LINKING ACID-BASE BALANCE WITH NITROGEN REGULATION IN THE DECAPOD CRUSTACEAN, *CARCINUS MAENAS*

by

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ABSTRACT

As one of the most successful invasive species in the marine environment around the globe, the green crab *Carcinus maenas* possesses efficient regulatory mechanisms to quickly acclimate to environmental changes. The most important organs in this process are the nine pairs of gills that not only allow for osmoregulation, but have been shown to be involved in ammonia excretion and respiratory gas exchange. To date, however, little is known about the gills' contribution to acid-base regulation that might become increasingly important in a "future ocean scenario" whereby surface ocean pH is predicted to drop by up to 0.5 units by the year 2100.

The present thesis aims to characterize the green crab gills' role in acid-base regulation and how it is linked to ammonia excretion. Briefly, green crabs were exposed to hypercapnia in order to challenge organismal acid-base homeostasis and characterize their compensatory response to this stressor. While osmoregulating green crabs were capable of fully compensating for the resulting extracellular respiratory acidosis after being exposed to 0.4 kPa pCO_2 for 7 days, osmoconforming green crabs were observed to only partially compensate for the accompanying drop in hemolymph pH after acclimation to 1% CO_2 for 48 hours. Perfusion experiments on isolated green crab gills showed that different gills contributed to the excretion of H^+ in an individual pattern, which was mirrored by their capability for ammonia excretion, indicating that NH_4^+ is an important component of branchial acid excretion. Experiments on gill mRNA expression and pharmaceutical effects on isolated gills identified distinct epithelial transporters to play significant roles in branchial acid-base regulation: Rhesus-like protein, basolateral bicarbonate transporter(s), cytoplasmic V-(H^+)-ATPase, Na^+/H^+-exchanger, basolateral

Na^+/K^+-ATPase, cytoplasmic and membrane bound carbonic anhydrase, and basolateral K^+-channels. Regarding the latter, the present work provides the first sequence-based evidence for a potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel (CmHCN) capable of promoting NH_4^+ transport in the green crabs' gill epithelium, and further demonstrates its direct involvement in branchial acid-base regulation. This highly conserved protein is a potentially important novel key-player in acid-base regulation in all animals.

Interestingly, the observed principles linking acid-base to ammonia regulation in the decapod crustacean gill epithelium resemble many observations previously made in vertebrates. The data of the present thesis therefore provides valuable information for general acid-base regulation, while contributing substantially to our understanding of acid-base regulation in invertebrates.

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DEDICATION

Had anyone told me 15 years ago, that one day I would be as close as putting a “Dr.” in front of my name as I am now, I simply would have declared that person crazy.
Had anyone told me 10 years ago when I embarked on this journey to become a marine biologist, that I would end up with spineless little critters instead of Humpback whales and Bottlenose dolphins, I would have given him or her a more than doubtful look.
Had anyone told me 5 years ago that I would be a marine biologist in the heart of the Canadian prairies, I would have never even had heard of Winnipeg in my life.

Yet, here I am, in “friendly Manitoba”, the home of Winnie-the-Pooh, coming to realize that I am at the end of this winding road for now, on top of this mountain that I started to climb what feels like a lifetime ago. I was – and am - living my dream. I would not be in this place of my life had it not been for my beloved mother, friend and soulmate, Dolores Fehsenfeld. Through her death, she taught me everything about life.

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LIST OF ABBREVIATIONS

AE	HCO_3^-/Cl^- exchanger (anion exchanger)
AER	Anisosmotic extracellular regulation
αCO_2	Solubility coefficient for CO_2
Ala	Alanin
ANOVA	Analysis of Variance
CA-1	Membrane bound carbonic anhydrase
CA-2	Cytoplasmic carbonic anhydrase
cAMP	Cyclic adenosine monophosphate
cDNA	Copy- Desoxyribonucleic acid
cNMP	Cyclic nucleotide monophosphate
CT	Total carbon
DIC	Dissolved inorganic carbon
DMSO	Dimethyl sulfoxide
GO-terms	Gene ontology terms
HAT	V-(H^+)-ATPase
HCN	Hyperpolarization activated cyclic nucleotide-gated K^+-channel
HEA	High environmental ammonia
HEK293	Human embryonic kidney 293 cell line
IC	Ion chromatography
IC_{50}	Inhibitor constant
IPCC	Intergovernmental Panel on Climate Change
I_{SC}	Short-circuit current

K_{IR}	Inward-rectifying K^+-channel
K_V	Voltage-gated K^+-channel
Leu	Leucin
NBC	Na^+/HCO_3^--cotransporter
NBS	Northern Balance Scale for pH
NHE	Na^+/H^+-exchanger
NKA	Na^+/K^+-ATPase
NKCC	$Na^+/K^+/2\ Cl^-$-cotransporter
qPCR	Quantitative polymerase chain reaction
Pa	Pascal (1 Pa = 7.5 x 10^{-3} torr = 9.87 x 10^{-6} atm = 10 x 10^{-6} bar)
pCO_2	CO_2 partial pressure
pK1	First dissociation constant (for H_2CO_3)
Rbs-3	Ribosomal gene 3
RCP 8.5	Representative Concentration Pathways scenario 8.5 as predicted by the Intergovernmental Panel on Climate Change)
Rh	Rhesus-like protein
RNA	Ribonucleic acid
SEM	Standard error mean
SID	Strong ion difference
TAL	Thick ascending limb

CHAPTER 1:

GENERAL INTRODUCTION AND LITERATURE REVIEW

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DW helped editing the manuscript.

1.1. The importance of acid-base homeostasis in aquatic decapod crustaceans

Maintaining acid-base balance is fundamental for all living organisms, including decapod crustaceans (Henry and Wheatly, 1992). Only slight disturbances in the concentration of acid-base equivalents resulting in shifts of pH in the intra- or extracellular fluids may impair properties of essential proteins and their regulation (i.e. enzymes; Somero, 1986, respiratory proteins; Riggs, 1988; Truchot, 1975c) and ultimately lead to a disruption of basic physiological functions. Consequently, securing whole animal acid-base homeostasis not only includes the maintenance of a constant intra- and extracellular pH, but also needs mechanisms in place for their re-adjustments after an acid-base disturbance. Factors to disrupt acid-base homeostasis in decapod crustaceans might include a variety of intrinsic as well extrinsic parameters like the internal acid-load due to exercise (e.g. Booth et al., 1984; Rose et al., 1998), shifts in $CaCO_3$ handling during the moulting process (e.g. Mangum et al., 1985), and fluctuations of environmental parameters like salinity (e.g. Whiteley et al., 2001), temperature (e.g. McMahon et al., 1978; Whiteley and Taylor, 1990), pO_2/pCO_2 (e.g. De Fur et al., 1983; Urbina et al., 2013) and ammonia (Cheng et al., 2013; Martin et al., 2011). The regulation of acid-base balance in aquatic decapod crustaceans therefore is a complex interaction of physiological and biochemical processes including respiratory gas exchange, ion-regulation and overall adjustments of metabolism (Henry and Wheatly, 1992).

Most studies on acid-base regulation in decapod crustaceans to date concentrate on whole animal extracellular acid-base status and its responses upon disturbances (see section 1.2). Few studies have investigated acid-base regulation on a cellular level and

therefore, little is known about direct trans-branchial transport of acid–base equivalents in decapod crustaceans (see section 1.3). While most available acid-base related data has been collected on brachyuran hyper-regulating crabs (i.e. the green crab, *Carcinus maenas*, the blue crab, *Callinectes sapidus*, and the Chinese mitten crab, *Eriocheir sinensis*), the present chapter aims to provide a broader review of the currently available literature on acid-base regulation also in the other major members of decapod crustaceans, namely prawns (in this chapter referred to as synonym for penaeoid and sergestoid shrimp), Anomura, shrimp (caridaean shrimp), lobster, and crayfish (De Grave et al., 2009; figure 1.1).

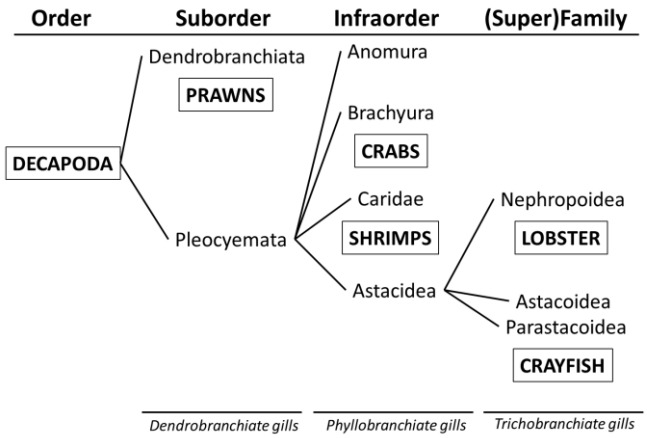

Figure 1.1. Overview of the different sub-groups of decapod crustaceans as discussed in this chapter. The nomenclature follows De Grave et al. (2009). To avoid confusion, members of the suborder Dendrobrachiata are referred to as "prawns" throughout the text, while "shrimps" solely refers to the infraorder Caridae.

1.1.1. Tissues involved in acid-base regulation

In crustaceans, anisosmotic extracellular regulation (AER), or the osmotic and ionic buffering of the extracellular fluid in order to maintain (acid-base) homeostasis, is mainly driven by the antennal glands, gut and gills (McNamara and Faria, 2012).

A tissue-specific inventory of epithelial membrane transporters then translates the changes of extracellular adjustments into the cell to ensure the intracellular maintenance of acid-base balance (Freire et al., 2008).

Antennal glands. Situated at the anterior end of the body at the base of the eyestalks, the paired antennal glands are mainly involved in the production (ultrafiltration) and ionic regulation of the urine to maintain extracellular water balance (Larsen et al., 2014). Therefore they can be regarded as analogues of the nephron of the vertebrate kidney, the major acid-base regulatory organ in mammals and other terrestrial vertebrates (Weiner and Verlander, 2013). Even though urine [Na^+], [K^+] and [Cl^-] are adjusted upon disturbance of (acid-base) homeostasis, antennal glands are believed to rather contribute to the regulation of divalent cations like Ca^{2+} and Mg^{2+} based on the respective clearance ratios (Wheatly, 1985).

Only a few studies have investigated the role of antennal glands in acid-base regulation in decapod crustaceans. In freshwater-acclimated *C. sapidus*, net urinary acid-base and ammonia efflux were negligible and did not change significantly when animals were exposed to hypercapnia (2% CO_2; Cameron and Batterton, 1978a). In the Dungeness crab, *Metacarcinus magister*, acclimated to dilute salinity (~ 20 ppt) the increase in antennal gland-mediated HCO_3^- efflux resulted in an increased alkalinisation of the urine, but was accompanied by an increase in HCO_3^- reabsorption over time, likely to assist in HCO_3^- accumulation in the hemolymph (Wheatly, 1985). However, in respect to the overall base output in response to dilute salinity acclimation, the antennal gland of *M. magister* contributed to only 10% at best (Wheatly, 1985). Also in the freshwater-acclimated euryhaline signal crayfish, *Pacifastacus leniusculus*, exposed to hyperoxia, an

initial extracellular acidosis resulted in an increase in HCO_3^- reabsorption from the urine to buffer hemolymph pH, but in parallel, an acidification of the urine was observed mainly due to increased ammonia (NH_4^+) excretion (Wheatly and Toop, 1989). Similar to the observations of hyposaline-acclimated *M. magister*, however, net H^+ efflux accounted for only 10% of the whole animal response in this crayfish. Interestingly, antennal glands of *P. leniusculus* show a significantly higher activity of carbonic anhydrase (CA), the enzyme involved in the hydration of CO_2 to form H^+ and HCO_3^-, compared to the gills (Wheatly and Henry, 1987). When acclimated to hypersaline conditions, however, CA activity was progressively reduced with increased salinity (up to 80% at ~25 ppt).

In conclusion, the existing data suggest an overall negligible involvement of antennal glands in acid-base regulation in decapod crustaceans.

Gut and gut diverticula. Besides the respective adjustment of urine flow, gut-mediated fluid absorption and secretion of digestive fluid have been shown to be ion-dependent in both, hypo- and hyper-regulating crustaceans and likely helps in the regulation of the hemolymph composition (Mantel and Farmer, 1983). Accordingly, crustacean gut epithelia have been shown to possess Na^+/K^+-ATPase (Chu, 1987; Chung and Lin, 2006; Mantel and Farmer, 1983). Even though being directly exposed to the environment and showing evidence for the capability to take up / excrete small ions like Na^+, Cl^-, K^+, Ca^{2+} and SO_4^- (Ahearn, 1978; Mantel and Farmer, 1983), a potential role of the gut in acid-base regulation has not been investigated to date.

In addition to the gut, the presence of an electrogenic, likely apically situated $2Na^+$:$1H^+$-exchanger in the hepatopancreas of lobster and freshwater prawns (Ahearn et al., 1990; 1994) would provide an important player for acid-base regulation in this tissue.

Similar as for the gut, however, a direct involvement for the hepatopancreas in maintaining acid-base homeostasis has not been investigated to date. Clearly, future studies need to be performed in order to characterize the potential roles of gut and hepatopancreas in crustacean acid-base regulation.

Gills. Similar to fish and cephalopods, the majority of the acid-base relevant ion regulatory apparatus of decapod crustaceans is located in their gill epithelia (Henry et al., 2012; Larsen et al., 2014 and references therein). Not only are the gills involved in respiratory and acid-base physiology, but they are the major organs also for ion regulation and ammonia excretion, therefore linking all of these regulatory processes (Freire et al., 2008; Henry et al., 2012).

All decapod crustaceans possess paired gills that are covered by a fine chitinous cuticle, lined by a single-layered epithelium and attached to a basal lamina (Freire et al., 2008). Depending on taxa, the number of paired gills, their location of attachment and the arrangement of the gill lamellae (phyllobranchiate, trichobranchiate, dendrobranchiate, figure 1.1) vary substantially, providing more or less gill surface amplification for ion and gas exchange processes between the external (environment) and the internal medium (hemolymph). For further details the reader is referred to the extensive descriptions by Taylor and Taylor (1992).

According to their different life-strategies (i.e. primary habitat / habitat changes), gill epithelia of decapod crustaceans exhibit specific characteristics that can vary even within the respective sub-/infraorder or superfamily. Acid-base status and regulation in decapod crustaceans has been shown to be linked to external salinity and NaCl regulation (e.g. in the freshwater crayfish, *Astacus leptodactylus* (Ehrenfeld, 1974), *C. sapidus* (Henry and

Cameron, 1982) and *E. sinensis* (Whiteley et al., 2001)), and therefore the tightness of the gill epithelium consequently might also affect the animals' capability for acid-base regulation. While the gill epithelia of strong hyper-regulators like *E. sinensis* (Weihrauch et al., 1999a) and freshwater crayfish (Wheatly and Gannon, 1995) represent a tight epithelium (conductance for ions <5 mS cm^{-2}), the epithelium of weak hyper-regulators like *C. maenas* (Weihrauch et al., 1999a) and the American lobster, *Homarus americanus*, is much leakier and allows for increased intercellular transport of ions (conductance 40-60 mS cm^{-2}). Gills of osmoconforming crustaceans like *M. magister* (Hunter and Rudy, 1975) or Red rock crabs, *Cancer productus* (Weihrauch et al., 1999a), in contrast are highly permeable for ions (conductance > 200 mS cm^{-2}) and therefore these species are very limited in their capability to osmoregulate.

Furthermore, specializations of gill epithelia can be seen at the ultrastructural level. Of the five different cell types found in decapod crustacean gill epithelia (thin cells, thick cells, flange cells, attenuated cells and pilaster cells; Freire et al., 2008), thin cells can be found in all gills of osmoconforming crabs as well as the most anterior 4-6 pairs of gills of hyper-regulating crabs like *C. maenas* (Compere et al., 1989), *C. sapidus* (Copeland Fitzjarrell, 1968) and *E. sinensis* (Barra et al., 1983). Some thin cells can also be observed in the gill epithelium of lobsters (Haond et al. 1998). Due to their low height (1-5 μM), a lack of extensive membrane folding and low number of mitochondria, thin cells are generally believed to be associated with respiratory epithelia, and therefore have been considered to play an increased role in acid-base regulation rather than osmoregulation (Freire et al., 2008). In some hyper-regulating crabs like *C. maenas* (Compere et al., 1989) and *C. sapidus* (Copeland and Fitzjarrell, 1968), thin cells can also

be found surrounding the thick cells (also called ionocytes due to their supposed major role in ion transport) in the most posterior (osmoregulatory) pairs of gills, therefore indicating that acid-base regulatory properties might not be solely associated with the anterior gills in these species. To date, however, the direct site for acid-base regulation in euryhaline brachyuran gills has not been confirmed, while osmoregulation has been demonstrated to be associated predominantly with the posterior gills (Henry et al., 2012) and ammonia with both anterior and posterior gills (Weihrauch et al., 1999a).

The gill epithelia of lobster (Haond et al.,1998), prawns (Bouaricha et al., 1994), shrimp (Freire and McNamara, 1995) and freshwater crayfish (Morse et al., 1970) on the other hand are more homogeneous and possess so-called flange cells that exhibit features of both thin and thick cells of crabs, and are therefore believed to incorporate both respiratory/acid-base and ion regulatory functions (Freire et al., 2008).

Even though most pilaster cells in the epithelia of crabs and crayfish seem to inherit a mainly stabilizing function for the intra-lamellar septum, they are the exclusive sites for the V-(H^+)-ATPase in *E. sinensis* indicating an additional role for these cells in acid-base regulation in this species (Freire et al., 2008).

1.1.2. Pre-adaptation through life strategies?

The estimated 14,000 species of aquatic decapod crustaceans can be found in nearly all water bodies of the world. As described earlier, the capability of inhabiting specific water bodies mainly depends on their regulatory capacity due to the characteristics of the gill epithelium as well as the general permeability of their carapace. Acid-base regulatory capabilities are therefore likely correlated with life history, genetic pre-disposition and physiological plasticity (Melzner et al., 2009). As metazoans with a relatively high

metabolic rate and level of activity, a high capacity to adjust body fluid pH (also dependent on the relatively large fluid volume), and relatively less expressed calcified structures compared to other marine calcifiers like corals, echinoderms and molluscs, (decapod) crustaceans are believed to cope better with changes in their environment than other marine invertebrates (Wittmann and Pörtner, 2013).

The following sections are intended to roughly characterize the basic life-strategies for key-species of the major decapod crustacean groups that will be discussed in more detail concerning their acid-base regulatory capabilities in the subsequent sections.

Prawns. While most Dendrobranchiata (Penaeidae) are euryhaline hyper-/hypo-osmoregulators found in the marine environment (e.g. *(Marsu)Penaeus japonicus* (Cheng et al., 2013); (*Lito)Penaeus vannamei* (Liu et al., 2015)), some species of Sergestidae are found in freshwater. Many species inhabit deep (offshore) waters, while most Penaeidae are mainly found in shallow inshore tropical and subtropical waters and estuaries. Some prawn species are known to bury in mud bottoms during the daytime, challenging their acid-base homeostasis as described below (Tavares and Martin, 2010).

Anomura. While king crabs (Lithodoidea) like the southern king crab, *Lithodes santolla*, are sub-tidal species that can be found between 5 and 700 m depth in temperate waters (Urbina et al., 2013), hermit crabs (Paguroidea) like *Pagurus bernhardus* (De la Haye et al., 2011) and Porcelain crabs (Galatheoidea) like *Petrolisthes cinctipes* (Carter et al., 2013) are commonly found in the intertidal zone, potentially being trapped in rocky tide pools experiencing large spatial and temporal changes in abiotic parameters as discussed below.

Crabs. With over 6,700 species in 93 families, brachyuran crabs constitute to *ca.* 50% of all decapod crustaceans (Ng et al., 2008). Accordingly, all imaginable life-strategies and habitat uses are exhibited by this infraorder. Some of the most thoroughly investigated crabs are the osmoconforming *M. magister*, the weak hyper-osmoregulating *C. maenas* and the closely related *C. sapidus*, as well as the strong hyper-osmoregulating *E. sinensis*. Like prawns and Anomura, they are oftentimes trapped in tide-pools (Truchot and Duhamel-Jouve, 1980) and some species bury in the sediment (Bellwood, 2002).

Shrimp. Besides Brachyuran crabs, (palaemonid) shrimps are the most diverse of the decapod groups with a great inter- and intra-specific variability in osmoregulatory capabilities. While most species can be found in freshwater and are strong hyper-regulators (i.e. genus *Macrobrachium*), some species inhabit estuarine (brackish) and even marine waters (e.g. genus *Palaemon*, *Palaemonetes*) and are hyper-/hypo-osmoregulators (Freire et al., 2003; McNamara and Faria, 2012). Some shrimp species are associated with the intertidal zones and therefore more shallow waters (e.g. *Crangon crangon*, Almut and Bamber, 2013), or are amphidromous and occupy those habitats during their early life stages (e.g. the northern prawn, *Pandalus borealis*, Hammer and Pedersen, 2013; *Macrobrachium olfersii*, Freire and McNamara, 1995; McNamara and Lima, 1997). Other shrimps are deep-water dwellers (e.g. adult *P. borealis*, Hammer and Pedersen, 2013).

Lobster. Lobsters, especially the commercially important lobsters of the genus *Homarus* and the Norway lobster, *Nephros norvegicus*, have traditionally been considered to be osmoconforming, stenohaline (salinity > 25 ppt) and limited to coastal and offshore habitats down to 700 m (Chapman, 1980; Cooper and Uzmann, 1980; Dall, 1970). Recent

research, however, identified them to be present also in estuarine and intertidal regions where they experience short-term fluctuations in salinity and other abiotic parameters (Charmantier et al., 2001). Additionally, the reproduction of lobsters seems to be dependent on higher water temperatures that lead to the animals' migration into different habitats and consequently, their exposure to different environmental conditions that potentially challenge acid-base homeostasis (Charmantier et al., 2001).

Crayfish. Crayfish belong to the only decapod superfamily that is almost entirely found in freshwater (Reynolds et al., 2013). However, many crayfish species depend on the connection to the ocean in order to breed and therefore have a limited capability to osmo- and ionregulate (Pequeux, 1995). A remarkable number of crayfish build complex burrows in which they spend most of their life (Crandall and Buhay, 2008; Guiasu, 2002). Others are defined as stream or lake/pond/large river dwellers, or are obligate cave-dwellers (Crandall and Buhay, 2008). Due to the very different chemistry of freshwater (low total osmolarity of ~4 mOsm/mainly $CaCO_3$ *vs.* 1000 mOsm/mainly NaCl in marine environments), ion regulation in crayfish is challenged and they maintain a lower, yet still hyper-osmotic extracellular ionic concentration and a lower carapace permeability for ions and water compared to marine decapod crustaceans (Wheatly and Gannon, 1995).

1.1.3. Challenging acid-base homeostasis

As (opportunistic) omnivores, all decapod crustaceans experience regular internal acid-loads due to the catabolism of proteins and the resulting build-up of extracellular ammonia (mostly NH_4^+ at physiological pH; Weiner and Verlander, 2013) that can affect extra- as well as intracellular acid-base homeostasis (Larsen et al., 2014). The response of

decapod crustaceans upon exercise (functional/internal hypoxia) on the other hand results in a build-up of lactate and CO_2 in the hemolymph, therefore delivering H^+ and challenging extracellular acid-base regulation (Henry et al., 1994; see below).

Besides these intrinsic sources of acid-base disturbances, many of the mainly benthic aquatic decapods experience regular fluctuations of the abiotic parameters pH, pCO_2/pO_2, salinity and temperature in their surrounding environment. In intertidal zones, estuaries and water bodies with restricted connection to the well buffered open ocean like the Baltic Sea, naturally recurrent elevated pCO_2 (hypercapnia, ~234 Pa *vs.* normal levels of 39 Pa), changes in pH (7.5-8.2) and temperature (3.3-18.7°C), as well as varying salinity (14.5-21.5 ppt) challenge acid-base homeostasis on a regular basis. Additionally, decapods in these shallow water environments are prone to be trapped in tide pools, where they experience even more drastic changes in all abiotic parameters (Truchot, 1988), including extremely low levels of oxygen (hypoxia; Truchot and Duhamel-Jouve, 1980). In extreme cases, these tide pools fall dry so that decapods are exposed to air. As mentioned above, many decapod crustacean species hide in burrows and caves or bury in the sediment to avoid predators (Larsen et al., 2014). With only limited water circulation around them, metabolic ammonia builds up around the animals, consequently exposing them to high environmental ammonia (HEA, Weihrauch et al., 2004b), another challenging factor for acid-base homeostasis mainly for osmoconforming crabs like *M. magister* (Martin et al., 2011). Furthermore, pH has been shown to decrease to as low as 6.5 within the first few centimeters of sand and mud substrates, accompanied by elevated CO_2 levels of up to 1,600 Pa (Widdicombe et al., 2011).

Besides these naturally occurring challenges for acid-base homeostasis, global climate change and its impacts on acid-base regulation of decapod crustaceans and other invertebrates has become of greatest concern (Whiteley, 2011). On the one hand, the anthropogenic increase of atmospheric pCO_2 and its oceanic uptake will result in a decrease of the surface ocean pH of up to 1.4 units by the year 2300, termed ocean acidification (IPCC, 2013). Even though crustaceans are predicted to be less sensitive to ocean acidification than other invertebrates, still one third of all investigated species in a current meta-analysis by Wittmann and Pörtner (2013) were negatively affected at an environmental pCO_2 of 851 and 1,370 μatm (scenario RCP8.5.; Meinshausen et al., 2011). On the other hand, the increase of ocean surface temperature of up to 3°C by the year 2100 as predicted by the Intergovernmental Panel on Climate Change (Collins et al., 2013) might pose an additional challenge for acid-base homeostasis in decapod crustaceans, and even to shifts in whole ecosystem ecology and animal distributions (Walther et al., 2002). As a result, so-called dead zones and oxygen minimum zones (zones of low or depleted oxygen) are markedly increasing due to anthropogenic pollution and the resulting increase in algal blooms, and likely also due to a decrease in water circulation resulting from global warming (Mora et al., 2013). In combination, ocean acidification and warming negatively affected the included crustacean species' growth and potentially their survival, but had no severe effects on calcification (Harvey et al., 2013).

1.2. Whole animal acid-base homeostasis and regulation

1.2.1. Hemolymph acid-base status

In decapod crustaceans, acid-base homeostasis on the whole animal level is described best by the carbonate system characteristics of the extracellular fluid. A disturbance of acid-base homeostasis can be of metabolic (shifts in aerobic/anaerobic metabolism and production of organic acids) or respiratory (shifts in respiratory CO_2) origin and leads to a decrease (acidosis) or increase (alkalosis) in hemolymph pH if not compensated for. Typically, these fluctuations are depicted in a Davenport diagram as shown in figure 1.2 (Davenport, 1974).

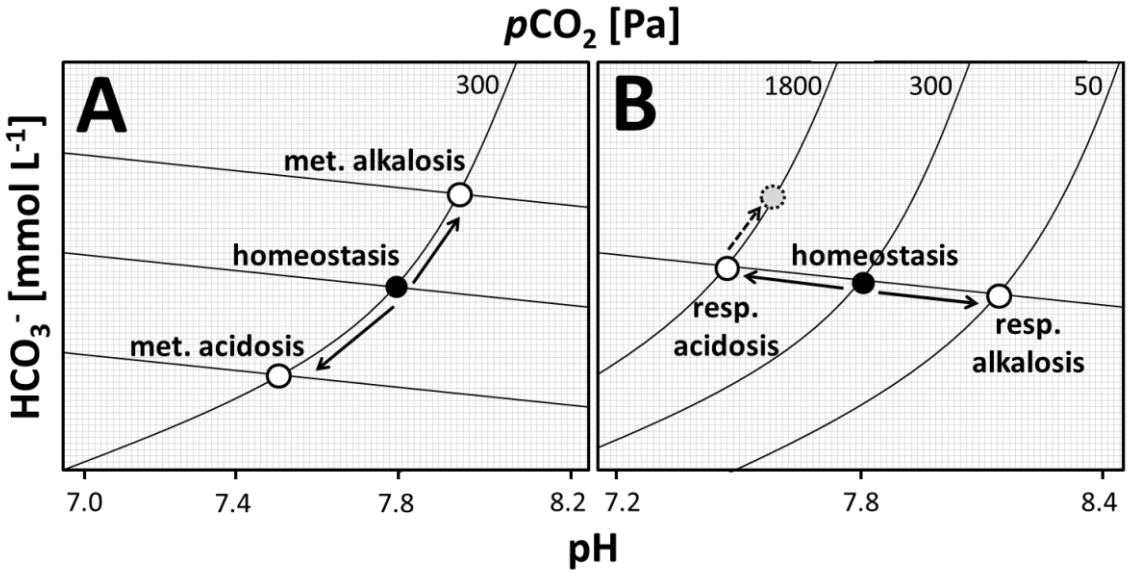

Figure 1.2. Davenport diagram for disturbances in acid-base homeostasis. (A) metabolic and (B) respiratory components of acid-base disturbance. Indicated values are fitted roughly to represent an average decapod crustacean as listed in tables 1.1.-1.7. Diagrams are reproduced according to Davenport (1974). Filled circles, acid-base homeostasis; open circles, status after disturbance. Horizontal lines indicate the non-bicarbonate buffer lines. The dashed line indicates a potential partial compensation by accumulation of HCO_3^- (grey circle; see text for details).

Besides a relatively small contribution of non-carbonate buffers like the respiratory pigments and other proteins (i.e. in *C. maenas*, Appelhans et al., 2012), adjustments in the hemolymph carbonate speciation allows for the buffering of the extracellular fluid upon an acid-base disturbance, according to following equation:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

Due to the low solubility of O_2 in water, aquatic animals need to establish a high flow rate of the external medium across their major gas exchange surfaces in order to ensure sufficient oxygenation. Aquatic decapod crustaceans are therefore very restricted in adjusting ventilation rates in order to regulate pCO_2. With a pK value of 6.2, extracellular CO_2 is mainly dissociated into H^+ and HCO_3^- at average physiological pH. Therefore, acid-base disturbances may be counteracted using primarily ion regulatory mechanisms at the gills (Henry et al., 2012; Truchot, 1988).

Tables 1.1-1.7 give an overview of hemolymph acid-base characteristics of decapod crustaceans under control conditions, as well as changes induced by various stressors as described in section 1.1.3 and below. Due to the vast number of publications on acid-base disturbances, I do not claim for the list to be complete, but rather tried to give representative examples for as many different species as possible. In case multiple studies were available for the same species, I attempted to include the most relevant paper that was comparable to the other studies. Data was partly extracted from graphs and values transformed into the units as depicted in the tables where applicable. If significantly different, values are given for the time point of maximum effect as well as from the end of the incubation period.

Table 1.1. Physiological, whole animal response of decapod crustaceans upon exposure to air.

Species	Group	Reference	Condition	Water salinity [ppt]	Water/air temp [^{0}C]	Hemolymph pH	Hemolymph pCO_2 [Pa]	Hemolymph HCO_3^- [mM]	Hemolymph TCO_2 [mM]	Lactate [mM]
Lithodes santolla	2	Urbina et al., 2013	control	30	12	7.65	-	-	-	-
			50 h air	30	12	6.78	-	-	-	-
Pachygrapsus crassipes	3	Burnett and McMahon, 1987	control	32	23	7.8	306	-	5	1.0
			12 h air	N/A	23	7.7	1300	-	19	0.5
Eurytium albidigitum	3	Burnett and McMahon, 1987	control	32	23	7.7	270	-	4	1.0
			8 h air	N/A	23	7.3	1500	-	9	0.5
Hemigrapsus nudus	3	Burnett and McMahon, 1987	control	32	11	7.80	330	-	9	-
			4 h air	N/A	11	7.95	530	-	15	-
Neohelice granulata	3	Luquet and Ansaldo, 1997	control	"BW"	20	7.82	280	-	-	-
			6 h air	N/A	20	7.75	310	-	-	-
Carcinus maenas	3	Simonik and Henry, 2014	control	32	10	7.70	-	-	7.8	1.1
			6 h air	N/A	10	7.57	-	-	9.9	7.1
Carcinus maenas	3	Truchot, 1975b	control	35	15	7.82	150	-	3.86	-
			9 h air	N/A	15	7.59	680	-	9.67	-
			115 h air	N/A	15	7.79	590	-	13.79	-
Cancer productus	3	DeFur and McMahon, 1984	control	32-35	10	7.84	320	-	10	0.9
			4 h air	N/A	10	7.62	510	-	13	9.0
Scylla serrata	3	Varley and Greenaway, 1992	control	35	25	7.70	400	-	8	1.25
			6 h air	N/A	25	7.58	740	-	10	1.04
			7 d air	N/A	25	7.79	830	-	19	1.04
Homarus gammarus	5	Whiteley and Taylor, 1990	control	32-35	15	7.8	130	4.1	-	0.41
			3 h air	32-35	10	7.6*	330*	6.6*	-	2.12*
			24 h air	32-35	20	7.8	800*	18.2*	-	3.24*
Homarus gammarus	5	Taylor and Whiteley, 1989	control	35	15	7.78	400	9.0	-	1.8
			14 ha air	N/A	15	7.64*	1200	15.8	-	6.2
Austropotamobius pallipes	6	Taylor and Wheatly, 1981	control	0	15	7.90	400	6.9	-	0.55
			4 h air	N/A	15	7.45*	1130*	6.9	-	8.28*
			24 h air	N/A	15	7.79	1040*	15.4*	-	0.57

Group definitions: (2) Anomura, (3) crabs, (5) lobster, and (6) crayfish. Where available from the study, asterisks indicate significant differences.

Table 1.2. Physiological, whole animal response of decapod crustaceans upon changes in environmental pO_2 (hyper- or hypoxia).

Species	Group	Reference	Condition	Water pO_2 [kPa]	Water salinity [ppt]	Water temp [^{0}C]	Hemolymph pH	Hemolymph pCO_2 [Pa]	Hemolymph HCO_3^- [mM]	Hemolymph TCO_2 [mM]	Lactate [mM]
Carcinus maenas	3	Burnett and Johansen, 1981	control	19.3 ?	35	10	7.85	450	-	13	-
			66 h	2.7-3.3	35	10	8.05*	170*	-	7*	-
			control	19.3 ?	17	10	7.95**	425	-	15.9**	-
			22 h	2.7-3.3	17	10	8.29*	95*	-	6.2*	-
Carcinus maenas	3	Hill et al., 1991	control	21	30	10	7.86	240	6.52	8.2	1
			30 min	0	30	10	8.09*	190	-	8.1	3
			12 h	0	30	10	7.84	190	3.17*	5	18*
Palaemon elegans	4	Taylor and Spicer, 1991	control	19.3 ?	32	10	7.79	-	-	5.4	0.55
			6 h mod	4	32	10	7.93*	-	-	1.9*	5.00*
			6h sev	1.3	32	10	7.95*	-	-	5.3	0.73
Palaemon serratus	4	Taylor and Spicer, 1991	control	19.3 ?	32	10	7.8	-	-	5.1	0.6
			6 h mod	4	32	10	8.0*	-	-	1.5*	6.7*
			6 h sev	1.3	32	10	8.1*	-	-	4.9	1.2
Astacus leptodactylus	6	Dejours and Beekenkamp, 1977	control	3.2	0	13	7.85	330	5.9	-	-
			44 d (hyper)	80	0	13	7.65	800	9	-	-
Pacifastacus leniusculus	6	Wheatly et al., 1991	control	-	0	12	7.83	330	7	-	-
			6 h (hyper)	67	0	12	7.55*	800	10*	-	-
			48 h (hyper)	67	0	12	7.73	730	13*	-	-
Austropotamobius pallipes	6	Wheatly and Taylor, 1981	control	19.3	0	15	7.90	405	-	7.5	0.40
			24 h low $	8.4	0	15	7.96	230	-	5.0	0.49
			24 h mod $	4.8	0	15	7.98	195	-	4.0	0.97
Orconectes rusticus	6	Wilkes and McMahon, 1982	control	16.5	0	15	7.78	480	-	5.84	-
			24 h	6-7.3	0	15	7.98*	140*	-	2.30*	-
			6 d	6-7.3	0	15	7.90*	270*	-	3.20*	-

Group definitions: (3) crabs, (4) shrimp, and (6) crayfish. Asterisks indicate significant differences, where available. Double asterisks indicate values significantly different from the other control. Question marks indicate a value taken from a cross-reference within the paper.

Table 1.3. Physiological, whole animal response of decapod crustaceans upon changes in environmental pCO_2 (hypercapnia).

Species	Group	Reference	Condition	Water pH	Water pCO_2 [kPa]	Water salinity [ppt]	Water temp [^{0}C]	Hemolymph pH	Hemolymph pCO_2 [Pa]	Hemolymph HCO_3^- [mM]	Hemolymph TCO_2 [mM]
Callinectes sapidus	3	Henry et al., 1981	control	-	-	25	26	7.78	270	-	5
			48 h	-	2	25	26	7.61	2100	-	20
Callinectes sapidus	3	Cameron, 1978	control	-	0.04	-	-	7.96	530	-	10
			15 min	-	1	-	-	7.65	1470	-	12
			44 h	-	1	-	-	7.80	1200	-	19
Carcinus maenas	3	Appelhans et al., 2012	control	8.06	0.066	15	13	7.82	380	8	-
			10 weeks mod	7.84	0.126	15	13	7.83	490	10	-
			10 weeks sev	7.36	0.351	15	13	7.81	550	12	-
Carcinus maenas	3	Truchot, 1975a	control	7.8	0.04	35	17	7.79	180	3.9	-
			24 h	7.4	0.31	35	17	7.71	730	13.7	-
Necora puber	3	Spicer et al., 2007	control	7.98	0.02	34	15	7.90	190	6.6	-
			24 h	7.30	0.08	34	15	7.82	360	10.8	-
			24 h	6.70	1.11	34	15	7.97	1190	27.4	-
			24 h	6.05	6.04	34	15	7.59	4520	55.9	-
Necora puber	3	Small et al., 2010	control	7.85	0.074	35	17	7.73	190	2.63	-
			30 d sev	6.69	1.250	35	17	7.53	880	8.66	-
Metacarcinus magister	3	Hans et al., 2014	control	8.1	0.049	32	14	7.93	133	4.9	-
			7 d	7.4	0.330	32	14	8.01	402*	14.9*	-
Metacarcinus magister	3	Pane and Barry, 2007	control	7.90	-	34	10	7.82	265	6	-
			90 min	7.08	1.013	34	10	7.42*	1000*	8*	-
			24 h	7.08	1.013	34	10	7.75	900*	18*	-
Chionoecetes tanneri	3	Pane and Barry, 2007	control high O_2	7.85	-	34	3	7.8	240	5	-
			24 h, at high O_2	7.08	1.013	34	3.5	7.4*	535*	5	-
			control low O_2	7.08	-	34	3	8.00	240	9.5	-
			24 h, at low O_2	7.08	1.013	34	3.5	7.65*	665*	11.5	-
Pandalus borealis	4	Hammer and Pedersen, 2013	control	8.1	0.052	35	7	7.76	250	5.1	-
			16 d	6.9	0.920	35	7	7.60	1000*	13.0*	-
Palaemon elegans	4	Dissanayake et al., 2010	control	8.0	0.10	32	15	7.70	-	?	-
			14 d	7.5	0.31	32	15	7.55	-	Δ1.0	-
			30 d	7.5	0.38	32	15	7.80	-	Δ1.8	-
Palaemon serratus	4	Dissanayake et al., 2010	control	8.0	0.12	32	15	7.7	-	?	-
			14 d	7.5	0.32	32	15	7.7	-	Δ1.2	-
			30 d	7.5	0.37	32	15	8.2	-	Δ2.4	-

(Caption for table 1.3) Group definitions: (3) crabs, and (4) shrimp. Asterisks indicate significant differences, where available. Question marks indicate unavailable data; accordingly, differences in hemolymph HCO_3^- are given as the difference in comparison to controls.

Table 1.4. Physiological, whole animal response of decapod crustaceans upon changes in environmental temperature.

Species	Reference	Condition	Water salinity [ppt]	Water temp [^{0}C]	Hemolymph pH	Hemolymph pCO_2 [Pa]	Hemolymph HCO_3^- [mM]	Lactate [mM]
Carcinus maenas	Truchot, 1973	control	20	15	7.75	335	6.6	-
		4 d	20	5	7.90	225	7.3	-
		4 d	20	10	7.75	280	6.5	-
		4 d	20	21	7.63	425	5.9	-
		4 d	20	26	7.53	545	5.5	-
Carcinus maenas	Howell et al., 1973	control	32	15	7.79	625	13.2	-
		7 d	32	5	8.07	305	16.7	-
		7 d	32	10	7.86	590	16.0	-
		7 d	32	20	7.77	560	10.2	-
Callinectes sapidus	Howell et al., 1973	control	32	15	7.77	320	5.9	-
		7 d	32	5	8.03	465	18.8	-
		7 d	32	10	7.80	425	9.2	-
Callinectes sapidus	Cameron and Batterton, 1978	control	0	10	7.94	280	10.35 $^{\$}$	-
		7-14 d	0	27	7.74	400	7.60 $^{\$}$	
Metacarcinus magister	McMahon et al., 1978	control	32	12	7.84	225	5.83	1.5
		10 d	32	7	7.91	235	7.42	1.4
		10 d	32	17	7.73	250	4.47	1.5

All studies have been conducted on brachyuran crabs. $^{\$}$values are for total carbon.

Table 1.5. Physiological, whole animal response of decapod crustaceans upon changes in environmental salinity.

Species	group	Reference	Condition	Water pH	Water pCO_2/pO_2 [kPa]	Water salinity [ppt]	Water temp [^{0}C]	Hemolymph pH	Hemolymph pCO_2 [Pa]	Hemolymph HCO_3^- [mM]
Callinectes sapidus	3	Henry and Cameron, 1982	control	-	-	35	-	7.75	320	4.2
			24 h	-	-	12	-	7.84	400	9.0
Eriocheir sinensis	3	Truchot, 1992	control	8.1	0.06	35	15	7.89	280	7.9
			3 d	8.4	0.06	0	15	8.02*	495*	16.0*
			control	8.4	0.06	0	15	7.96	425	12.2
			24 h	8.1	0.06	35	15	7.96	240*	8.0*
Eriocheir sinensis	3	Whiteley et al., 2001	control	-	-	35	12	7.76	460	7.1
			6 h	-	-	12	12	7.50*	290	4.0
			24 h	-	-	12	12	7.94	310	9.0
Necora puber	3	Whiteley et al., 2001	control	-	-	35	12	7.82	300	7
			6 h	-	-	24	12	7.80	500	9
Carcinus maenas	3	Truchot, 1981	control	8.19	0.039	35	15	7.85	215	5.5
			48 h	7.85	0.040	12	15	8.08*	200	8.0*
			24 h	8.19	0.039	35	15	7.65*	190	3.0*
Pacifastacus leniusculus	6	Wheatly and McMahon, 1982	control	-	-	0	15	7.95	365	8.47
			48 h	-	-	8	15	8.00	180	5.73
			48 h	-	-	16	15	7.96	140	4.76*
			48 h	-	-	32	15	7.83*	90	2.83*

Group definitions: (3) crabs, and (6) crayfish. Asterisks indicate significant differences, where available.

Table 1.6. Physiological, whole animal response of decapod crustaceans upon exercise.

Species	Group	Reference	Condition	Water salinity [ppt]	Water temp [^{o}C]	Hemolymph pH	Hemolymph pCO_2 [Pa]	Hemolymph TCO_2 [mM]	Lactate [mM]
Metacarcinus magister	3	McDonald et al., 1979	control	27	8	7.94	230	8.3	0.69
			20 min	-	-	7.52	430	-	9.90
Callinectes sapidus	3	Booth et al., 1984	control	31	20	7.67	470	5.7	2
			30 min	31	20	7.24	800	3.6	15
Carcinus maenas	3	Hamilton and Houlihan, 1992	control	31	15	7.83	210	5.2	3.9
				31	15	7.76*	250	4.3	9.7*
Homarus americanus	5	Rose et al., 1998	control	32	14	7.78	268	4.80	0.20
			30 min slow	32	14	7.64*	507*	5.51*	1.55*
			30 min fast	32	14	7.42*	603*	5.74*	1.29*

Group definitions: (3) crabs, and (5) lobster. Asterisks indicate significant differences, where available.

Table 1.7. Physiological, whole animal response of decapod crustaceans upon a combination of different environmental stressors.

Species	Group	Reference	Condition	Water pH	Water pCO_2/pO_2 [kPa]	Water salinity [ppt]	Water/air temp [^{0}C]	Hemolymph pH	Hemolymph pCO_2 [Pa]	Hemolymph HCO_3^- [mM]	Hemolymph TCO_2 [mM]	Lactate [mM]
Metapenaeus joyneri	1	Dissanayake and Ishimatsu, 2011	control	8.2	0.04	32	15/20/25	7.65	-	-	-	-
			10 d HC	8.2	1.00	32	15	7.73	-	-	-	-
			10 d HC+temp	8.2	1.00	32	20	7.79	-	-	-	-
			10 d HC+temp	8.2	1.00	32	25	7.80 (2 d)	-	-	-	-
Hyas araneus	3	Harms et al., 2014	control	8.2	0.045	32	5	8.05	250	2.8	-	-
			10 weeks HC mod	7.8	0.100	32	5	8.00	350*	4.2	-	-
			10 weeks HC sev	7.6	0.190	32	5	7.75*	430*	4.0	-	-
			control + temp	8.2	0.037	34	10	7.98	210	1.4	-	-
			10 weeks mod HC+temp	7.9	0.095	34	10	7.95	430*	5.1*	-	-
			10 weeks Sev HC+temp	7.5	0.204	34	10	7.70*	430*	3.0	-	-
Necora puber	3	Rastrick et al., 2014	(0) control	-	0.041	35	10	7.84	290	6.6	-	1.0
			(1) 2 weeks temp	-	0.041	35	15	7.89	300	7.1	-	1.0
			(2) 2 weeks HC	-	0.101	35	10	7.87	380	9.2*	-	1.0
			(3) 2 weeks temp+HC	-	0.101	35	15	7.95	370	11.0*	-	1.0
			control: 3 h air (0)	-	0.041	N/A	10	7.3**	1100**	7.0	-	8.0**
			3h air of (1)	-	0.041	N/A	15	7.3	1600	12.0	-	7.5
			3h air of (2)	-	0.101	N/A	10	7.5	1100	14.0	-	9.0
			3h air of (3)	-	0.101	N/A	15	7.3	2100	14.0	-	10.0
Cancer productus	3	DeFur et al., 1980	control	-	18	32	10	7.89	280	-	9.0	-
			4 h air	-	-	N/A	10	7.62	1100	-	13.6	-
			24 h hyperoxia	-	61-68	32	10	7.80	1100	-	20.0	-
Chionoecetes tanneri	3	Pane and Barry, 2007	control high O_2	7.85	-	34	3	7.8	240	5	-	-
			24 h HC, at high O_2	7.08	1.013	34	3.5	7.4*	535*	5	-	-
			control low O_2	7.08	-	34	3	8.00	240	9.5	-	-
			24 h HC, at low O_2	7.08	1.013	34	3.5	7.65*	665*	11.5	-	-

Group definitions: (1) prawns, and (3) crabs. Asterisks indicate significant differences, where available. Double asterisks indicate values significantly different from the other control. HC, hypercapnia; mod, moderate; sev, severe.

Under control conditions, all decapod crustaceans maintain their hemolymph pH typically between 7.7 and 8.0. Hemolymph pCO_2 levels, however, can vary quite substantially between crustacean species and are typically low (*ca.* 200-500 Pa) due to the almost complete dissociation to H^+ and HCO_3^- at physiological pH, but high enough likely to enable diffusion out of the body along the gradient between the extracellular fluid and the environment (*ca.* 40 Pa; Henry et al., 2012; Melzner et al., 2009). Prawns like *P. japonicas* seem to be an exception: These decapod crustaceans have a slightly lower than average hemolymph pH (7.5) and accordingly, a higher pCO_2 (*ca.* 600 Pa; Cheng et al., 2013). Similar to hemolymph pCO_2, levels of hemolymph HCO_3^- have also been observed to vary between species, and typically average between 4-9 mmol L^{-1}. As indicated in tables 1.1-1.7, control levels for hemolymph [HCO_3^-] as high as 14 mmol L^{-1} have been observed in some studies, but these values have to be treated with caution as they may indicate that animals were in pre-moult rather than intermoult stages (see 1.2.2).

Exposure to air (table 1.1). The primary consequence for most aquatic decapod crustaceans of emerging from water is the collapse of their gills, physically impairing gas exchange processes. Many crabs therefore retain branchial water in their gill chambers, likely to facilitate CO_2 diffusion (Burnett and McMahon, 1987). Due to the higher solubility of O_2 in air compared to water, however, some decapod crustaceans like the striped shore crab, *Pachygrapsus crassipes* (Burnett and McMahon, 1987), or *C. maenas* (Simonik and Henry, 2014) are capable of extracting O_2 from the air, and voluntarily move out of the water to offset acid-base disturbances resulting from other stressors like hypoxia (Wheatly and Taylor, 1979).

Generally, exposure to air results in a pronounced respiratory acidosis with a pH drop of 0.1-0.2 units (in crabs) up to 0.5-0.7 units (in Anomura and crayfish), a 2-5 fold increase in hemolymph pCO_2 and a significant 2-3 fold elevation of hemolymph HCO_3^- in all investigated decapod crustaceans (table 1.1).

It seems, however, that there are marked species-specific differences in compensating for the experienced acidosis. While some crabs (DeFur and McMahon, 1984; Simonik and Henry, 2014), lobsters (Taylor and Whiteley, 1989; Whiteley and Taylor, 1990) and crayfish (Taylor and Wheatly, 1981) seem to switch partly to anaerobic metabolism and therefore experience an additional metabolic acidosis with a pronounced increase in hemolymph lactate levels, other crabs like *Eurytium albidigitum* (Luquet and Ansaldo, 1997) and *P. crassipes* (Burnett and McMahon, 1987) seem to undergo a metabolic depression (*E. albidigitum*) or maintain or even increase their aerobic metabolism (*P. crassipes*). Additionally, some crab species were observed to increase their strong ion difference (SID) *via* ion exchange processes at the gill in response to emersion (Burnett and McMahon, 1987; Luquet and Ansaldo, 1997; Truchot, 1979), which is interpreted to help offset the experienced acidosis (Stewart, 1978). Consequently, *C. maenas* (Truchot, 1975b), the mangrove crab, *Scylla serrata* (Varley and Greenaway, 1992), *Homarus gammarus* (Whiteley and Taylor, 1990), the white-clawed crayfish, *Austropotamobius pallipes* (Taylor and Wheatly, 1981), *P. crassipes* and the purple shore crab, *Hemigrapsus nudus* (Burnett McMahon, 1987), are capable of fully compensating for the pH drop resulting from the experienced acidosis, while *E. albidigitum* is not (Burnett and McMahon, 1987).

Hyper-/hypoxia (table 1.2). Due to its low solubility in water compared to air, oxygen has to be considered one of the limiting factors in the aquatic environment (Dejours, 1975). Hence, only subtle changes in water pO_2 result in immediate alterations of ventilation rates in aquatic decapod crustaceans in order to be able to maintain aerobic metabolism (Jouve-Duhamel and Truchot, 1983; Truchot, 1988). Consequently, hyperventilation as observed in moderate hypoxic conditions simultaneously leads to an increase in branchial CO_2 excretion and therefore respiratory alkalosis (elevated pH and lower HCO_3^-), while reduced ventilation in a moderate hyperoxic environment ultimately leads to accumulation of hemolymph pCO_2 and hence a respiratory acidosis (lower pH and higher HCO_3^-), as can be seen in table 1.2. In shrimp and *C. maenas*, the increase in hemolymph lactate during moderate hypoxia and anoxia, respectively, suggests that in these decapod crustaceans a metabolic component seems to be present that might explain the reduced levels of total carbon/HCO_3^-, but did not affect the actual increase in hemolymph pH (Taylor and Spicer, 1991). Interestingly, when the same shrimp species *Palaemon elegans* and *P. serratus* were exposed to a severe hypoxia (< 2 Pa), lactate levels stayed constant and total carbon was not affected (Taylor and Spicer, 1991).

Hypercapnia (table 1.3). In contrast to hypoxia, exposure to elevated environmental pCO_2 does not drive a ventilation response in decapod crustaceans due to its very similar solubility in air and water (Henry et al., 2012; Jouve-Duhamel and Truchot, 1983). Nonetheless, exposure to hypercapnia leads to a respiratory acidosis marked by a rapid drop in hemolymph pH of up to 0.4 units and substantial increases in pCO_2 (2-4 fold) in all investigated decapod crustaceans (table 1.3). Elevated extracellular pCO_2 is believed to be maintained in order to ensure an outwardly directed CO_2 gradient for the diffusion-

based excretion of metabolic CO_2 (Melzner et al., 2009). Most decapod crustaceans are capable of fully compensating for the respiratory acidosis by accumulating HCO_3^- in their hemolymph to buffer excess protons, likely *via* active ion regulatory processes at the gill. While some species are capable of maintaining or even increasing their metabolic rate in response to hypercapnia (*C. maenas*, Appelhans pers. communication), others experience a metabolic depression (e.g. *M. magister*, Hans et al., 2014; *P. borealis*, Hammer and Pedersen, 2013).

Temperature (table 1.4). It has been shown for poikilotherm animals such as decapod crustaceans that temperature correlates inversely with hemolymph pH in order to maintain extracellular H^+/OH^- ratios to ensure a constant net charge of proteins (Howell et al., 1973; Truchot, 1973). In parallel, hemolymph pCO_2 seems to generally stay constant/increase only slightly with increasing temperature, while $[HCO_3^-]$ and/or total carbon (C_T) decreases more drastically. The authors of the respective studies (Cameron and Batterton, 1978b; Truchot, 1973) attributed the changes in C_T to active regulation of HCO_3^- *via* ion exchanges at the gills in order to compensate for the acidosis, rather than solution of the carapace or passive processes alone (Henry et al., 2012).

Salinity (table 1.5). Generally, acclimation to low salinity results in a metabolic alkalosis in all investigated decapod crustaceans, characterized by an increase in hemolymph pH and relatively stable pCO_2, but a significant increase in $[HCO_3^-]$. Conversely, when freshwater crayfish (Wheatly and McMahon, 1982), freshwater Chinese mitten crabs (Truchot, 1992) or brackish water acclimated green crabs (Truchot, 1981) were acclimated to full-strength seawater, they developed a metabolic acidosis characterized by a decrease in hemolymph pH and HCO_3^-. In this case, however, an additional slight

respiratory alkalosis (decrease in pCO_2) was observed as well, likely compensating for the metabolic acidosis. Throughout the time course of different salinity acclimations, however, species can exhibit specific alterations to this general pattern. An initial respiratory acidosis for example was observed in *C. maenas* upon acclimation to dilute salinity before switching to the expected metabolic alkalosis (Truchot, 1981), and in *E. sinensis* a transient respiratory acidosis marked by a spontaneous drop in pH was only present at 6 days (Truchot, 1992).

While Henry and Cameron (1982) attributed the observed increase in hemolymph [HCO_3^-] following acclimation of *C. sapidus* to dilute salinity to the additionally observed change in the strong ion difference (anisosmotic extracellular regulation), no equivalent observation was made in brackish-water acclimated *E. sinensis* in the study by Whiteley et al. (2001). In contrast, Truchot (1981, 1992) suggested isosmotic intracellular regulation in the cells leading to overall metabolic adjustments to be responsible, resulting in either a measureable efflux of base or acid into the environment. These observations reveal the complex nature of acid-base disturbances upon different salinity acclimations and consequently, the reasons for the observed metabolic alkalosis and acidosis, respectively, are not yet fully understood.

Exercise (table 1.6). Hemolymph lactate levels are typically held lower than 1 mmol L^{-1} and negligible in undisturbed decapod crustaceans, but can increase more than 10-fold in crabs that experience a metabolic acidosis due to exercise. Furthermore, the experienced acidosis is characterized by an immediate drop in pH of up to 0.4 units and a 2-fold increase in hemolymph pCO_2, therefore also resembling characteristics of a respiratory acidosis. Interestingly, exercised lobsters seem to be able to avoid anaerobic metabolism

during exercise for the most part and experience primarily a respiratory acidosis without the substantial rises in hemolymph lactate observed in other crustaceans (Rose et al., 1998).

In *M. magister* (McDonald et al., 1979), *C. maenas* and *C. sapidus* (Booth et al., 1984), the proton concentration in the hemolymph was observed to be lower than could be expected from the accumulated lactate, given that both are produced in equimolar quantities during glycolysis (Hochachka and Mommsen, 1983). Due to the observed drastic increase in ammonia excretion, Booth et al. (1984) concluded that at least part of the protons are excreted as NH_4^+ *via* ion exchange processes at the gill epithelium.

Combined stressors (table 1.7). As is clear by the previous sections, extracellular acid-base regulation in response to environmental disturbances can be quite complex despite some common principles. While the discussed studies isolated one stressor at a time and investigated its effects on the respective species' acid-base characteristics, environments are rarely that "simple" and a combination of simultaneous stressors seems much more likely, especially in tide pools (Truchot, 1988) in the face of the ongoing global climate change (IPCC, 2013).

For example, when combined with an increase in water temperature, hypercapnia resulted in a respiratory alkalosis in the velvet swimming crab, *Necora puber*, that was not observed when crabs where exposed to either one of the stressors alone (Rastrick et al., 2014). Even though the increase in HCO_3^- upon hypercapnia and high temperature acclimation rendered the crabs more resistant to short periods of subsequent emersion, they still experienced the same magnitude of acidosis and recovery from these stressors was significantly attenuated. In a different example, prior acclimation of green crabs to

hypercapnia enabled them to avoid an uncompensated hypercapnic acidosis that was induced by low environmental alkalinity in normocapnic-acclimated animals (Truchot, 1984). In *C. productus*, the respiratory acidosis usually observed following exposure to hyperoxia was not observed in crabs that were first exposed to air (DeFur et al., 1980). Finally, *Metapenaeus joyneri* exposed to a combination of elevated temperature and hypercapnia no longer experienced an acidosis as to be expected by studies on other decapod crustaceans, but exhibited an alkalosis (Dissanayake and Ishimatsu, 2011).

Even though studies on combined environmental stressors are sparse, the existing data indicates alarming differences in the acid-base responses of decapod crustaceans in comparison to single-stressor studies. Therefore, it would be desirable for future research to focus on a more holistic and realistic approach.

1.2.2. Calcification, $CaCO_3$ and moulting

Due to their hard and inflexible exoskeleton, decapod crustaceans depend on a series of moults in order to grow. During the different pre-, inter- and postmoult stages that compose the complex moult cycle (Mangum et al., 1985), the connectives between the living tissue and the extracellular cuticle are loosened and water uptake ensures the shedding of the old and the expansion of the new carapace. The exoskeleton contains the majority of the organismal $CaCO_3$ that is mobilized during the moult in order to soften this structure and either excreted into the environment or stored in gastroliths for the new exoskeleton (Ahearn et al., 2004). Generally, decapod crustaceans experience a pronounced pre-moult alkalosis (increase in hemolymph HCO_3^-) in order to compensate for a concomitant acidosis of mainly metabolic origin (increase in hemolymph lactate) after successful exuviation (Mangum et al., 1985). Interestingly, this HCO_3^- seems not to

originate from mobilization of the exoskeletal stores. Even though an early study by Robertson (1960) detected a seemingly HCO_3^--correlated increase in hemolymph [Ca^{2+}] and [Mg^{2+}] in premoult *C. maenas*, later studies on the blue crab *C. sapidus* did not detect a change in hemolymph [Ca^{2+}] but observed a decrease in [Cl^-] instead, indicating a direct Cl^-/HCO_3^--exchange with the environment as the source of the extracellular HCO_3^- (Cameron and Wood, 1985; Cameron, 1978; Henry et al., 1981; Mangum et al., 1985). As a response to air exposure, however, crayfish (Wheatly and Gannon, 1995), the anomuran porcelain crabs *Petrolisthes laevigatus* (Lagos and Cáceres, 2008) and *P. violaceus* (Vargas et al., 2010), as well as sub-populations of the brachyuran crab *Cyclograpsus cinereus* (Lagos et al., 2014) and *Neohelice* (*Chasmagnathus) granulata* (Luquet and Ansaldo, 1997) were able to mobilize exoskeletal Ca^{2+}/HCO_3^- stores in response to the acid-base disturbance.

Interestingly and in contrast to other invertebrate marine calcifiers like mussels (Beniash et al., 2010; Michaelidis et al., 2005) and corals (Langdon et al., 2000), calcification of the carapace in response to hypercapnia (ocean acidification) seems to increase in the red rock cleaner shrimp *Lysmata californica* (Taylor et al., 2015), the prawns *(Lito)Penaeus occidentalis* and *P. monodon* (Wickins, 1984), female Anomuran Red king crab, *Paralithodes camtschaticus* (Long et al., 2013b), as well as the crab *C. sapidus*, the lobster *H. americanus* and the prawn *Penaeus plebejus* (Ries et al., 2009). The robustness of the crustacean carapace is believed to be due to its increased amount of calcite, the less soluble form of $CaCO_3$ (Taylor et al., 2015), as well as its complete coverage with a relatively thick organic epicuticle (Ries et al., 2009) and the crustaceans' generally high capability for acid-base regulation. While an increase in calcification might sound

advantageous, it potentially has negative effects on the crustaceans' moulting frequency (Wickins, 1984) and crypsis/predator defense (Taylor et al., 2015). The metabolic investment and possible allocation of energy resources due to an increased calcification might also lead to other negative impacts in these crustaceans that might reduce the overall fitness (i.e. metabolic depression and reduced and growth; Taylor et al., 2015). An increase in calcification as well as an increase in metabolic costs in premoult was already observed in very early life stages (zoea I larval stage) of *P. camtschaticus* (Long et al., 2013a) and the brachyuran great spider crab, *Hyas araneus* (Schiffer et al., 2013).

Only a few decapod crustaceans like *N. puber*, however, might also exhibit a dissolution of their exoskeleton in response to high levels of ocean acidification (Spicer et al., 2007). Also in late European lobster larvae, exposure to hypercapnia resulted in significantly lower carapace mass and mineralization in response to hypercapnia (Arnold et al., 2009), as well as a delay in the first moult cycles (Keppel et al., 2012).

1.2.3. Development and early life stages

Only few studies to date have investigated the effect of stressors known to result in disturbance of acid-base homeostasis for adults, on earlier life-stages and the development of decapod crustaceans. The major focus of studies regarding this topic has been the exposure to a future ocean scenario with elevated pCO_2 and the resulting ocean acidification. Evidence is nonetheless growing for negative impacts of hypercapnia already in the early and putatively more vulnerable life stages, even though sensitivity seems to be highly variable between species.

When exposed to environmental hypercapnia (140 Pa) for 3-10 days, all developmental stages (embryos, larvae and juveniles) of *P. cinctipes* (Anomura) were affected regarding

their metabolic performance (Carter et al., 2013). Surprisingly, embryos seemed to be the most sensitive stage and had a clearly lower metabolic rate. Larvae seemed to cope relatively well with hypercapnia by switching from lipid to protein metabolism which was evident by the increase in the C:N ratio. Finally, similar to larvae, juveniles did not seem to be directly impacted by exposure to hypercapnia but had a lower protein content (Carter et al., 2013). A different scenario is observed regarding the early life stages in *P. camtschaticus*. Even though female fecundity was not effected in this species, hypercapnia-exposed embryos showed clear morphological malformations and longer hatching times that led to abnormal larvae with a decreased survival rate (Long et al., 2013a). Also in juveniles of this species, lower survival and growth rates were observed and in addition, their condition index decreased (Long et al., 2013b). Accordingly, exposure of juvenile crayfish *A. pallipes* to low pH resulted in lower survival and growth (Haddaway et al., 2013).

Similar to what was observed in Anomura, also in the intertidal, osmoconforming brachyuran crab *M. magister* and the deep-water Tanner crab, *Chionoecetes bairdi*, exposure to high pCO_2 resulted in a decreased survival rate of larvae (Descoteaux, 2014; Miller, 2015). One of these studies also observed a prolonged hatching time and delay of the development of the different larval stages for *M. magister*, even though larval size was not affected (Miller, 2015).

In addition, larval and juvenile European lobster, *Homarus gammarus*, exposed to long-term hypercapnia showed severe morphological abnormalities, even though effects were not increased upon simultaneously elevated temperatures. The authors concluded hypercapnia had potential negative effects in lobsters: impaired respiration (puffy or

curled carapace), reduced ability to find food (bent rostrum) or sexual partners (walking legs, claw and antenna), and lowered swimming ability (tail-fan damages; Agnalt et al., 2013). Interestingly, the closely related American lobster showed more severe negative responses in larvae upon–acidification including deficiencies in growth and moulting (Keppel et al., 2012) that were not observed in *H. gammarus*, while embryos of the Norway lobster conversely seemed less vulnerable (Styf et al., 2013).

Generally, the present data indicate that hypercapnia might have direct and indirect consequences for the species' overall survival and fitness. In the majority of cases, decapod crustacean development through early life stages appears to be impaired which may lead to an increase in mortality. Interestingly, there is also evidence for significant parental effects (Carter et al., 2013) supporting the hypothesis that life history and genetic pre-disposition (Melzner et al., 2009) might indeed play an important role in the coping potential of specific species.

1.3. Gill epithelial acid-base regulation

1.3.1. Gill epithelial transporters involved in acid-base regulation

While the numerous studies on acid-base homeostasis in aquatic decapod crustaceans have focussed on describing the whole animal acid-base status in response to diverse environmental stressors as described above, to date only a few investigations have commented on the actual regulatory mechanisms involved. These few studies indicate that the high acclimation potential of decapod crustaceans in response to environmental changes can be attributed mainly to ion exchange processes in the gill epithelium (see

above). As the major site for osmoregulation and ammonia excretion (reviewed by Henry et al., 2012; Larsen et al., 2014; Weihrauch et al., 2004b), the gills possess many epithelial membrane transporters that are likely also involved in acid-base regulation. Indirect evidence was drawn from the observation that changes in hemolymph acid-base equivalents (H^+/HCO_3^-) were accompanied by changes in the strong ion difference (Na^+/Cl^-) when decapod crustaceans were exposed to air and dilute salinity (Burnett and McMahon, 1987; Ehrenfeld, 1974; Henry and Cameron, 1982; Luquet and Ansaldo, 1997; Truchot, 1979), or when carbonic anhydrase, the enzyme responsible for the hydration of CO_2 to H_2CO_3 and the subsequent dissociation into H^+ and HCO_3^- and *vice versa*, was blocked (Burnett et al., 1981; Henry and Cameron, 1983; Henry et al., 2003). While the crustacean gill epithelium has been subject to many investigations of membrane transporters involved in osmoregulation and ammonia excretion (for reviews see Henry et al., 2012; Larsen et al., 2014; Weihrauch et al., 2004b), hardly anything is known about the respective mechanisms for acid-base regulation. Figure 1.3 represents the current working models for general acid-base regulation, as well as ammonia excretion and osmoregulation in the model organism *C. maenas*.

Trans-branchial active NaCl transport in moderate hyper-osmoregulators such as *C. maenas* (Riestenpatt et al., 1996) and *N. granulata* (Lucu and Siebers, 1987; Onken et al., 2003) is fairly well characterized. As can be seen in figure 1.3.A, basolateral Na^+/K^+-ATPase and Cl^--channels, as well as apical $Na^+/K^+/2Cl^-$-cotransporter supported by apical and basolateral K^+-channels are key-players in this osmoregulatory mechanism. A number of studies also suggested an alternative pathway for NaCl uptake *via* apical Na^+/H^+- and HCO_3^-/Cl^- -exchangers, linked to the actions of a carbonic anhydrase (Henry

et al., 2003; Lucu, 1990; Onken et al., 2003; Tresguerres et al., 2008), therefore directly linking NaCl transport to the transport of acid-base equivalents. In *N. granulata*, however, a basolateral Na^+/H^+-exchanger seems to promote intracellular Na^+ uptake in exchange for H^+ rather than being situated apically (Tresguerres et al., 2008).

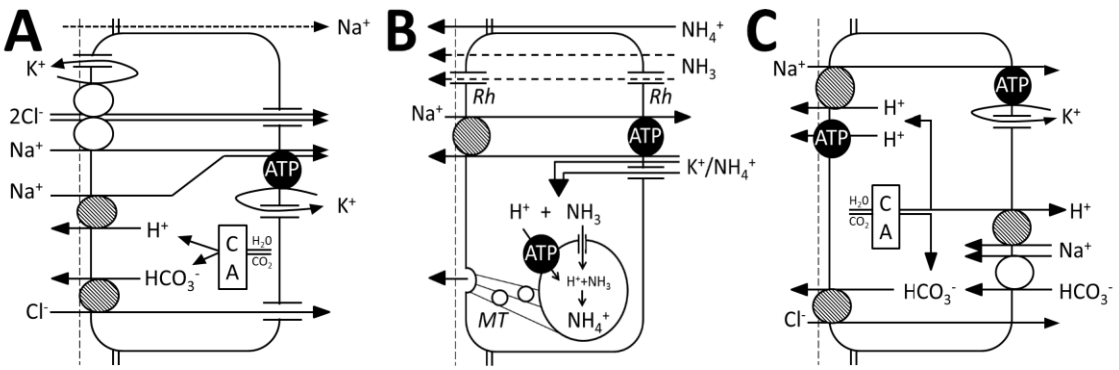

Figure 1.3. Current working models for branchial ion transport in decapod crustaceans. (A) osmoregulation, (B) ammonia excretion in the weak hyper-osmoregulator *Carcinus maenas* and (C) general hypothetical model for acid-base regulation in crustaceans. Models have been developed based on (A) Onken et al., 2003, (B) Weihrauch et al., 2002 and (C) Freire et al., 2008. ATP, ATPase; CA, carbonic anhydrase; MT, microtubule network; Rh, Rhesus-like protein. Potential overlaps in the transporter inventory of all models are basolateral Na^+/K^+-ATPase, basolateral K^+-channels and potential apical Na^+/H^+-exchanger. (A) and (C) also share a potential apical HCO_3^-/Cl^--exchanger. Light dashed lines indicate the cuticle covering the apical membrane.

Overlapping with the model for NaCl transport, basolateral Na^+/K^+-ATPase and $Cs^{+/}Ba^{2+}$-sensitive K^+-channels have also been shown to be involved in ammonia excretion by the gills of *C. maenas* (Weihrauch et al., 1998; Weihrauch et al., 2004b; figure 1.4.B). Additionally, a cytoplasmic V-(H^+)-ATPase and a functional microtubule network have been hypothesized to promote ammonia excretion over the apical membrane *via* NH_3 trapping and transport in acidified vesicles in this species (Weihrauch et al., 2002), potentially linking ammonia excretion with acid-base regulation.

In comparison to the models for osmoregulation and ammonia excretion, the hypothetical model for acid-base regulation (figure 1.3.C) is much more speculative. A study by Siebers et al. (1994) identified carbonic anhydrase and the basolateral Na^+/K^+-ATPase to be involved in branchial acid-base regulation. In fish, the Na^+/K^+-ATPase generates the electrochemical gradient over the basolateral membrane that is then the major driving force for the excretion of H^+ *via* apical Na^+/H^+-exchangers in acid excretory epithelial cells (Choe et al., 2005; Edwards et al., 2002). A recently identified basolateral Na^+/HCO_3^--exchanger in the squid *Sepioteuthis lessoniana* (Hu et al., 2014) has also been postulated to be important for acid-base regulation in the euryhaline crab *N. granulata* (Tresguerres et al., 2008). A strictly apical distribution of V-(H^+)-ATPase, as hypothesized in the only available model for crustacean acid-base regulation (Freire et al., 2008), has only been identified in freshwater (and terrestrial) crustaceans (Tsai and Lin, 2007), including the red crab *Dilocarcinus pagei* (Weihrauch et al., 2004a) and *E. sinensis* (Onken and Putzenlechner, 1995; Tsai and Lin, 2007), as well as many freshwater fish (Gilmour and Perry, 2009), to generate an electrochemical gradient over the apical membrane to drive Na^+ uptake (Larsen et al., 2014; Weihrauch et al., 2001). While an apical V-(H^+)-ATPase seems unlikely to be present in sea- and brackish-water acclimated decapod crustaceans for osmoregulatory purposes and due to the high buffering capacity of the environment, the involvement of an apical V-(H^+)-ATPase cannot be excluded.

1.3.2. Genetic responses to acid-base disturbance

Two recent microarray and transcriptomic studies have identified changes in (mRNA) expression levels of gill epithelial transporters upon environmental disturbances that helped identify some of the candidate genes involved in acid-base regulation.

Interestingly, exposure to hypercapnia did not seem to elucidate a typical stress-response in posterior gills of osmoregulating green crabs. Applying microarray and quantitative real-time experiments, Fehsenfeld et al. (2011) observed generally only subtle changes in mRNA expression levels among over 4400 genes in *C. maenas*, and did not identify any changes in heat-shock proteins resembling direct indicators for stress. Instead, the data suggested an increased contribution of vesicular membrane transport, indicating that the proposed vesicular transport for active ammonia excretion (Weihrauch et al., 2002; figure 1.3.B) might indeed contribute to $H^+(NH_4^+)$ excretion and therefore acid-base regulation in this species. Additionally, most of the annotated common ion transporters of the gill epithelium were not differentially expressed with the exception of a significant up-regulation of a calcium-activated chloride channel and a transcript most similar to the mammalian hyperpolarization activated nucleotide-gated potassium channel, as well as the down-regulation of a Cl^-/HCO_3^- exchanger of the SLC4 family. Interestingly, these genes were also affected by acclimation of green crabs to dilute salinity (Towle et al., 2011).

A different picture is generated in the branchial response of *H. araneus* upon exposure to different levels of environmental pCO_2 combined with varying temperatures (Harms et al., 2014). While Na^+/K^+-ATPase was up-regulated following exposure to hypercapnia alone and hypercapnia in combination with temperature, mRNA levels of V-(H^+)-ATPase

and carbonic anhydrase were only significantly elevated upon moderate and severe hypercapnia (Harms et al., 2014). Additionally, changes in genes involved in metabolism indicated an enhanced aerobic metabolism in response to moderate hypercapnia, while severe hypercapnia induced a metabolic depression. Specifically, decreased trehalose metabolism of the gills seems to be a common response of hypercapnia as well as temperature acclimation in *H. araneus*.

Similar to the response of *C. maenas* (Fehsenfeld et al., 2011), analysis of the Gene Ontology terms (GO-terms; project for the consistent descriptions of gene products across databases; http://geneontology.org/page/documentation) indicated a restructuring of the gill epithelium and/or the cytoskeleton upon hypercapnia in *H. araneus* (Harms et al., 2014), a phenomenon that can also be observed upon acclimation to dilute salinity in posterior gills of *C. maenas* (Compere et al., 1989). In contrast to *C. maenas*, however, gill epithelia of *H. araneus* seem to undergo a pronounced stress response that includes the increase of expression for transcripts involved in intracellular oxidative stress defense, including a number of peroxidases.

1.4. Linking acid-base with ammonia regulation

Even though ammonia excretion in decapod crustaceans has been the focus of many studies, the potential importance of ammonia regulatory patterns in respect to acid-base regulation has not been acknowledged to date. Generally, ammonia exists in a pH-dependent equilibrium between the weak base NH_3 and its acidic form NH_4^+. With a pKa of 9.15, most ammonia is present as NH_4^+ at physiological pH (Weiner and Verlander, 2013). Due to its physical properties, ammonia (and therefore ammonia

excretion) might therefore very well contribute to acid-base homeostasis as an additional hemolymph buffer beside the carbonate system. Being the primary waste product of protein catabolism, NH_3/NH_4^+ levels are ultimately linked to the overall metabolic rate of the organism. As mentioned earlier, metabolic rates of decapod crustaceans are individually adjusted when experiencing external stressors that simultaneously also affect acid-base homeostasis. In response to hypoxia for example, metabolism and hemolymph ammonia decreased significantly in *N. norvegicus* (Hagerman et al., 1990). A similar response was seen in *M. magister* when exposed to hypercapnia and included also a significant decrease in ammonia excretion rates (Hans et al., 2014). As a recently identified key-player in branchial ammonia excretion, Rhesus proteins have been shown not only to mediate NH_3, but also to act as CO_2 channels in human red blood cells (Endeward et al., 2008; Kustu and Inwood, 2006; Musa-Aziz et al., 2009; Soupene et al., 2002; Soupene et al., 2004) and fish gills (Perry et al., 2010). Branchial ammonia excretion possibly promoted by the Rhesus-like protein, and CO_2 excretion (and hence acid-base regulation) might therefore be linked in the decapod crustaceans gill.

1.5. The green crab, *Carcinus maenas*

Originating from European waters, covering the Baltic Sea to the Azores, green crabs (or shore crabs) became one of the most successful marine invaders on the planet during the last few decades (Lowe et al. 2000). Nowadays *C. maenas* can be found on the East and West coast of the USA and Canada, West and South Africa and Australia where they threaten the existing natural ecosystem communities as well as commercial fisheries (Cameron and Metaxas, 2005; Jamieson et al., 1998; Lafferty and Kuris, 1996; Miron et

al., 2005). As a benthic predator and keystone species in coastal regions, green crabs are regularly confronted with environmentally unfavourable conditions due to periodic fluctuations in abiotic parameters (Thomsen et al., 2010), being trapped in tide-pools (Truchot, 1988) or by burying in the sediment (Bellwood, 2002; Weihrauch et al.,1999). As in other aquatic decapod crustaceans, the capability of *C. maenas*' to adjust to these environmental challenges is based on the efficient ion- and acid-base regulatory apparatus located in its gill epithelia (Henry et al., 2012). As can be seen in figure 1.4, green crabs possess 9 pairs of gills of which the posterior gills 7-9 are found to be mainly involved in osmoregulatory functions (Cieluch et al., 2004; Siebers et al., 1982). Based on their morphological differences and lower activity of Na^+/K^+-ATPase compared to the 3 posterior pairs of gills, anterior gills 1-6 are thought to be mainly involved in respiratory and excretory processes rather than NaCl uptake in this decapod crustacean species (Compere et al. 1989; Freire et al. 2008; Henry et al. 2012; Pequeux 1995). Interestingly, ammonia excretion has been shown to be equally efficient in the posterior as well as anterior gills (Weihrauch et al., 1999a). In conclusion, *C. maenas* has been characterized as a euryhaline weak hyper-osmoregulator (Compere et al., 1989; Freire et al., 2008) with a moderately leaky gill epithelium (Riestenpatt et al., 1996).

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Figure 1.4. The green or shore crab, *Carcinus maenas*. Internal anatomy and position of the gills. Dark blue, branchial flabella ("gill raker"); light blue, anterior gills 2-6; orange, posterior gills 7-9. Anterior gill 1 is positioned dorsally and usually not visible without removing the other anterior gills.

Numerous studies on ammonia excretory mechanisms and ion regulation in whole animals, as well as the level of the gill, have contributed to our understanding of regulatory patterns in green crabs (recently reviewed by Henry et al., 2012; Larsen et al., 2014; Weihrauch et al., 2009). Additionally, *C. maenas* has been the focus of many studies on whole animal acid-base balance of which fundamental investigations have mainly been performed by Jean-Paul Truchot in the 1970s and 1980s (e.g. Truchot, 1973, 1975a, 1975b, 1976, 1978, 1979, 1981, 1986). However, hardly anything is known about branchial acid-base regulation and how it might be linked to general ion and ammonia regulation in green crabs and decapod crustaceans in general.

As a model organism, green crabs provide the opportunity for a holistic approach to investigate acid-base and ammonia regulatory patterns. Their size allows for monitoring changes in whole animal homeostasis by simply analyzing the hemolymph. Regarding regulatory processes, the gill must be of special focus as it combines ion-, ammonia- as

well as acid-base regulatory pathways and therefore allows for the investigation of possible links and trade-offs between these processes. The technique of gill perfusion as established by Siebers et al. (1985) in this respect provides a suitable technique to measure fluxes of ammonia and acid-base equivalents across the isolated gill. Furthermore, applying ion flux and electrochemical studies on split gill lamellae in Ussing chambers allows for the more specific investigation of transport processes across the isolated gill epithelium. Lastly, a published EST database (Towle and Smith, 2006) and recent transcriptome (Verbruggen et al., 2015) help in identifying important genes involved in the regulatory processes.

1.6. Thesis objectives and chapter outline

The present thesis aims to contribute to the understanding of (branchial) acid-base regulation in the decapod crustacean *C. maenas* in general and the linkage of acid-base regulation to ammonia excretion in particular. As indicated in the previous section, my holistic approach implemented the diverse available techniques to investigate the links between whole animal acid-base status, contribution of the individual gills, as well as the branchial transporter inventory on the level of the gill epithelium.

While the current chapter (chapter 1) provides an overview of the current available literature and knowledge of decapod crustacean acid-base and ammonia homeostasis and regulation, the remaining chapters 2-4 present original research projects that deal with different aspects of this topic:

In chapter 2, I aimed to identify general underlying principles of acid-base regulation by challenging osmoregulating green crabs with hypercapnia. I investigated changes in

hemolymph composition, mRNA levels of distinct gill transporters as well as the capabilities of isolated gills to regulate acid-base equivalents and ammonia excretion.

Having observed distinct changes in mRNA expression of gill epithelial transporters as well as an individual and highly specific contribution of each gill to extracellular acid-base regulation in chapter 2, chapter 3 consequently aimed to further identify branchial key-transporters in the gill epithelium to participate in ammonia, H^+ and CO_2 excretion across the gill epithelium. This was accomplished by isolated gill perfusion experiments applying transporter-specific pharmaceuticals. In contrast to chapter 2, chapter 3 was performed on osmoconforming crabs in order to account for very basic underlying mechanisms. Equivalent to chapter 2, I additionally exposed osmoconforming green crabs to high environmental pCO_2 to elucidate potential differences in the capacity for acid-base regulation in comparison to the osmoregulating crabs. I recorded a time series for hemolymph characteristics of osmoconforming green crabs during short-term acclimation to hypercapnia and compared the results to the response observed in the osmoregulating *C. maenas* of chapter 2.

Based on the results of the inhibitor experiments on isolated gills in chapter 3, I was interested in the particular role of so far uncharacterized gill epithelial potassium channels in acid-base and ammonia regulation in osmoregulating green crabs in chapter 4. Specifically, I identified a candidate gene of the potassium/sodium hyperpolarization-activated nucleotide gated channel family (HCN) in the green crabs' transcriptome, and described its potential role in gill acid-base and ammonia regulation applying physiological and molecular techniques. Finally, chapter 5 summarizes the findings of the present thesis.

CHAPTER 2:

DIFFERENTIAL ACID-BASE REGULATION IN VARIOUS GILLS OF THE GREEN CRAB *CARCINUS MAENAS*: EFFECTS OF ELEVATED ENVIRONMENTAL $p\mathbf{CO_2}$

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(Fehsenfeld and Weihrauch, 2013)

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My contribution:

I conducted all experiments, analyzed the data and wrote the manuscript.

Other author's contribution:

DW helped with the design of the study and provided financial support, as well as laboratory equipment.

2.1. Abstract

Euryhaline decapod crustaceans possess an efficient regulation apparatus located in the gill epithelia, providing a high adaptation 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*, acclimated to diluted seawater. In gill perfusion experiments, all investigated gills 4 - 9 were observed to increase 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 specimens exposed to elevated pCO_2 levels for at least 7 days, mimicking a future ocean scenario as predicted by 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.

2.2. Introduction

Crustaceans, especially when inhabiting the highly variable intertidal zones of marine systems, are exposed to severe fluctuations in abiotic parameters of their environment (Thomsen et al., 2010). In order to keep homeostasis to maintain basic systemic functions, active acid-base regulation allows these animals to cope with fluctuations in the surrounding seawater pH and / or pCO_2. Elevated environmental pCO_2 (hypercapnia) results in an increased extracellular CO_2 partial pressure in the animal, enabling excretion of metabolic CO_2 along its positive diffusion gradient (Melzner et al., 2009). However, this increase would lead to an acidification of extracellular fluids if not compensated for (Dejours and Beekenkamp, 1977; Thomsen and Melzner, 2010; Truchot, 1975). While less active and / or sessile animals are not able to directly compensate for the acid load (Langenbuch and Pörtner, 2002) and respond with metabolic depression (Seibel and Walsh, 2003), several active species with high metabolic rates and pH sensitive respiratory pigments regulate extracellular pH (pH_e) by actively accumulating bicarbonate. The resulting modulation of the extracellular carbonate system compensates for an imminent pH drop 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., 2012, Spicer et al., 2007; 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 between different gills (8 to 9 pairs in total, depending on which species) which leads 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., 1999a) 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 H_2CO_3 and therefore subsequently to HCO_3^- and protons 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 Red 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 and Putzenlechner, 1995; Weihrauch et al., 2004a). In general, acid-base regulation through the gills is thought to be 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 and Perry, 2009). Studies on isolated gills of the crab, *Neohelice (Chasmagnathus) granulata*, 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

(Tresguerres et al., 2008). However, to date only CA and NKA have been shown to participate in acid-base regulation in *C. 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 and Weihrauch, 2001), the transporter inventory in decapod crustacean gill epithelia and their functional interactions are not fully understood at present.

Studies on acid-base regulation and on the ability of different species to counteract pH disturbances have become increasingly important in the context of the predicted future ocean scenario. 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 by the year 2100, and a change of up to 1.4 units until the year 2300, respectively (Caldeira and Wickett, 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).

In the present study, whole animal experiments, gill perfusion studies, and gene expression analysis of control and elevated environmental pCO_2 exposed osmoregulating green crabs have been performed in order to characterize general acid-base regulatory patterns. In order to identify key branchial transport proteins 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 active posterior gills 7, 8 and 9) were investigated simultaneously.

2.3. Material and Methods

2.3.1. Animals

Male green crabs were obtained from Bamfield Marine Sciences Center BC, Canada and kept at the animal holding facility of the University of Manitoba, Winnipeg, Canada in aerated 1200 L tanks with artificial seawater adjusted to a salinity of 32 ppt at 14 °C (Seachem Marine Salt ®) until experimentation.

Green crabs were acclimated to diluted seawater of a salinity of 10 ppt (Seachem Marine Salt®) in aerated 120 L aquaria for a minimum of 7 days before exposure to elevated pCO_2 (IKS Aquastar; iKS Computer Systeme GmbH, Germany). Each aquarium contained 8 animals marked with nail polish for identification. Animals were fed *ad libitum* with squid once a week. Water parameters (pH according to Northern Balance and Scale (pH_{NBS}), total dissolved inorganic carbon (DIC) and pCO_2) of the seawater in the aquaria were assessed daily and the full water body exchanged every one to two days. The pH was measured with the pH/ATC electrode 300729.1 (Denver Instruments, Goettingen, Germany) connected to a pH-ISE meter model 225 (Denver Instruments, Goettingen, Germany), while DIC was measured using a Corning 965 carbon dioxide analyzer. Seawater pCO_2 was then calculated using the Excel add-in CO2SYS (Lewis and Wallace, 1998) and the appropriate parameter and constants (K1, K2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) $KHSO_4$ dissociation constant after Dickson (1990), NBS scale [mol/kg H_2O]; table 2.1).

After acclimation to a salinity of 10 ppt at a pH of 7.7 ± 0.0 and a pCO_2 of 53.5 ± 2.1 Pa, animals were either assigned as control animals, or exposed to elevated pCO_2 for a minimum of 7 days (324.3 ± 20.0 Pa). The IKS Aquastar® was used to control the CO_2 influx in the experimental tanks to reach a set pH of 7.0. After 7 days, elevated pCO_2 exposed animals were then used for ammonia excretion measurements and in gill perfusion experiments. Only fasted animals (48 h after feeding) were used in the experiments.

Table 2.1. Water parameters of the 120 L acclimation tanks.

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

Green crabs were acclimated to either control or high pCO_2 diluted seawater.

2.3.2. Hemolymph analysis

In order to assess the carbonate system parameters and ionic composition of the hemolymph of *C. 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 total CO_2 (C_T) were measured immediately at 4 °C as described above, before storing the samples at -80 °C for liquid 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 (table 2; for the detailed method see Weihrauch et al., 1998).

To determine the ionic composition of the hemolymph, samples (diluted 1:40) were analyzed for cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) and anions (Cl^-, SO_4^{2-}) by liquid ion chromatography (Metrohm 850 Professional IC). The cation eluent was 1.7 mmol L^{-1} nitric acid and 0.7 mmol L^{-1} dipicolinic acid (column: Metrosep C4 250/4.0) and the anion eluent was 3.6 mM Na_2CO_3 (column: Metrosep A Supp 5 150/4.0). 0.1 mmol L^{-1} H_2SO_4 served as the anion suppressor. The flow rate was set to 0.7 ml min^{-1}.

Results of the IC for control animals were compared to other studies (Siebers et al., 1982; Weihrauch et al., 1999a; Winkler et al., 1988) and adjusted where 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.

Table 2.2. Hemolymph composition of control and high pCO_2 acclimated *Carcinus maenas* specimen.

	pH	pCO_2	HCO_3^-	Ammonia	Cl^-	Na^+	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}
Control animals	7.9 ± 0.02	263 ± 17	6.6 ± 0.4	156 ± 24	319 ± 6	259 ± 5	6.9 ± 0.2	5.9 ± 0.5	6.9 ± 0.2	2.6 ± 0.1
High pCO_2 exposed animals	7.9 ± 0.02	344 ± 21	7.9 ± 0.6	218 ± 17*****	316 ± 9	277 ± 9	8.1 ± 0.2*****	6.0 ± 0.3	6.6 ± 0.5	2.5 ± 0.1
Perfusion saline	7.9	254	7	100	280	260	8	5	7	-

Asterisks indicate significant difference relative to control values. All values represent mean ± SEM with n = 4 – 6. The dash indicates that SO_4^{2-} was not adjusted in the perfusion solution.

2.3.3. Whole body ammonia excretion

To determine the ammonia excretion rate of intact animals, fasted (48 h) green crabs acclimated to diluted seawater (salinity = 10 ppt) for 7 days were transferred to small buckets holding 2 L of aerated diluted seawater (controls). 10 mL water samples 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, diluted seawater 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 diluted seawater. 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).

2.3.4. Tissue preparation

For isolation of tissues for quantitative gene expression (qPCR) and gill perfusion experiments, one control or elevated pCO_2 exposed crab was placed on ice for 15 min

The perfusion solution was composed according to the results from the carbonate system and ion chromatographic analysis, complemented by literature values (see 2.3.2, table 2.2) and applied at a perfusion speed of 128 ± 0.1 µL/min using a peristaltic pump (Sci 323 Watson-Marlow Bredel Pump, Falmouth Cornwall, England). The perfusion solution contained (in mmol L^{-1}): 260 NaCl, 5 $CaCl_2$, 7 $MgCl_2$, 8 KCl, 7 $NaHCO_3$, 0.1 NH_4Cl, 0.3 glucose, 0.1 gluthation, 0.5 glutamine. The pH was set to 7.9, respectively. Each perfusion sequence was composed of five consecutive steps in which the pH of the

external medium was changed every 30 minutes: The initial control phase (pH 7.80 ± 0.02, control bathing solution) was followed by a low pH step (pH 7.44 ± 0.02 of the bathing solution). In the third step another control phase was applied (pH 7.81 ± 0.02), followed this time by a high pH step (pH 8.62 ± 0.02 of the bathing solution). As the fifth step, each perfusion sequence was ended by a third control phase (pH 7.83 ± 0.03, control bathing solution; figure 2.1). pH was manipulated using 0.1 mmol L^{-1} NaOH or HCL, respectively. Immediately after each step the pH of the perfusate was measured, including parallel beakers without gills to monitor the pH drift of the solution only. Following the whole perfusion sequence, samples were frozen at -80 °C until ammonia concentration measurement (including parallel solutions without gills as background reference).

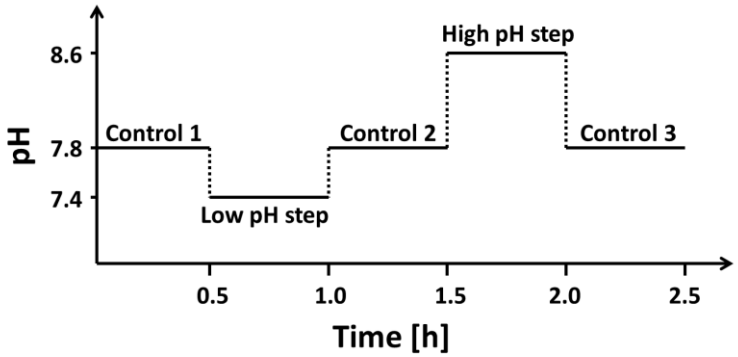


Figure 2.1. Experimental scheme of the gill perfusion. Each perfusion experiment was composed of five consecutive steps that were applied for 30 min each. A first control step (bathing solution = pH 7.8) was followed by a low pH step in which the pH of the bathing solution was lowered by 0.4 units to 7.4. After a second control phase, a high pH step was applied, increasing the pH of the bathing solution by 0.8 units to pH 8.6. A third control phase ensured that the gill was still functioning.

2.3.6. 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 DN*ase* treatment (DN*ase* 1, Invitrogen), RNA was tested by PCR (40 cycles), using crab specific primers CrabRbS3F1 / CrabRbS3R1 (Table 2.3) targeting the ribosomal protein RbS-3 (figure 2.2) and evaluated by gel electrophoresis, ethidium bromide staining and UV visualization. RNA was considered DNA-free 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. Primers employed in quantitative real-time PCR targeted sodium-hydrogen exchanger (NHE), Rhesus-protein (Rh), V-(H^+)-ATPase subunit B (HAT), anion [Cl^-/HCO_3^-] exchanger (AE), sodium-potassium ATPase α-subunit (NKA), sodium-bicarbonate co-transporter (NBC), membrane bound (CA-1) and cytoplasmic carbonic anhydrase (CA-2), produced a single PCR product of the predicted size and were quantified using the imager Biorad Versadoc 4000 MP and Image Lab^{TM} 3.0 software. For verification PCR products (both forward and reverse strands) were sequenced at the Robarts Research Institute (London, Ontario, Canada). In order to assess absolute quantity of gene expression, a standard curve for the respective genes was generated in the same run, 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. The absolute quantity of gene expression of the control samples (701.8 ± 77.0 fg) *vs.* the exposed samples (761.5 ± 85.4 fg) was stable and showed no difference in expression for this gene (Student's t-test with p = 0.61). Primer sequences and annealing temperatures for all real-time PCRs are listed in Table 2.

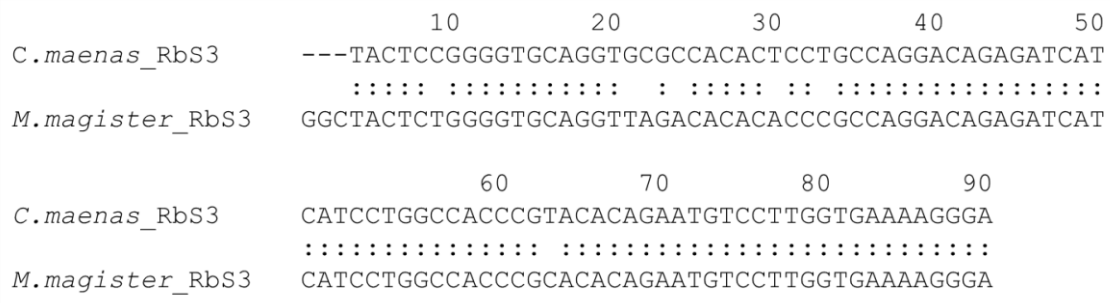


Figure 2.2. Alignment of the RbS-3 sequence of *Carcinus maenas* with *Metacarcinus magister* (*M. magister* ribosomal protein S3 mRNA, partial cds; GenBank accession JF276909.1).The sequences showed 89.2% identity on the level of single base pairs that translated into 100% identity on the amino acid level (data not shown).

2.3.7. 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 (H_0 = 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, Student's 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 Kruskal-Wallis-Test for more than one mean, respectively. All results were considered significant at $p < 0.05$.

2.4. Results

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

Hemolymph composition. While hemolymph HCO_3^-, Cl^-, SO_4^{2-}, Na^+, Ca^{2+} and Mg^{2+} levels did not change in elevated pCO_2 acclimated green crabs, a significant increase was observed for K^+ and ammonia concentrations (Student's t-test with $p = 0.004$ and 0.045, respectively). Hemolymph pH remained constant at 7.9 ± 0.3, while C_T and therefore calculated pCO_2 increased by *ca.* 30% (from 263.1 ± 17.0 Pa to 344.4 ± 20.6 Pa; table 2.2).

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 (Student's t-test with $p = 0.02$).

2.4.2. Gill perfusion experiments

General observations. After only one gill passage, C_T (and therefore calculated 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 2.3).

Table 2.3. Primer sequences employed in quantitative real-time PCR.

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

Transcript	GenBank acc. no.	Primer sequence	Amplicon size (bp)	Annealing temp [°C]
Ribosomal gene S3 (**RbS-3**)	JF276909.1	Forward: GTCCCTTTTCACCAAGGACA Reverse: CAAGGCCAAACTCAACAGGTT	160	60
Sodium hydrogen exchanger (**NHE**)	U09274.1	Forward: TTCGAGGGCTTCAGTGAGTT Reverse: TAAGGAAGCCCCAGATGATG	124	60
Rhesus-like protein (**Rh**)	AF364404.2	Forward: GGTGGTCTCGTGACAGGTTT Reverse: TTGTGACCCTCATCCTCCTC	119	60
V-H^+-ATPase, subunit B, K form (**HAT**)	AF189779.2	Forward: ACCCAGATCCCCATCCTTAC Reverse: AGAGAAGGCAGCACGTTGAT	149	60
SLC4A1 (anion exchanger Cl^-/HCO_3^-; (**AE**)	CX994129.1	Forward: TGATGCCAGTCAAACACCAT Reverse: AGCAAAGAGCTGCTGGAGAC	138	60
Sodium-potassium ATPase α-subunit (**NKA**)	AY035550.1	Forward: CAGGCTTGGAAACTGGAGAG Reverse: AGCATCCAGCCAATGGTAAC	137	60
Sodium-bicarbonate co-transporter (**NBC**)	DN202373.1	Forward: TTGCCACTTGATTTTGAGCAA Reverse: CAGCACAATATCCCAGTGGAA	90	60
Membrane-bound carbonic anhydrase **(CA-1)**	EU273944.1	Forward: GGTCTGGCAGTACTGGGTGT Reverse: AGCCTTGAGTGGGTACATGG	138	60
Cytoplasmic carbonic anhydrase **(CA-2)**	EU273943.1	Forward: CGCTCAGTTCCACTTCCA Reverse: ACATCTCAGCATCCGTCA	213	60

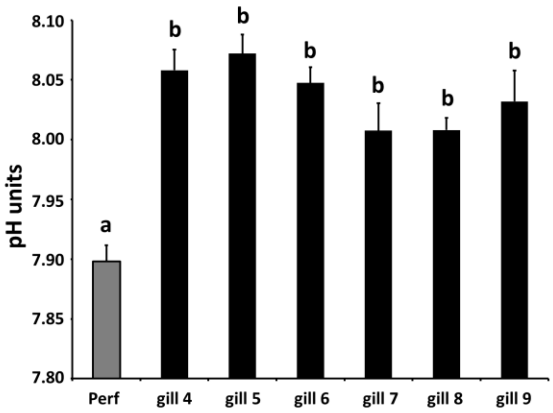


Figure 2.3. Regulation of the pH of individual gills after one gill passage in perfusion experiments. All gills (black bars) up-regulated the pH in the perfusate significantly compared to the initial pH of 7.9 of the perfusion solution (perf, grey bar; Student's t-test with $p < 0.01$).

Figure 2.4.A shows the equivalent proton decrease in the perfusate of control animals in the different steps of the experiment as described in figure 2.1. Under control conditions (control 1, figure 2.1), anterior gill 4 was most efficient in regulating the hemolymph pH and excreted 3.8 fold as many protons per mg gill tissue 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 extracellular medium (perfusate enriched with 100 μM ammonia) showed the same pattern for the single gills as the decrease in proton concentration (figure 2.4.B). 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).

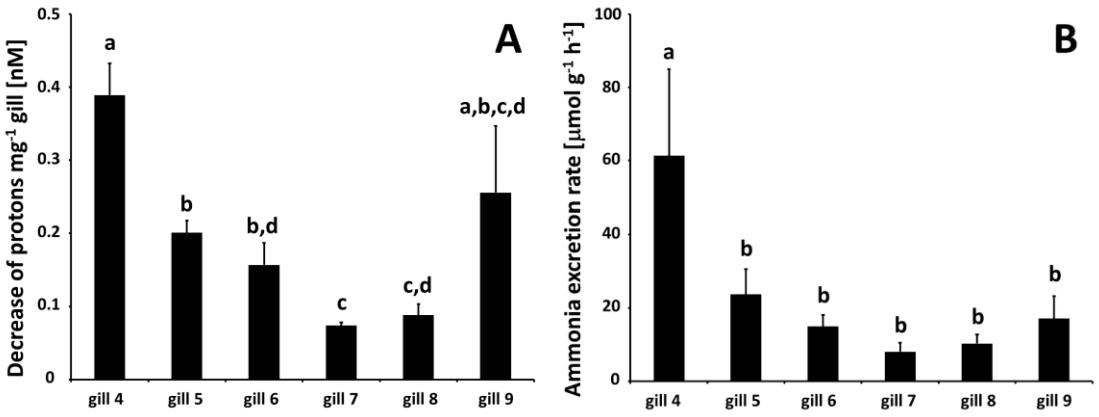


Figure 2.4. Proton and ammonia excretion patterns by isolated perfused gills of control *Carcinus maenas*. (A) Decrease of hemolymph proton concentration [H^+] (equivalent to an increase of pH) after one gill passage in gill perfusion experiments. (B) Ammonia excretion rates of control *C. maenas* after one gill passage in gill perfusion experiments. Small letters a, b, c, d indicate significant differences within the individual gills of control crabs ((A) Kruskal-Wallis test with $p < 0.05$, $n = 4 – 6$; (B) ANOVA with $p < 0.05$, $n = 3$-5).

Low and high pH challenge. Figure 2.5 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. For example while gill 5 in control animals showed a significant decrease of proton excretion in the low pH perfusion step compared to the control pH perfusion step, gill 5 of elevated pCO_2 exposed green crabs significantly increased proton excretion rates in comparison to control animals. As a result, the proton excretion rate of gill 5 of high pCO_2 acclimated animals in the low pH perfusion step was not significantly different from proton excretion rates in the control

pH perfusion step. 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 2.6). 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.

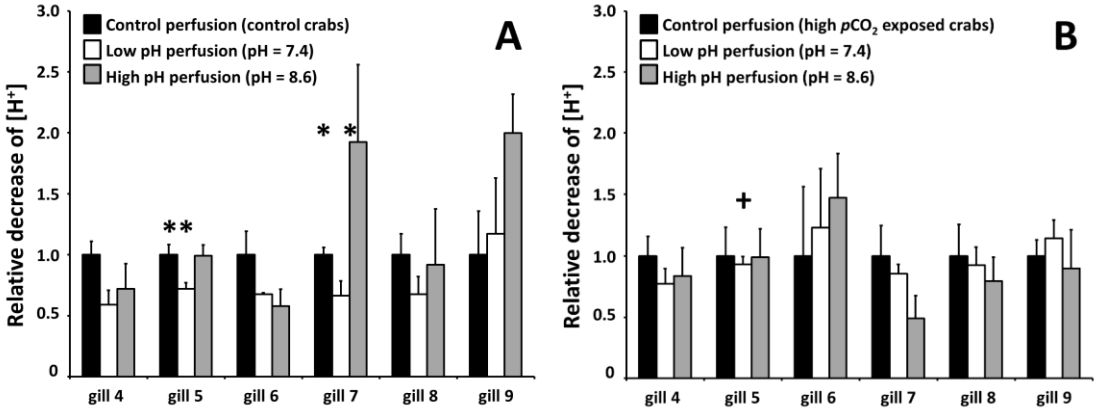


Figure 2.5. Relative decrease of protons in the perfusate during gill perfusion of control (A) and high pCO_2 exposed green crabs (B). All displayed values have been calculated employing the respective preceding control step in the perfusion. Asterisks denote significant differences regarding different steps of the perfusion sequence of control animals only. '+' denotes significant differences between control and high pCO_2 exposed crabs (Student's t-test with $p < 0.05$). Note: gill 4 control step *vs.* low pH step $p = 0.09$; control gill 4 *vs.* high pCO_2 exposed gill 4 $p = 0.09$. Values are given as means ± SEM, n = 3 - 6.

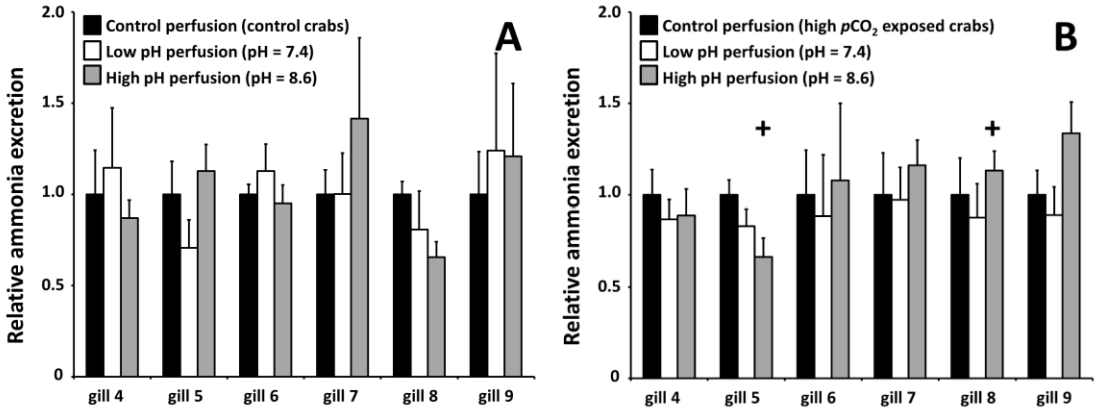


Figure 2.6. Relative ammonia excretion rates of all gills during gill perfusion of control (A) and high pCO_2 exposed green crabs (B) after only one gill passage in the different experimental phases. Low pH and high pH values are related to the preceding control step in the perfusion, respectively. '+' denotes significant differences between control and high pCO_2 exposed crabs (Student's t-test with $p < 0.05$). All values are given as means ± SEM, n = 3 - 6.

2.4.3. Quantitative real-time PCR

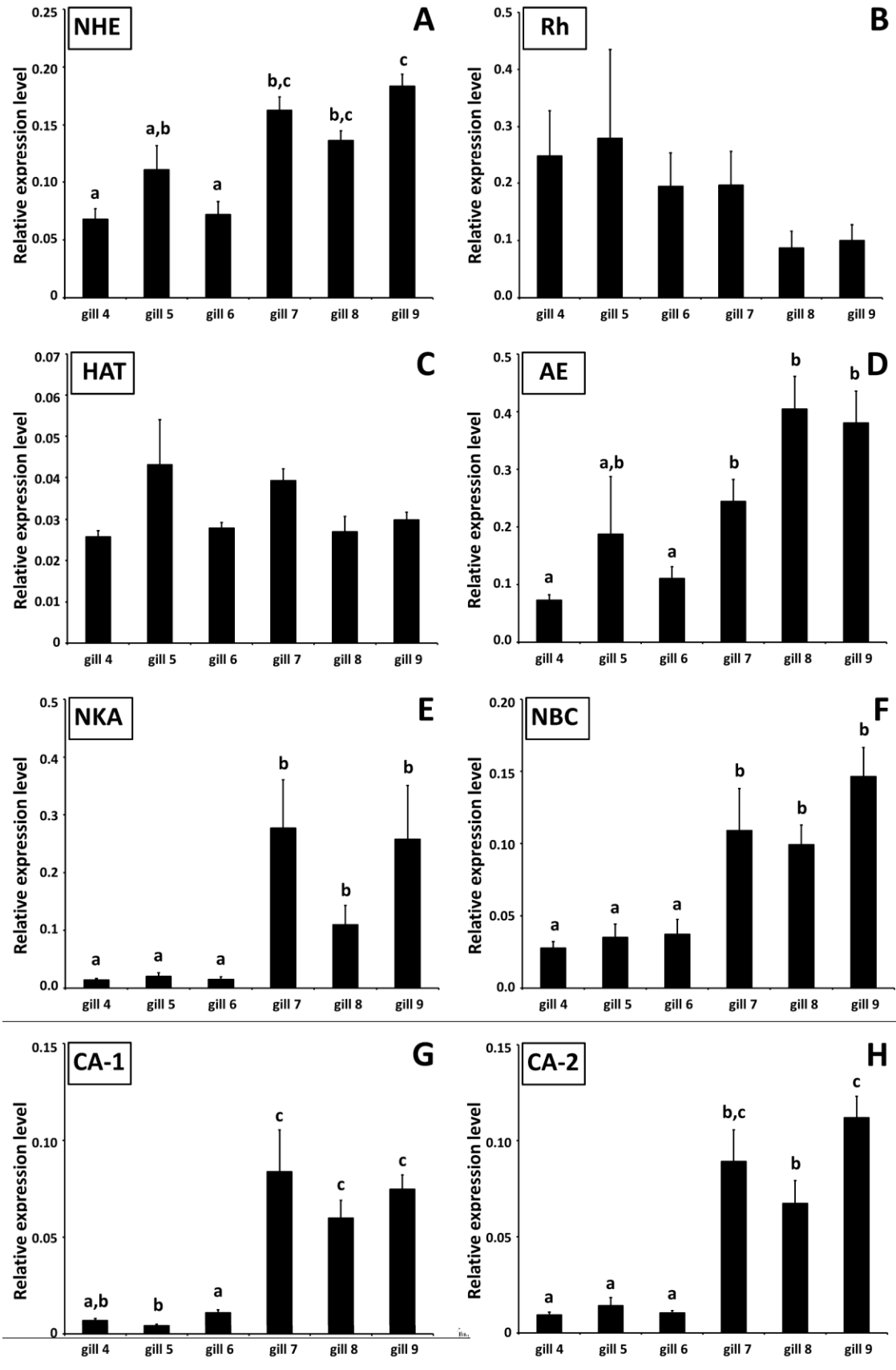
Relative expression levels of transporters. The highest overall relative expression levels were observed for the genes encoding the Rhesus-like protein (Rh) and the anion [Cl^-/HCO_3^-] exchanger (AE). While the V-(H^+)-ATPase (subunit B; HAT) had low expression in all gills, a number of other genes were differentially expressed in the different gills (figure 2.7).

The sodium-hydrogen exchanger (NHE; figure 2.7.A) and AE (figure 2.7.D) 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; figure 2.7.E), sodium-bicarbonate co-transporter (NBC, figure 2.7.F), membrane bound (CA-1; figure 2.7.G) and cytoplasmic carbonic anhydrase (CA-2; figure 2.7.H), was clearly restricted to the posterior gills and hardly detectable in anterior gills. In contrast, Rh was identified to be the only tested transcript that exhibited a trend for a higher expression level in the anterior gills (figure 2.7.B).

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 expression of almost all transcripts (NHE, Rh, HAT, NKA, NBC, CA-1, CA-2) compared to gill 7 and 9 (figure 2.7).

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 gills upon exposure to elevated pCO_2 (figure 2.8). While gill 6 and 8 displayed a general trend to up-regulate genes (significantly in case of the NKA in gill 6; trend in case of CA-1 in gill 8), expression levels of NHE, Rh, HAT and AE in gills 4 and 5 were 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. NKA showed a significant up-regulation in anterior gill 6. NBC exhibited slight up-regulation in gills 6, 7 and 8. CA-1 (membrane associated) was up-regulated in gill 4 (significantly, Student's 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.

(Following page) **Figure 2.7. Relative gene expression levels of specific transporters involved in acid-base regulation.** (A) sodium-hydrogen exchanger (NHE), (B) Rhesus-like protein (Rh), (C) V-(H^+)-ATPase (HAT), (D) anion exchanger HCO_3^-/Cl^- (AE), (E) sodium-potassium ATPase (NKA), (F) sodium-bicarbonate co-transporter (NBC), (G) carbonic anhydrase membrane bound (CA-1) and (H) cytoplasmic (CA-2) in the different gills 4-9 of control green crabs. Letters a,b,c denote significant differences (Students t-test with $p < 0.05$). Values represent means + SEM with $n = 3 – 6$.


NHE
A
Relative expression level
0.25
0.20
0.15
0.10
0.05
0
a
a,b
a
b,c
b,c
c
gill 4
gill 5
gill 6
gill 7
gill 8
gill 9
Rh
B
0.5
0.4
0.3
0.2
0.1
0.0
HAT
C
0.07
0.06
0.05
0.04
0.03
0.02
0.01
AE
D
b
b
NKA
E
NBC
F
CA-1
G
a,b
b
CA-2
H
b,c

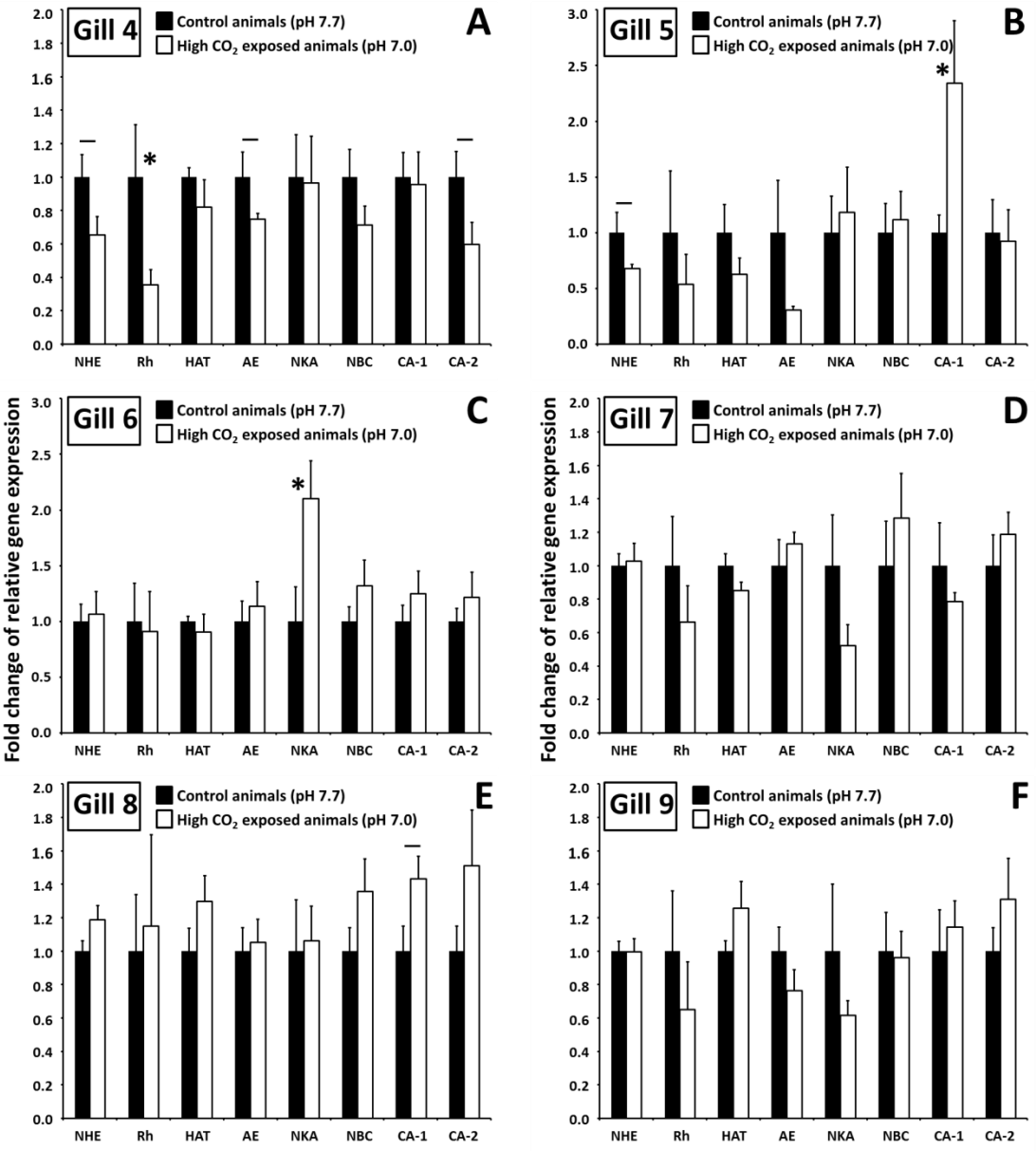


Figure 2.8. Quantitative real-time PCR of important transporters in control and hypercapnia-acclimated green crabs in gills 4-9. Fold change of relative mRNA expression of sodium-hydrogen exchanger (NHE), Rhesus-like protein (Rh), V-(H+)-ATPase (HAT), anion exchanger HCO_3^-/Cl^- (AE), sodium-potassium ATPase (NKA), sodium-bicarbonate co-transporter (NBC), carbonic anhydrase membrane bound (CA-1) and cytoplasmic (CA-2) of green crabs acclimated to control (54 Pa) and elevated pCO_2 (324 Pa). Gene expression levels have been related to the housekeeping gene RbS-3 and standardized to control expression levels of the respective transcript. Asterisks denote significant differences in gene expression between control and high pCO_2 exposed animals (Student's t-test with $p < 0.05$). Vertical bars indicate tendencies with $p < 0.15$ but > 0.05. The graph represents means + SEM (n = 4 - 6).

2.5. Discussion

As a benthic predator in coastal regions, *C. maenas* often is confronted with environmentally unfavorable conditions. For example, green crabs of the Baltic Sea are subject to a highly fluctuating habitat regarding abiotic parameters like pH, mainly due to upwelling processes (Thomsen et al., 2010). Green crabs originating from the Bamfield region for instance, encounter changing salinities according to the variation of freshwater influx (rain, rivers) into Barkley Sound (http://www.bms. bc.ca/information/ geoinfo.html). Additionally, brachyuran crabs bury in the sediment during the day for protection, and therefore are exposed to high environmental ammonia and hypercapnia in the interstitial fluids of their burrows (Bellwood, 2002; 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 and Duhamel-Jouve, 1980). Being the main regulatory organs in decapod crustaceans, the gills have been extensively discussed to play the major role in the response to diluted salinity (e.g. 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., 1999a; Weihrauch et al., 2004b). 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 et al., 2008; Towle and 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 *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.

2.5.1. Whole body acid-base regulatory patterns

C. maenas exhibits an increase in hemolymph pCO_2 levels when acclimated to an environment with elevated pCO_2 levels, thereby ensuring diffusion of CO_2 out of the body along its gradient (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 (e.g. Appelhans et al., 2012; Hayashi et al., 2004; Larsen and Jensen, 1997; Larsen et al., 1997; Spicer et al., 2007; Truchot, 1986). In fresh- and seawater fish, Cl^- has been identified to act as counter-ion for HCO_3^- and its hemolymph concentration is correspondently lowered in a 1:1 stoichiometry (e.g. (Hayashi et al., 2004)). In general, this modulation of the extracellular carbonate system can be seen in active species with high metabolic rates like fish, crustaceans and cephalopods, in order to compensate for a metabolic acidosis (Appelhans et al., 2012; Gutowska et al., 2009; Hu et al., 2011; Larsen et al., 1997; Spicer et al., 2007). Appelhans et al. (2012) showed a significant increase in hemolymph bicarbonate also for *C. maenas* when acclimated to 350 Pa pCO_2 for 3 months, but not in animals acclimated to 120 Pa pCO_2. However, in the present study, only a slight non-significant increase in hemolymph bicarbonate could be observed (table 2.2).

Additionally, ammonia levels in the hemolymph are significantly increased in elevated pCO_2 acclimated crabs. A recent study by Martin et al. (2011) showed that the marine Dungeness crab, *Metacarcinus magister*, is able to tolerate high ammonia levels (5-fold of controls) in its hemolymph over a period of at least 14 days when exposed to high environmental ammonia. The data in the current study suggests that higher levels of hemolymph ammonia (elevated by 85% compared to control animals) can also be tolerated for an extended time period by green crabs. To keep steady state levels constant at a new higher level, ammonia excretion rates increased significantly to 262% of controls, likely in order to secure sufficient excretion of this potentially toxic substance. Elevated environmental pCO_2 (hypercapnia) might lead to an increase in metabolic ammonia. Metabolic ammonia is mainly the result of amino acid metabolism and has been shown to increase due to an increased amino acid turnover because of the elevated energy demands in crabs when exposed to low salinity (reviewed by Henry, 1995; Weihrauch et al., 2009). It is therefore likely that in acclimation to varying environmental pCO_2, amino acids may be utilized as an energy source to counteract pCO_2 disturbance, leading to the observed increase in hemolymph ammonia and whole animal ammonia excretion. In this case, modulation of the active ammonia excretion would be necessary to ensure the new steady state at a lowest possible level in order to not harm the animal.

Both, an increase in extracellular ammonia as well as potassium have been shown to increase intracellular pH (pH_i) in adult rat carotid body glomus cells (Wilding et al., 1992). Excess ammonia may therefore also play a role as an additional pH buffer system to counteract pH disturbances in the green crabs' hemolymph.

Additionally, K^+ levels in the hemolymph increased significantly. It has been demonstrated in leech glial cells that pH_i is dependent on the membrane potential (Deitmer and Szatkowski, 1990). The authors show that the membrane potential itself is altered by extracellular K^+ concentrations; if extracellular K^+ is high, the membrane is hyperpolarized and pH_i increases, mainly due to electrogenic $Na^+/2HCO_3^-$ co-transport. The increase in extracellular K^+ in elevated pCO_2 acclimated green crabs might therefore be important to keep pH_i stable in a high pCO_2 environment. As a potential candidate to participate in this process, the hyperpolarization activated nucleotide-gated potassium channel (HCN2) should be taken into consideration in future studies. Gene expression of this channel has been identified to be significantly down-regulated in green crabs upon elevated pCO_2 acclimation in a recent study by Fehsenfeld et al. (2011).

2.5.2. Gill acid-base regulatory capacities

General observations. All investigated gills (pairs 4-9) of control crabs lowered pCO_2 in the perfusate significantly after only one gill passage, while simultaneously decreasing bicarbonate levels and elevating the pH (figure 2.3). Interestingly, different gills seemed to exhibit different patterns regarding this characteristic. Anterior gills seemed to be more efficient in elevating the pH than the posterior gills, with anterior gill 4 having the highest proton excretion rate (figure 2.4.A). This observation supports the hypothesis that the anterior gills are indeed the main players for branchial acid-base regulation, while the posterior gills are involved in both processes, osmoregulatory ion uptake and - with a lower tissue specific capacity - acid-base regulation. Additionally, the results from the current study revealed that individual gills should be considered

separately and might even be more differentiated concerning their contribution to osmo- and acid-base regulation, as had been previously discussed (Fehsenfeld et al., 2011).

Ammonia excretion rate patterns of single gills followed their pattern of proton excretion, suggesting that both processes are linked. Again, gill 4 exhibited the highest ammonia excretion rate, followed by gill 5 (figure 2.4.B). Ammonia excretion is based on at least two processes in euryhaline crabs, the passive diffusion of NH_3 and to a greater extent active ammonia excretion, most likely *via* exocytosis of ammonium loaded vesicles (Towle and Weihrauch, 2001; Weihrauch et al., 1998). Because a higher proton excretion rate of gill epithelial cells (as seen in gill 4) 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 mechanism. Ammonia excretion *via* ammonia trapping has been suggested to occur in gills of freshwater and seawater teleost fish (Hwang et al., 2011; Nawata et al., 2010; Weihrauch et al., 2009; Wright and Wood, 2009) and freshwater planarians (Weihrauch et al., 2012). The observed similar pattern of proton and ammonia excretion by all individual gills strengthens the hypothesis that both excretory processes are closely linked. It needs to be 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 generated by the gill epithelium itself (Martin et al., 2011; Weihrauch et al., 1998, 1999a). An exception is observed in gill 8, in which metabolically produced ammonia was particularly high.

Acclimation of green crabs to elevated environmental pCO_2 had no effect on either proton or ammonia excretion rates of any of the isolated gills. However, whole body ammonia excretion rates were observed to increase to 262% of control levels in elevated pCO_2 acclimated green crabs. It has to be considered though that the ammonia concentration in the applied perfusion solution resembled control levels, while hemolymph ammonia in elevated pCO_2 acclimated crabs was increased and therefore excretion due to passive diffusion would be facilitated. An increased ammonia excretion rate has also been shown in other invertebrates acclimated to elevated seawater pCO_2 like the blue mussel, *Mytilus edulis* (Thomsen and Melzner, 2010), and the sea urchin, *Strongylocentrotus droebachiensis* (Stumpp et al., 2012), acclimated to 405 and 285 Pa pCO_2, respectively, although the observed increase in green crabs as well as sea urchins was much lower compared to blue mussels (~60% vs. ~90%). While the increased whole body ammonia excretion rate might be due to the increased hemolymph ammonia concentration and therefore facilitated passive diffusion out of these animals, an additional ammonia excretion pathway for active ammonia excretion might have been stimulated by elevated environmental pCO_2. Thus, different additional regulatory mechanisms regarding ammonia excretion might be accomplished, presumably including an increased ammonia excretion through the antennal glands. Even though these organs play a minor role in ammonia excretion in crabs (Regnault 1987), an increased role in response to hypercapnia exposed crabs cannot be excluded.

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 2.5.A). The anterior gills seemed to be more vulnerable to this external pH disturbance (especially gill 5) than the posterior gills. The opposite 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. It has to be considered, however, that the gills produce metabolic CO_2 which itself might contribute to the H^+ gradient when hydrated by carbonic anhydrases. This process might be increased in the mitochondria rich posterior gills in response to elevated bath pH. The results show again the potentially high differentiation between the different gills and their function, but also indicate 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, either in a more acidic or alkaline medium (figure 2.5.B). This implies that acclimation processes are taking place that enable the crabs to counteract the external pH fluctuations more efficiently than control animals to leave them less vulnerable.

Even though branchial proton excretion was identified to be effected by lower and higher environmental pH values in anterior gills of control crabs, ammonia excretion levels of both anterior and posterior gills remained stable (figure 2.6.A), likely to ensure the elimination of the potentially toxic substance. For so far unexplained reasons, ammonia excretion rates in gill 5 and 8 were significantly affected in elevated pCO_2 acclimated crabs (lower and higher ammonia excretion rate, respectively; figure 2.6.B).

2.5.3. Gene expression of transcripts involved in acid-base balance and osmoregulation

For the majority of the analyzed transcripts, a higher level of expression was observed in the posterior gills (figure 2.7). As the posterior gills are thought to be mainly involved in osmoregulatory processes, and animals in the current study were acclimated to diluted seawater and were therefore hyper-regulating their hemolymph osmolality (Henry et al., 2003; Shaw, 1961; Zanders, 1981), the high expression levels of the Na^+/K^+-ATPase (NKA), sodium-bicarbonate co-transporter (NBC) and cytoplasmic carbonic anhydrase (CA-2) most likely resembles an osmoregulatory response, as shown in previous studies (Henry et al., 2003; Serrano and Henry, 2008; Siebers et al., 1982; Towle et al., 2011). Most genes with altered expression levels upon acclimation to low salinity in the microarray study of Towle et al. (2011) underwent the changes during the initial 48 hours. No pronounced changes were detected after 7 days, implicating that by the time point of exposure to hypercapnia in the present study, the salinity acclimation response would be completed.

Hemolymph osmoregulation in the green crab is accomplished primarily by the active transport of Na^+ and Cl^- across the posterior gills (Riestenpatt et al., 1996; Siebers et al., 1982). Confirming enzyme activity measurements of the Na^+/K^+-ATPase in the study by Siebers et al. (1982), mRNA expression of this pump in posterior gill 8 in the current study was indeed observed to be significantly higher than in gill 4. Due to a lower NKA activity in osmoregulating crabs, gill 6 is not considered to be associated with the posterior osmoregulatory active gills in *C. maenas* (Siebers et al., 1982). However, this gill shows a significant increase in NKA expression in elevated pCO_2 acclimated green

crabs (figure 2.8.C). This indicates that osmoregulation and acid-base regulatory processes are linked: both depend on an energizing NKA as shown by Siebers et al. (1982).

Expression patterns of NHE have not yet been shown in detail in the gills of a crustacean, nor has this transporter been localized in the gill epithelium. However, low and high expression levels in the anterior and posterior gills, respectively, indicate a role for this cation exchanger in osmoregulation as previously suggested for crabs (reviewed by Freire et al., 2008; Towle and Weihrauch, 2001) and freshwater fish (Evans et al., 2005; Perry et al., 2003). Gill perfusion and inhibitor experiments by Tresguerres et al. (2008) suggested the involvement of a basolateral NHE in the crab *N. granulata* acclimated to low salinity when perfused with increased hemolymph [HCO_3^-]. Different isoforms of NHE (1-3) were suggested to be involved in acid-base balance of euryhaline and seawater fish (e.g. *Fundulus heteroclitus*, *Myoxocephalus octodecimspinosus*; Claiborne et al., 1999; Edwards et al., 2005). Seawater adapted *F. heteroclitus* showed a significant increase in NHE3 protein levels following hypercapnia, likely involved in the observed increase in H^+ excretion (Edwards et al., 2005). However, to date only one NHE isoform was described for *C.maenas*, with closest homology to human NHE3 (Towle et al., 1997), and the present study did not identify a significant change in NHE expression levels in response to elevated pCO_2 in *C.maenas* (figure 2.8).

Interestingly, the carbonic anhydrase isoforms CA-1 and CA-2 did not show a prominent response upon long-term exposure to elevated pCO_2 levels in the gills (figure 2.8), despite their apparent dominant role in branchial osmoregulation in crabs as they have been shown to undergo 3-fold (CA-1) and a rapid 100-fold (CA-2) increase upon low

salinity acclimation in posterior gills (Serrano and Henry, 2008). However, an exception was observed in gill 4 where CA-2 showed a trend to be down-regulated ($p = 0.07$), and in gill 5, where a significant up-regulation of CA-1 was identified (figure 2.8.B). The lack of response of both CA isoforms in most gills might be explained by the high enzyme kinetics reported for these enzymes (Henry et al., 2003), warranting high flexibility upon a moderate pH-stress.

The sodium bicarbonate co-transporter (NBC), which has been shown to play a role in osmoregulation in a recent microarray study in *C. maenas* (Towle et al., 2011) and showed a high expression level only in the osmoregulatory active posterior gills in the current study (figure 2.7.F), showed no response to elevated environmental pCO_2 (figure 2.8). This transporter therefore might not play a crucial role in CO_2 regulatory processes, which is an interesting and unexpected finding. Regarding the involvement in acid-base balance, the NBC has been shown to mediate both, Na^+ and HCO_3^- movement into the blood in fish gills (Evans et al., 2005). It has also been discussed to be indirectly involved in ammonia excretion processes in fish (Wright and Wood, 2009). Considering bicarbonate as the main buffering component in the hemolymph to counteract pH disturbances, this buffer molecule likely participates in an acid-base regulatory response. However, NBC expression levels were also identified not to be altered upon elevated seawater pCO_2 in the microarray study on green crabs by Fehsenfeld et al. (2011) verifying data shown in the current study.

A second bicarbonate transporter, the anion exchanger (AE), has been discussed to be involved in acid-base regulation in crustaceans, in order to achieve electroneutrality since Cl^- typically is the counter-ion of HCO_3^- during the extracellular pH regulatory reaction

in seawater and freshwater fish (e.g. Hayashi et al., 2004; Larsen and Jensen, 1997). 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). When gills of *N. granulata* were perfused with a bicarbonate enriched perfusion solution, a significant increase in the transepithelial potential difference, as well as a switch from acid to base secretion, was only observed in the presence of Cl^-. In the same study, inhibitor experiments suggest the AE to be localized in the apical gill membrane (Tresguerres et al., 2008). However, biochemical characterization of this transporter is still lacking. Observing no change in expression levels of these two bicarbonate transporter genes NBC and AE in any of the investigated gills in the present study, is a puzzling finding that needs to be addressed in future studies.

Apical V-(H^+)-ATPase (HAT) has been identified to potentially play a role in acid-base regulation of low salinity acclimated *N. granulata* (Tresguerres et al., 2008). However, HAT in the gills of *C. maenas* is likely more associated with cytoplasmic vesicles than the apical membrane of the gill epithelium and plays an important role in active ammonia excretion, but is not involved in osmoregulatory processes as indicated by a lack of a response of the branchial transepithelial potential difference (PD_{te}) after blocking V-(H^+)-ATPase with 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 2.8), indicating that changes observed in ammonia excretion rates are likely due to alterations in the passive transbranchial movement rather than active ammonia transport across the gill epithelium or to the activation of existing transporters.

The Rhesus-like protein (Rh) was the only gene that exhibited a higher expression level in the anterior than in the posterior gills (figure 2.7.B). Rh was down-regulated in the anterior gills 4 and 5 in pCO_2 exposed green crabs and simultaneously gill 5 was observed to have a decreased ammonia excretion rate in those animals (figures 2.8.A and 2.8.B, respectively). Additionally, low environmental pH resulted in a decrease of the proton excretion rate in gill 5 (figure 2.5.A). The Rhesus-like protein discovered in crustaceans including *C. 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 *M. magister* (Martin et al., 2011). In human red blood cells (Endeward et al., 2008), the Rhesus-like protein (RhAG) 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 a CO_2 and ammonia channel in *Danio rerio* gills in response to hypercapnia. The Rhesus-like protein might therefore be a very important link between CO_2 regulation and ammonia excretion.

2.6. Conclusions

The results of the present study clearly show that exposure to altered environmental pCO_2 levels that mimic a near future scenario has significant effects on *C. maenas* at 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 an important role in acid-base regulation.

An important question still remaining, is how gills sense their actual acid / base status in order to counteract the disturbances as described above. It has been shown that the evolutionary conserved signalling enzyme soluble adenylyl cyclase (sAC) acts as a sensor of acid-base status in dogfish (Tresguerres et al., 2010). This enzyme produces the second messenger cAMP upon activation by HCO_3^- and is expressed in the gill epithelium, which makes it a suitable candidate as a sensor in crustaceans. 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 and 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.

CHAPTER 3:

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

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Other author's contribution:

DW helped with the design of the study and provided financial support, as well as laboratory equipment.

3.1. Abstract

The present study investigated acid-base regulatory mechanisms in seawater-acclimated green crabs, *Carcinus maenas*. 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 pCO_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, V-(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 cytoplasmatic localized V-(H^+)-ATPase, as well as potentially basolateral bicarbonate transporters (likely a Na^+/HCO_3^--cotransporter). In both cases, H^+ accumulated in the hemolymph and CO_2 excretion over 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.

3.2. Introduction

Over the last few decades, green crabs 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 (Cameron and Metaxas, 2005; Jamieson et al., 1998; 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 (Bellwood, 2002; Thomsen et al., 2010; Truchot and Duhamel-Jouve, 1980; Weihrauch et al., 1999a).

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; Freire et al., 2008; Henry et al., 2012; Pequeux, 1995). 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 $Na^+/K^+/2Cl^-$-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* (Lucu and Siebers, 1987; Onken et al., 2003; Riestenpatt et al., 1996). Basolateral Na^+/K^+-ATPase and Ba^{2+}/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 V-(H^+)-ATPase and a functional microtubule network (Weihrauch et al., 1998, 2004b). 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.

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, 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^- and *vice versa*, 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* (Burnett et al., 1981), the blue crab, *Callinectes sapidu*s (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^--cotransporters 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 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* (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 pCO_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.

3.3. Material and Methods

3.3.1. 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.

3.3.2. Acclimation to high environmental pCO_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 pCO_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 pCO_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).

3.3.3. 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 μl 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 glutathion, 0.5 glutamine, based on results from ion chromatography performed on hemolymph of full strength seawater-acclimated green crabs (see chapter 2). 100 μmol L^{-1} NH_4Cl was only added to the perfusion solution, not the bathing solution, to be consistent with *in vivo* conditions (Weihrauch et al., 1998). The pH of the perfusions solution was adjusted to 7.9 with 1 mol L^{-1} NaOH immediately before each respective perfusion step to ensure low variability in this parameter.

The perfusion protocol consisted of three consecutive steps (figure 3.1): 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.

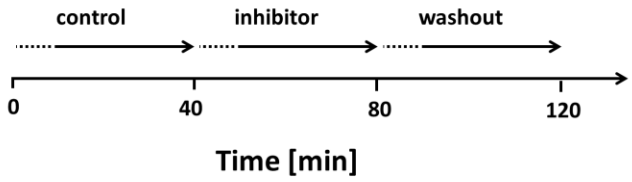

Figure 3.1. Perfusion scheme for inhibitor experiments on isolated anterior gill 5 of seawater-acclimated *Carcinus maenas*. Gills were perfused with a perfusion solution mimicking the ionic composition of hemolymph of seawater-acclimated (32 ppt) green crabs. A control step of 40 min was performed before the inhibitor was applied for the subsequent 40 min, followed by a 40 min wash-out period to ensure that the gill was still alive. All steps included a 10 min equilibration period (dashed lines) after which the perfusate was then collected for 30 min.

The inhibitors and their concentrations were chosen as follows, according to the available literature: 100 μmol L^{-1} tenidap (bicarbonate transporters, i.e. Na^+/HCO_3^--cotransporter, NBC; Ducoudret et al. 2001), 20 μmol L^{-1} KM91104 (V-(H^+)-ATPase; Kartner et al., 2010), 5 mmol L^{-1} ouabain (Na^+/K^+-ATPase, NKA; Weihrauch et al., 1998), 100 μmol L^{-1} amiloride (Na^+/H^+-exchanger, NHE; Siebers et al., 1982), 12 mmol L^{-1} $BaCl_2$ (K^+-channels; Schirmanns and Zeiske, 1994) and 1 mmol L^{-1} acetazolamide (carbonic anhydrase; 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).

3.3.4. 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 CO_2 (C_T) was measured using a Corning 965 carbon dioxide analyzer (Olympic Analytical Service, UK). pCO_2 and HCO_3^- 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 NH_3 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:

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

Accordingly, gill excretion rates for CO_2, HCO_3^- and ammonia were assessed based on the difference of the respective concentration in the perfusate compared to the initial perfusion solution for each step.

3.3.5. Maximum likelihood analysis of Na^+/H^+-exchangers

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., 2013). 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.

3.3.6. Statistics

All statistical analyses were performed using the software Past3 (Hammer et al., 2001). All data sets were first 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 or tested with non-parametric tests. Concerning parametric testing, a Student's t-test or paired t-test was applied comparing two means, whereas one-way ANOVA was applied comparing multiple data sets. In cases of non-parametric testing, a Mann-Whitney U-test was applied for comparison of two means, while the Kruskal-Wallis test with pairwise Mann-Whitney comparisons was used for the comparison of multiple means. All results with $p < 0.05$ were considered significant.

Table 3.1. GenBank accession numbers and description of Na^+/H^+-exchangers as used to generate the NHE gene tree (see figure 3.5).

Species	Accession no.	description
Caenorhabditis elegans	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
Homo sapiens	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
Aedes aegypti	AAM63432.1	Na^+/H^+ exchanger (NHE-3)
	XP_001654568.1	AAEL002051-PA (NHE-7,9)
	ACJ02512.1	Na/H exchanger 8
Carcinus maenas	AAC26968	sodium/hydrogen exchanger
Hyas araneus	-	Contig HaCtg18509[(1)]
	-	Contig HaIso05788 (predicted NHE-8)[(1)]

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

3.4. Results

3.4.1. Whole organism response of *C. maenas* to high pCO_2 exposure

The transfer of green crabs into the experimental units (inside, smaller tanks) caused a shift of hemolymph pH (not significant), pCO_2 (significant, repeated-measures ANOVA with Tukey's pairwise comparisons, $p < 0.05$, $n = 5$) and $[HCO_3^-]$ (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, pCO_2 and HCO_3^- levels remained slightly lower than observed in control animals before the transfer but remained constant over time (figure 3.2).

Green crabs exposed to high pCO_2 (1% CO_2) rapidly accumulated CO_2 and HCO_3^- in their hemolymph (figure 3.2). A significant increase in both hemolymph parameters was observed after only 6 hours and levelled off at a 4-fold increased level for pCO_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 pCO_2 crabs after 48 hours.

Hemolymph ammonia increased significantly from 93 ± 0.8 μmol L^{-1} in control animals to 406 ± 45 μmol L^{-1} in high pCO_2 crabs, as did the whole animal ammonia excretion rates (46 ± 10 versus 175 ± 34 nmol g^{-1} h^{-1}).

3.4.2. 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 3.3). Compared to green crabs acclimated to brackish-water (10 ppt, data from chapter 2), *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.

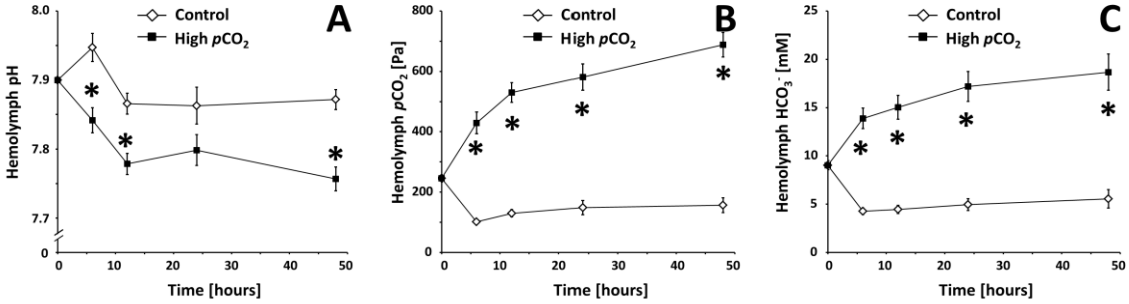

Figure 3.2. Time series of changes in hemolymph acid-base parameters of seawater (32 ppt) acclimated *Carcinus maenas* during the first 48 hours of exposure to elevated environmental pCO_2 (hypercapnia; 1% CO_2). Changes in (A) hemolymph pH, (B) hemolymph pCO_2, and (C) hemolymph HCO_3^- in high pCO_2 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 pCO_2 animals (Student's t-test with $p < 0.05$, n = 5-7).

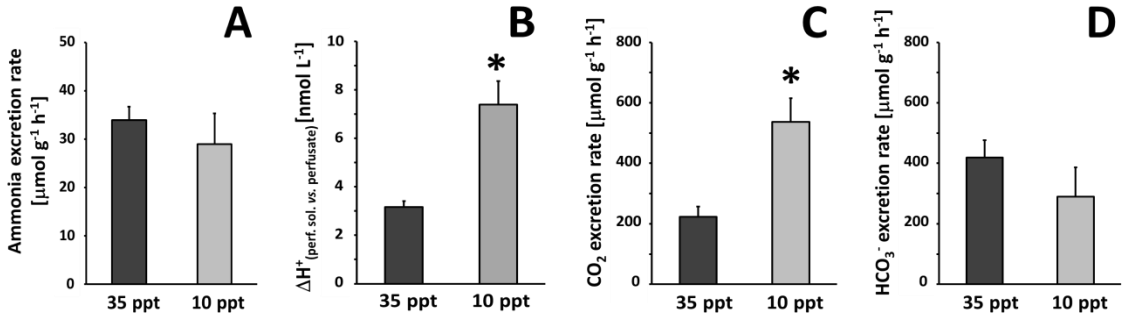

Figure 3.3. Comparison of the control perfusion step of osmoconforming and osmoregulating *Carcinus maenas*. Anterior gill 5 was perfused for 30 min with the respective perfusion solution mimicking the ionic composition of their hemolymph. Changes in the respective parameters hemolymph ammonia (A), H^+ (B), CO_2 (C) and HCO_3^- (D) have been calculated based on the loss in the perfusate. 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. Protons are expressed as ΔH^+ to account for changes in chemical properties of the perfusate (Stewart, 1978).The bigger $\Delta[H^+]$, the more loss in the perfusate equivalent to more excretion. Values for brackish-water acclimated green crabs are taken from chapter 2). Asterisks denote significant differences between gills of different salinity acclimations (Student's t-test with $p < 0.05$, n = 4 – 7).

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 3.4). Blocking potentially basolateral situated

bicarbonate transporters resulted in an accumulation of $[H^+]$ as well as CO_2 in the perfusate (i.e. perfusate concentration exceeding the concentration in the perfusion solution resulting in negative fold-change values; figure 3.4.A, B). Inhibiting V-(H^+)-ATPase basolaterally led to a similar level of accumulation of protons in the hemolymph as tenidap (figure 3.4.A). In contrast to tenidap, however, CO_2 excretion still took place but was drastically reduced (*ca.* 70%, figure 3.4.B). Basolateral bicarbonate transporters seemed not to be involved in ammonia excretion in anterior gill 5, whereas it was significantly reduced applying basolateral KM91104 (V-(H+)-ATPase; figure 3.4.B). It should be noted that basolaterally applied KM91104 might not only affect basolateral V-(H^+)-ATPase, but by diffusion across 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 $[H^+]$ (figure 3.4.A), apical application of KM91104 additionally resulted in a significant decrease of CO_2 excretion (figure 3.4.B).

Also all of the other basolaterally applied inhibitors significantly increased perfusate $[H^+]$ (figure 3.4.A). While blocking the Na^+/K^+-ATPase by ouabain and K^+-channels with $BaCl_2$ 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 $[H^+]$.

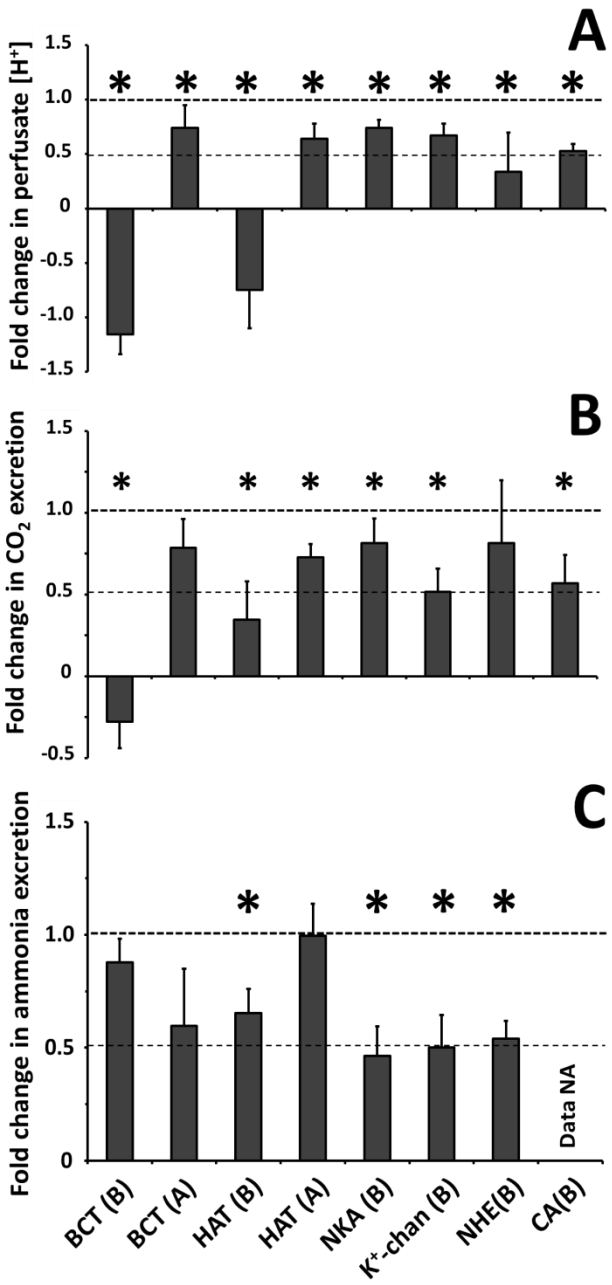

Figure 3.4. Relative changes in perfusate [H^+], CO_2 and ammonia excretion rates of isolated anterior gill 5 of seawater acclimated *Carcinus maenas* during gill perfusion applying inhibitors. B, basolateral; A, apical; BCT, bicarbonate transporter; HAT, V-(H^+)-ATPase; NKA, Na^+/K^+-ATPase; chan, channel; NHE, Na^+/H^+-exchanger. Inhibitors were: 100 μmol L^{-1} tenidap (BCT), 20 μmol L^{-1} KM91104 (V-(H^+)-ATPase), 5 mmol L^{-1} ouabain (NKA), 12 mmol L^{-1} $BaCl_2$ (K^+-channels), 100 μmol L^{-1} amiloride (NHE), 1 mmol L^{-1} acetazolamide (CA). Asterisks denote significant changes in comparison to the excretion rate during control perfusion (bold dashed lines; paired t-test with $p < 0.05$, $n = 4 - 8$). The light dashed lines indicate a 50% inhibition.

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 3.4.B). Besides the above mentioned pronounced effects on CO_2 excretion when blocking Na^+/HCO_3^--cotransporter and V-(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 V-(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 3.4.C).

3.4.3. Maximum likelihood analysis of Na^+/H^+-exchangers (NHEs)

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 3.5, the 24 sequences for NHEs (*Caenorhabditis elegans* NHX1-9, *Homo sapiens* NHE1-9, *Aedes aegypti* NHE-3,8,7/9, *Hyas araneus* 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*. However, node support for the second group including both *H. araneus* NHE isoforms is not strong. The NHE of *C. maenas* 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.

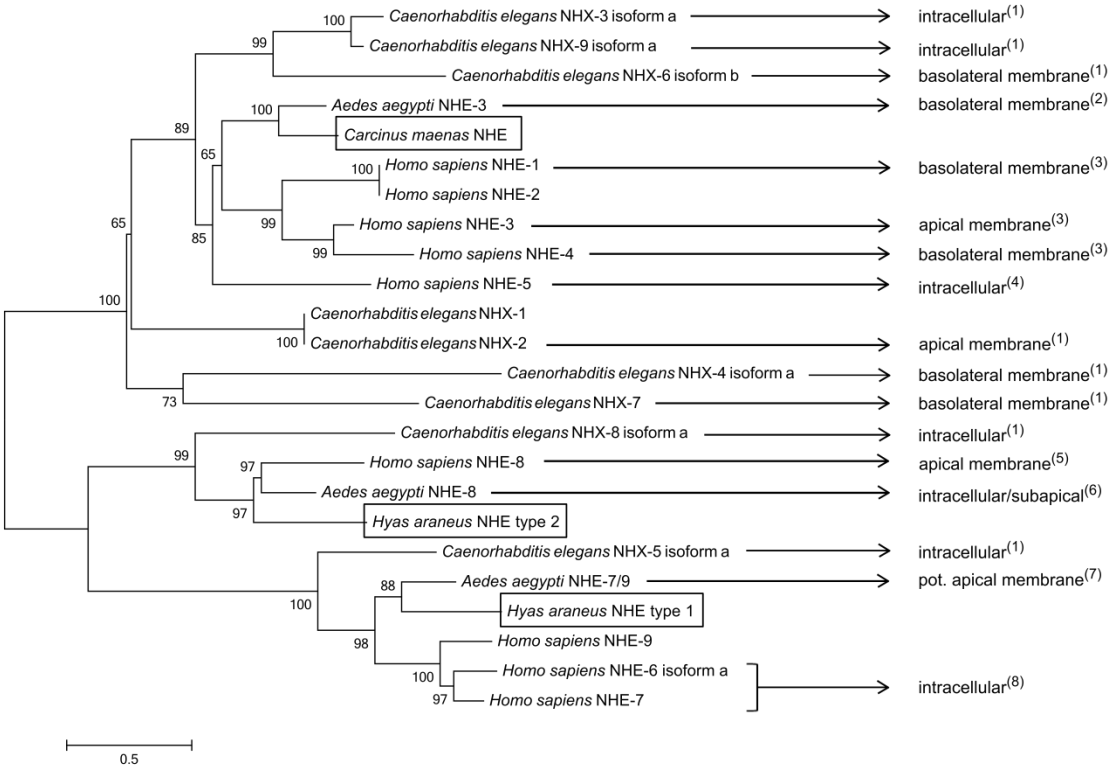

Figure 3.5. Maximum likelihood tree (unrooted) of Na^+/H^+-exchangers. The tree with the highest log-likelihood (-8093.0660) is shown and drawn to scale. The scale bar indicates the number of amino acid substitutions per site. Numbers beside branches represent bootstrap values in % (1000 replicates). 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 3.1.

3.5. Discussion

3.5.1. 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 pCO_2 (48h, 1% CO_2 = 1 kPa = 7.5 mmHg) with significantly increased hemolymph pCO_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% CO_2 = 0.4 kPa = 3 mmHg; Appelhans et al. 2012 (10 weeks exposure); chapter 2 (7 days exposure)) and the marine Dungeness crab, *Metacarcinus magister* (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 pCO_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 (chapter 2). 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 pCO_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 Japanese amberjack, *Seriola quinqueradiata*, and the Japanese flounder, *Paralichthys olivaceus*. In contrast, blood pH in star-spotted dogfish *Mustelus manazo* 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 $[NH_4^+]$ 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 and it is possible that complete restoration of hemolymph pH would have occurred on a longer time frame.

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 chapter) compared to

osmoregulating/ hypercapnia-acclimated crabs (chapter 2). 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* (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* when acclimated to 0.24 and 0.4 kPa pCO_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 high pCO_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 pCO_2 acclimation, independent of the initial hemolymph ammonia concentration (this chapter, chapter 2) and the level of external pCO_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 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.

3.5.2. 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 CO_2 than the anterior gill epithelia of osmoconforming green crabs, but not in respect to ammonia excretion (figure 3.3.B, C). This indicates that the adjustment of the excretion of acid-base equivalents indeed plays a direct role in osmoregulatory processes. Additionally, the fact that anterior gills are capable of increasing proton and CO_2 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 (chapter 2). 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*.

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

3.5.3.1. Opening remarks

The major aim of the present study was to increase our limited understanding and fragmentary knowledge of 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 subsequent interpretations need to be treated with caution. 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* 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, I chose the inhibitors used in this chapter 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.

3.5.3.2. Bicarbonate transporters

Unfortunately, no specific inhibitors for either Na^+/HCO_3^--cotransporters, or Cl^-/HCO_3^--exchangers, are currently available. Even though tenidap has been shown to inhibit Na^+/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 Cl^-/HCO_3^--exchanger (Ducoudret et al., 2001).

The application of tenidap in the present study identified potential basolateral bicarbonate exchanger(s) (i.e. a Na^+/HCO_3^--cotransporter and/or a Cl^-/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 ± 71 $mScm^{-2}$ 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 and Pequeux, 1990; 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, Na^+/HCO_3^--cotransporter as well as Cl^-/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; chapter 2). Even though mRNA levels of the anion exchanger were 3-fold higher than for the Na^+/HCO_3^--cotransporter, anterior gills still exhibited a significant mRNA expression level of the Na^+/HCO_3^--cotransporter comparable to mRNA levels for gill V-(H^+)-ATPase (chapter 2). While the Cl^-/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 Na^+/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 Na^+/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 $Na^+/K^+/2Cl^-$ cotransporter, basolateral Na^+/K^+-ATPase, K^+-and Cl^--channels; Riestenpatt et al., 1996), a similar distribution and function of a basolateral Na^+/HCO_3^--cotransporter can therefore be postulated in the green crab. Recently, a basolateral Na^+/HCO_3^--cotransporter has also been identified to be of high importance in acid-base regulation in the squid *Sepioteuthis*

lessoniana (Hu et al., 2014). Interestingly, a basolateral Na^+/HCO_3^--cotransporter as well as an apical Cl^-/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 [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 Na^+/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.

3.5.3.3. V-(H^+)-ATPase

In contrast to freshwater-acclimated crustaceans like the Chinese mitten crab, *Eriocheir sinensis* (Henry et al., 2012; Onken and Putzenlechner, 1995) 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* (Weihrauch et al.,

2004a) is higher in posterior gills, it tends to be more abundant in anterior gills of *C. maenas* (chapter 2; Weihrauch et al., 2002). 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 V-(H^+)-ATPase in the apical membrane due to the fusion of the V-(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.

3.5.3.4. 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^+ (Lignon, 1987; Skou, 1960; Weihrauch et al., 1998). In elasmobranchs and teleost fish, Na^+/K^+-ATPase plays an important role in acid-base balance (Gilmour and Perry, 2009; Perry and Gilmour, 2006). Generating an electrochemical gradient over the basolateral membrane by pumping 3 Na^+ out of the cell in exchange for only $2K^+$, Na^+/K^+-ATPase is the major driving force for the excretion of H^+ *via* apical Na^+/H^+-exchanger in acid excretory epithelial cells (Choe et al., 2005; Edwards et al., 2002). 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.

3.5.3.5. 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* and *Petrolishtes cinctipes* (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 NH_4^+. 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 NH_4^+ 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 3.5, Na^+/H^+-exchangers exhibit a complex evolutionary history. Even though it appears that the included sequences divide into two monophyletic groups node support for this distinction is not strong. 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 shows highest similarity 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* (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.

3.5.3.6. 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_{CO2}, figures 3.6, 3.7).

3.5.4. Hypothesized mechanism for proton excretion across the anterior gill epithelium of seawater-acclimated *C. maenas* (figure 3.6)

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 across 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 V-(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/potentially resulting from ammoniogenesis) 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* 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 V-(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 V-(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.

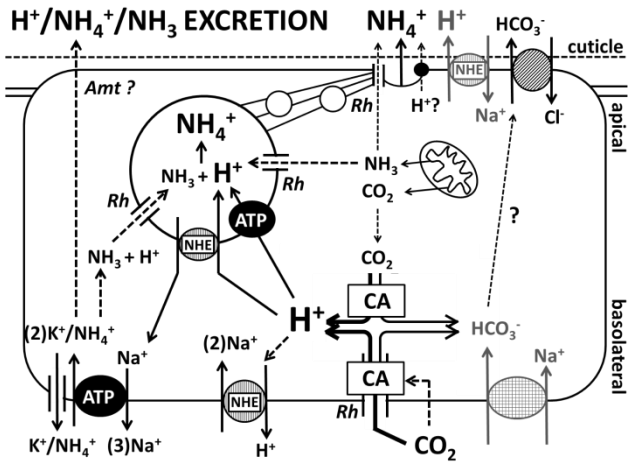

Figure 3.6. Hypothetical model for the regulation of proton and ammonia excretion in the anterior gill epithelium of seawater-acclimated *Carcinus maenas*. Key-players have been identified in perfusion experiments on isolated gill 5 by applying inhibitory pharmaceuticals for the respective components. 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. The mitochondrion indicates a potential intracellular, metabolic source for CO_2 and NH_3. 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 V-(H^+)-ATPase).

3.5.5. Hypothesized mechanism for CO_2 excretion across the anterior gill epithelium of seawater-acclimated *C. maenas* (figure 3.7)

As described earlier, CO_2 possibly enters the cell across 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_{CO2} across 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_{CO2} 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 Na^+/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 pCO_2 and translating into a decrease of CO_2 excretion rates. HCO_3^- might potentially be delivered by the basolateral Na^+/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 Na^+/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 pCO_2 and translating to a net decrease of CO_2 excretion across the epithelial membrane.

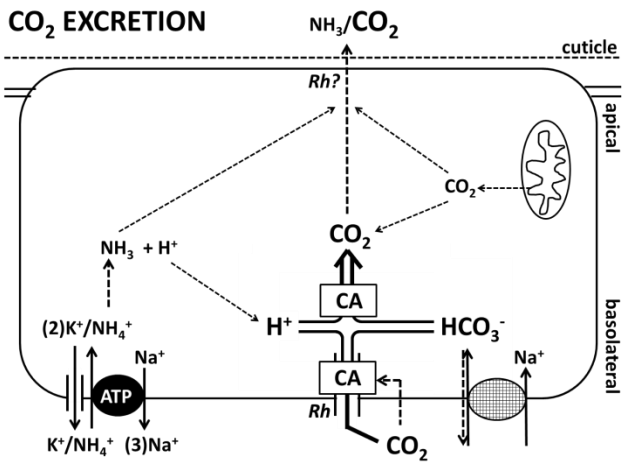

Figure 3.7. Hypothetical model for the regulation of CO_2 excretion in the anterior gill epithelium of seawater-acclimated *Carcinus maenas*. Key-players have been identified in perfusion experiments on isolated gill 5 by applying inhibitory pharmaceuticals for the respective components. Rh, Rhesus-like protein; CA, carbonic anhydrase; ATP, ATPases (basolateral Na^+/K^+-ATPase).

3.5.6. Hypothesized mechanism for NH_3/NH_4^+ excretion across the anterior gill epithelium of seawater-acclimated *C. maenas* (figure 3.6)

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, NH_3/NH_4^+ excretion does not seem to depend on HCO_3^- entering the cell *via* the basolateral Na^+/HCO_3^--cotransporter.

As applying KM91104 basolaterally is believed to target the vesicular V-(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 V-(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 NH_3/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 over 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 V-(H^+)-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.

3.6. 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 chapter 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.

CHAPTER 4:

THE ROLE OF AN ANCESTRAL HYPERPOLARIZATION ACTIVATED CYCLIC NUCLEOTIDE-GATED K^+-CHANNEL IN BRANCHIAL ACID-BASE REGULATION IN THE GREEN CRAB, *CARCINUS MAENAS*

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My contribution:

I conducted all experiments, analyzed the data and wrote the manuscript.

Other author's contribution:

DW helped with the design of the study and provided financial support, as well as laboratory equipment.

4.1. Abstract

Potassium channels play a crucial role not only for neurophysiological functions, but also in osmoregulatory ion uptake and ammonia excretion in the gills of decapod crustaceans. While branchial K^+-transport in Brachyuran crabs has been studied by applying pharmaceuticals (Ba^{2+}/Cs^+) and measuring ion fluxes in gill perfusion experiments and split gill lamellae, little is known of the actual nature of these channels in crustaceans.

Based on the identification of a hyperpolarization activated cyclic nucleotide-gated potassium channel (HCN) in the transcriptome of the green crab, *Carcinus maenas*, in the present study, quantitative real-time PCR revealed the ubiquitous expression of this channel in this species. Even though mRNA expression levels in the cerebral ganglion were found to be approximately 10-times higher compared to all other tissues, the posterior gills still expressed significant levels of HCN, suggesting an important role for this transporter in branchial ion regulation.

Applying the more general K^+-channel inhibitor Ba^{2+} as well as the HCN-specific blocker ZD7288 in gill perfusion experiments and in electrophysiological studies on the split gill lamellae revealed the presence of at least two different K^+/NH_4^+-transporting structures in the branchial epithelium of *C. maenas*. Furthermore, HCN mRNA levels in posterior gill 7 decreased significantly in response to the respiratory and/or metabolic acidosis that was induced by acclimation of green crabs to high environmental pCO_2 and ammonia, respectively. Consequently, the present study provides the first evidence for HCN-promoted NH_4^+ epithelial transport to be involved in both, branchial acid-base and ammonia regulation in an invertebrate.

4.2. Introduction

Potassium channels form a diverse family of ubiquitously expressed transmembrane proteins that are critical for a number of important physiological functions (Tian et al., 2014). In particular, K^+-fluxes are essential for the membrane excitability of neurons and muscles (Choe, 2002). In the crustacean gill epithelium K^+-channels have been identified to participate in the establishment of an electrochemical gradient across the basolateral membrane that is primarily generated by a basolateral Na^+/K^+-ATPase (Riestenpatt et al., 1996). Furthermore, apical K^+-channels seem to co-localize with apical $Na^+/K^+/2Cl^-$-cotransporter (NKCC) to permit K^+ circulation and thereby an uptake of Na^+ and Cl^- from the environment in hyper-regulating crustaceans (Onken et al., 1991; Onken et al., 2003; Riestenpatt et al., 1996). Due to the presence of an apical NKCC and K^+-channel as well as the accompanying basolateral Cl^--channel, the moderately leaky gill epithelium of *C. maenas* has been compared to the thick ascending limb of the mammalian kidney (TAL; Riestenpatt et al., 1996). The apical K^+-channel present in the TAL is a member of the inwardly rectifying K^+-channels K_{IR} (Kir1.1) and promotes apical efflux of K^+ to energize the apical NKCC. Additionally, Kir4.1/5.1 has been identified to be co-localized basolaterally with the Na^+/K^+-ATPase in the kidney's distal tubule, providing a basolateral back flow of K^+ out of the cell into the body fluids in order to maintain the electrochemical gradient (Hibino et al., 2010), as can also be postulated for crustacean gills.

While Ba^{2+} is generally regarded as an unspecific blocker of K^+-currents, recent studies suggest a mode of inhibition specifically for K^+-channels of the K_{IR}-family, and not delayed-rectifier or ATP-induced K^+-currents, by Ba^{2+} concentrations of up to

100 μmol L^{-1} (Franchini et al., 2004). Ba^{2+} as applied in gill perfusion or split gill lamella experiments in *C. maenas* did indeed influence the short-circuit current and trans-epithelial conductance in decapod crustacean gills (Henry et al., 2012; Larsen et al., 2014). Hence it can be hypothesized that at least part of the K^+-current in the crabs' gill epithelium is produced by K_{IR}-like channels, similar to what is observed in the vertebrate kidney. Based on the incomplete and complex di-phasic response of the short-circuit current (I_{SC}) upon application of Ba^{2+}, however, several studies on decapod gill epithelia indicate the participation of more than one kind of K^+-channel (Onken et al., 2003; Riestenpatt et al., 1996).

In addition to their role in osmoregulation, basolateral K^+-channels also participate in branchial ammonia excretion in *C. maenas*, with NH_4^+ substituting for K^+ due to its similar size and charge (Lignon, 1987; Skou, 1960; Weihrauch et al., 1998). Apical application of Cs^+ (another unspecific inhibitor for K^+-channels), however, did not affect branchial ammonia excretion in this species. Furthermore, branchial ammonia regulation in *C. maenas* has been observed to be closely linked to acid-base regulation (chapter 2), but the specific role of K^+-channels in this context is not clear.

Interestingly, a K^+-channel of the hyperpolarization activated cyclic nucleotide-gated channel family (HCN2) has recently been shown to be involved in acid-base and ammonia regulation in the collecting duct of the mammalian kidney (Carrisoza-Gaytán et al., 2011). While HCN2 has been mainly associated with pacemaker activities to date (DiFrancesco, 1993), Carrisoza-Gaytán and collegues showed that this gene is also expressed in the basolateral epithelium of rat kidney cells and capable of promoting NH_4^+

transport across the membrane, therefore participating in mammalian renal pH homeostasis.

The present study aims to investigate the role of K^+-channels in branchial acid-base regulation of the osmoregulating green crab *C. maenas*, and particularly a HCN-like protein as identified in the transcriptome. By application of two different K^+-channel inhibitors, Ba^{2+} and the HCN-specific ZD7288, acid-base and ammonia regulatory properties of the gill epithelium were characterized in respect to K^+ and NH_4^+ transport. A potential role for K^+-channels, and specifically for HCN, was also evaluated.

4.3. Material and Methods

4.3.1. Identification and gene tree analysis of CmHCN

In order to identify potential candidates for the hyperpolarization activated cyclic nucleotide-gated potassium channel family in *C. maenas* (CmHCN), the partial amino acid sequence for the hyperpolarization-activated ion channel of *Cancer borealis* (GenBank accession AAZ80094.3) was used as assembly scaffold for a recently generated transcriptome for *C. maenas* (Verbruggen et al., 2015) using the basic local alignment search tool (BLAST) feature in Geneious© version 7.1 (Biomatters Limited, Auckland, New Zealand). Accordingly, potential candidates for the basic voltage-gated (K_V) and inward-rectifying (K_{IR}) K^+-channels were identified in the *C. maenas*' transcriptome using the sequences for the ATP-dependent inward-rectifying potassium channel KIR4.1 and 5.1, as well as the potassium voltage-gated channel subfamily H member 6 isoform 1 of *Homo sapiens*.

For the gene analysis, the protein sequences retrieved from the NCBI protein database as listed in table 1 were aligned with the MUSCLE algorithm (Edgar 2004) as provided by MEGA 6.06 using the default settings (Tamura et al. 2013).

Table 4.1. Information on the potassium channels as included in the maximum likelihood analysis depicted in figure 4.1.

Species	(Infra)Class	Accession no.	description
Homo sapiens	Mammalia	NP_001185.3	HCN (2)
		NP_110406.1	K_V (H6 isoform 1)
		AAB07046.1	K_{IR} 4.1
		AAF73240.1	K_{IR} 5.1
Mus musculus	Mammalia	NP_032252.1	HCN (2)
		NP_001032801.1	K_V (H6)
		BAA92432.1	K_{IR} 4.1
		BAA34723.1	K_{IR} 5.1
Danio rerio	Teleostei	XP_686078.4	HCN
		NP_998002.1	K_V (H6)
		NP_001092204.1	K_{IR} 4.1
		NP_001265731.1	K_{IR} (J2a)
Strongylocentrus purpuratus	Echinoidea	NP_001028182.1	HCN
		XP_011669414.1	K_V (H7 isoform X1)
		XP_789112.1	K_{IR} (12)
Aedes aegypti	Insecta	XP_001658860.1	HCN (AAEL008056-P)
		XP_001653553.1	K_V (AAEL001542-PA)
		AGA61793.1	K_{IR} (1)
Drosophila melanogaster	Insecta	AAX78393.1	HCN (DMIH-A1B1C1)
		NP_476713.1	K_V (seizure, isoform A)
		CAC87638.1	K_{IR}
Daphnia pulex	Branchiopoda	EFX81385.1	HCN (DAPPUDRAFT_2754)
		EFX90436.1	K_V (DAPPUDRAFT_39704)
		EFX88146.1	K_{IR} (DAPPUDRAFT_311738)
Carcinus maenas	Malacostraca	GBXE01087917.1	HCN (Transcriptome contig)
		GBXE01103936.1	K_V (Transcriptome contig)
		GBXE01116654.1	KIR (Transcriptome contig)
Cancer borealis	Malacostraca	AAZ80094.3	HCN (HAIC)
Caenorhabditis elegans	Chromadorea	NP_001122688.1	K_V (UNC-103, isoform h)
		NP_509138.2	K_{IR} (IRK-2)

Accession numbers are according to GenBank and specific descriptions based on the NCBI protein database. HCN, potassium/sodium hyperpolarization activated cyclic nucleotide-gated channel; K_V, voltage-gated potassium channel; K_{IR}, inwardly-rectifying potassium channel.

The best protein model for the gene analysis was chosen according to the test function provided in MEGA 6.06 (Tamura et al., 2013). Subsequently, a gene tree was constructed in MEGA 6.06 applying the maximum likelihood method using the gamma-distributed LG-model with invariant sites (LG+I+G). A matrix of pairwise distances estimated using a JTT model (Jones et al., 1992) was used to automatically generate initial tree(s) for the heuristic search by applying Neighbor-Joining and BioNJ algorithms and subsequently selecting the topology with the superior log-likelihood value. Evolutionary rate differences among sites were modelled by discrete Gamma distribution (2 categories). All positions with less than 95% site coverage were eliminated (i.e. fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position). Bootstrap analyses were performed with 1000 replicates.

4.3.2. Animals

For gill perfusion experiments, male green crabs were caught in traps in the Kiel Fjord (Baltic Sea) at the pier in front of the Helmholtz Centre for Ocean Research (GEOMAR, Kiel/Germany; west shore building, 54°19.8'N; 10°9.0'E) at 3-4 m depth in May 2012 (collection permit issued Sep 2009, file number V313-72241.121-19). Animals were kept in *ca.* 250 L flow-through tanks in a climate chamber at the GEOMAR, directly fed by water from the Kiel Fjord (salinity = 14.3 ± 0.0 ppt, temperature = 17.2 ± 0.4°C, pH_{NBS} = 7.96 ± 0.03, pCO_2 = 96 ±7 Pa), until gill perfusions were performed (see below). Green crabs were fed *ad libitum* with blue mussels, *Mytilus edulis*, twice per week. Animals were fasted for a minimum of 2 days before experiments.

For quantitative real-time PCR and Ussing chamber experiments, male green crabs were caught in Barkley Sound at the opening of the Pipestem Inlet (Vancouver Island, BC,

Canada) in summer 2013 under the auspices of the Department of Fisheries and Oceans collection permit XR-235-2013 and transported back to Winnipeg, MB, Canada. They were kept at the Animal Holding Facility of the University of Manitoba in aerated 1200 L tanks with artificial seawater adjusted to a salinity of 32 ppt at 14 °C (Seachem Marine Salt®) until experimentation. For acclimation to dilute salinity (11 ppt), 8 animals were transferred into a 120 L tank for a minimum of 7 days and fed *ad libitum* with squid once a week. Water was exchanged at least every second day and always on the day after feeding. Green crabs were fasted for a minimum of 2 days prior to experiments.

For subsequent acclimation of green crabs to high environmental ammonia (HEA) for 7 days, the brackish water of the 120 L tanks was enriched with 1 mmol L^{-1} NH_4Cl. Water was exchanged on a daily basis and animals were not fed during HEA-acclimation.

4.3.3. Quantitative real-time PCR

Gene expression analysis was performed on tissues obtained from animals held at University of Manitoba. Anterior and posterior gills, cerebral ganglion, heart, skeletal muscle, antennal gland and hypodermis of osmoregulating *C. maenas* (11 ppt) acclimated to control conditions and high environmental ammonia (1 mmol L^{-1} NH_4Cl, 7 d), were harvested from animals acclimated as described above, while samples for hypercapnia-acclimated green crabs (400 Pa) were used as obtained in chapter 2. Tissue samples representing anterior and posterior gills were generated by pooling equal tissue amounts of the respective gills prior to isolating the RNA (gills 4 and 5 for anterior gills and gills 7, 8 and 9 for posterior gills). Changes in mRNA expression levels for *C. maenas* HCN (CmHCN) were investigated in gill 7 only in accordance with Fehsenfeld et al. (2011)

who observed most differentially expressed gene transcript in this gill of *C. maenas* in response to hypercapnia.

Briefly, total RNA was isolated under RNase-free conditions using TRIZOL (Invitrogen, Carlsbad, CA, USA). Following DN*ase* I (Ambion™, Thermo FisherScientific, Pittsburgh, PA, USA) treatment, regular PCR was performed to ensure the absence of DNA traces (RbS-3 primers; forward: 5' GTCCCTTTTCACCAAGGACA; reverse: 3' CAAGGCCAAACTCAACA GGTT). Subsequently, DNA-free RNA was transcribed with the iScript cDNA Synthesis Kit (Biorad, Mississauga, ON, Canada). Quantitative real-time PCR (qPCR) was performed on 20 ng cDNA and a final primer concentration of 0.4 μmol L^{-1} in 15 μl reactions with the SSO FastEvaGreen Supermix (Biorad). The gene specific primers for CmHCN were designed based on the identified transcriptome contig (forward: 5' GTCTTCATGCGCATCT TCAA; reverse: 5' – GTTGATTGCCAC CCAAGAGT) and resulted in the expected specific 123 bp fragment. The primers were verified to produce a single band in the qPCR by adding a melting curve analysis following the regular qPCR. In every run, a negative control (no template) was included to verify that the assay was not contaminated, as well as one of the standards treated as an unknown sample to ensure homogenous performance. A standard curve was included in each qPCR run based on a dilution series of known quantities of the respective gel-extracted gene fragment (0.01 fg - 100 fg; QIAquick Gel Extraction Kit, Qiagen). Additionally, the housekeeping gene ribosomal RbS-3 was used for internal normalization of hypercapnia-related tissues in accordance with chapter 2. No housekeeping gene (i.e. RbS-3, arginine kinase) with constant mRNA expression levels could be identified for HEA-acclimated green crabs. Hence, absolute quantification was

solely assessed using the included standard curve. In this case, a standard curve based on diluted cDNA samples was performed to ensure comparable amplification efficiency (PCR-fragments: 99.4 ± 0.2% (n = 3); cDNA templates: 100.5 ± 0.7% (n = 3)).

4.3.4. Perfusion experiments on isolated posterior gill 7

For gill perfusion experiments in Kiel, hemolymph ammonia, hemolymph carbonate system parameters, as well as hemolymph cation concentrations (Na^+, K^+ and Ca^{2+}; flame photometer analysis performed by Thomas Stegmann, Institute of Physiology, Kiel University) of control green crabs were assessed for the adjustment of the perfusion solution as described in chapter 2 to account for *in vivo* conditions of the Baltic Sea individuals. Accordingly, the perfusion solution consisted of (in mmol L^{-1}): 340 NaCl, 9 $CaCl_2$, 7 $MgCl_2$, 9 KCl, 14 $NaHCO_3$, 0.13 NH_4Cl, 0.5 glutamine, 0.5 glucose, 0.1 glutathione. The pH (NBS scale) was adjusted to 7.9. Posterior gills 7 were perfused according to the procedure described in chapter 2, following the technique of Siebers et al. (1985). Each perfusion consisted of a control step, followed by the application of the respective inhibitor and a third wash-out step to ensure the survival of the gill tissue. Each perfusion step allowed for 10 minutes adjustment of the gill after which the perfusate was collected for 30 minutes. Inhibitors were administered individually throughout the 40 minutes of the second perfusion step. In order to block basolateral K^+-channels, $BaCl_2$ was applied basolaterally at a concentration of 12 mmol L^{-1} (Schirmanns and Zeiske, 1994). The inhibitor ZD7288 (4-Ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride) was applied basolaterally at a concentration of 10 μmol L^{-1} to specifically block the K^+-current generated by the identified HCN, according to Carrisoza-Gaytán et al. (2011). Gill ammonia excretion

rates were calculated based on the difference in the perfusate compared to the perfusion solution. The proton concentration in the perfusate was calculated based on the pH, while perfusate pCO_2 and HCO_3^- were calculated using the equations and constants following Truchot (1976). An increase in perfusate $[H^+]$, CO_2 and/or $[HCO_3^-]$ was considered equivalent to a decrease in excretion.

4.3.5. Ussing chamber experiments on split gill lamellae (posterior gill 7)

Ussing chamber experiments were performed at room temperature in Winnipeg, Canada. In accordance with gill perfusion experiments, single gill lamellae of posterior gill 7 were split according to Schwarz and Graszynski (1989) and mounted in the EM-CSYS-6 Ussing chamber system (Physiologic Instruments, San Diego, USA) and specific sliders (P2308), providing a circular aperture of 1 mm that resulted in an open epithelial surface area of 8×10^{-3} cm^2. Silicon grease was used to seal the edges of the preparation in order to prevent potential edge damage. Preparations were aerated throughout the experiment. Symmetrical conditions (i.e. identical media on both sides of the epithelium) were used in order to account for only active ion transport, with the following saline being applied to both the apical and basolateral side (in mmol L^{-1}): 248 NaCl, 5 $CaCl_2$, 4 $MgCl_2$, 5 KCl, 2 $NaHCO_3$, 5 HEPES, 2 glucose (after Riestenpatt et al., 1996). The pH_{NBS} was adjusted to 7.9 with 0.1 M NaOH. Two pairs of Ag/AgCl electrodes were connected to the preparation *via* agar bridges (3% agarose in 3 mol L^{-1} KCl): one pair served to monitor the trans-epithelial potential difference PD_{te} (data not shown), while the second pair served as current electrodes to short-circuit the PD_{te} with an automatic clamping device (VCC 600, Physiologic Instruments, San Diego, USA). The short-circuit current I_{SC} (as a measure of ion/charge movements over the gill epithelium; Riestenpatt et al., 1996) was

recorded using the Acquire Analyze software (version 2.2). All electrodes were referenced, and the voltage of all electrodes set to 0 before the tissue was introduced into the set up.

Dose-response curves for $BaCl_2$ and ZD7288 were obtained to determine the inhibitor constant (IC_{50}) at which the respective inhibitor decreased the short circuit current (I_{SC}) by 50%, as well as the maximum change in I_{SC} (ΔI_{max}). The concentration of each basolaterally applied inhibitor was increased in a step-wise fashion as soon as I_{SC} levelled off for one concentration (after *ca.* 10-15 min) and then stayed constant for > 15 s.

4.3.6. Statistics

All statistical analyses were performed with PAST3 (Hammer et al., 2001). Data sets were first tested for normal distribution (Shapiro–Wilk test) and homogeneity of variances (F-test) in order to qualify for parametric statistical analyses. If one or both requirements were not met, data was log transformed. In cases where log transformation did not result in a data set appropriate for parametric analyses, non-parametric analyses were applied. For comparison of single means, a paired t-test or Mann-Whitney U-test was applied, while ANOVA or Kruskal-Wallis with pairwise Mann-Whitney comparisons was used to compare multiple means. All results with $p < 0.05$ were considered significant. Graphs were generated using the software Inkscape, version 0.48 (https://inkscape.org/). The dose-response curves were analyzed by GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

4.4. Results

4.4.1. Identification of CmHCN and gene tree analysis

A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 1.7367)). The rate variation model ([+I]) allowed for no sites to be evolutionarily invariable. All positions with less than 95% site coverage were eliminated resulting in a total of 251 positions in the final dataset.

For each of the three different K^+-channels (HCN, the basic voltage-gated ion channels K_V and the inward-rectifying potassium channel K_{IR}), a respective contig could be identified in the transcriptome of *C. maenas* as generated by Verbruggen et al. (2015). Together with other invertebrate and vertebrate sequences, the K^+-channel families appear to form two monophyletic families in the maximum likelihood analysis (figure 4.1), however, node-support values are not high and it can be argued that all three groups are separated. HCN seems to form a subfamily of the voltage-gated K^+-channels. Furthermore, the gene tree analysis revealed that the *C. maenas* proteins grouped with the respective other arthropod K^+-channels. While four different HCNs were found in mammals (for clarity only HCN2 was included, as relevant for the discussion), arthropods seem to express only one HCN. A similar observation was made for the K_{IR}-related proteins of the arthropods, which seemed to resemble KIR5.1 channels as found in mammals and fish.

The only two reasonably long identified contigs (resulting in an open reading frame >100 amino acids) in the green crab's transcriptome resembling HCN, GBXE01087917.1 (326 amino acids) and GBXE01153810.1 (148 amino acids), aligned to different regions of the HCN sequence of *C. borealis* (positions 291-712 *vs.* 66-213, respectively) and

HCN2 of *H. sapiens* (positions 360-659 *vs.* 180-299, respectively). Only GBXE01087917.1 contained the specific motifs for voltage-gated potassium channels (PLN03192) and the cyclic nucleotide-monophosphate binding domain (cAMP / cNMP / Crp) as determined by NCBI protein BLAST tool and was therefore chosen for further analysis. The partial CmHCN amino acid sequence was highly conserved and resembled the human HCN2 with 69% identity and 83% overall conserved domains (figure 4.2). The major binding site for ZD7288 was identified as Ala-425 and Leu-432 as recently described by Cheng et al. (2007).

CmHCN was observed to be ubiquitously expressed in all analyzed tissues, including muscular tissues (heart, skeletal muscle), nerves (cerebral ganglion) and potential ionregulatory tissues (hypodermis, antennal gland, anterior and posterior gills). While the cerebral ganglion exhibited the highest mRNA expression levels (*ca.* 10-fold compared to the other tissues), posterior gills still exhibited relatively high levels of the transcript compared to all other tissues tested (figure 4.3).

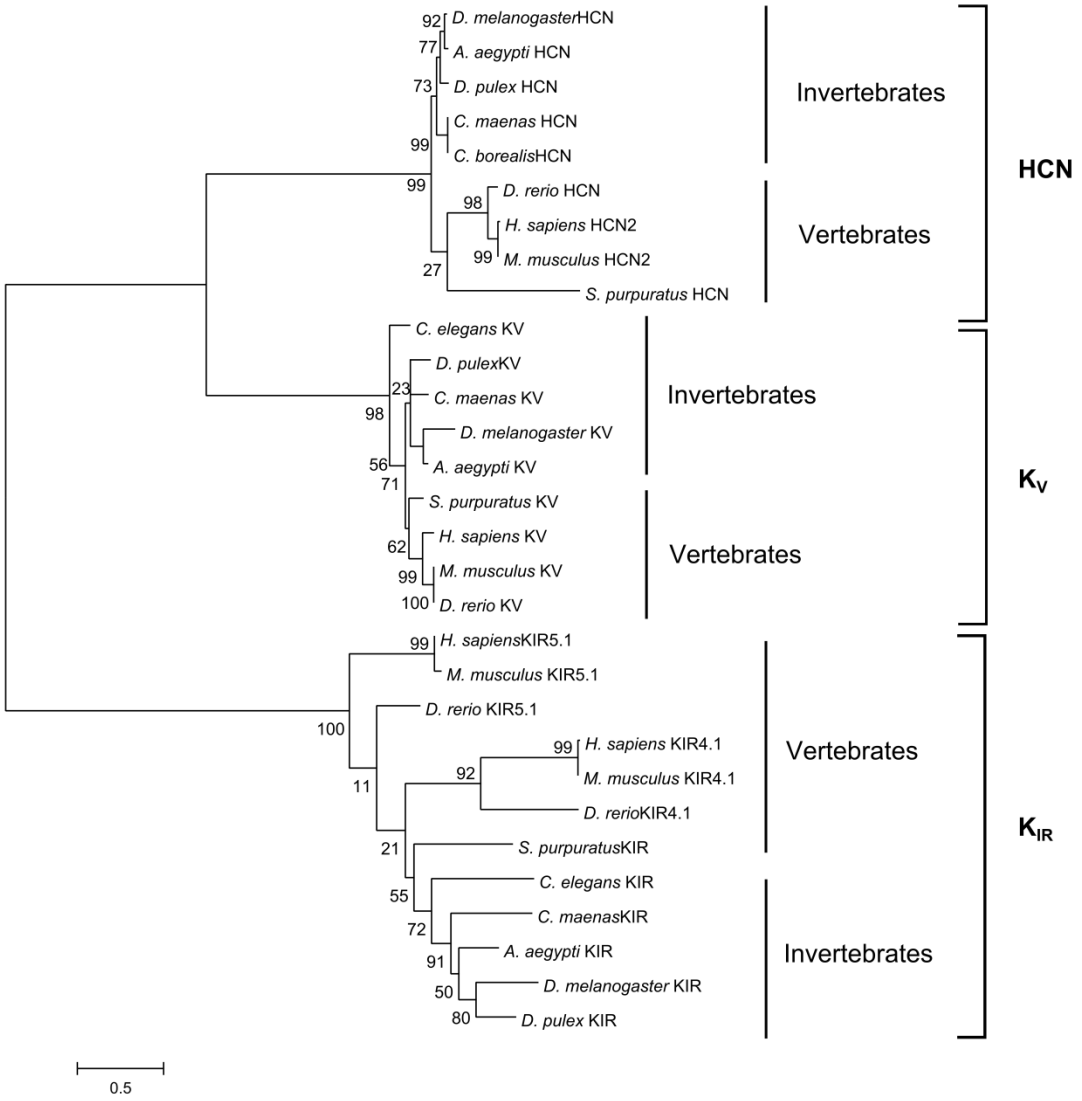

Figure 4.1. Maximum likelihood tree (unrooted) of voltage-gated (K_V and HCN) and K_{IR}-like potassium channels in vertebrates and invertebrates. The maximum likelihood analysis was based on the LG matrix-base model (Le and Gascuel, 2008) as implemented in MEGA6 (Tamura et al., 2013). The tree with the highest log-likelihood (-8003.4087) is shown and drawn to scale so that the branch lengths represent the number of substitutions per site (scale bar). Numbers beside branches represent bootstrap values (1000 replicates) in %. HCN, hyperpolarization activated cyclic nucleotide-gated potassium channel; K_V, voltage-gated potassium channel; K_{IR}, inward-rectifying potassium channel.

Figure 4.2. ClustalW alignment of *Carcinus maenas* HCN and *Homo sapiens* HCN2 protein. "*", single, fully conserved residue; ":", conservation of strong groups; ".", conservation of weak groups as designated by Biology WorkBench (version 3.2, http://seqtool.sdsc.edu/CGI/BW.cgi). Indicated in the sequence are the voltage-dependent potassium channel motif PLNO3192 (underlined), the Crp-motif for the predicted cNMP/cAMP binding site (grey box), and the binding sites Ala425 and Leu432 for the inhibitor ZD7288 (black boxes) after Cheng et al. (2007).

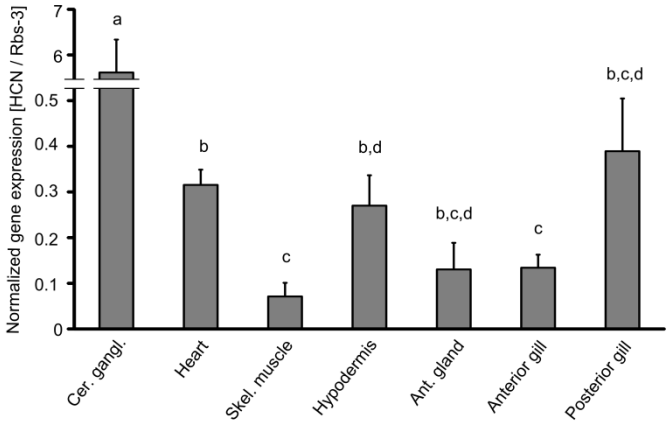

Figure 4.3. Normalized tissue expression of CmHCN in *Carcinus maenas* acclimated to brackish-water (11 ppt). mRNA expression was normalized to the ribosomal gene RbS-3. Values are given as mean ± s.e.m. Significant differences are indicated by lower case letters (Kruskal-Wallis test with pairwise Mann-Whitney, $p < 0.05$, $n = 4$-5).

4.4.2. Effects of K^+-channel inhibitors on branchial acid-base regulation, ammonia excretion and the short circuit current

The excretion of ammonia and all measured acid-base equivalents (H^+, CO_2, HCO_3^-) were significantly affected by both, Ba^{2+} and the HCN-specific ZD7288 in posterior gill 7 of osmoregulating green crabs when applied basolaterally (figure 4.4). The degree of inhibition, however, varied with both inhibitors. While branchial ammonia excretion decreased to a greater extent with the application of Ba^{2+} (*ca.* 70% *vs.* 40% with ZD7288; figure 4.4.A), the excretion of protons (as equivalent to accumulation of protons in the hemolymph) decreased more drastically under the influence of ZD7288 (1.3-fold; figure 4.4.B). The effect of Ba^{2+} on hemolymph [H^+] was small, yet significant. In regard to CO_2 excretion, the application of both inhibitors resulted in a significant, *ca.* 1.1-fold increase of pCO_2 in the perfusate (equivalent to a 1.1-fold decrease in excretion; figure 4.4.C). The most pronounced difference between Ba^{2+} and ZD7288 was observed in relation to HCO_3^-. While ZD7288 resulted in a small, but significant increase of [HCO_3^-] (5%) in the perfusate, Ba^{2+} resulted in the opposite response, namely a 5% increase in perfusate [HCO_3^-] (reduced excretion, figure 4.4.D).

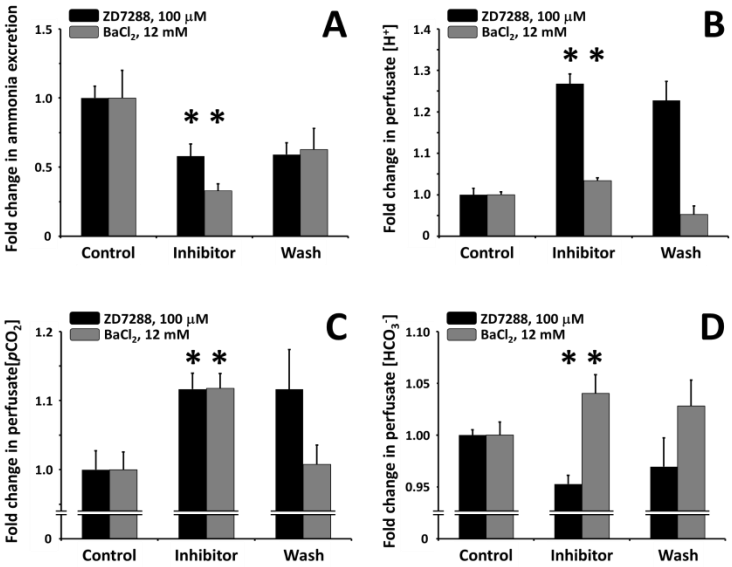

Figure 4.4. Changes in ammonia and acid-base excretory patterns by isolated perfused posterior gill 7 of brackish-water (14 ppt) acclimated *Carcinus maenas* following application of inhibitors for potassium channels. During individual experimental runs, either inhibitor was applied: HCN-specific inhibitor ZD7288 (black bars; 10 µmol L^{-1}), or the more universal inhibitor $BaCl_2$ (grey bars; 12 mmol L^{-1}). (A) Ammonia excretion rates, (B) perfusate $[H^+]$, (C) perfusate pCO_2 and (D) perfusate $[HCO_3^-]$ during control perfusion, subsequent application of the respective basolateral inhibitor and the following wash step. Values are given as mean ± s.e.m. Asterisks denote a significant difference between the control and the inhibitor step (paired t-test with $p < 0.05$, $n = 6$).

When applied basolaterally on split gill lamellae (gill 7) in Ussing chamber experiments, both inhibitors reduced the initial short-circuit current (inside negative, -280 ± 18µA cm^{-2}; n=19) in a dose dependent manner, fitted by the Hill equation as calculated by GraphPad Prism (figure 4.5). Even though a desired plateauing of the dose-response curve was not achieved, application of basolateral $BaCl_2$ was stopped at 20 mmol L^{-1} due to potential secondary effect on ion fluxes in response of the altered osmolarity of the basolateral saline. The inhibitor constant IC_{50}(BaCl2) was accordingly determined to be 2.6 mmol L^{-1}, as well as ΔI_{max} = -57.1% ≈ 194.0 µA cm^{-2} at 20 mmol L^{-1}(figure 4.5.A). At higher concentrations, I_{SC} became positive due to the

passive ion fluxes and was disregarded for the analysis. ZD7288 was clearly the more potent inhibitor and reached its maximum effect at *ca.* 100 nmol L^{-1} with ΔI_{max} = -34.33% ≈ 116.8 μA cm^{-2} and $IC_{50(ZD7288)}$ = 2.4 x 10^{-7} mmol L^{-1}(figure 4.5.B).

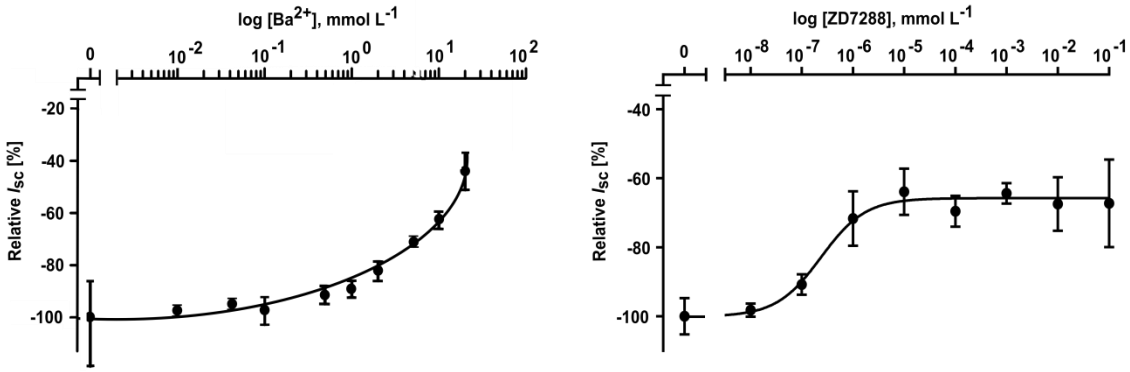

Figure 4.5. Dose-response curves of the potassium channel inhibitors $BaCl_2$ and ZD7288 in respect to the short-circuit current I_{SC} in Ussing-chamber experiments on split gill lamellae of *Carcinus maenas*. The graph shows percentage change in I_{SC} with respective inhibitor concentrations relative to the baseline I_{SC} (no inhibitor) set to 100% to eliminate within-sample variation. All experiments were performed on split gill lamellae of posterior gill 7 of brackish-water acclimated green crabs (11 ppt) under symmetrical conditions. IC_{50} for $BaCl_2$ = 2.6 mmol L^{-1} (n = 3 from 1 crab); IC_{50} for ZD7288 = 2.4 x 10^{-7} mmol L^{-1} (n = 3-7 for ZD7288, 3 crabs used).

4.4.3. CmHCN in response to environmental stressors

mRNA levels of CmHCN in posterior gill 7 were significantly down-regulated in green crabs exposed to either 7 days of high environmental pCO_2 (hypercapnia, 400 Pa; *ca.* 50% reduction) or 7 days of elevated environmental ammonia (1 mmol L^{-1} NH_4Cl HEA, *ca.* 80% reduction; figure 4.6).

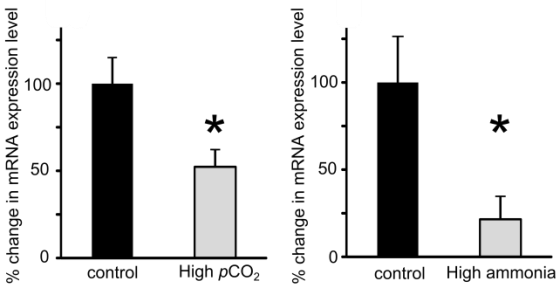

Figure 4.6. Gene expression changes of CmHCN in posterior gill 7 of brackish-water acclimated *Carcinus maenas* (11 ppt) exposed to chronic (7 day) environmental stressors. mRNA expression for high pCO_2 acclimated animals was normalized to the ribosomal gene RbS-3 prior to calculation of relative gene expression changes. For the gills in HEA acclimated animals, gene expression could not be normalized to a housekeeping gene but was evaluated applying a standard curve and related to the starting quantity. Values are given as mean ± s.e.m. Asterisks denote significant differences compared to control gene expression (Student's t-test, $p < 0.05$, $n = 4$).

4.5. Discussion

4.5.1. Potential K^+-permeable structures in the gill epithelium of *C. maenas*

In studies to date, the presence of K^+-channels and/or general K^+-permeable structures in the gill epithelium of decapod crustaceans, has only been verified by ion flux and short-circuit current studies on split gill lamellae following application of the inhibitors Ba^{2+} and Cs^+ (Onken et al., 1991, 2003; Riestenpatt et al., 1996). While Ba^{2+} is believed to specifically block K^+-channels of the K_{IR} family (inward rectifying) in mammals when applied in micromolar concentrations (Franchini et al., 2004), high concentrations seem also able to target voltage-gated K^+-channels of the K_V family (Hibino et al. 2010). Due to its similar radius in comparison to K^+, Ba^{2+} is able to enter the channel pore and bind to the selection filter motif (SF4)-domain of the respective K^+-channels, therefore competing with K^+ and blocking its binding site more potently because of its dual positive charge (Rossi et al., 2013). As figure 4.1 shows, representatives for all K_{IR}, K_V

and HCN channels can be found throughout the animal kingdom, including green crabs. To my best knowledge, however, the specific nature of K^+-promoting membrane channels in the crustacean gill has not been investigated to date. The potential HCN of *C. maenas* (CmHCN), as described in the present study, therefore provides the first (partial) sequence-based characterization of a K^+/NH_4^+ channel in a decapod crustacean and documents its involvement in branchial acid-base and ammonia regulation.

Importantly, figure 4.2 reveals that CmHCN exhibits the respective residues (Ala-425 and Leu-432) for ZD7288 binding (Cheng et al., 2007). Even though the latter residue differs from the mammalian Ile-432, Cheng et al. (2007) showed that substitution of Ile with Leu did not shift the IC_{50} significantly.

4.5.2. Molecular characterization of CmHCN

As seen by the gene tree analysis the high level of conservation of the HCN sequence between arthropods and vertebrates in general (indicated by the short branch lengths; figure 4.1), and *C. maenas* and *H. sapiens* specifically (high level of amino acid alignment; figure 4.2), is impressive. A similar level of conservation between invertebrate and vertebrate proteins is found in other important membrane transporters like the α-subunit of the Na^+/K^+-ATPase, one of the most important epithelial proteins (*C. maenas vs. H. sapiens*, 79% identity and 89% overall conserved domains). Consequently, HCN can be hypothesized to maintain a significant role as well as a similar function in the course of evolution regarding epithelial ion transport among various phyla.

In collecting ducts of rat renal cortex and medulla - structures that are responsible for both, ammonia and acid-base regulation in mammals (Weiner and Verlander, 2013) -

HCN2 has recently been observed to transport both K^+ and NH_4^+ (Carrisoza-Gaytán et al., 2011). As the major ion, ammonia and acid-base regulatory organ (Larsen et al., 2014), the crustacean gill resembles many of the transport processes that are observed in the mammalian kidney (Riestenpatt et al. 1996; chapter 3). It is therefore not surprising, that relatively high mRNA expression levels of HCN can be found in the osmoregulatory posterior gills of *C. maenas* in addition to its high expression in the cerebral ganglion and therefore likely involvement in nerve excitation. Interestingly, the difference of CmHCN mRNA levels between anterior and posterior gills mirror a trend equivalent to what is seen for Na^+/K^+-ATPase (NKA) activity in these tissues (*ca.* 2 *vs.* 11 μmol P_i mg $protein^{-1}$ h^{-1}), emphasizing the postulated close relationship between basolateral NKA and K^+-channels in mammalian (Hibino et al., 2010) as well as invertebrate epithelia (Riestenpatt et al., 1996).

Furthermore, the observed down-regulation of the mammalian renal HCN2 mRNA transcript in response to chronic metabolic acidosis in rats (Carrisoza-Gaytán et al., 2011), was also observed in the present study in the gills of *C. maenas* in response acclimation to high environmental ammonia (HEA). Similar to rats experiencing an ammonia load, green crabs also exhibit a metabolic acidosis in response to HEA (data not shown). In addition, a role for CmHCN in general acid-base regulation is suggested by its significant down-regulation in response to a respiratory acidosis (chapter 2) caused by exposure to hypercapnia, as also observed in the present study. Even though HCN homologs have been cloned from several invertebrate species and their hyperpolarization-activated current characteristics have been observed to closely resemble mammalian HCNs (Biel et al., 2009), their potential function has so far only been associated with

chemo-sensitivity in the fruit fly *Drosophila melanogaster* (Chen and Wang, 2012) and signal transduction in olfactory neurons of the spiny lobster *Panulirus argus* (Gisselmann et al., 2005).

The present study therefore is the first to provide evidence for a function of a conserved (ancestral) HCN as a key-player in acid-base and ammonia regulation in an invertebrate.

4.5.3. Characterization of K^+-transport across the gill epithelium

The results of the gill perfusion experiments as well as Ussing chamber experiments presented evidence for at least two different K^+/NH_4^+-promoting structures in branchial, basolateral epithelial membranes of *C. maenas*. These might include at least one (potentially K_{IR}-like) Ba^{2+}-sensitive K^+-channel, as well as the identified ZD7288-sensitve CmHCN.

More evidence for the potential function of the respective channels in branchial acid-base and ammonia excretion arises from the observed decrease of ammonia, proton and CO_2 excretion following application of both basolaterally applied inhibitors, Ba^{2+} and ZD7288, in gill perfusion experiments (posterior gill 7). Interestingly, HCO_3^- excretion is induced by ZD7288 but decreased with Ba^{2+}, therefore suggesting that the observed differences in excretion rates of ammonia, proton and CO_2, are not solely based on differences in the effective concentration of each inhibitor. Importantly, ZD7288 also significantly reduced ammonia excretion rates, indicating that the CmHCN indeed promotes NH_4^+ transport. These results therefore support my earlier findings that claimed a linkage between acid-base and ammonia regulatory processes in the gill epithelium of this decapod crustacean (chapter 2).

The effect of both inhibitors on overall epithelial ion movements and the resulting trans-epithelial current was further characterized by Ussing chamber experiments on split gill lamellae. While both inhibitors blocked I_{SC} in a dose-dependent manner, it seemed that the observed inhibitor constant (IC_{50}) for ZD7288 differed substantially from values observed for diverse tissues from other animals. While in the present study IC_{50} for ZD7288 was observed to be extremely low (2.4×10^{-7} mmol L^{-1}), the next smallest observed IC_{50} value was that of 3×10^{-4} mmol L^{-1} in sinoatrial node cells isolated from the guinea pig heart (BoSmith et al., 1993), but IC_{50} values of up to 41×10^{-3} mmol L^{-1} were observed in mammalian HCN1-4 when expressed in HEK293 cells or *Xenopus laevis* oocytes (Shin et al., 2001).

Regarding $BaCl_2$, however, the observed IC_{50} closely resembled the values of a previous study on the green crabs' posterior gill epithelium by Riestenpatt (1995). The IC_{50} of the present study of basolaterally $BaCl_2$ accounts for 2.6 mmol L^{-1}, a value that is well comparable to the one observed by Riestenpatt (1995) for apically applied $BaCl_2$ (3.2 mmol L^{-1}) and very similar to the IC_{50} of $BaCl_2$ as observed in the squid giant axon (2.8 - 13 mmol L^{-1} depending on external Ba^{2+} concentration; Armstrong and Taylor 1980). In vertebrates, however, IC_{50} values for a $BaCl_2$-related blockage of KIR2.x-channels in zebrafish ventricular myocytes tended to be significantly lower (1.8-132 μmol L^{-1}; Hassinen et al. 2015) than the observed values for the green crab. The observation that the IC_{50} of ZD7288 is so much lower as in vertebrates and the converse being true for $BaCl_2$ suggests that even though the sequences seem to be fairly well conserved, epigenetic factors may lead to different biomolecular functions. The level of

methyl-induced polarization by substitution of threonine to serine in the S4 binding site for K^+/Ba^{2+} for example, resulted in reduced Ba^{2+}-binding capacities (Rossi et al., 2013). Both inhibitors in this study were applied basolaterally based on: (1) the observation that apical CsCl (10 mmol L^{-1}), even though it significantly reduced I_{SC} (Riestenpatt et al., 1996), had been shown to have no substantial effect on ammonia excretion in *C. maenas* (Weihrauch et al., 1998) and (2) CmHCN appears to be an ortholog to the HCN2 channel identified in basolateral membranes of the vertebrate kidney (Carrisoza-Gaytán et al., 2011). However, it cannot be excluded that ZD7288 targeted the respective structure in the apical membrane due to its strong lipophilic character and subsequent diffusion into the cell (Gasparini and DiFrancesco, 1997; Harris and Constanti, 1995). A predominantly intracellular presence and action of ZD7288 would also explain why the effect of this inhibitor as observed in gill perfusion experiments was not reversed in the wash step in the gill perfusion experiment.

The observed effects of both inhibitors in gill perfusion as well as Ussing chamber experiments strengthen the results of earlier studies on split gill lamellae of *C. maenas* (Riestenpatt, 1995) and the Chinese mitten crab, *Eriocheir sinensis* (Onken et al., 1991), that indicated (at least) two different populations of K^+-channels to promote transcellular K^+ (and NH_4^+) excretion based on the observed incomplete and complex di-phasic response of the I_{SC} when Ba^{2+} was applied to the apical and basolateral membrane.

4.6. Conclusions

In conclusion, the present study strongly indicates the involvement of a basolateral HCN-like potassium channel in branchial acid-base and ammonia regulation in the decapod

crustacean *C. maenas*. Besides the observed Ba^{2+}-sensitive K^+-channel(s), this novel candidate gene promotes NH_4^+ transport across the gill epithelium as shown by my pharmacological experiments on isolated gills applying the HCN-specific inhibitor ZD7288. Interestingly, similar features (i.e. the potential dual transport capabilities of K^+ and NH_4^+ and mRNA expression changes in response to metabolic acidosis) have been observed for the HCN2 channel expressed in the mammalian kidney, indicating a conserved function of this gene for basic homeostatic regulation throughout the animal kingdom. The high level of conservation of the amino acid sequence between invertebrates and vertebrate HCNs highlights the potential importance of this gene as a key-player in acid-base and ammonia regulation in vertebrates as well as invertebrates.

CHAPTER 5:

FINAL DISCUSSION AND CONCLUSIONS

Setting out on this four year PhD journey, I was eager to revolutionize the world of crustacean acid-base physiology. Soon enough, I came to realize that this time frame would not even allow for a complete investigation of the mechanisms involved for only one of these amazing creatures, the green crab *Carcinus maenas*. Which aspect of acid-base regulation I ever focussed on demonstrated how complex *C. maenas'* acid-base regulatory machinery is. This includes the individual participation of each single gill in acid-base regulation and its link to ammonia excretion as in chapter 2, the involved branchial epithelial transporter inventory as described in chapter 3, and the highly conserved and specific candidate gene for acid-base and ammonia regulation, CmHCN, as characterized in chapter 4. It is therefore not surprising that *C. maenas* is so effective in adapting to a changing environment and why it is one of the most successful invasive species in recent times (Lowe et al., 2000).

Yet, the present thesis contributes substantially to our knowledge of acid-base regulation in moderate hyper-osmoregulating decapod crustaceans and provides avenues for future research on this topic also in other marine invertebrates. As hypothesized, acid-base homeostasis and regulation in green crabs is a complex interaction of gill epithelial transporters that at the same time are involved in osmoregulation and ammonia excretion (Larsen et al., 2014). With the help of the resulting adjustments in branchial excretion and uptake of acid-base equivalents, *C. maenas* is capable of effectively counteracting extracellular acid-base disturbances, for example by accumulation of hemolymph HCO_3^-

in order to buffer potential drops of pH resulting from increasing hemolymph pCO_2 levels (chapter 2 and 3).

5.1. The role of the gill in branchial acid-base and ammonia regulation

Even though the gill has long been accepted as the major regulatory organ in decapod crustaceans (Henry et al., 2012; Larsen et al., 2014), hardly any investigations have focussed directly on its role in acid-base regulation. While a branchial, intracellular regulation was postulated by Henry and Cameron (1982) and Truchot (1981, 1992) to play a role in the crustaceans' acclimation to dilute salinity, these and other earlier studies mainly focussed on the extracellular characteristics of acid-base disturbances rather than explaining the regulatory mechanisms behind these adjustments. In contrast, the present thesis aimed to elucidate these acid-base regulatory mechanisms at the level of the gill and gill epithelium, and therefore contributes to our knowledge of the branchial contribution to the maintenance of extracellular acid-base homeostasis.

One of the most important findings of the present thesis regarding the gill was the direct correlation of each individual gill's capability for H^+ excretion with its capacity for ammonia excretion (chapter 2). Interestingly, H^+/NH_4^+ excretion seems to be more efficient in anterior gills, the gill cluster that previously has been associated mainly with respiratory and excretory processes (Compere et al., 1989; Freire et al., 2008; Henry et al., 2012; Pequeux, 1995; Weihrauch et al., 1998). The fact that in isolated gill perfusion experiments, the perfusate pH did not change drastically when gills were subjected to different pH in the bathing solution demonstrates the important role of the gill to "buffer" and "translate" changes of the environment into the crabs' extra- and intracellular

compartments so that these spaces are kept relatively stable. Gills of green crabs that were acclimated to hypercapnia prior to gill perfusion were slightly more efficient in keeping the extracellular perfusate pH stable than control animals. Interestingly, anterior gills of osmoregulating green crabs (11 ppt) generally excreted more H^+ and CO_2 but less ammonia than anterior gills of osmoconforming green crabs (chapter 3), without undergoing major structural changes (Compere et al., 1989). This suggests three implications: First, the regulation of acid-base equivalents plays a direct role in osmoregulation; second, even if not to the same extent as posterior gills, anterior gills do contribute to osmoregulation *via* regulation of acid-base equivalents; and third, the adjustment of acid-base regulatory mechanisms in anterior gills are likely based on changes in mRNA levels of the present transporters (or their activity), instead of the whole epithelium being restructured as observed for posterior gills of osmoregulating green crabs (Compere et al. 1989) and *Neohelice (Chasmagnathus) granulata* (Luquet et al. 2002).

5.2. Membrane transporters involved in acid-base regulation in *C. maenas*

It seems that at the level of membrane transporters (mRNA expression), only subtle changes and adjustments are necessary to equip the green crabs' gill epithelium for an environmental pH/$p$$CO_2$ challenge, as indicated in the recent microarray study by Fehsenfeld et al. (2011) and in this thesis.

I identified a number of gill epithelial transporters that had only been associated with osmoregulation or ammonia excretion in the past, to also be involved in branchial acid-base regulation. The analysis of changes in mRNA expression levels of the respective candidate transporters in response to hypercapnia-acclimation of osmoregulating *C. maenas* identified the Rhesus-like protein, Na^+/K^+-ATPase (NKA) and membrane-bound carbonic anhydrase (CA) to play a significant role in this process. Interestingly, these changes are again mainly associated with the anterior gills, strengthening their apparent role in acid-base regulation.

The involvement of these proteins was also confirmed by the perfusion experiments on isolated gills applying inhibitors as performed in chapter 3. Additionally, results from these isolated gill perfusion experiments verified the involvement of the (cytoplasmic) V-(H^+)-ATPase and basolateral K^+-channels in branchial acid-base regulation. As these membrane proteins have been associated particularly with ammonia excretion in this species (Weihrauch et al., 1998, 2002), this finding provides additional evidence for ultimate mechanistic linkage of both of these processes. Regarding H^+ and CO_2 excretion across the anterior gill epithelium, a potential basolateral bicarbonate transporter (likely Na^+/HCO_3^--exchanger, NBC) played the most pronounced role in addition to the above-mentioned V-(H^+)-ATPase. NBC and a potential basolateral and possibly intracellular Na^+/H^+-exchanger (NHE) as described in chapter 3 have not been discussed in any regulatory function in *C. maenas'* gills to date even though they seem to be slightly down-regulated in response to dilute salinity (Towle et al., 2011) and involved in acid-base regulation in *N. granulata* (Tresguerres et al., 2008).

As the major result of this first comprehensive study of mechanisms for branchial acid-base regulation (chapter 3), I presented an updated, more detailed model for the regulation of the acid-base equivalents H^+, CO_2 as well as ammonia across the gill epithelium of *C. maenas*. The suggested mechanisms might not only be valid for *C. maenas*, but represent basic principles for general acid-base regulation that could be transferred to other invertebrates and/or acid-base regulatory tissues.

5.2.1 CmHCN - a highly conserved, new candidate membrane transporter in green crab gills involved in acid-base and ammonia regulation

In addition to the investigation of known gill epithelial transporters as performed in chapter 2 and 3, chapter 4 provides first the sequence-based evidence for a K^+-channel of the sodium/potassium hyperpolarization activated cyclic nucleotide-gated channel family and its involvement in acid-base and ammonia regulation in *C. maenas*.

The high level of conservation of this novel candidate gene CmHCN (up to 89%) between invertebrates and vertebrates (and specifically between *C. maenas* and *Homo sapiens*) indicates that this protein might be important for trans-epithelial ion movements throughout the animal kingdom.

Quantitative real-time PCR as performed in chapter 4, demonstrated the significant down-regulation of this transcript in response to environmental challenges like elevated pCO_2 and NH_4^+ levels, indicating that it might be a key-transporter in both branchial acid-base and ammonia regulation. Interestingly, together with the observed capability of CmHCN for NH_4^+ transport, the results closely resemble those observed for rat HCN2 in the mammalian kidney (Carrisoza-Gaytán et al., 2011) and indicate a conserved function for this protein in whole body acid-base and ammonia regulation.

5.3. The link between branchial acid-base regulation and ammonia excretion

In the mammalian kidney, acid-base regulation has been shown to be closely correlated to ammonia regulation (Weiner and Verlander, 2013). Renal ammoniagenesis taking place in the proximal tubule of the kidney and likely most other renal epithelial cells served as the major source for both HCO_3^- and NH_4^+, therefore playing an important role in acid-base as well as ammonia homeostasis (Weiner and Verlander, 2013). Interestingly, the transporter inventory in the gill epithelium of *C. maenas* (basolateral Na^+/K^+-ATPase, Cl^-- and K^+-channels, as well as apical $Na^+/K^+/2Cl^-$-cotransporters and K^+-channels) resembles much of what is also observed in the thick ascending limb of the Henle's loop in the mammalian kidney (Lucu and Siebers, 1987; Riestenpatt et al., 1996; Weiner and Verlander, 2013). As I point out in chapter 3, the green crabs' gill epithelium might resemble a combination of mechanisms also observed in other parts of the mammalian kidney, such as the collecting duct. It is therefore only logical to assume that also in the crustacean gill epithelium, regulation of ammonia and acid-base equivalents are closely linked, as they are in the mammalian kidney. While no conclusive studies have been performed on this subject, in the present thesis, I provide strong evidence for the close correlation of acid-base and ammonia regulation in a decapod crustacean from the whole animal level down to the single gill and gill epithelium, and the transporters within.

First of all, osmoregulating (chapter 2) as well as osmoconforming (chapter 3) green crabs exposed to hypercapnia significantly increased hemolymph ammonia levels and ammonia excretion, indicating that: (1) NH_3/NH_4^+ speciation of ammonia might contribute to the buffering capacity of the hemolymph, and (2) NH_4^+ excretion might be a

major contributor to H^+/acid secretion in order to restore extracellular acid-base homeostasis. Furthermore, on the level of the single gill, the pattern of individual gills in respect to H^+ excretion was closely mirrored by the excretion pattern for ammonia, indicating that NH_4^+ contributes significantly to the overall acid excretion of this organ. Predominantly being associated with respiratory and excretory functions in contrast to the osmoregulating posterior gills (Compere et al., 1989; Freire et al., 2008), anterior gills (especially gill 4) seemed to contribute to a greater extent to the overall H^+/NH_4^+ excretion than the posterior gills. Consequently, most of the gill epithelial transporters in anterior gill 5 identified to either directly or indirectly promote the excretion of acid-base equivalents, were also found to be involved in ammonia excretion across the gill epithelium of *C. maenas* as described in chapter 3.

5.4. Conclusions and future directions

In conclusion, the present thesis is the first comprehensive study on the mechanisms of acid-base regulation in *C. maenas*. The investigated basic principles for general acid-base regulation of the crustacean gill epithelium might well be transferable and help our understanding of acid-base and ammonia regulation in other marine (invertebrate) species and/or tissues.

Four years dedicated to this research and studying these processes in *C. maenas* made me realize just how complex gill epithelial ion transport is. The highly sophisticated mechanisms that this small organ accomplishes are fascinating. Even after four years of research, I have only touched the surface of acid-base regulation in crustaceans. Consequently, I am highly interested in further investigations of acid-base regulation at

the level of the gill epithelium, including hormonal control and cell signalling pathways. Specifically, I would like to better characterize the HCN channel and verify its role in acid-base and ammonia regulation in a vertebrate physiological context, i.e. in fish that possess both a kidney and gills. Furthermore, resulting from my microarray study (Fehsenfeld et al., 2011), several transcripts observed to be differentially expressed in response to hypercapnia in *C. maenas* might prove to be interesting for future characterization. Foremost, this includes the calcium-activated chloride channel as a direct novel ion-transporter that has not been investigated to date. Additionally, several differentially expressed genes (a multispanning endomembrane protein of the transmembrane 9 superfamily, a tetraspanin8-like protein and a putative Integrin-alpha-7) indicate that in response to hypercapnia, a restructuring of the posterior gills might take place similar to what is observed in response to dilute salinity acclimation (Compere et al., 1989). Hence, it would be interesting to apply microscopic techniques to look into a potential change of gill morphology in response to hypercapnia. Finally, the most up-regulated transcript in both the hypercapnia study (Fehsenfeld et al., 2011) as well as the dilute salinity study by Towle et al. (2011) was identified as a potential Syntaxin-binding protein and might be involved in vesicular trafficking to the apical membrane. This might therefore be a very interesting candidate gene to follow up on due to its potential involvement in the hypothesized acidified vesicle-dependent excretory pathway for ammonia and consequently also for acid-base equivalents.

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