Measuring intestinal fluid transport *in vitro*: Gravimetric method versus non-absorbable marker

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Abstract

The gut sac is a long-standing, widely used *in vitro* preparation for studying solute and water transport, and calculation of these fluxes requires an accurate assessment of volume. This is commonly determined gravimetrically by measuring the change in mass over time. While convenient this likely under-estimates actual net water flux (J_v) due to tissue edema. We evaluated whether the popular *in vivo* volume marker [^{14}C]-PEG 4000, offers a more representative measure of J_v *in vitro*. We directly compared these two methods in five teleost species (toadfish, flounder, rainbow trout, killifish and tilapia). Net fluid absorption by the toadfish intestine based on PEG was significantly higher, by almost 4-fold, compared to gravimetric measurements, compatible with the latter under-estimating J_v. Despite this, PEG proved inconsistent for all of the other species frequently resulting in calculation of net secretion, in contrast to absorption seen gravimetrically. Such poor parallelism could not be explained by the absorption of [^{14}C]-PEG (typically <1 %). We identified a number of factors impacting the effectiveness of PEG. One was adsorption to the surface of sample tubes. While it was possible to circumvent this using unlabelled PEG 4000, this had a deleterious effect on PEG-based J_v. We also found sequestration of PEG within the intestinal mucus. In conclusion, the short-comings associated with the accurate representation of J_v by gut sac preparations are not overcome by [^{14}C]-PEG. The gravimetric method therefore remains the most reliable measure of J_v and we urge caution in the use of PEG as a volume marker.

Keywords: gut sac, PEG 4000, polyethylene glycol, teleost, water.

Introduction

The *in vitro* gut sac preparation, involving longitudinal sections of the intestine ligated at one end and filled and emptied at the other *via* a catheter, is a long standing and popular method for the study of intestinal transport that is still used to address current physiological questions (Hamilton, 2014). With relative technical ease gut sacs permit the simultaneous, straightforward measurement of intestinal solute and water fluxes, and can also be modified to enable the monitoring of electrophysiological parameters (Grosell et al. 2005). Introduced in its everted form by Wilson and Wiseman in 1954 using segments of the mammalian small intestine, the first published investigations employing this methodology with teleost models appeared 10 years later (House and Green, 1963; 1965; Smith, 1964). This approach has since been used extensively to investigate various aspects of fish physiology, finding particular application in the study of osmoregulation (Collie and Bern, 1982; Veillette et al. 1995; Grosell et al. 2005) and ecotoxicology (Grosell et al. 1999; Nadella et al. 2006; Ojo and Wood, 2007).

Over the course of an experiment the concentrations of solutes within the gut sac will change, not only as they are transported by the epithelium, but also as a result of water movement into or out of the sac. Calculating the net flux of a solute therefore requires a sufficiently accurate measurement of fluid volume within the sac. This change in volume is most commonly obtained gravimetrically and involves lightly blotting the outer surface of the sac before weighing. Subsequently, the change in overall mass during the experiment is representative of the change in mucosal fluid volume and used to calculate fluid transport rate, J_v (Collie and Bern, 1982; Grosell et al. 1999; 2005; Marshall et al. 2002).

One shortcoming associated with the gravimetric method is that the blotting process alone can change the mass of the gut sac (House and Green, 1965), which can substantially influence the recorded volume. Irregularities may therefore arise depending on the consistency of the blotting method used. Secondly, one of the most significant issues is tissue edema (Parsons and Wingate, 1961; Smith, 1964; Jackson and Cassidy, 1970), due to the absence of functioning vasculature leading to the consequent intra-epithelial accumulation of fluid (Lee 1961; 1963; LeFevre et al. 1970; Levine et al. 1970). The gravimetric method is therefore likely to underestimate actual J_v since only fluid that has actually traversed the outer layers of the tissue is being measured. These issues have therefore led to the consideration of non-absorbable volume markers as an

alternate method of calculating volume change that should, in theory, provide a more accurate representation of fluid transport.

Polyethylene glycol (PEG) is an inert, synthetic polymer commonly used as a volume marker. High molecular weight PEGs (3350-4000) are considered to be poorly absorbed by the intestine (Schedl, 1966; Krag et al. 1975; Winne and Gorig, 1982; Furuichi et al. 1984; Schiller et al. 1997; Grosell and Genz, 2006), and easily determined in biological fluids by direct assay (Malawer and Powell, 1967) or labelling the PEG molecule with a radioisotope such as ^{3}H or ^{14}C (Wingate et al. 1972; Krag et al. 1975). The latter offers the greatest sensitivity and practical advantage, hence radiolabelled PEG has become a common choice in studies of gastrointestinal physiology, such as gut transit and digestibility in ruminants (Till and Downes, 1965; Pickard and Stevens, 1972), drinking rates in fish (Shehadeh and Gordon, 1969; Scott et al. 2006; Genz et al. 2008), and is widely regarded as a suitable volume marker for studies of intestinal absorption *in vivo* (Jacobson et al. 1963; Schedl, 1966; Maddrey et al. 1967; Schiller et al. 1997).

Using a volume marker such as PEG in gut sacs should eliminate errors concerning the intra-epithelial accumulation of fluid and inconsistencies associated with blotting. However, examples of volume markers being successfully used in gut sac preparations are scarce and appear limited to ^{14}C-inulin (Aull, 1966) and more recently ^{14}C-PEG 4000 (Genz and Grosell, 2011; Grosell and Genz, 2006; Grosell and Taylor, 2007; Genz et al. 2011). Although considered reliable *in vivo*, to the best of our knowledge there has not been any prior, direct validations of PEG as a volume marker for the gut sac technique. Furthermore, despite a large number of published studies using PEG as a reference marker some investigators have raised doubts about its reliability compared to more direct, gravimetric methods suggesting that volume markers such as PEG may not distribute homogeneously (Worning and Amdrup, 1965; Bunce and Spraggs, 1982; Ando et al. 1986), becoming trapped in the overlying mucus layer (Kirsch and Meister, 1982), and may also be appreciably absorbed (Sutton et al. 2001; Wood and Grosell, 2012). However, because two to three water molecules can be bound per PEG repeat unit (Antonsen and Hoffman, 1992), the effective molecular size of PEG is increased in aqueous solution, and this is considered to reduce potential interactions with the mucus layer, as well as absorption across the epithelium (Schiller et al. 1997). In contrast to this view, the lone pair of electrons on the oxygen atom of the PEG repeat unit (CH_2CH_2O) offers numerous potential hydrogen bond acceptors for

adhesion to the ionised sialic acid residues of mucus (Shojaei and Li, 1997). Furthermore, PEG adsorbs to glass and polypropylene (Crouthamel and Van Dyke, 1975; Gulliford et al. 1987) thus introducing another potential source of error.

The present study set out to directly compare the performance of [^{14}C] PEG 4000 alongside the traditional gravimetric method of measuring fluid transport by gut sacs from five different species of marine teleost; Gulf toadfish (*Opsanus beta*), European flounder (*Platichthys flesus*), rainbow trout (*Oncorhynchus mykiss*), mummichog killifish (*Fundulus heteroclitus*) and blue tilapia (*Oreochromis aureus*). All of these species are popular comparative models that have been frequently used for a variety of *in vivo* and *in vitro* investigations, including gut sacs. We hypothesised that the gravimetric method would under-estimate fluid transport relative to PEG. Furthermore, with the apparent lack of consensus in the literature on the performance of PEG, experiments were designed to evaluate PEG as a non-absorbable volume marker. If PEG could be validated as reliable this could lead to a standardised methodology for gut sacs, and the promise of much more accurate and representative measurements of fluid transport *in vitro.*

Materials and Methods

Experimental animals

Toadfish (n = 15, body mass 27-95 g) were obtained as by-catch from local shrimp fishermen around Biscayne Bay, Florida, U.S.A. and transferred to the aquarium facilities at the University of Miami, Rosenstiel School of Marine and Atmospheric Sciences. Upon arrival fish received a prophylactic treatment against ectoparasites, as previously described (McDonald et al. 2003), and were subsequently held in tanks receiving a continuous flow of filtered sea water (34-37 ppt, temperature 22-26 °C; from Bear Cut, Florida). Killifish (n = 6) were supplied by Aquatic Research Organisms Ltd. (Hampton, NH, U.S.A.) and shipped to the aquarium facilities at the University of Miami where they were maintained under similar seawater conditions to the toadfish for at least 2 weeks prior to use. Toadfish were fed frozen squid twice weekly but food was withheld for 48 hours prior to experiments. Killifish were fed a daily ration of AquaMax pellets (Purina, MN, U.S.A.) and food withheld for at least 24 hours before experiments.

European flounder (n = 15, mean body mass 390 ± 30 g) were obtained from local fishermen in Flookburgh, Cumbria, U.K. and transported to the marine aquarium facilities at the Department of Biosciences, University of Exeter, where they were held in flowing, aerated artificial seawater, made with commercial marine salts (Tropic Marin), as part of a recirculating seawater system maintained at 33-35 ppt and 11-13 °C. Food was typically withheld for at least 72 hours prior to experimentation, otherwise flounder were maintained on a diet of fresh ragworm (*Nereis virens*) fed once per week. Freshwater rainbow trout were obtained from Houghton Springs Fish Farm (Dorset, U.K.) and 6 of these fish (210 ± 8 g body mass) were acclimated to seawater (~28 ppt) over the course of 5 days by the addition of marine salts. The fish were then maintained at this salinity for a further 7 days prior to experimentation. During this 12 day period water temperature was 13.2 ± 0.3 °C and food withheld throughout. Tilapia (n = 8, mean body mass 255 ± 15 g) were obtained from the Institute of Aquaculture, University of Stirling (Stirling, U.K.) and transported to the marine aquarium facilities at the University of Exeter, where they were held in flowing, aerated artificial seawater maintained at 29.5 ± 0.2 ppt and 22.3 ± 0.2 °C. Food was withheld for one week prior to experimentation, otherwise tilapia were maintained on a commercial pellet diet (Skretting, Cheshire, U.K.) fed 3 times per week.

All of the experimental procedures performed in this study were approved by the Institutional Animal Care and Use Committee (Miami, U.S.A.) and the University of Exeter Ethical Committee under a U.K. Home Office licence in accordance with the Animals (Scientific Procedures) Act 1986 (Exeter, U.K.).

General experimental approach

Fish were euthanized by an overdose (300 mg l^{-1}) of buffered tricaine methane sulfonate (MS-222; Pharmaq Ltd.) followed by destruction of the brain. The abdominal cavity was opened and the entire intestine, from the stomach to the rectum was removed and bathed in serosal saline (Table 1), while the sacs were constructed. After carefully removing the attached vasculature and connective tissue, a short, 5 cm length of polyethylene tubing (1.19 mm ID; 1.70 mm OD), heat-flared at one end, was tied into the anterior section with a double silk ligature (US 2/0 or 3/0) immediately distal to the pyloric cecae (where present) or pyloric sphincter. This catheter was then used to flush the entire intestine through with mucosal saline (Table 1).

For the toadfish and flounder intestine, sacs were made from 2-3 cm lengths of the anterior, mid and posterior segments of the intestine. With little in the way of regional distinctions, the mid portion of the intestine was arbitrarily taken as the middle third of the intestine, with the posterior section taken as 2-3 cm proximal to the rectal sphincter. In contrast, the intestine of the rainbow trout, being a farmed fish and having consumed a commercial pellet diet, was surrounded by significant fatty deposits that engulfed the numerous pyloric cecae of the anterior intestine. It was therefore decided to not use the anterior segment; as well as being difficult to effectively blot, determination of surface area including all attached cecae, would have been problematic. Also, due to the size of fish, a single gut sac was made using the posterior portion of the intestine. Before doing so the fatty deposits around the intestine were gently stripped away with a pair of blunt forceps along with any connective tissue and blood vessels. For tilapia a single sac was made in a similar fashion from the anterior segment of the intestine only. Preparations from the killifish were constructed as reported previously (Genz and Grosell, 2011), where the entire intestine (less the very fragile distal portion) formed a single gut sac, with smaller bore polyethylene tubing (0.58 mm ID; 0.96 mm OD) employed for filling and emptying.

Once prepared each sac was filled with approximately 0.15-1.5 ml of mucosal saline (Supplemental Table) containing 0.1-0.25 µCi/ml (Specific activity = 13.0-15.4 mCi/g) of [^{14}C] PEG 4000 (Amersham Biosciences). The experiments with killifish were performed under symmetrical conditions hence the serosal saline listed in Table 1 was used to fill the sacs. The volume injected was determined by weighing the injecting syringe to the nearest 0.1 mg before and after filling the sac. A representative 'initial' sample (~300 µl) was obtained after gently flushing the saline back and forth three times to ensure complete mixing with the residual fluid within the sac. Once filled the catheter was tightly sealed. To simultaneously measure fluid transport gravimetrically each sac preparation was blotted with a lint-free tissue and weighed to the nearest 0.1 mg. The incubation was then commenced by placing the sac into a vial containing 6-20 ml of continuously aerated serosal saline (Table 1) in a water bath at 23-25 °C (toadfish, killifish and tilapia), or 11-13 °C (flounder and trout). After 1-3 hours the sac was removed from the vial (after recording the precise duration to the nearest minute), carefully blotted and re-weighed before the sac was emptied of its contents to obtain the 'final' sample.

Samples of the mucosal saline were taken at the beginning and end of the incubation period and analyzed for [^{14}C] PEG activity where 10-20 µl of sample was added to 0.5 ml deionised water followed by 4.5 ml scintillation cocktail (Emulsifier-Safe™, PerkinElmer in Exeter or EcoLume™, MP Biomedicals in Miami). Each sample was prepared in triplicate and counted on a LKB-Wallac 1214 RackBeta Scintillation Counter (Turku, Finland) or Beckman Coulter LS6500 Scintillation Counter (Brea, CA, U.S.A.) at Exeter, and a TmAnalytic BetaTract 6895 or Packard Tri-Carb 2100TR at Miami. Counts were corrected for background activity and quenching as appropriate. Divalent ion analysis was also performed on the initial and final samples of the mucosal saline. Magnesium was determined by atomic absorption spectrophotometry with a Varian SpectrAA-220 (Miami), Pye Unicam SP9 (Exeter) or by ion chromatography (Dionex ICS1000; Exeter). Sulfate was measured by a Dionex ion chromatography system, either a DX-120 (Miami) or ICS1000 (Exeter).

To measure the surface area of each sac, the ligatures were cut away and the sac opened longitudinally and spread out flat before placing a piece of clear acetate sheet on top and tracing the sac outline. This acetate outline was cut out and weighed to the nearest 0.1 mg. The gross surface area of the sac could then be calculated by comparing the mass of the sac tracing with another piece of acetate sheet of known surface area.

Calculating net fluid transport

The final volume (V_f) of the sac was calculated based on the activity of [^{14}C] PEG in the 'final' sample of mucosal saline recovered at the end of the incubation in relation to the 'initial' sample as follows:

$$V_f = (A_i \,/\, A_f) \times V_i \qquad (1)$$

where, A_i and A_f represent the activity (cpm) of [^{14}C] PEG in the initial and final samples of the mucosal saline, respectively. V_i is the initial volume (ml), which was injected into the sac at the beginning of the incubation. The initial and calculated final volume of the sac was then used to calculate the rate of net fluid transport (J_v):

$$J_v = (V_i - V_f) \,/\, SA \,/\, t \qquad (2)$$

where, SA is the surface area of the sac (cm^2) and t is time (h), and net fluid transport expressed as µl cm^{-2} h^{-1}. Net fluid transport based on the gravimetric method was simply calculated based on equation 2 but substituting V_i and V_f for the initial and final mass (g) of the sac, M_i and M_f, respectively.

Entry of PEG into the serosal saline.

As a volume marker PEG should not be absorbed over the course of an incubation. One way to assess this was to measure [^{14}C] PEG activity appearing in the serosal saline and this was routinely conducted as part of all gut sac experiments directly comparing gravimetric and [^{14}C] PEG methods. At the start of the incubation an 'initial' 0.5 ml sample of the serosal saline was taken. Similarly, at the end of the incubation a 'final' 0.5 ml sample was taken. These samples were then counted and the fraction of [^{14}C] PEG being absorbed by the gut sac and entering the serosal saline was calculated as a proportion of the total activity initially injected into the sac. The rate of [^{14}C] PEG appearance in the serosal saline was also subsequently used to estimate the permeability of the tissue to PEG 4000, P^{PEG} (cm s^{-1}) based on Fick's Law:

$$P^{PEG} = J_{ms}^{PEG} / C_m^{PEG} \qquad (3)$$

where, J_{ms}^{PEG} is the unidirectional flux of PEG from the mucosal to serosal saline (µg cm^{-2} s^{-1}) and C_m^{PEG} is the mucosal concentration of PEG (µg cm^{-3}).

PEG as a volume marker.

Changes in [^{14}C] PEG activity must accurately reflect actual changes in volume, therefore to assess how well [^{14}C] PEG detects changes in volume a working stock of mucosal saline was made and 0.5 ml aliquoted into a series of 1.5 ml micro-centrifuge tubes arranged in triplicate. To each triplicate set a volume of deionised water was added (0, 25, 50, 100, 200 or 400 µl) to provide precisely controlled degrees of dilution of the [^{14}C] PEG. After brief vortex mixing samples from each tube were prepared for counting as detailed above.

The change in volume was calculated based on the change in activity of [^{14}C] PEG and related to the actual volumes of deionised water added.

The following experiments set out to evaluate the performance of [^{14}C] PEG relative to the gravimetric method and were carried out using gut sacs from the flounder only.

Using unlabelled PEG as a 'carrier'. Gulliford et al. (1987) reportedly overcame the problem of adsorption by using a larger concentration of unlabelled PEG as a 'carrier', alongside [^{14}C] PEG. To verify this, the above experiment assessing the ability of [^{14}C] PEG to detect volume change, was repeated using 65 µg ml^{-1} of unlabelled PEG 4000, added as a 50 % solution (w/v) (Sigma Fluka) to the working stock of [^{14}C] PEG. This concentration of unlabelled PEG was approximately 10 times greater than the concentration of [^{14}C] PEG already present in the saline (6.5 µg ml^{-1}). To test whether the presence of unlabelled PEG would help to improve the performance of [^{14}C] PEG as a volume marker in gut sacs themselves another set of experiments were conducted as described above with the flounder, directly comparing fluid transport rates measured by the gravimetric method and [^{14}C] PEG, plus unlabelled PEG.

Interactions of PEG with the intestinal mucosal layer. Previous studies have suggested PEG is a poor volume marker as it does not distribute homogeneously within the intestine (Worning and Amdrup, 1965; Ando et al. 1986), and may become trapped in the overlying mucus layer (Kirsch and Meister, 1982). To measure the extent to which PEG adheres to the mucus layer, at the end of the incubation the ligatures were cut away and the sac opened longitudinally. The mucosal surface was then lightly blotted to remove any surface fluid before being gently scraped with a clean scalpel blade into a pre-weighed scintillation vial, weighed and then counted. The activity of [^{14}C] PEG in this gross sampling of the mucosal layer (consisting of mucus and overlying epithelial cells) was expressed as a proportion of the total activity initially injected into the sac.

Data presentation, supplementary information and statistical analysis

Data are presented as mean ±SE. Additional information on measurements taken from the gut sacs including the volume of mucosal saline (V_i) relative to sac mass (M_i), sac surface area (SA) and the change in sac volume ($V_i - V_f$) are presented as an accompanying Supplementary Table. Proportion data were arcsine transformed prior to analysis. After testing for approximate normality and equality of variance, paired t-tests were used to make direct comparisons of J_v measured gravimetrically with the [^{14}C] PEG method for each individual species. For the toadfish and flounder only, differences in J_v, the % [^{14}C] PEG entering the serosal saline, and P^{PEG} between the anterior, mid and posterior sections of the intestine were assessed by one-way ANOVA, using the General Linear Modelling (GLM) procedure. Where appropriate post-hoc, pairwise comparisons were made using Tukey simultaneous tests. For data failing to meet the assumptions of normality and equal variance, Mann-Whitney, Wilcoxon signed rank and Kruskal-Wallis tests were performed as non-parametric equivalents to the two-sample t-test, paired t-test and one-way ANOVA, respectively. Any necessary post-hoc comparisons following Kruskal-Wallis tests were made using Dunns procedure. The results of all tests were accepted as statistically significant at P <0.05. Statistical analysis was carried out using SigmaStat 3.5.

Results

Measuring fluid transport: Gravimetric versus [^{14}C] PEG

Based on the gravimetric method, net fluid transport (J_v) by gut sacs from anterior, mid and posterior sections of the toadfish intestine were not significantly different. Overall, these values for J_v were positive, indicative of net fluid absorption at a mean rate of 1.43 ± 0.44 µl cm^{-2} h^{-1}. A direct comparison of the gravimetric and PEG methods revealed that net fluid absorption was significantly higher based on [^{14}C] PEG (Fig 1A). Subsequent comparison of the two estimates of J_v for each individual gut sac did not reveal a significant linear relationship between the two methods for any segment, but highlights that J_v based on [^{14}C] PEG was in general far in excess of the corresponding measurement made gravimetrically (Fig. 1B).

For the flounder, net fluid transport obtained gravimetrically was also not significantly different between segments, with a fluid absorption rate of 4-6 µl cm^{-2} h^{-1}. Simultaneous measurements of fluid transport using

[^{14}C] PEG did not display the same consistency as the toadfish, ranging from apparent net secretion in the anterior intestine, to increasingly positive (absorptive) fluid fluxes in the mid and posterior segments (Fig. 2A). Based on the gravimetric method, fluid transport by the posterior intestine of the rainbow trout and anterior intestine of the tilapia also revealed average net absorption, at rates comparable to the flounder and toadfish (Fig. 2B). However, there was very poor agreement with the simultaneous measurements based on [^{14}C] PEG for the trout and tilapia. By comparison, under symmetrical conditions, the killifish intestine demonstrated very robust rates of fluid absorption, averaging 19 µl cm^{-2} h^{-1} when measured gravimetrically, but this was almost 40 % lower with [^{14}C] PEG (Fig. 2B).

Entry of PEG into the serosal saline

The proportion of [^{14}C] PEG recovered in the serosal saline at the end of each incubation was found to range from 0.1 to 0.6 % of injected [^{14}C] activity in toadfish, flounder and tilapia. Notably, there were no significant differences in the fraction of [^{14}C] PEG appearing in the serosal saline or PEG permeability between the anterior, mid and posterior segments of the intestine for either the toadfish or flounder (Table 2). For the killifish the amount of [^{14}C] PEG entering the serosal saline was 1.0 ± 0.3 %, and for the rainbow trout posterior intestine this was higher still at 1.5 ± 0.3 %. For J_v based on PEG there was a significant inverse relationship with the fraction of [^{14}C] PEG detected in the serosal saline (r = -0.882, P = 0.020) and P^{PEG} (r = -0.925, P = 0.008), for the trout only. Given the inconsistencies between J_v measured gravimetrically and by [^{14}C] PEG for the flounder, killifish and tilapia, the proportion of [^{14}C] PEG entering the serosal saline and the permeability of the tissue to PEG would expectedly be associated with an under-estimate of J_v, yet no similar significant correlations were detected for any of these species.

PEG as a volume marker

Away from the gut sac, [^{14}C] PEG generally over-estimated actual volume changes of 50 to 450 µl (Fig. 3). However, it was rather puzzling to find that it actually under-estimated volume when used to detect smaller changes (0 to 50 µl). The inclusion of unlabelled PEG as ‘carrier’ clearly had a beneficial effect on the performance of [^{14}C] PEG, not only making it much more reliable at smaller volumes but also reducing some of

the variation that was apparent with [^{14}C] PEG alone, particularly when larger volume changes were taking place (Fig. 3).

Using unlabelled PEG as a 'carrier'

With confidence that [^{14}C] PEG could now more accurately detect changes in volume in the presence of unlabelled PEG (Fig. 3), this combination of labelled and unlabelled PEG was subsequently put to the test in gut sacs from flounder and another direct comparison with the gravimetric method was made. Figure 4 shows that the calculation of net fluid transport based on [^{14}C] PEG did not improve, rather it deteriorated and yielded rates that were consistently negative (i.e. net secretion) across all sections of the intestine, in contrast to net absorption shown by the gravimetric method. In addition, Table 2 shows that when using unlabelled PEG 4000 as a carrier, the proportion of [^{14}C] PEG detected in the serosal saline was slightly, but significantly higher than seen previously for the posterior sections of the intestine (but not the anterior or mid). Similarly, the permeability of these posterior gut sacs to PEG also increased significantly in the presence of unlabelled PEG to 1.3×10^{-7} cm s^{-1}, compared to just 0.2×10^{-7} cm s^{-1} with [^{14}C] PEG alone.

Interactions with the mucosal layer

After 2 hours incubation within the flounder gut sacs the proportion of [^{14}C] PEG associated with the scraped mucosal layer appeared to be unaffected by the amount of unlabelled PEG present (Fig. 5). However, compared to more distal segments there was approximately 3 times more [^{14}C] PEG within this surface layer from the anterior section, accounting for around 2.5-3 % of labelled PEG initially present within the whole gut sac (Fig. 5).

Discussion

The gravimetric method of determining net water transport by the *in vitro* gut sac preparation was hypothesized to produce significant under-estimates when compared to [^{14}C] PEG, a popular non-absorbable volume marker. However, following direct evaluation of these two methods, we found [^{14}C] PEG to be inconsistent and unreliable for most species. The average rates of net fluid absorption by gut sacs measured

directly using the gravimetric method from all five species compared favourably with previous studies under similar conditions (Mainoya and Bern, 1982; Marshall et al. 2002; Grosell et al. 2005; Bucking et al. 2009). Net fluid absorption measured simultaneously by [^{14}C] PEG was on average 2-7 times higher in the toadfish intestine (Fig. 1A). These findings support the hypothesis that the gravimetric method does indeed have the tendency to under-estimate J_v, although there was no clear parallel relationship between the two methods (Fig. 1B). Even so, with < 1 % of [^{14}C] activity entering the serosal saline and low relative permeability (Table 2), this suggests PEG might be a suitable volume marker, but remarkably only for this particular species. PEG did not perform at all well in gut sac preparations from flounder, trout, killifish and tilapia where it typically displayed little to no consistency with the gravimetric method (Fig. 2). Indeed, recent work by Wood and Grosell (2012) corroborates this finding for the killifish, where the reported flux ratio of PEG 4000: water by gut sacs was ~0.3, meaning that had fluid transport been reported based on PEG it would have under-estimated the volume change by ~70 %. By comparison, in the present study with the killifish J_v was 40 % lower using PEG than with the gravimetric method (Fig. 2B).

Appearance of PEG in the serosal saline

In many cases PEG greatly under-estimated J_v, indicating apparent and substantial net fluid secretion, in complete contrast to the corresponding gravimetric measurement. From an open, *in vivo* perfusion of rat jejunum, [^{14}C] PEG 4000 was also found to significantly under-estimate J_v when directly compared with the gravimetric method (Sutton et al. 2001). This was explained by an "appreciable absorption" of the marker, although Sutton and co-workers do not report the appearance of PEG in either the circulation or urine. In the present study some of the marker may well have been absorbed because the final activity of [^{14}C] PEG recovered in the mucosal saline from a large number of gut sac preparations was found to be less than what was present initially. However, the levels of [^{14}C] detected in the serosal saline for the toadfish, flounder and tilapia was on average ≤ 1 % of the total activity initially injected into the sac (Table 2). For the killifish and rainbow trout the appearance of [^{14}C] PEG in the serosal saline was higher (though still a low proportion overall) at 1.0-1.5 %. Even if these relatively large fractions of [^{14}C] PEG were to have been retained within the gut sac, in terms of fluid transport this represents only 0.50 ± 0.14 and 0.60 ± 0.17 μl cm^{-2} h^{-1}, respectively, and

clearly cannot account for the considerable differences seen in J_v between the two methods (Fig. 2B). The permeability of the seawater-adapted killifish intestine to PEG 4000, previously measured using the gut sac methodology, ranged from 1-3 × 10^{-7} cm s^{-1} (Wood and Grosell, 2012), agrees very well with the value of 1.2 × 10^{-7} cm s^{-1} obtained here (Table 2). These findings on the fractional absorption of PEG, and its permeability across the teleost intestine, corroborate one of the most widely cited benefits of the high molecular weight PEGs – that they are very poorly absorbed (Jacobson et al. 1963; Till and Downes, 1965; Schedl, 1966; Krag et al. 1975; Winne and Gorig, 1982; Furuichi et al. 1984; Schiller et al. 1997).

PEG as a volume marker

Clearly, the appearance of PEG in the serosal saline cannot account for the discrepancies in fluid transport compared to the gravimetric method, which led to consideration of other potential sources of error which could explain the reduction of [^{14}C] PEG activity. Given the lack of consistency in terms of measuring fluid transport, compared with the gravimetric method, it was not surprising to find that [^{14}C] PEG on its own did not perform ideally as a volume marker (Fig. 3). A similar issue had previously been ascribed to adsorption (Crouthamel and Van Dyke, 1975), and presently we recorded substantial changes in [^{14}C] PEG activity after storage in a range of different materials (Table 3). Given that such a large reduction in activity (6 to 15 %) can potentially take place in the absence of the gut sac might go some way to explaining the reduction in [^{14}C] PEG activity and subsequent under-estimates of J_v. However, while representing a potential source of error this cannot resolve why [^{14}C] PEG was successful for the toadfish but not any other species, especially given the consistency with which the gut sac methodology and analytical methods were employed between our two laboratories.

The issue of adsorption becomes particularly significant when attempting to measure very small changes in [^{14}C] PEG activity. Gulliford et al. (1987) recommended using unlabelled PEG as a ‘carrier’ alongside [^{14}C] PEG and its inclusion did indeed improve the performance of the latter as a volume marker (Fig. 3). Unfortunately, this new found confidence in PEG did not extend to its application to flounder gut sacs which revealed over-whelming net secretion, not just in the anterior intestine as shown in Fig. 2A, but across all sections of the intestine, in direct contrast to consistent net absorption measured gravimetrically (Fig. 4).

Attempts to offset the problem of adsorption were therefore unsuccessful and surprisingly revealed a substantial deterioration in the performance of [^{14}C] PEG. Of note, since the osmotic impact of the additional unlabelled PEG (≈ 0.016 mmol l^{-1}) would have been negligible, this result suggests other issues, aside from adsorption, are at work when higher concentrations of PEG are used in conjunction with the intestine.

An additional source of variation related to the ability of PEG to accurately represent changes in sac volume may have arisen from insufficient mixing with the residual fluid initially present in the gut sac. For example, in the present study the time taken to flush the sac in order to obtain a well-mixed, representative 'initial' sub-sample was estimated to have been no more than 30 seconds. Using [^{14}C] PEG 4000, Karasov and Diamond (1983) measured the adherent fluid volume in everted 'sleeves' of mouse intestine finding that it took at least 2 minutes for PEG to completely equilibrate with the adherent fluid space. Furthermore, [^{3}H] water rapidly enters and equilibrates with the water space in mucus compared with [^{14}C] PEG 4000 (Lukie, 1983). Hence, it is possible that there was insufficient time for PEG to equilibrate with the residual volume of fluid in the sac, compared with water, hence introducing an additional source of variation into the calculation of J_v. The erratic rates of fluid transport (based on PEG), observed during *in vitro* perfusion of the everted Japanese eel (*Anguilla japonica*) intestine, compared to the steady rate of fluid absorption measured gravimetrically, were suggested to have been a manifestation of this issue (Ando et al. 1986).

Interactions with the mucosal layer

The very minor fractions of PEG appearing in the serosal saline cannot serve as conclusive proof that it remained completely unabsorbed. Having accounted for the problem of adsorption (Fig. 3) and confident there was minimal absorption into the serosal saline (Table 2), this led to consideration of whether the discrepancies observed with the PEG method could be attributed to its interactions with the epithelium. The overlying mucus layer is a vital physical barrier between the contents of the intestinal lumen and cell surface, and was considered a probable location for such an interaction. During *in vivo* perfusion of eel (*Anguilla anguilla*) intestine, Kirsch and Meister (1982) were forced to abandon both PEG and phenol red as volume markers, stating that they became progressively trapped in the mucus. In contrast, Skadhauge (1969), using ^{131}I-labelled phenol red during perfusion of eel intestine, found an even distribution between the perfusate and mucus.

Furthermore, Jacobson et al. (1963) incubated canine intestinal mucus, human gastric mucus and human serum protein with saline containing 4 % (unlabelled) PEG and found that after 3 hours the concentration of PEG in the test material had not changed. However, with such a high PEG concentration, combined with the relative lack of sensitivity of the turbidimetric assay (Till and Downes, 1965; Wingate et al. 1972), entry of the marker into the mucus may well have gone undetected.

Although scraping was a crude method for sampling the mucus, the amount of [^{14}C] PEG detected within the isolated mucosal layer of flounder intestine revealed significantly higher proportions in the anterior section compared to the mid or posterior (Fig. 5). Interestingly, the proximal regions of the flounder intestine possessed a visibly thicker mucus layer. This was evident in the wet mass removed from the anterior portion (29.9 ± 2.6 mg cm^{-2}), which was significantly greater by 2-3 fold than mid or posterior regions (14.7 ± 3.0 and 11.1 ± 2.0 mg cm^{-2}, respectively), suggesting more mucus was indeed present ($F_{2, 27}$ = 15.25, $P < 0.001$). For each individual segment there were no significant correlations between the proportion of [^{14}C] PEG associated with the mucosal layer and the mass of this layer removed, but across segments there was a clear, positive relationship (Fig. 6). This could help explain why J_v based on PEG from the anterior segment of the flounder manifested overwhelmingly as net secretion relative to more distal segments (Fig. 2A). This association may be related to the chemical nature of the mucus which has been shown to vary between different regions of the intestine in three other flatfish species (Murray et al. 1996), and these characteristics might alter the number of potential bonding sites for PEG (Shojaei and Li, 1997). Furthermore, the abundance of certain mucin residues may thus determine the extent to which PEG interacts with, or adheres to, the mucus layer. Indeed, one possible explanation why PEG behaved as a reasonable volume marker in the toadfish intestine, but not the other species examined, may be related to the histochemistry of the mucus, which also demonstrates species-specific differences among teleosts (Reifel and Travill, 1979; Murray et al. 1996). Despite being another potential source of error, the association of PEG with the mucus layer cannot completely account for the observed differences in J_v given that only 3 %, at most, of [^{14}C] PEG injected into the sac was detected in the mucosal layer (Fig. 5). This would translate to an approximate increase in calculated J_v from -4.14 ± 1.14 µl cm^{-2} h^{-1} to only -2.06 ± 1.09 µl cm^{-2} h^{-1}, compared to 2.00 ± 0.58 µl cm^{-2} h^{-1} obtained gravimetrically (Fig. 4).

Having addressed adsorption, accumulation of the labelled marker in the serosal saline (Table 2) and the mucosal layer (Fig. 5), this leaves sequestration by the tissue itself as a possibility. An unfortunate oversight for these experiments, and a limitation to their interpretation, is that after sampling the mucosal and serosal salines, and scraping the mucus layer, [^{14}C] PEG activity was not measured in the remaining tissue. Interestingly, exposure of isolated human cervical mucus to concentrations of PEG 3350 as low as 10^{-5} g ml^{-1} caused the mucin fibres to coalesce creating patchiness across the mucus gel surface (Willits and Saltzman, 2001). By comparison, the concentrations of [^{14}C] PEG used in the present study were 6.5 to 19.2 × 10^{-6} g ml^{-1} with [^{14}C] PEG alone, but increased ~10-fold to 7.2 × 10^{-5} g ml^{-1} with unlabelled PEG. It is possible that when PEG was presented to the gut sac (Fig. 4) this may have similarly disrupted the mucus layer allowing PEG to become sequestered within the epithelium. Notably, the use a higher PEG concentration did not translate to greater overall absorption. Relative to the preceding experiments with [^{14}C] PEG only, the presence of additional unlabelled PEG significantly increased its fractional absorption (from 0.1 to 0.5 %; W_{13} = 48.0, P = 0.011) and permeability (from 0.2 × 10^{-7} to 1.3 × 10^{-7} cm s^{-1}; W_{13} = 46.0, P = 0.005) in the posterior intestine only, not the anterior or mid segments (Table 2).

An alternative to [^{14}C] PEG as a non-absorbable marker could be to use Mg^{2+} and/or SO_4^{2-} which were both present in the mucosal saline (Table 1). The intestinal epithelium of marine teleosts displays very low permeability to both of these ions *in vitro* (Grosell and Taylor, 2007), and they have been used as a reliable proxy for intestinal fluid transport *in vivo* (Hickman, 1968; Genz et al. 2008). Despite this, a direct comparison of J_v measured gravimetrically from Figures 1 and 2 with calculations of J_v based on changes in the corresponding concentrations of Mg^{2+} and SO_4^{2-} in the mucosal saline reveals that these ions do not offer a suitable alternative to either PEG or the gravimetric method of measuring fluid transport *in vitro* (Table 5). The incompatibility of these two ions to serve as an alternative may well be related to their relatively low (but significant) rates of net absorption *in vitro* which have been reported for the teleost intestine when measured under *in vivo*-like conditions (Pelis and Renfro, 2003; Grosell and Taylor, 2007; Genz et al. 2011). In contrast to this absorption, under symmetrical experimental conditions, Mg^{2+} and SO_4^{2-} are both actively secreted on a net basis by the seawater-acclimated flounder and killifish intestine (Pelis and Renfro, 2003; Genz and Grosell, 2011), which may consequently explain the rather excessive estimates of J_v for the latter species (Table 5).

In summary, the gravimetric method was hypothesised to produce significant under-estimates of fluid transport by the *in vitro* gut sac preparation when compared alongside [^{14}C] PEG, a popular non-absorbable volume marker. Following direct evaluation of these two methods in gut sacs from a range of model teleosts only the toadfish intestine conformed to this hypothesis. However, for the other species investigated (flounder, rainbow trout, killifish and tilapia), PEG was an inconsistent volume marker displaying poor parallelism with the gravimetric method which could not be simply explained or accounted for by its (low rate of) absorption into the serosal saline. A number of alternative, complicating and interacting factors could potentially affect the ability of PEG to function as a reliable volume marker. One major issue was adsorption to the surface of sample tubes. While it was possible to circumvent this problem using unlabelled PEG this had an unexpected, deleterious effect on PEG-based J_v when used in gut sacs suggesting other factors were affecting its performance. There were found to be interactions with the gut sac itself, specifically the overlying mucus layer, where a direct correlation between the amount of adherent [^{14}C] PEG and the size of this mucosal layer was observed. Following on from this there is also the potential for sequestration of PEG within the tissue itself. The problems associated with the accurate representation of water transport by *in vitro* sac preparations of the intestine are not easily overcome by the non-absorbable marker [^{14}C] PEG. In conclusion, the gravimetric method represents the best option for measuring volume changes by gut sacs for most species. Given the uncertainties in its behaviour highlighted by this report we urge caution in the use of PEG as a volume marker and recommend a thorough evaluation of its performance before doing so.

Acknowledgements

We are grateful to Ian and Tony McClure, the local fishermen of Flookburgh, Cumbria (U.K.) for collecting the flounder used in this study, and to Jan Shears for assistance with fish husbandry at Exeter (U.K.). We thank Ray Hurley and Debbie Fretz in Miami (U.S.A.) for supplying the toadfish. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) grants BBS/S/A/2004/11078 and BB/F009364/1 to R.W.W., and National Science Foundation (NSF) grants IAB0743903 and 1146695 to M.G.

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Figure legends

Fig. 1: (A) A comparison of fluid transport rates, J_v (µl cm^{-2} h^{-1}) measured simultaneously by the gravimetric method (dark, shaded bars) and [^{14}C] PEG (grey, shaded bars) using gut sacs made from the anterior, mid and posterior sections of the toadfish intestine (n = 15, 6 and 7 for each section, respectively). Means labelled with an asterisk represent a statistically significant difference (paired analysis). Values are mean ± SE. (B) The relationship between J_v measured by the gravimetric method and [^{14}C] PEG for each section of the intestine. Positive values denote net absorption while negative values represent net secretion. The dashed line represents the ideal 1:1 relationship between the two methods.

Fig 2: (A) A comparison of fluid transport rates, J_v (µl cm^{-2} h^{-1}) measured simultaneously by the gravimetric method (dark, shaded bars) and [^{14}C] PEG (grey, shaded bars) using gut sacs made from the anterior, mid and posterior sections of the flounder intestine (n = 10 for each section). (B) A comparison of fluid transport rates, J_v (µl cm^{-2} h^{-1}) measured simultaneously by the gravimetric method (dark, shaded bars) and [^{14}C] PEG (grey, shaded bars) using gut sacs made from the rainbow trout (posterior intestine only, n = 6), tilapia (anterior intestine only, n = 8) and killifish (n = 6). Positive values denote net absorption while negative values represent net secretion. Means labelled with an asterisk represent a statistically significant difference (paired analysis). Values are mean ± SE.

Fig. 3: Evaluating PEG as a volume marker. The calculated volume change (µl) based on the change in activity of [^{14}C] PEG plotted against the actual volume change in a series of micro-centrifuge tubes containing a working stock of [^{14}C] PEG following precise dilution with a set volume of deionised water. The dark, filled circles represent volume changes in tubes containing [^{14}C] PEG only, the open circles represent volume change calculated by [^{14}C] PEG but in the presence of unlabelled PEG (uPEG). The solid line is the ideal 1:1 relationship between calculated and actual volume change. Values are mean ±SE, n = 3 for each data point.

Fig. 4: A comparison of fluid transport rates (µl cm^{-2} h^{-1}) measured simultaneously by the gravimetric method (dark, shaded bars) and [^{14}C] PEG, with unlabelled PEG (uPEG) as a carrier (grey, shaded bars), using gut

sacs made from the anterior, mid and posterior sections of the European flounder intestine. Positive values denote net absorption while negative values represent net secretion. Means labelled with an asterisk represent a statistically significant difference (paired analysis). Values are mean ±SE, n = 5 for each section.

Fig. 5: The proportion of [^{14}C] PEG initially injected into gut sacs that was detected in the scraped mucosal layer at the end of an incubation period in the presence of [^{14}C] PEG only (dark, shaded bars) and [^{14}C] PEG with unlabelled PEG (uPEG) as a carrier (grey, shaded bars). Gut sacs were made from the anterior, mid and posterior sections of the European flounder intestine. Values are mean ±SE, n = 5 for each section and treatment. Means labelled with different letters indicate a significant difference ($P < 0.05$).

Fig. 6: The relationship between the proportion of [^{14}C] PEG initially injected into gut sacs that was detected in the scraped mucosal layer at the end of an incubation period and the mass of mucosal layer removed (mg cm^{-2}). Gut sacs were made from the anterior, mid and posterior sections of the European flounder intestine. Values are mean ±SE, n = 10 for each section and treatment.

Tables

Table 1: The individual salts used to compose the mucosal and serosal salines. The concentration of each component salt is given in mmol l^{-1}. Measured osmolality is given in mOsm kg^{-1}.

	Mucosal salines			Serosal salines		
	Toadfish	**Flounder**	**Trout/tilapia**	**Toadfish**	**Flounder/trout/tilapia**	**Killifish**
NaCl	69.0-77.8	114.0	99.0	151.0	146.0	144.1
KCl	2.0-5.0	5.0	5.0	3.0	3.0	5.1
$MgCl_2.6H_2O$	27.0-30.0	15.0	15.0	-	-	-
$CaCl_2.6H_2O$	2.0-10.0	5.0	5.0	1.0	2.0	1.6
$MgSO_4.7H_2O$	70.0-77.5	40.0	60.0	0.9	0.9	0.9
$NaHCO_3$	1.0	1.0	1.0	5.0	8.0	11.9
Na_2HPO_4	-	-	-	0.5	0.5	2.9
KH_2PO_4	-	-	-	0.5	0.5	-
HEPES (Free acid)	-	-	-	5.5	4.0	-
HEPES (Na^+ salt)	-	-	-	5.5	4.0	-
Urea	-	-	-	4.5	-	-
D-Glucose	-	-	-	3.0-5.0	6.0	5.5*
L-Glutamine	-	-	-	-	6.0	-
pH	7.60	-	7.67	7.80**	7.80**	7.80**
Osmolality†	310-315	321	315	309	331	292

*Glucose was replaced by mannitol when used as the mucosal saline in the symmetrical killifish experiments.

**pH was adjusted to 7.80 with the addition of the appropriate HEPES buffer following equilibration of the serosal saline with 0.5 % CO_2 (O_2 balance). For the toadfish and killifish, the serosal salines were gassed with 0.3 % CO_2 (O_2 balance) and pH adjusted using NaOH or HCl as appropriate.

†Before each experiment the osmolality of each corresponding mucosal and serosal saline were matched to eliminate any transepithelial osmotic gradient.

Table 2: The proportion of [^{14}C] PEG detected in the serosal saline at the end of an incubation and the accompanying PEG permeability, P^{PEG} (cm s^{-1}) of gut sacs from the Gulf toadfish, European flounder, rainbow trout, tilapia and killifish in the presence of [^{14}C] PEG only, and when using [^{14}C] PEG with unlabelled PEG (uPEG) as a 'carrier'. Values are mean ± SE and sample sizes are given in parentheses.

Species/Section	**[^{14}C] PEG in serosal saline** (%)	P^{PEG} (× 10^{-7} cm s^{-1})
Toadfish		
Anterior (15)	0.30 ± 0.05	0.27 ± 0.05
Mid (6)	0.35 ± 0.07	0.27 ± 0.03
Posterior (7)	0.59 ± 0.30	0.46 ± 0.20
Flounder ([^{14}C] PEG only)		
Anterior (10)	0.28 ± 0.13	0.53 ± 0.24
Mid (10)	0.17 ± 0.03	0.31 ± 0.05
Posterior (10)	0.10 ± 0.02	0.17 ± 0.03
Flounder ([^{14}C] PEG + uPEG)		
Anterior (5)	0.39 ± 0.16	0.68 ± 0.31
Mid (5)	0.25 ± 0.14	0.54 ± 0.33
Posterior (5)	0.49 ± 0.21	1.27 ± 0.62
Trout		
Posterior (6)	1.50 ± 0.33	1.87 ± 0.38
Tilapia		
Anterior (8)	0.40 ± 0.06	1.37 ± 0.12
Killifish		
Anterior/mid (6)	0.99 ± 0.34	1.17 ± 0.35

Table 3: The change in activity (cpm) of [^{14}C] PEG after being aliquoted from a working stock (2689 ± 14 cpm) into a series of clean, dry tubes composed of a range of different materials. Values are mean ±SE, n = 3 for each material.

Material	**[^{14}C] PEG activity** (cpm)	**% Difference**
Teflon	2520 ±35	-6.3 ±1.3
Glass	2363 ±7	-12.1 ±0.2
Polystyrene	2252 ±30	-12.6 ±1.1
Polyethylene	2292 ±13	-14.8 ±0.5
Polypropylene	2275 ±10	-15.4 ±0.4

Table 4. A comparison of net fluid transport (J_v) by gut sacs measured indirectly using the changes in [Mg^{2+}] and [SO_4^{2-}] as surrogate non-absorbable markers of volume. For comparison J_v based on [^{14}C] PEG and the direct, gravimetric method are shown alongside. Significant differences in relation to the gravimetric method (following paired analysis) are represented by asterisks. Positive values denote net absorption while negative values represent net secretion. Values are mean ± SE, sample sizes are indicated in parentheses.

		Net fluid transport, J_v (µl cm^{-2} h^{-1})			
Species	**Region**	**Gravimetric**	**[^{14}C] PEG**	**Mg^{2+}**	**SO_4^{2-}**
Toadfish	Anterior (15)	0.68 ± 0.74	5.43 ± 1.73*	1.94 ± 2.30	3.75 ± 2.06
	Mid (6)	2.50 ± 0.50	8.32 ± 1.37*	6.10 ± 1.08*	6.93 ± 0.89*
	Posterior (7)	2.11 ± 0.31	4.78 ± 0.82*	1.86 ± 1.61	4.53 ± 1.20
Flounder	Anterior (10)	4.07 ± 0.69	-4.22 ± 1.56*	-3.29 ± 2.27*	-
	Mid (10)	6.44 ± 1.64	2.63 ± 3.07	4.07 ± 4.25*	-
	Posterior (10)	4.04 ± 1.39	6.28 ± 2.05	6.69 ± 3.14	-
Trout	Posterior (6)	4.49 ± 0.91	-1.88 ± 2.31*	-0.76 ± 2.14*	-0.69 ± 2.05*
Tilapia	Anterior (8)	5.89 ± 3.19	-3.52 ± 7.37	1.64 ± 4.61*	3.29 ± 4.91
Killifish	Anterior/mid (6)	18.87 ± 1.98	11.60 ± 3.82	35.38 ± 5.34*	28.61 ± 6.30

Figures

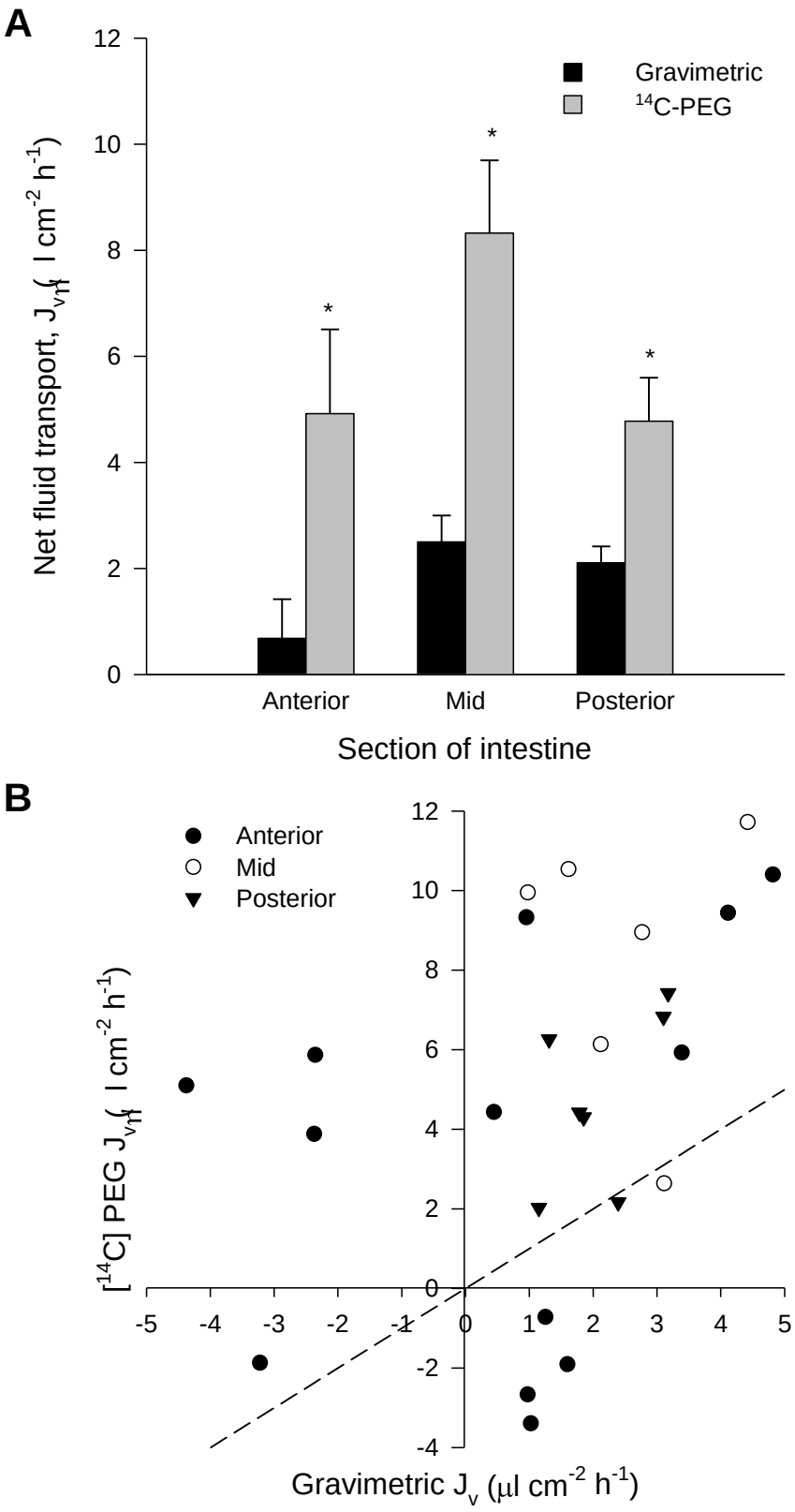

Figure 1.

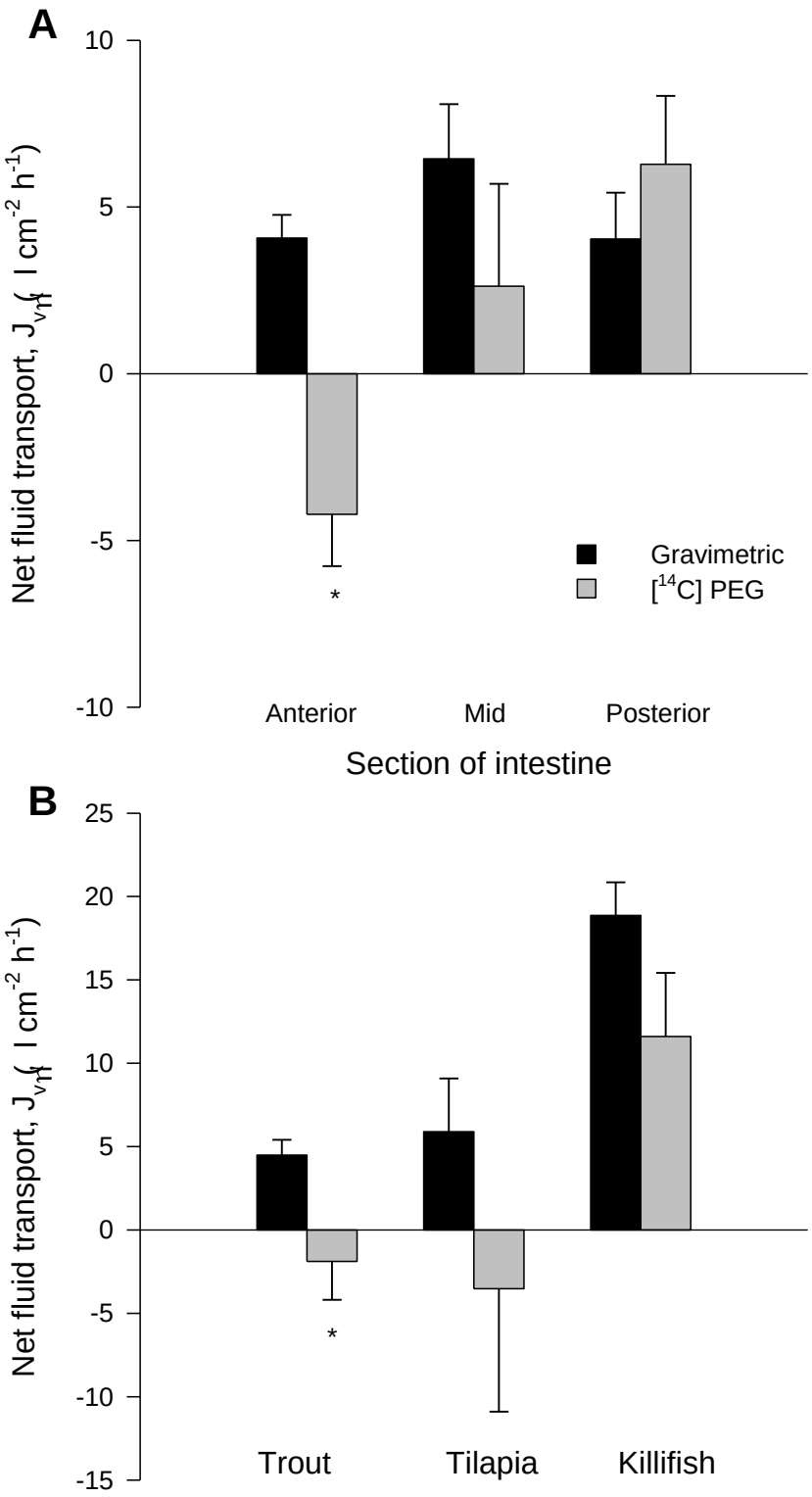
A
10
5
0
-5
-10
Net fluid transport, J_{vn} (l cm^{-2} h^{-1})
*
Gravimetric
[^{14}C] PEG
Anterior
Mid
Posterior
Section of intestine
B
25
20
15
10
5
0
-5
-10
-15
Net fluid transport, J_{vn} (l cm^{-2} h^{-1})
*
Trout
Tilapia
Killifish

Figure 2.

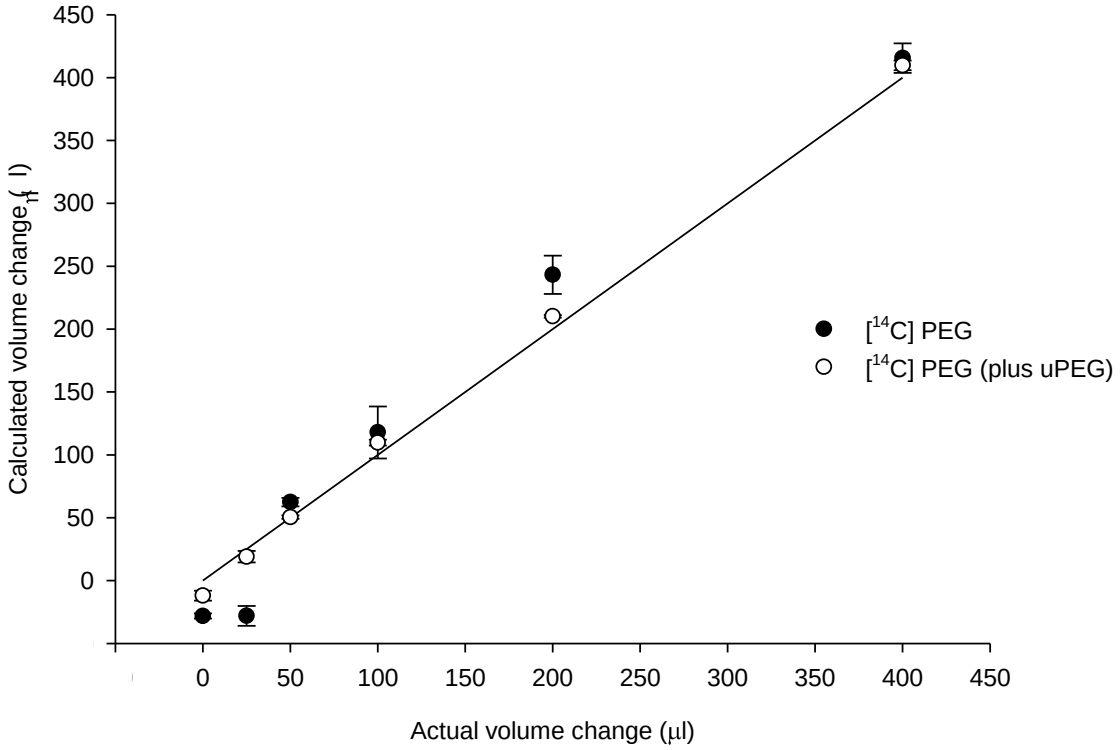
Calculated volume change (μl)
Actual volume change (μl)
[14C] PEG
[14C] PEG (plus uPEG)
0
50
100
150
200
250
300
350
400
450

Figure 3.

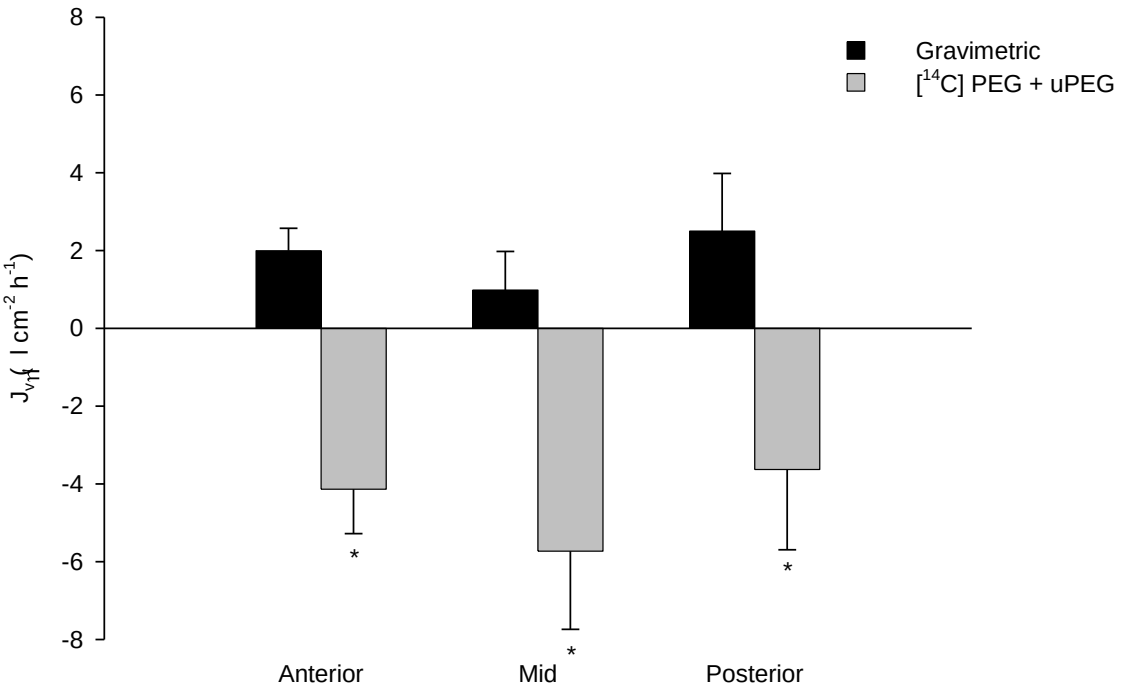
8
6
4
2
0
-2
-4
-6
-8
Gravimetric
[14C] PEG + uPEG
Anterior
Mid
Posterior
*
*
*

Figure 4.

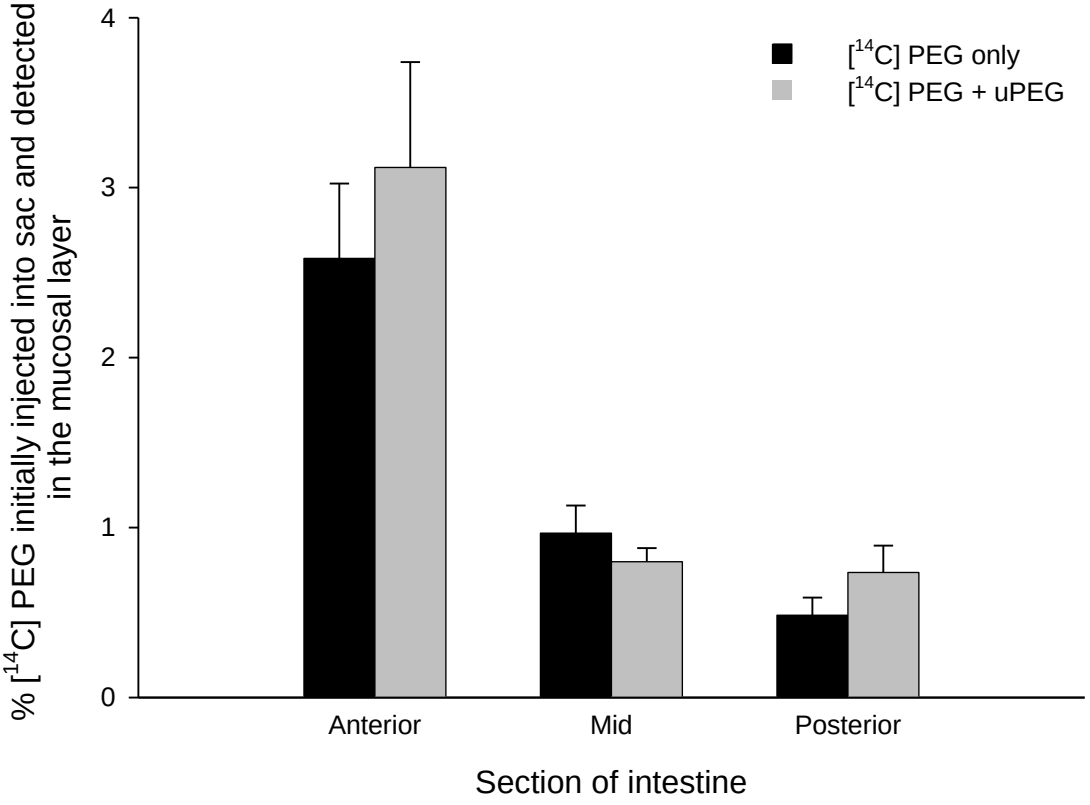
% [14C] PEG initially injected into sac and detected in the mucosal layer
4
3
2
1
0
[14C] PEG only
[14C] PEG + uPEG
Anterior
Mid
Posterior
Section of intestine

Figure 5.

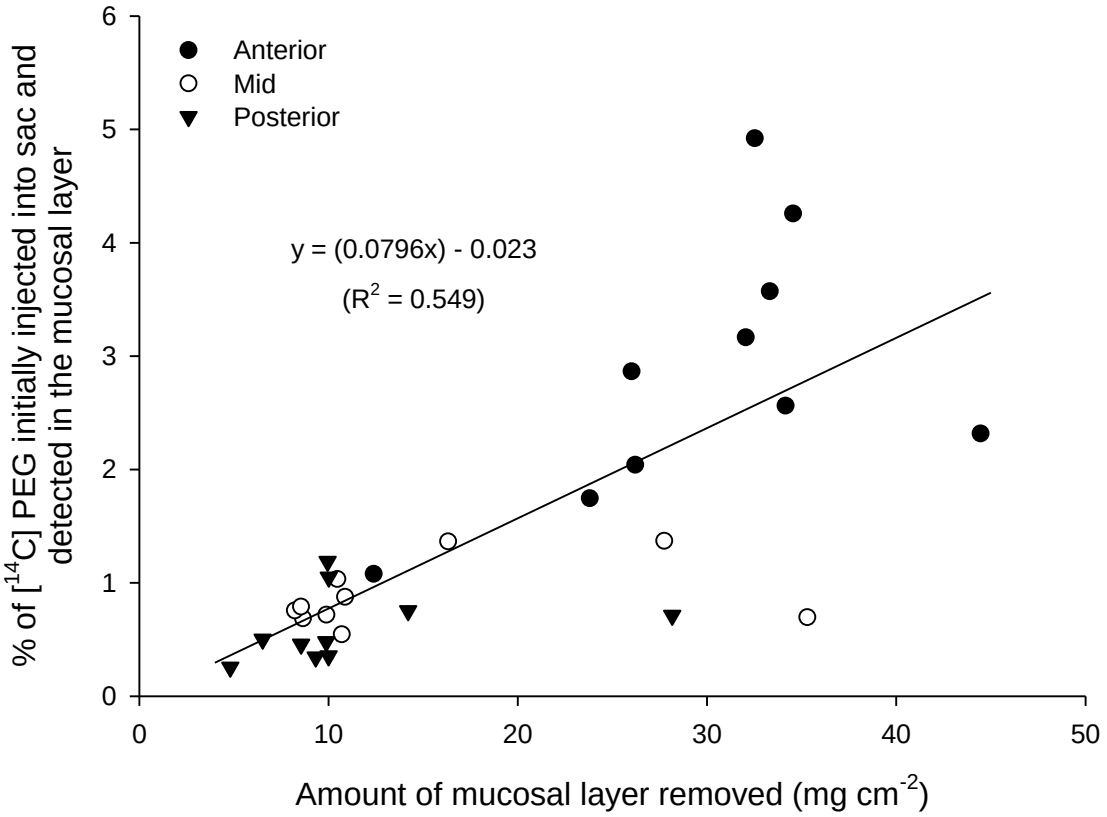
Anterior
Mid
Posterior
y = (0.0796x) - 0.023
(R^2 = 0.549)
% of [^{14}C] PEG initially injected into sac and detected in the mucosal layer
Amount of mucosal layer removed (mg cm^{-2})
0
1
2
3
4
5
6
0
10
20
30
40
50

Figure 6.

Supplemental Materials

Supplemental Table 1: Additional details on the initial volume of mucosal saline injected into each sac (V_i, ml), its relation to the overall initial sac mass (M_i, g) and sac surface area (SA, cm^2) are presented. The calculated change in volume (µl) is also given for both the gravimetric method and [^{14}C] PEG within gut sacs from the Gulf toadfish, European flounder, rainbow trout, tilapia and killifish, where a positive value indicates the volume of fluid absorbed from the sac and a negative value is indicative of net fluid secretion into the sac. Values are mean ± SE and sample sizes are given in parentheses.

Species/Section	Sac Measurements			**Δ Volume** ($V_i - V_f$, µl)	
	V_i (ml)	**V_i: M_i** (ml/g)	**SA** (cm^2)	**Gravimetric**	**[^{14}C] PEG**
Toadfish					
Anterior (15)	0.335 ± 0.043	0.485 ± 0.034	4.22 ± 0.47	9.0 ± 6.5	32.1 ± 13.6
Mid (6)	0.447 ± 0.032	0.639 ± 0.023	4.96 ± 0.39	31.3 ± 4.5	108.4 ± 17.4
Posterior (7)	0.473 ± 0.038	0.643 ± 0.027	5.10 ± 0.35	27.8 ± 2.5	64.8 ± 10.1
Flounder ([^{14}C] PEG only)					
Anterior (10)	0.761 ± 0.071	0.499 ± 0.023	5.23 ± 0.49	53.0 ± 11.0	-43.3 ± 15.8
Mid (10)	0.929 ± 0.076	0.618 ± 0.017	4.81 ± 0.30	83.5 ± 23.9	44.2 ± 38.5
Posterior (10)	1.052 ± 0.082	0.651 ± 0.013	6.03 ± 0.62	62.3 ± 21.4	120.3 ± 47.6
Flounder ([^{14}C] PEG + uPEG)					
Anterior (5)	0.660 ± 0.110	0.462 ± 0.034	5.48 ± 0.58	19.5 ± 5.6	-41.2 ± 8.7
Mid (5)	0.912 ± 0.063	0.644 ± 0.009	5.27 ± 0.52	10.1 ± 9.4	-62.1 ± 22.0
Posterior (5)	0.933 ± 0.052	0.662 ± 0.022	4.87 ± 0.47	24.2 ± 15.5	-35.1 ± 21.2
Trout					
Posterior (6)	0.632 ± 0.049	0.527 ± 0.031	6.84 ± 0.44	61.6 ± 13.9	-24.2 ± 34.1
Tilapia					
Anterior (8)	0.571 ± 0.047	0.643 ± 0.010	3.85 ± 0.29	21.6 ± 11.0	-30.4 ± 37.3
Killifish					
Anterior/mid (6)	0.188 ± 0.019	0.664 ± 0.012	1.76 ± 0.18	65.8 ± 9.7	43.4 ± 17.7

M_i - the initial mass of the filled sac which includes initial volume of mucosal saline injected (V_i), as well as sutures and filling catheter.
V_f - final volume within sac – see equations 1 and 2 in Materials and Methods for further details.
uPEG - unlabeled PEG 4000.