Differential acid-base regulation in various gills of the green crab *Carcinus maenas*: Effects of elevated environmental pCO_2

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Abstract

Euryhaline decapod crustaceans possess an efficient regulation apparatus located in the gill epithelia, providing a high acclimation potential to varying environmental abiotic conditions. Even though many studies focussed on the osmoregulatory capacity of the gills, acid-base regulatory mechanisms have obtained much less attention. In the present study, underlying principles and effects of elevated pCO_2 on acid-base regulatory patterns were investigated in the green crab *Carcinus maenas*. In gill perfusion experiments, all gills of control green crabs were observed to up-regulate the pH of the hemolymph by 0.1 – 0.2 units. Anterior gills, especially gill 4, were identified to be most efficient in the equivalent proton excretion rate. Ammonia excretion rates mirrored this pattern among gills, indicating a linkage between both processes. In specimen exposed to elevated pCO_2 levels for at least 7 days, mimicking a future ocean scenario as predicted until the year 2300, hemolymph K^+ and ammonia concentrations were significantly elevated, and an increased ammonia excretion rate was observed. A detailed quantitative gene expression analysis revealed that upon elevated pCO_2 exposure, mRNA levels of transcripts hypothesized to be involved in ammonia and acid-base regulation (Rhesus-like protein, membrane-bound carbonic anhydrase, Na^+/K^+-ATPase) were affected predominantly in the non-osmoregulating anterior gills.

Keywords: pH; gill perfusion; quantitative real-time PCR; ammonia; Rhesus-like protein

1. Introduction

During the last 150 years, a notable increase in atmospheric CO_2 occurred, elevating pCO_2 from 28 Pa (pre-industrial, ~1750; 1 Pa = 10 ppm) to 39 Pa by the year 2000 (IPCC 2007). Although large changes in atmospheric CO_2 have been recorded throughout earth history, this current anthropogenic caused increase in pCO_2 is much more rapid and severe than the cyclic changes of pCO_2 during the last 20 million years (Tripati et al. 2009). With increasing atmospheric pCO_2, a decrease in global surface ocean pH of between 0.3 to 0.5 units due to oceanic CO_2 uptake is predicted until the year 2100, and a change of up to 1.4 units until the year 2300, respectively (Caldeira 2005, IPCC 2007). The resulting changes in carbonate chemistry speciation, termed 'ocean acidification', may become a general stress factor modulating future marine and freshwater communities by differentially influencing the fitness of aquatic species (Doney et al. 2009, Fabry et al. 2008, Kroeker et al. 2010, Melzner et al. 2009). Elevated environmental pCO_2 results in an increased extracellular CO_2 partial pressure in the animal, as positive diffusion gradients of CO_2 have to be maintained in order to excrete metabolic CO_2 (Melzner et al. 2009). This can then lead to an acidification of extracellular fluids (Dejours & Beekenkamp 1977, Thomsen & Melzner 2010a, Truchot, 1975). However, several active, high metabolic species with pH sensitive respiratory pigments regulate extracellular pH (pH_e): active modulation of the extracellular carbonate system leads to bicarbonate accumulation and pH compensation while maintaining pCO_2 values sufficiently high for diffusive CO_2 flux out of the animal (fish: Larsen et al. 1997; crustaceans: Appelhans et al. (personal communication), Spicer et al. 2006; cephalopods: Gutowska et al. 2009; Hu et al. 2011).

In teleost fish, cephalopods, and decapod crustaceans, the majority of the acid-base relevant ion regulatory apparatus is located in gill epithelia. Structural and functional analysis of these organs in crustaceans suggests a specialization of gill arches which lead to the general differentiation of anterior and posterior gills (reviewed by Freire et al. 2008). Until recently, anterior gills were mainly associated with gas exchange while the posterior gills were linked to ion regulation. However, a clear distinction in functionality between both groups of gills can only be applied for NaCl absorption, while NH_4^+ excretion takes place in both gill types (Martin et al. 2011, Weihrauch et al. 2004b) and acid-base regulating properties, Ca^{2+} transport and NaCl secretion have not yet been localized (reviewed by Freire et al. 2008). Although carbonic anhydrase (CA) - the enzyme converting CO_2 to HCO_3^- and vice versa - was identified to be more active in posterior gills (Henry et al. 2003), another transporter associated with acid-base regulation, the V-(H^+)-ATPase, was demonstrated to be more abundant in anterior gills of *C. maenas* (Weihrauch et al. 2001). In contrast, V-(H^+)-ATPase in the freshwater acclimated Chinese mitten crab *Eriocheir sinensis* and the true freshwater crab *Dilocarcinus pagei* is predominantly present in the osmoregulatory active posterior gills and closely linked to the Cl^-/HCO_3^--exchanger in short-circuited gill lamellae (Onken & Putzenlechner 1995, Weihrauch et al. 2004a). In general, acid-base regulation through the gills is closely linked to osmoregulation. It is thought that net proton extrusion in gills of fish is primarily achieved via active (V-(H^+)-ATPase) and secondarily active ion transport molecules (e.g. sodium proton exchangers, NHE; sodium bicarbonate co-transporters, NBC), with a strong supporting role of carbonic anhydrases (CA) and Na^+/K^+-ATPase (NKA; Gilmour & Perry 2009). Studies on isolated gills of the crab *Neohelice (Chasmagnathus) granulata* (Tresguerres et al. 2008) suggest that basolateral

NHE and NKA, CA, and apical anion exchangers participate in a response stimulated by elevated hemolymph HCO_3^-, while CA, apical V-(H^+)-ATPase and basolateral HCO_3^--dependent co-transporters mediate the response to a low pH hemolymph. However, up to date only CA and NKA could be proven to participate in acid-base regulation in *Carcinus maenas* in the osmoregulatory active posterior gills (Siebers et al. 1994). Although models for osmoregulatory NaCl transport and for acid-base regulation in gills of euryhaline crabs have been postulated (reviewed by Freire et al. 2008, Towle & Weihrauch 2001), the transporter inventory in decapod crustacean gill epithelia and their functional interactions are not fully understood at present.

In the present study, whole animal experiments, gill perfusion studies, and gene expression analysis of control and elevated environmental pCO_2 exposed green crabs have been performed in order to characterize general acid-base regulatory patterns. In order to identify branchial key players that are affected by predicted anthropogenic induced changes in the marine pH and carbonate system, 6 functionally different gills (the osmoregulatory inactive anterior gills 4 and 5, the intermediate gill 6, as well as the osmoregulatory highly active posterior gills 7, 8 and 9) have been investigated simultaneously.

2. Material & Methods

2.1. Animals

Male green crabs *Carcinus maenas* were obtained from the Bamfield Marine Sciences Center BC, Canada and kept at the Animal Holding Facility of the University of Manitoba, Winnipeg,

97 Canada in aerated 1200 L tanks with artificial seawater adjusted to a salinity of 32 ppt at 14 °C
98 (Seachem Marine Salt ®) until experimentation.
99 Green crabs were acclimated to brackish water of a salinity of 10 ppt (Seachem Marine Salt®) in
100 aerated 120 L aquaria for a minimum of 7 days with a before exposed to elevated pCO_2 (IKS
101 Aquastar; iKS Computer Systeme GmbH, Germany). Each aquarium contained 8 animals marked
102 with nail polish for identification. Animals were fed *ad libitum* with squid once a week. Water
103 parameters (pH, total carbon C_T and pCO_2) of the seawater in the aquaria were assessed daily
104 and water exchanged every one to two days. The pH was measured with the pH/ATC electrode
105 #300729.1 (Denver Instruments, Goettingen, Germany) connected to a pH-ISE meter model 225
106 (Denver Instruments, Goettingen, Germany), while C_T was measured using the Corning 965
107 carbon dioxide analyser. Seawater pCO_2 was then calculated using the Excel add-in CO2SYS
108 (Lewis & Wallace 1989) and the appropriate parameter and constants (K1, K2 from Mehrbach
109 et al. (1973) refit by Dickson and Millero (1987)), $KHSO_4$ dissociation constant after Dickson
110 (1990), NBS scale [mol/kg H_2O]; table 1).
111 After acclimation to salinity of 10 ppt at a pH of 7.7 ± 0.0 and a pCO_2 of 53.5 ± 2.1 Pa, animals
112 were either used as control animals, or exposed to elevated pCO_2 for a minimum of 7 days
113 (324.3 ± 20.0 Pa). The IKS Aquastar® was used to control the CO_2 influx in the experimental
114 tanks to reach a set pH of 7.0. After 7 days, elevated pCO_2 exposed animals were then used for
115 ammonia excretion measurements and in gill perfusion experiments.

2.2. Hemolymph analysis

In order to assess the carbonate system parameters and ionic composition of the hemolymph of *Carcinus maenas*, samples were taken from 6 individuals (each for control and elevated pCO_2 exposed animals) by puncturing the arthrodial membrane at the base of a walking leg with a sterilized syringe. Samples were centrifuged for 5 min at 5000 rpm and 4 °C. The supernatant was transferred to new tubes and pH and C_T were measured immediately as described above, before storing the samples at -80 °C for ion chromatographic analysis and ammonia measurements. Hemolymph pCO_2 and $[HCO_3^-]$ were calculated using following equations:

$$(1)\ pCO_2 = C_T / (10^{pH - pK1} \times \alpha CO_2 + \alpha CO_2)$$

$$(2)\ [HCO_3^-] = 10^{pH - pK1} \times \alpha CO_2 \times pCO_2$$

with pK1 being the first dissociation constant of carbonic acid and αCO_2 being the solubility coefficient for carbon dioxide as described by Truchot (1976).

Total ammonia concentrations of all samples were measured using a gas-sensitive NH_3 electrode (Orion 9512 from Thermo Scientific, Cambridgeshire, England) connected to a digital mV / pH meter (for the detailed method see Weihrauch et al. 1998; table 2).

To determine the ionic composition of the hemolymph, samples were analyzed by ion chromatography (Metrohm 850 Professional IC) applying cation (Na^+, Ca^{2+}, Mg^{2+} and K^+) and anion standards (Cl^-, SO_4^{2-}). Results of the IC for control animals were compared to other studies (Siebers 1982, Weihrauch et al. 1999, Winkler 1987) and adjusted were applicable in order to compose the perfusion solution. Chloride concentrations had to be decreased in the

perfusion solution in comparison to the hemolymph because NaCl salt was used as the major Na^+ and Cl^- source.

2.3. Whole body ammonia excretion

To determine the ammonia excretion rate of intact animals, green crabs acclimated to brackish water (salinity = 10 ppt) for 7 days were transferred to small buckets holding 2 L of aerated brackish water (controls). 10 ml water sample were taken after 10, 40 and 70 minutes and frozen at -20 °C until analysis. To investigate the effect of exposure to elevated pCO_2 on whole animal ammonia excretion rates, brackish water acclimated animals were exposed to 324.3 ± 20.0 Pa pCO_2 as described above before being transferred to buckets holding 2 L of the respective high pCO_2 brackish water. At the end of each experiment, crabs were blotted dry and weighed. In order to determine the background diffusion of ammonia out of the buckets, the same set-up was performed with no animals in the bucket. Total ammonia concentrations of all samples were measured using a gas-sensitive NH_3 electrode (Weihrauch et al. 1998).

2.4. Tissue preparation

For isolation of tissues for quantitative gene expression (qPCR) and gill perfusion experiments, one control or elevated pCO_2 exposed crab were placed on ice for 15 min and euthanized by destroying its ventral ganglion by pressing a spike through the ventral side of the body wall at the height of the anterior tip of the tail. One set of the gill pairs 4 to 9 was transferred to RNAlater® (Ambion, #AM7024) and frozen at -80 °C until total RNA isolation and gene

155 expression analysis by qPCR, while the second set was collected for gill perfusion and placed in
156 petri dishes containing brackish water.

157 2.5. Gill perfusion

158 The perfusion protocol and set-up followed the previous study by Weihrauch et al. (1999).
159 Collected gills 4 – 9 were placed into 50 ml beakers filled with 30 ml of artificial seawater of a
160 salinity of 10 ppt, resembling the control tank water green crabs were acclimated in (bathing
161 solution). In parallel for each step, 50 ml beakers were filled with the respective bathing
162 solution, but without gills, in order to monitor the pH drift of the solution alone. Air stones
163 ensured optimal aeration in the beakers. The perfusion solution was composed according to
164 the results from the carbonate system and ion chromatographic analysis, complemented by
165 literature values (see 2.2, table 2) and applied at a perfusion speed of 128 ± 0.1 µl/min using a
166 peristaltic pump (Sci 323 Watson-Marlow Bredel Pump, Falmouth Cornwall, England). The
167 perfusion solution contained (in mmol L^{-1}): 260 NaCl, 5 $CaCl_2$, 7 $MgCl_2$, 8 KCl, 7 $NaHCO_3$,
168 0.1 NH_4Cl, 0.3 Glucose, 0.1 Gluthation, 0.5 Glutamine. The pH was set to 7.9, respectively. Each
169 perfusion sequence was composed of 5 consecutive steps that were applied for 30 min each.
170 An initial control phase (pH 7.80 ± 0.02, control bathing solution) was followed by a low pH step
171 (pH 7.44 ± 0.02 of the bathing solution). In the third step another control phase was applied
172 (pH 7.81 ± 0.02), followed this time by a high pH step (pH 8.62 ± 0.02 of the bathing solution).
173 As a fifth step, each perfusion sequence was ended by a third control phase (pH 7.83 ± 0.03,
174 control bathing solution; figure 1). Immediately after each step, pH of the perfusate and
175 bathing solution was measured, including parallel beakers without gills to monitor the pH drift

of the solution only. Following the whole perfusion sequence, C_T was assessed using the Corning 965 carbon dioxide analyser and samples frozen at -80 °C until ammonia concentration measurement (including parallel solutions without gills as background reference).

2.6. Quantitative real-time polymerase chain reaction analysis (qPCR)

RNA from the gill set 4 – 9 stored in RNAlater® (Ambion, #AM7024) at -80 C° was extracted under RNase-free conditions using TRIZOL reagent (Invitrogen, Carlsbad , CA, USA). Following DNase treatment (DNase 1, Invitrogen), RNA was tested by PCR (40 cycles), using crab specific primers CrabRbS3F1 / CrabRbS3R1 (Table 3) targeting the ribosomal protein RbS3 (figure 2) and evaluated by gel electrophoresis, ethidium bromide staining and UV visualization. RNA was considered DNA-uncontaminated by absence of a PCR product compared to a positive control (gill cDNA template). 0.9 μg of DNA free RNA was reversely transcribed into cDNA using the iScript cDNA synthesis kit (Biorad, Mississauga, Ontario, Canada). The quality of all generated cDNAs was again tested by a PCR employing the primer pair CrabRbS3F1 / CrabRbS3R1 PCR. All primers to be employed in quantitative real-time PCR targeting sodium-hydrogen exchanger (NHE), Rhesus-protein (Rh), V-type H^+-ATPase subunit B (HAT), anion [Cl-/HCO3-] exchanger (AE), sodium-potassium ATPase α-subunit (NKA), sodium-bicarbonate co-transporter (NBC), membrane bound (CA-1) and cytoplasmic carbonic anhydrase (CA-2), were ensured to produce a single PCR product of the predicted size and were quantified using the imager Biorad Versadoc 4000 MP and Image Lab™ 3.0 software. For verification PCR products were sequenced at the Robarts Research Institute (London, Ontario, Canada). Standard curves of the respective genes were generated employing a dilution series of known quantities (10 pg - 1 fg

cDNA) of the respective gel extracted PCR product (QIAquick Gel Extraction Kit, Qiagen) of the target gene. For the standard curve, a R^2 value of >0.99 was required. Real-time PCR assays were performed employing cDNA transcribed from 45 ng total RNA, 1 µmol L^{-1} of each primer and SSO FastEvaGreen Supermix (Biorad, Mississauga, Ontario, Canada) in a 15 µl assay. Single product PCR was verified performing a melting curve analysis. As the housekeeping gene for relative real-time PCR, RbS3 was used. Suitability of RbS3 was evaluated by quantitative PCR showing similar expression levels of the gene in all gills from control and elevated pCO_2 exposed crabs (data not shown). Primer sequences and annealing temperatures for all real-time PCRs are listed in Table 3.

2.7. Statistics

All statistical analysis was performed using the software PAST (http://palaeo-electronica.org/2001_1/past/issue1_01.htm; Hammer et al. 2001). Outliers were identified by Grubb's test. All data sets for comparison of means were first tested for normal distribution with the Shapiro-Wilk test (α = 5 %), followed by log-transformation in case the null-hypothesis (data is normally distributed) had to be rejected. Levene's test was performed to ensure homogeneity of variance. In case of normally distributed data and homogeneity of variance, t-test was performed to compare two means and ANOVA was performed to compare more than one mean. The permutation t-test (permutation N = 9999) as included in PAST was performed in case homogeneity of variance was not given. In case of non-normally distributed data (before and after log-transformation), Mann-Whitney-U-Test was applied to compare two means, and

the Kruskil-Wallis-Test for more than one mean, respectively. All results were considered significant in case $p < 0.05$.

3. Results

3.1. Whole animal characteristics of control and elevated pCO_2 acclimated green crabs

Hemolymph composition. While hemolymph Cl^-, SO_4^{2-}, Ca^{2+} and Mg^{2+} levels did not change in elevated pCO_2 acclimated green crabs, Na^+ and HCO_3^- levels slightly increased ($p_{Na} = 0.09$, $p_{HCO} = 0.11$). A significant increase was observed for K^+ and ammonia concentrations. Hemolymph pH remained constant at 7.9 ± 0.3, while pCO_2 increased by ca. 30% (Table 2).

Whole body ammonia excretion rates. Ammonia excretion rates increased significantly from 157.34 ± 39.63 to 411.40 ± 92.11 nmol g^{-1} h^{-1} in elevated pCO_2 acclimated green crabs (t-test with $p = 0.02$).

3.2. Gill perfusion experiments

General observations. After only one gill passage, pCO_2 of the perfusate was decreased, while [HCO_3^-] and the pH of the perfusate (hemolymph) was significantly increased by 0.11 – 0.17 units by all gills of control animals compared to the initial perfusion solution (data for pH shown in figure 3). Figure 4A shows the equivalent proton decrease in the perfusate of control animals in the different steps of the experiment as described in figure 1. Under control conditions (control 1, figure 1), anterior gill 4 was most efficient in regulating the hemolymph

pH and excreted 3.8 fold as many protons per mg gill as the most inefficient gill 7. Gills of animals exposed to elevated pCO_2 still showed the same pattern, but did not differ significantly from control animals (data not shown). Ammonia excretion rates determined by measuring the decrease of ammonia concentration in the external medium (perfusate enriched with 100 μM ammonia) showed the same pattern for the single gills as the decrease in proton concentration (figure 4). When ammonia gain in the apical bath was measured instead, higher concentrations of ammonia were observed in the bath than were calculated as the perfusate loss, indicating the metabolically produced ammonia by the gills themselves. Metabolic ammonia accounted for 44.0 ± 5.5 % of the total ammonia enrichment in the bathing solution in all gills except for gill 8, in which it accounted for 83.0 %. No effects of long-term acclimation of green crabs to elevated pCO_2 levels on ammonia excretion rates of the single gills could be observed (data not shown).

Low and high pH challenge. Figure 5 shows the capacity of perfused gills to regulate pH when challenged with low (7.4) or high pH (8.6) in the surrounding medium (bath). The data demonstrate that in general, gills of control animals were less efficient in responding to a change in the external medium than gills of green crabs that were acclimated to elevated pCO_2 prior to gill perfusion. While gill 5 in control animals showed a significant decrease of proton excretion in the low pH perfusion step, elevated pCO_2 exposed green crabs compensated this effect and significantly increased proton excretion rates to initial levels. Gill 4 exhibited the same trend. In contrast, gill 7 increased proton excretion significantly in the high pH perfusion step.

Ammonia excretion rates of gills of control green crabs were not altered in the low and high pH challenge of the gill perfusion (figure 6). However, in elevated pCO_2 acclimated green crabs, a decreased ammonia excretion rate was observed for gill 5, as well as an increased ammonia excretion rate in gill 8 regarding the high pH perfusion step (figure 6).

3.3. Quantitative real-time PCR

Relative expression levels of transporters. The highest overall relative expression levels were observed for the genes encoding the Rhesus-like protein (Rh) and the anion [Cl^-/HCO_3^-] exchanger (AE; figure 7). While the V-(H^+)-ATPase (subunit B) was equally low expressed in all gills, a number of genes were differently expressed in the different gill arches.

The sodium-hydrogen exchanger (NHE) and AE were identified to be expressed two to four fold higher in the posterior gills than in the anterior gills. Further, expression of the Na^+/K^+-ATPase (α-subunit; NKA), sodium-bicarbonate co-transporter (NBC), membrane bound (CA-1) and cytoplasmic carbonic anhydrase (CA-2), was clearly restricted to the posterior gills and hardly detectable in anterior gills.

In contrast, Rh was identified to be the only of the tested transcripts that exhibited the tendency to a higher expression level in the anterior gills.

Noteworthy regarding the anterior gills is that gill 5 showed higher expression levels of almost all transcripts (NHE, Rh, HAT, AE, NKA, CA-2) compared to gill 4 and 6. In contrast, regarding the posterior gills, gill 8 exhibited lower expressions of almost all transcripts (NHE, Rh, HAT, NKA, NBC, CA-1, CA-2) compared to gill 7 and 9.

Changes of relative expression patterns of transporters upon long-term exposure to elevated pCO_2 levels. Expression patterns of key transcripts in acid-base and ion regulation showed clear differences among different gill arches (figure 8) upon exposure to elevated pCO_2. While gill 6 and 8 displayed a general tendency to up-regulate genes (significantly in case of the sodium-potassium ATPase NKA in gill 6; tendency of CA-1 in gill 8), expression levels of NHE, Rh, HAT and AE in gills 4 and 5 were at least in tendency lower in elevated pCO_2 acclimated green crabs compared to control crabs. Interestingly, Rh also showed down-regulation in the posterior gills 7 and 9. The NKA showed a significant up-regulation in gill 6 and a down regulation (tendency) in the osmoregulatory active gills 7 and 9. NBC exhibited slight up-regulation in gills 6, 7 and 8. CA-1 (membrane associated) was up-regulated in gill 4 (significantly, t-test with $p < 0.05$) and gill 8, while CA-2 (cytoplasmic form) only shows slight up-regulation in gill 8 besides slight down-regulation in gill 4.

4. Discussion

As a benthic predator in coastal regions, *C. maenas* often is confronted with environmental unfavorable conditions. For example, shore crabs of the Baltic Sea are subject to a highly fluctuating habitat (Thomsen et al. 2010b). Additionally, crabs exhibit a burying behavior (Bellwood 2002) that lets them encounter high environmental ammonia or low pH eventually (Weihrauch et al. 1999). Living in the intertidal zone, green crabs are also often found in tide pools where they are exposed to high temperatures and high pH (Truchot & Duhamel-Jouve 1980). Being the main regulatory organs in decapod crabs, the gills are discussed to play a

major role in the response upon diluted salinity (Lovett et al. 2006, Siebers et al. 1982, Torres et al. 2007) and changes in environmental ammonia levels (Martin et al. 2011, Weihrauch et al. 1999). While studies on osmoregulatory NaCl transport have been focussed on the mitochondria rich posterior gills, acid-base regulation – considered to take place in the thin, mitochondria poor anterior gills (Compere et al. 1989) – has not been investigated in nearly as much detail (reviewed by Freire 2008, Towle & Weihrauch 2001).

The present study identified various key features in regard to acid-base regulation on both levels, the whole animal and on individual gills. It demonstrates clearly that each gill is highly specialized and that the simple distinction between anterior and posterior gills has to be treated with caution, as had already been suggested in a recent microarray study on the green crab by Fehsenfeld et al. (2011). The present data suggests that the green crab *C. maenas* possess distinct mechanisms to counteract pH disturbances, including regulation of extracellular HCO_3^-, K^+ and ammonia levels, and alterations in gene expression of distinct transcripts in the branchial tissues.

4.1. Whole body acid-base regulatory patterns

Carcinus maenas exhibits an increase in hemolymph pCO_2 levels when acclimated to an environment with elevated pCO_2 levels, likely in order to maintain a gradient to enable diffusion of CO_2 out of the body (Melzner et al. 2009). In order to counteract a resulting drop in pH of the body fluids due to the CO_2 load, fish and crustaceans increase the bicarbonate level in their hemolymph (Larsen et al. 1997, Truchot 1986). In general, this active modulation of the extracellular carbonate system can be seen in high metabolic species like fish, crustaceans and

320 cephalopods, in order to compensate for a metabolic acidosis (Gutowska et al. 2009, Hu et al.
321 2011, Larsen et al. 1997, Spicer et al. 2007). This trend has also clearly been observed in the
322 present study (see Results, table 2).
323 Additionally, ammonia levels in the hemolymph are significantly increased in elevated pCO_2
324 acclimated crabs. A recent study by Martin et al. (2011) showed that the decapod crab
325 *Metacarcinus magister* is able to tolerate high ammonia levels (5-fold of controls) in its
326 hemolymph over a period of at least 7 days when exposed to high environmental ammonia.
327 The data in the current study suggests that also in green crabs, higher levels of hemolymph
328 ammonia (elevated by 85 % compared to control animals) can be tolerated for an extended
329 time period. Since ammonia excretion properties are not compromised, this access ammonia
330 might therefore play a role as an additional pH buffer system to counteract pH disturbances in
331 the green crabs' hemolymph. To keep steady state levels constant at a new higher level,
332 probably due to an increased internal ammonia production, also ammonia excretion rates
333 increased significanty to 262 % of controls, likely in order to secure sufficient excretion of this
334 potentially toxic substance.
335 In addition, K^+ levels of the hemolymph increased significantly which might be explained by its
336 effect on the membrane potential on nerve cells.
337 Both, an increase in external ammonia as well as potassium have been shown to increase
338 intracellular pH (pH_i) in adult rat carotid body glomus cells (Wilding et al. 1992). Also, it has
339 been demonstrated in leech glial cells that pH_i is dependent on the membrane potential. The
340 membrane potential itself is altered by extracellular K^+ concentrations; if extracellular K^+ is high,
341 the membrane is hyperpolarized and pH_i increases, mainly due to electrogenic $Na^+/2HCO_3^-$ co-

342 transport (Deitmer & Szatkowski 1990). The increase in extracellular K^+ in elevated pCO_2
343 acclimated green crabs might therefore be indeed important to keep pH_i stable in a high pCO_2
344 environment. As a potential candidate to participate in this process, the hyperpolarization
345 activated nucleotide-gated potassium channel (HCN2) should be taken into consideration in
346 future studies. Gene expression of this channel has been identified to be significantly down-
347 regulated in green crabs upon elevated pCO_2 acclimation in a recent study by Fehsenfeld et al.
348 (2011).
349

350 **4.2. Gill acid-base regulatory capacities**

351 *General observations*. All investigated gills (pairs 4 – 9) of control crabs lowered pCO_2 in
352 the perfusate significantly after only one gill passage, while simultaneously decreasing
353 bicarbonate levels and elevating the pH (figure 3). Interestingly, different gills seemed to exhibit
354 different patterns regarding this characteristic. Anterior gills seemed to be more efficient in
355 elevating the pH than the posterior gills, with anterior gill 4 having the highest proton excretion
356 rate (figure 4A). This fact supports the theory that the anterior gills are indeed the main players
357 for branchial acid-base regulation, while the posterior gills are involved in both processes,
358 osmoregulatory ion uptake and - with a lower tissue specific capacity - acid-base regulation
359 (Cieluch et al. 2003, Siebers et al. 1982, Truchot 1979). Additionally, the results from the
360 current study revealed that individual gill arches have to be considered separately and are even
361 more differentiated than previously discussed (Fehsenfeld et al. 2011).

Ammonia excretion rate patterns of single gills followed their pattern of proton excretion. Again, gill 4 exhibited the highest ammonia excretion rate, followed by gill 5 (figure 4B). In solution, the non-ionic NH_3 and the ionic NH_4^+ exist in a pH dependent equilibrium and in the average physiological stage (hemolymph pH 7.8) ca. 98 % of total ammonia exists in the ionic form NH_4^+ (Cameron and Heisler, 1983). Ammonia excretion is based on at least two processes, the passive diffusion of NH_3 and to the greater extent an active ammonia excretion, most likely via exocytosis of ammonium loaded vesicles (Weihrauch et al. 1998, 2001). Because a higher proton excretion rate of gill epithelial cells would lead to a higher acidification of the boundary layer in the sub-cuticular space of the gill, it would thereby create a higher outwardly directed pNH_3 gradient, that potentially leads to enhanced ammonia excretion rates due to an ammonia trapping mechanisms, as seen in gill 4. Ammonia excretion via ammonia trapping has been suggested to be in place in gills of freshwater teleost fish (reviewed by Weihrauch et al. 2009, Wright & Wood 2009) and freshwater planarians (Weihrauch et al. 2012). The observed similar pattern of proton and ammonia excretion by all individual gills additionally strengthens the hypothesis that both excretory processes are closely linked.

It needs to be to mentioned though, that the measured increase of ammonia in the bathing solution in gill perfusion experiments was higher than the ammonia loss measured in the perfusate. This can be explained by metabolically produced ammonia by the gill epithelium itself (Martin et al. 2011, Weihrauch et al. 1998, 1999). An exception is observed in gill 8, in which metabolically produced ammonia was particularly high, due to so far unexplained reasons.

Acclimation of green crabs to elevated environmental pCO_2 had no effect regarding both, proton and ammonia excretion rates of any of the isolated gills. However, as whole body ammonia excretion rates are observed to increase in elevated pCO_2 acclimated green crabs to 262 % of control levels, an additional ammonia excretion pathway must have been stimulated by elevated environmental pCO_2. Thus, different additional regulatory mechanisms regarding ammonia excretion might be accomplished. This might include the differential regulation of ammonia excretion mechanisms of not yet identified proteins, e.g. a soluble adenylyl cyclase (sAC), that has been shown to play a role in acid-base regulation and sensoring in the elasmobranch gill (Tresguerres et al. 2010).

Low and high pH challenge. When exposed to a low pH of 7.4 in the bathing solution during gill perfusion, all gills of control crabs exhibited a decreased proton excretion rate, possibly due to the higher inwardly directed proton gradient over the gill membrane (figure 5A). The anterior gills seem to be more vulnerable to this external pH disturbance (especially gill 5) than the posterior gills. The contrasting response can be observed when the bath pH was increased to 8.6: in this scenario, mainly the posterior gills (significant in gill 7) responded with an increase in proton excretion compared to control levels, which most likely is facilitated by the, in this case outwardly directed $[H^+]$ gradient over the gill membrane. This again shows the potentially high differentiation between the different gills and their function, but also indicates that the clusters of either anterior or posterior gills may work together in distinct external disturbances of the pH. Gills of crabs acclimated to long-term elevated pCO_2 in contrast are observed to show no differences in proton excretion levels in comparison to the control perfusion step, neither in a more acidic nor alkaline medium (figure 5B). This implies that

405 acclimation processes are taking place that enable the crabs to counteract the external pH
406 fluctuations more efficiently than control animals to leave them less vulnarable.

407 Even though branchial proton excretion was identified to be effected by lower and higher
408 environmental pH values in anterior gills of control crabs, ammonia excretion levels of both,
409 anterior and posterior gills, remained stable (figure 6A). However, ammonia excretion rates in
410 gill 5 and 8 were significantly affected in elevated pCO_2 acclimated crabs (lower and higher
411 ammonia excretion rate, respectively; figure 6B).

412 **4.3. Gene expression of transcripts involved in acid-base balance and osmoregulation**

413 For the majority of the analyzed transcripts, a clearly higher level of expression was observed in
414 the posterior gills (figure 7). As the posterior gills are discussed to be mainly involved in
415 osmoregulatory processes, and animals in the current study have been acclimated to brackish
416 water and are therefore hyper-regulating their hemolymph osmolality (Shaw 1961, Zanders
417 1980, Henry et al. 2003), the high expression levels of the Na^+/K^+-ATPase (NKA), sodium-
418 bicarbonate co-transporter (NBC) and cytoplasmic carbonic anhydrase (CA-2) resemble an
419 osmoregulatory response, as shown in previous studies (Henry et al 2003, Serrano & Henry
420 2008, Siebers et al. 1982, Towle & Weihrauch 2001, Towle et al. 2011).

421 Hemolymph osmoregulation in the green crab is accomplished primarily by the active transport
422 of Na^+ and Cl^- across the posterior gills (Siebers et al., 1982, Riestenpatt et al. 1996). Confirming
423 enzyme activity measurements of the pump in the study by Henry et al (2003), mRNA
424 expression of NKA in posterior gill 8 in the current study was indeed observed to be significantly
425 higher than in gill 4. Due to a lower NKA activity in osmoregulating crabs, gill 6 is not considered

426 to be associated with the posterior osmoregulatory active gills in *C. maenas* (Siebers et al.
427 1982). However, this gill shows a significant increase in NKA expression in elevated pCO_2
428 acclimated green crabs (figure 8C). This indicates that osmoregulation and acid-base regulatory
429 processes are linked and depend on an energizing NKA as suggested by Siebers et al (1994).

430 Expression patterns of NHE have not yet been shown in detail in gills of a crustacean, neither
431 has this transporter been localized in the gill epithelium. However, low and high expression
432 levels in the anterior and posterior gills suggest indeed a role in osmoregulation as has been
433 suggested for crabs (reviewed by Freire 2008, Towle & Weihrauch 2001) and in gills of
434 freshwater fish (Perry et al. 2003, Evans et al. 2005). Although different isoforms of NHE were
435 identified to be involved also in acid-base balance of euryhaline and seawater fish (e.g.
436 *Fundulus heteroclitus*, *Myoxocephalus octodecimspinosus*; Edwards et al. 2005, Claiborne et al.
437 1999), the present study did not identify a significant change in NHE expression levels in
438 response to elevated pCO_2 in crabs (figure 8). However, it has to be noted that a tendency to
439 down-regulation was observed in the anterior gills 4 and 5 ($p = 0.13$ and $p = 0.12$, respectively).

440 Interestingly, also the carbonic anhydrase isoforms CA-1 and CA-2 which apparently play a
441 dominant role in branchial osmoregulation in crabs - they have been shown to undergo 3-fold
442 (CA-1) and a rapid 100-fold (CA-2) increase upon low salinity acclimation in posterior gills
443 (Serrano & Henry 2008) - did not show a prominent response upon long-term exposure to
444 elevated pCO_2 levels in the gills (figure 8). However, exceptions were observed in gill 4 where
445 CA-2 is down-regulated in tendency ($p = 0.07$), for gill 8 with a slight up-regulation in CA-1, and
446 in gill 5 where a significant up-regulation of CA-1 was identified (figures 8A and 8B,

447 respectively). The lack of response in most gills might be explained by the high enzyme kinetics
448 reported for these enzymes (Henry et al. 2003), warranting likely a high flexibility upon a
449 moderate pH-stress.

450 The sodium bicarbonate co-transporter (NBC), which has been shown to play a role in
451 osmoregulation in a recent microarray study in *C. maenas* (Towle et al. 2011) and showed a
452 strong expression level only in the osmoregulatory active posterior gills in the current study
453 (figure 7F), was not identified to participate in a response to elevated environmental pCO_2
454 (figure 8). Regarding the involvement in acid-base balance, the sodium-bicarbonate co-
455 transporter (NBC) has been proofed to mediate both, Na^+ and HCO_3^- exit into the blood in fish
456 gills (Evans et al. 2005). It has also been discussed to be indirectly involved in ammonia
457 excretion processes in fish (Wright & Wood 2009). Considering bicarbonate as the main
458 buffering component in the hemolymph to counteract pH disturbances, this buffer molecule
459 likely participates in an acid-base regulatory response. However, NBC expression levels were
460 also identified not to be altered upon elevated seawater pCO_2 in the microarray study on green
461 crabs by Fehsenfeld et al. (2011). No change in expression levels of this gene being observed in
462 any of the investigated gills is a puzzling finding that clearly needs to be addressed in future
463 studies.

464 The vacuolar V-(H^+)-ATPase (HAT) in the gills of *C. maenas* is associated rather with cytoplasmic
465 vesicles than the apical membrane of the gill epithelium and plays an important role in active
466 ammonia excretion, but is not involved in osmoregulatory processes as indicated by a lack of a
467 response of the branchial transepithelial potential difference (PD_{te}) after the application of the

V-(H^+)-ATPase inhibitor bafilomycin (Weihrauch et al. 2002). In the present study, the expression level of this gene was not affected by acclimation to elevated pCO_2 in any gill (figure 8), indicating that changes observed in ammonia excretion rates are likely due to alterations in the passive transbranchial movement rather than active ammonia transport over the gill epithelium or to the activation of existing transporters.

The anion exchanger (AE) has been identified to be one of the most down-regulated transcripts in gills of animals exposed to elevated seawater pCO_2 (Fehsenfeld et al. 2011). In order to achieve electroneutrality, Cl^- typically is the counter-ion of HCO_3^- during the extracellular pH regulatory reaction. Extracellular HCO_3^- accumulation, as has also been observed in the current study, is likely to be enabled by Cl^-/HCO_3^- exchangers (Larsen et al. 1997). However, up to date biochemical characterization of this transporter is still lacking. In the present study, AE is down-regulated in tendency in anterior gill 4 and gill 5 (figures 8A and 8B, respectively), and therefore might be involved in a regulatory acid-base response, eventually complementing CA-1 function.

The Rhesus-like protein (Rh) is the only of the investigated transcripts that exhibits a higher expression level in the anterior than in the posterior gills (figure 7B). Rh is down-regulated in the anterior gills 4 and 5 in pCO_2 exposed green crabs and simultaneously, gill 5 is observed to have a decreased ammonia excretion rate in those animals (figures 8A and 8B, respectively). Additionally, an effect of low environmental pH on proton excretion rates was observed in gill 5 (figure 4A). The Rhesus-like protein discovered in crustaceans including *Carcinus maenas* (Weihrauch et al. 2004b), shows high homology to the human Rhesus-like ammonia

transporter, and is most likely to be involved in ammonia excretion also in this species, as it has been suggested for the Dungeness crab *Metacarcinus magister* (Martin et al. 2011). In human red blood cells (Soupene et al. 2002), the Rhesus-like protein has been shown to not only participate in ammonia excretion, but also to act as a CO_2 channel. In fish, Perry et al. (2010) identified Rhbg and Rhcg1 to act as both, CO_2 and ammonia channel in *Danio rerio* gills in response to hypercapnia. Additionally, expression levels of rhesus proteins are significantly affected in response to high environmental ammonia in various species (reviewed by Wright & Wood 2009).

The Rhesus-like protein might therefore be a very important link between CO_2 regulation and ammonia excretion. The present study suggests that in crabs this link is mainly associated with the anterior gills.

5. Conclusions

The results of the present study show clearly that the exposure to altered environmental pCO_2 that mimic a near future scenario has an effect on the investigated decapod crab *Carcinus maenas* on the whole animal level. K^+ and ammonia concentrations were significantly elevated in the hemolymph, and might be involved in buffering extra- and intracellular pH. Additionally, the performance of single isolated gills was differentially affected in gill perfusion experiments as well as in regard to gene expression levels of Rhesus-like protein, membrane-bound carbonic

anhydrase and sodium-potassium ATPase. In general, anterior gills seem to be more affected than posterior gills, indicating their important role in acid-base regulation.

An important question still remaining to be solved, is how gills sense their actual acid / base status in order to counteract the disturbances as described above. It has been shown by Tresguerres et al. (2010) that the evolutionary conserved signaling enzyme soluble adenylyl cyclase (sAC) acts as a sensor of acid-base status in dogfish. This enzyme produces the second messenger cAMP upon activation by HCO_3^- and is expressed in the gill epithelium, which makes it a highly suitable candidate as a respective sensor in crustaceans as well. Additionally, crustacean hormones that have been identified to be involved in ion- and osmoregulation, like cAMP-activating dopamine and serotonin (reviewed by Morris 2001) or the crustacean hyperglycaemic hormone CHH (Chung & Webster 2006), might participate in sensing and maintaining acid-base homeostasis. The identification and characterization of sensors of crustacean's acid-base status is highly desirable and has to be focus of future investigations.

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702 Figures

703 **Figure 1.** Experimental scheme of the gill perfusion. Each perfusion experiment was composed
704 of 5 consecutive steps that were applied for 30 min each. A first control step (bathing solution =
705 pH 7.8) was followed by a low pH step in which the pH of the bathing solution was lowered by
706 0.4 units to 7.4. After a second control phase, a high pH step was applied, increasing the pH of
707 the bathing solution by 0.8 units to pH 8.6. A third control phase ensured that the gill was still
708 functioning.

709 **Figure 2.** Alignment of the RbS3 sequence of *Carcinus maenas* with *Metacarcinus magister* (*M.*
710 *magister* ribosomal protein S3 mRNA, partial cds; GenBank accession no. **JF276909.1**).The
711 sequences showed 89.2 % identity. When translated to the protein, 100 % identity of both
712 deduced sequences was achieved (data not shown).

713 **Figure 3.** Regulation of the pH of individual gills after one gill passage in perfusion experiments.
714 All gills (black bars) up-regulated the pH in the perfusate significantly compared to the initial pH
715 of 7.9 of the perfusion solution (perf, grey bar; t-test with $p < 0.01$).

716 **Figure 4**. (A) Decrease of hemolymph proton concentration [H^+] (equivalent to an increase of
717 pH) in control *Carcinus maenas* after one gill passage in gill perfusion experiments. (B)
718 Ammonia excretion rates of control *C. maenas* after one gill passage in gill perfusion
719 experiments. Small letters a, b, c, d indicate significant differences within the individual gills of
720 control crabs ((A) Kruskil-Wallis test with $p < 0.05$, $n = 4 – 6$; (B) ANOVA with $p < 0.05$, $n = 3 - 5$).

721 **Figure 5.** Relative decrease of the proton concentration [H^+] in the perfusate during gill
722 perfusion of control (A) and high pCO_2 exposed crabs (B) after only one gill passage in the
723 different experimental phases. All displayed values have been calculated employing the
724 respective preceding control step in the perfusion. * denotes significant differences regarding
725 different steps of the perfusion sequence of control animals only, while + denotes significant
726 differences between control and high pCO_2 exposed crabs (t-test with $p < 0.05$). Note: gill 4
727 control step vs. low pH step $p = 0.09$; control gill 4 vs. high pCO_2 exposed gill 4 $p = 0.09$. Values
728 are given as means ± SEM, $n = 3 - 6$.

729 **Figure 6.** Relative ammonia excretion rates of all gills during gill perfusion of control (A) and
730 high pCO_2 exposed crabs (B) after only one gill passage in the different experimental phases. All
731 gene expression levels are in relation to the housekeeping gene, RbS3. Low pH and high pH
732 values are related to the preceding control step in the perfusion, respectively. * denotes

733 significant differences between control and high pCO_2 exposed crabs (t-test with $p < 0.05$). All
734 values are given as means ± SEM, n = 3 - 6.

735 **Figure 7.** Relative gene expression levels of sodium-hydrogen exchanger (NHE), Rhesus-like
736 protein (Rh), V-(H^+)-ATPase (HAT), anion exchanger HCO_3^-/Cl^- (AE), Sodium-potassium ATPase
737 (NKA), sodium-bicarbonate co-transporter (NBC), carbonic anhydrase membrane bound (CA-1)
738 and cytoplasmic (CA-2) in the different gills 4 – 9 of control green crabs. Letters a,b,c denote
739 significant differences (t-test with $p < 0.05$). Values represent means + SEM with n = 3 – 6.

740 **Figure 8**. Quantitative real-time PCR sodium-hydrogen exchanger (NHE), Rhesus-like protein
741 (Rh), V-(H^+)-ATPase (HAT), anion exchanger HCO_3^-/Cl^- (AE), Sodium-potassium ATPase (NKA),
742 sodium-bicarbonate co-transporter (NBC), Carbonic anhydrase membrane bound (CA-1) and
743 cytoplasmic (CA-2) in gills 4 – 9 (A – F) of green crabs acclimated to control (54 Pa) and elevated
744 pCO_2 (324 Pa). Gene expression levels have been related to the housekeeping gene RbS3 and
745 standardized to control expression levels of the respective transcript. * denotes significant
746 differences in gene expression between control and high pCO_2 exposed animals. Vertical bars
747 indicate tendencies with $p < 0.15$ but > 0.05. The graph represents means + SEM (n = 4 - 6).

748

Tables

Table 1. Water parameters of the 120 L tanks green crabs were acclimated in to either control or high pCO_2 brackish water.

	control tank	high pCO_2 tank
salinity [ppt]	10	10
temperature [°C]	14	14
pH	7.7 ± 0.0	7.0 ± 0.0
C_T [mmol/kg SW]	664 ± 25	1013 ± 49
pCO_2 [Pa]	53.5 ± 2.1	324.3 ± 20.0

pH and total carbon (C_T) of the tanks was assessed every 1 to 2 days during the acclimation phase of *Carcinus maenas*. Partial pressure of CO_2 (pCO_2) was calculated applying the measured parameters and CO2SYS software (see Material and Methods for details). Under experimental conditions (high pCO_2), a drop in pH of 0.7 units could be observed, while pCO_2 increased 6-fold. Values are given as mean ± SEM.

Table 2. Hemolymph composition of control and high pCO_2 acclimated *Carcinus maenas* specimen.

	HCO_3^-	Ammonia	Cl^-	Na^+	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}
Control animals (pH 7.7)	6.6 ± 0.4	155.57 ± 24.37	319.1 ± 5.6	259.2 ± 4.8	6.9 ± 0.2	5.9 ± 0.5	6.9 ± 0.2	2.6 ± 0.1
High pCO_2 exposed animals (pH 7.0)	7.9 ± 0.6	218.41 ± 17.13*****	316.1 ± 9.0	276.7 ± 9.1	8.1 ± 0.2*****	6.0 ± 0.3	6.6 ± 0.5	2.5 ± 0.1
Perfusion saline	7	100	280	260	8	5	7	-

Bicarbonate was calculated as described in Material & Methods. Ammonia was measured using an ammonia selective electrode. Ions were assessed through ion chromatography. All concentrations are given in [mM] except for ammonia in [μM]. * indicates significant difference to control values. All values represent mean ± SEM with n = 4 – 6.

Table 3. Primer sequences employed in quantitative real-time PCR.

Transcript	GenBank Accession no.	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)	Annealing T [°C]
Ribosomal gene S3 (**RbS3**)	JF276909.1	GTCCCTTTTCACCAAGGACA	CAAGGCCAAACTCAACAGGTT	160	60
Sodium hydrogen exchanger (**NHE**)	U09274.1	TTCGAGGGCTTCAGTGAGTT	TAAGGAAGCCCCAGATGATG	124	60
Rhesus-like protein (**Rh**)	AF364404.2	GGTGGTCTCGTGACAGGTTT	TTGTGACCCTCATCCTCCTC	119	60
V-H^+-ATPase, subunit B, K form (**HAT**)	AF189779.2	ACCCAGATCCCCATCCTTAC	AGAGAAGGCAGCACGTTGAT	149	60
SLC4A1 (anion exchanger Cl^-/HCO_3^-) (**AE**)	CX994129.1	TGATGCCAGTCAAACACCAT	AGCAAAGAGCTGCTGGAGAC	138	60
Sodium-potassium ATPase α-subunit (**NKA**)	AY035550.1	CAGGCTTGGAAACTGGAGAG	AGCATCCAGCCAATGGTAAC	137	60
Sodium-bicarbonate co-transporter (**NBC**)	DN202373.1	TTGCCACTTGATTTTGAGCAA	CAGCACAATATCCCAGTGGAA	90	60
Membrane-bound carbonic anhydrase **(CA-1)**	EU273944.1	GGTCTGGCAGTACTGGGTGT	AGCCTTGAGTGGGTACATGG	138	60
Cytoplasmic carbonic anhydrase **(CA-2)**	EU273943.1	CGCTCAGTTCCACTTCCA	ACATCTCAGCATCCGTCA	213	60

Primer sequences are given as (5' → 3').