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Effects of natural history on osmoregulatory behaviors in two stream-dwelling frogs (Pseudacris cadaverina and P. regilla)

Heidy Lorena Contreras

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EFFECTS OF NATURAL HISTORY ON OSMOREGULATORY BEHAVIORS
IN TWO STREAM-DWELLING FROGS (PSEUDACRIS CADAVERINA
AND P. REGILLA)

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Heidy Lorena Contreras
March 2007
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Approved by:

Colleen Talbot, Ph.D., Chair, Biology

Anthony Metcalf, Ph.D.

David Polcyn, Ph.D.

Stanley Hillyard, Ph.D.
School of Dental Medicine, UNLV
ABSTRACT

Differences in osmoregulatory behaviors were studied in two stream-dwelling tree frogs (Pseudacris cadaverina and P. regilla) with different natural histories. Specifically, dehydration thresholds between each species were determined. Differences in ability to find a water source and osmotic preferences of hydrating solutions were also studied. It was found that P. regilla opportunistically exploit water while P. cadaverina absorbs water only when dehydrated to a specific degree. P. regilla seems to prefer dilute solutions to re-absorb water while P. cadaverina re-absorbs water from more concentrated solutions. Although there was a general preference for specific solutions, there were no differences in the frogs ability to find a water source. In general both species moved around the aquarium at random. Differences in pattern of movement were observed only between hydration states where hydrated P. regilla move around the aquarium more often than P. cadaverina.
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Amphibians, being greatly dependent on environmental moisture, have natural histories and life histories which are strongly influenced by the distribution and abundance of water. Although it is true that most species are limited in their distribution due to their dependence on freestanding water, some have successfully colonized arid terrestrial habitats. The physiological challenge of maintaining a stable internal water balance when an animal is found in habitats with low water availability is exacerbated by the animals' highly water-permeable skin (Churchill and Storey 1995). With a few exceptions, amphibians, as a group, have not evolved structures to greatly resist integumentary exchange of mass (Feder and Burggren, 1984). Their skin is a site for flux of water, heat, electrolytes and respiratory gases. Over evolutionary time physiological, behavioral, structural and metabolic adaptations have developed in some terrestrial anurans which have assisted in minimizing, or preventing, the rapid diffusion of important solutes (Churchill and Storey 1995).
Opportunistic absorption of water, storing large amounts of dilute urine in the bladder, reducing integumentary permeability and displaying water conservation responses when away from a water source are all adaptations which allow amphibians to exploit the terrestrial environment. These adaptations minimize dehydration by reducing the rate of evaporative water loss through the integument and maintaining the amount of water found in the organism (Pough et al. 1983; Brekke et al. 1991; Hillyard et al. 1998; Hillyard 1999).

One of the major consequences of dehydration is a decrease in body fluid volume (and pressure) and a concomitant increase in plasma osmolality. Disruption of stable concentrations of solutes in extra-cellular fluids within an organism may cause normal physiological functions to be impaired (Acher et al. 1997). A common mechanism employed by many animals to maintain proper hydration is the induction of behavioral responses associated with water absorption. In mammals it has been shown that an increase of only 2-3% in plasma osmolality, or a decrease of 10-15% of blood volume, will induce thirst behavior. A slight change in plasma osmolality therefore elicits behavioral responses to re-establish
homeostatic conditions. This type of behavioral response has been observed in mammals and other vertebrate groups that take in water orally. In contrast, frogs and toads do not normally drink orally when they become dehydrated, instead, they absorb water osmotically across their integument, often through a specialized region known as the seat patch (Townson, 1799; Krough, 1939; Nakashima and Kamishima 1990). The seat patch, or pelvic patch, is a specialized area of the ventral abdomen which extends out to the thighs of many anurans (Bentley et al. 1972). Since this area of the skin is highly vascularized, has increased surface area and overlies a large lymphatic space (Bentley et al. 1972; Yorio and Bentley 1977; Hillyard et al. 1998), osmotic uptake from the environment is highly efficient. It is believed that the pelvic patch is also used in evaluating the osmotic and/or ionic concentrations of possible hydrating sources. When anurans become dehydrated they will first initiate a search behavior called seat-patch-down (Hillyard et al. 1998). During this behavior the seat patch is continuously pressed on and lifted off the substrate. This occurs while the frog moves to different points on the hydrating source. Although the seat-patch comes into
contact with a solution, the volume of substrate absorbed is minimal. This allows the frog to "sample" the hydrating source, that is, it allows the frog to "taste" (Hillyard et al. 1998; Pers. Obs.). Once a "preferable" water source has been located a different behavior, the water absorption response (WR), is induced. During this behavior the hind limbs are abducted and the surface of the pelvic patch is completely pressed onto the substrate (Stille, 1958; Townson, 1975; Hillyard, 1991). Water moves down its osmotic gradient from the outside of the animal through the pelvic patch and into the animal. The rate of water movement is related to the surface area of the skin, the osmotic gradient, and the hormonally controlled water permeability of the skin (Hoffman and Katz 1999).

Usually, frogs found away from a water source are found in a posture termed the water conservation response (or in a burrow of some sort). In this response the animal will pull their hind and fore limbs close to their body therefore decreasing the amount of skin surface area that is exposed with to environment. This decreases the amount of water that is being lost to the environment. However, even in this posture, there may still be some
unavoidable evaporative water loss that may lead to dehydration. In order to counteract this effect, many terrestrial anurans have evolved the ability to store large amounts of dilute urine in their urinary bladder (Shoemaker and Bickler 1979; Jorgensen 1998). The dilute urine may be reabsorbed from the bladder when the animals are away from a permanent water source, offsetting the increase in osmotic concentrations of body fluids that occurred due to evaporative loss across the skin.

In anurans there seems to be a positive correlation between the tolerance for dehydration and the degree of terrestriality (Thorson and Svihla 1943; Ray 1958; Schmid 1965; Larson 1971; Young et al. 2005). That is, anurans that are highly terrestrial seem to tolerate higher levels of dehydration than anurans inhabiting mainly aquatic habitats. Additionally life history (degree of terrestriality) is also related to how dehydrated an amphibian must be before it initiates WR (Hillyard et al. 1998). *Bufo punctatus* is a small toad that can be found as far as 1km away from a permanent water source when foraging. It has been shown that this toad will consistently display the water absorption response when dehydrated by as little as 1% - 3.6%. The related species
B. cognatus, B. marinus and Scaphiopus couchii consistently display the water absorption response when dehydrated by 10% - 15% from their initial body weight (Maleek et al. 1999; Johnson and Propper 2000). The differences observed in the dehydration thresholds of B. punctatus and B. cognatus and S. couchii may be related to whether the animal drinks opportunistically or needs to suppress their water seeking behavior for long periods (Hillyard et al. 1998). Bufo cognatus and S. couchii are seasonally active toads which may stay in burrows until summer rainfall begins while B. punctatus does not burrow and seems to depend on opportunistic water absorption in order to forage large distances away from a permanent water source (Hillyard et al. 1998). Initiation of water absorption behaviors may also be under control by other factors such as the size of an animal. B. marinus is a large terrestrial toad that is always found near a permanent water source. Its low surface area to volume ratio seems to favor slower evaporative water loss (Cohen 1952; Tracy 1976), than that observed in smaller toads such as B. cognatus, B. punctatus and S. couchii, whose higher surface area to volume ratios favors more rapid evaporative water lost. Consistent water availability and
slow evaporative water loss might allow *B. marinus* to have a higher dehydration threshold before the water absorption response is initiated (Hillyard et al. 1998).

The terrestrial tree frogs (*Hyladinae*) can be found in a variety of habitats throughout the world and have evolved from bufonids rather than ranids (Noble, 1954). Some species within this family of frogs have evolved a low rate of evaporative water loss through their integument (Jameson 1966) and seem to lose water at rates one-third to one-half those of typical amphibians (Feder and Burggren, 1992). In North America, the genus *Pseudacris* (the chorus frogs) is widely distributed from the southern tip of Florida to northern Canada and from the east- to west-coasts (Moriarty and Cannatella, 2003). They occur in a variety of habitats and prefer to breed during the cold seasons laying their eggs near or in permanent or temporary bodies of water (Noble, 1954).

The California and Pacific Tree Frogs are two species of terrestrial amphibians in the genus *Pseudacris* found throughout the state of California. Although there has been some debate as to where each species should be placed in evolutionary history (Maxson 1975; Hedges 1986, Cocroft 1994; Da Silva 1997) recently both species, along with
other North American tree frogs, have been placed within the genus *Pseudacris* instead of the genus *Hyla* (Moriarty and Cannatella 2004). This recent change in genus will be noted in the present study.

*Pseudacris cadaverina*

*P. cadaverina*, the California Tree Frog, is a primarily nocturnal species that is mostly distributed in upland stream habitat (Cunningham 1964) in discontinuous cismontane populations from San Luis Obispo County to Bahia de Los Angeles, Baja California (Stebbins 2003). It is usually found near canyon streams and washes at elevations ranging from sea level to 1500m among rocks, quiet pools, and shade. Clustering of this species tend to occur by stream boulders and the large pools they create (Kay 1989). During the day *P. cadaverina* seeks shade and protection among rock crevices near water (Stebbins 2003).

Their breeding season usually lasts from March to May, although temperature and rainfall seem to influence the length of the season. Changes in season are not only correlated with changes in the overall density of frogs seen near the water but also with the locations where frogs may be found (Dole 1974). For example, surveys done
from mid August to January along a 500m stretch of boulder-strewn stream resulted in finding only 12 frogs, an average of one animal per census (Dole 1974). In late February and early March a much higher average of 9 frogs per census can be found in the same area. During this period, when reproductive activity is at its highest, frogs are found close to the water’s edge, rarely more than 20cm away, or in the water itself. From April through August, on the other hand, the animals were seen mainly on a cliff face or on large boulders as high as 2 m above the water.

Dole (1974) saw that most individuals typically occupied only a small portion of the total available habitat (stream). In areas where there was constant availability of water (the lower portion of the canyon being studied) and a suitable number of hibernating sites, individuals rarely moved more than 3 or 4 m from their original capture site (census were taken from July 1964 to February 1966). Individuals inhabiting the higher portion of the canyon were faced with limited supplies of water and hibernating sites. These individuals moved further distances (up to 75 m) from the higher canyon into the lower one in order to obtain the necessary resources.
Although these individuals would travel down to obtain the needed resources, and even sometimes to breed, they always returned to their former areas afterward.

Pseudacris regilla

Compared to P. cadaverina, P. regilla, the Pacific Tree frog is a generalist species both temporally and in their distribution (Stebbins 2003). They are active day and night and are found in a wide range of habitats including grasslands, chaparral, woodland, desert oases, agricultural regions, and even in residential areas; these habitats can occur from sea level to the tops of mountains. This species ranges from British Columbia, Canada to the tip of Baja California, México and eastward to west Montana and eastern Nevada. This species is chiefly a ground dweller (Stebbins 2003) and it is usually found among low plant growth near water (Pers. Obs.).

During late summer, fall and early winter these frogs may be found up to a mile away from breeding sites, hiding in areas having low moisture, such as logs or rocks (Jameson 1957). A particular set of environmental conditions, which according to Jameson (1957) are believed to be the first summer rains after a certain threshold of
reproductive hormonal production is reached, begin the onset of congregation in breeding sites during the months of January through August. Although the males seem to enter the ponds and remain there for several months, they do not stay for the entire breeding season.

*P. regilla* breeds between January and August when males move to permanent or semi-permanent ponds where breeding choruses are formed (Jameson, 1957). At these breeding sites advertisement calls may occur twenty-four hours a day during the height of the breeding season (Jameson 1956; Jameson 1957; Brenowitz and Rose 1999) the calls seem to come with greater intensity during the few hours after sunset (Pers. Obs.). This species has been found calling in pools formed by melting snow at 1200 meters in April and at 1800 meters in June. In elevations above 500 meters, males call as late as August, so it seems that the breeding season is determined by temperature as well as with altitude (Jameson 1957).

Breeding site distribution seems to be the limiting factor on the occurrence and size of the population. Semi-permanent ponds used by this species to breed generally contain a large amount of males in the shallower portions of the pond where vegetation cover is the heaviest.
(Jameson 1956). Females begin to move to the breeding sites a few weeks after the males. When the female enters the pond she will probably mate with a few males that are found near the point of where she entered.

*Pseudacris regilla* may have a homing response which allows them to return to the same breeding sites even when moved more than 1000 yards from the home (Jameson 1957). During the breeding season the home range of this species is relatively small and males are only found a couple of feet away from their calling site. However the activity during the non-breeding season ranges up to several hundred yards from the breeding site.

*Pseudacris cadaverina* and *P. regilla*

Although these two species seem to have an overlapping range in southern California and in northern Baja California (I. Phillipsen, Pers. Comm.), it has been reported (Stebbins 2003) that the two species are seldom found together in the same locality. However, I found these species to show a different pattern. Most localities within San Bernardino County containing *P. cadaverina* also contain *P. regilla* (Pers. Obs.). Although unclear, this sympatric association may have developed as
a response to recent droughts and specific selection of microhabitats by each species.

The ability to exploit different microhabitats might be associated with certain adaptations related to the prevention and/or response to dehydration in these frogs. As mentioned before, it has been shown that highly terrestrial species can tolerate high dehydration levels (Thorson and Svihla 1943). It has also been suggested that highly terrestrial frogs may absorb water opportunistically when foraging away from a permanent water source (Hillyard et al. 1998). Both of these adaptations may assist the animal in returning to a permanent body of water before reaching lethal levels of dehydration. On the other hand, frogs that are found near a permanent water source tolerate lower levels of dehydration and may absorb water only after they have become dehydrated (i.e. will not absorb water opportunistically) since they are typically able to locate a hydrating source very quickly once dehydrated.

Differences in the manner in which resources are exploited by *P. cadaverina* and *P. regilla* may therefore facilitate the sympatric association observed in San Bernardino County.
Organismal Osmoregulation

In mammals an increase of only 2-3% in plasma osmolality will induce thirst behavior. Osmoreceptors, or cells, that sense the changes in osmolality in the hypothalamus, signal the brain to induce drinking behavior (Acher et al. 1997). Changes in osmolality, in general, do not induce thirst; the increase in osmolality must be due to an increase in concentration of specific solutes for this to occur. For example, an increase in plasma osmolality caused by urea or glucose causes little or no effect on drinking behavior. On the other hand, an increase in plasma osmolality caused by NaCl or mannitol will readily induce drinking behavior (Robertson, ). This phenomenon is thought to be due to the manner in which the osmoreceptors in the hypothalamus work. NaCl and mannitol do not seem to permeate the osmoreceptor plasma membrane and an increase in extracellular concentration of these solutes creates a gradient between the outside and inside of the cell. In order to reach equilibrium, water diffuses out of the osmoreceptor cell, causing the cell to shrink. This leads to a signal to be sent to the brain that there is an increase in extra-
cellular osmolality. Urea and glucose, on the other hand, readily permeate the osmoreceptor cell membrane and are therefore unable to create the trans-cellular gradient needed to pull water out of the cell. Since water does not leave the cell there is no change in its conformation and therefore there is no signal sent to the brain of a change in plasma osmolality.

Changes in an organism’s blood volume and/or blood pressure also influence whether thirst is induced or inhibited. In mammals it has been shown that a decrease in plasma volume/pressure of 10-15% will induce drinking behavior (Koeppen and Stanton 2001). Changes in blood volume are sensed by low pressure baroreceptors found in the atria of the heart and pulmonary vessels. These baroreceptors sense the “fullness” of these compartments. When there is a reduction in blood volume these baroreceptors send signals through the afferent fibers of the vagus and glossopharyngeal nerves to the brain which leads to an induction of drinking behavior (among other physiological responses) and helps re-establish a “normal” blood volume (Kato et al. 1999). These same nerves transport signals to the brain from high-pressure baroreceptors found within the aortic arch and carotid
sinus whose function is to sense a decrease in blood pressure. Once again, thirst is induced in order to re-establish a “normal” blood pressure.

In mammals, it has also been shown that a decrease in blood pressure stimulates the renin-angiotensin-aldosterone system. In this system, low blood pressure leads to a decrease in renal tubular NaCl, which is sensed by the macula densa cells of the kidney nephron and induces the secretion of renin into the blood. Renin is an enzyme that converts angiotensinogen to angiotensin I, which is converted into angiotensin II by angiotensinogen converting enzyme in the respiratory capillaries. Although most studies done on the renin-angiotensin-aldosterone system focus on mammals, there are some studies which focus on the effects of Angiotensin II in amphibians. For example, Hoff and Hillyard (1991) found that the water absorption response could be induced in fully hydrated *B. punctatus* when Angiotensin II was injected peritoneally. Further studies have found that the renin-angiotensin-aldosterone system is employed by a wide range of anuran species to induce drinking behavior (Tran et al. 1992; Propper and Johnson 1994; Propper et al. 1995).
Structure and Function of the Amphibian Integument

There are several factors which may affect the manner in which mass transport through the integument is achieved in vivo, and therefore a general knowledge of the skin’s anatomy is beneficial. The integument of the frog consists of an external epidermis and an internal dermis, which are separated by a basement membrane of collagen fibers. The epidermis is the actual barrier between the inside of the organism and its environment (as reviewed in Talbot, 1993). Tight junctions between these cells create a high resistance barrier that prevents paracellular movement, forcing transport to occur through the cells. Tight junctions also induce differentiation in function between the apical and the basolateral membranes of the epithelial cells, thus allowing specific placement of the different proteins required in the two step transepithelial transport of solutes.

The dermis is a multi-layered structure, deep to the epidermis, containing the blood vessels, glands and pigment cells of the skin, but is mainly a sheet of connective tissue (Stebbins and Cohen, 1995). Water that is absorbed from the environment must move partly through
the dermis in order to be absorbed into the blood vessels and then transferred to the rest of the body. Water that is not taken up into the blood vessels continues to move through the dermis and into the lymph space found next to the inner side of the dermis. The lymph space is able to handle not only large volumes of liquid absorbed across the skin but also the fluid that is produced by ultrafiltration of blood across the cutaneous capillary walls (Hillyard and Larsen 2001). Lymph is ultimately returned to the circulatory system.

Anuran skin is a complex organ that contains regional variation in order to perform different physiological functions (Talbot, 1993). For example the pelvic patch, found in the lower abdomen and inner thigh, has a greater permeability to water than other regions of the skin (Bentley and Main 1972). This is done by increasing surface area in this part of the integument through skin folds and by increasing of blood flow through the capillaries to ensure that a local physical environment optimal for water uptake is created (Winokur and Hillyard 1992). Anuran skin, is therefore not a uniform tissue, but instead it is highly heterogenous.
Mechanisms of Ion and Water Transport

Ion transport through the cells making up epithelia is via both active and passive transport systems. In sodium absorbing epithelia, sodium enters the cell from the environment by passive transport through epithelial sodium channels in the apical membrane. Active transport of sodium by the Na⁺/K⁺ ATPase pump on the basolateral side of the epithelial cell not only moves Na⁺ from the cytoplasm to the extracellular fluid (completing transepithelial sodium transport) but also pumps K⁺ into the epithelial cell. The flow of K⁺ out of the cell through K⁺ leak channels on the basolateral membrane produces an inside negative resting potential, which helps create the negative membrane potential that allows Na⁺ to diffuse into the cell across the apical membrane. The driving force for the net flow of Na⁺ across the frog skin from the mucosal solution is therefore the active transport of Na⁺ out of the cell towards the serosal solution.
The Present Study

In order to colonize highly terrestrial habitats anurans must be able to withstand high levels of dehydration stress. Although individual factors such as fast evaporative water loss, extreme high/low temperatures and body size, seem to influence dehydration tolerance of an individual (Jameson 1966), interspecific differences are also apparent between anuran species inhabiting different environments (Hillman 1980). The current study is focused on differences in the behavioral responses to dehydration in Pacific and California Tree Frogs. Specifically, I will examine the dehydration level required in each species before water absorption behaviors are consistently induced (dehydration threshold). Interspecific ability to locate a water source, as well as preferences for a specific solutions (varying in solute concentration) will also be investigated.

_P. cadaverina_ and _P. regilla_ seem to have different ranges within their habitats. As previously discussed _P. regilla_ are active in a wide range of different types of habitats and have been found as far as a mile away from their breeding site. Adaptations such as high tolerance to dehydration and opportunistic water absorption may have
evolved in this species which allow it to generally exploit resources. This may help explain its wide distribution throughout different habitats. Although *P. cadaverina* are able to move large distances away from their breeding sites it seems that their movement is correlated with an available water source; movement occurs along the stream and not away from it. *Pseudacris cadaverina* seem to have specific microhabitat requirements especially related to water availability. Unlike *P. regilla*, which may be found almost anywhere, *P. cadaverina* prefers rocky areas within canyon streams at high elevations. Their seasonal movements suggest a relatively low adaptation to dehydration stress and we therefore might expect a relatively low dehydration tolerance. Since this species is always found near a water source, it is not expected to absorb water opportunistically, but instead to reabsorb water only after it has become dehydrated. According to these species natural histories we expect that *P. regilla* will consistently show water absorption behaviors at high dehydration levels while *P. cadaverina* will show these responses at lower levels; *P. regilla* will have a higher dehydration threshold compared to *P. cadaverina*. 
Previous studies have suggested that frogs have the ability to "taste" a water source and therefore determine whether it is osmotically preferable. It has been suggested that chemoreceptors on the pelvic patch allow them to detect whether a solution is highly concentrated (Koyama et al. 2001; Hillyard et al. 2004; Nagai et al. 2004). Osmoreceptors on the frogs feet, on the other hand, may allow the frogs to determine whether a water source is available. The second question in my study will try and discover whether these species are able to detect where a hydrating source is located and move towards it in a non-random fashion. Differences between P. regilla and P. cadaverina in their ability to differentiate between highly concentrated and dilute solutions will also be examined.
CHAPTER TWO
DEHYDRATION THRESHOLD IN THE CALIFORNIA AND PACIFIC TREE FROGS

Introduction

Amphibians, as a group, are highly dependent on freestanding water due to the high permeability of their skin. Although rapid evaporative water loss through the integument has largely limited amphibian distribution, some species have developed physiological, structural and/or metabolic adaptations which allow them to colonize more terrestrial environments (Thorson and Svihla 1943; Thorson 1955; Thorson 1956; Ray 1958; Schmid 1965). Behavioral responses to dehydration have been studied in amphibians due to their role in the exploitation of arid terrestrial habitats by many taxa. These behaviors, (i.e., the water conservation response) seem to be important not only in preventing rapid dehydration via the integument but also facilitate the detection of a hydrating source (i.e., seat-patch down response) and facilitate quick re-hydration (i.e., water absorption response). When dehydrated anurans encounter a potential rehydration source they display a behavior termed “seat-
patch-down” (SPD) where the pelvic patch is continuously pressed and lifted from the substrate, allowing the animal to detect whether a water source is present (Hillyard et al. 1998). Once water is found, the animal displays the water absorption response (WR) where the hind limbs are abducted and the pelvic patch is pressed onto the substrate so that water can be absorbed osmotically (Stille 1958). This response is maintained until the animal is hydrated and stable hydrated levels are reached.

Previous studies have shown that arid environments constrain normal activities and that environmental humidity affects amphibian behavior (Feder and Londos 1984; Seebacher and Alford 2002; Preest et al. 2003; Wisely and Golightly 2003). In spite of this, amphibians have colonized a variety of habitats: from strictly aquatic to highly terrestrial. These major differences in a species’ natural history strongly influences differences in behaviors associated with osmoregulation (Schmid 1965). Hillyard et al. (1998) observed that the water absorption behavior in toads was induced at different levels of dehydration in species faced with differing dehydration extremes throughout their natural histories. Species which were normally found near a permanent water source had
higher dehydration thresholds (showed WR consistently only after reaching higher specific levels of dehydration) than species which were known to travel large distances away from a hydrating source (which absorbed water opportunistically). Toads that were forced to suppress water-seeking behaviors during a large period of time during the year (due to aestivation) also had high dehydration thresholds before water absorption behavior was consistently induced.

Although Hillyard’s et al. review suggested possible differences in anuran behavior related to their specific natural histories, the two species being compared drastically differed in either size or mode of life. In this study we aimed to investigate the role that natural history plays on the osmoregulatory behaviors of two similar species: *Pseudacris regilla* and *P. cadaverina*. These two species are in the Hylidae family (subfamily: Hylinae) which include species that have evolved a low rate of evaporative water loss across their integument (Shoemaker et al., 1992). Although some species within this family secrete lipids to decrease water permeability of the skin (Amey and Grigg 1995) most, including *P. regilla* and *P. cadaverina*, do not employ this mechanism to
prevent high rates of water loss. Instead the integument is not uniform in its permeability over the animal’s body (Schmid 1965; Bentley and Main 1972); permeability of the skin is higher on the pelvic patch so that water may be absorbed more quickly during re-hydration.

The California Tree Frog, *P. cadaverina*, is primarily a nocturnal species that is mostly distributed in upland stream habitats (Cunningham 1964) in discontinuous cismontane populations from San Luis Obispo County to Bahia de Los Angeles, Baja California (Stebbins 2003). It is usually found near canyon streams and washes at elevations ranging from sea level to approximately 2,300 m where there are rocks, quiet pools, and shade. Individuals of this species tend to cluster by stream boulders and in the large slow pools they create (Kay 1989). During the day, this species of frog seeks shade and protection among rock crevices near water (Stebbins 2003) and rarely seems to move more than two meters away from a permanent body of water (Dole 1974). Unlike *P. cadaverina*, the Pacific Tree Frog, *P. regilla*, is active during the day and night (Stebbins 2003). It is found in a wide range of habitats including grasslands, chaparral, woodland, desert oases, agricultural regions, and even in
residential areas; these habitats can occur from sea level to ca. 3,500m. The distribution of *P. regilla* ranges from British Columbia, Canada to the tip of Baja California, México and eastward to west Montana and eastern Nevada. During late summer, fall and early winter *P. regilla* may be found up to a mile away from breeding sites, hiding in areas with low moisture, such as logs or rocks (Jameson 1956).

*Pseudacris regilla* and *P. cadaverina* are sister taxa (Moriarty and Cannatella 2004) and, although they seem to have an overlapping range in southern California and in northern Baja California, it has been reported (Stebbins 2003) that the two species are seldom found together in the same locality. However, personal accounts have shown a different pattern and most of the localities within San Bernardino County in California containing *P. cadaverina* also contain *P. regilla* (I. Phillipsen, Pers. Obs.). A decrease of available water in the environment might be the major source driving this sympatric association (Fellers and Drost 1993), but the sustainability of both species at these localities is probably explained by other factors. These two frogs may be able to coexist due to
differences associated with the species’ natural history so that interspecific competition is naturally reduced.

In this study the dehydration thresholds, that is, the level of dehydration where water absorption behavior is consistently induced for 80% of *P. cadaverina* and *P. regilla* individuals were studied. We expect that the observed behaviors will largely be influenced by the natural history of each species. Since *P. regilla* travel large distances away from a water source during the non-breeding season we expect that osmoregulatory behaviors will be induced at low levels of dehydration (i.e. they will be more opportunistic in obtaining water, when available). *P. cadaverina*, on the other hand, might require high levels of dehydration before behaviors are consistently induced, since it is normally only found a couple of meters away from a water source and does not rely on opportunistic water absorption (Jameson 1956; Jameson 1957; Dole 1974; Harris 1975). Other physiological processes and behaviors associated with osmoregulation may also differ between these two organisms, therefore, differences in time spent on moist substrate, time spent in the water absorption response once it has been initiated and re-hydration rates were
also measured. We expect *P. regilla* to spend less time in WR and have higher re-hydration rates when compared to *P. cadaverina*, since they are expected to reabsorb water opportunistically and not necessarily when dehydrated.

Materials and Methods

**Animals**

Adult Pacific (*P. regilla*) and California (*P. cadaverina*) Tree Frogs were collected from Mill Creek in Highland (San Bernardino County), California in October of 2003. All frogs were housed together in a 20 gallon glass terrarium containing soil, a store bought bark substrate, natural plant material, and two plastic containers filled with water. Granite rocks of 5-10 cm in height were placed in the terrarium. Frogs were able to rest on rocks, water, plants or moist soil ad libitum. Small sections of egg cartons were also placed in the terrarium so that frogs could burrow underneath. The frogs were kept on a 12L:12D light cycle at ca. 70 F and were fed live crickets once per week. All individuals were allowed to recover in this terrarium at least three days between dehydration trials.
**Observation Tank**

A glass 20 L aquarium was placed on supports and elevated above an angled mirror allowing observation of the bottom of the tank. The four sides of the aquarium were covered with window film (a sheet of thin, black, plastic covered with 2mm diameter pores 1 cm apart) that still allowed the observer to see inside the aquarium. The bottom of the tank was covered with white paper except for the area where a saturated tissue was placed. The mirror situated underneath the tank allowed for complete detection of the water absorption response, which was defined as a posture where the frog’s hind limbs were abducted and the pelvic skin was pressed onto the moist tissue (Stille 1958; Hillyard et al. 1998).

**Behavioral Experiments**

To allow for all individuals to start at a similar hydration level, all frogs were forced hydrated for half an hour before each trial by being individually placed in a closed plastic container filled with 1mM NaCl. After the hydration period excess water was removed from the frog’s skin by gentle swabbing and each frog’s weight was measured and recorded. Since frogs are able to re-absorb dilute urine that is stored in the bladder, pelvic
pressure was applied to expel urine and the frog's weight was once again recorded. This final weight was taken as initial standard weight (Ruibal, 1962).

After recording standard weight, each individual was dehydrated by a specific amount. Since the rate of dehydration has been shown to affect behavioral responses to evaporative water loss in some Hyla/Pseudacris species (Jameson 1966), frogs were dehydrated either: 1) rapidly (using a fan) or 2) slowly (without a fan). Rapid dehydration was induced by placing frogs in a small plastic container which had been thoroughly perforated (top, bottom, and all sides). The container was placed above a fan in order to apply constant ventilation and induce a high rate of evaporative water loss. Frogs were maintained in the container for periods of 15 min - 4 hrs until the desired of dehydration was reached. Slow dehydration was achieved by keeping frogs in a dry tank and allowing normal levels of evaporative water loss (1-13 hours). Dehydration was based on changes from the initial hydrated body mass (assuming that changes in mass are directly proportional to water loss), therefore dehydration was indicated as percent change from the standard weight. Five dehydration trials were conducted
for each tree frog species. Since previous studies had shown that P. regilla dehydrated by 10% from initial hydrated weight induced WR, P. regilla were nominally dehydrated to 3 ± 0.3, 5 ± 0.5, 8 ± 0.8, 10 ± 1.0 and 15 ± 1.5% of their standard weight while P. cadaverina were dehydrated to 5 ± 0.5, 8 ± 0.8, 10 ± 1.0, 15 ± 1.5 and 18 ± 1.8% of their standard weight. All frogs were tested in a blocked design, where each frog was randomly assigned to a specific dehydration level, and all individual were tested at all levels.

Observations

A fifteen-minute observation period commenced once a frog was placed on a saturated (1 ml of a 1mM NaCl solution) 11 x 11 cm tissue. During the observation period the following were recorded: 1) Time spent on the saturated tissue; 2) Whether WR was initiated; and 3) Time spent in the water absorption response. At the end of the fifteen-minute trial the frog was re-weighed; a weight gain indicated water absorption and a weight loss indicated further dehydration. After each trial, frogs were allowed to re-hydrate ad libitum with fresh 1mM NaCl solution.
A negative control for the water absorption response was conducted using forced hydrated frogs, performing fifteen-minute observations without dehydration. Frogs were then placed in a 10 L holding tank and allowed to re-hydrate.

**Statistical Analysis**

A chi squared test was used to determine if there were any intraspecific differences between rapidly and slowly dehydrated individuals with regards to the frequency in which water absorption behavior (WR) was displayed at each level of dehydration. Linear regression was used to test the relationship between percent dehydration and proportion of frogs displaying WR in both California and Pacific Tree Frogs. Differences in the species responses to increasing levels of dehydration were tested with an analysis of covariance (ANCOVA) where species of tree frog was the fixed variable, percent dehydration was the covariate and proportion of frogs in WR was the dependent variable. All percentage data were ArcSine transformed to normalize the distribution. Time spent on substrate, time in WR, average percentage weight change from initial standard weight and re-hydration rates were analyzed using a repeated measures ANOVA with level
of dehydration (0, 3, 5, 8, 10, 15 and 18%) as the within-subjects factor and species of frog (California vs. Pacific) as the between-subjects factor.

Results

The proportion of frogs showing WR did not significantly differ as a function of dehydration rate (p>0.05). Additionally, the effect of dehydration rate on any of the frog’s responses was tested with a repeated measures ANOVA design to specifically examine time spent on tissue and change in weight between quickly and slowly dehydrated trials. Both analyses showed that there was no significant mean differences in the frog’s behavior (p>0.05) between these two dehydration types; therefore the data (time spent on tissue, time in WR and %weight change during observation period) for the two rates of dehydration were pooled for each frog and the averages were used for the rest of the analysis. Analysis of the “frequency of frogs showing WR” included data for both dehydration rates, therefore each level of dehydration had a total of 18 observations (2 per individual).

The proportion of frogs, for both species, displaying water absorption behavior increased with increased
dehydration (Fig. 1). Water absorption response was initiated by both species of frog at relatively low levels of dehydration. Even though previous studies (Laura Paasch and Heidy Contreras, unpublished data) had suggested that naïve frogs would not initiate WR when fully hydrated, WR was observed in 8 of 18 (44%) of P. regilla trials and in 1 of 18 (5.6%) of P. cadaverina trials when frogs were fully hydrated. In P. regilla essentially in all of the trials (>80%) WR was shown at 8% dehydration. On the contrary, dehydration of almost 18% was required before WR was shown in 80% of P. cadaverina trials. This species rarely initiated WR when fully hydrated and the proportion of trials where the response was shown did not increase until frogs were dehydrated by 18% from their initial weight. When P. cadaverina were dehydrated to levels between 3-15%, the proportion of trials in which WR was displayed fell within 33-50% of the total observations. By 18% dehydration, WR was shown in 83% of the trials (15 out of 18 trials) sometime during the fifteen minute observation period (Fig. 1). Linear regression of the ArcSine transformed data suggested that 86% of the variability observed in the proportion of trials in which P. regilla showed WR was due to the level
of dehydration. The strong relationship between dehydration level and proportion of trials in which WR was shown was also present in P. cadaverina were 83% of the variability was due to dehydration level (Fig. 1). The pattern of response (slope of the regression lines) for these two species of frog was essentially the same (F = 4.121, df = 1, p = 0.07), but there were significant differences in the proportion of trials in which frogs showed WR at different dehydration levels between species (F = 19.74, df = 1, p=0.001); more P. regilla induced WR at all levels of dehydration when compared to P. cadaverina.

Time spent on a damp substrate was analyzed using a repeated-measure ANOVA to compare responses within and between California and Pacific Tree Frogs. Results showed a linear trend (F = 70.14, df = 1, p = 0.0001) in time spent on tissue as a function of dehydration level (Fig. 2). In general, at low levels of dehydration, there were a larger number of P. regilla individuals spending longer periods of time on the wet tissue compared to P. cadaverina. At 15% dehydration 78% of the Pacific Tree Frogs spent >12 minutes on the tissue whereas only 22% of P. cadaverina spent equal amounts of time on the wet
substrate. At 18% dehydration 89% of *P. regilla* spent >13 minutes on the tissue whereas 55% of *P. cadaverina* spent equal amounts of time on the tissue. It was not until *P. cadaverina* were dehydrated by 22% (from the initial hydrated weight) that all of the frogs spent the entire observation period on the wet surface during all of the trials. Although there are differences for each species in the amount of time spent on the wet tissue at each dehydration level, there was no significant interaction between dehydration level and species (*F*=0.82, *df*=6, *p*=0.56). This may be due to the low observed power (0.31) for this part of the analysis. Overall, significant mean differences (*p*<0.05) were observed as a function of dehydration level (*F*=12.52, *df*=6, *P*=0.0001) and, independently, as a function of species of frog (*F*=8.96, *df*=1, *P*=0.009). *Pseudacris regilla* spent more time on the saturated tissue (average amount of time spent on tissue regardless of dehydration level: 578 sec) than the *P. cadaverina* (average amount of time spent on tissue regardless of dehydration level: 384 sec).

For both species, approximately 95% of the time a frog spent on the saturated tissue was spent in the WR posture; once the frog stopped showing the response it
would typically move off of the tissue. In order to determine differences in the amount of time spent in WR between the two species, a repeated measure analysis of variance was again used where dehydration level and species of frog were the independent variables and time in WR was the dependent variable. All of *P. regilla* showed water absorption response when dehydrated above 10% from their initial hydrated weight. This level of response was not seen in *P. cadaverina* until frogs were dehydrated by 22% from their initial hydrated weight. Individual *P. regilla* displaying WR maintained the response on average, for at least 10 min (Fig. 3). As levels of dehydration increased not only did the number of individuals showing the response increase (up to 8% dehydration where all individual showed the response) but also the average time spent in this response increased, mainly due to an increase in the proportion of trials in which the response was shown (Fig. 3). *Pseudacris cadaverina*, on the other hand, were more variable in their response. At low levels of dehydration, time in WR ranged from < 1 min to > 12 min. As the degree of dehydration increased, the time in WR increased for the population, particularly at above 15% (Fig. 3). Significant differences in the time spent
in WR where observed between dehydration levels alone (regardless of species) \((F=15.5, \, df=6, \, p=0.0001)\) where time population spent in WR increased with dehydration. Overall time spent in WR also differed collectively between species (regardless of dehydration level and on whether WR was shown or not) \((F=9.38, \, df=1, \, p=0.007)\); \(P. \, regilla\) spent more time overall in the response than the \(P. \, cadaverina\). When only looking at individuals that showed the water absorption response at different dehydration levels, there were no significant differences in the time spent in the response \((F=1.264, \, df=6, \, p=0.281)\) (Fig. 4).

Individuals that did not initiate the water absorption response, or only engaged in it for short periods of time continued to lose water (mass) over the 15 min observation period in spite of water being available; this happened most often in frogs dehydrated by <5% of initial standard weight (Fig. 5). A repeated measures ANOVA showed that there was a significant \((F=34.92, \, df=1, \, p=0.0001)\) linear relationship between weight gain (water flux) and dehydration level; as dehydration levels increased there was an increase in weight gained \((F=9.148, \, df=6, \, p=0.0001)\). Since \(P. \, regilla\) displayed WR in almost
all of the trials by 8% dehydration, there was an overall net water gain at all dehydration levels >8% during the observation trial. *Pseudacris cadaverina* did not consistently show WR until 18% dehydration and therefore individuals not responding at lower dehydration levels continued to lose weight during the trial. Overall, there were no significant differences (F=0.808, df=1, p=0.382) in the weight change between these two species of frog during the observation period.

In both species of tree frogs there is a positive correlation between the amount of time spent in the water absorption response and the amount of weight gained during the trial. Interestingly, the specific re-hydration efficiencies, for each species, differ according to level of dehydration (Fig. 6). At lower levels of dehydration, *P. regilla* re-hydrate more quickly (y-intercept: 0.097% standard weight/min) than *P. cadaverina* (y-intercept: -0.087% standard weight/min). This is in agreement with, *P. regilla* earlier responding at lower levels of dehydration. As dehydration levels increase, *P. cadaverina* seems to increase in its effectiveness in re-hydration (slope: 0.017% standard weight/min) than *P. regilla* (slope: 0.007% standard weight/min). These data,
along with the data in Figure 3, suggest that the more an individual is dehydrated, the greater the amount of water gained during WR. These data also suggest that *P. regilla* is able to exploit hydrating substrates more efficiently when at lower levels of dehydration (benefiting opportunistic absorption of water) compared to *P. cadaverina*.

**Discussion**

Although there appear to be a variety of physiological/morphological adaptations (Geise and Linsenmair 1988; Roth 2005) and behaviors (Pough et al. 1983; Hoffman and Katz 1989; Andrade and Abe 1997; Navas et al. 2002) which decrease EWL, previous work has also suggested that anurans may be able to opportunistically exploit hydrating sources in order to compensate for high levels of dehydration (Brekke et al. 1991; Hillyard et al. 1998). For example, *B. punctatus*, which may move as far as 1 km away from a permanent water source, showed rehydration behaviors when dehydrated by 1 - 3.6% of initial standard weight (Brekke et al. 1991; McClanahan et al. 1995). On the other hand *B. marinus*, normally found near the water’s edge, needed to be dehydrated by 10-15% of
initial standard weight before consistently inducing water absorption behaviors (Hillyard et al. 1998). This led investigators to assume that anurans foraging away from a water source should exploit water opportunistically in order to maximize the time spent foraging away from a body of water. It is well known that foraging is largely restricted by environmental moisture in amphibians and that animals found in dry environments return to hydrating sources much more quickly than animals found in humid environments (Feder and Londos 1984). Opportunistic exploitation of hydrating substrates in organisms inhabiting dry areas therefore allows animals to forage for a longer period of time away from a permanent body of water. Water that’s absorbed opportunistically can be stored as dilute urine in the bladder and then be reabsorbed when the animal becomes dehydrated (Shoemaker and Bickler 1979; Jorgensen 1998). This diminishes the risk of desiccation and its harmful effects on locomotion (Preest and Pough 1989), metabolism (Churchill and Storey 1995), and other physiological functions while foraging and permits enough time for the animal to return to a permanent water source.
Our results show that *P. regilla*, a species known to travel large distances away from a permanent body of water, consistently initiated WR even when hydrated (Fig. 1). *Pseudacris cadaverina*, a species normally found less than two meters away from a stream’s edge, on the other hand, did not consistently initiate WR until higher dehydration levels were reached (at least 8% from initial standard weight). In both species, the proportion of frogs displaying WR increased as dehydration levels increased, but overall a higher proportion of *P. regilla* showed the response at lower levels of dehydration. In fact all individual frogs showed the response at least once when dehydrated by 8% (Fig. 3) and, all of the frogs showed the response during all trials once they had been dehydrated to 15% from their initial weight (Fig. 1). In contrast, some individuals within the *P. cadaverina* population did not even show WR once until they were dehydrated to 22% (Fig. 3).

As animals become more dehydrated during a foraging bout, we would expect that the time spent in the water absorption response would increase accordingly. Although previous studies (Contreras and Paasch *in preparation*) showed that *P. regilla* dehydrated to 10% from their
initial weight maintained WR consistently for the entire observation period (15 minutes), here we found that WR varied according to the level of dehydration and species of frog (Fig. 3). In general, as in previous studies (Brekke 1991), time spent in WR increased as dehydration levels increased for both species, but *P. regilla* spend more time in this behavior across most dehydration levels (except when dehydrated to 18%) when compared to *P. cadaverina* (Fig. 3). These results may be related to the difference in the proportion of frogs inducing this behavior at a specific dehydration level, and it is therefore important to also compare between only those individuals actually showing the response. When we do this, we see that there are no actual differences between those individuals showing the response, regardless of species, in the average amount of time spent in WR, except when the animals are fully hydrated (Fig. 4). Population differences therefore lie mainly in the proportion of frogs showing the response at different levels (Fig. 3). This helps explain differences in the proportion of weight change within each species at different dehydration levels. We see that fully hydrated *P. regilla* lost less weight during the observation period than *P. cadaverina*
(Fig. 5). This makes sense since 6 individuals showed the response in this species while only 1 *P. cadaverina* induced WR at this dehydration level; the other 8 individuals lost weight to the environment during the observation. All of *P. regilla* showed WR when dehydrated by 8% from their standard weight and we see a corresponding, pronounced, net weight gain for dehydration levels > 8%. Likewise, in *P. cadaverina* there is a pronounced weight gain when all of the frogs are displaying WR (at 18% dehydration).

Interestingly, there is also an underlying difference in re-hydration efficiency between these two species (Fig. 6). It’s been shown that movement of water across the amphibian skin is passive (Machin 1969; Hillyard 1976), and therefore an increase in the osmotic gradient between the animal and its environment alone will undoubtedly increase re-hydration rates. Although we do see an increase in re-hydration rates as dehydration levels increase in both species, *P. regilla* re-hydrates at a faster rate at lower levels than *P. cadaverina*. Differences in the rates of water uptake may be explained by differences in the species physiology; differences in vasotocin levels in the blood may cause differences in re-
hydration rates. Vasotocin (AVT) has been found to increase the number of aquaporins in the epidermal cells of the ventral skin of anurans (Acher 1997; Malvin 1993; Santos et al. 2000; Takahiro et al. 2003). Therefore, an increase in the concentration of AVT in the blood causes an increase in the number of aquaporins inserted into the skin. Differences between these two species in the amount of vasotocin in the circulatory system may be directly related to different adaptations according to the species natural history. *Pseudacris cadaverina* does not show water absorption behaviors until a high dehydration level has been reached. Therefore, there may be a slow and steady increase vasotocin to ensure that water is absorbed very rapidly once dehydration occurs. *Pseudacris regilla*, on the other hand, seems to absorb water opportunistically, even when fully hydrated or when dehydrated by very small amounts. In this species, vasotocin levels may be high at even low levels of dehydration. These adaptations could allow each species to exploit resources (water) very differently.

In conclusion, *P. regilla* absorb water opportunistically even when fully hydrated while *P. cadaverina* needs to reach a specific level of dehydration.
before WR is induced. The proportion of frogs inducing WR increases as dehydration level increases in both species, but there is an overall larger proportion of *P. regilla* showing WR at all dehydration levels. Although there are differences in the proportion of frogs showing WR at each dehydration level, there is no real difference between species in the time spent absorbing water once WR is induced. Therefore, we see no real differences in weight change (signifying water loss/gain) between species across most dehydration levels. Although the time spent in the response does not differ between species, there does seem to be an underlying difference in re-hydration efficiency. Re-hydration rates increase more rapidly in *P. cadaverina* as dehydration increases than in the Pacific Tree Frog. We propose that this may be related to vasotocin levels released when the animal becomes dehydrated, and that the corresponding concentration of this peptide released into the bloodstream is related to the natural histories of each species.
CHAPTER THREE
ABILITY OF FROGS TO FIND WATER
AND SALT PREFERENCES

Introduction

Amphibians, as the first vertebrates to bridge the gap between terrestrial and aquatic life, continuously encountered constraints related to this new way of life as they began to colonize land. One major hurdle they had to overcome, which still governs their ability to survive today, was dehydration due to evaporative water loss (EWL) across the integument. Highly terrestrial amphibians are known to have higher dehydration tolerances (Thorson 1955; Ray 1958; Schmid 1965). Unlike their aquatic counterparts, highly terrestrial species must have evolved mechanisms to deal with the physiological effects associated with a decrease in body water content, such as: 1) increased concentration of interstitial fluids, 2) increased blood viscosity and 3) low blood pressure (Hillman 1990). Along with tolerance to physiological extremes, terrestrial species also evolved skin which is regional in specialization which, in addition to decreasing evaporative water loss, allows re-hydration to
proceed more effectively. For example, the cutaneous osmotic permeability of the completely aquatic species *Xenopus laevis* is indistinguishable across dorsal, ventral pectoral and ventral pelvic regions of skin (Bentley 1972). On the other hand, pelvic permeability relative to the rest of their skin is much greater in the tree-frogs (Hylidae) (Yorio 1977), allowing this terrestrial species to quickly reabsorb water from any wet surface (soil, leaves, rocks, etc...) while foraging away from a permanent body of water. Therefore, while *X. laevis* is obligated to submerge in water in order to maximize its rate of re-hydration, the more terrestrial species is able to quickly re-hydrate from any water source that becomes available without the need to completely submerge in water.

Along with physiological and morphological adaptations for the maintenance of proper water balance, behavioral adaptations are also very important (Hillyard 1999). These osmoregulatory behaviors can be grouped into two categories: 1) prevention of/limiting the rate of dehydration and 2) re-hydration. When terrestrial anurans are found away from a water source they are often found in a posture termed “the water conservation response” (Pough 1983). In this response, the fore- and hind limbs are
brought close to the body, decreasing skin surface area exposed to the environment. This posture decreases cutaneous water loss and minimizes dehydration rate until conditions are better for them to find water. In the presence of moisture terrestrial anurans elicit the "water absorption response" (WR), in order to facilitate rehydration (Stille 1958). In this posture the hind limbs are abducted and the pelvic patch is completely pressed onto the substrate, increasing the skin surface area coming into contact with the hydrating source. Water moves down an osmotic gradient from the substrate into the animal through the integument on the pelvic patch. Since anurans must absorb water osmotically, the osmolarity of the fluid is important (Brekke 1991; Hillyard 2001). It is believed that the pelvic patch may not only serve as a site for quick re-absorption of water, but it may also play an important role in detecting water (on a substrate) and in determining if the quality of water is suitable for re-absorption (Brekke 1991; Hoff 1993; Nagai 2004). Previous work has suggested that before WR is induced, a behavior termed seat-patch-down (SPD) is displayed (Hillyard 1998) where the animal continuously presses and lifts the pelvic patch on a substrate. The amount of time
that the pelvic patch comes into contact with the substrate is minimal and effective for re-absorption. It is therefore believed that this behavior allows the animal to essentially "taste" the solution on the substrate so that it can determine whether it is osmotically preferable.

Elevated plasma osmotic concentrations seem to be the dominant factor eliciting seat-patch-down behavior (SPD) in terrestrial anurans when initially dehydrated (Hillyard 1998). Studies have suggested that osmoreceptors on the pelvic patch (and feet) allow anurans to detect the water potential of a hydrating source (Stille 1952; Brekke 1991) while chemoreceptors may help in discriminating between solutions containing different types of salts (Hoff 1993; Nagai 2004). Dehydrated Bufo punctatus placed on surfaces containing hyperosmotic (500mM) urea solutions do not induce water absorption behavior but show seat-patch-down behavior more frequently than when placed in less concentrated solutions (Brekke 1991). This suggest that they may able to detect the osmotoic pressure of the solution. When given hyperosmotic NaCl solutions (250mM), this toad avoids the substrate altogether (Hoff 1993).
However, when amiloride, a blocker of epithelial Na\(^+\) channels that are typically found in frog skin, is added to the 250 mM NaCl solution hydration behaviors are restored (Hoff 1993). Amiloride has been shown to prevent the detection of Na\(^+\) by blocking Na\(^+\) channels in the lingual epithelium of mammals and frogs (Heck GL 1984; Avenet 1988). Hoff and Hillyard’s (1993) results suggest that the amiloride sensitive Na\(^+\) channels found in anuran skin also serve a chemosensory function similar to those found in mammalian taste buds (Desimone 1984). This idea was supported by Nagai et al. (1999) who saw that in toads, time in WR on 250 mM NaCl solutions containing 10 \(\mu\)M amiloride was prolonged relative to the amount of time in WR when toads were placed on 250 mM NaCl without amiloride. Amiloride did not restore WR behavior by toads on 250 mM KCl.

The ability of anurans to use the pelvic patch for osmo- and chemoreception gives rise to several questions: What is an “adequate” hydrating source? Is this “adequate” hydrating source similar for all species? If not, why are there differences between species? In this study we hypothesize that a species’ terrestriality is
positively correlated with the concentration of the solution where WR is displayed; frogs that are highly terrestrial will show WR in more concentrated solutions. Species that forage away from a permanent body of water may face higher dehydration levels while foraging than those found near water. Since water moves passively across the integument (Machin 1969; Hillyard 1976) individuals dehydrated to a high level may re-hydrate from more concentrated solutions due to the higher osmotic concentration of their interstitial fluid. This adaptation seems especially advantageous for frogs normally found away from a permanent water source since the presence of dilute substrates may be limited and frogs may have to exploit more concentrated solutions such as puddles or other moist surfaces.

Tree frogs, a group of terrestrial anurans found in a variety of habitats throughout the world, provide a great model for answering these questions. Within this family of frogs, species vary widely in the distance in which they move away from a permanent water source. The California and Pacific Tree Frogs are two species of terrestrial amphibians in the genus *Pseudacris* (formerly *Hyla*; Moriarty 2004) found in California. The California
Tree Frog, *P. cadaverina*, is primarily a nocturnal species that is mostly distributed in upland stream habitats (Cunningham 1964) in discontinuous cismontane populations from San Luis Obispo County to Bahia de Los Angeles, Baja California (Stebbins 2003). It is usually found near canyon streams and washes at elevations ranging from sea level to 2,290m above sea level, where there are rocks, quiet pools, and shade. Individuals of this species tend to cluster by stream boulders and in the large slow pools they create (Kay 1989) and rarely seem to move more than two meters away from a permanent body of water (Dole 1974). Unlike the California Tree Frog, the Pacific Tree Frog, *P. regilla*, is active during the day and night (Stebbins 2003). It ranges from British Columbia, Canada to the tip of Baja California, México and eastward to west Montana and eastern Nevada. It is found in a wide range of habitats including grasslands, chaparral, woodland, desert oases, agricultural regions, and even in residential areas; these habitats can occur from sea level to 3,500m above sea level. Although mostly found in the dense vegetation surrounding streams and lakes during the breeding season, during late summer, fall and early winter *P. regilla* may be found up to a mile away from its
breeding sites, hiding in areas with low moisture (Jameson 1956; Jameson 1957).

Differences in these species’ natural history may suggest differences in their ability to locate a water source once dehydrated. *Pseudacris cadaverina*, since always found near a permanent water source, may be less proficient at locating a hydrating substrate when compared to *P. regilla*, which is likely to encounter high levels of dehydration while foraging away from a permanent water source. Since each species faces different dehydration levels in their natural environments “adequate” water sources might also differ for each species; *P. regilla* may utilize more concentrated solutions and *P. cadavérina* may utilize more dilute solutions. It is possible that when offered solutions varying in concentration, dehydrated *P. regilla* might be more general in their response and induce WR in various solutions whereas dehydrated *P. cadaverina* might be more specific (inducing WR only in dilute solutions). This study aims to determine if differences exist between *P. cadaverina* and *P. regilla* in the ability to locate water and in their preferences to concentrated/dilute solutions.
Materials and Methods

Animals

Collection/Maintenance. Adult Pacific (P. regilla) and California Tree Frogs (P. cadaverina) were collected from Mill Creek in Highland (San Bernardino County), California in October of 2003. All frogs were housed together in a 20 gallon glass terrarium containing soil, a store bought bark substrate, natural plant material, and two plastic 1 L containers filled with water. Granite rocks 5-10 cm in height were placed in the terrarium so that frogs were able to rest on rocks, water, plants or moist soil, ad libitum. Small sections of egg cartons were also placed in terrarium so that frogs could burrow underneath. The terrarium was located at CSUSB’s Animal House and frogs were kept on a 12L:12D_light cycle at ca. 70 F and were fed live crickets once per week. All individuals were allowed to recover in this terrarium at least three days between trials.

Preparation for Observation Trials. To control for initial hydration levels all frogs were forced hydrated for half an hour each trial by being individually placed in a small closed plastic container containing 1mM NaCl. After the hydration period, excess water was removed from
the frog’s skin by gentle blotting. Each frog’s weight was measured and recorded then pelvic pressure was applied to expel stored urine and the frog’s weight was once again determined. This final weight was labeled as initial standard weight (Ruibal, 1962). Frogs of each species were dehydrated to the minimum level in which all individuals display water absorption behavior: 15% for the Pacific Tree Frog and 22% for the California Tree Frog (Contreras and Talbot, in prep.). Dehydration was based on changes from initial standard weight, assuming that changes in mass were directly proportional to water loss. The level of dehydration is indicated as the percent change relative to the initial standard weight. Since previous studies showed that there was no difference in the response of these species to dehydration as a function of dehydration rate (Contreras and Talbot, in prep.), a relatively rapid dehydration protocol was used in these experiments. Frogs were individually placed in a small plastic container which had been thoroughly perforated (top, bottom, and all sides). The container was then placed above a fan to apply constant ventilation and induce a high rate of evaporative water loss. Frogs were
kept in the container for periods of 45 min - 4 hrs, until the desired level of dehydration was reached.

Detection of Water Source

Observation Tank. A 20L glass aquarium was placed on supports and elevated to allow visualization of the ventral surface of frogs above an angled mirror placed under the aquarium. The four sides of the aquarium were covered with window film (a sheet of thin, black plastic with regular 2 mm diameter pores). Although visibility was decreased, the observer could still see the frog inside. The bottom of the aquarium was divided into four equal quadrants (36cm x 14cm) which were further subdivided into 4cm x 4cm grids. During a typical trial, a dry tissue (8 x 8 cm) was placed on the corner of one quadrant, a tissue saturated with 1mM NaCl (since previous studies have shown positive results at this NaCl concentration) was placed on the corner of second quadrant and two quadrants were left empty. The location of the tissues was randomly positioned between trials. In the trials to examining behaviors in the absence of a water source, three quadrants were left empty with the remaining quadrant containing a dry tissue which was randomly moved between trials. Behavioral Observations. A dehydrated
frog was placed in a small opaque container which was placed at the center of the observation tank for one minute. The observation period began when the container was lifted and the frog was free to move from this initial point. Each animal was observed and the following was recorded: 1) direction/orientation of initial movement, 2) total time spent in each quadrant; 3) evidence of search behavior or water conservation behavior; 4) total time to find wet tissue. Frogs were observed until they found the wet tissue or for a maximum of twenty minutes. To determine whether dehydration influenced the frog’s behavior during these observation trials, frogs were forced hydrated and then placed in the observation tank fully hydrated and observed as above. To look for differences in the behavior of dehydrated frogs in the absence of water, frogs were dehydrated as above and then placed in a dry observation tank (three empty quadrants and one quadrant containing a dry tissue). Each individual was observed for twenty minutes.

For all of the trials, the frog’s movement was recorded on a data sheet containing a model of the aquarium, using the 4x4 cm grid as reference points. Each individual was tested three times for each of the
different trials. After the trials, frogs were placed in terrarium and were allowed a minimum of three days to recover.

**Statistical Analysis.** A one-sample t-test was used to determine if Pacific and California Tree Frogs showed a significant pattern of movement towards one area of the tank. Frogs were expected to spend equivalent amounts of time (25% of total time) in each quadrant if random movement was displayed. Each treatment was replicated three times for all frogs. The average amount of time spent in each quadrant was determined for all treatments, and these were used to calculate averages for the species. Species average time in a quadrant were converted to proportions by dividing the average time each frog spent on a quadrant by the average time spent searching for a water source. These data were ArcSine transformed. A repeated-measures analysis of variance (ANOVA) was used to examine possible differences between species in the amount of time spent in each quadrant for each treatment. In this analysis, time spent in a quadrant was the dependent variable, type of quadrant (dry, empty, and wet) was the fixed variable and species (California vs. Pacific Tree Frog) was the between subjects variable. A repeated-
measures ANOVA was also used to look for interspecific differences in time spent in each quadrant as a function of type of trial: hydrated vs dehydrated. In this analysis the dependent variable was amount of time spent in each quadrant, the fixed variable was quadrant type and the between subjects factor was type of trial (hydrated vs. dehydrated).

Salt Preferences

Observation Tank. A 61x71 cm plexi-glass tank was placed on supports and elevated above an angled mirror. A grid (6cm x 6cm squares) was created on the bottom of the tank. The tank was divided into six equal sections, where each section radiated out from the center of the tank and became progressively larger towards the outer walls (Appendix 2). A 10ml petri dish was placed in each section containing tissue paper saturated with different concentrations of NaCl (1, 50, 150, 200, 250 mM). The dishes were randomly arranged in a circle with each dish equidistant from the center of the tank and from each other. Salt solutions were replaced between trials.

Behavioral Observations. Pacific Tree Frogs were dehydrated by 15% from their initial standard weight and California Tree Frogs were dehydrated by 22% from their
initial standard weight. Before each trial, a frog was placed in a small opaque container at the center of the observation tank for a minute. The observation period began once the container was lifted and the frog was free to move. The frog’s movements were recorded over a twenty-minute observation period on a data sheet containing a model of the aquarium. During the observation period the following was recorded: 1) location of frog and whether it entered a solution and showed WR, 2) concentration of NaCl in which WR was initiated, 3) time spent on NaCl solution, 4) time spent in WR. Movements to different solutions were also recorded during the observation period. Frogs were weighed after each trial.

To determine if dehydration had an effect on the frog’s search behavior, trials were conducted on fully hydrated frogs. Frogs were forced hydrated (as described above) and then immediately placed into the observation tank. Observations were conducted in the same manner as above. Each frog was tested three times for each of the different trials (hydrated and dehydrated).

Statistical Analysis. The average amount of time spent on a particular solution, and in WR, was determined
for each individual frog for both hydrated and dehydrated trials. These were used to find the species average. To test for differences between species in these variables, a repeated-measures ANOVA was used. The fixed variable in these analysis was salt concentration and the between subjects factor was species. A contingency Ω test was used to determine if the number of frogs found on a particular solution was significantly different from expected. The number of frogs displaying WR on a particular solution was also tested in this manner.

Results

Detection of Water Source

Average "searching time for all P. cadaverina, regardless of whether water was found, was more than twice as long in fully hydrated versus dehydrated individuals (Table 1). Total searching time was taken as time to find wet tissue or 20 minutes (whichever was shorter). Therefore, differences in the overall observation time between treatments may be explained by differences in the proportion of frogs finding water. Dehydrated P. cadaverina found water in 96% of the trials while fully hydrated P. cadaverina found in only 46% of the trials.
The water conservation response (WCR) was displayed more often (8 trials) for a considerable amount of time (> 13 min) when frogs were hydrated than when they were dehydrated (none of the frogs displayed WCR). Hydrated frogs that did not continuously display WCR, were observed intermittently moving about the aquarium and showing WCR for short periods of time, while still others seemed to move around the aquarium non-stop (Putnam and Hillman, 1977). Frogs that did not find water during a trial were observed for a total of 1200 seconds, while those that found water usually found it, on average, in the first 493 seconds (Table 1). We can see that an increase in the proportion of frogs finding water therefore lowers the total observation time for each treatment (Fig. 7). Likewise, average observation time for all of P. regilla (regardless of whether water was found) was also almost twice as long in versus dehydrated frogs (Table 2); frogs found water in more trials when dehydrated (96%) than when they were hydrated (63%). Hydrated Pacific Tree Frogs moved around the tank more frequently than California Tree Frogs. Water conservation behaviors were observed for more than 13 minutes in only two trials, and more frogs seemed to move around the tank while showing WCR.
intermittently. Hydrated *P. regilla* took, on average, 385 seconds to find water. When dehydrated, there were no differences between species in the proportion of trials in which water was found. There were also no significant differences in the amount of time to find water (*F* = 0.87, *df* = 1, *p* > 0.05). Dehydrated *P. regilla* and *P. cadaverina* moved about the aquarium more quickly and water was found in less than 300 seconds in almost half of all the trials. In some trials, frogs moved directly toward the water but in general frogs, from both species, seemed to actively search the aquarium before finding water; e.g. they did not seem to be able to immediately sense a water source.

If frogs were randomly moving throughout the aquarium, they were expected to spend an equal proportion of time (25% of total time) in each quadrant. We found that this was the case for *P. cadaverina*, in both hydrated and dehydrated trials (*p* = 0.99 and *p* = 1, respectively), thus movement around the aquarium for this frog seemed to be random (Fig. 8). *Psuedacris regilla* also moved randomly throughout the aquarium in both hydrated (*p* = 0.99) and dehydrated trials (*p* = 1), but differences between treatments were observed in the proportion of time
spent in a quadrant \( (F = 5.38, \text{ df} = 3, p = 0.003) \). When fully hydrated \( P. \) regilla spent a higher proportion of time (46% of total observation time) in the quadrant containing the dry tissue whereas frogs that were dehydrated spent only 17% of total observation time in that same quadrant. Differences in proportion of time spent in the empty quadrants were also observed; hydrated frogs spent a lower proportion of time in these quadrants (43% of total observation time) than when dehydrated (66% of total observation time). Proportion of time spent in the quadrant containing the wet substrate was low in both treatments (Fig. 8).

No significant differences were observed between dehydrated \( P. \) cadaverina and \( P. \) regilla in the proportion of time spent in a particular quadrant \( (F = 2.12, \text{ df} = 3, p>0.05) \) when a hydrating substrate was available (Fig. 8). Instead, both species seemed to move randomly around the observation tank and quickly moved onto the water once an animal was approximately 1-2 cm away from the wet tissue. Interestingly, \( P. \) cadaverina spent similar proportions of time in both empty quadrants and in the quadrant containing the dry tissue. \( P. \) regilla, on the other hand, moved around the empty quadrants more often than in any
other type of quadrant (Fig 8). Hydrated trials followed the same pattern, where there were no significant differences in the proportion of time spent in a particular quadrant between species. Although there were no significant differences, an interesting pattern once again appeared. *P. regilla* spent relatively similar proportions of time in the empty quadrants and in the quadrant containing the dry tissue, *P. cadaverina* spent more time in the empty quadrants (Fig. 2).

When frogs from both species were dehydrated and placed in the aquarium with no available water, there were no significant differences in the amount of time spent in each quadrant (*p > 0.05*) in both species (Table 1). The constant movement, from one quadrant to the next, may help in explaining these results. In both species, movement around the aquarium was constant and there was no evidence of WCR throughout the observation period.

Salt Preference

Frogs were placed in the center of an observation tank and their pattern of movement was followed to determine if they would choose to move onto a substrate of a given salt content. *P. regilla* and *P. cadaverina* seemed to randomly enter solutions of different concentrations
during both hydrated and dehydrated trials. Unlike in the previous experiment, when dehydrated, both *P. regilla* and *P. cadaverina* only found water (regardless of concentration) in 63% of the total trials (N = 27 and N = 24, respectively). These results differ from previous studies which show that essentially 100% of dehydrated frogs display water absorption behaviors when presented with a dilute solution (Paasch and Contreras, *unpublished data*; Chapter 2).

The salt content of the solution most frequently visited differed between each species during both dehydrated and hydrated treatments. Dehydrated *P. cadaverina* were most frequently found on 150mM and 200mM NaCl solutions; frogs were found in each solution in 21% of the trials (Table 3). Dehydrated *P. regilla*, on the other hand, were most frequently found on 1mM and 50mM NaCl solutions (Table 4). Interestingly, the number of unique individuals entering the most dilute solutions (n=4-5) was double that of individual frogs entering the highest concentrated solutions (n=2). Hydrated Pacific Tree Frogs, once again, were most frequently found on the 1mM NaCl solution (22% of trials) and the number of unique frogs found on this solution (n=6) was triple that found
on the most concentrated solution (Table 4). Fully hydrated California Tree Frogs, on the other hand, were found most frequently found on the 250mM NaCl solution (17% of total trials); however only two unique frogs seemed to move onto the solution (Table 3).

Time spent on a substrate of a specific salt content significantly differed between species. In general, dehydrated *P. regilla* spent more time on solutions which were relatively dilute ($F = 2.63$, df = 5, $p = 0.03$) while *P. cadaverina* spent more time on a substrate with a more concentrated salt content. *P. regilla* that entered dishes spent approximately 10% of their time on the 1mM and 50mM NaCl solution whereas *P. cadaverina* spent less than 5% of the total observation time on these solutions (Figure 9). As NaCl concentrations increased, the proportion of time *P. regilla* spent in a dish with a particular solution decreased. The opposite trend seems to occur in *P. cadaverina*; the highest proportion of time was spent on more concentrated solutions (Figure 9). These trends were more apparent during hydrated trials. *Pseudacris regilla* spent about 18% of the total observation period on the 1mM NaCl solution, and the time spent in more concentrated solutions decreased as concentration increased.
Pseudacris cadaverina, on the other hand, spent about 15% on the 250mM NaCl solution, and the proportion of time spent on more dilute solutions decreased as NaCl concentrations decreased (Figure 9).

Although there were no significant differences in the number of frogs displaying WR in a particular solution between species during hydrated or dehydrated treatments, differences in the overall proportion of frogs showing WR (in any solution, regardless of NaCl concentration are apparent, particularly in hydrated frogs. When dehydrated, P. regilla showed WR in 63% of the total trials (N=27) whereas in P. cadaverina, WR was shown in 58% of the total trials (N=24); P. regilla showed WR in slightly more trials. When frogs were hydrated, P. regilla showed WR in 56% of the total trials but P. cadaverina showed WR in only 42% of the trials.

In most trials, if a frog entered a solution WR was immediately shown and the frog would not leave the solution voluntarily until the response was terminated. In general, more Pacific Tree Frogs showed WR in the lower concentrated solutions (1 - 100mM NaCl) than in higher concentrated solutions (150- 250mM NaCl). When analyzing the overall results for all individuals, regardless of
whether WR was displayed or not, we saw that *P. regilla* spent a higher proportion of time in WR while on less concentrated solutions than in more concentrated solutions whether hydrated or dehydrated (*F* = 4.82, df = 5, *p*=0.001) (Fig. 10). *P. cadaverina*, on the other hand, did not show a specific pattern when displaying WR; there was no difference in the number of unique frogs (or proportion of trials) showing WR at a specific concentration in both hydrated and dehydrated trials. The average proportion of time dehydrated *P. cadaverina* (regardless of whether WR was displayed or not) spent in this response did not differ when comparing between highly concentrated solutions (150 - 250mM NaCl), however differences were observed between the proportion of time spent in these concentrated solutions and the proportion of time spent in more dilute (1 - 100mM NaCl) solutions (Fig. 10). When *P. cadaverina* was hydrated, the average proportion of time spent in WR did not differ when found in 1 - 200mM NaCl solutions (i.e., they spent from 0 - <10% of total observation time in WR in these solutions).

Interestingly, around 15% of the total observation period was spent (on average) in the most concentrated solution (Fig. 10).
There was a difference in the proportion of time spent in WR within a particular dish depending on species \( (F = 2.7, \text{ df } = 5, \text{ } P = 0.03) \) when the animals are hydrated. However, this is due mainly to differences in the number of frogs displaying WR. Hydrated *P. regilla* showed WR more frequently (in the more dilute solutions) than *P. cadaverina*. Therefore, there was an increase in the average amount of time spent in WR while on these solutions for all of *P. regilla*, regardless of whether WR was displayed or not. When comparing the actual time spent in WR between individuals that actually showed the response, we see that the time spent in WR did not differ between species (Fig. 9 and 10). When dehydrated, we again see that differences between these two populations persist \( (F = 2.84, \text{ df } = 5, \text{ } p = 0.021) \), but again these differences are mainly due to differences in the proportion of frogs displaying WR on specific solutions. Again, when looking only at individuals that actually showed the response, the time spent in the response does not differ between species (Fig. 9 and 10).

Although each species differed in the overall proportion of trials in which WR was displayed, and in the salt content in which WR was induced most frequently,
there were no significant differences overall in the relative weight gained/lost during the observation period for both hydrated and dehydrated trials ($F = 1.56$, df = 2, $p = 0.232$) or between species ($F = 0.22$, df = 1, $p = 0.65$). Weight gain/loss was highly variable in both species for both treatments, but in general frogs displaying WR during the observation period gained water while those that did not show WR continued to lose water (Fig. 11).

Discussion

Previous studies have shown that there is a strong correlation between the natural history of anurans and their response to dehydration (Thorson 1943; Cohen 1952; Thorson 1955; Thorson 1956; Ray 1958; Schmid 1965; Bentley 1966; Jameson 1966; Larson 1971; Young 2005). It has been shown that natural populations of species that are frequently subjected to rapid dehydration display WR at lower dehydration levels relative to those which are less frequently subjected to these conditions (Brekke 1991; Propper 1994). This opportunistic display of WR assists in preventing high levels of dehydration when these species forage away from a permanent water source and
allows them to exploit more arid and terrestrial environments. The present study continues to support this idea. *P. regilla*, a species known to move up to a mile away from a permanent body of water while foraging, found water and showed WR more frequently even when hydrated compared to hydrated *P. cadaverina*, a species of tree frog normally found next to a permanent body of water (Table 1 and 2). Interestingly, hydrated *P. regilla* moved about the aquarium more often than *P. cadaverina*, and therefore spent less time in water conservation responses. In fact, *P. regilla* seemed to spend a considerable proportion of time in the quadrant containing a dry tissue rather than spending more time in just empty quadrants. When frogs were dehydrated, the proportion of time spent in the quadrant containing a dry tissue decreased, while the proportion of time spent in the empty quadrants increased (Fig. 8). These results suggest that frogs may have learned that the presence of a tissue signifies the availability of water, therefore when hydrated they continuously moved onto the tissue. When dehydrated, they visited the dry tissue, but once they discovered it was dry they did not re-visit it. Dehydrated, frogs seemed to move around the aquarium more frequently overall and water
was found more rapidly and more often. \textit{P. cadaverina} were more often found in the water conservation response. Differences in the species response may suggest different mechanisms to deal with dehydration. For example, it has been shown that \textit{P. regilla} absorb water opportunistically while \textit{P. cadaverina} don’t typically respond until they reach a specific dehydration level (Chapter 2). Therefore, it is beneficial for \textit{P. regilla} to continuously forage, rather than display water conservation behaviors, in order to find a water source so that it can frequently re-hydrate and prevent dehydration. \textit{Pseudacris cadaverina}, on the other hand, display water conservation behaviors in order to decrease the rate of evaporative water loss to prevent reaching threshold dehydration levels; water conservation behaviors decrease the amount of water being lost to the environment, so that frogs may stay away from a water source for a long period of time before needing to re-hydrate. \textit{Pseudacris regilla} prevents dehydration by constantly rehydrating, while \textit{P. cadaverina} prevents dehydration by decreasing rate of evaporative water loss via behavioral modifications.

Frogs may use osmoreceptors to determine whether a water source is available, but osmoreceptors may also be
used to determine whether a water source is appropriate for re-hydration (Brekke 1991; Hoff 1993; Hillyard 2004; Nagai 2004). When dehydrated tree frogs were given a series of concentrated NaCl solutions (1, 50, 100, 150 200, 250 mM), the proportion of trials in which WR was shown was much lower than what was previously observed in these species, when frogs were either offered only a dilute water source or directly placed on a dilute hydrating substrate (Paasch and Contreras unpublished, Chapter 2). In previous studies 95-100% of the dehydrated frogs found water and displayed water absorption behaviors. In the present study, dehydrated frogs (regardless of species) subjected to hydrating solutions differing in NaCl concentrations displayed WR in about 50% of the total trials. Hillyard (1999) showed that B. punctatus are able to determine the availability of an osmotically preferable water source prior to any skin contact. B. punctatus was able to not only detect moisture but also the presence of NaCl via a sensitive olfactory mechanism. This might help explain the reduced number of trials in which WR was shown in the present study.
We expected that dehydrated frogs given a choice of solutions of different salt content would differ in which solution WR was displayed depending on species; we expected the more opportunistic *P. regilla* to induce WR in highly concentrated solutions whereas *P. cadaverina* to induce WR in more dilute solutions like those near their permanent home. Differences in the proportion of frogs inducing WR on specific NaCl concentrations were definitely observed between species (Fig. 10), but not those that we had predicted. We believed that *P. regilla* would consistently induce WR in highly concentrated solutions since in the wild they would want to absorb water from any available water source (such as small puddles which may contain more salts than freshwater) while foraging away from a stream/pond. *Pseudacris cadaverina*, on the other hand, does not travel large distances away from the stream, and therefore only rehydrates from freshwater under natural conditions. Therefore, we expected that *P. cadaverina* would show a preference for more dilute solutions. However, *P. regilla* induced WR more frequently in lower concentrated solutions regardless of hydration state (Table 4), while *P. cadaverina* induced WR more frequently in more concentrated
solutions (Table 3). The actual time that individual frogs spent in WR did not differ between solutions; once WR was initiated, regardless of species or hydration state, the time spent in the response was relatively constant. However the average proportion of time that the species (regardless of whether individuals showed the response or not) spent in WR while on a specific solution differed between *P. regilla* and *P. cadaverina* (Fig. 9). Overall, *P. regilla* spent a greater proportion of time in WR in more dilute solutions, while *P. cadaverina* spent a higher proportion of time in WR when found in more concentrated solutions. A steep gradient between body fluids and ambient water allows for the quick uptake of water, therefore, showing water absorption behaviors on dilute solutions, regardless of hydration state, is beneficial. However, it is unclear as to why frogs may choose to re-hydrate from more concentrated solutions. Various studies have shown that toads will move away from highly concentrated solutions without displaying WR (Koyama 2001; Hillyard 2004; Nagai 2004) and will discriminate against highly concentrated NaCl solutions more rapidly than against solutions, of the same concentration, composed of different solutes. This is also true of *P. regilla*
(Paasch and Contreras, in preparation). So why does *P. cadaverina* spend a larger proportion of time in WR while on highly concentrated NaCl solutions in this study? The reasons may lie on the osmotic gradients between the animal and its environment. Normally, frogs found on concentrated solutions would continue to lose water since there is a steep osmotic gradient pulling water out of the animal. Osmoreceptors on the skin may therefore signal the brain so that proper behaviors may be shown in order to decrease the amount of water being lost. Even though, *P. cadaverina* primarily show WR on more concentrated solutions, they actually gained water (Fig. 11); therefore it appeared beneficial for them to stay on the solution. Osmoreceptors, therefore, may not have signaled the brain since water was not being lost. This response to such a high concentration seems extremely unusual, especially since previous studies have shown toads to reject a 250 mM NaCl solution (Kotinsky 2000). Interstitial fluids in *Scaphiopus couchii* are on average around 400mOsm during the month of June (Shoemaker, et al. 1969). Dehydration to 22% from initial standard weight might increase interstitial osmolalities to 488mOsm in this species. If the interstitial fluids of *P. cadaverina* are slightly more
concentrated than those observed in S. couchii, then dehydration to 22% from initial standard weight might create a gradient which allows re-hydration from a highly concentrated solution. Future work should therefore focus on the interstitial fluid concentration of these species in order to determine fluid osmolarities which may affect physiological and behavioral responses.

This study continues to support the idea that the natural history of a species has a strong effect on behavior associated with osmoregulation. We have seen that frogs which normally forage away from a permanent body of water will absorb water when hydrated if water is available more frequently than frogs that do not move away from a permanent body of water. Not only are water absorption behaviors induced more frequently in these species when hydrated, but there is a preference for more dilute solutions. These results make sense since water would not move osmotically into the hydrated animal unless it is at a lower osmotic concentration than the interstitial fluids of the frog. Species that do not show water absorption behaviors until dehydrated to a certain degree can therefore re-hydrate from more concentrated solutions since the osmotic concentration of the
interstitial fluid will be higher. In conclusion, differences between *P. cadaverina* and *P. regilla* response to dehydration can be attributed mainly to differences in the timing of the response: *P. regilla* prevent high levels of dehydration by re-hydrating whenever water is available (regardless of hydration state), while *P. cadaverina* consistently show water absorption behaviors once a certain level of dehydration has been reached.
FIGURE 1: Percentage of Pacific and California Tree Frogs showing WR at different dehydration levels as percentages of dehydration relative to their initial standard weight. If WR was initiated during any portion of the 15 min observation it was scored as a positive. A total of 9 Pacific and California Tree Frogs were used in each trial. Trials were done twice for each frog for n=18. Regression and linear equation from ArcSine transformed data (not shown) presented for each species of frog.
FIGURE 2: Average amount of time (sec) spent on a saturated (1mM NaCl) tissue in Pacific and California Tree Frogs at different dehydration levels. Data shown are the combined results for quickly and slowly dehydrated trials. Data as mean + SE for all trials (N = 18). Note that maximum response was capped at 15 min (900 seconds).
FIGURE 3: Average amount of time (sec) spent in WR in Pacific and California Tree Frogs at different dehydration levels. Data shown for all trials (N=18) whether WR was shown or not (i.e., t=0 of WR was not shown). The number of unique individuals (a) and the total number of trials (b) in which frogs showed WR at each dehydration level is specified (a,b). Data as mean + SE. Note that time was capped at 15min (900 sec). * = only one trial performed for that dehydration level (N=9).
FIGURE 4: Average amount of time (sec) spent in WR in Pacific and California Tree Frogs at different dehydration levels. Data shown only for frogs which induced WR during any portion of the 15min observation period. Of the two trials conducted for each individual the time used in analysis was either the time spent in WR for one observation or the mean if WR was observed in both trials. The number of unique frogs displaying WR at each dehydration level is shown. Data as mean ± SE.
FIGURE 5: Proportional change in weight for A) Pacific and B) California Tree Frogs at specific dehydration levels during fifteen minute observation period. Data shows weight change of all frogs (those that induced WR and those that did not). Negative numbers indicate weight loss and positive numbers indicate weight gain. There was no significant difference (p>0.05) in weight change as a function of species. Data shown are the combined results for quickly and slowly dehydrated trials. Data as mean + SE.
FIGURE 6: Re-Hydration rates (individual weight change (g) during time (sec) spent in water absorption response) for (A) *P. regilla* (B) *P. cadaverina*. Data shows is the combined results for quickly and slowly dehydrated trials and includes only individuals showing WR.
Figure 7: Average time (sec) P. cadaverina and P. regilla spent searching for water during hydrated (black) and dehydrated (gray) trials. Data shown for all of individual frogs (solid bars) as well as for frogs that found water during the observation period (dashed bars). Error bars represent standard error for the mean (black = all frogs; white = frogs that found water).
Figure 8: Proportion of time *P. cadaverina* (A) and *P. regilla* (B) spent in three different quadrants (empty or containing a dry or wet tissue) during hydrated (black) and dehydrated (gray) trials. Dashed lines represent expected proportion of time in each quadrant if animal randomly moved throughout aquarium.
Figure 9: Average proportion of time *P. cadaverina* (A) and *P. regilla* (B) spent on solutions with different NaCl concentrations (1, 50, 100, 150, 200, 250mM). *P. regilla* spent a larger proportion of time in solutions with lower concentrations (<100mM) when hydrated or dehydrated whereas *P. cadaverina* spent a larger proportion of time in more concentrated solutions
Figure 10: Average proportion of time *P. cadaverina* (A) and *P. regilla* (B) were found in WR on different solutions (1, 50, 100, 150, 200, 250 mM NaCl) during hydrated and dehydrated treatments. Numbers above bars represent unique individuals inducing WR in at least one of the trials. Since frogs could positively respond in all three trials, the percentage of trials in which frogs induced WR is shown in parenthesis. Total number of trials for *P. cadaverina* was N=24 and for *P. regilla* N=27 for both treatments.
Figure 11: Proportion of weight gained/lost (from initial standard weight) for *P. cadaverina* (black) and *P. regilla* (grey) during hydrated (solid) and dehydrated (dashed) treatments. Data shown are means (± SE) for frogs that showed WR and for those that did not show WR during the observation period.
APPENDIX B

TABLES
### Table 1: Values reported represent the mean (and SE) for all frogs, only frogs that found water and frogs that did not find water for all 3 treatments. Each treatment was repeated three times with 8 California Tree Frogs. *Includes time spent in 3 empty quadrants (Quads: 2, 3 and 4)"
Table 2: Values reported represent the mean (and SE) for all frogs, only frogs that found water and frogs that did not find water for all 3 treatments. Each treatment was repeated three times with 8 California Tree Frogs. *Includes time spent in 3 empty quadrants (Quads: 2, 3 and 4)
Psuedacris cadaverina

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<td>224 + 139 (3, 13%)</td>
<td>245 + 88 (2, 13%)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150mM</td>
<td>0</td>
<td>0</td>
<td>472 + 153 (3, 21%)</td>
<td>466 + 151 (3, 21%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 mM</td>
<td>176 (1, 4%)</td>
<td>97 (1, 4%)</td>
<td>247 + 50 (5, 21%)</td>
<td>253 + 63 (4, 17%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250mM</td>
<td>590 + 99 (2, 17%)</td>
<td>577 + 96 (2, 17%)</td>
<td>174 + 64 (3, 13%)</td>
<td>172 + 64 (3, 13%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Values reported are the means (and ± SE) of hydrated and dehydrated (22% from initial weight) California Tree Frogs found on a solution or in WR while excluding those who did not show response or were not found on that solution. A total of 8 frogs were used in three different trials. Numbers in parenthesis represents number of unique individuals who were found on the solution, or in WR in at least one of the trials. Since frogs could positively respond in all three trials, the percentage of the total 24 (3 trials x 8 individuals) are also shown for frogs found on a solution or in WR.
Table 4: Values reported are the means (and ± SE) of hydrated and dehydrated (15% from initial standard weight) Pacific Tree Frogs. A total of 9 frogs were used in three different trials. Numbers in parenthesis represents number of unique individuals found in a dish or in WR in at least one of the trials. Since frogs could positively respond in all three trials, the percentage of 27 (3 trials x 9 individuals) trials in which frogs were found in each dish or in WR is also shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (sec) to initiate search</td>
<td></td>
</tr>
<tr>
<td>Solutions (mM NaCl)</td>
<td>Time (sec) in Dish</td>
<td>Time (sec) in WR</td>
</tr>
<tr>
<td>1 mM</td>
<td>315 ± 32 (6, 22%)</td>
<td>291 ± 29 (6, 22%)</td>
</tr>
<tr>
<td>50 mM</td>
<td>200 ± 77 (4, 15%)</td>
<td>189 ± 117 (4, 15%)</td>
</tr>
<tr>
<td>100 mM</td>
<td>248 ± 86 (4, 15%)</td>
<td>181 ± 63 (4, 15%)</td>
</tr>
<tr>
<td>150 mM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>200 mM</td>
<td>296 (1, 4%)</td>
<td>294 (1, 4%)</td>
</tr>
<tr>
<td>250 mM</td>
<td>279 ± 83 (2, 7%)</td>
<td>360 (1, 4%)</td>
</tr>
</tbody>
</table>
REFERENCES


