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# Effect of environmental salinity manipulation on uptake rates and distribution patterns of waterborne amino acids in the Pacific hagfish

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ABSTRACT

Among vertebrates, hagfish are the only known iono- and osmoconformers, and the only species thus far documented to absorb amino acids directly across the skin. In the current study, short-term (6 h) manipulations of exposure salinities (75–125% seawater) were conducted to determine whether changes in osmotic demands influenced the uptake and tissue distribution of waterborne amino acids (alanine, glycine and phenylalanine), in the Pacific hagfish, *Eptatretus stoutii*. No changes in erythrocyte or muscle amino acid accumulation rates were noted, but the patterns of plasma amino acid accumulation were suggestive of regulation. Contrary to expectations, glycine transport across the skin in vitro was enhanced in the lowest exposure salinity, but no other salinity-dependent changes were demonstrated. Overall, this study indicates that uptake and distribution of amino acids varies with salinity, but not in a manner that is consistent with a role for the studied amino acids in maintaining osmotic balance in hagfish.



## 1. Introduction

Hagfish are unique among vertebrate animals in that both the ionic and osmotic concentrations of their extracellular body fluids conform to those of seawater (SW) (Bellamy and Chester-Jones, 1961; McFarland and Munz, 1958; Sardella et al., 2009). This strategy is likely beneficial to the animal as it will minimise diffusive ion and water movements, and the subsequent costs of restoring salt and water balance. There is, however, a complication associated with this approach in that intracellular osmotic concentrations must also balance with extracellular fluid osmolality. The retention of ions to balance intracellular osmolality is not favoured, owing to the inhibitory effects that high ion concentrations have on cellular processes (e.g. disruption of enzyme function; Hochachka and Somero, 2002). Instead, organic osmolytes are generally employed. Teleost and elasmobranch fish accumulate high concentrations of beta amino acids, such as taurine, to balance intracellular osmolality (Goldstein and Kleinzeller, 1987). In contrast, hagfish principally accumulate standard alpha amino acids, with proline and alanine being of particular importance (Cholette and Gagnon, 1973; Fincham et al., 1990).

The intracellular accumulation of alpha amino acids is not the only unusual feature of amino acid handling in the hagfish. These animals are unique among studied vertebrates in their ability to absorb amino acids directly from the water using branchial and cutaneous epithelia (Bucking et al., 2011; Glover et al., 2011a). Recent work indicates that the absorption of waterborne amino acids may be an adaptation that allows hagfish to maintain a basal level of nutrient uptake in quiescent periods between infrequent feeding activities (Glover et al., 2016). Under this scenario hagfish are proposed to absorb amino acids from SW, or from the interstitial water of sediments associated with hagfish burrows. This latter source may contain levels of free amino acids as high as 10 μM (Lee et al., 1992). However, the biological purpose of waterborne amino acid uptake remains unstudied.

There are many possible biological roles for amino acids once absorbed. They can be incorporated into protein, metabolised for energy, and/or utilised for osmotic balance (Cowey, 1994). In the current study we sought to determine whether a short-term experimental salinity manipulation would induce a change in the uptake and/or tissue distribution of waterborne amino acids in the Pacific hagfish, *Eptatretus stoutii* (Lockington, 1878). In hagfish (Cholette et al., 1970; Cholette and

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Gagnon, 1973), teleosts (Fugelli and Zachariassen, 1976), and elasmobranchs (Forster and Goldstein, 1976; Boyd et al., 1977), there is evidence that altering environmental salinity causes changes in intracellular amino acid concentrations, but whether this results in altered amino acid uptake at transport epithelia, is unknown. We hypothesised that changes in intracellular osmolyte demand would alter both uptake and distribution of waterborne amino acids, therefore directly linking cutaneous and branchial absorption to a role in osmotic balance. This is a hypothesis that has been examined in marine invertebrates, with some data supporting the concept of transport linked to osmotic demands (Neufeld and Wright, 1998), and other data refuting the theory (Silva and Wright, 1992). Should evidence for osmotic demand-linked transport exist in hagfish, it could suggest that the dependence on alpha amino acids in hagfish osmoregulation has led to the maintenance or development of this novel route of nutritive uptake in this primitive vertebrate.

## 2. Materials and methods

### 2.1. Animal collection and holding

Adult hagfish ($n = 75$; mass range 40–80 g) were collected (Fisheries and Oceans Canada permit XR107 2013) from Barkley Sound, off the west coast of Vancouver Island, Canada, using baited traps. Following capture, hagfish were transferred to outdoor tarpaulin- covered 500-L tanks, containing flow-through SW at 12 °C, located at Bamfield Marine Sciences Centre (BMSC). Hagfish were left, unfed, for at least one week prior to experimentation. All procedures were approved by the BMSC Animal Care and Use Committee (RS-13-11).

### 2.2. In vivo exposures

In vivo exposures were based on methods first described in Bucking et al. (2011). Hagfish ($n = 6$ for each salinity/amino acid combination) were placed into sealed 600-mL plastic containers consisting of 100% SW (in mM: Na, 492; K, 9; Ca, 12; Mg, 50; Cl, 539; pH 8.0), 75% SW (100% SW diluted with dechlorinated Bamfield tap water (in mM: Na, 0.30; K, 0.005; Ca, 0.14; Mg, 0.05; Cl, 0.23; pH 7.2)), or 125% SW (100% SW with artificial sea salt (Seachem Marine Salt, Madison, GA) added). Each container was individually aerated. The osmolalities (Wescor Vapro 5520, Logan, UT) of these waters averaged (±SEM) 725 (±5), 956 (±3) and 1161 (±39) mmol $kg^{-1}$ for the 75%, 100% and 125% treatments, respectively. To each container either glycine, L-alanine or L-phenylalanine were added from a freshly made stock solution to give an exposure concentration of 10 μM. This exposure concentration is based upon levels of free amino acids in sediment interstitial fluids (Lee et al., 1992), a potential source of amino acids to the hagfish integument. These three amino acids were chosen as all have been previously demonstrated to be absorbed directly from the water by *E. stoutii* (Bucking et al., 2011; Glover et al., 2011a, 2016). To each container radiolabelled amino acid was then added (10–20 μCi; [2.6 3H]-L-phenylalanine (Amersham); $^{3}$H–glycine (Perkin-Elmer); $^{3}$H-L-alanine (Perkin-Elmer)). Triplicate 1-mL water samples were taken at the start and the end of the exposures (6 h), and were counted on a liquid scintillation counter (LS6500; Beckman Coulter) following addition of 5 mL of Optiphase scintillation fluor (Perkin-Elmer). These values were used for the calculation of specific activity (SAct):

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

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

After 6 h, hagfish were removed from the containers, and euthanised by anaesthetic overdose (2 g $L^{-1}$ 3-aminobenzoic acid ethylester; MS222). Blood was withdrawn from the caudal sinus, and spun (5000 rpm, 5 min) to separate plasma and red blood cells. Thereafter, ~1 g samples of medial lateral body muscle and medial gut were removed, digested in 2 N HNO3 at 65 °C for 48 h, before all samples had 5–10 mL of UltimaGold AB scintillation fluor (Perkin-Elmer) added. Radiolabel was measured via a liquid scintillation counter (LS6500; Beckman Coulter), with quench correction based on the external standards ratio approach. Tissue (plasma, red blood cell, muscle and gut) amino acid accumulation was determined as follows:

$$\text{Tissue accumulation}\left(\mathrm{nmol\ g^{-1}\ h^{-1}}\right) = (\mathrm{cpm_t}/\mathrm{SAct})/\mathrm{W}/\mathrm{t} \quad (2)$$

where tissue radioactivity ($cpm_t$) was divided by specific activity (SAct; Eq. (1)), and then divided by sample weight (W) and time of exposure (t; 6 h).

### 2.3. In vitro skin transport assays

Uptake of amino acids across the skin was determined according to the protocol first described by Glover et al. (2011a). Hagfish were exposed to one of three salinities (75, 100 or 125% SW; 6 h; $n = 7$ per salinity) as described above, before being euthanised. A blood sample was removed from the caudal sinus, spun (5000 rpm, 5 min), and the resulting plasma analysed for osmolality (Wescor Vapro 5520, Logan, UT). Three skin sections were then removed from the anterior dorsal surface and each was placed into a separate modified Ussing chamber (one section randomly assigned to each amino acid: alanine, glycine or phenylalanine). This chamber consisted of a 20-mL plastic vial with an aperture cut in the lid. Skin was stretched across the vial opening, and the lid screwed into place. In the inside of the chamber was placed 10 mL of 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; $NaH_2PO4 \cdot H_2O$, 2.06; NaHCO3, 41; and glucose, 5; pH 7.6; Forster and Fenwick, 1994). Two ports were punched in the bottom of the vial, one allowing aeration via a needle bubbler, the other acting as a vent. The vial was then suspended upside-down in 150-mL beakers containing aerated filtered SW (75, 100 or 125% SW; see composition above), 10 μM of "cold" amino acid, and ~1 μCi $mL^{-1}$ of radiolabelled amino acid (see above). No attempt was made to adjust the osmolality of the serosal medium to match the exposure osmolality. Initial and final 1-mL samples of mucosal SW were taken for determination of specific activity (see Eq. (1)). Uptake was determined by accumulation in the skin (following digestion in 2 N $HNO_3$ at 65 °C for 48 h, and addition of 10 mL of UltimaGold AB scintillation fluor; Perkin-Elmer) and in the serosal Ringer (5-mL sample; 10 mL UltimaGold AB scintillation fluor) after 2 h:

$$\text{Uptake}\left(\mathrm{nmol\ \ cm^{-1}\ h^{-1}}\right) = (\mathrm{cpm_s}/\mathrm{SAct})/\mathrm{SA}/\mathrm{t} \quad (3)$$

where $cpm_s$ is accumulation of radioactivity in both the skin and 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).

### 2.4. Statistical analysis

The effects of salinity (75, 100 and 125% SW) and amino acid (alanine, phenylalanine and glycine) on tissue accumulation and skin uptake were determined via two-way analysis of variance (ANOVA), followed by a post hoc Tukey's test (SigmaPlot ver. 11.2). All data were considered significant at an alpha level of 0.05.

## 3. Results

Exposure of hagfish for 6 h to dilute seawater (75% SW) resulted in a decrease in plasma osmolality, while exposure to more concentrated

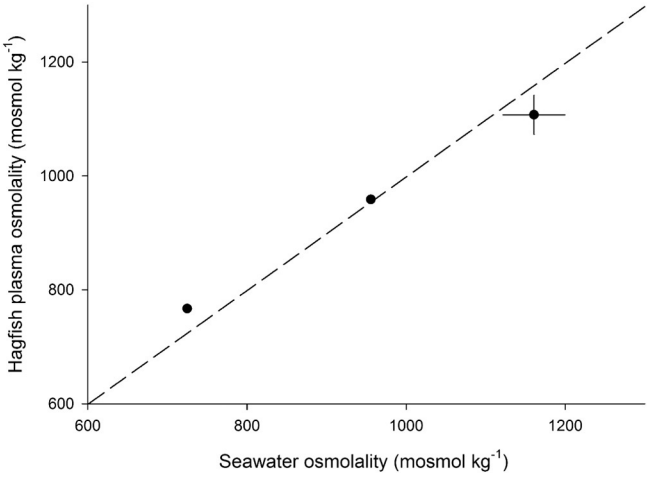


**Fig. 1.** Effect of environmental osmolality on plasma osmolality in Pacific hagfish following a 6 h exposure to one of three exposure salinities (75, 100 or 125% SW). Plotted values represent means ± SEM of seven replicates (note error bars are obscured at two lower osmolalities). The dotted line represents the isosmotic relationship.

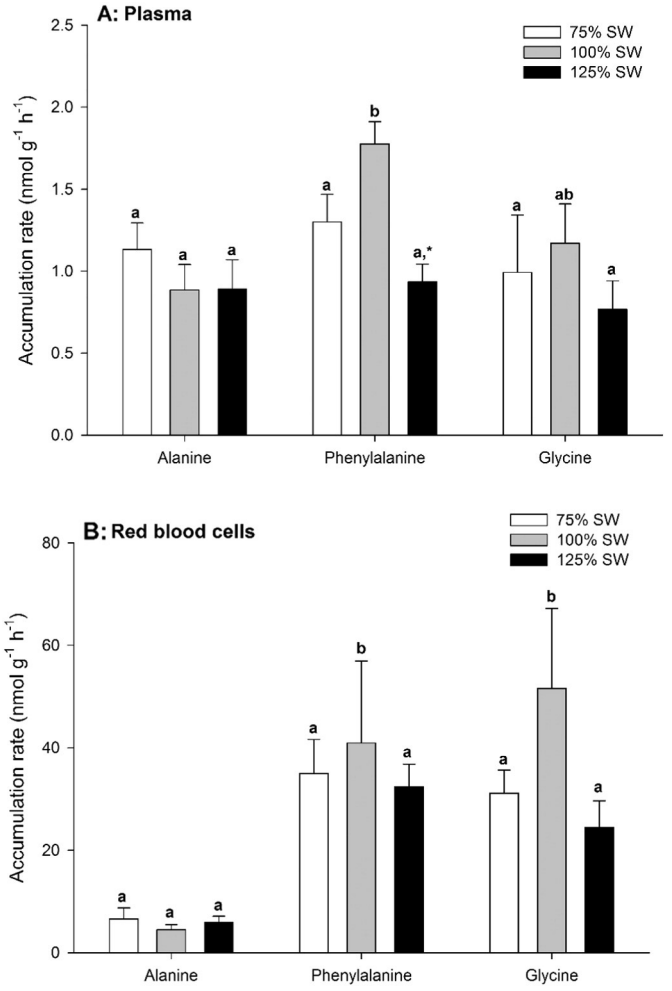


**Fig. 2.** Effect of environmental salinity (75, 100 or 125% SW; 6 h) on the accumulation of waterborne amino acid (alanine, phenylalanine or glycine) in plasma (A) or red blood cells (B) of Pacific hagfish. Plotted values represent means ± SEM of six replicates. Asterisk indicates a significant difference with respect to salinity within an amino acid, while bars sharing letters are not significantly different with respect to amino acids within a salinity. Statistical significance was determined by two-way ANOVA, followed by a post hoc Tukey's test at $\alpha = 0.05$.

seawater (125% SW) led to an increase in plasma osmolality (Fig. 1). The plasma osmolality lay very close to the isosmotic line (where plasma osmolality equals SW osmolality).

Accumulation of amino acids in hagfish plasma was shown to be both salinity- and amino acid-dependent (two-way ANOVA: salinity, $p = 0.041$; amino acid, $p = 0.042$; interaction, $p = 0.263$; Fig. 2A). The accumulation of phenylalanine in plasma was significantly lower in hagfish that had been exposed to 125% SW than those exposed in 100% SW. Phenylalanine accumulation in plasma was higher than that of alanine, but only in hagfish exposed to 100% SW. In contrast, there were no significant salinity-dependent effects on red blood cell amino acid accumulation (two-way ANOVA, $p = 0.233$; Fig. 2B). However, accumulation in red blood cells was amino acid-dependent (two-way ANOVA, $p < 0.001$), with alanine displaying a significantly lower accumulation relative to both phenylalanine and glycine in hagfish exposed to 100% SW. In fact, the rate of alanine accumulation was only 9 and 11% of the rates for phenylalanine and glycine, respectively. The interaction between the two factors (salinity and amino acid) was not significant (two-way ANOVA, $p = 0.500$).

The accumulation of amino acids in muscle tissue was also shown to be independent of salinity, with no significant differences in accumulation rates noted between the hagfish exposed to 75%, 100% or 125% SW (two-way ANOVA, $p = 0.071$; Fig. 3). Conversely, amino acid accumulation rates in muscle after a 6 h exposure were dependent on the amino acid examined (two-way ANOVA, $p < 0.001$). Muscle phenylalanine accumulation was significantly higher than that of both alanine and glycine in hagfish exposed to 100% SW. The interaction between salinity and amino acid was not significant (two-way ANOVA, $p = 0.069$).

In vitro transport of amino acids into and across isolated skin sections of hagfish exposed to the three different salinity conditions tested is displayed in Fig. 4. These data show a significant overall effect of salinity (two-way ANOVA, $p = 0.021$), owing to a significant 2.3-fold increase in glycine uptake in hagfish skin exposed to 75% SW, relative to the 100% SW control. Post hoc analysis showed that there were significant differences between alanine, phenylalanine and glycine with respect to transport across skin sections isolated from hagfish exposed to 100% SW. Phenylalanine and glycine rates of transport were significantly lower than those of alanine.

## 4. Discussion

Hagfish were challenged for 6 h to altered environmental salinity to determine the effect of osmolality on waterborne amino acid uptake and tissue accumulation. We hypothesised that changes in osmotic demand would induce changes in amino acid absorption and distribution if waterborne amino acids are directly utilised for osmotic balance. Specifically, we expected that exposure to 125% SW would result in an increase in amino acid uptake and accumulation in red blood cells and muscle as the hagfish sought to balance intracellular osmolality with elevated environment osmolality. Correspondingly, a decrease in osmotic demand at the lower tested salinity (75% SW) was expected to reduce waterborne amino acid uptake and tissue accumulation. This hypothesis was supported only by the data for plasma accumulation. For example, a decrease in plasma phenylalanine accumulation in 125% SW-exposed hagfish (Fig. 2A) could indicate an increase in absorption into tissues from the plasma. However, the lack of any significant changes in amino acid accumulation rates in red blood cells or muscle argues against a major role for waterborne amino acids in osmotic balance in the Pacific hagfish.

### 4.1. In vivo waterborne amino acid accumulation

The current study confirmed previous work indicating that *E. stoutii* is an osmoconformer (McFarland and Munz, 1958; Sardella et al., 2009), with plasma osmolality matching environmental osmolality (Fig. 1). Changes in plasma osmolality in hagfish are generated by water

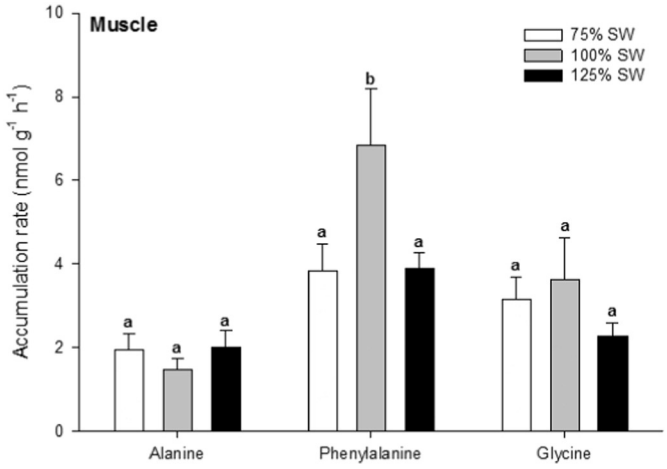


**Fig. 3.** Effect of environmental salinity (75, 100 or 125% SW; 6 h) on the accumulation of waterborne amino acid (alanine, phenylalanine or glycine) in muscle of Pacific hagfish. Plotted values represent means ± SEM of six replicates. Bars sharing letters are not significantly different with respect to amino acids within a salinity. Statistical significance was determined by two-way ANOVA, followed by a post hoc Tukey's test at $\alpha = 0.05$.

movement into, or out of, the plasma (Foster and Forster, 2007; Toop and Evans, 1993). Thus in the current study, exposure to 75% SW caused an increase in plasma volume, resulting in plasma dilution. Consequently, if the uptake of waterborne amino acids into the plasma continued unchanged relative to that in 100% SW hagfish, a decrease in plasma amino acid accumulation rate may have been expected (same amount of amino acid taken up into a greater volume). However, there were no significant changes in plasma amino acid accumulation rates in hagfish exposed to 75% SW. Similarly, in plasma of hagfish exposed to 125% SW, an increase in accumulation rate would have been predicted (same amount of amino acid taken up into a lesser volume), whereas the only significant effect was actually a decrease in phenylalanine accumulation (Fig. 2A).

There are two possible explanations for these patterns. The first is that the patterns of plasma amino acid accumulation were driven by fluxes to or from tissues depending on osmotic demands. However,

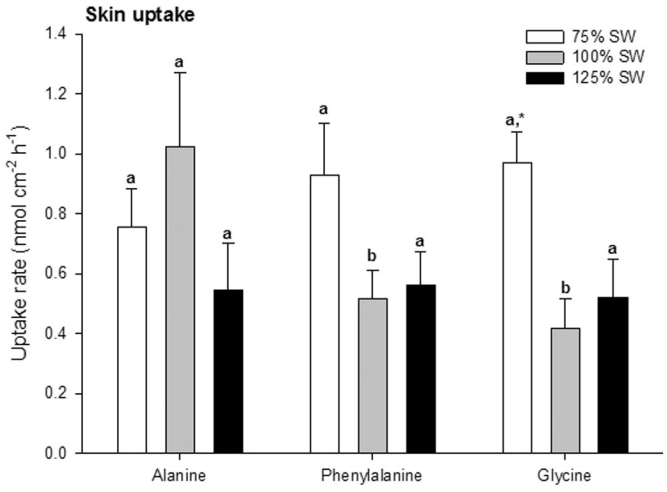


**Fig. 4.** Effect of environmental salinity (75, 100 or 125% SW) on the uptake of waterborne amino acid (alanine, phenylalanine or glycine) into and across isolated anterior skin of Pacific hagfish. Plotted values represent means ± SEM of seven replicates. Asterisk indicates a significant difference with respect to salinity within an amino acid, while bars sharing letters are not significantly different with respect to amino acids within a salinity. Statistical significance was determined by two-way ANOVA, followed by a post hoc Tukey's test at $\alpha = 0.05$.

the changes in plasma amino acids were not mirrored by decreased accumulation of amino acids in red blood cells or muscle of 75% SW-exposed hagfish or increased accumulation in these tissues of 125% SW-exposed animals. It is, however, possible that tissues other than those monitored in the current study may have exhibited altered amino acid accumulation rates on a salinity-dependent basis. The alternative explanation is that environmental salinity induced changes in the epithelial uptake of waterborne amino acids. This alternative explanation is not supported by the changes observed in skin transport in the current study (e.g. an increase in glycine transport at 75% SW). However, branchial transport is known to play a significant role in waterborne amino acid uptake (Glover et al., 2016), but was not measured in the current study. It is therefore possible that the regulation of plasma amino acid accumulation was facilitated by altered transport across the gill.

Overall, the lack of change in muscle and red blood cell accumulation rates of waterborne amino acids (Figs. 2B, 3), argues against an osmotic role for these absorbed substrates. It has been shown that acclimation to elevated salinities does result in an increase in the amino acid concentration of hagfish muscle cells, and vice versa with respect to exposure to dilute SW (Cholette et al., 1970; Cholette and Gagnon, 1973). Unlike the situation in plasma, where water movements primarily contribute to osmotic changes, in muscle cells around 80% of the altered cellular osmotic concentration is believed to occur via modification of cellular osmolytes such as amino acids (Cholette et al., 1970). Thus it is likely that the changes in cellular amino acid demand are not met by altered tissue accumulation of waterborne amino acids, and instead are met by amino acid sources already present in the animal.

The lack of change in red blood cell amino acid accumulation may, however, have a different explanation. In vitro studies show that hagfish erythrocytes respond to hypotonic exposure by swelling, and that there is no subsequent volume regulation response (Nikinmaa et al., 1993). This is distinct from teleost fish red blood cells, which show a strong volume regulation response (Thomas and Egee, 1998). Consequently, there are no changes in cellular amino acid permeability that would act to correct this osmotic imbalance (Fincham et al., 1990). This lack of regulation likely explains the salinity-independence of red blood cell amino acid accumulation in the current study.

While there were no salinity-dependent changes in tissue amino acid accumulation, there were differences in accumulation rates between amino acids. Previous studies examining waterborne amino acid uptake as a function of hagfish fed state showed that phenylalanine accumulated at a higher rate than alanine in most tissues (Glover et al., 2016), a pattern that was consistently observed in the current study in hagfish exposed to 100% SW. This is in contrast to total muscle amino acid concentrations, where phenylalanine has been shown to occur at a lower level than either alanine or glycine (Cholette and Gagnon, 1973; Hwang et al., 2002). This disconnect between accumulation rate and intracellular concentration lends support to the hypothesis that balancing intracellular osmolality is not the major role of waterborne amino acids. If this was the case then it might be expected that the amino acids with the greatest utility as an osmolyte (i.e. those present at the highest concentration), would be taken up at the greatest rate.

It is important to note that under isosmotic conditions hagfish do not drink (Morris, 1965). However, by analogy with hyper-regulating marine teleosts, increasing the salinity of the medium may induce drinking in hagfish, as a mechanism for balancing diffusive water loss. If this occurred, then it is possible that the patterns of amino acid uptake could reflect intestinal absorption (Glover et al., 2011b). However, in the current study the gut tissue was assayed for the presence of radiolabel, and the concentrations were significantly lower in the 125% SW exposed hagfish, than in the hagfish exposed to 100% SW (data not shown), suggesting drinking was unlikely to have occurred. The presence of low concentrations of amino acids in hagfish gut following in vivo exposure has been shown previously, and is likely a consequence of amino acids of waterborne origin being secreted into the bile, and then emptying into the intestine (Glover et al., 2016).

### 4.2. In vitro skin uptake

In an in vitro assay, an increase in glycine uptake was noted into and across the skin isolated from hagfish exposed to 75% SW (Fig. 4). This finding is inconsistent with the hypothesis that when exposed to a hypo-osmotic medium, the hagfish would have a reduced need to sequester intracellular amino acids, and thus would down-regulate transport. There were no other significant salinity-related effects on amino acid transport. As such it is unlikely that the transport of amino acids is regulated by the hagfish in order to match osmolyte demand.

The increase in glycine uptake in skin sections from hagfish exposed to dilute salinities is likely related to the decrease in medium sodium. Epidermal glycine transport has been previously shown to be sodium-dependent, with the removal of mucosal sodium strongly stimulating glycine transport (Glover et al., 2011a). Consequently, the effect on glycine uptake is likely to be a direct effect on transport, rather than an effect related to a homeostatic process. It is noteworthy that this effect is in contrast to the predicted effect of reduced sodium in the exposure medium. Most putative glycine transporters utilise the inwardly-favourable electrochemical gradient for sodium to facilitate glycine uptake (i.e. sodium co-transport; Bröer, 2008). With a reduction in available sodium to power transport, glycine uptake would be predicted to decrease. That glycine uptake does not adhere to this prediction has been attributed to allosteric modulation of the transporter (Glover et al., 2011a). The sodium-independence of alanine transport confirms previous reports in hagfish skin (Glover et al., 2011a), while it appears that phenylalanine transport is also sodium-independent, and as such is consistent with uptake of phenylalanine mediated via the mammalian System T transport family (Mariotta et al., 2012).

The current study lends support to previous work showing that epidermal alanine and glycine uptake in hagfish is mediated by distinct transporters (Glover et al., 2011a). In 100% SW, glycine uptake was significantly lower than that of alanine, while in 75% SW there was a significant increase in glycine uptake relative to the 100% SW control, while a similar effect was not observed for alanine. There has been less work characterising epidermal phenylalanine transport in hagfish, but in the current study the pattern of transport for this amino acid was distinct from that of alanine. In mammals, these two amino acids are known to share transport systems (e.g. b0,+, B0), or to be transported via distinct carriers (e.g. System A for alanine, System T for phenylalanine; Bröer, 2008). Although more work is required to characterise specific uptake pathways for amino acids across hagfish skin, the current study suggests independent carrier-mediated systems are present in the skin for all three amino acids examined herein.

## 5. Conclusion

Overall, the results present no compelling evidence to suggest that the Pacific hagfish takes up waterborne amino acids in order to support intracellular osmolality. Consequently, this nutritive uptake pathway is most likely to play a direct role in the supply of biosynthetic or energy substrates. In fact, it has been hypothesised that the ability of hagfish to absorb amino acids across the skin is an adaptation that facilitates a basal influx of nutrients from sediment interstitial waters, thus supplying hagfish with fuel to sustain long periods of starvation between periods of feeding (Glover et al., 2016).

While there was no evidence that amino acids were specifically sequestered in tissues to act as osmolytes, there was evidence that uptake of amino acids from the water was impacted by environmental salinity. This was provided by the lack of change in plasma amino acid concentration despite dilution and concentration of this compartment, and direct evidence of altered glycine transport across skin taken from hagfish exposed to different water salinities. This finding adds to the body of work showing that waterborne amino acid uptake is altered by environmental factors, such as hypoxia and feeding (Bucking et al., 2011; Glover et al., 2016).

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