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Adaptations to *in situ* feeding: novel nutrient acquisition pathways in an ancient vertebrate

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During feeding, hagfish may immerse themselves in the body cavities of decaying carcasses, encountering high levels of dissolved organic nutrients. We hypothesized that this feeding environment might promote nutrient acquisition by the branchial and epidermal epithelia. The potential for Pacific hagfish, *Eptatretus stoutii*, to absorb amino acids from the environment across the skin and gill was thus investigated. L-alanine and glycine were absorbed via specific transport pathways across both gill and skin surfaces, the first such documentation of direct organic nutrient acquisition in a vertebrate animal. Uptake occurred via distinct mechanisms with respect to concentration dependence, sodium dependence and effects of putative transport inhibitors across each epithelium. Significant differences in the absorbed amino acid distribution between the skin of juveniles and adults were noted. The ability to absorb dissolved organic matter across the skin and gill may be an adaptation to a scavenging lifestyle, allowing hagfish to maximize sporadic opportunities for organic nutrient acquisition. From an evolutionary perspective, hagfish represent a transitory state between the generalized nutrient absorption pathways of aquatic invertebrates and the more specialized digestive systems of aquatic vertebrates.

Keywords: amino acid; absorption; gill; integument; dissolved organic matter; feeding physiology

1. INTRODUCTION

The development of an impermeable integument serves multiple purposes in animals, including facilitation of homeostasis by minimizing exchanges between the internal and external environments. However, some aquatic animals retain permeable body surfaces capable of exchange with the environment. In nutrient-enriched habitats such as estuaries, these surfaces may permit the uptake of dissolved organic matter such as free amino acids. For example, a number of invertebrate phyla (including molluscs [1], annelids [2] and echinoderms [3]) have been documented to assimilate organic nutrients directly across the integument and/or the gill epithelia. These nutrients may be used directly for energy or used as osmolytes to counter osmotic changes induced by fluctuating environmental salinities [4]. In fish, the gill plays an important role in osmoregulation, but organic nutrient assimilation is restricted to the specialized epithelial surfaces of the digestive system [5], as it is in other vertebrates.

Hagfishes are thought to represent the oldest extant connection to the ancestral vertebrate [6], making them of significant interest for examining the evolutionary development of vertebrate traits. They also exhibit a number of characteristics considered to be specializations to their seafloor habitats. Feeding is one of these specializations. Hagfish may feed by burrowing into decaying carcasses and eating their way out from the inside [7]. This potentially exposes gills and skin to a nutrient-rich medium. Consequently, there is the opportunity for hagfish to absorb dissolved organic matter from the medium, similar to the scenario in estuarine invertebrates.

Hagfish are osmoconformers, maintaining a body fluid osmolarity in line with that of the seawater they inhabit. They do, however, use gill ion transporters for acid–base homeostasis [8]. As osmoconformers, hagfish have a reduced need to develop an impermeable skin epithelium to protect against ion and water exchanges. This, coupled with their utilization of a nutrient-rich feeding niche and evidence of organic nutrient acquisition in osmoconforming aquatic invertebrates [1–4], suggests that hagfish may be capable of using the gill and skin epithelia for organic nutrient absorption. This hypothesis was investigated in the Pacific hagfish (*Eptatretus stoutii*) using *in vitro* preparations to determine uptake characteristics of the amino acids L-alanine and glycine. This study showed, for the first time, the capacity for absorption of organic nutrients across a non-gastrointestinal epithelium in a vertebrate animal.

2. MATERIAL AND METHODS

(a) *Animal collection and euthanasia*

Pacific hagfish (*E. stoutii*) were collected in baited traps from Barkley Sound (Vancouver Island, Canada) and transported to flow-through seawater holding facilities at Bamfield Marine Sciences Center (BMSC). Animals were held in large (approx. 5000 l) tarpaulin-covered outdoor tanks, subjected to natural summer lighting and water temperature

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(12°C). Juvenile hagfish were designated as such on the basis of size (<30 g) and the presence of translucent skin. Hagfish were not fed following collection, and were euthanized in 3-aminobenzoic acid ethylester (MS-222, Sigma; 2 g l^{-1}) for collection of tissue for *in vitro* preparations.

(b) *Gill perfusion*

Eptatretus gills occur as rows of 10–13 paired pouches running laterally along the pharynx. In the gill pouch, radial water channels run countercurrent to blood-filled lamellae, providing a favourable surface for exchange of constituents (such as ions, gases, nutrients and waste products) between the blood and water [9]. All trials were performed on medial gill pouches after initial experiments demonstrated that L-alanine uptake rates were equivalent in anterior, medial and posterior pouches. Absorption was monitored using tritium-labelled L-alanine and glycine.

The gill perfusion protocol was modified from Forster & Fenwick [10]. Afferent and efferent water ducts from medial gill pouches were cannulated with PE50 cannula (Intramedic, Clay Adams), and secured in place with surgical silk. Initial trials with food colouring dissolved in hagfish ringer (see composition below) were used to test the integrity of the preparation and to demonstrate perfusion of the radial branchial water channels. The afferent cannula was connected to a peristaltic pump, and test solutions (see below) were perfused through each gill pouch at a mean rate of 5.9 ± 1.1 ml h^{-1}, for 3 h. Flow rate was determined by weighing efferent perfusates, collected over 30 min intervals. These flow rates are approximately 10 per cent of those expected in living hagfish [10], but were necessarily low to avoid cannula clogging, pouch swelling and the potential development of artefact transport pathways. Throughout perfusion, gills were immersed in 5 ml of aerated filtered seawater. The perfusion methodology is represented graphically in electronic supplementary material, figure S1.

For calculation of uptake, the initial perfusate fraction was discarded owing to the presence of non-labelled perfusate, which led to dilution of the isotope signal. Thereafter, the disappearance of isotope from each 30 min fraction was determined based on the difference in counts between afferent and efferent solutions. The uptake in each of the final five perfusate samples, representing the final 2.5 h of perfusion, was averaged, divided by gill wet weight and converted to an hourly rate, resulting in units of nanomoles per gram per hour.

(c) *Skin flux*

Amino acid flux across hagfish skin was tested using a modified Ussing-type chamber (see electronic supplementary material, figure S1). For adult skin, the flux chamber consisted of a 15 ml plastic scintillation vial, with an aperture cut out of the screw-top lid, such that the skin could be stretched across the aperture and secured in place by the lid. Ten millilitres of hagfish ringer was injected into the vial through a sample port at the base of the vial. Through a separate aperture at the base, a 16-gauge needle was inserted, which was connected to an airline for aeration. The skin chamber was then inverted and immersed in the radiolabelled solution. For juvenile preparations, the skin was stretched across a 0.5 ml centrifuge tube, with an aperture cut in the lid, such that the skin could be snap-locked into place. This chamber was similarly aerated, inverted and immersed in the radiolabelled solution. Preparations were shown to exclude food colouring dye placed in the external solution for at least 12 h, confirming integrity. Uptake was determined over 3 h, and expressed per unit surface area exposed (nmol cm^{-2} h^{-1}). Following the assay, skin was removed from the chambers and rinsed in isotope displacement solution (100 mM glycine, 100 mM L-alanine in filtered seawater) to remove any adsorbed isotope. Accumulation in the skin tissue and the serosal compartments was considered to represent absorbed amino acid. For all skin assays reported here skin was dissected from the medial dorsal region lateral to the gill openings.

(d) *Solutions*

Filtered seawater (in millimolar: Na^+, 492; K^+, 9; Ca^{2+}, 12; Mg^{2+}, 50; Cl^-, 539; pH 8.0) was used as the radiolabelled external medium, except for tests of sodium-dependence where sodium-free hagfish ringer (in millimolar: $C_5H_{14}NOCl$, 474; KCl, 8; $CaCl_2 \cdot 2H_2O$, 5; $MgSO_4 \cdot 7H_2O$, 3; $MgCl_2 \cdot 6H_2O$, 9; $KH_2PO_4 \cdot H_2O$, 2.06; $KHCO_3$, 41; glucose, 5; pH 7.6) was employed. Hagfish ringer containing sodium (in millimolar: NaCl, 474; KCl, 8; $CaCl_2 \cdot 2H_2O$, 5; $MgSO_4 \cdot 7H_2O$, 3; $MgCl_2 \cdot 6H_2O$, 9; $NaH_2PO_4 \cdot H_2O$, 2.06; $NaHCO_3$, 41; glucose, 5; pH 7.6 [10]) was used as the control for these experiments, to account for any differences in uptake between natural seawater and ringer. Hagfish ringer was used as the serosal medium in skin uptake studies.

For analysis of concentration dependence, amino acid levels of 10, 50, 100, 250, 500 and 1000 μM were used for both L-alanine and glycine. Radiolabel was added at a level of 1 μC_i ml^{-1} for ^{3}H-glycine (PerkinElmer, Boston, MA, USA) and 5 μC_i ml^{-1} for ^{3}H-L-alanine (PerkinElmer). Uptake of L-alanine (100 μM) and glycine (10 μM gill; 100 μM skin) was tested in the presence of tenfold excess levels of putative transport modifiers D-alanine and glycine (for L-alanine uptake assays) and L-alanine (for glycine uptake assays) to determine specificity of transport pathways.

(e) *Tissue digestion and radioisotope analysis*

Adult skin samples were digested in 5 ml 1 N HNO_3 (60°C, 48 h) before the addition of UltimaGold liquid scintillant (PerkinElmer). Juvenile skin was treated in a similar manner with a 1 ml acid digest. For gill efferent perfusates, a 1 ml aliquot was taken for each 30 min perfusate sample and added to 4 ml of ACS liquid scintillant (GE Healthcare, Buckinghamshire, UK). All samples were held in the dark for 12 h before counting (LS6500; Beckman Coulter, Fullerton, CA, USA). Automatic quench correction was performed based on the external standards ratio.

(f) *Skin histology*

Dorsal skin samples (1 × 1 cm, lateral to medial gill apertures) were taken from adult (130–175 g) and juvenile (11–14 g) hagfish for histological examination of skin thickness. Excised skin was fixed in neutral buffered formalin, dehydrated in an ethanol series and embedded in celloidin and Ralwax, before 7 μm sections were cut using a Leica rotary microtome. Sections were stained in double-strength Mayer's haemalum and eosin, and mounted in Eukitt.

(g) *Data analysis and statistics*

Concentration dependence data were assessed using SigmaPlot (v. 11.0; SPSS). All data (see §3; figure 1 and table 1)

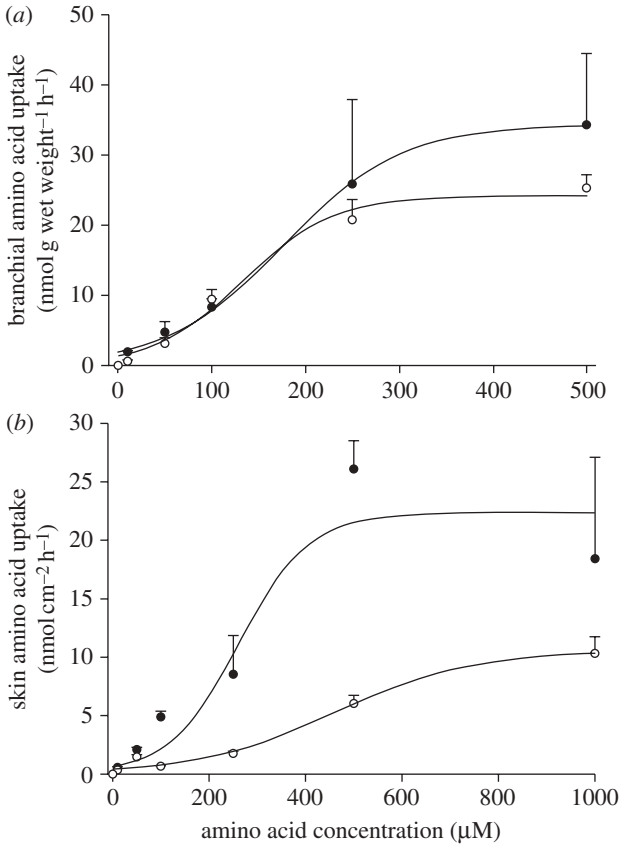

Figure 1. Concentration-dependent ^{3}H-L-alanine (black circles) and ^{3}H-glycine (white circles) uptake in (*a*) gill (nmol g^{-1} h^{-1}) and (*b*) skin (nmol cm^{-2} h^{-1}). Branchial uptake was determined via a gill perfusion method, while skin uptake was determined using a modified Ussing chamber (see §2). Values represent the mean ± s.e.m. of five to six observations. Fitted lines were modelled on raw data using SigmaPlot.

best fitted a sigmoidal distribution:

$$y = \frac{a}{1 + e^{-(x - x_0/b)}}.$$

Differences between uptake profiles were tested by comparing parameters of kinetic curves using *t*-tests, and in lieu of a multiple comparison adjustment a conservative degree of freedom assessment based on tested concentrations (rather than individual measurements) was applied [11]. One-way analysis of variance (ANOVA) followed by LSD *post hoc* tests or Student *t*-tests were used to determine other significant differences at an α level of 0.05. For percentage data, arcsine transformation was performed prior to analysis.

3. RESULTS

The amino acids ^{3}H-glycine and ^{3}H-L-alanine were absorbed by both the gill and skin of the Pacific hagfish. Uptake profiles across each of these epithelia did not conform to typical hyperbolic concentration-dependent Michaelis–Menten kinetics, but instead best fitted sigmoidal distribution curves (figure 1).

There was a large linear increase in uptake at glycine and L-alanine concentrations greater than 500 μM for gill preparations (not shown), indicative of a diffusive pathway of uptake. The parameters (see equation in §2) derived from the curve-fitting are displayed in table 1. The curves for glycine and L-alanine uptake across the gill were statistically indistinguishable from each other. Branchial uptake of glycine and L-alanine was sodium-dependent, with both showing a significantly reduced uptake in response to sodium substitution in the external medium (figure 2*a*). In the presence of tenfold excesses of D-alanine and glycine, L-alanine uptake was stimulated more than fourfold relative to L-alanine alone. Similarly, glycine uptake was also stimulated by tenfold excess of L-alanine, although the magnitude of this effect was greater (close to 25-fold stimulation).

Table 1. Parameters derived from sigmoidal curve-fitting of concentration-dependent L-alanine and glycine uptake across Pacific hagfish gill and skin. '*a*' denotes uptake rate range of curve (nmol g^{-1} h^{-1} for gill; nmol cm^{-2} h^{-1} for skin; note that owing to different units, statistical comparison between gill and skin was not possible for this parameter); '*b*' denotes slope at transition point; and x_0 denotes concentration of amino acid at the transition point (μM). See §2 for sigmoidal equation and statistical analysis.

treatment	*a*	*b*	x_0
gill–alanine	172 ± 34	63 ± 30	177 ± 55
gill–glycine	118 ± 7	42 ± 12[b]	125 ± 17[b]
adult skin–alanine	22 ± 3[a,c]	73 ± 46	262 ± 55[a]
adult skin–glycine	11 ± 1[a]	150 ± 30[b]	465 ± 44[a,b]
juvenile skin–alanine	47 ± 5[c]	71 ± 40	236 ± 49

[a]Significantly different ($p < 0.05$) compared with same parameter for the other amino acid in the same tissue.
[b]Significantly different ($p < 0.05$) compared with same parameter for the same amino acid in the other tissue.
[c]Significantly different ($p < 0.05$) between adult and juvenile.

Absorption profiles of glycine and L-alanine across the skin were largely distinct from each other, and also from the uptake parameters calculated for the gill. The kinetic values of juvenile skin compared with adult skin were similar (table 1), but the compartmental distribution of absorbed amino acid differed significantly. In adults, the majority (usually greater than 99%) of amino acid accumulated in the serosal compartment. Conversely, the majority (69–86%) of radiolabel accumulated in the skin tissue in juvenile hagfish. This effect was present at all tested amino acid concentrations. Histological examination discerned that the epidermis (86 ± 9 versus 159 ± 16 μm) and the skin as a whole (epidermis, dermis and subdermis; 308 ± 15 versus 793 ± 116 μm) were significantly thinner in juvenile hagfish than in adults (see electronic supplementary material, figure S2).

The effects of putative transport modifiers on skin amino acid uptake were quite distinct from those observed in the gill (figure 2*b*). Here, there was no significant effect of sodium on L-alanine, although sodium-free media increased glycine uptake. Excess glycine had no impact on L-alanine uptake, and *vice versa*. D-alanine at 1 mM significantly inhibited the absorption of 100 μM L-alanine with an uptake of 4.9 ± 0.5 nmol cm^{-2} h^{-1} dropping by 47 per cent to 2.6 ± 0.2 nmol cm^{-2} h^{-1}.

4. DISCUSSION

The current study discerned novel pathways of nutrient acquisition in an ancient vertebrate, with hagfish shown

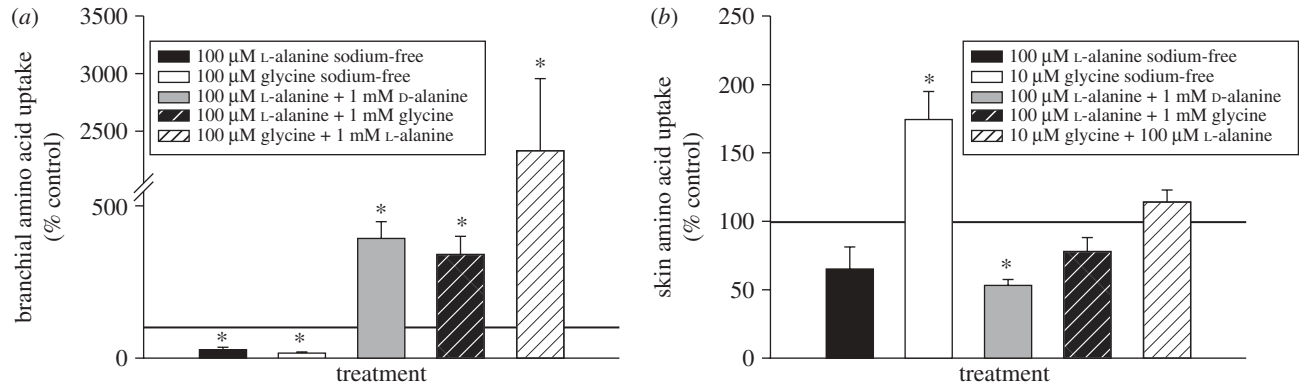

Figure 2. Effect of external medium manipulation on (*a*) branchial and (*b*) skin uptake of ^{3}H-L-alanine and ^{3}H-glycine. The horizontal black bar represents the control level of amino acid uptake. Sodium-free preparations (white bars) were conducted in sodium-free hagfish ringer and compared with normal hagfish ringer as controls, while the control for the other treatments was filtered seawater containing the level of test amino acid in the absence of putative uptake modifiers (see §2). Branchial uptake was determined via a gill perfusion method, while skin uptake was determined using a modified Ussing chamber (see §2). Plotted data represent the mean $\pm$ s.e.m. of five or six replicates. Significant differences (*) in uptake between control and test assays were assessed at the $\alpha = 0.05$ level using one-way ANOVA followed by an LSD *post hoc* assessment. Statistical analysis was performed on the raw data.

to be capable of absorbing amino acids directly from the aqueous medium via both the skin and the gill. The mechanisms of uptake appeared to differ between these two tissues, and qualitatively skin absorption also differed significantly between adult and juvenile animals. Gill and skin amino acid uptake may be a mechanism designed to maximize nutrient acquisition and represent an adaptation to a scavenging lifestyle.

(a) *Gill and skin amino acid uptake*

Branchial uptake pathways for glycine and L-alanine appeared to be shared. These amino acids are physicochemically and structurally similar. They possess similar isoelectric points (6.07 for glycine, 6.11 for alanine) and R groups (H for glycine, CH_3 for alanine), and these properties probably explain their capacity to share transport mechanisms. For example, both substrates exhibited sodium dependence (indicative of sodium co-transport), mutually impacted the other's absorption and displayed statistically indistinguishable kinetic uptake profiles. Co-transport with sodium is advantageous as animal cells maintain intracellular sodium at low levels to minimize perturbing effects on cellular components [12]. This ensures that sodium influx into the cell is favoured and allows amino acids to use this electrochemical gradient to traverse the cell membrane.

Glycine and L-alanine appeared to be largely absorbed through pathways across the skin that were distinct, both from each other and from those used in the gill. Glycine uptake, but not that of L-alanine, was sodium-dependent, although in this case removal of sodium from the assay medium caused an increase in uptake, as opposed to the decrease observed in the gill. This is probably an example of allosteric modulation (see discussion below). In contrast to the gill, excess levels of the other amino acids had no significant effect on uptake of the amino acid alone.

Taken together, sodium dependence, nonlinear uptake kinetics and the modification of uptake by other amino acids strongly suggest that amino acid uptake in hagfish gill and skin occurs via specific transport pathways, rather than solely by diffusion. The exception was at high amino acid levels (>500 µM) in the gill, where diffusive uptake dominated. In mammals, sodium-dependent (e.g. system A) and sodium-independent (e.g. system asc) transporters of both L-alanine and glycine are present [13]. There are also systems that recognize only one of these substrates (e.g. system Gly for glycine, system B^0 for L-alanine). Evidence also exists for both shared and distinct pathways for L-alanine and glycine absorption in the gut of teleost fish [14].

Uptake via a specific transport pathway usually yields a hyperbolic saturation curve characteristic of Michaelis–Menten kinetics. Multiple pathways with distinct kinetic properties will instead generate more complex curves [15], such as the sigmoidal relationships seen in the present study (figure 1). Further physiological and molecular characterization is required to identify the specific amino acid transport systems present in hagfish gill and skin.

Branchial uptake of L-alanine and glycine followed sigmoidal kinetics, but evidence suggested a single, shared pathway. This may indicate the presence of an allosteric transporter. The activities of such transporters are modified by the presence of allosteric ligands, which bind to the transporter and alter the relationship between substrate concentration and uptake rate. The result is a sigmoidal shape to the kinetic curve, rather than the characteristic hyperbolic shape of a simple Michaelis–Menten kinetic relationship (e.g. [16]). Another piece of evidence supports the hypothesis that an allosteric transporter may be responsible for glycine and L-alanine uptake in the hagfish gill. Homotrophic activation is a phenomenon whereby the presence of one transport substrate significantly stimulates the uptake of the substrate of interest. The large stimulations in branchial glycine uptake in the presence of L-alanine, and *vice versa*, may be evidence of a homotrophic activation effect (figure 2*a*). This effect has been observed previously in studies of piscine amino acid transport [17].

It is difficult to compare the quantitative importance of branchial and skin uptake owing to the complex kinetics

and the distinct mechanisms of determining uptake. However, skin uptake rates at a substrate concentration of 500 μM were 6.0 ± 0.7 and 26 ± 2 nmol cm^{-2} h^{-1} for glycine and L-alanine, respectively. Intestinal uptake for the same substrates at the same concentrations were 3.6 and 6 nmol cm^{-2} h^{-1} for glycine and L-alanine, respectively (C. N. Glover, C. Bucking & C. M. Wood 2010, unpublished data), rates equivalent to or less than those of the skin. Given the large surface area of the skin it is clear that direct assimilation from the ambient medium could play a significant role in organic nutrient acquisition.

(b) *Adult versus juvenile animals*

Quantitatively, the acquisition of L-alanine across the skin of juvenile hagfish was similar to adults, despite the much reduced epidermal thickness. Qualitatively, however, the majority of L-alanine accumulated in the skin of juveniles and in the serosal compartment of adults. Morphologically, adult hagfish skin is characterized as being very well vascularized [18]. The capacity of hagfish for cutaneous gas exchange has been subject to much speculation. While some estimates suggest that the skin may contribute as much as 80 per cent of total oxygen uptake [19], it is now considered that this is unlikely given the skin thickness and high dermal capillary oxygen tensions [9]. Instead, the significant blood supply to the skin is believed to be a consequence of the large synthetic requirements associated with slime production [20]. Reasons for the differences in short-term fate of transported amino acids (into skin of juveniles and serosal compartment of adults) require further investigation, but may relate to different metabolic demands between juvenile and adult hagfish.

(c) *Environmental and evolutionary context*

Many invertebrate animals absorb amino acids across external epithelia, specifically for roles in balancing intracellular osmolarity in response to changing environmental salinity [12]. Hagfish are stenohaline, and have little need to modify intracellular osmolarity. However, hagfish immersion in decaying carrion while feeding provides an opportunity for absorbing organic nutrients directly from the ambient medium. Hagfish are opportunistic feeders, and can survive several months between meals [21]—an attribute potentially due, in part, to their low metabolic rates [22]. It is important for hagfish to maximize the opportunity for nutrient acquisition when food is available. This appears to have been achieved, in part, by the capacity to absorb dissolved organic nutrients across their gill and skin, an ability not previously described in a vertebrate animal.

The phylogenetic placement of hagfish suggests that early aquatic vertebrates may have employed a digestive strategy whereby nutrient transporters were broadly distributed on epithelial surfaces, similar to the scenario in aquatic invertebrates. Coupled with the development of osmoregulatory strategies and the need for a reduced permeability for ions and water, a more specialized digestive system may have developed. This is suggestive of an evolutionary trade-off between nutrient absorption and the need to limit exchanges across permeable surfaces.

All procedures were approved by the BMSC animal ethics committee.

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