Skin permeability of the amphibious mangrove rivulus *Kryptolebias marmoratus* in response to emersion

by

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A Thesis
presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Masters of Science
in
Integrative Biology

Guelph, Ontario, Canada

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ABSTRACT

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The skin of the amphibious fishes is known to be a physiologically important tissue for maintaining homeostasis during emersion. I tested the hypothesis that skin permeability would be altered to maintain water balance during salinity acclimation and/or emersion. Osmotic permeability of the skin was assessed using $^3$H$_2$O fluxes, and bulk osmotic flow was quantified using a novel dye-dilution technique. No regional variability between the dorsal and ventral skin region was found, although fish more often positioned themselves on the ventral surface when emersed. Hypersaline acclimation resulted in a decrease in $^3$H$_2$O influx across the skin relative to hyposaline acclimation. Acute emersion (1 day) induced a decrease in bulk osmotic water loss in hypersaline-acclimated fish, whereas prolonged emersion (7 days) resulted in an increase in $^3$H$_2$O influx. These findings suggest that *K. marmoratus* alter skin permeability to maximize water uptake and minimize water loss while emersed in hypersaline conditions.
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ACKNOWLEDGEMENTS

First and foremost I would like to recognize Dr. Patricia Wright for her guidance, patience, and seemingly endless drive. Pat, you have been an amazing partner throughout this process and have instilled skills and qualities of a researcher that I value highly. You are a continuous motivation and inspiration, with an advisory style to be admired. Thank you for making my masters experience meaningful, productive, and transformative.

I would also like to recognize the members of the Wright lab for their academic support. In particular, I would like to recognize Andy Turko and his intellectual contributions to this work. Andy’s innovation and passion for his work have provided me a great model for thinking outside of the box. I look forward to seeing what you accomplish.

Finally, I would like to extend thanks to members of my personal life including friends and family who have been nothing but supportive throughout my time in Guelph. Namely, I would like to thank Jen Bernard and Kayla Deasley for their camaraderie on and off campus. I will also take this opportunity to formally thank my parents and loved ones on Prince Edward Island, who have been a tremendous foundational body. I’m sure GG has burned through a few sets of beads on my behalf. Finally, I would like to formally thank Dan Hamilton, who has been a tremendous motivator and reminder of what is to come.

To all, I sincerely thank you.
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INTRODUCTION

Amphibious fishes are a morphologically diverse group of semi-aquatic organisms living at the boundary between water and land (Sayer and Davenport, 1991). These fishes vary in their tolerance of terrestrial environments, as moving between aquatic and terrestrial habitats places burdens on physiological systems to maintain homeostasis (Sayer, 2005). Amphibious fishes have various adaptations to maintain respiration, ionic- and osmoregulation, and acid–base regulation out of water. My MSc thesis addressed osmoregulatory challenges in a model euryhaline, amphibious fish, the mangrove rivulus, *Kryptolebias marmoratus*. This species tolerates extended emersion and encounters variable salinities in its natural habitat, especially with changing seasons. My goal was to identify the mechanisms of water balance that *K. marmoratus* use when out of water on either hypo- or hypersaline substrates.

**Iono-Osmoregulation**

The main osmoregulatory epithelium in fishes is the gill. It functions as the primary exchange surface for ions and water between the internal blood plasma and the external environment. The dynamics of ion and water movement differ between the freshwater and seawater fishes due to differences in ionic and osmotic gradients (Isaia 1984; Payan, Girard and Mayer-Gostan 1984).

*Seawater fishes*. Seawater fishes maintain an internal osmotic concentration approximately one third that of their external environment. Generally, the blood plasma in marine fishes is ~400 mOsm, while their hypersaline environment is ~1000
mOsm (Evans 2008). This generates a strong osmotic gradient, favoring the outward passive diffusion of water toward the hyperosmotic environment. To maintain osmotic equilibrium, saltwater fish drink seawater and absorb water across the intestinal epithelium. The relatively low internal osmolality of saltwater fishes also generates electrochemical gradients that favor the movement of salts from the ambient water into the relatively hypoosmotic blood plasma. To oppose this diffusional gradient, saltwater fish actively excrete excess NaCl through mitochondrial rich cells, or ionocytes, in the gills. The kidney also aids in salt excretion, producing low volumes of urine rich in divalent ions (Krogh 1939; Evans 1980; Evans et al. 1999; Evans et al. 2005; Marshall and Grosell 2006; Evans 2008; Whittamore 2012; Edwards and Marshall 2013).

**Freshwater fishes.** Freshwater fishes have blood that is hyperosmotic to their ambient environment. Generally, the blood plasma of freshwater fish is ~300 mOsm while their ambient environment is ~1 mOsm (Evans 2008). In contrast to seawater fishes, freshwater fishes face a gradient favoring ion loss and must transport Na\(^+\) and Cl\(^-\) ions inward via active transport mechanisms in gill ionocytes. There is also an osmotic gradient across the gill that causes the passive uptake of water. Excess water uptake, as well as ion loss, is compensated for by the kidney, through NaCl reabsorption and the production of copious amounts of dilute urine. It is obvious how important a permeable surface is for compensatory diffusion and exchange to maintain osmotic and ionic balance in both saltwater and freshwater fishes (Krogh 1939; Evans, 1980; Evans et al. 2005; Marshall and Grosell 2006; Evans 2008; Whittamore 2012).
Terrestrial animals. Maintaining water balance is also a challenge for terrestrial animals. Evaporation of water directly from the surface of the skin accounts for 50-80% of total water loss in resting terrestrial animals (reptiles, birds, and mammals), followed by pulmonary evaporation occurring across respiratory surfaces (Kirschner 1991). Variation in evaporative water loss across the skin is dependent upon the animals’ environment, as individuals from more arid environments have adaptations to minimize water loss to cutaneous evaporation. Inhabiting burrows and high altitudes allow animals to decrease the stress of evaporative water loss, as these environments generally have higher relative humidity and are cooler and moister (Kirschner 1991). The skin of many reptiles is also adapted to arid habitats with a very low permeability to water to prevent evaporative water loss. Even scaleless aquatic sea snakes in air lose less water across the skin than a typical amphibian (Bennett and Licht 1975). Altering skin permeability to prevent evaporation and maintain water balance is thus obviously important to animals inhabiting terrestrial environments.

When amphibious fishes leave water or emerse, gill function may not be maintained (Graham, 1997). Thus, many fishes moving to land must employ an alternative exchange surface to maintain homeostasis of ions and water. Some amphibious fish use the cutaneous surface for ion and water regulation. Mitochondrial-rich cells similar to gill ionocytes have been discovered in the skin of the mudskippers Periophthalmus modestus (Yokota et al. 1997) and Periophthalmus cantonensis (Yokoya and Tamura, 1992), the ventral surface of the African lungfish (Strula et al. 2001), and the mangrove rivulus K. marmoratus (LeBlanc et al. 2010).
Skin ionocytes increase in size after emersion in *K. marmoratus* with no change in sodium efflux rate, suggesting the recruitment of the skin for ionoregulation (LeBlanc et al. 2010). In the amphibious swamp eel (*Synbranchus marmoratus*), cutaneous ion transport represents 75% of ionoregulation with no effect of emersion on sodium influx or efflux (Stiffler et al. 1986). Similar results were found in estivating lungfish (*Protopterus dolloi*), with no effect of long-term emersion on plasma osmolality or ion flux (Wilkie et al. 2007). Thus, there is evidence that the skin plays a critical role in maintaining ion gradients in fish out of water.

**Water flux**

The balance of water across the skin of amphibious fish is a topic that has not been thoroughly investigated. Many studies of water balance in fishes focus on whole body water flux experiments (Evans 1967; Potts et al. 1967; Rudy 1967; Evans 1969a,b; Potts and Rudy 1971; Loretz 1979; Wilkie et al. 2007; LeBlanc et al. 2010). Whole body fluxes incorporate drinking rates and exchange across the gill, kidney, and skin. Drinking during emersion in amphibious fishes, however, would not be possible. In addition, a terrestrial environment with a hypersaline substrate may result in decreased water content of both intra- and extracellular body compartments, resulting in dehydration via osmotic water loss (reviewed by Takei 2015). Because fish out of water are unable to drink to rehydrate these compartments, alternative sites of water exchange must be used.

The specific sites of water exchange and the cellular mechanisms involved in water flux have not been explored in amphibious fishes in detail. It is possible that the
skin is an important site for water balance in amphibious fish. In the African lungfish
(*P. dolloi*), chronic emersion (6 months) caused water efflux across the skin to
decrease by 60% (Wilkie et al. 2007), potentially as a means to conserve body water.
Although water flux across the dorsal skin was never measured, it was assumed that
water moved primarily across the ventral skin in this fish. This was because water
fluxes were comparable between the immersed and emersed groups, despite the
emersed fish being limited to only ventral contact with the water source. This
potentially high ventral permeability of the lungfish mirrors the highly permeable
pelvic patch common in anuran amphibians (McClanahan and Baldwin 1968; Bentley
and Main, 1972; Bentley and Yorio 1976). It is possible that *P. dolloi* and other
amphibious fishes use the ventral skin for water exchange during emersion and alter
skin permeability to water during prolonged emersion to prevent excessive water loss.
Lungfish are known to enter a metabolic depression during estivation, which may
account for the observed decrease in cutaneous water flux (Smith 1930; Janssens
1964). Many amphibious teleost fish do not enter an estivation during emersion and
could respond to emersion differently than the lungfishes. It is largely unknown how
teleost skin permeability is altered during emersion.

Water balance in amphibious fishes has also been studied by measuring tissue
water loss. In air-exposed (1 day) barred mudskippers (*Periophthalmus sobrinus*),
body mass decreased as a result of evaporative water loss (Gordon 1969). This
decrease however was relatively low compared to more terrestrially adapted
amphibians (Gordon 1969). A related species of mudskipper, *P. cantonensis* uses a
hierarchy of tissue water loss when exposed to desiccation pressures (Gordon et al.
1978). These fish lost the greatest proportion of water from the heart, followed by the blood and white muscle, with the liver and brain actually increasing in water content (Gordon et al. 1978). This strategy seems to utilize internal redistribution of water to maintain a hydrated central nervous system. Low rates of water loss were found in the amphibious Chilean clingfish *Sicyases sanguineus* (Gordon 1970). The gut has been suggested as an osmoregulatory surface in this latter species, as they ingest large volumes of water before extended emersion (Marusic et al. 1981). *S. sanguineus* retain intestinal water for up to 6 hours, but by 1 day water was no longer in the intestine, suggesting it’s use for water balance.

Taken together, previous studies suggest that amphibious animals have adaptations to maximize water balance and prevent water loss during emersion. Although much is known about amphibian integumentary function, no work has examined water flux across amphibious fish skin in isolation. Whole body experiments do not allow us to examine the relative contribution of the skin to overall osmoregulation. How do different skin regions differ in terms of skin permeability? And how does cutaneous water permeability change with acclimation to a terrestrial habitat, particularly under hypersaline conditions when fish are unable to replace water loss by drinking?

*Measures and mechanisms of water flux.* The movement of water is driven by osmotic gradients. The movement of ions down chemical and/or electrical gradients generates changes in osmotic potential, thus creating osmotic gradients. Water movement across biological membranes is often quantified with two measures of membrane permeability: the diffusional permeability (unidirectional flux measured
with tracers) and hydraulic conductivity (bulk water flux in an osmotic experiment) (Danity and House 1966; Kirschner 1991). Tracers are substances that can be tracked through an organism or other biological system, such as radiolabelled water (tritiated water: $^3$H$_2$O). Diffusional permeability is a measure of water movement as water molecules obey Fick’s law of diffusion. Water movement, here, is solely due to diffusion, as diffusional permeability experiments are conducted under isosmotic conditions. Hydraulic conductivity measures bulk water movement as water moves down osmotic gradients and obeys fluid dynamics (Isaia 1984). The literature surrounding the permeability of biological membranes to water is often inconsistent with regard to terminology and technique. Hydraulic conductivity, for example, is often referred to as “osmotic permeability” as it will be in the current study. Such variation requires that terms are explicitly defined to avoid inappropriate comparisons. In the current study, water flux down an osmotic gradient was examined, and the osmotic permeability of amphibious fish skin was calculated. Although tracer experiments were used to measure unidirectional water flux, diffusional permeability could not be quantified, as an osmotic gradient was always present.

Water movement has been examined at the cellular level (between the intra- and extracellular fluid), as well as across the whole body or specific epithelia (Kirschner 1991). Water may move down osmotic gradients through epithelia by transcellular or paracellular pathways (Brandner 2007; Kawedua et al. 2007).

*Transcellular.* Water molecules can move across a cell membrane by simple diffusion, that is, directly between the lipid molecules that make up a phospholipid
bilayer (Zeuthen 2010). Water may also move freely though transmembrane protein channels incorporated directly into the cellular membrane (facilitated diffusion). These channels were discovered and termed aquaporins (Agre et al. 1993). Aquaporins make up a superfamily of membrane protein channels (Edwards and Marshall, 2013), and orthologs of these proteins are widespread throughout both the plant and animal kingdoms with the teleost fish clade having the largest variety (Cerdà and Finn 2010). Aquaporins exist in the membrane as homotetramers, made up of four monomeric channels that provide a route for the movement of water down the osmotic gradient.

There is evidence that aquaporin isoforms have specialized roles within tissues. Aquaporin-3 expression in the gill has been found to change with salinity acclimations (increased expression in freshwater; decreased in seawater) in European sea bass (*Dicentrarchus labrax*) (Giffard-Mena et al. 2007), the silver sea bream (*Sparus sarba*) (Deane and Woo 2006) and the European eel (*Anguilla anguilla*) (Cutler and Cramb 2002). These results suggest that aquaporin-3 specifically, and broadly the transcellular route via aquaporins, is important for osmoregulation in fish and responds to changes in the environment by altering membrane permeability. It is unknown how an aerial environment and the risk of water loss would influence the transcellular route in the skin of amphibious fishes.

Water moving against a transepithelial osmotic gradient (or ‘uphill’ water movement) has been observed in classic studies of water movement across amphibious skin (Reid 1892; Koefoed-Johnsen and Ussing, 1953; House 1964). Many studies focusing on uphill water movement examine the intestinal lumen during
digestion (Curran, 1960; Skadhauge 1969, Genz et al. 2011; Whittamore 2012), but little is known about this property in skin. Uphill water movement is accomplished by means of cotransporters: proteins that move water alongside solutes. These proteins use downhill ion gradients to move water molecules against uphill osmotic gradients (Zeuthen 2010). Water molecules can also become engulfed within a membrane protein, and during this protein’s conformational change to transport target solutes, water is secondarily moved (Whittamore 2012). As in the intestine, it is possible that water cotransport is important to cutaneous water dynamics.

**Paracellular.** Tight junctions are regions in the paracellular space where membrane associations are made between neighboring cells. The tight junction complex proteins are used to regulate paracellular transport (Tsukita and Furuse, 1999; Kolosov et al. 2013). The major protein constituents of the tight junction complex are the claudins, occludins, tricellulins, junctional adhesion molecules (JAM), and zonula occludens (ZO) proteins (Chasiotis et al. 2012; Gonzalez-Mariscal et al. 2003). More specifically, claudin proteins are often tissue specific and, along with occludin and tricellulin proteins, dictate passage through the paracellular space (Gunzel and Yu, 2013). The JAM molecules function primarily to traffic larger cells like lymphocytes (Gonzalez-Mariscal et al. 2003). Finally, the ZO proteins provide a link between the cell membrane and the cytoskeleton, creating support for the complex protein assemblage (Bauer et al. 2010).

The claudin family is extensively studied in fish. There have been a total of 56 claudin proteins discovered in a single fish species (*Fugu rubripes*; Loh et al. 2004). Some of these isoforms are comparable to mammals, while some are the result of
genetic duplication events. In general, high concentrations of tight junction proteins are found in the gills of hyperosmotic fish (to prevent the excessive paracellular diffusion of ions) and low numbers of tight junction proteins are found in ‘leaky junctions’ of hypoosmotic fish (to allow for ease of Na\(^+\) paracellular diffusion) (Evans et al. 2005; Kolosov et al. 2013).

Tight junction proteins have been discovered in the skin of multiple fish (pufferfish *Takifugu rubripes*: Loh et al. 2004; green spotted puffer *Tetraodon nigroviridis*: Bagherie-Lachidan et al. 2009; zebrafish *Danio rerio*: Kumai et al. 2011; goldfish *Carassius auratus*: Chasiotis and Kelly 2012; carp *Cyprinus carpio*: Syakuri et al. 2013) though little work has been done to detail their role in water balance. In the fully aquatic *T. nigroviridis*, it has been found that salinity has a significant effect on tight junction tightness through the mRNA expression of claudin-3, claudin-8 and claudin-27 in the skin (Bagherie-Lachidan et al. 2008; Bagherie-Lachidan et al. 2009). Under these conditions there was also an upregulation of claudin proteins, reducing skin and gill permeability. Yet, in the European eel (*A. anguilla*), claudin-27 gene expression declined in the gills after seawater acclimation (Kalujnaia et al. 2007). It appears that there is some degree of species specificity to claudin regulation in response to salinity changes.

To date, there is no information on how epithelial tight junctions or water flux across the skin may be altered in amphibious fish out of water. LeBlanc et al. (2010) discovered that hypersaline seawater (45 ‰) acclimated *K. marmoratus* exhibited a decrease in water efflux, while freshwater (1 ‰) acclimated fish demonstrated an increase in water efflux after 9 days of air exposure. Changes in water efflux could be
related to branchial, cutaneous or renal responses and involve either paracellular or transcellular flux. LeBlanc et al. (2010) suggested the potential role of tight junction proteins in osmoregulation in emersed fish, although this has not been explored. Interestingly, body water content significantly increased by ~1% after 11 days in emersed *K. marmoratus* (Litwiller et al. 2006), suggesting that fish may actually take up water from a moist substrate during emersion.

**Objectives and Hypothesis**

I designed my experiments under the assumption that tritiated water ($^3$H$_2$O) would travel across the skin identically to unlabeled water. I also assumed that $^3$H$_2$O would move through both paracellular and transcellular routes, while $^{14}$C-PEG-4000 would move exclusively through the paracellular route due to its relatively large molecular size. I questioned whether there would be regional differences in permeability in *K. marmoratus*’ skin, because in preliminary observations, *K. marmoratus* appeared to “sit” on their ventral surface when air exposed. Thus, the ventral surface should be adapted for water uptake from the moist substrate. A porous and water permeable ventral surface has also been described in other amphibious organisms to facilitate water balance.

I hypothesized that the permeability of the skin would be altered through paracellular and transcellular mechanisms during emersion to facilitate water balance. This hypothesis predicts the following outcomes:

i. In hypersaline-acclimated fish, emersion will result in an increase in water influx to combat evaporative water loss, as well as a decrease in water
efflux to oppose the osmotic gradient and maintain internal body water. In freshwater-acclimated fish, water influx and efflux will remain comparable to immersed fish, as evaporative water loss will be opposed by passive water uptake due to the osmotic gradient.

ii. Bulk osmotic flow of water across the skin (efflux) will be lower in hypersaline-acclimated fish exposed to air relative to control fish in water, as the skin will be tightened to prevent water loss. Inward bulk osmotic flow (influx) will be greater in hyposaline-acclimated fish exposed to air relative to control fish in water because water uptake will combat evaporative loss and any osmotic water loading may be compensated for by the renal system.

iii. In both hypersaline- and freshwater-acclimated fish, emersion will induce a tightening of the skin’s cellular junctions (paracellular) to maintain body water content. Thus, $^{14}$C-PEG-4000 influx and efflux will decrease in emersed compared to immersed fish.

iv. Aquaporins are ubiquitous in animal tissues and facilitate transcellular water flux. Inhibition of aquaporins will reduce water flux regardless of salinity, but will have a proportionally larger impact where water flux rates are higher.
METHODS

Experimental Animals

Adult mangrove rivulus, *Kryptolebias marmoratus* (0.18 g ± 0.01) were acquired from a colony housed under constant conditions (25°C, 15 ‰, pH 8, 12L:12D cycle) in the Hagen Aqualab at the University of Guelph. The DAN strain, originally native to Belize, was used in these experiments. All experiments were conducted following the guidelines of the Animal Utilization Protocol 2239 at the University of Guelph.

Experimental Design

Four series of experiments were conducted. The first series examined emersion behavior to see if certain regions of the skin are selected for moist substrate contact. The second series aimed to identify any differences in $^3$H$_2$O fluxes across the dorsal and ventral regions of the skin. The third series was designed to examine whether air exposure and hyper- or hypo-salinity acclimation had effects on the overall osmoregulatory function. In the third series, $^3$H$_2$O and $^{14}$C-PEG-4000 fluxes were used to distinguish the relative importance of the transcellular ($^3$H$_2$O) and paracellular (PEG) transport routes through the skin. The third series also involved designing a novel method to identify the degree of osmotic water movement that occurred across the skin, after acclimation to different salinities and emersion treatments. In the fourth series, experiments were performed to examine the effects of HgCl$_2$, a known aquaporin channel blocker, on water flux across isolated *K. marmoratus* skins (Savage and Stroud 2007).
**Series 1: Body orientation during emersion.** Behavioral analysis of the body position of fish out of water (1 day) was conducted using serial still photography. A GoPro camera (Original HD Hero, CA, USA) was mounted above emersed fish in containers lined with moistened filter paper. Experiments were originally conducted using a smaller (15 cm³) emersion environment. The experiments were repeated using a larger (700 cm³) emersion environment. The small arena was used to parallel leaf litter or insect galleries inside of logs that *K. marmoratus* inhabit during dry seasons (Taylor et al. 2008), while the larger arena mimicked the crab burrows that *K. marmoratus* frequently travel between on land (Taylor et al. 1995). Photos were captured every five seconds for two hours and body position in contact with the substrate was recorded. Photos were coded based on how fish were orientated (dorsal, ventral, lateral, dorsolateral, or ventrolateral). These values were then converted into time (%) to determine how long fish maintained skin-substrate contact per skin region.

**Series 2: Regional variation in skin permeability.** To determine if regional differences in permeability of the skin existed, *in vitro* experiments were carried out using isolated pairs of dorsal and ventral skins from the same individual in an Ussing chamber, following a protocol similar to that of Cooper et al. (2013). Briefly, fish were euthanized and the skin was carefully dissected. The skin was scraped free of muscle with a blunt instrument while being bathed in serosal saline solution. The serosal saline consisted of (mmol/L) 125 NaCl, 2 KCl, 1 MgSO₄, 5 NaHCO₃, 2
CaCl$_2$, 1.25 KH$_2$PO$_4$, and 5.55 glucose and the pH was adjusted to 7.5 (Cooper et al. 2013). The skin was then sandwiched between the two halves of the Ussing chamber (~0.6 mL each). The skin was then left for one hour to stabilize (Cooper et al., 2013) in saline (serosal side) or an environment bath (mucosal side; water of the appropriate salinity). The serosal solution was aerated with a humidified 0.5% CO$_2$/O$_2$ balance gas mix to mimic the relevant gaseous environment of the blood plasma, while the mucosal solution was aerated with humidified air. During the one-hour stabilization period, a dye test was performed using food colouring (Club House, London ON, Canada; water, propylene glycol, tartrazine, citric acid, and sodium benzoate) to ensure that there was no leakage. At the end of this stabilization period, the chamber was fully rinsed and replenished with the appropriate solutions. Triplicate samples of these solutions were collected from the chamber system to analyze and subtract as background. $^3$H$_2$O (Perkin Elmer Ltd., Ontario, Canada) was added to either the serosal or mucosal side (1 µCi/mL), mixed by gently pipetting, and triplicate samples (25 µL) were taken at $t = 0$ min from both the mucosal and serosal chambers. Preliminary experiments showed that $^3$H$_2$O flux across the skin was linear up to 30 minutes (Figure 1), so a 10-minute flux period was chosen. Immediately following this flux, the chamber was rinsed and replenished with unlabeled serosal/mucosal solutions. $^3$H$_2$O was then added to the opposite side and samples were taken as before. The direction of flux was randomized, providing both influx and efflux data on each skin without an effect of order. Following the bidirectional fluxes, an additional dye test was performed to ensure the skin had not been compromised during the experimental period. The serosal/mucosal saline samples were mixed with
aqueous counting scintillant (5 mL; 667 ml toluene, 333 ml Triton X-100, 4 g 2,5-diphenyloxazole, and 0.2 g 1,4-bis[5-Phenyl-2-oxazolyl]ben- zene; 2,2′ -p-
Phenylene-bis[5-phenyloxazole]; Sigma) and subsequently counted in a scintillation counter (Beckman Coulter LS6500 Multi-Purpose Scintillation Counter, California, USA).

**Series 3: Influence of salinity and air exposure on skin permeability.** To determine the potential effects of salinity and emersion on water transport through the skin, fish were divided into four experimental conditions: hypersaline water in water, hypersaline water in air, hyposaline water in water, and hyposaline water in air. These salinities are also ecologically relevant, as *K. marmoratus* populations have been found at and between these extremes in the wild (0.3 ‰: Wright, unpublished data; 45 ‰: Frick and Wright 2002). Fish were transferred from brackish water (15 ‰) to 0.3 ‰ or 45 ‰ and were acclimated for one week prior to experiments. These salinities represented osmotic differences of ~380.63 mOsm in the freshwater treatments and ~1373.27 mOsm in the hypersaline treatment. Air-exposed fish were emersed for 1 day in an aerial environment of high humidity as previously described (Ong et al. 2007). The air-exposure time was chosen because of the reported acute nature of claudin upregulation, (Kwong et al. 2013) and preliminary evidence of cutaneous claudin protein expression in *K. marmoratus* (F. Galvez, P. Wright and S. Kelly, unpublished data). An additional hypersaline emersion group with a seven-day aerial exposure period was also tested to determine the effects of prolonged air exposure. Following their respective acclimations, fish from each of the five
experimental treatments were euthanized and the skins were mounted in Ussing chambers, as described above.

Unidirectional water flux was measured through a dorsal section of the skin in either the mucosal-to-serosal (influx) or serosal-to-mucosal (efflux) direction. $^3$H$_2$O fluxes were sampled after 10 minutes as described above. Following the $^3$H$_2$O flux, each half of the chamber system was thoroughly rinsed with their respective solutions and replenished. The paracellular permeability marker $^{14}$C-PEG-4000 was added to the same side as $^3$H$_2$O had been previously added (1 µCi/mL). As before, background samples were collected, and the aerated chamber system was left for 1 hour. Final samples were taken ($t = 60$ min) and a dye test was used to ensure skin viability was maintained. An hour-long flux period was chosen for $^{14}$C-PEG-4000 due to the larger molecular mass and size of PEG-4000 compared to $^3$H$_2$O. Experiments on isolated tissues have also measured $^{14}$C-PEG-4000 over hourly periods previously (Genz and Grosell 2011; Wood and Grosell 2012).

To determine if the skin tissue held on to trace amounts of radionucleotide between influx and efflux experiments, digestion of skin and subsequent scintillation counter analysis was performed. Methods followed a modified protocol of Wood and Laurent (2003), where tissues were submerged in 1N HNO$_3$ and left at 60 °C for 1 day with occasional vortexing. Samples were then centrifuged at 500 x g for 5 minutes and 25 µL of supernatant was mixed with scintillation cocktail and counted.

Transepithelial potential (TEP) was also measured during flux experiments. Electrodes were constructed using 4% agar bridges, 0.5 mol/L KCl, and ferric chloride treated silver wires (A-M Systems, Washington, USA; Cooper et al. 2013).
The electrode bridges were placed on either side of the skin while a modified BNC cable was submerged in the KCl solution, completing the electrical circuit. The signal was recorded in mV using a pH meter (Orion 520, Thermo Fisher Scientific Inc., Massachusetts, USA).

To assess the distribution of claudin proteins in the skin, immunohistochemical staining was used. Staining procedures followed closely the methods of Chasiotis and Kelly 2008 and Bui and Kelly 2014. Briefly, tissues were dissected and fixed in Bouin’s fixative for 1-4 hours. Tissues were then dehydrated in a series of ethanol rinses (70-100%), followed by cleaning with xylene. Tissues were then embedded in paraffin and sectioned (5 µm) on a Leica RM 2125RT manual rotary microtome (Leica Microsystems Inc., Richmond Hill, ON, Canada). Sections were then placed on glass slides and deparaffinized with xylene. Tissue slides were rehydrated with an ascending series of ethanol rinses (100-50%) Heat-induced epitope retrieval (HIER) was accomplished by repeatedly heating samples while being bathed in sodium citrate buffer followed by washes in phosphate buffered saline (PBS). Samples were then outlined with a hydrophobic pen and quenched for 30 minutes in 3% hydrogen peroxide. Slides were rinsed with distilled water and washed with Kodak Photo-Flo 200 in PBS, TritonX-100 in PBS, and antibody dilution buffer (ADB) in PBS sequentially. Slides were incubated overnight in primary antibodies for Na⁺-K⁺-ATPase (NKA; mouse anti-NKA; 1:10 dilution) and claudin-10e (rabbit anti-Cldn; 1:10 dilution). Claudin 10e was chosen because of its localization in the skin of a teleost fish as well as its potential role in osmoregulatory function (Bui and Kelly 2014). Following this primary incubation, slides were rinsed
in PBS containing 0.05% Triton X-100 and probed for one hour at room temperature with secondary antibody for NKA and Cldn (fluorescein isothiocyanate (FITC)-labeled goat anti-mouse in 1:500 dilution and tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit in 1:500 dilution, respectively (Jackson ImmunoResearch Laboratories, Inc., USA). All antibodies were diluted in PBS containing 0.05% Triton X-100, 10% goat serum and 0.1% BSA. Slides were then rinsed in a series of PBS washes containing 0.05% Triton X-100 and Kodak PhotoFlo 200 before being carefully wiped and mounted with Molecular Probes ProLong Antifade (Invitrogen Canada Inc., Canada).

The relative influence of the osmotic gradient on water movement across _K. marmoratus_ skin was determined using a novel dye-dilution method. First, green food dye (Club House, London ON, Canada) solutions (1%) were made using either mucosal (hypersaline groups) or serosal (hyposaline groups) saline. This skins from fish previously acclimated to hypo- or hypersaline water (control, emersion: 1 day) were mounted in the Ussing chamber as previously described. In hyposaline-acclimation experiments, the serosal saline was replaced with the 1% food dye serosal saline solution. In hypersaline acclimation experiments, 45 ‰ seawater was replaced with the 1% food dyed hypersaline solution. Duplicate samples (100 µL) of equal volume were collected at t = 0 min from both sides of the Ussing chamber to maintain hydrostatic equilibrium across the skin. The system was then left for 1 hour to allow for water to move via the osmotic gradient across the skin. Following this time period, replicate well-mixed 100 µL samples were taken (t = 60 min). Food dye
solutions represented the environment into which water would move osmotically, thus diluting the colour and reducing the absorbance of the sample.

To determine the optimal wavelength at which to measure the absorbance of the diluted samples, a spectral scan of the 1% dye solution was conducted on a SpectraMax® 384 Plus microplate reader (Molecular Devices, Sunnyvale CA, USA). The scan yielded multiple peaks in absorbance with a suggested maximal absorbance value of 190 nm (Figure 2). Measuring samples at this wavelength, however, yielded a large degree of error. This was because 190 nm was just at the minimal sensitivity of the microplate reader. Therefore, 425 nm was used as the optimal absorbance wavelength and a standard curve of various dye dilutions consistently yielded a R² of >0.98 (Figure 3). The experimental 100 µL samples were loaded into a 96 well plate and read at 425 nm. Absorbance values obtained from the samples were compared to a standard curve to determine how much water moved osmotically across the skin over the one-hour period (Figure 4). These values were precise down to the µL range. This technique allowed me to measure true bulk water movement across the skin as a result of ecologically-relevant osmotic gradients.

Series 4: Influence of HgCl₂ on ³H₂O flux. Skins were mounted in the Ussing chamber setup as previously described. All HgCl₂ treated ³H₂O fluxes were performed in the mucosal to serosal (influx) direction. An initial ³H₂O flux was taken over 10 minutes as above, followed by a 30-minute incubation in HgCl₂ enriched solutions of serosal saline and hypersaline water (0.03 or 3 mol/L). These solutions were refreshed following the incubation and used for the post-incubation flux media.
This protocol allowed for the comparison of pre- and post-incubation $^3$H$_2$O flux rates of the same skin to quantify the effect of HgCl$_2$ treatment.

Calculations and Statistical Analysis

Analytical procedures to calculate $^3$H$_2$O flux rate ($J_{^3H2O}$) followed the methods and formulae detailed in Pärt et al. (1999). The $^3$H$_2$O flux formula is represented as below:

$$J_{^3H2O} = \frac{\Delta \text{dpm}}{T \times A \times SA}$$  \hspace{1cm} (1)

where $\Delta \text{dpm}$ is change in disintegrations per minute, $T$ is time (s), $A$ is area (cm$^2$), and $SA$ is specific activity. Specific activity was calculated using Equation 2 below:

$$SA = \frac{(dpm_i + dpm_f)}{2 \times Volume}$$  \hspace{1cm} (2)

where the initial ($dpm_i$) and final ($dpm_f$) disintegrations per minute values per sample volume (µL) are averaged and divided by sample volume. A PEG permeability (cm/sec) formula similar to Gilmour et al. 1998 is detailed below:

$$P - PEG = \frac{\Delta \text{dpm} \times Volume}{T \times A \times SA \times 3600}$$  \hspace{1cm} (3)

where $\Delta \text{dpm}$ is change in disintegrations per minute, Volume is sample volume, $T$ is time (h), $A$ is area (cm$^2$), $SA$ is specific activity as calculated in Equation 2, and 3600 to convert hours to seconds.
Data were analyzed using either a two-way repeated measures analysis of variance (ANOVA), one-way ANOVAs, or when appropriate Student’s T-tests in SigmaPlot 11 (Systat Software, San Jose, CA).

RESULTS

Emersion Behaviour

Fish exposed to an aerial environment for one day spent significantly more time exposing their ventral/ventrolateral skin surfaces to the moist substrate than dorsal, dorsolateral or lateral regions. This was true for both the 15 cm$^3$ emersion environment ($P<0.004$; Figure 4A) as well as the 700 cm$^3$ emersion environment ($P=0.001$; Figure 4C). In the larger environment, the ventral skin was exposed 42% of the time, while dorsal and dorsolateral regions were never exposed to the moist surface. Fish most often maintained substrate contact of the ventral region while simultaneously exposing their lateral side to a wall of the container (deemed “ventrolateral”). This ventrolateral body orientation occurred ~57% of the time. In the more confined environment (15 cm$^3$), the ventral and ventrolateral surfaces were most often exposed to the moist substrate, followed by the dorsal, lateral, and finally the dorsolateral. When dorsal and ventral categories are combined (lateral data omitted), greater ventral substrate contact was apparent in both emersion environments ($P<0.004;0.001$; Figure 4B,D respectively).
Regional Variability in Skin Water Flux

$^{3}\text{H}_2\text{O}$ flux across the skin of *K. marmoratus* showed no regional variability (Figure 5). Dorsal and ventral skins from the same individuals were not significantly different ($P=0.680$). $^{3}\text{H}_2\text{O}$ influx and efflux rates across the same skins were also not significantly different ($P=0.729$).

Effects of Salinity and Acute Air Exposure on Skin Permeability

Water flux. Salinity had an effect on water influx rates in control fish in water. Freshwater-acclimated fish had a higher rate of $^{3}\text{H}_2\text{O}$ influx compared to the hypersaline-acclimated fish ($P=0.026$). There was, however, no effect of one-day of air exposure in either salinity ($P=0.204$; Figure 6A). Water efflux rates were not significantly affected by salinity ($P=0.207$), or one-day of emersion ($P=0.281$; Figure 6B).

PEG permeability. Inward PEG permeability (mucosal to serosal $^{14}\text{C}$-PEG-4000 flux) was independent of both salinity ($P=0.081$) and acute air exposure ($P=0.187$; Figure 7A). As with inward PEG permeability, outward PEG permeability (serosal to mucosal $^{14}\text{C}$-PEG-4000 flux) was independent of both salinity ($P=0.220$) and 24-hour emersion ($P=0.210$; Figure 7B)

Effects of Chronic Air Exposure on Skin Permeability

Water flux. The emersion period was extended to more closely match the protocol of LeBlanc et al. (2010) who examined whole body water flux in *K. marmoratus*. Water influx was significantly higher in fish acclimated to hypersaline
water (7 days) and then exposed to air for 7 days ($P=0.031$; Figure 8A) compared to control fish in water. There was no significant difference in $^3$H$_2$O influx between 45‰ fish in water and fish that had been air exposed for only one day ($P=0.906$). There were no significant effects of either acute or prolonged emersion on $^3$H$_2$O efflux rates (Figure 8B).

**PEG permeability.** Both inward and outward PEG permeability were found to be independent of air exposure of seven days (Figure 8C, D). There was no significant difference in $^{14}$C-PEG-4000 influx ($P=0.115$) or $^{14}$C-PEG-4000 efflux ($P=0.620$) between fish in water or fish air exposed for 7 days.

*The Effect of Salinity and Acute Emersion on Bulk Osmotic Flow*

Osmotic water flux (mucosal to serosal) did not differ between freshwater-acclimated fish in water and those air-exposed for one day ($P=0.335$; Figure 9). In freshwater fish, the osmotic influx was between 11 and 16 µL over a one-hour period. In hypersaline-acclimated fish, osmotic water flux (serosal to mucosal) significantly decreased in fish exposed to air for 1 day ($P=0.042$; Figure 10). In the control group in water, ~21 µL of water was lost across the skin, but this flux decreased to ~9 µL after one day out of water.

*IHC expression*

Qualitatively, both hypersaline and freshwater-acclimated fish showed general staining of Cldn-10e and NKA in the epidermis and underlying muscle tissue. Cldn-10e staining appeared more prominent in the muscle tissue than in the skin, appearing
more diffuse in the skin. NKA-positive cells were irregular in shape and can be seen in the skin near mucous cells. Although not quantifiable, NKA-positive cell abundance and cell size appeared greater in hypersaline-acclimated fish (Figure 11).

*Transepithelial Potential (TEP)*

Hypersaline-acclimated fish exhibited a more negative TEP than freshwater-acclimated fish.

*HgCl₂ inhibition of aquaporins*

³H₂O influx rates across isolated *K. marmoratus* skins were not significantly affected by 30-minute incubation in either 0.03 or 3 mmol/L HgCl₂ (*P*=0.509 n=5, 0.417 n=3 respectively; data not shown). Prior to incubation in 0.03 mmol/L HgCl₂, influx was 0.42 µL/cm²/min (± 0.09), while post-incubation influx was 0.49 µL/cm²/min (± 0.13). Prior to incubation in 3 mmol/L HgCl₂, influx was 0.21 µL/cm²/min (± 0.04), while post-incubation influx was 0.33 µL/cm²/min (± 0.12).
Figure 1. Time series of $^3$H$_2$O movement (dpms) across isolated *K. marmoratus* skin, indicating linear transfer of $^3$H$_2$O over 10 minutes. All data are means ± SEM (N=4).
$R^2 = 0.9275$
**Figure 2.** Spectral scan of 1% food dye solution showing peaks of optical density (absorbance) with a suggested optimal wavelength at 190 nm. This suggested reading value is indicative of the minimum sensitivity of the machine, rather than an optimal reading value. A more optimal peak in absorbance at 425 nm was chosen for dye-dilution sample reading, resulting in minimal error.
**Figure 3.** Standard curve of absorbance values from diluted 1% green food dye solution. Samples were diluted using 12.5 µL of pure water and all samples were read at a 425 nm wavelength. Absorbance values from bulk osmotic flow experiments were used to obtain a volume of water moved via the osmotic gradient using similar standard curves.
Absorbance = -0.0125x + 1.3103

\[ R^2 = 0.9993 \]
Figure 4. Behavioural observation of intact *K. marmoratus* held out of water for 1 day. The time (\%) spent with various skin regions in contact with the moist filter paper substrate in 15 cm$^3$ polystyrene wells (A, combined data B) or 700 cm$^3$ plastic containers (C, combined data D). All data are means ± SEM (N=5: bracketed values). Groups that do not share a common letter are significantly different as determined by One Way ANOVAs, while a * symbol indicates significant differences as a result of a paired T-test ($P<0.004$: 15 cm$^3$; $P<0.001$: 700 cm$^3$).
Figure 5. $^{3}\text{H}_2\text{O}$ influx and efflux rates (µL/cm$^2$/min) across the isolated dorsal and ventral skins of *K. marmoratus* acclimated to brackish water (15‰). All data are means ± SEM (N=7: bracketed values). Data analyzed using a Two Way ANOVA ($P<0.05$) yielding no statistical significance.
Influx

Efflux

$^{3}\text{H}_{2}\text{O Flux (µL/cm}^2\text{/min)}$

Dorsal

Ventral

(7)

(7)

(7)

(7)

$\square$ Dorsal

$\blacksquare$ Ventral

0.5

0.4

0.3

0.2

0.1

0

(7)

(7)
Figure 6. $^3$H$_2$O influx (A) and efflux (B) rates in the isolated skin of *K. marmoratus* acclimated to 0.3 and 45 ‰ water for 7 days and either kept in water or air exposed for 1 day. All data are means ± SEM (N=6-11: bracketed values). An * symbol indicates statistical significance as a result of a two-way ANOVA and Holm-Sidak post hoc test ($P<0.05$).
A. 

$^{3}\text{H}_2\text{O}$ Influx (µL/cm$^2$/min) 

- Water
- Air

B. 

$^{3}\text{H}_2\text{O}$ Efflux (µL/cm$^2$/min) 

- Water
- Air
Figure 7. PEG permeability (P-PEG, cm/s) of the skin to $^{14}$C-PEG-4000 influx (A) and efflux (B) in the isolated skin of *K. marmoratus* acclimated to 0.3 and 45 ‰ water for 7 days and either kept in water or air exposed for 1 day. All data are means ± SEM (N=4-11: bracketed values). All data were analyzed using a two-way ANOVA ($P<0.05$).
Figure 8. $^3$H$_2$O flux (A: influx, B: efflux) and PEG permeability (P-PEG, cm/s) of the skin to $^{14}$C-PEG-4000 flux (C: influx, D: efflux) in the isolated skin of *K. marmoratus* acclimated to 45‰ water for 7 days and either kept in water or air exposed for 7 days. All data are means ± SEM (N=5-11: bracketed values). An * symbol indicates statistical significance as a result of a t-test ($P<0.05$).
A. 

\[ ^{3}\text{H}_2\text{O} \text{ Influx (µL/cm}^2\text{/min)} \]

B. 

\[ ^{3}\text{H}_2\text{O} \text{ Efflux (µL/cm}^2\text{/min)} \]

C. 

\[ \text{P-PEG}_{\text{influx}} \text{ (cm/s} \times 10^{-6}) \]

D. 

\[ \text{P-PEG}_{\text{influx}} \text{ (cm/s} \times 10^{-6}) \]
Figure 9. Bulk osmotic flow of water across the isolated skin of *K. marmoratus* (mucosal to serosal) acclimated to 0.3 ‰ water for 7 days followed by 24-hours of water or air exposure. Data are means ± SEM (N=5: bracketed values) and were analyzed using a t-test.
Osmotic Water Influx (µL)

Water: (5) 10 µL
Air: (5) 15 µL

0.3 %
**Figure 10.** Bulk osmotic flow of water across the isolated skin of *K. marmoratus* (serosal to mucosal) acclimated to 45 ‰ water for 7 days followed by 24-hours of water or air exposure. All data are means ± SEM (N=4-5: bracketed values). An * indicates statistical significance as a result of a t-test (*P*<0.05).
Osmotic Water Efflux (µL)

Water

Air

(4)

(5)

45 %

*
Figure 11. Representative images of fluorescein-stained (FITC – green) and tetramethylrhodamine-stained (TRITC – red) skins of *K. marmoratus* acclimated to their respective salinities for 7 days and emersion time was 1 day. A: 0.3 ‰ in water, B: 0.3 ‰ air, C: 45 ‰ in water, D: 45 ‰ air. FITC staining localizes to Na⁺/K⁺-ATPase rich cells while the TRITC staining is specific for claudin 10e. 20x magnification; scale bar = 100µm. S: Skin; M: Muscle; Arrowhead: mucous cells.
Table 1. Transepithelial potential (TEP) data (mV) from the isolated skin of *K. marmoratus* acclimated to 0.3 and 45 ‰ water for 7 days and either kept in water or air exposed for 1 day. All data are means ± SEM (N=6-12).
<table>
<thead>
<tr>
<th>Salinity (‰)</th>
<th>Emersion (days)</th>
<th>TEP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0</td>
<td>-5.26 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>-5.79 ± 0.5</td>
</tr>
<tr>
<td>45</td>
<td>0</td>
<td>1.68 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.31 ± 0.28</td>
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DISCUSSION

Cutaneous water movement has been identified in other amphibious organisms, yet this study is the first to quantify water flux dynamics in isolated skin preparations of fish out of water. Unlike previous studies in amphibians and lungfish, I found no regional differences in \(^3\)H\(_2\)O flux. I have shown that acclimation to a hypersaline environment induced changes in skin permeability, decreasing water influx across the skin. I also developed a novel technique for assessing the bulk osmotic flow through the skin \textit{in vitro}. This technique is highly sensitive and I was able to quantify \(\mu\)L differences in water movement over an hour due to an osmotic gradient. It appears that exposure to air alters the properties of the skin. Acute emersion (1 day) in air induced a decrease in bulk osmotic water loss in hypersaline-acclimated fish, whereas more prolonged emersion (7 days in air) increased \(^3\)H\(_2\)O influx. These data suggest that to maintain water homeostasis on a hypersaline substrate on land, \textit{K. marmoratus} increases water influx and decreases water efflux across the skin. Such changes would help maintain water balance during the dry season when water sources evaporate completely or become increasingly hypersaline. \textit{K. marmoratus} are known to enter rotting mangrove logs in the dry season and may spend weeks inside aerial chambers hollowed out by insects (Taylor et al. 2008). Along with metabolic water, cutaneous water transport may be a critical source of water, as they are unable to drink to replace fluid loss.

Regional Water Permeability and Emersion Behaviour

In this study, I expected that \textit{K. marmoratus} would exhibit regional differences in skin permeability, having a water permeable ventral surface to allow for water uptake
during emersion. I showed that paired $^3$H$_2$O fluxes across isolated dorsal and ventral skins from the same individual did not differ. The lack of regional differences does not support previous studies in amphibious fishes and amphibians (Wilkie et al. 2007, McClanahan and Baldwin 1968; Bentley and Main, 1972; Bentley and Yorio 1976; Yorio and Bentley 1977). The ventral pelvic patch in anuran amphibians facilitates water uptake across the skin from terrestrial substrates during periods away from water and is 10-20 times more permeable to water than the dorsal skin (Yorio and Bentley 1977; Bentley and Main 1972). In the only other study of cutaneous water balance in a fish out of water, Wilkie et al. (2007) also found an appreciable flux of $^3$H$_2$O across the ventral surface in African lungfish *P. dolloi*. Here, it was assumed that $^3$H$_2$O was moved between internal and external environments solely through ventral surface contact. Equivalent fluxes across both dorsal and ventral body regions in *K. marmoratus* suggest that an increased ventral permeability is not a shared trait among all amphibious fishes. The lungfishes are considered basal relatives to modern day tetrapods. It is possible that this enhanced ventral permeability for water exchange on land is a trait that appeared after the divergence of the teleost fishes.

My experiments of regional variability were carried out on fish acclimated to control conditions i.e., a brackish (15‰) aquatic environment. It is possible that increased water permeability across the ventral surface is a response to emersion. Further experimentation would be needed to assess whether ventral permeability increases only after fish have been chronically air exposed. In the current study, the size of the available fish limited experimentation on both dorsal and ventral skins from the same individual. Concerted effort is required to grow larger fish in larger containers in future work.
In preliminary observations, I found that *K. marmoratus* often exposed the ventral surface to a moist substrate during emersion. In follow up experiments, behavioural analysis of body position out of water further supported this observation. *K. marmoratus* were most often found “sitting” on the ventral and ventrolateral skin. This finding was true for both smaller confined chambers (15 cm$^3$) and larger more open chambers (700 cm$^3$). The lack of regional variability in skin permeability implies that greater ventral/ventrolateral skin-substrate contact is a result of one or a combination of other physiological and/or behavioural factors.

The finding that *K. marmoratus* spent more time on their ventral surface can be explained in different ways. Selection of an upright body orientation may be due to a righting reflex, which animals use to maintain proper body orientation. More simply, sitting on the ventral surface may prevent obstruction of the dorsally set eyes in this species. Ventral/ventrolateral-substrate contact may also be support for thigmotaxis in *K. marmoratus* as a predator avoidance technique. Thigmotaxis, or wall-following behavior, has been noted in multiple fish species (Champagne et al. 2010; Sharma et al. 2009). When fish are introduced to a novel or stressful environment, they show a preference for the periphery of the arena. This diminishes their “domain of danger” (Hamilton 1970) or surrounding area from which a predator could attack. This explanation for greater ventral/ventrolateral substrate contact is supported by observational data of *K. marmoratus* readily seeking shelter upon emersion (Taylor et al. 1990). Minimizing surface area for evaporative water loss may also be an explanation for the greater ventral/ventrolateral exposure to the substrate. *K. marmoratus* communally inhabit moist rotting logs that have been galleried by insects (Taylor 2008), providing maximal contact,
probably along dorsal and ventral surfaces, with a moist substrate. This may be a strategy to limit exposed surface area and minimize evaporative water loss during emersion.

*Unidirectional Water Flux*

*Methodological issues.* Two problems that researchers encounter when measuring permeability via unidirectional fluxes in *in vitro* systems have been poor mixing (unstirred boundary layers) and solute/solvent back-flux (Isaia 1984, Loretz 1979). Unstirred boundary layers here are regions of laminar flow adjacent to the skin where molecules move only by diffusion (Dainty and House 1966a,b). If present, unstirred boundary layers would gradually increase the diffusional distance of $^3$H$_2$O across the skin. The formation of unstirred layers significantly decreased diffusional water flow in isolated frog skins (Dainty and House 1966a,b). Solute/solvent back-flux occurs when interacting solutions are in equilibrium, and the movement of molecules is bidirectional. Both may arise in a closed system, and could artificially decrease measured flux across a membrane. In the current study, both of these potential problems were minimized. Constant aeration of the Ussing chamber system provided adequate mixing and prevented the formation of significant boundary layers. Back-fluxes were minimized by using a short, 10-minute flux period, where water flux rates were in the linear part of the curve (Figure 1).

Rates of influx and efflux were performed on different individuals to minimize the potential contamination from leftover radioactivity within the Ussing chamber system. When skin tissue was dissolved and counted after the initial 10-minute flux, it had significantly higher radioactivity (dpms) than control skins that had not been used in
flux experiments (data not shown). It is possible that residual radioactivity within the skin could artificially increase rates of flux in subsequent experiments. This was avoided by using separate individuals for influx and efflux measurements.

Effect of Salinity. Regardless of salinity acclimation, overall rates of water flux across the isolated skin of *K. marmoratus* were comparable to amphibians. A study of four amphibian species reported that rates of inward water transport across ventral skins were between 0.3 and 1.3 µL/cm²/min, whereas across dorsal skins the values were between 0.0016 and 0.1 µL/cm²/min. (Yorio and Bentley 1977). My values were in the same range as the ventral skin of amphibians, but at the lower end (0.15-0.50 µL/cm²/min). Slightly lower permeability of *K. marmoratus* skin is possibly due to the presence of scales in the dermis, retained in the isolated skin preparations in this study. To my knowledge, no other studies have examined rates of water flux across isolated fish skin, thus little comparison can be made.

In the current study, salinity significantly affected rates of $^3$H$_2$O influx. When *K. marmoratus* were acclimated to hypersaline conditions, water influx across the skin was significantly reduced relative to fish acclimated to hyposaline conditions. Intact Mozambique tilapia, *Oreochromis mossambica*, acclimated to double strength seawater had $^3$H$_2$O turnover rates one third that of freshwater-acclimated fish, suggesting a greater permeability for water uptake in freshwater-acclimated fish (Potts et al. 1967). When a number of freshwater, marine, and euryhaline aquatic fish species were compared, influx of $^3$H$_2$O was higher in all freshwater compared to saltwater species (Evans 1969b). Motais et al. (1969) also found that both *A. anguilla* and the European flounder *Platichthys flesus* acclimated to freshwater had greater osmotic and diffusional inflow of
water than saltwater-acclimated individuals (Motais et al. 1969). Thus, acclimation to hyposaline conditions appears to induce an increase in water permeability in fishes, while hypersaline acclimation results in a decrease in water permeability. My work identifies the specific contribution of the skin in reducing osmotic water permeability in hypersaline conditions, a strategy that may be important in preventing osmotic water loss across the skin.

$^3$H$_2$O efflux rates in isolated skin were not affected by salinity acclimation (1 or 7 days) in my study. In intact *K. marmoratus*, $^3$H$_2$O efflux was significantly lower (two- to threefold) in fish acclimated to 45 ‰ relative to 0.3 ‰ for 1 month (LeBlanc et al. 2010). It is highly possible that in these experiments, whole body water efflux included water elimination by the kidneys, as freshwater fish are known to excrete relatively large volumes of urine. In other studies in fish where whole body $^3$H$_2$O flux was determined, $^3$H$_2$O fluxes were identical across environmental salinity (*Xiphister atropurpureus* Evans 1967; *Pholis gunnellus* Evans 1969a). As well, in isolated anuran amphibian skin, external salinity had no effect on water efflux across the skin (Koefoed-Johnsen and Ussing 1953). As with other fish and amphibian species, outward water flux across the isolated *K. marmoratus* skin remains relatively constant regardless of salinity.

Adjustments to osmotic permeability have been attributed to both the transcellular and paracellular routes in other animals. A 97% reduction in aquaporin 3 transcript abundance after saltwater acclimation in the European eel *A. anguilla* (Cutler and Cramb 2002) has been suggested as a cause of the 11-fold reduction in osmotic permeability after saltwater acclimation in this species (Motais and Isaia, 1972). However, I found no effect of HgCl$_2$ on $^3$H$_2$O water flux, suggesting that water may move across the skin.
through alternative transcellular pathways or via the paracellular route. Salinity acclimation has also been found to influence the expression of claudins 3, 4, 6, 10d, 10e, 27a, and 30 variably. Changes in expression of claudins have been hypothesized to be important in osmoregulation in fishes (Tipsmark et al. 2008a; Tipsmark et al. 2008b, Bui and Kelly 2014). The IHC images of claudin in *K. marmoratus* skin did not reveal any obvious differences in expression due to salinity or emersion acclimations. More work is needed to quantify any possible molecular changes in *K. marmoratus* skin using Western blot methodology.

*Effect of emersion.* My hypothesis predicted that hypersaline acclimation with emersion would result in an increase in water influx, while hyposaline-acclimated fish would have $^3$H$_2$O influx rates similar to immersed fish. The data provide support for both of these predictions. $^3$H$_2$O influx rates of both hypo- and hypersaline-acclimated fish were not influenced by 1 day of emersion, while prolonged emersion (7 day) resulted in a significant increase in cutaneous $^3$H$_2$O influx in fish exposed to a hypersaline substrate. This finding suggests that *K. marmoratus* has some adaptive mechanism to allow for water uptake in an environment with compounded stresses of osmotic and evaporative water loss. These results suggest that water is moving actively, occurring “uphill” against the osmotic gradient. Active water transport through amphibian skin was first suggested by Reid (1892) after observing “the passage of fluid across the living skin by virtue of its own unaided activity”. Other studies of water movement through amphibian skin using a similar experimental setup as the current study have found similar results (Keofoed-Johnsen and Ussing, 1958; House 1964). Uphill water movement has been reported in the intestines of marine teleosts and is thought to be a means to absorb pure
water from imbibed seawater (Curran, 1960; Skadhauge 1969, Genz et al. 2011; Whittamore 2012). It has been proposed that ion uniporters and active ion transporters, such as Na⁺/K⁺-ATPase are the vectors of uphill water movement (Zeuthen and Stein, 1994; Zeuthen 2010). This idea is supported by the interdependency observed between NaCl and water movement across bullfrog skin (Huf et al. 1951; as cited in Koefoed-Johnsen 1953). LeBlanc et al. (2010) observed significantly larger Na⁺/K⁺-ATPase rich-cells in the epidermis of *K. marmoratus* after hypersaline acclimation and 7 days of emersion, in agreement with IHC images in this study (see below). Thus, *K. marmoratus* may use active ion transporters in the skin to move water against the gradient to maintain water balance.

My hypothesis predicted that hypersaline acclimation would result in a decrease in water efflux, while hyposaline-acclimated fish would have ³H₂O efflux rates comparable to immersed fish. Unlike ³H₂O influx, short or long-term emersion failed to induce significant differences in water efflux in either hypo- or hypersaline-acclimated fish. In whole body water efflux experiments in *K. marmoratus*, 9 days out of water on a moist hypersaline surface resulted in a marked decrease in ³H₂O efflux (LeBlanc et al. 2010). In the freshwater African lungfish *P. dolloi* and *P. annectens*, prolonged air exposure from 6-8 months also resulted in a lower whole body water efflux (Wilkie et al. 2007; Patel et al. 2009). Whole body flux experiments, however, do not provide specific information regarding cutaneous exchange. Although ³H₂O efflux was unaltered by emersion in *K. marmoratus* experiments, bulk osmotic loss of water under similar conditions was decreased. The reason for the discrepancy between ³H₂O efflux and bulk loss of water (efflux) is unknown (see below).
**Bulk osmotic flow**

I designed a novel technique to measure the bulk flow of water across the skin of *K. marmoratus*. In freshwater acclimation experiments, the serosal saline was replaced with a 1% green food dye serosal saline solution. In hypersaline acclimation conditions, 45‰ seawater was also replaced with a 1% green food dye hypersaline solution. Well-mixed samples from both sides of the Ussing chamber were taken at t = 0 and t = 60. The dyed solutions were hyperosmotic to the un-dyed solutions, thus gaining water osmotically and becoming dilute over the hour. Absorbance values obtained from the samples were compared to a standard curve to determine how much water moved osmotically across the skin in the one-hour period. These values were precise in the µL range.

Previously, water flow across isolated tissues has been measured gravimetrically (House and Green 1965; Loeschke, Bentzel, and Csaky, 1970; Collie and Bern 1982; Cornell et al. 1994; Scott et al. 2008; Wood and Grosell 2012). One disadvantage of this method is the potential mass gain by the cellular absorption of water across the entire tissue. Here, the tissue may weigh more without any transmembrane movement of water. In a similar manner, incomplete drying of the gut sac or trapped water in either sealed end of the preparation may artificially skew perceived water movement. In my new technique, these two sources of error are eliminated. Gravimetric analysis also requires tedious attention to leaks and frequent handling of the preparation. My technique for measuring osmotic water flow requires minimal handling, and the presence of any leaks becomes known immediately with appearance of dye in the un-coloured chamber.
Bulk water flow across isolated skin preparations from freshwater- and hypersaline-acclimated fish responded differently in fish previously exposed to air. One day of air exposure in 45 ‰ but not 0.3 ‰ acclimated fish resulted in a significant reduction in the bulk water flow across the skin compared to controls. Overall water flow was reduced by ~97 µL/cm²/hr to the environment or a ~43 % decrease. Thus, hypersaline acclimation in concert with short-term air exposure is likely an osmotically stressful environment for these amphibious fish. Studies of amphibians during metamorphosis found that integument permeability decreases as they adopt a more terrestrial life. Reductions in ³H₂O turnover rates (internal to external media) during metamorphosis have been discovered in the freshwater African clawed frog *Xenopus laevis* (Schultheiss, Hanke and Maetz 1972) and green frog *Rana clamitans* (Mackey and Schmidt-Nielsen 1969 as cited in Schultheiss, Hanke and Maetz 1972). These findings in amphibians, along with a decreased bulk water efflux in *K. marmoratus* imply that entering a dehydrating environment results in a decrease in skin permeability, potentially to conserve water.

It is surprising that a reduction in bulk osmotic water efflux after 1 day of emersion was not mirrored in the ³H₂O efflux data, where there was no significant effect of acute emersion. Bulk osmotic water flux experiments were preformed immediately after mounting isolated skins in the Ussing chamber, while ³H₂O fluxes were given 1-hour to stabilize prior to sampling. It is possible that this stabilization period causes air-exposed skins to revert to their prior aquatic permeability, resulting in the lack of difference in osmotic permeability experiments. Preliminary experiments, however, showed no significant difference between ³H₂O fluxes beginning at t = 0 or t = 60
minutes. The bulk osmotic water efflux represents net flux across the skin over 1 hour, whereas $^3$H$_2$O efflux is only in one direction over 10 minutes. Also, $^3$H$_2$O flux experiments only allow for the measurement of the tritium-labeled water molecules that move across the skin, while the bulk osmotic water flux experiments allow for the measurement of all transported water molecules. Further experiments are required to appropriately compare the data from these two techniques.

One day of air exposure in hyposaline-acclimated fish did not result in a significant difference from the control group in water. Therefore, freshwater-acclimated fish deal with a gain of water across the skin from the external environment of ~88-119 µL/cm$^2$/hr. To maintain water balance, the kidney likely voids this water load. The lack of change in freshwater bulk osmotic flow agrees with $^3$H$_2$O and $^{14}$C-PEG-4000 flux data in hyposaline fish, as there was no observed effect of short-term emersion.

**Transepithelial Potential**

TEP values collected in the current study fall in line with previous measurements in isolated *K. marmoratus* skins. Cooper et al. (2013) recorded TEP values in isolated *K. marmoratus* skins of -4.19 mV and 1.45 mV for 1 and 15 ‰ respectively. The values presented here follow this trend, with fish acclimated to 0.3 ‰ water having a more negative TEP value of -5.26 mV and hypersaline-acclimated fish having a TEP of 1.68 mV. These values are reflective of the difference in membrane potential as a result of ionic gradients across the skin. Here, a greater ionic gradient would generate a greater TEP and vice versa. These measurements were used as a secondary test of membrane
viability, as a torn skin would allow for mixing of the serosal saline and saltwater to yield a TEP of 0 mV.

**Paracellular permeability**

$^{14}$C-PEG-4000 flux. The polyether compound polyethylene glycol 4000 was used to assess paracellular permeability changes. Studies examining the movement of PEG largely use cultured tissues (Wood, Gilmour, and Pärt 1998; Van Itaille et al. 2008; Chasiotis, Wood, and Kelly 2010). In my study, PEG permeability (P-PEG) calculated from $^{14}$C-PEG-4000 efflux across *K. marmoratus* skin was between $8.14 \times 10^{-7}$ and $5.85 \times 10^{-5}$ cm/s, while PEG permeability from $^{14}$C-PEG-4000 influx was between $7.92 \times 10^{-7}$ and $2.40 \times 10^{-5}$ cm/s. PEG permeability (inward and outward) of the mummichog *F. heteroclitus* intestine was in the lower range of values I measured in *K. marmoratus* skin ($1-3 \times 10^{-7}$ cm/s; Wood and Grosell 2012). PEG permeability of the isolated skin of *K. marmoratus* was not significantly affected by salinity or air exposure. These data suggest that the paracellular route remains relatively unaltered with exposure to strong osmotic gradients and the stress of evaporative water loss. It has been found previously that hypersaline acclimation (35 ‰) decreased tight junction “leakiness” (leak conductance) in the opercular epithelium of *O. mossambicus* (Kultz and Onken 1993). As well, freshwater acclimation has been found to promote an increased PEG flux, or loosening of the paracellular space in cell cultures of rainbow trout *Oncorhynchus mykiss* gills (Gilmour et al. 1998; Wood et al. 1998). In some of my experiments sample size was relatively low (n=4). Greater sample size may reveal a clearer relationship in *K. marmoratus* skin. It has been proposed, however, that there is no comparable relationship
between PEG flux and water movement, supported by a lack of alteration to PEG permeability across various osmotic pressure experiments (Wood and Grosell 2012). The authors suggested that there is a separation between the paracellular water pathway and the paracellular PEG pathway.

**Immunohistochemistry.** Although only qualitatively examined, the skin of hypersaline-acclimated fish showed notably more Na\(^+/\)K\(^+-\)ATPase-positive cells in the epidermis than in freshwater-acclimated fish. This finding is consistent with the work of LeBlanc et al. (2010) on *K. marmoratus* skin, and other studies that show Na\(^+/\)K\(^+-\)ATPase cells increasing in size after hypersaline acclimation (Uchida et al. 2000; McCormick et al. 2003; Scott et al. 2008). As discussed above, skin Na\(^+/\)K\(^+-\)ATPase-positive cells may also provide a route for active water movement inward against the osmotic gradient through water cotransport. Claudin 10e staining in the skin was quite diffuse, only allowing for the conclusion that Cldn10e is present in the skin of *K. marmoratus*. Tight junction proteins, including Cldn10e, may be important in mediating the decreased water influx and decreased bulk flow of water observed in hypersaline acclimated fish. Recently, hypersaline acclimation has been found to increase Cldn10e mRNA expression in the skin of *T. nigroviridis* (Bui and Kelly 2014). As well, unpublished data has identified claudins 6, 10d, and 10e in the gills and skin of *K. marmoratus* through Western blot analysis (Galvez, Bui, Wright, and Kelly unpublished). In addition, a ‘honeycomb-like’ staining pattern was observed using whole mount confocal microscopy. This pattern localizes these claudin proteins to the junctions between neighbouring cells, and hints at their potential role in the tightening/loosening of
the paracellular space. Further studies are required to understand the role of claudin proteins in skin permeability in *K. marmoratus*.

**Summary and Conclusions**

My data provide evidence that salinity and emersion alter the permeability of *K. marmoratus*’ skin to water. In hypersaline-acclimated fish, bulk osmotic efflux was found to significantly decrease after only 1 day in air, while $^3$H$_2$O influx was increased after 7 days in air. These findings together suggest that *K. marmoratus* limit water loss to an osmotic gradient and increases water uptake, potentially through water cotransport across the skin. As well, hypersaline acclimation resulted in a lower $^3$H$_2$O influx across the skin, further supporting a reduction in skin permeability in an osmotically dehydrating environment. It is unclear whether skin permeability is altered through changes in the transcellular or paracellular route, as $^{14}$C-PEG-4000 fluxes were unaffected by both salinity and emersion acclimations and HgCl$_2$ had no effect on water flux. Overall my findings suggest that *K. marmoratus* alter skin permeability to maximize water uptake and minimize water loss while emersed in hypersaline conditions.
GENERAL DISCUSSION

Future directions for this work should address the role of water cotransport through active ion transporters and uniports on overall water balance. It is currently unknown what degree of total water movement can be attributed to secondary active transport. This form of transport is responsible for ~30% of total water movement in a mammalian intestinal lumen (Zeuthen et al. 2001), and may be an important mechanism of osmoregulation in amphibious fish. Illustrating an interdependence between ion and water fluxes, as well as linking changes in water flux to changes in uniport/ion transport protein abundance in the skin may link water transport and ion regulatory proteins.

Hormonal control of water balance is another area of research that remains unexplored in *K. marmoratus*. Neurohypophysial hormones such as vasotocin as well as the steroid hormones aldosterone and cortisol have been recognized to affect membrane permeabilities and subsequent movement of water (Goldenberg and Warburg 1982; Alvarado and Johnson 1966; Bentley and Yorio 1976; Yorio and Bentley 1978; Pärt et al. 1999; Hasegawa 2003; Chasiotis, Wood, and Kelly 2010). Assessing hormone concentrations and hormone receptor distribution and their influences on skin permeability could shed light onto how water is managed as a whole in amphibious organisms out of water. Circulating concentrations of hormones in the blood should first be assessed following salinity and emersion acclimations. Intraperitoneal injections of hormones and subsequent osmotic and/or diffusional water permeability measures (via the isolated skin technique) would help us to understand the influence of hormones on skin permeability specifically. If injection was not possible in the relatively small *K. marmoratus*, hormone-enriched baths may be adequate for hormone delivery.
A quantification of drinking rate and urine production in *K. marmoratus* would be valuable in examining whole body water balance and how these organisms acclimate to hyper-/hyposaline acclimation. This may indicate how severe the osmotic challenge is for hypersaline fish out of water and away from a drinking source. Drinking rate could be determined by quantifying the ingestion of an inert marker such as yttrium oxide as used in digestibility studies (Storebakken et al. 1999; Hung et al. 1999). Following salinity and/or emersion acclimation, fish would be left to drink in a yttrium oxide labeled bath. Higher drinking rate would be indicated by greater imbibed yttrium/ytterbium in the gut, quantified using ICP spectrophotometry. Using a radiolabelled impermeant would allow for scintillation counting of the gut contents for quantification. To quantify urine production, catheterization of large fish may be necessary.

More intensive investigation of regional variability could also be conducted. In the current study, dorsal and ventral variability was only examined in fish acclimated to 15 ‰ in an aquatic environment. It is still unknown if acclimation to 0.3 or 45 ‰ water or emersion induces changes in regional permeability in *K. marmoratus*. To test this, the flux of $^3$H$_2$O across paired dorsal and ventral skins from *K. marmoratus* should be conducted following prolonged emersion in hypo- and hypersaline environments.

Further study of regional variability or general skin permeability could focus on epithelial lipid content. In hylid tree frogs (*Agalychnis dacnicolor*, *Hyla arenicolor*, *H. squirrellia*, and *H. femoralis*), lipid content was significantly higher in the dorsal skin, which was less permeable to water as well as unresponsive to permeability stimulating hormones (Yorio and Bentley 1977). Changes in skin permeability with salinity/emersion acclimation may be explained by changes in skin cholesterol content. Cholesterol has
been found to affect osmotic water flux in isolated gills due to its stiff structure within cell membranes (Redwood and Hayden 1969). The steroid ring of the cholesterol molecule increases the rigidity of a phospholipid bilayer by decreasing the space between bilayer constituents. The resulting decrease in membrane fluidity limits the spaces through which water can travel (Finkelstein 1976). Cholesterol depleting agents have been found to increase gill water weight gain (Redwood and Hayden 1969). In the same study, a specific cholesterol-complexing aqueous pore-forming antibiotic resulted in a dramatic permeabilization of gill arches to osmotic water flux. It is possible that the mechanism for altering water flux across *K. marmoratus* skin is the integration of cholesterol, or other hydrophobic lipid molecules, into epithelial cell membranes. In an amphibious fish species (*Periophthalmodon schlosseri*), low permeability of the skin to NH$_3$ flux was attributed to increased tissue cholesterol and phosphatidylcholine content (Ip et al. 2004). These membrane constituents stabilize membranes, decreasing membrane fluidity and thus permeability. Exposure to increased environmental NH$_3$ induced an increase in tissue cholesterol and saturated fatty acid content, presumably to decrease the permeability of the skin to NH$_3$ influx. These results highlight the importance of tissue lipid content on amphibious skin permeability specifically. It is likely that skin permeability to water flux is also affected by tissue lipid content in *K. marmoratus*. This proposed work would generate a holistic profile of water balance in this euryhaline amphibious fish.
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