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ORIGINAL PAPER

Determining the functional role of waterborne amino acid uptake in hagfish nutrition: a constitutive pathway when fasting or a supplementary pathway when feeding?

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Abstract Hagfish are unique among aquatic "vertebrates" in their ability to absorb amino acids directly from the water via skin and gill epithelia, but it is unknown whether this phenomenon extends beyond a few studied substrates; what effect fed state has on absorption; and what functional role this may play in hagfish nutrition. Using in vivo and in vitro transport assays, uptake and tissue distribution of the waterborne amino acids L-alanine, L-lysine, and L-phenylalanine were examined as a function of fed state. All three amino acids were shown to be taken up from the water (lysine and phenylalanine for the first time). Following immersion in radiolabelled solutions for 24 h, phenylalanine was the amino acid that accumulated at the highest levels in almost all tissues, with the highest accumulation noted in red blood cells and bile, followed by gill and liver. In general, tissues of fed hagfish displayed a significantly reduced phenylalanine accumulation compared to tissues of hagfish fasted for 3 weeks. An in vitro assay showed that phenylalanine was transported across the skin at the highest rate, with the uptake of lysine occurring at the lowest rate. Feeding status had no significant effect on in vitro transport. These data indicate that dissolved organic nutrients are a significant source of nutrition to hagfish, and may be relatively more important during periods of fasting than during periods of feeding when immersed in decaying carcasses.

Keywords Cutaneous · Feeding · Nutrients · Starvation · Transport · Uptake

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Introduction

Hagfish are unique among aquatic "vertebrate" animals in that they are able to utilise their skin and gill epithelia to absorb nutrients directly from the water (Glover et al. 2011a; Bucking et al. 2011; Schultz et al. 2014). It has been suggested that hagfish, as osmoconformers, have a reduced need to use the integument as a barrier minimising ionic exchange, and thus, can utilise these epithelia as absorptive surfaces without compromising osmotic homeostasis (Glover et al. 2013). However, the specific role that waterborne amino acid uptake plays in hagfish nutrition is unknown. Two functions can be hypothesised. The first suggests that gill and skin amino acid uptake enables a hagfish to maximise nutrient absorption when engaged in opportunistic scavenging (Glover et al. 2011a). Hagfish burrow into decaying carcasses (Glover and Bucking 2015), a spatially and temporally variable food source, for which there is significant competition among benthic fauna (Davies et al. 2006). Having surfaces capable of exploiting the nutrient-rich soup present inside carrion, in addition to

the gastrointestinal surface for absorption of ingested food, is therefore likely to be advantageous for hagfish nutrition.

The second hypothesis suggests that the cutaneous and branchial surfaces could function as a constitutive nutrient absorption pathway. Hagfish may go significant periods between feeding, with laboratory studies indicating survival of fasting periods in excess of 9 months (Tamburri and Barry 1999). Between periods of feeding, many hagfish species burrow in muddy substrates (Fernholm 1974; Martini 1998). The presence of organic substrates in marine sediments, and/or the presence of low levels of dissolved nutrients in surrounding water may facilitate nutrient absorption in between meals. This would allow the gut and associated digestive functions to lie quiescent, but still allow acquisition of organic substrates, albeit at a relative low rate. If the main biological role of branchial and cutaneous nutrient absorption is constitutive nutrient uptake, then these pathways may be relatively more important in fasting than in fed hagfish. Conversely, if the main function of waterborne nutrient uptake is to maximise absorption during feeding in decaying carrion, then an upregulation of cutaneous and branchial transport might be observed in fed animals relative to fasted ones. Such a response would mirror that of other animals that may fast for long periods. For example, the Burmese python shows significant upregulation in sugar and amino acid transport, which is accompanied by changes in the mass of the gut (Cox and Secor 2008). The atrophy of tissues and quiescence of transport activities during fasting, and their subsequent upregulation during feeding, is believed to be a mechanism that reduces overall energy costs in animals with periodic feeding strategies (Cox and Secor 2008). To date, however, there has been no investigation on how fed state affects waterborne amino acid uptake in hagfish.

Another significant knowledge gap regarding the uptake of waterborne nutrients in hagfish is the extent to which different substrates are absorbed. To date the only amino acids shown to be taken up from the water by hagfish are glycine and L-alanine (Glover et al. 2011a; Bucking et al. 2011). Amino acids differ in their physicochemical properties and essentiality, factors that could significantly influence their ability to be absorbed by the skin and gills. For example, both glycine and alanine are non-polar, non-essential amino acids. There have been no studies examining the uptake of essential, or of charged or polar, amino acids. Furthermore, although amino acids act as building blocks in protein synthesis and as energy substrates, some have other important functions, including osmotic balance (Currie and Edwards 2010), and acting as precursors for other biologically important entities (e.g. hormones, nucleotides; Cowey 1994). These different biological roles could influence the uptake rates and distribution patterns of absorbed amino acids. For example, owing to a putative role in cytoprotection, a significantly greater proportion of absorbed glycine is accumulated in the brain following in vivo exposure of Pacific hagfish to this amino acid under conditions of anoxia (Bucking et al. 2011).

In the current study, the ability of the Pacific hagfish (*Eptatretus stoutii*) to absorb the amino acids L-alanine, L-lysine and L-phenylalanine from seawater was examined. These three amino acids differ in terms of their essentiality and key physicochemical properties. For example, while alanine is a non-essential amino acid (at least in teleost fish), lysine and phenylalanine are essential (NRC 1993). Phenylalanine and alanine are both non-polar, while lysine is basic and positively charged at seawater pH values. Despite this, all three amino acids tested share similar lipid solubilities, with log octanol: water partition coefficients (log K_{ow}) of −2.85, −3.05, −1.38 for alanine, lysine and phenylalanine, respectively. Using whole animal in vivo transport assays, and in vitro isolated skin transport assays, the uptake of these amino acids was examined. While this is the first study to examine the ability of phenylalanine and lysine to be taken up from the water by hagfish, alanine has previously been characterised as a transportable substrate (Glover et al. 2011a). In addition, the effect of fed state on in vivo and in vitro waterborne uptake and distribution were assessed to delineate the potential functional role (constitutive uptake in between feeding bouts, or a supplementary pathway during feeding inside decaying carrion) of this process in hagfish.

Materials and methods

Animal collection, holding and feeding

Pacific hagfish (*E. stoutii*) were collected by baited trap in Barkley Sound on the west coast of Vancouver Island, British Columbia, and transferred to 500 L holding tanks receiving flow-through natural seawater (12 °C) at the Bamfield Marine Sciences Centre (BMSC). Hagfish were collected under Fisheries and Oceans Canada permits (XR107 2012), and all manipulations were approved by the BMSC Animal Care and Use Committee (RS-12-8). "Fasted" hagfish were maintained without feeding for three to four weeks prior to experimentation. "Fed" hagfish were transferred from holding tanks and immersed in vigorously aerated 100 L tanks containing partially blended slurry of North Pacific hake (*Merluccius productus*) in seawater. Hagfish were left immersed for between 16 and 24 h, before being removed from feeding tanks, and employed directly in experiments as fed animals.

In vivo amino acid transport assays

Individual hagfish (n = 36, consisting of 18 fed and 18 fasted animals; overall mean (±SEM) mass of 67 ± 4 g) were placed in aerated, lidded plastic containers (600 mL) containing filtered seawater (in mM: Na, 492; K, 9; Ca, 12; Mg, 50; Cl, 539; pH 8.0). Hagfish were placed in chambers and left for ~10 min. Any slime produced from handling (2–3 instances) was then removed and seawater topped back up to 600 mL, before either L-phenylalanine, L-lysine, or L-alanine was added to provide a final concentration of 10 µM. Each chamber was also spiked with 10–20 μC_i of respective radiolabelled amino acid ([2,6 ^{3}H]-L-phenylalanine (Amersham); ^{3}H-L-lysine (Perkin Elmer); ^{3}H- L-alanine (Perkin Elmer)). Triplicate water samples were taken initially, and at the completion of the exposure, for the determination of specific activity:

$$\text{Specific activity}\left(\text{cpm nmol}^{-1}\right) = A/[\text{AA}] \quad (1)$$

where A is the average activity (cpm mL^{-1}) of the radiolabelled amino acid stock solution, and [AA] is the amino acid concentration (nmol mL^{-1}) of the stock solution.

Hagfish were left immersed for 24 h, after which they were rinsed in clean fresh seawater, euthanised by anaesthetic overdose (2 g L^{-1} 3-aminobenzoic acid ethyl ester; MS222), and weighed. Blood was then extracted from the caudal sinus, and centrifuged (5000 rpm, 5 min) to separate plasma and red blood cells, before a longitudinal ventral incision was made, exposing the internal organs. Bile was extracted from the gall bladder using a needle and syringe, and the following tissues were removed: liver, heart, tongue, gill and gut. The gut was sluiced of any contents using clean seawater. A section of muscle from the anterior ventral surface was removed, as was a series of 3–4 slime glands from the mid-body, before the brain was removed from the cranial cavity. Small sections of anterior (near the head), medial (midpoint of the body) and posterior (near tail) skin (~1 cm^2) were taken from the ventral surface. All tissues were weighed, and somatic indices were calculated (tissue-specific total weight divided by total animal weight, multiplied by 100) for those tissues where the entire tissue was extracted (gill, gut, heart, liver, tongue, bile, brain).

Thereafter, ~1 g subsamples of gill, liver, gut and tongue were dissected and placed in 20 mL plastic vials, to which was added 1–2 mL of 2 N HNO_3 for tissue digestion. For the heart, brain, muscle, slime gland, and red blood cell samples, the whole sample was digested in 1–2 mL 2 N HNO_3. All tissue digests were heated at 65 °C for 48 h, and vigorously shaken every 12 h. Bile and plasma samples were not acid-digested. To all tissue samples 5–10 mL of scintillation fluor (UltimaGold; Perkin Elmer) was added, while 5 mL of fluor (Optiphase; Perkin Elmer) was added to water samples. All samples were then assessed for radioactivity using a liquid scintillation counter (LS6500; Beckman Coulter), with quench correction based on the external standards ratio. Total accumulation (nmol g^{-1}) was determined as follows:

$$\text{Total accumulation}\left(\text{nmol g}^{-1}\right) = \left(\text{cpm}_\text{f} - \text{cpm}_\text{i}/\text{SAct}\right)/\text{W}_\text{wb} \quad (2)$$

where the differences between initial (cpm$_i$) and final (cpm$_f$) radioactivities in exposure waters were converted to amino acid concentrations by dividing by specific activity (SAct; see Eq. 1), and then divided by hagfish whole body mass (W_{wb}). Tissue-specific accumulation rates (nmol g^{-1} h^{-1}) were calculated similarly:

$$\text{Tissue accumulation}\left(\text{nmol g}^{-1}\text{h}^{-1}\right) = \left(\text{cpm}_\text{t}/\text{SAct}\right)/W_t/t \quad (3)$$

where tissue radioactivity (cpm$_t$) was divided by specific activity (SAct; Eq. 1), and then divided by tissue weight (W_t) and time of exposure (t; 24 h).

In vitro amino acid transport assays

Fed or fasted hagfish were euthanised, and sections of anterior dorsal skin were removed and placed in a modified Ussing chamber system for the assessment of transmural amino acid transport, based on a previously published protocol (Glover et al. 2011a). Briefly, skin was stretched across an aperture cut into a lid of a 15 mL plastic scintillation vial, inside of which was 10 mL of continuously aerated hagfish Ringer (in mM: NaCl, 474; KCl, 8; $CaCl_2{\cdot}2H_2O$, 5; $MgSO_4{\cdot}7H_2O$, 3; $MgCl_2{\cdot}6H_2O$, 9; $NaH_2PO_4{\cdot}H2O$, 2.06; $NaHCO_3$, 41; and glucose, 5; pH 7.6). This vial was suspended in a beaker containing 150 mL of aerated filtered seawater, to which was added "cold" (i.e. unlabelled) amino acid (final concentration: 10 µM of either L-phenylalanine, L-lysine, or L-alanine) and radiolabelled amino acid (~1 μC_i mL^{-1}). After 2 h, a 1 mL sample of the Ringer was removed and assayed for ^{3}H radioactivity, as described above. Uptake rates (nmol cm^{-2} h^{-1}) were calculated as follows:

$$\text{Uptake}\left(\text{nmol cm}^{-2}\text{h}^{-1}\right) = \left(\text{cpm}_\text{s}/\text{SAct}\right)/\text{SA}/t$$

where cpm$_s$ is the accumulation of radioactivity in the serosal medium divided by specific activity (SAct; Eq. 1) to give accumulated amino acid (nmol). This was then divided by surface area (SA; cm^{-2}), and time (t; 2 h). The surface area was the amount of skin surface exposed to the medium

Table 1 Somatic indices (mean ± SEM) for various tissues in fasted and fed hagfish ($n = 6$)

Tissue	Fasted	Fed
Gill	4.29 ± 0.14	4.22 ± 0.17
Gut	6.60 ± 0.28	8.30 ± 0.36[a]
Heart	0.18 ± 0.02	0.15 ± 0.21
Liver	2.71 ± 0.16	2.40 ± 0.09[a]
Tongue	5.10 ± 0.12	4.53 ± 0.13[a]
Bile	0.35 ± 0.02	0.32 ± 0.03
Brain	0.07 ± 0.01	0.05 ± 0.01

[a] Significant differences within a tissue between fed and fasted treatments (two-way ANOVA, post hoc Tukey's test; $\alpha = 0.05$)

through the aperture in the scintillation vial lid (1.76 cm^2; Glover et al. 2011a).

Statistical analysis

All data were tested for normality and homogeneity of variance, using Kolmogorov–Smirnov and Levene's test, respectively. If data failed these tests they were transformed. Subsequently, all amino acid data were subjected to two-way ANOVA with fed state and amino acid as the two factors, followed by a Tukey's post hoc test. Differences between tissue somatic indices were also assessed via two-way ANOVA followed by post hoc Tukey's test, with tissue and fed state as the two factors. All data were deemed significant at α level of 0.05.

Results

There was no significant difference in hagfish mass between fed and fasted groups (one-way ANOVA, $p = 0.195$). However, fed hagfish had significantly greater relative gut mass than hagfish that had been fasted (Table 1). In contrast relative tongue and liver masses in fed hagfish were reduced compared to fasted hagfish. Although both fed and fasted hagfish had bile in gall bladders, in fasted hagfish this was much darker in colour than the bright yellow observed in fed animals.

Based on disappearance of amino acid from the bathing medium, hagfish accumulated between 25 (fasted alanine) and 93 (fasted phenylalanine) nmol g^{-1} over a 24 h exposure (Fig. 1). Phenylalanine was the only amino acid that exhibited a difference in accumulation rate with fed state, with a decrease in fed animals. This resulted in the significantly higher accumulation rate of phenylalanine (approximately twofold) relative to lysine and alanine in fasted hagfish, returning to a rate that was statistically indistinguishable from that of the other amino acids in fed hagfish.

Skin tissues displayed similar patterns of amino acid accumulation, regardless of the region along the body surface from which the skin was sourced (Fig. 2a, c). In all conditions alanine displayed the lowest accumulation rates (significantly so for all but fed anterior skin), while phenylalanine and lysine rates of accumulation were indistinguishable. There were no fed state-dependent effects. The other directly exposed transport epithelium, the gills, exhibited high rates of phenylalanine accumulation in

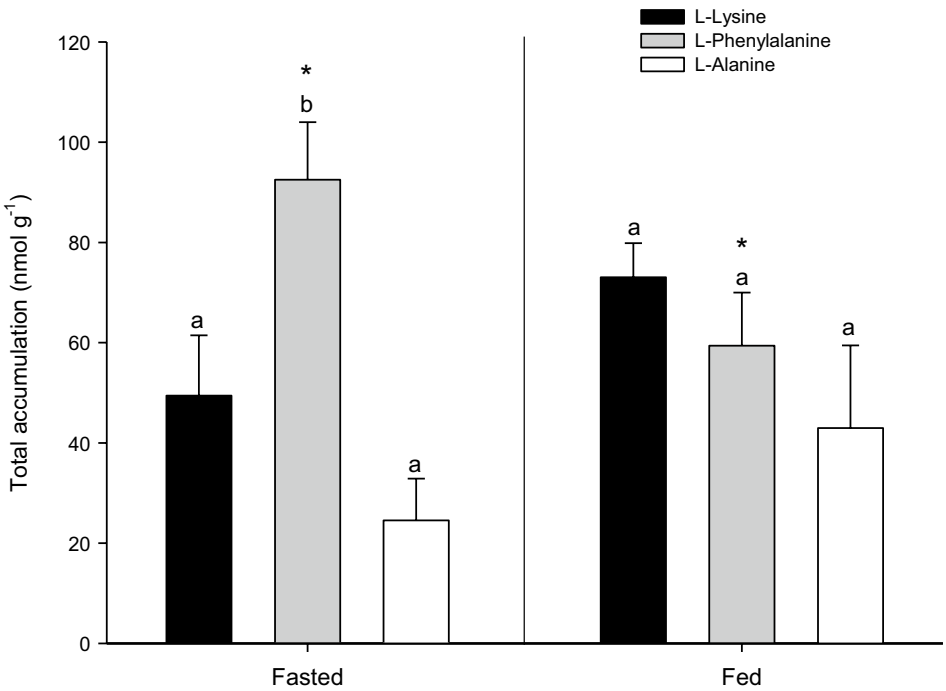

Fig. 1 Total amino acid accumulation (nmol g^{-1}) in fasted versus fed hagfish following a 24 h immersion in 10 µM lysine, phenylalanine or alanine. *Plotted points* represent means (±SEM) of 6 replicates. *Bars with shared letters* indicate no differences in amino acid accumulation rate within a feeding treatment, while *asterisks* indicate significant differences between accumulation rates of the same amino acid between fed and fasted animals (two-way ANOVA, post hoc Tukey's test; $\alpha = 0.05$)

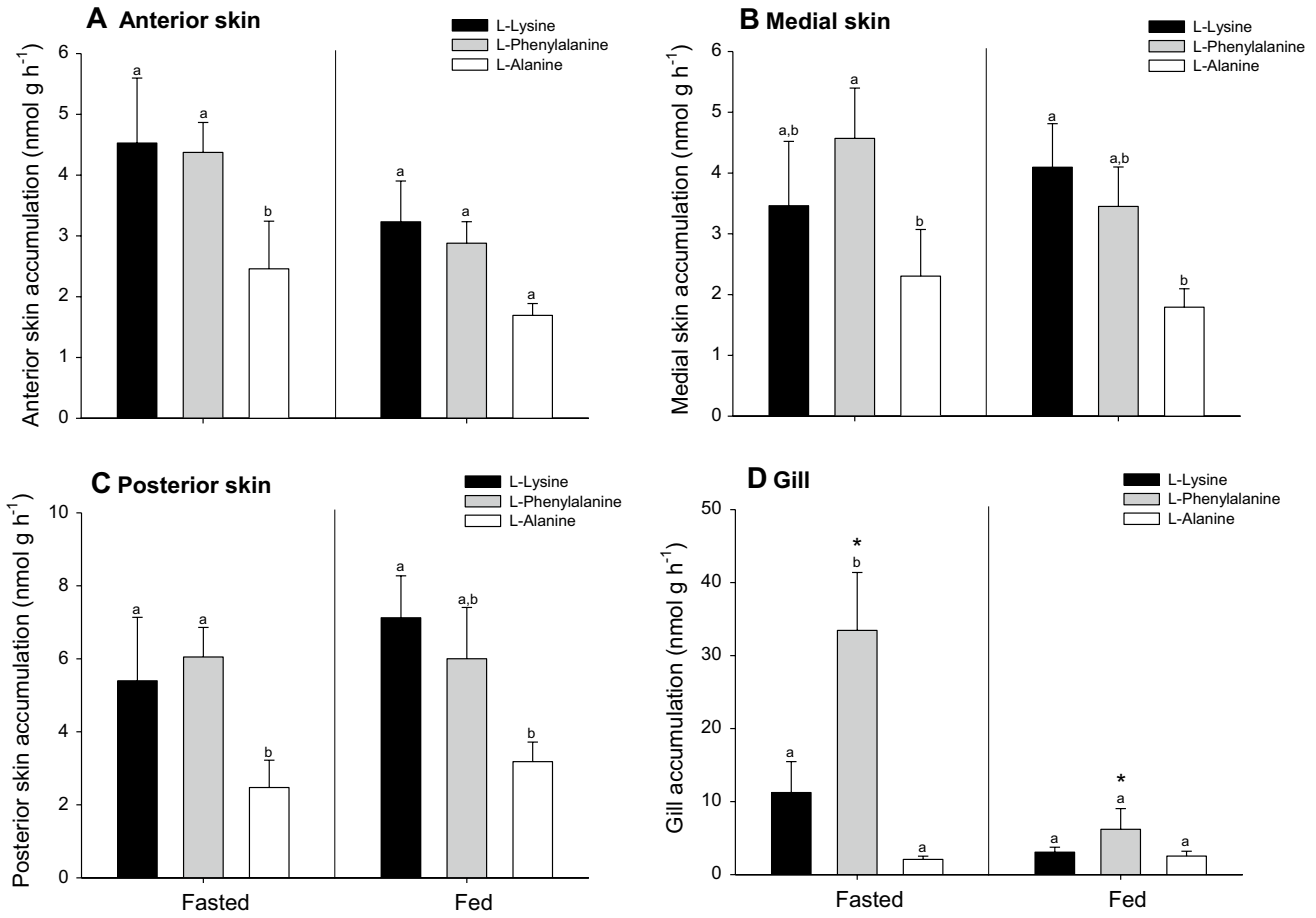

Fig. 2 Tissue-specific amino acid accumulation rates (nmol g^{-1} h^{-1}) for anterior skin (**a**), medial skin (**b**), posterior skin (**c**) and gill (**d**) in fasted versus fed hagfish following a 24 h immersion in 10 μM lysine, phenylalanine or alanine. *Plotted points* represent means (±SEM) of 6 replicates. *Bars with shared letters* indicate no differences in amino acid accumulation rate within a feeding treatment, while *asterisks* indicate significant differences between accumulation rates of the same amino acid between fed and fasted animals (two-way ANOVA, post hoc Tukey's test; $\alpha = 0.05$)

fasted hagfish, with values more than threefold and 16-fold those of lysine and alanine, respectively (Fig. 2d). These rates were also approximately fivefold those of phenylalanine accumulation in the skin. In the fed animals, phenylalanine accumulation fell significantly to rates equivalent to those for the other two tested amino acids.

Blood components showed similar patterns of amino acid accumulation. Phenylalanine accumulation rates were significantly higher than those of alanine and lysine in fasted animals, and in the fed condition these rates decreased significantly in both plasma and red blood cells (Fig. 3). Note, however, that red blood cells accumulated all amino acids at rates at least an order of magnitude greater than plasma. For fasted hagfish, the ratio of red blood cell to plasma accumulation rates was 58, 13 and 4, for phenylalanine, lysine and alanine, respectively.

The patterns of amino acid accumulation in muscle tissues (body muscle, tongue, and heart) are shown in Fig. 4. In both body muscle and tongue of fasted hagfish, amino acids accumulated in the order: phenylalanine > lysine > alanine. In body muscle this relative relationship was maintained, just at lower rates for all three amino acids, albeit only the decline in phenylalanine accumulation was significant relative to fasted hagfish. In the tongue muscle amino acids showed a similar decline, however, the result of the significant decline in phenylalanine between the two fed states was no significant difference in accumulation rates between all three amino acids. The heart showed a similar but distinct pattern of accumulation (Fig. 4c). Phenylalanine accumulated at a significantly higher rate than alanine and lysine in both fasted and fed hagfish, but the rate of phenylalanine accumulation was significantly lower in fed animals.

Fasted hagfish showed a significantly higher rate of phenylalanine accumulation in the liver than lysine and alanine, and this rate was also higher than that of fed

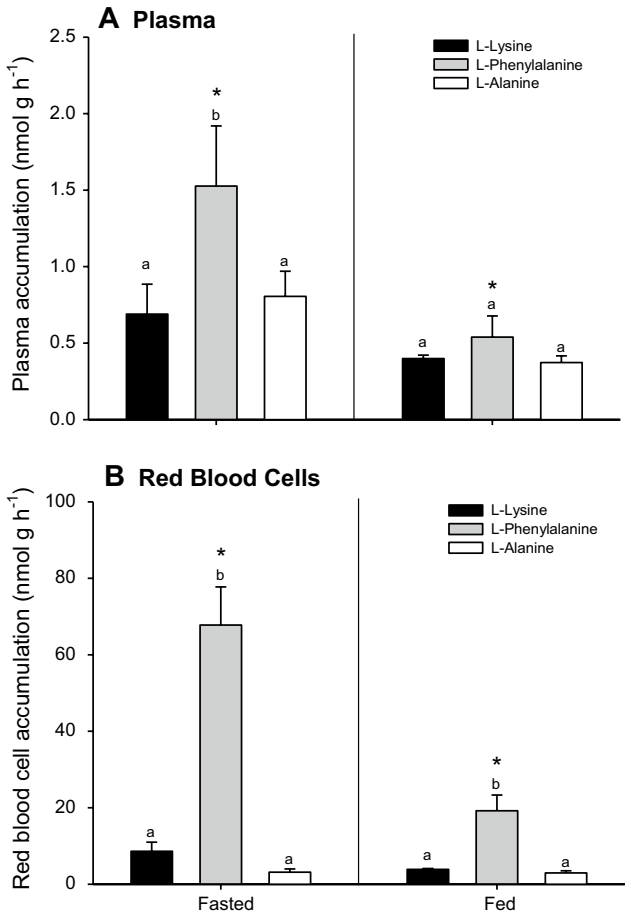

Fig. 3 Tissue-specific amino acid accumulation rates (nmol g^{-1} h^{-1}) for plasma (**a**) and red blood cells (**b**) in fasted versus fed hagfish following a 24 h immersion in 10 μM lysine, phenylalanine or alanine. *Plotted points* represent means (±SEM) of 6 replicates. *Bars with shared letters* indicate no differences in amino acid accumulation rate within a feeding treatment, while *asterisks* indicate significant differences between accumulation rates of the same amino acid between fed and fasted animals (two-way ANOVA, post hoc Tukey's test; $\alpha = 0.05$)

animals (Fig. 5a). The magnitude of phenylalanine accumulation in the bile of fasted animals (~80 nmol g^{-1} h^{-1}; Fig. 5b) was the highest of all tissues. Despite a decline to ~50 nmol g^{-1} h^{-1} in fed hagfish, this rate of accumulation was still significantly higher than those of lysine and alanine. The pattern of gut amino acid uptake was identical to that for bile, albeit at a much lower rate of accumulation (Fig. 5c). The rate of brain phenylalanine accumulation was significantly greater than that of alanine, but not lysine, in fasted animals (Fig. 5d). A significant decline of phenylalanine bioaccumulation in the brain occurred in fed animals such that there were no significant differences in accumulation rates among all amino acids. With feeding, slime glands exhibited significantly reduced accumulation rates of both lysine and phenylalanine, but not alanine (Fig. 5e). In the fasted condition, the rate of phenylalanine accumulation was significantly greater than that of lysine, which in turn was significantly greater than that of alanine. Only the accumulation of phenylalanine in the slime gland was significantly higher than alanine accumulation in fed hagfish.

In vitro transport assays showed that phenylalanine was taken across the skin at a level significantly higher than lysine and alanine (Fig. 6). There were no statistically significant effects of feeding on transport rate, but in the skin assays from fed hagfish phenylalanine uptake was elevated relative to lysine.

Discussion

Pacific hagfish absorb amino acids from the water

The Pacific hagfish was able to absorb amino acids directly from the water. Previous work has shown that both glycine and alanine are taken up by hagfish skin and gills (Glover et al. 2011a; Bucking et al. 2011), and thus, the current study extends this finding to phenylalanine and lysine. Glycine and alanine are non-polar amino acids. Although no studies of amino acid essentiality have been conducted for hagfish, in teleost fish these are both considered non-essential (NRC 1993). Both phenylalanine and lysine are essential amino acids in teleost fish, with the former classified as non-polar and the latter being positively charged at seawater pH. Uptake of amino acids from the water, therefore, appears to occur regardless of either nutritional need or physicochemical properties of the individual amino acid, at least over the small subsample of amino acids studied thus far.

The extent of amino acid uptake from the water was significant. Based on the disappearance of amino acids from the exposure medium, and depending on fed state and the specific amino acid examined, a remarkable 28–75 % of the amino acids in the exposure chambers were absorbed in 24 h of exposure. In addition to their roles in protein synthesis, and as an energy substrate, amino acids are believed to be especially important in hagfish due to their osmoregulatory function (Currie and Edwards 2010). As marine osmoconformers, hagfish largely balance intracellular osmolality by accumulating amino acids. While marine teleost fish do the same, the magnitude of this effect is greater in hagfish owing to the higher extracellular fluid osmolality. Furthermore, while teleost fish primarily use β-amino acids, such as taurine as intracellular osmolytes, hagfish utilise standard α-amino acids (Fincham et al. 1990; Cholette and Gagnon 1973). This likely contributes to the high levels of free amino acids in hagfish muscle and red blood cells (Bellamy and Chester-Jones 1961). This is also reflected in amino acid uptake rates. For example, in hagfish red blood cells,

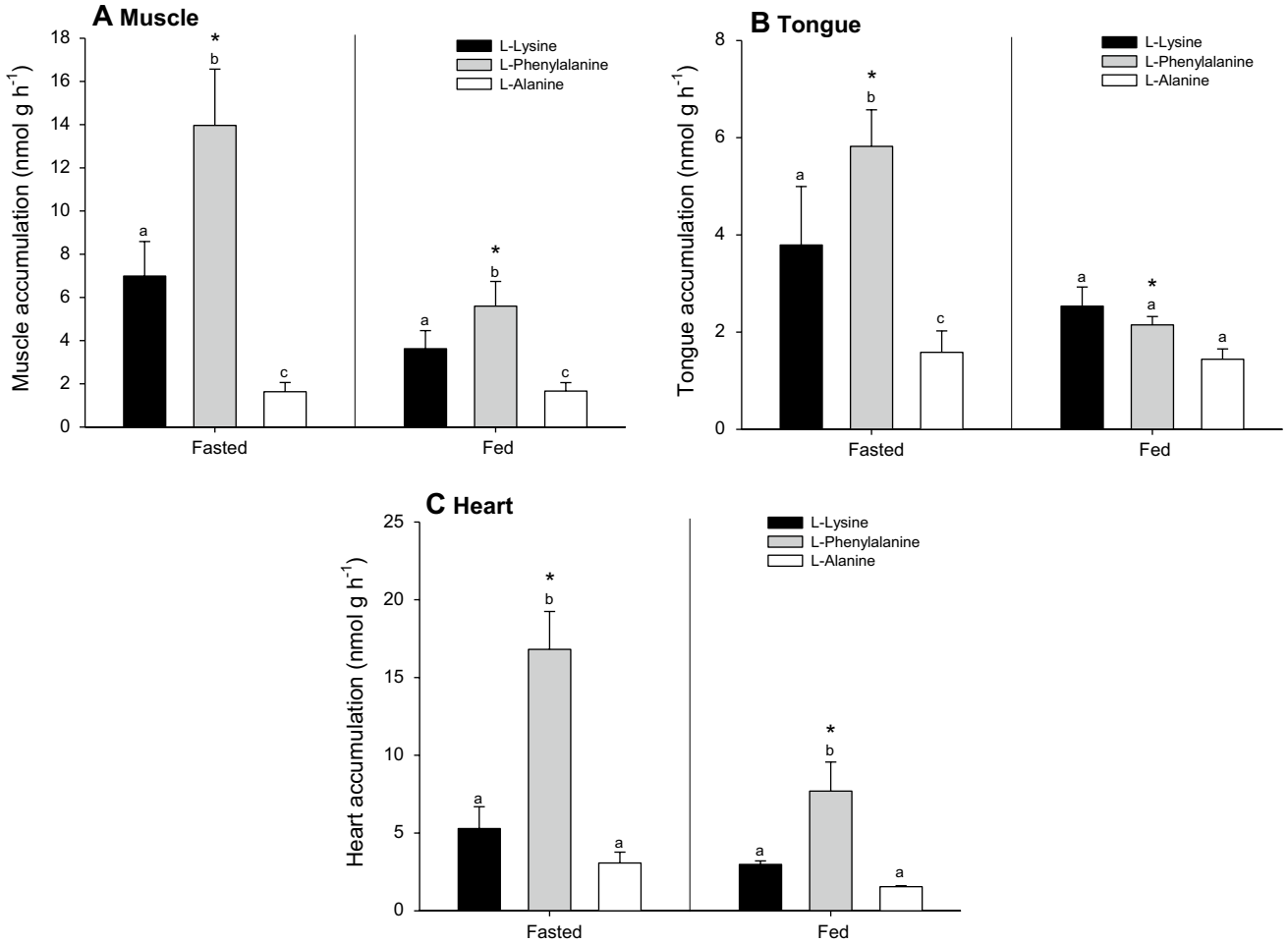

Fig. 4 Tissue-specific amino acid accumulation rates (nmol g^{-1} h^{-1}) for muscle (**a**), tongue (**b**), and heart (**c**) in fasted versus fed hagfish following a 24 h immersion in 10 μM lysine, phenylalanine or alanine. *Plotted points* represent means (±SEM) of 6 replicates. *Bars with shared letters* indicate no differences in amino acid accumulation rate within a feeding treatment, while *asterisks* indicate significant differences between accumulation rates of the same amino acid between fed and fasted animals (two-way ANOVA, post hoc Tukey's test; $\alpha = 0.05$)

the rate of alanine uptake is 10000 times that of mammalian red blood cells (Fincham et al. 1990). The high biological demand for, and utility of, amino acids in hagfish, is thus likely responsible for their significant capacity for waterborne amino acid absorption.

Total accumulation (Fig. 1) was based on disappearance of amino acid from the medium, and as such could have overestimated the rates at which amino acids were truly absorbed by the animal. For example, hagfish are well known for their ability to produce copious amounts of slime (Fudge et al. 2015). Thus, binding of amino acids to slime on the body surface could have decreased water levels but prevented uptake. However, slime was removed from hagfish prior to the assay, and the presence of slime has also been shown not to impede amino acid (alanine) transport across the skin (Glover et al. 2015). In fact, a rough calculation indicates that uptake based on amino acid disappearance may underestimate true uptake. Assuming that muscle comprises 50 % of hagfish body mass (Emdin 1982) the estimated total amino acid uptake based on tissue appearance was greater than uptake based on medium disappearance, by an average of 16 %.

It is important to note that, in contrast to marine teleost fish and lampreys, hagfish do not drink. Morris (1965), investigating this phenomenon found that a minority of hagfish did swallow seawater, but this was attributed to experimental artefact. This conclusion fits with the predicted physiology of salt and water balance. As osmoconformers, the osmolarity of hagfish body fluids is matched closely to that of their environment (e.g. Bellamy and Chester-Jones 1961), eliminating the need to balance osmotic concentration by drinking, as in marine teleost fish. This was supported in the current study by the low levels of amino acids accumulating in the gut

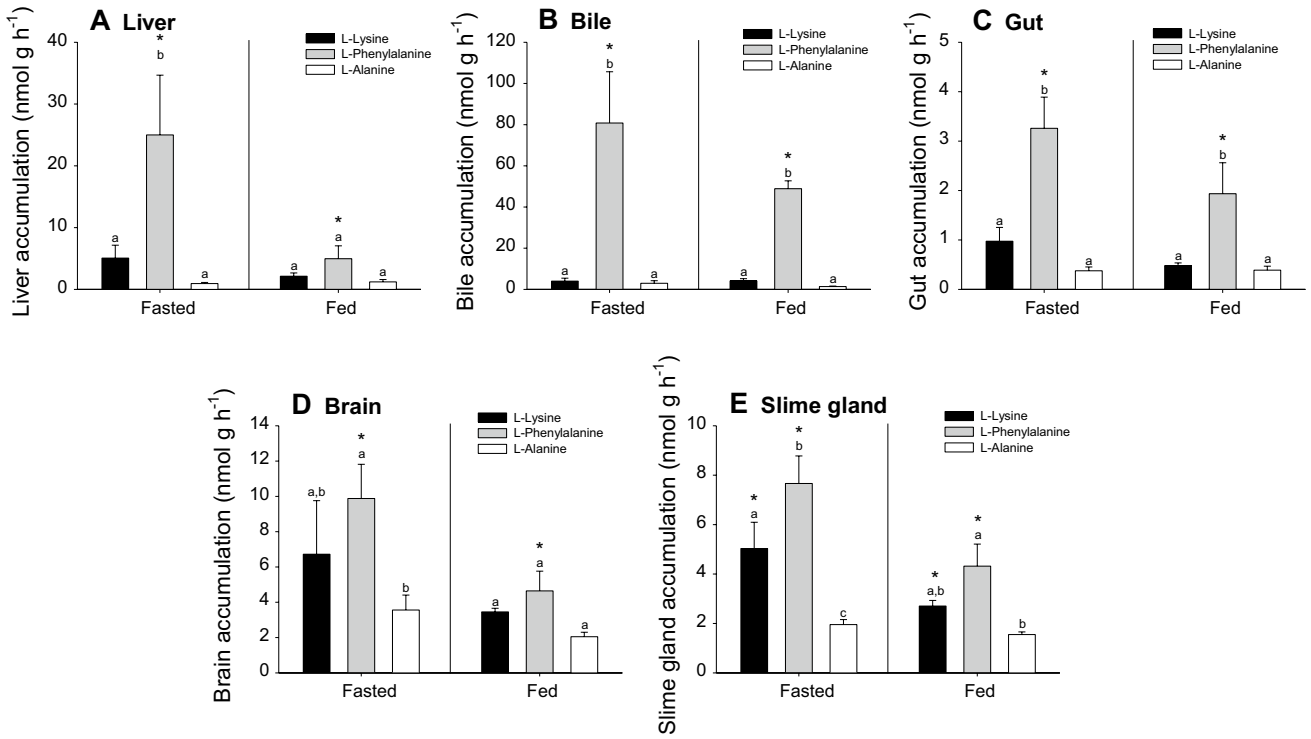

Fig. 5 Tissue-specific amino acid accumulation rates (nmol g^{-1} h^{-1}) for liver (**a**), bile (**b**), gut (**c**), brain (**d**), and slime gland (**e**) in fasted versus fed hagfish following a 24 h immersion in 10 μM lysine, phenylalanine or alanine. *Plotted points* represent means (±SEM) of 6 replicates. *Bars with shared letters* indicate no differences in amino acid accumulation rate within a feeding treatment, while *asterisks* indicate significant differences between accumulation rates of the same amino acid between fed and fasted animals (two-way ANOVA, post hoc Tukey's test; $\alpha = 0.05$)

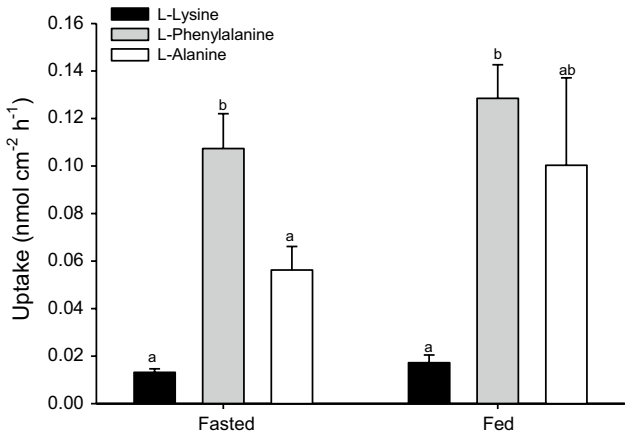

Fig. 6 *In vitro* amino acid uptake rates (nmol cm^{-2} h^{-1}) across anterior dorsal skin sections collected from fasted or fed hagfish. *Plotted points* represent means (±SEM) of 6 replicates. *Bars with shared letters* indicate no differences in amino acid uptake rates within a feeding treatment (two-way ANOVA, post hoc Tukey's test; $\alpha = 0.05$). There were no significant differences between fasted and fed states

tissue, relative to accumulation rates in epithelia directly exposed to radiolabelled amino acids. For example, in fasting hagfish the lysine accumulation rate in the skin was 3.5–5.4 nmol g^{-1} h^{-1}, in the gill was 11.2 nmol g^{-1} h^{-1}, but in the gut was only 1.0 nmol g^{-1} h^{-1}. The osmoregulatory strategy of hagfish, coupled with the relative lack of amino acid accumulation in this tissue, strongly suggests that drinking of the medium, and uptake of the amino acids via the gut, is highly unlikely to account for the appearance of amino acids in hagfish tissues.

Amino acid uptake and tissue distribution

Previous work has demonstrated that the uptake of alanine and glycine from seawater in hagfish is saturable and subject to competitive inhibition, indicating the presence of specific amino acid transporters (Glover et al. 2011a). It is therefore likely that the uptakes of phenylalanine and lysine observed in the current study are also transporter-mediated. To date the exact nature of the amino acid transporters in the gill and skin has not been characterised, but evidence suggests a mixture of different systems are present (Glover et al. 2011a). On the basis of shared substrates and sodium-independence, studies of gut and red blood cell amino acid absorption have both hypothesised the presence of an "asc system" transporter in hagfish that facilitates alanine uptake (Fincham et al. 1990; Glover et al. 2011b). In red blood

cells lysine transport is achieved by a putative "system y^+" transporter (Young et al. 1991). The current study is the first to characterise phenylalanine transport in any hagfish tissue, and further studies of sodium-dependence and assessment of competing substrates would be required to identify a candidate transport system for this amino acid. In mammals, phenylalanine transport is achieved by a number of transport systems, including "system T", "system L", "system $B^{0,+}$" and "system $b^{0,+}$" (Bröer 2008).

There have been few studies that have examined the amino acid profiles in hagfish tissues. It has, however, been documented that there is a large difference between erythrocyte and plasma amino acid concentrations, and that the extent of this difference is strongly amino acid-dependent (Cholette and Gagnon 1973; Fincham et al. 1990). Alanine, for example, has been reported to have a 219-fold higher concentration in red blood cells than in plasma (Fincham et al. 1990). The equivalent value for lysine was 11, while phenylalanine displayed an intermediate value of 63 (Fincham et al. 1990). In the current study, the relative distribution of newly accumulated lysine and phenylalanine between red blood cells and plasma in fasted hagfish closely matched the study of Fincham and colleagues (based on total amino acid concentrations), with ratios of 13 and 58, respectively. However, the red blood cell: plasma ratio for alanine was 4, less than 2 % of that noted in the previous investigation. This could be a consequence of the two different approaches. The study of Fincham et al. (1990) measured actual levels of amino acids in hagfish under natural conditions. The current study examined only radiolabelled amino acid following a 24 h environmental exposure to elevated concentrations. Thus, the variations in relative alanine distribution between the two studies could relate to differences in rates of compartmental label exchange with existing "cold" pools between essential (phenylalanine and lysine) and non-essential (alanine) amino acids, and/or may reflect a compensatory response to elevated alanine.

Muscle tissue is the only other hagfish tissue in which the profile of amino acids has been examined. Hwang et al. (2002) showed that proline was the amino acid with the highest concentration in muscle cells of the Japanese hagfish (*Eptatretus burgeri*). Among the amino acids examined in the current study, concentrations of alanine were greater than those of lysine, which in turn were greater than those of phenylalanine (Hwang et al. 2002). Similar patterns for alanine and phenylalanine were observed in the earlier work of Cholette and Gagnon (1973) in the Atlantic hagfish (*Myxine glutinosa*). The study of Hwang et al. (2002) also examined total muscle amino acid concentration. This measure is more closely related to that employed in the current study, as in our study the radiolabel could have been incorporated into proteins, and thus, does not necessarily represent only free amino acids. Total amino acid contents were in the relative order of lysine > alanine > phenylalanine (Hwang et al. 2002). In the current study, this order (based on newly accumulated amino acid and not total concentration) was phenylalanine > lysine > alanine. Whether this represents differences in handling between species, an effect of the experimental approach (see above), or other factors, is unknown.

One potential factor that could contribute to differences between the current and previous studies is the use of radiolabel and the essentiality of phenylalanine. Phenylalanine is required for the production of tyrosine, itself a precursor for many important amine signalling molecules (e.g. dopamine, serotonin, thyroxine and catecholamines; Fernstrom and Fernstrom 2007). In this study, it is not possible to distinguish between phenylalanine and any of its products as the tritium label is located on the benzyl side chain, and thus, follows the metabolic pathway. It is therefore possible that phenylalanine accumulation may represent the accumulation of tyrosine or other metabolites.

Effect of fed state

Immersion of hagfish in fish slurry induced several minor changes in somatic indices, indicative of changes in fed state (Fig. 1). Most notably, there was an increase in relative gut mass, suggestive of morphological, and potentially functional, changes associated with digestive requirements. This is a commonly observed response in teleost fish (e.g. Blier et al. 2007), but has not been previously measured in hagfish.

With the exception of a decrease in lysine accumulation in the slime gland, the only impact of fed state on hagfish amino acid uptake and tissue distribution in vivo was a decrease in phenylalanine accumulation in all studied issues, apart from the skin. These tissue-specific changes mirrored an overall decrease in total phenylalanine uptake, suggesting that the decline in tissue phenylalanine accumulation rate reflects the reduced uptake of this amino acid from the water. In *Xenopus* oocytes (Taylor et al. 1996), elevated exposure to amino acids, as might occur during feeding in hagfish, results in down-regulation of the "$B^{0,+}$ transport system" (capable of transporting phenylalanine), but not the "asc system" (thought to be the major transporter family involved in alanine uptake in hagfish; see above). If identical feeding-related changes in transport system activities occurred in the hagfish skin and/or gill, they would produce a pattern very similar to that observed in the current study (decreased phenylalanine uptake, unchanged alanine uptake).

The relatively minor changes in amino acid handling between fed and fasted hagfish, may relate to the extent of fasting period. In the current study, hagfish were fasted

for 3 weeks. In captivity, *E. stoutii* are capable of surviving fasting periods of 9 months (Tamburri and Barry 1999). Cholette and Gagnon (1973) noted that starvation of up to 66 days produced no changes in serum amino acid profiles in *M. glutinosa*. It is therefore possible that extended fasting would provide a greater challenge to nutrient reserves, and could thus generate a more significant effect on amino acid uptake and tissue distribution.

In vitro versus in vivo

Patterns of amino acid uptake differed depending on whether in vitro or in vivo techniques were employed. Skin transport assays revealed higher rates of phenylalanine (seven to eightfold) and alanine (four to sixfold) transport relative to those of lysine, and also showed that fed state had no impact on transport (Fig. 6). In in vivo assays in fasted hagfish the rate of phenylalanine disappearance from the bathing medium was elevated by about twofold relative to the approximately equivalent uptake rates of the other two studied amino acids. However, in fed hagfish in vivo, all amino acid transport rates were statistically indistinguishable, following a decrease in the rate of phenylalanine absorption (Fig. 1). The key difference between these assays is that during in vivo studies amino acids could be taken up by either the skin or the gill, whereas only cutaneous transport was examined in vitro. This suggests that branchial absorption might be a particularly important route of amino acid uptake, a suggestion that has also been made for waterborne nickel uptake in hagfish (Glover et al. 2015). That the gill is a potentially important site of uptake is supported by the pattern of accumulation in the gill (Fig. 2d). Branchial amino acid accumulation was high and it mimicked the pattern of disappearance, at least in terms of a greatly reduced phenylalanine uptake in fed fish. It is also important to note that skin uptake assays represent initial rate conditions, whereas in vivo assays were conducted over 24 h, and may reflect the additional influences of metabolism and excretion of amino acids on overall accumulation pattern. It also pertinent to note that factors, such as cutaneous blood flow (Cooper et al. 2012) and neural and humoral regulatory feedback mechanisms (Edwards et al. 2015), which may play important roles in modifying skin transport in vivo, are missing in vitro. These differences could contribute to the differences observed between the two experimental approaches used. Nevertheless, both in vivo and in vitro assays indicate that all three tested amino acid substrates are capable of being absorbed directly from seawater.

Waterborne amino acid uptake: an adaptation to feeding or to fasting?

The functional importance of waterborne amino acid uptake in hagfish is unknown, but two hypotheses are proposed (see "Introduction"). Amino acid uptake could serve to supplement feeding, permitting hagfish to maximise nutrition by accessing nutrients dissolved in the decaying body cavities of carrion. Alternatively, amino acid uptake from the water could serve as a constitutive nutrient pathway permitting hagfish to attain nutrition directly from organic-rich sediments or from dissolved amino acids in seawater. Although results varied by experimental approach, examined tissue and amino acid identity, where there was an effect of feeding in the current study it was to decrease amino acid absorption. Such a pattern is the opposite of that observed in species that fast for extended periods, and then feed (e.g. Cox and Secor 2008). This argues against a role for waterborne amino acid uptake in feeding within decaying carcasses, and instead suggests that the major role of skin nutrient absorption is to maintain some nutritive uptake in between feeding bouts. Such a strategy, in conjunction with low resting metabolic rate (Forster 1990), might facilitate lengthy periods of fasting (Tamburri and Barry 1999).

Kinetic characteristics of uptake may also offer insight into the potential role of the skin in nutrition. Transport affinities, which represent the substrate concentrations required to give half maximal transport rates, would be expected match the environmental levels of substrate. Levels of free amino acids in seawater range from the tens to hundreds of nanomoles per litre, depending on depth, analytical method and seasonal factors (Lu et al. 2014). However, levels of total free amino acids in interstitial waters of sediments, which could be sources of nutrition to burrowing hagfish, can exceed 10 μM (Lee et al. 1992). Transport affinities for branchial and cutaneous amino acid (alanine and glycine) uptake are in the range of 125–465 μM (Glover et al. 2011a). That these values are an order of magnitude higher than the levels likely to be found in constitutive feeding scenarios (water column or sediment), argues against the conclusions drawn in the current study. Unfortunately, there are no measures of available amino acids inside decaying carcasses, but it is likely that these are in the range or even higher than the amino acid uptake affinities determined. Consequently, further research is required to confirm the functional role of waterborne amino uptake acid in hagfish.

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