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A review of reductionist methods in fish gastrointestinal tract physiology

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ABSTRACT

A holistic understanding of a physiological system can be accomplished through the use of multiple methods. Our current understanding of the fish gastrointestinal tract (GIT) and its role in both nutrient handling and osmoregulation is the result of the examination of the GIT using multiple reductionist methods. This review summarizes the following methods: *in vivo* mass balance studies, and *in vitro* gut sac preparations, intestinal perfusions, and Ussing chambers. From Homer Smith's initial findings of marine fish intestinal osmoregulation in the 1930s through to today's research, we discuss the methods, their advantages and pitfalls, and ultimately how they have each contributed to our understanding of fish GIT physiology. Although *in vivo* studies provide substantial information on the intact animal, segment specific functions of the GIT cannot be easily elucidated. Instead, *in vitro* gut sac preparations, intestinal perfusions, or Ussing chamber experiments can provide considerable information on the function of a specific tissue and permit the delineation of specific transport pathways through the use of pharmacological agents; however, integrative inputs (*e.g.* hormonal and neuronal) are removed and only a fraction of the organ system can be studied. We conclude with two case studies, i) divalent cation transport in teleosts and ii) nitrogen handling in the elasmobranch GIT, to highlight how the use of multiple reductionist methods contributes to a greater understanding of the organ system as a whole.

1. Introduction

Integration of any physiological system within an organism naturally complicates our ability to understand the functioning of that system. Gastrointestinal physiology is no different as interpretation of *in vivo* studies can be confounded by: endogenous neural and hormonal pathways, the influence of microbial communities and endogenous secretions, and muscular contractions initiating unpredictable or unknown movement of digesta along the length of the gastrointestinal tract (GIT). The reductionist *in vitro* approach might simply view the GIT as an open tube from mouth to anus with a single layer of epithelial cells that functions to add (*e.g.* stomach acid secretion) or remove (*e.g.* intestinal absorption of glucose) substances from the lumen of the gut. These additions and removals ultimately aid in breakdown of food and subsequent provision of nutrients or energy for the organism. Indeed, most of the major advances in GIT physiology have been heavily reliant on cellular, tissue, and organ-based studies that in essence remove the confounding noise associated with the integrated role the organ plays in whole animal physiology. The reductionist provides a focused interpretation of the role single cells or regions of the gut may play in nutrient transport, which are then pieced together to provide a holistic understanding of how the GIT may be involved in regulating whole animal nutrient balance.

In fish, many of the *in vivo* approaches that have been adopted in mammals (such as fistula's or extra corporeal loops) are not possible, so much of our understanding of GIT function is related to *in vivo* mass balance studies alongside *in situ* or *in vitro* tissue preparations. Herein we will review the main approaches that have been used to develop our current understanding of fish GIT physiology. Importantly, the examples we use focus on both nutrient absorption and osmoregulation in the fish GIT. Indeed, one of the first major breakthroughs in fish gut physiology was the demonstration of teleosts drinking seawater for osmoregulatory purposes (Smith, 1930); a role that is now well-established in the literature, with the described mechanisms the result of a number of reductionist type studies being contextualised to form a whole. In brief, imbibed water is first desalinated by the relatively short region of the oesophagus (Hirano and Mayer-Gostan, 1976; Parmalee and Renfro, 1983); subsequent handling of ions in the intestine of the GIT results in formation of carbonate crystals first observed by Shehadeh and Gordon (1969) and later described as a critical component of water balance in

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marine teleosts (Taylor and Grosell, 2006; Wilson and Grosell, 2003). These and other reductionist studies have provided us with an understanding of both the reason and, as importantly, the mechanisms behind water balance in marine teleosts. These reductionist studies have also been used to develop a broader understanding of biogeochemical cycles such as estimating the contribution marine teleosts have toward the marine inorganic carbon cycle (Wilson et al., 2009); contributions that may be impacted by rising temperature, carbon dioxide and ocean acidification as a result of global climate change (Perry et al., 2010; Heuer et al., 2012; Heuer and Grosell, 2014). Levels of biological organisation that are far removed from the reductionist approach.

In this review we will expand on a variety of commonly used approaches in fish GIT physiology, all of which have been developed from the mammalian literature. We will briefly focus on divalent cation handling in teleosts and nitrogen balance in elasmobranchs as two case studies where reductionist techniques have been particularly well-utilised. While each technique has contributed significantly to our understanding of GIT function in fish, we recognise they are not without their pitfalls and we discuss challenges and limitations associated with each in regard to data interpretation.

2. *In vivo* preparations

Early studies of GIT function were accomplished using *in vivo* mass balance models with a 'substrate in' minus 'substrate out' approach to determine substrate absorption. While studies of this nature appear in human and other mammalian models in literature from the 18th century, major advances in fish physiology did not come about until the work of Homer Smith in the 1930s. Like his earlier counterparts, Smith began his investigations with 'input *vs* output' experiments to discover the role of the intestine in salt and water handling (Smith, 1930). By applying phenol red to tank water and observing its subsequent accumulation within the GIT, Smith determined that marine adapted fishes such as the sculpin, (*Myoxocephalus sp.*) and eel, (*Anguilla* sp.) drink their environment (Fig. 1A). The volume of water absorbed was quantified by collecting the red digestive fluids and measuring the amount of added water required to return the colouration to that of the surrounding tank water. Further, it was noted that as the water was absorbed along the tract, the phenol red became more concentrated. Fluids were then collected from different GIT regions where it was determined that monovalent ions were removed from the fluids at earlier segments (indicative of absorption), while divalent ions remained the same or even increased (indicative of exclusion and/or secretion) (Hickman Jr., 1968; Shehadeh and Gordon, 1969; Smith, 1930). Thus, with these simple *in vivo* experiments Smith became the first to note that marine fish drink seawater to maintain fluid balance, thereby designating a role for the fish intestine in osmoregulation.

Direct measurement of GIT fluids within specific segments is still employed to determine ion/water transport (*e.g.*Bogevik et al., 2009; Bucking and Wood, 2009; Bucking and Wood, 2007, 2006; Hansen et al., 2008; Koven et al., 1997; Wood and Eom, 2019). Generally, upon dissection the tract is ligated with sutures at either end of a designated section to restrict fluid movement and permit the collection of segment-specific fluids. The changing content of these fluids allows for inferences to be drawn regarding substrate and/or water movement. These measurements are often paired with *in vivo* measurements of plasma composition, as employed by Phillips (1944). In his study, Phillips manipulated the diet of brook trout (*Salvelinus fontinalis*), noting an increase in plasma salt content with elevated dietary sodium chloride concentrations (0.91 g/kg-1.82 g/kg) (Fig. 1B). These studies also revealed limitations in ion handling/homeostasis as fish fed extreme salt diets (3.64 g/kg) experienced edema and eventual mortality (Phillips, 1944). Similar limits in salt-load processing were discovered regarding branchial acquisition of salts wherein exposing fish to a 3–5% salt bath caused an increase in plasma salt-load that was differentially handled depending upon the concentration or duration of exposure (Phillips, 1944) (Fig. 1C).

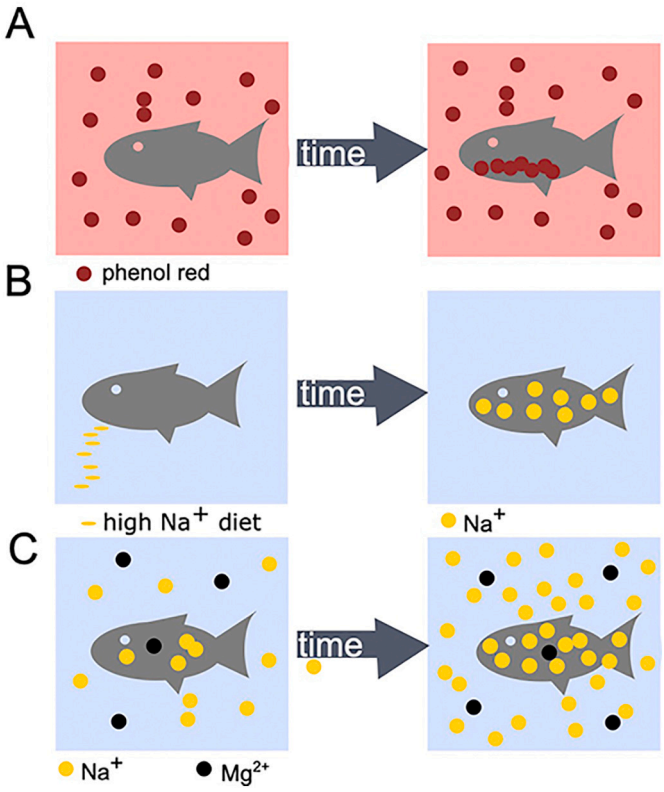

Fig. 1. Schematic representation of *in vivo* experiments. A) Smith (1930) added phenol red (red circles) to the tank water to determine that saltwater fish drink. Given time, the phenol red accumulated within the GIT of the fish. The imbibed volume was calculated as the amount of water added to the gut contents, resulting in a return of the concentrated red colour to that of the original tank water. B) Altering the dietary composition, can alter the plasma composition of the fish. In this image, representing experiments conducted by Philips (1944), the addition of high sodium food (yellow ovals) to the diet results in increased sodium (yellow circles) content inside the fish. C) Altering the environmental ionic composition can alter the plasma composition of the fish. In this image, the addition of sodium (yellow circles) to the fish tank results in heightened sodium content inside the fish. Magnesium (black circles) however, is instead primarily secreted by the fish and remains unchanged in the plasma. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Today, aquaculture operations have benefitted from *in vivo* studies in which diet is altered and nutrient uptake measured by appearance of substrates in the plasma/whole body content to determine absorption efficiency and how that may relate to changes in growth (*e.g.*Ambardekar et al., 2009; Azaza et al., 2009; Cai et al., 2015; Dabrowski et al., 2010; Hansen et al., 2008; Krogdahl et al., 2005, to name a few). These techniques ensure fine-tuning during regular operations of the aquaculture facility to improve conversion efficiency, yield at harvest and ultimately profit margin in the industry.

2.1. Limitations and advantages

While a plethora of knowledge on fish GIT function has been amassed using *in vivo* methods, there are some limitations that accompany these methods. The coarse nature of these measurements fails to account for the minutiae of digestive processes that enhance or prevent transport of substances across the epithelia. Specific endogenous contributions (*e.g.* digestive enzymes, biliary secretions, existing fluids) to the GIT, or loss of acquired substrate through urinary excretion cannot be accounted for. For example, Bucking and Wood (2007) demonstrated cation secretion

in the anterior section of rainbow trout (*Oncorhynchus mykiss*) intestine. They postulated these cations were derived from biliary, pancreatic, and intestinal secretions. Yet, without direct measurements of each potential source, these hypotheses remain unconfirmed. Furthermore, the role of sub-cellular components in nutrient absorption and the many possible transport proteins responsible for the movement of various ions and water cannot be identified or characterized under these types of experiments. Therefore, significant advances in our understanding of GIT transport physiology came as a result of isolating these systems from the animal to conduct *in vitro* investigations.

Despite these shortcomings, it is clear that a lot of information can be gleaned from *in vivo* experiments. The animal remains in relatively natural condition, and measurements can be made at the whole animal level. Intact circulatory systems will provide appropriate oxygenation, deliver hormonal signals, and remove acquired substrates from the area to maintain appropriate gradients for the movement of nutrients. Additionally, neuronal inputs and peristaltic movements persist, as do endogenous secretions. The interaction of all organ systems can provide a holistic picture of digestive processing that can fluctuate depending upon the animal's current metabolic state (*i.e.* energy expenditure *vs* reserves). One example of *in vivo* experiments leading to increased physiological understanding is whole body nitrogen handling in marine elasmobranchs. In one *in vivo* study conducted by Wood et al. (2007), dogfish were fed and GIT fluid samples were obtained over the course of 360 h. Differential urea handling was observed along the length of the GIT with stomach fluid urea (145 mM) and chyme content (310 mM) significantly elevated compared to that of the food source (4.2 mM). Interestingly, stomach fluid and chyme urea concentrations were regulated below that of the plasma (438 mM). This *in vivo* investigation suggested the urea was utilised to equilibrate the osmotic composition of the fluid and the authors proposed the possibility of intestinal secretion of urea *via* the ornithine-urea cycle (OUC) in order to regulate the osmotic composition of the chyme (Wood et al., 2007); a query that required further reductionist approaches to understand.

3. Gut sac preparations

Gut sac preparations are an *in vitro* technique where transport rates are either measured across the entire excised organ, or the intestine is divided into multiple segments to assess the transport capacity of each section. One of the earliest publications of this technique was within the rat intestine (Fisher and Parsons, 1949) and little has changed in terms of the method. To prepare a gut sac, the tissue of interest (*i.e.* stomach or intestine) is excised from the animal's body cavity. Following gentle rinsing with a physiologically relevant saline (*e.g.* Ringer's or Cortland's) the organ is tied off at either end with suture after being filled with saline containing the substrate of interest. The filled sac is suspended in a bath of aerated saline and the movement of the substrate is measured over a defined period of time. This movement is relative to the amount of tissue present, which can be determined as tissue mass (g), protein content (mg protein per g tissue), or surface area (cm^2). These metrics are then used to calculate flux, denoted by J, as follows:

$$J = \frac{(C_i \times V_i) - (C_f \times V_f)}{A \times T} \quad (1)$$

where C denotes the substrate concentration at the initial (i) or final (f) sampling point, V represents the total volume of the gut sac or serosal medium (depending on whether measuring disappearance or appearance), A denotes the tissue area, and T represents the flux time. This calculation is modified for radiolabelled substrates whereby the specific activity (the radioactivity per mole of substrate) is incorporated in C as follows:

$$C = \frac{R}{V_s \times SA} \quad (2)$$

where R represents the measured radioactivity, V_s is the sample volume that yields said amount of radioactivity, and SA represents the specific activity of the solution. This calculation for C can then be utilised in Eq. (1).

The measurement of substrate movement can be quantified in any one or all three of the following ways: mucosal disappearance, tissue accumulation, or serosal appearance. Often the substrate itself can influence which metric is utilised. For instance, acquired glucose will be rapidly converted into glucose-6-phosphase upon cell entry to maintain inward directed gradients for this important energy molecule (Bell et al., 1990). Thus, measurements of glucose disappearance from the mucosa are possible; however, the metabolization potential could alter tissue accumulation or serosal appearance measurements. Non-metabolizable analogs for glucose such as 2-deoxy-D-glucose or 3-*O*-methylglucose can provide a means to measure transport using methods other than mucosal disappearance. Substrate movement, whether mucosal disappearance, serosal appearance, or tissue accumulation, is often measured using radiolabelled substrates (*e.g.* $^{45}Ca^{2+}$ or ^{14}C-urea). Radioactive isotopes are highly sensitive and permit the direct and easy measurement of a labelled molecule of choice. This can occur at the level of the cell, tissue, or the whole body where, in the context of the GIT, researchers can measure the movement of the radiolabelled substrate across the tissue and beyond where the sequestration of radiolabel in different tissues can provide insight into the assimilation of that molecule. The position of the isotope on the molecule of interest can also provide insight into the metabolism of that substrate. For example, as glucose is metabolized the different carbon molecules (C1-C6) have different fates and are released as CO_2 (C3, C4), enter the tricarboxylic acid cycle in the C-1 position of acetate (C2 and C5) or in the C-2 position of acetate (C1, C6) (Kawagoe et al., 2016). Thus, the incorporation of radiolabel into a new molecule can illustrate the metabolism of the parent molecule. However, the use of radiolabelled isotopes has limitations, including isotope half-life (*e.g.* ~9.5 min ^{27}Mg and ~21 h for ^{28}Mg), making it prohibitive and/or costly to use in gut preparations. Availability of appropriate isotopes can also hinder research, for example the lack of radiolabelled ammonia consigns *in vitro* studies investigating ammonia transport across the GIT to using ^{14}C-methylamine as a proxy for ammonia. While research has shown similarities in terms of transport proteins used (Nawata et al., 2010), structural differences will impact transport kinetics (discussed below); however, these proxy molecules can still provide valuable insight into the mechanisms of GIT transport.

To conduct transport assays using radiolabelled substrates, the gut sac is prepared as above and radiolabelled substrate is added to the serosal or mucosal saline for the duration of the flux (Fig. 2). Upon termination of the flux period, the tissues are rinsed in baths containing elevated concentrations of the substrate of interest to displace any adsorbed radiolabelled substrate on the outer surface. The tissues can be scraped to separate the epithelia from the underlying muscle and connective tissue, and each tissue can then be digested in acid (*e.g.* 2 N HNO_3) at high heat (65 °C) for 2 days. Following digestion, scintillation cocktail is added when using beta-emitting isotopes, and samples are placed into a liquid scintillation counter which measures the radioactivity as counts per minute (CPM). When using higher energy gamma-emitting isotopes, scintillant is not necessarily required and the radioactivity (CPM) is measured using a gamma counter. In the case of tissue samples, a correction may need to be applied to account for radioactive 'quench' resulting from the tissue itself. To apply the quench correction a quench curve must be created by digesting a known tissue mass in HNO_3 as described above, to obtain tissue/mL of acid. From the digested tissue stock, a linear curve of increasing concentrations is created (*e.g.* 0, 20, 50, 200. 500 mg tissue) with an equal volume of known radioactivity added to each. As the tissue content increases, the amount of measurable radioactivity will often decrease in a linear fashion as the signal is quenched by the opaqueness of the increasingly concentrated tissue

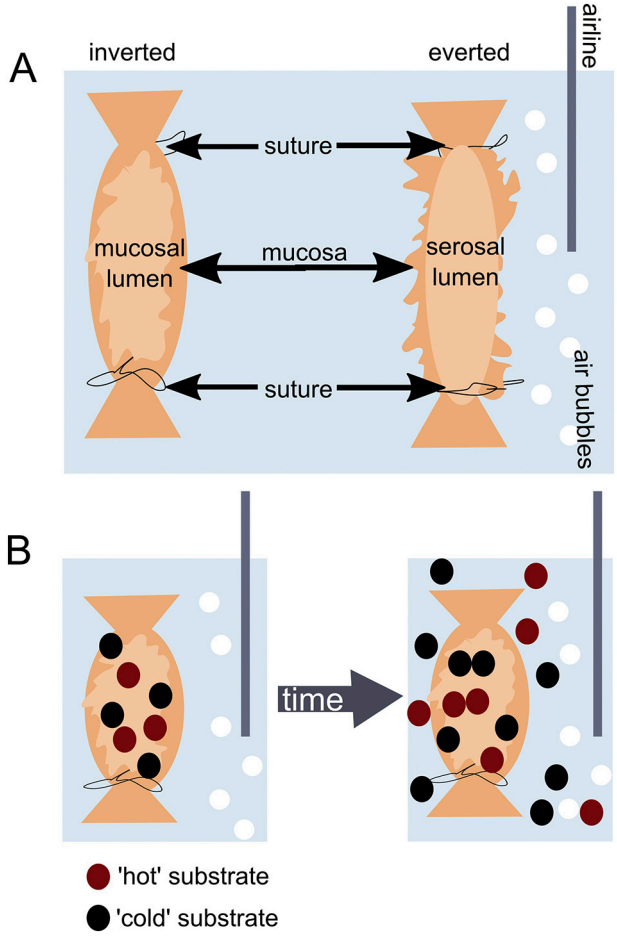

Fig. 2. Schematic representation of gut sac preparations A) portrayal of both the inverted (*i.e.* mucosa inside the sac as would present *in vivo*) and everted (*i.e.* mucosa facing outward) preparations. B) Demonstration of the gut sac technique using radiolabelled substrates. A known ratio of hot:cold substrate is added to the gut lumen. Over time, the substrate can be transported into the gut tissue or the surrounding serosal fluid for measurement of transport rates. Note: radiolabelled substrates are not always required.

solution. The equation of the line obtained from the relationship is then used to back-calculate the counts to what they would be without the confounding presence of the tissue.

3.1. Limitations and advantages

It is difficult to assess the true surface area of a gut sac preparation, given the fine structures of the GIT including: stomach rugae, folds, villi, microridges, and/or microvilli. Researchers may underestimate the surface area of the transport surface because these morphologies are not easily quantified. Some authors have considered these limitations and employed conversions to better account for gut surface area in their calculations. For example, Liew et al. (2013) calculated the surface area of the gut sac in relation to the kilogram of the animal as a whole to convert flux rates to a whole organism basis (rate per kilogram of body mass). Another limitation is the unstirred boundary layers created by tying off the ends of the gut sac and preventing lumenal mixing.

Researchers must also design experiments to be of an appropriate duration so as to get measurable differences when treatments are applied. For example, preliminary experiments might investigate transport rates over different time periods to establish when transport is saturated. By selecting a shortened time frame, the researcher will ensure transport is unsaturated and any increases in transport rate can now be discerned. An appropriate experimental duration is also important to ensure tissue viability as well as to minimize backflux, wherein the transport direction is reversed and the substrate returns to the lumen either *via* transcellular or paracellular means. By restricting the experimental time, the potential for intracellular buildup and the reversal of concentration gradients is lessened.

Additionally, serosal appearance can be difficult to accurately measure using gut sacs owing to the large volume of bathing media typically used; however, using an everted gut sac can help overcome this shortfall (Fig. 2A). The shift toward everted gut sac experiments did not occur until the 1950s and 1960s for fish research, and were based upon the original works of Wilson and Wiseman (1954) and the refined technique of Karasov and Diamond (1983) for mammals. The same gut sac protocol, as described above, is followed; however, upon excision, the intestine is everted using a glass rod. In this instance the internal environment ("serosa") is a much smaller volume and serosal appearance can be readily measured. In addition, the exposure of the intestinal epithelium to the aerating solution can minimize unstirred boundary layers and better oxygenate the tissue. Yet, this method is not without disadvantages. The mucosal volume is greatly amplified and the requisite substrate amount is likewise increased. The aerating solution, often 95% O_2:5% CO_2, also provides far greater O_2 concentrations to the lumenal tissues than the typical *in vivo* environment. Furthermore, the act of everting the organ can damage muscle fibres or lumenal mucosal tissue (Smith, 1964), which could distort measurements. This method is also not appropriate for animals such as Chondrichthyans (cartilaginous fish) with an intestinal spiral valve composed of invaginated folds that make eversion impossible. For both inverted and everted gut sac preparations, the issue arises of altered physiology with manual handling leading to a number of aphysiological conditions including the removal of mucus, irritation and mucus production, and/or potential release of enzymes. Furthermore, the act of organ excision itself removes *in vivo* circulation, as well as hormonal and neuronal inputs.

However, both inverted and everted gut sac preparations permit segment-specific analyses as multiple sacs can be created along the length of the tract to highlight differences not possible using whole animal *in vivo* preparations. Further advantages include a relatively simple experimental preparation. The reduced mucosal volume of an inverted gut sac results in reduced substrate requirements while the larger volume of serosal media, combined with its ease of access, permits successive sampling from the serosa. The application of stimulants and/or inhibitors for putative transporters can help elucidate the specific cellular pathways involved (see "modifiers of solute transport"). Given these many benefits, the gut sac approach has been extensively utilised to investigate many aspects of fish GIT function, including osmoregulation (Collie and Bern, 1982; Hoogenboom et al., 2020; Veillette et al., 1995; Weinrauch et al., 2020), nutrient absorption (Glover et al., 2011; Weinrauch et al., 2019; Weinrauch et al., 2018a, 2018b), ion regulation (Glover et al., 2016; Glover et al., 2015, 2003; Glover and Goss, 2020), and ecotoxicology (Grosell et al., 1999; Nadella et al., 2006; Ojo and Wood, 2007).

4. *In situ* intestinal perfusion

Another favoured method used to investigate GIT transport is *in situ* intestinal perfusion, which combines elements of the gut sac with that of the *in vivo* approach. In these experiments, a catheter can be implanted into the junction between the stomach and anterior intestine, alongside a stomach drain catheter to collect imbibed water. A rectal catheter is then inserted to permit posterior collections, while a caudal blood catheter is implanted to facilitate measurement of changes in blood parameters (*e.g.* Wilson and Grosell, 2003) (Fig. 3). Flux rate calculations incorporate the volume of perfusate using the following equation:

$$J_{net} = \frac{\left([X]_{perf}\right) - \left([X]_{if} + [X]_{ppt}\right)}{M \times T}$$

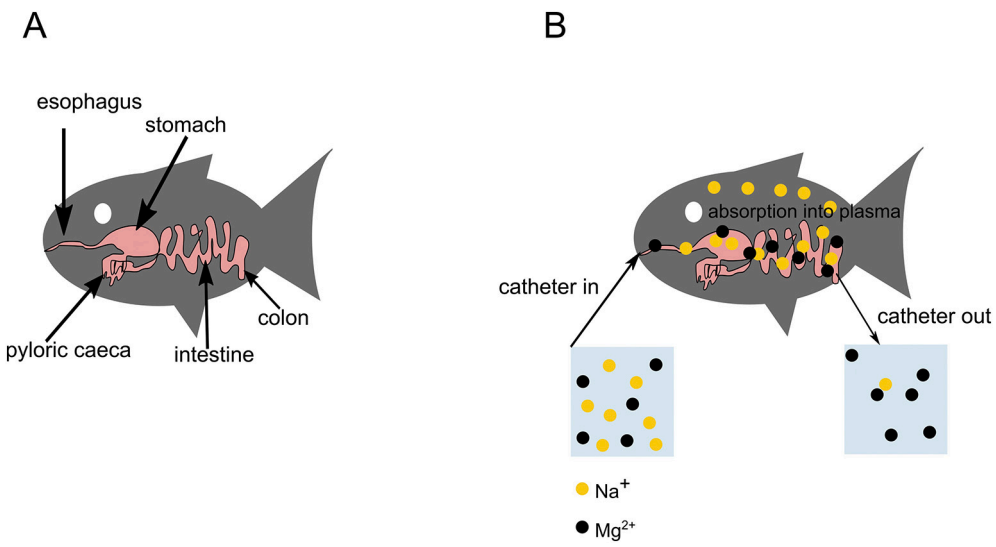

Fig. 3. Schematic representation of *in vivo* perfusion set up. A) denotes the structures of the fish GIT. B) The fish GIT is perfused with saline of the researcher's choice; in this case high Na^+ (yellow circles) and Mg^{2+} (black circles). As the perfusate moves along the tract, ions can be absorbed (*e.g.* Na^+) or excluded (*e.g.* Mg^{2+}) and excreted *via* the anal catheter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

where, J_{net} represents net flux rate, X_{perf} denotes the quantity of substrate added to the perfusate, X_{if} denotes the total amount intestinal fluid, while X_{ppt} represents any precipitates collected during the perfusion, M is the fish mass, and T represents the time over which the perfusion occurred.

4.1. Limitations and advantages

During such perfusions fish are maintained under anaesthesia, allowing intact animal functioning with the possibility to directly measure changes in intestinal ion/molecule/water movement. With this method the entire intestinal length can be examined, or as recently shown, sections may be assessed for luminal accumulation of CO_2 (Wood and Eom, 2019). Importantly, the rate at which solutions are perfused into the intestine can be supraphysiological and may not reflect the natural state. There are variations to this method where the entire gut is excised and the intestine and its vasculature are cannulated to permit continual flow through the tube (*e.g.*Bucking et al., 2011) (Fig. 4). With these experiments, stagnant build-up of substrates, which can hamper gradient transport, are no longer a worry, thus promoting a more natural rate of uptake.

5. Ussing chambers

While *in vivo* studies can be used to examine transport mechanisms in the context of the whole organism, and *in vitro* gut sac and perfusion

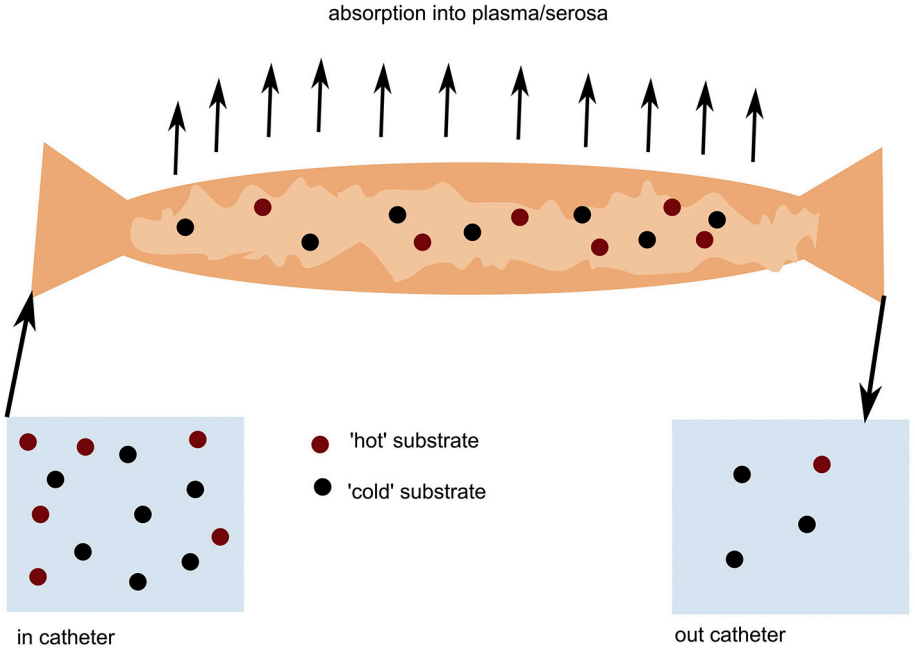

Fig. 4. Diagram of an *ex vivo* intestinal perfusion. The intestine is cannulated at either end and a fluid containing a known ratio of cold:hot substrate moves unidirectionally by way of a peristaltic pump. The intestine will transport the substrate into the tissue or serosal/plasma and the amount of transport can be calculated based upon appearance in the serosa or as the difference between in the 'in' and 'out' fluid. Note: radiolabelled substrates are not required.

preparations utilize whole organs, Ussing chambers allow for the examination of transport mechanisms across epithelial tissues. Hans H. Ussing, a "founder of epithelial transport" (Larsen, 2009; Lindemann, 2001), first published his bi-chamber design in 1949 (Ussing, 1949). Using radiolabelled isotopes (^{24}Na and ^{38}Cl) he examined the 'influx' and 'outflux' (now referred to as 'efflux') of sodium and chloride, and measured the potential difference across isolated frog skin mounted between two parallel plates (tissue holders). To eliminate diffusional forces the bi-chamber bathed both sides of the tissue in isotonic saline solution, allowing Ussing to show that sodium influx exceeded sodium efflux, and concluding it was due to active uptake. In a follow-up study with Karl Zerahn (Ussing and Zerahn, 1951) they introduced a modified chamber that allowed for the calculation of true active uptake across the epithelium. This new design passed an external current through the tissue to short-circuit (clamped to 0.0 mV to eliminate electrical gradients) the transepithelial potential difference (TEPD), ensuring both sides of the skin had the same potential. In combination with the isotonic saline solution (to eliminate chemical gradients), the short-circuit ensured any transport of ions across the tissue would be due to active transport, and the resultant current running through the short-circuit would equal all the net transport processes. Ussing and Zerahn discovered the current flowing through the short-circuited frog skin equaled the net active transport of sodium, with influx being the dominant direction of transport, creating a positive environment which attracts chloride and repels potassium (Ussing and Zerahn, 1951). These experiments led to an understanding of the active transport of sodium and ultimately expanded the field of transport research.

The fundamental electrophysiological concepts of Ussing's chamber are still applied in many fields of study including cystic fibrosis research, pharmaceutical drug development, food absorption efficiency studies in the aquaculture industry, and comparative investigations of terrestrial and aquatic animals (see case studies). Transepithelial voltage (V_{te}), or active transport potential, is generated as ions are actively transported across an epithelium (Frömter, 1979). TEPD (also denoted as TEP or PD) is the sum of all membrane potentials across an epithelium. TEPD is linked to tissue permeability, with higher TEPD values indicating greater unidirectional ion transport (Bach et al., 1997; Söderholm et al., 1998). Within an Ussing chamber set-up, TEPD can also be used as an indicator of tissue viability and epithelial function, with low initial TEPD readings significantly linked to impaired barrier function, leakage, and epithelium damage within human intestinal tissues (Söderholm et al., 1998; Wallon et al., 2005).

Transepithelial resistance (R_{te}, also denoted as TER or TEER) can be used as an indicator of the "tight" or "leaky" structure of a membrane (*i.e.* tissue integrity or permeability) (Bach et al., 1997; Söderholm et al., 1998; Srinivasan et al., 2015). R_{te} is the result of ion movement across cell membranes:

$$\frac{1}{R_{te}} = \frac{1}{R_a + R_b} + \frac{1}{R_s}$$

where transepithelial resistance (R_{te}) depends on the transcellular resistance of the apical (R_a) and basolateral (R_b) membrane and the paracellular (R_s) resistance (Hou, 2019). Therefore, a "tight" membrane with fewer paracellular spaces exhibits higher TER, while a "leaky" membrane will show lower TER because more ions move through the paracellular pathways rather than ion channels (Hou, 2019; Srinivasan et al., 2015).

Short-circuit current (I_{sc}) is the flow of current required to maintain V_{te} across the epithelium at 0.0 mV, and is an indicator of the net ion transport (*i.e.* the greater the epithelial transport, the greater the current required to maintain 0.0 mV) (Clarke, 2009). Higher I_{sc} values are indicative of active membranes exhibiting increased ion transport. The I_{sc} values of weatherloach (*Misgurnus anguillicaudatus*) intestine mounted in Ussing chambers were higher in the anterior region compared to the posterior; this corresponded to morphological and functional attributes indicative of an active digestive and absorptive zone. The lower I_{sc} in the posterior intestine corresponded with less active transport and was in line with the posterior's role in respiratory gas exchange (Wilson et al., 2013) When I_{sc} is used in conjunction with inhibitors, insight can be gained into the activity and regional distribution of ion channels across a membrane. Bumetanide, which inhibits basolateral Na^+-K^+-2Cl^--cotransporter (NKCC1), and amiloride, which inhibits the epithelial Na^+ channel (ENaC) were used on mammalian colonic tissue mounted in Ussing chambers. The basolateral application of bumetanide inhibited NKCC1 in the early distal colon, and the apical application of amiloride inhibited ENaC in the late distal colon. These transporter inhibitions were evidenced by a reduction in I_{sc}, consistent with reduced active transport across the colonic tissue (Tang et al., 2017). However, the researchers discovered that I_{sc} was stimulated (increased) in the early distal colon after the application of bumetanide. This led to the discovery that the I_{sc} current recorded in the late distal colon was due to active Cl^- absorption rather than Na^+ or K^+, and was not dependent on ENaC-mediated Na^+ absorption, as was previously thought.

To investigate ion channel behaviour, voltage-clamp and the current-clamp techniques can be used on tissues mounted in Ussing chambers. These techniques essentially allow ion flow to be recorded as electrical current (Halliwell et al., 1994). The voltage clamp technique "clamps" or holds the membrane potential at a known voltage and the resulting ion-generated current is recorded (Polder et al., 2005). Many membrane-bound ion channels are voltage-gated and only open within a certain range of membrane voltage (*i.e.* membrane potential). By subjecting tissue to various "clamped" voltages it is possible to gain insight into the ion channel activity of the membrane under specific currents. If a membrane is held at a hyperpolarized level (inside the cell becomes more negative) and only a slight current is recorded, it can be concluded that hyperpolarization would not lead to an increase in membrane ion permeability. However, holding the same tissue at a depolarized level (inside the cell becomes less negative) would result in a stronger current, and thus an increase in ion permeability. The current-clamp technique varies by only briefly inserting a current into a membrane rather than maintaining a prolonged voltage, and then recording the change to the membrane potential (Polder et al., 2005). This latter technique may be favourable for researchers concerned with membrane rupture which can occur during voltage-clamping if high voltage is sustained for too long (Robello and Gliozzi, 1989; Genco et al., 1993).

Another primary function of the Ussing chamber is to quantify the flux of ions or molecules across tissue mounted between two chambers, where influx (J_{ms}) represents the movement from the mucosal (*i.e.* apical or lumenal) to serosal (*i.e.* basolateral or blood) side, and efflux (*i.e.* accumulation) (J_{sm}) represents movement from the serosal to the mucosal side of the tissue. Net flux (J_{net}) is the subtraction of J_{sm} from J_{ms} so that resulting positive values indicate a net influx and negative values indicate a net efflux:

$$J_{net} = J_{ms} - J_{sm} \quad (4)$$

Several methods can be used to quantify the resultant movement of ions and molecules that occur across the epithelium, including ion chromatography and flame photometry for ions and charged molecules, scintillation counting for radiolabelled substrates (*e.g.* ^{14}C or ^{3}H labelled amino acids), and gamma counting for higher energy radioactive isotopes (*e.g.* $^{22}Na^+$ or $^{125}I^-$). Samples collected from either side of the Ussing chamber throughout an incubation period can be compared to an initial control sample of the saline solution to determine the net flux of analytes of interest across the tissue (Fig. 5). For the quantification of specific molecules, researchers can use a radioactive tracer specific to the molecule under investigation (*e.g.* $^{45}Ca^{2+}$; Flik et al., 1990) and place it into the "hot-side" of the Ussing chamber to subsequently quantify its movement to the opposite "cold-side". Control samples collected from both the hot- and cold-side immediately following the addition of the tracer can be compared to post-incubation samples to determine flux:

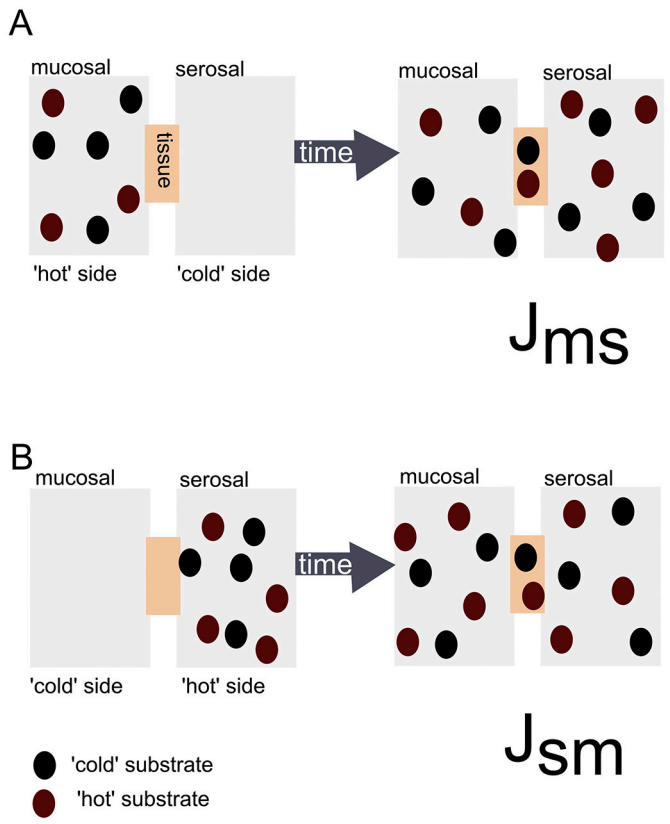

Fig. 5. Representation of intestinal Ussing chamber preparations. A) Demonstrates mucosal to serosal flux (J_{ms}) wherein hot substrate is added to the mucosal side of the tissue and over time appears in the tissue itself or the serosal chamber. Flux rate can be measured as serosal appearance and/or mucosal disappearance.
B) Demonstrates serosal to mucosal flux (J_{sm}) where hot substrate is applied to the serosal chamber and flux rate is measured as serosal disappearance and/or mucosal appearance of the substrate of interest. Note: radiolabelled substrates are not necessarily required.

$$J_{hc} = V \times \left(\frac{(A_f - A_i)}{SA \times S \times T} \right) \quad (5)$$

where: J_{hc} is the flux of the radiolabelled tracer from the hot-side to the cold-side (mmol h^{-1} cm^2), V is the total volume of solution in one side of the chamber (mL), A_f is the post-incubation activity of the cold-side (CPM ml^{-1}), A_i is the initial activity of the cold-side (CPM ml^{-1}), SA is the initial specific activity of the hot-side (CPM $mmol^{-1}$), S is the aperture of the tissue holder mounted between the chambers (cm^2), and T is the length of the incubation (h). By using a paired experimental design and placing the radiolabelled tracer into the mucosal side of the chamber in one set-up, and the serosal side of a second set-up, the J_{hc} (Eq. (5)) from the mucosal to the serosal side (J_{ms}), as well as the serosal to the mucosal side (J_{sm}) can be used to determine net flux (J_{net}), as calculated above (Eq. (4)).

A common metric of analysis for both gut sac and Ussing chamber preparations is to examine the uptake kinetics of the molecule of interest. Using the equation first developed by Michaelis and Menten (1913), the rate of substrate uptake is measured over increasing substrate concentrations.

$$v = \frac{J_{max} * [S]}{K_m + S} \quad (3)$$

where, v is the rate of reaction, J_{max} is the maximal reaction rate, [S] is the substrate concentration, and K_m represents the substrate concentration at which half- maximal reaction rate is achieved (a proxy for transporter affinity). The maximal reaction rate is observed as a plateau where the system becomes saturated (*i.e.* all available modes of transport are currently moving substrate at maximal capacity). The only means by which J_{max} can be increased is *via* an increase in the number of apically expressed transport proteins responsible for the movement of the substrate. The second metric obtained from kinetic analysis, K_m, can be altered as a result of allosteric modification of the transport protein. These parameters (J_{max} and K_m) can be used for comparisons across substrates, tissues, and organisms. Notably, fish often function at reduced temperatures compared to mammals, meaning intestinal transport processes are slower and lead to a higher affinity for each substrate (Bakke et al., 2010). There are some instances where the kinetics will not conform to the hyperbolic Michaelis-Menten form. For example, nutrient uptake in the Pacific hagfish (*Eptatretus stoutii*) is known to conform to sigmoidal kinetics, which is indicative of multiple transport pathways with differing affinities and capacities. This can be the result of cooperativity between subunits or substrates, allosteric modification of the transporters, or even the binding of the substrate to intestinal mucus (Glover et al., 2011; Glover and Goss, 2020; Weinrauch et al., 2018a, 2019).

Ussing chambers can also be used to measure the flux of acids (H^+) and bases (HCO_3^-) (Ficara et al., 2003). A pH-stat titration system can be used to calculate the base secretion rates of a tissue by recording the volume of acid added to the bathing solution to maintain a constant pH. The base secretion rates are calculated based on the concentration of the acid titrant and the rate of addition (Grosell and Genz, 2006). The same system can also be used to measure the reverse: the acid secretion rates calculated by the concentration of the base titrant and rate of addition (Genz and Grosell, 2011; Wilson et al., 2013). To determine whether ammonia moves across a tissue in its gaseous (NH_3) or ionized (NH_4^+) form, a pH-stat system can record the current across the short-circuited tissue, as well as the pH of the bathing solution. If NH_3 efflux occurs, the TEPD would not change but the lumenal fluid alkalinity would increase. If NH_4^+ efflux occurs, a change in current and voltage would be recorded, and lumenal fluid acidity would increase. If both NH_3 and NH_4^+ occurs, a change in current and voltage would be recorded, but the pH would remain stable (Rabbani et al., 2018).

5.1. Limitations and advantages

There are limitations to the Ussing chamber system, such as non-specific identification of transporter pathways, time-limited tissue viability, and intraspecific variations which can make data interpretation difficult. The small surface area of the tissue holder apertures can also be limiting; depending upon the experimental organism, a small tissue sample may represent only a minimal fraction of the organ under investigation, yet these small tissue sections are used to extrapolate conditions within the larger organ. Therefore, to gain a holistic understanding, it is prudent to qualify conclusions based on Ussing chamber data within the context of additional *in vitro* data, such as gut sac or prefusion preparations, molecular mRNA expression studies, and/or *in vivo* experiments.

There is also the potential for tissue damage or leakage when dissecting and mounting tissues if holes or tears occur. Tilapia (*Oreochromis sp.*) and dogfish (*Squalus sp.*) intestinal tissue preparations may require stripping the mucosal layer from the underlying serosal tissue prior to flux experiments (Anderson et al., 2015; Flik et al., 1990); damaged cells may prevent the passage of solutes, and holes can result in the unregulated flow of solutes and a decrease in recorded open circuit potential and R_{te} across the tissue (Gordon et al., 1990). Damage to the edge of the tissue (edge-effect) can occur if too much pressure is applied when the tissue is mounted between the two plates of the tissue holder and can extend up to 0.7 mm from the tissue holder into the preparation; this can result in enlarged intracellular spaces and provide additional pathways for leakage (Dobson Jr and Kidder 3rd, 1968; Gordon et al., 1990;

Higgins et al., 1975). Edge-effect is more substantial when the aperture and tissue samples are small (< 1 cm^2) and the damaged edge comprises a significant percentage of the exposed tissue; when possible the largest aperture and tissue samples should be used (Dobson Jr and Kidder 3rd, 1968; Helman and Miller, 1973). To mitigate the edge-effect, Stockmann et al. (1999) created a container for small tissue biopsy (0.05 cm^2) that prevents edge damage while maintaining accurate R_{te}, I_{sc}, and flux rate. This container creates the "tight" seal Hans Ussing (1949) required for his chambers while not damaging the tissue edge of very small samples. It should also be noted that care should be taken when loading the tissue onto the holders so as not to stretch the tissue and disturb the villi-crypt axis and underlying structures and affect the R_{te} of the tissue (Polentarutti et al., 1999; Stockmann et al., 1999; Sjöberg et al., 2013).

As discussed above in reference to the gut sacs, the *in vitro* Ussing chamber experimental set-up can also reduce *in vivo* enzymatic, hormonal, peristaltic, or microbial influence and/or control over the transport of molecules. This can be problematic when investigating the mechanisms behind molecular transport if a driving piece of the puzzle is missing. Also, *in vivo* influx is generally accompanied by subsequent movement of the molecule away from the site of influx through the circulatory system, meaning solutes transported down *in vivo* concentration or electrical gradients would not be impeded by stagnant accumulation. Therefore, a major disadvantage to current commercial Ussing chamber designs is a lack of serosal flush; meaning accumulation of the products transported across the mounted tissue could hamper subsequent transport if the gradients are reduced, or if a negative feedback loop is activated. To our knowledge, no commercial perfusion system is currently available; however, Li et al. (2004) have provided schematic drawings of an "in-house" continuously perfused chamber design.

On the other hand, the many advantages of the Ussing Chamber system include the small aperture of the commercial tissue holders allowing usage of tissues from small organisms (*e.g.* Killifish, *Fundulus heteroclitus*) (Genz et al., 2011) or biopsy samples (Stockmann et al., 1999). Alternatively, when used to examine animals such as the North Pacific spiny dogfish (*Squalus acanthias suckleyi*), the small aperture allows for multiple flux experiments using a single animal. An adult dogfish (> 1 kg) can yield 14–15 individual intestinal spiral valve folds, and each fold can generally be split into two portions, allowing for paired experimental designs. Thus, 28–30 non-repetitive flux experiments can be run on a single dogfish, reducing the requirement for wild-captured animals while yielding copious data. The volumes of the commercial chambers can also be smaller (< 5 mL) relative to whole organ perfusions or gut sac preparations of larger fish (> 5 mL), reducing the volume of reagents and/or labelled tracers required for flux experiments. When used in conjunction with perfusions or gut sac preparations, the reductionist approach to the Ussing chamber system can yield specific information on transport rates, as well as the effectiveness of inhibitors and promoters, giving insight into potential cellular transport pathways and support microscopy and molecular expression research.

6. Modifiers of solute transport

Solute movement across the GIT can be modified by many components including: ions/molecules of similar size and charge that compete or block relevant transporters (*e.g.* cobalt as a calcium substitute), pharmacological agents that directly (*e.g.* Diltiazem or Trifluoperazine for L-type calcium channels) or indirectly (*e.g.* ouabain for sodium-potassium ATPase (NKA)) affect a transport route, dietary components (*e.g.* amino acids), or gut contributions (*e.g.* mucus). While the alteration of solute movement may initially seem to have a negative connotation, researchers have co-opted these properties to learn more about transport routes as putative transport pathways can be determined through the use of specific inhibitors or stimulators. These inhibitors and stimulators can be applied to one or both sides of an Ussing chamber setup, or to the serosal or mucosal saline in a gut sac or intestinal perfusion. The subsequent passage of molecules can then be promoted, blocked, or unchanged to provide insight into probable transport pathways for the substrate under investigation.

In the instances where pathways of absorption are shared between molecules of similar physicochemical properties, the presence of one molecule can affect the absorption of another. Shared transport is particularly evident where substrates utilize promiscuous transporters such as amino acids (*i.e.* transport largely dependent upon charge) or metals (*i.e.* transporters for micronutrients of similar size and charge). Thus, when two substances are competing for the same transport pathway, the presence and concentration of each substance will affect the transport rate of itself and its competitor. For instance, using everted gut sacs, Berge et al. (1999) demonstrated interactive effects between two amino acids, arginine and lysine, in the Atlantic salmon (*Salmo salar*). Namely, arginine consistently inhibited lysine uptake whereas lysine could either stimulate or inhibit arginine transport depending upon the relative concentration of each amino acid.

Dissimilar molecules can also influence intestinal uptake rates, for example, using intestinal perfusions Glover and Hogstrand (2002) revealed altered zinc absorption in the gut of freshwater rainbow trout (*Oncorhynchus mykiss*) when amino acids were added to the luminal solution. Specifically, it is suggested that histidine could either maintain zinc's solubility (thereby promoting uptake), or a zinc-histidine chelation complex could form and be transported by a new pathway. Solute transport may also be affected by gut contributions such as mucus. Research on the rainbow trout demonstrated increased metal uptake when mucus was present (Ojo and Wood, 2007). This was likely owing to the binding of the charged metals to the mucus layer. The acidic microclimate next to the gut epithelia then favoured release from the mucus, leading to elevated uptake rates (Bakke et al., 2010). Given the naturally complex intestinal environment, the effects of these modifiers of solute transport should be considered when conducting GIT transport research.

7. Case Studies

The GIT contributes to multiple aspects of an animal's homeostasis (*e.g.* digestion and nutrient absorption, acid-base balance, and water-mineral balance). Herein we provide two case studies (divalent cations and nitrogen transport) illustrating how a combination of the aforementioned techniques has ultimately advanced our understanding of the fish GIT and its role in osmotic homeostasis.

7.1. Divalent cations in the fish GIT

From the initial *in vivo* experiments of Smith (1930), it was determined that divalent cations (*i.e.* Mg^{2+} and Ca^{2+}) were not absorbed along the GIT but were excreted instead. Yet, these cations are integral to many physiological processes, including: activators and/or modulators of many enzymes including ATP-dependent transporters; potential secondary messenger in the regulation of hormone actions; and, primary components in hard structures including bones and scales (reviewed in: Bijvelds et al., 1998; Flik and Verbost, 1993). Therefore, researchers have investigated the possibility that dietary routes of uptake contribute to cation acquisition, in addition to the known branchial routes of uptake (Baldisserotto et al., 2004; Bjornsson and Nilsson, 1985; Hossain and Furuichi, 1999; Shearer and Åsgård, 1992; Shim and Ng, 1988; Sundell and Bjornsson, 1988). By removing/altering cation content from feed, it has been determined that these substrates are necessary to sustain fish health at the whole animal level. Although magnesium is largely excluded in the intestinal fluid, studies have revealed a 30–70% absorption of calcium (Bjornsson and Nilsson, 1985; Hickman Jr., 1968; Shehadeh and Gordon, 1969). More recently, dietary acquisition of these cations were examined in rainbow trout (*O. mykiss*) using both segmental cation disappearance within the tract, and cation appearance in the plasma (Bucking and Wood, 2007). The results from these studies

suggest the stomach, a GIT segment heretofore thought to be primarily involved in digestion and not absorption, is actually responsible for ~50% of dietary calcium uptake and ~90% of dietary magnesium uptake in this freshwater fish (Bucking and Wood, 2007). While these whole animal studies provide insight into dietary cation regulation, the mechanisms of uptake were not discernable but studies conducted on gut-sac preparations or Ussing chambers have provided more information on the specific mechanisms of transport.

Ussing chamber studies in tilapia (*O. mossambicus*) utilised ouabain (a pharmacological inhibitor of NKA; see *"modifiers of solute transport"*) to examine the potential role of NKA in intestinal calcium and magnesium transport (Flik et al., 1993; Flik et al., 1990). The addition of ouabain to the serosal chamber led to significant reductions in both calcium and magnesium influx. Similar findings were obtained when sodium was removed from the saline solution; thus, it was determined that calcium and magnesium uptake from the intestinal lumen of *O. mossambicus* were sodium-dependent. The evidence from these studies suggested involvement of NKA as well as Na^+/Ca^{2+} or Na^+/Mg^{2+} exchangers, but further molecular studies (not discussed in detail in this review), such as gene cloning, oocyte expression systems, immunohistochemical or *in situ* hybridization analyses would be necessary to confirm the transporters involved. Competitive inhibitors of calcium transport, lanthanum and cobalt (relatively non-specific calcium channel blockers), were applied to the mucosal saline of gut sacs in experiments conducted on Pacific hagfish (*E. stoutii*) at a 10× concentration of the radiolabelled substrate to ensure effective competition. While these inhibitors did not alter calcium transport in the hagfish gut, the authors indicate that this may be the result of the concentrations tested (*i.e.* testing inhibition at low substrate concentrations on a high-capacity transport system) (Glover and Goss, 2020). Other studies of inhibitors on gut calcium uptake in seawater acclimated rainbow trout have likewise shown no effect (Klinck et al., 2012), yet the same inhibitors do significantly alter calcium acquisition rates in isolated Atlantic cod (*Gadus morhua*) enterocytes (Larsson et al., 1998). Although these are different species which may lead to disparate results, it may also be the result of the method employed wherein the gut sac has additional components (*e.g.* gut enzymes, mucus) contributing to the effectiveness (or lack thereof) of the inhibitor. One study from Genz et al. (2013) examined the rate of calcium flux across the intestine of the lake sturgeon (*Acipenser fulvenscens*) acclimated to differing external calcium environments using both gut sac preparations and an Ussing chamber setup. While the magnitude and direction of flux calculated in this study agreed with previous results from this species acclimated to low (0.1 mM) environmental calcium (Allen et al., 2011), opposing fluxes were determined within the study itself depending upon method. Lake sturgeon acclimated to normal (0.34 mM) and elevated (2.26 mM) external calcium environments demonstrated a positive net flux in gut sac preparations yet a negative net flux using the Ussing chambers. The authors attribute this difference to the different methods employed, with gut sacs directly measuring net flux and the Ussing flux determined from serial samples calculating both influx and efflux over time. Perhaps, akin to the hagfish study, the lower calcium levels did not show a difference as the tested concentration was below that typically seen by the transport system; yet, when the system is functioning at a relevant or elevated concentration, transporter kinetics are affected and the method employed may impact the results obtained.

The use of multiple methods has substantially furthered our understanding of divalent cation homeostasis in the intestine. Although the gut acquires divalent cations, the original findings of exclusion are still true (Smith, 1930). The elevated concentrations of magnesium and calcium in GIT fluids, combined with their propensity for precipitation with phosphate and carbonate, have been co-opted by seawater fish as a mechanism for osmoregulation. This integral finding was decades in the making and began with the experiments of Shehadeh and Gordon (1969) where they first noted the presence of so-called 'mucus tubes' within the intestines of seawater-acclimated rainbow trout. These 'mucus tubes' increased in prevalence with increasing environmental salinity, leading to the formation of the hypothesis that this was a route for divalent cation excretion. Wilson and Grosell (2003) built upon this hypothesis by utilizing *in vivo* whole animal experiments, gut perfusions, and Ussing chambers. The intestine of the European flounder (*Platichthys flesus*) was perfused with one of three calcium-containing solutions: normal calcium (5 mM), high calcium (20 mM), or normal calcium in a highly buffered condition (to limit calcium carbonate precipitation). The high calcium condition led to a near 60% increase in bicarbonate excretion and 7× increase in carbonate precipitates, while calcium carbonate precipitation was limited in the highly buffered saline. This suggested the fish were able to match bicarbonate secretion rates in order to prevent excessive calcium uptake. This was further supported by *in vivo* data where fish were housed in seawater containing elevated concentrations (7× normal levels) of calcium. Despite a high calcium content in the imbibed water, plasma calcium concentrations remained unchanged, but led to decreasing plasma total CO_2 (tCO_2). The authors suggested the decrease in tCO_2 was the result of elevated bicarbonate secretion and the need to hydrate endogenous CO_2. The Ussing chamber experiments corroborate these findings where the serosal application of CO_2 led to increased bicarbonate secretion (Wilson and Grosell, 2003). Furthermore, the Ussing chamber experiments demonstrated a role for pH, with lower pH values leading to elevated bicarbonate secretion rates, leaving electrophysiological measurements untouched. Overall, these experiments demonstrate that the seawater teleosts are capable of matching intestinal bicarbonate secretion rates with calcium content to limit cation uptake and maintain internal calcium homeostasis, but simultaneously promote water absorption by limiting osmolyte numbers *via* calcium carbonate precipitation (Wilson and Grosell, 2003). It is clear that our advancing knowledge of fish GIT cation transport for diet and osmoregulation is the result of a combination of reductionist approaches.

7.2. Nitrogen homeostasis in the elasmobranch GIT

The body of literature concerning the role of nitrogen in aquatic animals has also benefited from the conglomerate of reductionist methods. Nitrogen is essential for somatic processes, but nitrogen requirements for some animals go beyond somatic obligations and play a critical role in osmoregulation, as it does for ureosmotic cartilaginous fishes (Chondrichthyans). These animals retain high concentrations of urea (> 250 mM) to help balance the osmotic pressure of their marine environments while retaining almost negligible ammonia concentrations (< 250 uM) (Smith, 1936; Wood et al., 2010). Historically, research investigating nitrogen balance and homeostasis in Chondrichthyans focused on excretion across the gills and kidney (Boylan, 1972; Boylan, 1967; Fines et al., 2001; Hays et al., 1977; Morgan et al., 2003b; Morgan et al., 2003a; Pärt et al., 1998; Schmidt-Nielsen et al., 1972; Walsh and Smith, 2010). More recently, focus has shifted to the GIT as the main site of dietary nitrogen acquisition (Anderson et al., 2015; Anderson et al., 2012, 2010; Hoogenboom et al., 2020; Liew et al., 2013; Wood et al., 2019; Wood et al., 2007); thus, research has begun to provide insight into the acquisition and retention of a critical metabolite, rather than primarily examining the excretion of a waste product.

Marine elasmobranchs have been described as "nitrogen-limited" due to infrequent feeding habits, continual loss of urea primarily across branchial routes, and the requirements of nitrogen for somatic and osmotic processes (Wood, 2001). Studies focused on *S. a. suckleyi* have revealed these elasmobranchs are capable of regulating their internal nitrogen concentrations both during active digestion and between meals (*i.e.* periods of fasting) (Hoogenboom et al., 2020; Kajimura et al., 2008; Liew et al., 2013; Wood, 2001; Wood et al., 2019; Wood et al., 2010). An *in vivo* study that fit *S. a. suckleyi* with indwelling stomach tubes for controlled force-feedings, showed urea excretion (which comprises >90% of total nitrogen excreted) did not change following a meal, while ammonia (which comprises <3% of nitrogen excreted) increased only

slightly (Wood et al., 2005). A second feeding study allowing the dogfish to feed voluntarily showed a post-prandial decrease (~39%) in urea excretion (Wood et al., 2007). A concomitant study on prolonged fasted dogfish showed that 56 days without food did not affect the rate of urea excretion in these animals, nor urea concentrations in the plasma, while ammonia excretion was negligible and plasma ammonia decreased significantly (Kajimura et al., 2008; Wood et al., 2010). When challenged with increased prandial urea, as would occur when feeding upon other Chondrichthyans, the plasma urea and ammonia concentrations also did not change (Hoogenboom et al., 2020). Collectively, these studies indicate strong homeostatic regulation of internal nitrogen concentrations regardless of metabolic state.

To further investigate homeostatic mechanisms of whole-animal nitrogen balance and quantify the uptake of nitrogen from the GIT, *in vitro* gut sacs were made from both fed and fasted dogfish cardiac and pyloric stomachs, intestinal spiral valve, and colon (Anderson et al., 2010; Hoogenboom et al., 2020; Liew et al., 2013; Wood et al., 2019). These studies showed that nitrogen, in the form of both ammonia and urea, is taken up across all sections of the GIT of fed dogfish (Liew et al., 2013; Wood et al., 2019). This was confirmed when individual spiral valve folds from fed dogfish were mounted in Ussing chambers and a net influx of ^{14}C-urea was shown to occur from the lumenal to the basolateral side (Anderson et al., 2015). Only spiral valves from fasted dogfish showed a net efflux, or accumulation, of urea within the lumen; this trend also held true for the intestine and colon of fasted little skate (*Leucoraja erinacea*) (Anderson et al., 2010; Liew et al., 2013). Subsequent work using Ussing chambers also showed ^{14}C-urea accumulated on the lumenal, or apical, side of fasted dogfish (Anderson et al., 2015). This sequential reductionist approach showed the intestine to be the main site of nitrogen acquisition along the GIT, while showing the colon to be resistant to the loss of urea (Anderson et al., 2010). It has also opened the door for further investigations into the specific transport pathways responsible for nitrogen transport, some of which have shown mRNA expression of a urea transporter (UT) and two Rhesus (Rh) glycoprotein ammonia transporters (Rhbg and Rhp2) along the GIT (Anderson et al., 2010; Nawata et al., 2015; Hoogenboom et al. unpublished).

The *in vitro* gut sac and Ussing chamber data concerning nitrogen movement raised an interesting question: why would a nitrogen-limited animal allow nitrogen to accumulate in the lumen of the intestine during periods of fasting when nitrogen-intake is not occurring? The answer may lie within the intestinal microbiome. *In vivo* samples of intestinal digestive fluids and epithelial tissue showed the presence of urease, likely microbial in origin, in both fed and fasted *S. a. suckleyi* and spotted ratfish (*Hydrolagus colliei*) (Wood et al., 2019). Mucosal saline samples collected after 3 h incubation of *in vitro* gut sacs of the two stomachs and intestine also showed the presence of urease (Weinrauch et al., 2020). This lead to the proposal that urease likely plays an important role in nitrogen homeostasis in Chondrichthyans; urease may convert accumulated urea into ammonia for subsequent synthesis of other nitrogenous products, such as amino acids (Wood et al., 2019). This nitrogen recycling may not be solely for the benefit of the host, but also the overall health and sustenance of the GIT microbiome in elasmobranchs, as proposed by Hoogenboom et al. (2020) and previously shown in other vertebrate groups (Singer, 2003). Thus, it is clear that these varied reductionist approaches work together to form new hypotheses and understandings of physiological systems.

8. Conclusions

As with most reductionist approaches no single method tells the whole story. Rather, our understanding of gut function in fishes is a combination of methods employed alongside a comparative assessment of functionality from other vertebrate groups. Importantly, we cannot rely on any single method as each has their own set of advantages and disadvantages. In understanding the physiology of the GIT in fishes it is worth noting that reductionist methods will likely underrepresent the role of the microbiome in GIT physiology. Furthermore, removal of any tissue from the organism will result in changes in fluid chemistry with gasses likely being the most immediate and dramatic. Only recently were the first *in vivo* levels of pCO_2 in the fish gut reported (Wood and Eom, 2019), suggesting that epithelia tissue in the intestine of the rainbow trout and goldfish is faced with significant levels of CO_2 that are influenced by feeding and salinity; levels of CO_2 not normally employed in isolated gut tissue experiments such as the gut sac or Ussing chambers. Furthermore, techniques not discussed herein can significantly add to our understanding of fish GIT function. The development of isolated cell cultures, while costly and time-intensive, can yield consistent and reproducible results. The external physicochemical environment which bathes the cells can be accurately controlled and provide a more realistic environment, especially pertaining to the gasses discussed above. In the last decade, an enterocyte cell line (RTgutGC) has been verified as an appropriate model for transport studies (Kawano et al., 2011) and provides an exciting new reductionist approach for the fish physiologists to employ. Knowledge of gut physiology in fishes has been enhanced using the reductionist approach, and as advances are made in measuring techniques so will our ability to further refine our understanding of gut physiology.

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