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Drinking and water permeability in the Pacific hagfish, *Eptatretus stoutii*

Chris N. Glover[1,2,3] · Chris M. Wood[2,4,5] · Greg G. Goss[2,3]

Abstract Hagfish are osmoconformers, maintaining an internal osmolality that matches their seawater habitats. Hagfish would, therefore, appear to have no physiological need to drink, but previous studies are equivocal regarding whether drinking in hagfish occurs. The current study addressed this knowledge gap, by examining drinking and water permeability in the Pacific hagfish, *Eptatretus stoutii*. One-third of analysed hagfish were shown to accumulate radiolabelled drinking rate markers (tritiated inulin and polyethylene glycol-4000) in their gut tissues; however, this was attributed to the presence of markers in the blood perfusing the digestive tract, following absorption through paracellular pathways at the gill. No accumulation of marker was observed in hagfish subjected to more dilute (75% seawater) or more concentrated (125% seawater) media. Diffusive water efflux, measured by tritiated water washout, was shown to be very high, with 50% of body water exchanged within 14 to 16 min, depending on exposure salinity. In full-strength seawater, the total exchangeable pool of water was 78% of hagfish mass. We conclude that hagfish do not drink, and their high water permeability is likely to result in rapid osmotic equilibration under circumstances where perturbations may occur.

Keywords Drinking · Hagfish · Osmoconforming · Salinity · Water permeability

✉ Chris N. Glover
cglover@athabascau.ca

1 Faculty of Science and Technology and Athabasca River Basin Research Institute, Athabasca University, 1 University Drive, Athabasca, AB T9S 3A3, Canada

2 Bamfield Marine Sciences Centre, Bamfield, BC, Canada

3 Department of Biological Sciences, University of Alberta, Edmonton, Canada

4 Department of Zoology, University of British Columbia, Vancouver, Canada

5 Department of Biology, McMaster University, Hamilton, Canada

Introduction

Hagfish are unique among vertebrates in that they are osmoconformers, maintaining an extracellular fluid composition that closely mimics that of their marine habitat (Bellamy and Chester-Jones 1961; Sardella et al. 2009; Glover et al. 2017). Osmoconforming in a stenohaline marine animal offers several advantages. It reduces the costs of salt and water balance, and therefore contributes towards a very low resting metabolic rate (Forster 1990), an important adaptation to a resource-poor benthic feeding environment (Martini 1998; Glover and Bucking 2016). Osmoconforming also reduces the need to utilise the integument as a barrier limiting ion and water exchange, instead allowing the skin and gill epithelia to adopt physiological roles, such as nutrient absorption (Glover et al. 2011, 2015, 2016; Schultz et al. 2014), waste excretion (Clifford et al. 2015), and acid–base regulation (Clifford et al. 2014).

Another benefit of osmoconforming is that it alleviates the need to drink. Marine teleosts, which are hypo-osmotic to their environments, must drink constantly to replace water lost via diffusion (Takei 2015). While hagfish are not faced with diffusive water loss, they do lose small amounts of water through the production of urine (Riegel 1998; Tait et al. 2009), and drinking has been considered as one possible mechanism by which hagfish could gain water and thus balance these losses (Morris 1965; Rankin 2002). To

date, however, studies of drinking in hagfish are equivocal. Morris (1965) used a dye-based approach combined with a surgical technique, and found that some Atlantic hagfish (*Myxine glutinosa*) swallowed seawater (SW), and others did not. In those animals that imbibed, the changes to the composition of the swallowed SW were inconsistent with a role in osmotic balance, and thus, drinking was considered to be an experimental artifact (Morris 1965). In the Pacific hagfish (*Eptatretus stoutii*), employing a method that avoided surgical intervention, McFarland and Munz (1965) were able to extract fluid from the guts of animals placed in both full-strength and dilute SW, and concluded that drinking had occurred. In a study of a closely related species, *Eptatretus burgeri*, dye-based drinking rate markers were measurable in gut fluids, but the calculated drinking rate was significantly lower than that of marine teleosts measured by a similar technique (Kobayashi et al. 1983).

An alternative to drinking is to have high water permeability, allowing exchange between the animal and the environment to correct any osmotic imbalance. Some authors have noted that hagfish plasma is slightly hyperosmotic to SW (Morris 1965; Cholette et al. 1970; McInerney 1974), which would provide a gradient for water influx, sufficient to replace renal losses (Rankin 2002). Supporting this, hagfish are highly permeable, with water exchange rates significantly greater than those of teleost fish. For example, *E. stoutii* was shown to have a water influx rate constant more than 12-fold greater than that of a marine teleost fish (staghorn sculpin, *Leptocottus armatus*) tested under similar conditions (Rudy and Wagner 1970). However, the rates of water influx for the teleost fish in this study were higher than those measured by other authors (e.g. Evans 1969), suggesting that the value for hagfish water permeability may have been overestimated. Nevertheless, the high permeability of hagfish to water is likely to play an important role in osmotic balance. This is demonstrated by data that show the presence of slime, which will increase the diffusive barrier (Shephard 1994), causing hagfish to become more hyperosmotic to their environments (McFarland and Munz 1958), presumably because of an inability to correct renal water loss.

Given the high permeability of hagfish to water, and the finding that under normal SW salinities there are no changes in the composition of imbibed water consistent with an osmotic role for drinking, the occurrence and/or role of drinking in hagfish remains enigmatic. The current study aimed to examine both drinking and water permeability (diffusive water efflux as assessed by the washout of tritiated water) in the Pacific hagfish, *E. stoutii*. Drinking was investigated using radiolabelled markers. These offer advantages over previously used dye-based techniques in that they offer greater sensitivity and avoidance of false positives associated with the presence of coloured components in the gut (Evans 1968; Dall and Smith 1977). To investigate how drinking and water permeability may change with osmotic challenge, these parameters were also examined in hagfish placed in more dilute (75% SW), or more concentrated (125% SW) media.

Materials and methods

Animal collection and maintenance

Pacific hagfish (*Eptatretus stoutii*) were collected via baited traps from Barkley Sound (GPS co-ordinates 48°50′58, 125°08′02) on the west coast of Vancouver Island, Canada, under permit XR 202 2016 from Fisheries and Oceans Canada. They were then transported to 250-L outdoor holding tanks, receiving flow-through SW at 12 °C, at Bamfield Marine Science Centre (BMSC). Hagfish were held, unfed, for 2–3 weeks before experimentation. All experiments were conducted under a University of Alberta Animal Use Protocol (AUP 00001156), and by approval from the BMSC Animal Care and Use Committee.

Drinking rate

Initially, two different drinking markers were tested: [methoxy-^{3}H]-inulin (Perkin Elmer; MW = 5500; n = 3, mean hagfish mass ($\pm$ SEM) = 62 $\pm$ 7 g) and [1-2-^{3}H]-polyethylene glycol-4000 (PEG; American Radiolabelled Chemicals; MW = 4000; n = 6; mean hagfish mass = 70 $\pm$ 9 g). Hagfish were placed in sealed 1250-mL plastic containers with 1000 mL of filtered 100% SW (in mM: Na, 492; K, 9; Ca, 12; Mg, 50; Cl, 539; pH 8.0), and supplied with gentle aeration, before 3–5 μCi of the markers were added. Initial and final 1-mL water samples were taken for determining radiolabel activity. Hagfish were left in the dark, undisturbed, for 6 h (overnight), before being gently removed from the chambers, and euthanised by anaesthetic overdose (2 g L^{-1}; pH-neutralised 3-aminobenzoic acid ethylester (MS222) in 100% SW). For all hagfish, blood samples (~1 mL) were taken from the caudal sinus, but for those hagfish exposed to ^{3}H-PEG, a blood sample was also taken from the dorsal aorta, following a longitudinal ventral body wall incision exposing the digestive tract. Ligatures were then tied at the distal and proximal ends of the gut. The proximal ligature was placed just posterior to the bile duct. The entire gut was then excised, split into three approximately even portions, and weighed, taking care not to lose any fluids. Each individual gut section was then digested in 10 mL of 2 N HNO$_3$ at 65 °C for 72 h, before 10 mL of scintillation fluor (ACS, GE Healthcare) was added to 2 mL samples of the digest, and the samples were counted on a liquid scintillation counter (LS6500; Beckman Coulter).

Drinking rate was also assessed in hagfish exposed to 75% SW (filtered 100% SW diluted with dechlorinated BMSC tap water (in mM: Na, 0.30; K, 0.005; Ca, 0.14; Mg, 0.05; Cl, 0.23; pH 7.2); $n=6$; mean hagfish mass $=67\pm5$ g), and 125% SW (filtered 100% SW with artificial SW salts added; Instant Ocean; $n=6$, mean hagfish mass $=62\pm6$ g). These studies were conducted as detailed above, except for the difference in exposure salinity (and anaesthetic solution salinity, which was matched to exposure salinity). In these tests, only sinus blood samples were collected, and only the ^{3}H-PEG marker was used. Hagfish were transferred directly from their holding salinity (filtered 100% SW) into exposure chambers.

A 'limit of blank' was determined by scintillation counting of gut digests of hagfish that had not been exposed to radiolabel. All counts were initially subjected to a quench correction via the external standards ratio approach. If the quench-corrected counts of gut digests of hagfish exposed to the drinking rate markers were higher than the value of the mean plus three times the standard deviation of the quench-corrected counts of the blanks (Armbruster and Pry 2008), then drinking was considered to have occurred. Drinking rate was then calculated as follows:

$$\text{Drinking rate} \left(\mu L\ g^{-1}\ h^{-1}\right) = \left(cpm_t \times cpm_w^{-1}\right) \times W^{-1} \times t^{-1}$$

where cpm_t is the quench-corrected tissue counts per minute (sum of all three gut sections), cpm_w is the counts per minute per μL of exposure water, W is hagfish mass (g), and t is time (h).

Blood samples were centrifuged ($5000\times g$, 2 min), and plasma removed and weighed. A 20-μL aliquot of plasma was taken for assessment of plasma osmolality (Wescor Vapro 5520), and the rest was added to 2 mL of scintillation fluor (ACS, GE Healthcare), and counted (LS6500; Beckman Coulter). Plasma accumulation of radiolabel ($\mu L\ mL^{-1}\ h^{-1}$) was calculated as described above, except mass was replaced by plasma sample volume in mL.

Diffusive water efflux

Individual hagfish were placed in aerated, 1250-mL sealed plastic chambers, each containing 1000 mL of filtered 100% SW and 10 μCi of tritiated water (Perkin Elmer). Hagfish were left in these chambers for 6–8 h to ensure tissue equilibration of radiolabelled and "cold" water. Triplicate 1-mL water samples were taken for assessment of radiolabel activity. Hagfish were then gently removed from chambers via a net, and rinsed for approximately 1 min in a clean, unlabelled, 100% SW water bath to minimise carry over of label to the efflux chamber. Hagfish were then gently placed in clean 1250-mL sealed plastic chambers containing 1000 mL of unlabelled 75% ($n=6$; mean hagfish mass ($\pm$S.E.M.) $=47\pm4$ g), 100% ($n=6$; mean hagfish mass $=49\pm7$ g) or 125% ($n=6$; mean hagfish mass $=33\pm3$ g) SW. Triplicate 1-mL samples were then taken at times 2, 5, 10, 30, 60, 120, and 360 min following introduction to the efflux chamber, to measure the appearance of label in the water. None of the hagfish produced slime at any stage of the experiment.

Water samples were added to 5 mL of scintillation fluor and counted as described previously. Efflux was calculated as:

$$\text{Efflux} \left(mL\ g^{-1}\right) = \left(cpm_a \times cpm_i^{-1}\right) \times W^{-1}$$

where cpm_a is the counts per minute per mL appearing in the water, cpm_i is the counts per minute per mL of the initial incubation medium, and W is hagfish mass (g). The relationship between efflux of water from the hagfish and time allowed calculation of the total exchangeable water pool (maximal efflux) and the time to achieve half maximal efflux. Based on the maximal efflux for each hagfish, the unexchanged water (tritiated water remaining in the hagfish) was calculated (maximal water efflux (mL) minus cumulative water efflux (mL) at a given time point). When the unexchanged water values were subjected to a natural logarithm transformation and plotted against time, the slope of the linear portion of the curve (over the first 10 min) was used to determine the initial rate of body water efflux per minute.

Statistical analysis

All data were subjected to tests of normality and homogeneity of variance, using the Kolmogorov–Smirnov and Levene's test, respectively. If data failed these tests, nonparametric approaches were used. Differences between rates of drinking marker accumulation in plasma, and between parameters of water efflux in different salinities, were assessed via a one-way ANOVA, followed by a Tukey's post-hoc test. Differences in drinking rates between markers, and between plasma accumulation in central and sinus blood, were assessed by a Mann–Whitney U test. All data were deemed significant at an α level of 0.05, and are reported as means $\pm$ S.E.M (N).

Results

Of the 21 hagfish used for assessment of drinking rate, only 7 accumulated radiolabel in their gut (Table 1). All 7 of these hagfish were exposed to drinking markers in 100% SW, and no hagfish in either 75% SW or 125% SW accumulated markers above the level of the blank. This situation occurred despite the fact that hagfish exposed to different salinities exhibited clear alterations in plasma

Table 1 Apparent "drinking" parameters in Pacific hagfish

Drinking marker	Salinity	Number of hagfish	Number of "drinking" hagfish	"Drinking" volume per hagfish (μL)	"Drinking" volume per "drinking" hagfish (μL)	"Drinking" rate per hagfish (μL g^{-1} h^{-1})	"Drinking rate" per "drinking" hagfish (μL g^{-1} h^{-1})
^{3}H-inulin	100% SW	3	3	12 ± 6	12 ± 6	0.03 ± 0.01	0.03 ± 0.01
^{3}H-PEG	75% SW	6	0	0	0	0	0
	100% SW	6	4	2 ± 1	4 ± 1	$0.01 \pm 0.00*$	$0.01 \pm 0.00*$
	125% SW	6	0	0	0	0	0
Total		21	7	3 ± 1	8 ± 3	0.01 ± 0.00	0.02 ± 0.01

* Significant ($\alpha = 0.05$) difference relative to the ^{3}H-inulin treatment group, as determined by the Mann–Whitney U test
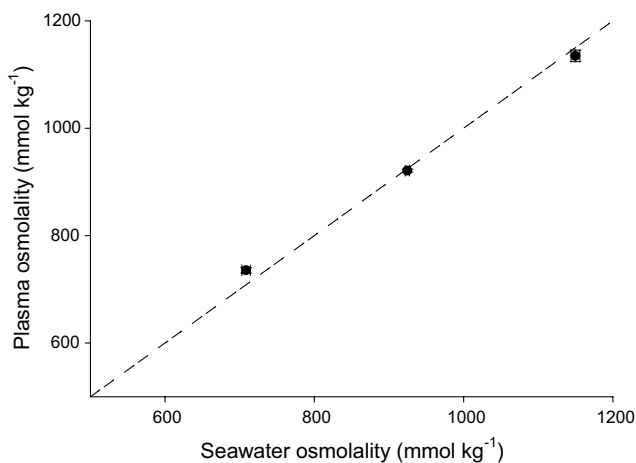

Fig. 1 Effect of environmental osmolality on plasma osmolality in Pacific hagfish following a 6-h exposure to ^{3}H-PEG in one of three salinities (75, 100, or 125% SW). *Plotted values* represent means $\pm$ S.E.M. of six replicates (note that error *bars* are obscured by the *plotted points*). The *dotted line* represents the isosmotic relationship
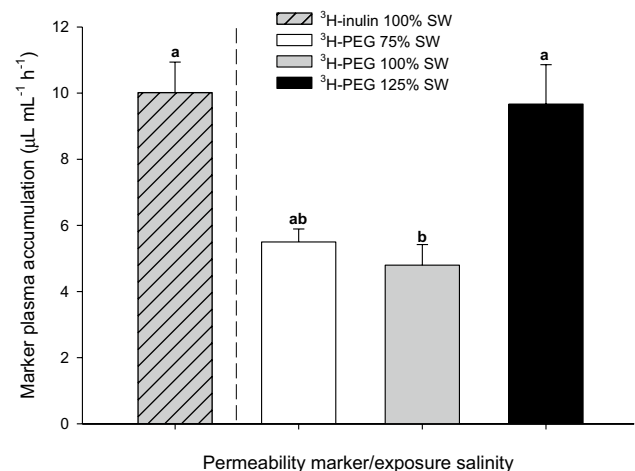

Fig. 2 Accumulation of drinking rate markers ^{3}H-inulin or ^{3}H-PEG in caudal sinus plasma of Pacific hagfish following a 6-h exposure in one of three salinities (75, 100, or 125% SW). *Plotted values* represent means $\pm$ S.E.M. of six (^{3}H-PEG) or three (^{3}H-inulin) replicates. *Bars sharing letters* are not significantly different ($\alpha = 0.05$), as determined by one-way ANOVA, followed by Tukey's post-hoc test

osmolality that conformed to those of the exposure salinity (Fig. 1). Hagfish exposed to the marker ^{3}H-inulin appeared to "drink" more than those hagfish exposed to the marker ^{3}H-PEG (12 ± 6 vs. 2 ± 1 μL), but this effect was not statistically significant ($p = 0.227$). Overall, those hagfish that appeared to "drink" imbibed 8 ± 3 μL, a value that fell to 3 ± 1 μL, if all hagfish (those that "drank" and those that did not) were included in the calculation. When accounting for hagfish mass and time of exposure, the apparent "drinking rate" of "drinking" hagfish was 0.02 ± 0.01 μL g^{-1} h^{-1}, and for all hagfish was 0.01 ± 0.00 μL g^{-1} h^{-1} (Table 1).

All hagfish exposed to drinking markers did, however, exhibit accumulation of radiolabel in plasma (Fig. 2). Hagfish exposed to ^{3}H-inulin in 100% SW accumulated 10.0 ± 0.9 μL mL^{-1} h^1, a significantly higher concentration than hagfish exposed in the same salinity but to ^{3}H-PEG (4.8 ± 0.6 μL mL^{-1} h^1; $p = 0.027$). Hagfish exposed to ^{3}H-PEG in 75% SW displayed marker accumulation

equal to that in 100% SW (5.5 ± 0.4 μL mL^{-1} h^1), but hagfish exposed to 125% SW exhibited ^{3}H-PEG accumulation rates significantly higher than those exposed at 100% SW (9.7 ± 1.2 μL mL^{-1} h^1; $p = 0.015$; Fig. 2).

In hagfish exposed to ^{3}H-PEG in 100% SW, plasma samples were taken from both the caudal sinus and centrally, from the dorsal aorta. Plasma from the central circulation displayed significantly higher rates of marker accumulation than caudal sinus samples (19.7 ± 5.6 vs. 4.8 ± 0.6 μL mL^{-1} h^1; $p = 0.024$; Fig. 3).

Diffusive water efflux from hagfish increased with time, with this pattern being independent of exposure salinity (Fig. 4a). From these curves, the total exchangeable water pool and the time taken to exchange half of this pool could be determined. The exchangeable pool was significantly lower in hagfish in 75% SW, than in hagfish in 100 or 125% SW (0.66 ± 0.01 vs. 0.78 ± 0.02, and 0.81 ± 0.03 mL g^{-1}, respectively; ANOVA, $p < 0.001$; Table 2). Conversely, the
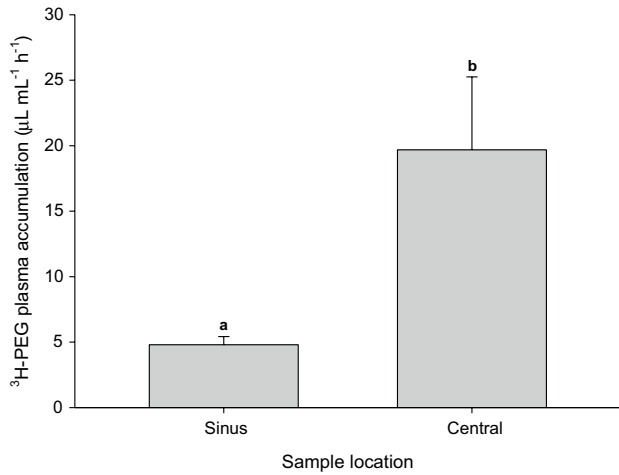
Fig. 3 Accumulation of ^{3}H-PEG in central or caudal sinus plasma of Pacific hagfish following a 6-h exposure in 100% SW. Plotted values represent means ± S.E.M. of six replicates. *Bars* sharing *letters* are not significantly different ($\alpha = 0.05$), as determined by the Mann–Whitney *U* test
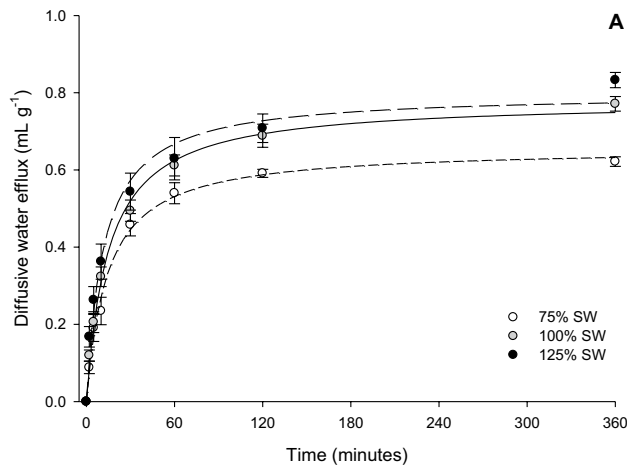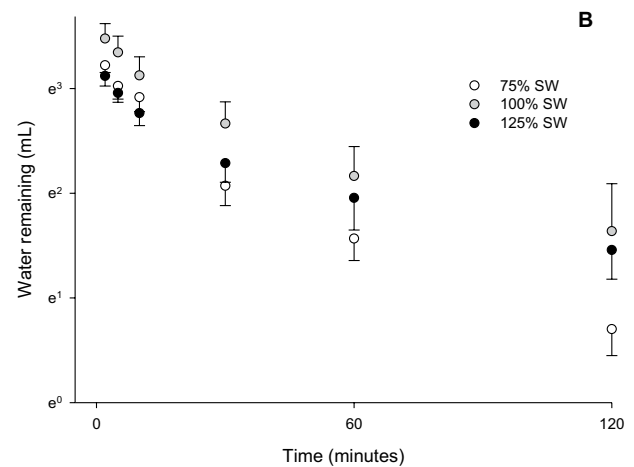
time to achieve a 50% efflux of body water was not significantly different between the three salinities (ANOVA, $p = 0.863$; Table 2), with all exhibiting a time of between 14.1 and 16.0 min (Table 2). Based on disappearance of tritiated water from the animal, hagfish exposed to all three salinities showed similar trends of water efflux (Fig. 4b). Based on slopes of the relationship between water remaining in the animal and time (over the first 10 min), all three groups displayed the initial rates of water efflux between 953 and 961 µL min^{-1}; which were statistically indistinguishable (Table 2; ANOVA, $p = 0.818$). For comparison to the previous research (Rudy and Wagner 1970), mass-specific water efflux rates at 5 min for hagfish in the current study were 2287 ± 420, 2469 ± 324, and 3157 ± 417 µL g^{-1} h^{-1}, at 75% SW, 100% SW, and 125% SW, respectively (Table 2), with no significant differences as a function of salinity (ANOVA, $p = 0.300$). Water efflux rates at the time where half the total body water pool was exchanged were 1505 ± 300 (75% SW), 1618 ± 227 (100% SW), and 2153 ± 610 (125% SW) µL g^{-1} h^{-1} (Table 2), and again,

Fig. 4 Mass-specific diffusive water efflux (**a** mL g^{-1}; based on appearance of tritiated water in exposure chamber) and retention of tritiated water (**b** mL; calculated remaining internal pool based on total efflux) in Pacific hagfish exposed to one of three salinities (75, 100, or 125% SW). *Plotted values* represent means ± S.E.M. of six replicates

Table 2 Water efflux parameters in Pacific hagfish as a function of salinity

	Salinity		
	75% SW	100% SW	125% SW
Exchangeable water pool (ml g^{-1})	0.66 ± 0.01[a]	0.78 ± 0.02[b]	0.81 ± 0.03[b]
Time to achieve half maximal water efflux (T$_{0.5}$; min)	15.8 ± 2.9[a]	16.0 ± 2.1[a]	14.1 ± 2.7[a]
Initial rate of water efflux (µL min^{-1})	961 ± 10[a]	953 ± 7[a]	956 ± 10[a]
Mass-specific efflux rate at 5 min (µL g^{-1} h^{-1})	2287 ± 420[a]	2469 ± 324[a]	3157 ± 417[a]
Mass-specific efflux rate at T$_{0.5}$ (µL g^{-1} h^{-1})	1505 ± 300[a]	1618 ± 227[a]	2153 ± 610[a]

Values sharing letters within a row are not significantly different ($p = 0.05$) as determined by one-way ANOVA, followed by Tukey's post-hoc test

no significant salinity-related differences were discerned (ANOVA, $p = 0.484$).

Discussion

Do hagfish drink?

In the current study, drinking rate markers were present in the digestive tracts of one-third of hagfish investigated. Depending on whether the drinking rate calculation was based on all hagfish, or only those that showed accumulation, the mean apparent "drinking rate" averaged 0.01 or 0.02 $\mu L\ g^{-1}\ h^{-1}$, respectively. In a previous study in *Myxine glutinosa*, Morris (1965) showed similar variability with respect to the presence of the drinking rate marker phenol red in hagfish guts (5 of 13 animals), but those hagfish that did swallow water were shown to do so at a much higher rate than those in the current study (2.7 $\mu L\ g^{-1}\ h^{-1}$, average of all hagfish). Analysis of the gut fluid indicated that its composition was highly variable and unable to be explained in terms of a role in osmotic balance (Morris 1965). It was therefore suggested that drinking was likely an artifact of the surgical method used to prevent fluid escaping the gut (Morris 1965). In a study using the appearance of dye in the gut fluid as a proxy of drinking rate, the hagfish *E. burgeri* was shown to imbibe water at a mean rate of 0.52 $\mu L\ g^{-1}\ h^{-1}$ (Kobayashi et al. 1983). This rate was higher than that measured in an elasmobranch fish (banded dogfish, *Triakis scyllia*; 0.03 $\mu L\ g^{-1}\ h^{-1}$), but was lower than those for all the marine teleost fish examined in the same study (range 0.75 to 12.7 $\mu L\ g^{-1}\ h^{-1}$; Kobayashi et al. 1983). Consequently, it was concluded that hagfish do not drink (Kobayashi et al. 1983), consistent with the finding of Morris (1965). That the apparent rates of "drinking" in the current study are more than an order of magnitude lower than those measured previously, coupled with arguments presented below, lends support to this conclusion.

The conclusion that hagfish do not drink is, however, in conflict with the outcomes of one previous study. In Pacific hagfish, McFarland and Munz (1965) showed that fluid accumulated in the guts of animals placed in full-strength SW. They also observed that when exposed to dilute SW, a reduced volume of gut fluid resulted. In teleost fish, it is well established that under conditions where the animals are hyperosmotic to their surroundings, and osmotic water influx is occurring across gill and skin epithelia, drinking is greatly reduced or stops (Takei 2015). The decrease in drinking rate in dilute SW measured by McFarland and Munz (1965) was consistent with this prediction, and hinted at a role for drinking in osmotic balance. This finding is contrary to the current study where no accumulation of radiolabel in the gut could be detected in either dilute or concentrated SW (Table 1). However, it is possible that drinking was a function of the methodological approach used.

In the McFarland and Munz (1965) study, the authors collected gut fluid by applying posteriorly directed pressure to the midsection, which resulted in a jet of fluid being expelled from the anus. It is therefore possible that at least a component of this fluid was urine, which is excreted via the anus following collection at the terminus of the archinephric ducts (Weinrauch et al. 2016). This is supported by evidence of a significant decrease in gut fluid calcium and magnesium relative to the exposure medium, which is consistent with the known capacity of the archinephric duct to resorb these ions (Alt et al. 1981). It was also noted that not all hagfish contained a dye marker used to determine that the source of the gut fluid was the external medium (McFarland and Munz 1965). It is therefore possible that a component of the drinking measured by these investigators may have been urine rather than swallowed SW.

It is important to note that that in the current study, not even the placement of hagfish in a concentrated medium, a manipulation that increased plasma osmolality (Fig. 1), was able to induce drinking (Table 1). In fish species regularly exposed to environments that vary in salinity, similar exposures to hyperosmotic media will induce drinking, through the stimulus of feedback pathways such as the renin-angiotensin system (Nobata et al. 2013). However, fish that rarely, if ever, naturally encounter salinity changes, have little need for such a response. As such, stenohaline species such as hagfish appear not to possess mechanisms that induce drinking (Kobayashi et al. 1983), even at the expense of dehydration.

Methodological considerations

As noted above, methodology is a key factor influencing the different outcomes in studies that have examined drinking in hagfish. Morris (1965) employed a surgical technique to ensure that any imbibed water did not exit through the digestive tract. However, surgery will cause stress, which, in turn, can lead to increased drinking, at least in teleost fish (e.g., Best et al. 2003; Grosell et al. 2004; Smith et al. 2007). All previous works examining drinking in hagfish have been conducted by dissolving low concentrations of dye (phenol red or carmine particles) into SW, and measuring appearance in the gut fluid (McFarland and Munz 1965; Morris 1965; Kobayashi et al. 1983). However, the extraction of dye can be complex, and measurement can be confounded by the presence of coloured secretions (e.g., bile and chyme) that interfere with colorimetric determinations (Evans 1968; Dall and Smith 1977). For this reason, radiolabelled techniques, such as those employed in the current

study, are generally favoured for drinking rate assessment (Evans 1968).

However, radiolabelled approaches also have their limitations. In the current study, the drinking rate markers were both beta emitters. These are subject to quenching in tissues, a phenomenon that reduces their signal, and therefore makes them more difficult to detect relative to background (Dall and Smith 1977). As a consequence, it is possible that the method employed in the current study may have failed to detect the presence of small volumes of imbibed sea water in the hagfish gut, while the previous dye-based techniques may have overestimated "drinking". However, it is unlikely that any underestimation in the current study would be so significant as to impact the conclusion that hagfish do not drink. Nevertheless, the use of impermeable gamma-emitting markers, which produce stronger signals unimpeded by quenching (Dall and Smith 1977), may be of future value.

Drinking marker permeability

Markers of drinking have the characteristics of being inert and impermeable, meaning that they should only be detected in the gut following swallowing, and that their concentration should reflect the rate of drinking (Evans 1968; Dall and Smith 1977). However, both ^{3}H-inulin and ^{3}H-PEG were found in the plasma of all studied hagfish, independent of whether the marker was also found in the gut tissues. This indicates that the markers are capable of being absorbed directly from the water, likely by the gill or skin epithelia. Indeed, PEG-4000 is used in fish as a marker of branchial paracellular permeability (e.g. Robertson and Wood 2014; Robertson et al. 2015). The appearance of drinking rate markers in the blood is, therefore, likely to explain the low rate of drinking measured in the current study. Whole gut tissue was taken for analysis of drinking rate, and this therefore included blood perfusing the tissue. Although the gut is not heavily vascularised in hagfish (Jansson and Falkmer 1998), the presence of radiolabel within the blood would likely have been sufficient to lead to detection of marker in gut tissues. Supporting this, a simple regression analysis between plasma marker concentration and "drinking rate" for all hagfish exposed in 100% SW (i.e., the exposure group where a value could be ascribed for drinking), yielded a positive linear relationship with an r^2 value of 0.86 (data not shown). Furthermore, taking values for plasma volume (red cell space) for the gut of *E. stoutii* measured by Forster and colleagues (2001), and plasma marker concentration from the current study, a rough calculation was conducted to predict gut marker accumulation, assuming the presence of marker arises from blood perfusion of the gut. This analysis yielded an average predicted "drinking" value for 100% SW hagfish

that closely matched the measured gut accumulation in these animals (0.013 μL g h^{-1} predicted, 0.014 μL g h^{-1} measured).

The presence of "drinking" only in 100% SW may, therefore, be explained by factors that relate to gut blood flow. For example, the relatively higher blood volumes of hagfish in dilute salinities (e.g., Cholette et al. 1970) may have diluted the marker in hagfish assayed in 75% SW, while under conditions of hyperosmotic stress (125% SW), where blood volume is limited, a decline in gut blood flow could lead to a decrease in marker. Although in the current study no dilution of the marker in plasma of hagfish placed in 75% SW was observed, this could be due to the sampling location of the blood (see below).

There are several notable findings regarding the appearance of drinking rate markers in the plasma of hagfish. First, inulin was shown to accumulate in plasma at higher concentrations than PEG, indicating that inulin is more permeable to hagfish epithelia. Theoretically, the higher molecular weight of inulin (5500) should result in lower permeability, relative to the smaller PEG (MW 4000). However, similar relative patterns of permeability have been observed for these markers in the intestine of rats (Ghandehari et al. 1997), and in the urinary bladder epithelium of SW-adapted rainbow trout (Beyenbach and Kirschner 1976). This pattern is thought to relate to the more compact structure of inulin, which allows it to more easily access paracellular pathways (Ghandehari et al. 1997). Second, concurrent sampling of sinus blood and central circulation blood showed that there was a greater accumulation of ^{3}H-PEG in the central circulation, relative to plasma collected from the subcutaneous sinus. The central and the sinus circulations are connected, with the subcutaneous sinus being joined to the central circulation via anastomoses between lymph vessels and blood vessels in the skin (Lomholt and Franko-Dossar 1998). Differences in properties between these two compartments have been noted previously. For example, the haematocrit of sinus blood is usually considerably lower than that of central blood (Forster et al. 1989). The higher concentration of marker in the central circulation suggests that most of the marker has gained access to the blood via the gills, and has remained 'trapped' centrally rather than being evenly distributed throughout the blood. Markers such as PEG may, therefore, be of considerable future value in examining the relationship between the central and sinus circulations in hagfish.

Water permeability

The Pacific hagfish was shown to have a very high water permeability, confirming the findings of Rudy and Wagner (1970). Using a technique based on water influx over 5 min, these authors calculated an influx rate of 2287 μL g^{-1} h^{-1}

in *E. stoutii*. The current study examined water permeability in the efflux direction, and when calculated over a 5-min time interval, a value of 2469 μL g^{-1} h^{-1} was recorded, in close agreement with Rudy and Wagner ([1970]).

The diffusive water efflux values for hagfish are considerably higher than those observed for teleost fish. Based on the initial slope (first 10 min) of the relationship between the amount of radiolabelled water remaining in 100% SW hagfish and time, these animals turn over 176% of their total body water in an hour. In freshwater fish, diffusive water efflux occurs at a rate between 43 and 45% per hour (Liew et al. [2013]), while marine teleosts display hourly efflux rates in the order of 20% (Motais et al. [1969]). Water turnover rates in the current study therefore indicate that hagfish are approximately four and eightfold more permeable to diffusive water flux than freshwater and marine teleost fish, respectively.

Among the water efflux parameters measured in the current study, the only one that varied as a function of water salinity was the total exchangeable body water pool, which was significantly lower in hagfish where water efflux was examined in 75% SW, relative to hagfish examined in either 100% or 125% SW (Table 2). When placed in dilute SW, hagfish are faced with an immediate osmotically driven diffusive influx of water (e.g., Cholette et al. [1970]). This would act to dilute the radiolabelled water, leading to a lower calculated exchangeable water pool value, at least over the short period of exposure to hypotonic water in the current study (6 h). This work therefore provides indirect evidence of volume loading in hagfish, supporting previous studies. For example, hagfish exposed to dilute salinities take around 48 h to return mass (and thus volumes) back to starting values (Toop and Evans [1993]), while there is also evidence for a lack of regulation of cellular transport to correct changes resulting from water influx (Nikinmaa et al. [1993]; Glover et al. [2017]).

Despite an increase in exposure salinity (125% SW), hagfish water efflux characteristics remained unchanged. This suggests that hagfish have little capacity to regulate these exchanges, a finding consistent with previous research (Cholette et al. [1970]; Sardella et al. [2009]; Toop and Evans [1993]). It is notable that the total exchangeable pool of water in hagfish exposed in 100% SW was 78.5 mL 100 g^{-1}. This matches closely to the previous estimates of hagfish water content (74.6%), based on drying whole hagfish to a constant weight (McCarthy [1976]).

Conclusion

The current study provided no compelling evidence for the occurrence of drinking in the Pacific hagfish (*Eptatretus stoutii*). The appearance of radiolabelled drinking markers in gut tissue was likely due to the presence of these markers in blood perfusing the gut. However, hagfish were shown to exhibit very high permeability to water, confirming previous work. This high permeability is likely a function of the osmoconforming strategy of hagfish, which minimises net flux between body fluids and the environment, and thus reduces the need for the skin to act as a diffusive barrier (Glover et al. [2013]). This, in turn, has allowed hagfish to take advantage of skin and gill epithelia for the purposes of nutrient acquisition, a function that is largely incompatible with epithelia that serve as barriers. Furthermore, if any osmotic imbalances do occur (e.g. loss of water through urine), then the high permeability of hagfish integument will allow these imbalances to be corrected, without the need to drink.

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