University of Nevada, Reno

Regulation of Seat-patch Water Potentials in Anurans

A thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in Biology

by

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Abstract

Decades of research on water exchange in frogs have assumed that blood osmotic potential drives water uptake. However, more recent reports have suggested an intermediate control of the water potential partially separate from the osmotic potential of blood. Some reports have speculated that seat-patch may act as a compartment of water exchange between the blood and the environment. Thus, I have studied the water potential of the seat-patch and blood to evaluated their roles in water uptake in frogs. The water potentials of seat-patch and blood were measured for six anuran species, *Xenopus laevis, Lithobates pipiens, L. catesbeiana, Anaxyrus boreas, Pseudacris cadaverina* and *P. hypochondriaca*. Seat-patch water potentials were inferred from changes in body mass between frogs and different aquatic environments whose water potentials were manipulated by adjusting the osmolality of sucrose. Rates of water exchange by frogs were plotted against the osmotic potentials of environmental solutions. The x-intercepts of these graphs were taken to be the point at which water exchange is zero. The x-intercept marks the point where the water potential of the frog seat-patch is equal to the water potential of the environment. Seat-patch water potentials were compared across species. The osmotic potential of blood was also measured for all species at several levels of body hydration. These water potentials of blood were compared to the seat-patch water potentials. More aquatic species had seat-patch water potentials that were less negative than those of terrestrial species, and those seat-patch water potentials were different from the water potentials of their blood. More terrestrial species had more negative seat-patch water potentials that were similar to their blood water potentials. These findings indicate

a physiological mechanism in anurans to control water potential of seat-patches that has not previously been reported in the literature.

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Introduction

Amphibians have been extremely successful in colonizing a diverse range of habitats ranging from fully aquatic, to fully terrestrial, and even arboreal. Some habitats are very xeric and can potentially lead to lethal desiccation, so some amphibians employ physiological and behavioral mechanisms to prevent water loss and increase water uptake. Understanding the successes of amphibians in environments differing in degree of terrestriality requires understanding the efficacy of their adaptations for water uptake in different hydric environments.

Regardless of other morphological, physiological, or behavioral hydroregulatory adaptations, nearly all frogs must maintain a moist skin to survive (Duellman and Trueb 1994). Unlike other terrestrial vertebrates, anurans (frogs and toads) do not have cornified skin layers or scales to protect underlying tissue skin layers. One way for anurans to cope with being exposed to water loss is to compensate by absorbing water through their skin. Terrestrial frogs are able to absorb water at rapid rates through a highly vascularized area of skin called the "seat patch." The seat patch is associated with both behavioral and physiological adaptations for water uptake. The behavioral adaptations consist of body posturing, so that the seat patch is oppressed against a wet environmental surface from which water can be absorbed. Physiological adaptations of the seat patch include control of rates of water uptake via antidiuretic hormones, which can influence the permeability of the seat patch (largely by unknown mechanisms that likely includes changes of skin conductance and seat-patch water potential).

Because frogs use a wide range of habitats where access to free-standing water differs, species should vary in their ability to regulate their water uptake. Fully aquatic frogs would benefit from a seat-patch water potential that is closer to that of water as a way to prevent overly rapid water uptake which would ablate the need to urinate excessively. On the other hand, highly terrestrial species would benefit from a seat-patch water potential that is much lower than that of the environment to facilitate rapid water uptake when in contact with a moist environment (e.g., muddy soil or standing water).

Here, I test whether seat-patch water potentials are correlated with terrestriality of several frog species differing in their ecological habit. Thus, I hypothesize that more aquatic species can adjust their seat-patch water potentials to be less negative and that more terrestrial species can adjust their seat-patch water potentials to be more negative.

Background Information

Hydrobiology and Hydroregulation

The physiology of amphibians is well studied, but the literature on seat-patch water potentials of anurans is sparse. The water potential of the seat-patch has been measured in at least two species of anurans. The seat-patch water potential of the Northern leopard frog, *Lithobates pipiens*, was measured as approximately -200 kPa when the frog is at 95% of its standard body mass (the mass of a fully hydrated frog with an empty bladder), and -750 kPa when the frog is injected with antidiuretic hormone (Tracy and Rubink 1978). Tracy's experiment used carbowax as the known osmotic potential solution, but

commercial sources of carbowax do not have dependably uniform molecular weights, and thus it is difficult to depend on calculated water potential of solutions of carbowax. Here I used sucrose, because sucrose solutions can easily be made to have a known osmotic potential.

The second species that has had its seat-patch water potential measured is the cocoon-forming frog, *Cyclorana australis*, which was determined to be between -210 and -345 kPa (Tracy et al. 2007). Since the water potential of the seat patch has been measured in only two species, more data are required to survey the extent to which the water potentials of anuran seat patches differ, and to determine the extent to which frogs have control of those water potentials separate from control of the water potential of the frog's blood. The literature is also lacking in how seat-patch water potentials are related to the habitat use of anuran species. To understand the physiological processes affecting the water potential of the seat patch, separate from the osmotic potential of blood, requires a review of how anurans manage water uptake, storage and loss of body water. The water budget of an anuran is made of three components; influx, efflux and the difference between these two flows is the change in water storage.

In anurans, both the body tissues and the urinary bladder can store water, but water storage largely occurs in the urinary bladder. The urinary bladder is considered a true storage compartment for water because dilute urine can nearly always be found in the bladder. This volume of dilute urine is usually not expelled into the environment unless

the frog is disturbed in some way (Hillman et al. 2009). Water stored in the bladder can be mobilized into the blood in times of dehydration (Jorgensen 1991, 1994, 1997). Movement of water from the urinary bladder back into the body fluids is partly controlled by antidiuretic hormones (Ewer 1952). Species that are more likely to experience water loss (more terrestrial species) tend to store more water in their urinary bladder (Bentley 1971).

Anuran water influx is particularly interesting in that most frogs do not drink water. Water influx in frogs comes from the preformed water in the food they eat, metabolic reactions that produce water, and cutaneous uptake of water. The influx from preformed water in food is generally less than 1% body mass/day (Hillman et al. 2009). Water gained from aerobic metabolism is less than 0.1% body mass/day (Hillman et al. 2009). The only significant water influx comes from cutaneous uptake in essentially all anuran species.

Water efflux in amphibians includes water losses through fecal waste, urination, and evaporation. Water lost through fecal waste is estimated to be between 0.01 – 0.06% body mass/day (Hillman et al. 2009). Voiding urine can be a major loss of water because the bladder can hold a large percentage of the body mass. The amount of urine stored in the bladder of well-hydrated *Cyclorana australis* accounted for 125 - 136% of standard body mass (Tracy et al. 2007). Evaporative water loss is made up of both water lost through respiratory surfaces and cutaneous surfaces. Pulmonary water loss generally makes up about 10% of the total evaporative water loss in those anuran species that have

high cutaneous resistances to evaporative water loss, while it only makes up approximately 0.5% in species with low resistances to evaporative water loss (Shoemaker and McClanahan 1975). Because pulmonary evaporative water loss accounts for such a small amount of the whole, evaporative water loss is commonly calculated as being essentially entirely cutaneous.

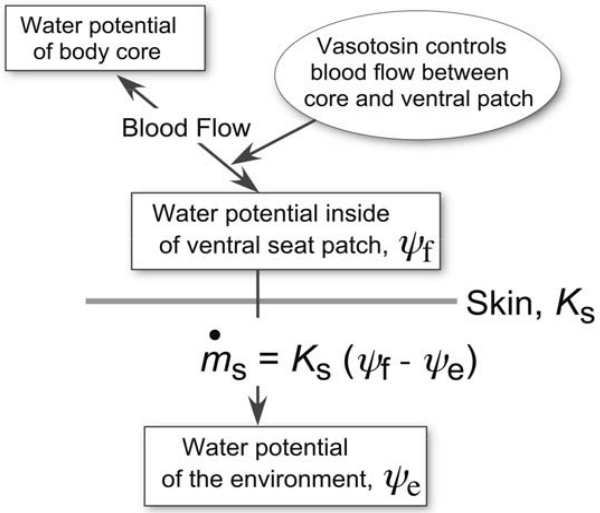
In amphibians, the lymphatic system returns water, which has entered the interstitial fluid space, back to the blood. This loss from the capillaries occurs when fluids in the blood pass through capillary walls into the interstitial fluid space due to the hydrostatic pressure of the circulatory system. This fluid is called lymph, and it is generally osmotically similar to blood (Reynolds et al. 2009). Lymph has lower protein concentrations, and lacks blood cells. The solute concentrations of lymph and blood are similar because the capillary walls are largely permeable to the solutes contained in blood. Lymph moves from the interstitial fluid into interconnected subcutaneous sacs, which are separated by connective tissue walls with controllable one-way valves (Hillman et al. 2009). The lymph is then returned to the blood through two pairs of lymph hearts. One pair is located anterior to the third vertebra the other pair is located laterally to the urostyle. Lymph must be returned to the blood by the lymph hearts because frogs will die due to a loss of plasma volume, and an increase in hematocrit, if the lymph hearts do not function (e.g., if they are experimentally removed; Hillman et al. 2009). There are also interspecific differences in the arrangement of the lymph sacs (Carter 1979) and in the ability to alter lymph movement to counter short-term fluctuations in hydration (Hillman and Withers 1988).

In summary, water influx in anurans that are not fully aquatic is comprised almost entirely of water absorbed through the ventral seat patch, and efflux is mainly water lost through cutaneous evaporative water loss. A deeper look into the major mechanisms that affect water uptake and water loss will provide a better understanding of how anurans manage these components of their water economy.

Water Uptake

Cutaneous uptake in anurans is driven by the differences in the water potentials of the surrounding environment and that of the amphibian (Box 1). Water uptake occurs when the water potential of the amphibian is less than the water potential of the environment. The rate of water uptake is further influenced by the hydric conductivity of the skin absorbing water (Tracy 1982). In more terrestrial species, the seat patch is usually highly vascularized, and in some species, it contains adaptations to increase surface area. In more aquatic species, the seat patch is less specialized (Hillman et al. 2009).

Box 1. Processes of liquid water exchange between the environment and the blood of a frog



water exchange between a frog and its environment. Water exchange ($\dot{m}_s$) is driven by the water potential difference between the skin of the seat patch and the environment ($\psi_f - \psi_e$) and controlled, in part, by the conductance of the skin (K_s). The water potential of the seat patch is controlled by the antidiuretic hormone, arginine vasotocin (AVT) providing a second control of water exchange. The water potential of the blood within the core of the animal can be isolated from the water potential of the seat patch as a means to control water exchange with the environment.

In more terrestrial species, the rate of water uptake through the seat patch is known to change depending on the hydration level of the frog (Christensen 1974, Tracy and Rubink 1978). Hydration level influences plasma circulation and antidiuretic hormones, which alter the presence of aquaporins in the skin. Aquaporins are a family of water conducting channels (Preston and Agre 1991) that provide the mechanism by which animal tissues can rapidly move water through cells of the body. In the seat patch, water first enters through the apical membrane when an antidiuretic hormone, arginine vasotocin (AVT), causes aquaporins (type 2 and/or type 3) to become inserted into the apical membrane of the skin cells. Water then diffuses across the basolateral membrane through constitutively expressed aquaporins (type 3) and into capillaries through another type of aquaporin (type 1) (Hillman et al. 2009). The distribution of aquaporins in the seat patch among anurans species seems to vary based upon the habitat in which the species lives. Terrestrial, scansorial, semiaquatic and fully aquatic species all have aquaporin – 3, while more terrestrial and scansorial species have combinations of both aquaporin – 2 and 3 (Suzuki et al. 2006). All have aquaporin -1, which allows water to move into the capillaries. Differences in aquaporins present in the seat patches may lead to differences in seat-patch water potentials of frogs.

Water Loss

Cutaneous evaporative water loss depends on both the humidity of the surrounding environment, and the resistance of the frog skin itself to evaporative water loss. Most anurans have little to no cutaneous resistance to evaporative water loss. In species with low cutaneous resistance, the rate of evaporative water loss is equal to the rate by which

water is lost from a free water surface. This is because, unlike mammals and birds, the outer layers of skin in anurans are living cells (Drewes et al. 1977). To keep this outer layer of cells alive, anurans must maintain a moist skin mostly by secreting mucous onto their skin. Therefore, there is always a high cost in terms of evaporative water loss.

Despite their low resistance to cutaneous water loss, anurans can modify this water loss behaviorally and in some species physiologically. One behavior that anurans may use in minimizing the exposed surface area is to modify body posture. For example, anurans exhibit a water conserving posture in which the pelvic area is appressed against some surface, the fore and hind limbs are appressed against the trunk of the body, and the head is usually angled downward towards the surface on which the frog sits (Heatwole et al. 1969). Some species burrow during dry seasons, which can allow them to escape cutaneous water loss almost entirely. Species in several clades of tree frogs have glands that produce a lipid that is spread over the exterior of their bodies (Gomez et al. 2006). This lipid coating greatly reduces cutaneous water loss. Herein, the research was confined to species that do not produce a lipid barrier to water loss, and so they have evaporative water loss rates similar to that of a free water surface.

Anuran Ecology

Not all anurans have the same physiological adaptations to manipulate the rate of water uptake or the rate of water loss. It is thought that these interspecific differences are due to adaptations to different habitats. For this study, species were placed into nine ecological groups ranging from aquatic to terrestrial to arboreal based on the characteristics outlined

by Hillman et al. (2009). These categories are made up of nine groups that range from aquatic to terrestrial to arboreal. I studied species from five of these groups. These groups were aquatic, semi-aquatic, semi-terrestrial mesic, semi-terrestrial xeric and scansorial. For example, aquatic anurans are those species that spend the majority of their lives underwater and possess adaptations for a fully aquatic life. These adaptations include a functional lateral line system and webbed toes. These species also appear to have few specialized mechanisms for water uptake. Semi-aquatic species are species that live primarily in water or maintain a close proximity to a water source. Semi-aquatic species also tend to lack any adaptations for facilitated water uptake due to their proximity to water.

Semi-terrestrial species are species that spend a considerable amount of time on land, but lay eggs in an aquatic environment. This group can be split into a mesic group and a xeric group. Semi-terrestrial mesic species spend the majority of their lifetimes in a terrestrial habitat away from free water, but they live in mesic environments such as leaf litter in forests or surrounding marsh areas. Semi-terrestrial xeric species lack the ability to burrow, but make up for this inability to reduce evaporative water loss with the capacity to absorb water quickly. Scansorial species tend to possess modifications of the digital tips to enable climbing and grasping. Not all species in this group live in trees though and most do have adaptations for rapid water uptake.

I studied the seat-patch water potentials of six species, which differ in habitat use. The African clawed frog, *Xenopus laevis*, is a fully aquatic species while the American

bullfrog, *Lithobates catesbeiana*, is a model of the semi-aquatic species. The semi-terrestrial mesic species is the Northern leopard frog, *Lithobates pipiens*, and the semi-terrestrial xeric species is the Western toad, *Anaxyrus boreas*. Finally, two species were scansorial species. The first is the Pacific chorus frog, *Pseudacris hypochondriaca*, which is a chorus frog that can be found both in trees and under logs. The second is the California tree frog, *Pseudacris cadaverina*, which is a chorus frog that is often found basking in sunlight on rocks next to streams. These six species provide a group of species that differ widely in their use of habitat types.

Materials and Methods

Seat-patch water potential

The seat-patch water potential was inferred from experiments manipulating environmental water potential to find those environments with which frogs do not exchange water – i.e., when the water potential of the environment and seat patch are equal. To do this, frogs were catheterized to remove any water stored in their bladder. Some species would urinate when handled, but these species were still catheterized to ensure an empty bladder. The body mass of the frog with an empty bladder was then recorded as the standard body mass. The frogs were then dehydrated in a wind tunnel until they came to 90% of their standard body mass (the fully-hydrated body mass).

Plastic containers were used to hold the sucrose solutions used as the environments for experimental frogs (outer container) and a smaller container was used to secure the frog from movements (inner container). A metal, mesh screen platform was placed in the outer container so that the screen sat roughly 2.5 cm above the bottom of the container.

The inner containers used to hold the frogs in the sucrose solutions were round plastic containers with air holes cut in the lid. The inner containers had the flat bottoms removed and replaced with a metal, mesh screen (Figure 1).

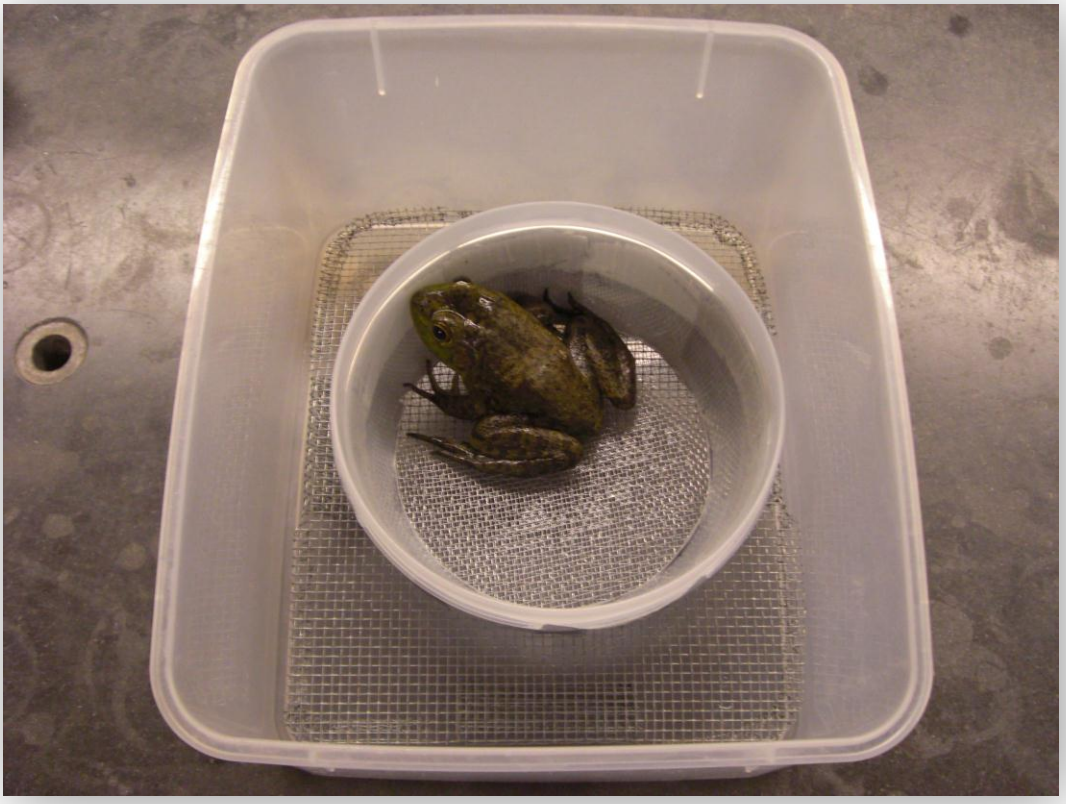


FIGURE 1. Experimental setup to measure seat-patch water potential in anurans. The outer container held a sucrose solution while the inner container (with bottom cut out) held the anuran (Lid not shown in figure).

The containers used for smaller frogs were petri dishes with holes drilled into the bottom to allow sucrose solution to flow in. The inner containers were placed on the metal screen platform. Then enough of the sucrose solution was added so that the solution level was a

few millimeters above the metal screen platform. A dehydrated frog was then placed in the inner container so that the frog was partly submerged in sucrose solution. The body mass of the frog was then recorded every ten minutes for one hour (unless more data were still needed due to a frog urinating). Before recording the body mass, each frog was lightly patted dry. The mean change in body mass reflects the net water exchange by influx and efflux. The water influx into the frog can be obtained by adding the CEWL to the net water exchange. This calculated water influx was then plotted against water potential of the environment (sucrose solutions), and the x-intercept is the point at which no water is exchanged because the water potential of the seat patch is equal to the water potential of the sucrose solution. The x-intercept for each individual frog was then calculated and was used to determine the standard deviation around the calculated seat-patch water potential for each species. The x-intercept represents the water potential at which (on average) no water is exchanged, and thus, the water potential of the frog's seat patch. The x-intercept for each individual frog was then calculated and was used to determine the standard deviation around the calculated seat-patch water potential for each species. Individual regressions that had an r^2 less than 0.6 or had a positive trend in water uptake were removed from both the mean and standard deviation. A t-test was then used to test for differences among seat patch water potentials of the six species.

Cutaneous Evaporative Water Loss

Cutaneous evaporative water loss during the water uptake experiment was measured by using frog models made from 3% agar. Negative molds of frogs were made by pouring dental alginate on live frogs that had been given MS222 (Tricaine) as an anesthetic to

minimize their movements while the mold was being made (See Appendix A). Alginate sets in less than one minute, so no harm is caused to the frog. Plaster of Paris was then poured into the alginate mold to create a positive mold of the frog. A latex paint was then painted onto the plaster of Paris mold in several layers to make a durable, reusable negative mold. A 3% agar was then made and poured into the latex negative mold and allowed to set The agar frog model was then coated with fingernail polish approximately where the frog would have been in contact with the sucrose solution. The agar frog model was then placed in the same experimental apparatus for measuring water uptake except the level of liquid was kept just below the platform so the agar frog model did not come in contact with it. This was done to keep the relative humidity roughly the same as in the experiment measuring seat-patch water potential. The change in mass of the agar frog model was then recorded every ten minutes for an hour. The frog model was not removed from the container in which the model was measured, so both the mass of the frog model and the container were measured at each time interval as a way to minimize damage to the agar frog that could occur due to handling. These data were then used to correct the measurements taken while recording water uptake. The mean cutaneous evaporative water loss from the agar models for one species was then added to the corresponding species' water uptake measurements.

Osmotic Potential of Anuran Blood

Blood was collected from frogs that were fully hydrated and dehydrated by 5%, 10%, 15% and 20% from their standard body mass. Frogs were first catheterized, and then allowed to dehydrate in a wind tunnel to the desired body mass. *Xenopus* were

dehydrated osmotically by placing them in a sucrose solution of -600 kPa. This was done since they appeared to be unable to endure dehydration in air. Blood samples were collected from the abdominal midline vein using a 23-gauge hypodermic needle. The blood sample was then transferred to the 25 μL sample device used by the freezing point osmometer (Advanced Instruments 3MO). Blood osmotic potential was measured for blood collected from the midline abdominal vein. This site was used because blood collection from this vein could be done at all frog hydration levels, whereas collecting lymph from the lymphatic system requires time to allow lymphatic fluid to accumulate after pressure is applied to the lymph sack so that lymph is not pumped from the sack. This made it difficult to acquire a lymph sample at any specific hydration level (see Appendix B).

The osmotic potentials of blood for each species at 100% and 90% hydration were compared using a t –test to determine if there was a significant difference between these hydration levels. This comparison was made to show that 90% hydration is an appropriate hydration level to use to make comparisons with (i.e. different than a fully hydrated frog). For each species, the water potential of the seat-patch and the osmotic potential of blood at 90% hydration were compared using a t-test.

Results

Cutaneous Evaporative Water Loss

The agar frog models were the same size, shape and posture of the living frogs from which molds were made. The models were easily manipulated so that cutaneous evaporate water loss (CEWL) could be measured from them. In addition, it was easy to apply nail polish to imitate areas of the frog from which there should be no water loss (areas in contact with experimental solutions). It is important to note that the CEWL data reported here are the CEWL for the species while in this particular experimental setup and not the CEWL for these species under normal circumstances. To find the net water influx, the CEWL (Table 1) was added to the net water exchange.

TABLE 1. The mean cutaneous evaporative water loss (CEWL) of six anuran species in the experimental setup. Data were collected using agar frog models (3% agar). The CEWL reported here are for species while in the experimental setup, not under normal circumstances.

Species	Mean CEWL (mg/min)	%Body mass lost/ h
Xenopus laevis	-0.42 ± 0.02	0.88
Lithobates catesbeiana	-4.7 ± 0.93	0.17
Lithobates pipiens	-2.8 ± 0.42	0.32
Anaxyrus boreas	-2.5 ± 0.16	0.18
Pseudacris cadaverina	-0.54 ± 0.15	0.58
Pseudacris hypochondriaca	-0.53 ± 0.06	0.63

Seat-patch Water Potential

There were differences in the seat-patch water potentials among the frogs (Figure 2, 3; See Appendix C for water exchange values for each species).

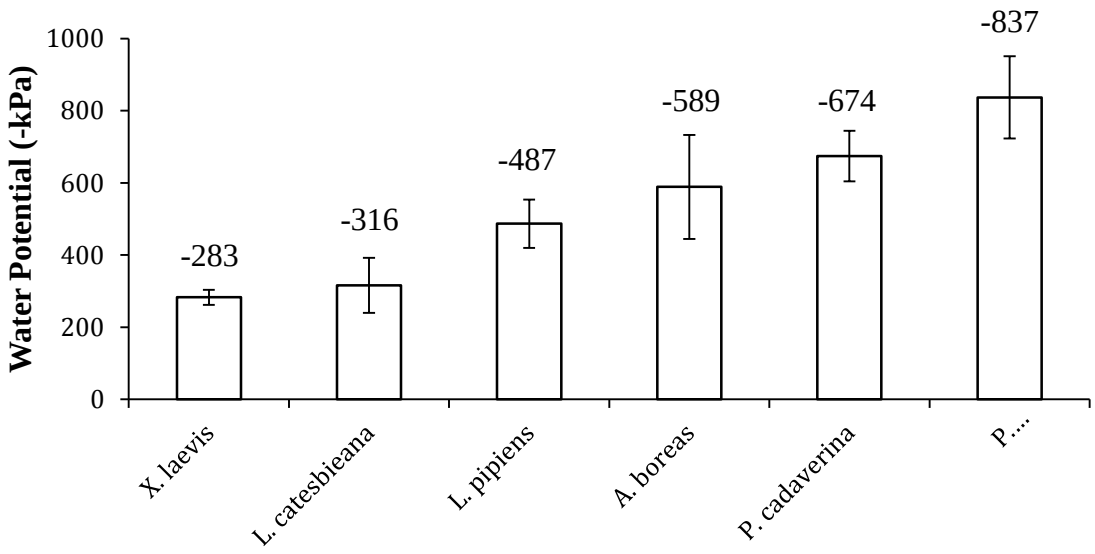


FIGURE 2. The seat-patch water potential of six anuran species dehydrated to 90% standard body mass.

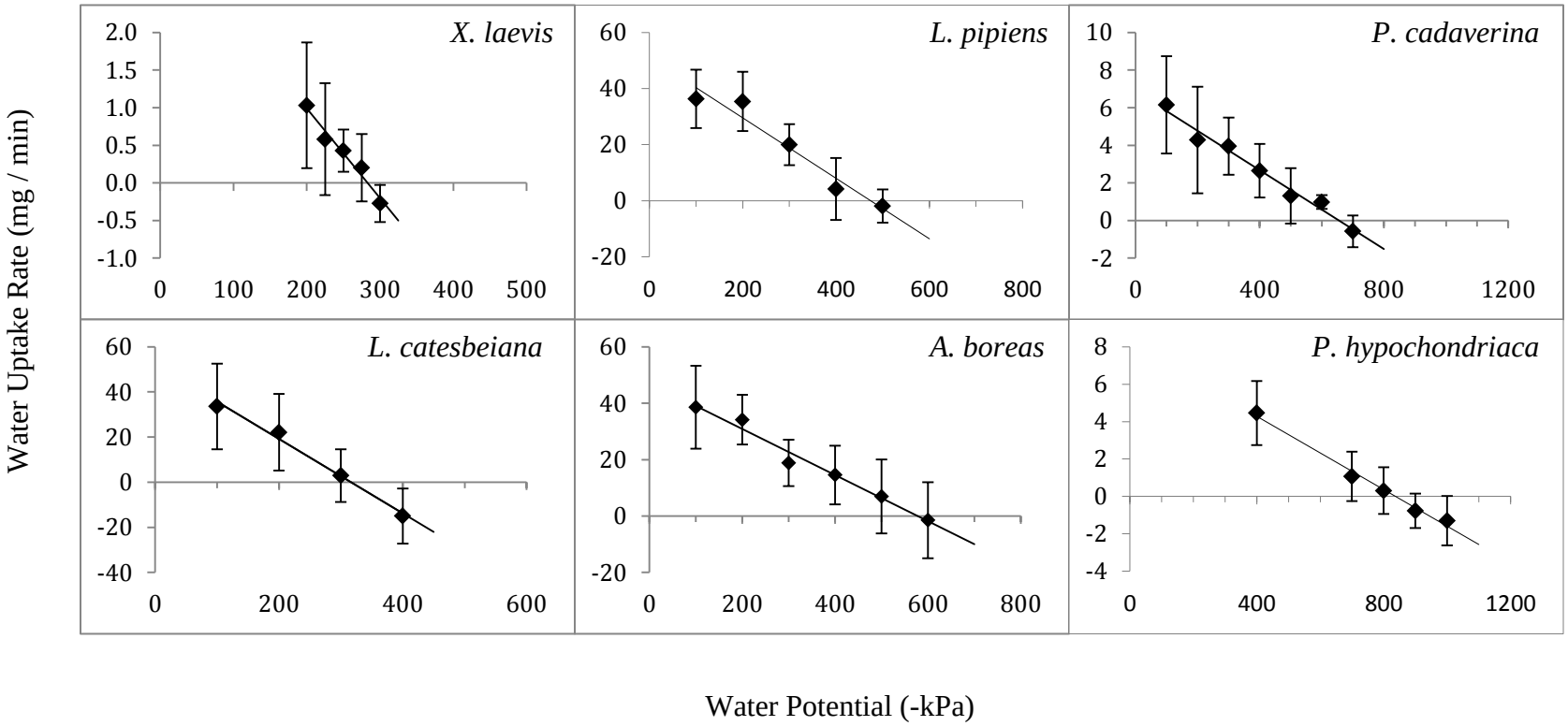


FIGURE 3. The rate of water exchange for six anuran species exposed to different environmental water potentials (sucrose solutions). The x-intercept is taken to be point at which no net water exchange occurs and is therefore where the seat-patch water potential is equal to the environmental water potential.

Osmotic Potential of Anuran Blood

The osmotic potential of blood was determined for all six species at hydration levels ranging from 100% hydration to 80% hydration (see Appendix D). Blood osmotic potentials for *X. laevis* were only collected for hydrations between 100% and 90% because this species rarely experiences dehydration in nature, and it appears unable to endure dehydration. Below are the data for fully hydrated frogs and frogs that were dehydrated to 90% standard body mass (Table 2). We report the data at these two hydration levels because the osmotic potential of fully hydrated frogs provides us with a baseline to compare with published values and the osmotic potential of frogs dehydrated to 90% standard body mass provides a value to compare to the seat patch water potential of frogs dehydrated to 90% standard body mass. All blood osmotic potentials at 90% hydration were significantly lower (more negative) than at 100% hydration.

TABLE 2. Osmotic potentials of blood in six anuran species while at 100% and 90% standard body mass (the mass of a fully hydrated frog with an empty bladder). The osmotic potential of blood was measured using a freezing point osmometer.

	Osmotic Potential (-kPa)	
Species	Blood (100%)	Blood (90%)
Xenopus laevis	581 ± 23	700 ± 29*
Lithobates catesbeiana	480 ± 32	580 ± 13*
Lithobates pipiens	593 ± 22	697 ± 31*
Anaxyrus boreas	590 ± 7	685 ± 11*
Pseudacris cadaverina	635 ± 35	707 ± 13*
Pseudacris hypochondriaca	710 ± 44	840 ± 41*

* denotes a statistical difference between 90 and 100% hydration

Comparisons Among Seat-patch Water Potentials of Different Anurans

There were several similarities and differences among seat-patch water potentials for each species (Table 3, See Appendix E for a complete table of statistics). The fully aquatic species, *X. laevis*, and the semi-aquatic species, *L. catesbeiana*, had statistically similar seat-patch water potentials ($t_{19} = 1.11$, $P > 0.2$), but were statistically different from all other species ($P < 0.001$, see Appendix E for all comparison test statistics). The seat-patch of the semi-terrestrial mesic species, *L. pipiens*, was statistically different from the semi-terrestrial xeric species ($t_{32} = 2.59$, $P < 0.02$), and statistically different from both of the arboreal species (*L. pipiens vs. P. cadaverina* $t_{21} = 6.08$, $P < 0.001$, *L. pipiens vs. P. hypochondriaca* $t_{28} = 10.41$, $P < 0.001$). The semi-terrestrial xeric species, *A. boreas*, had a seat-patch water potential that was statistically similar to the arboreal species, *P. cadaverina* ($t_{23} = 1.48$, $p > 0.1$), but it was statistically different from the second arboreal species, *P. hypochondriaca* ($t_{30} = 5.28$, $p < 0.001$). The arboreal species, *P. cadaverina* and *P. hypochondriaca*, had statistically different seat patch water potentials from each other ($t_{19} = 3.45$, $p < 0.01$).

TABLE 3. Comparisons between anuran seat patch water potentials. Species are ordered by their habitat use; species between habitat types are considered to be semi-"habitat type". Comparisons were made using t-tests.

Aquatic	Semi-aquatic	Semi-terrestrial	Arboreal
X. laevis =	L. catesbeiana ≠	L. pipiens ≠	A. boreas ≠ P. hypochondriaca ≠ = P. cadaverina

Anuran Seat-patch Water Potential vs. Blood Osmotic Potential

For frogs at 90% hydration level, a t-test was used to compare blood osmotic potential with seat-patch water potential (Figure 4). In the more aquatic species, *X. laevis* and *L. catesbeiana*, and the semi-terrestrial species, *L. pipiens*, the seat-patch water potential was significantly different from the blood osmotic potential (*X. laevis:* $t_{12} = 29.71$, $P < 0.001$, *L. catesbeiana:* $t_{18} = 8.33$, $P < 0.001$, *L. pipiens:* $t_{21} = 5.54$ $P < 0.001$). In the more terrestrial species, *A. boreas*, the seat-patch water potential was not significantly different than the blood osmotic potential ($t_{24} = 1.86$, $P > 0.05$). Both of the arboreal species, *P. cadaverina* and *P. hypochondriaca*, had seat-patch water potentials that were not significantly different from the blood osmotic potential ($t_8 = 0.78$, $P > 0.4$ and $t_{21} = 0.08$, $P > 0.9$ respectively) (see Appendix F for complete table of statistics).

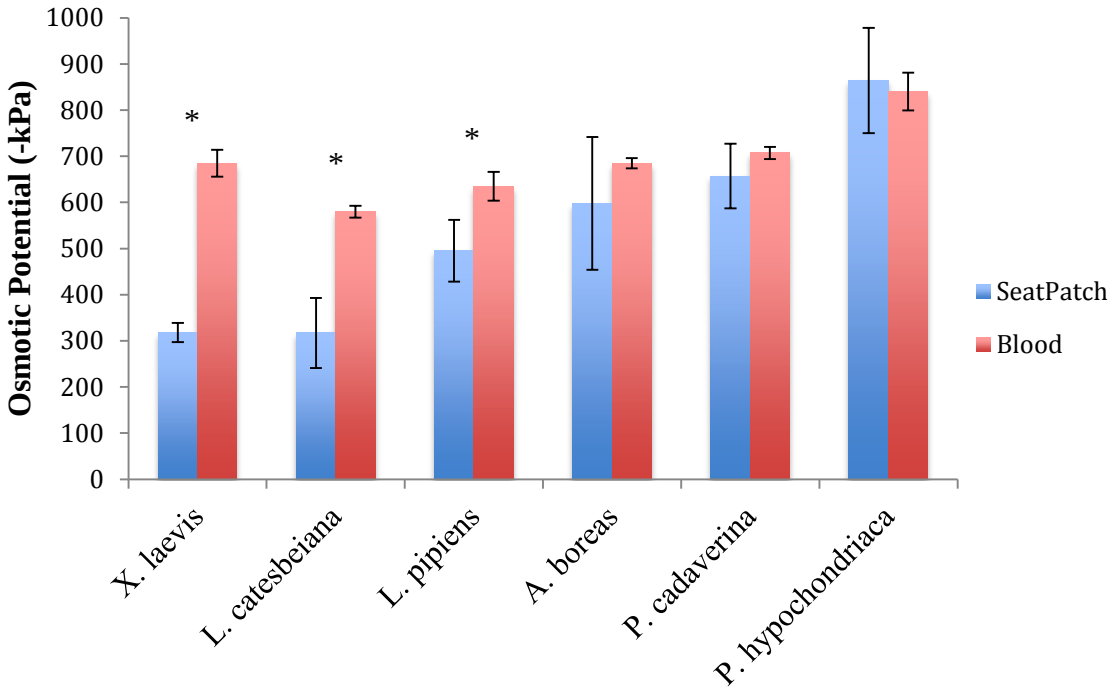


FIGURE 4. Comparisons between the seat patch water potentials and the blood osmotic potentials of six anuran species (* denotes statistical significance). See Appendix G for data on seat-patch and blood osmotic potentials.

Discussion

Seat-patch water potentials varied among the six anuran species I studied and those variations in water potentials correlated with terrestriality of the species. The more aquatic species, *X. laevis* and *L. catesbeiana,* had similar seat-patch water potentials that were lower (less negative) than the other species. The semi-terrestrial mesic species, *L. pipiens,* had a seat-patch water potentials between the aquatic species and the more terrestrial species. The semi-terrestrial xeric species, *A. boreas,* had a mean seat-patch water potential that was not significantly different from *P. cadaverina*. *P. cadaverina* had a seat-patch water potential that was lower (more negative) than *P. hypochondriaca,* which had the most negative seat patch water potential.

The differences in seat-patch water potential and blood osmotic potential correlated with the terrestriality of those species. Among the aquatic and the semi-terrestrial mesic species, osmotic potentials of the blood and the seat-patch were statistically different. Among the semi-terrestrial xeric and the scansorial species, osmotic potentials of the blood and the seat-patch were statistically similar.

The blood osmotic potentials recorded in this thesis research fall reasonably within the ranges of blood osmotic potentials recorded for other species. The blood osmotic potentials of five well-hydrated terrestrial/fossorial species ranged from -499 kPa in *Neobatrachus aquilonius* to -726 kPa in *Bufo viridus* (Cartledge et al. 2006, Cartledge et al. 2008, Degani et al. 1984, McClanahan 1972). The blood osmotic potential in fully

hydrated species that I studied ranged from -480 kPa in *L. catesbeiana* to -710 kPa in *P. hypochondriaca*. Comparing blood osmotic potentials of dehydrated frogs in this report to those in the literature is difficult because reports in the literature do not present osmotic potentials for frogs at levels of dehydration comparable to those in this report.

Our results suggest that more aquatic species are able to control their seat-patch water potentials to be lower (less negative) than the osmotic potential of their blood, but that more terrestrial species are unable to do so. This ability to control the water potential of the seat-patch is potentially beneficial to more aquatic species, as it would allow them to slow influx of water, thus reducing the need to urinate. The opposite is potentially true for the more terrestrial species, having a high water potential would allow these species to absorb water readily when dehydrated.

Frogs might be able to regulate the seat-patch water potential to a level different from their blood via aquaporins located in the seat-patch. The types and locations of aquaporins in frogs are receiving increased study. In anurans, aquaporins have been studied in the kidney, urinary bladder and the seat patch, Aquaporin (AQP) h-3 is found in the pelvic skin of all anurans regardless of their habitat types (aquatic, semi-terrestrial, terrestrial and arboreal). AQP h-2 is found in the urinary bladder of species across all habitat types as well (Suzuki et al. 2006, Ogushi et al. 2010). In contrast, the pelvic skin of aquatic and “semi-terrestrial” species lack the AQP h-2, but AQP h-2 is found in the pelvic skin or more terrestrial and arboreal species (Suzuki et al. 2006, Ogushi et al. 2010). However, the semi-terrestrial species in Suzuki et al. 2006 is *L. catesbeiana* and

should possibly be called semi-aquatic. Overall, more terrestrial species have two types of aquaporins in the seat patch while more aquatic species have only one type.

In this study more aquatic species had a lower seat-patch water potential than more terrestrial species. This difference is consistent with what is known about the distribution of aquaporins in frogs. This could explain why the more terrestrial species have higher seat-patch water potentials than the more aquatic species in our experiment.

Despite the presence of AQP h-3 in the seat-patch, the permeability of the seat patch of the fully aquatic species, *X. laevis*, is unaffected by antidiuretic hormones such as AVT (Bentley 1969). Interestingly, compared to other species the sequence of AQP h-3 in *X. laevis* has an additional eleven amino acid terminal segment, called a CT tail (Ogushi et al. 2010). This unique aquaporin, named AQP x-3, has lost its functionality as an aquaporin, and it does not affect membrane permeability while the CT tail is present. When AQP x-3 has the CT tail experimentally removed, it regains its functionality as an aquaporin (Ogushi et al. 2010). This would suggest that individuals of *X. laevis* lack functional aquaporins when they are well hydrated, and this would allow them to slow excessive water uptake. Thus, when individuals of *Xenopus* are well-hydrated, we expect that they would experience slow water uptake in fresh water. In stagnant waters where the environmental water potential is not zero, but some negative value, we would expect even slower water uptake. On the other hand, when *Xenopus* individuals are dehydrated, they appear to be able to activate AQP x-3 aquaporins (through a process that is not currently known) to change their seat-patch water potential to be more negative than that of the environment. Hence, *X. laevis* that activate AQP x-3 would be able to rehydrate.

For more terrestrial species, the water potential of the terrestrial substrate from which water is obtained, plays a large role in their water balance. The water potential of soil changes depending on the composition of the soil, and how much water is in the soil (Tracy 1976). More terrestrial species would benefit from having a higher seat-patch water potential because this would allow absorption of water from soils with a wider range of water potentials. Semi-terrestrial mesic species would benefit from having intermediate seat-patch water potentials as they spend a majority of their time sitting on moist substrates. Semi-terrestrial mesic species would still have a range of soil water potentials from which to uptake water while on short forays away from water. It appears that more terrestrial species possess both AQP h-3 and AQP h-2 types, but a closer look at the molecular diversity of aquaporins present in the seat-patch in more terrestrial species is warranted.

To summarize, more aquatic species appear to have lower seat-patch water potentials and these seat-patch water potentials are different from the osmotic potential of their blood. This would be advantageous for these aquatic species because water is generally readily available, and there is no physiological need for a constant uptake of water. The low seat-patch water potential of aquatic species is likely due to them having only the aquaporin type AQP h-3 present in the seat-patch. More terrestrial/arboreal species appear to have higher seat-patch water potentials that are similar to the osmotic potential of their blood. These groups would likely benefit from having a high seat-patch water potential that allows them to take up water over a greater range of soil water. The high seat-patch water potentials seen in more terrestrial groups is likely due to the presence of both AQP h-3

and AQP h-2 in the seat patch.

One limitation of this study is its ability to statistically test how seat-patch water potential relates to anuran habitat type. Each habitat type was represented by one or two species, although several individuals of each species were studied. Because most habitat types were represented by only a single species, this study is limited in the ability to draw conclusions about how the seat-patch water potential is related to habitat type. Here, the individual frog is the unit of replication when greater generalizations could be made, if the unit of replication was species. Hence, additional work surveying more species (ideally species that are similar in ecological habit, but different in phylogenetic relatedness), would allow for a better understanding of the relationship between seat-patch and ecological habit of the species. For example, several aquatic species could be compared to characterize the group, and then those species could be compared to a group of semi-aquatic species, a group of semi-terrestrial mesic species, and so on. The scale needed for this group by group comparison exceeded the scope of the research reported here (6 species vs. dozens of species). The scope of research reported here provides a preliminary platform to assess the extent to which seat-patch water potentials differ among species that differ in ecological habitat, and deeper delving into this question will follow with studies adding more species to the survey.

An unexplained observation in the research reported here was the ability of three species, *X. laevis*, *L. catesbeiana* and *P. hypochondriaca* to maintain a relatively constant water uptake rate while immersed in different water potentials. This seems an impossible feat

under the assumption of constant physiological parameters in the frogs. For example, the rate of water exchange across a permeable membrane is described with the following equation: $M = A_v K_{frog} (\Psi_{frog} - \Psi_e)$. The rate of water exchange, M, is affected by the area of skin in contact with the environmental surface, A_v, the conductance of the membrane in contact with the environmental surface, K_{frog}, and the difference between the water potential of the frog and the environment, $(\Psi_{frog} - \Psi_e)$. One could imagine that the hydric conductance and seat-patch water potential of the frog should be a constant, but these parameters must change in these three species so that the rate of water uptake is maintained as a constant (Figure 5). Evaluating the mechanism of that change, or those changes, is a logical next step in investigating the physiology of water uptake in frogs.

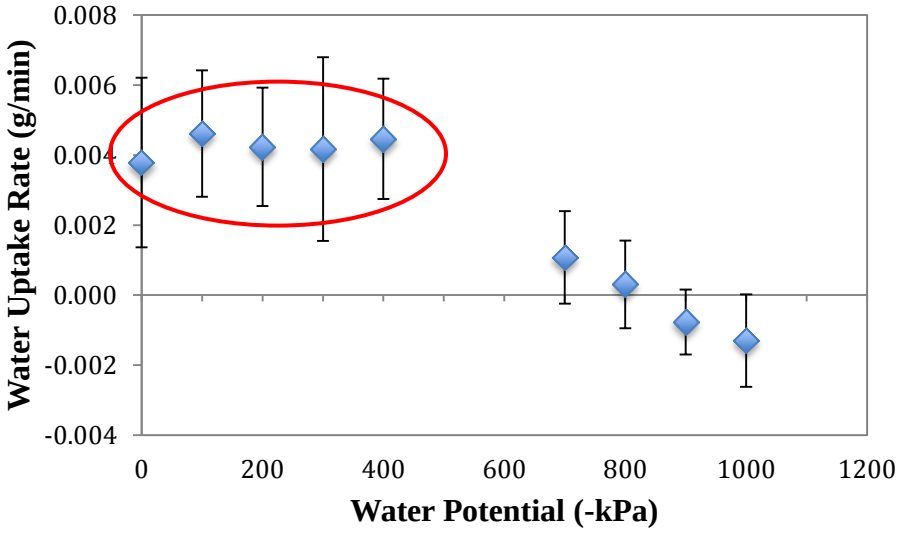


FIGURE 5. Mean water uptake rates of *P. hypochondriaca* in sucrose solutions differing in water potential. The points encircled in red indicate that the physical/physiological properties of the frogs appear to change so that water uptake rate can remain a constant in spite of a different environmental water potential.

Ultimately, this study indicates a complexity of physiological machinery and flexibility that was previously unknown. In addition, the study indicates that much remains to be learned about the physiological mechanisms by which frogs control water exchange with the environment. For example, it appears that some species have the ability to modify their water uptake in different environments in ways that optimize water uptake respective to the ecological habitat of the frogs. Furthermore, the observations in Figure 6 suggest that the frogs have the ability to sense water exchange rates and modify skin conductance (less likely) or seat-patch water potential separate from the seat-patch water potentials reported for species here. Understanding the still-unknown mechanisms to water exchange in aquatic and terrestrial frogs ultimately will help us understand the adaptations associated with the invasion of land by the earliest terrestrial amphibians.

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Appendices

Appendix A. Use of MS-222 on anuran species

The anesthetic of choice for this experiment was MS-222 (tricaine sulfonate). The amount of MS-222 given to each species is summarized in Table A-1. Generally, frogs were anesthetized within 10-15 minutes or less of administering MS-222. After the frog was used to make an alginate mold, it was placed on a paper towel that was on a metal screen platform that was submerged in water so that the water level was a few millimeters above the surface. The paper towel was also manipulated so that the frog's snout was elevated above the water level. The frogs were monitored and usually recovered fully within 20 minutes or less.

TABLE A-1. Anesthetic MS-222 (tricaine sulfonate) concentrations used to anesthetize six anuran species.

Species	MS222 Concentration (g/L)
X. laevis	3
L. catesbeiana	5
L. pipiens	3
A. boreas	3
P. cadaverina	3
P. hypochondriaca	4

Appendix B. Collection of lymph vs. blood collection from the midline abdominal vein

The lymph collection technique described by Reynolds et al. (2009) was initially attempted to collect body fluids with an osmotic potential similar to that of blood. This technique was first attempted on *L. pipiens* and *L. catesbeiana* due to their larger size. It proved extremely difficult to allow enough time for lymphatic fluid to build up while at

the same time collecting a lymph sample at a particular hydration level (i.e. 90% standard body mass). The second problem was that two of the species used in this research, *P. cadaverina* and *P. hypochondriaca*, were less than 10 g and some individuals were less than 5 g. It has been noted that there would be an insufficient amount of lymph for analysis (>0.1ml) in small frogs (Reynolds et al. 2009). The technique used to collect blood osmotic potentials was blood collection from the midline abdominal vein. In some species, *L. pipiens* for example, the midline abdominal vein could be seen easily near the surface of the skin. The midline abdominal vein in other species, such as *A. boreas*, could not be seen due to coloration on the abdomen but could still be easily found as it lies nearly in the center of the abdomen. Collecting blood was preferential over collecting lymphatic fluids since blood could be collected when the frog was precisely at a given hydration level. The amount of blood collected was usually less than 1ml as the freezing point osmometer required a sample no larger than 25 µl. We successfully used this method on frogs ranging from < 5g to > 100g in this study.

The method for collecting small volumes of blood from *Xenopus laevis* described by Mashoof et al. was attempted but met with limited success. The method also describes using MS-222, which may affect the osmolality of blood. Since using an anesthetic may alter the osmolality of the fluid being measured, this technique did not meet the needs of the study. Hence, the midline abdominal vein was used for *Xenopus laevis* as well.

Appendix C. Rates of water exchange with sucrose solutions for six anurans species

TABLE C-1. Mean net water exchange rates (mg/min) between six anuran species and sucrose solutions differing in water potential. These rates have been adjusted to account for cutaneous evaporative water loss.

Solution Ψ (-kPa)	X. laevis	L. catesbeiana	L. pipiens	A. boreas	P. cadaverina	P. hypochondriaca
0	1.437±0.836	29.8±19.0	43.0±10.4	41.9±14.7	5.48±2.59	3.79±2.42
100	1.129±0.743	33.6±17.0	36.3±10.6	38.6±8.8	6.15±2.83	4.62±1.80
200	1.030±0.281	22.1±11.6	35.4±7.3	34.2±8.3	4.28±1.53	4.23±1.69
225	0.581±0.432					
250	0.428±0.247					
275	0.200±0.507					
300	-0.273±0.574	2.9±12.2	20.0±11.1	18.8±10.4	3.95±1.42	4.17±2.62
400		-14.9±13.9	4.20±5.9	14.6±13.1	2.65±2.07	4.46±1.71
500			-1.9±5.9	6.9±13.5	1.31±0.37	
600				-1.4±12.0	0.98±0.84	
700					-0.57±0.64	1.08±1.32
800						0.31±1.25
900						-0.77±0.93
1000						-1.29±1.32

Appendix D: Data of Blood Osmotic Potentials Between Species at Different Hydration Levels

TABLE D-1. The blood osmotic potentials (kPa) for six anuran species dehydrated to 75-100 percentage of their standard body mass. Table extends two pages.

Species	100	95	90	85	80	75
X. laevis	-553.88	-694.62	-712.78			
	-551.61	-646.95	-687.81			
	-599.28	-644.68	-681.00			
	-608.36		-683.27			
	-585.66		-658.30			
	-585.66		-646.95			
			-728.67			
L. catesbeiana	-474.43	-533.45	-581.12	-578.85	-712.78	
	-490.32	-537.99	-565.23	-592.47	-705.97	
	-490.32	-522.10	-597.01	-617.44	-703.70	
	-528.91	-547.07	-592.47	-594.74	-669.65	
	-519.83	-524.37	-576.58	-583.39	-676.46	
	-503.94	-517.56	-569.77	-590.20	-681.00	
	-478.97				-662.84	
	-488.05					
	-478.97					
	-460.81					
	-433.57					
	-419.95					
L. pipiens	-592.47	-592.47	-572.04	-733.21	-808.12	-837.63
	-594.74	-592.47	-628.79	-719.59	-769.53	-853.52
	-558.42	-649.22	-637.87	-690.08	-837.63	-837.63
	-587.93	-628.79	-646.95	-696.89	-833.09	-848.98
	-601.55	-671.92	-674.19	-710.51	-724.13	-785.42
	-631.06	-646.95	-646.95	-785.42	-726.40	-783.15
	-585.66	-685.54	-640.14	-724.13	-746.83	-871.68
						-846.71
						-837.63

Species	100	95	90	85	80	75
A. boreas	-587.93	-644.68	-676.46	-724.13	-905.73	
	-594.74	-662.84	-683.27	-719.59	-885.30	
	-587.93	-660.57	-676.46	-771.80	-726.40	
	-581.12	-660.57	-685.54	-771.80	-749.10	
	-601.55	-635.60	-692.35		-753.64	
	-587.93	-649.22	-701.43		-703.70	
		-612.90	-694.62		-728.67	
		-612.90	-669.65		-719.59	
		-615.17				
		-610.63				
P. cadaverina	-640.14	-637.87	-721.86	-844.44	-932.97	
	-628.79	-635.60	-696.89	-839.90	-923.89	
	-599.28	-628.79	-701.43	-855.79	-939.78	
	-562.96	-637.87		-858.06	-908.00	
		-696.89		-821.74		
				-812.66		
				-908.00		
				-910.27		
P. hypochondriaca	-712.78	-783.15	-780.88	-794.50	-937.51	
	-692.35	-794.50	-771.80	-810.39	-955.67	
	-703.70	-780.88	-851.25	-957.94	-803.58	
	-721.86	-767.26	-835.36	-957.94	-808.12	
	-717.32	-778.61	-880.76	-976.10	-814.93	
	-603.82	-830.82	-894.38	-962.48	-830.82	
		-796.77	-848.98			
		-812.66	-864.87			
			-848.98			

Appendix E. Statistics for seat-patch water potential comparisons between species

TABLE E-1. Statistical comparisons between the seat-patch water potentials of anuran species using a t-test.

Species vs. Species	P value	t-test value	df
P. hypochondriaca vs. P. cadaverina	$p < 0.01$	3.45	19
P. hypochondriaca vs. A. boreas	$p < 0.001$	5.28	30
P. hypochondriaca vs. L. pipiens	$p < 0.001$	10.41	28
P. hypochondriaca vs. L. catesbeiana	$p < 0.001$	14.23	26
P. hypochondriaca vs. X. laevis	$p < 0.001$	12.59	19
P. cadaverina vs. A. boreas	$p > 0.1$	1.48	23
P. cadaverina vs. L. pipiens	$p < 0.001$	6.08	21
P. cadaverina vs. L. catesbeiana	$p < 0.001$	10.43	19
P. cadaverina vs. X. laevis	$p < 0.001$	14.16	12
A. boreas vs. L. pipiens	$p < 0.02$	2.59	32
A. boreas vs. L. catesbeiana	$p < 0.001$	6.42	30
A. boreas vs. X. laevis	$p < 0.001$	5.53	23
L. pipiens vs. L. catesbeiana	$p < 0.001$	6.55	28
L. pipiens vs. X. laevis	$p < 0.001$	7.80	21
L. catesbeiana vs. X. laevis	$p > 0.2$	1.11	19

Appendix F. Statistics for comparisons between seat-patch and blood osmotic potentials

TABLE F-1. Statistical comparisons between the seat-patch water potentials and the blood osmotic potentials of anuran species using a t-test.

Species	P value	t-test value	df
X. laevis	$p < 0.001$	29.71	12
L. catesbieana	$p < 0.001$	8.33	18
L. pipiens	$p < 0.001$	5.54	21
A. boreas	$p > 0.05$	1.86	24
P. cadaverina	$p > 0.4$	0.78	8
P. hypochondriaca	$p > 0.9$	0.08	21

Appendix G. Data for seat-patch water potentials and blood osmotic potentials for six anuran species

TABLE 4. Comparison of the seat-patch water potential to the osmotic potential of blood in six anuran species while at 90% standard body mass (the mass of a fully hydrated frog with an empty bladder). Seat-patch water potentials were measured by recording changes in body mass while rehydrating in sucrose solutions that varied in water potential. The osmotic potential of blood was measured using a freezing point osmometer.

	Water Potential (-kPa)		
Species	Seat-patch(90%)	Blood(90%)	Blood(100%)
Xenopus laevis	318 ± 21	700 ± 29	581 ± 23
Lithobates catesbeiana	317 ± 76	580 ± 13	480 ± 32
Lithobates pipiens	495 ± 67	697 ± 31	593 ± 22
Anaxyrus boreas	598 ± 144	685 ± 11	590 ± 7
Pseudacris cadaverina	657 ± 70	707 ± 13	635 ± 35
Pseudacris hypochondriaca	864 ± 114	840 ± 41	710 ± 44