

The skin of fish as a transport epithelium: a review

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Abstract The primary function of fish skin is to act as a barrier. It provides protection against physical damage and assists with the maintenance of homeostasis by minimising exchange between the animal and the environment. However in some fish, the skin may play a more active physiological role. This is particularly true in species that inhabit specialised environmental niches (e.g. amphibious and air-breathing fish such as the lungfish), those with physiological characteristics that may subvert the need for the integument as a barrier (e.g. the osmoconforming hagfish), and/or fish with anatomical modifications of the epidermis (e.g. reduced epithelial thickness). Using examples from different fish groups (e.g. hagfishes, elasmobranchs and teleosts), the importance of fish skin as a transport epithelium for gases, ions, nitrogenous waste products, and nutrients was reviewed. The role of the skin in larval fish was also examined, with early life stages often utilising the skin as a surrogate gill, prior to the development of a functional branchial epithelium.

Keywords Skin · Integument · Epidermis · Gas exchange · Ammonia excretion · Nutrient absorption · Ionoregulation

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Introduction

The epidermal surfaces of animals play an integral role in homeostasis. A universal application of the integument is as a barrier, critical for the maintenance of a gradient between the concentrations of ions, gases, nutrients and/or metabolic substrates inside the animal, and those outside (Chuong and Chang 2002). The importance of this role differs as a function of animal phylogeny and environment. Most soft-bodied marine invertebrates, for example, are osmoconformers with limited capacities for ionoregulation. In these animals, the integument will clearly have a reduced role as a barrier, with exchange of ions and water between the organism and the environment limited, at least compared to that of aquatic vertebrates and invertebrate osmoregulators such as the Crustacea. In these latter groups, the integument is often highly modified to emphasise the barrier function. This may involve the development of a thickened impermeable epithelium, the presence of keratinised scales (e.g. marine reptiles) or feathers (e.g. marine birds), or an exoskeleton (Chuong and Chang 2002). In terrestrial organisms, the barrier role of the integument is critical for minimising exchanges such as evaporative water loss, particularly important for animals isolated from reliable sources of water (Lillywhite 2006). However, the development of reduced cutaneous permeability incurs a lost opportunity. Given that the skin is exposed directly to the environment, it is a potential surface for acquisition of resources such as nutrients and oxygen. It is also a surface across which metabolic waste, such as ammonia and urea, could be readily exchanged without the need for specialised excretory products and/or systems.

The skin of all fish consists of two components, an epidermis and a dermis. The epidermis is the outermost

section and ranges anywhere from two cell layers thick (e.g. in larval fish) to more than ten, with the thickness ultimately being determined by factors such as species, age, and body region (Elliott 2000). Scales of fish are distinct to those of other scaled animals. They are mineralised, not keratinised, and are usually dermal in origin, meaning that they are overlain by epidermal layers (Hawkes 1974). An exception to this is the skin of sharks, in which the scales protrude through the epidermal surface (Meyer and Seegers 2012). Another distinct feature of fish skin is that it is considered a metabolically active tissue, in contrast to the skin of terrestrial vertebrates that often contains keratinised layers that primarily function to prevent evaporative water loss (Lillywhite 2006).

This review examines the evidence for active roles of fish skin as a transport epithelium. In general, these functions overlap with the other epithelia that are exposed to the environment, the gill and the gut, and consist of gas exchange, ion homeostasis (including acid–base regulation), nitrogenous waste excretion, and nutrient acquisition. These functions of the skin appear to be particularly important for early life stages of fish, where the epidermal surface compensates for the absence of a fully functional branchial epithelium. The other common theme that emerges is the importance of the skin in amphibious and air-breathing fish, where the lack of emersion in water is likely to impair gill function.

Gas exchange

By virtue of its large surface area, small epithelial diffusion distances, direct contact with the ambient medium, and high rates of blood perfusion, the gill is an ideal transport surface (Feder and Burggren 1985). For this reason, the uptake of oxygen and excretion of carbon dioxide are physiological functions that are principally ascribed to the branchial epithelium. As a diffusive process, gas exchange is largely dependent on four key variables: partial pressure gradient, surface area, diffusion distance, and the diffusion constant (essentially a measure of the gas permeability of the diffusive barrier). The first three are well known to be under physiological control in the gills. Traditionally, it has been considered that the diffusion constant is not subject to physiological regulation, but recent evidence that two families of gill transport proteins (Rhesus (Rh) glycoproteins and aquaporins) may facilitate the diffusion of at least two respiratory gases (ammonia and carbon dioxide) is starting to challenge this view (Virkki et al. 2001; Nawata and Wood 2008; Cerda and Finn 2010; Perry et al. 2010). Regardless, under certain scenarios the function of the gill can be compromised and, with transient or adaptive modifications, the skin can make a significant contribution to

gas exchange. Earlier surveys of the literature note that the skin contributes anywhere from 5 to 73 % of total oxygen uptake (Feder and Burggren 1985; Martin 1995). The contribution is generally greatest in air-breathing fish, but the phenomenon is present even in fish that remain wholly aquatic.

Respiration in air is favourable relative to that in water. The higher concentration of oxygen in this medium ensures a large partial pressure gradient from the environment into the animal. Furthermore, the reduced density of air relative to water can cause collapse of the delicate gill structure in species that lack specific structural modifications to the gill, thus reducing effective exchange area (e.g. Sacca and Burggren 1982). Consequently, under conditions of hypoxia, or owing to environmental desiccation, many fish species will utilise the air as a respiratory medium. Under this scenario, cutaneous gas exchange may become more important. In an elegant demonstration of the role of the skin as a compensatory respiratory tissue, Nonnotte and Kirsch (1978) showed that in the shanny (*Blennius pholis*) the skin increased its contribution to oxygen uptake only when the gills were also emersed.

Structural modification as an adaptation to cutaneous gas exchange is a common theme, with some authors suggesting oxygen uptake via the skin may be restricted to regions of the body surface that are scaleless (Park et al. 2003). Supporting this, in a survey of coral reef fishes Nilsson et al. (2007) noted that air breathing was present only in those species that exhibited a scaleless integument. However, fish scales are usually found underneath the epidermal layer (Hawkes 1974) and are therefore unlikely to impede gas exchange. Instead, it is the increased capillarity of the epidermal and dermal layers of the skin, a feature that often co-occurs with scalelessness (e.g. Nilsson et al. 2007; Park et al. 2003), which is likely to be more important in facilitating cutaneous gas exchange.

Increased vascularity of the cutaneous surface is not, however, restricted to scaleless species. In mudskippers, increased capillarity is observed in skin, particularly those regions that are most likely to be exposed to the air (Park 2002; Zhang et al. 2003; Park et al. 2006). This is an adaptation that increases the surface area of the skin that is capable of exchange and/or reduces diffusive path length, thereby facilitating gas transport. For some fish species, perfusion of the integument is also flexible. The pearl blenny (*Entomacrodus nigricans*; Graham et al. 1985) and the black prickleback (*Xiphister atropurpureus*; Daxboeck and Heming 1982) display dilation of epidermal blood vessels in response to aerial exposure, presumably a mechanism for enhancing transepithelial gas exchange. The mangrove rivulus (*Kryptolebias marmoratus*) is reported to show an initial stress-related vasoconstriction followed thereafter by increases in perfusion (Wright

2012). This latter response is associated with enhanced endothelial cell production in the caudal fin following emersion (Cooper et al. 2012), indicating angiogenesis as an acclimation response to promote cutaneous gas exchange. Indeed, the presence of epidermal capillaries in this species reduces the diffusive distance between the environment and the blood to as little as 1 μm (Grizzle and Thiagarajah 1987), thus contributing to the effective use of cutaneous respiration (Ong et al. 2007).

Given the advantages of a small diffusive distance for gas exchange, it is not surprising that cutaneous oxygen consumption is particularly important in early life stages. Cutaneous respiration accounts for greater than 80 % of total oxygen requirements in salmonid early life stages (Rombough and Ure 1991; Wells and Pinder 1996), a proportion that is maintained until 23–28 days post-hatch in rainbow trout (Fu et al. 2010). At this stage, there are a number of factors that favour a switch away from the skin as the primary respiratory surface. The epidermis starts thickening, increasing the diffusive path length, and the yolk sac area decreases. At the same time body mass increases, decreasing the surface area to volume ratio (Wells and Pinder 1996). Concomitantly, the development of gill lamellae results in an increase in branchial surface area and a decrease in diffusive distance (Wells and Pinder 1996; Rombough and Moroz 1997).

Little is known regarding gas exchange in early life stages of viviparous fish, but, as for the better-studied oviparous species, the lack of a well-developed gill is likely to result in the majority of oxygen uptake being localised to the integument. Consistent with this hypothesis, late-gestation embryos are thought to increase integumental diffusive area by expansion of well-vascularised fin regions as metabolic oxygen demand increases (Wourms et al. 1988).

Gills are advantageous as respiratory organs, owing in part to their capacity to be actively ventilated. Movement of fresh, oxygenated water across the gills (e.g. via buccal and opercular movements) reduces any unstirred water layer. As oxygen is consumed, the layer immediately overlying the respiratory surface becomes depleted of oxygen and the gradient for diffusion is dissipated. In some environments, therefore, where there is an absence of appreciable water flow and/or locomotion may be compromised, the unstirred water layer may be a significant impediment to cutaneous respiration. Using an isolated skin method, Takeda (1996) showed that increased oxygen uptake was associated with increased ventilation of the skin, confirming this. This corresponds well with the general hypothesis that cutaneous respiration is associated with favourable environmental conditions and becomes less important when environmental conditions (e.g. flow, dissolved oxygen content) deteriorate (Feder and Burggren 1985).

While cutaneous oxygen consumption has been documented in many fish across a number of habitats, it is only in a few species that the skin can be considered to function as a true respiratory tissue. In studies of more than a dozen species inhabiting both marine and freshwaters, only four (black bullhead, *Ictalurus melas*; shanny, *B. pholis*; European plaice, *Pleuronectes platessa*; European flounder, *Platichthys flesus*) exhibited uptake rates that exceeded the oxygen demands of the skin itself (Kirsch and Nonnotte 1977; Nonnotte and Kirsch 1978; Nonnotte 1981; Fig. 1). For the other species examined, oxygen uptake was not sufficient to exceed the metabolic demands of the skin. In larval Atlantic salmon, the large surface area to volume ratio appears sufficient to meet metabolic demand by local diffusion across the integument alone, likely subverting the need for a specialised gas transport epithelium (Wells and Pinder 1996). However, shortly after hatching, and before the gills take over the majority of oxygen uptake, it has been suggested that the skin of the yolk sac may function as a true respiratory organ and supply internal tissues that are isolated from the general skin surface as body size increases (Wells and Pinder 1996).

The skin may play a more important role in carbon dioxide excretion than in oxygen uptake. Carbon dioxide diffuses through tissue and water more rapidly than oxygen (Dejours 1981). Consequently, the fish is less dependent on blood perfusion of the skin and dissipation of a boundary layer by ventilation of the skin surface than it is on the transport of oxygen. This indicates that relative to oxygen, carbon dioxide will be excreted more efficiently. This is supported by experimental data. For example, transfer rates for carbon dioxide across the skin of the lamprey (*Geotria australis*) were 1.5-fold higher than for oxygen (Potter et al. 1997). In aerially exposed galaxiid fish (inanga, *Galaxias maculatus*; Canterbury mudfish, *Neochanna burrowsius*), the cutaneous contribution to carbon dioxide excretion may be up to double that across the gill (M.A. Urbina, A.S. Meredith, C.N. Glover and M.E. Forster unpublished data). It is noteworthy, however, that in the dogfish (*Scyliorhinus canicula*), the skin makes only a small contribution to both total carbon dioxide (4 %) and total oxygen (5 %) exchange (Toulmond et al. 1982), emphasising that the exchange of gases across the skin will depend on species and factors such as skin thickness.

Overall, it can be concluded that cutaneous gas exchange will be favoured in fish that: have a high surface area to volume ratio; are exposed to environments that are well oxygenated (i.e. air or flowing water); that have reduced diffusion distances, primarily achieved by high vascularisation and physiologically mediated changes to skin perfusion. Consequently, species that are small, with elongate body form, and capable of aerial respiration (e.g. galaxiid fish, Urbina et al. 2011; Martin et al. 1993; mangrove rivulus, Ong et al. 2007) are prominent among fish that respire cutaneously.

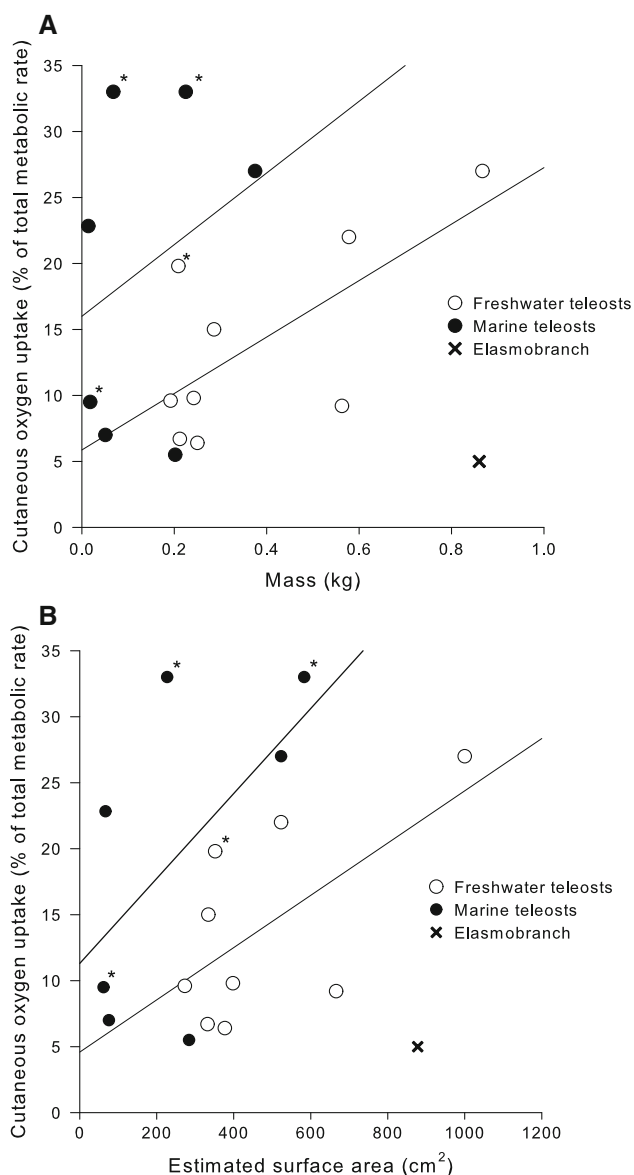


Fig. 1 Cutaneous oxygen uptake as a percentage of total metabolic rate in fish as a function of **a** mass or **b** surface area. Teleost data were compiled from Kirsch and Nonnotte (1977), Nonnotte and Kirsch (1978), Nonnotte (1981), and Steffensen et al. (1981). Elasmobranch data were from Toulmond et al. (1982). All experiments were conducted at 13 °C except for the dogfish which was performed at 15 °C. For panel **a**, the equation of the freshwater teleost regression line is $y = 21.39x + 5.87$ ($r^2 = 0.4667$) and the marine teleost regression line is $y = 27.16x + 15.99$ ($r^2 = 0.091$). For panel **b** the equation of the freshwater teleost regression line is $y = 0.02x + 4.58$ ($r^2 = 0.3808$) and marine teleost regression line is $y = 0.03x + 11.31$ ($r^2 = 0.3347$). The elasmobranch was not analysed in the regressions, but is included for comparison. Asterisk indicates the oxygen uptake exceeded the oxygen demand of the tissue itself

Ion transport

The skin surface of fish has a well-recognised role in salt and water homeostasis. As a barrier between the animal

and the environment, the cutaneous epithelium is important for isolating compartments that differ in their osmotic and/or ionic compositions (see Marshall and Grosell 2005; Glover 2007; Hwang et al. 2011). Both marine and freshwater teleost fish, for example, maintain osmotic concentrations that differ from that of their habitats, and the skin is an important barrier in reducing ion and water fluxes (Ghioni et al. 1997). Elasmobranch fish are isosmotic with the marine environment. This is achieved by maintaining ion concentrations that are only moderately higher than those of teleosts, but with very high levels of urea. In this scenario, the skin is likely to play an important role in reducing ion and urea diffusion.

However, the skin can also play a more active role in ion regulation. In the late 1970s and early 1980s, it was shown that certain skin regions of seawater-acclimated specimens of mummichog (operculum; *Fundulus heteroclitus*), long-jawed goby (lower jaw; *Gillichthys mirabilis*), shanny (whole body, but especially pectoral fins; *B. pholis*), and Mozambique tilapia (operculum; *Oreochromis mossambicus*) were capable of ion transport (Degnan et al. 1977; Karnaky et al. 1977; Marshall 1977; Nonnotte et al. 1979; Foskett and Scheffey 1982). All of these isolated skin studies demonstrated an active net efflux of chloride ions, which was localised to the mitochondria-rich (MR) “chloride” cells using the vibrating probe technique (Foskett and Scheffey 1982). This initiated many years of research utilising these and other (e.g. mudskipper pectoral skin; Yokota et al. 1997) isolated skin epithelium preparations as surrogate models for investigating branchial ion transport processes (Marshall and Bellamy 2010). The fine details of this active chloride efflux into the MR cell crypts, with sodium shunted through the paracellular pathway, have now been extensively investigated in literally hundreds of studies. Isolated skin epithelia preparations have been of significant value in understanding active sodium chloride excretion in marine fish, as they are ideal for in vitro Ussing chamber-style, vibrating probe and scanning ion-selective electrode technique (SIET) analyses that are impossible with intact gills. Overall, the skin ion transport process appears to be equivalent to that occurring through the gills, where the bulk of the MR cells are usually located.

The utility of these skin preparations as in vitro model systems for determining ion transport mechanism has been the focus of most studies. Sometimes overlooked, however, is the importance that these cutaneous transport processes may themselves have in ionic homeostasis in the intact fish. Marshall and Bern (1980) calculated that 23 % of the net chloride efflux occurred via the skin of *Gillichthys*, while in the shanny the skin accounted for 65 % of the total chloride efflux (Nonnotte et al. 1979). The difference in the magnitude of these contributions likely represents the

relatively larger area of skin known to possess MR cells in the latter species. As with the shanny, the marbled swamp eel (*Synbranchus marmoratus*) has cells resembling gill ionocytes, complete with apical microridges, in the general epidermis, suggesting a relatively large proportion of skin with the ability to transport ions (Stiffler et al. 1986). This is also reflected in the high estimated contribution of the skin to total ion uptake in this species (75 %; Stiffler et al. 1986). Interestingly, the intact mummichog actively takes up sodium from freshwater, but is dependent on dietary chloride and takes up almost no chloride from the water (Patrick et al. 1997). All of the very small amount of chloride absorption that does occur can be explained by active chloride uptake at the opercular epithelia, yet there is no active sodium uptake in this preparation in freshwater-acclimated specimens (Wood and Marshall 1994; Marshall et al. 1997; Burgess et al. 1998; Wood and Laurent 2003). Later molecular work confirmed that the mRNA responses of opercular epithelial transporters to freshwater transfer were very different from those of the gill transporters (Scott et al. 2005). However, these differences were not seen after transfer to seawater (Scott et al. 2008).

This example illustrates the point that while these model skin preparations have been invaluable in replicating the properties of the seawater gill, they have been markedly less successful in replicating freshwater gill ion transport processes. Indeed, the bulk of research has been restricted to preparations from seawater-acclimated specimens incubated in vitro under Ussing conditions (i.e. with isotonic saline on both mucosal and serosal surfaces). To our knowledge, there has been only one report of a cephalic skin preparation from a freshwater-acclimated teleost that actively takes up both sodium and chloride (and indeed calcium as well, see below) when bathed with mucosal freshwater and serosal saline. This was from the Nile tilapia, *Oreochromis niloticus* (Burgess et al. 1998). Taking the transepithelial potential and unidirectional fluxes into account, the observed flux ratios for all three ions significantly exceeded the respective criteria for active uptake predicted by the Ussing flux ratio equation (Fig. 2). Nevertheless, the actual uptake fluxes of sodium and chloride were very small, and the density of MR cells on the opercular epithelia of the tilapia was only about 1/40th of that on the mummichog epithelia.

Overall, the literature suggests that the role of the skin in ion homeostasis may be more significant in seawater than in freshwater settings. In the guppy *Poecilia reticulata*, seawater transfer stimulates the development of MR cells in the skin (Schwerdtfeger and Bereiter-Hahn 1978), again suggesting that this tissue has a more important role in ionoregulation in marine habitats. A recent study examined changes in the mRNA expression of the basolateral sodium pump (sodium/potassium ATPase; NKA) in inanga skin, to

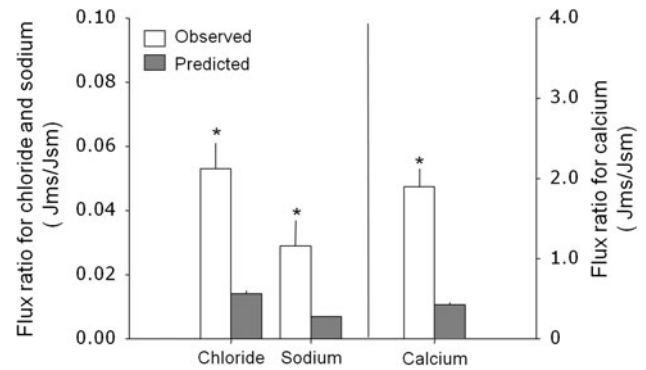


Fig. 2 Observed unidirectional flux ratios (*open bars*) in comparison to flux ratios predicted by the Ussing criterion (*hatched bars*, taking into account ionic concentrations, activity corrections, and transepithelial potential) for the active transport of chloride, sodium, and calcium in the uptake direction from mucosal freshwater to serosal saline of isolated opercular epithelium preparations from freshwater-acclimated Nile tilapia (*Oreochromis niloticus*). For all three ions, the asterisks indicate significant differences ($P < 0.05$, paired t test), indicating that the uptake is active. Plotted values represent mean \pm SEM ($n = 6$). Data are from Burgess et al. (1998)

determine if this key mediator of ion transport was responsive to salinity change in skin tissue (Urbina et al. 2013). The presence of different alpha subunit isoforms was noted, and although changes that mimicked isoform switching in the gill were observed, these narrowly eluded statistical significance. This study utilised whole skin tissue and thus a more selective study focussing specifically on ion-transporting regions of the skin may yield evidence of changes in the ion transport entities relevant to meeting ionoregulatory requirements at different salinities.

The relative importance of the skin for ion transport in aerially exposed fish has also been the subject of investigation. In air, gills are unlikely to be functional for ion transport, owing to decreased surface area due to lamellar collapse. However, many of these emersed species maintain themselves in moist environments, meaning that the skin, especially the ventral surface, may have access to small amounts of water that can be used to regulate osmotic and ionic balance. In the mangrove rivulus, clusters of MR cells are found throughout the skin surface in numbers equivalent to those of the gill and after 9 days of aerial exposure these ionocytes increase in area (LeBlanc et al. 2010). These cells were associated with the transport of sodium ions and water across the cutaneous surface, and the maintenance of salt (chloride, and to a lesser extent, sodium) and water balance. In the marbled swamp eel, emersion had no significant impact on cutaneous sodium influx or efflux (Stiffler et al. 1986), and in lungfish (*Protopterus dolloi*) a similar pattern was observed. In this latter species, equivalent influx and efflux rates for chloride, sodium, and water in aquatic versus emersed fish were recorded (Wilkie et al. 2007). The main locus for exchange

in both media was the ventral skin, a surface known to contain MR cells (Sturla et al. 2001). In the marbled goby (*Oxyeleotris marmorata*) and the mudskipper (*Periophthalmodon schlosseri*), calcium influxes were decreased upon emersion, but owing to the concomitant decrease in calcium efflux at the gill, these exchanges were sufficient to maintain calcium homeostasis (Fenwick and Lam 1988). Together, these data indicate that the skin performs an important role in ion regulation in emersed species.

As alluded to above, the transport of ions across the skin of fish is not restricted to monovalent ions. Calcium has also been shown to traverse the cutaneous epithelium. In fact, in rainbow trout skin isolated from the cleithrum bone, calcium, but not sodium or chloride, was actively transported from mucosal freshwater (Marshall et al. 1992). In opercular epithelia from mummichogs, calcium was actively absorbed by preparations from both freshwater and seawater specimens, with higher rates in the former estimated to represent 20–46 % of whole animal uptake rates in vivo (Marshall et al. 1995; Verbost et al. 1997). In vivo studies on freshwater trout directly confirmed the importance of the cutaneous route of absorption in this species, with approximately 50 % of calcium uptake attributed to the skin in a partitioned study (Perry and Wood 1985). This value was much greater than the 1–7 % contribution estimated by McCormick et al. (1992) based on their in vitro examination of calcium uptake in the opercular epithelium of the Nile tilapia, although Burgess et al. (1998) later reported much higher rates for this same preparation.

Similar to its importance in gas exchange, the skin also plays a critical role in ion regulation of larval fish. As the gill is considered the primary site of ionic transport in fish, ionoregulatory requirements prior to the development of a functional gill will need to be met by the skin. In the rainbow trout, for example, this means that the skin takes the primary responsibility for ion regulation until around 15–16 days post-hatch (Fu et al. 2010). As with adult fish that use the skin as an uptake surface, this is achieved primarily by MR cells that resemble those of the adult gill. The presence of these ionocytes has been noted on various regions of the larval integument (Varsamos et al. 2005), but the densest populations of these cells usually occur on the yolk sac membrane (e.g. Bodinier et al. 2010). This property has led to significant use of the yolk sac membrane as a surrogate model for investigating ionocyte function, utilising both in situ and in vitro techniques (e.g. yolk ball, Kaneko et al. 2002; SIET, Shen et al. 2011). While the capacity of the larval fish integument to regulate ions is sufficient for basic homeostasis, in most fish species it appears less competent than the later-developing gills in dealing with salinity changes that may occur in the environment. For example, high mortality has been reported for sea bream embryos subjected to salinity changes prior to

gill development (Bodinier et al. 2010). Similar trends (lower survival of early life stages in the face of salinity challenge) have also been seen in Nile tilapia (Fridman et al. 2012) and ayu (*Plecoglossus altivelis*; Iguchi and Takeshima 2011).

Nitrogenous waste excretion

Ammonia is an end product of metabolism that exists in solution as both a respiratory gas (NH_3) and as a cation (NH_4^+). These two forms are readily interconvertible, but the latter greatly predominates at physiological pH. For purposes of this review, the term ‘ammonia’ will be used to refer to total ammonia, and NH_3 and NH_4^+ will be used when specifically referring to the gaseous and cationic forms, respectively.

Owing to the toxicity of both forms, ammonia must be rapidly and efficiently excreted or converted to a less toxic product (Randall and Ip 2006). Most fish are fully aquatic and therefore any ammonia generated internally generally does not accumulate within the body, but rather diffuses to the environment, down partial pressure and/or electrochemical gradients. The epithelium best suited as a locus for ammonia excretion is the gill, by virtue of the properties that also endear it to gas exchange (see above).

Current models of branchial ammonia excretion in fish incorporate recent findings regarding the role of the Rh glycoproteins. Ammonia diffuses out of red blood cells and through the pillar cells, a process mediated by the Rh protein Rhag. It then moves through the basolateral membrane of the epithelial cell facilitated by Rhbg, and finally across the apical membrane into the environment via a third Rh protein, Rhcg (Nakada et al. 2007b; Shih et al. 2008; Weihrauch et al. 2009; Wright and Wood 2009; Wright and Wood 2012). NH_4^+ binds to the Rh channels, but is deprotonated so that NH_3 passes through the channels (Nawata et al. 2010b). Upon exiting on the apical side, the NH_3 is immediately “trapped” by combination with a proton (supplied by either an apical sodium/hydrogen exchanger (NHE) or a proton pump coupled to a sodium channel), thereby reforming NH_4^+ . This proton trapping acts to maintain the partial pressure gradient facilitating continued diffusive ammonia excretion and provides a loose coupling of ammonia excretion to sodium uptake in a metabolon (Wright and Wood 2009, 2012; Hwang et al. 2011). This general mechanism appears to hold in both freshwater and seawater, although in the latter environments it is hypothesised that other mechanisms may also be operative, at least in some studied species (Wright and Wood 2012). These alternative mechanisms include paracellular diffusion, basolateral transport of NH_4^+ (as a K^+ substitute) via NKA and/or the sodium/potassium/chloride

transporter (NKCC), and even NH_4^+ passage through an apical NHE (Randall et al. 1999; Nakada et al. 2007a; Nawata et al. 2010a, b; Cooper et al. 2013).

There are, however, some scenarios that significantly impede the excretion of ammonia. For example, in very alkaline waters, the limited availability of protons is likely to exclude proton trapping, resulting in equilibration of the diffusive gradient for ammonia (Randall and Ip 2006). Fish exposed to air will also lack the ability to excrete ammonia (either as an ion or as a gas) owing to both the lack of water and the possible collapse of the gills. In species that are regularly exposed to air, such as the aestivating lungfish, ammonia is often converted to urea, an end product that is less toxic than ammonia and therefore able to be stored in the body tissues until water becomes available again (Wood et al. 2005a, b). In fish where the use of the gill may be compromised by environmental factors (i.e. high pH or aerial exposure), the skin has been shown to perform a role in nitrogenous waste excretion.

Wood (1993) surveyed the literature available at the time regarding the quantitative role of the skin versus the gills in ammonia and urea excretion. These were largely divided chamber studies, many of which were confounded by the possibility of nitrogenous waste excretion through the kidney and digestive tract. However, the overall conclusion was that while the gills predominated for the excretion of both waste products, in both freshwater and seawater, the relative contribution of the skin tended to be greater in the marine fish, especially for ammonia. There has been little work on this topic in the intervening period.

Fish that emerge may be faced with gill collapse and reduced water availability. As ammonia excretion is diffusive, the accumulation of ammonia in the surface water layer will dissipate the diffusive gradient and will preclude further excretion. Some air-breathing species have solved this problem by actively excreting ammonia. In the gill of the giant mudskipper, where the mechanism of active ammonia excretion has been best described, this is thought to be achieved by the exit of NH_4^+ via the apical NHE, energised by the action of the basolateral NKA (Randall et al. 1999). There is also evidence from the climbing perch (*Anabas testudineus*, Tay et al. 2006) that active excretion of ammonia may occur across the integument. Ammonia excretion continues across the skin despite the level of ammonia in the water contacting the cutaneous surface being in excess of 10 mM. In both aqueous and aerial exposures conducted with this species, the skin contributes around 50 % of total ammonia excretion, with the magnitude of excretion increasing with length of the terrestrialisation period (Tay et al. 2006).

Another mechanism believed to assist with ammonia excretion in emerged fish is volatilisation from the skin surface. In the weatherloach (*Misgurnus anguillicaudatus*)

the majority of gaseous ammonia loss appears to occur across the gut, a known respiratory surface in this species; however the skin is also thought to play a role (Tsui et al. 2002). Weihrauch et al. (2009) have provided a potential mechanistic model to explain this phenomenon. Volatilisation is achieved by the alkalisation of the skin surface, which drives the $\text{NH}_3/\text{NH}_4^+$ equilibrium to favour the formation of the NH_3 gas. As the partial pressure builds, ammonia is volatilised from the skin surface. Volatilisation has also been described for the aerially exposed mangrove rivulus, which excretes around 40 % of its nitrogenous waste as gaseous ammonia across the skin (Frick and Wright 2002). This is again accompanied by alkalisation of the skin surface, which results in a 30-fold increase in ammonia partial pressure (Litwiller et al. 2006). However, the mechanism behind the alkalisation of the skin surface remains unclear, as direct secretion of base does not seem to be involved (Cooper et al. 2013). It is hypothesised that volatilisation may be accompanied by active ammonia excretion in these species to move ammonia from the blood to the cutaneous surface (Litwiller et al. 2006). Notably, Rhcg proteins are localised in the apical membranes of NKA-rich cells in the skin of the rivulus (Wright and Wood 2009; Cooper et al. 2013). Volatilisation via the skin is an effective strategy for allowing emersion for significant periods of time, without incurring ammonia toxicity.

There are a number of scenarios that can elevate plasma ammonia levels in fish and exacerbate the need for ammonia excretion. For example, under conditions of high environmental ammonia, its diffusive influx via the gills can greatly increase ammonia burden. Following the discovery that various Rh proteins were expressed in the skin of rainbow trout (Nawata et al. 2007), it was further shown that these cutaneously expressed ammonia transporters were upregulated following high environmental ammonia in trout (Nawata et al. 2007), mangrove killifish (Hung et al. 2007), and pufferfish (*Takifugu rubripes*; Nawata et al. 2010a). Increasing the number of ammonia transporters in high environmental ammonia may seem counterproductive in that it could possibly exacerbate inward ammonia diffusion, but by analogy with the pufferfish gill (Nawata et al. 2010a), selectively upregulating the apical transporter with linkage to an active transport mechanism in a limited population of epithelial cells is likely to minimise diffusive ammonia influx while maximising active excretion. Upregulation of Rh proteins has also been observed after inhibition of ammonia excretion with waterborne HEPES (Nawata and Wood 2008) and loading via ammonia infusion in trout (Nawata and Wood 2009). Together, these data suggest a role for the skin as a 'rescue' pathway under conditions where the capacity for gill ammonia excretion is compromised. Indeed, recent studies on trout indicate that the functional capacity of the skin for

ammonia excretion increases under such circumstances (A.M. Zimmer, C.J. Brauner, and C.M. Wood unpublished data). Similarly, in cold-acclimated goldfish, the increase in interlamellar cell mass effectively reduces the gill surface area, therefore potentially compromising ammonia excretion. Under this scenario, Smith et al. (2012) showed that extrabranchial ammonia excretion (presumably representing skin) plays a relatively greater role in excretion.

Another important role of the skin in helping to maintain ammonia excretion and avoid toxicity during high environmental ammonia exposure may be to secrete acid. This is an approach employed by some air-breathing fish that inhabit water-filled burrows. The resulting reduction in environmental pH helps to maintain diffusive NH_3 excretion. For example, the slender lungfish rapidly acidifies its freshwater environment to below pH 5 when exposed to 30 mmol L^{-1} total ammonia (Wood et al. 2005b). The extent to which this occurs via the skin versus via the gills is unknown, but in the giant mudskipper exposed to the same ammonia level, most of the acid was secreted by the head region. In both mudskipper (Ip et al. 2004) and lungfish (Loong et al. 2007), the skin actually decreased its ammonia permeability, a response hypothesised to prevent NH_3 backflux. This has been attributed to an increased cholesterol and fatty acid content (Ip et al. 2004).

As discussed previously, in larval fish the skin takes on many of the critical roles of the gill, compensating for the delayed development of this tissue post-hatch. In larval fish Rh proteins have been localised to the skin (Nakada et al. 2007a; Shih et al. 2008; Braun et al. 2009). SIET and morpholino gene knockdowns have shown that these Rh proteins are associated with ammonia excretion across the skin of larval zebrafish (Shih et al. 2008; Kumai and Perry 2011; Shih et al. 2011). In larval trout, ammonia excretion occurs predominantly through the rear of the fish (i.e. via the skin) early in development and then transitions to the gills as development proceeds (A. M. Zimmer, P.A. Wright and C.M. Wood unpublished results). This pattern overlaps with that of sodium uptake and is separate from the transition in oxygen uptake (cf. Fu et al. 2010). This is indicative of a functioning Rh metabolon that links both ammonia excretion and sodium uptake and suggests that it may primarily function in ion homeostasis. Supporting this hypothesis is the fact that, to date, there is no evidence for a role of the skin in ammonia excretion in marine fish larvae, where sodium uptake is of minimal importance.

A small number of fish species display ureotelism as adults, relying on urea as their principal nitrogenous waste product. This includes fish that are facultatively ureotelic, often those that are aerially exposed for long periods of time. Here the utilisation of the less toxic nitrogenous end product permits storage of waste in the absence of water. The lungfishes are an excellent example of such species.

These animals are normally ammoniotelic when in water. However, after a period of aestivation/terrestrialisation, during which nitrogenous waste builds up as urea in the body fluids, re-emersion of three species of lungfishes (*Protopterus aethiopicus*, *P. dolloi*, *P. annectans*) led to a rapid, massively elevated urea excretion (Smith 1930; Wood et al. 2005a; Hung et al. 2009). At least in the latter two species, the urea excretion accounted for much of the nitrogen excretion that had been suppressed during the 21- to 33-day period of emersion. Furthermore, it occurred in a biphasic pattern (Fig. 3). The first phase appeared to be a combination of branchial, renal, and cutaneous fluxes, but the second larger phase occurred mostly through the skin (Wood et al. 2005a). This appears to be mediated by a cutaneous urea transporter, evidenced by a 2.5-fold increase in transporter expression levels over a time course closely matching that of the second phase of urea excretion (Hung et al. 2009).

Hagfish are primarily ammoniotelic, but may feed on meals that are high in urea (Braun and Perry 2010). Furthermore, while feeding inside decaying carcasses (see below), they may be exposed to high levels of ammonia (Bucking et al. 2011). Consequently, ammonia and/or urea may diffuse down a gradient into the animal across the gill. Studies have shown that hagfish are capable of excreting ammonia across the skin (Braun and Perry 2010), likely a mechanism of reducing circulating ammonia once feeding has finished. The skin of hagfish is also permeable to urea, albeit at rates around 25 % of those for ammonia when measured in vitro (Braun and Perry 2010). These cutaneous

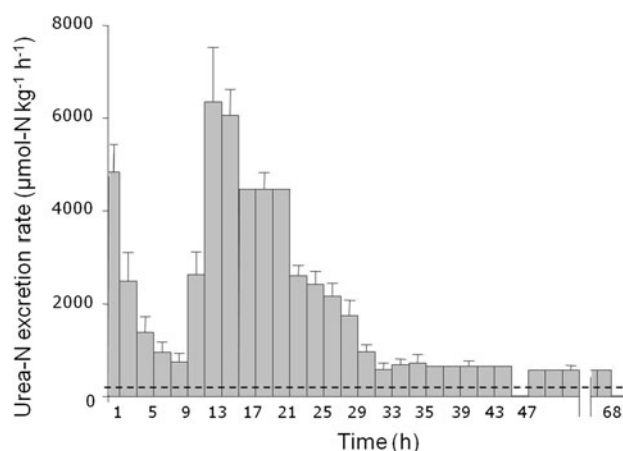


Fig. 3 Rates of urea-N excretion in the slender lungfish (*Protopterus dolloi*) during return to aquatic conditions after 30 days of aestivation. Note the biphasic pattern and sustained elevation of urea-N excretion. The second larger phase is mainly due to urea excretion across the skin. The dashed horizontal line represents the control rate of urea-N excretion in aquatic animals ($25 \mu\text{mol kg}^{-1} \text{h}^{-1}$). The total nitrogen washout is equivalent to 16 days of nitrogen waste production. Plotted values represent mean \pm SEM ($n = 7$). Data are from Wood et al. (2005a)

transport processes are likely to be an effective adaptive strategy for a feeding behaviour that exposes the fish to a potentially toxic environment (Braun and Perry 2010). As seen below, the skin of hagfish is also likely to play a role in the feeding strategy itself.

Nutrient absorption

All fish acquire nutrients through the diet, assimilating both organic (e.g. carbohydrates, lipids, proteins) and inorganic (e.g. trace elements) nutrients via the gastrointestinal epithelium (Bakke et al. 2011). Nutrient concentrations in the diet are likely to greatly exceed the levels in ambient water. For example, levels of dissolved amino acids in marine waters range between 3 and 1,400 nM; although levels are higher in freshwaters owing to enhanced primary productivity and allochthonous input, they do not exceed 5,000 nM (Münster 1993). Concentrations of dissolved carbohydrates in aquatic systems are in the same order of magnitude (0.4–5,000 nM; Münster 1993). As a consequence, there appears to be limited need or opportunity for fish to take up dissolved organic nutrients via the skin.

Although a similar argument holds for aquatic invertebrates, the presence of integument nutrient transport has been well documented in this group. In fact, more than a dozen phyla have been demonstrated to absorb amino acids directly from the ambient medium via the gill and/or the integument. This phenomenon is generally considered to be restricted to marine osmoconformers (Gomme 2001), although there is evidence that some osmoregulating freshwater invertebrates may also have this capacity (e.g. Eaton and Thomas 1999). In freshwater species and marine osmoregulators such as teleost fish, the greater demand for reduced permeability to limit diffusive ion exchange is likely to supersede any advantages achieved by having a surface permeable to nutrients. Those marine osmoconformers that do display nutrient absorption across the integument are generally filter-feeding organisms or those that rely on sporadically available and/or low-quality food sources. As such, the advantage of having an additional digestive surface likely outweighs any issues associated with higher integument permeability in these species.

There is one known exception to the rule that fish species do not utilise the skin as a nutrient acquisition surface. Grover Stephens, in his 1968 review of integument nutrient absorption (Stephens 1968), commented that the hagfish *Eptatretus* was capable of absorbing amino acids from seawater. Hagfish are unusual in that they are the only vertebrate that does not regulate the exchange of monovalent ions (e.g. sodium, chloride, potassium) and water between their extracellular fluids and the environment (Currie and Edwards 2010). Their ability to absorb organic

nutrients via their skin is therefore consistent with the hypothesis that integument permeability is a key factor limiting organic nutrient uptake in other fish. Furthermore, hagfish have a feeding behaviour that may expose them to greatly elevated levels of dissolved nutrients. When large carcasses reach their seafloor habitats, hagfish can burrow inside the animal (Martini 1998), encountering high concentrations of organic substrates.

However, it is only recently that data confirming the ability of the hagfish skin to absorb nutrients have been published (Glover et al. 2011a; Bucking et al. 2011). Employing an Ussing-type chamber, the transmural influx of the tritiated amino acids alanine and glycine across the skin of the Pacific hagfish (*Eptatretus stoutii*) was examined. Influx was shown to be saturable and the kinetic properties of uptake were distinct for each amino acid, indicative of specific transport pathways for amino acids across hagfish skin (Glover et al. 2011a). In vivo studies, where hagfish were immersed in radiolabelled glycine, showed a significant accumulation of the amino acid within hagfish tissues (Bucking et al. 2011), confirming the in vitro observations.

Based on the uptake kinetic characteristics of these amino acids, the hagfish integument can be considered a high affinity, low capacity absorptive route in comparison to the low affinity, high capacity route of the hagfish gut (Glover et al. 2011b). This fits with the relative nutrient levels associated with each pathway of uptake (i.e. higher levels via the gut, lower levels via the skin). In concert with their low metabolic rates (Forster 1990), this may be an adaptation facilitating long periods between feeding. However, relative to invertebrate integumentary uptake, the capacity and the affinity of hagfish skin for glycine and alanine are low. Affinity constants (K_m 's) for marine invertebrates usually range from around 1 to 100 μM (Wright and Manahan 1989), compared to an estimated affinity in the order of 200–500 μM for hagfish skin (Glover et al. 2011a). The capacity of hagfish skin to take up glycine was an order of magnitude lower ($0.2 \text{ nmol cm}^{-2} \text{ min}^{-1}$; Glover et al. 2011a) than that of the sand dollar, *Dendraster excentricus* (Stephens et al. 1978), a species considered to exhibit among the highest capacities for nutrient absorption (Wright and Manahan 1989). This rate value does not, however, account for the total surface area available to the animal for transport. Given that all regions of *Eptatretus stoutii* skin show the ability to absorb amino acids (Fig. 4), the relatively large surface area available for nutrient absorption may compensate for a relatively low uptake capacity.

Interestingly, in contrast to adult skin, where amino acids were rapidly moved through the skin into serosal compartments, in vitro studies of juvenile skin showed that the tissue itself retained the majority of amino acid (Glover

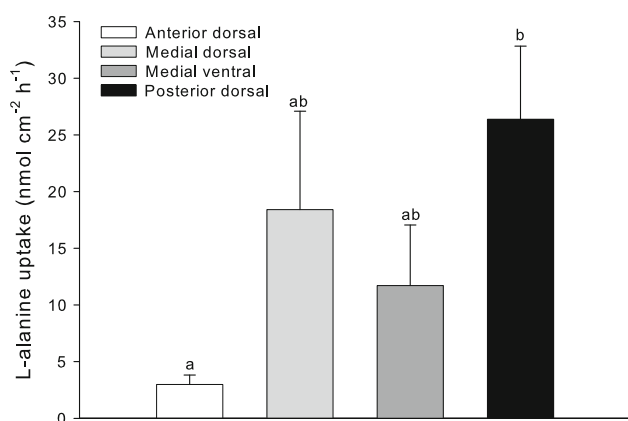


Fig. 4 Transmural uptake (mucosal to serosal) of [³H]-L-alanine (1 mM) across the isolated skin of adult hagfish (*Eptatretus stoutii*) collected from various body regions. Transport was assessed using a modified Ussing chamber (see Glover et al. 2011a). The plotted values represent the mean \pm SEM ($n = 4-6$). Bars sharing letters are not significantly different ($P < 0.05$) according to a one-way ANOVA, followed by a post hoc LSD test

et al. 2011a). This may indicate life stage-dependent utilisation of the absorbed nutrient. In juveniles, amino acids may be a metabolic substrate fuelling slime production, whereas amino acids may have other anabolic uses in adults. It has been suggested, for example, that glycine may have an important neuroregulatory role in adult hagfish exposed to anoxia associated with burrowing and feeding behaviour (Buckling et al. 2011). This hypothesis is supported by an increase in brain glycine sourced directly from the water in anoxic hagfish (Buckling et al. 2011).

The ability of hagfish skin to take up other dissolved organic nutrients is not known. Evidence from invertebrates suggests that both carbohydrates (e.g. Welborn and Manahan 1990) and free fatty acids (e.g. Hoffmann and Wulf 1993) are capable of being absorbed across integument surfaces, so it is likely that hagfish skin also has this ability.

By virtue of an osmoconforming strategy that reduces the need for low skin permeability, a relatively undifferentiated gut (Glover et al. 2011b), and a temporally sporadic feeding behaviour, hagfish are well placed to use the skin for nutrient transport. Whether hagfish are exceptional among fish in this regard is unknown. However, under conditions where skin permeability is high, and/or digestive function is compromised, and/or elevated exposure to dissolved nutrients occurs, then skin absorption may be present. As discussed above, larval fish, for example, utilise the skin for key branchial processes until the gill becomes functional. Similarly, the guts of larval fish are usually not fully functional before the yolk sac is depleted (Wilson and Castro 2011), and therefore the skin may act as an important early pathway for organic nutrient absorption. Cutaneous uptake of organic nutrients may also be

promoted by environments highly enriched in these substrates. Hydrothermal vents are one such habitat, where dissolved free amino acids can reach levels as high as 25 μ M (Klevenz et al. 2010), potentially facilitating uptake of these nutrients by the skin of species that are known to inhabit such environments (e.g. *Eptatretus stickrotti*; Møller and Jones 2007).

Although the digestive tract accounts for almost all organic nutrient absorption, the supplementary role of the fish gill in inorganic micronutrient absorption is well described (Bury et al. 2003). However, to date there have not been any studies that have demonstrated the passage of metal ions across the skin of fish. As some metal micronutrients traverse epithelia via ion mimicry (e.g. copper for sodium; zinc for calcium), fish that utilise the skin for ion homeostasis (see above) may also have the capacity for metal micronutrient absorption.

Some evidence does show that fish skin can accumulate metal nutrients. Sharks, in particular, have a well-documented ability to sequester metals, including nutrients such as manganese and zinc, in their skin tissue (Jeffrey and Teyssie 2006). In teleost fish, dose-dependent accumulation of zinc in skin has been noted, with this nutrient specifically associated with scales in the mummichog (Sauer and Watabe 1984). This association is presumably owing to the physicochemical mimicry of calcium (Bury et al. 2003). In the guppy *Poecilia reticulata* zinc exposure was associated with increased epidermal mucus production (Gheorghiu et al. 2009). Evidence from the gut of rainbow trout suggests that mucus may be able to facilitate zinc absorption at nutritionally important levels (Glover and Hogstrand 2002). Critically, it remains to be determined whether metal micronutrient accumulation in the skin represents that which has been taken up directly from the water, or whether this is a storage and/or detoxification route and thus represents metal absorbed from the gill and/or gut.

Perspectives

The common view is that the skin of fish is a relatively inert surface. It plays a passive role as a barrier against exchanges between the body and the environment, has key functions in behaviour (Elliott 2000), and acts as an attachment surface for the various roles of body mucus (Shephard 1994). However, it is becoming increasingly apparent that the skin can make significant contributions to transport functions usually ascribed to the gill and the gut of fish.

The skin appears to play a role in oxygen uptake and carbon dioxide excretion in teleost fish, and although air-breathing species are the prototype cutaneous respirers,

even wholly aquatic species may exchange these gases across the skin. Many of the adaptations that facilitate gas exchange across the skin of fish are also useful for both ion transport and nitrogenous waste excretion. These two processes are potentially linked in fish skin through the presence of cells in the cutaneous surface that possess transporters known to facilitate these exchanges in the gills of fish. Finally, although only a single confirmed example exists of a fish that uses its body surface as a nutrient transport epithelium, there are other scenarios under which this phenomenon may exist, making it a particularly interesting area for future research.

The increasing awareness of the role of the skin in fish homeostasis also raises questions as to whether the skin may also be a locus of toxic impact. For example, many metal toxicants exert their impacts via interference with ion homeostasis (Wood 2012). If the skin of fish is a significant pathway for ion absorption, then this could either act to increase the sensitivity of fish to waterborne metals or could alternatively act as a supplementary pathway that may decrease sensitivity.

Another avenue worthy of further investigation is the interplay between skin and the gill with respect to functional plasticity. The gill of fish participates in multiple homeostatic processes. However, changes in the gill that favour one function may often impair other processes. For example, increased branchial permeability in freshwater fish will increase oxygen transport, but at the same time will exacerbate ion and water exchanges with the environment, a trade-off known as the osmorepiratory compromise (e.g. Matey et al. 2011). The utilisation of the skin as a physiologically active surface may play a significant role in compensating for such functional conflicts, permitting spatial separation of distinct transport processes. Similar epithelial crosstalk has been observed between the gut and gill of fish with respect to ion metabolism (e.g. Franklin et al. 2005).

Clearly, there is much that remains to be elucidated regarding the exact mechanisms by which substrates such as gases, ions, and nutrients traverse the cutaneous epithelium, and the relative importance of this pathway for homeostasis. However over the last decade, the application of advanced techniques has significantly furthered our understanding of transport processes across the skin. SIET, for example, has allowed measurements of ions at a single cell level (e.g. Shen et al. 2011), while the use of ion-specific fluorescent dyes in combination with confocal fluorescence microscopy and suitable model species also has significant promise (Esaki et al. 2007). The application of modern cellular and molecular techniques such as quantitative PCR (e.g. Hung et al. 2009), immunohistochemistry (e.g. Hiroi et al. 2008), and translational knockdowns (e.g. Kumai and Perry 2011) are increasingly

providing an enhanced insight into the structural framework underlying skin transport functions.

Fortunately, there are a number of species that seem particularly amenable for investigating the physiological roles of skin. Fish such as the hagfish, inanga, shanny, lungfish, and mangrove rivulus, for example, have demonstrated abilities to transport multiple substrates across their cutaneous surfaces. This capacity is facilitated by extreme behaviours and physiological strategies for dealing with environmental stressors, consistent with Krogh's principle ("For many problems there is an animal on which it can be most conveniently studied"; Krebs 1975), and bestowing these species with model organism status.

Another potential model system for examining cutaneous transport is the larval fish skin epithelium. In early life stages, there are multiple functions attributed to the gill and the gut of adults that are likely to be initially carried out through the skin. For example, there is a distinct lack of study on the role of the skin in nutrient uptake in larval fish. Such investigation may provide fundamental insight into the evolutionary development of the digestive tract, but also find application in aquaculture settings.

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