CHAPTER FIFTEEN

GAS EXCHANGE AND ACID-BASE BALANCE

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Introduction

Neohelice granulata regularly leaves the water and travels beyond the high tide mark to forage on terrestrial resources such as plants of *Sporobolus*, climbing on them to feed on their soft parts. Early physiological studies have shown that crabs of this species maintain an active rate of oxygen consumption ($\dot{M}O_2$) for several hours during air exposure (Dezi et al. 1987, Santos et al. 1897). These terrestrial excursions require physiological and structural adaptations for an efficient aerial exchange of respiratory gases, which is essential for maintaining the elevated aerobic metabolism required for locomotion.

In water, gills efficiently suffice crabs' respiratory needs, thanks to their large surface area and short diffusion distance between water and hemolymph. Together with high ventilation rates, these features allow for efficient O_2 uptake, despite the relatively low O_2 concentrations found in aquatic environments. Besides, the large CO_2 capacitance of water facilitates CO_2 excretion, resulting in low hemolymph CO_2, ensuring a relatively high pH, but with low [HCO_3^-] and buffering capacity (Dejours 1994).

When an aquatic animal is exposed to air, gills may lose structural support and therefore collapse, resulting in reduced surface area for gas exchange. Although the high O_2 concentration and capacitance in the air may allow still meeting O_2 uptake requirements, despite the smaller respiratory surface area, CO_2 excretion is impaired and may lead to respiratory acidosis. Within brachyuran crabs, there is a high degree of terrestriality and a wide range of air-breathing capabilities, ranging from exclusively aquatic, to intertidal, and to fully terrestrial species. Species living in the low intertidal are exposed to air only during the lowest tides; the large gills of these species are not structurally reinforced and collapse in air (Gray 1957, Taylor and Butler 1978), with a subsequent decrease in O_2 uptake and activity during emersion (Taylor and Wheatly 1979, Burnett and McMahon 1987). In contrast, intertidal species such as *Carcinus maenas* can remain active during short periods of emersion, by taking up O_2 from the water retained within the branchial chamber. However, they suffer a marked reduction in venous pO_2, as the branchial chamber water becomes depleted of O_2 (deFur 1988).

The most active amphibious crabs, the so-called bimodal breathing crabs, can move between water and land freely. During emersion, most of the O_2 uptake takes place across modified parts of the inner walls of the branchiostegite, the branchiostegal lungs. Their gills are reduced and thickened, and excrete CO_2 and nitrogen to the water retained in the branchial chamber, also exchanging ions for acid-base and salt regulation (Henry 1994).

Finally, land crabs such as the Christmas Island crab *Gecarcoidea natalis*, have even more reduced gills and perform most of their uptake and CO_2 excretion across the branchiostegal lungs. Unlike aquatic and amphibious crabs, in which the carbonic anhydrase (CA) enzyme is restricted to the gills, land crabs also have abundant cell membrane-bound CA in the branchiostegal lung epithelium, which allows for efficient CO_2 excretion to air (reviewed in Farrelly and Greenaway 1994, Henry 1994).

Neohelice granulata is an active amphibious crab with unique adaptations for breathing air. Their gills are medium-sized (~600mm^2g^{-1})

[Luquet et al. 2000], not much smaller than gills from intertidal crabs that display low levels of activity during air exposure (Santos et al. 1987, Luquet et al. 2000). However, during this exposure *N. granulata* retains a large volume of water within the branchial chamber (~88% of the total capacity) [Luquet et al. 2000], thus preventing gills from collapsing and providing an O_2 store and CO_2 sink. Moreover, recent work has identified branchiostegal lungs as a potential additional site for O_2 uptake during emersion (Halperin et al. 2000, 2002). Besides, *N.granulata* re-circulates the branchial water over the carapace, which presumably helps to oxygenate it while also ventilating excess CO_2 (Halperin et al. 2000). However, this might exacerbate water evaporation, limiting the duration of land excursions, unless the crab was able to reload the branchial chamber with water, in tide or rain pools. This chapter describes the morphological and physiological adaptations that make *N. granulata* an active amphibious species.

Functional morphology of respiratory organs

N. granulata possesses eight phylobranchiate gills in each branchial chamber, but the first two gills are greatly reduced. As extensively described in crustaceans (reviewed by Taylor and Taylor 1992), the anterior gills (1 to 5 in this species) are respiratory, while the three most posterior gills are predominantly ion-regulatory (Genovese et al. 2000, 2004, Luquet et al. 2000, 2002b). Each gill lamella is a flattened sac formed by a simple epithelium with varying thickness according to the gill function. The respiratory epithelium consists of thin, squamous principal cells with wide lateral expansions ~2µm thick, covered by a ~1.3µm thick cuticular layer (Fig. 15.1), and it does not change with acclimation salinity (Genovese et al. 2000).

Pillar cells from opposite sides abut in the middle of the lamellar hemocoel. The lamellae of both respiratory and ion-regulatory gills have dorsal marginal channels lined with a very thin (attenuated) epithelium, i.e., 0.5 to 1.0µm in gill 3 (Fig. 15.1B), and 0.5 to 1.5µm in gill 8. The cuticular layer of the marginal channel, especially in the dorsal part of the gill, is slightly thickened (up to 3µm) [Luquet et al. 2000]. The reinforced structure and the thinner epithelium of the marginal channels allow gas exchange when the gills collapse in the absence of enough water in the branchial chamber and would cause most of the hemolymph to be contained in the gill marginal areas during emersion. Gills 4 and 5 are mostly lined by a thin respiratory epithelium, but also present a low cubic epithelium bordering part of the dorsal and medial regions of the lamellae.

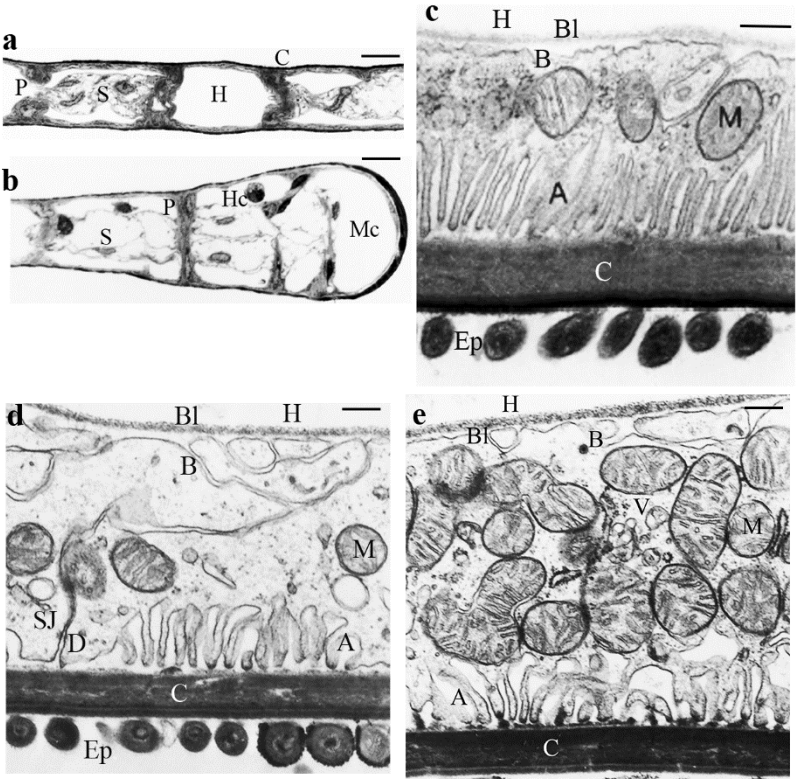

Figure 15.1; a, b. Longitudinal semi-thin sections of gill 3 of *Neohelice granulata*. Proximal (a) and distal (b) zone of a lamella showing respiratory epithelium; a connective septum separates the hemolymphatic space in two; c. Ultrastructure of gill 3 showing infoldings of the apical membrane and scarce mitochondria do not associated with the basolateral membrane; d. Ultrastructure of the type-I cell of gill 5 showing few mitochondria, a long septate junction, and a band desmosome; e. Type-II cell of gill 5 demonstrating high density of mitochondria not associated with basolateral infoldings. A, apical folds; B, basolateral membrane; Bl, basal lamina; C, cuticle; D, band desmosomes; E, epithelium; Ep, epibionts; H, hemolymph; Hc, hemocyte; M, mitochondrion; Mc, marginal channel; S, connective septum; Sj, septate junction. Scale bars: 15µm for a and b, and 1µm for c, d, and e. From Luquet et al. (2000), and Genovese et al. (2004).

Based on electron microscopy images, Genovese et al. (2004) identified two types of principal epithelial cells in these gills, and renamed them as “medial gills”. Briefly, type-I cells show few mitochondria loosely associated with the basolateral membrane that interdigitate with the flanks of pillar cells (Fig. 15.1D). The thin, respiratory epithelium lining the ventral part of the medial gills lamellae, is probably made of flattened type-I cells and pillar cells. Type-II cells are evident at light and electron microscopy, because they are thicker and darker than type-I cells, due to a notably high density of mitochondria. These cells, characteristically show numerous vesicles beneath the apical membrane folds. The basolateral membrane is not conspicuously infolded, neither associated with mitochondria (Fig. 15.1E).

Posterior gills (6 to 8) are involved in active ion transport for acid-base and osmotic regulation functions (Luquet et al. 2000a, Onken et al. 2003, Tresguerres et al. 2003, 2008). Posterior gill lamellae are mostly lined by a cuboidal epithelium, whose thickness ranges from 6 to 12.5µm, and decreases from the dorsal to the ventral zone. An attenuated tissue always lines the marginal channel. Electron micrographs show that the basolateral membranes of both thick principal cells (ionocytes) and pillar cells of these gills are extensively interdigitated, with large numbers of mitochondria tightly packed between these membranes (Genovese et al. 2000, 2004, Luquet et al. 2000) [Fig. 15.2]. Both the thickness and the ultrastructure of this epithelium vary with salinity, as explained in Chapter 14 Volume II.

The branchiostegal lungs of terrestrial and amphibious crabs are lined with a very thin epithelium and cuticle, with a close spacing to the respiratory medium contained in the branchial chamber, therefore being favourable for gas diffusion (Taylor and Taylor 1992). Besides, the surrounding vasculature is highly developed, which ensures adequate hemolymph perfusion for gas exchange (Farrelly and Greenaway 1993, 1994). The morphology of the branchiostegal respiratory epithelium varies from smooth, in species with large branchial chambers, to highly invaginated or evaginated, in species with smaller branchial chamber volumes (Díaz and Rodriguez 1977, Taylor and Taylor 1992, for a review). The combination of chamber size and branchiostegal epithelial morphology maximizes the total respiratory surface area. The histology and ultrastructure of the branchiostegite of *N. granulata* have been described by Halperin et al. (2000, 2002) [Fig. 15.3].

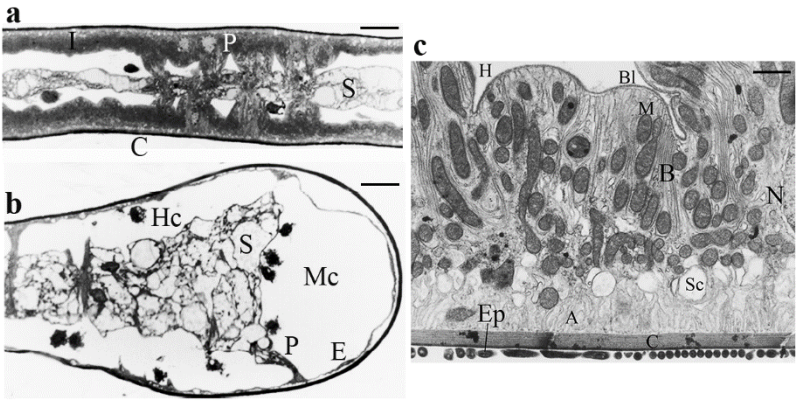

Figure 15.2; a, b. Longitudinal sections of ion regulatory gills of *Neohelice granulata*. Proximal (a) and distal (b) zone of a lamella showing a thick epithelium composed of ionocytes and pillar cells. The epithelium of the marginal channel is attenuated. Semi-thin sections stained with toluidine blue. Scale bars: 13µm; c. Ultrastructure of gill 6 showing infoldings of the basolateral membrane tightly associated with mitochondria. A, apical folds; B, basolateral membrane; Bl. basal lamina; C, cuticle; E, epithelium; Ep, epibionts; H, hemolymph; Hc, hemocyte; I, ionocytes; M, mitochondrion; Mc, marginal channel; N, nucleus; P, pillar cell; S, connective septum. Electron micrographs were double-stained with uranyl acetate and lead citrate. Scale bar: 2.5µm. From Luquet et al. (2000), and Genovese et al. (2004).

The branchial chamber lumen is separated from the branchiostegal circulation by the inner branchiostegal wall, which is formed by an attenuated epithelium covered by a thin cuticle. The branchiostegal respiratory epithelium has a few simple folds and lobulated projections, which moderately increase the respiratory surface area (Halperin et al. 2000, 2002). The mean diffusion distance between the lumen of the branchial chamber and the hemolymph (epithelium plus cuticle), is 1.6µm with a minimum of 0.5µm.

The space between the inner and outer branchiostegite walls is filled by a spongy connective tissue bearing large polymorphic cells that are stained by the periodic acid–Schiff (PAS) reaction, indicating abundant glycogen, glycoproteins, and/or glycolipids. The branchiostegal vasculature includes major vessels and large sinuses near the external integument, and smaller vessels and thin sheet-like sinuses delimited by fibrillar connective tissue located directly beneath the inner respiratory wall. Hemolymph most likely flows from the thoracic sinus through the major vessels, which progressively narrow, until they reach the respiratory sinuses, where gas exchange between hemolymph and the branchial chamber lumen takes place. The inner and outer branchiostegal walls are connected by striated muscle strands, which probably contract when the crab is immersed to reduce hemolymph flow to the branchiostegite, and relax during emersion, to increase the perfusion rate, thus favouring the respiratory function of the branchiostegite.

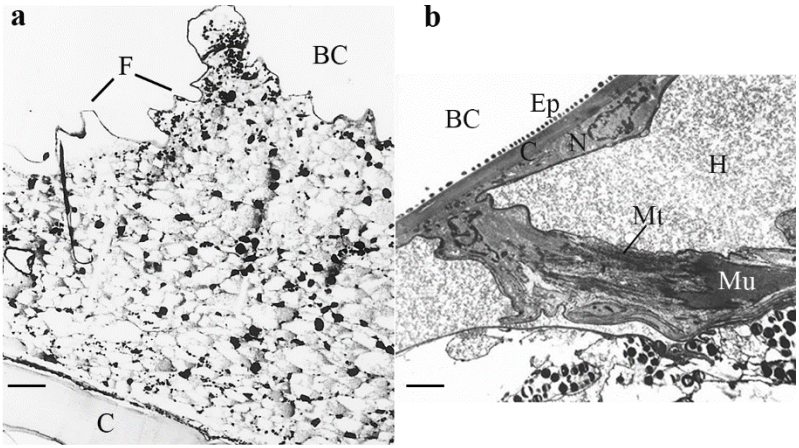

Figure 15.3. Cross-section of the branchiostegite of *Neohelice granulata*; a) general view showing inner wall folds lining the branchial chamber. The thick cuticle corresponds to the external body wall. Masson´s trichrome. Scale bar: 45µm; b) ultrastructure of branchiostegite. Under the inner cuticle, the attenuated epithelium is attached to striated muscle through microtubules. Hemolymphatic lacunae are evident. Electron micrographs were double-stained with uranyl acetate and lead citrate. Scale bar: 2µm. BC, branchial chamber; C, cuticle; E, epithelium; Ep, epibionts; F, folds; H, hemolymphatic lacunae; Mu, muscle; Mt, microtubules. From Halperin et al. (2000, 2002).

The exopodite of the second maxilla of decapod crustaceans is modified into a ventilatory appendix called the scaphognathite, which pumps water or air through the gills. As explained in detail below, adjustments to its beating frequency (Fsc) is a major mechanism for ventilatory control in *N. granulata*.

Ventilatory responses during emersion

When *N. granulata* is immersed in well-aerated brackish water (12psu), venous pO_2 is ~4KPa, pCO_2 is ~0.24KPa, pH is ~7.75, and $[HCO_3^-]$ is ~6.5mmol L^{-1} (Luquet and Ansaldo 1997, Luquet et al. 1998, Halperin et al. 2000) [Table 24.1]. Emersion barely affects heartbeat rate (F_H), and induces a 40-50% reduction in Fsc (Luquet et al. 1998), together with a slight decrease in the O_2 uptake rate ($\dot{M}O_2$) [Luquet et al. 1998, Halperin et al. 2000]. However, the low density and high O_2 content of air allow for relatively stable venous pO_2 even after 6h of air exposure (Luquet and Ansaldo 1997). On the other hand, CO_2 excretion rate ($\dot{M}CO_2$), is significantly reduced by up to ~80% after 4h of emersion (Luquet et al. 1998, Halperin et al. 2000), with a significant reduction in gas exchange ratio (R: $\dot{M}CO_2$/ $\dot{M}O_2$). These results demonstrate that CO_2 excretion is more challenging than O_2 uptake during air-breathing, as described for other bimodal breathing crabs (reviewed by deFur 1988 and Henry 1994).

After emersion, there is an immediate decrease in Fsc despite the nearly unchanged internal pO_2 (Luquet et al. 1998). This suggests the existence of a very sensitive mechanism that senses hemolymphp O_2 and slows down Fsc. A similar decrease in Fsc has been reported for intertidal crabs, while subtidal ones exhibit a marked increase in Fsc that is associated with internal hypoxia (deFur 1988, for a review). Intriguingly, inhibition by acetazolamide of carbonic anhydrase (which is essential for $\dot{M}CO_2$), prevents the emersion-induced Fsc decrease (Luquet et al. 1998). This suggests the presence of a second Fsc regulatory mechanism, which is "turned on" upon elevations in internal pCO_2: the increase in Fsc would enhance CO_2 ventilation and compensate for carbonic anhydrase inhibition. Supporting this idea, after 4h of emersion, when the effect of acetazolamide is fading, there is an increase in $\dot{M}CO_2$ resulting in a decrease in internal pCO_2, and also Fsc starts to decrease (Luquet et al. 1998). It should be mentioned that before and during all the experiments described in this section, crabs were not disturbed and remained mostly inactive. Particularly, respirometry was conducted in small chambers, which allow very little locomotion; therefore, the obtained $\dot{M}O_2$ and

$\dot{M}CO_2$ values are representative of *N. granulata* resting metabolism. Future experiments should be performed in active conditions, to analyze the ventilatory and metabolic adjustments that enable this species to forage in intertidal and supratidal areas.

Evidence for bimodal breathing

To test whether *N. granulata* is a bimodal breather (Henry 1994), Halperin et al. (2000) withdrew the water from the branchial chamber during air exposure, a condition that most likely happens during long-term land excursions in the wild due to evaporation. Similar to previous experiments (Luquet et al. 1998), control crabs with or without water in their branchial chambers, experienced a reduction in $\dot{M}O_2$, $\dot{M}CO_2$, and R, also accumulating CO_2 in their hemolymph during emersion. On the other hand, venous pO_2 was not affected by emersion in control crabs but significantly increased in crabs deprived of gill chamber water after 240min of air exposure.

Table 15.1. Respiratory and acid-base variables recorded in *Neohelice granulata,* immersed and along time of emersion. F_H, heart rate; Fsc, ventilatory rate (scaphognathite beating rate); pO_2, O_2 partial pressure; pCO_2, CO_2 partial pressure; CCO_2, total CO_2 content; $\dot{M}O_2$, O_2 uptake rate; $\dot{M}CO_2$, CO_2 excretion rate. SID, strong ion difference ($[Na^+] – [Cl^-]$). Data from [1]Luquet and Ansaldo (1997), [2]Luquet et al. (1998), [3]Halperin et al. (2000).

Emersion time (min)	0	15	60	120	240
F_H (beats min^{-1})[2]	133	144	128	118	105
Fsc (beats min^{-1})[2]	232	150	140	122	115
pO_2 (Kpa)[1,3]	4.0-4.3	3.60	3.46	3.20	3.5-4.3
pCO_2 (Kpa)[2]	0.24	0.30	0.46	0.43	0.46
CCO_2 (mmol L^{-1})[2]	6.5-8.0	10.0	11.6	11.7	11.4-13.0
pH[2]	7.74	7.65	7.71	7.72	7.70
$\dot{M}O_2$ (mmol kg^{-1} h^{-1})[2,3]	5.92	3.21	2.71	--	3.13
$\dot{M}CO_2$ (mmol kg^{-1} h^{-1})[2,3]	7.30	2.38	1.58	--	1.50
SID (meq L^{-1}][2]	25	34	60	87	70

Treated crabs, also showed higher hemolymph total CO_2 content (CCO_2) associated with a greater reduction in R than the controls at 240min of air exposure. These results confirm that O_2 uptake in *N. granulata* is not impaired during emersion and that the water inside the gill

chamber is not an essential source of O_2. Moreover, the elevated venous pO_2 in crabs deprived of gill chamber water, indicates more efficient O_2 uptake through the branchiostegite, after long periods of air exposure. However, the water in the gill chamber is an essential sink for metabolic CO_2 excretion. These results indicate that *N. granulata* is a bimodal breather, because it can exchange respiratory gases through both air and water, across two distinct respiratory organs (Henry 1994).

Acid-base regulation during emersion

The impairment of $\dot{M}CO_2$ and subsequent elevation of hemolymph pCO_2 results in respiratory acidosis immediately after emersion, which *N. granulata* compensates *via* metabolic H^+ secretion into the branchial chamber water, and HCO_3^- accumulation in hemolymph (Table 15.1). Aquatic animals typically excrete H^+ in exchange for external Na^+ (Larsen et al. 2014); in *N. granulata* the external sink for H^+ and source of Na^+ is the water inside the branchial chambers (Luquet and Ansaldo 1997).

Similar to other amphibious crabs exposed to air (Burnett and McMahon 1987) and also to hypercapnic terrestrial crabs (Burnett 1988), *N. granulata* experiences an increase in hemolymph strong ion difference (SID) during emersion, which mirrors the pH recovery (Luquet and Ansaldo 1997, Luquet et al. 1998). Both $[Na^+]$ and $[Cl^-]$ increase immediately after emersion and reach maximum levels about 1h after. However, $[Na^+]$ increases more than $[Cl^-]$, and $[Na^+]$ remains elevated throughout emersion, while $[Cl^-]$ returns to control values about 2h after emersion. The resulting increase in SID from ~25mM in control crabs to ~9mM in emersed crabs is essential for hemolymph pH compensation (c.f. Stewart 1978).

While terrestrial crabs dissolve $CaCO_3$ from the carapace as a source of CO_3^{2-}/HCO_3^- that buffers pH (Burnett, 1988), $[Ca^{2+}]$ in the hemolymph of *N. granulata* does not change during emersion (Luquet and Ansaldo 1997). This indicates that the increased hemolymph $[HCO_3^-]$ is not derived from the carapace, but instead is most likely derived from gill HCO_3^- absorption (see below).

The responses listed above were observed in *N. granulata* acclimated to brackish water. Crabs acclimated to hypersaline water (41psu) also show an initial CCO_2 accumulation and respiratory acidosis, but stabilize venous pCO_2 and pH after 1h of emersion (Mougabure Cueto 1998). However, the hemolymph $[Na^+]$ and $[Cl^-]$ responses are different in *N. granulata* acclimated to high salinity: $[Cl^-]$ significantly decreases 1h after emersion, and $[Na^+]$ increases slightly, but not significantly throughout the

emersion period (Mougabure Cueto 1998). These results are similar to the increase in hemolymph SID and pH compensation exhibited by crabs acclimated to brackish water; however, the compensatory mechanisms must be different. Such difference might be due to a masking effect from ionic regulation: while *N. granulata* from brackish water must actively uptake NaCl for hyper-ionic regulation, *N. granulata* acclimated to 41psu salinity must actively excrete NaCl for hypo-ionic regulation (Luquet et al. 2002a) [see Chapter 14 Volume II for details about morphological and physiological adaptations in these crabs]. Upon air exposure, high-salinity acclimated crabs seem to up-regulate Cl^- excretion more than Na^+ excretion, which may instead happen, or in addition to up-regulation of Na^+/H^+ exchange, and HCO_3^- absorption. However, the cellular mechanisms involved and regulatory pathways are unknown and deserve further investigation.

Branchial cellular mechanisms for acid-base regulation

The gills of aquatic animals are responsible for the majority of blood or hemolymph acid-base regulation, by actively excreting H^+ or HCO_3^- to the surrounding water across specialized cells. In animals living in hypo-osmotic environments (i.e., brackish and freshwater), acid-base regulation and ionic regulation are linked through the exchange of H^+ for Na^+ and HCO_3^- for Cl^- (reviewed by Henry et al. 2012, Larsen et al. 2014, and Fehsenfeld and Weihrauch 2017) [see Chapter 14 Volume II]. As a result, it is rather difficult to study acid-base regulatory mechanisms independently from ion-regulatory mechanisms. Furthermore, the environmental [NaCl] imposes thermodynamic constraints, whereby secreting H^+ and HCO_3^- in a dilute NaCl medium, such as oligohaline or freshwater environments, requires more energy (and different ion-transporting proteins) compared to brackish and seawater (Kirschner 2004).

The general morphology and histology of *N. granulata* acclimated to different environments, as well as the mechanisms for NaCl absorption and secretion, are described in Chapter 14 Volume II. Here, we summarize our knowledge about cellular mechanisms for acid-base regulation in gills from *N. granulata* acclimated to 2psu salinity. To this respect, immunohistochemical evidence revealed a high abundance of two key ion-transporting enzymes in *N. granulata* gills: the Na^+/K^+-ATPase (NKA), and the V-type H^+-ATPase (VHA) [Tresguerres et al. 2008, Fig. 15.4A, B]. NKA and VHA are present in the cuboidal principal cells that form the majority of the gill lamella surface, and also in the pillar cells that connect

opposing hemi-lamella. However, while NKA is present in the well-developed invaginations of the cell basolateral membrane (Fig.15.2C, see also Chapter 14), VHA has a more diffuse subcellular localization, and in some cells, it is concentrated along the apical membrane in contact with the subcuticular space (Fig. 15.4A). However, it remains unclear whether *N. granulata* gills have two ion-transporting cell subtypes that differ in the VHA subcellular localization, or if VHA insertion into the apical membrane is locally regulated in each cell.

Experiments with isolated and perfused gills provided functional information about cellular acid-base regulatory mechanisms in *N. granulata* (Tresguerres et al. 2008). Under controlled conditions, isolated gills performed net H^+ excretion. This basal rate of H^+ excretion was significantly stimulated when the pH of the perfusate (the medium simulating the hemolymph), was reduced from 7.75 to 7.45. This acidic condition also caused an increase in the transepithelial potential (Vte, a measure of ion transport) that was prevented (or reduced) by acetazolamide, by the VHA inhibitor bafilomycin, the HCO_3^- transporter inhibitor DIDS, and in Na^+-free medium. Altogether, these results indicate the presence of a pH sensing mechanism that becomes activated during acidosis and stimulates apical VHA for enhanced H^+ secretion and Na^+ absorption, coupled to HCO_3^- absorption (Fig. 15.4C). This model matches the responses observed in *N. granulata* during emersion (see above), suggesting that it is a physiologically relevant mechanism, by which gills control hemolymph acid-base status during land excursions. However, other factors that cannot be studied in the isolated gill preparation, such as hormones or control of circulation, cannot be ruled out.

Isolated *N. granulata* gills can be induced to switch from net H^+ secretion to net base secretion by elevating the perfusate and bath [HCO_3^-] from 2.5 to 12.5mM (Tresguerres et al. 2008). Similar to acid-stimulated H^+ excretion, HCO_3^--stimulated base excretion was associated with an increase in Vte; alkalinizing the saline from 7.75 to 7.81 with Tris-base, to match the effect of elevated HCO_3^- on pH, did not induce an increase in Vte, thus confirming that $HCO_3^-$was the stimuli and not the pH. The HCO_3^--stimulated Vte was prevented (or reduced) by ouabain, acetazolamide, amiloride, and Cl^--free conditions, while bafilomycin, DIDS, and SITS had no significant effects. Altogether, these results suggest that base secretion across *N. granulata* gills depends on NKA, carbonic anhydrase, basolateral Na^+/H^+ exchangers, and apical anion exchangers (Fig.15.4C). The *in vivo* physiological relevance of this mechanism is unclear, but it could be important when *N. granulata* returns to water after a prolonged land excursion, and it needs to excrete the

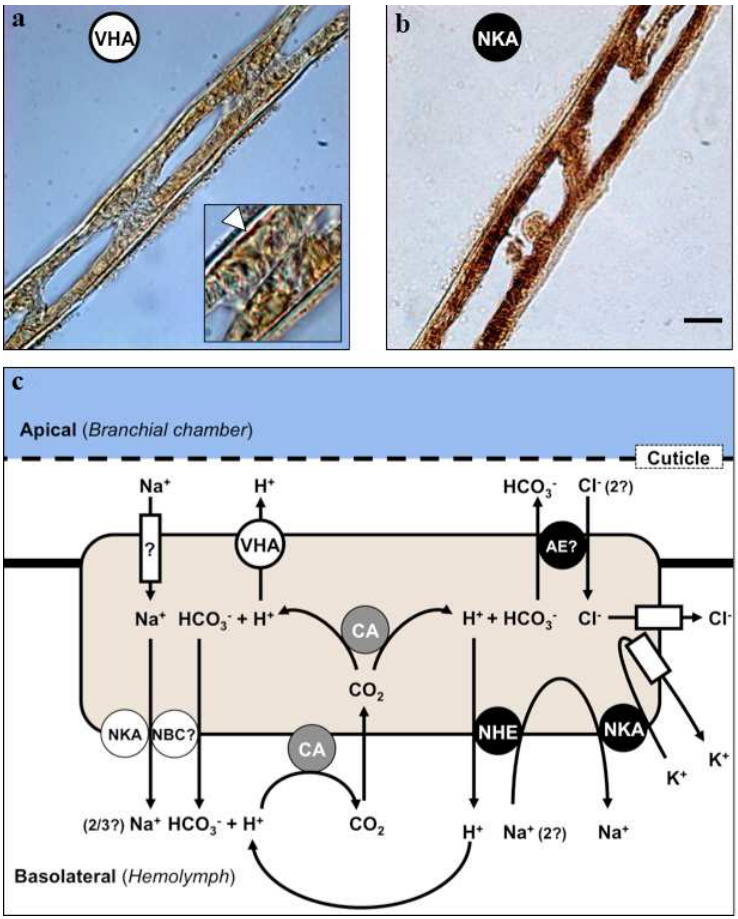

Figure 15.4. Cellular mechanisms for acid-base regulation in *Neohelice granulata* gills; a, b. Immunolocalization of V-type H^+-ATPase (VHA) and Na^+-K^+-APase (NKA) in gills of *N. granulata* acclimated to 2psu salinity. The inset in (a) points out to VHA labelling in the apical domain of a pillar cell (arrowhead). Scale bar: 10 µm; c. Cellular model proposed for branchial acid-base regulation. The left side of the diagram shows the mechanism for H^+ excretion and HCO_3^- absorption, and the right side shows the mechanism for HCO_3^- excretion and H^+ absorption. Both systems are illustrated in a single cell; however, the existence of cell subtypes cannot be ruled out. The molecular identity and stoichiometry of the ion-transporting proteins are unknown; a question mark (?) indicates the lack of direct evidence for the involvement of the corresponding transporter. AE, anion exchanger; CA, carbonic anhydrase; NBC, Na^+/HCO_3^- cotransporter; NHE, Na^+-H^+ exchanger. Modified from Tresguerres et al. (2008).

excess HCO_3^- accumulated in hemolymph during metabolic compensation of respiratory acidosis.

The identity of the H^+ and HCO_3^- sensing mechanisms are unknown, but candidates include H^+ sensing G-protein coupled receptors and HCO_3^--sensing soluble adenylyl cyclase (sAC) [reviewed in Tresguerres et al. 2010, 2011, 2014]. Supporting the latter statement BLAST searches indicate the presence of sAC-like genes in crustaceans.

Future Prospects

The unique physiological adaptations for bimodal breathing, make *N. granulata* an exciting model species for comparative studies, as well as for studying the evolution of crab terrestriality. The roles of branchiostegal lungs and gills in resting *N. granulata* acclimated to brackish water, for both O_2 uptake and CO_2 excretion during emersion, are clearly established, as well as the hemolymph NaCl, pH, and HCO_3^- responses associated with acid-base balance. However, future studies should investigate the responses during the increased activity of *N. granulata*, and also in crabs acclimated to either more dilute or more concentrated mediums. Isolated and perfused gills, as well as studies with Ussing chamber (Onken et al. 2003), can provide important functional evidence about the cellular mechanisms for acid-base and ionic regulation. These mechanisms can improve our understanding of crabs' ability to conquer different environments, as well as to provide a framework for predicting potential effects of pollutants on *N. granulata* fitness.

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List of abbreviations

AE, anion exchanger; CA, carbonic anhydrase; CCO_2, total CO_2 content; DIDS (4,4'-Diisothiocyanatostilbene-2,2'-disulfonate), anion transport inhibitor; F_H, heartbeat rate; Fsc, scaphognathite beating frequency; $\dot{M}CO_2$, rate of carbon dioxide excretion; $\dot{M}O_2$, rate of oxygen uptake; NBC, Na^+/HCO_3^+cotransporter; NHE, Na^+-H^+ exchanger; NKA, Na^+/K^+-ATPase; PAS, periodic acid–Schiff; pO_2, partial pressure of oxygen); R, gas exchange ratio ($\dot{M}CO_2$/ $\dot{M}O_2$); sAC, soluble adenylyl cyclase; SID, strong ion difference ($[Na^+] - [Cl^-]$); SITS, bicarbonate transport inhibitor; VHA, V-type H^+-ATPase; $\dot{V}O_2$, rate of oxygen consumption; Vte, transepithelial potential

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