A survey of ionoregulatory responses to extended exercise and acute hypoxia in freshwater Amazonian and Southern Ontarian teleosts: *Investigating the osmorespiratory compromise*

By

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TITLE: A survey of ionoregulatory responses to extended exercise and acute hypoxia in freshwater Amazonian and Southern Ontarian teleosts: *Investigating the osmorespiratory compromise.*

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ABSTRACT

The osmorespiratory compromise is the trade-off between high gill permeability for oxygen uptake and low gill permeability for conservation of ions in fish. The fundamental purpose of this study was to examine facets of the osmorespiratory compromise in freshwater fish under conditions of extended exercise and acute hypoxia, in light of previous research identifying very different gill morphometric and ionoregulatory modifications in the hypoxia-tolerant Amazonian oscar (*Astronotus ocellatus*) and the hypoxia-intolerant rainbow trout (*Oncorhynchus mykiss*). A technique using [^{3}H]polyethylene-4000 ([^{3}H]PEG-4000) for branchial paracellular permeability measurement was developed, and then applied to investigate the osmorespiratory compromise during extended swimming. Methods were developed to overcome the challenges of renal [^{3}H]PEG-4000 loss, respirometer surface adsorption, and freshwater drinking of the chemical. In both trout and oscar, corrections were employed for these sources of error – leading to findings that in both species, branchial [^{3}H]PEG-4000 permeability was not rectified and freshwater drinking was quite high. In both species, during an 8-h swim (1.2BL/s), oxygen consumption rate increased by 75-90%; drinking rate remained high but did not increase. Branchial paracellular permeability increased by 61% during exercise in trout but remained constant in oscar. The methods developed here can be widely applied to future studies of branchial paracellular permeability.

Unidirectional fluxes (by ^{22}Na) of sodium, and net fluxes of potassium, ammonia, and urea were observed during a 2-h nomoxia:2-h hypoxia (30% O_2 saturation):2-h normoxic recovery protocol – to identify adaptive trends across phylogenies and/or

environments in North and South American teleosts. Strategies for coping with hypoxia appeared to be environmentally, rather than phylogenetically linked, since both the oscar (perciform) and the tambaqui (*Colossoma macropomum* – characiform) displayed characteristic permeability reduction (of apparent transcellular origin); both frequently encounter severe hypoxia in their natural habitat. Two North American perciforms, pumpkinseed (*Lepomis gibbosus*) and bluegill sunfish (*Lepomis macrochirus*) which live in less hypoxic environments, increased branchial ion leakage as in the hypoxia-intolerant trout. Four Amazonian tetra species (all characiformes: *Paracheirodon axelrodi, Hemigrammus rhodostomus, Moenkausia diktyota, Hyphessobrycon bentosi rosaceus*) which experience intermediate hypoxia in their native Rio Negro presented variable responses. Finally, during a 4-h swim at1.2BL/s, branchial ion fluxes were not reduced but elevated in oscar, indicating that ionoregulation in this species occurs primarily transcellularly, and that adaptive strategies to one manifestation of the osmorespiratory compromise (hypoxia) may not apply to another (exercise).

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Hmm, well if I ever *do* make it to the Oscars, looks like I've already got a speech.

THESIS FORMAT:

This thesis is written in the 'sandwich' format, with four chapters, as approved by the members of my supervisory committee. The first chapter is a general introduction and overview of my project, while the second and third chapters are manuscripts in preparation for submission to peer-reviewed scientific journals. The final chapter revisits this project's objectives and outlines major findings and applications, as well as their relevance to this area of physiology.

Chapter 1*: General introduction; background and thesis objectives.*

Chapter 2*: A methodological investigation of PEG-4000 handling during rest and exercise in the rainbow trout (*Oncorhynchus mykiss*) and the Amazonian oscar (*Astronotus ocellatus*).*

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***Comments*:** This study was conducted by LMR under the supervision of CMW. The experiments on oscars in Brazil were facilitated with much help from DK, AB, VFA-V, and ALV. The latter studies were performed in the labs of AB, VFA-V, and ALV.

Chapter 3*: A survey of ionoregulatory aspects of the osmorespiratory compromise during acute environmental hypoxia in tropical and temperate teleosts.*

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Chapter 4*: Summary of findings and conclusions.*

TABLE OF CONTENTS

LIST OF FIGURES

Figure 2.9. Relative [^{3}H]PEG-4000 uptakes normalized for weight (g) and water concentration (cpm/mL) as compared between carcass and ECFV (extracellular fluid volume) for non-cannulated, gut-excised stationary and swimming (1.2BL/s, 15cm/s) trout at 18˚C. Means are ±S.E.M. (N=7 for stationary carcass, 6 for stationary ECFV; N=14 for swimming). Means sharing the same letter are not significantly different (P>0.05), as determined by a paired t-test.

Figure 2.10. Branchial [^{3}H]PEG-4000 permeability changes between non-cannulated stationary and swimming juvenile trout at 18˚C. Means are ±S.E.M. (N=7 for stationary; N=14 for swimming). Means sharing the same letter are not significantly different (P>0.05) as determined by a paired t-test.

Figure 2.11. Division of total [^{3}H]PEG-4000 cpm in cannulated Amazonian oscar in efflux experiments, as an indicator of flux location at ~28˚C. Means are ±S.E.M. (N=5). Means sharing the same letter are not significantly different (P>0.05) as determined by a paired t-test performed on arcsin values.

Figure 2.12. Various physiological clearance rates in cannulated stationary Amazonian oscars in [^{3}H]PEG-4000 efflux experiments at ~28˚C. Means are ±S.E.M. (N=5). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way ANOVA followed by Tukey *post hoc* test.

Figure 2.13. [^{3}H]PEG-4000 permeability in stationary, cannulated efflux and influx trials in Amazonian oscars at ~28˚C. Means are ±S.E.M. (N=5). No significant differences.

Figure 2.14. Metabolic rate – as measured by oxygen consumption – changes with 4 h swimming at 1.2BL/s (15cm/s) at ~28˚C, in Amazonian oscars. Means are ±S.E.M. (N=4 for stationary; N=23 for swimming). Means sharing the same letter are not significantly different (P>0.05) as determined by a paired t-test.

Figure 2.15. Relative [^{3}H]PEG-4000 uptakes normalized for weight (g) and water concentration (cpm/mL), comparing uptake estimated from ECFV (extracellular fluid volume) and carcass counts in stationary and swimming influx trials in Amazonian oscars at ~28˚C. Means are ±S.E.M. (N=9 for all except swimming ECFV where N=7). No significant differences.

Figure 2.16. Drinking rate, as estimated by excised gut [^{3}H]PEG-4000 cpm in Amazonian oscars at ~28˚C, FW. Means are ±S.E.M. (N=4 for stationary, N=9 for swimming). No significant differences.

Figure 2.17. Tissue [^{3}H]PEG-4000 amount (mL) in Amazonian oscars based on tissue cpm, corrected for weight (g) and normalized for water concentration (cpm/mL) at ~28˚C. Means are ±S.E.M. (N=8 for all except stationary gut trials where N=4). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way ANOVA on ranks followed by Dunn's *post hoc* test.

Figure 2.18. [^{3}H]PEG-4000 permeabilities during with swimming, as measured via influx experiments in the Amazonian oscar at ~28˚C. Means are ±S.E.M. (N=9 for all except swim ECFV (extracellular fluid volume) where N=7). No significant differences.

Figure 2.19. [^{3}H]PEG-4000 changes based on water cpm loss during influx swimming trials, (A) in rainbow trout at 18˚C. Means are ±S.E.M. (N=20). (B) in Amazonian oscars at ~28˚C. Means are ±S.E.M. (N=10). (C) for the purpose of predicting 'theoretical' uptake – versus actual ECFV (extracellular fluid volume) and carcass uptakes. Means are ±S.E.M. (N=20 for trout water, N=10 for oscar water; N=14 for trout ECFV and carcass; N=7 for oscar ECFV, N=9 for oscar carcass). Means sharing the same letter within species are not significantly different (P>0.05) as determined by a one-way ANOVA on ranks followed by Dunn's *post hoc* test.

Figure 3.1. Flux changes during 2-h:2-h:2-h normoxia:hypoxia:recovery protocol for the Amazonian oscar at ~28˚C, with (A) 30% O_2 saturation and (B) 10% O_2 saturation. Means are ±S.E.M. (N=6). Letters denote significance (P<0.05) as determined by a one-way repeated measures ANOVA, with a Holm-Sidak *post hoc* test (B) 10% O_2 sat. Means are ±S.E.M. (N=6). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA with a Holm-Sidak *post hoc* test.

Figure 3.2. Rainbow trout flux changes during 2-h:2-h:2-h protocol (~35% average O_2 sat throughout the 2-h hypoxic period) at 18˚C. Means are ±S.E.M. (N=5). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

Figure 3.3. Na influx changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak post hoc test.

Figure 3.4. Na efflux changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

LIST OF TABLES

LIST OF ABBREVIATIONS

MO_2: Oxygen consumption

PO_2: Partial pressure of oxygen

FW: Fresh water

JNa in/out/net: Na flux in/out/net

GFR: Glomerular filtration rate

UFR: Urine flow rate

ECFV: Extracellular fluid volume

DR: Drinking rate

PEG-4000: Polyethylene glycol, M.W. 4000

CHAPTER ONE: General Introduction

Ionoregulation in Freshwater Fish

The Father of Comparative Physiology, August Krogh, has been named as putting forth 'Krogh's Principle;' the notion that: "For many problems there is an animal on which it can be most conveniently studied" (Krogh, 1929; Krebs, 1975). While this may appear intuitive now, Krogh's involvement with comparative animal studies allowed him to appreciate how gaining an adequate understanding of complicated principles in basic systems can have value for applications to more complex environments and physiology. Due to his significant contributions to physiology, particularly in the field of fish physiology, fundamental concepts of ion regulation in both sea and freshwater teleosts have been established and further developed since. In fact, three physiologists – August Krogh, Homer Smith, and Ancel Keys – were among the first to study ion differences between fish and their environment and the homeostatic mechanisms that govern them. In the case of marine teleosts, the external environment contains ~60% more NaCl per liter than their internal environment, rendering them 'hypotonic' relative to the water (Smith, 1932; Evans, 2008). As a result of this strong tissue-environment ion gradient, these fish run a constant risk of dehydration and/or salt overload, and therefore maintain homeostasis by drinking the water around them and producing small quantities of relatively concentrated urine (Smith, 1932). Conversely, freshwater fish encounter the opposite challenge: as their plasma has a ~ 300x greater osmotic concentration than the surrounding environment with the bulk of the osmotic pressure being contributed by Na and Cl. This tends to cause overhydration and a net loss of salts – compensated for by

active branchial ion uptake, diet, and excretion of large volumes of dilute urine (Smith, 1932; Evans, 2008). As the gills are a crucial site of ion exchange, determining factors or environments that influence branchial permeability is of the utmost importance for understanding ionoregulation in teleost species.

True to the Krogh Principle, foundational work on fish from the 1930s has led to considerable advances in this field. Since then, it has become possible to identify and clone specific channels and transporters on both the apical (water-facing) and basolateral (internally-facing) gill epithelia involved in homeostatic regulation of internal ion concentrations, acid-base balance, and nitrogenous waste excretion.

Oxygen uptake Mechanics and the Osmorespiratory Compromise

Besides ionoregulation, perhaps the most obvious function of the gill is its role in oxygen uptake. In order to maximize oxygen absorption from the water, blood flows through the gill lamellae – which comprise the majority of the gill filament's surface area– counter-currently to water (Hills and Hughes; 1970; Hughes and Morgan, 1973). This means that when water flows from the buccal cavity over the filaments, it is in a direction opposite to that of deoxygenated blood flow such that oxygen can be continually taken up via diffusion, while always maintaining a high environment-plasma oxygen gradient. Interestingly enough, in rainbow trout, Booth (1978) reported that only 58% of all secondary lamellae are perfused with blood under resting conditions. This is believed to allow for increased lamellar recruitment – and therefore increased oxygen uptake – when necessary. This becomes particularly important during hypoxia (Fisher et al., 1969)

and exercise (Randall et al., 1967). Nevertheless, increasing gill efficiency in oxygen uptake is not without its costs, which will be described presently.

Under conditions wherein elevated oxygen uptake is difficult or restricted, morphological and physiological alterations can be enacted to increase gill functional surface area (Nilsson, 1986; Postlethwaite and McDonald, 1995). However in doing so, ion loss across the effectively-larger gill also increases; this has consequently been termed the 'osmorespiratory compromise' (Nilsson, 1986; Postlethwaite and McDonald, 1995). This increased surface area can be accomplished several main ways, the first of which is via increased lamellar recruitment. Recruitment itself is regulated by a number of factors, such as the contraction of what are known as 'pillar cells' within the lamellae; these are stacked, contractile cells internally linking both lamellar epithelia and capable of contracting or expanding (Booth, 1978; Nilsson, 2007). Secondly, increasing blood pressure (via increased cardiac output during swimming, or vasodilation and vasoconstriction of afferent and efferent gill vasculature respectively) can enhance lamellar perfusion and recruitment (Booth, 1978; Soivio and Tuurala, 1981). This is exacerbated by increased levels of circulating catecholamines, which also elevate blood pressure and oxygen permeability at the gill, and vasodilate gill blood vessels (Randall et al., 1972; Booth, 1978; Holbert et al., 1979; Gonzalez and McDonald, 1992). Both stress – handling and otherwise (Mazeaud and Mazeaud, 1981; Gonzalez and McDonald, 1992) – and exercise have been demonstrated to induce elevations in circulating catecholamine levels in salmonids, further decreasing vascular resistance to gill blood flow (Stevens and Randall, 1967; Randall et al., 1972). While these actions permit greater capacity for gas

exchange, elevated blood pressure can also distort renal and branchial epithelial tight junctions thereby facilitating elevated rates of filtrational ion loss (Gonzalez and McDonald, 1994). Another mechanism employed to facilitate oxygen uptake involves reducing the diffusion distance between blood and water. This may occur by shunting of blood from embedded or peripheral bypass channels to the more central lamellar vasculature, and/or to the thinning effect of internal stretch (due to higher blood pressure) on the diffusion barrier which likewise decreases the diffusion distance for ion loss (Holeton and Randall, 1967; Randall et al., 1972; Booth, 1978; Isaia, 1984; Gonzalez and McDonald, 1992).

Thus, it has been observed that the greater the increase in oxygen consumption a species experiences, the greater its relative rate of ion loss (Randall et al., 1972; Gonzalez and McDonald, 1994). *Nevertheless*, loss – specifically of Na – increased 1.6-10x more than oxygen consumption during exercise in rainbow trout, indicating that this is being caused by something more than the factors which change oxygen uptake (Gonzalez and McDonald, 1992). While increased transepithelial hydrostic pressure due to elevated blood pressure may have an effect, its contribution is thought to be relatively minimal (Potts and McWilliams, 1989; Gonzalez and McDonald, 1992). Rather the more likely culprit is an increase in ionic (e.g. Na) permeability, which can occur via two distinct pathways.

Routes of ion transport across complex epithelia such as gills are referred to as either ‘transcellular’ (i.e. through cells), or ‘paracellular’ (i.e. between cells). Whereas transcellular pathways encompass ion channels and pumps occupying the cell membranes

of the branchial epithelial cells permitting intracellular ion movement, paracellular permeability is subject to regulation by tight junctions between the epithelial cells. In the case of Na loss due to the osmorespiratory compromise, it was observed that external Na concentration did not affect ion loss, thereby ruling out carrier-mediated exchange diffusion or 'leaky' pumps – two possible transcellular routes (Gonzalez and McDonald, 1992). In fact, it is now widely believed that most diffusive Na loss occurs primarily via paracellular flux from the branchial surface, and may be exacerbated by hydrostatic filtration with increased blood pressure. This may result in the bulging of tight junctions and reduction of their integrity thereby facilitating increased ion loss (McDonald et al., 1989; Gonzalez and McDonald, 1992; 1994). Similar changes in tight junction 'tightness' can be observed during such challenges as sea/freshwater acclimation, or changes in external calcium concentration (Spry and Wood, 1989; Brown and Davis, 2002; Henriksson et al., 2008; Rajasekara et al., 2008).

The Brazilian Amazon versus Southern Ontario: An Extreme Environment

When one thinks about the Amazon jungle in the heart of South America, the first thing that comes to mind is probably not 'extreme environment.' Thriving, fruitful, flourishing, abundant – perhaps… but a *survival challenge*? In fact, both are equally accurate.

As rich and full of life as the Brazilian Amazon is, it is also considered an extreme environment – particularly for aquatic species. In addition to being very dilute due to draining nutrient-poor forest soils, these waters also contain large amounts of decaying plant matter – resulting in high dissolved organic carbon, low pH, and most importantly:

extreme hypoxia (Furch, 1984; Baldisseroto, 2008). In fact, due to cyclical effects of photosynthesis, respiration, and organic decomposition, oxygen saturation can range seasonally and even diurnally from 'supersaturated' during the day to almost anoxic at night (Val, 1996). Várzea lakes – jungle floodplains – often face the most severe hypoxia, reaching daytime *maximum* saturation values of 0.5mg/L at 28˚C; nevertheless, these areas are crucial for feeding and reproduction among a wide variety of species (Saint-Paul and Soares, 1987). Igarapés (small rivers and streams) are also hypoxic sites, but to a lesser extent (Val, 1996). Thus, studying teleost ionoregulatory responses to hypoxia is not only interesting but also incredibly relevant – particularly for species of the Brazilian Amazon, which is where much of the research in this thesis took place.

Even in the more local region of Southern Ontario however, water oxygen levels are seen to oscillate with seasonal temperature changes and potential freezing of lakes. When lakes freeze in the winter, ice and snow can prevent penetration of light, and consequently block photosynthetic oxygen replenishment via photosynthesis (Magnuson et al., 1985). Although hypoxic stress is experienced among these indigenous species with much less frequency and variable severity, this still illustrates the fact that hypoxic stress is a ubiquitous challenge among aquatic environments (Nurnberg, 2004; Farwell et al., 2007).

Osmorespiratory Compromise Responses in two Model Species

The two model organisms upon which the present study was based are the Amazonian oscar (*Astronotus ocellatus*) and the rainbow trout (*Oncorhynchus mykiss*). The oscar, a native of the Amazon River basin, is accustomed to frequent bouts of

hypoxia and thus relies on its ability to tolerate this unavoidable oxygen shortage and ensure homeostatic cellular conditions via metabolic depression and downregulation of aerobic metabolism, in favour of upregulated anaerobic glycolysis (Almeida-Val et al., 2000; Richards et al., 2007). Metabolic depression is considered the most widespread strategy for coping with hypoxia (Nilsson and Ostlund-Nilsson, 2008) and may in conjunction with an increase in activity of LDH – a glycolytic enzyme – with body mass, explain why larger oscar (which have a lower mass-specific metabolic rate) are more hypoxia-tolerant than smaller oscar (Almeida-Val et al., 2000; Sloman et al., 2006). In fact, these fish can depress their rate of ATP consumption during hypoxia by 40-70% (van Waversveld et al., 1989; Johansson et al., 1995; van Ginneken et al., 1996; Muusze et al., 1998). However, this species also exhibits a most distinct ionoregulatory adaptation to hypoxia – a marked reduction in branchial permeability (Wood et al., 2007; 2009). This strategy is reminiscent of the 'channel arrest' hypothesis proposed for the reduction of metabolic rate in brain and liver tissues of other extremely hypoxia-tolerant species as the Crucian carp (*Carassius carassius*) (Hochachka, 1986; Boutilier and St-Pierre, 2000; Boutilier, 2001). Under low-oxygen conditions, unidirectional fluxes of Na and tritiated water, and net fluxes of other moieties (K, ammonia, urea, osmotic water flux) are markedly reduced. Importantly however, the flux of the paracellular marker [^{3}H]-polyethylene glycol-4000 (PEG-4000) remains unchanged. At the same time, the mitochondrial rich cells (MRCs) involved in ionoregulation are recessed and covered over by pavement cells (PVCs), which contribute to gas exchange – preventing ion loss without restricting oxygen uptake (Wood et al., 2007; 2009; Matey et al., 2011). Thus in

this instance, decreasing permeability along the transcellular axis becomes very important as it acts as the primary site of ion loss reduction. In contrast, no changes appear to occur paracellularly (Almeida-Val et al., 2000; Richards et al., 2007; Wood et al, 2007; 2009; Scott et al., 2008; Matey et al., 2011). In fact, the oscar is so well-adapted that it can even survive complete anoxia for up to 6 h at 28˚C (Almeida-Val et al., 2000); this is certainly an excellent model organism in which to study adaptations to hypoxia.

Conversely, the trout is a complementary model species because of its tremendous hypoxia *in*-tolerance (Holeton et al., 1967). Under hypoxic conditions, it responds in a similar manner to that of the oscar in that both species increase their mucous cell surface area to allow oxygen uptake while preventing cation loss due to the negatively charged mucous (McDonald, 1983; Scott et al., Wood et al., 2009). Also, both experience an increase in oxygen transfer factor (a measure of the relative ability of the gill for gas exchange); this is transient for the oscar, but lasting in the trout (Randall et al., 1967; Scott et al., 2008). Beyond this, rainbow trout appear ill-adapted for hypoxia: over the course of an 8-h exposure to severe hypoxia (~80torr), the trout displayed a triphasic response (Iftikar et al., 2010). During the first hour, bi-directional Na flux increased, an effect thought to be due to greater lamellar perfusion and a 30% increase in the number of exposed MRCs; by the fourth hour, flux rates were corrected back to control levels, probably due to oxygen starvation of the transport processes in conjunction with increased mucous production and a decrease in MRC numbers to below control (though their surface area remained high due to swelling) (Iftikar et al., 2010). However, from the fourth to the eighth hour, flux rates were elevated again, accompanied by prolonged

increases in MRC surface area (Iftikar et al., 2010). The stimulatory effect of acidosis may have contributed to this effect – that is during hypoxia, catecholamines cause the extrusion of protons from red blood cells in order to increase blood O_2 transport capacity, resulting in increased Na influx since this is coupled to acid efflux at the gills (Wood, 1988; Walsh et al., 1998; Thomas et al., 1991). At all times, however, Na efflux was observed to be greater than influx and resulted in net ion loss (Iftikar et al., 2010).

On the other hand, rainbow trout are considered to be excellent swimmers and are very well adapted for exercise – a different facet of the osmorespiratory compromise. During an extended swimming challenge, trout are seen to experience net ion loss (again, Na is of particular importance as its efflux increased by 70% within the first hour) which is corrected to below baseline within just three hours (Wood and Randall, 1973; Gonzalez and McDonald, 1992, 1994; Posthlethwaite and McDonald, 1995). Although this increased ion loss is attributable to enhanced lamellar perfusion and recruitment, the means by which it is corrected remain unclear (Wood and Randall, 1973).

On the topic of ion loss reduction, it is very important to note that recent evidence has been published supporting a selective reduction in passive Na loss at the gill under ion-poor conditions (in zebrafish, *Danio rerio*) (Kwong et al., 2013). More specifically, it was observed that elevated paracellular gill permeability does not necessarily coincide with increased passive Na loss, due to the proposed action of claudin-b (claudins are major transmembrane proteins regulating paracellular permeability) (Tsukita and Furuse, 2000; Kwong et al., 2013). In dilute environments, claudin-b is strongly expressed in transport epithelia (i.e. the gill and kidney), where it co-localizes with Na/K-ATPase

(involved in Na uptake); Kwong et al. (2013) hypothesized that claudin-b forms a selective paracellular barrier against Na loss without changing any other aspects of tight junction permeability. Consequently, this must be taken into consideration before assuming that all observable paracellular permeability changes automatically result in Na efflux changes, or conversely, before attributing differences in Na efflux unaccompanied by paracellular permeability alterations to exclusively transcellular routes.

Thesis Objectives

The overarching aim of this research was to study the osmorespiratory compromise under conditions of extended exercise and hypoxia, with the ultimate goal being the development of techniques for measuring permeability changes observed during this phenomenon. The practical consequence is that further studies can not only benefit from *results* obtained here, but can also use and improve upon *methods* established by this work. To fulfill this goal, the following specific objectives were proposed:

a) To establish and validate a technique for measuring branchial paracellular permeability with the paracellular flux marker [^{3}H]PEG-4000, that is not confounded by i) drinking of the chemical from the external medium, ii) renal loss of the chemical to the external medium, or iii) 'loss' of the chemical to surface adsorption in the flux chamber. Such a method is contingent upon PEG showing equal bidirectional permeability across the gill.

b) To use the technique from (a) to characterize paracellular permeability changes over an 8 h period of extended exercise in the rainbow trout and Amazonian oscar.

c) To develop and test a hypoxia 'rapid screening protocol,' wherein fish are subjected to 2-h normoxia: 2-h hypoxia: 2-h normoxic recovery. This protocol was designed to provide a fast and uniform means of differentiating permeability changes across a wide range of species exposed to this stressor.

d) Using the protocol developed in (c), this project aims to classify species' ionoregulatory responses to the osmorespiratory compromise, in order to identify any trends associated with phylogeny and/or environment.

Experimental Measurements

By evaluating a particular set of flux parameters in this study it was possible to compare results with previous research, and so obtain a more complete perspective of cellular processes and ionoregulatory changes under experimental conditions. For a particular ion or molecule moving across the gill, flux (or "flow per unit area") (Bird et al., 1960) can be measured in terms of net or unidirectional fluxes. 'Net flux' (J_{net}) quantifies the total rate of movement of the substance, and is the net sum of the two directional fluxes: influx (J_{in}) and efflux (J_{out}), signs considered. Influx is a positive value and indicates the rate of passage across the gills *into* the organism (i.e. uptake), while efflux is negative and refers to passage out of the organism (i.e. loss or excretion). Naturally, the larger the active surface area, the greater potential there exists for flux across it.

Na fluxes were observed using ^{22}Na, as well as cold Na measurements as a general indicator of a species' ability to maintain ionic homeostasis under a particular stressor. K , ammonia, and urea flux were also considered, as they are indicators of

transcellular permeability (Lauren and McDonald, 1985; Wood et al., 2009; Wright and Wood, 2009; McDonald et al., 2012). As nitrogenous waste products, ammonia and urea excretion rates also serve as a measure of metabolic rate and protein turnover.

Also, the radiolabeled chemical [^{3}H] polyethylene glycol ([^{3}H]PEG-4000) was used to study filtration and clearance parameters, uptake rates, and paracellular gill permeability. PEG is typically employed to monitor glomerular filtration rate (GFR) since it is filtered out of the plasma but not reabsorbed by the kidney or permeable in the bladder (Beyenbach and Kirschner, 1976). Nevertheless, it is appropriate for use as a marker of gill paracellular flux as it is effectively confined to the extracellular space (Beyenbach and Kirschner, 1976; Curtis and Wood, 1991; Scott et al., 2004; Sloman et al., 2004). This chemical can also be used as a drinking rate marker based on its appearance in the gut (Blewett et al., 2012).

Major Hypotheses

This study was divided into two major sections, both dealing with the osmorespiratory compromise: an exercise challenge, and a hypoxia challenge. The aim of the first was to assess the nature of branchial permeability changes in the trout and oscar during exercise, and specifically to determine whether the observed correction in branchial Na loss in trout during prolonged swimming likely occurs via a paracellular or transcellular pathway. It was hypothesized that during extended swimming,

a) both the trout and oscar would show negligible drinking of the external medium at rest and minimal amounts during exercise; and,

b) exercise would be accompanied by increased paracellular permeability (and therefore theoretical Na loss) in the trout, but not in the oscar.

The first hypothesis was proven false in that both species demonstrated substantial drinking rates at rest which did not significantly increase with exercise. However, the second hypothesis was confirmed: the trout *did* increase paracellular permeability over an 8-h swim, whereas the oscar showed no such increase but instead produced data suggesting elevated transcellular permeability with exercise.

For the second portion of this project, the goal was to use the rapid screening protocol to classify the hypoxic responses of a variety of North and South American teleosts as to whether they were more similar to oscars (i.e. permeability reducers) or trout (i.e. permeability increasers), and to identify possible phylogenetic and/or environmental trends within these. It was hypothesized under a protocol of 2-h:2-h:2-h normoxia:30% O_2 saturation, hypoxia:normoxic recovery, that:

a) oscar and trout would show characteristic response patterns of decreased and increased permeability, respectively; and

b) common adaptive strategies would be observed across species – whether according to common lineage or a shared environment.

In fact, both of these hypotheses were confirmed – among species tested from both Canada and Brazil, trends in hypoxic response were observed based on their environment, with the oscar and trout on either end of the tolerance spectrum.

This study has made significant contributions to the techniques by which tight junction modifications and therefore gill paracellular permeability can be studied using

[^{3}H]PEG-4000, and the various corrective measures that must be put in place for its effective use. It has moreover provided a deeper understanding of both North and South American species' adaptive strategies to different components of the osmorespiratory compromise, and the roles which phylogeny and environment play in this.

CHAPTER TWO. Regulation of gill permeability in rainbow trout (*Oncorhynchus mykiss*) and Amazonian oscar (*Astronotus ocellatus*) at rest and during extended exercise.

Abstract

Using freshwater rainbow trout (*Oncorhynchus mykiss*) as a model system, this study developed a technique to measure the paracellular permeability of the gills, and then applied it to investigate the osmorespiratory compromise during extended swimming. [^{3}H]PEG-4000 – a paracellular marker typically used as an indicator of extracellular fluid volume and glomerular filtration rate – was applied to measure gill paracellular flux. [^{3}H]PEG-4000 permeability was compared between efflux trials (i.e. injection into the fish and depuration measurements) and influx trials (i.e. dosage of water and uptake measures). However, these methods introduced several novel challenges. Firstly, bladder cannulation so as to collect urine separately was necessary (in efflux trials where the fish was loaded with [^{3}H]PEG-4000) because of the inherent capacity of [^{3}H]PEG-4000 for renal filtration. Drinking of [^{3}H]PEG-4000 from the surrounding water introduced the potential for overestimation of branchial uptake in influx trials so gut excision became necessary, and back-calculations to account for this were implemented. Uptake estimates were made based on accumulation of [^{3}H]PEG-4000 cpm in the whole carcass and the estimated ECFV of the fish (which were similar), but could not be determined based on the disappearance of [^{3}H]PEG-4000 cpm from the water. The latter provided a gross overestimation of [^{3}H]PEG-4000 cpm uptake, likely attributable to surface adsorption within the respirometer. By employing the various corrective measures

developed during this study, [^{3}H]PEG-4000 permeability of the gills was found to be equal in the efflux and influx directions (i.e. not rectified).

The[^{3}H]PEG-4000 influx method was then used to study paracellular permeability changes during extended exercise (1.2BL/s – body lengths per second – or 15cm/s) in the rainbow trout (*Oncorhynchus mykiss*) and Amazonian oscar (*Astronotus ocellatus*), two model species chosen because of previous comparative studies indicating differences in their osmorespiratory compromises during exercise. At rest, branchial paracellular permeability was approximately 3-fold greater in urinary cannulated trout (1.15 ±0.25 x10^{-4} mL/20g/s [N=25]) than in urinary cannulated oscars (0.43 ±0.07 x10^{-4} mL/20g/s [N=15]), despite a 10˚C higher temperature in the latter (28˚C vs 18˚C). Both species elevated their rates of O_2 consumption by 75-90% during extended exercise. Drinking did not change from rest to swimming in either species, but was a substantial component of PEG uptake. Branchial [^{3}H]PEG-4000 permeability increased significantly by 61% in trout, yet remained unchanged in oscar during extended exercise. Thus the osmorespiratory compromise responses differ during exercise as well as during hypoxia in the two species. The methods developed in the present study can be widely applied to measure branchial paracellular permeability changes in future studies.

Introduction

Under conditions requiring improved oxygen uptake such as hypoxia or exercise, the functional surface area of the gill increases via increased lamellar perfusion; this occurrence, however, may result in elevated ion loss across the gills and has consequently been named the 'osmorespiratory compromise' (Nilsson, 1986; Postlethwaite and McDonald, 1995). Previous studies of this phenomenon under hypoxic conditions have been performed in both the Amazonian oscar (*Astronotus ocellatus*) and rainbow trout (*Oncorhynchus mykiss*) by Wood et al. (2007, 2009), Iftikar et al. (2010), and Matey et al. (2011). Indeed their distinctly different gill morphometric and ionoregulatory responses during hypoxia form the basis for this project's species comparison during extended exercise.

During an 8-h continuous sub-maximal swim experiment conducted in rainbow trout, Wood and Randall (1973) reported flux changes in a number of ions, focusing particularly on Na. Throughout the first hour of swimming, Na efflux was seen to increase by 70%; however, by the third hour, it was corrected to below baseline. This was suggested to be at least partially attributable to the hormone prolactin, which is a key player in ionoregulation and specifically facilitates survival in ion-poor water by altering tight junction morphology (paracellular permeability) (Potts and Evans, 1966; Wood et al., 1973; Postlethwaite and McDonald, 1995; Manzon, 2002). It must be noted that over the 8-h exercise period, Na influx was unchanged; in fact, plasma Na levels tended to increase due to an apparent reduction in blood volume (Wood and Randall, 1973). The observed increase in efflux unaccompanied by any corresponding influx changes under

this protocol is consistent with Na loss caused by greater lamellar perfusion seen during the osmorespiratory compromise, rather than Na transport mechanisms such as exchange diffusion (which is associated with bi-directional changes). During a similar exercise regime imposed on juvenile rainbow trout by Posthlethwaite and McDonald (1995) however, a 3-fold increase in Na influx was observed – so previous findings have been variable. In any case, due to the extensive Na loss observed with exercise across all of these studies, gill permeability can be confidently assumed to have increased; this is a finding consistent with the nature of the osmorespiratory compromise.

Routes of ion transport and therefore permeability changes across complex epithelia (such as gills) can be divided into either the transcellular or paracellular pathway. It is important to differentiate between these two routes in order to determine whether permeability changes occur because of tight junction modifications between epithelial cells (paracellular), or because of ion channel/pump changes (transcellular). However, gill permeability changes, particularly along the paracellular route, can be very difficult to measure accurately, especially during exercise. Using radiolabeled [^{3}H] polyethylene glycol ([^{3}H]PEG-4000, a para- and extra-cellular marker), changes in gill paracellular permeability can be observed based on branchial fluxes between the fish and external medium. Nevertheless, several possible sources of error must be considered. For example, [^{3}H]PEG-4000 permeability might be rectified – i.e. not bidirectionally equal; this has never been examined in intact fish. Additionally, drinking of the external medium could potentially occur, which would elevate apparent [^{3}H]PEG-4000 influx. Similarly, renal depuration by glomerular filtration would elevate apparent [^{3}H]PEG-4000 efflux.

Drinking can be accounted for by gut excision or back-calculation based on observed drinking rates, and renal flux can be accounted for by urinary cannulation or back-calculations for glomerular filtration rate (GFR). As cannulation is potentially stressful for the fish and would inhibit swimming performance, one goal of this study was to circumvent this by alternative methods, if possible. Thus, the first objective of this study was to develop a method for paracellular permeability measurement using [^{3}H]PEG-4000, and to account for the associated sources of error involved with its use.

A second goal was to apply the techniques developed through this work to compare paracellular permeability changes with exercise in the Amazonian oscar and rainbow trout. Previous studies have compared the osmorespiratory compromise experienced under hypoxia in these species, during which they display opposing ionoregulatory responses (Wood et al., 2007; 2009; Iftikar et al., 2010; Matey et al., 2011). Specifically, in trout, gill permeability to ions and other substances (e.g. ammonia) increases during hypoxia (the traditional osmorespiratory compromise), whereas in the oscar, gill permeability to these same moieties decreases (a novel ionoregulatory compromise that does not appear to impair respiratory gas exchange, Scott et al., 2008). While the ionoregulatory responses of the trout to exercise have been well-studied (see above), it has never been conclusively shown whether the increased gill permeability is of paracellular or transcellular origin. The ionoregulatory responses to exercise in Amazonian oscars have never been examined, but its unusual reduction in gill permeability during hypoxia appears to be of transcellular origin (Wood et al., 2009).

The following specific hypotheses were tested: (i) both the trout and the oscar would show negligible drinking of the external medium at rest and minimal amounts during exercise, such that corrections for the influx of [^{3}H]PEG-4000 by drinking would be small; (ii) both would exhibit much larger renal than branchial losses of [^{3}H]PEG-4000, and therefore this would have to be taken into account; (iii), branchial permeability to [^{3}H]PEG-4000 would be bidirectionally equal, once corrections for uptake by drinking and efflux by glomerular filtration were implemented; and (iv) the trout would exhibit an increased paracellular permeability during exercise, whereas the oscar would exhibit either an unchanged or decreased paracellular permeability during exercise.

Materials and Methods

Fish Husbandry

Rainbow trout

Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 20-30g and of ~12 cm in length were obtained from Humber Springs trout farm (Orangeville, ON) and held for at least one week of acclimation prior to use, at ~18˚C in 500-L flow-through tanks containing Hamilton dechlorinated tap water (moderately hard: $[Na^+]$=0.6mM, $[Cl^-]$=0.8mM, $[Ca^{2+}]$=0.9mM, $[Mg^{2+}]$=0.15mM, $[K^+]$=0.05mM; pH ~8.0). Fish were fed every other day with a 10% body mass ration of 5-point Martin's dried commercial trout pellet feed (Martin Mills Inc., Elmira, ON) and starved 48 h prior to experimentation. They were also subjected to a 12-h:12-h light:dark daily photoperiod. All procedures were approved by the McMaster University Animal Research Ethics board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

Amazonian oscar

Juvenile Amazonian oscars (*Astronotus ocellatus*) of similar size and weight as the trout (20-30g, ~12cm) were obtained from Sítio dos Rodrigues (Km 35, Rod.AM-010, Brazil) and moved to the Ecophysiology and Molecular Evolution Laboratory of the Instituto Nacional de Pesquisas da Amazônia (INPA), in Manaus, AM. Fish were held in 500-L flow-through tanks, at ~28˚C. The holding and experimental water was typical Amazonian soft water obtained from a well on INPA property ($[Na^+]$=35μM, $[Cl^-]$=36μM, $[Ca^{2+}]$=18μM, $[Mg^{2+}]$=4μM, $[K^+]$=16μM; pH ~ 6.5). Fish were fed daily with commercial pellets (Nutripeixe Tr 36, Purina Co, São Paulo, SP) and also subjected to a natural 12-h:12-h light:dark daily photoperiod. After ~4 weeks, fish were air-shipped

from Manaus (AM) to Cassino (RS) and allowed to acclimate to lab conditions there ($[Na^+]$=0.48mM, $[Cl^-]$=0.55mM, $[Ca^{2+}]$=0.33mM, $[Mg^{2+}]$=0.08mM, $[K^+]$=0.02mM; pH 7.0; 12-h:12-h light:dark photoperiod) for one week prior to experimentation. The feeding regime was the same as in Manaus. This transport to a lab with a radioisotope permit for [^{3}H] was necessary to meet Brazilian national radioisotope usage regulations.

[^{3}H]PEG-4000 Details

Radiolabeled [^{3}H]polyethylene glycol-4000 (PEG-4000) is a marker of paracellular flux (Pezron et al., 2002; Wood et al., 2009) and the principal chemical used throughout this study. Radioisotope stock was obtained from Sigma Aldrich (St. Louis MO USA), and 1μCi was used in each experiment in a manner to be described below.

Urinary Cannulation Procedure

This procedure followed the methods described by Wood and Randall (1973), where fish were anesthetized in nominal 0.1g/L MS-222 (Syndel Laboratories Ltd, Vancouver BC); pH correction was unnecessary as pH remained within the 7-8 range. Fish were fitted with internal urinary bladder catheters (see Wood and Patrick, 1994) (Clay-Adams PE10 tubing, with PE60 sleeves attached by VetBond veterinary glue). For trout, the procedure was performed on an operating table which allowed continual water flow across the gills; this was not necessary in the hypoxia-tolerant oscars. These PE60 sleeves were then anchored to the body wall by silk sutures and the fish revived by flushing fresh water across the gills.

Rainbow trout were then transferred to an aerated Tupperware holding container with either 2440mL (‘efflux’ experiments) or 500mL (‘influx’ experiments) of

dechlorinated Hamilton tap water for a 24-h acclimation period, during which urine flow was monitored to ensure catheter patency. Movement within the flux chambers was restricted by a custom-made plastic canvas made of craft material (Darice, Strongsville ON) stitched into a suspended holding chamber within the apparatus using dental tape (mint-waxed Sunstar GUM, Guelph ON). This prevented the fish from turning around and thereby tangling its catheter, yet at the same time permitted a relatively high volume in the flux container.

For 'efflux' experiments, fish in which the urinary catheter was working were lightly anesthetized again after this 24-h 'recovery/test' period and injected into the caudal hemal arch with 1μCi [^{3}H]PEG-4000 dissolved in 1mL Cortland saline. Another 16-h 'recovery' period ensued where fish could acclimate and [^{3}H]PEG-4000 could equilibrate throughout the extracellular space. At the end of this time, a water sample was taken before replacement of the 2440 ml with fresh dechlorinated water, after which an 8-h experimental period was immediately begun.

For influx experiments, 1 μCi [^{3}H]PEG-4000 was directly administered to the holding water after the 24-h acclimation period (hence the reason for the lower experimental water volume).

For Amazonian oscars, the protocol was modified for 'efflux' experiments; fish were injected with [^{3}H]PEG-4000 simultaneously with cannulation before transfer to containers filled with 600mL dechlorinated Cassino tap water. This represented the beginning of the 16-h 'recovery' period. Again, this was followed by the 8-h experimental period. For 'influx' experiments, fish were also placed in 600mL fresh

water for 16 h, then the water was changed and [^{3}H]PEG-4000 added to mark the beginning of the 8-h period. This modification was employed because it was more difficult to maintain patent urinary catheters in oscars over a longer period.

Experimental Design

Cannulation Trials

After the 16-h recovery period during which UFR was monitored to ensure catheter patency, the 8-h experimental was commenced – marked by a water change (for both efflux and influx experiments) and [^{3}H]PEG-4000 dosage to the water (for influx experiments). Initial water samples were taken 15 min from dosage to allow for thorough mixing via aeration, and water samples were subsequently taken every two hours afterwards; urine flow was collected continuously until the experiment was terminated. The same protocol was performed in both trout and oscar.

Stationary versus Swimming Trials

Based on results from the experiments outlined above, it was deemed unnecessary to cannulate the fish used in the following swimming experiments (see Results) and non-cannulated stationary influx experiments. Fish were transferred to 3.2-L (32 x 6cm) Blazka respirometers and left to acclimate with aeration for 30 min before [^{3}H]PEG-4000 was added to the water. At this time, trout were then either swum or left stationary (fish were too large to turn around in the respirometry chamber, so fish in both trials were oriented in the same direction). Swim speed was 1.2 body lengths per second (BL/s), as this is well within the optimal scope of salmonid migratory swim speeds (which ranges from 0.6-2BL/s) and is recommended by previous researchers (Ware 1978; Trump and

Leggett, 1980; Posthlethwaite and McDonald, 1995). Oscars were swum at the same speed since they were of comparable size to the trout and previous swimming data were unavailable in published literature. However, in oscar swimming experiments, the first 4-h period of the trial was stationary and the second 4-h period involved exercise. In both species, an initial water sample was taken 15 min after radioisotope dosage (to ensure mixing) and then every 2 h until experiment termination at 8 h.

Oxygen consumption measurements were also taken to establish that this speed is in fact effective in elevating metabolic rate. This was accomplished by periodically closing the respirometry system without aeration and measuring initial and final partial pressures of oxygen over 10-minute intervals with (for trout) a Clarke-type oxygen electrode (Cameron Instruments, Port Aransas Texas) connected to a 1900 A-M Systems Polarographic Amplifier digital dissolved oxygen meter manufactured by A-M Systems (Carlsborg, WA, USA) for trout experiments, or a WTW Oxi325 Oximeter (Weilheim, Germany) for oscar experiments. Oxygen consumptions were then calculated as outlined below, taking into account fish mass, time, chamber volume, and other variables.

Tissue Analyses

At the end of the 8-h experimental period for *all* trials, fish were sacrificed by a MS-222 overdose and a terminal blood sample was taken by caudal puncture for measurement of plasma [^{3}H]PEG-4000 concentration. When calculating permeability and [^{3}H]PEG-4000 flux across the gills during influx experiments, it is important to also consider another possible route of [^{3}H]PEG-4000 entry - via drinking. Although it was initially assumed based on previous work (Shehadeh and Gordon, 1969; Hirano, 1974),

that drinking would be minimal in stationary freshwater trout, further experiments indicated that this was not the case – at least under these experimental conditions. Thus, drinking was corrected for by excising the stomach and intestine – tied at both ends to prevent loss of contents (Blewett et al., 2013). Analysis of the [^{3}H]PEG-4000 content of the gastrointestinal tract permitted calculation of drinking rate, and an estimate of how much of whole body [^{3}H]PEG-4000 uptake was due to drinking (see below).

From here, the carcass itself (containing all tissues and organs except the gut) was placed in a 50-mL Corning^{TM} centrifuge tube (Sigma Aldrich, St. Louis MO USA) with 15mL of 1N nitric trace metal grade acid, HNO_3. The gut was also carefully excised so as to keep its contents intact, and transferred to a 15-mL Corning^{TM} tube to which 5mL of 2N nitric acid was added. Samples were then sealed and incubated at 65°C for 48 h, after which time they were centrifuged and 1mL of each supernatant transferred to 20-mL scintillation vials. Perkin-Elmer (Waltham, MA, USA) Ultima Gold scintillation fluid was added to tissue samples (5mL fluor:1mL 1N nitric acid+tissue), and Perkin-Elmer Opti-phase scintillation fluid was added to water samples (5mL fluor: 5 mL water). Plasma samples were collected by centrifuging terminal blood samples and adding 100µL of supernatant (plasma) to 4.9mL water, and again adding 5mL Opti-phase fluor. [^{3}H]-PEG-4000 radioactivity (measured in β emissions) was determined using a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin-Elmer, Waltham, MA, USA). Plasma, carcass, and gut results were quench-corrected with quench curves constructed from various amounts of tissue digest or plasma, using the external standard ratio method for

quench correction. All samples were standardized to a common counting efficiency, which was that of the water samples.

Calculations

Oxygen Consumption

Oxygen consumption (MO_2) values, measured in μmol/g/h, were calculated using the following equation:

$$MO_2 = \frac{P_{O2\,i} - P_{O2\,f}}{\Delta t} \times V \times S_C \times S_{Cc}$$ (Brauner et al., 1992; Clarke and Johnson, 1999) (1)

In the above equation, $P_{O2\,i}$ and $P_{O2\,f}$ are the initial and final partial pressures of oxygen (mmHg). Δt represents the total time of the experiment (hours), and V is the volume of the respirometer (L) minus the weight of the fish (kg). The solubility coefficient S_C is 1.8925μmol/L/mmHg for 18˚C FW (trout) and 1.5906μmol/L/mmHg for 28˚C FW (oscars) (Boutilier et al., 1984), and S_{Cc} represents the mass scaling coefficient used to normalize the metabolic rate per 20g fish – calculated as:

$S_{Cc} = 10^{0.79 \log (20/\text{weight(g)})}$ (Clarke and Johnson, 1999). (2)

This removes the effect of body mass on metabolic rate and allows for comparison between fish of different sizes.

Clearance Rates and [^{3}H]PEG-4000 Flux Parameters

For cannulated fish, urinary [^{3}H]PEG-4000 excretion was calculated as the product of the urine volume over 8 h and the measured urine [^{3}H]PEG-4000 concentration (cpm/mL). As [^{3}H]PEG-4000 depuration from the branchial and renal pathways was monitored, gill clearance rates and urinary clearance rates (the latter

equivalent to glomerular filtration rates, GFR, due to no [^{3}H]PEG-4000 reabsorption) were calculated.

GFR was calculated based on Curtis and Wood (1991) as:

$$GFR = \frac{\text{PEG cpm 8-h loss to urine}}{\text{Mean 0-8 h plasma cpm/mL}} \times \frac{1}{W} \times \frac{1}{\Delta t} \quad (3)$$

Where W is weight (g), t is time (h), and the PEG urine cpm and mean plasma cpm/mL are based upon sample radioactivity. Although plasma data were only available from terminal blood samples, initial plasma [^{3}H]PEG-4000 concentration was determined using the findings of Munger et al. (1991) based on [^{14}C]inulin research, that there exists 193mL of ECFV (i.e. equivalent to plasma) per kg tissue in rainbow trout. Since no oscar ECFV data were available and for the purpose of comparison, this value was used in the oscar as well. Thus, the estimated ECFV total cpm per fish at 8 h is the fish weight (kg) multiplied by plasma cpm/mL and this 193mL/kg factor. For fish in efflux experiments the initial total ECFV cpm is therefore *this* value, plus the total 8-h cpm loss from the fish. The total can then be divided by fish weight (kg) and 193mL/kg to find the initial (0h) plasma concentration. This allowed us to compute the mean plasma cpm/mL from 0-8 h.

Next, gill plasma clearance rate was calculated as:

$$\text{Gill plasma clearance rate} = \frac{\text{PEG cpm 8-h loss to water}}{\text{Mean 0-8 h plasma cpm/mL}} \times \frac{1}{W} \times \frac{1}{\Delta t} \quad (4)$$

In order to find total PEG counts in the carcass, counts were measured for a 1-mL sample of tissue digest (gut extracted) to which 15mL of 1N HNO_3 had been added (5mL was added to gut samples). Total tissue cpm was found as:

Total tissue cpm = measured cpm*(W + mL acid added) (5)

Drinking Rate

The drinking rate was calculated based on gut [^{3}H]PEG-4000 counts using a modification of the equation put forth by Blewett et al. (2013) and subsequently scaled to a 20g fish for comparison with permeability data (Clarke and Johnson, 1999):

$$DR = \frac{\text{Total gut cpm}}{\text{Mean 0-8 h water cpm/mL}} \times \frac{1}{\Delta t} \times S_{Cc} \quad (6)$$

Where $S_{Cc} = 10^{0.79 \log (20/\text{weight(g)})}$ (Clarke and Johnson, 1999). (7)

Now having found the drinking rate, one can back-calculate the actual total ECFV or carcass count based on how much [^{3}H]PEG-4000 was consumed orally during influx or efflux experiments.

Actual Total cpm= Calculated Total cpm – (mL drunk x Mean 0-8 h water cpm/mL) (8)

This calculation was implemented in all trials where the gut was not excised (i.e. trout cannulation experiments – before it was known that stationary fish drink).

Renal Depuration Rate

In addition to drinking, another factor for which it may be important to make correction is renal [^{3}H]PEG-4000 depuration. Indeed, in the past, [^{3}H]PEG-4000 has primarily been used as a renal glomerular filtration rate marker (e.g. Beyenbach and Kirschner, 1976; Curtis and Wood, 1991) (as opposed to an indicator of branchial permeability, as in this study). As the results will show, urinary cannulation was determined to be unnecessary during influx experiments because in the relatively short experimental period, the amount of [^{3}H]PEG-4000 (taken up by branchial influx) which

was depurated via the urine was very small – nevertheless, it was still possible to predict this minimal urine loss for greater accuracy.

In this case, urine [^{3}H]PEG-4000 loss must be estimated on an hourly basis: first, the expected hourly amount of [^{3}H]PEG-4000 taken up to the ECFV (H1 uptake) is the total terminal ECFV count (cpm) divided by 8. From here using the GFR estimate from equation 3:

$$\text{H1 Loss} = \frac{\text{H1 uptake (cpm/h)} \times \text{GFR (mL/kg/h)}}{193\text{mL/kg}} \quad (9)$$

Thus, based on the expected [^{3}H]PEG-4000 amount circulating in the plasma during hour 1, GFR (basically the rate at which plasma is filtered) can be used with the amount of plasma itself to calculate how much [^{3}H]PEG-4000 is lost during this time. Similarly:

$$\text{H2 Loss} = \frac{([\text{H1 uptake}] - [\text{H1 Loss}] + [\text{H2 uptake}])\ (\text{cpm/h}) \times \text{GFR (mL/kg/h)}}{193\text{mL/kg}} \quad (10)$$

This pattern extends up to the eighth hour, and hourly losses can be summed to determine the total renal loss. This can then in turn be added back to the total [^{3}H]PEG-4000 counts in the ECFV or carcass. When comparing total 8-h [^{3}H]PEG-4000 cpm lost to the urine, predicted percentages (i.e. (8-h Loss)/(ECFV total counts)x100) can be compared to actual percentage loss during cannulation trials.

Permeability

Paracellular permeability was calculated using a modification of the formula from Wood et al. (1998):

$$\text{Permeability} = \frac{\text{Net appearance of PEG (cpm)}}{\text{Mean PEG (cpm/mL)}} \times \frac{1}{\Delta t\ (\text{h})} \times \frac{1}{3600(\text{sec/h})} \quad (11)$$

This yields permeability in units of mL/s per fish, which is then multiplied by the aforementioned scaling coefficient S_{Cc}, which again equals $10^{0.79 \log (20/\text{weight(g)})}$ (Clarke and Johnson, 1999) and thus scales permeability data in terms of mL/20g/s – an average-sized fish in these experiments.

For efflux experiments (recall, the fish had been injected with [^{3}H]PEG-4000), so Net Appearance of [^{3}H]PEG-4000 (cpm) in equation 11 was measured from water [^{3}H]PEG-4000 concentration multiplied by the flux chamber volume, whereas Mean PEG cpm/mL was considered to be the average plasma concentration (cpm/mL) over the 8-h experiment). For influx experiments (where the fish was either stationary or swimming in water that had been dosed with [^{3}H]PEG-4000), net appearance of [^{3}H]PEG-4000 in the fish can either be calculated based on calculated ECFV uptake counts, or directly measured from the digested carcass. Data from both of these methods will be shown throughout the Results for comparative purposes.

Unfortunately, attempting to estimate permeability based on [^{3}H]PEG-4000 uptake via disappearance from the water led to gross overestimations (likely due to radioisotope loss via adherence to the respirometer surface itself) and therefore proved extremely unreliable. This was identified in both oscar and trout experiments and will be elaborated upon further in the Discussion.

Statistics

All data have been expressed as the means $\pm$ 1 SEM, where N = sample size. All statistical testing was carried out using the program Sigma Plot 10.1, with Sigma Stat Integration 3.0. Figure legends denote the specific statistical test performed in each case,

but in general, comparisons between experimental data points were evaluated using a paired or un-paired t-test (comparing between two data sets) or One-Way Analysis of Variance (ANOVA) followed by a Fisher LSD *post hoc* test (for multiple data sets). If the normality test was failed, a Kruskal-Wallis ANOVA on ranks analysis was executed prior to a Tukey or Dunn's *post hoc* test. Data being compared were considered statistically significant if $P<0.05$ and were represented graphically as such by different letters or asterisks above significant sets of data. Means not sharing the same letters are significantly different.

Results

The following Results section will be organized, first, by presenting data obtained through studying urine parameters, drinking rate and branchial paracellular ([^{3}H]PEG-4000) flux in the rainbow trout, followed by a mirrored study in the Amazonian oscar. All methodological development tests, troubleshooting, and initial corrections were performed in the rainbow trout, then applied in the oscar.

Methodological Development in Rainbow Trout

Various parameters were measured by monitoring [^{3}H]PEG-4000 counts and fluxes over 16-h recovery (from urinary cannulation) periods and immediately subsequent 8-h experimental periods. It is important to note that for cannulated fish, efflux experiments refer to trials in which the fish themselves were caudally injected with [^{3}H]PEG-4000, and branchial and/or renal effluxes of [^{3}H]PEG-4000 were monitored throughout the recovery and experimental periods, whereas influx experiments were those in which[^{3}H]PEG-4000 uptake into the fish from dosed water was observed over the 8-h trial. A key goal of these comparisons was to establish whether or not [^{3}H]PEG-4000 permeability was rectified – i.e. whether or not it showed the same permeability in the influx and efflux directions at rest, as this would dictate the approach that could be used in the subsequent swimming trials. A second goal was to determine to what degree it was necessary to correct for fluxes of [^{3}H]PEG-4000 that did not cross the gills but rather passed by renal or drinking routes.

Figure 2.1 displays the urine flow rate in the trout used in this study, measured to ensure catheter patency following cannulation.

Urine flow rates measured via cannulation were similar in both influx and efflux trials, during the 16-h recovery (from cannulation) period beginning immediately after cannulation and extending into the subsequent 8-h experimental period. For reference, similar UFR values (~0.004mL/g/h) reported by Wood and Randall (1973) in mature trout of 180-350g are indicated by the dashed horizontal line.

Again as detailed in the Materials and Methods section, in efflux experiments, fish were injected with [^{3}H]PEG-4000 at the beginning of the 16-h recovery period. The radiolabeled marker was allowed to depurate during that time and the subsequent 8-h experimental period. Figure 2.2 displays the division of [^{3}H]PEG-4000 flux from cannulated fish used in *efflux* experiments during the 8-h time course of the experimental period, where the observed GFR was 10.46±1.93mL/kg/h and UFR was 6.93±1.21mL/kg/h.

Of the total[^{3}H]PEG-4000 effluxed over 8 h, approximately 60% of counts were lost renally as opposed to branchially (i.e. the percentage of [^{3}H]PEG-4000 fluxed to the water) during efflux trials. However, for influx experiments where the holding water of the fish was dosed with [^{3}H]PEG-4000 instead of the fish itself, Figure 2.3 shows the complementary division of [^{3}H]PEG-4000 counts between the urine and the fish, where in this case plasma GFR was 10.46±3.33mL/kg/h, and UFR was 4.69±1.11mL/kg/h.

In contrast to Figure 2.2 – which shows the majority of injected [^{3}H]PEG-4000 leaving the fish via the renal route – Figure 2.3 shows significantly less [^{3}H]PEG-4000 appears in the urine as compared to what is taken up branchially and appears in the ECFV. Total carcass cpm were statistically similar to but slightly higher than total ECFV

cpm – therefore the ECFV value is displayed in the graph as it is the more conservative estimate of gill uptake. In fact, only 15% (maximum) of all radioisotope taken up at the gills is lost to the urine over 8 h, which is near the predicted value of 9.7% based on an observed GFR of 10.46mL/kg/h.

Next, Figure 2.4 compares [^{3}H]PEG-4000 amounts in both the carcass and extracellular fluid volume (ECFV) for cannulated and non-cannulated trials (non-cannulated fish had the gut excised to avoid artificially inflating carcass counts via drinking after it became known that this was occurring in stationary fish). Note that for stationary trout, the observed drinking rate was 4.6 x 10^{-5}mL/20g/s, or 8.3 mL/kg/h.

As seen above, gut-excised influx fish showed slightly lower [^{3}H]PEG-4000 amounts in both the carcass and ECFV. Also note that carcass and ECFV [^{3}H]PEG-4000 cpm were statistically similar within all experimental trials, as expected.

Actually, this can also be seen in Figure 2.5, which compares permeability between efflux and influx trials in the stationary, cannulated trout – where influx permeability is measured based on whole body [^{3}H]PEG-4000 cpm in both the carcass and ECFV. Note that permeability has been scaled to a 20g fish (Clarke and Johnson, 1999), which is experimentally relevant considering the average size of the cohort used during these treatments. The overall mean permeability in resting trout for these three estimates was 1.15 ±0.25 x10^{-4} mL/20g/s (N=25).

According to this graph [^{3}H]PEG-4000 influx permeability (calculated via both methods) is not statistically different than that of efflux for resting fish, as supported by the data shown in Figure 2.4. The findings that [^{3}H]PEG-4000 permeability is

bidirectionally equal and so can be measured in either direction, the importance of gut excision for accurate influx permeability measurements, and the negligible need for urinary catheterization during 8-h influx experiments – all set the stage for the subsequent swimming tests.

The second part of the [^{3}H]PEG-4000 work conducted in trout comprised a series of stationary versus swimming (at 1.2BL/s, or 15cm/s) influx experiments, where the holding water of a Blazka respirometer was dosed with [^{3}H]PEG-4000 and uptake was measured. Fig. 2.6 displays changes in metabolic rate as estimated by oxygen consumption measurements over an 8-h swim period, scaled to display hourly oxygen consumption per 20g fish for non-cannulated individuals (Clarke and Johnson, 1999).

It is evident from Figure 2.6 that swimming significantly increases metabolic rate as indicated by oxygen consumption throughout the entire 8-h period, and this is therefore an appropriate swim speed for further tests. The mean overall MO_2 at rest was 173±13 µmol/20g/h (N=18) and during exercise was 302±9 mL/20g/h (N=72), an elevation of 75%. It should be noted that there is no evidence of stress in the control group over the 8-h period, which would be indicated by significantly high oxygen consumption rates that decrease with time (i.e. acclimation).

During swimming experiments where fish were not cannulated it was not possible to measure GFR, but urine [^{3}H]PEG-4000 loss was still accounted for, as GFR could be estimated based on a predicted 30% GFR increase based on the data of Hofmann and Butler (1979) for an MO_2 increase of 75%. This corresponds to a 12.6% renal [^{3}H]PEG-4000 loss.

Another possible source of error to be accounted for is drinking of the external medium. During initial stationary cannulated trials, drinking was observed to be surprisingly high. However, by excising the gut during subsequent non-cannulated stationary and swimming trials this would prevent overestimation of [^{3}H]PEG-4000 uptake due to drinking, and would also show whether or not the drinking rate increases with swimming. This is seen in Figure 2.7.

Although it was initially assumed – based on previous work (Shehadeh and Gordon, 1969; Hirano, 1974) – that drinking would be negligible in stationary freshwater trout, Figure 2.7 indicates that this is not the case based on radioisotope counts in the excised gut, at least under these experimental conditions. In fact, drinking is not seen to increase during swimming at all, as predicted. Figure 2.8 shows the amount of [^{3}H]PEG-4000 taken up orally under both stationary and swimming conditions compared to what is gained branchially.

With swimming the carcass:gut [^{3}H]PEG-4000 proportion trends to increase – indicating that with exercise, the amount taken up to the carcass (i.e. via the gills) increases whereas gut uptake does not.

Nevertheless, the substantial amounts of [^{3}H]PEG-4000 taken up via drinking whether stationary or swimming would lead to artificially inflated influx permeability measurements, as radioisotope lost from the water is actually being sequestered in the gut (between 22-38%). Again, this is why the gut was excised during non-cannulated stationary and swimming trials: to prevent overestimation of branchial uptake and to observe drinking rate changes with swimming. Back-calculations were performed for

drinking in the preliminary cannulated stationary trials where the gut had not been excised. Intuitively, drinking must be accounted for during influx experiments because the water has been dosed with radioisotope. However, it must also be accounted for in efflux experiments as drinking causes the amount of injected [^{3}H]PEG-4000 lost branchially to appear lower; nevertheless, the water concentration of [^{3}H]PEG-4000 is so low that this correction is negligible, non-significant and only included for the sake of accuracy. Figure 2.9 confirms an increase in [^{3}H]PEG-4000 uptake with swimming, now with the gut excised.

In the previous figure, [^{3}H]PEG-4000 cpm in the carcass and ECFV were individually weight-corrected, then normalized for the average water cpm/mL over the 8-h experimental period. There is a significant increase in amounts of [^{3}H]PEG-4000 taken up by both the carcass and ECFV during swimming, and none of this increase being sequestered in the gut.

Thus it is with this in mind that [^{3}H]PEG-4000 permeability in non-cannulated, gut-*excised* stationary and swimming (1.2BL/s) trout can be accurately compared.

Figure 2.10 shows a significant increase from stationary to swimming gill [^{3}H]PEG-4000 permeability based on both ECFV and carcass cpm, averaging a 61% increase. The fact that calculations were based on similar cpm between both the ECFV and the carcass attest to the accuracy of this observation. These results, supported by the previously outlined data, indicate that the paracellular permeability of the gills increases during an 8-h period of extended exercise in the trout.

Amazonian oscar

Experiments conducted in Amazonian oscars (*Astronotus ocellatus*) were performed in Cassino, RS. As fish used here were of approximately equal length as the trout (~ 12 cm) and weight (20-30g) in Hamilton, ON, the same swim speed of 1.2BL/s (15cm/s) was also applied. As in the rainbow trout, significantly more of the injected [^{3}H]PEG-4000 depurates renally than across the gills, as seen in Figure 2.11 (compare with Figure 2.3).

This flux division indicates that – although it was considerably more difficult to cannulate and maintain a patent catheter throughout the full experiment in the oscar than in the trout – the catheters were in fact patent throughout the 8-h period.

Also measured throughout this 8-h period were plasma gill clearance, glomerular filtration rate (GFR), and urine flow rate (UFR).

Data obtained from the stationary efflux trials appeared more reliable than those collected from stationary influx trials, which had a much higher standard error – and were furthermore more relevant as these efflux fish were directly injected with the radioisotope (as opposed to measuring uptake from the water and subsequent urinary depuration of influx fish). These showed a gill plasma clearance rate, GFR, and UFR of 1.1, 12.6, and 1.5mL/kg/h – respectively – and were similar to trout findings.

[^{3}H]PEG-4000 fluxes in efflux and influx trials with stationary cannulated oscars are displayed in Fig. 2.13, where the very minor [^{3}H]PEG-4000 loss to the urine in influx trials has been corrected for as in the trout experiments. Drinking corrections were rendered unnecessary by gut excision.

As in the trout, ECFV and carcass counts are statistically similar, again confirming the extracellular nature of this radioisotope and its validity as a paracellular marker. It is also seen in Figure 2.13 that [^{3}H]PEG-4000 permeability is bi-directionally equal in the oscar, as it was in the trout (calculated using the carcass *and* ECFV methods for determining influx permeability). The overall mean permeability in resting oscar for these three estimates was 0.43 ±0.07 x10^{-4} mL/20g/s (N=15), which is significantly lower than in trout (1.15 ±0.25 x10^{-4} mL/20g/s [N=25]). Consequently, further trials were all influx experiments (i.e. the holding water was dosed with [^{3}H]PEG-4000 as opposed to the fish), using non-cannulated fish and gut excision.

At this point it is important to note that non-cannulated stationary and swimming oscars, all trials began with a 4-h stationary flux period followed by either 4 h of continued rest or 4 h of swimming at 1.2BL/s (15cm/s). The latter experiments are therefore termed 'Swimming 4/4'. It was not possible to separate [^{3}H]PEG-4000 data from the 4-h stationary period and the 4-h swim period for these trials.

Figure 2.14 shows the effect of 4 h of extended exercise on metabolic rate as compared to stationary fish.

The previous graph shows that a 4-h swim period *does* in fact provoke a significant increase (90%) in metabolic rate measured by MO_2 for the oscar. In these experiments, for the purpose of investigating paracellular changes in this species and drawing comparisons, complementary experiments using [^{3}H]PEG-4000 (a paracellular flux marker) were performed to those conducted on rainbow trout – except that in this case, the gut was excised in *every* trial to account for drinking.

Fig. 2.15 compares [^{3}H]PEG-4000 tissue cpm upon sacrifice for subjects of stationary and swimming experiments, by ECFV (as calculated from plasma counts) and carcass amounts of radioisotope.

Note that there is no significant difference in tissue [^{3}H]PEG-4000 cpm between stationary and swimming fish in Figure 2.15. Additionally, like the rainbow trout, the Amazonian oscar does not appear to increase its drinking rate with swimming when gut counts are measured in non-cannulated individuals (Fig. 2.16).

As confirmed in the previous figure, drinking rates clearly do not increase for oscar during swimming as it goes from 1.73 ±0.94x10^{-5} mL/20g/s (3.11 ±1.69 mL/kg/h) (N=4) to 1.82 ±0.50x10^{-5} mL/20g/s (3.28 ±0.90 mL/kg/h) (N=9). Additionally, neither stationary nor swimming drinking rate is significantly different between the oscar and trout, whose DR changes from 4.62 ±1.50x10^{-5} mL/20g/s (8.32 ±2.70 mL/kg/h) (N=7) to 3.00 ±0.63x10^{-5} mL/20g/s (5.40 ±1.13 mL/kg/h) (N=14) with swimming. No changes in oscar DR with exercise is supported even further by data seen in Figure 2.17.

This shows that as in trout, oscar carcass cpm comprise the majority of the [^{3}H]PEG-4000 taken up – however, this ratio does not increase with swimming. Also, mL/g values are generally comparable to the trout, as is percent radioisotope allocation (81% and 88% carcass:gut in the oscar; 62% and 78% carcass:gut in the trout).

Finally when comparing the effect of swimming (once again, keep in mind that the permeability measurement takes into account a previous 4-h rest period) on [^{3}H]PEG-4000 permeability against stationary fish, there are no significant changes.

Figure 2.18 clearly shows that stationary permeability (where [^{3}H]PEG-4000 uptake is measured both as the carcass cpm and the ECFV cpm) is statistically similar to that of fish swimming at 1.2BL/s (15cm/s) for 4 h after a 4-h rest period. Therefore extended swimming does not appear to have any significant impact on paracellular [^{3}H]PEG-4000 permeability in the oscar.

Discussion

The overarching purpose of this project was to study the osmorespiratory compromise and specific adaptations to it in the rainbow trout (*Oncorhynchus mykiss*) and the Amazonian oscar (*Astronotus ocellatus*), by comparing paracellular gill permeability at rest and throughout a period of extended swimming. Thus, it was first necessary to develop and evaluate an experimental protocol – the methodology of which will be valuable for researchers carrying out further studies in this area – in order to measure their branchial paracellular permeability during rest and exercise.

Rainbow Trout

The paracellular marker [^{3}H]PEG-4000 is primarily used as a means of measuring such renal parameters as GFR (Beyenbach and Kirschner, 1976; Curtis and Wood, 1991) and ECFV (Beyenbach and Kirschner, 1976); also, it is frequently used as an *in vitro* paracellular permeability marker for cultured gill epithelia (Kelly and Wood, 2002; Wood et al., 2002). In order to establish its legitimacy for determining gill paracellular permeability *in vivo*, however, it was first necessary to prove that it has bidirectionally equal permeability across the gills (and in fact it *was* proven, for both rainbow trout and oscar). By employing urinary cannulation, it was possible to measure [^{3}H]PEG-4000 influx (water to fish) and efflux (fish to water) [^{3}H]PEG-4000 permeability. It is difficult for fish to swim naturally with implanted urinary catheters, and even more difficult to keep the catheters patent when the fish is exercising in the swim tunnel, prompting the question: 'Can it be avoided in swimming experiments?' It was the aim of this study to

measure permeability based on *influx* experiments (where cannulation becomes potentially unnecessary if renal depuration is low) as opposed to *efflux* experiments.

Throughout the 16-h recovery period from cannulation (plus simultaneous [^{3}H]PEG-4000 injection, in the case of efflux experiments) and the subsequent 8-h experimental period, urine flow rates were comparable to earlier values reported by Wood and Randall (1973) for the same species, being only slightly higher – this perhaps due to smaller fish size in the current study and therefore a higher surface area:volume ratio. UFRs were also similar across both treatments. This simply establishes that the urinary catheters used here were patent, and thus gill flux measurements would not be confounded by renal [^{3}H]PEG-4000 depuration into the water.

In terms of answering the question as to whether or not cannulation can be avoided, Figure 2.2 shows that for trout (injected with [^{3}H]PEG-4000) in efflux experiments, renal flux comprises almost 60% of [^{3}H]PEG-4000 loss, where the remaining 40% is branchial. However, for fish in influx experiments, Figure 2.3 argues that urine [^{3}H]PEG-4000 flux is substantially lower than the amount taken up to the ECFV (a slightly more conservative measure than carcass uptake). Therefore low urine cpm excretion during influx experiments indicates that for a period of 8 h, renal excretion of [^{3}H]PEG-4000 which has been taken up at the gills is negligible. Therefore, influx permeability measurements can be taken without the complication of accounting for the urinary excretion of [^{3}H]PEG-4000 *back* into the water (although back-calculations *were* implemented for the sake of accuracy). In short, since [^{3}H]PEG-4000 permeability is equal in both directions, urinary cannulation can be avoided by performing only influx

experiments in further trials – thus facilitating permeability measurements during swimming.

Figures 2.4 and 2.5 confirm the extracellular nature of this chemical by comparing amounts of [^{3}H]PEG-4000 in both the carcass and the ECFV, the latter based on the product of the measured plasma concentration and the estimated ECFV as a function of body mass (Munger at al., 1991). This ECFV calculation was used for fish in influx and efflux experiments and then for stationary versus swimming (both influx) fish. Since carcass and ECFV counts were similar within all treatments, this a) supports the choice of [^{3}H]PEG-4000 as an appropriate extracellular marker, and b) provides *two* means of estimating total [^{3}H]PEG-4000 uptake counts during influx experiments – an additional assurance of accuracy in permeability measurements. This is especially valuable, as [^{3}H]PEG-4000 uptake cannot be estimated based on disappearance from the water in influx experiments; surface adsorption to the respirometer produces grossly overestimated values. As shown in Figure 2.19, the theoretical [^{3}H]PEG-4000 uptake during swimming based on water counts would be approximately 100 000cpm over an 8-h period, a 100-fold overestimation. As the same respirometers were used in the matching Amazonian oscar treatments, the same problem was also identified in those trials.

Nevertheless, as ECFV and carcass counts were almost identical within all treatments, this additional measure of [^{3}H]PEG-4000 uptake would not be necessary. Interestingly, in the trout, ECFV estimates were consistently (slightly) higher than those of the carcass in both cannulated and non-cannulated trials, whereas carcass estimates of permeability were consistently higher in the oscar – as the disparity in ECFV versus

carcass estimates for both species was minimal this could simply be coincidence, or it may be due to inter-specific body composition and physiological differences [recall that the predicted extracellular fluid volume of 193mL/kg was developed for trout (Munger et al., 1991) and applied to both species, as no value was available for oscar].

This being established, [^{3}H]PEG-4000 permeability was finally calculated for fish in both influx experiments (based on both ECFV and carcass uptake) and efflux experiments and found to be similar in all treatments (Figure 2.5). Consequently, [^{3}H]PEG-4000 permeability *is* bidirectionally equal across the gills, as has previously been observed in cultured rainbow trout gill epithelial membranes by Wood and Part (1997) and Wood et al. (1998). Therefore, by simply measuring [^{3}H]PEG-4000 influx permeability during the swimming trials it was possible to avoid cannulation and its attendant problems entirely.

Swimming [^{3}H]PEG-4000 Trials.

The next comparisons to be drawn were those between stationary and swimming fish. As detailed in the Materials and Methods, fish were swum (or left stationary, as a control) at 1.2BL/s (~15cm/s), a pace well within the normal scope of salmonid migratory swim speeds (ranging from 0.6-2BL/s) and the actual discrete value recommended by previous researchers as the 'optimal swim speed' (Ware, 1978; Trump and Leggett, 1980; Posthlethwaite and McDonald, 1995). For non-cannulated experiments, the gut was excised because high drinking rates were observed during the cannulated trials. This prevented overestimation of [^{3}H]PEG-4000 uptake, and allowed for comparison of drinking rates between stationary and swimming fish based on [^{3}H]PEG-4000 cpm in the

gut. As drinking had previously been found to be negligible in freshwater trout (Shehadeh and Gordon, 1969; Hirano, 1974) and numerous studies since then have assumed the same, the high drinking rate observed during this study was somewhat surprising. Exceptions to this minimal freshwater drinking include newly hatched alevin (Tytler et al., 1990; Fuentes and Eddy, 1997), and perhaps drinking association with active feeding (Ruohonen et al., 1997; Kristiansen and Rankin, 2001). However, drinking rates observed for starved adults in *this* study were 4.6×10^{-5} mL/20g/s (8.3 mL/kg/h) in stationary trout, and 3.0×10^{-5} mL/20g/s (5.4 mL/kg/h) in swimming trout. Al-Reasi (2012) has recently observed a similarly high drinking rate in zebrafish (*Danio rerio*) of 2.3 ± 0.5 mL/kg/h. While this freshwater drinking cannot yet be completely explained, stress is one factor which has been suggested to increase drinking and may therefore partially contribute to this observation (Eddy, 1981; Mazeaud and Mazeaud, 1981). Although this study predicted that negligible stationary drinking rates would remain relatively unchanged with swimming, Figure 2.7 indicates that *already*-substantial rates of drinking instead do not significantly change at all with swimming (at 1.2BL/s). This was also confirmed by observing the percent of [^{3}H]PEG-4000 apportioned to the gut as compared to the rest of the carcass in Figure 2.8 – it comprises an unexpectedly large proportion in resting fish which is actually lessened upon swimming, due to an apparent increase in carcass uptake capacity. That being said, it is very important to note that this increased carcass:gut [^{3}H]PEG-4000 ratio upon swimming provides supportive evidence for increased gill paracellular permeability under conditions of osmorespiratory compromise. As previously detailed, drinking artificially increases [^{3}H]PEG-4000 permeability values as the cpm

supposedly being taken up across the gills are actually being taken into the gut by drinking, but back-calculations (for previously completed cannulation experiments), gut excisions (for all stationary and swimming trials), and urinary corrections (based on GFR) provided methods to correct for these sources of error.

Metabolic changes observed during exercise in rainbow trout supported the choice of 1.2BL/s as an ideal extended swim speed. There are two important points to note about the information displayed in Figure 2.6. First, the significantly elevated oxygen consumption between stationary and swimming fish at all time points establishes that this level of exercise *was* effective at raising metabolic rate by 75% during the entire swim trial, although this pace did not visibly appear difficult for the fish to maintain. Resting MO_2 is also consistent (when divided by 20, since values are reported here in μmol/20g/h for straightforward comparison with permeability data) with values observed in previous studies in juvenile trout. For instance, Alsop and Wood (1997) measured resting oxygen consumption as 5μmol/g/h in 10-20g trout, and Gonzalez and McDonald (1992) recorded 4.3μmol/g/h in 7-16g fish. Secondly, the fact that there is no significant reduction between resting (control) fish at 0h and any time points measured thereafter – i.e. after hours of settling in the respirometer – indicates that handling stress was minimal during these experiments.

Finally, branchial [^{3}H]PEG-4000 permeability could be calculated and compared between stationary and swimming trout, and a significant increase (61%) in permeability by averaging both carcass and ECFV measurements is observed. This was also supported by data from Figures 2.5 (displaying [^{3}H]PEG-4000 tissue amounts in both trials) and 2.9

– detailed above, which provide convincing evidence that swimming tends to increase paracellular permeability. This offers valuable insight into the objective to characterize routes of ion loss during extended exercise in this species; nevertheless, to conclusively establish that the later ion loss *correction* observed by Wood and Randall (1973) is a paracellular change, future studies might consider comparing paracellular changes seen here with transcellular changes during exercise and over shorter intervals across the 8-h period. While the commonly-held assumption is that Na passively moves paracellularly down the concentration gradient in freshwater species, recent work has also shown a degree of transcellular regulation of Na transport in trout under hypoxia (Iftikar et al., 2010), and certainly in oscar (Wood et al., 2007, 2009).

Lately, evidence has been published supporting a selective reduction in passive Na loss at the gill under ion-poor conditions (in zebrafish, *Danio rerio*) (Kwong et al., 2013), yet permeability to PEG-400 (i.e. a smaller paracellular marker) actually increased. More specifically, it was seen that increased paracellular gill permeability does not necessarily coincide with increased passive Na loss, due to the proposed action of claudin-b (claudins are major transmembrane proteins regulating paracellular permeability) (Tsukita and Furuse, 2000; Kwong et al., 2013). In dilute environments, claudin-b is strongly expressed in transport epithelia (i.e. the gill and kidney), where it co-localizes with Na/K-ATPase (involved in Na uptake); Kwong et al. (2013) hypothesized that claudin-b forms a selective paracellular barrier against Na loss without changing any other aspects of tight junction permeability. This is supported by the previous findings of McDonald and Rogano (1986), who saw that trout which were adapted to hard water but held in ion-poor

conditions showed net branchial Na loss with no corresponding change in mannitol clearance (a measure of branchial paracellular permeability). Thus based on the behaviour of paracellular Na flux at the gills compared with these known paracellular flux *markers*, it is possible that [^{3}H]PEG-4000 permeability is not entirely representative of Na permeability – future studies may also consider using ^{22}Na in conjunction with the [^{3}H]PEG-4000 protocol presented here.

Amazonian oscar

Stationary [^{3}H]PEG-4000 Trials

In order to compare results with those of the rainbow trout, parallel cannulation and stationary/swimming experiments were performed for the Amazonian oscar in Rio Grande, RS, Brazil (except that here the gut was excised and measured in *all* trials to account for drinking). The only difference was that all fish were subjected to a 4-h rest period, followed by a 4-h swim period (in exercised fish) or rest period in controls. This particular length of time was chosen for two reasons: primarily because this species is not known for its proficiency in extended exercise – in fact, it was difficult to motivate them to swim at all, let alone for 4 h. Secondly, Wood et al. (2007) found that there is only a 7-h maximal window of time for oscar before ^{22}Na backflux occurs, which was used for an identical swimming trial to be described in Chapter 3.

In these experiments, given that individuals used were also the same size as trout, the same swim speed was employed (1.2BL/s, or 15cm/s). However, oscar are definitely not as well-adapted as trout for extended swimming, and – although they were only exercised for 4 h as opposed to 8 h in the trout because of this – they appeared to show

visible difficulty in maintaining this speed. Nevertheless, oscars exhibited almost the same increase of oxygen consumption (Figure 2.14) as seen in the trout. In fact, both species showed comparable resting metabolic rates as well, despite the ~10°C higher temperature in oscar. Applying a standard Q10 value of 2.0 (Small et al., 1966; Paranjape, 1967; Teal and Carey, 1967; Widdows, 1973), this suggests that aerobic metabolic rate in the oscar is only about half that of the trout.

As evidence that urinary catheters were overall functional in the oscars, Figure 2.14 shows a renal loss of [^{3}H]PEG-4000 equivalent to 95% of the amount injected, and only 5% via the branchial route. This correlates very closely with findings in oscars by Wood et al. (2009) where gill [^{3}H]PEG-4000 clearance comprises 5% of urinary clearance. Note that this renal value is higher than the measurements made in trout, of ~60% renal loss and 40% branchial, but is more comparable to values obtained by Curtis and Wood (1991) who saw a renal loss of 88% in cannulated trout.

Again it was necessary to establish the same preliminary facts, that: a) urinary catheters were patent, b) [^{3}H]PEG-4000 is an appropriate paracellular marker, and c) its permeability is bidirectionally equal across the gills. Figure 2.12 displays measured urine parameters obtained by [^{3}H]PEG-4000 use in conjunction with cannulation. Wood et al. (2009) found a gill plasma clearance rate, GFR, and UFR of 0.4, 9, and 3mL/kg/h – respectively. They saw that gill clearance was only "about 5% of the simultaneously measured clearance of [^{3}H]PEG-4000 via the urine." The present data for the stationary efflux trials showed a gill plasma clearance rate, GFR, and UFR of 1.1, 12.6, and 1.5mL/kg/h – respectively.

When compared between influx and efflux in stationary oscar, [^{3}H]PEG-4000 is once again proven to be bidirectionally permeable across the gills (Fig. 2.14), as in trout (calculated using the carcass *and* ECFV methods for determining influx permeability). Once again, this has the same implication; specifically that cannulation is not necessary for stationary/swimming trials provided that renal loss is back-calculated, nor is direct radioisotope injection necessary, as permeability can be measured in the influx direction, so only the water itself needs to be dosed. It is also noteworthy that Figure 2.13 shows that while mean permeabilities measured for the resting, cannulated oscar (0.43 ±0.07 x10^{-4} mL/20g/s [N=15]) are significantly lower by almost 3-fold than those seen in the trout under the same conditions (1.15 ±0.25 x10^{-4} mL/20g/s [N=25]). This difference occurs despite the 10°C higher temperature (28°C vs 18°C) in the oscar experiments than in trout experiments. The Q10 for a process based on physical diffusion (~1.2-1.6) (Motais and Isaia, 1972) is likely lower than that used earlier for O_2 consumption (~2 or higher) (Krogh, 1916; Small et al., 1966; Steffensen, 2002). Nevertheless, this difference emphasizes the much lower gill paracellular permeability in the oscar, likely an adaptation to the ion-poor, frequently challenging conditions that exist in its natural habitat.

Swimming [^{3}H]PEG-4000 Trials

Stationary and swimming experiments performed in the oscars all began with a 4-h stationary flux period, followed by another 4-h rest or swimming phase, due to the inability of these fish to maintain this pace for any longer period. Once again, due to inherent interaction between the respirometer and [^{3}H]PEG-4000 used in the experiment,

quantification of [^{3}H]PEG-4000 loss from the water resulted in an enormous overestimate of [^{3}H]PEG-4000 uptake (Fig. 2.19A-C), so data from these two periods could not be separated. Nevertheless, enough information is presented for both stationary and 'swimming 4/4' fish that valid conclusions may still be confidently drawn based on these findings.

As in trout, Figure 2.15 illustrates the suitability of [^{3}H]PEG-4000 as an extracellular marker in both stationary and 'swimming 4/4' oscar because carcass and ECFV uptake cpm for the oscar are equal. These data show no difference in whole body cpm between stationary and swimming fish (unlike the trout), indicating that the gills do not increase their ability to take up [^{3}H]PEG-4000 paracellularly with exercise.

In terms of drinking, the oscar has a high drinking rate at rest that does not change with exercise – a pattern already seen in the trout, although drinking rate is lower in oscars than trout (where the mean stationary and swimming DR for oscar and trout are $1.79 \pm 0.43 \times 10^{-5}$ mL/20g/s [N=13] and $3.54 \pm 0.65 \times 10^{-5}$ mL/20g/s [N=21], respectively). Possible reasons that drinking rate might be lower may include, a) oscar appeared – in general – to be less stressed during all treatments than the trout, and b) oscar ion loss is known to be tightly regulated via the transcellular pathway during hypoxia, so perhaps ion leakage is not commonly encountered; also, drinking to replace ions would be a fairly ineffective strategy in any freshwater environment, particularly in the oscar's dilute native waters (Wood et al., 2009).

This raises a potentially confounding issue in the oscar experiments. Owing to radioisotope licensing issues, these experiments were performed in oscars that had been

transported from their native Manaus, AM to Cassino, RG and acclimated there. The most notable change was a higher water calcium concentration in Cassino (details in Materials and Methods). This is especially important as calcium is involved in tight junction integrity and therefore high external concentrations may reduce paracellular permeability by 'tightening' tight junctions (Spry and Wood, 1989) and thereby inhibiting paracellular (i.e. [^{3}H]PEG-4000) flux Brown and Davis, 2002; Rajasekaran et al., 2008). Nevertheless, water calcium concentrations in Rio Grande (0.3mM) were still lower than those observed in the trout experiments (0.9mM) in Ontario.

Figure 2.17 shows that the ratio of [^{3}H]PEG-4000 in the carcass:gut remains constant for both stationary and swimming fish, and thereby supports what is seen in Figure 2.18 – that [^{3}H]PEG-4000 permeability in the oscar (which is significantly lower than that of the trout), does not increase during the swimming challenge imposed by this protocol (unlike the trout). There is not even a slight elevation to indicate that paracellular permeability increases are potentially being masked by the initial 4-h stationary period. In fact, for the sake of calculation, assume that the permeability of the first *4-h stationary* period for swimming trials (in which *8-h* permeability values are 9.8×10^{-5} and 1.10×10^{-4} mL/20g/h – by ECFV and carcass, respectively) is equal that of the *full 8-h* permeability in stationary (i.e. control) experiments (1.17×10^{-4} and 1.48×10^{-4} mL/20g/h respectively). In that case, the calculated permeability for the swimming portion itself would be 7.3×10^{-5} and 7.2×10^{-5} mL/20g/h by ECFV and carcass, respectively. This may actually indicate slightly lower paracellular permeability during swimming. Moreover, previous work (Wood et al., 2007; 2009; Matey et al., 2011) as well as ^{22}Na

experiments in Chapter 3 provide convincing evidence that the oscar alters its branchial ion permeability primarily via the transcellular pathway, as opposed to the paracellular route – under osmorespiratory compromises imposed by both swimming and hypoxic challenges.

Conclusion

Recall that in the Introduction, four specific hypotheses were being tested. Firstly, that both the trout and the oscar would show negligible drinking of the external medium at rest and minimal amounts during exercise, such that corrections for the influx of [^{3}H]PEG-4000 by drinking would be small. In fact, this was proven false – both species exhibited considerably (and comparably) high drinking rates at rest, which did not significantly change with exercise. Gut excisions were performed to correct [^{3}H]PEG-4000 uptake values for this, however, and prevent overestimation. The second hypothesis was that both species would exhibit much larger renal than branchial losses of [^{3}H]PEG-4000, which was observed during efflux experiments. This was corrected for by urinary cannulation, but in influx experiments was deemed unnecessary since 8 h was not sufficient for significant renal depuration of [^{3}H]PEG-4000 taken up from the external water. Thirdly, it was hypothesized that branchial permeability to [^{3}H]PEG-4000 would be bidirectionally equal in both the Amazonian oscar and rainbow trout. This third hypothesis was also confirmed, as [^{3}H]PEG-4000 permeability was not rectified in either species after drinking and renal corrections were applied. Lastly, it was predicted that paracellular permeability would increase in the trout during exercise but remain unchanged or decreased in oscar – which was also observed as trout branchial [^{3}H]PEG-4000 permeability significantly increased and did not change in the oscar.

In summary, this research has led to two major findings. Firstly, gill paracellular permeability – as measured by [^{3}H]PEG-4000 was elevated during sustained exercise in the rainbow trout, whereas no change was observed in the Amazonian oscar. Secondly,

this particular methodology has brought to light some of the challenges and potential problems associated with [^{3}H]PEG-4000 use as a branchial permeability marker, including: renal loss, overestimation based on water loss due to 'stickiness' of the chemical (i.e. surface adsorption to the respirometer), and most importantly, drinking in freshwater fish. By incorporating corrective measures to control for these, this technique can be applied to measure paracellular permeability and tight junction modifications associated with any number of factors – from sea-water acclimation to manipulation of temperature, pH, water oxygen saturation, external ammonia, and contaminant levels.

Figure 2.1. Urine flow rates over 16-h recovery and 8-h experimental periods in cannulated trout, at 18˚C. Means are ±S.E.M. (N=20 for efflux experiments; N=10 for influx experiments). No significant differences.

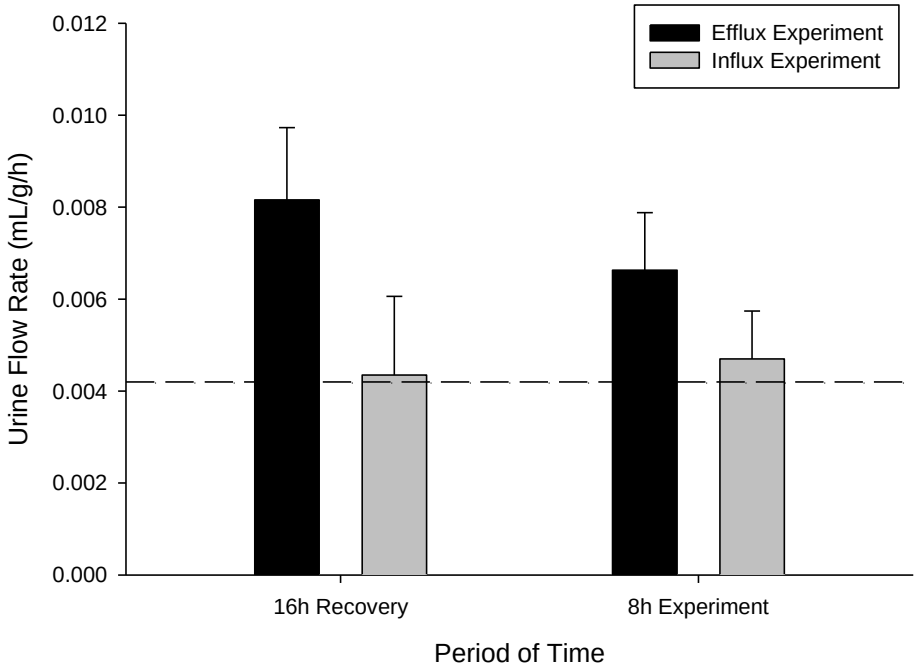

Efflux Experiment
Influx Experiment
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Urine Flow Rate (mL/g/h)
16h Recovery
8h Experiment
Period of Time

Figure 2.2. Division of depurated [^{3}H]PEG-4000 cpm (%) to the urine or to the water (presumably across the gills) in cannulated trout used in efflux experiments during the 8-h experimental period at 18˚C. Means are ±S.E.M. (N=15). Means sharing the same letter are not significantly different (P>0.05) as determined by a paired t-test performed on arcsin values.

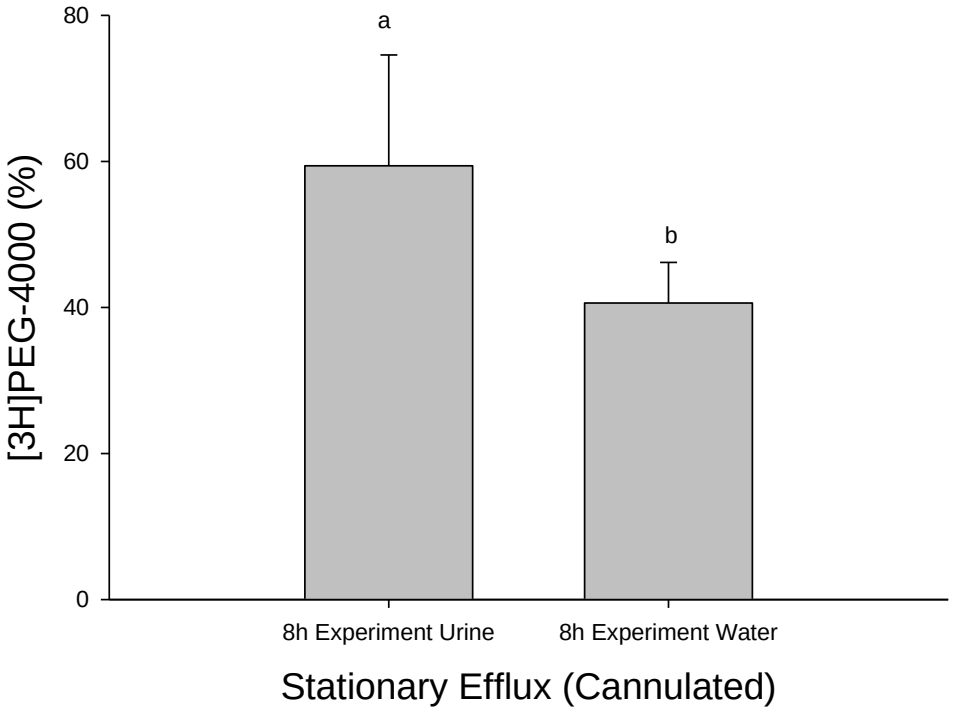

80
60
40
20
0
[3H]PEG-4000 (%)
a
b
8h Experiment Urine
8h Experiment Water
Stationary Efflux (Cannulated)

Figure 2.3. Division of PEG cpm (%) in cannulated trout used in influx experiments during the 8-h experimental period at 18˚C. Means are ±S.E.M. (N=15). Means sharing the same letter are not significantly different (P>0.05) as determined by a paired t-test performed on arcsin values.

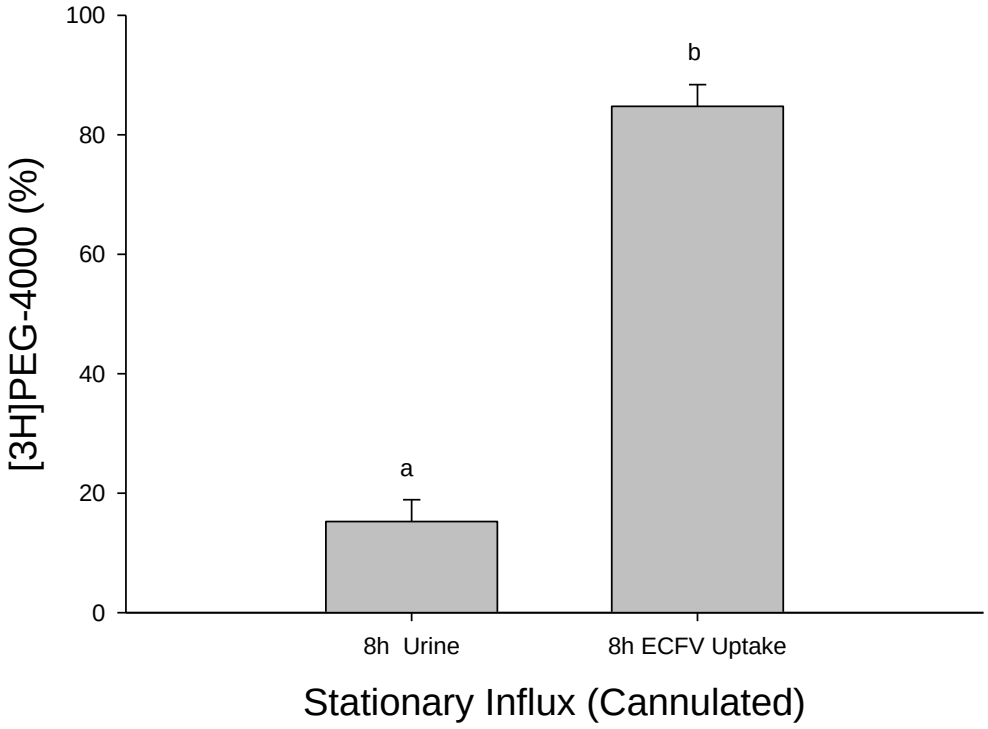

100
80
60
40
20
0
[3H]PEG-4000 (%)
a
b
8h Urine
8h ECFV Uptake
Stationary Influx (Cannulated)

Figure 2.4. [^{3}H]PEG-4000 cpm normalized for weight (g) and concentration (cpm/mL) as compared between carcass and ECFV (extracellular fluid volume) for stationary trout at 18˚C. "Cann" refers to cannulated fish, whereas "Gut Ex" means gut-excised. Means are ±S.E.M. (N=22 for efflux; N=7 for influx). No significant differences.

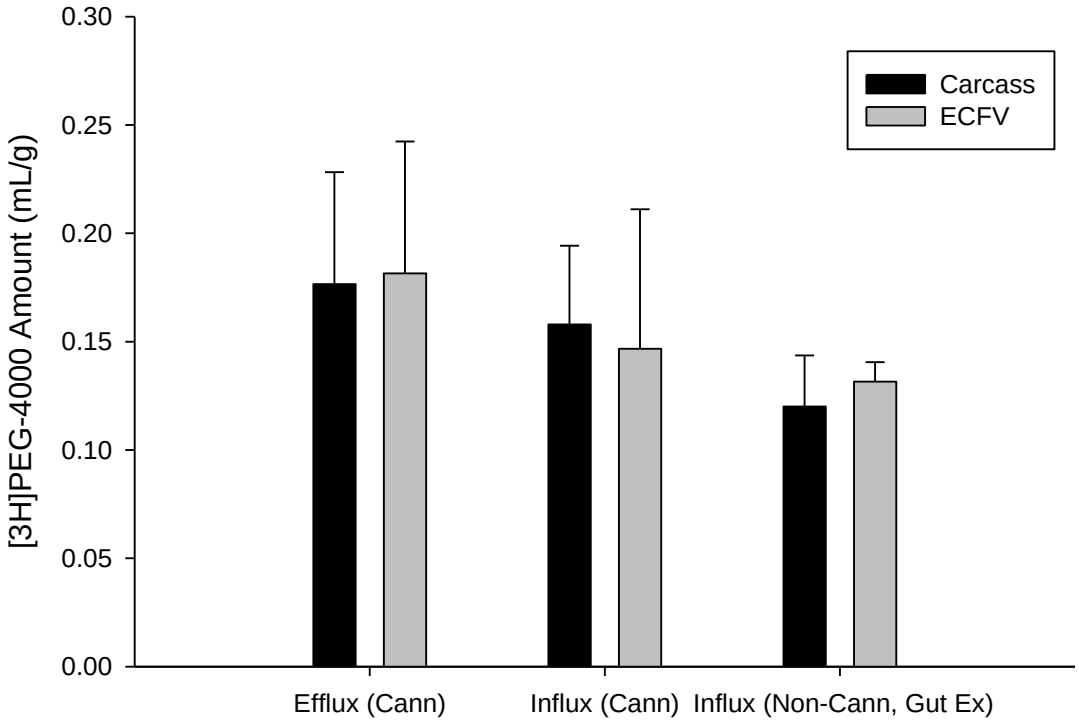

0.30
0.25
0.20
0.15
0.10
0.05
0.00
[3H]PEG-4000 Amount (mL/g)
Carcass
ECFV
Efflux (Cann)
Influx (Cann)
Influx (Non-Cann, Gut Ex)

Figure 2.5. Gill permeability to [^{3}H]PEG-4000 in stationary, urinary cannulated trout at 18˚C. Means are ±S.E.M. (N=8 for influx ECFV (extracellular fluid volume), 7 for influx carcass; N=10 for efflux). No significant differences.

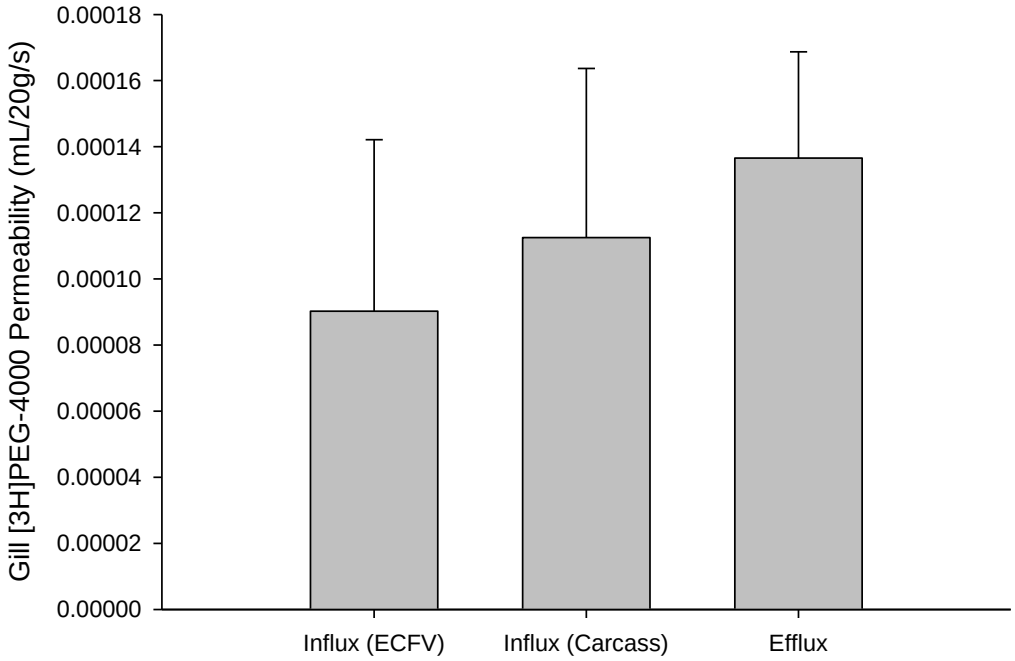

Gill [3H]PEG-4000 Permeability (mL/20g/s)
0.00018
0.00016
0.00014
0.00012
0.00010
0.00008
0.00006
0.00004
0.00002
0.00000
Influx (ECFV)
Influx (Carcass)
Efflux

Figure 2.6. Metabolic rate changes in juvenile trout during an 8-h swim at 1.2BL/s (15cm/s) in Blazka respirometers at 18˚C. Means are ±S.E.M. (N=14). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way ANOVA on ranks followed by Holm-Sidak *post hoc* test.

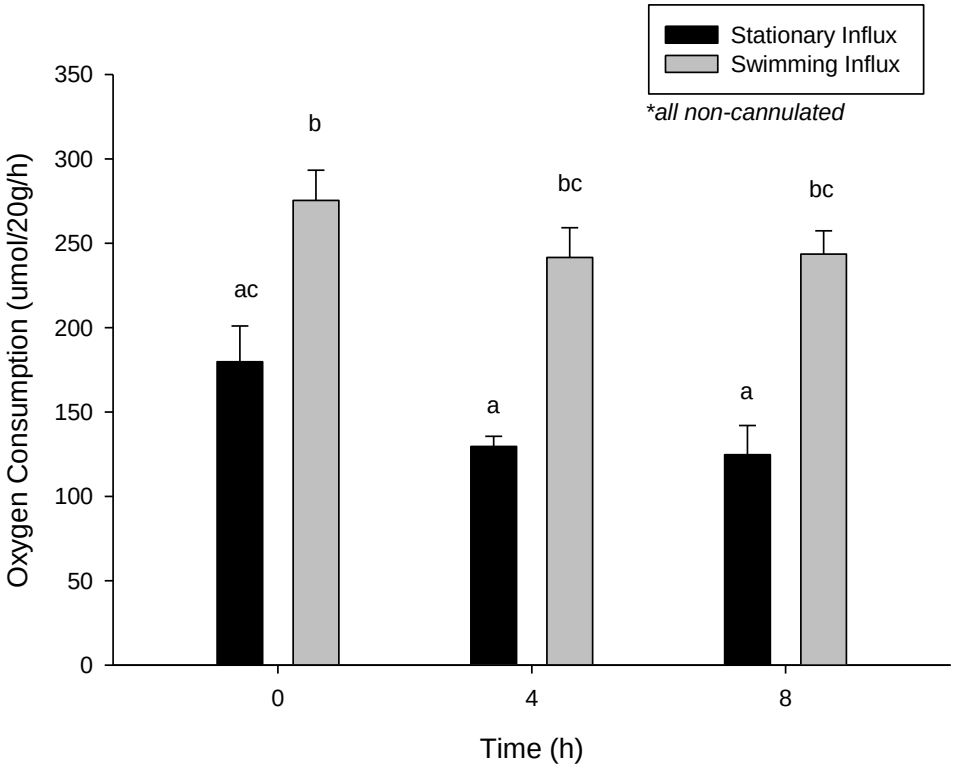

Stationary Influx
Swimming Influx
*all non-cannulated
Oxygen Consumption (umol/20g/h)
350
300
250
200
150
100
50
0
ac
b
a
bc
a
bc
0
4
8
Time (h)

Figure 2.7. Drinking rates in non-cannulated trout based on excised gut [^{3}H]PEG-4000 cpm in stationary and swimming trout at 1.2BL/s (15cm/s) in Blazka respirometers at 18˚C. Means are ±S.E.M. (N=7 for stationary; N=14 for swimming). No significant differences.

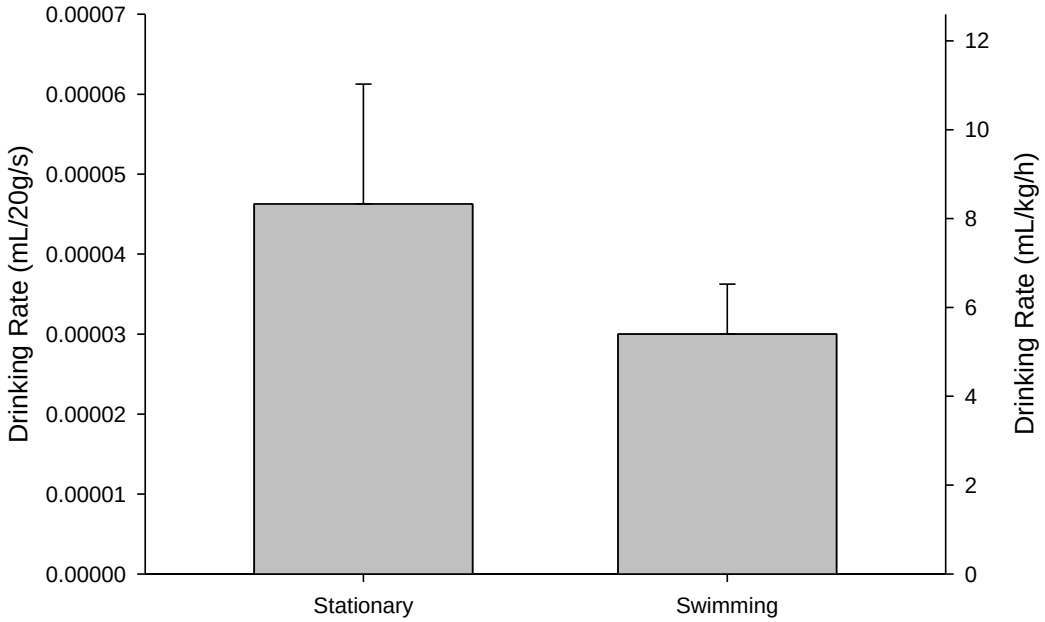

0.00007
0.00006
0.00005
0.00004
0.00003
0.00002
0.00001
0.00000
Drinking Rate (mL/20g/s)
12
10
8
6
4
2
0
Drinking Rate (mL/kg/h)
Stationary
Swimming

Figure 2.8. Relative [^{3}H]PEG-4000 uptakes normalized to weight and to the external water concentration (mL/g) compared between the carcass (via branchial uptake) and the gut (via drinking) in trout at 18˚C. Means are ±S.E.M. (N=6 for stationary; N=14 for swimming). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way ANOVA on ranks followed by Dunn's *post hoc* test.

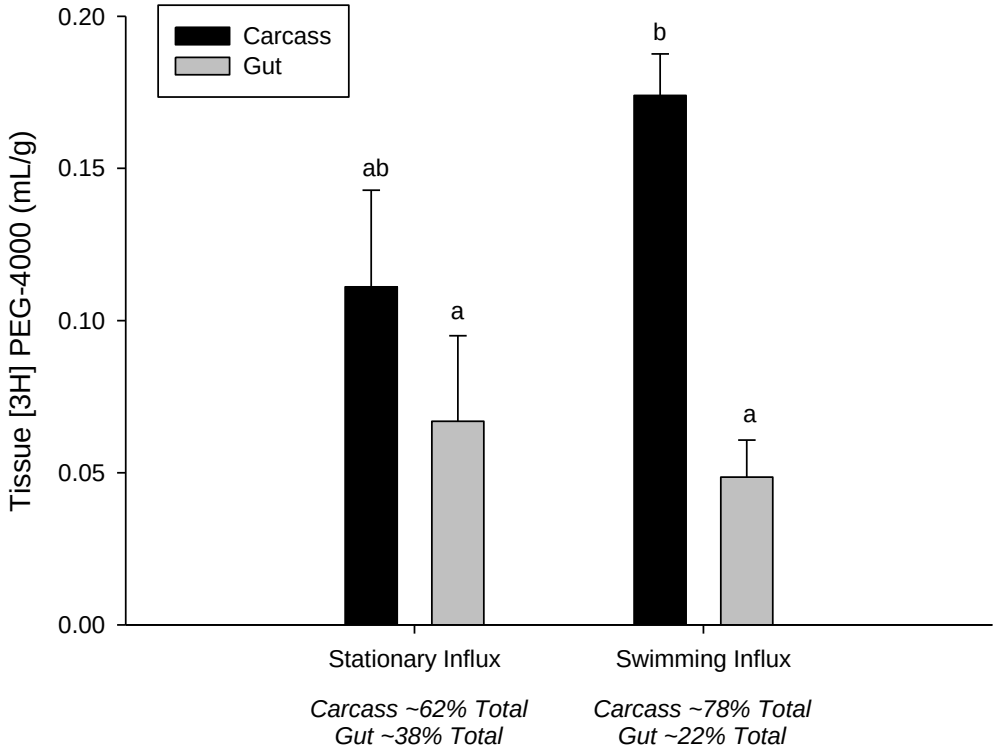

Carcass
Gut
Tissue [3H] PEG-4000 (mL/g)
0.20
0.15
0.10
0.05
0.00
ab
a
b
a
Stationary Influx
Swimming Influx
Carcass ~62% Total
Gut ~38% Total
Carcass ~78% Total
Gut ~22% Total

Figure 2.9. Relative [^{3}H]PEG-4000 uptakes normalized for weight (g) and water concentration (cpm/mL) as compared between carcass and ECFV (extracellular fluid volume) for non-cannulated, gut-excised stationary and swimming (1.2BL/s, 15cm/s) trout at 18˚C. Means are ±S.E.M. (N=7 for stationary carcass, 6 for stationary ECFV; N=14 for swimming). Means sharing the same letter are not significantly different (P>0.05), as determined by a paired t-test.

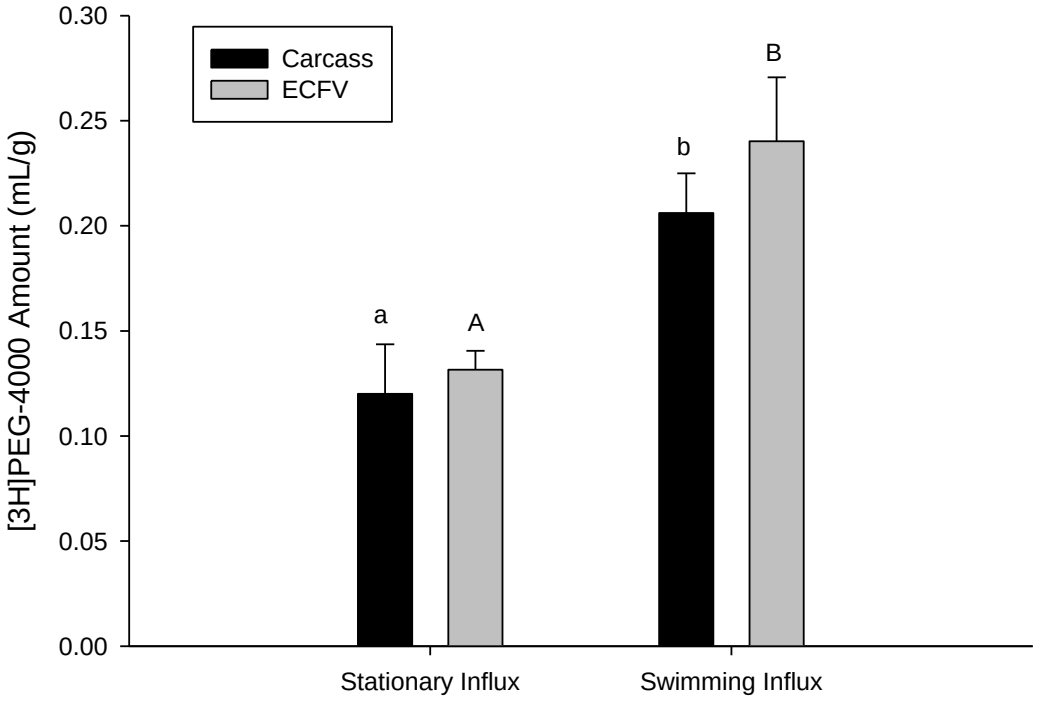

0.30
0.25
0.20
0.15
0.10
0.05
0.00
[3H]PEG-4000 Amount (mL/g)
Carcass
ECFV
a
A
b
B
Stationary Influx
Swimming Influx

Figure 2.10. Branchial [^{3}H]PEG-4000 permeability changes between non-cannulated stationary and swimming juvenile trout at 18˚C. Means are ±S.E.M. (N=7 for stationary; N=14 for swimming). Means sharing the same letter are not significantly different (P>0.05) as determined by a paired t-test.

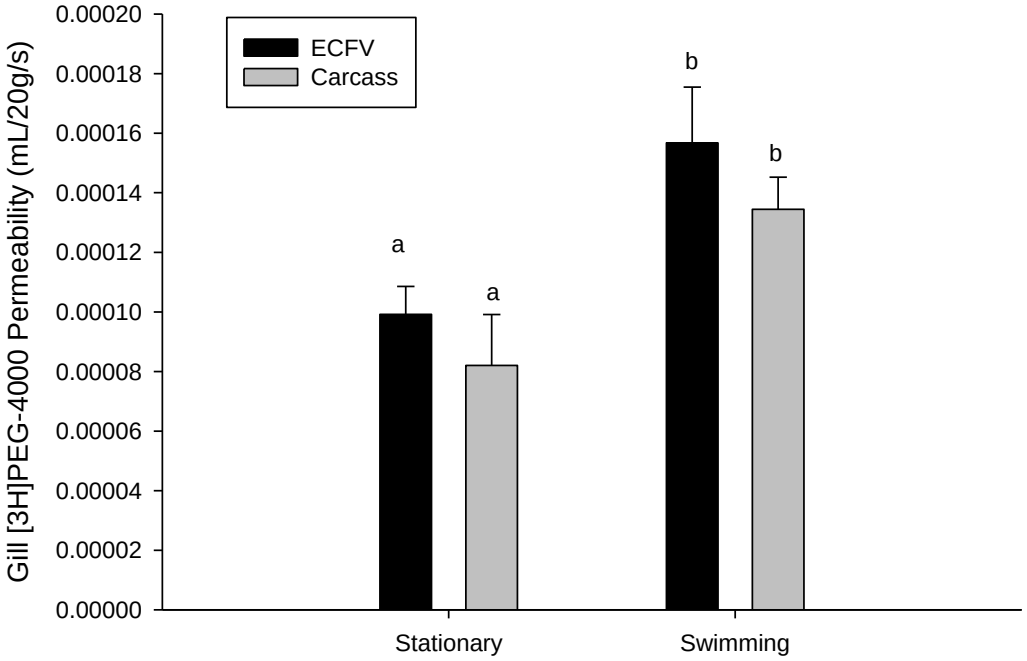

Gill [3H]PEG-4000 Permeability (mL/20g/s)
0.00020
0.00018
0.00016
0.00014
0.00012
0.00010
0.00008
0.00006
0.00004
0.00002
0.00000
ECFV
Carcass
a
a
b
b
Stationary
Swimming

Figure 2.11. Division of total [^{3}H]PEG-4000 cpm in cannulated Amazonian oscar in efflux experiments, as an indicator of flux location at ~28˚C. Means are ±S.E.M. (N=5). Means sharing the same letter are not significantly different (P>0.05) as determined by a paired t-test performed on arcsin values.

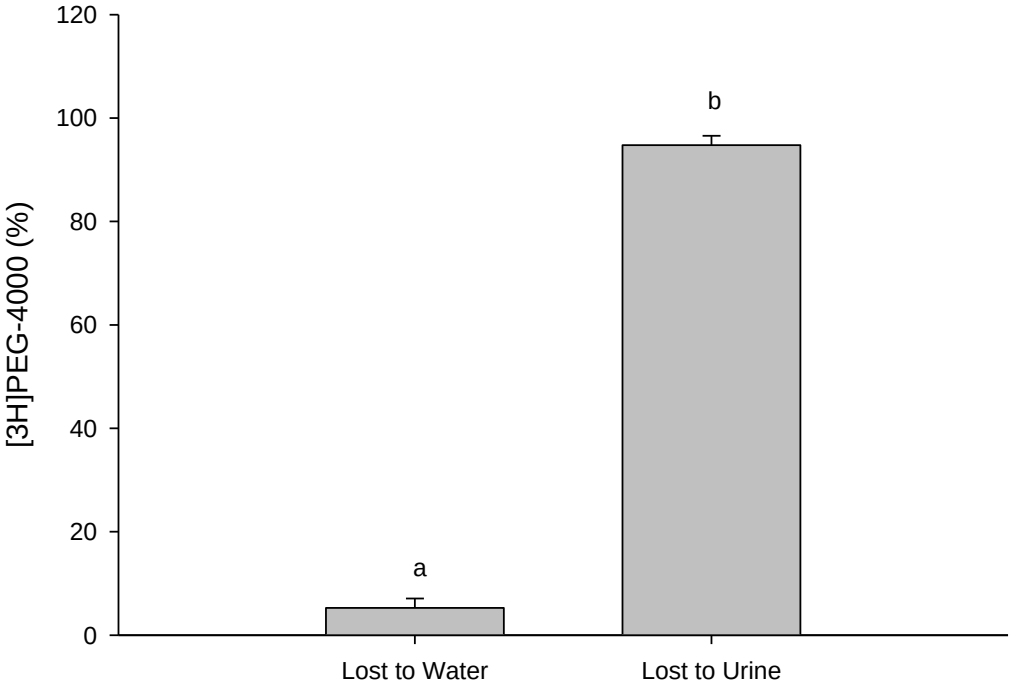

120
100
80
60
40
20
0
[3H]PEG-4000 (%)
a
b
Lost to Water
Lost to Urine

Figure 2.12. Various physiological clearance rates in cannulated stationary Amazonian oscars in [^{3}H]PEG-4000 efflux experiments at ~28˚C. Means are ±S.E.M. (N=5). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way ANOVA followed by Tukey *post hoc* test.

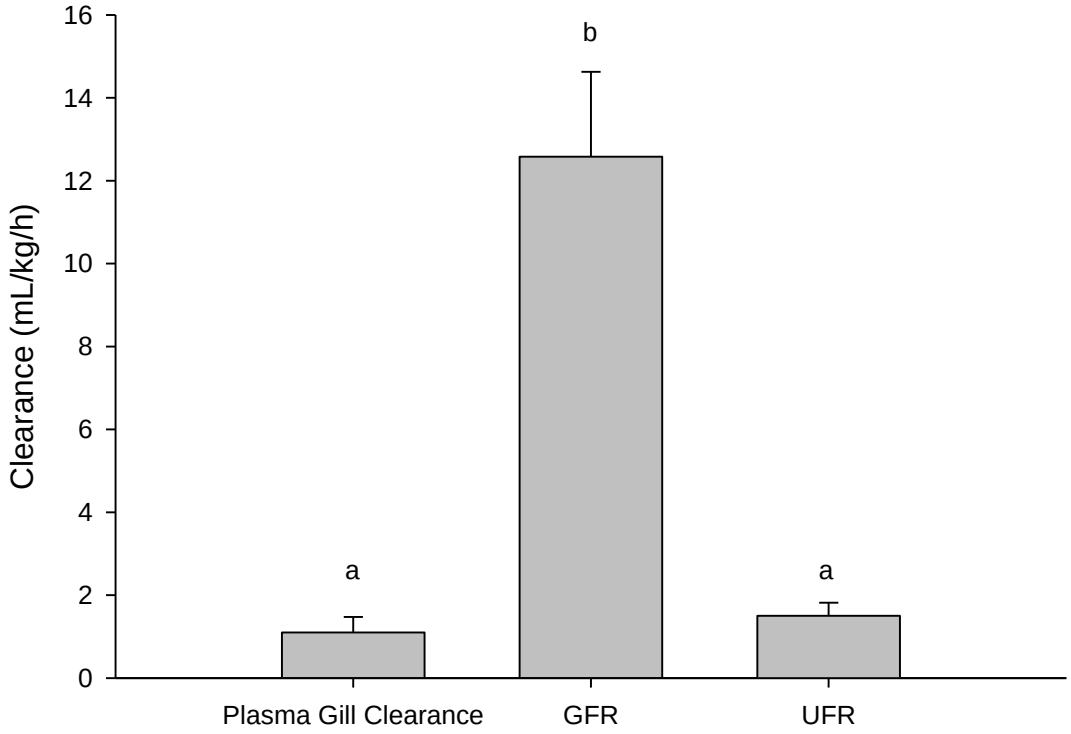

16
14
12
10
8
6
4
2
0
Clearance (mL/kg/h)
a
b
a
Plasma Gill Clearance
GFR
UFR

Figure 2.13. [^{3}H]PEG-4000 permeability in stationary, cannulated efflux and influx trials in Amazonian oscars at ~28˚C. Means are ±S.E.M. (N=5). No significant differences.

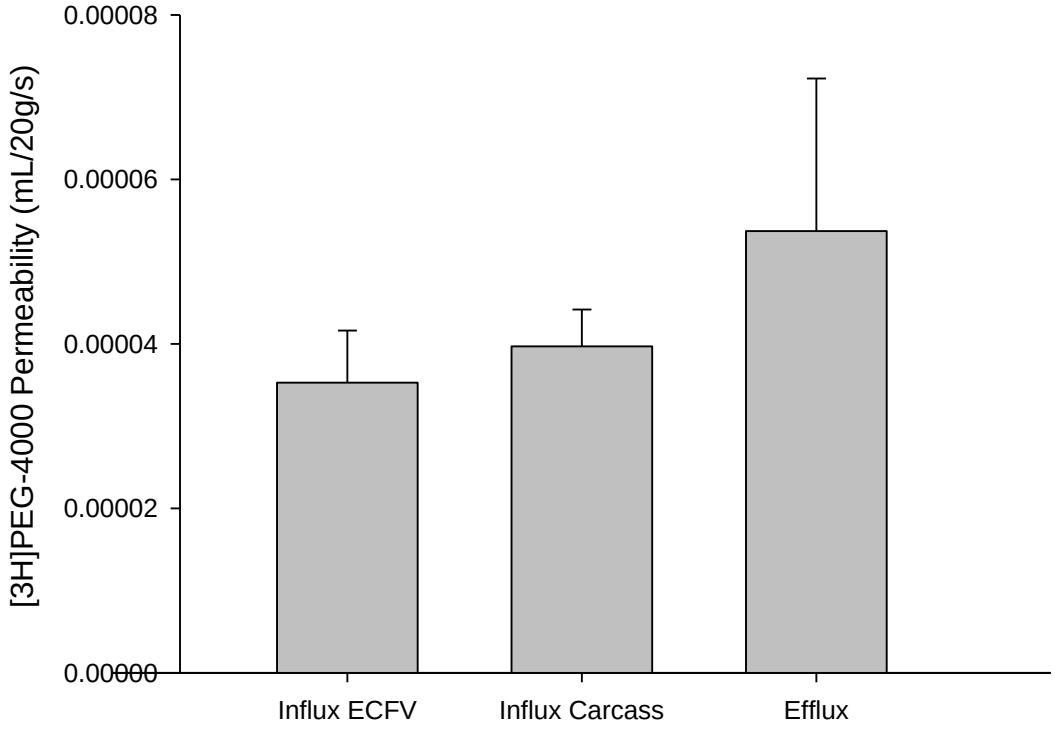

0.00008
0.00006
0.00004
0.00002
0.00000
[3H]PEG-4000 Permeability (mL/20g/s)
Influx ECFV
Influx Carcass
Efflux

Figure 2.14. Metabolic rate – as measured by oxygen consumption – changes with 4 h swimming at 1.2BL/s (15cm/s) at ~28˚C, in Amazonian oscars. Means are ±S.E.M. (N=4 for stationary; N=23 for swimming). Means sharing the same letter are not significantly different (P>0.05) as determined by a paired t-test.

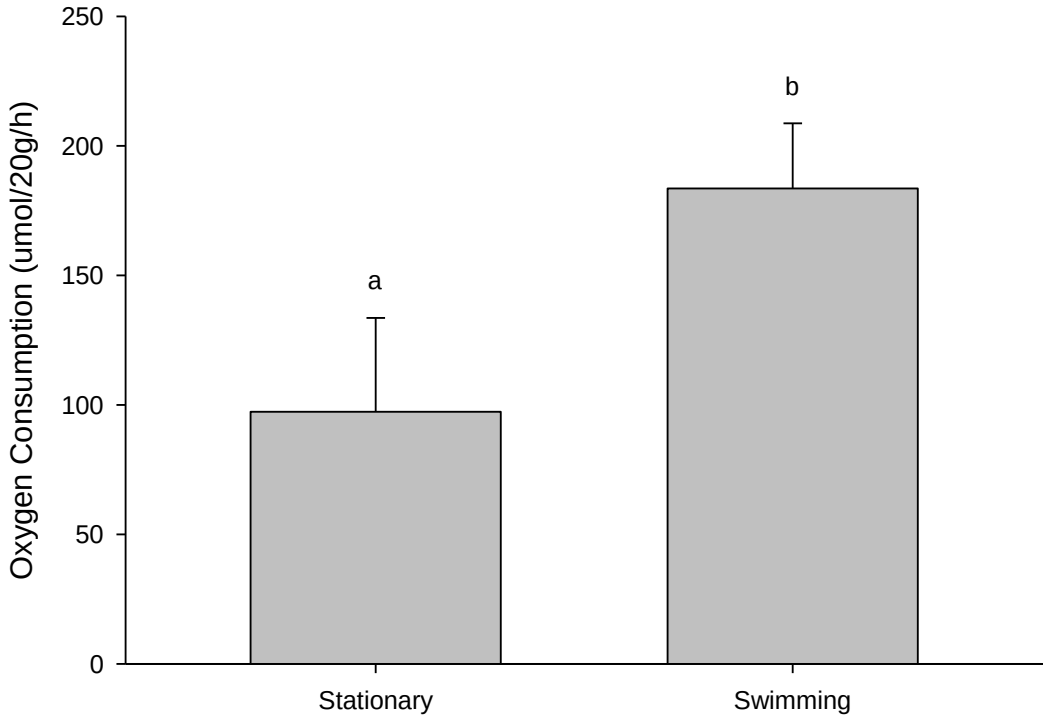

Oxygen Consumption (umol/20g/h)
250
200
150
100
50
0
a
b
Stationary
Swimming

Figure 2.15. Relative [^{3}H]PEG-4000 uptakes normalized for weight (g) and water concentration (cpm/mL), comparing uptake estimated from ECFV (extracellular fluid volume) and carcass counts in stationary and swimming influx trials in Amazonian oscars at ~28˚C. Means are ±S.E.M. (N=9 for all except swimming ECFV where N=7). No significant differences.

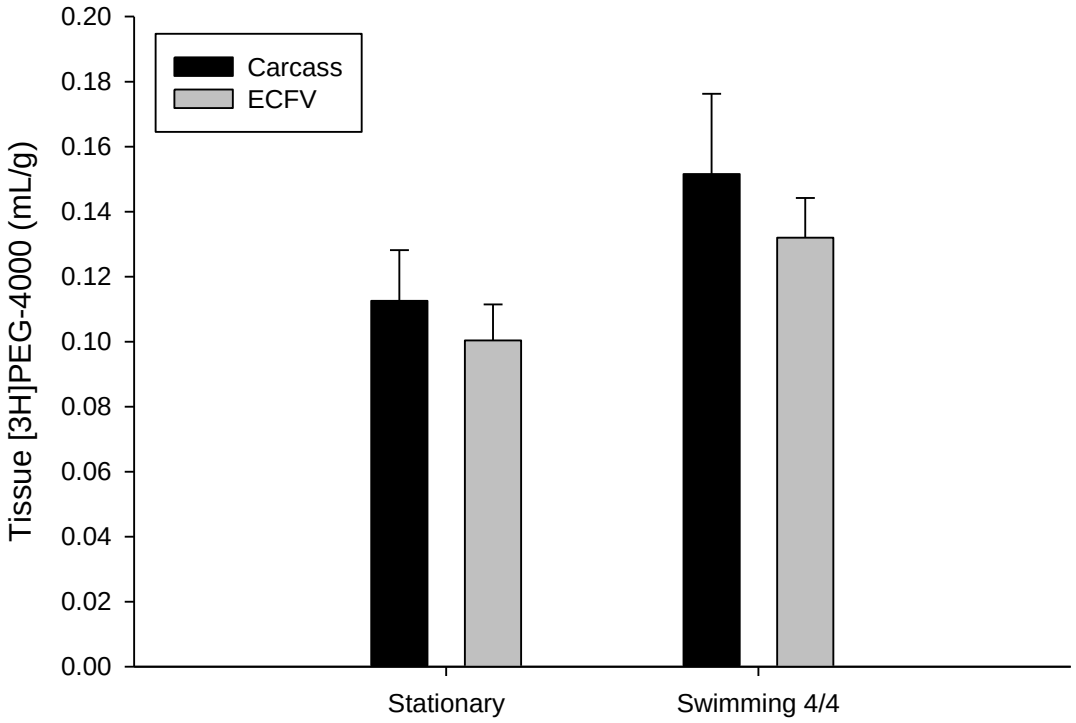

Carcass
ECFV
Tissue [3H]PEG-4000 (mL/g)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Stationary
Swimming 4/4

Figure 2.16. Drinking rate, as estimated by excised gut [^{3}H]PEG-4000 cpm in Amazonian oscars at ~28˚C, FW. Means are ±S.E.M. (N=4 for stationary, N=9 for swimming). No significant differences.

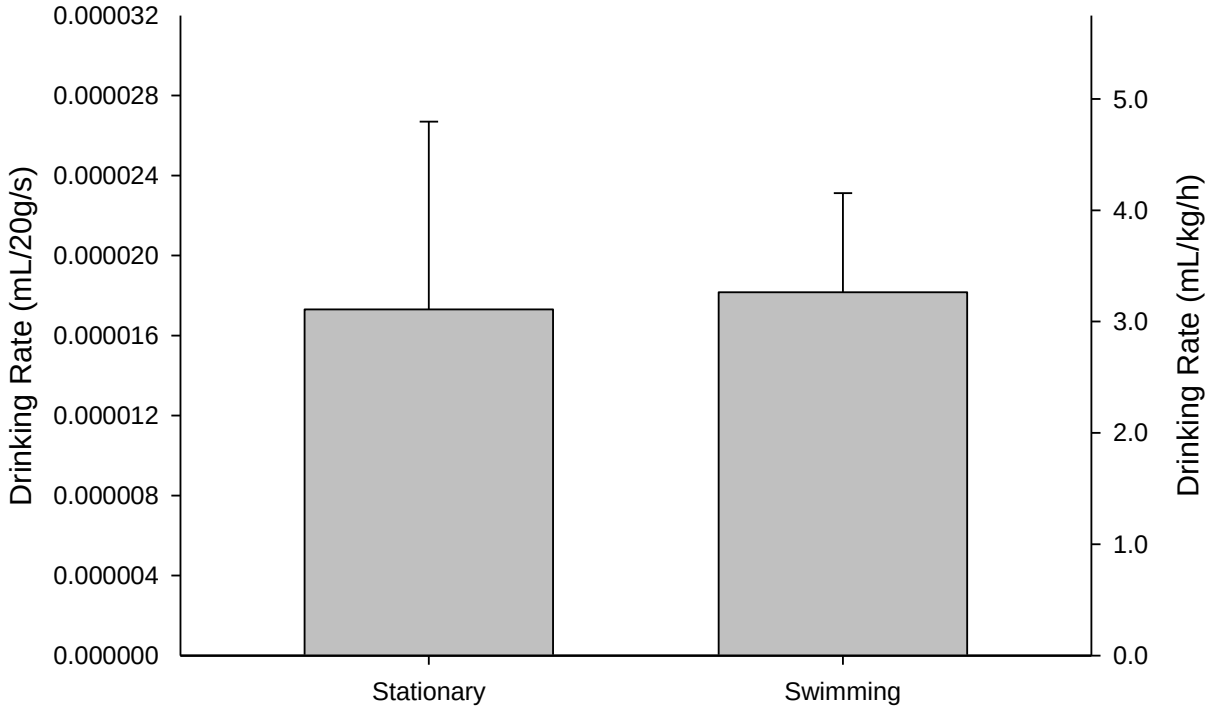

0.000032
0.000028
0.000024
0.000020
0.000016
0.000012
0.000008
0.000004
0.000000
Drinking Rate (mL/20g/s)
5.0
4.0
3.0
2.0
1.0
0.0
Drinking Rate (mL/kg/h)
Stationary
Swimming

Figure 2.17. Tissue [^{3}H]PEG-4000 amount (mL) in Amazonian oscars based on tissue cpm, corrected for weight (g) and normalized for water concentration (cpm/mL) at ~28˚C. Means are ±S.E.M. (N=8 for all except stationary gut trials where N=4). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way ANOVA on ranks followed by Dunn's *post hoc* test.

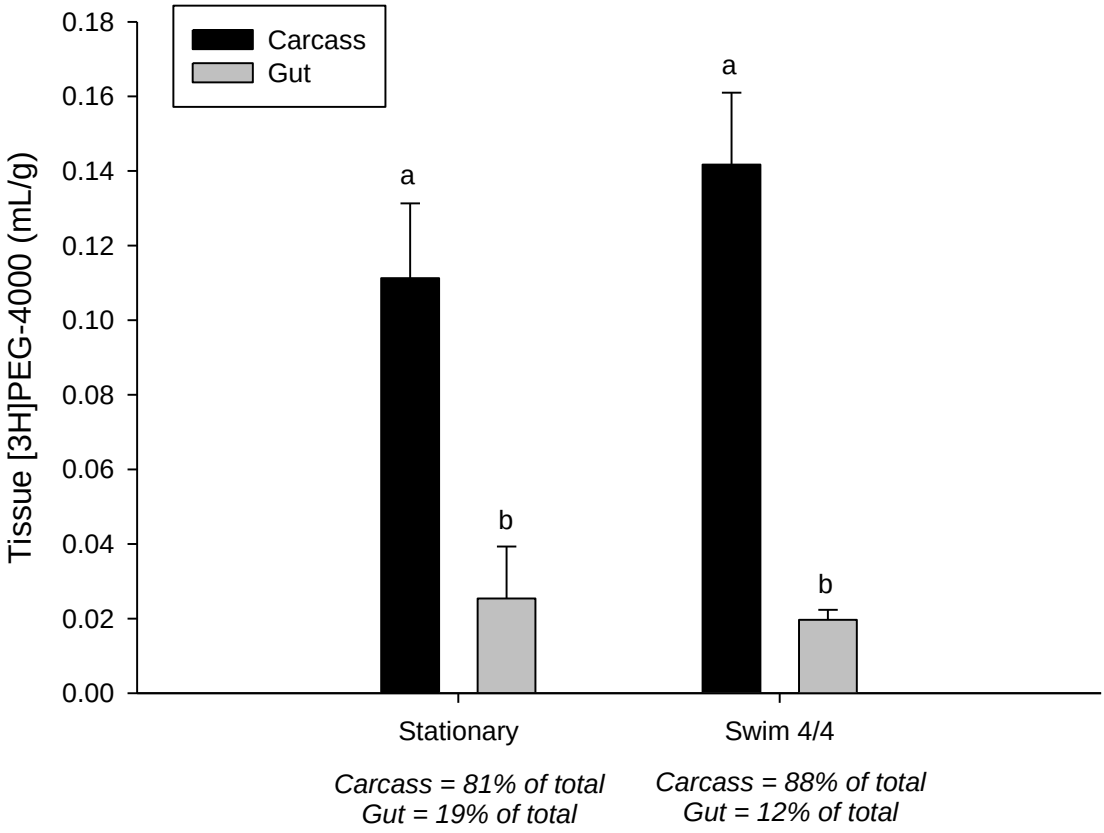

Carcass
Gut
Tissue [3H]PEG-4000 (mL/g)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
a
b
a
b
Stationary
Swim 4/4
Carcass = 81% of total
Gut = 19% of total
Carcass = 88% of total
Gut = 12% of total

Figure 2.18. [^{3}H]PEG-4000 permeabilities during with swimming, as measured via influx experiments in the Amazonian oscar at ~28˚C. Means are ±S.E.M. (N=9 for all except swim ECFV (extracellular fluid volume) where N=7). No significant differences.

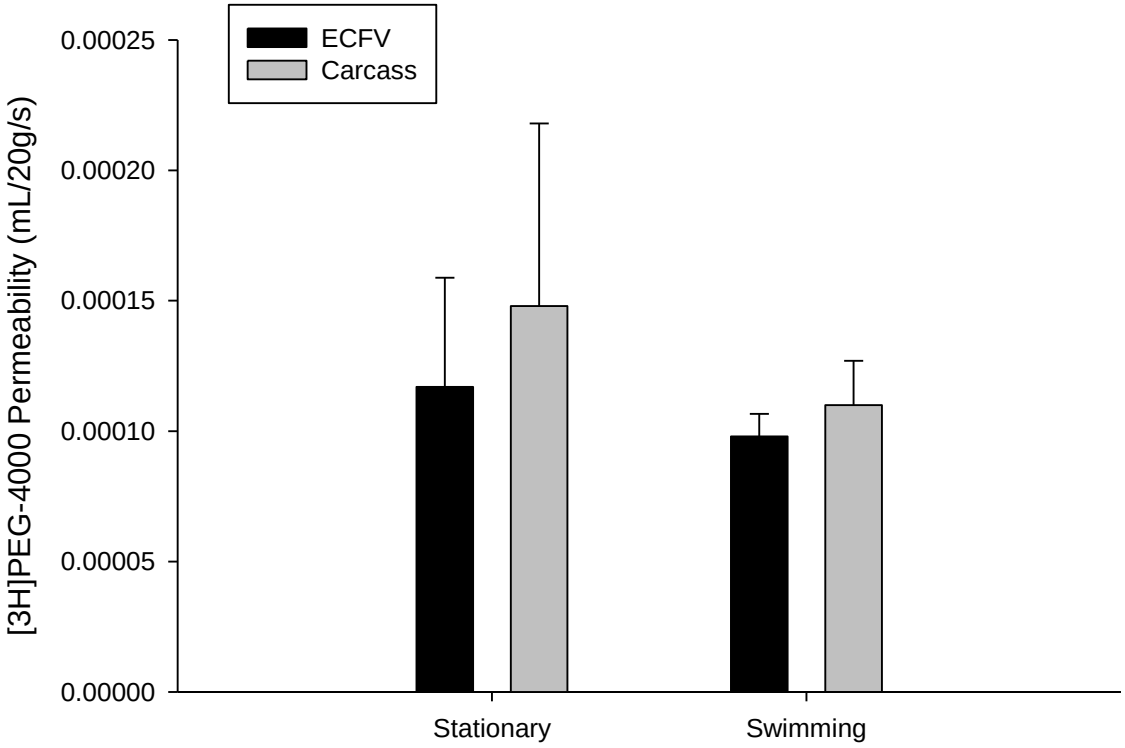
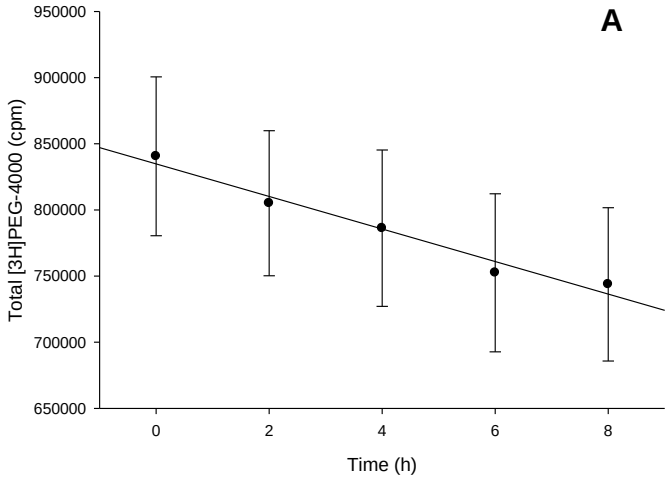
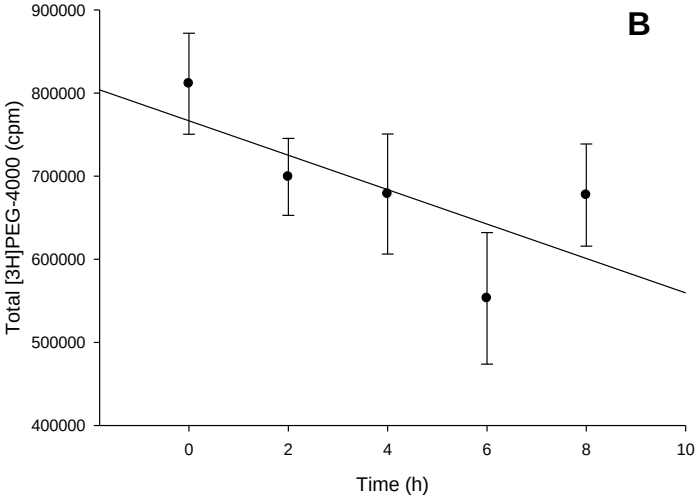
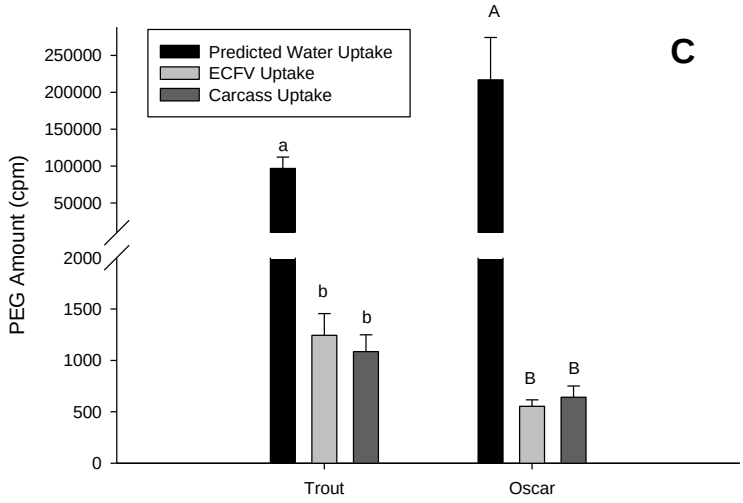

ECFV
Carcass
[3H]PEG-4000 Permeability (mL/20g/s)
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
Stationary
Swimming

Figure 2.19. [^{3}H]PEG-4000 changes based on water cpm loss during influx swimming trials, (A) in rainbow trout at 18˚C. Means are ±S.E.M. (N=20). (B) in Amazonian oscars at ~28˚C. Means are ±S.E.M. (N=10). (C) for the purpose of predicting 'theoretical' uptake – versus actual ECFV (extracellular fluid volume) and carcass uptakes. Means are ±S.E.M. (N=20 for trout water, N=10 for oscar water; N=14 for trout ECFV and carcass; N=7 for oscar ECFV, N=9 for oscar carcass). Means sharing the same letter within species are not significantly different (P>0.05) as determined by a one-way ANOVA on ranks followed by Dunn's *post hoc* test.


A
Total [3H]PEG-4000 (cpm)
950000
900000
850000
800000
750000
700000
650000
0
2
4
6
8
Time (h)


B
Total [3H]PEG-4000 (cpm)
900000
800000
700000
600000
500000
400000
0
2
4
6
8
10
Time (h)


C
Predicted Water Uptake
ECFV Uptake
Carcass Uptake
PEG Amount (cpm)
250000
200000
150000
100000
50000
2000
1500
1000
500
0
a
b
b
A
B
B
Trout
Oscar

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CHAPTER THREE: A survey of ionoregulatory aspects of the osmorespiratory compromise during acute environmental hypoxia in tropical and temperature teleosts.

Abstract

This study investigated the ionoregulatory responses to acute hypoxia among various South and North American teleosts, in order to identify trends in the osmorespiratory compromise across phylogenies and/or environments. ^{22}Na was used to quantify unidirectional Na flux rates, and net Na, K, ammonia, and urea flux rates were also measured during a 2-h nomoxia:2-h hypoxia (30% O_2 saturation) :2-h normoxic recovery protocol.

The oscar (an Amazonian perciform) displayed a characteristic reduction in branchial permeability during hypoxia in which Na bidirectional flux rate, as well as ammonia and K flux rate, decreased. The trout (a North American salmoniform) at the opposite end of the hypoxia-tolerance spectrum exhibited an increase in bidirectional Na fluxes, and no change in K and ammonia fluxes. Of the other species studied, the tambaqui (an Amazonian characid which shares the same extremely hypoxic environment with the oscar) also displayed the permeability reduction strategy, whereas the bluegill sunfish (a perciform related to the oscar but found in typically non-hypoxic North American waters) more closely resembled the trout hypoxic response. Four Brazilian tetra species, which are subject to intermediate levels of hypoxia in their native Rio Negro habitat presented varied hypoxic responses, as did the pumpkinseed sunfish. Thus adaptive strategies to hypoxia do *not* appear to show a phylogenetic linkage but may well be related to a species' native environment. Pcrit was compared in several species, but did

not appear to be a diagnostic indicator of hypoxia tolerance. Finally, flux parameters were monitored in the oscar during extended swimming (4 h at 1.2 body lengths/sec) to determine whether it would show the same response to this facet of the osmorespiratory compromise. Rather than displaying a reduced branchial permeability, both Na and K loss rates were significantly elevated and ammonia excretion was not shut down. The oscar appears better adapted for the familiar osmorespiratory stress of hypoxia than the unfamiliar stress of extended exercise.

Introduction

Studying teleost ionoregulatory responses to hypoxia is not only interesting but also very environmentally relevant – particularly for species of the Brazilian Amazon, which is where the majority of this project took place. For species living in the floodplains and flooded jungles of the Amazon, the capacity to withstand and adapt to broad changes in oxygen availability is crucial – as this area experiences extreme PO_2 variability on a daily basis with fluctuating patterns of photosynthesis and organic decay (Saint-Paul, 1984; Saint-Paul and Soares, 1987). Even in the more local region of Southern Ontario, water oxygen levels are seen to oscillate with seasonal temperature changes and freezing of lakes – creating a degree of hypoxic stress for indigenous species and illustrating the fact that hypoxia is a ubiquitous challenge in all aquatic environments (Nurnberg, 2004).

In circumstances where oxygen uptake from the aquatic environment becomes difficult or restricted, branchial alterations can be employed which will allow functional surface area of the gill to be increased (Nilsson, 1986; Postlethwaite and McDonald, 1995). By implementing these changes, however, branchial ion loss from the hyper-osmotic fish to the hypo-osmotic environment also increases – which has consequently been termed the 'osmorespiratory compromise' (Nilsson, 1986; Postlethwaite and McDonald, 1995). Two conditions where this can most clearly be seen are with extended exercise and during hypoxia.

The nature of this project in terms of its species comparison during the osmorespiratory compromise was based on previous studies by Wood et al. (2007, 2009),

Scott et al. (2008) Iftikar et al. (2010), and Matey et al. (2011) of the Amazonian oscar (*Astronotus ocellatus*) and rainbow trout (*Oncorhynchus mykiss*) during hypoxia. In that case, these two fish displayed completely opposite reactions in terms of gill ionoregulation.

In the hypoxia-tolerant oscar, a 'channel arrest' method of coping with hypoxia was observed throughout an 3-4 h acute exposure at PO_2~10-20 torr (Wood et al., 2009). Although paracellular permeability as indicated by flux of the radiolabeled paracellular marker [^{3}H]polyethylene glycol (PEG-4000) did not change, both Na influx and efflux immediately decreased (resulting in no net Na flux change). Similarly, the net fluxes of K, ammonia (note: *not* ammonia production, as plasma concentrations increased), urea, and tritiated water also decreased, and later branchial Na/K-ATPase activity also fell (Wood et al., 2007, 2009). This observed reduction in ion loss is thought to be effected via transcellular channel closure in mitochondrial rich cells (MRCs), which is achieved by pavement cell (PVC) coverage (Wood et al., 2009; Matey et al., 2011). In fresh water, MRCs comprise less than 15% of the branchial epithelium and play a central role in gill ionoregulation, whereas PVCs comprise more than 85% and contribute to gas exchange (Matey et al., 2011). Thus, during hypoxia MRCs were reduced, recessed, and covered by PVCs – an adaptive mechanism which prevents ion loss without restricting gas exchange (Matey et al., 2011). On the whole, the oscar is accustomed to frequent bouts of severe hypoxia in its natural environment and thus relies on its ability to ensure ionoregulation by these adaptive gill morphological changes in the face of unavoidable oxygen shortage. It copes with the latter via metabolic depression and downregulation of aerobic

metabolism – accompanied by upregulated glycolysis (Almeida-Val et al., 2000; Sloman et al., 2006; Richards et al., 2007; Scott et al., 2008; Wood et al., 2007; 2009).

In the hypoxia-intolerant trout, a comparable level of hypoxia (PO_2 ~10 – 20 torr) is rapidly fatal. However, throughout extended and less acute (4+h, PO_2~80- 110 torr) hypoxia challenges, no reductions in branchial ion loss, ammonia flux, or Na/K-ATPase activity were observed (Iftikar et al., 2010). Specifically, a net Na loss was observed as unidirectional Na influx and efflux rates both increased, with efflux remaining consistently greater than influx (Iftikar et al., 2010). In contrast to the oscar, MRC number and surface area actually increased during hypoxia, exacerbating branchial ion leakage (Iftikar et al., 2010). Unlike the oscar, trout generally do not inhabit hypoxic environments and therefore do not *tolerate* hypoxia but rather attempt to maintain oxygen uptake by increasing effective gill permeability and surface area, thereby suffering maladaptive increases in ion leakage, and loss of ionoregulatory homeostasis.

Based on these two very different species, there appear to be two approaches to the osmorespiratory compromise during acute hypoxia. The oscar controls ionoregulation and reduces gill ion fluxes, while the trout sacrifices ionoregulation, allowing gill ion fluxes to increase. This raises the question as to whether these two strategies are widespread in different species of fish, and whether alternate or intermediate patterns also occur. Furthermore, does the pattern of ionoregulatory responses during hypoxia correlate with phylogeny or environment?

With this background in mind, a rapid normoxia-hypoxia-normoxia recovery test protocol was devised to help identify the two strategies, and was then applied to a range of other Brazilian species which were available opportunistically on two research trips to the Rio Negro region of the Amazon basin – *Paracheirodon axelrodi* (cardinal), *Hemigrammus rhodostomus* (hemigrammus), *Moenkausia diktyota* (moenkausia), *Hyphessobrycon bentosi rosaceus* (rosaceus) and *Colossoma macropomum* (tambaqui) - as well as two local centrarchid species endemic to Ontario: *Lepomis gibbosus* (pumpkinseed sunfish) and *Lepomis macrochirus* (bluegill sunfish). Of the aforementioned teleosts, *P. axelrodi, H. rhodostomus, H. rosaceus, M. diktyota,* and *C. macropomum* are all members of the characidae family; whereas *A. ocellatus* and the centrarchidae are perciformes (Branson and Moore, 1962; Trapani, 2001). In terms of environmental similarities among these species, the oscar and tambaqui are frequently exposed to severe hypoxia upon entering seasonally flooded South American jungles for feeding and reproduction, whereas the tetras (cardinal, hemigrammus, moenkausia, and rosaceus) tend to be found in small rivers and streams (igarapés) and thereby experience less severe hypoxia (Muusze et al., 1998; Almeida-Val et al., 2000; Crampton et al., 2008). Of course, sunfish (the two centrarchids studied) are native to North American bodies of water – which can still experience seasonal hypoxia but with much less frequency and variable severity (Farwell et al., 2007). Observing the hypoxic responses of several North and South American teleosts allows for comparison and classification of broader aspects of the osmorespiratory compromise across a range of fish lineages and environments.

In order to study this, ^{22}Na was used to monitor changes in unidirectional Na fluxes (active Na influx, passive Na efflux) as well as net Na fluxes at the gills, together with measurements of the net fluxes of K , ammonia, and urea-N, as these are indicators of transcellular permeability (Lauren and McDonald, 1985; Wood et al., 2009; Wright and Wood, 2009; McDonald et al., 2012). Being nitrogenous waste products, ammonia and urea also serve as indicators of metabolic rate and protein turnover. These parameters were all measured during a 2-h control period, the subsequent 2-h hypoxic period, and the 2-h normoxic recovery period.

Another aspect of this project was an inter-specific assessment of Pcrit, or critical oxygen tension. Pcrit represents the partial pressure of oxygen at which the rate of oxygen consumption starts to decrease, indicating that an organism which was previously an oxyregulator is now becoming an oxyconformer (Yeager and Ultsch, 1989). Pcrit provides an assessment of an organism's capacity (independent of need, as this can be adaptive or maladaptive) for oxygen uptake at varying levels of oxygen saturation in the water. This study overlays species' hypoxic ionoregulatory responses with Pcrit, obtained experimentally or from the literature, in order to gain further insight into the adaptive mechanisms employed.

Finally, as previous studies have compared the Amazonian oscar with rainbow trout under hypoxia (for which the oscar is better-adapted), the next step was to compare these two species during *another* facet of the osmorespiratory compromise: exercise, where the trout is potentially better-adapted. Thus, this portion of the study involved measuring the aforementioned flux parameters during extended swimming, using

respirometry procedures similar to those developed in Chapter 2 to allow for comparison with previous trout exercise data.

During this project, the following specific hypotheses were tested: i) Amazonian oscar and rainbow trout will display distinctive and different ionoregulatory responses to hypoxia under this rapid screening protocol similar to those previously recorded for each species under more prolonged challenge and recovery scenarios (e.g. Wood et al.2007, 2009; Iftikar et al., 2010) ; ii) a particular species' adaptive response to hypoxia will correlate with its environment and the regularity with which it faces this challenge, as opposed to phylogeny; iii) while Pcrit is a measure of an organism's capacity for oxygen uptake at varying saturations, it will not necessarily correlate with ionoregulatory strategies during hypoxia; and iv) while very well-adapted to hypoxia, the Amazonian oscar will be not be adapted for extended exercise and will therefore not employ its characteristic permeability reduction response during a swim challenge.

Materials and Methods

Fish Husbandry

Rainbow Trout, Pumpkinseed and Bluegill Sunfish

Juvenile rainbow trout (*Oncorhynchus mykiss*) of weight 20-30 g and length ~12 cm were obtained from Humber Springs trout farm (Orangeville, ON) and held for a minimum of one week of acclimation prior to use, at ~18˚C in 500-L flow-through tanks containing Hamilton dechlorinated tap water (moderately hard: $[Na^+]$=0.6mM, $[Cl^-]$=0.8mM, $[Ca^{2+}]$=0.9mM, $[Mg^{2+}]$=0.15mM, $[K^+]$=0.05mM; pH ~8.0). Fish were fed every other day with a 10% body mass ration of 5-point Martin's dried commercial trout pellet feed (Martin Mills Inc., Elmira, ON). Wild-caught (Lake Opinicon, ON) mature pumpkinseed and bluegill sunfish (*Lepomis gibbosus* and *Lepomis macrochirus*) (70-90g;15-20cm) were also held under the same lab conditions and fed a combination of blackworms (*Lumbriculus variegatus*), beef liver, and squid daily at 2-3% body weight. All fish were subjected to a 12-h:12-h light:dark daily photoperiod and starved at least 24 h prior to experimentation. Post-experimentation, fish were allowed to recover under previous holding conditions so they could be used in other experiments. All procedures were approved by the McMaster University Animal Research Ethics board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

Amazonian oscar, Tambaqui, and Rosaceus Tetra

Juvenile Amazonian oscars (*Astronotus ocellatus*) (20-30g, ~12cm) and tambaqui (*Colossoma macropomum*) (10-25g, ~10cm) were obtained from Sítio dos Rodrigues (Km 35, Rod.AM-010, Brazil) and moved to the Ecophysiology and Molecular Evolution Laboratory of the Instituto Nacional de Pesquisas da Amazônia (INPA), in Manaus, AM.

Mature rosaceus tetra (*Hyphessobrycon bentosi rosaceus*) (0.5-0.8g) were wild-caught from the Rio Negro and maintained under lab conditions at INPA for at least one month before use. Oscar and tambaqui were held in 500-L flow-through tanks and rosaceus in 50-L non-flow-through tanks, at ~28˚C. The holding and experimental water was typical Amazonian soft water obtained from a well on INPA property ($[Na^+]$=35μM, $[Cl^-]$ =36μM, $[Ca^{2+}]$=18μM, $[Mg^{2+}]$=4μM, $[K^+]$=16μM; pH 6.5). Fish were fed daily with commercial pellets (Nutripeixe Tr 36, Purina Co, São Paulo, SP) and starved at least 24 h prior to experimentation. They were also subjected to a natural 12-h:12-h light:dark daily photoperiod. All procedures were in compliance with Brazilian and INPA animal care regulations.

Cardinal, Hemigrammus, and Moenkausia tetra

Experiments were performed on board a research vessel (the Ana Clara, from Manaus) moored in the Rio Negro approximately 50 km northeast of Barcelos, Amazonas, Brazil. Cardinal tetra (*Paracheirodon axelrodi*), hemigrammus (*Hemigrammus rhodostomus*), and moenkausia (*Moenkausia diktyota*) of approximately 0.3-0.5g were collected locally using baited minnow traps and held in large aerated tanks of Rio Negro water ($[Na^+]$, $[Cl^-]$ = 20uM, $[K^+]$, $[Ca^{2+}]$ = 10μM, pH 4.5-5, 30-35˚C), and starved for a period of 3-7 days prior to experimentation. In other experiments directed at Na and ammonia transport mechanisms (unpublished results), all parameters were stable after three days of starvation. On the research vessel fish were exposed to ambient conditions; that is, a natural photoperiod of 12-h:12-h light:dark and water temperature ~30-35˚C.

^{22}Na Details

In Brazil, ^{22}Na (NaCl) manufactured by New England Nuclear (Dupont, Boston, MA) was used throughout flux experiments. In Hamilton, ^{22}Na (NaCl) was obtained from Eckert and Ziegler Isotope Products Inc (Valencia, CA). In both locations for all experiments, 1μCi total was added during each 2-h flux period in a manner to be described.

Experimental Design

2-h Normoxia, 2-h-Hypoxia, 2-h Normoxia Recovery Protocol

All fish were transferred to aerated Tupperware containers and allowed to settle for 30 minutes prior to experimentatin. Wild-caught tetra (*Paracheirodon axelrodi, Hemigrammus rhodostomus,* and *Moenkausia diktyota*) were held at ~30-35˚C in 60-mL Rio Negro water; captive tetra (*Hyphessobrycon bentosi rosaceus*), Amazonian oscar (*Astronotus ocellatus*), and tambaqui (*Colossoma macropomum*) were held at ~28˚C in 60-mL (tetra) and 500-mL (oscar and tambaqui) INPA well-water. Rainbow trout (*Oncorhynchus mykiss*) and sunfish (*Lepomis gibbosus,* and *Lepomis macrochirus*) were held at ~18˚C in 1000-mL dechlorinated Hamilton tap water. Containers were dosed with ^{22}Na and allowed to mix for 15 minutes; at this point a 2-h normoxic flux period was started and hourly water samples (5 mL) were taken. After this, the holding water was changed with minimal disturbance to the fish, dosed with ^{22}Na again, and nitrogen was bubbled through to deoxygenate it to the required level: 30% O_2 saturation (~48 torr) in all species but the rainbow trout, which were brought to 45% O_2 saturation (~72 torr). Trout consumed oxygen so rapidly that aeration was necessary every half hour to raise

saturation back to 45%, so average saturation over the entire 2 h period was actually ~35% (~56 torr). After a 15-minute mixing period, the 2-h hypoxic flux period started, again with hourly water sampling. Finally after the hypoxic period, a 2-h recovery period was instituted – water was again changed with minimal disturbance, and dosed with ^{22}Na, then maintained at normoxic levels. After a 15-minute mixing period, the 2-h normoxic recovery flux period was begun, with hourly water sampling. Post-experiment, larger fish were anaesthetized, weighed and returned to regular holding conditions, while smaller fish were sacrificed by MS-222 overdose and weighed.

Water PO_2 was measured using a Clarke-type oxygen electrode (Cameron Instruments, Port Aransas Texas) connected to a 1900 A-M Systems Polographic Amplifier, manufactured by A-M Systems (Carlsborg, WA 98324, USA) in Canada, and a WTW Oxi325 Oximeter (Weilheim, Germany) in Brazil.

Pcrit Trials

Pcrit is defined as the partial pressure of oxygen at which oxygen consumption begins to decline, and oxygen regulators become oxygen conformers – it is frequently used as an indicator of an organism's capacity for hypoxic tolerance (Yeager and Ultsch, 1989). Pcrit trials were conducted in the tambaqui, pumpkinseed, and bluegill sunfish. Tambaqui were allowed to settle for 30 min in glass jars with 850mL aerated INPA well-water; sunfish were held in Tupperware containers with 2L aerated dechlorinated Hamilton tap water for this settling period. Air bubbles were removed and closures sealed with Parafilm; O_2 electrode holes were sealed when readings were not being taken. Measurements were taken every 5 minutes while the fish consumed the O_2 in the

respirometer until approximately 20 torr (~12% O_2 sat) in sunfish and 10 torr (~6%) in tambaqui, after which time experiments were terminated and aeration begun again. Total time for a typical trial was between 1.5-2.5h. Pcrit itself was calculated with the program designed by Yeager and Ultsch (1989).

Swimming Trials

Amazonian oscars were transferred from holding conditions to 3.2-L (32 x 6cm) Blazka swimming respirometers identical to those used in Chapter 2, and allowed to settle with aeration for 30 minutes, after which time ^{22}Na (1μCi) was added to the water. After a 15-min mixing period an initial water sample (5mL) was taken, and subsequent samples were taken every 2 h afterward until experiment termination at 6 h. In these experiments, 0-2 h was considered an extended acclimation period – from 2-6 h fish either remained stationary or were swum at 1.2 body lengths per second (BL/s); that is, ~15cm/s. This value was previously observed to be effective in raising oscar metabolic rate by 90% over a 4-h swim period (Chapter 2). After completion of the experiment, fish were allowed to recover in normal holding conditions.

Water Analyses

Water samples taken in every trial were measured for ^{22}Na. In Brazil, this was done by scintillation counting of subsamples using Ultima Gold scintillation fluid (Perkin-Elmer, Waltham, MA, USA; 5-mL fluor: 5-mL water) with analysis on either a Triathler portable counter (Hidex, Helsinki, Finland) for the experiments on the research vessel Ana Clara, or a Beckman LS6500 counter (Beckman Coulter, Fullerton, CA, USA) at the INPA laboratory. Tests showed that quenching was constant so correction was

unnecessary. In Canada this was done by gamma counting using a Perkin-Elmer Wizard 1480 3" Auto Gamma Counter (Shelton, CT, USA). Separate subsamples were also measured for cold ion concentrations (Na, K), as well as ammonia, and urea-N as detailed below.

Na and K concentrations were measured via atomic absorption spectrophotometry in Canada using a SpectrAA 220FS Atomic Absorption Spectrometer (Varian Canada Inc, Missisauga, ON) and for the experiments in Brazil at the INPA laboratory using a AAS Avanta 932 Plus, GBC (Hampshire, IL, USA), whereas on the research vessel Ana Clara , Na and K concentrations were measured by flame photometry, using the Analyser Instrumentação Analítica 910 Digital Flame Photometer (São Paulo, BR). Water total ammonia and urea concentrations were measured colourimetrically by the salicylate hypochlorite assay (Verdouw et al., 1978) and the diacetyl monoxime assay (Rahmatullah and Boyde, 1980), respectively.

Calculations

Oxygen Consumption

Oxygen consumption (MO_2) values in the Pcrit experiments, measured in μmol/g/h, were calculated using a using the following equation (Brauner et al., 1992):

$$MO_2 = \frac{P_{O2\,i} - P_{O2\,f}}{\Delta t} \times V \times S_C \times S_{Cc} \qquad (1)$$

In the above equation, P_{O2i} and P_{O2f} are the initial and final partial pressures of oxygen (mmHg). Δt represents the total time of the experiment (hours), and V is the volume of the respirometer (L) minus the weight of the fish (kg). The solubility coefficient, S_C, in fresh water is 1.8925μmol/L/mmHg for 18˚C and

1.5906μmol/L/mmHg for 28˚C (Boutilier et al., 1984), and S_{Cc} represents the mass scaling coefficient (Clarke and Johnson, 1999) used to normalize the metabolic rate per g fish, calculated as:

$$S_{Cc} = 10^{0.79 \log (1/\text{weight(g)})} \quad (2)$$

This normalization to one gram removes the effect of body mass on metabolic rate and allows for comparison between fish of different sizes with the Yeager and Ultsch (1989) Pcrit determination software.

Flux Calculations

As hourly water samples were taken throughout the three experimental periods (control, hypoxia, and recovery), flux rates were available for the first hour as well as the second. There were no systematic differences, therefore values displayed in the Results are the average of these two one-hour flux rates, for each period.

Na fluxes were measured using ^{22}Na disappearance from water. Na influx was calculated as:

$$JNa_{in} = \frac{\Delta cpm \times V}{\text{Average SA} \times W \times t} \quad (3)$$

where V is the effective volume of the container (mL), W is the weight of the fish (g), and Δt is the duration of the flux period (h). Also, average specific activity (SA) was determined by averaging initial and final specific activities, which compare ^{22}Na cpm and total water Na over that time period:

$$\text{Initial (or Final) SA} = \frac{\text{Initial (or final) cpm}}{\text{Initial (or final) Na}} \quad (4)$$

Net Na flux was calculated from the change in the total concentration of Na in the water as measured by atomic absorption or flame photometry:

$$JNa\ net = \frac{\Delta Na \times V}{W \times \Delta t} \quad (5)$$

and Na efflux was calculated by difference as:

$$JNa\ out = JNa\ net - JNa\ in \quad (6)$$

Only the net flux of K could be measured by using atomic absorption or flame photometry since radioactive K was not used. Thus net fluxes for K, ammonia, and urea were all calculated according to formula 5.

Statistics

Data have been expressed as the means $\pm$1 SEM, where N = sample size. All statistical testing was carried out using the program Sigma Plot 10.1 with Sigma Stat Integration 3.0. Figure legends denote the specific statistical test performed in each case, but in general, comparisons between experimental data were evaluated using a paired t-test (comparing between two sets), Repeated Measures Analysis of Variance (ANOVA), or One-Way ANOVA – followed by a Holm-Sidak *post hoc* test (for multiple sets). If the normality test was failed, a Kruskal-Wallis ANOVA on ranks analysis was executed prior to a Tukey *post hoc* test. The t-test was used during exercise trials to determine the significance of changes between control oscar and swimming oscar, whereas Repeated Measures ANOVA tests identified changes between the control, hypoxic, and recovery periods for species under the rapid screening protocol. Repeated Measures ANOVA's could be used during these experiments to observe changes over time since the same fish

were monitored continuously throughout all three periods. A One-Way ANOVA (followed by the Tukey *post hoc* test) allowed for comparison between measured Pcrit values for tambaqui, pumpkinseed, and bluegill sunfish. In all cases, differences were considered statistically significant for $P \leq 0.05$, and were represented in bar graphs by different letters above control/hypoxic/recovery bars (Figures 3.1-3.8), or by asterisks above control/swimming bars (Figure 3.11).

Results

The following experiments were conducted both at McMaster University on native fish and during two field trips to the state of Amazonas, Brazil on species obtained from the Rio Negro. All fish were subjected to 2-h control (normoxic) fluxes with ^{22}Na followed by 2-h of 30% O_2 saturation (unless stated otherwise), and then a 2-h normoxic recovery period. As the characteristically different response patterns of oscar *versus* trout to acute hypoxia were the basis for this study, it was first necessary to determine whether they could be detected under the 2-h-2-h-2-h protocol that was developed for the purpose of rapid screening.

Amazonian oscar

Individual oscars (~12cm, 20g) were subjected to the 30% O_2 saturation test, as well as an additionally more severe 10% O_2 saturation test as pictured in Figure 3.1, panels A and B.

At both severities, the same trends are seen in Figures 3.1A and B – decreased Na bi-directional flux, ammonia flux, K net flux, and even (already-low) urea-N flux during hypoxia, all of which are corrected during recovery.

Rainbow trout

Preliminary trials showed that trout responded poorly when exposed to 30% O_2 saturation for the full 2-h hypoxic period (individuals showed loss of equilibrium and in some cases subsequent death), so this species was instead tested at a nominal 45% O_2 saturation (72 torr). As explained in Methods, the mean exposure was to an O_2 saturation of ~35% (~56 torr), Trout results are seen in Figure 3.2.

In contrast to the oscar, the rainbow trout elevated its Na flux rates during hypoxia – influx, efflux, and net Na flux rates all increased during the 2-h hypoxic period and were later corrected during the recovery phase. It is important to note that also unlike the oscar, ammonia flux rates did *not* change throughout the experiment, and K flux rates tended to become more negative.

Now that the (predicted) responses of these two key species had been observed with the 2-h:2-h:2-h normoxia:hypoxia:recovery protocol, the ionoregulatory responses of the other North and South American species listed in the Introduction were recorded. It should be noted that these tests were performed at both 30% and 10% O_2 saturation for some of the species tested (the oscar and both types of sunfish); however, only the 30% results are being displayed to facilitate comparison across all species tested. 10% saturation data showed similar but more pronounced trends except in the bluegill sunfish, where mortality occurred in 4/5 fish subjected to it. In the following figures, the responses for each flux parameter are shown separately for all species, with the oscar responses on the left and the trout responses on the right as points of reference.

Na Influx

Figure 3.3 shows that the tambaqui response to hypoxia is in fact very similar to that documented in the oscar, in terms of Na influx rates. The hemigrammus, moenkausia, and pumpkinseed sunfish show no significant changes in Na influx over the hypoxic and recovery periods, whereas the cardinal tetra, rosaceus, and bluegill sunfish appear to more closely resemble the trout to varying degrees in their substantially elevated Na influx during hypoxia; this is subsequently corrected during recovery. It should be noted that for

Na influx (and other flux comparisons), North American species (pumpkinseed, bluegill, and trout) were seen to have generally lower flux rates than South American species (oscar, tambaqui, and tetra).

Na Efflux

Figure 3.4 displays Na flux from another perspective – that is, in terms of passive Na loss under this protocol.

The tambaqui is seen to significantly reduce Na efflux, which in this case does not recover after the hypoxic challenge is ended. However the rest of the species tested under this protocol did not appear to show the Na efflux depression that is characteristic of the oscar and tambaqui. Indeed several species (rosaceus, moenkausia, bluegill) instead reacted in a similar way to the rainbow trout, exhibiting an elevation of Na efflux rate.

Na Net Flux

The Brazilian species of rosaceus and moenkausia tetra behaved in terms of net Na flux more like the trout than other Brazilian species from the Rio Negro (Fig. 3.5) They show a significant increase to negative Na flux rates during hypoxia which is subsequently lessened during the recovery period. Na net flux changes in the other species were generally not significant.

Ammonia Efflux

Though not significant, the tambaqui shows a trend towards a decreased ammonia net flux with hypoxia as in oscar, a response which is also displayed by the rosaceus and moenkausia, as well as by both the pumpkinseed and the bluegill (Fig. 3.6). Among the other species, there were no obvious changes in ammonia flux, as in trout.

K Net Flux

In the tambaqui, net rate of K loss decreased significantly during hypoxia, and even further during normoxic recovery, whereas in rosaceus, it tended to increase during hypoxia, and then decreased significantly during normoxic recovery (Fig. 3.7). There were no clear trends in K net flux responses in other species.

Urea-N Efflux

Urea-N flux rates (these are urea flux rates multiplied by two; that is, flux rates per N molecule rather than per molecule of urea) varied greatly amongst species, with very high rates in cardinals and moenkausia (Fig. 3.8). There were few responses to acute hypoxia, namely that a significant decrease in urea-N excretion occurred in the bluegill sunfish between hypoxia and recovery.

One way to display changes in nitrogenous waste production taking into account both urea and ammonia during hypoxia, is to show changes in the ratio of urea-N:ammonia-N excretion.

As seen in Figure 3.9, the ratio of nitrogenous waste production in terms of urea:ammonia excretion was seen to significantly change in the tambaqui, rosaceus, and bluegill. In the tambaqui and rosaceus, this ratio increased from control to hypoxia whereas in the bluegill it remained constant throughout these periods but decreased during recovery.

Pcrit

Pcrit was measured for several of the aforementioned species using a program developed by Yeager and Ultsch (1989). Note that the 30% oxygen saturation level at which the rapid screening experiments were performed is 48 torr.

As can be seen in Figure 3.10, oxygen consumption rates are fairly constant at higher oxygen saturations (above the determined Pcrit value). However, below the Pcrit oxygen consumption decreases in a linear fashion. To provide a complete perspective, experimentally-determined Pcrits from this study (for the tambaqui, pumpkinseed, and bluegill) are compared with values from existing literature (for oscar and trout) in Table 3.1.

Seen in Table 3.1, most of the species tested appeared to have Pcrits within a fairly well-defined range; nevertheless, the three experimentally-determined values (tambaqui and both sunfish species) were significantly different from one another according to a One-Way ANOVA followed by a Tukey *post hoc* test. When including the literature values for the trout and oscar (Table), rainbow trout and bluegill sunfish appear to have Pcrits slightly higher than the other species. It should be noted that in the table, bluegill Pcrit is seen to be 101.7±8.3 torr; however, the variability of initial oxygen consumption measurements (made at PO_2>120 torr) indicate that fish may have been stressed at this time, artificially elevating Pcrit – if measurements above 120 torr are excluded, bluegill values become 80.8±7.8 torr. As seen in Table 3.1, several values are available for the oscar, ranging from 16 torr to 70 torr.

Applications to Exercise – Amazonian oscar

This experiment examined the question of how the oscar behaves in an ionoregulatory capacity during extended exercise, to evaluate whether or not it exhibited the same branchial permeability reduction as during acute hypoxia. After a 2-h acclimation period, fish were either allowed to remain at rest (control) or forced to swim at 1.2 BL/s for 4 h in Blazka respirometers.

Relative to simultaneous resting controls, extended exercise resulted in significant increases in both Na efflux and K net flux rates in oscars (Fig. 3.11) in contrast to responses during acute hypoxia (cf. Fig. 3.1A,B). Na influx rate tended to increase and net Na flux rate became more negative during exercise (Fig. 3.11), although these changes were not significant. Ammonia efflux rate did not change, again in contrast to the reduction seen during acute hypoxia (cf. Fig. 3.1A,B). Urea-N fluxes could not be measured accurately due to the high volume of the swimming respirometers.

Discussion

Flux Parameters

As previously outlined, the characteristically different ionoregulatory responses to hypoxia of the Amazonian oscar (*Astronotus ocellatus*) and the rainbow trout (*Oncorhynchus mykiss*) were the foundation for the present survey of the responses of other species. The goal was to identify trends as a function of phylogeny and/or environment. By focusing on the following flux parameters (Na influx, efflux, and net flux; ammonia, urea-N, and K net flux) it was possible to compare these findings with previous literature and also gain insight into ion movement and gill permeability changes. The unidirectional and net fluxes of Na in particular were measured because of its importance as the major cation in the extracellular fluid. Regulation of Na therefore provides a general indicator of ionoregulation during the osmorespiratory compromise. Ammonia flux is also a valuable indicator, as it serves not only as a transcellular permeability marker due to its transport through Rh proteins on the branchial cell membranes (Wright and Wood, 2009), but it also provides information regarding N-metabolism and protein turnover (Alsop and Wood, 1997). K is another indicator of transcellular permeability; while some paracellular flux of K cannot be ruled out, it is likely negligible as intracellular K concentrations are approximately 100-fold higher than in plasma – therefore, most of the loss at the gills is likely transcellular (Lauren and McDonald, 1985; Iftikar et al., 2010). Lastly, urea-N was also measured during this study. Although fish tested here are not ureotelic, urea may still serve as an indicator of both transcellular permeability (because of its transport through UT proteins in the gill

membranes (McDonald et al., 2012), as well as N-metabolism and protein turnover (Wood, 1993; Alsop and Wood, 1997).

In earlier studies, the Amazonian oscar's response to hypoxia has been documented as a marked decrease in gill permeability, apparently of transcellular origin, via MRC reduction, recession, and coverage by PVCs (Wood et al., 2007; 2009; Matey et al., 2011), resulting in decreased net K flux, net urea-N flux, net ammonia flux, and decreased Na influx and efflux rates, with no change in net Na balance. Rainbow trout are known to display the opposite response: a marked increase in gill permeability (again possibly of transcellular origin because MRCs expand with PVC contraction), increases in both unidirectional Na fluxes, increases in net Na loss, increases in net K loss, and no reduction in ammonia excretion rate (Iftikar et al., 2010; Matey et al., 2011). This being the case, the first goal was to determine if the 2-h normoxia: 2-h hypoxia (30% O_2 sat): 2-h recovery protocol used in this study could produce the same effects. In general, the results from this rapid screening protocol supported the two categories of response, with unidirectional and net Na fluxes and ammonia fluxes, proving to be the more useful indicators. Net K and urea-N fluxes were not diagnostic as few species showed significant changes under this protocol.

Amazonian oscar

Figures 3.1A and B display the findings using this 2-h;2-h-2-h hypoxic challenge protocol in the oscar at both 30% and 10% oxygen saturation, respectively, and agree with previous flux studies (Wood et al., 2007, 2009). In accord with these studies, these effects are more pronounced during the more severe challenge. The current results

therefore confirm the reduction in gill permeability during hypoxia which was interpreted as a the result of a "channel arrest" strategy (i.e. PVCs paving over receding MRCs) from the morphological studies of Wood et al. (2009) and Matey et al. (2011) .This adaptive strategy allows for restriction of ion loss from these 'ionoregulatory' cells without apparent inhibition of oxygen uptake (Scott et al., 2008).

Rainbow Trout

When rainbow trout were tested at an (average) O_2 saturation of 35%, they also showed the predicted outcome: a significant increase in both Na influx and efflux. Ammonia flux did not change significantly, in accord with previous observations by Iftikar et al., 2010. While the decreased ammonia excretion rate seen in oscar has been attributed to a decreased transcellular permeability (as opposed to decreased production), the typical trout response is to instead increase ammonia production rates under conditions of stress or exercise (Wood, 1988, 2001; Randall and Tsui, 2002).

Species Survey

Na Fluxes

Based on Figs. 3.3-3.5, it appears that tambaqui are the only other species of those tested that show a permeability reduction (i.e. a similar response to oscar), at least in terms of Na. During hypoxia, both influx and efflux of Na are significantly lowered and stay that way during recovery as well. Although tambaqui are of a different order than the oscar, they live in the same environment and face hypoxic challenges of similar severity and frequency; thus, it is logical that this adaptation could have arisen twice. Tambaqui are also uniquely adapted to hypoxia as they employ 'surface respiration,' and even

rapidly expand their lower lip morphology to facilitate this (Saint-Paul, 1984; Florindo et al., 2006). On the other hand, species such as the cardinal, rosaceus, moenkausia, and the centrarchids appear to vary in their responses but tend to more closely resemble the trout with generally elevated Na flux rates during hypoxia. Notably, the Na flux rates for fish tested in Ontario were lower than those of Amazonian species; this is likely due in part to physiological differences, but may also be owing to the fact that the sunfish and trout were tested in much 'harder' (Hamilton, ON) water than what was available in Manaus, AM for the remaining species. While this would not have a drastic impact on the overall concentration gradient for ionic interchange with the environment, the difference in calcium concentrations may have impacted gill permeability. Calcium (Ca) is known to play a role in tight junction integrity, and therefore higher external concentrations may have reduced branchial permeability (Spry and Wood, 1989) in the North American fish while lower external Ca may have allowed for greater leakage in the South American species. Consequently if ion efflux rates are being elevated for the Amazonian fish, it seems logical that influx rates are likely also increased to compensate for and maintain ionic homeostasis. The higher temperature (by 10˚C) in Brazil *versus* Ontario may also have been a factor, tending to elevate both passive leakage and active uptake rates.

Ammonia and K Fluxes

Significant or non-significant reductions in ammonia excretion were observed during hypoxia in most of the species in this survey (oscar, tambaqui, rosaceus, moenkausia, pumpkinseed, and bluegill), whereas the remainder (cardinal, hemigrammus, and trout) showed no obvious changes. Without measuring plasma ammonia

concentrations, it is not possible to conclusively state whether this decrease is due to lowered transcellular permeability or an inhibition of protein turnover (i.e. lowered metabolism) – however, concomitant changes in ion flux rates provide some indications.

For the tambaqui, a simultaneous decrease in both ammonia and K flux, as well as Na efflux during hypoxia was observed, indicating that the channel arrest strategy first seen in the perciform oscar likely occurs in a characid of the same environment. Thus, a transcellular inhibition response to hypoxia does not appear to be phylogenetically confined, but rather spans across lineages whose members face similar challenges. However, this phenomenon was not identified in any of the other species so this adaptation does not appear among other characiformes under this protocol. While the other tetra showed varying results in terms of ammonia excretion, a significant reduction during hypoxia occurred only in the rosaceus with a similar pattern seen in moenkausia. Therefore, in these two species this was likely a metabolic effect (Boutilier, 2001) rather than a result of gill morphology/permeability changes. This conclusion is reinforced by the increases in Na efflux and net flux in these two species during hypoxia. The concomitant increase in Na influx rate seen in rosaceus at the time of this ammonia net flux inhibition provides evidence against Na/ammonia coupling previously observed in other Rio Negro species – this includes the oscar, although said coupling was seen to disappear during hypoxia (Wood et al., 2009). Thus, while these two tetra species are still capable of survival in hypoxic areas, the fact that their response more closely resembles that of the trout than of the oscar, tambaqui, or even cardinal and hemigrammus indicates that the rosaceus and moenkausia are perhaps less tolerant than other Amazonian species.

Thus the characid hypoxia response appears to range from the tambaqui (which encounters frequent and extreme hypoxia in the Amazonian floodplains) to the smaller tetra (which have variable responses and reside in less severe rivers and streams).

Finally, looking at the two sunfish species, both species display decreased ammonia excretion rates during hypoxia which quickly recover afterwards. In the bluegill, there is a corresponding trend toward concomitant increased K loss during hypoxia and recovery, whereas this is less defined in the pumpkinseed. Similarly, significant Na flux rates also significantly increased during hypoxia in the bluegill but not the pumpkinseed. In fact, pumpkinseed sunfish were seen to be the more hypoxia-tolerant species, showing less marked changes provoked by hypoxia as well as a lower Pcrit, findings supported by previous research on gene expression of glycolytic and metabolic enzymes compared among sunfish species (Farwell et al., 2007; Davies et al., 2011), as well as greater survival capacity at lower oxygen saturation levels. When both species of sunfish were subjected to a 10% O_2 saturation challenge as opposed to 30%, no mortality was observed in the pumpkinseed; rather, this more severe hypoxia simply enhanced effects seen during the 30% O_2 sat challenge (as it did in the oscar, which of course showed its own adaptation), although trends remained unchanged. However, 4/5 bluegill sunfish subjected to this challenge died, again bearing witness to the degree of metabolic and ionic distress caused by this level of hypoxia. Based on differing tolerance levels and species-specific ionoregulatory responses to hypoxia, this raises the question of whether there may exist species-specific strategies within centrarchids for coping with low environmental oxygen.

Urea-N Efflux

In most of the species subjected to this hypoxia challenge, urea-N excretion remained relatively constant (Figure 3.8). Being ammoniotelic, these fish rely primarily on ammonia diffusion into the environment to eliminate metabolic nitrogenous waste, lacking ornithine urea cycle (OUC) activity as adults and excreting only minimal, baseline amounts of urea by uricolysis or arginolysis (that is, breaking down of uric acid and arginine, respectively) (Gregory, 1977; Wood, 1993; Wright et al., 1995; Randall and Tsui, 2002). Until fairly recently, the OUC was not even thought to exist within teleost fish; nevertheless, although urea comprises less than 20% of all nitrogenous waste excretion, OUC enzyme activity *has* been detected in teleosts to varying degrees in early life stages (Wright et al., 1995). In this study, significant changes in urea flux (measured as urea-N; that is, per N atom within each urea molecule rather than per molecule of urea) are observed only in bluegill sunfish. Here, urea is significantly decreased from hypoxia to recovery (other species display similar trends) – again likely a reflection of energy conservation coincident with decreased metabolic rate rather than transcellular permeability changes.

Figure 3.9 succinctly consolidates these changes in nitrogen metabolism by showing the ratio of urea-N to ammonia-N excretion rates. Species displaying significant changes during the hypoxia and nomoxic recovery regime were the tambaqui, rosaceus, and bluegill sunfish – but for different reasons. For the tambaqui, the urea:ammonia nitrogen excretion rate increased with hypoxia – likely due to the large but non-significant suppression in ammonia excretion occurring during hypoxia and a very slight

attendant increase in urea. The ratio was also elevated in the rosaceus during hypoxia, this time due to significantly decreased ammonia excretion during this period without any substantial change in urea. Lastly, the bluegill displayed a different trend by leaving this ratio constant during hypoxia but decreasing it during the recovery phase. In this case, ammonia excretion increased significantly during the recovery period but was also overshadowed by a significant *decrease* in urea production during this phase. It should be noted that the species-specific ratios displayed in Figure 3.9 are generally consistent with previous literature recording that teleosts excrete 80-90% of their nitrogenous waste as ammonia and only 10-20% as urea (Wood, 1993; Wright, 1995).

Summary of Significant Changes

The purpose of this work was to expand this species survey 'opportunistically' and identify any possible phylogenetic and/or environmental trends. Phylogenetic relationships are illustrated in Figure 3.12, overlain by the type of ionoregulatory response to hypoxia which was exhibited.

For hypoxia-tolerant species such as the oscar (perciform) and tambaqui (characid) which commonly experience severe hypoxia in their native Amazonian floodplains, the capacity for channel arrest is clearly seen. In the relatively hypoxia-tolerant tetra (characids) of the Rio Negro, ionoregulatory responses to hypoxia range from variable responses to increased permeability. The rainbow trout is of the salmoniform order and therefore unrelated to any of the perciformes or characiformes; it too displays an increased permeability with hypoxia, a challenge which it probably rarely experiences, and therefore is not well adapted for. The two species of sunfish studied

(perciformes) – although more closely related to the oscar than any of the other species used in this project – do not typically encounter severe hypoxia in their native North American waters. While the pumpkinseed sunfish is more hypoxia-tolerant and displays a 'variable' ionoregulatory response to this challenge, the bluegill resembles the trout in its capacity for maladaptive ion leakage. Thus, it was observed that hypoxic response strategies do *not* appear to be phylogenetically related at all but may well be environmentally linked, according to a species' frequency and severity of exposure to this challenge in its natural habitat.

Pcrit

An organism's 'Pcrit' value (or critical oxygen tension) can be a valuable measure of its hypoxia tolerance, or at least an indicator of its reliance upon oxygen at varying levels of saturation (Yeager and Ultsch, 1989). More specifically, it is defined as the partial pressure of oxygen at which oxygen consumption begins to decline, and oxygen regulators become oxygen conformers (Ultsch, 1974; Beckenbach, 1975; Yeager and Ultsch, 1989). As outlined in the Results, experimentally determined Pcrits for tambaqui and both sunfish, augmented by existing data for the oscar and trout (Table 3.1), could be divided into two groups. Species with low Pcrits were the tambaqui and pumpkinseed sunfish (~30-40torr), which is reasonable since both of these species are known to be hypoxia tolerant. Higher Pcrits were seen in the rainbow trout and bluegill sunfish (~60-100torr, or ~60-80 torr if initial 'stressed' oxygen consumption measurements are excluded for the bluegill). Indeed, *these* two species were the only ones to also display mortality at lower oxygen saturations. A high Pcrit for these species is logical, as they do

not appear to employ adaptive mechanisms for dealing with hypoxia and are clearly not well-equipped to handle it.

Although one would intuitively assume that the oscar should have a low Pcrit, literature values for the oscar range from as low as 16 torr (De Boeck et al., in prep) to as high as 70 torr (Sloman et al., 2006). However, the Sloman et al. (2006) study and the study of Wood et al. (2007), comparing 15g to 200g fish, agreed that larger oscars tended to be more hypoxia-tolerant and have lower Pcrits than smaller ones, which may be a contributing factor to this broad range of available Pcrit data. As fish used in *this* study were on the smaller side, it is reasonable to assume that their Pcrits would be towards the upper side of this range. Nevertheless it *is*, in fact, quite possible that oscars of any size could have a high Pcrit and yet be well-adapted to hypoxia; this is supported by Scott et al. (2008), who observed that the oscar shows a somewhat higher Pcrit than other similarly hypoxia-tolerant Amazonian species. Recall, Pcrit is simply the oxygen pressure at which oxygen consumption begins to decline – and part of the oscar's response is to shut down oxygen consumption in favour of anaerobic glycolysis (Almeida-Val et al., 2000; Richards et al., 2007). Thus a correlation need not necessarily exist between Pcrit and hypoxia tolerance, particularly if a species' hypoxic *strategy* involves depressing aerobic metabolism (i.e. oxygen consumption). If Pcrit only serves as a measure of oxygen uptake and transport capacity and is influenced by such factors as adaptive mechanisms and even feeding (Wang et al., 2009), perhaps a different endpoint may yield a better measure of adaptedness and ability to handle this challenge – for instance, survival time at a certain PO_2. This is again supported by the succinct conclusion of Scott

et al. (2008): "While the Pcrit of oscar is at or above that of many other fish, their hypoxia/anoxia tolerance is exceptional… which suggests that Pcrit is not a reliable sole indicator of hypoxia tolerance in fishes."

Applications to Exercise – Amazonian oscar

Having established two basic ionoregulatory response patterns to the osmorespiratory compromise induced by hypoxia – permeability reduction and increased leakage – these findings can now be tied in with another ionoregulatory challenge. Since the oscar is the consummate example of channel arrest during hypoxia, this begs the question: what happens during exercise? As was seen in Chapter 2, there are no paracellular permeability changes with extended swimming in *A. ocellatus*; but what happens to the transcellular pathway? Using radiolabeled ^{22}Na, it was observed that this species' strategy for hypoxia adaptation does *not* translate to a similar response to exercise. In Figure 3.11, the oscar is actually behaving more like the trout, in that its Na efflux and net K loss are significantly increased with swimming!

Recall that Wood and Randall (1973) observed a 70% increase in Na efflux during the first hour of swimming in trout (excellent swimmers); indeed, increased Na loss with increased oxygen consumption due to the osmorespiratory compromise has been observed in many rainbow trout studies (Gonzalez and McDonald, 1992; 1994; Postlethwaite and McDonald, 1995). However, over a 4-h swim in oscars (relatively poor swimmers) there is a 97% increase in Na efflux (atypical compared to the oscar hypoxic response) and a 200% increase in K efflux. As K is considered a marker of transcellular flux, this suggests that transcellular permeability is increasing during exercise, in contrast to decreasing

during hypoxia (Wood et al., 2009). In this case, however, oscar are not as well-adapted for extended swimming and the relatively unfamiliar challenge appears to be producing the opposite osmorespiratory compromise effect to that of hypoxia. Ammonia flux (another transcellular marker excreted primarily via Rh proteins) (Wright and Wood, 2009) also does *not* appear to be shut down in the oscar during swimming – likely a result of increased metabolism and protein turnover during swimming, and a lack of transcellular permeability inhibition.

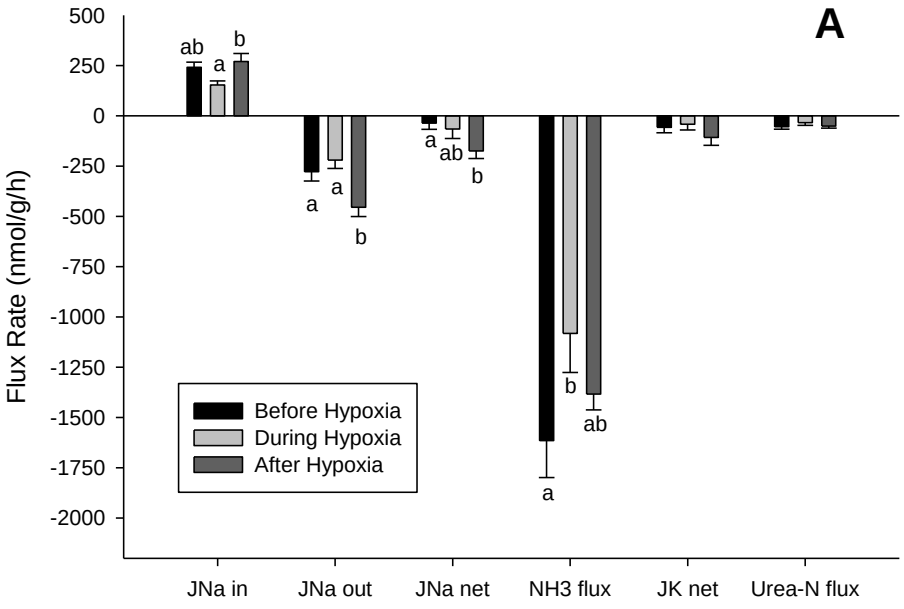
This conclusion, based on Na, K, and ammonia flux data, supports the original observation by Wood et al. (2007, 2009) that the oscar's primary means of adjusting ionic permeability during stress is via alteration of the transcellular pathway, but the alterations during exercise are opposite those during hypoxia. At the same time, there appear to be minimal concomitant changes in gill paracellular permeability in the oscar, in contrast to the increase observed in trout during exercise (Chapter 2).

Conclusion

At the beginning of this study, four specific hypotheses were raised. Firstly, using the 2-h:2-h;2-h rapid screening protocol, the hypoxic responses of the Amazonian oscar and rainbow trout would correspond to those previously observed. This was confirmed, as the oscar were seen to decrease branchial permeability when subjected to this study's rapid screening protocol whereas trout branchial permeability increased. Secondly, it was proposed that a species' environment would determine its adaptive response (as opposed to phylogeny) – this too was verified as unrelated species sharing a similar environment showed parallel ionoregulatory changes during this hypoxic treatment. More specifically, although the two species of sunfish tested were more closely related to the perciform oscar, it was the characid tambaqui (exposed to frequent and severe hypoxia) that displayed a characteristic permeability reduction, whereas the sunfish (relatively unfamiliar with hypoxia) and several other Rio Negro characids did not show this adaptation and often more closely resembled rainbow trout. Species living in 'intermediate' environments showed variable responses. The third hypothesis proposed that while Pcrit is a measure of oxygen uptake *capacity*, it does not necessarily correlate with hypoxic tolerance and may be affected by adaptive mechanisms. It was seen from both experimentally-determined data and available literature that this is the case; Pcrit does not appear to be a reliable sole indicator of hypoxia tolerance in fishes. Finally, the final hypothesis tested was that the Amazonian oscar – although well-equipped to deal with hypoxia – would not employ a permeability reduction during extended exercise, due to the fact that it does not normally perform extended exercise. This was confirmed, and

indeed transcellular permeability appeared to increase with swimming in this species, as indicated by increased Na and K efflux rates.

Figure 3.1. Flux changes during 2-h:2-h:2-h normoxia:hypoxia:recovery protocol for the Amazonian oscar at ~28˚C, with (A) 30% O_2 saturation and (B) 10% O_2 saturation. Means are ±S.E.M. (N=6). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA, with a Holm-Sidak *post hoc* test. (B) 10% O_2 sat. Means are ±S.E.M. (N=6). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA with a Holm-Sidak *post hoc* test.


A
Flux Rate (nmol/g/h)
500
250
0
-250
-500
-750
-1000
-1250
-1500
-1750
-2000
ab
a
b
a
a
b
a
ab
b
a
b
ab
Before Hypoxia
During Hypoxia
After Hypoxia
JNa in
JNa out
JNa net
NH3 flux
JK net
Urea-N flux

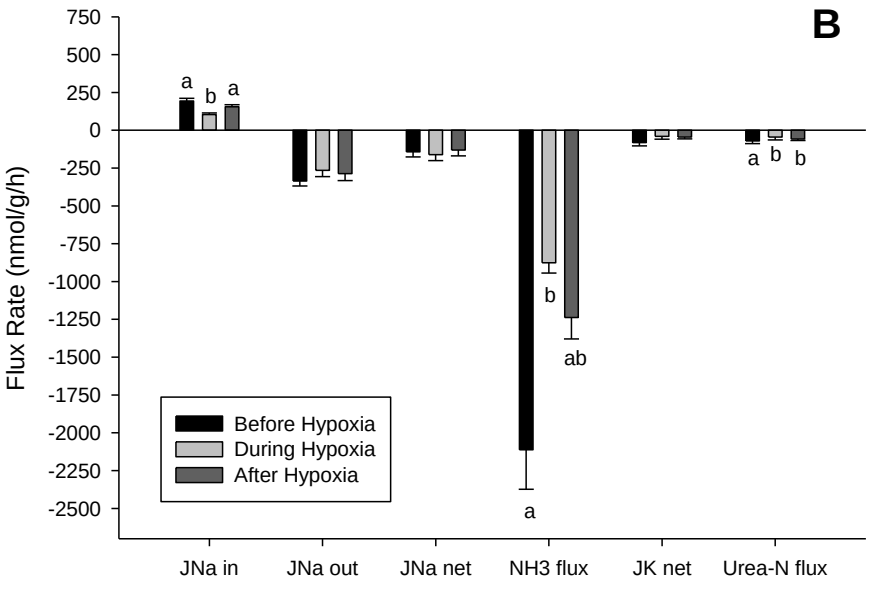

B
Flux Rate (nmol/g/h)
750
500
250
0
-250
-500
-750
-1000
-1250
-1500
-1750
-2000
-2250
-2500
a
b
a
b
ab
a
a
b
b
Before Hypoxia
During Hypoxia
After Hypoxia
JNa in
JNa out
JNa net
NH3 flux
JK net
Urea-N flux

Figure 3.2. Rainbow trout flux changes during 2-h:2-h:2-h protocol (~35% average O_2 sat throughout the 2-h hypoxic period) at 18˚C. Means are ±S.E.M. (N=5). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

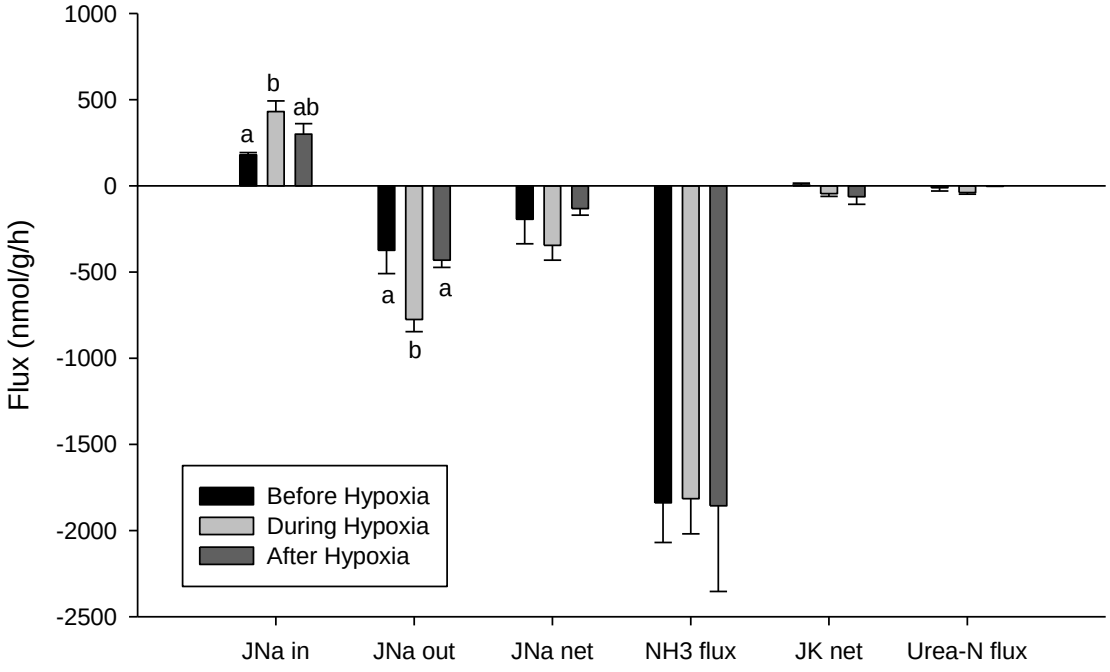

1000
500
0
-500
-1000
-1500
-2000
-2500
Flux (nmol/g/h)
a
b
ab
a
b
a
Before Hypoxia
During Hypoxia
After Hypoxia
JNa in
JNa out
JNa net
NH3 flux
JK net
Urea-N flux

Figure 3.3. Na influx changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak post hoc test.

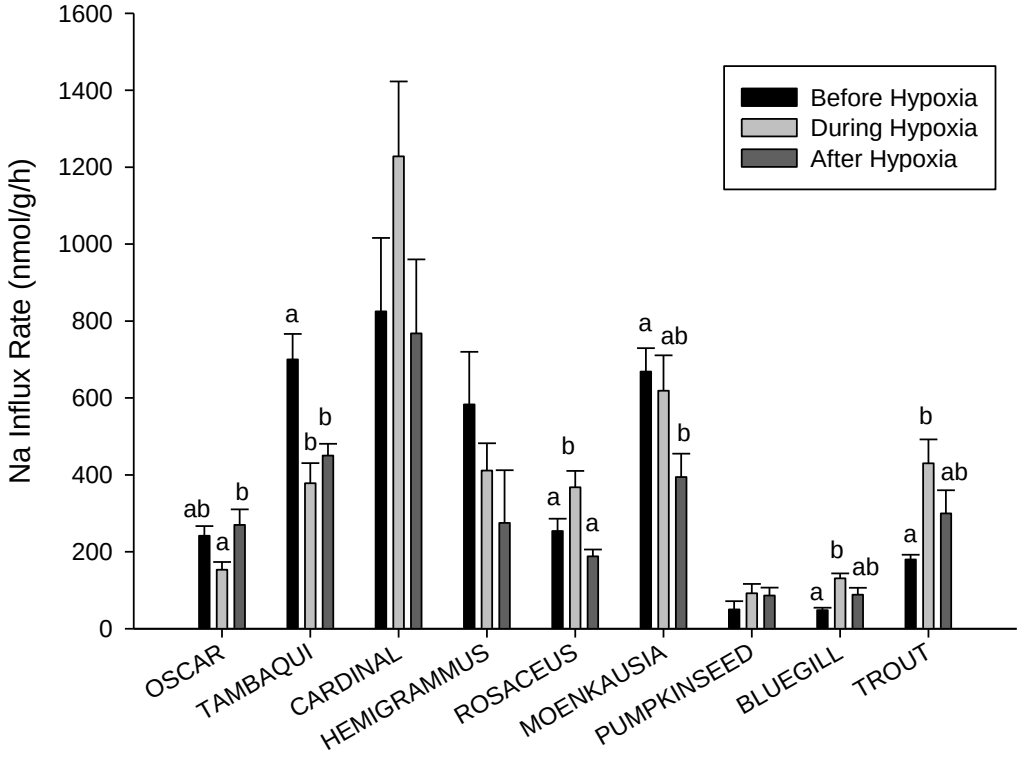

Na Influx Rate (nmol/g/h)
1600
1400
1200
1000
800
600
400
200
0
Before Hypoxia
During Hypoxia
After Hypoxia
ab
a
b
a
b
b
a
b
b
a
b
a
a
ab
b
a
b
ab
a
b
ab
OSCAR
TAMBAQUI
CARDINAL
HEMIGRAMMUS
ROSACEUS
MOENKAUSIA
PUMPKINSEED
BLUEGILL
TROUT

Figure 3.4. Na efflux changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

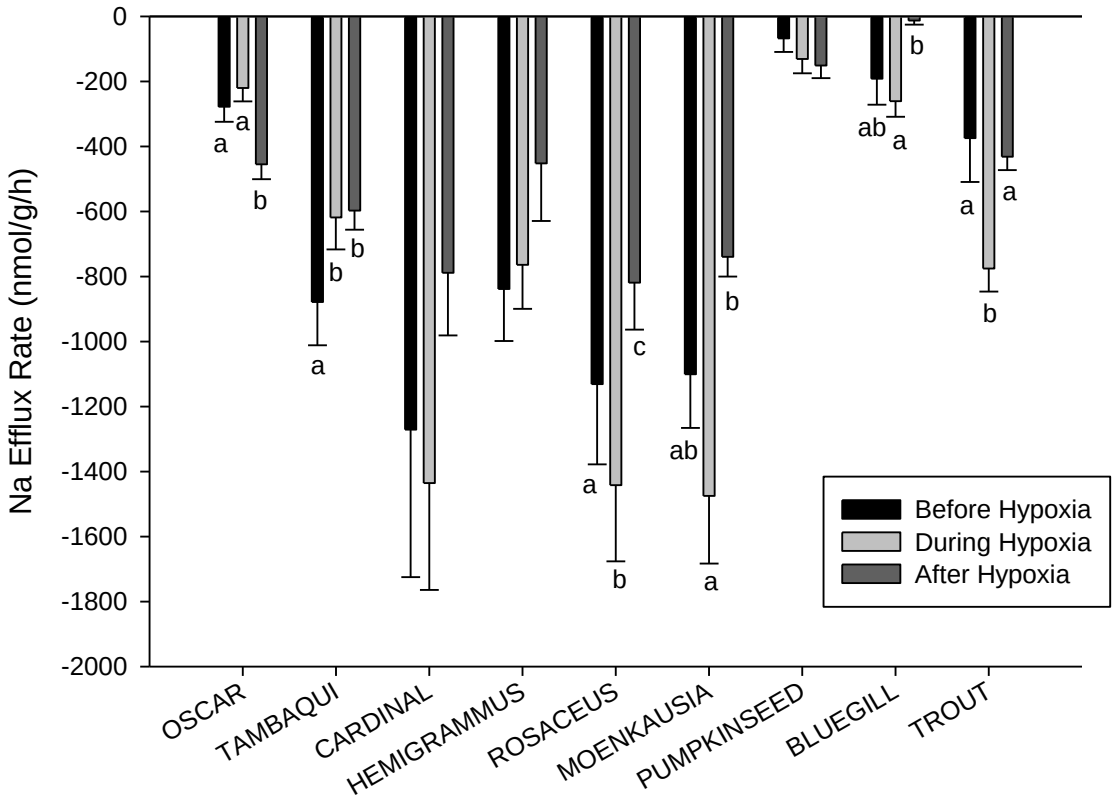

Na Efflux Rate (nmol/g/h)
0
-200
-400
-600
-800
-1000
-1200
-1400
-1600
-1800
-2000
a
a
b
a
b
b
a
a
b
a
b
c
ab
a
b
ab
a
b
a
b
OSCAR
TAMBAQUI
CARDINAL
HEMIGRAMMUS
ROSACEUS
MOENKAUSIA
PUMPKINSEED
BLUEGILL
TROUT
Before Hypoxia
During Hypoxia
After Hypoxia

Figure 3.5. Na net flux changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

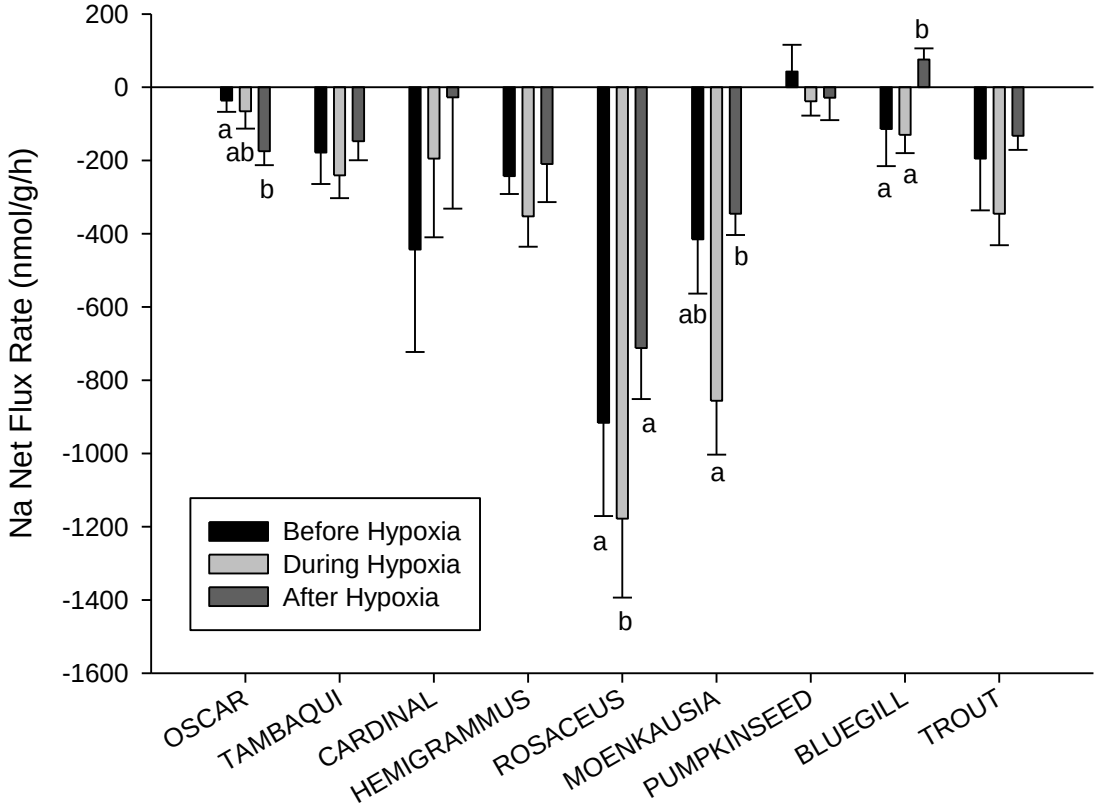

Na Net Flux Rate (nmol/g/h)
200
0
-200
-400
-600
-800
-1000
-1200
-1400
-1600
Before Hypoxia
During Hypoxia
After Hypoxia
a
ab
b
a
b
ab
a
b
a
b
a
a
b
OSCAR
TAMBAQUI
CARDINAL
HEMIGRAMMUS
ROSACEUS
MOENKAUSIA
PUMPKINSEED
BLUEGILL
TROUT

Figure 3.6. Ammonia excretion rate changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

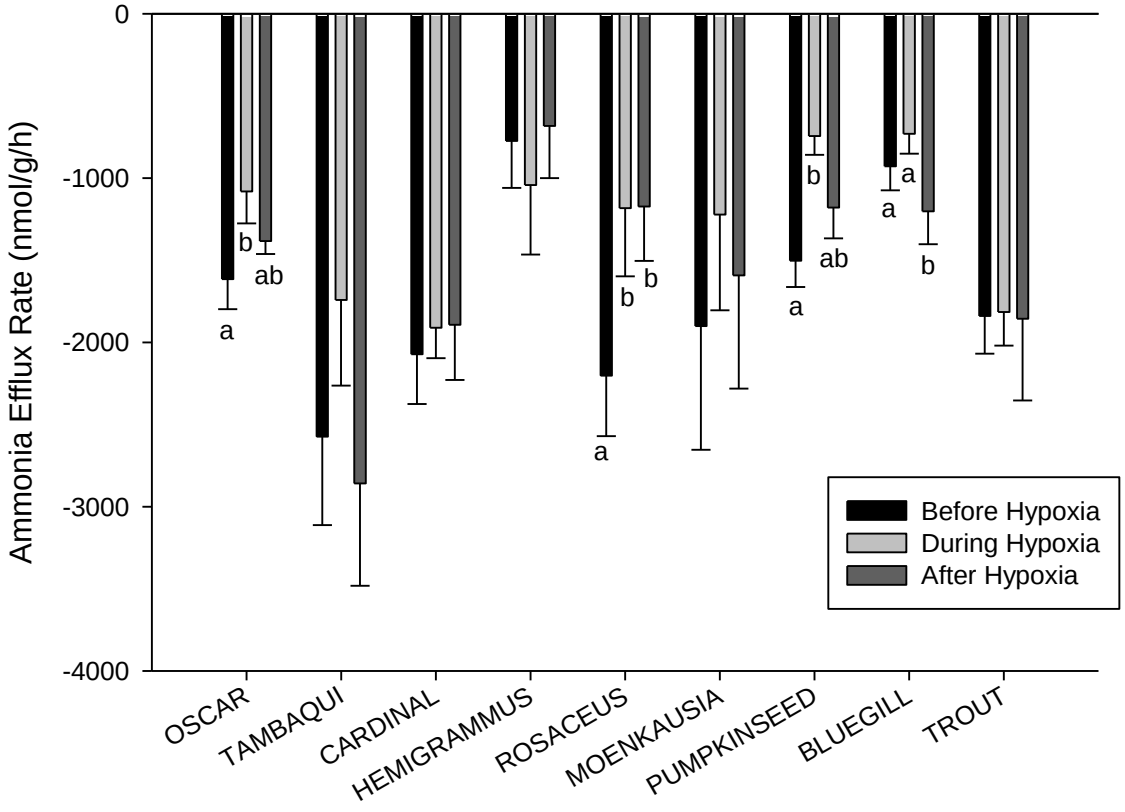

Ammonia Efflux Rate (nmol/g/h)
0
-1000
-2000
-3000
-4000
a
b
ab
b
b
a
b
ab
a
b
a
Before Hypoxia
During Hypoxia
After Hypoxia
OSCAR
TAMBAQUI
CARDINAL
HEMIGRAMMUS
ROSACEUS
MOENKAUSIA
PUMPKINSEED
BLUEGILL
TROUT

Figure 3.7. K net flux changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

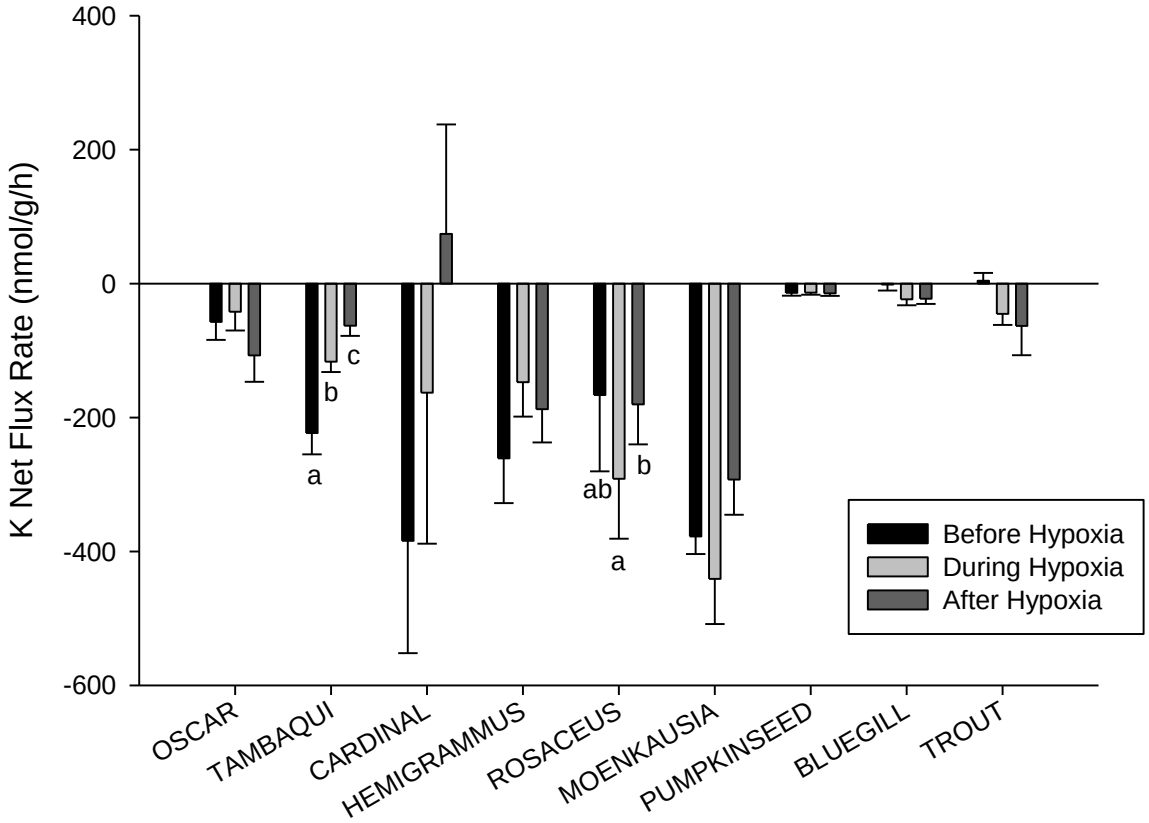

400
200
0
-200
-400
-600
K Net Flux Rate (nmol/g/h)
a
b
c
ab
a
b
Before Hypoxia
During Hypoxia
After Hypoxia
OSCAR
TAMBAQUI
CARDINAL
HEMIGRAMMUS
ROSACEUS
MOENKAUSIA
PUMPKINSEED
BLUEGILL
TROUT

Figure 3.8. Urea-N excretion rate changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

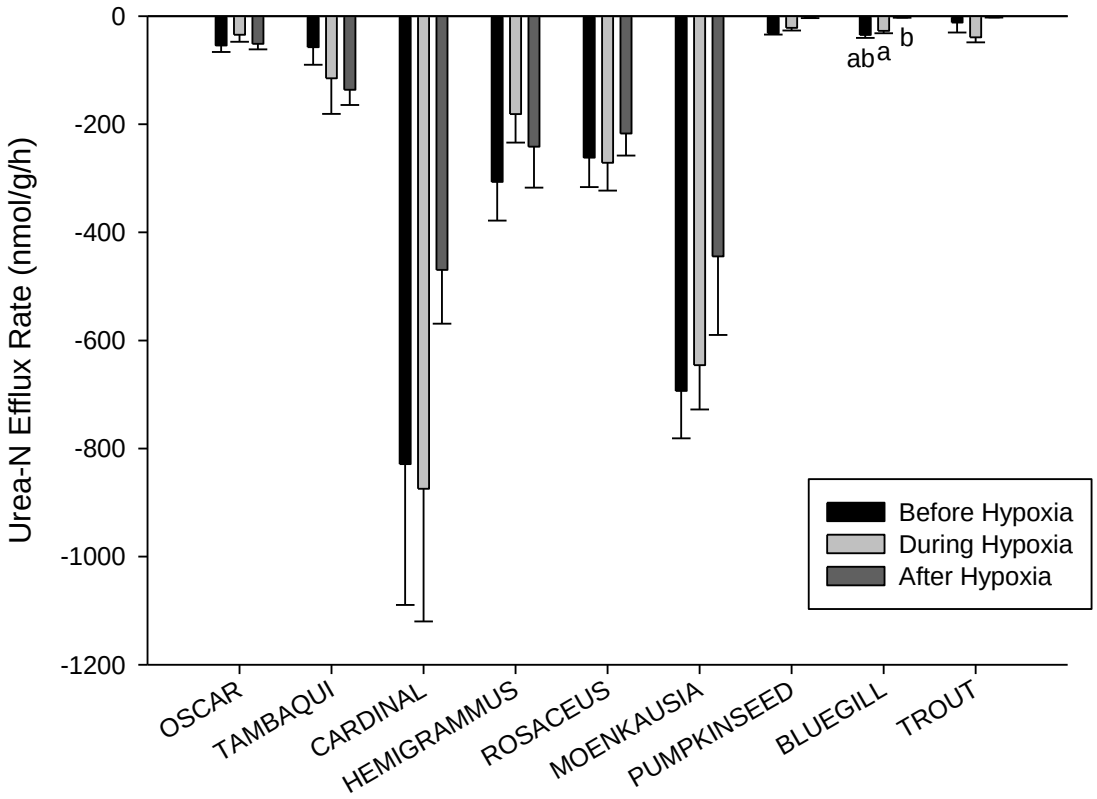

Urea-N Efflux Rate (nmol/g/h)
0
-200
-400
-600
-800
-1000
-1200
ab
a
b
Before Hypoxia
During Hypoxia
After Hypoxia
OSCAR
TAMBAQUI
CARDINAL
HEMIGRAMMUS
ROSACEUS
MOENKAUSIA
PUMPKINSEED
BLUEGILL
TROUT

Figure 3.9. Urea-N:Ammonia-N ratio changes during 2-h:2-h:2-h protocol (30% O_2 sat) at ~28˚C (Brazilian species) or 18˚C (Sunfish). Means are ±S.E.M. (N=6 for Brazilian species, N=5 for Sunfish). Means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

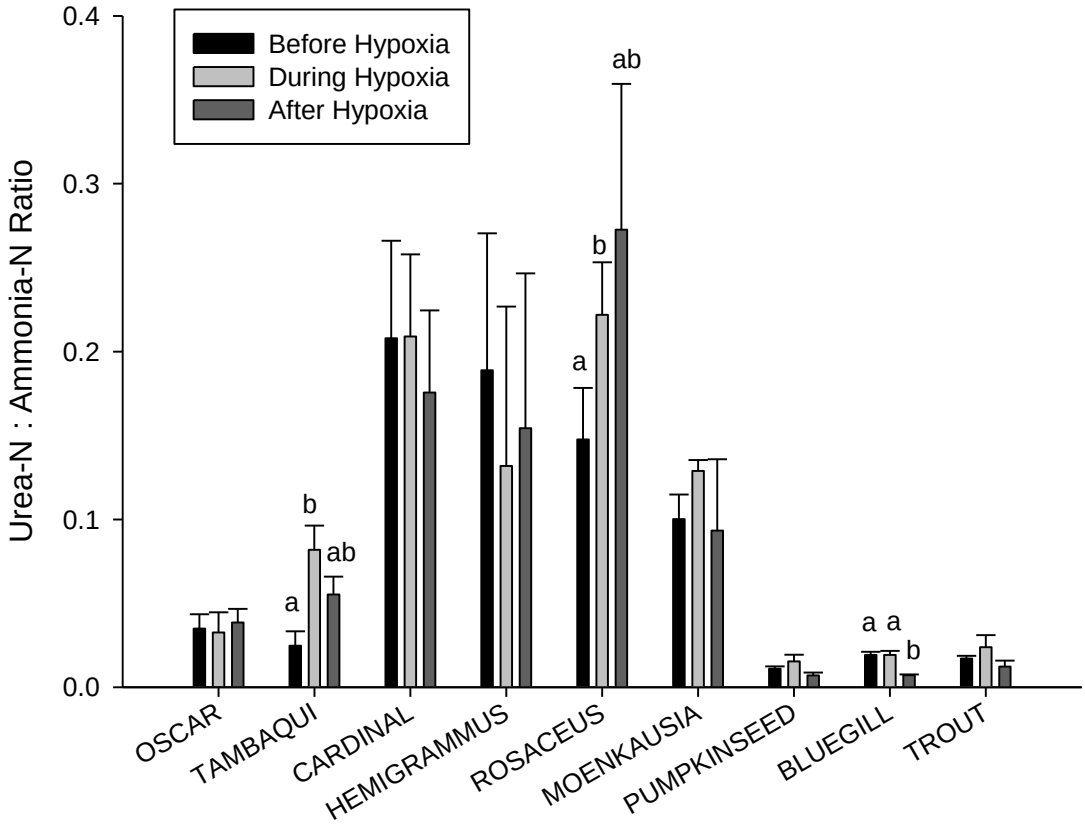
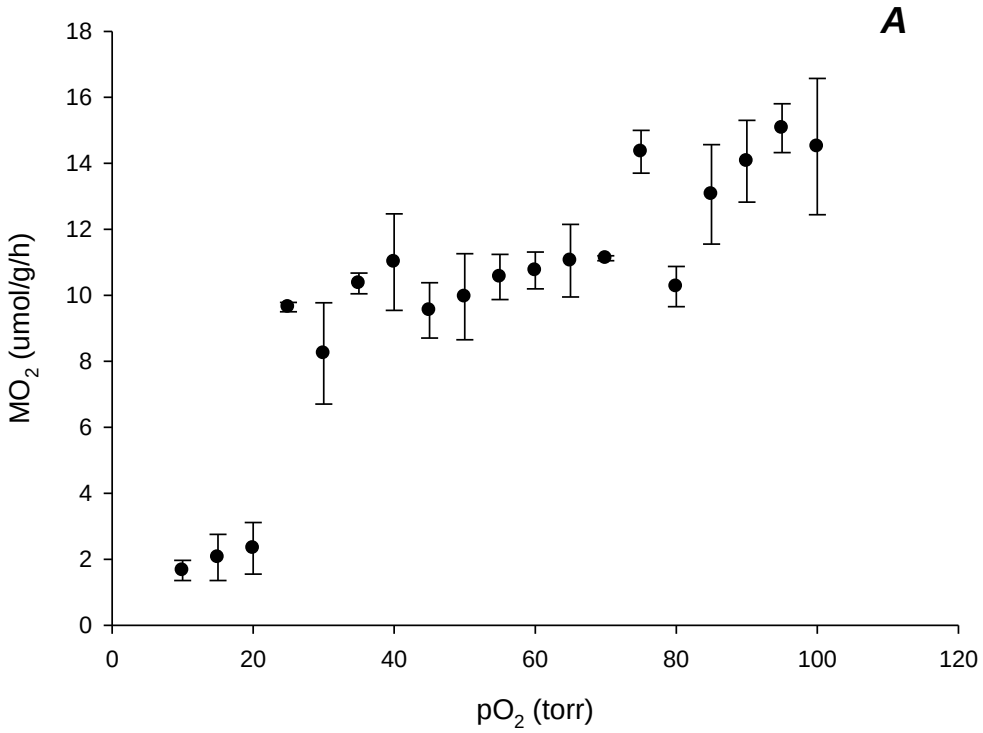
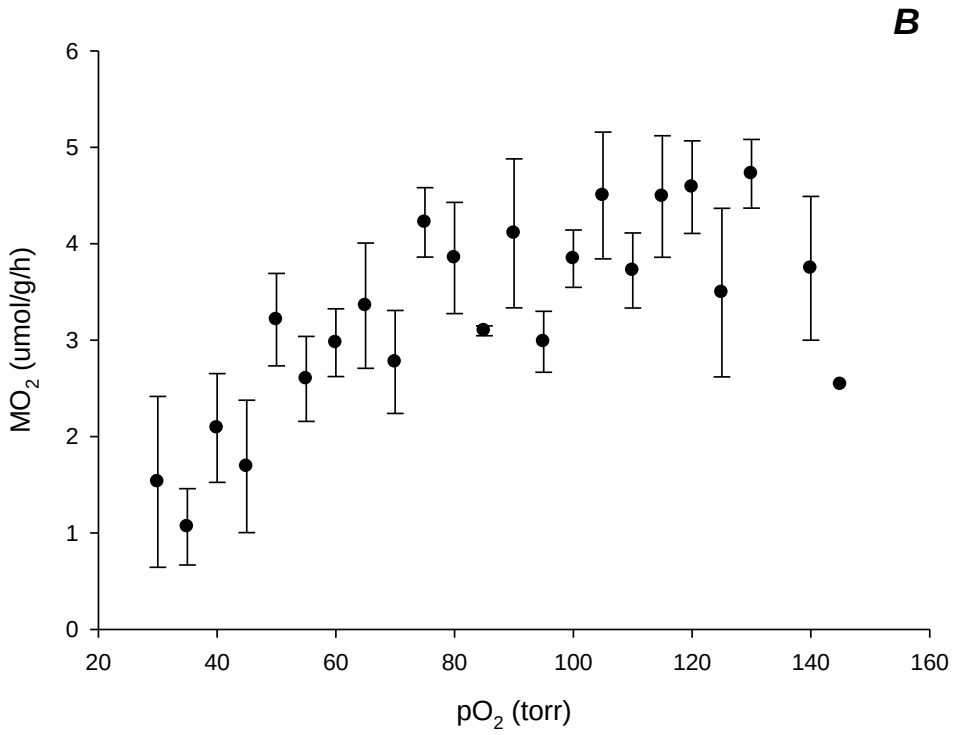

Before Hypoxia
During Hypoxia
After Hypoxia
Urea-N : Ammonia-N Ratio
0.4
0.3
0.2
0.1
0.0
a
b
ab
a
b
ab
a a
b
OSCAR
TAMBAQUI
CARDINAL
HEMIGRAMMUS
ROSACEUS
MOENKAUSIA
PUMPKINSEED
BLUEGILL
TROUT

Figure 3.10. Oxygen consumption rates across a range of oxygen saturations, useful for determining critical oxygen tension (Pcrit) by Yeager and Ultsch (1989) protocol. Metabolic rates are grouped in 5 torr pO_2 increments with means ±S.E.M, for A) tambaqui (N=4, Pcrit=28.6±7.9 torr), B) pumpkinseed sunfish (N=5, Pcrit=41.9±4.8 torr), and C) bluegill sunfish (N=5, Pcrit=101.7±8.3 torr).


A
MO2 (umol/g/h)
pO2 (torr)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120


B
MO2 (umol/g/h)
pO2 (torr)
0
1
2
3
4
5
6
20
40
60
80
100
120
140
160

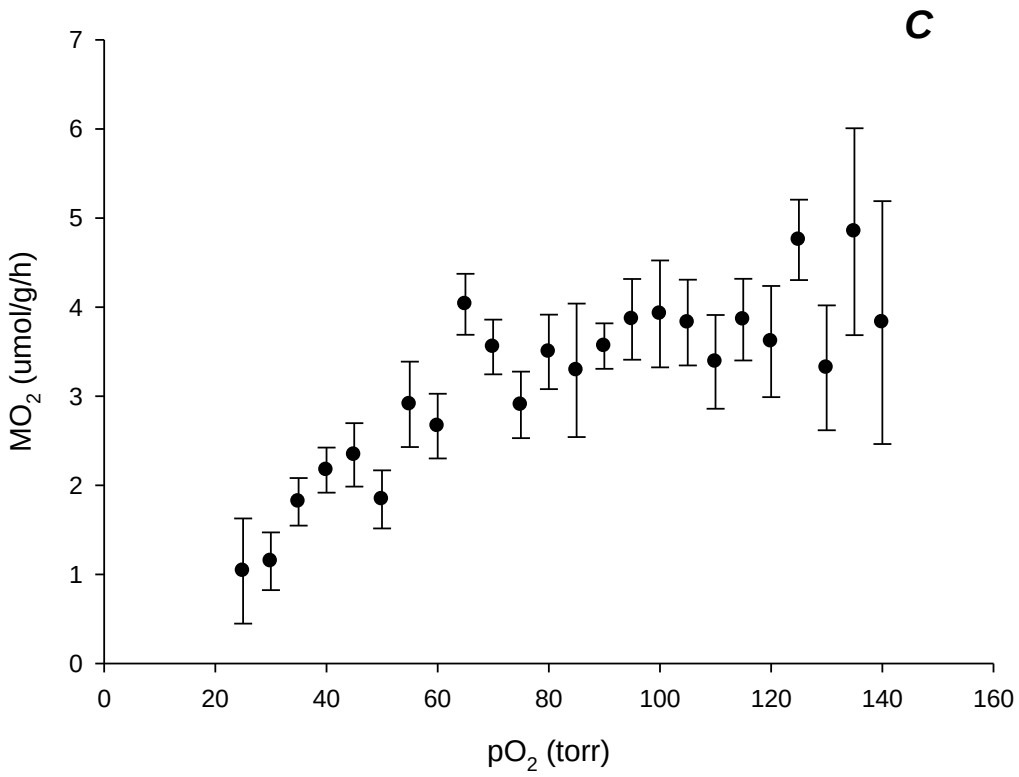

C
7
6
5
4
3
2
1
0
MO2 (umol/g/h)
0
20
40
60
80
100
120
140
160
pO2 (torr)

Table 3.1. Critical oxygen tension (Pcrit) values based on Yeager and Ultsch (1989) protocol in the present species survey, with additional values reported in the literature. Means are ±S.E.M. (N=4 for experimentally-determined values in the tambaqui, N=5 for pumpkinseed and bluegill). (Experimentally-measured) means sharing the same letter are not significantly different (P>0.05) as determined by a one-way repeated measures ANOVA followed by a Holm-Sidak *post hoc* test.

Species	Pcrit
Tambaqui (*Colossoma macropomum*)	28.6±7.9 torr (A)
Pumpkinseed sunfish (*Lepomis gibbosus*)	41.9±4.8 torr (B)
Bluegill sunfish (*Lepomis macrochirus*)	101.7±8.3 torr (C)
Rainbow Trout (*Oncorhynchus mykiss*)	63 torr (McIntyre, 2011)
Amazonian oscar (*Astronotus ocellatus*)	50-70 torr (Sloman et al., 2006) 46 torr (Scott et al., 2008) 16-27 torr (De Boeck et al., in prep)

Figure 3.11. Changes in flux parameters between rest and swimming (1.2BL/s, 15cm/s) in Amazonian oscar at ~28˚C. Means are ±S.E.M. (N=12-16). Asterisks denote significance ($P<0.05$) as determined by a paired t-test.

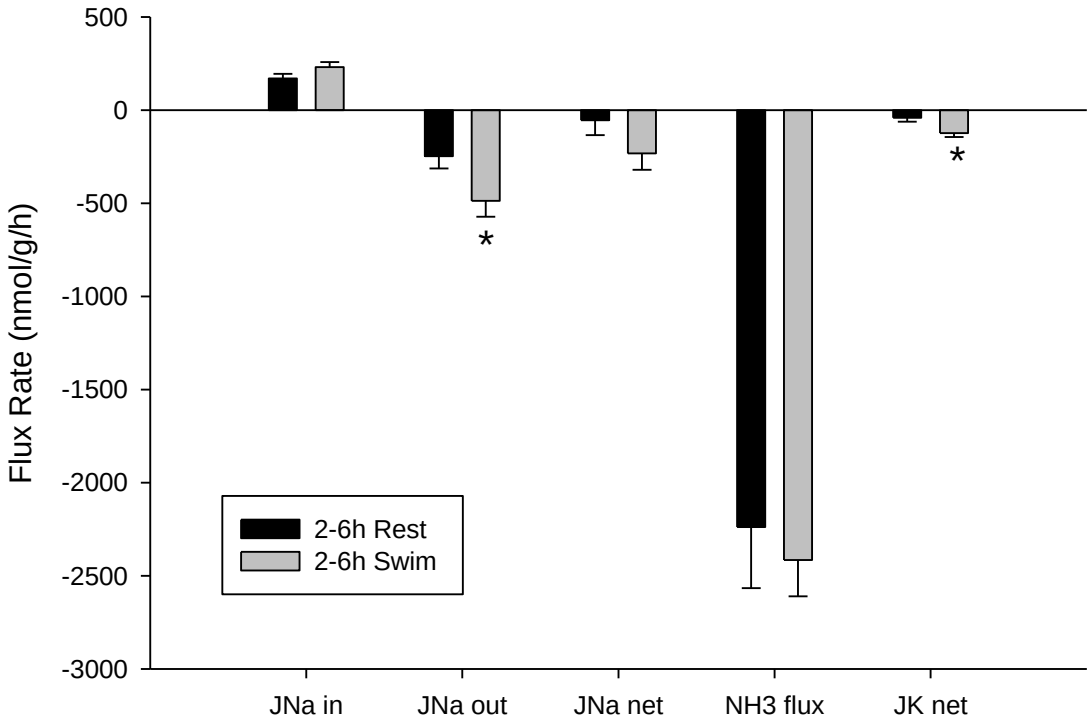
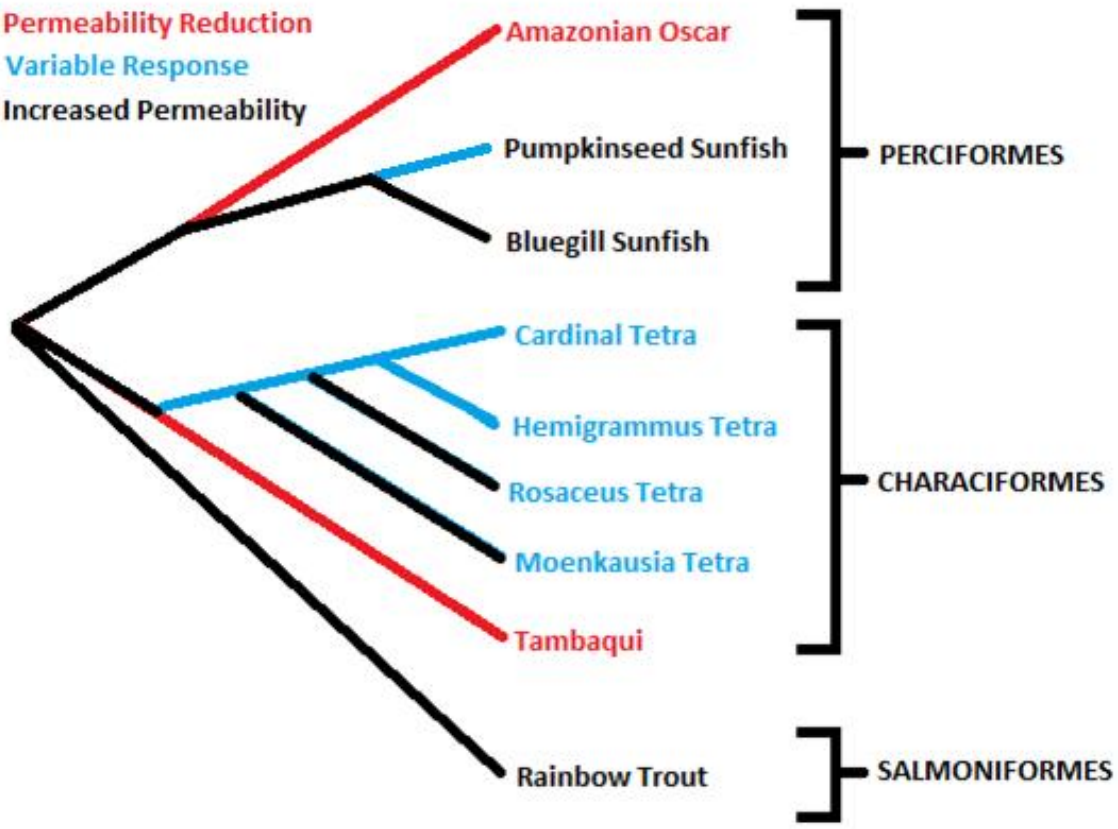

500
0
-500
-1000
-1500
-2000
-2500
-3000
Flux Rate (nmol/g/h)
2-6h Rest
2-6h Swim
*
*
JNa in
JNa out
JNa net
NH3 flux
JK net

Figure 3.12. Phylogenetic tree of species within the hypoxia species survey and their respective hypoxic response (Adapted from Lennard et al., 2006). Red lines indicate a permeability reduction strategy, blue lines show variable responses, and black lines represent increased permeability.


Permeability Reduction
Variable Response
Increased Permeability
Amazonian Oscar
Pumpkinseed Sunfish
Bluegill Sunfish
PERCIFORMES
Cardinal Tetra
Hemigrammus Tetra
Rosaceus Tetra
Moenkausia Tetra
Tambaqui
CHARACIFORMES
Rainbow Trout
SALMONIFORMES

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CHAPTER FOUR: Summary of findings and conclusions

Research Basis

The overarching purpose of this research was to examine the osmorespiratory compromise under both extended exercise and acute hypoxia, whilst examining species' adaptations to it. Chapter 2 presented a comparison between the rainbow trout (*Oncorhynchus mykiss*) and Amazonian oscar (*Astronotus ocellatus*) during long-term swimming, founded upon previous work by Wood and Randall (1973), Posthlethwaite and McDonald (1995), Gonzalez and McDonald (1992, 1994), and Iftikar et al. (2010) for trout, and Wood et al. (2007, 2009) and Scott et al. (2008) for oscar, and Matey et al. (2011) for both species. Next, Chapter 3 introduced results from an 'opportunistic' interspecific survey of hypoxic responses for a variety of North and South American teleosts. Altogether, this study has provided further insight into the osmorespiratory compromise, as well as how the responses are linked to physiology, phylogeny, and the environment.

Thesis Objectives: Revisited

As was outlined in Chapter 1, there were four main thesis objectives for this project; these were:

a) To establish and validate a technique for measuring branchial paracellular permeability with the paracellular flux marker [^{3}H]PEG-4000, that is not confounded by *i)* drinking of the chemical from the external medium, *ii)* renal loss of the chemical to the external medium, or *iii)* 'loss' of the chemical to surface adsorption in the flux chamber.

Such a method would be contingent upon PEG showing equal bidirectional permeability across the gill.

b) To use the technique from **(a)** to characterize paracellular permeability changes over an 8-h period of extended exercise in the rainbow trout and Amazonian oscar.

c) To develop and test a hypoxia 'rapid screening protocol,' wherein fish are subjected to 2-h normoxia: 2-h hypoxia: 2-h normoxic recovery, as a fast and uniform means of differentiating branchial permeability changes across a wide range of species exposed to this stressor.

d) Using the protocol developed in **(c)**, to classify species' ionoregulatory responses to the osmorespiratory compromise for identification of any trends associated with phylogeny and/or environment.

These objectives were adequately met in this study, as will be demonstrated presently.

Swimming and Paracellular Permeability

Although previous work had compared the ionoregulatory responses of rainbow trout and Amazonian oscar in terms of hypoxic response, it was the aim of this work to evaluate them during endurance exercise. This achieved the goals of investigating the ionoregulatory profile of the oscar during swimming, as well as more closely examining routes of permeability changes in trout. Work conducted during this portion of the study has brought to light two major findings: primarily that extended swimming increased branchial paracellular permeability (as measured by [^{3}H]PEG-4000) in the rainbow trout, whereas no change was observed in the Amazonian oscar. Secondly, this particular

technique has revealed some of the challenges and potential problems of using [^{3}H]PEG-4000 as a branchial permeability marker: renal loss, overestimation based on loss to the water due to respirometer surface adsorption, and chiefly, drinking in freshwater fish. By implementing corrections to control for these, this methodology can be further applied to measure paracellular permeability and tight junction modifications caused by a host of other factors.

Hypoxia Species Survey

The primary goal of Chapter 3 was to identify strategies for hypoxia tolerance among a variety of North and South American freshwater teleosts, and to characterize phylogenetic and/or environmental trends among them. As in the exercise study and previous research, rainbow trout (hypoxia-intolerant salmoniformes) were seen to increase gill permeability during this challenge. In contrast, the Amazonian oscar (hypoxia-tolerant perciformes) displayed a distinct permeability *inhibition* – known to be due to transcellular permeability reduction (Wood et al., 2007, 2009; Iftikar et al., 2010; Matey et al., 2011). Other perciform species were examined (pumpkinseed and bluegill sunfish), as were several types of characiformes (tambaqui and cardinal, hemigrammus, rosaceus, and moenkausia tetra), but only the tambaqui – which are routinely exposed to hypoxic stresses comparable to that of the oscar – showed a similar permeability inhibition, thus leading to the conclusion that adaptive strategies for hypoxia tend to correlate strongly with environmental conditions rather than phylogeny. Pcrit was also measured during this study but is so strongly affected by adaptive strategies to hypoxia that it is therefore a better measure of oxygen uptake capacity than of hypoxia tolerance.

In order to tie Chapters 2 and 3 together, branchial flux parameters in Amazonian oscar were then compared using ^{22}Na during the fairly unfamiliar challenge of extended exercise. While no paracellular changes had been observed (Chapter 2), swimming *did* appear to cause an increase in transcellular permeability (Chapter 3) as opposed to the inhibition seen with hypoxia. This indicated that, while very well-adapted to hypoxia, the Amazonian oscar was not adapted for extended exercise and therefore did not employ its characteristic permeability reduction strategy during this challenge.

Future Directions

Perhaps one of the most important lessons learned over the course of this work has been that answering one question always seems to raise two more. While the research carried out during this study can prompt further investigations in a number of directions, it may be most prudent to focus upon several key areas.

In terms of the exercise responses, although it was determined that elevated ion loss by trout during swimming *does* occur paracellularly (and potentially transcellularly), it was not conclusively proven that this is the route by which leak 'correction' occurs (seen in Wood and Randall, 1973; Gonzalez and McDonald, 1992; Postlethwaite and McDonald, 1995). Thus, it would be beneficial to study [^{3}H]PEG-4000 permeability changes at *intervals* in the trout (i.e. every 2 h for 8 h) to determine whether this correction is seen for [^{3}H]PEG-4000 flux as well as for Na. The role of prolactin (a hormone known to alter tight junction morphology) also remains relatively unknown in this phenomenon although this hormone has been suggested to play a role in extended swimming (Potts and Evans, 1966; Wood et al., 1973; Postlethwaite and McDonald,

1995; Manzon, 2002). Finally, the interspecific comparison portion of the project could yield clearer results if oscar were subjected to the swim test *without* an initial 4-h rest period. One benefit of developing methodology for measuring branchial permeability in Chapter 2 is its wide range of applications for measuring permeability changes during manipulation of environmental conditions including temperature, pH, water oxygen saturation, salinity, external ammonia, and contaminant concentrations. It would be especially relevant to this field of study to observe paracellular permeability changes in trout during acute hypoxia.

The second part of this research studied hypoxic responses at the uniform hypoxic level of 30% O_2 saturation across the species survey to allow for widespread comparison, but it would be valuable to approach this from a different angle by studying the same flux parameters at equivalent *severities* instead. This could be accomplished by measuring these parameters at a relative species-specific oxygen saturation level; that is, a percent of some endpoint (i.e. Pcrit, percent survival at a certain oxygen saturation, oxygen 'LC50'). Another interesting route to explore would be to investigate potential *disadvantages* of the transcellular permeability inhibition strategy seen in species facing regular, severe hypoxia (oscar and tambaqui). For instance, is this inhibition also preventing proton movement out of the gills? Would its long-term use create an acid-base imbalance? What are the potential advantages to conversely increasing MRC surface area and retracting PVCs in trout during hypoxia – is this simply an adverse reaction to attempted oxygen uptake maintenance, or is increasing active ion uptake a secondary response to compensate for greater passive losses?

In any case, the findings shown here have served to further advance physiological understanding of the osmorespiratory compromise and the factors that interact to influence a species' adaptive capacity for relevant environmental challenges. Hopefully the research itself and methods developed therein will facilitate future investigation in this field.

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