

Review

Cultured gill epithelia as models for the freshwater fish gill

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Abstract

We review recent progress in the development of models for the freshwater teleost gill based on reconstructed flat epithelia grown on permeable filter supports in primary culture. Methods are available for single-seeded insert (SSI) preparations consisting of pavement cells (PVCs) only from trout and tilapia, and double-seeded insert (DSI) preparations from trout, containing both PVCs (85%) and mitochondria-rich cells (MRCs, 15%), as in the intact gill. While there are some quantitative differences, both SSI and DSI epithelia manifest electrical and passive permeability characteristics typical of intact gills and representative of very tight epithelia. Both preparations withstand apical freshwater exposure, exhibiting large increases in transepithelial resistance (TER), negative transepithelial potential (TEP), and low rates of ion loss, but there is only a small active apical-to-basolateral “influx” of Cl^- (and not of Na^+). Responses to various hormonal treatments are described (thyroid hormone T3, prolactin, and cortisol). Cortisol has the most marked effects, stimulating Na^+, K^+ -ATPase activity and promoting active Na^+ and Cl^- influxes in DSI preparations, and raising TER and reducing passive ion effluxes in both epithelia via reductions in paracellular permeability. Experiments using DSI epithelia lacking Na^+ uptake demonstrate that both NH_3 and NH_4^+ diffusion occur, but are not large enough to account for normal rates of branchial ammonia excretion, suggesting that Na^+ -linked carrier-mediated processes are important for ammonia excretion in vivo. Future research goals are suggested.

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1. Introduction

The fish gill is arguably one of the most complex transport epithelia in the biological world. This single epithelium is designed to simultaneously transport respiratory gases, major and minor electrolytes, acid–base equivalents, nitrogenous wastes, and water, and is probably the principal organ of homeostasis for all of these. To achieve this diversity of function, the epithelium covers hundreds of filaments and thousands of lamellae. The structure is of exquisite beauty under the microscope, and comprises over 50% of the total surface area of the animal. However, this architectural heterogeneity has rendered the gill epithelium completely refractory to the sort of rigorous electrophysiological and radioisotopic flux analyses pioneered by Ussing

[1,2] and Koefoed Johnson et al. [3]. To overcome this problem, physiologists have searched for surrogate epithelia that perform the same functions as the gill. Cranial epithelia from several marine teleosts have been discovered which appear to faithfully duplicate the ionic transport functions of the gill in seawater. Indeed, most of what we currently know about the mechanisms of active salt excretion in marine fish can be traced back to pioneering work on the opercular epithelia of seawater-adapted killifish [4,5] and tilapia [6], and the jawskin of the seawater-adapted goby [7]. These surrogate models contain an abundance of ion-transporting mitochondria-rich cells (MRCs), often called “chloride cells”. The epithelia can easily be dissected away from underlying tissue, yielding flat sheet-like preparations that survive well in vitro in the absence of blood flow. Such epithelia can be mounted in an Ussing chamber, allowing exposure of the apical and basolateral surfaces to asymmetrical media as in vivo (e.g. apical seawater, basolateral physiological medium).

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Physiologists have searched largely in vain for a comparable surrogate for the freshwater gill which can be used to study active salt uptake from freshwater, passive salt losses, and accompanying electrical and permeability characteristics. Preparations investigated to date include opercular epithelia of freshwater-adapted tilapia [8–10] and freshwater-adapted killifish [10–13], and the cleithral epithelium of the freshwater-adapted rainbow trout [14], all of which contain freshwater-type MRCs. Overall, these preparations have proven quite useful for characterizing active Ca^{2+} uptake, and specifically localizing it to the MRCs. However, when bathed in apical freshwater, the epithelia take up Cl^- only at very low rates and Na^+ often not at all.

An entirely different approach is to use primary cell culture techniques and “reconstruct” flat gill epithelia under a controlled culture regime in such a way that they can be exposed to asymmetrical media as in vivo (i.e. apical freshwater, basolateral physiological medium). The advantages that such an approach may confer are numerous and include the opportunity to, work with a “living” system at different stages of development, examine changes in gill cell/epithelial function during conditions of chronic experimental manipulation or manipulate cell-type presence by addition or removal during culture preparation. The use of such experimental protocols is limited when using more traditional freshly isolated epithelia. Our group has been gradually developing this approach over the past decade. The present article reviews progress to date and future perspectives in the context of other developments in the field. Early progress was reviewed by Pärt and Bergstrom [15].

2. Cultured gill cells from freshwater fish

Pärt et al. [16] developed the first procedures for growing, in primary culture, gill epithelial cells from freshwater rainbow trout (*Oncorhynchus mykiss*), and all further progress has stemmed from modifications of this original methodology. In brief, the approach consists of harvesting epithelial cells by trypsinization and plating them in culture flasks, using Leibowitz L-15 media supplemented with 2 mmol l^{-1} glutamine, 5% (v/v) foetal bovine serum (FBS), and antibiotics. All procedures are performed using sterile technique in a laminar flow hood. After 24 h, the medium is changed, washing away all non-attached cells (~65%), leaving only pavement cells (PVCs, also called “respiratory cells”) or their progenitors which rapidly divide to form colonies. On day 4, the antibiotics are removed, and by days 6–9, the cells are usually close to confluence in the flask. [^3H]thymidine incorporation studies demonstrate that mitotic activity is maximal at this time. Light, fluorescence, and electron microscopy reveals that the confluent carpet consists only of PVCs with their characteristic “finger-print” microridges on the apical surface, as seen in vivo

[17] and in explant cultures of trout gill tissue [18–20]. All other cell types (most importantly MRCs) have disappeared since they fail to attach. Avella et al. [21] have developed comparable methods for growing PVCs in primary culture from the gills of a seawater fish, the sea bass (*Dicentrarchus labrax*).

These PVCs grown on plastic support have been used for a variety of different studies, some of which have been recently reviewed [22]. These include investigations on cell volume regulation and intracellular ions [15,23,24], the mechanisms of intracellular pH regulation [20,25,26], the extent of electrochemical coupling via gap junctions between cells [27], protein synthesis and its associated costs [28], phase I and phase II metabolism for biotransformation and detoxification of steroids and lipophilic xenobiotics [29–31], and various types of toxicological testing [23,28,32,33,34].

Recently, O'Donnell et al. [35] succeeded in patch-clamping cultured PVCs from freshwater rainbow trout grown on plastic support. In particular, they characterized a large-conductance (372 pS, “maxi”) Cl^- channel on the apical surface of the cultured cells. The channel was activated by patch excision, and exhibited an open probability that was sensitive to membrane potential in a bell-shaped fashion, a relatively high permeability (81% that of Cl^-) to HCO_3^- , and sensitivity to blockade by DPC, Zn^{2+} and stilbene derivatives such as DIDS. All of these characteristics, apart from DPC inhibition, were very different from those of a low-conductance (8 pS) Cl^- channel characterized in the apical membranes of cultured PVCs from the sea bass [36]. The latter appeared similar to a low-conductance Cl^- channel in MRCs of the opercular epithelium of seawater killifish [37], and was postulated to be involved in the outward transport of Cl^- [36]. In contrast, O'Donnell et al. [35] hypothesized that the maxi- Cl^- channel of trout PVCs was involved in the inward transport of Cl^- , perhaps masquerading as an apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger.

However, the presence of a solid plastic support on the basal surface of these cultured PVCs is a serious limitation, because transepithelial transport cannot be studied. Furthermore, since the L-15 culture medium bathing the apical surface of the cells essentially duplicates the extracellular fluid that would bathe the basolateral surface in vivo, and freshwater is not present, physiological polarization is questionable. Indeed, studies on intracellular pH regulation in this system have concluded that the mechanisms characterized are likely basolateral “housekeeping mechanisms” that have migrated in culture, and that the absence of apical freshwater may have prevented expression of the true apical mechanisms (Na^+ -acid and Cl^- -base exchange linkages) thought to be involved in transepithelial transport in vivo [20,25,26]. Similarly, O'Donnell et al. [35] noted that the supposedly apical maxi- Cl^- channel could equally well be a basolateral Cl^- channel that had relocated in culture.

3. Cultured gill epithelia from freshwater fish

In mammalian epithelial physiology, the problem of questionable cell polarity was overcome long ago [38,39] through the development of techniques for growing epithelia on permeable filter supports (“inserts”). These inserts are now marketed commercially. The basolateral surface of the epithelium attaches to the highly permeable filter through which it receives nutrition from an underlying well filled with culture medium. Since the upper part of the insert is physically separated from the well, the apical surface of the epithelium can be exposed to either the same medium (“symmetrical” conditions) or to an entirely different medium (“asymmetrical” conditions). Transport between the two media and the electrical characteristics [e.g. transepithelial potential (TEP), transepithelial resistance (TER)] of the epithelium can be easily studied under precise conditions. Avella and Ehrenfeld [40] and Avella et al. [41] have developed culture methods for growing such an epithelium on permeable filter support from the gills of a seawater fish, the sea bass. The approach is based on modification of earlier flask culture methods for PVCs of this species [21], and yields an epithelium which appears to consist entirely of PVCs.

For freshwater fish, two techniques are now available for growing “reconstructed” gill epithelia on permeable supports from freshwater rainbow trout, yielding two different types of epithelia; the reader is referred to Kelly et al. [42] for full methodological details. The first approach, which involves cells from only one fish, is the “single-seeded insert” (SSI) technique developed by Wood and Pärt [43]. This yields an epithelium consisting entirely of PVCs arranged in typically about four horizontally overlapping cell layers (range=2–7) as in vivo [44]. PVC internal morphology is similar to that seen in vivo with few mitochondria, abundant rough endoplasmic reticulum, and numerous tight junctions and desmosomes between adjacent cells. Those on the outer surface have the characteristic “fingerprint” microridges on their apical membranes and a prominent glycocalyx (see Refs. [43,45] for electron micrographs). Recently, the technique has been adapted for growing comparable PVC epithelia from freshwater tilapia (*Oreochromis niloticus*) [46].

In brief, in the SSI method, the PVCs are first cultured in flasks exactly as described above until they approach confluence at the time of maximum mitotic activity (6–9 days). At this point, the cells are harvested by trypsinization, washed, and replated onto the abovementioned filter inserts. In our experience, the cell culture inserts which work best are the Falcon brand marketed by Becton Dickinson (Franklin Lakes, NY, USA), consisting of cyclopore polyethylene terephthalate material with a pore density of 1.6×10^6 pores cm^{-2} . Inserts of different diameter can be used with equal success, depending on experimental needs. The epithelia are then allowed to develop for approximately 6 days by which time they reach a stable condition suitable for experimenta-

tion. During this period, the antibiotic-free medium (L-15 + 2 mmol l^{-1} glutamine + 5% FBS as above) is changed every 48 h, and the TER is monitored every 24 h using a chopstick electrode system with an EVOM voltohmmeter designed for monitoring filter cultures (WPI, Sarasota, FL, USA).

More recently, Fletcher et al. [47] developed the “double-seeded insert” (DSI) technique, which involves sequential seeding of freshly trypsinized gill cells from two separate fish directly onto the same filter (again cyclopore polyethylene terephthalate membranes) on successive days. Twenty-four hours after the first seeding, the filter is thoroughly washed to remove the bulk of the cells that have not attached, along with mucous material and debris. At this time, before the new set of cells and media are added, the neoformed PVC layer can easily be seen under phase-contrast microscope. The procedure is then repeated, and non-attached material is again washed away at 48 h. The culture medium is the same as used for SSI, with antibiotics present up until either 48 or 96 h, and TER measurements are made on successive days to track the development of the epithelium. There is no phase of initial flask culture, so the overall procedure is faster than the SSI method, yielding an epithelium with stable electrical characteristics suitable for experimentation in 6–9 days.

This DSI method yields a more complex epithelium consisting of both PVCs (about 85%) and MRCs (about 15%) in the approximate proportions in which they occur in the gill in vivo [48,49]. The MRCs themselves will not attach to the filter on the first seeding, but enough PVCs attach to form a “lawn”, and this allows the MRCs added on the second seeding to nest in this lawn. The PVCs have identical morphology to that seen in SSI epithelia, and again are arrayed in overlapping layers. The MRCs, easily identified by fluorescent mitochondrial stains such as rhodamine 123, DASPEI, or DASPMI, occur singly or in clusters, are voluminous, open apically to the external environment, form deep tight junctions with neighbouring PVCs, and exhibit ultrastructural morphology (numerous granular mitochondria, a dense tubular system; see Ref. [47] for electron micrographs) similar to that seen in MRCs of freshwater gills in vivo [17]. No “accessory cells” or very shallow junctions typical of seawater MRCs [49,50] are seen.

4. Electrical characteristics and functional polarization of cultured gill epithelia

As soon as the cultured epithelium becomes confluent on the filter (generally 24–48 h after final seeding), TER rises significantly above background, and then increases dramatically in a sigmoidal fashion, reaching a more or less stable plateau by days 6–7 (Fig. 1). TER is thereafter stable for at least 3 days, and experiments are generally performed during this period of electrical stability. While there is

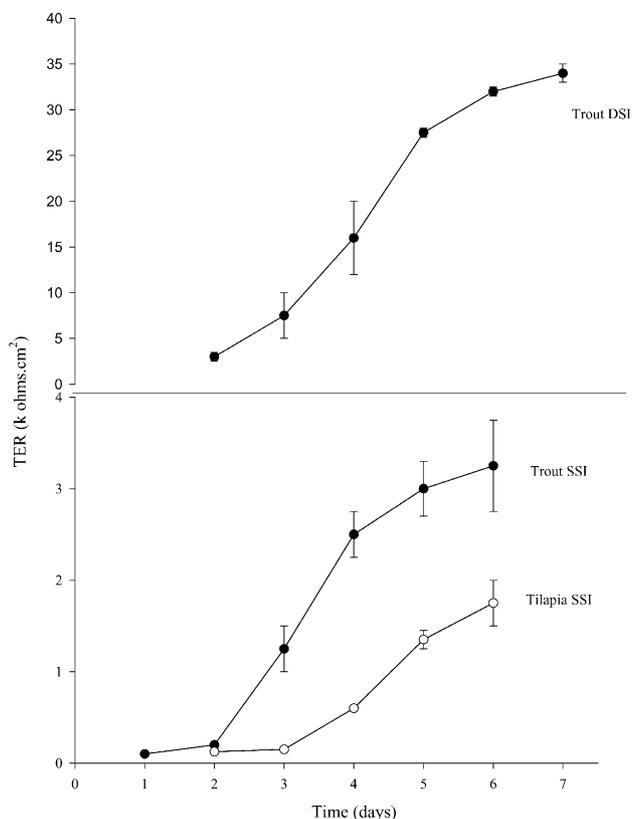


Fig. 1. The TERs of reconstructed gill epithelia increase in a sigmoidal fashion to a plateau after 6–7 days in culture, with generally higher values in trout DSI preparations (upper panel, data from Ref. [47]) than in trout SSI preparations (lower panel, data from Ref. [43]), with smaller values in tilapia SSI preparations (lower panel, data from Ref. [46]). Means \pm 1 S.E. ($N=14-20$).

considerable variability among different batches of fish and seasons, the absolute plateau TER is almost always higher in trout DSI preparations than in trout SSI preparations, whereas in tilapia (for which only SSI preparations are currently available), plateau TER is generally in the lower range of trout SSI values (e.g. Fig. 1).

While it is tempting to interpret the increase in TER as a “growth curve”, available evidence indicates that this is not the case. At least for trout DSI preparations, there is no relationship whatsoever between TER and cell numbers in the epithelium (Fig. 2) or between cell numbers and protein content. Rather, it would appear that once confluence is reached, cell numbers and size stay more or less constant, and what changes is junctional tightness. This conclusion is in accord with the demonstration [51] of a correlation between tight junction formation and increased TER in cultured mammalian epithelia, and is directly supported by the fact that epithelial permeability to a paracellular channel marker, polyethylene glycol-4000 (PEG-4000), decreases as TER increases in cultured trout gill epithelia. There is in fact a positive linear relationship between epithelial conductance (the inverse of TER) and PEG-4000 permeability in both SSI [45,52] and DSI preparations [53]. Interestingly, there is also

a positive relationship between the number of MRCs present and TER [47]. At least in part, this explains the higher TER in DSI relative to SSI preparations. This observation is surprising as TER typically decreases with MRC density in seawater fish cranial epithelium models (e.g. Ref. [54]). However, it suggests that in the absence of accessory cells, which traditionally contribute “leaky” junctions in the seawater gill [55,56], the freshwater MRCs contribute tighter junctional complexes, and/or smaller transcellular conductances than do PVCs alone. The TER values occurring in the plateau phase are amongst the highest ever recorded for an epithelium in culture (Table 1), and clearly classify both SSI and DSI preparations as “tight” epithelia.

Upon exposure to apical freshwater, TER increases dramatically within a matter of a few minutes (Fig. 3). The extent of the increase under these asymmetrical conditions depends to some extent on starting TER: those preparations having the lowest TER in the presence of apical L-15 exhibit the largest relative increases when exposed to apical freshwater, and vice versa. Thus, SSI preparations exhibit the largest increases (up to 22-fold; e.g. Refs. [43,46]), whereas DSI preparations exhibit smaller increases, or when starting at very high TER, no rise at all [53]. The maximum TER appears to be about 30,000 Ω cm² regardless of whether conditions are symmetrical or asymmetrical, or whether the epithelium is SSI or DSI. To our knowledge, there are no records of TER in the intact gill of freshwater fish, though measurements exist for a number of gill surrogates in vitro (Table 1). These also exhibit an increase in TER when the apical medium is switched from isotonic saline to freshwater, and suggest that the absolute values for the surrogates in apical freshwater are in the same general range as the cultured gill epithelia. Clearly, the intact gill must be very tight electrically, as traditionally assumed but never proven [57,58].

It is often further assumed that gill paracellular permeability is reduced by junctional tightening when euryhaline fish move into freshwater, thereby minimizing net ion losses [55,59,60], but this is not supported by experiments with cultured trout gill epithelia. Thus, the increase in TER accompanying apical freshwater exposure is accompanied by an increase (typically about twofold) rather than a decrease in PEG-4000 permeability in both SSI [45,52] and DSI epithelia [53]. The relationship between epithelial conductance and PEG-4000 permeability is reset to a higher intercept [52]. A more than compensating decrease in transcellular conductance explains the overall increase in TER observed, and therefore, Na⁺ and Cl⁻ effluxes exhibit little change initially. This has been interpreted as a closure of ion channels on the apical membrane in response to external dilution. Possibly, the rapid synthesis of phosphatidylethanolamine which occurs at this time, as demonstrated by the incorporation of apically applied ³²P into gill lipid, may represent a change in apical membrane structure which facilitates this closure [61]. Another phenomenon that may be consistent with reduced permeability in these

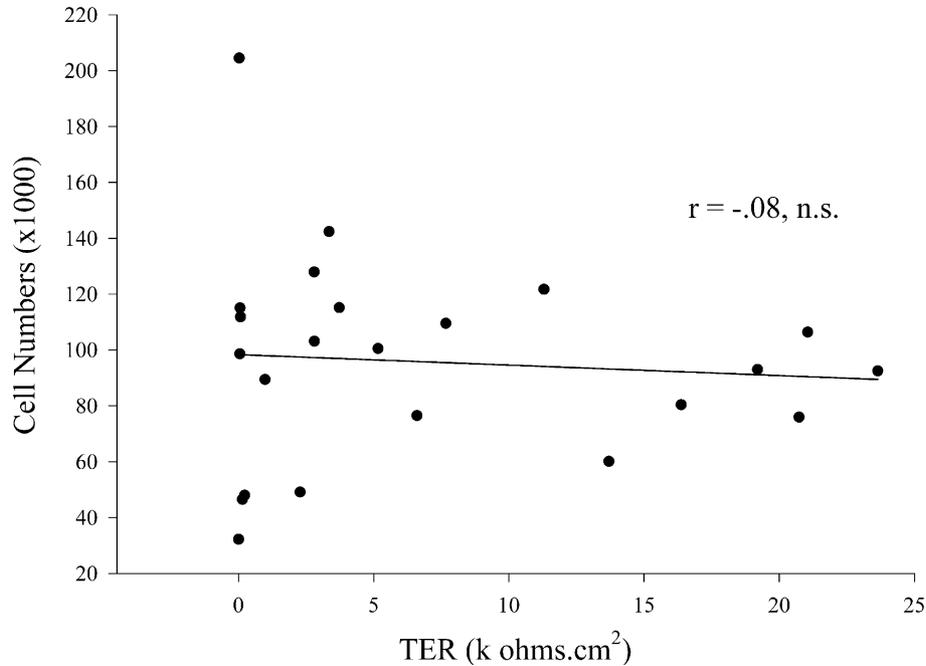


Fig. 2. There is no relationship between TER and cell numbers (growth surface 0.9 cm^2) in trout DSI preparations (C.M. Wood, B. Eletti, and P. Pärt, unpublished data). $N=24$.

preparations, and therefore cannot be discounted, is the potential removal of membrane proteins from the apical domain. Interestingly in tilapia SSI preparations, both PEG-4000 permeability and ion effluxes decrease upon initial exposure to apical freshwater, so in this species, junctional tightening does occur, and both paracellular and transcellular conductance probably decrease [46].

Table 1
Representative transepithelial resistance ($\Omega \text{ cm}^2$) of various epithelia

Preparation	Apical culture medium or saline	Apical freshwater	Ref.
<i>Cultured freshwater gill epithelia</i>			
Trout SSI	2000–8000	10,000–20,000	[43,45,52]
Tilapia SSI	1000–4000	8000–15,000	[46]
Trout DSI	6000–30,000	20,000–30,000	[47,53]
<i>Gill surrogates</i>			
Trout cleithral epithelium	–	10,600	[14]
Mozambique tilapia opercular epithelium	260	3700	[8]
Nile tilapia opercular epithelium	169	1052	[10]
Killifish opercular epithelium	294	660	[11]
<i>Traditional “tight” epithelia in culture</i>			
Toad A6 kidney cells	5000	–	[95]
Rat alveolar cells	2250	–	[96]
<i>Traditional “leaky” epithelia</i>			
Caco-2 intestinal cells	258	–	[97]
Flounder kidney cells	23	–	[98]

The epithelia can sustain apical freshwater exposure with approximately stable electrical characteristics (Fig. 3) and permeability for about 3 h, and are able to fully recover upon return to apical L-15 media. Even after 48 h of apical freshwater, TER remains significantly higher than under symmetrical conditions. However, over this longer time period, there is a progressive deterioration of barrier function, manifested as falling TER, less negative TEP (see below), increasing PEG-4000 permeability, increasing Na^+ and Cl^- losses, changing relationships between PEG-4000 permeability and transepithelial conductance, and between ion effluxes and conductance, and ultrastructural changes in the surface layer of cells [43,45,52,53,62]. Both transcellular and paracellular permeability increase over time.

The switch from symmetrical to asymmetrical conditions provides a convincing demonstration that the cultured epithelia are functionally polarized. When freshwater is applied to the basolateral rather than the apical side of trout SSI epithelia, TER declines to levels not significantly different from zero within 3 h, and shows no recovery upon restoration of apical L-15 media (Fig. 3). Exactly the same response is seen with trout DSI epithelia [53]. Clearly, the epithelia are set up so as to tolerate dilute conditions only on their apical side.

There appears to be some variation in TEP amongst different studies, at least for trout SSI preparations under symmetrical conditions. In some reports [43,47,52,63], TEP was not significantly different from zero when L-15 media was present on both surfaces (also in tilapia SSI epithelia [46]). However, in others [62,64], there was a small but significantly positive TEP (+1 to +3 mV, with reference to

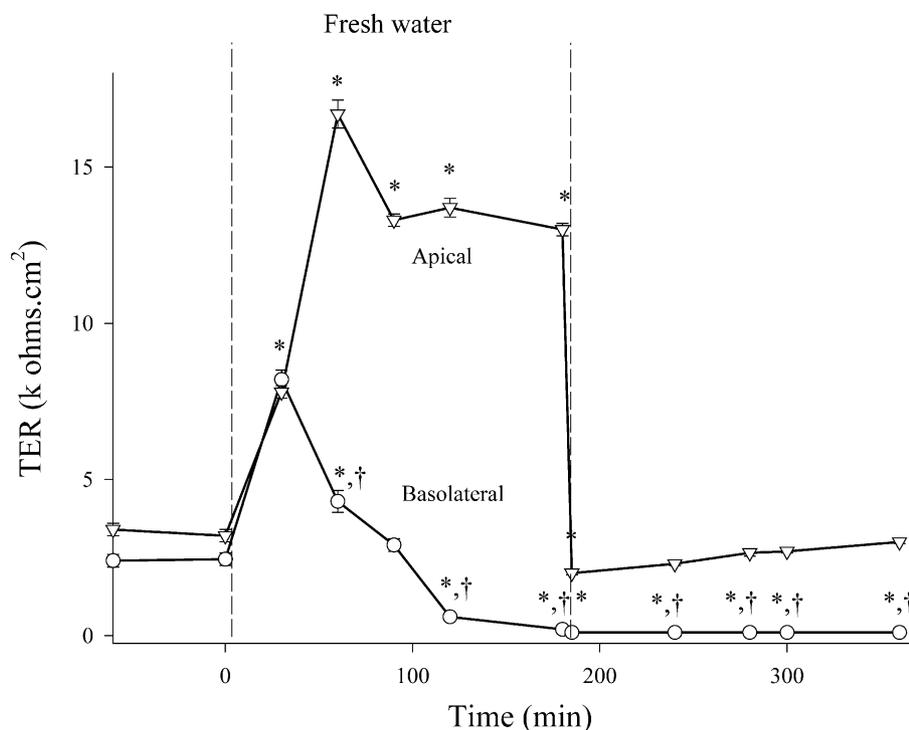


Fig. 3. Cultured branchial epithelia are functionally polarized, as illustrated by this comparison of the responses in TER of trout SSI preparations acutely exposed to either apical freshwater or basolateral freshwater. Note the rapid increase and stabilization of the TER with apical freshwater, and eventual recovery upon restoration of apical culture medium, whereas TER collapses with basolateral freshwater exposure, and never recovers with restoration of basolateral culture medium. Asterisks indicate significant differences ($P \leq 0.05$) relative to pre-exposure values; daggers indicate significant differences ($P \leq 0.05$) between the two treatments (M. Fletcher, M.J. O'Donnell, C.M. Wood, and P. Pärt, unpublished data). Means \pm 1 S.E. ($N=10$).

the apical surface as 0 mV) under these conditions. In yet another study [65], TEP was significantly negative (-1 to -5 mV) under symmetrical conditions. At least in the latter study, the potential appeared to be attributable to electrogenic transport, because it was inhibited by vanadate (a blocker of P-type ATPases) and by a combination of potassium cyanide and iodoacetate (blockers of metabolism). This would imply active anion uptake (apical-to-basolateral) or cation extrusion (basolateral-to-apical) under symmetrical conditions, but the needed fluxes would be so small (around 1.5 nequiv $\text{cm}^{-2} \text{h}^{-1}$) as to be virtually unmeasurable. There is an obvious need for further investigation to clarify the situation.

Trout DSI epithelia exhibit significantly positive TEPs under symmetrical conditions. Mean values range from $+1.9$ mV [47] up to $+9.0$ mV [63] as a function of TER; the slope of the relationship indicates that a net electrogenic transepithelial flux (anion extrusion or cation uptake) of about 15 nequiv $\text{cm}^{-2} \text{h}^{-1}$ would be needed to explain the potential. While this is larger than needed to explain the negative potential observed in SSI epithelia [65], it would still be very difficult to detect by standard flux measurement techniques. Using chamber short-circuit analysis would be an ideal way to attack the problem. Avella and Ehrenfeld [40] and Avella et al. [41] have applied this approach to demonstrate that the very positive potential ($+13$ to $+30$ mV) observed across the cultured epithelium of the marine

sea bass is due to electrogenic Cl^- transport at a net rate of 60 – 130 nequiv $\text{cm}^{-2} \text{h}^{-1}$.

Upon exposure to apical freshwater, TEP becomes highly negative, typically averaging about -11 mV, with occasional values as low as -35 mV, in both SSI and DSI preparations of trout [43,47] as well as in SSI preparations of tilapia [46]. These values are similar to those observed in euryhaline teleosts in vivo when they move into freshwater, and are usually interpreted as mainly diffusion potentials due to a higher passive permeability to Na^+ than to Cl^- [59,66]. Nernstian analysis suggests that the same explanation applies in the cultured epithelia [43]. At least in DSI epithelia, the greater the TER, the more negative is the TEP, though the slope of the relationship can account for only a small portion of the TEP. Thus, a small negative electrogenic component appears to be superimposed on a large negative diffusion potential [47].

5. Ion transport by cultured gill epithelia

Our ultimate objective in developing cultured gill epithelia from freshwater teleosts was to produce a model system which duplicates the intact gill in terms of ion transport, but this has yet to be fully realized. In terms of “passive” characteristics (i.e. TER, TEP, PEG-4000 permeability, diffusive efflux rates of Na^+ and Cl^-), absolute values in trout

SSI preparations appear to be very close to the best estimates available for the gills of freshwater trout in vivo [43,45]. Furthermore, the largely passive fluxes of Na^+ and Cl^- appear to be fully conductive, accounting for the bulk of the measured conductance. However, in terms of active Na^+ and Cl^- uptake, measured using standard radiotracer (^{22}Na , ^{36}Cl) techniques similar to those pioneered by Ussing [1,2] and Koefoed Johnson et al. [3], the cultured SSI epithelium is no better than the surrogate gill models derived from various cranial epithelia of freshwater fish, as described in the Introduction. Under symmetrical conditions, unidirectional ion flux rates are approximately equal in both directions, so there is little net flux, and upon acute exposure to apical freshwater, Na^+ and Cl^- “influx rates” (apical-to-basolateral fluxes) amount to no more than a few percentages of “efflux rates” (basolateral-to-apical fluxes)—that is, the drop in influx is approximately proportional to the drop in apical Na^+ or Cl^- concentration. Under such conditions, the most reliable indicator to determine whether active transport occurs is calculation of the Ussing–Teorell flux ratio criterion [2,67]. Here the measured ratio of unidirectional fluxes (J_{in} , J_{out}) is compared to that predicted from the measured TEP and measured activities of the ion in question on the apical (A_{Ap}) and basolateral (A_{Bl}) sides of the epithelium:

$$\frac{J_{\text{in}}}{J_{\text{out}}} = \frac{A_{\text{Ap}} e^{(-zFV/RT)}}{A_{\text{Bl}}} \quad (1)$$

where z is the ionic valence; V is the measured TEP in volts; and F , R , and T have their usual thermodynamic values. With some reservations [68], none of which are likely operative here, a significant discrepancy between measured and predicted ratio is indicative of active transport.

When applied to both trout and tilapia SSI preparations in a number of studies [46,52,62–64], the flux ratio criterion has consistently indicated a small but significant active “influx” of Cl^- alone under asymmetrical conditions (apical freshwater), with a tendency for active “efflux” of Na^+ . This is a surprising result in view of the fact that SSI epithelia are composed only of PVCs, whereas the MRCs are generally thought to be the sole sites of active Cl^- uptake in the intact freshwater gill [69,70]. It does, however, concur with the finding of a high-conductance Cl^- channel located on the apical membranes of freshwater trout gill PVCs in flask culture [35]. Interestingly, this same pattern (a very small active “influx” of only Cl^- from apical freshwater) is also seen when various cranial epithelia are mounted in Ussing chambers in vitro (see Introduction). However, the latter do contain freshwater-type MRCs.

In some ways, this “problem” of the SSI preparations (active uptake of Cl^- in the absence of MRCs) is analogous to that for the cultured preparation of seawater gill cells from the sea bass where a pure PVC epithelium performs active basolateral-to-apical extrusion of Cl^- [40,41], and the PVCs express apical Cl^- channels in flask culture [36]. This result directly opposes current models [5,11,56,60] which

consider Cl^- transport to be the exclusive function of seawater-type MRCs in vivo. When the trout SSI preparation is mounted under symmetrical conditions, analogous to those used for the sea-bass epithelium, it too actively extrudes Cl^- , and also Na^+ , albeit at low rates [62]. Clearly, the situation is confusing, but it could be argued that the fundamental limitation of all these cultured epithelia is that they consist of only PVCs, whereas “normal” transport as in the intact gill might well require both MRCs and PVCs. Currently popular models suggest that Na^+ , at least in part, is taken up across PVCs via a Na^+ channel energized by the electrogenic actions of an apical H^+ -ATPase, while Cl^- is taken up by MRCs through an apical $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism [69–72].

For this reason, we developed the trout DSI preparation which incorporates both cell types [47]. Absolute flux rates of Na^+ and Cl^- tend to be lower in both directions under all conditions than in SSI preparations, reflecting the lower diffusive conductance (i.e. higher TER) of DSI epithelia. Despite the 15% complement of MRCs, DSI epithelia exhibit flux ratios very similar to those of SSI preparations when exposed to apical freshwater. Thus, the active “influx” of only Cl^- , and not Na^+ , occurs from apical freshwater. However, under symmetrical conditions, there is a tendency for active Na^+ “influx” but passive Cl^- movement [47,63], different from the pattern seen in SSI preparations. There is also a vigorous active uptake of Ca^{2+} (measured with ^{45}Ca) which is in accord with the abundance of MRCs and current theories as to the importance of MRCs in Ca^{2+} transport (see Introduction). Curiously, however, an equally marked active extrusion of Ca^{2+} occurs under asymmetrical conditions, again indicating that not all the correct cues for transport of the correct polarity are present.

Acute freshwater exposure is likely not a “natural” phenomenon for a fish living in saline waters, and if it occurred, would undoubtedly be accompanied by internal dilution. In our cultured gill epithelia, immediately before asymmetrical experimentation, apical media are acutely replaced with freshwater. By mimicking more realistic freshwater exposure conditions, with the final goal of activating normal ion transport, it will be interesting to fully examine various modifications of media replacement that ultimately lead to asymmetrical culture conditions. For example, progressive, gradual dilution of the apical medium during the 6-day period when the epithelium is actually developing or, conversely, manipulating basolateral media to simulate internal dilution. Recent experiments (B. Zhou, S.P. Kelly, and C.M. Wood, unpublished observations) indicate that trout DSI preparations tolerate the former of these media “manipulation regimes” well, with TER continuing to develop and rise in a normal fashion down to an apical dilution consisting of only 25% L-15. Modifications of this gradual exposure regime, and others, may provide further improvements to epithelial performance in the future.

6. Hormonal effects on cultured gill epithelia

In vivo, dramatic endocrine changes occur when fish move from saline waters into freshwater [11,73], so hormonal support of the cultured epithelium may be very important in establishing “normal” ion transport and permeability in freshwater. The cultured gill epithelial system is well suited for the study of hormone effects on gill cell/epithelia physiology as culture media can be readily manipulated. This can facilitate the examination of, transient or long-term hormone effects under defined conditions, isolated or combined hormone regimes or even a “bioassay-type” approach where cells/epithelia may respond contrarily to fluids/extracts derived from fish in different physiological states [46]. In the sea-bass PVC epithelium, arginine vasotocin, prostaglandin E₂, and β -adrenergic agents are stimulatory to Cl⁻ “outflux”, while α -adrenergic agents are inhibitory [41]. In cultured freshwater gill epithelia, three hormones have been investigated in some detail to date. While cortisol appears to be the most promising overall, tests with thyroid hormone (T3) and prolactin have also yielded very useful information.

Kelly and Wood [64] evaluated T3, because in freshwater fish, elevations in circulating T3 levels are normally associated with exposure to ionoregulatory toxicants, smoltification, and seawater entry [73,74]. When trout SSI epithelia are grown in the presence of physiological concentrations (10 ng ml⁻¹) of T3, there is a decrease in TER and an increase in unidirectional Na⁺ and Cl⁻ flux rates, but no change in PEG-4000 permeability under symmetrical conditions, suggesting a specific effect in increasing transcellular conductance. However, there is no activation of active Na⁺ or Cl⁻ “influx”. The presence of T3 significantly elevates Na⁺,K⁺-ATPase activity of the PVCs, but there is no improvement in freshwater performance—that is, the pattern of only slight active Cl⁻ “influx” and Na⁺ “efflux” continues, there is no protective effect on TER or PEG-4000 permeability, and indeed, net ion losses to apical freshwater are actually greater overall. The effects of T3 on DSI epithelia where MRCs are also present have yet to be evaluated.

Prolactin, the traditional “freshwater-adapting hormone” [73,75], has been tested on both SSI and DSI epithelia from trout. Gilmour et al. [45] found no effects on SSI epithelia, but worked at a concentration that was probably too low (0.2 ng ml⁻¹) relative to typical circulating levels in rainbow trout (10–30 ng ml⁻¹ [76]). However, at 10–50 ng ml⁻¹ prolactin applied throughout the period of culture, Kelly and Wood [63] found distinct effects on SSI epithelia, but only when freshwater was present on the apical surface, in accord with the “freshwater-adapting” function of this hormone. These include increased TER and decreased Na⁺ efflux rates, with unchanged PEG-4000 permeability, suggesting a specific effect in reducing transcellular conductance, perhaps by closing ion channels. There is no effect on the Na⁺,K⁺-ATPase activity of the PVCs. Thus, the actions of prolactin under asymmetrical conditions are very different from those of T3 [64]. Prolactin effects are similar in DSI epithelia, but

with the additional response of increased Na⁺,K⁺-ATPase activity (presumably an MRC response), but again there is no activation of Na⁺ influx [63].

Cortisol is widely recognized to play dual roles in euryhaline fish, promoting both adaptation to seawater and adaptation to dilute freshwater [73]. At least in part, the latter action is explicable by promotion of MRC proliferation and increased Na⁺,K⁺-ATPase activity within individual MRCs [73,77,78], but there also appears to be a promotion of H⁺-ATPase activity [71] which is present in both PVCs and MRCs in freshwater trout, but only in PVCs in freshwater tilapia [72]. With this background in mind, we have examined cortisol effects on SSI epithelia (PVCs only) from both trout [62] and tilapia [46], with preliminary studies on trout DSI epithelia (PVCs plus MRCs).

Responses of trout and tilapia SSI epithelia to cortisol are qualitatively identical. When the epithelia are grown in the presence of physiological levels of cortisol (10–1000 ng ml⁻¹), there are dramatic dose-dependent increases in TER and TEP, and accompanying decreases in PEG-4000 permeability, indicative of junctional tightening and decreased paracellular permeability. Unidirectional Na⁺ and Cl⁻ fluxes are actually lower, but there is a clear changeover from active Na⁺ and Cl⁻ “efflux” under symmetrical conditions in the absence of cortisol to active Na⁺ and Cl⁻ “influx” in the presence of cortisol. However, there is no increase in Na⁺,K⁺-ATPase activity. When these epithelia are exposed to apical freshwater, cortisol is unable to activate Na⁺ “influx” from freshwater in preparations from either species (indeed, small active “effluxes” continue) so the pattern of a small active “influx” of Cl⁻ alone persists. Typical changes in TER, TEP, and PEG-4000 permeability are seen, but dose-dependent differences in all parameters still occur—that is, the response to freshwater exposure is more marked in cortisol-supported epithelia. Most importantly, the junctional tightening caused by cortisol persists, so net Na⁺ and Cl⁻ “efflux” rates are greatly reduced, an important adaptation to freshwater which has been overlooked in previous in vivo studies. Indeed, when homologous serum taken from stressed tilapia (endogenous cortisol = 497 ng ml⁻¹) is substituted for the normal FBS during culture of tilapia SSI preparations, the same effects are seen in comparison to preparations cultured with homologous serum from unstressed tilapia (cortisol = 7 ng ml⁻¹) [46].

When cortisol is applied basolaterally to trout SSI epithelia which are already mature, the TER response takes about 48 h to develop, suggesting that cortisol is acting relatively slowly [62]—that is, via genomic receptors [79]. Two types of genomic corticosteroid receptors have now been cloned in fish, the traditional glucocorticoid-type receptor [80], and more recently a mineralocorticoid-type receptor [81]. In tilapia SSI preparations, available evidence strongly suggests that cortisol-stimulated junctional tightening in PVCs is mediated through the former, because the response is duplicated by a glucocorticoid agonist (dexamethasone) and blocked by a glucocorticoid receptor antag-

onist (RU486) [46]. Sloman et al. [82] have reported that the cortisol-mediated proliferation of MRCs which occurs during exposure of trout to ion-poor freshwater is mediated through mineralocorticoid receptors. Therefore, PVCs and MRCs may have different corticosteroid receptor types.

The application of cortisol to trout DSI epithelia has yielded encouraging results (S.P. Kelly and C.M. Wood, unpublished data). These preparations exhibit the same dose-dependent increases in TER and decreases in paracellular permeability as SSI preparations, and the effects are sustained during apical freshwater exposure (Fig. 4). However, DSI epithelia also respond to cortisol with an increase in Na^+, K^+ -ATPase activity, and preliminary observations suggest that they may exhibit active Na^+ and Cl^- uptake in the presence of cortisol (500 ng ml^{-1}) by the flux ratio criterion. Further work is needed to clarify the nature of these responses, but they offer great scope for future development of the cultured epithelium as a model for ion transport in the freshwater gill.

An important area to pursue in this regard is the potential interactive effects of hormones. To date, we have looked for these only in SSI epithelia, and not in any great detail. Nevertheless, cortisol clearly exerts interactive effects on T3 responses, most importantly enhancing the stimulation of Na^+, K^+ -ATPase activity caused by T3 alone, even though cortisol alone is ineffective in this regard [64]. Prolactin exerts a subtle effect on the influence of cortisol on the response to apical freshwater exposure (S.P. Kelly and C.M. Wood, unpublished observations). Initially, when prolactin is combined with cortisol, elevations in TER and reductions in PEG-4000 permeability are no greater than those caused by

cortisol alone, but after 24 h freshwater exposure, the responses are sustained to a greater extent in the additional presence of prolactin, the “freshwater adapting hormone”. We anticipate that interactive effects may prove to be much greater in DSI epithelia where MRCs are also present.

7. Cultured gill epithelia as models for branchial ammonia excretion

Freshwater teleosts excrete more than 80% of their total nitrogenous waste as ammonia, via the gills, but the mechanisms by which this occurs remain highly controversial (see Refs. [83,84] for recent critical reviews). The potential transporter-mediated mechanisms are thought to be linked directly or indirectly to active Na^+ influx, while the others rely on the simple diffusion of NH_3 and/or NH_4^+ along partial pressure and electrochemical gradients, respectively. Much of the uncertainty stems from the fact that is impossible to accurately measure or manipulate the water and blood chemistry defining these gradients *in vivo* because of the complex geometry of the intact gill with respect to blood and water flow. However, these procedures can be performed quite easily with cultured flat epithelial preparations. Therefore, we have employed the trout DSI epithelium to examine the diffusive components of transepithelial ammonia flux, exploiting the fact that in the absence of hormonal support, there is no active Na^+ “influx” [85]. The pH and ammonia concentrations on the two sides of the epithelium were adjusted so as to produce different P_{NH_3} gradients for NH_3 diffusion and electrochemical driving forces for NH_4^+

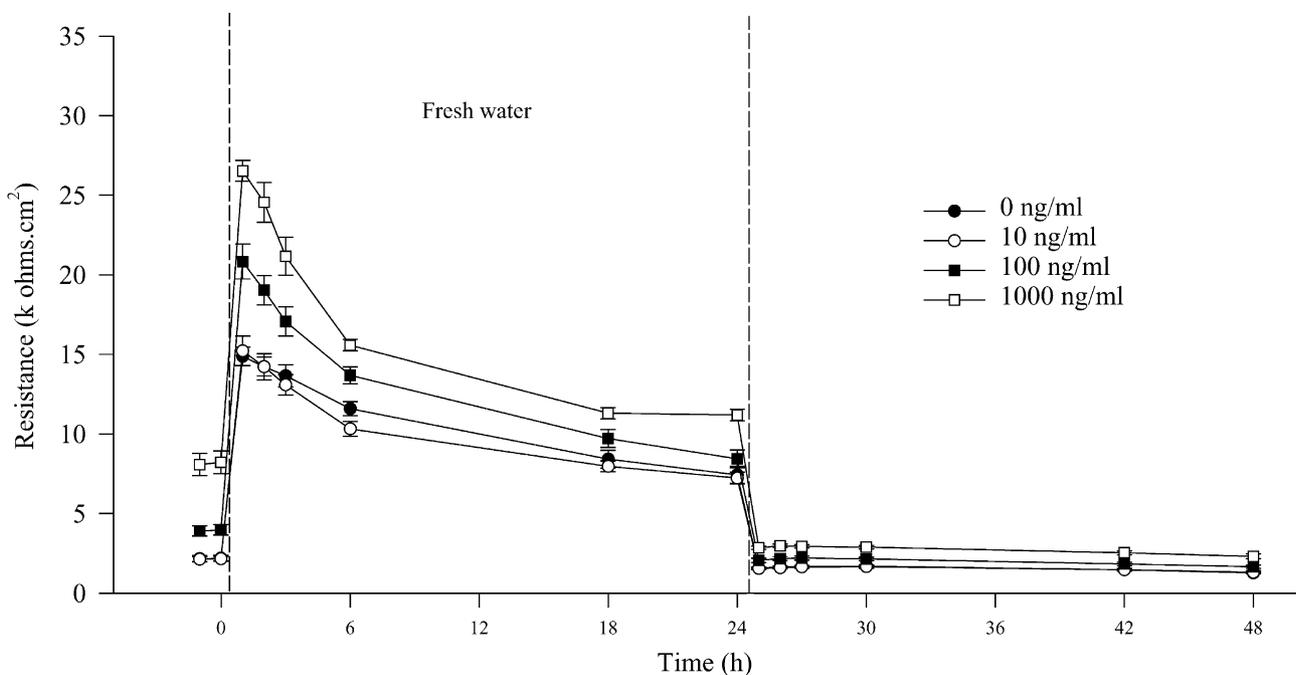


Fig. 4. When DSI epithelia are allowed to develop in the presence of cortisol, there are concentration-dependent elevations of TER, and these differences are sustained during 24 h exposure to apical freshwater (S.P. Kelly and C.M. Wood, unpublished data). Means \pm 1 S.E. ($N=10$).

diffusion under symmetrical and asymmetrical conditions, and the study also exploited the natural range of variability in TER (i.e. conductance) amongst preparations.

The conclusions of this analysis are that NH_3 diffusion clearly occurs across the preparation, total ammonia flux tracking the P_{NH_3} gradient in a linear fashion (Fig. 5). However, ammonia flux is much higher under asymmetrical conditions, a difference correlated with the higher PEG-4000 (paracellular) permeability in the presence of apical freshwater. The slopes of the relationships are the same between symmetrical and asymmetrical conditions, so NH_3 permeability is unchanged. The difference is explained by a significant ammonia flux even at zero P_{NH_3} gradient (i.e. positive intercept to the line). This is due to the diffusion of the charged form (NH_4^+) which occurs only under asymmetrical conditions and which tracks conductance in a linear fashion. NH_3 permeability can be calculated from the slopes of the lines in Fig. 5 and is about $1.5 \times 10^{-4} \text{ cm s}^{-1}$, a fairly typical value for epithelia, whereas NH_4^+ permeability is about an order of magnitude lower. The estimated P_{NH_3} gradient operating from blood-to-water in vivo is only about 65 μTorr and is marked in Fig. 5. Under these conditions, the combined NH_3 and NH_4^+ fluxes would amount to about $26 \text{ nmol cm}^{-2} \text{ h}^{-1}$, or about 20% only of measured in vivo values, with NH_4^+ making the larger contribution. Indeed, if the measured ammonia excretion in vivo occurred entirely by NH_3 diffusion as argued by some authors [86,87], NH_3 permeability of the fish gill would have to be about two orders of magnitude higher than the value of Fig. 5, which seems unlikely.

Overall, the contribution of these studies to clarifying the current controversies [83,84] are threefold. First, NH_4^+ diffusion cannot be dismissed as quantitatively unimportant. Second, while NH_3 diffusion certainly occurs, its quantitative importance may have been greatly overestimated in the past. Finally, diffusion alone provides ammonia flux rates substantially lower than those measured in vivo. These conclusions provide impetus to re-examine potential Na^+ -coupled carrier mechanisms for ammonia excretion in vivo. Furthermore, the question should be revisited in vitro once a cultured gill preparation with normal Na^+ “influx” from freshwater has been developed.

8. Future perspectives

The opportunities for further development and exploitation of cultured epithelial preparations of the freshwater gill are immense. We here briefly list future directions that we think are particularly important.

- (i) Cultured branchial epithelia from a wider range of freshwater teleosts, both stenohaline and euryhaline, should be explored to see if their potential for active transport from apical freshwater is better than in trout or tilapia epithelia.
- (ii) Cultured branchial epithelia have already been used in aquatic toxicology [28,33,34], but with recent legislative moves against live animal testing and in favour of in vitro testing in many jurisdictions, they

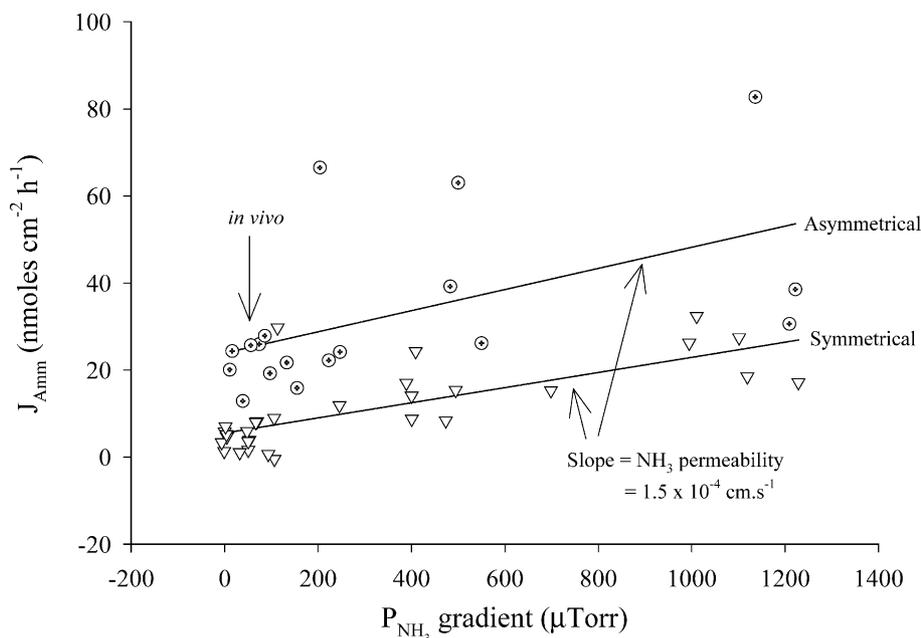


Fig. 5. Ammonia flux (J_{Amm}) from basolateral-to-apical media is linearly related to the corresponding P_{NH_3} gradient in trout DSI preparations, indicating that NH_3 diffusion occurs under both symmetrical ($r=0.74$, $N=31$, $P \leq 0.05$) and asymmetrical conditions ($r=0.53$, $N=18$, $P \leq 0.05$). The slopes of the two lines are identical, representing an NH_3 permeability of about $1.5 \times 10^{-4} \text{ cm s}^{-1}$, but the intercept is significantly higher ($P \leq 0.05$) under asymmetrical conditions (apical freshwater) due to a significant permeability to NH_4^+ . The arrow marks the estimated P_{NH_3} gradient operating in vivo, at which there would be a combined NH_3 and NH_4^+ diffusive flux of only about $26 \text{ nmol cm}^{-2} \text{ h}^{-1}$, or about 20% of measured in vivo values (data from Ref. [85]).

will prove increasingly valuable. This is especially true for toxicants which attack the transport or barrier properties of the gill, and for those, such as metals and many organics, where there is a need to present the toxicant to the *in vitro* system in natural freshwater so as to preserve its speciation. Important future goals will be to develop techniques by which cultured epithelia can be stored and stockpiled for tests on demand, and for new culture methods where epithelia with multiple cell types can be grown from individual fish, thereby duplicating the interindividual variability which is so valuable in whole animal testing.

- (iii) A wider range of hormones (e.g. growth hormone, IGF-1, neurohypophysial peptides, catecholamines), and hormone combinations should be evaluated for their potential to activate normal ion transport in DSI epithelia. These tests may be most profitably done in combination with gradual freshwater exposure regimes to achieve more realistic freshwater exposure conditions.
- (iv) Now that essential trace metals such as copper and zinc are known to be actively taken up through the freshwater gill, with evidence of Cu passing through the Na⁺ pathway [88] and Zn through the Ca²⁺ pathway [89], cultured branchial epithelia may be profitably employed to examine the mechanisms involved.
- (v) Patch-clamp analysis of cultured PVCs on plastic support has already proven to be informative [35,36]. If this can be done in cultured DSI epithelia *in situ*, it offers the prospect of studying apical channel activity of both PVCs and MRCs in relation to the composition of apical and basolateral media (e.g. tonicity, hormonal status).
- (vi) Vibrating probe analysis was the technique which fundamentally proved the localization of active Cl⁻ current to the MRCs in opercular epithelial models for the seawater gill [6,90]. This approach applied to DSI epithelia, especially with modern improvements [91], may help illuminate the respective functions of MRCs and PVCs in the freshwater gill.
- (vii) The increasing availability of cDNA probes and antibodies for epithelial transporters has started to clarify the location of certain processes in the intact freshwater gill (e.g. Refs. [72,92]). These approaches should prove very useful in cultured epithelia.
- (viii) Cell culture systems containing just a few cell types are ideally suited for new genomic and proteomic techniques designed to screen for alterations in expression of thousands of mRNAs and even more proteins [93]. For example, the effects of apical freshwater exposure or basolateral hormonal manipulation in just the PVCs could easily be examined in SSI preparations, and the additional responses contributed by MRCs in DSI preparations.
- (ix) For proteomic analysis, it will be essential to remove bovine proteins (i.e. FBS) from the growth media, hopefully by replacement with homologous fish serum. This has already proven to be possible for sea bass [40] and tilapia [46] preparations, though early trials suggested that frozen trout serum was toxic [16].
- (x) Techniques have recently been developed for separating subtypes of MRCs and PVCs from freshwater trout gills [94]. If these can be used to preferentially seed filter cultures with selected cell types, they offer the possibility of a finer scale analysis in virtually all of the above approaches.

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