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ORIGINAL PAPER

# Characterisation of L-alanine and glycine absorption across the gut of an ancient vertebrate

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**Abstract** This study utilised an in vitro technique to characterise absorption of two amino acids across the intestinal epithelium of Pacific hagfish, *Eptatretus stoutii*. Uptake of L-alanine and glycine conformed to Michaelis–Menten kinetics. An uptake affinity ($K_m$; substrate concentration required to attain a 50% uptake saturation) of 7.0 mM and an uptake capacity ($J_{max}$) of 83 nmol $cm^{-2}$ $h^{-1}$ were described for L-alanine. The $K_m$ and $J_{max}$ for glycine were 2.2 mM and 11.9 nmol $cm^{-2}$ $h^{-1}$, respectively. Evidence suggested that the pathways of L-alanine and glycine absorption were shared, and sodium dependent. Further analysis indicated that glycine uptake was independent of luminal pH and proline, but a component of uptake was significantly impaired by 100-fold excesses of threonine or asparagine. The presence of a short-term (24 h) exposure to waterborne glycine, similar in nature to that which may be expected to occur when feeding inside an animal carcass, had no significant impact on gastrointestinal glycine uptake. This may indicate a lack of cross talk between absorptive epithelia. These results are the first published data to describe gastrointestinal uptake of an organic nutrient in the oldest extant vertebrate and may provide potential insight into the evolution of nutrient transport systems.

**Keywords** Amino acid · Glycine · Alanine · Absorption · Hagfish · Nutrient transport

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## Introduction

Hagfish are a model organism of considerable utility for the study of nutrient transport. As the oldest extant vertebrate (Bardack 1998), hagfish offer the opportunity to examine the evolution of nutrient transport pathways. Furthermore, hagfish are unique among vertebrates in that they are capable of absorbing dissolved organic nutrients across multiple epithelia: the gastrointestinal tract, the epidermis and the gill (Glover et al. 2011). The latter two surfaces may be exposed to elevated levels of dissolved nutrients via a feeding behaviour that involves immersion of the hagfish within the body cavities of dead and dying animals that fall to the sea floor (Martini 1998). Hagfish therefore represent a system permitting the examination of functional diversification and plasticity between epithelial surfaces with multiple and overlapping roles in homoeostasis. However, to date there are no published reports detailing nutrient transport characteristics in hagfish gut.

The digestive system of hagfish is considered primitive. They have no stomach, and the intestine is a relatively simple tube, with no gross morphological indications of

regional differentiation. A number of key physiological roles are located in the gut of hagfish, including haematopoietic (Fänge 1998), immunological (Uzzell et al. 2003) and endocrine (Thorndyke and Falkmer 1998) functions. The presence of a columnar epithelium (Barrington 1945), proteolytic enzymes (Nilsson and Fänge 1970) and lipase (Vigna and Gorbman 1979) are indicative of a role in nutrient absorption.

Coupled with the lack of research specifically on the hagfish gut, very little is known regarding epithelial transport in hagfish. Although these animals are osmoconformers, they do appear to have a rich assortment of ion transporters, thought to function primarily in acid–base homoeostasis (Claiborne et al. 2002). This also suggests the capacity to utilise ion gradients to drive nutrient transport processes, as has been described for glucose uptake in the hagfish renal epithelium (Floge et al. 1984). Recent evidence has shown that both the gill and skin are capable of absorbing amino acids directly from the water. In gill, the bulk of glycine and L-alanine uptake occurred via a shared, sodium-dependent transporter, while the transport of these amino acids across the skin appeared to occur primarily through distinct pathways (Glover et al. 2011).

This study sought to investigate nutrient absorption across the intestinal epithelium in hagfish for the first time. To allow direct comparison with previous studies on nutrient absorption across the skin and gill (Bucking et al. 2011; Glover et al. 2011), L-alanine and glycine were selected as substrates for examination of intestinal nutrient transport in the gut of the Pacific hagfish (*E. stoutii*).

## Materials and methods

### Animals

Pacific hagfish (*E. stoutii*; Lockington) were collected from Barkley Sound (Vancouver Island, Canada) using baited traps. Animals were maintained at Bamfield Marine Sciences Centre (BMSC) in 5,000 L tarpaulin-covered outdoor tanks receiving flow-through seawater, and subjected to natural summer lighting and water temperature (12°C). Hagfish were not fed following collection and were left for at least a week prior to experimentation. All procedures were approved by the BMSC animal ethics committee.

### Gut sac technique

Following euthanasia (3-aminobenzoic acid ethylester (MS-222); Sigma; 2 g $L^{-1}$), a lateral incision was made exposing the gastrointestinal tract. The entire tract was removed and gently flushed with hagfish Ringer (see composition below). Three 2–5 cm sections were cut from each intestine and randomly assigned to different treatments. This ensured that all data comprising a treatment mean were sourced from distinct individuals, thus avoiding pseudoreplication. Final *n* values of 6 or 7 were achieved for all manipulations (see figure captions for specific *n* values). Analysis showed that there was no regional differentiation of amino acid uptake (see "Results") validating this approach. Each section was formed into a gut sac, following a method applied previously in marine teleosts (Glover et al. 2003). Briefly, one end of the sac was sealed with suture silk, and into the other end a short flared length (~5 cm) of cannula (PE50; Intramedic; Clay-Adams, Parsippany, NJ, USA) was secured in place with more silk. Filtered seawater containing radiolabelled amino acid (see below) was introduced into the sac via the cannula. The sac was filled until firm but pliant to the touch (0.6–2.8 mL, depending on size). The cannula was then heat sealed and the gut sac immersed in 15 mL of aerated hagfish Ringer ('serosal' compartment). After 2 h, the gut sac was removed, drained and split open laterally. In similar teleost fish gut sac preparations, inorganic nutrient absorption was in the linear range at this time point, thus permitting calculation of initial uptake rate kinetics (Nadella et al. 2006b). The mucosal surface of the sac was flushed for 30 s with a cold displacement solution (1 M glycine, 1 M L-alanine in filtered seawater) to displace adsorbed isotope. The mucosal surface was scraped with a glass microscope slide, and a simplified determination of gut surface area made assuming the gut to be a straight tube (Glover et al. 2003). Radiolabel accumulating in the gut tissue and the serosal solution was considered to be absorbed (Nadella et al. 2006b).

### Gut sac solutions and manipulations

Mucosal gut sac solutions consisted of filtered seawater (in mM, $Na^+$, 492; $K^+$, 9; $Ca^{2+}$, 12; $Mg^{2+}$, 50; $Cl^-$, 539; pH 8.0). The exception was testing the sodium dependence of amino acid uptake, where sodium-free hagfish Ringer (in mM, $C_5H_{14}NOCl$ (choline chloride), 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 used as the mucosal solution in an experiment which compared this treatment to gut sacs with otherwise identical mucosal hagfish Ringer containing sodium (in mM, 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; Forster and Fenwick 1994). Hagfish Ringer (with sodium) was used as the serosal solution in all gut sac manipulations.

For investigation of concentration-dependent amino acid uptake, the following levels of L-alanine or glycine were tested: 0.5, 1, 2.5, 5 and 10 mM. Radiolabel was added to

mucosal solutions at levels of 5 $\mu C_i\ mL^{-1}$ for [$^3$H]-L-alanine (Perkin-Elmer, Boston, USA) and 1 $\mu C_i\ mL^{-1}$ for [$^3$H]-glycine (Perkin-Elmer, Boston, USA). To characterise the uptake pathway, the effects of putative transport inhibitors were tested. These solutions contained 0.5 mM of the tested $^3$H-labelled amino acid and 100-fold higher levels of D-alanine or glycine (for [$^3$H]-L-alanine treatments) or L-alanine (for [$^3$H]-glycine treatments). [$^3$H]-glycine uptake (at 500 μM in the mucosal solution) was investigated further in separate experiments utilising 100-fold excess levels (50 mM) of amino acids (proline, asparagine or threonine) known to share uptake pathways with glycine in mammalian systems (Bröer 2008). The impact of lowered pH on glycine uptake was also tested, with hagfish Ringer pH dropped from pH 7.6 to 6.0 using dilute hydrochloric acid. Preliminary samples from two animals indicated that this is the approximate gut fluid pH of unfed hagfish.

### Effect of waterborne glycine on intestinal glycine uptake

Individual hagfish (2-L aerated glass containers) were exposed to glycine-spiked filtered seawater to give a final glycine concentration of 1 mM. After 24 h, the animals were removed, euthanised and gut sacs prepared as described previously. Uptake was assessed at two glycine concentrations in the assay medium: 500μM and 10 mM. Results were compared to hagfish treated in an identical manner, but without the addition of waterborne glycine.

### Tissue digestion and radioisotope analysis

Gut tissue samples were digested in 1 N $HNO_3$ (60°C, 48 h) before the addition of UltimaGold liquid scintillant (Perkin-Elmer, Boston, MA, USA). To aqueous samples, ACS (GE Healthcare, Buckinghamshire, UK) liquid scintillant was added. Following scintillant addition, 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.

### Statistical analysis

Concentration-dependent uptake kinetics of amino acids were analysed using SigmaPlot (ver. 11.2; Systat Software, Chicago, IL, USA). Data were fitted to a two-parameter hyperbolic model, consistent with Michaelis–Menten kinetics. Tests of statistical significance were performed using one-way ANOVA followed by post hoc LSD analysis where appropriate. Percentage data were arcsine transformed prior to analysis.

## Results

Statistical analysis was unable to discern any differences between nutrient transport characteristics on the basis of gut region. Sacs sourced from the posterior intestine displayed uptake rates equivalent to 85 ± 10% (glycine) and 88 ± 15% (L-alanine) of those measured in the anterior gut sacs. All subsequent analyses treated data as being independent of the section of the gut from which the sac was sourced.

L-alanine and glycine both demonstrated concentration-dependent uptake patterns across *E. stoutii* gut sacs. Uptake was best characterised by hyperbolic Michaelis–Menten kinetics (Fig. 1). For L-alanine, the maximal rate of uptake ($J_{max}$) was 83 ± 20 nmol $cm^{-2}\ h^{-1}$, and the L-alanine concentration required to give half this maximal rate ($K_m$) was 7.0 ± 3.2 mM. The corresponding values for glycine were 11.9 ± 2 nmol $cm^{-2}\ h^{-1}$ and 2.2 ± 1.0 mM, respectively.

The uptake of both amino acids was largely sodium dependent (Fig. 2). Replacement of sodium in the mucosal Ringer with choline and potassium reduced L-alanine uptake at 1 mM to 36% of its value in the presence of sodium. Similarly, glycine uptake at 1 mM was reduced to half its value in sodium-free Ringer relative to sodium-containing Ringer.

The addition of D-alanine at levels 100-fold in excess of L-alanine in the mucosal medium significantly inhibited L-alanine uptake to 43% of that in the absence of D-alanine (Fig. 3a). The effect of 100-fold excess of glycine on L-alanine absorption was even greater with uptake dropping to 30%. The reciprocal effect (100-fold excess L-alanine on glycine absorption) also resulted in a significant reduction

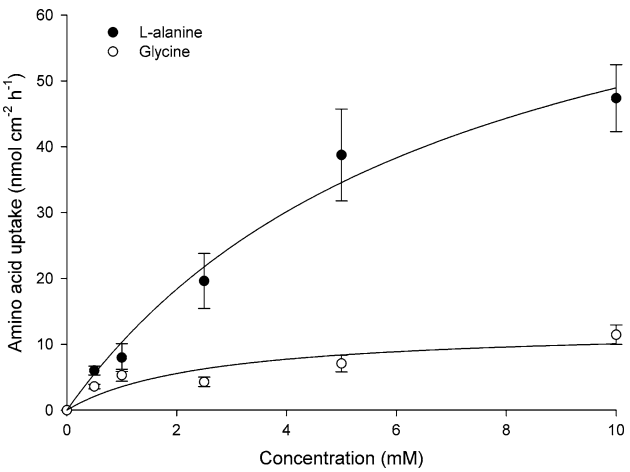


**Fig. 1** Concentration dependence of L-alanine (*black*) and glycine (*white*) uptake (nmol $cm^{-2}\ h^{-1}$) across intestinal gut sacs of hagfish. *Plotted values* represent the mean ± SEM of six preparations. Curves were fitted using SigmaPlot (ver. 11.2; Systat)

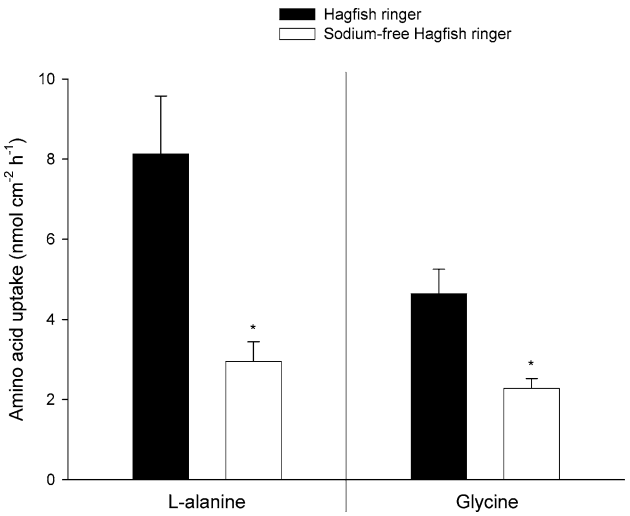


**Fig. 2** Sodium dependence of L-alanine and glycine uptake (at 1 mM; nmol $cm^{-2}$ $h^{-1}$) across intestinal gut sacs of hagfish. *Plotted values* represent the mean ± SEM of six preparations. Statistically significant differences (*) between hagfish Ringer containing sodium and sodium-free hagfish Ringer were determined by one-way ANOVA at an α-level of 0.05

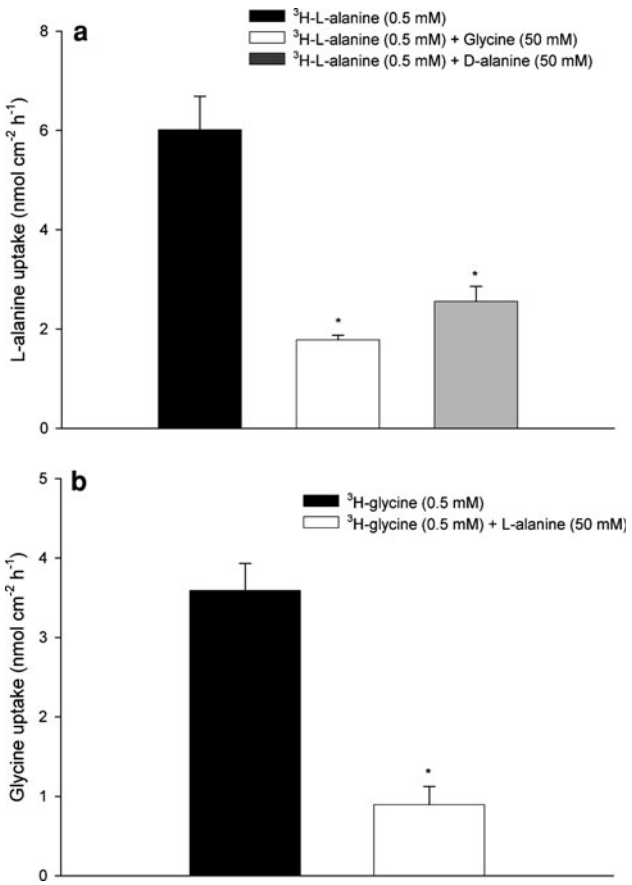


**Fig. 3** Effect of putative amino acid uptake inhibitors on L-alanine (**a**) and glycine (**b**) uptake (nmol $cm^{-2}$ $h^{-1}$) across intestinal gut sacs of hagfish. Levels of $^{3}$H-labelled amino acids were 0.5 mM, with putative inhibitors added at 50 mM. *Plotted values* represent the mean ± SEM of six preparations. Statistically significant differences between test amino acid alone and treatments with putative inhibitors (*) were determined by one-way ANOVA, followed by a post hoc LSD test where appropriate at an α-level of 0.05

in uptake compared to inhibitor-free controls, with a 75% decrease in glycine uptake recorded (Fig. 3b).

One hundredfold excess levels of proline had no impact on glycine uptake (Fig. 4), while the lowering of saline pH from 7.6 to 6.0 also had no effect. Conversely the addition of threonine significantly inhibited glycine uptake with values dropping from 0.92 ± 0.25 to 0.31 ± 0.07 nmol $cm^{-2}$ $h^{-1}$. Asparagine also significantly reduced glycine uptake from the control level to 0.34 ± 0.11 nmol $cm^{-2}$ $h^{-1}$.

To assess whether gut amino acid uptake could be modified by the presence of waterborne amino acids, hagfish were exposed for 24 h to waterborne glycine (1 mM). Intestinal sacs removed from these exposed animals showed glycine uptake characteristics identical to those obtained from guts of time-matched control animals (Fig. 5).

## Discussion

### Concentration-dependent kinetics of amino acid uptake

The absorption of both glycine and L-alanine across the hagfish gut conformed to Michaelis–Menten kinetics. Absorption was sodium dependent and inhibited by putative uptake competitors. Similar findings have been previously described in marine teleost fish (Balocco et al. 1993; Storelli and Verri 1993; Bogé et al. 2002), although a key difference also exists. In general, teleost fish exhibit affinity constants that are lower than those described here for hagfish (L-alanine $K_m$ = 7.0 mM; glycine $K_m$ = 2.2 mM), representing a higher affinity for amino acid absorption. For example, using seawater eel membrane vesicle preparations with an inside-negative membrane potential, Storelli and Verri (1993) reported uptake affinities in the range of 0.12–0.59 mM for naturally occurring amino acids. Organic nutrient transport affinities for teleost fish are also generally higher than those for mammals (Bakke et al. 2011). In this respect, intestinal nutrient transport in hagfish resembles that of hagfish erythrocytes with properties more closely aligned to mammals than other fish (Young et al. 1994).

The higher affinity (lower $K_m$) of teleost fish transport is believed to be partly a compensation offsetting the lower inherent transport rates (a "$Q_{10}$" effect related to the lower environmental temperatures of poikilotherms) and the reduced surface area of fish intestinal tracts (Karasov et al. 1985). Higher affinities may not fully compensate for the relatively reduced uptake capacity of fish intestine, but are

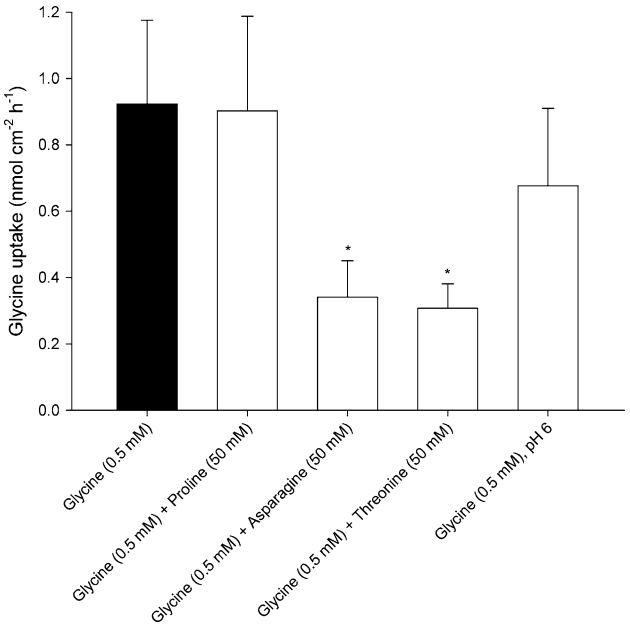


**Fig. 4** Effect of proline, asparagine, threonine and reduced pH on glycine uptake (nmol $cm^{-2}$ $h^{-1}$) across intestinal gut sacs of hagfish. Glycine was present at a concentration of 0.5 mM, with other amino acids added at 50 mM. *Plotted values* represent the mean ± SEM of seven preparations. Statistically significant differences between test amino acid alone and treatments with putative inhibitors (*) were determined by one-way ANOVA, followed by a post hoc LSD test where appropriate at an α-level of 0.05

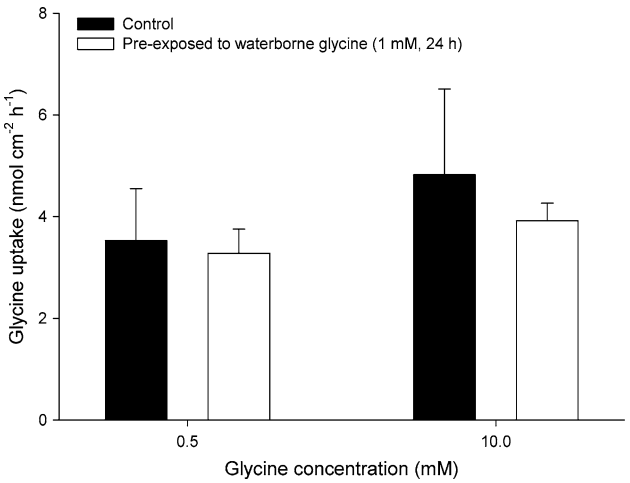


**Fig. 5** Effect of 24-h pre-exposure to waterborne glycine (1 mM) on glycine uptake (nmol $cm^{-2}$ $h^{-1}$) across intestinal gut sacs of hagfish. *Plotted values* represent the mean ± SEM of six preparations. No significant differences were determined (one-way ANOVA at an α-level of 0.05)

considered adequate to meet their comparatively lower metabolic needs (Karasov et al. 1985). Hagfish have very low metabolic rates (Forster 1990), inhabit environments similar to many studied teleosts and yet display amino acid uptake affinities that appear kinetically more similar to mammals than fish. The low affinity of uptake may reflect the capacity of the gills and skin of Pacific hagfish to also absorb amino acids and thereby supplement gastrointestinal nutrient uptake (Glover et al. 2011). Although simple Michaelis–Menten kinetics could not be applied to gill and skin absorption in that study, both these tissues clearly exhibited higher uptake affinities than the gut in the present study. This characterises the intestine of hagfish as a low-affinity amino acid uptake system. Low-affinity gastrointestinal uptake and high-affinity branchial uptake is a pattern observed for metal micronutrient absorption in teleost fish (e.g. Glover and Hogstrand 2002; Nadella et al. 2006b) and reflects the relatively low levels of substrate in the water, and the comparatively high levels in the chyme (Nadella et al. 2006a).

It is interesting to note that the uptake affinity of glycine uptake was higher (i.e. lower $K_m$) than that of L-alanine. Recent evidence suggests that the uptake of glycine across the gut of Pacific hagfish is stimulated by hypoxia (Bucking et al. 2011), a condition expected to be commonly encountered in feeding environments. The comparatively higher affinity of glycine uptake relative to that of L-alanine may be reflective of the well-known importance of glycine in hypoxia tolerance response (Nilsson and Lutz 1991; Gundersen et al. 2005).

## Putative transport systems

A saturable uptake profile, and demonstration of inhibition by amino acids with similar physicochemical characteristics, suggests that gastrointestinal absorption in hagfish was achieved via specific membrane transporters. There are multiple amino acid transporter families that are capable of translocating L-alanine and glycine across epithelia (see Bröer 2008). These include transporters that recognise both L-alanine and glycine as substrates (systems A, $B^0$, asc, PAT), and those that discriminate between these amino acids (e.g. system ASC for L-alanine, system Gly for glycine). A significant component of L-alanine and glycine absorption across the hagfish gut appeared to be achieved by a shared pathway and was sodium dependent. This suggested that uptake was mediated, at least in part, by a system A (SLC38A), $B^0$ (SLC6A) or PAT (SLC36A) transporter. In mammalian systems, these transporter families all transport L-alanine and glycine, are sodium dependent and are expressed in the intestinal epithelium (Bröer 2008). However, not all uptake could be inhibited by removal of sodium and the addition of 50-fold excess inhibitors, suggesting that more than one pathway may be in operation at least at lower amino acid levels.

Glycine absorption was further probed using a variety of amino acids thought to share uptake pathways in mammalian tissues: proline (system PAT; SLC36A), asparagine (system A; SLC38A) and threonine (system asc; SLC7A;

Bröer 2008). The proton dependence was also investigated as some systems known to transport glycine are also pH sensitive (e.g. PAT). The results clearly showed that glycine uptake was unimpacted by pH or proline, likely eliminating a role for the PAT system in hagfish intestinal amino acid absorption. Threonine significantly inhibited glycine uptake, suggesting the presence of system asc transporter. This is consistent with the inhibition observed in the presence of elevated L-alanine, which is also a known substrate of this system in mammals. However, threonine or L-alanine failed to completely inhibit glycine uptake suggesting a role for other transport systems. Significant inhibition in the presence of 100-fold excess of asparagine suggests that system A may also facilitate glycine uptake.

The assignment of uptake patterns to amino acid transport systems is based on the assumption that properties of these transporters are conserved through the vertebrate lineage. Evidence from teleost fish suggests that amino acid transporters in lower vertebrates may exhibit distinct properties (Bakke et al. 2011), indicating that further studies may be required to justify this assumption. Molecular characterisation would be of particular value, and would also provide further perspective in the understanding of the evolution of amino acid transport families (Sundberg et al. 2008).

### Lack of evidence for epithelial cross talk

In the current study, there was no evidence of epithelial cross talk, where the presence of elevated levels at one epithelium (skin/gill) was capable of altering the transport properties at another epithelium (gut). Such cross talk has been observed previously for metal and ion nutrients in teleost fish gill and gut. For example, elevated dietary calcium leads to a reduction in gill-mediated calcium absorption in rainbow trout (Franklin et al. 2005). The lack of any significant effect might be a function of the low metabolic rate of hagfish (Forster 1990), whereby turnover of transporter numbers or isoforms might be expected to be slower than that in animals with higher metabolic rates. It is also possible that cues other than the presence of the amino acid in water are required for induction of epithelial regulation. It is known, however, that amino acids are key chemoattractant molecules in hagfish (Tamburri and Barry 1999), and thus the presence of these alone might have been expected to be sufficient to promote a change from a quiescent digestive physiology.

### Environmental context

This is the first study to characterise the absorption of organic nutrients across the intestine of hagfish. Results suggest that components of the uptake pathway are shared with higher vertebrates. Whether the hagfish transport pathways are representative of nutrient absorption pathways of the protovertebrate or embody a derived condition resulting from the unusual and specialised feeding behaviour of carrion immersion (and the presence of extra-intestinal pathways for maximisation of nutrient uptake) is a matter for debate.

If hagfish are to maximise the nutritional value of a limited nutrient resource, then significant physiological changes in nutrient metabolism might occur with feeding. In many respects, the feeding behaviour of hagfish is akin to that of terrestrial "sit and wait" predators such as the python. It has been shown that nutrient absorption rates and capacities in fed Burmese pythons increase in the order of 25-fold above fasting levels (Secor and Diamond 1995). A snapshot of intestinal amino acid uptake in unfed hagfish may therefore not be representative of absorptive function during the processing of a meal. The next step is an examination of how these transport properties change during actual feeding.

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