might be targeted by one inhibitor. Furthermore, inhibitors might have an effect on apical membranes even when applied basolaterally if they are able to diffuse into the cell, especially when they are dissolved in DMSO which is known to increase of membrane permeability. A recent study by de Ménorval et al. (2012),

however, showed that only concentrations of >10 vol% (2.74 mol%) of DMSO led to severe membrane damage in isolated Chinese hamster lung fibroblast cells, a concentration more than 10x higher than applied in the present study.

Despite the limitations of inhibitors applied on the isolated gill in perfusion experiments, the present data provides valuable information on potential key-players in branchial acid-base regulation as a basis for future studies. Where ever possible, we chose the inhibitors used in this study according to the available literature on decapod crustaceans. In gill perfusion experiments equivalent to those conducted in the present study, as well as electro-physical observation on split gill lamellae, most of the inhibitors are fairly well characterized (i.e. dose-response curves, effects on gill conductance, trans-epithelial voltage and short-circuit current) and the respective studies led to reasonable conclusions concerning the gill epithelial transporter inventory in decapod crustaceans that were taken into consideration for the present study.

Bicarbonate transporters

Unfortunately, no specific inhibitors for either $\text{Na}^+/\text{HCO}_3^-$ -cotransporters, or $\text{Cl}^-/\text{HCO}_3^-$ -exchangers, are currently available. Even though tenidap has been shown to inhibit $\text{Na}^+/\text{HCO}_3^-$ in *X. laevis* oocytes (Madhok 1995; Ducoudret et al. 2001) and found to be a better inhibitor (reversible effects, more potent, fewer side-effects) than 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), the experiments in that study were conducted in the absence of Cl^- and therefore not accounting for effects on a potential $\text{Cl}^-/\text{HCO}_3^-$ -exchanger (Ducoudret et al. 2001).

The application of tenidap in the present study identified potential basolateral bicarbonate exchanger(s) (i.e. a $\text{Na}^+/\text{HCO}_3^-$ -cotransporter and/or a $\text{Cl}^-/\text{HCO}_3^-$ -exchanger) to be key-

players for acid-base regulation in *C. maenas*. The data of the current study suggests a basolateral presence of at least one bicarbonate transporter. Even though hardly any effect was observed for the apical application of tenidap, results have to be treated with caution. The apical side of the gill epithelium is covered with a cuticle that has a high conductance *C. maenas* ($583 \pm 71 \text{ mS cm}^{-2}$ under symmetrical conditions, i.e. identical crabs saline on both sides; Onken and Riestenpatt 2002), but has also been shown to be cation-selective. In particular, specific pores in the epicuticle seem to discriminate between particles of different charge and size (Lignon 1987). The lacking response of apical tenidap (and KM91104 as described below) might therefore indicate that the relatively big molecule simply cannot cross the selective cuticle and therefore does not reach the apical membrane, similar to what has been observed for amiloride (Na^+/H^+ -exchanger; Onken and Riestenpatt 2002; Weihrauch et al. 2002).

A candidate for both, $\text{Na}^+/\text{HCO}_3^-$ -cotransporter as well as $\text{Cl}^-/\text{HCO}_3^-$ -exchanger, has been shown to be expressed in the gill epithelium of osmoregulating *C. maenas* with a higher mRNA expression level in posterior gills (2-3-fold; Fehsenfeld and Weihrauch 2013). Even though mRNA levels of the anion exchanger were 3-fold higher than for the $\text{Na}^+/\text{HCO}_3^-$ -cotransporter, anterior gills still exhibited a detectable mRNA expression level of the $\text{Na}^+/\text{HCO}_3^-$ -cotransporter comparable to mRNA levels for gill $\text{V-(H}^+)\text{-ATPase}$ (Fehsenfeld and Weihrauch 2013). While the $\text{Cl}^-/\text{HCO}_3^-$ -exchanger was significantly upregulated in posterior gills of osmoregulating green crabs in response to hypercapnia (ca. 1.1-fold, Fehsenfeld et al. 2011), the $\text{Na}^+/\text{HCO}_3^-$ -cotransporter responded to dilute salinity acclimation (ca. 1.3-fold upregulation; Towle et al. 2011).

In the thick ascending loop (TAL) of Henle in the mammalian kidney, a electroneutral $\text{Na}^+/\text{HCO}_3^-$ -cotransporter has been observed to transport HCO_3^- and Na^+ from the blood into the cell (Krapf 1988). Based on the identified similarity of the transporter inventory between the mammalian TAL and the gill epithelium of *C. maenas* (apical $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -cotransporter, basolateral Na^+/K^+ -ATPase, K^+ - and Cl^- -channels; Riestenpatt et al. 1996), a similar distribution and function of a basolateral $\text{Na}^+/\text{HCO}_3^-$ -cotransporter can therefore be postulated in the green crab. Recently, a basolateral $\text{Na}^+/\text{HCO}_3^-$ -cotransporter has also been identified to be of high importance in acid-base regulation in the bigfin reef squid (*Sepioteuthis lessoniana* (Férussac, 1831 in Lesson, 1830–1831), Hu et al. 2014). Interestingly, a basolateral $\text{Na}^+/\text{HCO}_3^-$ -cotransporter as well as an apical $\text{Cl}^-/\text{HCO}_3^-$ -exchanger have been postulated to be involved in acid-base regulation in the osmoconforming crab *N. granulata* (Tresguerres et al. 2008). Tresguerres et al. show in this study that symmetrically (i.e. identical in the apical and basolateral saline) elevated $[\text{HCO}_3^-]$ in experiments on split gill lamellae resulted in a switch from branchial acid- to base secretion, as well as a DIDS-insensitive increase in the trans-epithelial voltage that depended on the presence of Cl^- . In contrast, a decrease of pH in the perfusion solution lead to an increase in acid excretion that was Na^+ -dependent and DIDS-sensitive while also leading to an increase in the trans-epithelial voltage. Hence, the effect of tenidap as observed in the present study might likely be explained by tenidap mainly targeting a basolateral $\text{Na}^+/\text{HCO}_3^-$ -cotransporter that would provide the major carbonic anhydrase-independent HCO_3^- source for the epithelial cell. A potential apical anion exchanger on the other hand would provide a way for HCO_3^- out of the cell and accordingly, its down-regulation as seen in response to hypercapnia (Fehsenfeld et al.

2011) would likely help to retain HCO_3^- to buffer the experienced (extracellular) acid-load.

V-(H⁺)-ATPase

In contrast to freshwater-acclimated crustaceans like the Chinese mitten crab (*Eriocheir sinensis* (Milne-Edwards, 1853), Onken and Putzenlechner 1995; Henry et al. 2012) the V-(H⁺)-ATPase in *C. maenas* is not involved in osmoregulatory processes in the gills, but contributes to active ammonia excretion in osmoregulating green crabs (Weihrauch et al. 2002). While expression of V-(H⁺)-ATPase in the freshwater-acclimated *E. sinensis* (Onken and Putzenlechner 1995) and the true freshwater crab (*Dilocarcinus pagei* (Stimpson, 1861), Weihrauch et al. 2004a) is higher in posterior gills, it tends to be more abundant in anterior gills of *C. maenas* (Weihrauch et al. 2002, Fehsenfeld and Weihrauch 2013). Due to the cytoplasmic distribution of V-(H⁺)-ATPase in gill epithelial cells of brackish-water acclimated green crabs (Weihrauch et al. 2002), these authors postulated its presence in the membrane of vesicles. Acidification of intracellular vesicles via the V-(H⁺)-ATPase is believed to trap ammonia in these vesicles in the form of NH_4^+ to then be transported to the apical membrane for exocytosis, a process that can be blocked by colchicine, taxol and thiabendazole (Weihrauch et al. 2001). This hypothesis of an ammonia transport in acidified vesicles is supported by the results of the present study as ammonia as well as H^+ excretion is inhibited by the V-(H⁺)-ATPase blocker KM91104 (Kartner et al. 2010). A primarily apical distribution of V-(H⁺)-ATPase as shown for the posterior gills of the freshwater crab *D. pagei* (Weihrauch et al. 2004a) and as also seen in the gills of many freshwater fish (Gilmour and Perry 2009) seems unlikely for seawater and brackish-water acclimated crustaceans as it is not needed to generate an

electrochemical gradient for the uptake of Na^+ (Weihrauch et al. 2001). The rather small observed effect on H^+ and CO_2 excretion of apically applied KM91104 therefore may be attributed to only a transient presence of $\text{V}-(\text{H}^+)$ -ATPase in the apical membrane due to the fusion of the $\text{V}-(\text{H}^+)$ -ATPase carrying vesicles. As mentioned earlier, however, it cannot be excluded that the relatively large molecule KM91104 simply cannot cross the selective cuticle and therefore does not reach the apical membrane.

Na^+/K^+ -ATPase and K^+ -channels

As suggested by the present data, basolateral Na^+/K^+ -ATPase and K^+ -channels are essential not only for the excretion of ammonia, but also for acid-base equivalents. This is not surprising as both transporters have been shown to directly promote NH_4^+ (and therefore H^+) entry from the hemolymph into gill epithelial cells by substituting NH_4^+ for K^+ (Skou 1960; Lignon 1987; Weihrauch et al. 1998). In elasmobranchs and teleost fish, Na^+/K^+ -ATPase plays an important role in acid-base balance (Perry and Gilmour 2006; Gilmour and Perry 2009). Generating an electrochemical gradient over the basolateral membrane by pumping 3 Na^+ out of the cell in exchange for only 2 K^+ , Na^+/K^+ -ATPase is the major driving force for the excretion of H^+ via apical Na^+/H^+ -exchanger in acid excretory epithelial cells (Edwards et al. 2002; Choe et al. 2005). As noted earlier, inhibiting both the Na^+/K^+ -ATPase and basolateral K^+ -channels in brackish-water acclimated green crabs resulted in a significantly reduced ammonia excretion across the branchial epithelia (Weihrauch et al. 1998), a response also seen in seawater-acclimated green crabs in this study. Even though to be treated with caution due to the differences in the applied perfusion solution as explained earlier, Siebers et al. (1994) identified Na^+/K^+ -ATPase to also be involved in pH regulation in acid-base regulation.

Na⁺/H⁺-exchanger

While a potential electrogenic Na⁺/H⁺-exchanger (2Na⁺/1H⁺) has been identified to be present in crustacean gills (Shetlar and Towle 1989), its localization is not clear.

Inhibitor experiments on isolated gills and spilt gill lamellae of osmoconforming crabs like *Cancer antenarius* (Stimpson, 1856) and *Petrolishtes cinctipes* (Stimpson, 1871) (Hunter and Kirschner 1986) as well as *C. maenas* (Weihrauch et al. 1998) indicated an apical distribution for this transporter. Follow-up studies, however, showed that effects of amiloride on ammonia excretion resulted from the inhibitor's interference with the cuticle and the effect of this drug on a potentially apically localized Na⁺/H⁺-exchanger remains unknown (Onken and Riestenpatt 2002; Weihrauch et al. 2002). While Siebers et al. (1994) observed no differences in fluxes of acid-base equivalents across the gill epithelium of brackish-water acclimated *C. maenas* when the Na⁺/H⁺-exchanger was inhibited by apically and basolaterally applied amiloride, these results have to be treated with caution not only because of the potential interference of the cuticle, but also due to the composition of the perfusion solution. First of all, Siebers et al. (1994) applied symmetrical conditions (identical media on both sides of the epithelium) with diluted seawater as the bathing and perfusion solution that did not contain any NH₄⁺. Additionally, the pH was buffered to 8.1 with 0.75 mmol L⁻¹ TRIS basically eliminating actual changes of free acid-base equivalents. However, a basolateral Na⁺/H⁺-exchanger has been identified to be involved in acid-base regulation of gill epithelia in brackish-water acclimated crabs *N. granulata* (Tresguerres et al. 2008), promoting intracellular Na⁺ uptake in exchange for H⁺ that is excreted into the hemolymph. A similar phenomenon has also been observed in the TAL where NH₄⁺ has been shown to

substitute for the H^+ in basolateral NHE-4 to be transported into the blood in exchange for Na^+ (Bourgeois et al. 2010; Weiner and Verlander 2013).

As can be seen in figure 4, Na^+/H^+ -exchangers exhibit a complex phylogeny. Even though a clear distinction of the included sequences into two monophyletic groups can be made, both groups include basolateral, intracellular and apical transporters and hence provide no clear separation of NHE with potentially different functions per clade. For a more detailed analysis on evolutionary origins of eukaryotic NHEs the reader is referred to Brett et al. (2005). The most widely expressed isoform of Na^+/H^+ -exchanger to be found in virtually all vertebrate membranes, NHE-1, has also been shown to be present in the basolateral TAL and likely plays a more universal role as housekeeping gene for pH and volume regulation (Bianchini et al. 1995; Landau et al. 2007). In *A. egyptii*, NHE-3 is associated with the basolateral membrane of the malpighian tubules, the midgut and the ion-transporting sector of the gastric caeca (Pullikuth et al. 2006). As can be seen in the maximum likelihood analysis, the identified NHE of *C. maenas* falls into the same monophyletic group as NHE-1 (*H. sapiens*) and sisters to the NHE-3 of *A. aegypti*. In addition to the therefore likely basolateral presence of NHE in *C. maenas* gill epithelium due to its similarity to *A. aegypti* NHE-3, an expression in the cytoplasm is also possible. In the transcriptome of *H. araneus*, two isoforms of NHE have been identified. Interestingly, they show highest similarity to the intracellularly expressed NHE-8 of *A. aegypti* (Piermarini et al. 2009), but also align with the potentially apical isoform NHE-7/9 of *A. aegypti* (Blaesse et al. 2010) and the apical NHE-8 of *H. sapiens* (Goyal et al. 2003). Recently, an apical NHE was identified in antennal glands of the semi-terrestrial decapod crustacean *Ocypode stimpsoni* (Ortmann, 1897) (Tsai and Lin 2014).

Nehrke and Melvin (2002) observed an expression of Na^+/H^+ -exchanger (NHX-3) in the cytoplasm (vesicular membranes) of the hypodermis of the soil-dwelling nematode *C. elegans*. Furthermore, in the human nervous system, a Na^+/H^+ -exchanger (NHE-5) is associated with vesicles (Lukashova et al. 2013). The similarity of *H. araneus* NHE to both membrane and cytoplasmic isoforms of NHEs strengthens the hypothesis of an additional, not yet identified vesicle-associated form of Na^+/H^+ -exchanger in *C. maenas* in addition to the likely basolateral and potential apical presence of this transporter.

Carbonic anhydrase

The results for CA in the present study have to be treated with caution as the effects described are only observable in the third perfusion step and not immediately following the application of the inhibitor. This may be due to the fact that the inhibitor acetazolamide only slowly penetrates the membranes, and therefore can only exhibit its full effect after a longer application (Holder and Hayes 1965; Teppema et al. 2001).

In the gills of *C. maenas*, two isoforms of branchial CA have been identified, a cytoplasmic and a membrane bound isoform (Boettcher et al. 1990; Serrano and Henry 2008). In teleost fish, mainly the cytosolic isoform of CA has been identified to be present in the gill epithelium, while elasmobranch gills express both the cytosolic and membrane-bound isoforms (Gilmour and Perry 2009). Also in the kidney of fish both the cytosolic and membrane bound CA isoforms play a role in acid-base regulation (Gilmour and Perry 2009). In osmoconforming seawater-acclimated green crabs, CA activity is similar in all gills with a high expression of the membrane-bound isoform, whereas upon acclimation to brackish-water carbonic anhydrase activity increases 8-fold mainly due to an increase of the cytoplasmic pool (Henry et al. 2003; Serrano and Henry 2008). Data

from the present study suggests the involvement of both isoforms, but indicates a potentially more important role for membrane-bound carbonic anhydrase as the primary source for basolateral CO_2 entry into the epithelial cell by generating a partial pressure gradient for CO_2 (ΔP_{CO_2} , figures 5, 6).

Hypothesized mechanism for proton excretion across the anterior gill epithelium of seawater-acclimated *C. maenas* (figure 5)

The major source of intracellular protons in the gill epithelium in the proposed model is the basolateral entry of CO_2 into the epithelial cell through diffusion, or possibly through a basolateral Rhesus-like protein as discussed in Weihrauch et al. (2004b), and its immediate conversion to H^+ and HCO_3^- by a membrane-bound carbonic anhydrase (Serrano and Henry 2008) generating a gradient for CO_2 over the membrane. The major excretory pathway for protons into the environment then is *via* acidified vesicles or directly across the apical membrane *via* an apical Na^+/H^+ -exchanger or $\text{V}-(\text{H}^+)$ -ATPase from the fused vesicle membrane. The excess HCO_3^- formed in this reaction may leave the cell apically *via* a potential anion exchanger. Inhibiting (membrane-bound) CA leads to the weakening of the CO_2 gradient over the basolateral membrane and ultimately less CO_2 entering the cell. Less intracellular CO_2 results in lower intracellular H^+ but higher hemolymph H^+ which translates into the observed decrease in H^+ excretion rates. Additionally, intracellular metabolically produced CO_2 might indirectly serve as another source of H^+ due to the cytoplasmic CA-mediated hydration to H_2CO_3 and subsequent dissociation to H^+ and HCO_3^- . Taking into account the contribution of the cytoplasmic carbonic anhydrase, less of the intracellular (metabolic) CO_2 might be present in the dissociated form when CA is blocked, therefore again lowering intracellular H^+

concentrations which would consequently lead to a decreased proton excretion *via* vesicular ammonia trapping and/or direct excretion across the apical membrane as suggested in the present study.

As an additional proton source, NH_4^+ (weak acid) enters the epithelial cell *via* a basolateral Na^+/K^+ -ATPase (supported by K^+ -channels; Skou 1960; Weihrauch et al. 1998). Alternatively, NH_4^+ might be directly excreted across the apical membrane, possibly *via* ammonia transporters (Amts), which have recently been identified to be expressed in gills of decapod crustaceans (Transcriptomes of *H. araneus*, *Procambarus clarkii* (Girard, 1852) and *C. maenas*, Weihrauch pers. comm.). Inhibiting basolateral Na^+/K^+ -ATPase eliminates this direct pathway, therefore providing less intracellular NH_4^+/H^+ and leading to its accumulation in the hemolymph, therefore resulting in the direct reduction of H^+ excretion *via* acidified vesicles or directly *via* the apical membrane. A similar effect is observed when basolateral K^+ -channels are inhibited: the resulting build-up of intracellular K^+ will lead to an decreased electrochemical gradient for K^+ and thereby indirectly inhibit the NH_4^+ transporting Na^+/K^+ -ATPase.

Intracellular protons are pumped into vesicles mainly *via* a $\text{V}-(\text{H}^+)$ -ATPase, and possibly also by a vesicular Na^+/H^+ -exchanger (see paragraph above). Within the vesicles, H^+ traps NH_3 by forming NH_4^+ so that proton excretion *via* this potentially major H^+ excretory pathway is closely linked to ammonia excretion as hypothesized by Weihrauch et al. (2002). Targeting this hypothesized vesicular $\text{V}-(\text{H}^+)$ -ATPase and Na^+/H^+ -exchanger with the inhibitors KM91104 and amiloride, respectively, prevented H^+ from entering these vesicles and consequently is not able to be excreted *via* this pathway, explaining the observed decrease in H^+ excretion. Alternatively a basolateral Na^+/H^+ -

exchanger would provide a way for (excess) H^+ out of the cell into the hemolymph and might help to regulate hemolymph acid-base balance.

When the H^+ (NH_4^+) loaded vesicles containing $V-(H^+)$ -ATPase reach the apical membrane, they fuse and release their contents into the environment. This fusion is hypothesized to provide the (transient) presence of an apical $V-(H^+)$ -ATPase in the apical membrane under control conditions, which might promote an additional, vesicular-independent way for proton excretion. This is supported by the observed decrease of H^+ excretion as a result of apical application of KM91104 to block $V-(H^+)$ -ATPase in the present study.

Blocking a basolateral bicarbonate transporter (likely a Na^+/HCO_3^- -cotransporter) should lead to the accumulation of HCO_3^- in the hemolymph. To prevent an extracellular alkalosis, this excess hemolymph HCO_3^- would need to be buffered by H^+ , potentially provided by basolateral Na^+/H^+ -exchanger from the (excess) intracellular pool of H^+ , therefore resulting in a decrease of H^+ excretion as it was indeed observed in the present study when tenidap was applied basolaterally.

Hypothesized mechanism for CO_2 excretion across the anterior gill epithelium of seawater-acclimated *C. maenas* (figure 6)

As described earlier, CO_2 possibly enters the cell over the basolateral membrane by membrane diffusion or an identified branchial Rhesus-like protein (Weihrauch et al. 2004b). The proposed immediate hydration of CO_2 to H_2CO_3 (catalysed by CA) and its dissociation into H^+ and HCO_3^- generates a ΔP_{CO_2} over the basolateral membrane so that CO_2 fluxes are directed from the hemolymph into the epithelial cell. By inhibiting this potential membrane-bound carbonic anhydrase (Boettcher et al. 1990, Serrano and Henry

2008) and therefore the rapid dissociation of CO_2 into H^+ and HCO_3^- , the establishment of a ΔP_{CO_2} across the basolateral membrane would be prevented, therefore leading to the accumulation of CO_2 in the hemolymph instead (equal to a decrease in CO_2 excretion rates, as observed in the present study). Additionally, a cytoplasmic carbonic anhydrase (Boettcher et al. 1990, Serrano and Henry 2008) provides CO_2 from HCO_3^- (entering by a basolateral $\text{Na}^+/\text{HCO}_3^-$ -cotransporter) binding to (excess) intracellular H^+ , likely generated from the high protein metabolism of the gill. When blocked, less intracellular CO_2 is generated which consequently results in less CO_2 potentially being directly excreted across the apical membrane *via* diffusion and/or facilitating channels (i.e. Rhesus-like protein). The decrease of CO_2 excretion (translating into an increase in hemolymph CO_2) observed when Na^+/K^+ -ATPase and K^+ -channels are blocked basolaterally can be attributed to the indirect effect of the resulting accumulation of H^+ in the hemolymph as described above. The excess hemolymph H^+ would be buffered by HCO_3^- and form CO_2 , hence increasing hemolymph $p\text{CO}_2$ and translating into a decrease of CO_2 excretion rates. HCO_3^- might potentially be delivered by the basolateral $\text{Na}^+/\text{HCO}_3^-$ -cotransporter switching its direction due to the change in concentration gradients for Na^+ over the basolateral membrane.

Finally, when a basolateral bicarbonate transporter (likely $\text{Na}^+/\text{HCO}_3^-$ -cotransporter) is blocked a major CO_2 -independent intracellular source for HCO_3^- is eliminated and instead, HCO_3^- accumulates in the hemolymph. This excess hemolymph HCO_3^- is buffered by H^+ (or potentially NH_4^+) and creates CO_2 , resulting in the observed increase in hemolymph $p\text{CO}_2$ and translating to a net decrease of CO_2 excretion across the epithelial membrane.

Hypothesized mechanism for $\text{NH}_3/\text{NH}_4^+$ excretion across the anterior gill epithelium of seawater-acclimated *C. maenas* (figure 5)

Generally, ammonia excretion as proposed in the hypothesized model of this study depends to a significant amount on NH_3 trapping in acidified vesicles. In contrast to H^+ and CO_2 excretion, however, $\text{NH}_3/\text{NH}_4^+$ excretion does not seem to depend on HCO_3^- entering the cell *via* the basolateral $\text{Na}^+/\text{HCO}_3^-$ -cotransporter.

As applying KM91104 basolaterally is believed to target the vesicular $\text{V}-(\text{H}^+)$ -ATPase (Weihrauch et al. 2001), the immediate result should be a decrease in ammonia excretion *via* the proposed acidified vesicles due to the lack of $\text{V}-(\text{H}^+)$ -ATPase mediated H^+ entry into the vesicles. When inhibiting the basolateral Na^+/K^+ -ATPase, a direct pathway for NH_4^+ (NH_3) to enter the cell is compromised, resulting in a the obvious reduction of $\text{NH}_3/\text{NH}_4^+$ excretion *via* acidified vesicles. Again, a similar effect is observed when basolateral K^+ -channels are inhibited, which grant the backflow of K^+ from the cytoplasm into the hemolymph and thereby allow the Na^+/K^+ -ATPase to maintain the cellular membrane potential and low cytoplasmic Na^+ concentrations to energize branchial NaCl transport processes. As demonstrated by Riestenpatt et al. (1996), blocking these basolateral K^+ -channels, and therefore indirectly the Na^+/K^+ -ATPase, consequently resulted in a reduced ion flux across the gill epithelium, demonstrated by the observed inhibition of the short-circuit current by *ca.* 85%. The pump would no longer be able to actively transport NH_4^+ , and thereby H^+ , into the cell. Additionally, NH_4^+ might also directly substitute for K^+ in the potentially bi-directional K^+ -channels identified in the basolateral membrane of the gills of *C. maenas* (Riestenpatt et al. 1996; Weihrauch et al.

1998) and therefore an additional direct way for ammonia to possibly enter the cell would be eliminated.

As discussed earlier, the Na^+/H^+ -exchanger (NHE) potentially promotes H^+ entry into acidified vesicles. Therefore its inhibition should be comparable to the inhibition of the $\text{V-(H}^+)\text{-ATPase}$ and would lead to the observed decrease of ammonia excretion *via* acidified vesicles. In addition, even though details on the localization of NHE in *C. maenas* are lacking, it can be speculated based on the maximum likelihood analysis that similar to *H. araneus* also *C. maenas* might express a basolateral (and/or apical) NHE, allowing for proton excretion into the hemolymph.

Conclusions

In conclusion, the present study is one of the first comprehensive studies on the mechanisms of acid-base regulation in invertebrates and specifically crustaceans. Understanding the underlying principles and mechanisms of acid-base regulation in the green crab might prove important to assess their potential and capability for further invasion of marine habitats especially in respect to a future ocean scenario. Additionally, the results of the present study elucidated basic principles for general acid-base regulation in invertebrates that might well be transferable to other marine species and/or other acid-base regulatory tissues. However, the application of inhibitors on isolated gill perfusion experiments as used in this study may exhibit certain limitations (i.e. specificity of the pharmaceuticals, potential side-effects) and therefore, follow-up studies would be required to verify the obtained data. Nonetheless, the present study provides a solid basis for deepening studies in the future.

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Tables

Table 1. GenBank accession numbers and description of Na⁺/H⁺-exchangers as used to generate the NHE gene tree (see figure 4).

Species	Accession no.	Description
<i>Caenorhabditis elegans</i>	NP_510622	NHX-1
	NP_495614	NHX-2
	NP_001023735	NHX-3 isoform a
	NP_001024562	NHX-4 isoform a
	NP_001024720	NHX-5 isoform a
	NP_001022214	NHX-6 isoform a
	AAM18109	NHX-7
	NP_001021728	NHX-8 isoform a
	NP_001023627	NHX-9 isoform a
<i>Homo sapiens</i>	NP_003038.2	sodium/hydrogen exchanger 1
	Q9UBY0.1	sodium/hydrogen exchanger 2
	AAI01670	sol. car. fam. 9/sodium/hydrogen exchanger (NHE-3)
	NP_001011552	sodium/hydrogen exchanger 4 precursor
	Q14940	sodium/hydrogen exchanger 5
	NP_001036002	sodium/hydrogen exchanger 6 isoform a precursor
	NP_115980	sodium/hydrogen exchanger 7 isoform 2 precursor
	Q9Y2E8	sodium/hydrogen exchanger 8
	BAD69592	sodium/proton exchanger NHE9
<i>Aedes aegypti</i>	AAM63432.1	Na ⁺ /H ⁺ exchanger (NHE-3)
	XP_001654568.1	AAEL002051-PA (NHE-7,9)
	ACJ02512.1	Na/H exchanger 8
<i>Carcinus maenas</i>	AAC26968	sodium/hydrogen exchanger
<i>Hyas araneus</i>	-	Contig HaCtg18509 ⁽¹⁾
	-	Contig HaIso05788 (predicted NHE-8) ⁽¹⁾

Specific descriptions for each locus are from on the NCBI protein database. ⁽¹⁾retrieved from the transcriptome generated by Harms et al. (2013).

Figure captions

Figure 1. Time series of changes in hemolymph acid-base parameters of seawater-acclimated (32 ppt) *Carcinus maenas* during the first 48 hours of exposure to elevated environmental $p\text{CO}_2$ (hypercapnia; 1% CO_2). Changes in (A) hemolymph pH, (B) hemolymph $p\text{CO}_2$, and (C) hemolymph HCO_3^- in high $p\text{CO}_2$ crabs (filled squares) versus control crabs (open diamonds). Hemolymph was drawn through a hole in the carapace sealed with dental dam at 0, 6, 12, 24 and 48 hours. Asterisks denote significant differences between control and high $p\text{CO}_2$ animals (two-way ANOVA, $p < 0.05$, $n = 5-6$), whereas letters denote differences either within control (upper case, $n = 4$) or high $p\text{CO}_2$ (lower case, $n = 6$) animals over time (repeated-measures one-way ANOVA, $p < 0.05$).

Figure 2. Comparison of the control perfusion step of osmoconforming and osmoregulating *Carcinus maenas*. Anterior gill #5 was perfused for 30 min with the respective perfusion solution mimicking the ionic composition of their hemolymph. Changes in the respective parameters hemolymph ammonia (A), H^+ (B), CO_2 (C) and HCO_3^- (D) have been calculated based on their loss in the perfusate (= perfusion solution collected after one passage through the gill) in comparison to the initial perfusion solution. Protons are expressed as ΔH^+ to also account for potential shifts in chemical properties of the perfusate (Stewart 1978). The bigger ΔH^+ , the less H^+ remained in the perfusate after one passage through the gill, indicating a net excretion of H^+ . For CO_2 and HCO_3^- excretion rates the calculation was based on the measured pH and total carbon as described in the material and methods. Values for brackish-water acclimated green crabs

are taken from Fehsenfeld and Weihrauch (2013). Asterisks denote significant differences between gills of different salinity acclimations (Student's t-test with $p < 0.05$, $n = 4-7$).

Figure 3. Relative changes in perfusate $[H^+]$, CO_2 and ammonia excretion rates of isolated anterior gill #5 of seawater acclimated *Carcinus maenas* during gill perfusion applying inhibitors. Negative values indicate an accumulation of the respective component in the perfusate (= perfusion solution after one passage through the gill) compared to the initial perfusion solution, i.e. perfusate concentrations exceeded concentrations in the initial perfusion solution. Positive values between 0-1 indicate a less pronounced loss of the respective component (equivalent to lower excretion, or smaller ΔH^+ in case of protons) in the perfusate in comparison to the perfusion solution. B, basolateral; A, apical; BCT, bicarbonate transporter; HAT, V- (H^+) -ATPase; NKA, Na^+/K^+ -ATPase; chan, channel; NHE, Na^+/H^+ -exchanger; CA, carbonic anhydrase. Inhibitors were: 100 $\mu\text{mol L}^{-1}$ tenidap (BCT), 20 $\mu\text{mol L}^{-1}$ KM91104 (V- (H^+) -ATPase), 5 mmol L^{-1} ouabain (NKA), 12 mmol L^{-1} $BaCl_2$ (K^+ -channels), 100 $\mu\text{mol L}^{-1}$ amiloride (NHE), 1 mmol L^{-1} acetazolamide (CA). Asterisks denote significant changes in the inhibitor step compared to the control perfusion (bold dashed lines; paired t-test with $p < 0.05$, $n = 4 - 8$). The light dashed lines indicate a 50% change.

Figure 4. Maximum likelihood tree (unrooted) of Na^+/H^+ -exchangers. The tree with the highest log-likelihood (-8093.0660) is shown and drawn to scale. The scale bar indicates the number of amino acid substitutions per site. Numbers beside branches represent bootstrap values in % (1000 replicates). Upper case numbers represent the

respective reference: (1) Nehrke and Melvin 2002; (2) Pullikuth et al. 2006; (3) Weiner and Verlander 2013; (4) Lukashova et al. 2013; (5) Goyal et al. 2003; (6) Piermarini et al. 2009; (7) Blaesse et al. 2010 and (8) Linz and Busch 2003. GenBank accession numbers are provided in table 1.

Figure 5. Hypothetical model for the regulation of proton and ammonia excretion in the anterior gill epithelium of seawater-acclimated *Carcinus maenas*. Key-players have been identified in perfusion experiments on isolated gill #5 by applying inhibitory pharmaceuticals for the respective components. Dashed arrows with a question mark indicate potential direct excretion of H^+/NH_4^+ across the apical membrane. Thickness of arrows indicate the estimated importance for the respective pathway. Grey transporters seem only relevant for H^+ -excretion. Rh, Rhesus-like protein; CA, carbonic anhydrase; NHE, Na^+/H^+ -exchanger; Amt, ammonia transporter; ATP, ATPases (basolateral Na^+/K^+ -ATPase, vesicular / apical $\text{V}-(\text{H}^+)$ -ATPase).

Figure 6. Hypothetical model for the regulation of CO_2 excretion in the anterior gill epithelium of seawater-acclimated *Carcinus maenas*. Key-players have been identified in perfusion experiments on isolated gill #5 by applying inhibitory pharmaceuticals for the respective components. Dashed arrows with a question mark indicate possible direct excretion of NH_4^+ across the apical membrane. Thickness of arrows indicate the estimated importance of the respective pathway. Rh, Rhesus-like protein; CA, carbonic anhydrase; ATP, ATPases (Na^+/K^+ -ATPase).

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