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Exploring the versatility of the perfused crustacean gill as a model for transbranchial transport processes

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ABSTRACT

The study of transbranchial ion and gas transport of water-breathing animals has long been a useful means of modeling transport processes of higher vertebrate organs through comparative physiology. The molecular era of biological research has brought forward valuable information detailing shifts in gene expression related to environmental stress and the sub-cellular localization of transporters; however, purely molecular studies can cause hypothetical transport mechanisms and hypotheses to be accepted without any direct physiological proof. Isolated perfused gill experiments are useful for testing most of these hypotheses and can sometimes be used outright to develop a well-supported working model for transport processes relating to an animal's osmoregulation, acid-base balance, nitrogen excretion, and respiratory gas exchange as well as their sensitivity to pollutants and environmental stress. The technique allows full control of internal hemolymph-like saline as well as the ambient environmental fluid compositions and can measure the electrophysiological properties of the gill as well as the transport rates of ions and gases as they traverse the gill epithelium. Additives such as pharmaceuticals or peptides as well as the exclusion of ions from the media are commonly used to identify the importance of specific transporters to transport mechanisms. The technique can also be used to identify the penetrance, retention, and localization of pollutants within the gill epithelium or to explore the uptake and metabolism of nutrients directly from the ambient environment. While this technique can be applied to virtually any isolatable organ, the anatomy and rigidity of the decapod crustacean gill make it an ideal candidate for most experimental designs.

1. Introduction

Water-breathing animals possess gills or gill-like organs that act as a direct interface between their hemolymph or blood and the ambient environment. Gills are generally multifunctional, performing the majority of an animal's respiratory gas exchange, osmoregulation, acid-base balance, and excretion of nitrogenous end products while also acting as a major toxicological interface for metals and pollutants (Callaghan et al., 2016; Claiborne et al., 2002; Goss et al., 1992; Henry et al., 2012; Larsen et al., 2014). While the anatomical and vascular design of these organs can differ dramatically (Fig. 1), these physiological functions are largely conserved with some exceptions such as freshwater insects that use non-branchial epithelia such as their integument rather than their gill-like organs for most regulatory functions (Graham, 1990, 1988; Griffith, 2017; Weihrauch et al., 2012a). Investigating how these physiological responsibilities are regulated, their responses to environmental change, and their limitations are critical to understanding aquatic life and is beneficial to the fields of aquaculture and conservation given the importance of the gills to the animals' sensitivity to water quality. The field of branchial transport physiology is also important to the field of comparative physiology as the branchial epithelium of fishes and crustaceans have conserved features relating to the Na^+/Cl^- transporting nephrons of the mammalian kidney (Mount, 2014) and the resorption/dissolution of vertebrate bones (Luquet, 2012; Roer, 1980).

While modern molecular and immunohistochemical techniques can be applied to virtually any biological system, many physiological methods cannot. Current literature often favours the accumulation of large amounts of molecular information with few direct measurements of a live system's transport capabilities under physiological conditions – evidence that is fundamental to developing transport mechanisms or to evaluate responses to environmental change. Perfusion techniques

Abbreviations: P_{NH3}, Partial pressure of NH_3; P_{CO2}, Partial pressure of CO_2; P_{O2}, Partial pressure of O_2.

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permit a living organ's physiological activities to be monitored outside of the animal by passing artificial blood or hemolymph-like salines through its vasculature while bathing it in a controlled environmental saline. The technique offers full control over the composition of internal and external salines making it a versatile means of measuring baseline branchial physiology as well as the effects of an additive such as pharmaceuticals and hormones (see table 1). The excised organ maintains its structural integrity allowing the gill to be studied with its cells still in their native anatomical and physiological associations and preventing intracellular factors from becoming disrupting as they would in a tissue homogenate, isolated cell/organellar extraction, or membrane vesicle preparation (Towle, 1993).

While theoretically any organ may be perfused, the relative difficulty in achieving properly cannulating their vasculature and ensuring adequate distribution of blood or hemolymph across the tissue varies substantially. For example, the nephrons embedded within the mammalian kidney act as the functional unit of NaCl and osmoregulatory transport and are studied due to their importance to basal human physiology as well as pathophysiology. While several successful efforts to isolate and perform measurements on nephrons have been achieved the organ's delicate nature and small size makes these experimental setups rather complex (Casellas and Navar, 1984). Ideal tissues must have clear afferent and efferent vessels within a size range suitable for cannulation with enough extra tissue to properly seal the organ with adhesives or a pressurized clamp (Fig. 2). The tissue must be able to withstand a certain degree of rigidity to minimize compromising its structural integrity from physical handling as well as the pressure of fluid delivered through a peristaltic pump or a gravity-fed drip. Non-ideal features of some biological systems have been circumvented using 'semi-systemic' preparations in place of isolated organ perfusion; however, the experimental method's value suffers as a result. For example, perfusion of an entire fish's head in place of an individual

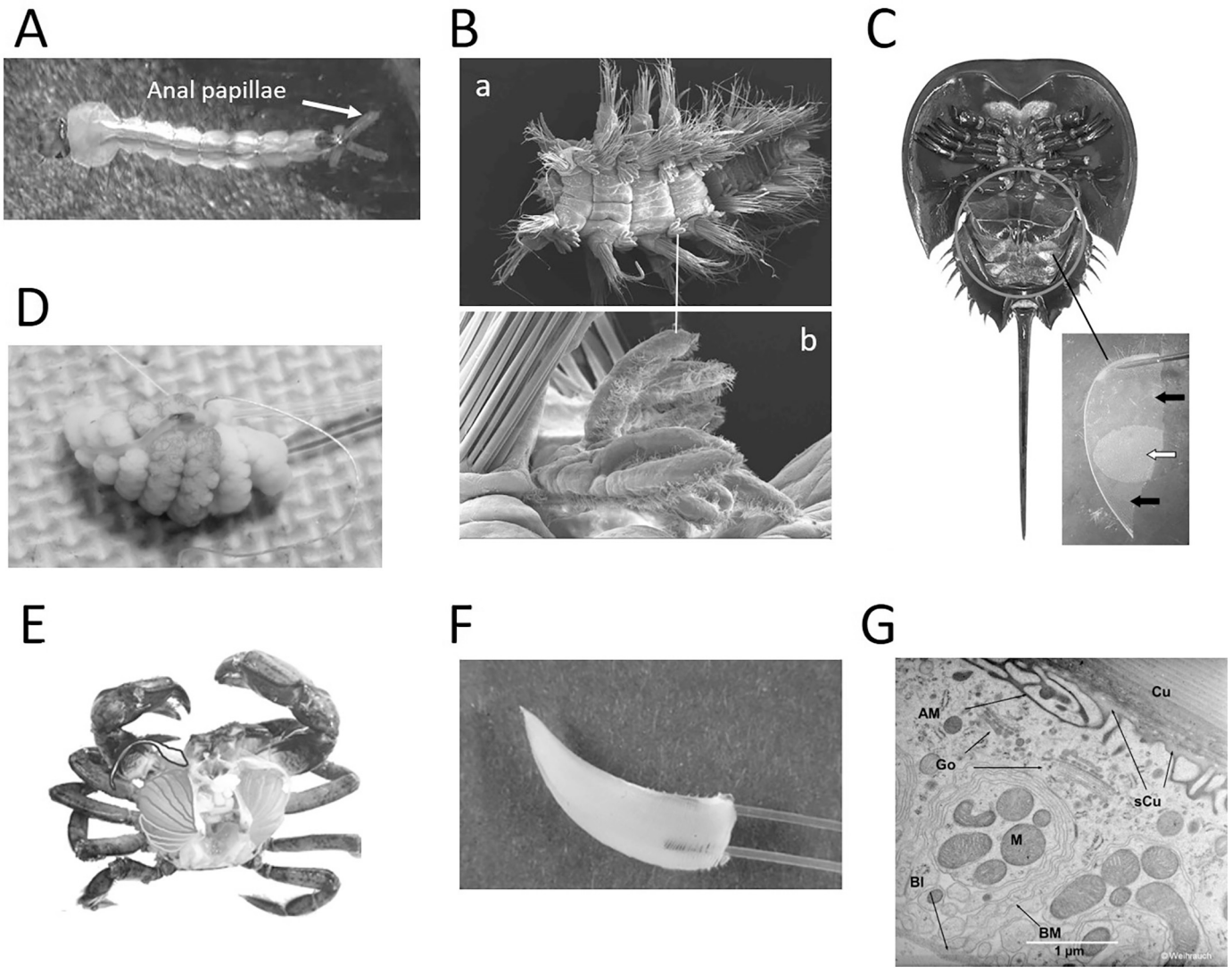

Fig. 1. Various gill types found in invertebrates. A, anal papillae of the freshwater living mosquito larva of *Aedes agyptii* (modified after Clark et al., 1999). B, gills of the marine polychaete *Eurythoe complanata*. a, gills localized to base of the animals' parapodia; b, magnification of gill (with thanks to H. Meyer and G. Purschke, more detailed information can be found under Purschke et al. (2017)). C, book gills (encircled) from the horseshoe crab *Limulus polyphemus*. Insert: single intact gill lamella displaying a distinct central mitochondria-rich area (white arrow) surrounded by thin peripheral mitochondria-poor areas (black arrows) Hans et al. (2018)). D, cannulated, perfused gill from *Octopus vulgaris*. Dark branches indicate infused dye visible shortly after perfusion (more detailed information can be found under Hu et al. (2017)). E, the green crab *Carcinus maenas*, bare of carapace showing epipod of first maxilliped (encircled black on the left side), anterior gills (encircled grey on the left side), posterior gills (encircled white on the left side; with thanks to Sandra Fehsenfeld). F, cannulated phyllobranchiate posterior gill of *C. maenas*. G, Electron micrograph of the posterior gill epithelial cells of *C. maenas*. AM, apical membrane; Bl, basal lamina; BM, basolateral membrane; CP, clathrin-coated pit; Cu, cuticle; Go, Golgi apparatus; M, mitochondria; Mt, microtubules; rER, rough endoplasmic reticulum; sCu, subcuticular space (Weihrauch et al. (2002).

Table 1
Selection of species whose perfused gills have bene documented and the objectives targeted. References: [1]Siebers et al., 1994, [2]Gocha et al., 1987, [3]Siebers et al., 1985, [4]Lucu et al., 1989, [5]Niyogi et al., 2016, [6]Blewett et al., 2015, [7]Onken and Graszynski, 1989, [8]Mo et al., 2003, [9]Luquet et al., 2002, [10]Burnett and Towle, 1990, [11]Pierrot et al., 1995b, [12]Spanings-Pierrot et al., 2000, [13]Eckhardt et al., 1995, [14]Martinez et al., 1998, [15]Lohrmann and Kamemoto, 1987, [16]Lucu and Siebers, 1987, [17]Postel et al., 1998, [18]Böttcher et al., 1991, [19]Silvestre et al., 2004, [20]Rathmayer and Siebers, 2004, [21]Weihrauch et al., 1998, [22]Weihrauch et al., 1999, [23]Weihrauch et al., 2002, [24]Martin et al., 2011, [25]Fehsenfeld and Weihrauch, 2016b, [26]Fehsenfeld and Weihrauch, 2015, [27]Hans et al., 2014, [28]Fehsenfeld and Weihrauch, 2013, [29]Allen et al., 2020, [30]Onken and McNamara, 2002, [31]Zanders and Rojas, 1996, [32]Tresguerres et al., 2008, [33]Onken and Graszynski, 1989, [34]Drews and Graszynski, 1987, [35] Onken et al., 2000.

Objective	Species	Literature
PD_{TE}	*Carcinus maenas*	1,3, 4,16
	Chasmagnathus granulatus	9
	Pachygrapsus marmoratus	11, 12
	Dilocarcinus pagei	30
	Eriocheir sinensis	33
	Uca rapax	31
	Ucides cordatus	14
$Cl^-/Na^+/HCO_3^-$ tracer	*Carcinus maenas*	4, 16, 18, 26
	Callinectes sapidus	10, 15
	Chasmagnathus granulatus	9,32
	Eriocheir sinensis	2, 8, 20
	Ucides cordatus	14
Nitrogen	*Cancer pagurus*	22
	Carcinus maenas	4, 21, 22, 23, 25,26, 28
	Eriocheir sinensis	22
	Metacarcinus magister	24, 27
	Xenograpsus testudinatus	29
Acid-base	*Carcinus maenas*	25,26, 28
	Metacarcinus magister	27
	Xenograpsus testudinatus	29
Zinc	*Carcinus maenas*	5
Nickel	*Carcinus maenas*	6
Cadmium	*Carcinus maenas*	17
	Eriocheir sinensis	19
Hormones/cAMP	*Pachygrapsus marmoratus*	12, 13
	Callinectes sapidus	15
	Eriocheir sinensis	35

delicate teleost gill is viable (e.g. Campbell et al., 1999; Johansen and Pettersson, 1981; Verbost et al., 1987); however, the results can be difficult to interpret due to the flow of hemolymph through extra-branchial organs that may not remain as viable as the gill over time. Other non-branchial tissues were also perfused to investigate osmoregulatory processes including perfusion of the NaCl secreting rectal glands of elasmobranchs (Hayslett et al., 1974). These experiments provided valuable information related to salt-excreting glands and tissues such as the salt glands of seabirds (Hildebrandt, 2001) and reptiles (Babonis et al., 2009), but failed to further our understanding of salt-absorbing tissue such as the thick ascending limb of the loop of Henle in the mammalian kidney that critically manage volume homeostasis (Mount, 2014). Following August Krogh's principle (Krogh, 1929), researchers eventually found organs that were relatively easy to perfuse that mimicked our target modeling of the mammalian kidney – the perfused gill of osmoregulating crustaceans.

2. Value of crustaceans as model organisms of molecular transport

The study of crustacean physiology offers several benefits, both as an isolated study species and to the field of comparative physiology given their representation within the animal kingdom and their broad habitat range. Invertebrates account for approximately 95% of species in the animal kingdom most of which are members of the insect or crustacean families (Wilson, 1987). In addition to their high species representation, crustaceans occupy virtually all known habitats including waters of all salinities, hydrothermal vents, the deserts of Australia, and even vase-like leaves of bromeliad plants (Allen et al., 2020; Bishop, 1963; Diesel, 1989; Morris, 2002; Morris and Greenaway, 1990; Ng et al., 2000; Rogers et al., 2012). The wide ecological range of crustaceans means that even extreme levels of environmental challenges can be applied to crustaceans without using unrealistic conditions that an animal may never see in nature (*e.g.* extreme desiccation in *Holthuisana transversa* (Greenaway and MacMillen, 1978; Linton and Greenaway, 1995) or extreme hypercapnia in *Xenograpsus testudinatus* (Allen et al., 2020)). Most importantly, while crustaceans may be evolutionarily distant from higher vertebrates, their transport physiology is often largely conserved permitting the use of their transport epithelia as comparative models including branchial representation of osmoregulation and acid-base/nitrogen equivalent transport processes of the mammalian kidney (Henry et al., 2012; Mount, 2014) or the use of hypodermis to model the bi-directional Ca^{2+} transport that occurs during vertebrate bone resorption and deposition (Luquet, 2012; Roer, 1980). These types of studies even resulted in the discovery of the Na^+/K^+-ATPase within peripheral leg nerves of the European green shore crab, *Carcinus maenas*, by Skou, 1960, which received the Nobel Prize in 1961 due to its widespread importance to the scientific community.

Most branchial studies focus on either teleost fishes or crustaceans – both of which rely almost unanimously on transbranchial ion and gas transport to maintain their physiological homeostasis (Claiborne et al., 2002; Evans and Cameron, 1986; Gilmour and Perry, 2009; Goss et al., 1992; Henry et al., 2012; Larsen et al., 2014). Gills can have substantially different structures and anatomy (Fig. 1), however, their functions and transport mechanisms are largely conserved. While it is possible to perfuse both teleost and crustacean gills their ability to produce accurate and detailed results are substantially different. Teleost gills are innervated and have blood-flow patterns that are more complicated than that of crustacean gills making it difficult to successfully isolate and cannulate the tissue without causing damage or resulting in incomplete perfusion of its vasculature. In comparison, the anatomy and vasculature of brachyuran crab gills (see below) allows them to be easily dissected from the animal, cannulated, and sealed in a matter of minutes at which point perfusing them with nutrient-enriched saline can keep them alive for upwards of 6-hours (Fig. 2C; Siebers et al., 1985).

3. Anatomy and vasculature of decapod crustacean gills

While the crustacean family as a whole is too diverse to generalize, higher crustaceans (i.e. *Brachyura*, *Malacostraca*) have a semi-closed vascular system that directs hemolymph from their single-chambered heart through anterior and posterior arteries (Mcgaw and Reiber, 2015; McGaw and Reiber, 2002). These arteries feed distinct sections of the animal's body and eventually pool into sinuses composing the open-portion of the crustacean circulatory system that is then perfused through the gills before returning to the heart (Mcgaw and Reiber, 2015; McGaw and Reiber, 2002). This hemolymph flow pattern results in the gills being burdened with the metabolic end products of extra-branchial tissues while also being the main organ that is in direct contact with the surrounding environment (Freire et al., 2008; Henry et al., 2012). As hemolymph enters the gill's efferent vessel it moves across each lamella towards the afferent vessel making use of a counter-current flow that maximizes the gradients between the hemolymph and surrounding water. This process maximizes the potential for O_2 intake and release of CO_2 along their partial pressure gradients (P_{O2} and P_{CO2}) while allowing ion-transport processes to occur in parallel (Freire et al., 2008). The hemolymph exits the lamella into the afferent vessel where its back-flow is prevented by valves (Taylor and Taylor, 1986) as it exits the gill becoming post-branchial hemolymph that is slightly more alkaline and low in CO_2 and ammonia.

Crustacean gills may be phyllobranchiate (*i.e.* leaf-like lamellae), trichobranchiate (*i.e.* hair-like lamellae), or dendrobranchiate (*i.e.* multi-branched lamellae) in relation to their increasingly complex

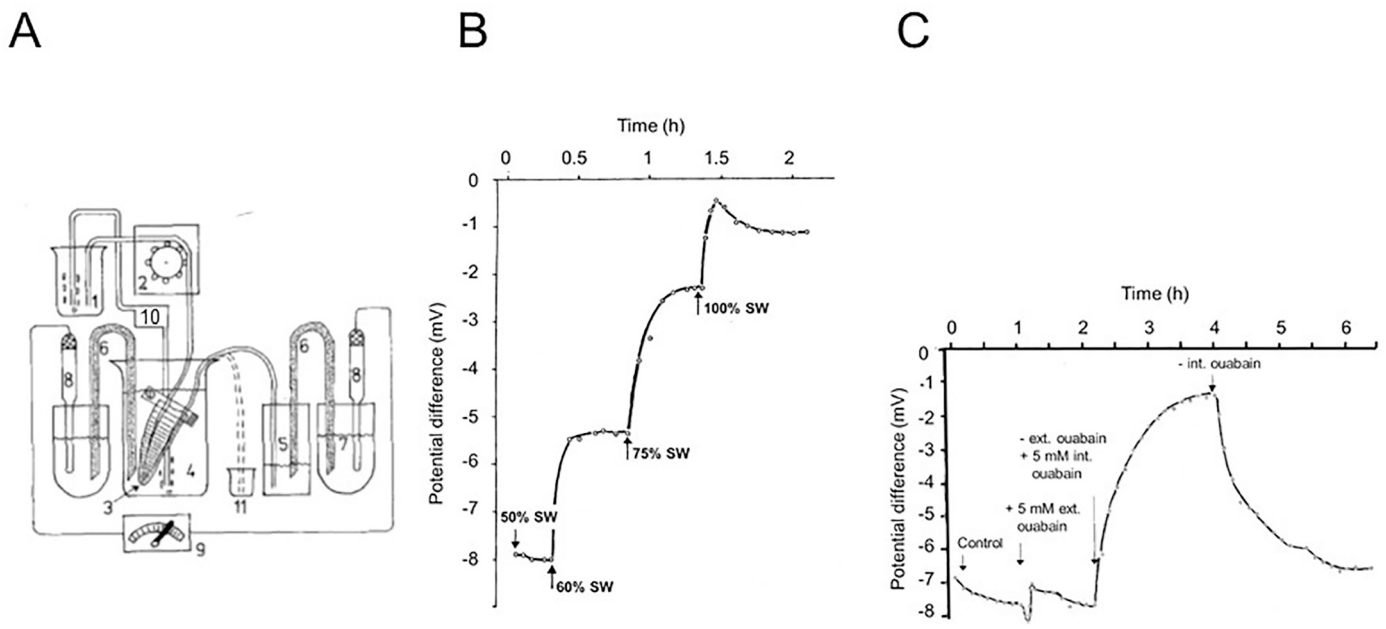

Fig. 2. Measurements of the transepithelial potential difference (PD_{TE}) across the perfused posterior gills of *Carcinus maenas*. A, Schematic set-up of the design to measure the PD_{TE} over the isolated perfused gill. 1, aerated external media; 2, peristaltic pump; 3 gill; 4, external bath (aerated); 5, small collecting beaker for perfusate; 6, agar bridge (in 3% KCl); 7, saturated KCl; 8, electrodes; 9, millivolt meter; 10, aeration. B, PD_{TE} measured over the isolated perfused posterior gill 7 of *C. maenas* acclimated to 10 ‰ S. Gills were perfused and bathed symmetrically with 50, 60, 75, or 100% seawater. C, PD_{TE} measured over the isolated perfused posterior gill 7 of *C. maenas* acclimated to 10 ‰ S., perfused and bathed with 50% seawater. Changes of PD_{TE} were recorded under the influence of the Na^+/K^+-ATPase inhibitor ouabain. Images are modified after Siebers et al. (1985).

structure as occurs in brachyuran crabs, malacostracan lobsters, and penaeid shrimps, respectively. While these different types of gills have unique structures they are relatively similar in most aspects of their epithelial characteristics including their absence of innervations and musculature and have lamellae composed of a single cell-layered epithelium covered by a thin cuticular layer along its apical membrane that provides the gill with structural support while also functioning as a molecular sieve (Roer and Dillaman, 1984). The phyllobranchiate gills of brachyuran crabs are the most thoroughly studied of the crustacean gills and possess distinct anterior and posterior gills that are primarily related to respiratory gas exchange and ionoregulatory transport, respectively. The anterior gills, usually gills 1–6, are characterized by their thin (ca. 1–4 μm) epithelia making them suitable for respiratory gas exchange whereas the posterior gills, usually gills 7-9, display thin (ca. 1–4 μm) and thick (ca. 10–20 μm) areas. The thick "patches" are composed of mitochondrial and Na^+/K^+-ATPase-rich cells with substantial basolateral infoldings to increase the surface area available for transport and are increasingly present in crabs acclimated to dilute salinities (Fig 1G; Barra et al., 1983; Compere et al., 1989; Goodman and Cavey, 1990; Siebers et al., 1982; Towle et al., 2001; Wang et al., 2012; Weihrauch et al., 2004). In fact, marine osmoconforming species are only believed to possess thin respiratory epithelia due to their reduced need for NaCl transport (Freire et al., 2008). While these gill types are specific to brachyuran crabs, similar cell types are present in the dendrobranchiate gills and trichobranchiate gills of penaeid shrimps and lobsters or crayfish, respectively, in the form of respiratory and ionoregulatory zones of each gill filaments instead of specific gills (Dickson et al., 1991; Dunel-Erb et al., 1997). The paired nature of crustacean gills along the left and right side of the animal allows for a direct comparison of the control and treated gill's transport rates (or other measurements) avoiding hindrance in data discrepancy caused by inter-individual factors that may be prevalent in experiments grouping transport rates of separate control and treatment gills.

4. Osmoregulation

Osmoregulation is vital to an organism's survival as it ensures that vital fluids have sufficient concentrations of electrolytes and maintains cellular volume homeostasis. Osmotic homeostasis is heavily dependent on the transepithelial movement of Na^+ and Cl^- making the study of these ion's transport mechanisms extremely valuable. While molecular techniques can provide detailed information relating to a transport mechanism it cannot develop a hypothetical transport mechanism without direct investigation and measurement of the ion's physical movement across the intact epithelium, making perfusion experiments highly valuable.

Perfused gills of crustaceans have been highly influential in the mechanistic study of osmoregulation. Since the perfused gill maintains the tissue's structural integrity, it permits the study of NaCl transport through electrophysiological measurements. By using a microvoltmeter connected to Ag/AgCl reference electrodes and agar bridges (3% agar in 3 mol l^{-1} KCl) immersed in saturated KCl that connects the internal and external media that represent a hemolymph-like solution and environment-like solution, respectively (Siebers et al., 1985; Fig. 2A), it is possible to measure the transepithelial potential difference (PD_{TE}; Fig. 2B; refer to Table 1 for more references) across gills of isolated and perfused hyper-regulating crab gills. The PD_{TE} represents the capacity of the tissue to actively transport charged molecules (mostly Na^+ or Cl^-) while also providing an indication of the integrity of the isolated tissue based on its ability to maintain its electrochemical gradients as is demonstrated in Fig. 2C. The capacity for active transport was found to be a common trait of the posterior phyllobranchiate gills of osmoregulatory active decapod crabs inhabiting dilute media (Freire et al., 2008). In terms of viability, these methods have demonstrated that the posterior gills of *C. maenas* were able to generate a control-like PD_{TE} for over 6 hours despite transient exposure to the Na^+/K^+-ATPase inhibitor ouabain (Siebers et al., 1985) – a process that has also been noted in *Pachygrapsus marmoratus* (Pierrot et al., 1995a). Measuring the perfused gill's viability over lengthy perfusions is an important validation step in any experimental design; however, measuring PD_{TE} cannot indicate

viability in osmoconforming marine species as their NaCl transport is virtually abolished (Henry et al., 2012).

Direct measurement of Na^+ and Cl^- fluxes across the perfused crab gill using radiotracers (e.g. $^{22}Na^+$, $^{36}Cl^-$, see Table 1) are a viable means to investigate osmoregulatory processes, especially when paired with pharmacological or molecular inhibitors of specific transporters to the basolateral membrane. Caution must be taken when applying pharmaceutical or molecular inhibitors to the apical epithelium of the gill due to cuticle. The cuticle is a non-biologically active porous barrier primarily composed of a mixture of Ca^{2+}, Mg^{2+}, and structural chitin-protein matrixes (Roer, 1980; Roer and Dillaman, 1984). While its presence gives the gills greater rigidity than those of teleost fishes, its porous nature means large molecules can produce false results due to steric blocking of the pores as occurs upon application of apical amiloride which appears to cause inhibition of an NHE when in reality it only affects the cuticle cation permeability (Onken and Riestenpatt, 2002; Weihrauch et al., 2002). Pairing these studies with the knowledge gained from measurements of the area-dependent short circuit current (I_{SC}) and conductance (G_{TE}) of the isolated split half-lamella mounted in modified micro Ussing chambers (see Table 2), as well as enzyme activity assays, immunohistochemistry, and gene-expression analysis (Henry et al., 2012; Riestenpatt et al., 1996; Serrano et al., 2007; Towle et al., 1997; Towle and Weihrauch, 2001; Tsai and Lin, 2007) a current working model for branchial osmoregulatory NaCl uptake of moderate hyper-regulating crustaceans was developed (Fig. 3A). This transport model is heavily reliant on the basolaterally localized Na^+/K^+-ATPase to energize NaCl uptake by creating electrochemical gradients that secondarily energize apically localized $Na^+/K^+/2Cl^-$ cotransporter to promote Na^+ and K^+ uptake from the environment. Absorbed K^+ is recirculated via apical K^+ channels, ensuring the $Na^+/K^+/2Cl^-$ cotransporter continuously functions. Apical Cl^- uptake is believed to occur through the electroneutral exchange of HCO_3^- for Cl^- by anion exchangers (AE) while cytosolic Cl^- moves into the hemolymph space through basolateral Cl^- channels due to the negative cell potential of the gill. The negative PD_{TE} generated by chloride's movement into the hemolymph further promotes Na^+ uptake via paracellular pathways of the moderately leaky epithelium (see Table 2; Riestenpatt et al., 1996; Weihrauch et al., 1999) into the hemolymph. Apically localized electrogenic $2Na^+/H^+$-exchangers (NHEs; Kimura et al., 1994; Towle et al., 1997) also facilitate uptake of Na^+ from the environment in exchange for H^+ as a counterion (Fig. 3A). As the Na^+/H^+ and Cl^-/HCO_3^- exchangers depend on H^+ and HCO_3^- as counterions, respectively, carbonic anhydrase plays an important role in maintaining osmoregulatory activity (Henry et al., 2003; Henry and Cameron, 1983). This mechanism has also been shown to be sensitive to cAMP levels by adding the cyclic nucleotide to the hemolymph-like saline of perfused gills of hyper-regulating blue crabs, *Callinectes sapidus* (Lohrmann and Kamemoto, 1987). This sensitivity is believed to be linked to cAMP activation of the Na^+/K^+-ATPase which is also sensitive to the presence of several hormones, presumably through similar mechanisms (see below; (Eckhardt et al., 1995; Genovese et al., 2006; Halperin et al., 2004). As a whole, comparison of this working model to that of the thick ascending limb of the loop of Henle in the mammalian kidney (Mount, 2014) reveals a high degree of similarity, confirming that the crustacean gill is an excellent model to explore NaCl transport mechanisms in a much younger evolutionary species.

Gill perfusions can also be used in more-or-less the same fashion to investigate adaptations of crustaceans to freshwater – a challenging environment whose low ionic content posed a major barrier of entry during the terrestrialization of ancestral species (Giribet and Edgecombe, 2019). While crayfish are often used as a model for freshwater osmoregulation of decapod crustaceans, their trichobranchiate gills are often small and difficult to perfuse (Barradas et al., 1997; Croghan et al., 1965); however, for some brachyuran crustaceans such as the holoimnic rainbow crab *Dilocarcinus pagei* (Onken and McNamara, 2002; Weihrauch et al., 2004) and the highly invasive Chinese mitten crab *E. sinensis*, (Rudnick et al., 2005), strong-hyperregulators capable of permanently inhabiting freshwater, working models for branchial osmoregulatory mechanisms have been developed (Fig. 3B). In contrast to gills of moderately hyper-regulating crustaceans, the freshwater branchial epithelium is energized by two ion pumps - the basolateral Na^+/K^+-ATPase, and the apically localized V-type H^+-ATPase - to drive transepithelial NaCl uptake (Freire et al., 2008; Larsen et al., 2014; Onken, 1996; Onken and Graszynski, 1989; Onken and Putzenlechner, 1995; Pequeux et al., 1984; Tsai and Lin, 2007; Weihrauch et al., 2001, 1999). In freshwater crabs, the apical V-type H^+-ATPase is expressed in specific cells that are likely electrically-coupled via gap junctions to mitochondria-rich cells that possess a high abundance of the Na^+/K^+-ATPase. Low intracellular $[Na^+]$ and a high negative cell potential drives Na^+ into the cytoplasm via apical Na^+ channels and potentially an electrogenic Na^+/H^+ exchanger (Towle et al., 1997; Weihrauch and Towle, 2000). Cytoplasmatic Na^+ then leaves the cell through the basolateral membrane in exchange for K^+ via the Na^+/K^+-ATPase like the previously described mechanism for moderate hyper-regulating crabs. Chloride uptake in freshwater crustaceans primarily occurs within the V-type H^+-ATPase rich cells through apical Cl^-/HCO_3^- exchange and the ion's subsequent passage from the cytosol to the hemolymph through basolateral Cl^- channels (Fig. 3B). Like osmoregulatory processes of hyper-regulating crabs, the carbonic anhydrase plays a pivotal role in providing counterions for Na^+ and Cl^- exchange with the additional responsibility of providing protons for the V-type H^+-ATPase in freshwater epithelia. Much like the osmoregulatory mechanism of hyper-regulating crustaceans, freshwater epithelia are sensitive to cellular cAMP levels presumably through activation of the Na^+/K^+-ATPase (Onken et al., 2000; Riestenpatt et al., 1994). Electrophysiological measurements on either perfused or split-gills of freshwater crustaceans have also indicated that their gill epithelia must be significantly tighter at the paracellular levels than brackish and seawater-dwelling crustaceans as indicated by their low conductance (G_{TE}) measured over the gills (ca. 4 mS cm^{-2} for *E. sinensis*; ca. 4 and 18 mS cm^{-2} for distal and proximal gill lamella of *D. pagei*, see Table 2; Onken and McNamara, 2002; Weihrauch et al., 1999). This improved epithelial tightness prohibits the uptake of Na^+ via the paracellular pathway, but also severely mitigates the passive loss of the ions to the

Table 2
Electrophysiological measurements of various crustaceans' branchial epithelia and their acclimatory conditions. Short circuit current (I_{SC}) represents the tissue's net ion transport. Conductance (G_{TE}) of the gill represents the ion-tightness of the tissue's paracellular pathways where high values are ion-leaky and low values are ion-tight. References: [1]Weihrauch et al., 1999, [2]Riestenpatt et al., 1996, [3]Onken and McNamara, 2002, [4]Onken and Putzenlechner, 1995, [5]Postel et al., 2000, [6]Lucu and Towle, 2010.

Species/tissue	Salinity (‰)	Isc (μA cm^{-2})	G_{TE} (mS cm^{-2})	Literature
Cancer pagurus Anterior gills	35		282	1
Cancer pagurus Posterior gills	35		253	1
Carcinus maenas Anterior gills	10		62	1
Carcinus maenas Posterior gills	10	-375	45	1,2
Dilocarcinus pagei Distal lamella	Tap water	-59	3.8	3
Dilocarcinus pagei Proximal lamella	Tap water	+41	18	3
Eriocheir sinensis Anterior gills	0.5		4	1
Eriocheir sinensis Posterior gills	Tap water	-88	3.6	1,4
Idotea baltica Endopodite	20	-445	84.1	5
Homarus americanus Epipodite	20	-185.4	55.2	6

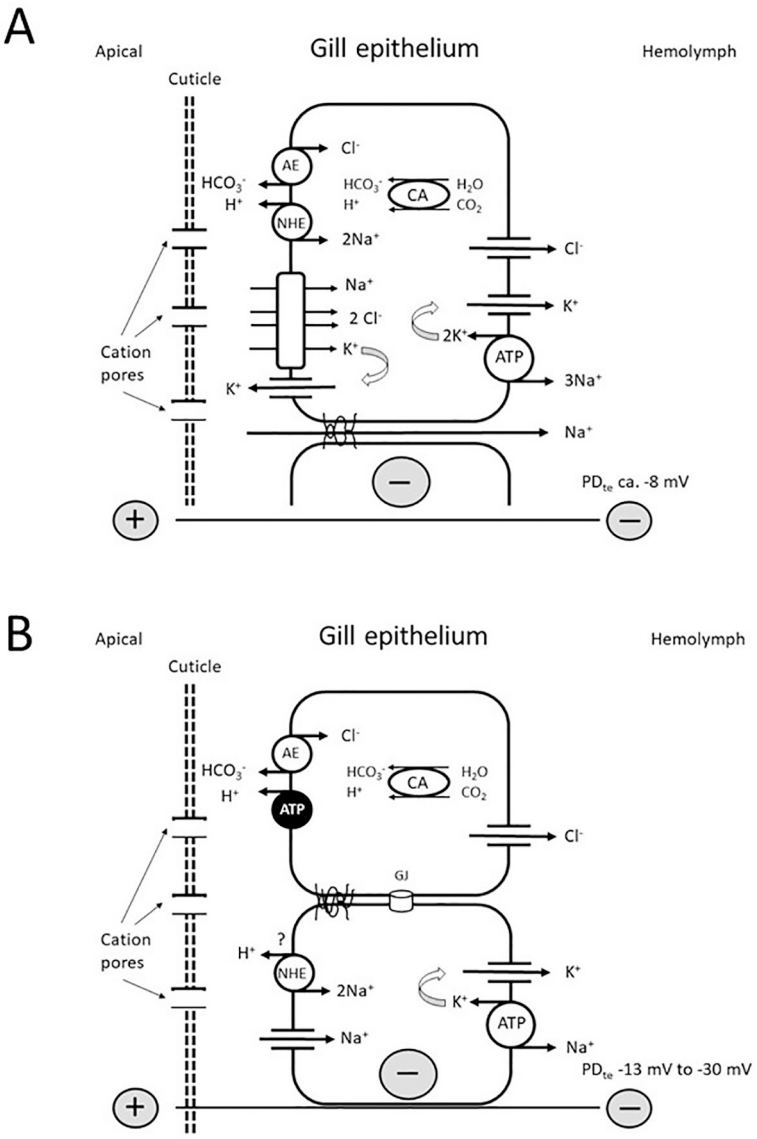

Fig. 3. Working models for the branchial NaCl uptake mechanism in a moderate hyperregulating crab (A), and freshwater dwelling crab (B). AE, anion exchanger; CA, carbonic anhydrase; NHE, sodium/proton exchanger; GJ, gap junctions. For details regarding to the mechanism refer to the text.

ion-poor freshwater environments along their chemical gradient.

5. Ammonia excretion

Amino acid catabolism is a fundamental process used by living cells to produce to maintain both basal and enhanced metabolic activity (McGaha et al., 2012). As amino acids are broken down, nitrogenous end-products are released as ammonia whose accumulation at even sub-millimolar levels can cause toxic effects including potentially lethal dysfunction of the nervous system, pH homeostasis, and ionoregulatory functions (Ip and Chew, 2010; Larsen et al., 2014; McKenzie et al., 2003; Wilkie, 1997; Young-Lai et al., 1991). As such, all animals must have an effective means of either excreting or detoxifying ammonia resulting in considerable dedication to uncovering related mechanisms and how life history and environmental constraints can drive evolutionary changes (Andrikou et al., 2019; Larsen et al., 2014; Weihrauch et al., 2017; Weihrauch et al., 2012b; Wright, 1995). Water-breathing animals take advantage of ammonia's water solubility to excrete nitrogenous end-products directly as ammonia across their branchial epithelium. Direct excretion of ammonia is beneficial to animals as energy is not required to detoxify ammonia by conversion to less toxic molecules such as urea or uric acid, which are common strategies of water-limited terrestrial species (Wright, 1995; Wright and Wood, 2012).

Shortly after the discovery of branchial ammonia excretion by Smith, 1929 in fish, research began to study how aquatic animals' excretion rates and hemolymph ammonia levels respond to environmental change. By the 1980s and 1990s, transbranchial transport mechanisms for ammonia began to develop in a holistic sense (Wilkie, 1997). In a simplified sense, these mechanisms presumed that passive diffusion of NH_3 gas along its partial pressure gradient (P_{NH3}; see reviews by Evans and Cameron, 1986; Kormanick and Cameron, 1981) was the main mechanism of ammonia excretion for most animals. Excretion of NH_4^+ was known to occur and was hypothesized to be due to NH_4^+ substitution for K^+ in transporters such as the Na^+/K^+-ATPase (Claiborne et al., 1982; Mallery, 1983), $Na^+/K^+/2Cl^-$ cotransporters (Evans and More, 1988), and K^+-channels due to the similar size and charge of the molecules or by Na^+/NH_4^+ exchange by an NH_4^+-accepting Na^+/H^+-exchanger (Cameron and Heisler, 1983; Krogh, 1938; Maetz and García Romeu, 1964); however, its contribution to total ammonia excretion was presumed to be much lower than the diffusion of NH_3 despite almost

all ammonia existing as NH_4^+ at physiological pH (pKa = 9.2-9.8; Cameron and Heisler, 1983). While most of the studies used to develop these hypothetical models provided crucial information, they were often based on whole animal manipulation which cannot distinguish the fine details occurring within the branchial epithelium.

The use of the perfused crustacean gill to study transepithelial ammonia transport allowed for much more detailed hypothetical transport models to be produced (Fig. 4). Targeted inhibition of specific K^+-transporters such as the Na^+/K^+-ATPase (Fehsenfeld and Weihrauch, 2016a; Weihrauch et al., 2002) and K^+-channels (Weihrauch et al., 1998) by ouabain and Cs^+, respectively, applied to the basolateral epithelium of the crustacean gill provided direct evidence that NH_4^+ is collected from the hemolymph and into the cytosol *via* K^+-substitution. In fact, the Na^+/K^+-ATPase is now known to mediate mass transport of NH_4^+ across the basolateral membrane of the gill epithelium (Weihrauch et al., 2017; Weihrauch and Allen, 2018) and can even be synergistically stimulated by the presence of NH_4^+ (Leone et al., 2017). In addition, the perfused gill revealed that ammonia excretion is severely impaired by disruption of the microtubule network leading to the discovery of vesicular trafficking of ammonia as a means of excretion (Fehsenfeld and Weihrauch, 2015; Weihrauch et al., 2002) where NH_3 is trapped within vesicles that are acidified by either the V-type H^+-ATPase or Na^+/H^+-exchangers (Fehsenfeld and Weihrauch, 2015; Weihrauch et al., 2002). These vesicles are believed to momentarily detoxify ammonia by removing it from the cytosol and eventually excrete ammonia by exocytosis, explaining how ammonia may be excreted independent of unfavorable environmental conditions (Fig. 4; Weihrauch and Allen, 2018). This process is presumably the main mechanism that allows gills of animals like the Dungeness crab, *Metacarcinus magister*, to actively excrete ammonia despite the presence of an 18-fold hemolymph-directed ammonia concentration gradient at rates that can rival the gill's excretion under ammonia-free conditions (Martin et al., 2011; Weihrauch et al., 1999). Since the discovery of this mechanism in crustaceans, it is now confirmed to be present in other systems including the insect gut (Weihrauch, 2006) and nematodes (Adlimoghaddam et al., 2015).

In addition to their use as a tool for testing hypothetical models, perfused crustacean gills can also be used to identify putative ammonia transporters and their importance to the net excretory process. For example, originally identified as a putative ammonia transporter in the nephrons of rat kidneys (Carrisoza-Gaytán et al., 2011), the highly conserved hyperpolarization-activated cyclic nucleotide-gated K^+-channel (HCN) has now been identified as an important ammonia transporter in the perfused gills of *C. maenas* through specific inhibition by ZD7288 in comparison to the generic Ba^{2+} inhibition of K^+-channels (Fehsenfeld and Weihrauch, 2016b). While few studies have taken advantage of this model system, it allows the transporter's relevance to be investigated within the intact excretory system rather than homologous expression of transporters suitable expression systems such as *Xenopus laevis* oocytes (Westhoff et al., 2002) or yeasts (Marini et al., 2000) given that a mode of inhibition or suppression of the target transporter exists (e.g. pharmaceuticals, gene knock-outs/ins, etc.).

6. Acid-base balance: H^+, CO_2, and HCO_3^- transport

Maintaining an organism's intra- and extracellular acid-base status is an essential physiological process as even slight changes in pH can reduce or even abolish enzymatic processes (Somero, 1986). Regulation of acid-base status prevents changes in fluid pH due to metabolic input (i.e. production of CO_2, H^+, OH^-, NH_3/NH_4^+) as well as environmental challenges. Elevation of environmental CO_2, termed hypercapnia, can dramatically affect an animals' acid-base status as environmental CO_2 rapidly diffuses across lipid bilayers. Furthermore, hypercapnia offsets the outwardly directed diffusive CO_2 partial pressure gradient (ΔP_{CO2}) animals are believed to rely on for CO_2 excretion (Melzner et al., 2009). Since CO_2 can be reversibly hydrated to form carbonic acid (H_2CO_3), elevated environmental or extracellular P_{CO2} will also reduce pH upon dissociation of H_2CO_3 into H^+ and HCO_3^- (and potentially further dissociation of HCO_3^- into H^+ and CO_3^{2-}). This process is the basis of how anthropogenic carbon emissions are driving ocean acidification, which is expected to induce severe challenges in aquatic organisms' physiology and energy allocation (IPCC, 2019). Unlike air-breathing animals that predominantly rely on altering their rate of respiration to manage their acid-base status, water-breathing animals rely on ion-transport as they must maintain high respiration rates to extract sufficient oxygen from surrounding waters limiting their respiratory flexibility (Gilmour and Perry, 2009; Henry and Wheatly, 1992; Perry and Gilmour, 2006). Therefore, many of the mechanisms underlying acid-base regulation in

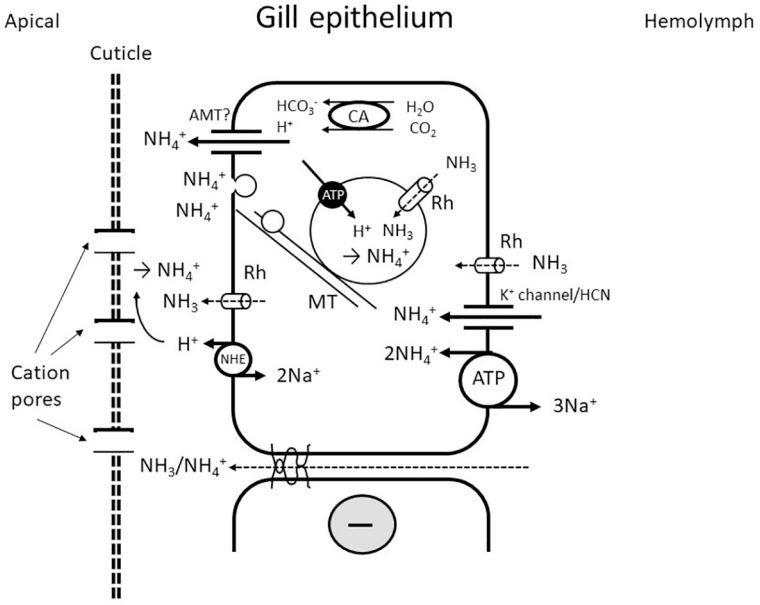

Fig. 4. Working model for the branchial ammonia excretion mechanism in a moderate hyperregulating crab. AMT, ammonia transporter; CA, carbonic anhydrase; HCN; hyperpolarization-activated cyclic nucleotide–gated channels; MT, microtubule network; NHE, sodium/proton exchanger; Rh, Rhesus-like protein. For details regarding to the mechanism refer to the text.

water-breathing animals relate to their osmoregulatory mechanisms as H^+ and HCO_3^- are common counter-ions for Na^+ and Cl^- transport, respectively (Kirschner, 1979; Mantel and Farmer, 1983; Truchot, 1983). These mechanisms include direct H^+-excretion, manipulation of the carbonate buffering system, and occasionally shifts in non-carbonate buffering systems to regulate their acid-base status (i.e. NH_3/NH_4^+; Evans et al., 2005; Fehsenfeld and Weihrauch, 2017; Henry et al., 2012; Weihrauch and Allen, 2018). Perfused gill preparations allow researchers to study the baseline acid-base regulatory capacity of the branchial epithelium by replicating native conditions as well as investigation of the animal's ability to respond to acute or chronic acid-base challenges such as those predicted to occur due to climate change (IPCC, 2019), or where poor water-quality develops as is common when animals are buried within the sediment (McGaw, 2005), or occupying tide pools (Pfister, 2007) and mangrove environments (Kathiresan and Bingham, 2001).

The general acid-base exploratory perfusion experiment involves bathing the gills in an apical medium representing the environment (*e.g.* 35 ppt. salinity, pH 8.0–8.2, 40 Pa P_{CO2}, 2.5 mmol l^{-1} [HCO_3^-], and 0 mmol l^{-1} [NH_4Cl]) while the internal perfusate is a simple saline similar in composition to the animal's hemolymph. Measurement of acid-base parameters pre- and post-passage of the hemolymph-like saline through the tissue allows the acid-base compensatory actions of the gill to be determined. Acid flux can be monitored using changes in pH as a proxy (Allen et al., 2020; Fehsenfeld and Weihrauch, 2015; Hans et al., 2014) or more accurately by acid-titration (Clifford et al., 2018). Combining changes in pH with measurements of total carbon can be used to mathematically determine changes in CO_2 and HCO_3^- levels using dissociation and solubility constants of the carbonate system in an appropriate species (often those of *C. maenas* determined by Truchot (1976)) and a modified version of the Henderson-Hasselbalch equation. It is worth noting that one should also determine environmental levels of the carbonate equilibrium, which require different equations provided by CO_2SYS software (Pierrot et al., 2006) and constants supplied therein. Changes in non-carbonate buffering systems such as ammonia can similarly be measured although solutions may require deproteinization if amino acids are present before use of either an ISE ammonia electrode (Weihrauch et al., 1998) or suitable plate-assay (Holmes et al., 1999; Verdouw et al., 1978). Performing these measurements provides a well-rounded base-line acid-base transport profile of the gill allowing for the addition of pharmaceuticals or molecules that target key acid-base regulatory transporters to determine their relevance to each process.

Since ion-transport processes are the main means of physiological regulation in water-breathing species (*i.e.* Na^+/H^+ exchangers, Cl^-/HCO_3^- exchangers, transport of NH_4^+ by K^+-transporters…) it can be difficult to interpret what transport is predominantly related to osmoregulation versus acid-base regulation. Using osmoconforming species such as marine crustaceans is a useful means of circumventing this issue as their NaCl transport rates are minimal or negligible in comparison to hyper- or hypo-regulating animals (Henry et al., 2012; Henry and Wheatly, 1992). Since the lack of NaCl transport causes PD_{TE} to be essentially zero, an alternative means of monitoring the perfused gill's viability over time is to apply reversible inhibitors where a return toward baseline measurement can be performed by removing the inhibitor from the saline (Fehsenfeld and Weihrauch, 2015).

These types of experiments have developed hypothetical working models of transbranchial acid-base balance such as those of Fehsenfeld and Weihrauch (2015) displayed in Fig. 5. The model describes the involvement of ion transporters such as the basolateral Na^+/K^+-ATPase, EIPA-sensitive Na^+/H^+ exchangers, and tenidap-sensitive Na^+/HCO_3^- cotransporters as well as vesicular trafficking of ammonia (Fig. 4) in acid-base regulation of seawater-acclimated posterior gills of *C. maenas*. Carbonic anhydrase, which is further discussed below, assists these transporters in the excretion of acid-base equivalents by providing a relatively constant availability of H^+ and HCO_3^- (Burnett et al., 1985; Burnett and Mcmahon, 1985; Gilmour and Perry, 2009; Henry et al., 2003; Henry and Cameron, 1982a; Perry and Gilmour, 2006). Most of the current gaps in these models concern the transporters within the apical gill epithelium, which are difficult to accurately target due to the crustacean cuticle using the perfused gill method (see below).

Transbranchial movement of CO_2 and its effects on the carbonate equilibrium is also crucial to maintaining an animal's acid-base homeostasis. Transport of CO_2 is influenced by carbonic anhydrase activity, the enzyme responsible for the rapid and reversible hydration of CO_2, which occupies a different physiological role in dilute and seawater due to its relation to osmoregulation. In dilute media, carbonic anhydrase plays an important role in both acid-base and osmoregulation where it provides a constant source of H^+ and HCO_3^- used as counterions for Na^+ and Cl^- uptake, respectively (Henry and Cameron, 1983; Neufeld et al., 1980; Towle et al., 1976). As salinity increases, the demand for

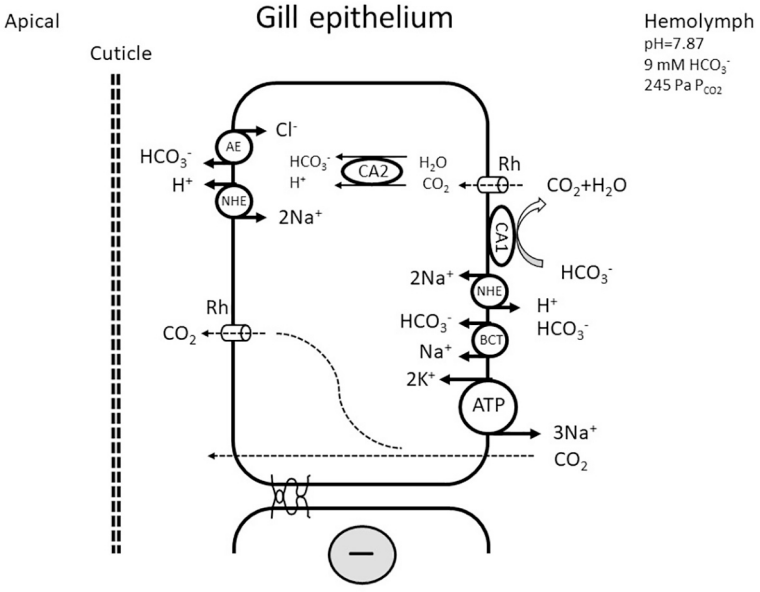

Fig. 5. Working model for the branchial acid-base regulatory mechanism in a moderate hyperregulating crab (*C. maenas*). AE, anion exchanger; BCT; bicarbonate cotransporter; CA, carbonic anhydrase; NHE, sodium/proton exchanger; Rh, Rhesus-like protein. For details regarding to the mechanism refer to the text.

osmoregulatory NaCl transport decreases allowing the role of carbonic anhydrase in acid-base regulation to be studied without osmoregulatory input (Burnett, 1984; Burnett et al., 1985; Burnett and Mcmahon, 1985). As HCO_3^- is dehydrated, CO_2 is capable of diffusing across the basolateral epithelia – possibly facilitated by CO_2-accepting Rhesus proteins (Endeward et al., 2008) – where it is believed to make its way across the cytosol and through the apical epithelia along its partial pressure gradient (Melzner et al., 2009).

The study of CO_2 excretion using crustacean gills offers an additional benefit as it lacks the extracellular carbonic anhydrase activity found in vertebrates (Henry and Cameron, 1982b). The carbonate system within vertebrate blood is kept in equilibrium by extracellular carbonic anhydrase activity, meaning that as metabolic CO_2 is released from non-excretory tissues it is rapidly hydrated within the extracellular space as HCO_3^- and a H^+. As extra-branchial tissues of crustaceans produce metabolic CO_2 it diffuses into the hemolymph space where the lack of carbonic anhydrase causes CO_2 hydration to occur at a naturally slow pace creating a carbonate disequilibrium. As hemolymph has a relatively short transit time to reach the gills (Aldridge and Cameron, 1979), most of this carbon will still exist as CO_2 and can rapidly permeate the gill epithelia for excretion. Indeed, these results were supported in part by the apparent lack of influence by carbonic anhydrase over whole animal CO_2 excretion rates upon their exposure to acetazolamide and Diamox (Aldridge and Cameron, 1979; Cameron, 1979; Henry and Cameron, 1983; McMahon et al., 1984). The low permeability of these carbonic anhydrase inhibitors across most tissue's basolateral epithelium (Henry and Cameron, 1983) meant that cytosolic carbonic anhydrase remained active. As a result, whole animal exposure to carbonic anhydrase inhibitors and the lack of extracellular carbonic anhydrase activity led to the belief that carbonic anhydrase was not involved in CO_2 excretion of crustaceans.

If this CO_2 excretion system was accurate, CO_2 release should occur independent of the hemolymph's transit time through the gill as most CO_2 diffuses in the first moments of entering the gill (Randall, 1982). The perfused gill technique was used to control the flow-rate of hemolymph-like saline through the tissue revealing that CO_2 excretion was dependent on transit-time in a fashion that could be abolished by adding carbonic anhydrase inhibitors to the perfusion saline (Burnett, 1984; Burnett et al., 1985; Burnett and Mcmahon, 1985). Shortly after this discovery, the addition of a membrane-impermeable dextran-bound inhibitor of carbonic anhydrase was used to provide evidence that the basolateral crustacean gill epithelium contains a hemolymph-acting membrane-bound carbonic anhydrase similar to the mechanisms used by mammalian lungs (Klocke, 1978; Swenson, 1984) demonstrating the potential use of perfused gills to provide at least partial details on the localization of a target (Burnett and Mcmahon, 1985).

As previously mentioned, decapod crustaceans are a versatile group of animals that can be used to model almost any environmental situation due to their wide berth of habitats. However, since most research has traditionally focused on transbranchial osmoregulation our detailed understanding of other regulatory processes is largely focused on euryhaline crustaceans such as *C. maenas* (Fehsenfeld and Weihrauch, 2015) and *C. sapidus* that may not accurately describe the systems used by stenohaline osmoconforming crustaceans. Less detailed studies have begun to characterize the transport of acid-base equivalents across the gills of some untraditional crustacean species such as marine swimming crabs *Portunus trituberculatus* (Ren et al., 2015) and *Metacarcinus magister* (Hans et al., 2014) as well as the shallow vent-endemic crab *X. testudinatus* (Allen et al., 2020); however, a push for a thorough mechanistic study on freshwater or strictly marine species would be beneficial for comparative studies (Fehsenfeld and Weihrauch, 2017; Henry and Wheatly, 1992). This is especially important in the study of crustaceans themselves given that regulatory mechanisms can be quite different based on a species life history. For example, the gills of osmoconforming marine crabs are characterized by ion-leaky paracellular pathways (Weihrauch et al., 1999). In theory, an ion-leaky paracellular pathway across the gill epithelium would increase the rate that environmental stressors can enter the animal's extracellular space (*i.e.* H^+ during hypercapnia or NH_4^+ upon exposure to high environmental ammonia) while also easing passive loss of compensatory molecules. The reduced need for NaCl transport machinery, and therefore potentially less H^+/HCO_3^- transporter abundance, could also be detrimental when faced with an acute environmental challenge in marine crustaceans. Despite these seemingly detrimental characteristics perfusion of marine osmoconformer gills have found that they are readily capable of restoring acid-base status through accumulation of HCO_3^- (Allen et al., 2020; Fehsenfeld and Weihrauch, 2015; Hans et al., 2014) and can even maintain active ammonia excretion against a 16-fold inwardly-directed ammonia gradient for several hours (Martin et al., 2011). In the field of CO_2 excretion, the crustacean gill remains an interesting model as recent studies have found that in the shallow hydrothermal vent crab, *Xenograpsus testudinatus*, CO_2 excretion is maintained despite an immense hemolymph-directed P_{CO2} gradient. This raises the fundamental question as to whether or not non-diffusive and active transport of CO_2 can occur in uniquely adapted extremophiles (Allen et al., 2020). Further investigations into CO_2 excretory mechanisms and acid-base regulation of the perfused crustacean gill will help identify aquatic animals' responses to climate change and ocean acidification.

7. Metal toxicity/toxicology

Understanding the toxicological influence of trace metals and pollutants is a major avenue of research that can help mitigate the consequences of anthropogenic activity and conserve the health of our aquatic ecosystems. Identifying the site and route of entry of a toxicant as well as its retention and ability to enter the circulation of organisms is fundamental to identify the consequences of pollution. Toxicants generally enter aquatic organisms across thin epithelia by either diffusion or uptake by ion-transporters through ion-substitution (Pedersen and Bjerregaard, 2000; Rainbow, 1997). As the gill epithelia of water-breathing animals are in direct interaction with the environment, thin enough to permit respiratory gas exchange, and packed with ion transporters they are the major route of entry for trace metals and toxins (Henry et al., 2012; Rainbow, 1997). For example, the usual route of entry for trace metals such as cadmium (Cd^{2+}), zinc (Zn^{2+}), lead (Pb^{2+}), and copper (Cu^{2+}) are through Ca^{2+}-uptake pathways whereas silver (Ag^+) and copper enter the gill epithelium through Na^+/H^+ exchangers (Bianchini and Wood, 2003; Brooks and Mills, 2003; Martins et al., 2011b; Martins et al., 2011a; Morris and Greenaway, 1992; Niyogi et al., 2016; Pedersen and Bjerregaard, 2000). Despite gills accounting for less than 1% of a crab's total mass they accumulate and retain about 33% of trace mercury (Laporte et al., 1997) and 43% of Cu^{2+} (Martins et al., 2011b) in animals after several days of exposure. Furthermore, even though gills readily collect trace metals from the environment they also act as a barrier of entry to the hemolymph space with only trace amounts being released across the basolateral epithelium into the hemolymph space (Blewett et al., 2015; Laporte et al., 2002).

The perfused gill is a considerably powerful model for investigating toxicant uptake as well as the potential for the molecule to enter circulation in aquatic organisms. Monitoring the gills' transepithelial potential difference (PD_{TE}) can be used to monitor the gill's viability while monitoring potential effects of the toxicant and/or inhibitory additive on the tissue's osmoregulatory machinery (Pedersen and Bjerregaard, 2000). Gills have been perfused by toxicologists with radiotracers (e.g. ^{109}Cd, ^{63}Ni, ^{64}Cu, ^{65}Zn) permitting sensitive monitoring of the trace metals uptake, retention, its ability to enter the hemolymph space, and the gill's ability to efflux the molecule if possible (Blewett et al., 2015; Laporte et al., 2002; Martins et al., 2011b; Niyogi et al., 2016; Nørum et al., 2005; Pedersen and Bjerregaard, 2000). Further supplementing these experiments employing pharmaceuticals or additives such as lanthanum that have provided valuable information regarding the

mechanism of uptake, particularly in relation to Ca^{2+} transports (Ca^{2+} channels, Ca^{2+}/Na^{+} exchangers, Ca^{2+}-ATPase; Pedersen and Bjerregaard, 2000). In fact, experiments on perfused gills have indicated that the rate of uptake of some trace metals is almost the same as the whole animals' uptake when extrapolated for time (Laporte et al., 2002; Martins et al., 2011a, 2011b) confirming the organ as the dominant site of uptake.

Once the site and mechanism of toxicant uptake have been roughly characterized it is often beneficial to investigate how environmental conditions may affect the toxicant's interactions with the animal. For example, reduced salinity usually increases the effects of trace metals as freshwater hyper-osmoregulators have a greater magnitude of ion-transport machinery in place compared to brackish- and marine-dwelling species (Henry et al., 2012; Martins et al., 2011b; Niyogi et al., 2016). The increased need to collect environmental Ca^{2+} greatly increases the accidental uptake of trace metals such as Cd^{2+} whose uptake reduces overall branchial respiratory and osmoregulatory capacity (Engel and Fowler, 1979; Nørum et al., 2005; Pedersen and Bjerregaard, 2000; Sunda et al., 1978) and, in the case of Cd^{2+} and Cu^{2+}, can even inhibit the Na^{+}/K^{+}-ATPase (Martins et al., 2011a; Postel et al., 1998). As global change increases the temperature and acidity of aquatic environments their inhabitants will be faced with increased energetic demands associated with maintaining homeostasis – especially at vulnerable early life-stages or during molting processes e.g. in crustaceans (Nørum et al., 2005). For example, the gills of post-molt crabs dramatically increase their uptake of ambient cadmium by 40-times while their efflux remains unchanged which may dramatically increase the animal's risks given the already strenuous act of molting (Nørum et al., 2005). On the other hand, some trace metals such as cadmium and mercury respond to pH shifts by changing their chemical speciation resulting in impaired gill efflux and increased uptake, respectively (Laporte et al., 1997; Pedersen and Bjerregaard, 2000); however, no study to date has used hypercapnia-mediated pH challenges which may differ in their effects on the animal. These factors will become especially important if they influence the ionized state of metals and toxins in addition to the direct effects on the animal's physiology and metabolism (Engel and Fowler, 1979; Sunda et al., 1978). These changes will likely affect the toxicity of pollutants and trace metals; however, little research has focused on these future conditions. The high degree of control over environmental and extracellular fluid compositions required to complete these studies is offered by the perfused gill methodology, permitting the ability to study the acute and chronic effects of toxicants in gills of unexposed and pre-exposed animals, respectively.

8. Molting-related transport physiology

Decapod crustaceans are often known as the masters of calcification – capable of forming and dissolving their exoskeletons through their growth and molt-cycle in a similar fashion to how vertebrates bones undergo deposition and solubilization (Luquet, 2012). This process is dependent on several transport processes including mass bi-directional mobilization of Ca^{2+}, Mg^{2+} and CO_3^{2-}, development of pH-sensitive microenvironments to dissolve and solidify the exoskeleton (Ziegler et al., 2004), manipulation of tissue water-content, and elimination of metabolic end-products (H^{+}, CO_2, NH_3/NH_4^{+}) produced by strenuous exercise during ecdysis (Mangum et al., 1985). These processes are largely regulated by the epithelia lining the exoskeleton called the hypodermis as well as the branchial epithelia; however, the latter is virtually unstudied in relation to molt cycles.

Transbranchial Ca^{2+} transport involves a mixture of Ca^{2+}-ATPases, Ca^{2+}/Na^{+} exchangers, and Ca^{2+} channels whose importance has largely been determined as a secondary result of studies focused on trace metal toxicity (see above section), only one of which considered the importance of molt-cycles (Nørum et al., 2005). Using the isolated perfused gill technique Nørum et al. (2005) demonstrated that following ecdysis (molt stage $A_{1\text{-}2}$), the gills of postmolt crabs increase their Ca^{2+} intake by 12-fold as a result of active Ca^{2+} transport compared to intermolt (C_4) crabs. While this study demonstrates that major changes occur across the post-molt crustacean gill, coverage of C_4 and $A_{1\text{-}2}$ does not cover the entirety of the molt cycle (early pre-molt $D_{0\text{-}1}$, late premolt $D_{2\text{-}4}$, post-molt A_1-C_3, and intermolt C_4; Roer and Dillaman, 1984). There is a substantial gap in how gills change throughout the molt-cycle despite the cycle's importance to crustaceans, especially considering its importance in the early life-stages of crustaceans when the molt-cycle is renewed more frequently.

While an understanding of the general transport processes and baseline factors governing molt-related branchial ion transport must come first, there is also a lack of information relating the sensitivity of these processes to environmental change. Investigating how molt-related transport processes are affected by changes in environmental pH is an especially interesting topic given that oceanic pH will be significantly reduced in the future due to ocean and freshwater acidification and rising P_{CO2} (IPCC, 2019) affecting calcification through either acid-degradation or disruption of the alkaline micro-environment involved in shell deposition (Orr et al., 2005). Additionally, the bioavailability of carbonate within the water is expected to decrease substantially, which could also complicate calcification (Orr et al., 2005). To date, a single study has investigated the pH-sensitivity of transbranchial Ca^{2+}-transport in crustaceans, where perfused gills of post-molt *Carcinus meditteraneus* reduced Ca^{2+} influx by about 50% in response to marine pH being reduced from 8.1 to 7.5 (Lucu, 1994). These results infer that crustacean Ca^{2+} homeostasis could be challenged as early as the year 2100 when similar pH values are roughly expected to occur (IPCC, 2019). In the future, similar studies focusing on molt-related changes in transbranchial Mg^{2+} transport and the epithelium's elimination of metabolic end-products or water flux would be beneficial as, to the authors' knowledge, their fields remain uninvestigated. These investigations could be achieved by passing either radio-isotopes or 'hot' ions ($^{41}Ca^{2+}$, $^{28}Mg^{2+}$, $^{14}HCO_3^{-}$, $^{3}H_2O$, or 14C-methylamine) or using their 'cold' equivalents through the perfused gill although use of 'cold' Ca^{2+} and Mg^{2+} may prove difficult to detect due to their potentially low basal rate of transport during some molt stages. Use of the scanning-ion electrode technique (SIET) may be an interesting means of measuring 'cold' ion fluxes across perfused gills; however, the technique is significantly more powerful in dilute media and may be unsuitable for detection in concentrated seawater.

Using the isolated perfused gill technique to study molt-cycle related transport processes is, while conceptually promising, challenged by molt-cycle changes in the gill's structural integrity. While the cuticle poses some challenges to perfusion experiments (see below), its presence significantly increases the gill's structural integrity and physical manipulability. During pre-molt, crustaceans partially dissolve their cuticle until they eventually construct a new soft cuticle that must harden (Luquet, 2012; Roer, 1980; Roer and Dillaman, 1984) making it difficult to set up the gill perfusion without harming the tissue. Furthermore, it can be reasonably difficult to identify the current molt-stage of a crustacean. This becomes especially important as minor shifts in the cycle have been detected even within minutes (Williams et al., 2009; Williams et al., 2004) following ecdysis posing difficulties in accurately performing experiments.

9. Branchial nutrient uptake

Breakdown of foodstuff provides animals with energy and nutrients that are essential to survival and can impact an animal's ability to maintain physiological homeostasis by either generating the required fuel for ion-transporting pumps and by providing the ions used by the pumps and transporters (Bakke et al., 2010; Taylor and Grosell, 2006). While gills are well documented to play a pivotal role in strong ion transport they also contribute to the uptake of trace metals (Bury et al., 2003) and, at least in the case of the Pacific hagfish (*Eptatretus stoutii*),

nutrient absorption (Glover et al., 2016, 2011). While these studies have largely focused on the gills of fishes, recent studies have also suggested that crustaceans use their gills to accumulate nutrients from their surrounding environments due to their ability to uptake amino acids when isolated and perfused (Blewett and Goss, 2017). Specifically, amino acid uptake was substantially higher within the posterior ionoregulatory gills suggesting it may be connected to ionoregulatory transport processes where it could influence volume homeostasis in addition to being a small source of nutrition.

Branchial amino acid uptake is a novel and largely unexplored topic in crustacean physiology and may be advantageous for burrowing or emersed crabs. Many crabs are benthic bottom feeders feasting on detritus or burying in the seafloor for extended durations (McGaw, 2005), direct amino acid-uptake by the gills may allow crabs to maintain a basal degree of nutrient availability while buried especially when freshly molted crabs are waiting for their new exoskeleton to solidify. Furthermore, recent evidence suggests that in the semi-terrestrial thick crab, *Helice formosensis,* alanine may be important upon emersion when the crab recycles urine through its branchial chamber. During urine production most of the free amino acids are reabsorbed by the antennal gland; however, alanine remains in the urine at high concentrations and may be recollected by the gills for later use or as a temporary means of detoxifying nitrogenous end-products (Allen et al., pers. communication). Further investigation using perfused gills would be beneficial to develop an understanding of when branchial amino uptake is biologically relevant and upregulated.

10. Hormonal influence

Hormones and neuroendocrine factors play an enormous role in the regulatory responses of animal systems; however, their roles in crustaceans transbranchial transport processes are poorly characterized. The perfused crustacean gill technique is an especially valuable method for studying the regulatory effects of neuroendocrine factors as it allows for the delivery of a controlled amount of a peptide to the intact organ's epithelia without systemic input. In crustaceans, the study of neuroendocrine control over transbranchial transport processes has almost exclusively focused on osmoregulatory NaCl transport processes of hyper-regulating crabs as their transport mechanisms are reasonably well-characterized. While this section of the review focuses heavily on the study of dopamine, it is important to note that several other neuroendocrine factors have known effects on transbranchial NaCl transport including serotonin (5-hydroxytrytamine, 5-HT), the crustacean hyperglycaemic hormone (CHH), and octopamine (Genovese et al., 2006; Kamemoto and Oyama, 1985; Mo et al., 2003; Morris, 2001; Spanings-Pierrot et al., 2000).

Perfusion of posterior gills of hyperregulating crabs such as *E. sinensis* and *C. sapidus* with dopamine caused an increase in cytosolic levels of cAMP upon interacting with basolaterally localized dopamine receptors (Trausch et al., 1989) that subsequently affected the crabs' Na^+ and Cl^- transport processes (Bianchini and Gilles, 1990; Kamemoto and Oyama, 1985; Mo et al., 2003, 1998). These findings are mirrored at the whole animal level upon exposing seawater crabs to dilute salinities where circulating levels of dopamine more than doubles within an hour of exposure (Zatta, 1987) and branchial cAMP levels increase by up to 135% after 24-h (Sommer and Mantel, 1991) in *C. maenas*. Since dopamine and several other neuroendocrine factors operate through modulation of cAMP levels, several studies have adopted to simplify the method through the addition of the membrane soluble equivalent of cAMP, dibutyryl-cAMP (db-cAMP), or pharmaceuticals that affect the turnover rate of cyclic nucleotides (theophylline and forskolin; Bianchini and Gilles, 1990; Genovese et al., 2006; Halperin et al., 2004) instead of the peptides, which are capable of similarly altering NaCl transport levels (Bianchini and Gilles, 1990; Lohrmann and Kamemoto, 1987; Mo et al., 2003, 1998; Riestenpatt et al., 1994). Several electrophysiological and transport studies have been used to identify the mechanisms neuroendocrine factors and cAMP utilize to alter NaCl transport across the gill epithelium of *E. sinensis*. In brief terms of Na^+ flux, elevated cAMP was originally believed to increase Na^+ uptake through stimulation of the Na^+/K^+-ATPase; however, current literature has indicated that cAMP directly increases the affinity of apical Na^+ channels as well as their abundance (Riestenpatt et al., 1994), indicating the process involves more than phosphorylation and activation of the Na^+/K^+-ATPase. Interestingly, some research has even produced conflicting results, demonstrating that, while the gills of *E. sinensis* have a positive correlation between cAMP and Na^+ uptake, the gills of *C. maenas* may have a negative one. Acclimation of *C. maenas* to dilute salinity induces elevation of branchial cAMP; however, Na^+/K^+-ATPase activity is rapidly reduced suggesting that while Na^+ transport is linked to cAMP in both freshwater and brackish water-dwelling crabs, their mechanisms may be diametrically opposed (Lucu and Flik, 1999). More recent studies have found that in *Chasmagnathus granulatus* – a crab capable of hypo- and hyper-regulating - dopamine may be able to stimulate Na^+ transport in both directions in a dose-sensitive fashion (Genovese et al., 2006; Halperin et al., 2004). In addition to Na^+ uptake, cAMP has been found to increase uptake of Cl^- through a combination of basolateral Cl^- channel activation and, at least in freshwater *E. sinensis*, stimulation of the apical H^+-ATPase and Cl^-/HCO_3^- exchangers (Bianchini and Gilles, 1990; Mo et al., 2003; Riestenpatt et al., 1994). Both processes may also differ in terrestrial crustaceans such as *Gecarcinus natalis* and *Birgus latro* where serotonin, not dopamine, is responsible for modulating NaCl transport (Morris, 2001). While this short summary cannot be considered a thorough review of osmoregulatory neuroendocrine control, further investigation is clearly required to develop a well-rounded mechanistic model.

The studies of neuroendocrine factors are further complicated by the multifunctionality of most peptides. For example, while the crustacean hyperglycaemic hormone (CHH) was originally recognized as a regulator of glycogen mobilization (Keller et al., 1985; Sedlmeier, 1985) it is now well documented to regulate osmoregulatory NaCl transport (Eckhardt et al., 1995; Spanings-Pierrot et al., 2000), modulates aggressive behaviour (Aquiloni et al., 2012), reproduction, and molting (Webster et al., 2012) in a range of crustaceans. With the high number of neuroendocrine peptides and the evidence that species- and dose-specific interactions can have highly variable results, there is a great deal of room for future experiments to explore. There is also a current lack of knowledge of how transbranchial processes outside of osmoregulation are affected by neuroendocrine factors despite their presumably high impact on them. The perfused gill remains perhaps the best way to study the effects of neuroendocrine factors on a specific organ at several doses and will expectedly continue to shed light on this area of research.

11. New concepts and concerns

While the isolated perfused gill technique has been used to study transport processes since the mid-1950s (Koch et al., 1954), it remains an interesting and valuable technique whose combination with modern techniques can expand its applicability or circumvent its limitations. The primary limitation of the perfused crustacean gill is that large additives including most pharmaceuticals cannot reliably be applied to the apical epithelium due to the cuticle. There is a significant chance of producing false-positive results upon addition of large molecules and pharmaceuticals to the apical epithelium as changes in transport can be caused by unspecific steric-inhibition of cuticular pores rather than inhibition of the target transporter itself (Onken and Riestenpatt, 2002). Therefore, any results obtained in this manner must be cross-checked by applying the pharmaceutical to the isolated cuticle ensuring the free passage of the drug through methods such as a modified Ussing chamber and a detection method of the pharmaceutical. While it is not an easy task, pairing perfusions with gene knock-down or knock-out/in manipulations would allow the influence of transporters that cannot be

targeted by pharmaceuticals (e.g. Rhesus proteins), targets whose inhibition is fairly unspecific (e.g. Na^+/H^+ exchangers, Cl^-/HCO_3^- exchangers, most ion-channels), or apically-localized targets whose inhibition by pharmaceuticals is hindered by the cuticle. Under a similar precedent, gills may also be perfused under tightly controlled experimental conditions for various lengths of time permitting their direct ion-transport processes and then stored for downstream investigation of mRNA expression changes by quantitative PCR which, to the author's knowledge, has yet to be performed. Such experiments would require a set of control 'time-zero' or 'un-perfused' gills that are stored immediately upon excision in either an RNA preserving solution or homogenized in Trizol to preserve the crab's initial state. These sorts of experiments are an interesting and unexplored means of using perfused gills for toxicological studies to study the effects of a toxicant on mRNA expression of target genes. These gills may also be used for histochemical studies related to the toxicant by passing fixative through the perfused gill at the end of the experiment to identify cellular responses to the toxicant (i.e. mobilization of vesicles or translocation of transporters) or localization of the toxicant itself within the tissue using techniques such as autometallography (Laporte et al., 2002). Including respiratory proteins (i.e. hemocyanin) in the perfusion saline has also yet to be investigated despite its potential effects on acid-base balance both by its native buffering capacity and its effect on respiratory gas exchange.

In addition to applying new techniques to the perfused gill technique, it is also critical to optimize the technique itself, especially the set-ups that relate to gas transports (e.g. CO_2). An often-overlooked portion of perfusion experiments involves the selection of proper tubing and performing an appropriate tubing-control to account for potential absorption or release of molecules through the tubing itself. While polyethylene tubing is commonly used due to its flexibility and low cost it is not gas impermeable and can result in tube-length dependent changes in ammonia and CO_2 levels in the saline. Silicone-based tubing is highly gas permeable and should never be used in perfusion experiments. While less flexible and harder to manipulate FEP-lined polyethylene and nylon tubing are practically inert and do not generally affect the saline compositions based on the manufacturer's specifications. Furthermore, studies focusing on acid-base balance or gas transport should use a setup that is as gas-tight as possible including the use of gas-tight syringes (e.g. Hamilton needles and syringes) to sample perfusate from septum-sealed vials ensuring CO_2 and NH_3 degassing is kept to a minimum. While these conditions are rarely considered in most current literature, they should be used whenever possible so long as they are relevant to the experiment's overall question or hypothesis. Finally, while it is always valuable to compose hemolymph-like salines as close as possible to the natural state, a perfect match is virtually impossible, especially when considering the impact of hormones, respiratory proteins, or immune components. Fortunately, as research continues and our knowledge concerning the effects and importance of different additives expands, this issue will improve over time and should be resolved.

12. Conclusion

Isolated perfused crustacean gills have brought forward essential information in regards to transbranchial osmoregulation, acid-base balance, ammonia excretion, metal toxicity, hormone regulation, and more recently branchial nutrient uptake that is valuable to the study of crustaceans themselves as well as to vertebrate systems such as the teleost gill or mammalian kidney through comparative physiology. While this method is best described using the gills of decapod crustaceans, researchers have recently begun adopting the technique to study other systems including the study of ammonia excretion in the gills of the common octopus, *Octopus vulgaris* (Hu et al., 2017), and osmoregulation across gills of the California mussel, *Mytilus californianus* (Neufeld and Wright, 1995). These studies have further demonstrated the technique's broad flexibility in invertebrate systems despite their gills' unique structures (Fig. 1). The isolated perfused gill technique offers full manipulability of environmental and extracellular fluid compositions and can maintain gill viability for more than 6-hours as tested for crustacean gills (Fig. 2; Siebers et al., 1985). Its uses extend from identifying ion and gas transport capabilities of the tissue to verifying hypotheses developed from molecular experiments to understanding the role of hormones. While the molecular era of biological sciences has offered an immense amount of crucial knowledge in the fields of transport physiology, it has led to the development of many hypothetical transport models that are rarely verified physiologically. The perfused crustacean gill not only offers a tried and true method to verify such hypotheses but may be combined with molecular techniques allowing users to incorporate both the molecular and physiological processes to be studied simultaneously.

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Declaration of Competing Interest

The authors declare no conflicting interests.

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