

Evolving views of ionic, osmotic and acid–base regulation in aquatic animals

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ABSTRACT

The regulation of ionic, osmotic and acid–base (IOAB) conditions in biological fluids is among the most fundamental functions in all organisms; being surrounded by water uniquely shapes the IOAB regulatory strategies of water-breathing animals. Throughout its centennial history, *Journal of Experimental Biology* has established itself as a premier venue for publication of comparative, environmental and evolutionary studies on IOAB regulation. This Review provides a synopsis of IOAB regulation in aquatic animals, some of the most significant research milestones in the field, and evolving views about the underlying cellular mechanisms and their evolutionary implications. It also identifies promising areas for future research and proposes ideas for enhancing the impact of aquatic IOAB research.

KEY WORDS: CO₂, Comparative physiology, Gill, pH, Ionocyte, Acidosis, Alkalosis

Introduction

Journal of Experimental Biology (JEB) has been communicating studies on comparative physiology since its very origin (Crew et al., 1923). Maintaining ionic, osmotic and acid–base (IOAB) homeostasis is essential for all organisms, and aquatic animals (see Glossary) have evolved diverse strategies to regulate the IOAB status of their biological fluids in response to metabolic and environmental challenges. This makes IOAB regulation by aquatic animals exceptionally well suited for comparative physiology studies.

All biochemical reactions are affected to some degree by ionic strength and pH. Ionic composition and gradients determine the transmembrane and transepithelial transport of molecules, water fluxes can lead to disruptive cell swelling or shrinking, and pH affects the function of proteins and enzymes. ATP hydrolysis produces H⁺ and thus cellular work is the most significant (and a continuous) challenge to acid–base (A–B) homeostasis. The majority of the H⁺ produced is consumed by mitochondrial oxidative phosphorylation (Hochachka and Mommsen, 1983), which establishes additional links between oxygen availability and A–B regulation (Tresguerres et al., 2020). However, living surrounded by water imposes interlinked advantages and restrictions on aquatic animals that are not experienced by air breathers. Chiefly, aquatic animals must maintain permeability with the surrounding water to sustain gas exchange, waste excretion and nutrient acquisition, which makes them prone to diffusive fluxes

of gases, ions and water that can challenge their IOAB homeostasis. Additionally, the lower content of O₂ and the higher solubility of CO₂ in water relative to air increase the energetic cost of O₂ uptake, decrease the HCO₃[−] buffering capacity of biological fluids and virtually rule out the ventilatory regulation of CO₂ excretion as an A–B regulatory strategy in aquatic animals. Instead, they largely compensate for an acidosis by actively excreting H⁺/NH₄⁺ in exchange for external Na⁺ and for an alkalosis by excreting HCO₃[−] in exchange for external Cl[−]. These processes take place across the gills and skin and are mechanistically similar to IOAB regulation in the mammalian nephron. However, the IOAB conditions in aquatic environments can be highly diverse and variable, which drove the evolution of correspondingly diverse and dynamic physiological mechanisms to maintain IOAB homeostasis. For example, Na⁺ and Cl[−] concentrations can range from ~500 mmol l^{−1} in seawater down to <10 μmol l^{−1} in freshwater, which imposes thermodynamic constraints on the IOAB regulatory mechanisms used by aquatic animals in each water body and an intrinsic link between systemic A–B and ionic regulation in some freshwater species. Estuarine animals face changes in salinity resulting from tides and changes in freshwater input, and animals that migrate between seawater and freshwater experience even more pronounced changes. The levels of O₂, CO₂, pH and ammonia can dramatically change owing to the photosynthetic and respiratory activity of organisms, organic matter decomposition, upwelling and algal blooms. This induces hypoxia, hyperoxia, hypercapnia (see Glossary) and high environmental ammonia for periods of time ranging from minutes to days or weeks, or can be cyclical. Furthermore, aquatic animals cannot escape anthropogenic impacts on water bodies that affect IOAB homeostasis, including changes in salinity regimes owing to landscape disruption and ice melting, CO₂-induced acidification, heavy metal pollution, hypoxic zones and other disturbances caused by agriculture run-off (Fig. 1).

The main goal of this Review is to convey the progression of knowledge about IOAB regulation in aquatic animals. This synopsis is divided into five arbitrarily defined and overlapping eras (Fig. 2), beginning with a brief summary of knowledge before JEB (Box 1) and highlighting some of the outstanding work published in JEB throughout its centennial history (with our apologies for the many omissions). For practical reasons, the focus is on NaCl transport and H⁺ and HCO₃[−] excretion in adult aquatic animals; additional details can be found in the exhaustive reviews cited throughout this article and in other recent reviews about NaCl uptake in freshwater animals (Lee et al., 2022), sessile crustaceans (Sundell et al., 2019), cephalopods (Hu et al., 2015) and freshwater insects (Silver and Donini, 2021), and about the pH/temperature α-stat hypothesis (Wang and Jackson, 2016) and the osmorepiratory compromise (Wood and Eom, 2021).

JEB is launched in 1923

The first JEB article to discuss aquatic IOAB regulation was a review on the factors that affect the migration of anadromous fishes,

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Glossary

Aquatic animal

An ectothermic water-breathing animal that lives surrounded by water most of the time; excludes air-breathing fishes and aquatic mammals.

Divided chamber (Van Dam chamber)

Apparatus that separates the anterior and posterior ends of a fish to differentiate the contributions of the gills versus the gastrointestinal tract and the kidneys to gas and ion transport processes.

Extracellular fluid

Aqueous solution that bathes cells within an organism, such as blood plasma, interstitial fluid, endolymph or hemolymph.

Euryhaline

An aquatic organism able to tolerate a wide range of salinities.

Hypercapnia

Elevated CO₂ level, also known as hypercarbia.

Hyperionic

Higher ionic concentration relative to another solution (typically, the surrounding water).

Hyperosmotic

Higher osmotic pressure relative to another solution (typically, the surrounding water).

Hypo-ionic

Lower ionic concentration relative to another solution (typically, the surrounding water).

Ionocyte

An epithelial cell specialized for ion transport.

Iso-osmotic

Same osmotic pressure relative to another solution (typically, the surrounding water).

Pavement cell

Squamous-cuboidal cells that comprise the majority of the fish gill epithelium. They are generally assumed to passively mediate the passive exchange of respiratory gases; however, the existence of pavement cell subtypes with other specialized functions cannot be ruled out.

Stenohaline

An aquatic animal that tolerates a relatively narrow range of environmental salinity.

List of abbreviations

A–B	acid–base
ASIC	acid-sensing ion channel
CA	carbonic anhydrase
cAMP	cyclic adenosine monophosphate
CFTR	cystic fibrosis transmembrane regulator (ABC35)
cGMP	cyclic guanosine monophosphate
DIDS	4,4'-di-isothiocyano-2,2'-disulfonic stilbene
FW	freshwater
IOAB	ionic, osmotic and acid–base
MR	mitochondrion-rich
MRC	mitochondrion-rich cell
NCC	Na ⁺ /Cl [−] cotransporter (slc12a3)
NCKX	K ⁺ -dependent Na ⁺ /Ca ²⁺ exchanger (slc24)
NHE	Na ⁺ /H ⁺ exchanger (slc9)
NKA	Na ⁺ /K ⁺ -ATPase (ATP1)
NKCC	Na ⁺ /K ⁺ /2Cl [−] cotransporter (slc12a1 and a2)
PD	pendrin (slc26a4)
PNA	peanut lectin agglutinin
qPCR	real-time PCR
Rh	Rhesus
RT-PCR	reverse transcriptase PCR
sAC	soluble adenylyl cyclase (adcy10)
SEM	scanning electron microscope/microscopy
SITS	4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid
slc	solute carrier
SW	seawater
TEM	transmission electron microscope/microscopy
VHA	V-type H ⁺ -ATPase

including salinity, pH and CO₂ (Chidester, 1924). This article posed the question of whether, by disrupting O₂ transport, the pH/CO₂ of the water could affect fish movement and distribution to a greater extent than salinity, and recommended that 'true field ecologists' included a pH-measuring apparatus in their toolkit. However, another view argued that environmentally relevant pH variations were too small to have a significant effect on aquatic animals (Saunders, 1926). Other JEB articles continued to document the effects of salinity changes on fluid composition (Bateman, 1932, 1933; Pantin, 1931) and on the respiratory rate of aquatic invertebrates (Beadle, 1931; Löwenstein, 1935), and the ionic and osmotic properties of hagfish blood (Bond et al., 1932) and trout eggs (Gray, 1932). Experiments on excised hearts showed the importance of urea for shark cardiac function (Simpson and Ogden, 1932), and back-to-back articles provided a comprehensive comparison of the mechanisms of urine formation in aquatic crustaceans and molluscs (Picken, 1936, 1937). These studies greatly contributed to establishing the osmoregulatory patterns described in modern textbooks: marine invertebrates and hagfish are iso-osmotic (see Glossary) with seawater, marine fishes actively excrete NaCl to remain hypo-ionic (see Glossary) to seawater, but there are osmoregulatory differences between bony and cartilaginous fishes. Moreover, freshwater animals actively take up NaCl and excrete a dilute urine to remain hyper-ionic and hyperosmotic (see Glossary).

Tonometer experiments described that, as observed in vertebrates, CO₂/H⁺ decreased the affinity of squid hemocyanin for O₂; however, the opposite effect was seen for horseshoe crab and whelk hemocyanins (a 'reversed Bohr effect'; Redfield et al., 1926), and a JEB article reported a biphasic response for crab hemocyanin (Hogben, 1926). A few years later, a seminal study described the effects of CO₂/pH on fish blood–O₂ binding in unprecedented detail. It confirmed differences between active and sluggish species, concluded that the effect of CO₂/pH on fish blood O₂ binding was more complicated than in mammalian blood and proposed a molecular mechanism whereby elevated CO₂ sequentially inactivated each of the heme groups that bind O₂ (Root, 1931). The diverse effects of A–B on blood–O₂ transport are a reminder that, although mammalian physiology can provide valuable guidance, gross extrapolations to aquatic animals are not advisable owing to the unique physiological mechanisms that have evolved in the many distinct aquatic taxa and environments.

A new wave of research established the sites and mechanisms for ion absorption and excretion using whole-animal experiments combined with dissection, perfusion, ligation and divided-chamber (see Glossary) techniques. It was deduced that the osmoregulatory strategy of marine bony fishes involved seawater drinking, intestinal water and NaCl absorption, branchial NaCl excretion and renal handling of divalent ions (Smith, 1930). It was noted that freshwater animals absorb NaCl from dilute solutions and proposed that fish and crabs took Na⁺ in exchange for NH₄⁺ and Cl[−] in exchange for HCO₃[−] across their gills (Krogh, 1938). Influential JEB articles described the ion-transporting properties of frog skin (Francis and Pumphrey, 1933) and of different portions of the insect larval gut (Koch, 1938; Wigglesworth, 1933a,b, 1938). Studies at the cellular level were pioneered by the histological identification of the fish gill 'chloride cell' (Keys and Willmer, 1932) and the histochemical identification of

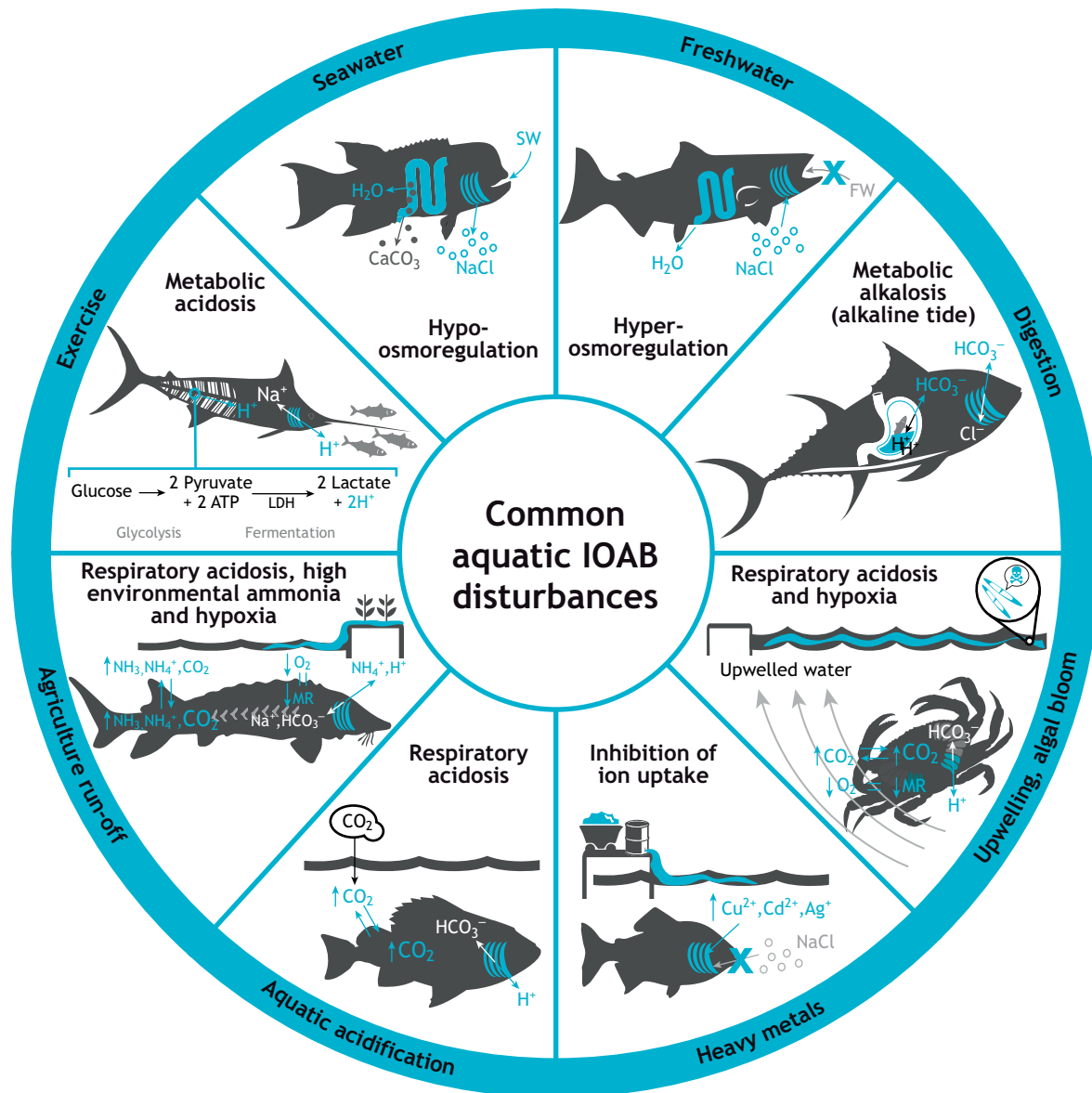


Fig. 1. Common ionic, osmotic and acid–base (IOAB) disturbances experienced by aquatic animals. Other disturbances include hypoxia, hyperoxia, postprandial acidic tide (see Box 5), feeding inside decomposing carcasses (equivalent to agriculture run-off), nighttime in densely populated environments such as kelp forests, coral reefs and mangroves (equivalent to upwelling and algal blooms), and absorption of human-generated sulfuric and nitric acids into freshwater (FW). MR, metabolic rate; SW, seawater.

Cl[−]-absorbing cells in anal papillae of freshwater larval insects and gills of crustaceans (Koch, 1934). These fundamental concepts were compiled in August Krogh's classic work 'Osmotic regulation in aquatic animals' (Krogh, 1938).

The overwhelming majority of JEB articles during the period originated from laboratories in the UK. Like many other normal aspects of society, JEB's publication output was severely disrupted by World War II (WWII). The few articles on aquatic IOAB regulation during the war years continued to document the effects of hypotonic environments on the fluid composition of invertebrate species (Beadle and Cragg, 1940; Ellis, 1939; Panikkar, 1941; Robertson, 1939), invertebrate muscular and ciliary function (Wells and Ledingham, 1940; Wells et al., 1940) and salmon sperm activation (Ellis and Jones, 1939). These types of studies continued in the post-war era, in some cases using more accurate methods to measure ions in minute volumes

of fluid (Ramsay, 1949, 1950; Robertson, 1949). In the USA, an iconic textbook for medical students synthesized the basics of A–B physiology (Davenport, 1947); it included detailed explanations of metabolic and respiratory challenges and adjustments using the 'pH–bicarbonate diagram' (today known as the Davenport diagram).

The 1950–1980 leap

As the devastating effects of WWII began to settle, there was a vigorous ramping up of technology, industry and research together with an explosion of new tools. Radioisotopes, such as ²²Na⁺ and ³⁶Cl[−] (generated as byproducts in nuclear fission reactors), were utilized to measure ion fluxes with high precision and accuracy. Radiotracer fluxes coupled with electrophysiological measurements in the Ussing chamber allowed for the identification and measurement of ionic currents across the skin of amphibians (Ussing, 1949). Later on,

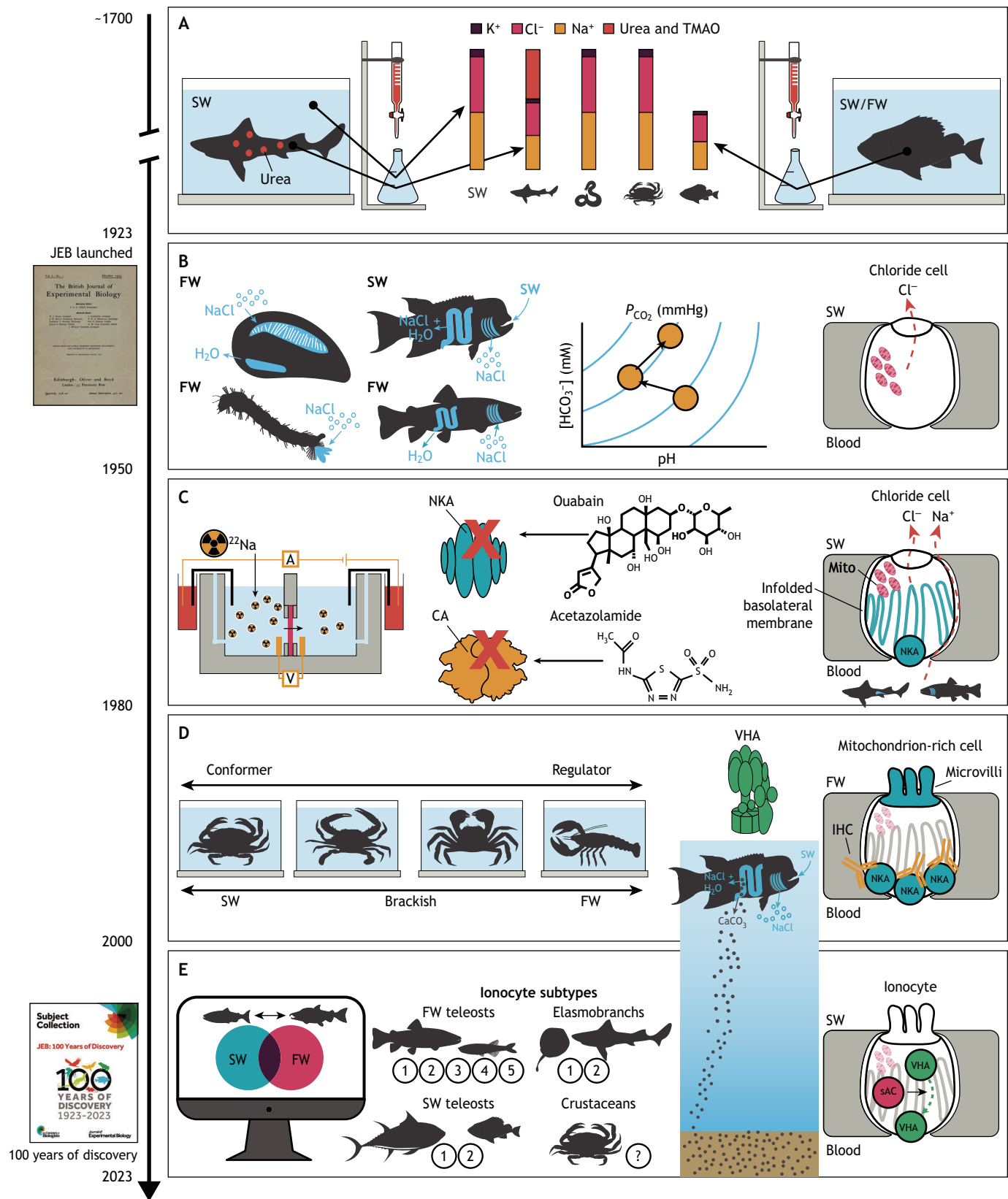


Fig. 2. See next page for legend.

the short-circuit current would be used as a proxy for net transepithelial ion transport (reviewed in Kirschner, 2004) across other flat ion-transporting epithelia, including the opercular membrane

of bony fishes, split gill lamellae of decapod crabs, cultured epithelial cells growing on permeable supports, segments of the fish gastrointestinal tract and the clam mantle.

Fig. 2. Selected research milestones of IOAB regulation of aquatic animals.

(A) Before JEB, differences in osmolyte composition were recognized among animals living in seawater (SW) and freshwater (FW). TMAO, trimethylamine-N-oxide. (B) Between 1923 and 1950, basic osmoregulatory strategies were identified (left), Davenport created his iconic diagram (middle) and the chloride cell was discovered (right). (C) The 1950–1980 leap produced the Ussing chamber (left), and the discovery of Na^+/K^+ -ATPase (NKA) and carbonic anhydrase (CA) and of specific drugs for functional studies (middle). Chloride cells were recognized to have numerous mitochondria (Mito), and the NaCl -excreting mechanism in teleost gills and the shark rectal gland was described (right). (D) Subsequent studies described crustacean osmoregulatory strategies (left) and marine teleost intestinal osmoregulation (middle image extending down into E) and its link with biomineralization. The V-type H^+ -ATPase (VHA) was discovered (middle). Microscopy techniques allowed ultrastructural and immunohistochemistry (IHC) studies of chloride cells (which were renamed ‘mitochondrion-rich cells’, right). (E) Between 2000 and 2023, molecular biology advances enabled mRNA network analyses to study responses to environmental challenges such as migrations between FW and SW (left). The importance of ichthyocarbonates for ocean biogeochemical cycles was recognized (middle). Ion-transporting cells were named ‘ionocytes’. Gill ionocyte subtypes were characterized, as were mechanisms that dynamically regulate ion transport, including VHA translocation to the basolateral membrane triggered by the activity of soluble adenylyl cyclase (sAC) (right).

Many paradigms, including the DNA double-helix structure and mitochondrial function, originated during the 1950s and 1960s and revolutionized biological research. A large number of enzymes were biochemically characterized, including carbonic anhydrase (CA) (Meldrum and Roughton, 1933) and Na^+/K^+ -ATPase (NKA) (Skou, 1957), and the drugs acetazolamide (Miller et al., 1950) and ouabain (Schatzmann and Räss, 1965) were developed to inhibit their respective activities *in vitro* and *in vivo*. Pharmaceutical companies developed the loop diuretics thiazide, furosemide and bumetanide, and the K^+ -sparing diuretic amiloride; these inhibit renal NaCl absorption and were used to treat high blood pressure (Jucker, 1976). Although the target proteins were not known at the time, these drugs were quickly adopted for investigating NaCl transport mechanisms in aquatic animals. Decades later, thiazide, bumetanide and amiloride would help identify Na^+/Cl^- cotransporters (NCCs), $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters (NKCCs) and Na^+/H^+ exchangers (NHEs). The stilbene compounds SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid) and DIDS (4,4'-di-isothiocyano-2,2'-disulfonic stilbene) were used to evaluate Cl^- -transporting mechanisms; however, their poor specificity made it difficult to discriminate between anion exchangers, Cl^- channels and other off-target effects.

The production of commercial transmission electron and scanning electron microscopes (TEM and SEM, respectively), coupled with the optimization of biological sample processing, enabled the visualization of the cellular ultrastructure during the 1960s and 1970s. Antibodies, fluorochromes and epifluorescence microscopy began to be used to detect proteins in tissues and cells (reviewed in Brandtzaeg, 1998); however, it would take until the 1990s for these techniques to be used for studying aquatic IOAB regulation.

The synergistic use of these novel concepts and tools triggered a rapid leap in knowledge. Robust CA and NKA activities were found to be a nearly universal characteristic of ion-transporting epithelia. Electrophysiology and radiotracer experiments coupled with drugs identified ion-transport mechanisms at the apical and basolateral membranes. TEM revealed that the ‘chloride cell’ of fish gills contained numerous large mitochondria and a tubular system (Doyle and Gorecki, 1961) that was later shown to be connected with the basolateral membrane (Philpott, 1966). Because these cells

transport other ions in addition to Cl^- , they were eventually renamed ‘mitochondrion-rich’ cells (MRCs). ‘Denser’ and ‘lighter’ MRC subtypes were noticed, the former being more abundant in seawater and having an apical pit (Doyle and Gorecki, 1961). Research during the subsequent decades (e.g. Laurent and Dunel, 1980) found a variety of MRC subtypes and other ion-transporting cells (collectively referred to as ‘ionocytes’; see Glossary) in different aquatic animals and environments.

During this era, JEB published hundreds of articles about aquatic IOAB regulation, especially on NaCl uptake by freshwater insects, crustaceans and fishes. This is reflected in the analysis of more frequently used words in JEB article titles during the 1960s and 1970s, which prominently feature ‘sodium’, ‘chloride’, ‘ionic’, ‘transport’, ‘pH’, ‘gills’, ‘osmotic’, ‘trout’, ‘goldfish’, ‘crayfish’, ‘crab’ and ‘malpighian’ (Franklin, 2023). Typical experimental approaches entailed measuring ionic fluxes, water–blood potential difference and composition of physiological fluids, sometimes in conjunction with inhibitors or exposure to environmental or metabolic challenges. These studies helped solidify the general concept of independent Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange in insect larvae, crustaceans and fish, as well as the link between ion uptake and A–B regulation in freshwater.

The presence of NKA enzymatic activity in gill extracts together with the inhibition of Na^+ uptake by ouabain and lack of inhibition by external K^+ indicated that basolateral NKA was the driving force for Na^+ uptake (Maetz, 1971). This would be confirmed using TEM observations of [^3H]ouabain binding in the basolateral membrane of freshwater gill MR-ionocytes (Kamaky et al., 1976). The pathway for Na^+ entry from freshwater into fish and crab gill cells was inhibited by amiloride (Kirschner et al., 1973); however, the NHEs (slc9 family) were yet to be characterized at a biochemical (Murer et al., 1976) and molecular level (Sardet et al., 1989). Whether Na^+ was taken up in exchange for metabolic H^+ or NH_4^+ was the subject of a healthy scientific debate that began to have some closure in the 2000s with the identification of ammonia-transporting Rhesus (Rh) channels (Wright and Wood, 2009; Box 2).

Inhibition of Na^+ uptake upon exposure to acidic pH (Maetz, 1973; Shaw, 1960) led to the realization of the potential damaging effects of acid release from coal mining and of acid rain on IOAB regulation (see Ultsch et al., 1981). A few years later, another link between anthropogenic pollution and aquatic IOAB regulation would be established, as scientists discovered the effects of metals on gill apical transporters involved in Na^+ and Ca^{2+} uptake, paracellular NaCl efflux and NKA activity (reviewed in Paquin et al., 2002).

All aquatic animals take up Cl^- in exchange for internal HCO_3^- through a mechanism that is generally inhibited by SITS (Perry et al., 1981). However, Cl^- uptake demonstrated high inter-individual variability and species-specific sensitivity to acetazolamide (Kerstetter and Kirschner, 1972), indicating a large influence of the internal milieu and diverse internal HCO_3^- sources. Inhibition of active Cl^- uptake by thiocyanate and anion-stimulated ATPase activity in gill plasma membranes were interpreted to reflect an apically located $\text{Cl}^-/\text{HCO}_3^-$ ATPase that energized Cl^- uptake (de Renzis, 1975). However, the anion-stimulated ATPase activity was later explained by mitochondrial contamination (Siebers et al., 1990), and the mechanisms that energize Cl^- uptake across the gills of freshwater teleosts remain unidentified.

In seawater fish gills, NKA was originally thought to be in the apical pit of the chloride cells and to directly pump Na^+ into seawater (Maetz, 1971). However, TEM and [^3H]ouabain binding assays determined that, just like in freshwater fishes, NKA was in

Box 1. The state of knowledge before JEB

The noxious effect of intracellular acidification was observed as early as the 17th century, when Isaac Newton noticed that vinegar (a weak acid) eliminated the spontaneous beating of isolated eel heart fragments (see Putnam and Roos, 1997). The impacts of ionic, osmotic and acid–base (IOAB) conditions on cardiac function were subsequently studied on frog hearts and organically expanded into molluscs, sharks and rays (McClendon, 1916; Mines, 1911). Generally, it was reported that acids and bases had opposing effects on heart beating; however, there was considerable interspecific variability that was attributed to differential interactions of H^+ and OH^- with the various levels of Na^+ , Cl^- , K^+ , Mg^{2+} and Ca^{2+} in the fluids of each animal.

Intracellular and extracellular fluid (see Glossary) composition was measured in a variety of aquatic animals using techniques such as change in mass, freezing point depression and ion titration. Notable work at the Stazione Zoologica di Napoli elucidated the osmolarity and salt concentrations in blood and tissues of a remarkable number of marine invertebrates and fishes, and identified basic ionoregulatory and osmoregulatory patterns (Frederiq, 1901). Foundational studies included the finding that sharks have ‘colossal quantities’ of urea (Staedeler and Frerichs, 1858) and that hagfish blood osmolarity was comparable to that of seawater (Lee, 1902). Mortality associated with fish in dilute water was proposed to occur as a result of impaired cellular functions resulting from salt loss across the gills (Ringer, 1884).

Some fundamental concepts emerged during this era, including the effects of CO_2 and pH on hemoglobin– O_2 binding (Bohr et al., 1904), the importance of the CO_2/HCO_3^- system in blood gas transport and pH buffering (summarized in Henderson, 1928), and the interrelationships between blood A–B balance and mammalian lung and kidney function (Davies et al., 1920; Haldane, 1921). Fish blood– O_2 binding was found to be much more sensitive to CO_2 than that of mammalian blood (Krogh and Leitch, 1919), and it was proposed that the ability of some fishes to rapidly increase their blood ‘alkaline reserve’ allowed them to maintain O_2 uptake from water with elevated CO_2 and decreased pH levels (Powers, 1922a,b).

the basolateral membrane (Karnaky et al., 1976). The enigma was solved through a series of functional studies using radiotracer fluxes, intracellular ion measurements, electrophysiology, and ouabain and furosemide treatment of live eels (Silva et al., 1977a), Ussing chamber-mounted killifish opercular membranes (Karnaky et al., 1977) and isolated and perfused shark rectal glands (Silva et al., 1977b). The model is among the most elegant in epithelial physiology: NKA drives electroneutral Na^+ and Cl^- cotransport from the blood into the cell, and because it also kicks the Na^+ back out, it acts as a chloride pump that actively loads up the cell with Cl^- . The negative intracellular electrical potential extrudes Cl^- into seawater or the rectal gland lumen, which in turn allows Na^+ to be extruded paracellularly down its electrochemical gradient (Silva et al., 1977b). The vibrating-probe technique would confirm that this mechanism takes place in MR-ionocytes (Foskett and Scheffey, 1982), and the model would be further perfected with the molecular, functional and localization characterization of the NKCC, the cystic fibrosis transmembrane regulator (CFTR) Cl^- channel and K^+ channels (reviewed in Evans, 2010; Box 3).

In the area of A–B regulation, experiments on rainbow trout identified the inverse relationship between plasma HCO_3^- accumulation and Cl^- loss during exposure to hypercapnia (Lloyd and White, 1967). A JEB article revealed that exposure to hypercapnia resulted in an equivalent increase in blood CO_2 that maintained the gradient for CO_2 excretion, instead of engaging the mechanisms to blow off excess CO_2 observed in air-breathing animals (Cameron and Randall, 1972).

Box 2. Ammonia excretion

Owing to its relevance for human health, mammalian renal physiologists quickly benefited from the molecular revolution through genomics, transcriptomics and access to genetic knockout mice, leading to major breakthroughs that served as inspiration for aquatic IOAB regulation research. The characterization of ammonia-transporting Rh channels from the mammalian kidney (Marini et al., 2000) and the model involving a functional coupling between apical RhCG with VHA in α -intercalated cells for NH_4^+ -trapping (Eladari et al., 2002) reinvigorated research on the mechanisms of ammonia excretion. Much of the foundational work on Rh proteins in aquatic animals has been published in JEB, including the first report of an Rh-like cDNA (from crab gills, Weihrauch et al., 2004) and functional studies on live fish (Hung et al., 2007), fish-gill cultured cells (Tsui et al., 2009) and mosquito larval anal papillae (Durant et al., 2016). JEB also published timely Review articles about the roles of Rh proteins in ammonia excretion in fish (Wright and Wood, 2009) and invertebrates (Weihrauch and Allen, 2018). More recently, methylammonium permease/ammonium transporters (Mep/Amt) were proposed to facilitate the entry of NH_4^+ from the hemolymph into mosquito anal papilla epithelial cells for its subsequent excretion through apical Rh channels (Chasiotis et al., 2016). Mep/Amts are not found in vertebrates, but may play important roles in ammonia excretion in other aquatic invertebrates. Even more recently, a JEB article identified hippocampus-abundant transcript 1 (DrHiat1) as a novel transporter that contributes to ammonia excretion in zebrafish larvae and potentially in other vertebrates (Zhouyao et al., 2022). In another exciting development, hagfish were shown to actively excrete NH_4^+ against a large gradient using apical NHEs in the gill ionocytes (Clifford et al., 2022b). But unlike other mechanisms in which NHEs acidify the boundary layer to promote NH_4^+ -trapping, hagfish NHE seems to directly excrete NH_4^+ in place of H^+ . Moreover, exposure to high environmental ammonia induced the disappearance of Rhcg from the ionocytes’ apical membrane, a mechanism that would restrict the inward diffusion of NH_3 while hagfish feed inside decomposing carcasses (Clifford et al., 2022b).

1980–early 2000s: an increased focus on organ and cellular studies

Studies on fish tissues

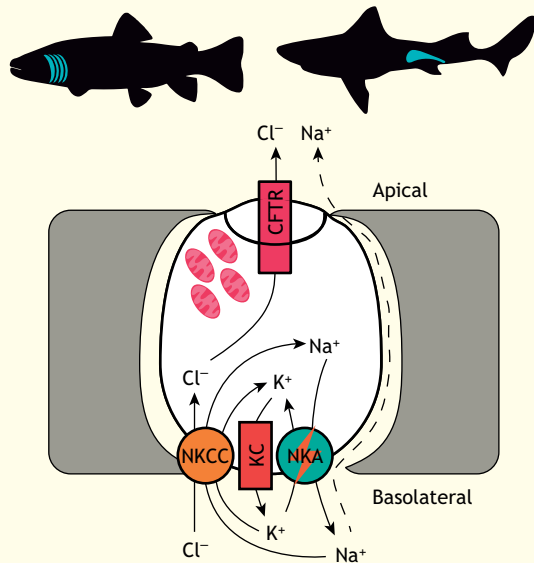
The teleost opercular membrane and the shark rectal gland enabled studies on the effects of hormones, neuropeptides and signaling molecules on ion transport without confounding effects from the rest of the animal (Epstein et al., 1983; Foskett et al., 1983). Similar studies on marine fish intestines identified the furosemide-sensitive co-transport of Na^+ , K^+ and Cl^- across the apical membrane (Musch et al., 1982), the differential effects of the second messengers cAMP and cGMP on ion absorption (Rao et al., 1984), and the links between HCO_3^- excretion and NaCl and water absorption (Ando and Subramanyam, 1990). The flounder urinary bladder revealed thiazide-sensitive apical electroneutral Na^+, Cl^- co-transport (Stokes, 1984), which would eventually lead to the initial identification and cloning of an NCC (Gamba et al., 1993). It was clear that IOAB regulation in aquatic animals was achieved by the concerted action of multiple ion-transporting proteins with species- and tissue-specific differential expression and regulation.

Attempts to study ion transport across fish gills independently from the rest of the animal relied on perfused head and isolated arch preparations (Perry et al., 1984a). However, this approach fell out of use midway through the 1980s, probably owing to technical limitations that affected reproducibility between studies. Nonetheless, it generated interesting conclusions, such as the potential partitioning of Cl^-/HCO_3^- and Na^+/H^+ exchanges between gill filaments and lamellae (Avella et al., 1987) and the rapid differential adrenergic regulation of ion uptake (Perry et al., 1984b). Other studies used SEM coupled with NaCl uptake kinetic analysis to correlate ultrastructural changes in

Box 3. NaCl excretion

NaCl secretion by fish gills and rectal glands is accomplished by the coordinated action of Na^+/K^+ -ATPase (NKA), $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter 1 (NKCC) and a K^+ channel (KC) located in the basolateral membrane of NaCl-excreting ionocytes, cystic fibrosis transmembrane regulator (CFTR)-like Cl^- channels located in the apical membrane, and paracellular transport of Na^+ . The elucidation of the ion-transporting proteins responsible for transcellular Cl^- excretion was the result of numerous studies, notably the functional characterization and localization of NKCC in dogfish rectal gland (Lytle et al., 1992) and Atlantic salmon gills (Pelis et al., 2001), and of a CFTR-like Cl^- channel in primary cultures of opercular epithelium (Marshall et al., 1995) followed by the subsequent cloning and characterization of CFTR in killifish gill and opercular epithelia (Singer et al., 1998). The co-localization of NKA, NKCC and CFTR in the same gill ionocyte was first reported in mudskipper (Wilson et al., 2000a). Further evidence for the roles of these proteins in NaCl excretion was provided by increased immunoreactive signals observed upon transfer of goby from freshwater to brackish and seawater – especially for CFTR (McCormick et al., 2003). More recent research has characterized the role of claudin tight junction proteins in the paracellular excretory pathway for Na^+ in gills of seawater-acclimated killifish (Marshall et al., 2017).

The cellular mechanism of NaCl excretion originally described in shark rectal gland and teleost gills is assumed to apply to all animal NaCl-excreting organs, including the nasal, lacrimal and tongue glands of birds and reptiles. Interestingly, CFTR immunolocalization in salmonid gill ionocytes remains unsuccessful even after attempts using homologous antibodies (Takei et al., 2014). Moreover, the CFTR ortholog of sea lamprey has unique structural and functional characteristics compared with those of more derived vertebrates (Cui et al., 2019), raising the possibility of an alternative apical Cl^- channel in the NaCl excretory mechanism of some fishes.



apical ionocyte surface area with blood IOAB regulation in response to environmental and metabolic challenges. This approach identified a ‘morphometric regulation’, whereby ion transport rates were modulated by differential changes in the apical surface area of the ionocyte subtypes through the development of microvilli and the retraction of adjacent pavement cells (see Glossary), resulting in increased ionocyte exposed apical membrane surface (Cameron and Iwama, 1987; Goss et al., 1992, 1994; Perry and Laurent, 1989).

The biochemical discovery of the V-type H^+ -ATPase (VHA) in the fungus *Neurospora crassa* (Bowman and Bowman, 1982) led to another re-evaluation of the mechanisms for IOAB regulation. The physiological roles that were ascribed to VHA during the 10 years following its discovery have been documented in an iconic JEB Special Issue (Harvey and Nelson, 1992). This early research focused on eukaryotic endocytic vacuoles and organelles, and also investigated the plasma membrane of specialized cells in the mammalian kidney, the frog skin and the gut of insects. Subsequently, the VHA proved to be a critical driving force for IOAB regulation in freshwater, for HCO_3^- excretion in seawater and for many other A–B-related cellular processes in a variety of aquatic animals.

The need to visualize larger tissue areas and to establish the cellular localization of ion-transporting proteins led to the adoption of immunohistochemistry. To our knowledge, its first use in aquatic IOAB regulation research was to study the localization of VHA in trout gills; this research was published in JEB (Lin et al., 1994). Other landmarks include the original immunolocalization of NKA in fish gills using the $\alpha 5$ monoclonal antibody (Witters et al., 1996), the co-immunolocalization of multiple proteins in tilapia, trout and mudskipper (Wilson et al., 2000a,b), and the visualization of distinct NKA- and VHA-rich ionocytes in freshwater and seawater Atlantic stingray (Piermarini and Evans, 2000).

The idea that VHA may play a role in IOAB regulation in freshwater fishes (Avella and Bornancin, 1989) and the first evidence supporting it (Fenwick et al., 1999; Lin and Randall, 1991; Lin et al., 1994; Sullivan et al., 1995) were published in JEB during the 1990s. These studies concluded that VHA in the apical membrane of the pavement cells in gill lamella excreted H^+ and energized Na^+ uptake via an apical Na^+ channel, similar to the role of MR-ionocytes in frog skin and toad urinary bladder and to mammalian renal intercalated cells. However, pharmacological VHA inhibition also depressed Cl^- uptake (Fenwick et al., 1999), and VHA was additionally present throughout gill filament cells (Lin et al., 1994). Furthermore, freshwater Atlantic stingray gill cells had VHA in their basolateral membrane, which suggested a role in H^+ absorption and energizing HCO_3^- excretion to seawater (Piermarini and Evans, 2001; Box 4).

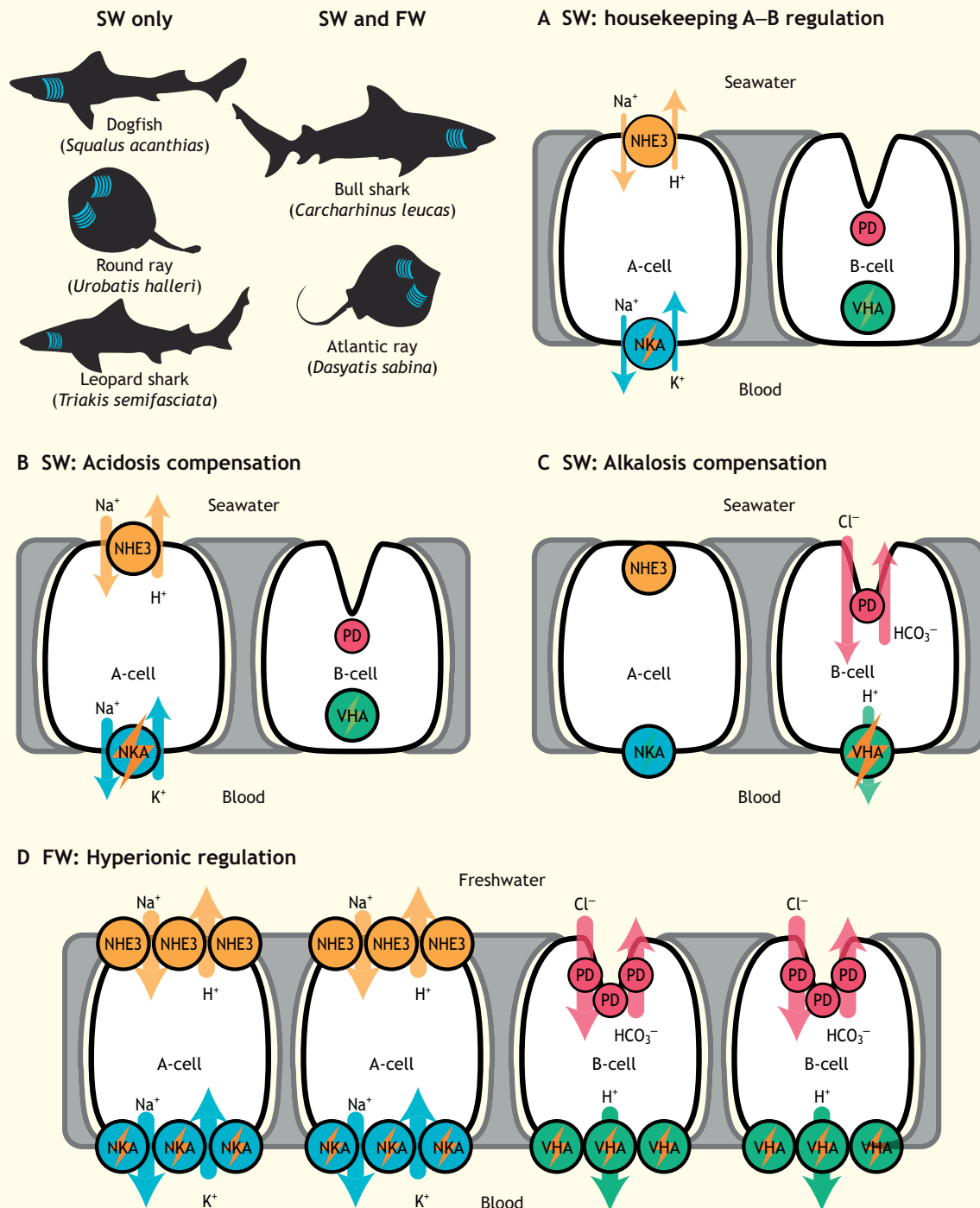
Other emerging experimental approaches were to isolate and culture gill cells for histological, TEM and biochemical studies (e.g. Hootman and Philpott, 1978; Perry and Walsh, 1989) and to measure intracellular pH regulation in live cells using ratiometric dyes (Pärt and Wood, 1996). To address the issue of cell polarity loss and to study transepithelial NaCl uptake, pavement cell primary cultures were grown on semi-permeable supports (Wood and Pärt, 1997) and ‘double seeded’ with MR-ionocytes (Fletcher et al., 2000). Although these preparations have not yet been able to adequately mimic the active transepithelial ion-transporting mechanisms of gills, they have proven helpful for studying ammonia excretion, paracellular permeability, hormonal regulation and the effects of pollutants (reviewed in Bury et al., 2014).

Studies on crab gills

A flurry of functional and molecular studies published in JEB during the 1990s and early 2000s greatly increased our understanding of ion transport across the posterior gills of decapod crustaceans. These gills are specialized for ion transport, can be isolated and perfused with relative ease, and their hemilamellae can be mounted in Ussing chambers. The cellular mechanisms underlying branchial ion transport in crustaceans have been extensively reviewed (Freire et al., 2008; Henry, 2001; McNamara and Faria, 2012). As

Box 4. Mechanisms for IOAB regulation in elasmobranch gill ionocytes

A synthesis of the literature on Atlantic stingray, bull shark, dogfish, leopard shark and round ray reveals a holistic picture of blood IOAB regulation in elasmobranchs. (A) In seawater, the acid-excreting (A)-ionocytes clear excess metabolic H^+ from the blood using basolateral NKA and apical Na^+/H^+ exchanger 3 (NHE3). (B) The A-ionocytes can be further activated during exercise and hypercapnia. (C) The base-excreting (B)-ionocytes are normally inactive; however, the postprandial alkalosis triggers the translocation of VHA to the basolateral membrane and of pendrin (PD) to the apical membrane, which activates HCO_3^- excretion and H^+ absorption. These mechanisms are mediated by pre-existing NKA, NHE3, VHA and PD protein, are modulated by local A–B sensors – such as soluble adenylyl cyclase (sAC) – and possibly by central adrenergic pathways, and are readily reversible. (D) Elasmobranchs that migrate from seawater to freshwater experience a reversal of ionic and osmotic fluxes. The short-term compensatory response involves shutting down rectal gland $NaCl$ excretion and activation of gill A- and B-type ionocytes to actively take up $NaCl$. In the longer term, there is increased NKA, NHE3, VHA and PD protein abundance, as well as increased numbers of A- and B-ionocytes. The size of the arrows is qualitatively proportional to the magnitude of the ionic fluxes. Intracellular and extracellular carbonic anhydrases, sAC, basolateral Cl^- channels and other important components of these mechanisms are not shown for the sake of simplicity.



environmental salinity decreases, gill epithelial tightness, NaCl uptake capacity and CA activity increase in association with a switch in ion-transporting mechanisms that provides clues about the evolution of hyperosmotic regulation during the invasion of freshwater environments. The gills of osmoconforming marine crabs show apical Na^+/H^+ and Cl/HCO_3^- exchange utilizing H^+ and HCO_3^- generated by cytosolic CA as substrates and powered by basolateral NKA. Similarities with hagfish (Evans, 1984), hemichordates and cephalochordates (Sackville et al., 2022) support the hypothesis that the original function of gill ion transport was A–B regulation and that this was later co-opted for hyperosmotic regulation. Weak hyperosmoregulators (e.g. *Carcinus maenas*) that live in brackish water (10–35 psu) absorb most NaCl through apical NKCC and basolateral NKA and an associated paracellular Na^+ flux (Riestenpatt et al., 1996). Moderate hyperosmoregulators (e.g. *Neohelice granulata*) use similar mechanisms in brackish water (Onken et al., 2003), and may additionally engage apical VHA to energize NaCl uptake in more diluted environments (1–2 psu) (Genovese et al., 2005). However, an inability to reduce paracellular permeability and the ensuing leak of NaCl precludes these animals from living in freshwater permanently. Strong hyperosmotic regulators (e.g. *Eriocheir sinensis*) have tight gill epithelia and can live in freshwater with <0.5 psu. They use apical VHA coupled with cytosolic CA activity to energize Na^+ uptake across a channel, or an electrogenic $2\text{Na}^+/\text{H}^+$ exchanger and Cl^- uptake via an anion exchanger. At the basolateral membrane, NKA energizes the movement of both Na^+ and Cl^- from the ionocyte into the hemolymph; under open-circuit conditions, the Na^+ and Cl^- currents short-circuit each other and allow for transepithelial NaCl uptake (Onken, 1999; Onken and Putzenlechner, 1995). The red freshwater crab *Dilocarcinus pagei* uses a similar mechanism to *E. sinensis*; however, Na^+ and Cl^- absorption take place in opposite hemilamellae (Onken and McNamara, 2002), which implies a regional partitioning of ionocyte subtypes that deserves further attention.

Some crab species are able to excrete NaCl across their gills to hypo-osmoregulate (Luquet et al., 2002; Martinez et al., 1998) through an unknown NaCl excretion mechanism, although gene upregulation during acclimation to concentrated seawater suggests the involvement of NKA, NKCC and VHA (Luquet et al., 2005). Interestingly, the outside positive transepithelial potential difference (Luquet et al., 2002) and the lack of CFTR in invertebrates indicate a mechanism that is fundamentally different from that of vertebrates. Clearly, IOAB regulation in crustacean gills would benefit from future studies investigating the cellular and subcellular localization of ion-transporting proteins and potential ionocyte subtypes associated with IOAB regulation.

Ion transport across isolated crab gill preparations is rapidly regulated by eyestalk extract, hormones, neuropeptides and second messengers, and by changes in hemolymph osmolarity, pH and $[\text{HCO}_3^-]$ (Onken, 1996; Riestenpatt et al., 1994; Siebers et al., 1985; Tresguerres et al., 2003, 2008) (Table S1). This type of regulation, which is too fast to involve changes in gene expression, is likely to play an essential role for the crab (and other animals) *in vivo* during sudden salinity changes encountered during tides, in estuaries and in tide and rain pools.

Studies on biomineralization

The comparative focus fostered by JEB catalyzed a vigorous exchange of ideas among subdisciplines that helped identify mechanistic links between IOAB regulation and multiple other physiological processes in aquatic animals. Among these, biomineralization stands out because

of its critical importance for global carbon cycling and an urgency to understand the impacts of climate change on aquatic life beyond those predicted by geochemical models that tend to dismiss biological regulation. Research published in JEB characterized links between A–B regulation, H^+ excretion and the formation of the crab carapace and fish otoliths (Cameron, 1985; Cameron and Wood, 1985; Payan et al., 1997). These studies, together with research on corals (Furla et al., 2000; Tambutté et al., 1996), indicated that a significant portion of the dissolved inorganic carbon in biomineralized structures was sourced from metabolically generated CO_2 , whereas the rest comes from environmental HCO_3^- . Although not surprising (after all, terrestrial vertebrates build their bone using CO_2 and HCO_3^- derived from mitochondrial respiration and blood plasma), these mechanisms go against the mainstream biogeochemical view that ocean acidification impairs biomineralization owing to the associated decrease in environmental CO_3^{2-} availability (NOAA PMEL Carbon Program, 2023). Importantly, our ability to forecast potential effects of ocean acidification on biomineralization is strictly linked to the generation of further knowledge about the underlying cellular and physiological mechanisms, both general and species-specific. This is an urgent topic that is uniquely suited for comparative IOAB experimental physiologists.

Research during the 1980s and 1990s identified a novel source of biomineralization resulting from intestinal IOAB regulation in marine bony fishes that was proposed to have geological significance (Walsh et al., 1991). This prediction would be confirmed 20 years later, and ichthyocarbonates would be estimated to contribute ~15% of oceanic carbonate production (Wilson et al., 2009) and >70% of mud carbonates in mangroves (Perry et al., 2011). This biological process is the result of cellular IOAB regulatory processes that allow bony fishes to stay hydrated in seawater (reviewed in Grosell and Oehlert, 2023), and also deserves more consideration by oceanographers and marine biogeochemists.

2000–2020s: the rise of molecular biology

The new century saw an explosion in the utilization of molecular techniques to clone cDNAs encoding ion-transporting proteins, determine their tissue expression and quantify their mRNA abundance in response to salinity or A–B challenges. Initially, these tasks relied on degenerate reverse transcriptase PCR (RT-PCR), northern blots and semi-quantitative RT-PCR, but they later transitioned to expressed sequence tag libraries, mRNA microarrays and real-time PCR (qPCR). Although these techniques were time consuming and expensive, they had the benefit of a requirement for *a priori* hypotheses that guided experimental design and interpretation of results. Early in the 2000s, a new experimental trend started whereby animals were exposed to an IOAB challenge and changes in mRNA abundance were interpreted as evidence for the involvement of the encoded proteins in the underlying homeostatic mechanisms. Among the first novel findings, this approach identified the existence of multiple NKA α -subunit isoforms in rainbow trout gills and a switch in isoform expression during salinity transfer (Richards et al., 2003b). This JEB paper stimulated research in multiple other fish species, including studies on Atlantic salmon that improved mRNA quantification accuracy with the use of qPCR and used mRNA *in situ* hybridization (Madsen et al., 2009), western blotting and immunohistochemistry (McCormick et al., 2009) to establish the cellular localization of the NKA isoforms.

Studies on elasmobranch gills

The presence of only two ionocyte subtypes in elasmobranch gills coupled with an exclusive role of the gills in A–B regulation in

seawater greatly facilitates studies at the whole-animal and cellular levels. Furthermore, elasmobranchs are amenable to comparative studies on ionic and osmotic regulation owing to the presence of stenohaline and euryhaline species (see Glossary; Box 4); they undergo a metabolic blood acidosis upon exhaustive exercise (Richards et al., 2003a), and they naturally experience a pronounced postprandial blood alkaline tide (Wood et al., 2005; Box 5). The discovery that pendrin (slc26a4) is an HCO_3^- -excreting anion exchanger in renal β -intercalated cells that express basolateral VHA (Royaux et al., 2001) initiated a series of studies that would resolve many questions about IOAB regulation in elasmobranch gills. First, pendrin was identified in base-excreting (B)-ionocytes from both marine and freshwater Atlantic stingrays (Piermarini et al., 2002). The apical localization of pendrin and basolateral localization of VHA in freshwater was a clear indication of their role in Cl^- uptake and HCO_3^- excretion for hyper-ionic regulation; however, both pendrin and VHA demonstrated a diffuse cytoplasmic localization in marine stingrays. A few years later, the induction of metabolic alkalosis in dogfish was found to cause the translocation of VHA to the basolateral membrane of B-ionocytes (Tresguerres et al., 2005), a mechanism that was dependent on functional microtubules (Tresguerres et al., 2006a) and CA (Tresguerres et al., 2007), and was essential for compensation of the blood alkalosis. Later, soluble adenylyl cyclase (sAC) was identified as the enzyme that sensed the blood alkalosis and triggered the translocation of VHA to the basolateral membrane (Tresguerres et al., 2010) and pendrin to the apical membrane (Roa et al., 2014) during a natural postprandial blood alkalosis. More recently, experiments on isolated round ray gill cells demonstrated that alkalosis and sAC-dependent activation

of B-cells does not require central hormonal or neurotransmitter input (Roa and Tresguerres, 2016). The widespread presence of CA, sAC, anion exchangers and VHA in base-excreting epithelial cells suggests an evolutionarily conserved mechanism for sensing and counteracting a blood alkalosis (Tresguerres, 2014). The gills of both freshwater and marine elasmobranchs also have acid-excreting (A)-ionocytes that co-express NKA and NHE3 (Choe et al., 2005, 2007; Reilly et al., 2011) and take up Na^+ in exchange for H^+ in a manner analogous to mammalian renal proximal tubule epithelial cells. These cells become inactivated during the alkaline tide (Roa and Tresguerres, 2019), and NKA and NHE3 abundances increase during transfer from seawater to freshwater, but not during short-term (hours) hypercapnia (Choe and Evans, 2003; Piermarini and Evans, 2000). Interestingly, both A- and B-cells become activated upon migration of euryhaline elasmobranchs from seawater to freshwater to mediate NaCl uptake for blood hyperionic regulation. Could the evolutionary invasion of freshwater environments have been facilitated by an activation of A- and B-ionocytes cells owing to more frequent bursts of active swimming, exposure to hypercapnia and postprandial alkaline tides?

Studies on freshwater bony fishes

Teleost fishes have more ionocyte types than elasmobranchs, and the mechanisms for IOAB regulation are accordingly more complex. This article briefly reviews the state of knowledge about freshwater rainbow trout and zebrafish; further details about these and other species can be found in a number of recent exhaustive reviews (e.g. Hiroi and McCormick, 2012; Hwang and Chou, 2013; Kwong et al., 2014; Shih et al., 2023).

Early in the 2000s and inspired by rabbit renal research, PNA^+ and PNA^- ionocyte subtypes were identified in freshwater trout gills based on their ability to bind peanut lectin agglutinin (PNA) (Galvez et al., 2002; Goss et al., 2001). These ionocytes were proposed to correspond to the denser and lighter chloride cell subtypes discovered in the 1960s and to the chloride MR and pavement MR cells observed in the 1980s. The idea that PNA^+ ionocytes mediated $\text{Cl}^-/\text{HCO}_3^-$ exchange and PNA^- ionocytes mediated Na^+/H^+ exchange was very attractive; however, the picture turned out to be much more complicated than that. For starters, PNA binding by fish ionocytes is restricted to rainbow trout, and even then, the PNA^+ ionocyte localization pattern throughout the gill epithelium is quite variable among studies (Brannen and Gilmour, 2018; Ivanis et al., 2008; Tresguerres et al., 2006b). Moreover, PNA^+ ionocytes express NHE2 and NHE3 (Ivanis et al., 2008), which implies a role in Na^+/H^+ exchange that was originally proposed for the PNA^- cells. Some of these discrepancies could be explained by the presence of PNA^+ ionocyte subtypes; for example, one may express basolateral NKCC1 and apical NHE3 and play a role in Na^+/H^+ exchange and ammonia excretion (Dymowska et al., 2014; Hiroi and McCormick, 2012), another may take up Cl^- through apical NCCs (Brannen and Gilmour, 2018), and yet another may express still elusive apical $\text{Cl}^-/\text{CO}_3^-$ exchangers in a metabolon with cytosolic CA and basolateral VHA (Tresguerres et al., 2006b). This hypothesis could be tested by immunolocalizing NCC and slc26a6, which is the most promising candidate for a $\text{Cl}^-/\text{HCO}_3^-$ exchanger in trout (Boyle et al., 2015; Leguen et al., 2015). The PNA^- ionocytes are proposed to take up Na^+ through apical acid-sensing ion channels (ASICs; Dymowska et al., 2014) energized by apical VHAs. However, the frog skin and the mammalian renal collecting duct exemplify that the Na^+ channel and VHA do not necessarily have to be in the same cell (Larsen et al., 2014). Could apical VHA be present in the pavement cells, as originally proposed

Box 5. Roles of the fish gut in IOAB physiology

The osmoregulatory strategy of marine bony fishes involves drinking seawater, absorbing NaCl across the esophagus and NaCl and water across the intestine, and excreting excess NaCl and divalent ions through the gills and kidneys, respectively (Smith, 1930). In addition to absorbing NaCl and water, the intestinal epithelium of marine bony fishes uniquely excretes large amounts of HCO_3^- , resulting in luminal alkalization that precipitates with Ca^{2+} and Mg^{2+} to form carbonates that reduce the osmotic pressure of the intestinal fluid, thus promoting water absorption (Grosell, 2010; Grosell and Oehlert, 2023).

In addition, many vertebrates excrete HCl into their stomach to aid with digestion. This mechanism involves excretion of H^+ into the stomach lumen by H^+/K^+ -ATPases in the apical membrane of the parietal cells, while the HCO_3^- is absorbed into the blood by basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchangers (Petrovic et al., 2002). Thus, food digestion can induce a blood alkalosis, especially in animals that feed infrequently and consume large meals. This phenomenon was originally identified in air-breathing vertebrates and is known as the postprandial 'alkaline tide' (Niv and Fraser, 2002). The first report of an alkaline tide in a fish was published in JEB in a study on dogfish (Wood et al., 2005). JEB also published evidence that the excess HCO_3^- was excreted across shark gills (Tresguerres et al., 2007), as well as reports of alkaline tides in freshwater (Buckling and Wood, 2008; Cooper and Wilson, 2008) and marine (Buckling et al., 2009) teleosts. However, marine teleosts may also excrete post-prandial HCO_3^- into the intestinal lumen, which is linked to the osmoregulatory strategy described above and might mask the alkaline tide (Buckling et al., 2009; Cooper and Wilson, 2008; Taylor and Grosell, 2009). Interestingly, freshwater killifish – which lack a true stomach – experience a post-prandial 'acidic tide' (Wood et al., 2010). The underlying mechanisms or physiological significance of this acidic tide remain unknown, but it was proposed to facilitate intestinal Cl^- absorption for systemic ionic and osmotic regulation and to enhance the offloading of O_2 from red blood cells to enhance aerobic ATP production to fuel intestinal digestion and nutrient and ion absorption.

(Sullivan et al., 1995; Wilson et al., 2000b), and still energize Na^+ uptake by the PNA^- ionocyte cells?

Fast generation times, year-round breeding and small size contributed to the emergence of zebrafish as a model system for vertebrate development during the 1990s, the sequencing of its genome in the 2000s (Grunwald and Eisen, 2002) and the adoption of zebrafish for studies IOAB regulation. The zebrafish embryo became an extremely useful model owing to the presence of abundant ionocytes in the yolk sac. These cells can be conveniently stained, quantified, probed with the scanning ion-selective technique and analysed for the effects of morpholino and CRISPR/Cas9 genetic manipulations before major complications are manifested. Current models propose at least four ionocyte subtypes in larval zebrafish skin: (1) VHA-rich (HR) cells with apical VHA, Rhcg1, NHE3 and a putative Na^+ channel and basolateral NKA (isoform $\alpha 5$); this cell type takes up Na^+ and H^+ and excretes NH_4^+ ; (2) NCC cells with apical NCC and basolateral NKA (isoform $\alpha 1$) that take up NaCl ; (3) NaR cells with apical Ca^{2+} channels and basolateral NKA (isoform $\alpha 5$) that take up Ca^{2+} ; and (4) KS cells with apical K^+ channels and basolateral NKA (isoform $\alpha 4$) that excrete K^+ for some unknown function (reviewed in Hwang and Chou, 2013). Similar to all other teleosts, the mechanisms for $\text{Cl}^-/\text{HCO}_3^-$ exchange remain elusive; however, one of these four ionocyte subtypes presumably expresses *slc26a3* and is responsible for Cl^- uptake and HCO_3^- excretion. In addition, *slc26a3* was also reported in non-NKA-rich cells (which make a fifth ionocyte subtype), and mRNA data suggested the presence of *slc26a4* and *slc26a6* in unidentified zebrafish larvae skin cells (Bayaa et al., 2009). It would be interesting to reassess the subcellular localization of these and some of the other transporters using multi-protein immunolocalization and confocal 3D optical reconstructions (e.g. Hiroi et al., 2008).

The IOAB regulatory mechanisms in zebrafish gills have not been explored to the same extent as those in larval skin. Zebrafish gills have HR cells that mediate H^+ excretion (Shih et al., 2022) and express *slc26s* (Perry et al., 2009), ASICs (Dymowska et al., 2015) and NHE3b (Yan et al., 2007; Zimmer et al., 2019). However, the presence of these proteins in specific ionocyte subtypes and their subcellular localizations are not clear. Recently, adult zebrafish were reported to activate a novel Na^+ uptake mechanism during short-term (2–8 h) exposure to low pH that is coupled to K^+ efflux and does not involve apical NHEs, Na^+ channels, NCCs or NH_4^+ -trapping (Clifford et al., 2022a). The identity of the apical transporter remains unknown, but mRNA evidence points to K^+ -dependent $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCKXs; *slc24a1-6*). It was proposed that this K^+ -dependent Na^+ uptake mechanism may gain ecophysiological relevance in the natural environment of zebrafish, which includes densely populated acidic shallow streams and stagnant ponds. Future studies will certainly examine whether other species inhabiting acidic waters use this mechanism, which may also inspire research on renal physiology and hypertension (Mrowka, 2023). Indeed, this mechanism has been deemed ‘among the most significant advances in the field of comparative osmoregulation over the past several decades’ (Perry and Wang, 2022).

Gill ionocyte subtypes in seawater bony fishes

The need to better understand the responses of marine fishes to environmental stressors (Fig. 1) sparked research to understand the molecular mechanisms for branchial H^+ and HCO_3^- excretion. The generalized pattern is that these NKA-rich ionocytes also express NHE3 and are responsible for excreting H^+ (Christensen et al., 2012; Hiroi and McCormick, 2012; Hsu et al., 2014; Seo et al.,

2013), a process that is favoured by the pronounced inwardly directed Na^+ gradient caused by the high concentration of Na^+ in seawater coupled with the activity of NKA. Upon exposure to hypercapnia, many seawater species are able to rapidly activate H^+ excretion to compensate for the ensuing blood acidosis. At least in European sea bass, the mechanism involves the widening of the ionocyte apical membrane with an associated increased availability of NHE3s exposed to seawater (Montgomery et al., 2022). Such a mechanism resembles the morphometric regulation of ion transport described in freshwater fishes during the 1980s, albeit at much faster time frames.

In addition, there is some evidence for the presence of separate VHA-rich ionocytes in gills from longhorn sculpin (Catches et al., 2006) and of VHA in NKA-rich ionocytes in red drum gills (Allmon and Esbaugh, 2017). VHA seemed to be present in the basolateral tubular system or in cytoplasmic vesicles even during exposure to extreme hypercapnia (Allmon and Esbaugh, 2017), which rules out a role in H^+ excretion across the apical membrane. Our immunofluorescence confocal microscopy images confirm these patterns in sardine (Fig. 3A) and rockfish (Fig. 3B,C). One type of ionocyte lacks NKA and has VHA in its cusp-like shape basolateral membrane (Fig. 3Ci–iii), whereas the other is the traditional ionocyte that has NKA in its heavily infolded basolateral membrane and has VHA also in the basolateral membrane or in cytoplasmic vesicles (Fig. 3Cii,iii). We tentatively propose that VHA in one or both ionocytes becomes activated during blood alkalosis in an analogous manner to VHA in the B-cells of elasmobranch gills. Confirming this hypothesis would require, once again, identifying the presumed apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger and establishing its cellular localization followed by functional studies following a blood alkalosis.

Why is there so much diversity in the gill ionocytes of teleosts compared with elasmobranchs and other aquatic animals? One reason may be related to the evolutionary history that includes an invasion of freshwater environments, a subsequent return to the sea for some species followed by multiple additional freshwater invasions (Halstead, 1985) that could have resulted in the selection of different branchial cellular mechanisms for IOAB regulation. Another potential reason is the great ecophysiological diversity of teleost fishes, which have disparate sizes, occupy a large range of freshwater and semi-terrestrial environments, and have diets with varied organic and ionic content and impacts on A–B physiology. Indeed, some teleosts experience a postprandial alkaline tide like elasmobranchs (Buckling et al., 2009; Cooper and Wilson, 2008), but others have an acidic tide (Wood et al., 2010; Box 5). In seawater, ionocyte function may have gained additional diversification owing to emerging interactions between IOAB transport both within each ionocyte and between gills and the gut (e.g. Taylor and Grosell, 2009). Another potential factor affecting ionocyte diversity might be differences in ammoniogenesis, gluconeogenesis and glycolysis in gill ionocytes that could favour apical H^+ excretion and Na^+ uptake via NHEs or VHA- Na^+ channels (Tseng et al., 2020). Moreover, there is also diversity in branchial and skin Ca^{2+} transport, which is not discussed in this Review but is an essential process for bony fishes. When we consider the potential combinations of all of these factors, the observed ionocyte diversity is less surprising.

Present and future: from omics to the environment

Next-generation sequencing has revolutionized research by enabling the relatively affordable analysis of whole genomes and extensive transcriptomes. However, even such powerful methods have limitations. Aquatic animals have unique physiologies enabled

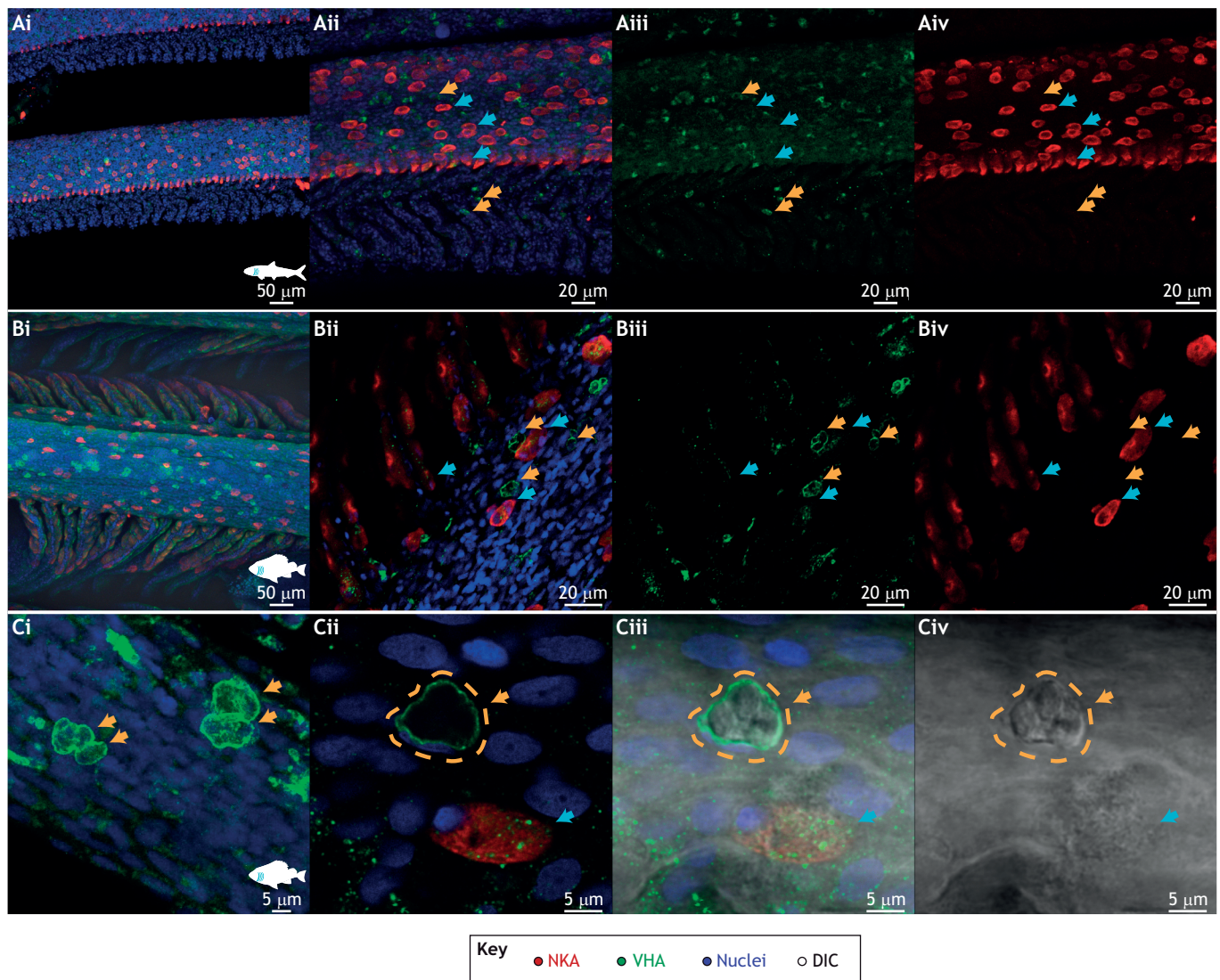


Fig. 3. Presence of ionocyte subtypes containing VHA in gills of marine teleosts. Whole-mount immunolocalization of NKA and VHA in gills from (A) Pacific sardine (*Sardinops sagax*) and (B,C) splitnose rockfish (*Sebastes diploproa*) reveal two ionocyte subtypes: a VHA-rich ionocyte that does not express NKA (yellow arrows), and an NKA-rich ionocyte that also expresses VHA (blue arrows). (C) Higher magnification images show that VHA is in the basolateral membrane of VHA-rich ionocytes, and throughout the cytoplasm or in the basolateral tubular system of NKA-rich ionocytes. Immunolocalization was performed following Kwan and Tresguerres (2022) and Montgomery et al. (2022) (see Supplementary Materials and Methods for more information).

by genes that do not have a counterpart in model species and thus lack functional annotation (Melzner et al., 2022). The mRNA levels do not always reflect protein levels (Gerbin et al., 2021), and whole-tissue transcriptomics analysis does not provide information about differential mRNA localization in cell types or about the subcellular localization of the encoded proteins. However, genomics and transcriptomics are superb for studying processes that depend on sequential switches of gene expression patterns (i.e. development), for processes for which the underlying physiological mechanism are already known, or for hypothesis generation followed by functional testing. Some recent examples include using mRNA network analysis to examine the effects of environmental stressors during freshwater–seawater transitions in salmonids (Monette and Velotta, 2022), single-cell transcriptomics to elucidate the molecular mechanisms of biomineralization in sea urchin larvae (Chang et al., 2021), and genomic analyses to investigate the molecular evolution of ionic regulation in natural populations spanning a salinity gradient (Velotta et al., 2022).

The growing interest in understanding the responses of aquatic animals to their dynamic environments and to anthropogenic stressors is leading to an expansion of experimental approaches used to study IOAB regulation in aquatic animals (Blewett et al., 2022; Zimmer et al., 2021). Many studies expose animals to extreme conditions to maximize compensatory responses and facilitate their detection and quantification; however, conclusions from such studies do not always apply to natural environments, nor are the compensatory responses necessarily proportional to the magnitude of the stressors. Additionally, many environmentally relevant physiological adjustments can occur very rapidly and do not rely on transcriptomic adjustments (Table S1). Thus, extrapolations from extreme experiments to the ‘real world’ should be performed with extreme caution.

Modern studies on IOAB regulation will require multidisciplinary collaborations with biogeochemists to measure and model environmental salinity, CO₂/pH, ammonia, temperature and O₂ levels with sufficient temporal and spatial resolution (e.g. Cyronak et al., 2020; Frieder et al., 2012). The expertise of aquatic IOAB physiologists

will be essential to assess the extent to which environmental variability challenges animal homeostasis and to evaluate compensatory responses. But although it is currently possible to efficiently measure blood ionic composition and osmolarity in wild aquatic animals (e.g. Dwyer et al., 2020), measuring other physiological variables will require the development of implantable sensors of blood A–B parameters and P_{O_2} , and of non-invasive biopsies to assess cellular physiology based on biochemical and cellular biomarkers. This endeavor will require collaborations with bioengineers to miniaturize sensors, amplifiers, processors and data loggers.

In addition, there will always be the need for experimental laboratory research on aquatic IOAB regulation. Much of our current knowledge has been generated from model species or from species with economic and ecological interest in developed countries, most times following Krogh's principle: 'For many problems there is an animal on which it can be most conveniently studied'. However, relatively little is known about aquatic animals from developing countries throughout the world. Perhaps we could learn something from plant biologists who, concerned about a growing trend for studies on the model species *Arabidopsis thaliana*, formulated 'No single organism (or technique) exists that can provide easy access to the diversity of hidden mechanisms that underlie all interesting and important physiological and biochemical problems' (Wayne and Staves, 1996).

Conclusions

This Review has attempted to convey that scientific research is a convoluted process. As time passes by, there is the risk of dismissing the nuances – much like a broken telephone game – potentially leading to oversimplification. The readers, and especially junior colleagues, are encouraged to exhaustively study the original literature, follow the trail of knowledge progression and to not automatically dismiss results that do not conform to current knowledge. Such a strategy is not only useful to better understand how the generation of scientific knowledge works, but can also help identify unresolved topics worth investigating through the lens of current paradigms and technologies (Romero, 2022).

Aquatic environments are extremely diverse both spatially and temporally. Moreover, the ocean and other aquatic ecosystems remain relatively unexplored and are increasingly threatened by anthropogenic activities that can affect IOAB homeostasis. Discovering physiological mechanisms underlying IOAB regulation and characterizing organismal responses and trade-offs will continue to be essential for satisfying human curiosity about how aquatic animals work, understanding and predicting how they will fare in rapidly changing environments, and identifying 'winners and losers' and informing conservation and policymaking agencies. Thus, IOAB research on aquatic animals should only gain more prominence during the upcoming decades. To fulfill this role, the aquatic IOAB physiology community should continue and increase our efforts to train the next generation of scientists in fundamental concepts as well as in emerging research areas and techniques.

Acknowledgements

We thank Drs Joshua Lonthair and Nicholas Wegner (NOAA Southwest Fisheries Science Center, La Jolla, CA, USA) for the Pacific sardine gill samples.

Competing interests

The authors declare no competing or financial interests.

Funding

M.T. was partially supported by National Science Foundation (NSF) grant IOS 1754994. G.T.K. was funded by the NSF Postdoctoral Research Fellowship in

Biology (award 1907334). A.W. was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Postdoctoral Fellowship.

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Table S1. Examples of rapid mechanisms of ionic, osmotic and acid–base regulation with established or potential ecophysiological relevance in aquatic animals.

<i>Organ</i>	<i>Mechanism</i>	<i>Function</i>
<i>Insect larvae</i>		
▪ Malpighian tubules and various gut segments	▪ Stimulation of ion transport and fluid secretion by serotonin and various peptides <i>via</i> Ca^{2+} , cAMP and cGMP ¹	▪ Ionic and osmotic regulation ▪ Alkalinization and acidification of anterior and posterior midgut lumen, respectively, for food digestion and nutrient absorption
<i>Crabs</i>		
▪ Gills	▪ Regulation of ion transport by the osmolality of the hemolymph ^{2,3,4}	▪ Modulation of NaCl absorption (and potentially, NaCl excretion) during tidal salinity changes and immersion in rain pools and tidal pools with concentrated seawater
<i>Hagfish</i>		
▪ Gills	▪ Subcellular translocation of NHE2 ⁵ , NKA ⁶ and VHA ⁶ ▪ Removal of Rhcg from the apical membrane ⁷	▪ Modulation of H^+ and HCO_3^- excretion during blood acidosis and alkalosis ▪ Prevent ammonia loading during feeding inside a decomposing carcass
<i>Elasmobranchs</i>		
▪ Rectal gland	▪ Activation of CFTR, NKA and/or NKCC by vasoactive intestinal peptide and adenosine <i>via</i> cAMP ⁸ ▪ Modulation of fluid secretion by blood A–B status ⁹	▪ Regulation of NaCl and fluid secretion upon NaCl load, feeding and exercise
▪ Gills	▪ Subcellular translocation of VHA and pendrin ^{10,11}	▪ Compensation of postprandial blood alkalosis
<i>Teleosts</i>		
▪ Larval skin	▪ Stimulation of NHE3b by cAMP/PKA ¹² and inhibition by H_2S ¹³ ▪ Stimulation of NCC by angiotensin ¹⁴ and cAMP/PKA ¹² ▪ β -adrenergic stimulation of HR cells ¹⁵	▪ Modulation of NaCl uptake and H^+ excretion
▪ Operculum	▪ Stimulation of NaCl excretion by cell shrinking and inhibition by cell swelling <i>via</i> β -integrin sensing and various kinases; modulation of CFTR, NKCCs and NKA ¹⁶	▪ Dynamic switch between excretion and absorption of NaCl and regulation of water permeability during tidal salinity changes
▪ Gills	▪ Morphological component of A/B ¹⁷ ▪ Widening of the apical pit of NKA & NHE3-rich ionocytes ¹⁸	▪ Acid–base regulation during hypercapnia
▪ Intestine	▪ Switch from fluid absorption to secretion <i>via</i> cAMP-stimulation of CFTR ¹⁶ ▪ Stimulation of NaCl absorption by HCO_3^- <i>via</i> sAC-dependent activation of NKCC2 and/or NKA ¹⁹	▪ Modulation of NaCl and fluid absorption and secretion and coordination with HCO_3^- excretion for the precipitation of ichthyocarbonates

¹Reviewed in (Silver and Donini, 2021), ²(Siebers et al., 1985), ³(Onken, 1996), ⁴(Tresguerres et al., 2003), ⁵(Parks et al., 2007), ⁶(Tresguerres et al., 2007b), ⁷(Clifford et al., 2022), ⁸(Epstein et al., 1983), ⁹(Wood et al., 2007), ¹⁰(Tresguerres et al., 2007a), ¹¹Roa et al 2017, ¹²(Kumai et al., 2014a), ¹³(Kumai et al., 2015), ¹⁴(Kumai et al., 2014b), ¹⁵(Kumai et al., 2012), ¹⁶reviewed in (Marshall, 2012); ¹⁷reviewed in (Goss et al., 1998), ¹⁸(Montgomery et al., 2022), ¹⁹(Tresguerres et al., 2010).

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Supplementary Materials & Methods

Methods for Figure 3

Immunostaining of Na⁺/K⁺-ATPase (NKA) and V-type H⁺-ATPase (VHA) in gill samples followed the methods described in (Kwan and Tresguerres, 2022; Montgomery et al., 2022). Pacific sardine (*Sardinops sagax*; n=6) and splitnose rockfish (*Sebastes diploproa*; n=5) were kept in flowthrough seawater aquaria and handled according to Institutional Animal Care and Use Committee protocol (#S10320) approved by the animal care committee at Scripps Institution of Oceanography, University of California San Diego.

NKA was immunodetected using a mouse monoclonal antibody against the α -subunit of chicken NKA (a5, Developmental Studies Hybridoma Bank, Iowa City, IA, USA; (Lebovitz et al., 1989)), and VHA was immunodetected using custom-made rabbit polyclonal or mouse monoclonal antibodies (GenScript, Piscataway, USA) against a highly conserved epitope within the VHA subunit B (AREEVPGRRGFPGY). The secondary antibodies were goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 (Invitrogen, Grand Island, USA).

Gill samples were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS, pH 7.4) on a shaker at 4°C overnight. On the following day, the samples were transferred immersed in 50% ethanol for 8 h, then stored in 70% ethanol at 4°C until processing. Immunohistochemistry preparation began by rehydrating the fixed samples in PBS + 0.1% tween (PBS-T) for 5 min, followed by rinses in ice-cold PBS containing 1.5 mg/mL NaBH₄ (6 x 10 min) to reduce autofluorescence. Samples were then incubated in blocking buffer (PBS-T, 2% normal goat serum, 0.02% keyhole limpet hemocyanin) for 1 h, then incubated with primary antibodies (NKA: 40 ng/mL; VHA: 6 μ g/mL in blocking buffer) (4°C overnight). On the next day, samples were washed in PBS-T, incubated with blocking buffer containing secondary antibodies (1:500) and the nuclear dye DAPI (1 μ g/mL) for 1 h. The stained gill samples were then washed in PBS-T, placed onto depressed glass slide fitted with a glass cover slip (No. 1.5, 0.17 mm), and whole-mount imaged using a Zeiss inverted confocal microscope (AxioObserver Z1 with LSM800; Oberkochen, Germany) and Zeiss ZEN 2.6 blue edition software. The immunostained gills were imaged with 20x (Plan-Apochromat 20x/0.8 M27) and 40x (Zeiss LD LCI Plan-Apochromat 40x/1.2 Imm Korr DIC M27) objective lenses, and uses optimized settings for Alexa Fluor 488 (to visualize VHA; excitation 493 nm at 0.5-1.0% laser power, emission 517 nm, detection 510–575 nm), Alexa Fluor 568 (to visualize NKA; excitation 577 nm at 0.2-1.0% laser power, emission 603 nm, detection 575–617 nm), and DAPI (to visualize nuclei; excitation 353 nm at 0.2% laser power, emission 465 nm, detection 410–470 nm). The Z-stacks were then analysed using orthogonal cuts across the X-Z or Y-Z planes, or presented as maximum intensity projection.