Acid-Base Regulation and Ammonia Excretion in the American Horseshoe Crab, *Limulus polyphemus*

by

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Abstract

Acid-base regulation is vital for animals and while the inorganic carbon system largely determines body fluid pH, another potentially valuable acid-base pair is ammonia (NH_4^+/NH_3). This study focuses on the American horseshoe crab (*Limulus polyphemus*), a phylogenetically ancient marine chelicerate with no published studies on its acid-base physiology. Physiological and molecular analyses indicate that Na^+/K^+-ATPase, Rhesus-protein (Rh), and carbonic anhydrase (CA) are involved in acid-base homeostasis and/or ammonia regulation. This likely occurs in the book gills, which consist of ultrastructurally distinct regions. The ventral half-lamella is ion-leaky and displayed high Rh-protein, cytoplasmic CA, and hyperpolarization-activated cyclic nucleotide-gated K^+ channel mRNA expression levels, suggesting a specialization in facilitated CO_2 and/or ammonia diffusion compared to the dorsal half-lamella. During hypercapnia acclimation, hemolymph acid-base status exhibited a compensated respiratory acidosis accompanied with signs of metabolic depression. Ammonia influx associated with high environmental ammonia acclimation was successfully counteracted, but induced modifications in acid-base homeostasis.

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A condensed form of this thesis is currently in the process of submission to the Journal of Experimental Biology. Dr. Horst Onken obtained most of the results for transepithelial conductance and potential difference of the horseshoe crab split gill lamellae. Dr. Jason Treberg performed most of the preparation for Na^+/K^+-ATPase enzyme and protein assays and also obtained some of the data, while Dr. Barbara Battelle collected brain tissue samples from adult animals at the Whitney Laboratory for Marine Bioscience in Florida. The remainder of the data were collected, analyzed, and presented in the final format by Stephanie Hans.

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List of Abbreviations

αCO_2	Solubility coefficient of CO_2
ANOVA	Analysis of Variance
CA-1	Membrane-bound carbonic anhydrase
CA-2	Cytoplasmic carbonic anhydrase
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CO_3^{2-}	Carbonate
C_T	Total dissolved inorganic carbon
DMSO	Dimethyl sulfoxide
fg	Femtogram
H_2CO_3	Carbonic acid
HAT	Vacuolar-type H^+-ATPase
HCN	Hyperpolarization-activated cyclic nucleotide-gated K^+ channel
HCO_3^-	Bicarbonate
HEA	High environmental ammonia
NBS	National Bureau of Standards pH scale
ng	Nanogram
NHE	Na^+/H^+-exchanger
NKA	Na^+/K^+-ATPase
Pa	Pascal
P_{CO2}	Partial pressure of CO_2
pH_i	Intracellular pH
p*K*	Dissociation constant
qPCR	Quantitative polymerase chain reaction
Rh	Rhesus protein
RNA	Ribonucleic acid
s.e.m.	Standard error of the mean

1. Introduction

1.1. Importance of pH regulation

All animals must maintain a stable intra- and extracellular fluid pH in order to continue normal physiological functions. At a typical intracellular pH (pH_i) near neutrality, the concentration of H^+ itself is not substantial compared to other ions such as Na^+, yet H^+ abundance determines the ionization state of charged molecules involved in a large variety of processes (Davis, 1958). For example, Davis (1958) found that at such pH, many non-excreted biosynthetic intermediates of metabolic pathways (e.g. glucose-6-phosphate and glyceraldehyde-3-phosphate in glycolysis) have at least one group that is ionized, which was most commonly either a phosphate or carboxyl group. Due to the significance of these intermediate molecules, the hypothesis put forth by the study suggests that one importance of maintaining a near neutral intracellular pH is to help trap valuable charged compounds within the cell's hydrophobic membrane.

As with intermediates in metabolic pathways, proteins are vital to cellular function and can also be highly sensitive to pH disturbances. The shape and stability of proteins determine their role and activity, and body fluid pH can critically affect the catalytic properties of these macromolecules (Somero, 1986). Two main theories regarding the ideal pH_i include the alphastat and pH-stat hypotheses. In order to understand the key difference between these theories, it must be kept in mind that the pH of water inversely varies with temperature by ~0.016 pH unit per °C; for example, a neutral pH of water at 25°C is 7.0, but at 37°C, it becomes 6.8 (Nattie, 1990). This inverse relationship applies to body fluid pH as well (Nattie, 1990; Reeves, 1972; Somero, 1986). According to the pH-stat hypothesis, cells maintain a constant pH_i

regardless of temperature (Somero, 1986), but this has implications on protein function given that the same pH_i over a range of body temperatures has varying concentrations of H^+, thus affecting the ionization state of proteins. Endothermic and ectothermic animals inhabiting a wide range of temperatures also appear to possess similar protein makeup but with variable pH_i despite the delicate pH-sensitivity of protein function (Nattie, 1990). The alphastat hypothesis helps to explain this phenomenon, where pH_i is suggested to be conserved near the temperature-dependent p*K* value of the important imidazole group of the protein's histidine residue to stabilize its fractional dissociation state (Reeves, 1972; Somero, 1986). The significance of this phenomenon is to set an optimal condition for numerous vital proteins in possession of histidyl residues, such as phosphofructokinase and lactate dehydrogenase, which are vital to glycolysis, as well as calmodulin, which is important for cyclic nucleotide metabolism, glycogen synthesis, and Ca^{2+} transport (Means et al., 1982; Somero, 1986; Wilson, 1977). Nevertheless, both theories stress the importance of tightly controlling pH_i as to continue proper cellular function.

A potential consequence of pH shifts in the extracellular fluid is a change in gas transport capacities. According to the Bohr effect, respiratory proteins such as hemoglobin and hemocyanin have evolved to possess gas-binding sites that are largely affected by pH in order to efficiently deliver much-needed O_2 to tissues (Riggs, 1988). If the animal's body fluid pH experiences a shift in either direction, not only will normal intracellular protein activity be impaired, but also crucial gas transport in circulating body fluids as well. Therefore, it is of upmost importance to establish body fluid pH within a narrow range through the regulation of acids and bases.

1.2. Carbon dioxide and ammonia as acid-base equivalents

1.2.1. Carbon dioxide

The inorganic carbon system is the main buffering system that is widely responsible for determining the pH of body fluids as well as seawater (Fabry et al., 2008). The inorganic carbon species include CO_2, carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}). The relationship between these inorganic carbon species is depicted as follows:

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+ \quad \text{(Eqn 1)}$$
$$\downarrow\uparrow$$
$$CO_3^{2-} + H^+$$

H_2CO_3 is a polyprotic acid and is therefore capable of donating more than one H^+ when fully dissociated. At full-strength seawater, the weak acid has a pK_1 of ~5.9 and pK_2 of ~9.0 (Dickson and Millero, 1987), and in such an environment, the estimated breakdown of inorganic carbon species is as follows: ~0.5% CO_2, ~88% HCO_3^-, and ~11% CO_3^{2-} (Fabry et al., 2008). Under a typical body fluid pH of approximately 7.2 to 7.8, the addition of CO_2, whether metabolic or environmental in origin, leads to the formation of H_2CO_3. This form of inorganic carbon quickly dissociates and results in excess H^+ within the solution; therefore, animals must efficiently control their CO_2 excretion to avoid shifts in body fluid pH.

1.2.2. Ammonia

In addition to metabolic CO_2, all animals also produce nitrogenous waste products from cellular amino acid metabolism and diverse modes of excretion can be found throughout the animal kingdom. The majority of nitrogenous waste products are either in

the form of ammonia, urea, or uric acid, and animals that predominantly excrete one particular molecule are respectively referred to as ammonotelic, ureotelic, or uricotelic (Larsen et al., 2014; Wright, 1995). Amongst the three forms, ammonia is the most toxic, yet also the least energetically costly to produce and is highly soluble in water; therefore, ammonotelism is often observed in aquatic animals due to the abundant availability of water for efficient dilution of body fluid ammonia (Larsen et al., 2014; Wright, 1995).

The term "ammonia" will henceforth refer to total ammonia, which includes both the gaseous/weak base (NH_3) and ionic/weak acid (NH_4^+) forms. The proportions of NH_3 and NH_4^+ within a given solution are determined by pH due to ammonia's pK_a of 9.2-9.8, depending on ionic concentration, temperature, and pressure (Cameron and Heisler, 1983; Eddy, 2005). Therefore, under a typical physiological pH, most of the ammonia within body fluids exists as NH_4^+ (Larsen et al., 2014). The hydrated ionic form of ammonia shares a similarity in ionic radius as K^+ and can therefore substitute K^+ as substrate in K^+-utilizing transporters (Choe et al., 2000; Skou, 1960). On the other hand, gaseous NH_3 is able to diffuse across cellular membranes and subsequently bind with H^+ to form NH_4^+, therefore potentially affecting intra- and extracellular pH (O'Donnell, 1997). A less-studied potential avenue of acid-base regulation is the use of ammonia as an acid (NH_4^+) and/or base (NH_3) equivalent. In invertebrates, perfused gills of the common octopus (*Octopus vulgaris*) maintain a basolateral (hemolymph) ammonia concentration of 250-300 µmol l^{-1}, suggesting that this compound is not only a waste product but also has a function in other processes, perhaps acid-base balance (D. Weihrauch, pers. communication). In another invertebrate example, a possible link was shown in the gills of green crabs (*Carcinus maenas*), where similar patterns of ammonia and H^+ excretory

capacities by 6 different investigated pairs of gills further indicated a relationship between the two processes (Fehsenfeld and Weihrauch, 2013).

1.3. Mechanisms of acid-base regulation

1.3.1. General mechanisms

Animals commonly encounter acidified conditions as a result of their own metabolic pathways, which produce acidic wastes such as CO_2 and ammonia. With respect to CO_2, terrestrial animals typically elevate their ventilation rate in order to expel CO_2 from their blood/hemolymph, but water-breathing animals face an additional challenge in that water holds less O_2 and is denser than air, therefore costing more expended energy to ventilate (Rahn, 1966; Truchot, 1988). Water-breathing animals also tend to have a lower outwardly directed diffusion gradient of CO_2 compared to terrestrial animals (Melzner et al., 2009; Rahn, 1966), further requiring other means of excreting CO_2 without relying solely on enhanced ventilation rate.

The first immediate response to alleviate the impact of any H^+ increase is the binding of H^+ by non-bicarbonate buffers such as proteins with histidine residues (e.g. respiratory proteins in extracellular fluids), which have functional groups that can be protonated (Melzner et al., 2009). However, this process is limited to the amount of proteins present for such function and may not be adequate depending on the scale of acidosis that the animal is experiencing. In addition, such resolve is only temporary and ultimately requires the actual removal of H^+ and/or acid equivalents from the body fluids in order to restore pH to normal levels. Certain calcifying animals (e.g. crabs) are also capable of dissolving the calcium carbonate stores within their shell/carapace in order to

increase circulating HCO_3^- levels as a means of elevating the body fluid alkalinity (deFur et al., 1980; Fabry et al., 2008; Hans et al., 2014); however, not all animals are equipped with such readily available internal stores. Acid-base balance can also be regulated *via* changes in metabolism; for example, gluconeogenesis and glycolysis are opposing pathways that could potentially affect pH_i depending on which process predominates at the time (Pörtner, 1989; Walsh and Milligan, 1989). Lastly, acid-base homeostasis can be reliably maintained by directly manipulating the acid-base ratio of the body fluid, which can be accomplished through ion regulation (Henry and Wheatly, 1992).

Many transporters are thought to partake in acid-base regulation through ion exchange, although the exact mechanism for seawater animals is not yet fully defined. One of the commonly studied pumps in ion regulation is the Na^+/K^+-ATPase (NKA), which actively creates an inwardly directed Na^+ gradient into the cytoplasm and has been shown to be involved in pH regulation in marine and brackish water animals (e.g. crustaceans; Fehsenfeld and Weihrauch, 2013; Fehsenfeld and Weihrauch, 2016a; Siebers et al., 1994). A proposed pathway for the pump's involvement in pH regulation in transport epithelia is the utilization of the Na^+ gradient in driving the movement of ions through secondarily active transporters such as Na^+/H^+-exchanger (NHE), which can ultimately excrete H^+ to the environment (Choe et al., 2002). Another pump of interest is the vacuolar-type H^+-ATPase (HAT), which uses ATP to transport H^+ across the apical membrane of freshwater organisms and is therefore directly capable of manipulating pH (Onken and Putzenlechner, 1995). HAT can also be localized in vesicles, where acidified vesicles may then be transported *via* the microtubule network and their content released through exocytosis to rid the cell of H^+ and/or acid equivalents (Weihrauch et al., 2001;

Weihrauch et al., 2002). Inhibitor studies targeting HAT in seawater-acclimated *C. maenas* showed a reduction in both H^+ and CO_2 excretion when KM91104, a specific inhibitor of HAT, was applied to isolated perfused gills and HAT was found to be cytoplasmic through immunocytochemical analysis (Fehsenfeld and Weihrauch, 2016a; Weihrauch et al., 2001). In addition, NKA and HAT have been shown to be indirectly activated by cAMP, suggesting that intracellular cAMP levels may also play a role in regulating acid-base balance (Amer and Kreighbaum, 1975; Butcher and Sutherland, 1962; Chibalin et al., 1992; Dames et al., 2006).

Passive CO_2 transport may be facilitated by channels such as the glycosylated Rhesus proteins (Rh-proteins; Perry et al., 2010), where the direction of movement is dependent on the partial pressure gradient and the localization of the protein. Multiple isoforms of Rh-protein have been identified across various animal taxa, including invertebrates, fish, and mammals (Adlimoghaddam et al., 2015; Han et al., 2006; Larsen et al., 2015; Nakada et al., 2007; Verlander et al., 2003, Weihrauch et al., 2012). Rh-proteins have been localized on the basolateral membrane, such as the fish Rhbg, and may allow the transport of CO_2 to/from the body fluid into the cell, while apically-localized isoforms, such as fish Rhcg2, may provide a direct exit of CO_2 towards the environment (Nakada et al., 2007; Perry et al., 2010). The reversible hydration of CO_2 that has been transported into the cytoplasm can be catalyzed by carbonic anhydrase (CA) to produce H^+ and HCO_3^- (Siebers et al., 1994). At least two invertebrate CA isoforms, cytoplasmic and membrane-bound, have been identified in various aquatic invertebrates such as brachyuran crabs and crayfish, and the membrane-bound isoform demonstrates a tendency for higher abundance than its cytoplasmic counterpart in osmoregulatory active

crustacean gills (Ali et al., 2015; Henry et al., 2003; Serrano and Henry, 2008; Serrano et al., 2007). While salinity studies on brachyuran crabs showed that the cytoplasmic isoform is greatly upregulated upon exposure to dilute seawater compared to the membrane-bound CA (Serrano et al., 2007; Serrano and Henry, 2008), precise differences in the role of each isoform in acid-base regulation are not yet fully understood but in general, given that H^+ and HCO_3^- affect pH, these products of CO_2 hydration can be utilized in acid-base extrusion/uptake as needed to maintain an optimal pH. For example, H^+ can be sourced to H^+ transporters such as HAT and NHE (Freire et al., 2008; Henry and Cameron, 1983; Henry et al., 2003; Whiteley et al., 2001), while HCO_3^- can be used by HCO_3^- transporters that facilitate the movement of HCO_3^- across membranes either in conjunction with Na^+ (e.g. Na^+/HCO_3^--cotransporter) or in exchange for Cl^- (Cl^-/HCO_3^--exchanger; Henry and Cameron, 1983; Henry et al., 2003). However, due to the nature of Na^+ and Cl^- as osmoregulatory ions, it was unclear whether HCO_3^- transporters still play a large role in acid-base regulation in osmoconforming marine animals. Recently, a basolateral application of an inhibitor targeting HCO_3^- transporters (tenidap) on the perfused gills of osmoconforming *C. maenas* highlighted the importance of HCO_3^- transporters in acid-base regulation (Fehsenfeld and Weihrauch, 2016a), lending support to the involvement of at least one of these transporters in the absence of active Na^+ and/or Cl^- uptake.

A rather novel avenue of mechanistic acid-base regulatory research has ventured into the role of hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN) in this process (Fehsenfeld and Weihrauch, 2016b). Typically, basolateral K^+ channels are vital for the function of the basolateral NKA as an exit route for K^+ ions that are actively

pumped into the cell in order to maintain the electrochemical gradient (Riestenpatt et al., 1996). Fehsenfeld and Weihrauch (2016b) showed that the use of an HCN-specific inhibitor (ZD7288) reduced H^+ and CO_2 excretion while promoting HCO_3^- excretion in *C. maenas*. HCN mRNA expression level was also significantly down-regulated following a weeklong acclimation to hypercapnia (400 Pa), suggesting that this protein is involved in acid-base regulatory processes.

Nevertheless, the predicted role of each transporter is speculative based on past studies on aquatic animals, and the exact mechanism of acid-base homeostasis in marine arthropods is still incomplete.

1.3.2. Ammonia

Previous studies on invertebrate ammonia excretory mechanisms have provided evidence supporting the involvement of the following key proteins: NKA, HAT, and Rh-proteins. As previously stated, NH_4^+ is able to replace K^+ as a substrate for NKA (Adlimoghaddam et al., 2015; Choe et al., 2000; Masui et al., 2002; Quijada-Rodriguez et al., 2015; Skou, 1960) and NKA-specific inhibitor studies employing ouabain induced a substantial reduction in transepithelial ammonia excretion in the skin of a freshwater leech and the gills of decapod crabs (Quijada-Rodriguez et al., 2015; Weihrauch et al., 1998; Weihrauch, 1999). The indirect participation of another pump, HAT, in ammonia excretion has been documented for all invertebrates studied so far, including freshwater planarians (Weihrauch et al., 2012), leeches (Quijada-Rodriguez et al., 2015), nematodes (Adlimoghaddam et al., 2015), insects (Chasiotis et al., 2016; Weihrauch, 2006), and decapod crabs (Weihrauch et al., 1998). In general, this pump creates a transmembrane

partial pressure gradient for NH_3 (P_{NH3}) by increasing the H^+ concentration on one side of a membrane. This allows NH_3 to move down its partial pressure gradient either by diffusion across the cell membrane (Martinelle and Häggström, 1993) or *via* NH_3 channels such as Rh-proteins (Larsen et al., 2014). Rh-proteins are expressed in all animals examined to date and the proteins' ammonia transport capabilities have been now been verified in invertebrates (Adlimoghaddam et al., 2015; Pitts et al., 2014; Quijada-Rodriguez et al., 2015), suggesting an important role of these proteins in ammonia excretory processes.

Lastly, Fehsenfeld and Weihrauch (2016b) not only began to investigate the role of HCN in invertebrate acid-base regulation, but also in ammonia excretion. Generally, K^+ channels can utilize NH_4^+ as substrate with 10-20% conductance for NH_4^+ compared to K^+ (Choe et al., 2000; Larsen et al., 2014). Fehsenfeld and Weihrauch (2016b) showed that the use of an HCN-specific inhibitor (ZD7288) reduced ammonia excretion by 40% in *C. maenas*. HCN mRNA expression level was also significantly down-regulated following a weeklong acclimation to HEA (1 mmol l^{-1} NH_4Cl), suggesting that this protein is involved in the process of ammonia excretion.

1.4. Implications of excess internal carbon dioxide and ammonia

1.4.1. Carbon dioxide

Despite the fact that animals naturally produce metabolic CO_2, any impairment in the excretion of this waste product can lead to negative consequences. Such incidence is termed respiratory acidosis, characterized by a pH decrease and accompanying increase in blood/hemolymph P_{CO2} and HCO_3^- concentration (Melzner et al., 2009). This could be

due to periods of breathing cessation or increased activity, the latter of which can also lead to metabolic acidosis. This type of acidosis is an acidification of the circulating body fluids accompanied with a decrease in HCO_3^- concentration, but occurs in the absence of P_{CO2} alterations (Melzner et al., 2009). The capacity to counter pH imbalance also varies according to the animal's activity level. Aquatic animals with high metabolic rates and/or living in highly variable habitats (e.g. fast-moving fish, intertidal invertebrates) show a tendency for higher tolerance to changes in internal CO_2 load given that they are accustomed to routine CO_2 fluctuations (Castellini and Somero, 1981; Fabry et al., 2008; Melzner et al., 2009; Seibel et al., 1997; Seibel and Walsh, 2003). On the other hand, animals living a less active lifestyle tend to exhibit lower capacities of pH compensation and are therefore assumed to be more vulnerable to the impacts of high internal CO_2 (Fabry et al., 2008; Melzner et al., 2009; Michaelidis et al., 2005).

Acidification of internal body fluids can also negatively impact calcifying animals, particularly those that are sedentary (e.g. corals, bivalves; Cohen and Holcomb, 2009; Melzner et al., 2009; Ries et al., 2009). Typically, these organisms incorporate inorganic carbon in the form of CO_3^{2-} with Ca^{2+} to form their $CaCO_3$ shell/exoskeleton, but as previously mentioned, acidic pH leads to a smaller proportion of CO_3^{2-} in the body fluids. Therefore, the process of calcification is vulnerable to acidification because of inadequate levels of CO_3^2, which can potentially decrease the hard protective layer and affect the animals' survival.

1.4.2. Ammonia

Much like CO_2, metabolically produced ammonia can have negative consequences on various physiological processes when its levels are not controlled within a tolerable range. NH_4^+ has the potential to enter intracellular locations meant for K^+ due to the similarity between the two ions (Choe et al., 2000; Skou, 1960). The gaseous NH_3 can cross cellular membranes and react with H^+, therefore disrupting any H^+-dependent processes, such as oxidative phosphorylation by mitochondria (O'Donnell, 1997). An excess of either form of ammonia can lead to numerous documented negative effects of ammonia toxicity amongst both invertebrates and vertebrates, including acid-base imbalance (Goldsmith and Hilton, 1992; Wilson and Taylor, 1992), interference with ion regulation (Young-Lai et al., 1991), and neurotoxicity (Butterworth, 2002; Marcaida et al., 1992). Aquatic animals are intriguing in that they generally tend to be more tolerant of this toxic waste compound and are capable of withstanding higher ammonia concentrations in the circulating body fluids in comparison to terrestrial mammals (Wright, 1995). However, efficient detoxification *via* immediate excretion of ammonia is still required to avoid reaching lethal concentrations.

1.5. Major extrinsic sources of acid-base disturbance

1.5.1. Naturally occurring causes

Certain animals (e.g. brachyuran crabs) bury in order to forage and/or as protection from potential danger, such as predators or adverse environmental conditions (Bellwood, 2002; Larsen et al., 2014), but this strategy comes at a physiological cost. When under the sediment, burying animals experience impaired water circulation while

still releasing CO_2 in addition to the CO_2 produced by various interstitial organisms (Widdicombe et al., 2011). As a result, the water immediately surrounding their gills or other breathing apparatuses likely has elevated P_{CO2} and creates an unfavourable environment for continued excretion of metabolic CO_2. Studies on the uppermost few cm of marine sandy and muddy substrates showed that such environments across various climates and seawater depths can be as high as P_{CO2} levels of 1600 Pa and pH of 6.5 (Widdicombe et al., 2011). Consequently, environmental P_{CO2} can considerably affect sediment-dwelling animals and must be efficiently counteracted to avoid acidification of the body fluids.

Low water ventilation encountered by burying marine animals can also impair ammonia excretion through reduced/lack of exchange with the environment. While nitrifying bacteria would typically help alleviate ammonia levels in the water through the conversion of ammonia into nitrite and nitrate, deeper sediment levels do not provide the oxic zone required for nitrification (Weihrauch et al., 2004; Widdicombe et al., 2011). Pelagic environments with favourable seawater conditions rarely exceed ammonia levels of 5 μmol l^{-1} (Koroleff, 1983), but studies investigating interstitial ammonia levels in the North Sea showed concentrations ranging between 100 and 400 μmol l^{-1}, and can reach as high as 2800 μmol l^{-1} depending on the sediment/water depth and nutrient influx (Weihrauch et al., 1999). Therefore, benthic and infaunal organisms are particularly at risk of encountering elevated environmental ammonia from their own metabolic processes.

Intertidal zones, such as estuaries, present a unique combination of stresses that have the potential to largely affect the acid-base status of organisms inhabiting those

environments. Being an area influenced by both freshwater and marine systems, exposure to fluctuations in pH, P_{CO2}, salinity, temperature, and nutrients (e.g. ammonia) often occurs throughout the day according to tide conditions, freshwater runoff, and wind patterns (Dames et al., 2000). Organisms living in these areas must thereby possess adaptations to cope with such stresses. In crustaceans, acclimation from freshwater to seawater has been shown to cause metabolic acidosis while the reverse direction of acclimation caused metabolic alkalosis (Henry and Cameron, 1982; Truchot, 1981; Truchot, 1992). These acid-base disturbances are predicted to be related to the interactions of H^+ with the process of adjusting the body fluid strong ion difference, interactions of HCO_3^- in osmoregulation, or a shift in the animal's overall metabolism due to cell volume regulation following environmental salinity stress (Henry and Cameron, 1982; Truchot, 1981; Truchot, 1992). Temperature can also vary in estuaries and, given the inverse relationship of temperature and pH, could lead to shifts in pH (Nattie, 1990); as a result, estuarine temperature fluctuations throughout the day may especially impact the acid-base status of ectothermic animals.

Oscillations in pH also depend on levels of photosynthetic activity, which may be intensified in sheltered coastal areas lacking sufficient water exchange with the open ocean (e.g. estuaries and lagoons; Middelboe and Hansen, 2007). Seawater alkalosis can occur during the day when dense aggregations of primary producers undergo peak photosynthesis and the consumption of inorganic carbon consequently leads to elevated seawater pH (Middelboe and Hansen, 2007). In contrast, acidification can take place at night while the organisms respire and release CO_2 (Middelboe and Hansen, 2007).

Animals sharing the same habitat as high densities of primary producers may then have to cope with diurnal fluctuations in pH.

1.5.2. Anthropogenic causes

An ongoing source of environmental CO_2 stress is known as ocean acidification, which is driven by excessive anthropogenic CO_2 emission through the burning of fossil fuels. As shown by equation 1, an increase in total seawater P_{CO2} subsequently acidifies the world's oceans. The current average surface seawater pH is approximately 8.1 with a P_{CO2} of ~40 Pa, but could reach pH 7.4 and P_{CO2} of ~200 Pa as predicted for the year 2300 according to continued "business as usual" emissions (Caldeira and Wickett, 2005). A hypercapnic environment can lead to increased diffusion of CO_2 into animals and result in an increase in body fluid P_{CO2} in order to maintain an outwardly directed gradient for CO_2 excretion (Fabry et al., 2008; Melzner et al., 2009). This elevated internal P_{CO2} also has an acidifying effect, which is more pronounced in organisms that lack counteractive mechanisms and/or adequate buffers (Melzner et al., 2009). Environmental hypercapnia has often been used to test the acid-base regulatory capacity of various animals and has resulted in a spectrum of responses, including full/partial hemolymph pH compensation by HCO_3^- accumulation (Hans et al., 2014; Michaelidis et al., 2005) and an upregulation of gene expression for transporters important for the process (Hu et al., 2013). Given the ongoing threat of ocean acidification to marine animals, the full extent of its effects must be determined for as many different marine taxa as possible.

Anthropogenic activity not only affects the carbonate system equilibrium of the world's oceans, but potentially nitrogen balance as well. For example, agricultural

drainage into coastal areas can lead to excess ammonia in the water from the leaching of fertilizers (Blann et al., 2009; Fisher et al., 1988), which could have implications on the excretion of this metabolic waste product by organisms that are exposed to such conditions. Animals utilizing an outwardly directed ammonia gradient for any portion of their ammonia excretory mechanism are at risk of encountering a reduction in gradient difference and may experience impaired ammonia excretion as a result. In addition, the conversion of ammonia into nitrite by nitrifying organisms requires O_2 and can ultimately deplete the O_2 content of bodies of water contaminated with fertilizer runoff (Blann et al., 2009).

The combination of nutrient over-enrichment through agricultural activity as well as rising seawater temperature associated with anthropogenic CO_2 release is thought to facilitate algal blooms by providing optimal conditions for reproduction (Paerl and Huisman, 2008). These microorganisms can overtake the water surface and obstruct sunlight from aquatic plants beneath the algal film, which eventually leads to plant death and the depletion of O_2 by bacteria that thrive from decomposing organic matter (Blann et al., 2009). As such, algal blooms are also associated with worldwide hypoxic zones, including the U.S. Atlantic coast as well as the Gulf of Mexico, and this secondary effect of anthropogenic-driven global climate change continues to threaten the survival of benthic animals in these regions (Blann et al., 2009; Doering et al., 1999; Glibert et al., 2001; Rabalais et al., 2001).

1.6. *Limulus polyphemus*

1.6.1. Background

While the majority of studies on aquatic organisms have investigated the mechanism of acid-base regulation and ammonia excretion in crustaceans, cephalopods, and teleost fish, no published study exists on either process for aquatic members of the subphylum Chelicerata to the author's knowledge. This is likely because the majority of this subphylum is composed of terrestrial arachnids, which release nitrogenous wastes predominantly in the form of guanine as a result of limited water availability (Anderson, 1966; Horne, 1969; Rao and Gopalakrishnareddy, 1962). An exception to the land-living lifestyle is the class Xiphosura, an example of which is the American horseshoe crab, *Limulus polyphemus.* The oldest horseshoe crab fossil dating back to 445 million years ago was first discovered in Manitoba, Canada, and horseshoe crabs have remained morphologically unchanged for 150 million years, earning them the nickname "living fossils" (Avise et al., 1994; Rudkin et al., 2008). *L. polyphemus* is currently widespread along coastal and estuarine areas of eastern U.S. and Mexico (Shuster, 1979), and sexually mature at ~10 years old with a lifespan of nearly 20 years (Shuster and Sekiguchi, 2003). Its eggs are mass-consumed annually as fuel for migrating shore birds (Botton et al., 1994) and the horseshoe crab is also of economic importance for the biomedical industry as its hemolymph is used for testing bacterial endotoxin contamination in medical products (Novitsky, 1984). When foraging for food, this benthic animal burrows through the sediment in search of prey, such as polychaete worms, bivalve molluscs, and crustaceans (Botton, 1984; Botton et al., 2003). Due to its protein-rich diet, it is hypothesized that *L. polyphemus* consequently produces high

amounts of nitrogenous waste predominantly in the form of ammonia as reported for all marine invertebrates investigated to date (Larsen et al., 2014). Adult *L. polyphemus* are also hyper-osmoregulators in dilute seawater, where they have been found to naturally occur in brackish water as diluted as 7 ppt with a temperature of up to 35°C (Anderson and Shuster, 2003; McManus, 1969; Robertson, 1970; Smith and Berkson, 2005). As osmoregulation and acid-base regulation may at least be partially linked through shared machinery (Henry and Wheatly, 1992), *L. polyphemus* makes for an interesting candidate for studying the latter process.

1.6.2. Book gills

Many representatives of ammonotelic aquatic animals, such as crustaceans and fish, predominantly regulate their acid-base homeostasis and excrete ammonia through the gills or gill-like structures (Evans et al., 2005; Freire et al., 2008; Weihrauch et al., 2009; Wright and Wood, 2009), which suggests that the book gills of *L. polyphemus* play a major role in these processes. In addition to maintaining internal homeostasis, *L. polyphemus* gills are remarkable because of their role in swimming, much like some aquatic isopod crustaceans (Alexander, 1988). Five pairs of gills can be found on the ventral posterior side of the horseshoe crab and each book gill is comprised of over 100 lamellae (Shuster, 1982; Fig. 1). An individual lamella consists of two single cell-layered epithelia (ventral and dorsal) that are separated by hemolymph space and supported by pillar cells to prohibit collapsing (Henry et al., 1996). There are ultrastructural differences within a single lamella, where a thick mitochondria-rich central patch (9 μm diffusion distance) is visibly surrounded by thin epithelia (4-5 μm diffusion distance; Henry et al.,

1996). Furthermore, within the central lamellar region, the ventral half-lamella is thicker than the corresponding dorsal half-lamella (Henry et al., 1996). The thick central region of the ventral epithelium is characterized by a high abundance of mitochondria and membrane infoldings as well as higher NKA activity compared to the dorsal and peripheral regions, all of which are indicative of an ideal site of active ion transport

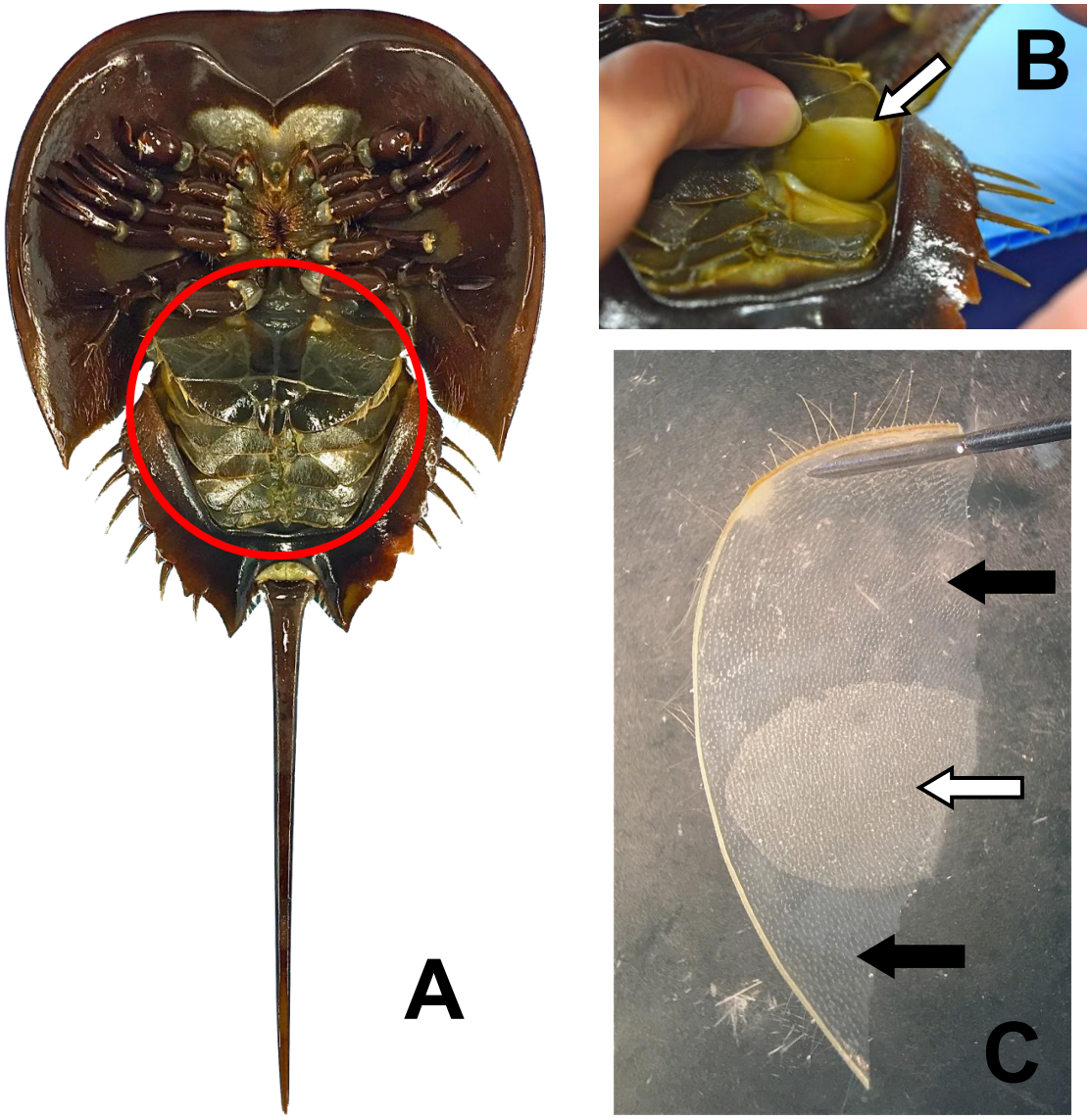

Fig. 1. Photographs of *L. polyphemus* book gills. (A) The ventral side of an adult male *L. polyphemus* (book gills are circled), (B) one book gill composed of over 100 lamellae (arrow), and (C) a single intact gill lamella showing the distinct central region (white arrow) surrounded by thin peripheral epithelium (black arrows). © Stephanie Hans.

(Henry et al., 1996). As a comparison to crustaceans, the occurrence of a thick, ion-transporting patch can also be found in the posterior gills of hyperregulating brachyuran crabs (Goodman and Cavey, 1990; Péqueux, 1995), which is surrounded by thin epithelium that is predicted to be primarily of respiratory function and therefore likely acid-base regulatory as well (Taylor and Taylor, 1992). This obvious difference became more pronounced with certain environmental stresses, such as a hyposmotic environment; in the blue crab (*Callinectes sapidus*) posterior gills, the mitochondria-rich patch increased over four-fold in size when seawater crabs were acclimated to freshwater, which further supports the significance of the patch in active ion transport (Aldridge and Cameron, 1982; Gilles and Péqueux, 1985). An intriguing observation was that *L. polyphemus* continued to exhibit a visually distinct central patch despite the lack of ongoing osmoregulation in seawater-acclimated animals (Henry et al., 1996), which were likely osmoconforming to the environment (Robertson, 1970). However, whether the degree of distinction is also affected by environmental stresses is not known.

Due to certain active acid-base regulatory and ammonia transport mechanisms found in fish and aquatic invertebrates (Chasiotis et al., 2016; Claiborne et al., 1982; Fehsenfeld and Weihrauch, 2013; Fehsenfeld and Weihrauch, 2016a; Nawata et al., 2010; Quijada-Rodriguez et al., 2015; Siebers et al., 1994; Weihrauch et al., 1998; Weihrauch et al., 1999; Weihrauch et al., 2012), an epithelium with the potential for high energy production could be presumed to have a major role in both processes. However, not only the thick salt-transporting epithelium is capable of regulating acid-base homeostasis and ammonia levels. Thin cells encompassing the perfused anterior gills of *C. maenas* were more efficient in elevating the artificial hemolymph pH than the mitochondria-rich

posterior gills (Fehsenfeld and Weihrauch, 2013). In terms of ammonia excretion, the thin epithelia of the anterior gills actually excreted ammonia at a higher rate than the thick, salt-transporting posterior gills (Weihrauch et al., 1998). In fact, both the anterior and posterior gills of the osmoconformer Dungeness crab (*Metacarcinus magister*), both of which exhibit ultrastructurally similar epithelia to the non-ion-transporting anterior gills of hyperregulating crabs, were highly suited for active ammonia excretion against a 16-fold inwardly directed gradient (Martin et al., 2011). These past studies indicate that although acid-base regulation, ammonia excretion, and osmoregulation share at least some of the same transporters, the former two processes do not rely entirely on the presence of salt-transporting cells and the possible importance of thin epithelia should not be overlooked.

1.6.3. Coxal glands

Another potentially important site of acid-base regulation and ammonia excretion is the coxal gland, which serves as an ultrafiltrating excretory organ similar to the crustacean antennal glands (Henry et al., 1996). The brachyuran crab antennal gland does not play a major role in ammonia excretion or ion transport, however; for example, *C. sapidus* antennal gland accounts for less than 2% of the total ammonia excretion and produced urine that is isosmotic to the hemolymph, therefore suggesting little to no ion modification of the excreted urine by the organ (Cameron and Batterton, 1978). Although the coxal glands in *L. polyphemus* are homologous to the crustacean antennal gland, several studies have suggested that coxal glands are set apart from decapod crustacean antennal glands in that they are capable of modulating the monovalent ion composition of

the urine more so than brachyuran crabs, even to the point of producing hyposmotic urine (Henry et al., 1996; Mangum et al., 1976; Towle et al., 1982). Given that certain transporters are shared between acid-base regulation, ammonia excretion, and ion regulation, it is highly possible that the coxal gland plays a role in all three processes in the horseshoe crab. However, limitations of the coxal gland must also be noted in that unlike the gills, the coxal gland is only capable of excretion rather than direct exchange with the environment.

1.7. Objectives

This study strives to gain first-time knowledge on basic acid-base regulatory and ammonia excretory mechanisms in a marine living fossil, *L. polyphemus*. To begin uncovering the involvement of various transporters and processes, the effects of pharmacological agents as well as environmental pH were observed. I strived to provide evidence that book gills are indeed important for acid-base and ammonia regulation, and further uncover differences between the various branchial regions. Lastly, I determined whether short-term acclimation to HEA and hypercapnia will induce changes in physiological aspects important to the fitness of this species.

2. Methods and Materials

2.1. Animals

L. polyphemus juveniles (Linnaeus, 1758; carapace width = 2-8 cm; weight = 1.2-43.4 g) were acquired from an aquarium store in Clearwater, Florida (Reefs2Go), while male adults (carapace width = 13.5-19 cm; weight = 322-951 g) were captured by the Whitney Laboratory for Marine Bioscience (St. Augustine, Florida, USA) from the Indian River Lagoon (Florida, USA). While not in use for experiments, animals were maintained at 22°C with a 12:12 h light-dark cycle in the Animal Holding Facility (University of Manitoba, Winnipeg, Manitoba, Canada) and a maximum of either 10 adults or 30 juveniles were held in 1200 L tanks filled with artificial seawater (SeaChem Marine Salt™, 32 ppt). Tanks were equipped with external filters and a UV-sterilization system, and a fine sand bed with crushed oyster shells was provided as substrate for juveniles while only crushed oyster shells were used for adults. Horseshoe crabs were fed *ad libitum* daily with raw shelled shrimp, squid, and smelt.

2.2. Experimental setup and analysis of seawater parameters

During experimental acclimations, all animals were maintained at 22°C and 32 ppt salinity. Six juveniles were held in a 70 L aquarium equipped with a filter and an air stone, while three adults were held in a 120 L aquarium at a time. The control acclimation aquarium was equipped with a degassing chamber to maximize aeration and reduce the seawater P_{CO2}. A full water change with fresh seawater was conducted every 1-2 days. Water salinity and temperature were analyzed daily, the former of which was measured with a refractometer. Ammonia concentration of aquarium water was measured using an

Orion™ 9512 ammonia gas sensing ISE electrode (Fisher Scientific, Ottawa, Ontario, Canada) with a pH/mV/temperature/ISE meter (Accumet™ Excel XL25, Fisher Scientific, Pittsburgh, Pennsylvania, USA) following the protocol described by Weihrauch et al. (1998).

To obtain the P_{CO2}, HCO_3^- concentration ($[HCO_3^-]$), and total alkalinity of seawater, the temperature and pH were measured using an Accumet™ pH/ATC electrode (Fisher Scientific, Ottawa, Ontario, Canada) connected to a pH-ISE meter model 225 (Denver Instrument, Bohemia, New York, USA), while total dissolved inorganic carbon (C_T) was analyzed using a Corning 965 TCO_2 Analyzer (Corning Limited Halstead, Essex, England). The pH, salinity, C_T, and temperature of the sample were entered into the CO2SYS Excel add-in (Lewis and Wallace, 1998) to calculate the P_{CO2}, $[HCO_3^-]$, and total alkalinity using the appropriate constants: K1, K2 after Mehrbach et al. (1973) refit by Dickson and Millero (1987), $KHSO_4$ dissociation constant after Dickson (1990), and NBS scale (mol kg^{-1} H_2O).

For hypercapnia acclimation, the IKS Aquastar (IKS ComputerSysteme GmbH, Karlsbad, Baden-Württemberg, Germany) provided continuous control of CO_2 injection into the aquarium to reach a P_{CO2} of 311 Pa and a pH of 7.4 (Table 1). A full water change was conducted every 1-2 days with pre-equilibrated high P_{CO2} seawater. Acclimation to high environmental ammonia (HEA) was achieved by enriching the aquarium with NH_4Cl to reach an average concentration of 965 µmol l^{-1} (Table 1). A full water change was carried out every 1-2 days with pre-equilibrated HEA seawater in order to minimize fluctuations in ammonia concentration. Prior to any experiment or

hemolymph collection, animals were starved for 2-4 days and all animals were acclimated for 7-9 days to their respective condition (control, HEA, or high P_{CO2}).

Table 1. Tank water parameters during acclimation of adult and juvenile *L. polyphemus* to control, high P_{CO2}, or high environmental ammonia (HEA) seawater.

Parameter	Control tank	High CO_2 tank	HEA tank
Salinity (ppt)	32	32	32
Temperature (°C)	22	22	22
Ammonia concentration (μmol l^{-1})	6.0 ± 0.5	---	965.2 ± 46.1
pH	8.1 ± 0.0	7.4 ± 0.0	---
P_{CO2} (Pa)	63.5 ± 3.0	310.6 ± 9.3	---
$[HCO_3^-]$ (mmol l^{-1})	2.27 ± 0.05	2.51 ± 0.13	---
Total alkalinity (mmol $kgSW^{-1}$)	2.67 ± 0.06	2.72 ± 0.05	---

2.3. Hemolymph parameters

Hemolymph was collected from the cardiac sinus of adults using a 1 ml syringe and 21 G needle. The sample was immediately centrifuged at 5000 x g and 4°C, and the supernatant was analyzed for pH, temperature, and total dissolved inorganic carbon (C_T) using the same methods as seawater samples (see above). The hemolymph P_{CO2} and $[HCO_3^-]$ were then calculated using the appropriate equations as well as αCO_2 and p$K1$ values obtained from Truchot (1976):

$$P_{CO2} = C_T/((10^{pH - pK1} * \alpha CO_2) + \alpha CO_2) \quad \text{(Eqn 2)}$$

$$[HCO_3^-] = 10^{pH-pK1} * \alpha CO_2 * P_{CO2} \quad \text{(Eqn 3)}$$

where αCO_2 is the solubility coefficient for CO_2 and pK_1 is the first dissociation constant of carbonic acid. The remaining hemolymph sample was frozen at -20°C until analyzed for ammonia concentration using the same method as previously described for tank water samples.

2.4. Whole animal ammonia excretion

All whole animal ammonia excretion experiments were conducted at 22°C in 32 ppt seawater with an air stone. The containers used for these experiments were loosely covered to reduce visual stress to the animals. To observe the effects of environmental pH and pharmacological agents on whole animal ammonia excretion, juveniles weighing 1.2-2.6 g were placed in 100 ml beakers containing 30 ml of seawater (SeaChem Marine Salt™) and exposed to the following conditions: pH 6.1 (10 mmol l^{-1} MES buffer), pH 9.0 (10 mmol l^{-1} Trizma® buffer), 0.1 mmol l^{-1} ouabain, 1 mmol l^{-1} acetazolamide (in 0.5% DMSO), 1 mmol l^{-1} colchicine, or 2.5 mmol l^{-1} theophylline. All solutions, including controls, used in the experiment utilizing acetazolamide also contained 0.5% DMSO, which did not affect ammonia excretion in the horseshoe crab. The pH of seawater solution containing pharmacological agents was adjusted to the same pH as control seawater (pH 8.2) using HCl or NaOH. The trials used in these experiments were conducted in the following order: equilibration period (2 h), treatment period (2 h), wash-out period (1 h), and control period (2 h). The equilibration period allowed the animals to release any extra ammonia from handling stress into the water prior to the start of the treatment period. A 15 ml water sample was taken at the end of all periods except the wash-out period. In order to rid the container of leftover ammonia from the previous period, seawater was gently siphoned out using airline tubing after each trial and the container was rinsed with fresh seawater. The process was repeated once more prior to starting the next time period.

The ammonia excretion rate by intact control animals were also compared to HEA and hypercapnia acclimated animals. In this set of experiments, juveniles weighing 14-43

g were placed in individual 14.5 x 9 cm containers with 300 ml of seawater and an air stone. Water samples (15 ml) were taken hourly for 2 h, along with a water change and rinse (as previously described) after each sample collection. The ammonia excretion rate of control crabs was measured while they were in control or HEA seawater, and then repeated after the same animals were acclimated to HEA for 7-9 days in order to determine the effects of HEA on whole animal ammonia excretion. For 7-9 day acclimated high CO_2 animals, the ammonia excretion rate by intact animals was determined while the animals were in high CO_2 seawater. Water samples from all whole animal ammonia excretion experiments were frozen at -20°C and later analyzed for ammonia concentration using the same method as other seawater samples. At the end of all experiments, animals were gently dried with paper towel and weighed.

2.5. $Na^+/K^+(NH_4^+)$-ATPase activity

Each sample consisted of whole lamellae collected from the anterior-most pair of book gills of three juvenile *L. polyphemus* and immediately frozen at -80°C. Determination of NKA activity and total protein concentration of the samples followed Cruz et al. (2013), with the exception of a higher ouabain concentration used (5 mmol l^{-1}), and a doubling of pyruvate kinase and lactate dehydrogenase concentrations to 10 and 8 IU ml^{-1}, respectively. The NKA assay protocol was modified from Gibbs and Somero (1989) and McCormick (1993), and utilized a UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, California, USA) at 20°C. Gill samples (approx. 90 mg) were homogenized at 9000 rpm for three 10 s periods using an Ultra-Turrax T25 homogenizer (IKA® Works Inc., Wilmington, North Carolina, USA) in 14 volumes of cold SEID

homogenization buffer composed of (pH 7.0): 150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, and 0.1% (w/v) deoxycholate. Homogenates were centrifuged at 4°C for 1 min at 5000 x g and the supernatant of each sample was collected for subsequent enzyme and protein assays.

The enzyme reaction buffer was buffered to pH 7.5 and included (in mmol l^{-1}): 10 NaCl, 5 $MgSO_4$, 50 imidazole, 3 ATP disodium salt, 2 phosphoenolpyruvate, and 0.2 NADH sodium salt. Pyruvate kinase was also added at 10 IU ml^{-1} as well as 8 IU ml^{-1} of lactate dehydrogenase. Either 10 mmol l^{-1} KCl or NH_4Cl was provided as substrate, and the NKA activity was calculated as the difference in the rate of change in absorbance at 340 nm in the absence (total ATPase activity) and presence of 5 mmol l^{-1} ouabain. Each gill sample was measured in triplicate and NKA activity was calculated using the NADH extinction coefficient of 6.2 mmol l^{-1} cm^{-1}. The protein concentration of each sample was determined using the Pierce™ BCA protein assay kit (Fisher Scientific, Ottawa, Ontario, Canada) with bovine serum albumin used as standards.

2.6. Ussing chamber

Ussing chambers (EM-CSYS-6, Physiologic Instruments, San Diego, California, USA) with 0.25 x 1.0 cm homemade tissue holders (0.25 cm^2 surface area; D. Weihrauch) were used to measure the transepithelial movement of ammonia across split gill lamellae when an *in vivo*-like gradient of 300 µmol l^{-1} (basolateral) to 0 µmol l^{-1} (apical) was applied. A total of six ventral epithelia and six dorsal epithelia (biological replicates) from three different adults were used in this experiment. Gill lamellae were collected from the left anterior-most book gill and immediately placed in chilled seawater

on top of ice. The thick chitinous edge of an individual lamella was held with fine forceps under a dissecting microscope while a 21G needle was inserted between the ventral and dorsal epithelia to separate the two half-lamellae. Another pair of fine forceps was inserted into the partially split area of the lamella, then used to tease apart the two sides of the lamella. In an intact animal, the dorsal side of the lamella is the side that faces the body while the ventral side faces away from the body (see detailed diagram in Henry et al., 1996).

Each half-lamella was individually mounted between a pair of lightly greased sliders while ensuring approximately equal surface area of both the thick central and thin peripheral regions. Each slider was gently inserted into one of the six chambers in the system, which allowed for simultaneous runs of three ventral and three dorsal half-lamella at a time. All chambers were temperature-controlled by an ARCTIC A10 refrigerated circulator (Fisher Scientific, Ottawa, Ontario, Canada) at 22°C. The chambers were filled with 4 ml of their respective solution: artificial seawater for the apical side and artificial hemolymph for the basolateral side. Artificial seawater was adjusted to pH 8.1 and composed of (in mmol l^{-1}): 430 NaCl, 10 $CaCl_2$, 37 $MgCl_2$, 10 KCl, and 2 $NaHCO_3$ (A. Winkler, Die Osmoregulation der Strandkrabbe Carcinus maenas (L.) in konstanten und fluktuierenden Salzgehalten, PhD thesis, Universität Hamburg, 1987). Artificial hemolymph was based on Robertson (1970), Smith et al. (2002), and the measured HCO_3^- concentration of non-acclimated animals prior to any experiments in this study. The solution was adjusted to pH 7.6 and consisted of (in mmol l^{-1}): 450 NaCl, 11.8 KCl, 9.9 $CaCl_2$, 31.9 $MgCl_2$, 14.1 $MgSO_4$, 4.4 $NaHCO_3$, 3.2 glucose, and 0.3 NH_4Cl. Tissues were allowed to equilibrate in the artificial solutions for 20 mins.

Following the equilibration period, chamber solutions were replaced with fresh solutions and allowed to incubate for 2 h, after which a sample from each solution was collected. Each sample was weighed to estimate its volume and frozen at -20°C until ammonia analysis, which followed the same protocol as aquarium seawater samples.

Electrophysiological parameters (V_{TE} and G_{TE}) of the ventral and dorsal half-lamellae were determined as described in detail in Onken and Siebers (1992) using the Lab Trax 4/16 (World Precision Instruments, Sarasota, Florida, USA) and VCC-600 voltage/current clamp (Physiologic Instruments, San Diego, California, USA). In this series of experiments, the central region of either the ventral (n=5) or dorsal epithelia (n=6) from three different adults was used. Experiments were conducted using symmetrical bathing solutions, which were artificial hemolymph of the same composition as previously listed for ammonia transport measurements. Gill lamellae were collected from the right anterior-most gills and were split by the same method. A custom made Ussing chamber was used, where a single half-lamella was mounted between two 1 mm^3 chamber compartments. Approximately 0.008 cm^2 of gill surface area was exposed to artificial hemolymph, which was set up in a flow-through system using gravity flow at a maximum rate of 125 bath volumes min^{-1}. To measure the transepithelial potential difference (PD_{TE}), Ag/AgCl electrodes were attached to the chamber compartments using agar bridges (3% agarose in 3 mol l^{-1} KCl) with the reference electrode on the basolateral side. To obtain the transepithelial conductance (G_{TE}) of the epithelium, small voltage pulses (1 mV) were generated across the membrane and the conductance was calculated from subsequent current deflections as measured by the voltage electrodes.

2.7. Quantitative real-time PCR

Tissue samples to be used in quantitative PCR consisted of two main groups: (1) the central and peripheral gill regions, coxal gland, and brain tissue samples, and (2) the ventral and dorsal branchial epithelia from control, HEA, and hypercapnia acclimated animals. Group 1 samples were collected in Florida, where adult male horseshoe crabs were anaesthetized by placing the animals on ice for 30 mins, then euthanized *via* dissection of the dorsal side with a Dremel and the removal of the brain and dorsal nerve cord. Tissue collection for group 2 was conducted in Winnipeg (Manitoba, Canada); adult males were anesthetized on ice for 30 mins, after which gill lamellae were collected from each individual and split into the ventral and dorsal halves.

All tissue samples were collected in Ambion™ RNA*later*™ Stabilization Solution (Fisher Scientific, Ottawa, Ontario, Canada) and later disrupted using a TissueLyser II (Qiagen, Toronto, Ontario, Canada). RNA was isolated using Ambion™ TRIzol™ reagent (Fisher Scientific, Ottawa, Ontario, Canada), and 0.91 μg and 0.27 μg of RNA from the first and second groups of samples (respectively) underwent DNase treatment (Invitrogen™ DNase I Amplification Grade, Fisher Scientific, Ottawa, Ontario, Canada). RNA samples were then tested for DNA contamination by employing polymerase chain reaction (PCR) with elongation factor 1-alpha (*EF-1α*) *L. polyphemus* specific primers designed based on published sequence (GenBank accession no.: U90051.1; Table 2), ensuring that no bands had formed when ethidium bromide-stained agarose gel electrophoresis was conducted and imaged using the Molecular Imager VersaDoc™ MP 4000 with Image Lab™ 3.0 software (Bio-Rad Laboratories, Mississauga, Ontario, Canada). DNA-free RNA samples were reverse-transcribed to

obtain cDNA using qScript™ cDNA Synthesis Kit (Quanta BioSciences, VWR, Radnor, Pennsylvania, USA).

Table 2. Specific primer sequences used in quantitative PCR. Na^+/K^+-ATPase α-subunit (NKA F/R), V-H^+-ATPase subunit B (HAT F/R), Rhesus protein (Rh F/R), cytoplasmic carbonic anhydrase (CA-2 F1/R1), hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN F1/R2), and RNA polymerase II (Pol2 F1/R1) were employed in quantitative polymerase chain reaction (qPCR) analyses, while elongation factor-1α (EF-1α F/R) was only used to test RNA purity (see Methods).

Transcript	Forward primer sequence (5' → 3')	Reverse primer sequence (3' → 5')	Amplicon size (bp)	Annealing temp. (°C)
NKA F/R	CTAGGTGGACTTGGAGAGCG	CCCACCAAACGCATATTACC	128	58
HAT F/R	CTGAATTCCTGGCCTACCAA	TCACGTGCAGCTGATACCTC	100	58
Rh F/R	GGCCTAGCTTTAACTCCGCT	GCAACACCTCCAGCTATGGT	178	58
CA-2 F1/R1	ACTTACCACCCCTCCCTGTT	CCCTATCACCTATCGGCAAA	191	58
HCN F1/R2	TCACTTGTTGCGTCAGTTCC	TCCGTCAGACAGACTTGTGG	207	58
Pol2 F1/R1	TCGTTTGGAGCACACAACTT	GAGAGATCCTTGTGGGGTCA	149	58
EF-1α F/R	GGCTACAATCCTGCCACTGT	GTTTGACCCTTGCGTTCAAT	122	58

Primers were designed based on published sequences of the respective gene and the *L. polyphemus* genome, and tested for the presence of a single amplicon of the correct product size using EconoTaq® DNA Polymerase (VWR, Radnor, Pennsylvania, USA) and a Mastercycler® pro S (Fisher Scientific, Ottawa, Ontario, Canada). The PCR products were imaged with gel electrophoresis, purified with E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, VWR, Radnor, Pennsylvania, USA), and sequenced at Robarts Research Institute (London, Ontario, Canada). The products were confirmed to code for the target gene by searching the sequence on GenBank using the BLAST algorithm and ensuring a similar match to previously published sequences, as well as an exact match to the published *L. polyphemus* genome. The optimal annealing temperature of the primers was then determined using gradient PCR.

The concentration of the extracted and amplified PCR product for each gene was measured using two different NanoDrop 2000C UV-Vis spectrophotometers (Fisher Scientific, Ottawa, Ontario, Canada) to ensure accuracy of concentrations. Standards of known concentrations, ranging from 0.01 fg cDNA μl^{-1} to 1 ng cDNA μl^{-1}, were prepared by conducting serial dilutions of the extracted and amplified PCR product, and were used alongside experimental cDNA samples in a MiniOpticon™ Real-Time PCR System (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Optimal quantitative PCR conditions were used in 10 µL assays with SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Mississauga, Ontario, Canada) and cDNA transcribed from either 23 or 9 ng of RNA (sample groups 1 and 2, respectively), which were incubated for 40 cycles. PCR products were verified to be single products by conducting a melting curve analysis. The results were log-transformed and adjusted to optimize for efficiency (range: 80-120%) and correlation (>0.99). All absolute values obtained for genes of interest in this study were normalized with an internal standard, RNA polymerase II (*Pol2*), which did not show significant difference in any of the tissues and/or treatments (data not shown).

Additional information regarding the Rh F/R primers should be noted. Although the PCR product resulting from a PCR reaction using these primers produced a single band and was confirmed to code for the single correct product *via* sequencing, a recent alignment of the partial Rh-protein sequence to two annotated Rh-protein isoforms on GenBank (XM_013920039.1 and XM_013917394.1) showed that a section of the partial sequence matches completely to one isoform while the remaining section matches to the second isoform. Given the tendency for one isoform to be abundantly expressed compared to the other (Adlimoghaddam et al., 2015), mRNA expression results for Rh-

protein in this study were likely composed predominantly of one isoform, but may also include expression levels of the second isoform as well.

2.8. Statistical analysis

All data sets are presented as mean ± s.e.m. and for all statistical tests, a P-value of <0.05 was considered significant. All data sets were analyzed for outliers (Grubbs test), normality (Shapiro-Wilk test), and homogeneity of variance (Levene's test) to determine whether the data set is parametric. Parametric two-sample data sets were tested with either Student's t-test or paired t-test, while Mann-Whitney test was performed for non-parametric data. Parametric data sets with more than two means were analyzed using one-way ANOVA with *post-hoc* Tukey's pairwise comparisons, while non-parametric sets were treated with Kruskal-Wallis test with *post-hoc* Mann-Whitney pairwise comparisons. All statistical analyses were carried out using the Paleontological Statistics (PAST) software (Hammer et al., 2001).

3. Results

3.1. Ammonia excretory mechanism

The effect of environmental pH on whole animal ammonia excretion was observed in juvenile *L. polyphemus via* acute exposure to acidic and basic seawater (Fig. 2). The ammonia excretion rate by intact animals under control conditions in seawater with a pH of 8.2 was 422.2 ± 36.4 nmol gFW^{-1} h^{-1}. When exposed to seawater adjusted to pH 6.1, ammonia excretion rate increased by 71% compared to animals exposed to control seawater, but a decrease of excretion by 26% was observed in animals exposed to seawater adjusted to pH 9.0.

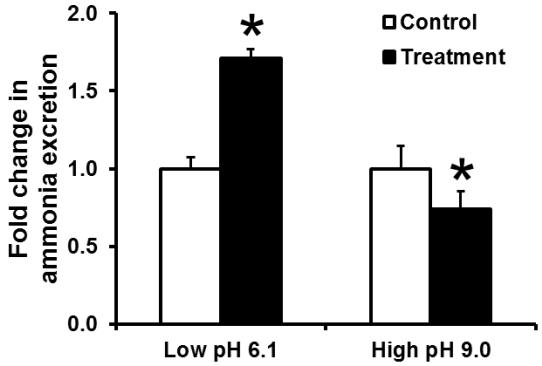

Fig. 2. The effect of environmental pH on whole animal ammonia excretion by juvenile *L. polyphemus*. White bars depict excretion rates under unbuffered control conditions (pH 8.2), while black bars depict excretion rates during exposure to either low (pH 6.1 with 10 mmol l^{-1} MES buffer) or high (pH 9.0 with 10 mmol l^{-1} Trizma base seawater pH. Data represent mean ± s.e.m. *Significant difference in excretion rate between control and treatment trials (two-tailed paired t-test; $P<0.05$; n=5-6).

In order to investigate the mechanism of ammonia excretion, various pharmacological agents were employed in experiments on intact juvenile *L. polyphemus* (Fig. 3). The whole animal ammonia excretion rate by juveniles during the control trial for each pharmacological experiment remained consistent with an average of 445.6 ±

25.7 nmol gFW^{-1} h^{-1} (n=22). When 0.1 mmol l^{-1} ouabain was used to inhibit the activity of Na^+/K^+-ATPase (NKA; Furriel et al., 2004; Prassas and Diamandis, 2008), a reduction in ammonia excretion by 11% can be observed in figure 3A (note: preceding test experiments employing 1 and 5 mmol l^{-1} ouabain were ethal to the animals). Application of 1 mmol l^{-1} acetazolamide, a drug that inhibits carbonic anhydrase (Coleman, 1967), decreased ammonia excretion by 21% (Fig. 3A). To investigate whether ammonia excretion depends on an intact microtubule network, 1 mmol l^{-1} colchicine was added to the media, which resulted in a trend (P=0.07) of increased excretion rate by 24% (Fig. 3A).

In order to evaluate whether ammonia excretion can be activated by intracellular cAMP levels, 2.5 mmol l^{-1} theophylline was employed. Theophylline inhibits phosphodiesterase, which can subsequently result in increased intracellular cAMP levels (Amer and Kreighbaum, 1975) and ultimately activating NKA as well as V-H^+-ATPase (HAT; Butcher and Sutherland, 1962; Chibalin et al., 1992; Dames et al., 2006). After addition of the drug, an increase in ammonia excretion by 19% was observed (Fig. 3B).

3.2. $Na^+/K^+(NH_4^+)$-ATPase activity

The inhibitor experiment employing ouabain suggested that NKA is involved in the *L. polyphemus* ammonia excretory mechanism. For verification, the activity of this enzyme was measured providing Na^+ and either K^+ or NH_4^+ as substrate for activation. When K^+ was provided, the NKA activity of whole gill homogenate was 17.0 ± 0.9 nmol ADP min^{-1} $mg\ protein^{-1}$ but was significantly lowered by 21% when K^+ was replaced by an equimolar concentration of NH_4^+ in the assay (data not shown).

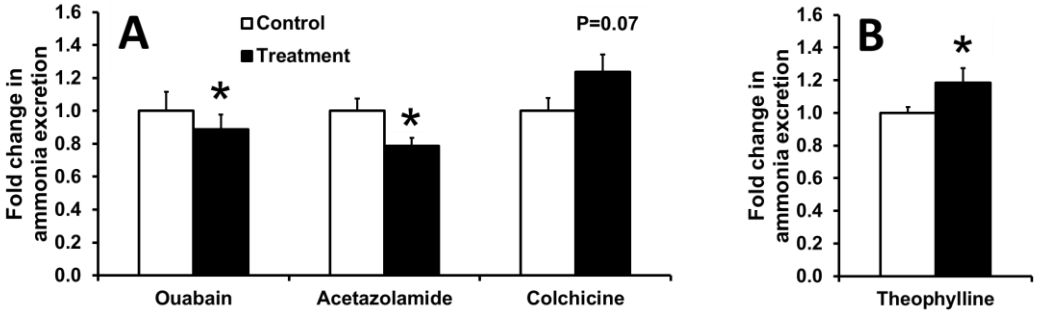

Fig. 3. The effect of various pharmacological agents on whole animal ammonia excretion by juvenile *L. polyphemus*. (A) Effect of inhibitors targeting Na^+/K^+-ATPase (0.1 mmol l^{-1} ouabain), carbonic anhydrase (1 mmol l^{-1} acetazolamide), and microtubule network (1 mmol l^{-1} colchicine). (B) Effect of theophylline (2.5 mmol l^{-1}), a phosphodiesterase inhibitor that indirectly increases intracellular cAMP. White bars depict excretion rates under control conditions, while black bars depict excretion rates during exposure to the indicated pharmacological agent. Data represent mean ± s.e.m. *Significant difference in excretion rate between control and treatment trials (two-tailed paired t-test; $P<0.05$; n=5-6). P-value is given for any comparison trending towards a difference from its respective control.

3.3. mRNA expression levels across tissues and within gill lamellae

To understand the animal's main site of ammonia excretion, mRNA expression levels of two key proteins of this process, Rh-protein and NKA, were determined in 4 different tissues including 2 sections of the gill: the central and peripheral regions, the former of which exhibited the thicker patch (Fig. 1). The relative mRNA expression level of Rh-protein in the central gill region was over 18-fold higher than that of the peripheral region, over 44-fold higher than in the coxal gland, and 950-fold higher compared to the levels found in the brain (Fig. 4A). The relative expression of NKA (α-subunit) in the central gill region was not significantly different from the peripheral gill region or the coxal gland, but the expression in the coxal gland was over 1.5-fold higher than the peripheral gill region (Fig. 4B). The relative NKA expression level in the brain was the lowest out of all investigated tissues (Fig. 4B).

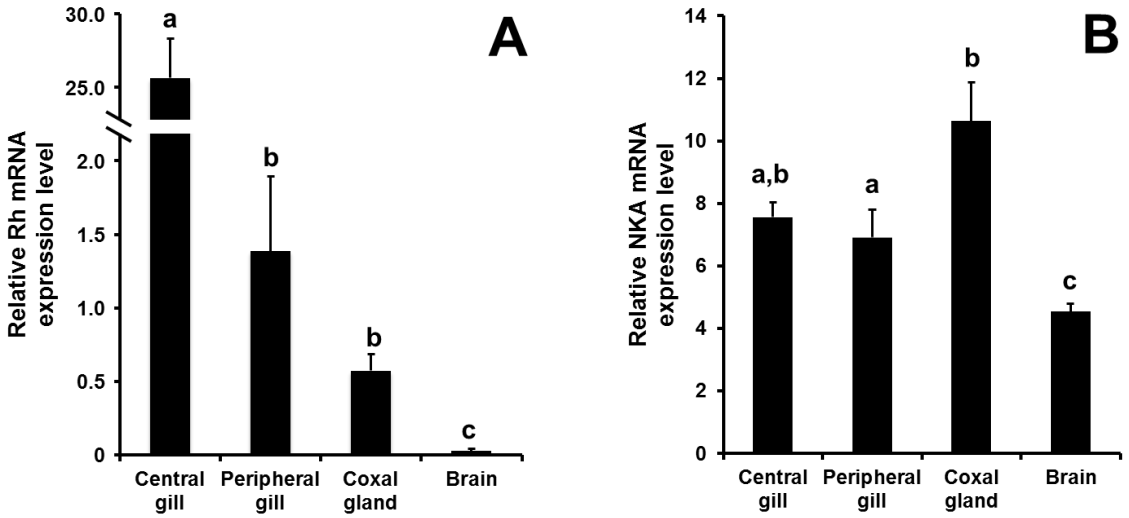

Fig. 4. Relative mRNA expression levels of (A) Rhesus protein (Rh) and (B) Na^+/K^+-ATPase α-subunit (NKA) in various tissues of adult *L. polyphemus*. Tissues include the central and peripheral gill regions, coxal gland, and brain, and all expression data are relative to RNA polymerase II. Data represent mean ± s.e.m. Significant differences between tissues of an individual gene are indicated by lowercase letters (Kruskal-Wallis test with *post hoc* Mann-Whitney pairwise comparison for Rh; log transformation and ANOVA with *post hoc* Tukey's pairwise comparison for NKA; $P<0.05$; n=5-6).

Expression levels of the Rh-protein indicated that the gills play a central role in ammonia excretion; therefore, in the following experiments, only the different branchial regions were analyzed for gene mRNA expression of additional genes including V-type H^+- ATPase subunit B (HAT), cytoplasmic carbonic anhydrase (CA-2), and hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN). The relative CA-2 and HCN expression levels were 11-fold and 3-fold higher, respectively, in the central region compared to the periphery; however, no significant difference was observed between mRNA expression levels of HAT (Fig. 5). Absolute mRNA expression levels of all genes for the central gill region are provided in table S1.

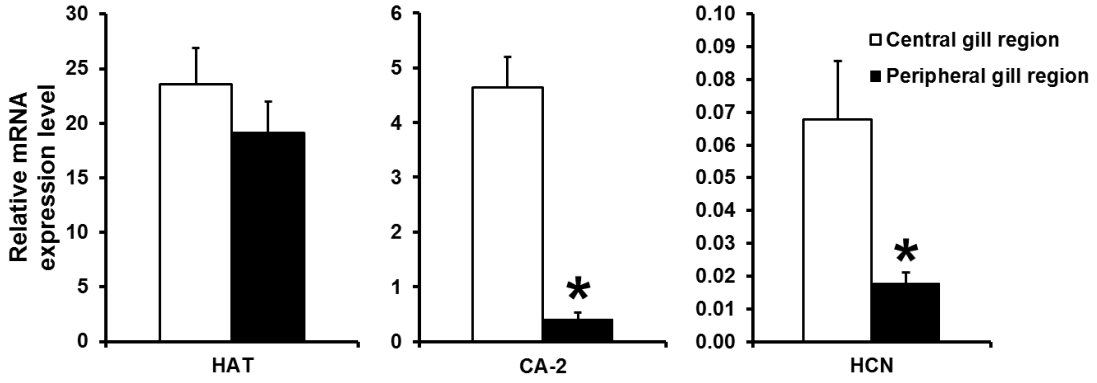

Fig. 5. Relative mRNA expression levels of additional genes involved in acid-base regulation in the central and peripheral gill regions. H^+-ATPase subunit B (HAT), cytoplasmic carbonic anhydrase (CA-2), and hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN) expression levels were standardized to RNA polymerase II for adult *L. polyphemus*. Data represent mean ± s.e.m. *Significant difference in relative mRNA expression between gill regions (two-tailed t-test for HAT; two-tailed Mann-Whitney test for CA-2; log transformation and two-tailed t-test for HCN; $P<0.05$; n=4-6).

3.4. Ventral versus dorsal branchial half-lamellae

To observe the ammonia transport function of the book gills in *L. polyphemus,* a preparation of the split gill lamella was employed to characterize both the ventral and dorsal epithelia of the lamella. Mounting the different half-lamellae into Ussing chambers has allowed the observation of lamellar properties relevant to ion transport, such as transepithelial conductance (G_{TE}), potential difference (PD_{TE}), and transepithelial ammonia fluxes. In adult *L. polyphemus*, a large significant difference in G_{TE} of the central region of the two half-lamellae was observed, where the dorsal half-lamella was found to have a much lower G_{TE} (0.20 ± 0.04 mS cm^{-2}) than the ventral half-lamella (145.40 ± 33.95 mS cm^{-2}; Fig. 6). However, PD_{TE} was very small and did not differ between the ventral (+0.1 ± 0.2 mV) and dorsal (+0.3 ± 0.4 mV) half-lamellae.

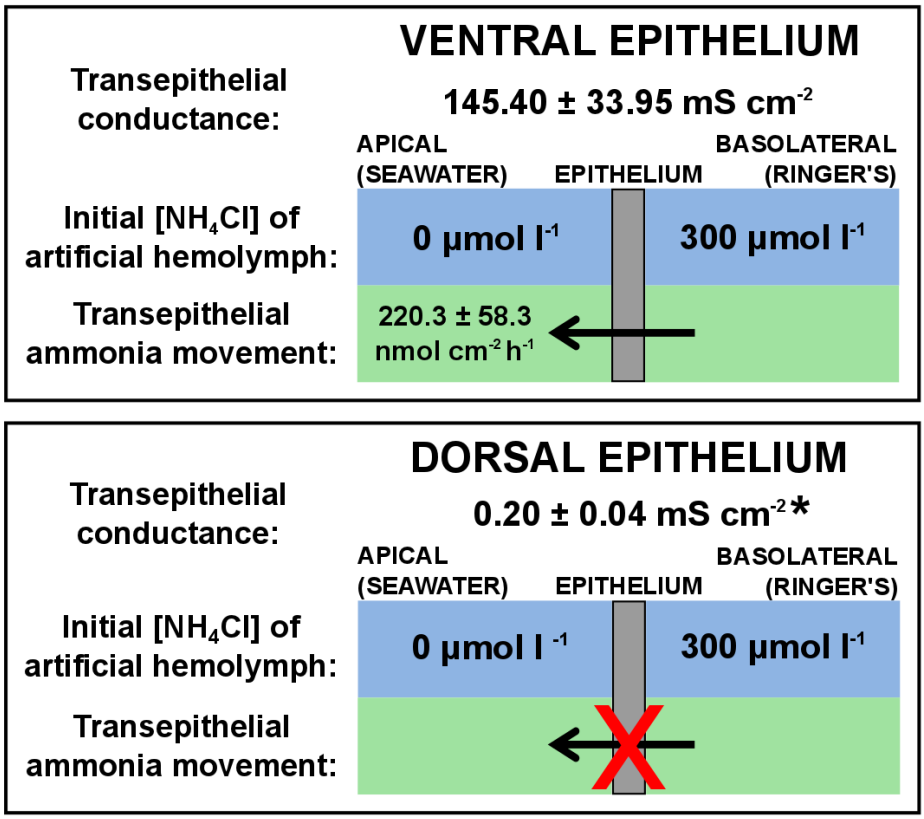

Fig. 6. Schematic of ventral and dorsal split gill lamellae of adult *L. polyphemus* with the corresponding transepithelial conductance and transepithelial ammonia movement. Ammonia-free artificial seawater was used as the apical solution while the basolateral side was exposed to *L. polyphemus* Ringer's solution enriched with 300 µmol l^{-1} NH_4Cl to mimic *in vivo* conditions. Data represent mean ± s.e.m. *Significant difference in transepithelial conductance between the ventral and dorsal epithelia (log transformation and two-tailed t-test; $P<0.05$; n=5-6).

When an ammonia gradient mimicking *in vivo* conditions (no NH_4Cl on apical side, 300 µmol l^{-1} NH_4Cl on basolateral side) was applied over the half-lamellae, transepithelial ammonia movement at a rate of 220.3 ± 58.3 nmol cm^{-2} h^{-1} was observed across the ventral epithelium as calculated by the loss of ammonia in the basolateral solution, while no net excretion could be detected across the dorsal epithelium (Fig. 6). The metabolic ammonia production by the gill half-lamellae was calculated using measurements of the initial ammonia concentration of both the apical and basolateral

solutions as well as the ammonia concentrations after the experimental period. The analysis revealed that in addition to the amount of ammonia loss in the basolateral side, the ventral half-lamella further enriched the apical side of the chamber at a rate of 83.5 ± 40.8 nmol total ammonia cm^{-2} h^{-1}. It is important to note that the basolateral release of ammonia cannot be excluded, but was difficult to determine due to an observed overall reduction of the ammonia concentration in the bathing solution facing the basolateral side. Interestingly, despite the low conductance and lack of net transepithelial ammonia excretion measured for the central region of the dorsal half-lamella, this tissue still produced metabolic ammonia. Of this, 98.5 ± 18.3 nmol cm^{-2} h^{-1} was released towards the apical side and 34.0 ± 16.2 nmol cm^{-2} h^{-1} towards the basolateral side. Combining the ventral and dorsal epithelia parameters to predict the overall function of the whole gill results in a transepithelial ammonia excretion rate of 186.3 nmol cm^{-2} h^{-1} and a total metabolic ammonia production of 216.1 nmol cm^{-2} h^{-1}.

At the molecular level, differences were observed in relative mRNA expression levels of Rh-protein, CA-2, and HCN between the two branchial half-lamellae, where the relative expression levels were 5-fold, 4-fold, and 4-fold higher, respectively, in the ventral epithelium compared to the dorsal epithelium (Fig. 7). However, relative mRNA expression levels of NKA and HAT did not significantly differ between the two sides of the branchial half-lamellae (Fig. 7). Absolute mRNA expression levels of all genes for the ventral epithelium are provided in table S2.

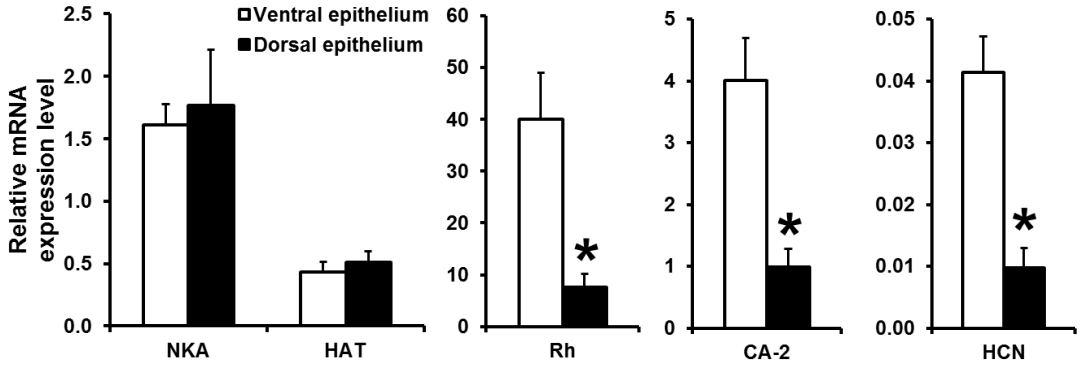

Fig. 7. Relative mRNA expression levels of genes involved in acid-base regulation in the ventral and dorsal gill epithelia. Na^+/K^+-ATPase α-subunit (NKA), H^+-ATPase subunit B (HAT), Rhesus protein (Rh), cytoplasmic carbonic anhydrase (CA-2), and hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN) expression levels were standardized to RNA polymerase II for adult *L. polyphemus*. Data represent mean ± s.e.m. *Significant difference in relative mRNA expression between ventral and dorsal epithelia of the respective gene (two-tailed Mann-Whitney test for Rh; two-tailed t-test for all other comparisons; $P<0.05$; n=5-6).

3.5. Effects of high P_{CO2} acclimation

Juvenile and adult *L. polyphemus* were acclimated to elevated P_{CO2} of 311 Pa to observe the effects of short-term (7-9 days) acclimation to hypercapnia on the animals' hemolymph parameters, ammonia excretion rates, and mRNA expression levels of proteins related to acid-base homeostasis. In control adult *L. polyphemus*, hemolymph pH averaged at 7.59 ± 0.04, while the P_{CO2} and HCO_3^- concentration were 164.1 ± 24.3 Pa and 2.5 ± 0.4 mmol l^{-1}, respectively. Following hypercapnia acclimation, a nearly 2-fold increase in hemolymph P_{CO2} with a corresponding 2-fold increase in HCO_3^- concentration was observed. However, there was no significant change in hemolymph pH (Table 3). Simultaneously, both the hemolymph ammonia concentration (183.8 ± 19.6 µmol l^{-1}) and whole animal ammonia excretion rate (170.2 ± 40.3 nmol gFW^{-1} h^{-1}) of hypercapnia acclimated animals were 43% lower than control animals (320.8 ± 36.9 µmol l^{-1} and 298.0 ± 31.7 nmol gFW^{-1} h^{-1}, respectively; Table 3).

Table 3. Hemolymph parameters of adult *L. polyphemus* following a 7-9 day acclimation to control, high P_{CO2} (311 Pa, pH 7.4), or high environmental ammonia (HEA; 965 µmol l^{-1} NH_4Cl) seawater. Data represent mean ± s.e.m. *Significant difference to control values (log transformation and two-tailed t-test for P_{CO2} comparison between control and HEA animals; two-tailed t-test for all other comparisons; P<0.05; n=5-6). P-value is given for any parameter trending towards a difference compared to control value.

Parameter	Control animals	High CO_2 animals	HEA animals
pH	7.59 ± 0.04	7.62 ± 0.05	7.47 ± 0.05 (P=0.09)
P_{CO2} (Pa)	164.1 ± 24.3	301.7 ± 33.7*	346.9 ± 51.8*
$[HCO_3^-]$ (mmol l^{-1})	2.47 ± 0.41	5.32 ± 0.20*	3.95 ± 0.34*
Ammonia concentration (µmol l^{-1})	320.8 ± 36.9	183.8 ± 19.6*	334.0 ± 28.3

Similar to control animals (Fig. 7), the relative mRNA expression level of Rh-protein, CA-2, and HCN in high P_{CO2} acclimated animals were at least 5-fold higher in the ventral half-lamella in comparison to the dorsal half-lamella (Fig. 8). However, no significant change in relative mRNA expression of any investigated protein was observed following the acclimation to elevated P_{CO2} seawater (Fig. 8).

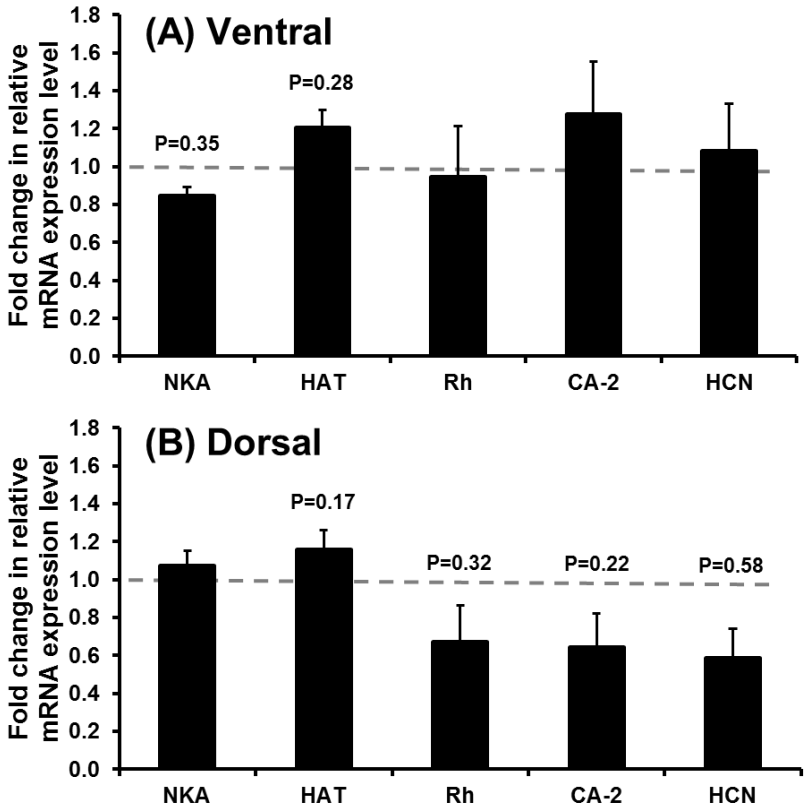

Fig. 8. Effect of hypercapnia acclimation on mRNA expression of genes involved in acid-base regulation. Expression levels of Na^+/K^+-ATPase α-subunit (NKA), H^+-ATPase subunit B (HAT), Rhesus protein (Rh), cytoplasmic carbonic anhydrase (CA-2), and hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN) relative to RNA polymerase II in the (A) ventral and (B) dorsal epithelia of hypercapnia acclimated adult *L. polyphemus* are compared in fold-change to control animals (gray dashed line). Data represent mean ± s.e.m. *Significant difference in relative mRNA expression between control and high P_{CO2} (311 Pa) acclimated animals (two-tailed Mann-Whitney test for HCN of ventral epithelium and HAT of dorsal epithelium; log transformation and two-tailed t-test for HCN of dorsal epithelium; two-tailed t-test for all other comparisons; $P<0.05$; n=4-6). P-values are given for any comparison that may visually appear to be significant.

3.6. Effects of high environmental ammonia (HEA) acclimation

Upon exposure to high environmental ammonia (HEA) of 965 μmol l^{-1} NH_4Cl for 7-9 days, *L. polyphemus* hemolymph pH slightly decreased to pH 7.47 ± 0.05 (P=0.09), while the hemolymph P_{CO2} and HCO_3^- concentration significantly increased to 346.9 ± 51.8 Pa and 4.0 ± 0.3 mmol l^{-1}, respectively (Table 3). No difference was found in the

hemolymph ammonia concentration between control animals (320.8 ± 36.9 µmol l^{-1}) and HEA animals (334.0 ± 28.3 µmol l^{-1}).

Control juveniles excreted ammonia at a rate of 298.0 ± 31.7 nmol gFW^{-1} h^{-1} when placed in ammonia-free seawater, but exhibited a significantly pronounced uptake of ammonia at a rate of 2393.3 ± 924.4 nmol gFW^{-1} h^{-1} immediately after placement in HEA seawater (1000 µmol l^{-1} NH_4Cl; Fig. 9). However, following a 7-9 day acclimation to 965 µmol l^{-1} NH_4Cl seawater, animals excreted ammonia at a rate of 331.6 ± 55.7 nmol gFW^{-1} h^{-1} while in HEA seawater, which did not differ from the excretion rate of control animals in ammonia-free seawater. When reintroduced to ammonia-free seawater, HEA acclimated animals excreted ammonia at a rate of 733.0 ± 99.6 nmol gFW^{-1} h^{-1} and was significantly higher than the excretion rate of control animals in ammonia-free seawater (Fig. 9).

HEA acclimation also induced changes in mRNA expression levels of genes that play a putative role in ammonia regulation. Following HEA acclimation, the ventral gill half-lamella exhibited a duplication in relative mRNA expression levels of Rh-protein compared to control levels, while in the dorsal half-lamella, a three-fold increase of the expression level was observed (Fig. 10). A similar change occurred in the relative CA-2 expression, where it was upregulated in both the ventral and dorsal half-lamellae after HEA acclimation. Within HEA animals, the ventral epithelium exhibited over 3-fold higher Rh-protein, CA-2, and HCN expression levels than the dorsal epithelium, similar to the pattern found in control animals. No effect of HEA acclimation was observed on NKA, HAT, and HCN mRNA expression levels in the ventral and dorsal half-lamellae (Fig. 10).

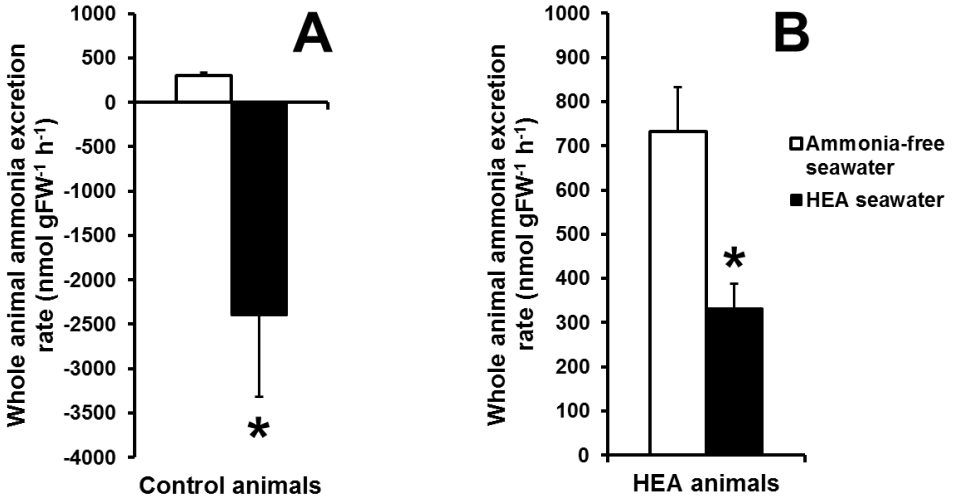

Fig. 9. Whole animal ammonia excretion by juvenile *L. polyphemus* following a 7-9 day acclimation to control (6 µmol l^{-1} total ammonia) or high environmental ammonia (HEA; 965 µmol l^{-1} NH_4Cl) seawater. Animals from each acclimation condition were exposed to ammonia-free water and HEA water (1000 µmol l^{-1} NH_4Cl), and the ammonia excretion rates were determined. Data represent mean ± s.e.m. *Significant difference in excretion rate between animals placed in ammonia-free seawater and HEA seawater within each short-term acclimation period (Kruskal-Wallis test with *post hoc* Mann-Whitney pairwise comparison; $P<0.05$; n=5-6).

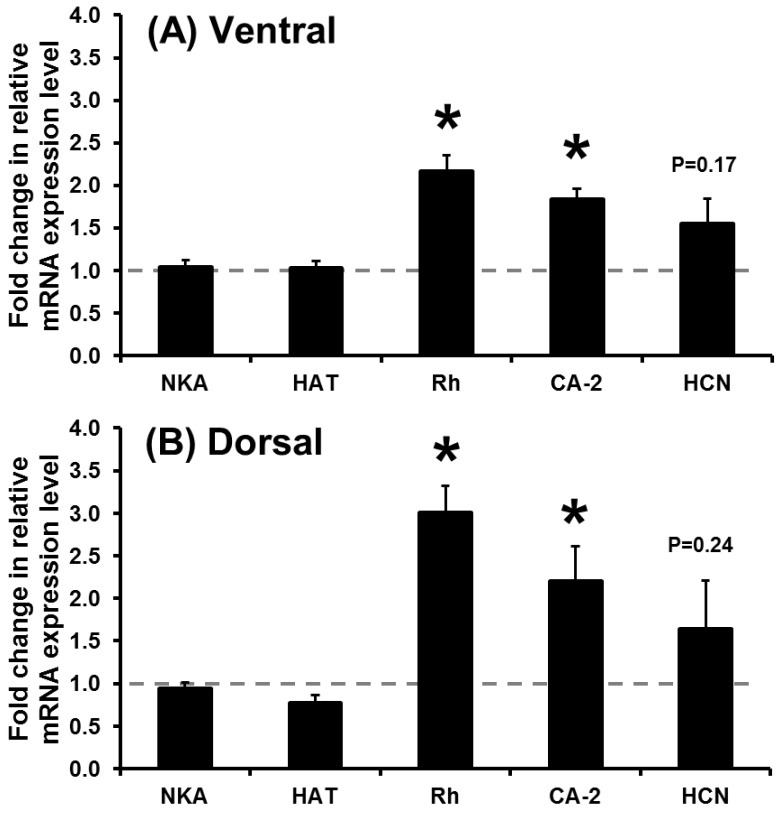

Fig. 10. Effect of high environmental ammonia acclimation on mRNA expression of genes involved in acid-base regulation. Expression levels of Na^+/K^+-ATPase α-subunit (NKA), H^+-ATPase subunit B (HAT), Rhesus protein (Rh), cytoplasmic carbonic anhydrase (CA-2), and hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN) relative to RNA polymerase II in the (A) ventral and (B) dorsal epithelia of high environmental ammonia (HEA) acclimated adult *L. polyphemus* are compared in fold-change to control animals (gray dashed line). Data represent mean ± s.e.m. *Significant difference in relative mRNA expression between control and HEA (965 μmol l^{-1} NH_4Cl) animals (two-tailed Mann-Whitney test for Rh of ventral epithelium; two-tailed t-test for all other comparisons; $P<0.05$; n=5-6). P-values are given for any comparison that may visually appear to be significant.

4. Discussion

4.1. Ammonia excretory mechanism

As a putative acid-base regulatory compound, inherent patterns of ammonia (NH_3/NH_4^+) regulation were investigated in *L. polyphemus*. Hemolymph ammonia concentration of adult horseshoe crabs (Table 3) were comparable to other marine invertebrates of similar size, such as the Dungeness crab (*Metacarcinus magister*; Hans et al., 2014; Martin et al., 2011) and the common octopus (*Octopus vulgaris*; D. Weihrauch, pers. communication). Ammonia excretion rates of juvenile North American horseshoe crabs (422 nmol gFW^{-1} h^{-1} in seawater pH 8.2) was higher than the rates measured for its Asiatic counterparts, *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* (~120 nmol gFW^{-1} h^{-1} and ~260 nmol gFW^{-1} h^{-1}, respectively; Hu et al., 2011), which may be representative of interspecific differences in physiology. Nevertheless, the excretion rate of *L. polyphemus* lies well within the range for ammonotelic marine brachyuran crabs in full-strength seawater (200-552 nmol gFW^{-1} h^{-1}; Durand and Regnault, 1998; Martin et al., 2011). Whilst other forms of nitrogenous waste products need to be investigated in future studies, one can deduce that, as with all other marine invertebrates studied thus far (reviewed in Larsen et al., 2014; Wright, 1995), *L. polyphemus* is also ammonotelic.

Acute exposure of juvenile *L. polyphemus* to acidic seawater (pH 6.1) evoked an increase in ammonia excretion rates (Fig. 2). Past studies investigating freshwater invertebrates, such as the giant river prawn *Macrobrachium rosenbergii* and the ribbon leech *Nephelopsis obscura*; Chen and Kou, 1996; Quijada-Rodriguez et al., 2015), reported a similar response when environmental pH was decreased by 3 pH units, indicating the occurrence of a pathway for apical NH_3-trapping as has been suggested for

the general excretion mechanism in the ammonia-excreting epithelia of freshwater animals (Larsen et al., 2014; Wright and Wood, 2009). Together with enhanced ammonia excretion in low pH environments, two Rh-proteins (potential NH_3 channels) were found to be expressed in the gills of *L. polyphemus*, which hint at apical NH_3-trapping. However, it is unlikely in marine animals that apical ammonia trapping plays a major role in the excretory process as the seawater carbonate buffering system would rapidly abolish any H^+ actively pumped across the apical epithelium (Fabry et al., 2008) rendering this excretory mechanism energetically futile. Instead, perhaps the animal exploits acid-base properties of ammonia to compensate for the environmental pH stress. Given that the ventral branchial half-lamella of *L. polyphemus* is leaky to ions (Fig. 6), an acidic environment may facilitate H^+ influx into the hemolymph space, which must be counteracted in order to maintain acid-base homeostasis. As NH_4^+ acts as an acid equivalent, increased ammonia excretion rates in *L. polyphemus* upon exposure to acidic environments may therefore serve to reduce the hemolymph acid load. In addition to H^+ influx, the leaky ventral epithelium might also allow the passive excretion of NH_3 through paracellular, transporter-independent pathways during increased ammonia excretion.

In the opposite regard, *L. polyphemus* exposed to alkaline environments decreased their ammonia excretion (Fig. 2). Under these conditions it is assumed that the horseshoe crab was losing acid equivalents to its surrounding environment due to the outwardly directed H^+ gradient. Reducing its ammonia excretion rate should allow the animal to retain acid equivalents, thus counteracting environmental stress.

Pharmacological agents were utilized in this study to further investigate the ammonia excretory mechanism of *L. polyphemus*. As the application of these drugs was not isolated to a specific tissue but rather to the entire intact animal, there is a possibility that: 1) the drugs were unable to penetrate the branchial cuticle and/or epithelia, but 2) could also affect general physiological processes that do not occur in the gills but rather in the rest of the body, which has secondary effects on ammonia excretion rates. Therefore, caution must be taken when interpreting the results of this experimental series.

Compromised accessibility may help to explain the relatively small effect of pharmacological agents used in this study on targets in the cuticle-protected apical membrane, and even more so on target transporters localized to the basolateral membrane of branchial tissue. An example of potentially compromised inhibition was observed when employing ouabain, an inhibitor of Na^+/K^+-ATPase (NKA; Furriel et al., 2004; Prassas and Diamandis, 2008), which significantly reduced ammonia excretion in *L. polyphemus* but only by 11% (Fig. 3A). NKA has been found to play an important role in the ammonia excretory mechanism of nearly all investigated aquatic animals so far, as shown for the gills of fish (Claiborne et al., 1982; Nawata et al., 2010) and crustaceans (Weihrauch et al., 1998; Weihrauch et al., 1999), anal papillae of mosquito larvae (Chasiotis et al., 2016), as well the skin, integument, and hypodermis of leeches/frogs, planarians, and nematodes, respectively (Adlimoghaddam et al., 2015; Cruz et al., 2013; Quijada-Rodriguez et al., 2015; Weihrauch et al., 2012). In fact, NKA can utilize NH_4^+ as substrate in place of K^+ (Choe et al., 2000; Skou, 1960) as also demonstrated in the current study on *L. polyphemus* NKA isolated from branchial tissue. Therefore, *L. polyphemus* NKA may very well be responsible for pumping NH_4^+ from the

hemolymph space into the cytoplasm of branchial epithelium cells. Interestingly, NKA activity (17 nmol ADP min $^{-1}$ mg protein $^{-1}$) observed in juvenile *L. polyphemus* using K^+ as substrate was lower than that found in an earlier study of adult *L. polyphemus* acclimated to the same salinity (~40 nmol ADP min^{-1} mg protein^{-1}; Henry et al., 1996). It should be noted that there was a noticeable temperature difference used during both enzyme assays; this study conducted all assays at 20°C while the investigation using adult animals (Henry et al., 1996) used 30°C. NKA activity is positively correlated with temperature within the 20-30°C range as shown for invertebrate and vertebrate NKA (e.g. sea urchin, carp; Leong and Manahan, 1997; Metz et al., 2003), and may explain the detected over 2-fold difference in activity level between this study and the results found by Henry et al. (1996). Further evidence of the participation of NKA derives from the observation that ammonia excretion rates increased after inhibition of phosphodiesterase by theophylline (Fig. 3B), which is similar to the freshwater leech (*N. obscura*; Quijada-Rodriguez et al., 2015) and the African clawed frog (*Xenopus laevis*; Cruz et al., 2013). This can ultimately lead to the activation of NKA and V-type H^+-ATPase (HAT) through increased intracellular cAMP levels caused by interrupted cAMP degradation, thus enhancing ammonia excretion (Amer and Kreighbaum, 1975; Butcher and Sutherland, 1962; Chibalin et al., 1992; Dames et al., 2006).

It is worthwhile to note that *L. polyphemus* appears to be very ouabain-sensitive in comparison to other investigated invertebrates. Exposure to 1 and 5 mmol l^{-1} ouabain was lethal within 2 hours of exposure, whereas the freshwater planarian (*Schmidtea mediterranea*) and the freshwater leech (*N. obscura*) tolerated 1.5 hours of 1 and 5 mmol l^{-1} ouabain, respectively (Quijada-Rodriguez et al., 2015; Weihrauch et al., 2012).

On the other hand, the marine polychaete (*Pareurythoe borealis*) was sensitive towards even lower ouabain concentrations than *L. polyphemu*s and exhibited fatality after a 1 hour exposure period to 0.1 mmol l^{-1} ouabain (D. Weihrauch, pers. communication). A possible explanation for such sensitivity to ouabain is the affinity of NKA to the inhibitor; for example, mammalian (dog, *Canis lupus familiaris*) NKA has been shown to exhibit a higher affinity for ouabain than the invertebrate counterpart (green crab, *Carcinus maenas*; Postel et al., 1998). Perhaps a similar phenomenon exists in the horseshoe crab, although future experimentation comparing the structure of mammalian and *L. polyphemus* NKA would be required to determine whether the protein's affinity to ouabain is indeed the cause of increased sensitivity of *L. polyphemus* to the drug.

To further characterize the ammonia excretory process in *L. polyphemus*, carbonic anhydrase (CA) was blocked by environmental application of acetazolamide (Fig. 3A). CA is predicted to provide H^+ for apical NH_3-trapping in freshwater organisms, which is made possible by the poorly buffered environment (Freire et al., 2008; Larsen et al., 2014; Shih et al., 2008; Wilson et al., 1994). Consequently, application of acetazolamide inhibited ammonia excretion in freshwater species such as leeches and planarians (Quijada-Rodriguez et al., 2015; Weihrauch et al., 2012). In freshwater animals, the transporters involved in the mechanism of apical NH_3-trapping are thought to be partially coupled with NaCl uptake, where active H^+ excretion across the apical cell membrane by HAT not only acidifies the apical unstirred boundary layer but also reduces the membrane potential to become more negative with respect to the environment, which promotes Na^+ influx through apical Na^+ channels (Hwang and Lee, 2007). CA has also been hypothesized to provide counter-ions for cation and anion exchangers involved in

Na^+ and Cl^- uptake in freshwater animals (Whiteley et al., 2001). Interestingly, however, CA does not appear to play a large role in ammonia excretion in brackish water-acclimated *C. maenas* as the addition of acetazolamide in the perfusion solution (artificial hemolymph) of isolated perfused gills caused an insignificant reduction in ammonia excretion rate by 18% (Weihrauch et al., 1998). A similar decrease in ammonia excretion was found in *L. polyphemus*, but as previously mentioned, acetazolamide was applied to the whole animal rather than directly as artificial hemolymph to an isolated gill, therefore imposing the risk of incomplete inhibition. Further experiments applying acetazolamide on Ussing chamber-mounted gill epithelia would determine the extent of CA involvement in *L. polyphemus* ammonia excretion.

CA is also essential to acid-base regulation in relation to the carbonate system given that the enzyme directly alters the proportions of the inorganic carbon species (Henry and Wheatly, 1992). Therefore, acetazolamide-induced reduction in ammonia excretion by *L. polyphemus* not only suggests the direct involvement of CA in ammonia excretion, but that acetazolamide may have also impaired CO_2 excretion and subsequently affected ammonia excretion. Any disruption in normal CO_2 excretion could lead to changes in the animal's acid-base status (Henry and Wheatly, 1992), and as ammonia can serve either as a weak acid (NH_4^+) or weak base (NH_3), ammonia and acid-base regulation are likely coupled to some extent (Boron, 2004). To determine whether acetazolamide indirectly inhibited the ammonia excretory mechanism of *L. polyphemus*, metabolic rate measurements of intact animals as an index of CO_2 production in the absence and presence of the drug are required in future studies.

In addition to the participation of NKA and CA, it was of interest whether the gills of the horseshoe crab also possess a vesicular microtubule-dependent ammonia excretory pathway, as suggested for *C. maenas* (Weihrauch et al., 2002) and a nematode (*Caenorhabditis elegans*; Adlimoghaddam et al., 2015). In *C. maenas*, it was assumed that rather than apical NH_3-trapping, NH_3 becomes trapped within acidified intracellular vesicles instead, and the NH_4^+-filled vesicles could then be transported to the apical membrane for exocytotic release (Weihrauch et al., 2002). To determine whether a similar mechanism is also in place in the horseshoe crab, animals were exposed to seawater dosed with colchicine, a drug known to displace key interactions in microtubule formation that ultimately prevents tubulin molecules from assembling into its straight structure (Ravelli et al., 2004; Wilson and Farrell, 1986). Surprisingly, exposure of *L. polyphemus* to colchicine caused a trend towards increased ammonia excretion (Fig. 3A) and it became apparent that vesicular NH_3-trapping may be utilized by this species in ammonia absorption, a phenomenon that has been previously shown in the midgut of the tobacco hornworm (*Manduca sexta*; Weihrauch, 2006). For a possible explanation, one has to keep in mind that ammonia might very well serve as a valuable acid-base equivalent. Depending on the need to excrete or uptake ammonia according to its acid-base homeostasis, *L. polyphemus* branchial cells presumably have the means to regulate ammonia transport to and from the hemolymph. The purpose of this ability is perhaps to maintain an optimal hemolymph concentration of approximately 300 µmol l^{-1} (Table 3) and such regulation of hemolymph ammonia concentration to a specific range was also observed in *O. vulgaris* (D. Weihrauch, pers. communication). When the perfused octopus gill was exposed to a variety of artificial hemolymph ammonia concentrations, it

exhibited either an excretion or retention of ammonia as needed to maintain the hemolymph concentration at 250-300 µmol l^{-1} (D. Weihrauch, pers. communication). Taking into account the leaky ventral epithelium of *L. polyphemus* gills (Fig. 6) and the outwardly directed ammonia gradient, a potentially substantial ammonia loss under control conditions may be partly countered *via* the shuffling of ammonia back towards the hemolymph using the microtubule network in order to retain ammonia for acid-base homeostasis.

4.2. Differential branchial regions

In the horseshoe crab, the site of acid-base regulation and ammonia excretion has not been previously determined, but was predicted to be the gills as is the case for many other non-mammalian marine animals (e.g. crustaceans and fish; Evans et al., 2005; Freire et al., 2008; Weihrauch et al., 2009). Results of mRNA expression levels across different tissues in *L. polyphemus* provide support for such prediction; Rh transcript levels were significantly more abundant in the gills compared to the coxal gland and brain, which is indicative of major CO_2/ammonia transport function of the tissue (Fig. 4A). However, it must be mentioned that Rh primers utilized in this study were recently found to bind to two Rh isoforms. As a result, the observed Rh expression levels should be considered as the sum of mRNA expression of the two isoforms. Previous studies on multiple Rh isoforms within the same species have shown that usually only one Rh isoform exhibits particularly high abundance; for example, *C. elegans* CeRhr-1 was 13-fold higher expressed in whole body homogenates compared to CeRhr-2 (Adlimoghaddam et al., 2015). It is then reasonable to assume that one isoform is likely

responsible for the majority of Rh transcript levels presented in the current study; however, future studies utilizing isoform-specific primers as well as protein quantification are required to determine the exact ratio of the two isoforms in each of the investigated tissues.

The ultrafiltrating coxal gland of *L. polyphemus* may serve as an acid-base and ammonia regulatory organ, secondary to the gills. In the present work, the coxal gland of seawater-acclimated adult animals expressed comparable levels of NKA to the central and peripheral regions of the gill (Fig. 4B). With NKA likely being a key player in these processes (e.g. Claiborne et al., 1982; Fehsenfeld and Weihrauch, 2016a; Perry and Gilmour, 2006; Siebers et al., 1994; Weihrauch et al., 1998), further investigation to determine the extent of coxal gland involvement should be conducted. However, it must also be kept in mind that the coxal gland may be at a disadvantage because it is not in direct contact with the environment; therefore, regulation is limited to secretion of the respective waste products rather than through exchange with the environment.

Within the gills of *L. polyphemus*, a previous study on ultrastructural differences between various gill regions suggested that the thick-celled, mitochondria-rich central region of the ventral epithelium possess the capacity for active ion transport due to its ultrastructural characteristics while the remaining peripheral branchial regions lacked such features (Henry et al., 1996). Even though acid-base regulation and ammonia excretion often have an active component (reviewed in Larsen et al., 2014), the abundance in mitochondria and membrane infoldings within the branchial tissues of marine brachyuran crabs does not appear to correlate with the capacity for branchial pH regulation and active ammonia excretion (Fehsenfeld and Weihrauch, 2013; Martin et al.,

2011; Weihrauch et al., 1999). Keeping in mind the potential for acid-base regulatory and ammonia excretory functions of both thick and thin-celled gill regions regardless of the presence of active ion transport characteristics, various branchial regions of adult *L. polyphemus* were investigated in this study.

L. polyphemus gill lamellae serve as a useful transport model system in that each lamella has a large surface area that can be split into the ventral and dorsal epithelia, opening up numerous possibilities in measuring ion transport and other parameters relating to acid-base and ammonia regulation. In this study, experiments utilizing split gill lamellae mounted in Ussing chambers showed strikingly opposing results between the two half-lamellae (Fig. 6). The central region of the ventral epithelium of adult animals exhibited moderately high ion permeability as shown by its conductance (G_{TE} = 145 mS cm^{-2}), a value that was medial to the leaky gills of a marine osmoconforming crab (*Cancer pagurus*; 253-282 mS cm^{-2}) and the moderately leaky gills of brackish acclimated hyperregulating *C. maenas* (41-62 mS cm^{-2}; Weihrauch et al., 1999). In contrast, the conductance of the central region of the dorsal epithelium (0.2 mS cm^{-2}) was even less permeable to ions than the tight branchial epithelium of both the anterior and posterior gills of the freshwater Chinese mitten crab (*Eriocheir sinensis*; 4 mS cm^{-2}). In *E. sinensis*, the high ion-resistance of the branchial epithelia is thought to be the crab's adaptation for reducing ion loss in an extremely hyposmotic environment (Weihrauch et al., 1999), but its function in the dorsal epithelium of *L. polyphemus* gill lamellae is so far unknown because animals exhibiting this phenomenon were not under osmotic stress. Under an *in vivo*-like internal to external ammonia gradient of 300 µmol l^{-1} NH_4Cl, the ventral epithelium excreted ammonia at a rate of 220 nmol cm^{-2} h^{-1} (Fig. 6). In contrast,

no net transepithelial ammonia excretion was observed in the dorsal epithelium (Fig. 6), which was an unexpected phenomenon given the outwardly directed ammonia gradient that promotes at least partial passive ammonia loss to the apical side. In the similarly low ion-permeable gills of *E. sinensis*, application of an outwardly directed gradient of 100:0 μmol l^{-1} still resulted in an excretion of ammonia, albeit at a slower rate than the ion-leaky gills of *C. pagurus* (Weihrauch et al., 1999). Interestingly, *L. polyphemus* dorsal epithelium was still metabolically active as the tissue continued to produce ammonia even more so than the ventral epithelium, although the reason behind this finding requires further investigation.

At the molecular level, the active pumps NKA and HAT were identified in *L. polyphemus* gills and mRNA expression levels revealed a uniform distribution of both genes regardless of branchial region (Figs. 4B, 5, 7), thereby suggesting a lack of difference in active ion transport utilizing these pumps. As previously mentioned, the role of HAT in apical NH_3-trapping as a means of acid excretion in species living in a highly buffered environment is less important than freshwater species that can take advantage of localized environmental pH manipulation to promote ammonia excretion (Fabry et al., 2008; Larsen et al., 2014; Quijada-Rodriguez et al., 2015; Weihrauch et al., 2009; Weihrauch et al., 2012; Wright and Wood, 2009). HAT may participate in vesicular NH_3-trapping in *L. polyphemus* where it serves to acidify vesicles to aid in shuttling ammonia back towards the hemolymph space rather than excreting the waste product to the environment (Fig. 3A). As for its role in acid-base homeostasis, HAT is presumably important given that its substrate directly determines pH, but given the fact that ammonia may also be a valuable acid-base equivalent, HAT may play a role in this process *via*

vesicular ammonia uptake. Future inhibitor experiments targeting HAT as well as enzyme activity and cellular localization of the pump in isolated branchial lamellae will help to determine its role in *L. polyphemus*.

With regards to NKA, results of this study are in contrast to findings by Henry et al. (1996), where the authors observed higher NKA enzyme activity in the central and ventral regions compared to the peripheral and dorsal regions, respectively. This may be explained by limitations of quantitative PCR results, where mRNA expression does not always correlate with enzyme activity as post-translational processes could affect the final abundance and/or activity of the protein. An example of a study where such discrepancy occurred was conducted on the blue crab (*Callinectes sapidus*), where NKA activity doubled in animals acclimated to dilute salinity while the mRNA expression level did not differ (Towle et al., 1976; Towle et al., 2001). It was then suggested by the authors that post-translational regulatory processes (e.g. subunit assembly, membrane trafficking, or cell signalling) are responsible for the increase in enzyme activity required to cope with a low salinity environment (Towle et al., 2001). Perhaps a similar occurrence took place in *L. polyphemus*, where post-translational regulatory processes (such as changes in intracellular cAMP to activate NKA; Fig. 3B) take precedence over mRNA transcript abundance regarding the control of protein production and/or activation.

However, if the mRNA expression data is indeed an accurate representation of protein abundance and subsequent enzyme activity of these active pumps in the animals used in the present study, and if the mitochondria-rich central region of the ventral epithelium indeed possesses similar enzyme activity levels as the rest of the branchial

regions, it would suggest that the capacity for active ion transport utilizing NKA and HAT is similar between all branchial regions of *L. polyphemus*. A couple of things must then be recognized; firstly, NKA is also a main transporter for NaCl uptake in hyperregulating animals in dilute seawater environments (Larsen et al., 2014). Given that: 1) *L. polyphemus* in this study were not actively osmoregulating, 2) the animals were not under any pH stress, and 3) the body fluid ammonia concentration of *L. polyphemus* (0.3 mmol l^{-1}; Table 3) is at a much smaller scale than inorganic osmolytes such as Na^+ (445 mmol l^{-1}; Robertson, 1970), acid-base and ammonia homeostasis under control conditions presumably require less energy and machinery than mass NaCl transport. Therefore, similar mRNA transcript levels and subsequent enzyme activity of NKA between different branchial regions are most likely adequate for basic acid-base and ammonia regulation in the absence of other more energy-demanding processes such as osmoregulation. Secondly, the uniformity in mRNA expression of two of the main active pumps may also point towards the possibility that other high energy-demanding processes utilize the abundance in mitochondria and membrane infoldings in the central region of the ventral epithelium, although these processes are so far undetermined.

Unlike the uniform expression pattern of NKA between tissues, *L. polyphemus* exhibited higher HCN mRNA expression levels in the central and ventral regions compared to the peripheral and dorsal regions, respectively (Figs. 5, 7). These findings were similar to results observed by Fehsenfeld and Weihrauch (2016b), where *C. maenas* HCN expression was slightly higher in the mitochondria-rich posterior gills that ultrastructurally resemble the central region of the ventral epithelium (Henry et al., 1996). The role of HCN in acid-base and ammonia regulation has not been extensively studied,

with the exception that branchial HCN expression was down-regulated in the green crab when animals were exposed to elevated environmental ammonia and P_{CO2} levels (Fehsenfeld and Weihrauch, 2016b). In this crab, basolateral localization within the branchial tissues was assumed due to the observed inhibition of ammonia excretion after basolateral blockage of the channel (Fehsenfeld and Weihrauch, 2016b). Also, in the mammalian kidney collecting duct, Carrisoza-Gaytán et al. (2011) showed the involvement of HCN2 in NH_4^+ transport and subsequently suggested a potential role in renal pH regulation. Perhaps much like in the mammalian kidney, *L. polyphemus* HCN can indeed transport NH_4^+ and are involved in acid-base and ammonia regulation, though further studies are required to confirm such theory.

Despite the lack of regional difference in NKA and HAT mRNA expression, the central region and ventral half-lamella of adult horseshoe crab gills showed high potential in acid-base and ammonia regulation through passive-mediated transport given its significantly high expression of Rh-protein and CA-2 compared to the peripheral region and dorsal half-lamella, respectively (Figs. 4A, 5, 7). Henry et al. (1996) also reported considerably higher enzyme activity of CA in the central region compared to the periphery, although this activity encompasses both the cytoplasmic and membrane-bound isoforms of CA, and at this time, the membrane-bound isoform of *L. polyphemus* CA has not been identified and its expression pattern needs to be investigated in future studies. As for Rh-protein, it has not been previously studied in *L. polyphemus* and although its localization is yet to be determined, basolateral and/or apical Rh-proteins have been suggested in the acid-base regulatory and ammonia excretory epithelia of other animals such as fish, mammals, and *C. elegans* (Adlimoghaddam et al., 2016; Han et al., 2006;

Nakada et al., 2007; Verlander et al., 2003). Basolaterally-localized Rh-proteins may accelerate metabolic CO_2/ammonia transport from the hemolymph into the cytoplasm, where CA-2 can hydrate CO_2 to produce HCO_3^- and H^+ as substrates for transporters involved in ammonia and acid-base regulation (Gilmour and Perry, 2009; Weihrauch et al., 2002). If one of the *L. polyphemus* Rh-protein isoforms is located on the apical membrane similar to Rhcg2 in fish gills (e.g. *Takifugu rubripes*; Nakada et al., 2007), it may directly provide facilitated transport of CO_2/ammonia into the environment if a partial pressure gradient is present. Nevertheless, the findings of substantially higher expression of Rh-protein and CA-2 in branchial cells of the central region and the ventral epithelium suggest that these epithelia are more specialized in facilitated diffusion of these metabolic waste products compared to the peripheral and dorsal regions, respectively. Cellular localization and functional expression of the identified Rh-proteins would determine where these proteins are situated and which of the possible substrates (CO_2, NH_3, and/or NH_4^+) may be transported by this channel.

4.3. Effects of elevated environmental ammonia and CO_2

As burying organisms (Botton, 1984), horseshoe crabs regularly encounter stagnant waters in their direct vicinity due to the limited water circulation within the sediment. Whilst buried, the animals continue to excrete metabolic wastes such as CO_2 and ammonia, which rapidly accumulate thus exposing them to unfavourable conditions. Excess levels of these compounds can also be caused by anthropogenic factors, such as ocean acidification and wastewater contamination (Caldeira and Wickett, 2005; Fisher et al., 1988). Such shifts in environmental parameters can alter the partial pressure and/or

concentration gradients of the waste products between the body fluid and the environment, and if this species regularly encounters high levels of environmental waste, it has likely developed efficient counteractive mechanisms to these stressors. To study the effects of these environmental challenges on acid-base homeostasis and ammonia regulation, juvenile and adult *L. polyphemus* were exposed to either hypercapnic seawater or high environmental ammonia (HEA) for a period of 7-9 days.

The hypercapnic conditions used in this study (P_{CO2} of 311 Pa, pH 7.4) are similar to the predicted future ocean scenario for the year 2300 (Caldeira and Wickett, 2005) in order to investigate how the horseshoe crab will fare with environmentally relevant elevation of CO_2. Hypercapnia acclimated adults experienced an increase in total environmental inorganic carbon and exhibited the typical signs of full-compensation for respiratory acidosis, which included a same-scale rise in hemolymph P_{CO2} and HCO_3^- concentration to maintain the same hemolymph pH (Table 3). Strikingly similar changes in the hemolymph carbonate system parameters were also observed in the Dungeness crab (*M. magister*) when acclimated to comparable seawater conditions (Hans et al., 2014), indicating that both species are capable of compensating for this level of environmental acidification. In addition to changes in hemolymph inorganic carbon parameters, the hemolymph ammonia concentration of adults and whole animal ammonia excretion rate of juveniles decreased by 43% after hypercapnia acclimation (Table 3). This correlated decrease in both parameters is suggestive of a passive component in the ammonia excretory process, possibly due to the leakiness of the ventral epithelium to ions (Fig. 6) and supported by the observed strong ammonia influx when exposed to HEA (Fig. 9). As suggested for *M. magister* and the peanut worm (*Sipunculus nudus*),

metabolic depression likely occurred in *L. polyphemus* during exposure to hypercapnic conditions (Hans et al., 2014; Langenbuch and Pörtner, 2008; Pörtner et al., 1998), although follow-up studies measuring O_2 consumption in the horseshoe crab are required as confirmation.

Interestingly, at the molecular level, high P_{CO2} acclimation caused no changes in branchial mRNA expression levels in all investigated genes (Fig. 8). This was in contrast to findings in the osmoregulating *C. maenas*, where mRNA levels for branchial Rh-protein was down-regulated while membrane-bound CA and NKA were up-regulated following hypercapnic acclimation of similar levels (Fehsenfeld and Weihrauch, 2013). Unlike *C. maenas*, a short-term acclimation to a hypercapnia level mimicking a near-future ocean scenario either did not give *L. polyphemus* adequate time to modify its mRNA expression pattern or was not detrimental to the animal so as to stimulate changes in mRNA transcript levels. Perhaps a long-term study conducted at similar P_{CO2} levels over several generations involving all life stages should be carried out to investigate whether the capacity of *L. polyphemus* to withstand this scale of hypercapnia stress can be maintained, which would also better represent the animal's response to future ocean acidification scenario.

To observe the possible effects of HEA, control juveniles were exposed to 1 mmol l^{-1} NH_4Cl seawater, where ammonia excretion plummeted to the point of influx (Fig. 9) likely because the high G_{TE} of the ventral epithelium allowed for immediate ammonia diffusion into the body fluids (Fig. 6). This indicates an inability for rapid upregulation of the animal's ammonia excretory mechanism to counteract the sudden influx of ammonia. However, when horseshoe crabs were previously acclimated to HEA

seawater for a minimum of 1 week, the animals exhibited normal excretion rates, thereby demonstrating an active ammonia excretion against an over 3-fold inwardly-directed gradient. During HEA acclimation, the animals apparently developed an increased efficiency in ammonia excretion that is required to continue releasing this nitrogenous waste even against an inwardly-directed gradient and maintaining an optimal hemolymph ammonia concentration of around 300 μmol l^{-1} (Table 3). Interestingly, mRNA expression analysis of the two major pumps usually involved in ammonia excretory processes (NKA and HAT) showed no change in abundance, which hints that at control mRNA levels, both pumps exhibit the plasticity to cope with this level of HEA challenge possibly due to increased protein translation/activity.

In addition to an active component of the ammonia excretory mechanism during HEA, other ways of coping with such environmental stress include a rapid conversion of ammonia into other less toxic nitrogenous waste forms, which has been observed in the swimming crab (*Portunus trituberculatus*; Liu et al., 2014). In *P. trituberculatus*, a 48-hour acclimation to 0.3 mmol l^{-1} NH_4Cl induced an increase in glutamine and urea levels in the hemolymph, and these changes were also accompanied by increased activity levels of enzymes important for the biosynthesis of glutamine and urea, including glutamate dehydrogenase, glutamine synthetase, and arginase. *L. polyphemus* may also use a detoxification strategy under HEA conditions to reduce the elevating hemolymph ammonia load into a tolerable range; as such, alternative nitrogenous wastes should be investigated in future studies.

The horseshoe crab's response to HEA acclimation was quite opposite to that of *M. magister* in that acclimation to 1 mmol l^{-1} NH_4Cl caused the Dungeness crab

hemolymph ammonia concentration to reach near environmental levels (Martin et al., 2011). Also, in *M. magister*, ammonia excretion rates ceased and Rh-protein mRNA expression in the posterior gills decreased (Martin et al., 2011). Unlike the Dungeness crab, *L. polyphemus* hemolymph ammonia concentration and ammonia excretion were unaffected by HEA acclimation, and Rh-protein expression was up-regulated instead (Table 3; Figs. 9, 10). Thus, based on these results, the two marine arthropods appear to have undertaken different strategies of coping with this level of HEA.

An increased efficiency in coping with HEA acclimation may have occurred at the cost of higher energy consumption due to active ammonia excretion, therefore producing more metabolic wastes such as CO_2. HEA acclimation may have also impaired CO_2 excretion itself as following HEA acclimation, adult *L. polyphemus* hemolymph acid-base status trended towards respiratory acidosis as characterized by a slight pH decrease and an accompanying significant P_{CO2} and [HCO_3^-] increase (Table 3). The mRNA expression of two proteins important for acid-base balance, Rh and CA-2, were significantly up-regulated after HEA acclimation in both the ventral and dorsal epithelia of adults (Fig. 10). The up-regulation of the Rh-protein may also enhance NH_3 and/or NH_4^+ transport (Caner et al., 2015) during active ammonia excretion in HEA animals. Given these results, HEA acclimation has induced modifications in acid-base regulation in *L. polyphemus* likely due to an increased demand for CO_2 excretion and/or impaired CO_2 excretion, which could be further investigated by measuring the animal's metabolic rate in future studies.

Despite high similarities in morphology, it is also possible that there may be physiological differences between the juveniles and adults used in this study. However,

both age groups exhibited similar patterns in ammonia regulatory response to hypercapnia, where reduced ammonia excretion by juveniles and a decrease in hemolymph ammonia concentration of adults occurred at the same scale of 43% (Table 3). In addition, both age groups showed active ammonia excretory capacities when challenged with HEA as seen by the lack of change in ammonia excretion and hemolymph ammonia concentration (Fig. 9; Table 3), which were tested on juveniles and adults, respectively. Regardless, future studies comparing the juveniles' and adults' response to environmental stresses would be beneficial in determining physiological differences between the two age groups, if any.

5. Conclusions and Future Directions

This project serves as a pilot study on a marine invertebrate species that is ideal for examining acid-base regulatory capacities, particularly regarding ion transport across the conveniently large gill epithelium. Considering the unchanging morphology of horseshoe crabs over 150 million years, it is possible that the mechanisms possessed by *L. polyphemus* are phylogenetically ancient and a comparison to more derived arthropods would be useful in observing how the mechanism of acid-base regulation has evolved in different taxa. Through studies utilizing pharmacological agents in *L. polyphemus*, NKA, CA, and elevated intracellular cAMP were shown to be involved in ammonia excretion. Animals seemed to maintain their hemolymph ammonia concentration at ~300 μmol l^{-1} possibly as a useful reservoir of this acid-base equivalent in order to counteract any acid-base-related disturbances, and the regulation of this parameter is likely to take place in the book gills. *L. polyphemus* was capable of excreting ammonia against a 3-fold

inwardly directed gradient if previously acclimated to such environment, which was interesting given that the mRNA expression of the active pumps usually involved in ammonia excretion (NKA and HAT) were not affected. Thus, the active component in establishing ammonia excretion against a gradient is so far undetermined and future studies would highly benefit from investigating such concept. The ventral and dorsal epithelia were highly differentiated; the former was leaky to ions, allowed transepithelial ammonia excretion during an outwardly directed ammonia gradient, and exhibited high mRNA expression of transporters likely involved in acid-base regulation (Rh, CA-2, and HCN). There remains a wide array of experiments that could be carried out using isolated gill lamellae, including: 1) the use of pharmacological agents to target transporters specifically at the gill epithelium rather than the whole animal, 2) testing various ammonia gradients to determine the gill's ammonia excretory capacities, 3) investigating the role/abundance of both Rh isoforms in different branchial regions, and 4) using antibodies to determine the localization of key transporters in acid-base regulation.

6. References

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Supplemental Tables and Figures

Table S1. Absolute values for mRNA expression levels of genes involved in acid-base regulation in the central gill region. Data are presented for Na^+/K^+-ATPase α-subunit (NKA), H^+-ATPase subunit B (HAT), Rhesus protein (Rh), cytoplasmic carbonic anhydrase (CA-2), hyperpolarization activated cyclic nucleotide-gated K^+ channel (HCN), and RNA polymerase II (Pol2) of control adult *L. polyphemus*. Data represent mean ± s.e.m.

	Starting fg of cDNA per ng of RNA
Gene	**Central gill**
NKA	0.39 ± 0.06
HAT	1.04 ± 0.17
Rh	1.23 ± 0.28
CA-2	0.32 ± 0.12
HCN	0.05 ± 0.02
Pol2	0.05 ± 0.01

Table S2. Absolute values for mRNA expression levels of genes involved in acid-base regulation in the ventral gill epithelium. Data are presented for Na^+/K^+-ATPase α-subunit (NKA), H^+-ATPase subunit B (HAT), Rhesus protein (Rh), cytoplasmic carbonic anhydrase (CA-2), hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN), and RNA polymerase II (Pol2) of control adult *L. polyphemus*. Data represent mean ± s.e.m.

	Starting fg of cDNA per ng of RNA
Gene	**Ventral epithelium**
NKA	2.91 ± 0.27
HAT	0.63 ± 0.07
Rh	68.61 ± 17.77
CA-2	6.66 ± 1.47
HCN	0.06 ± 0.01
Pol2	1.74 ± 0.35

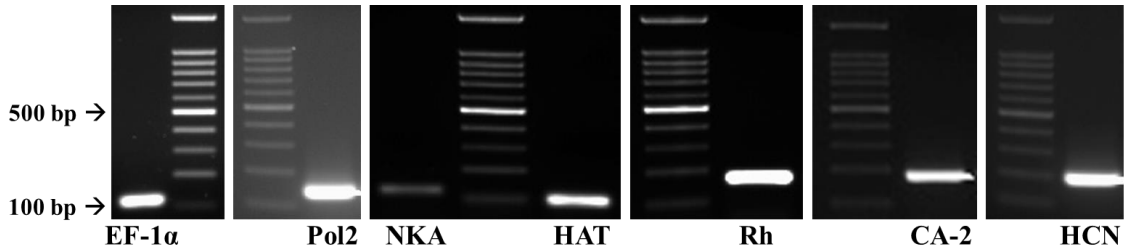

Fig. S1. Gel picture of specific primers for genes used in preparation for quantitative PCR. Genes include: elongation factor-1α F/R (EF-1α; 122 bp), RNA polymerase II F1/R1 (Pol2; 149 bp), Na^+/K^+-ATPase α-subunit F/R (NKA; 128 bp), H^+-ATPase subunit B F/R (HAT; 100 bp), Rhesus protein F/R (Rh; 178 bp), cytoplasmic carbonic anhydrase F1/R1 (CA-2; 191 bp), and hyperpolarization activated cyclic nucleotide-gated K^+ channel F1/R2 (HCN; 207 bp). Sequence size of DNA ladder is shown to the left of the figure.

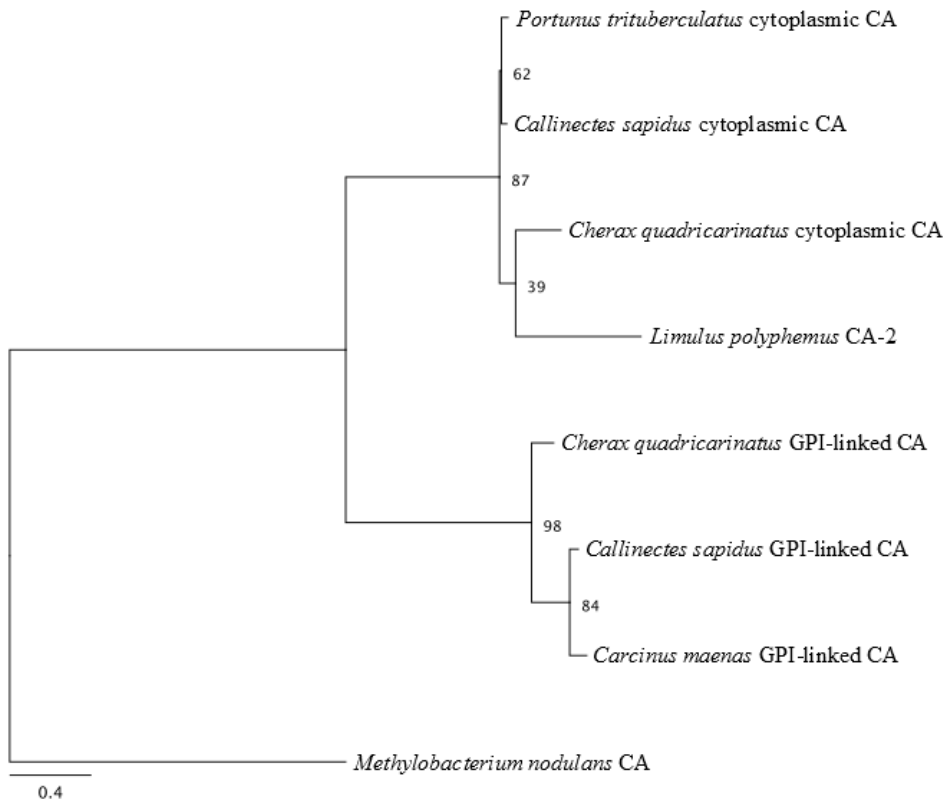

Fig. S2. Maximum likelihood tree showing the relationship of *L. polyphemus* cytoplasmic carbonic anhydrase (CA-2) partial sequence in comparison to cytoplasmic and membrane-bound carbonic anhydrase isoforms of arthropod representatives. GenBank protein accession numbers are as follows: swimming crab *Portunus trituberculatus* cytoplasmic CA (AFV46144), blue crab *Callinectes sapidus* cytoplasmic CA (ABN51213), freshwater crayfish *Cherax quadricarinatus* cytoplasmic CA (AIW68600), green crab *Carcinus maenas* GPI-linked CA (ABX71209), *Callinectes sapidus* GPI-linked CA (ABN51214), and *Cherax quadricarinatus* GPI-linked CA (AIW68601). The tree was rooted using *Methylobacterium nodulans* carbonic anhydrase (ACL55773). *L. polyphemus* partial sequence was based on a genome (GenBank accession no.: AZTN01039886) and the sequence was confirmed with mRNA sequencing using primers located within the partial sequence. The tree was created in RAxML version 7.2.8 using 500 bootstrap replicates and the GTR GAMMA nucleotide model. Node values represent the percent bootstrap support.