1-1-1998

Effects of sodium chloride and sodium gluconate concentration on hydration behavior and water transport in the red-spotted toad, Bufo punctatus

Polly Ann Sullivan
University of Nevada, Las Vegas

Follow this and additional works at: https://digitalscholarship.unlv.edu/rtds

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700   800/521-0600

EFFECTS OF SODIUM CHLORIDE AND SODIUM GLUCONATE

CONCENTRATION ON HYDRATION BEHAVIOR

AND WATER TRANSPORT IN THE

RED-SPOTTED TOAD,

BUFO PUNCTATUS

by

Polly Ann Sullivan

Bachelor of Science
University of Nevada, Las Vegas
1992

Master of Arts
University of Nevada, Las Vegas
1995

A thesis submitted in partial fulfillment
of the requirements for the degree of

Master of Science

in

Biological Sciences

Department of Biological Sciences
University of Nevada, Las Vegas
December 1998

UMI Number: 1393771

UMI Microform 1393771
Copyright 1999, by UMI Company. All rights reserved.

This microform edition is protected against unauthorized
copying under Title 17, United States Code.

UMI

300 North Zeeb Road
Ann Arbor, MI 48103

UNLV

Thesis Approval

The Graduate College
University of Nevada, Las Vegas

November 12 , 19 98

The Thesis prepared by

Polly Ann Sullivan

Entitled

Effects of Sodium Chloride and Sodium Gluconate Concentration on Hydration

Behaviour and Water Transport in the Red-Spotted Toad,

Bufo punctatus

is approved in partial fulfillment of the requirements for the degree of

Master of Science in Biology

Examination Committee Chair

Dean of the Graduate College

Examination Committee Member

Examination Committee Member

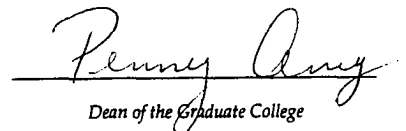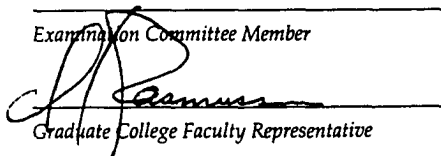_Graduate College Faculty Representative_

ii

ABSTRACT

Effects of Sodium Chloride and Sodium Gluconate Concentration
on Hydration Behavior and Water Transport in the
Red-spotted Toad, *Bufo punctatus*

by

Polly Ann Sullivan

Dr. Karin Hoff and Dr. Stan Hillyard,
Examination Committee Co-chairs
Professors of Biology
University of Nevada, Las Vegas

Toads hydrate from standing water or moist substrates by osmotic absorbtion across the ventral skin. On land toads adopt a distictive posture called the water absorption response (WR) to maximize contact with the substrate and facilitate water absorption. Experiments using hydration behavior on moist substrates and rehdration rate in standing water or salt solutions in dehydrated Red-spotted toads (*Bufo punctatus*) were used to demonstrate that: 1) toads can distinguish among NaCl concentrations, 2) NaCl facilitates water uptake across the skin, and 3) transport of the chloride anion facilitates sodium uptake and affects sodium detection by the toad. The results of these studies also strongly suggest that general osmotic mechanisms as well as epithelial sodium channels function in chemosensation across the amphibian skin and that the ventral skin and the feet may have different roles in chemosensation.

iii

TABLE OF CONTENTS

LIST OF FIGURES

vi

ACKNOWLEDGEMENTS

I would like to express my sincerest thanks to Dr. Karin Hoff for her continuous encouragement and support throughout the past several years. I have her to thank for my success. I would like to thank Dr. Stan Hillyard for the training in electrophysiology techniques and the help given to this thesis. I want to thank Dr Takatoshi Nagai for the time and attention given to teaching me appropriate research skills. I would like to thank Dr. Steven Carper for the excellent guidance as an advisor. I am very proud that I was a recipient of the NSF Women in Science and Engineering Research Awards and I thank Dr. Penny Amy for that honor. I would also like to thank all of the faculty and graduate students of the UNLV Department of Biology for being there when needed.

CHAPTER 1

INTRODUCTION AND HISTORICAL PERSPECTIVE

Unlike other vertebrates anuran amphibians (frogs and toads) do not drink orally, rather they absorb water osmotically across their skin (Bentley and Yorio, 1979). The study of water balance in terrestrial amphibians dates back as far as the 1790's (Townson, 1795). Townson noted that water balance for terrestrial amphibians was affected by a high rate of evapotranspiration across the skin. He also observed that in tree frogs (*Hyla arborea*) and frogs (*Rana tempora*) the water loss was countered by retention and reabsorption of water from the bladder and by absorption of water through the lower abdominal skin.

Behavior Associated with Water Uptake

Field observations of toad behavior related to the absorption of water through the skin of the lower abdomen by Stille (1952) led to a lab study of toad water uptake behavior by Stille (1958). It was observed that dehydrated toads adopt a distinct posture when placed on a moist surface that allows increased contact of the lower abdominal skin with the surface. He called this posture and related behavior the water absorption response. Compared to other areas of skin, the lower abdominal skin of many anuran species is adapted for higher rates of water uptake and is termed the pelvic patch or seat patch (McClanahan and Baldwin, 1969, Marrero and Hillyard, 1985).

1

Seat Patch Specializations for Water Uptake

The toad seat patch skin has specialized morphological features. The skin has small tubercles and channeling called epidermal sculpturing (Lillywhite and Licht, 1974) that allow increased surface area when the skin is pressed to a hydration surface (Hillyard, 1988). There are cutaneous attachments of the gracilis minor and a specialized cutaneous muscle, the abdominal crenator, underneath the seat patch that is thought to facilitate expansion and contraction of the seat patch skin (Winokur and Hillyard, 1992). The seat patch area also has higher vascularization than the pectoral area of toad ventral skin and, in dehydrated bufonids, is associated with increase of water. Capillaries may move the water which is absorbed across the skin into the circulatory system. Alternatively, the large lymphatic space in the proximity of the seat patch skin may also function in the transport of water absorbed across the skin to the general circulation (as reviewed by Jorgensen, 1997).

Specializations of Amphibian Skin for Active Sodium Transport

In addition to water uptake, the amphibian skin is able to actively absorb Na^+ and Cl^- from dilute pond water (Krogh, 1939) thereby allowing the frogs to maintain ionic as well as osmotic balance. Early studies of isolated frog skin showed that there is an electric current associated with transport of sodium ions across the skin (as reviewed by Jorgensen, 1997). This phenomena was generally referred to as electro-osmosis. Short circuit current measurement techniques facilitated the development of a model for active sodium transport across amphibian epithelium (Ussing and Zerahn, 1951, Koefeld-Johnson, Levi and Ussing, 1952). The model proposes that in frog skin, sodium ions enter the epithelial cells down an electro-chemical gradient across the apical membrane. The sodium entry step has been shown to be through amiloride blockable sodium ion channels (Lindemann and Van Driessche, 1977). The Na^+ is then actively

transported out of the cells by a Na^+/K^+ pump located in the basolateral membrane. Removal of sodium and subsequent potassium leakage out of the cell creates a favorable electrochemical gradient for further sodium diffusion inward and the chloride ion was thought to diffuse passively along the electro-chemical gradient (Koefeld-Johnsen and Ussing, 1958).

These physiological specializations for Na^+ and Cl^- absorption are present in the toad seat patch in addition to the specializations for water uptake. Recent studies of chloride suggest that Cl^- does not diffuse passively, but is actively transported by way of a Cl^--HCO_3 exchange across mitochondria-rich cells located at the apical surface of toad epithelium (Jensen, Sorensen, Larsen, and Willumsen, 1997).

The active sodium transport model was expanded to include transport of sodium ions through all of the cell layers of the toad epithelium (stratum granulosum, stratum spinosa and stratum basoteral) (Rick, Dorge, von Armin, Weigel and Thurau, 1981). These authors also demonstrated that the sodium ion transport across the apical membrane of toad skin was through amiloride blockable sodium ion channels.

Sodium Transport and Salt Taste in the Mammalian Tongue

The model for active Na^+ transport across amphibian skin (Ussing and Zerahn, 1951) has been applied to Na^+ transport across canine lingual epithelium in association with salt taste (DeSimone, Heck, Miersen and DeSimone, 1984), and in other mammals (Stewart 1997). In lingual epithelium the inward transport of sodium is increased by the cotransport of chloride through a paracellular pathway (Ye, Heck and DeSimone, 1991).

Sodium Transport and Salt Taste in the Amphibian Skin

Recent studies by Hoff and Hillyard (1993) suggest that a portion of the Na^+ chemosensory mechanism in toad skin is blockable by amiloride. That study suggests that the mechanism of Na+ transport across amphibian skin, like the tongue , may be a contributing factor to chemosensory transduction of this ion. However, not all Na^+ avoidance is eliminated by amiloride suggesting that there is also an amiloride insensitive component to Na^+ chemosensation. The amiloride insensitive component may be a paracellular route for Na^+ transport across the toad skin.

Studies of toad avoidance of hyperosmotic substrates has led to the investigation of possible similar chemosensory mechanisms in toad skin (Brekke, Hillyard and Winokur, 1991, Hoff and Hillyard, 1991). Brekke *et all.* (1991) showed that toads with feet covered by latex finger cots remained longer on hyperosmotic substrates than toads with bare feet. This supports observations by Stille (1952) that suggest that toads have a means of detecting osmotically favorable hydration sources with their feet. Further studies are needed to substantiate these observations.

Statement of the Problem and the Hypothesis

Behavioral and Physiological Effects of Salt Concentration

Beyond the avoidance of hyperosmotic solutions, little is known about hydration behavior of toads exposed to a wide range of solute concentrations that are in contact with their skin. The first set of experiments tests the hypothesis that the hydration behaviors of dehydrated toads, *Bufo punctatus,* presented with a range of hypoosmotic and hyperosmotic NaCl concentrations will differ; that is, that toads can discern (or taste) differences among solute concentrations.

A second set of experiments investigates whether NaCl concentration affects the rate of water uptake by dehydrated toads. It is known that antidiuretic hormone (ADH)

is released during dehydration (Nouwen and Kuhn, 1984) and that this hormone increases both Na+ transport and water permeability of the skin (Baker and Hillyard, 1991). These experiments test the hypothesis that sodium transport is coupled with water movement; that is, water gain will be augmented by the presence of salt in the hydration source and that amiloride, an inhibitor of transepithelial Na+ transport will reduce this augmentation.

From these two experimental approaches, I hope to describe the toads ability to detect and respond to Na^+ in its environment and also to describe a possible physiological benefit of the changes in hydration behaviors that relates to the toads ability to take up water.

Effects of Anions on Sodium Detection and Water Transport

Little is known about the cotransport of Na^+ and Cl^- across the toad skin. However, much is known about chloride transport across epithelium but the relative amount of paracellular verses transcellular chloride transport remains controversial. Ye, Heck and DeSimone (1991) noted that in canine lingual epithelium there is a paracellular shunt for chloride associated with the taste receptor cells. They showed that blockage of the movement across the tissue reduced the neural response for sodium, suggesting that the transport of Na^+ across the lingual epithelium is dependent in part on the cotransport of the chloride anion. In isolated toad epithelium, Jensen, Sorensen, Larsen, and Willumsen (1997) showed that in dilute media the active transport of chloride is driven by a $Cl^- - HCO_3$ exchange mechanism and is coupled to an active transport of Na+ through the $Na^+/K+$ pump. Both transport mechanisms are located on the apical membrane of mitochondrial rich cells. The dilute media was < 1 mM NaCl and the active transport is thought to preserve the chloride concentration in the toads.

This study tests the hypothesis that epithelial transport of Cl^- affects Na^+ transport, Na^+ detection and water transport in toads and that this difference is

discernible in toad hydration behavior and water uptake. The effects of Cl^- are examined by comparing behavioral response to equimolar concentrations of NaCl and the Na salt of the large, impermeant gluconate anion.

CHAPTER 2

EFFECTS OF SODIUM CHLORIDE CONCENTRATION ON HYDRATION BEHAVIOR AND WATER TRANSPORT IN RED-SPOTTED TOADS, *Bufo punctatus*

Introduction

Toads in the genus *Bufo* have a highly permeable region in the lower ventral skin termed a pelvic patch (McClanahan and Baldwin, 1969; Marrero and Hillyard, 1985). This highly permeable skin allows the toad to quickly recover water losses. The Red-spotted toad, *B. punctatus* displays a distinct posture when hydrating that maximizes contact of the permeable skin with the substrate. The hindlimbs are splayed as the pelvic patch is pressed to the moist surface. This behavior is called the water absorption response (WR) (Stille, 1958). Dehydrated individuals of this species can gain as much as 15% of their body weight in half an hour (Hoff and Hillyard, 1993a), while sitting in WR on a damp tissue.

Brekke *et al..*, (1991) showed that when dehydrated *Bufo punctatus* were placed on a tissue saturated with hyperosmotic urea solutions they would not initiate WR. The specialized skin of the pelvic patch of toads that enables rapid water uptake may also contain a mechanism that allows chemosensory discrimination of hydration surfaces (Hoff and Hillyard, 1993b). Further studies showed that dehydrated toads avoided surfaces saturated with hyperosmotic NaCl and KCl (Hoff and Hillyard, 1993b). This study also found that amiloride, a blocker of epithelial Na^+ channels, increased the frequency of initiation of WR behavior on NaCl but not on KCl or urea solutions. The

7

duration of the episodes of WR behavior was very brief so that the total time showing this behavior was not significantly increased during a five-minute observation period. These results suggest that toads initially evaluate an hydration surface with a sensory mechanism that includes an amiloride-sensitive transport pathway that is selective for Na^+ but that a commitment to maintain skin contact involves their ability to discern the osmolality of a hydration source by an amiloride-insensitive mechanism. A possible route of chemosensation may be through a salt sensitive mechanism in the skin of the lower leg whereby the solute wicks up the toad skin in a capillary action (Lillywhite and Licht, 1974) or by a sensory mechanism located in the bottom of the foot as suggested by Stille (1952).

Little is known about the mechanism that enables toads to discern differences among hyper and hypo -osmotic concentrations of different solutes such as NaCl, KCl and $CaCl_2$ that are in contact with their skin. We hypothesize that toads will behave differently to high and low concentrations of NaCl and that they will behave differently on NaCl as compared to KCl. In this study we examine the hydration behaviors shown by dehydrated toads, *Bufo punctatus*, presented with a range of NaCl concentrations (50, 100, 250 and 500 mM) as rehydration sources and compare them to behaviors shown when the toads are presented with deionized water.

A second experiment investigates whether NaCl concentration in a rehydration source is able to influence the rate of water uptake by dehydrated toads. It is known that antidiuretic hormone (ADH) is released during dehydration (Nouwen and Kuhn, 1984) and that this hormone increases both Na^+ transport and water permeability of the skin (Baker and Hillyard, 1991). If solute transport is coupled with water movement, we hypothesize that water gain will be augmented by the presence of salt in the hydration source and that inhibition of transepithelial Na^+ transport should reduce this augmentation. These hypotheses were tested by recording water weight gain by

dehydrated toads immersed in deionized water and NaCl solutions, in the presence and absence of amiloride.

From these two experimental approaches, we hope to characterize the ability of toads to detect Na^+ in their environment and to determine whether there is a physiological benefit to such a behavioral mechanism in terms of their ability to regain evaporative water loss.

Methods

Animals

Red-spotted toads, *Bufo punctatus*, were collected during the spring and summer of 1995, 1996 and 1997 from springs in the Spring Mountains in Clark County Nevada (Nevada Department of Wildlife Scientific Collection Permit # S14965). Experiments were conducted from May through November of the same years. Toads ranged in size from 4 to 21 grams in weight.

Toads were kept in 75 x 30 cm terraria containing local desert sand, large stacked rocks and pooled tap water that simulated the hydration environment of their natural habitat. The toads were kept on a 12:12 L:D cycle at room temperature (21 -24 °C) and were fed crickets two or three times a week. Toads were allowed to acclimate to

Dehydration Procedure

The renin-angiotensin system that regulates thirst and drinking in mammals consists of a series of hormonal and enzymatic interactions resulting in elevated levels of the peptide angiotensin II (AII) in the plasma and cerebral fluid (reviewed by Phillips, 1987). Intraperitoneal injection of AII into fully hydrated toads, *Bufo punctatus*, induced water absorption response suggesting that the thirst response in toads that induces water uptake through the skin is similar to the oral drinking response of

mammals (Hoff and Hillyard, 1991). Therefore, in these experiments all toads were dehydrated to insure a consistency of need for water by all specimens.

The bladder contents were emptied with a polyethylene cannula and a standard weight (the weight of a hydrated toad with urinary bladder empty (Ruibal, 1962) was recorded. Toads were placed in a dry 40 x 21 x 27 cm glass tank for two to four hours, until dehydrated by approximately 10% of their standard weight ($\bar{X} = 9.7\%$, range = 3.0 % to 24.3 %). Barometric pressure and relative humidity were recorded at the start of rehydration time and before and after each experimental trial. Changing barometric pressure (Hoff and Hillyard 1993a) and relative humidity (Hoff and Orgeron, in prep.) are known to affect hydration behaviors.

Toad and Substrate Selection

Toads were tested in random order but without repetition for each experimental treatment. Each toad was presented with the different substrates in random order for the behavioral experiments. In the water uptake experiments each animal was randomly chosen and used only once for each solution. No toad was used in an experiment more than once a day.

Data Collection and Analysis

Digital timekeepers were used to record the time of onset of each behavior to evaluate the duration of time spent in each behavior. Mean values for duration of hydration behaviors and weight change were compared by ANOVA using the Statview brand statistical software package (Abacus Concepts).

Hydration Behavior Experiments

The experimental process consisted of random selection of two toads, one to serve as a control with a substrate of de-ionized water and the other placed on a randomly selected substrate of 50, 100, 250 or 500 mM NaCl. Each behavioral experiment was conducted in 40 x 21 x 27 cm glass aquaria with dark paper or plastic

covered sides to prevent the toads from becoming frightened by the movements of the observer. An opaque divider placed across the middle created two observational cubicles which allowed experimental and control observations to be run simultaneously and in close proximity. The hydration posture of the toads was observed through the underside of the glass tank by use of an angled mirror placed beneath the tank. Substrates were presented by saturation of a 10 cm^2 Kimwipe brand tissue centered within each observational cubicle. Three ml of de-ionized water or salt solution was used to saturate the tissue for each trial.

The behavioral assay tracked the occurrence and duration of the hydration behaviors of toads when they were presented with water or with NaCl solutions. Several discrete postures and positions were defined (Figure 1, page 38):

OFF: *the toad is completely off of the saturated tissue.*

SPU (Seat patch up): the toad has one or more feet pressed against the tissue.

SPD (Seat patch down): the toad has at least a portion of the lower abdominal skin, the pelvic patch area, pressed against the tissue while placing hindlimbs parallel to the body.

WR (water absorption response): the toad has at least a portion of the lower abdominal skin, the pelvic patch area, pressed against the tissue while placing hindlimbs at an outward angle. This posture allows greater pelvic patch skin contact with the moist surface than in SPD.

We recorded the duration of each posture in each experimental trial and the mean for each substrate.

<u>Rehydration Experiments</u>

The purpose of this experiment was to evaluate water uptake rates in Red-spotted toads when forced to be in contact with an hydration source and to test whether certain substrates are more effective as hydration sources. The dehydrated toads were placed

in aqueous solutions of various NaCl concentrations for 20 minutes. To measure the water uptake, each toad was weighed before and after immersion. The weight change was assumed to be from water transport across the toad epithelium.

Each toad was placed in a straight sided widemouth glass jar 18 cm in height x 9 cm in diameter filled with 150 ml of substrate to a depth of approximately 4 cm. This depth submerged the lower abdominal area of the toad while allowing air access for the toad when in a comfortable sitting posture. For some very small toads the amount of liquid was reduced by up to 40 ml.

NaCl concentrations used in the rehydration experiment were the same as for the behavioral assays. The substrates were de-ionized water and 50 mM, 100 mM, 250 mM or 500 mM NaCl each without and with 10 μM amiloride. Amiloride is a specific blocker of epithelial Na+ channels and preliminary experiments with isolated *Bufo punctatus* skin have shown the blocker affinity (Ki) to be approximately 0.21 μM (Hillyard, Hoff and Sullivan, 1997). Each toad was immersed in the substrate for 20 minutes. The difference between the pre-experiment weight and the post experiment weight was the rehydration weight change. The mean value for percent weight change in each substrate is shown in Figure 4.

Electrophysiology Experiments

Toads that had been dehydrated for 1-2 hours were double pithed and spinal nerve #6 was dissected from the dorsal lymphatic space. The nerve was cut near the vertebral column and desheathed. The toad was then placed on its side so that the cut nerve could be placed over one of a pair of silver chloride recording electrodes. The nerve was covered with a mixture of paraffin oil and vaseline to prevent desiccation and the second electrode was placed in soft tissue adjacent to the nerve. The recording electrodes were connected to a Grass hi Z model HIP5 high impedance probe that also

received an iso-ground input from a silver chloride electrode inserted into a leg muscle of the toad.

Nerve activity was filtered within a bandwidth of 100-1,000 Hz and amplified with a Grass model 7P511J EEG preamplifier. Output from the preamplifier was monitored continuously on an oscilloscope and recorded on a Bio-Logic Model DTR 1205 digital audio tape recorder along with a 5 volt event marker to signal the onset and end of application of solutions to the skin. The signal was integrated with a time constant of 1.0 sec and the integrated signal was saved as files in a Sable Systems Data Acquisition Release 2.0 software package with a sampling rate of 0.05 seconds. The integrated response was calculated for specified time periods before and after the application of test solutions.

Solutions were presented continuously to the surface of the skin by gravity feed through silicon rubber tubing. The region of skin to be perfused was selected on the basis of the sensitivity of the response of nerve #6 to gentle mechanical stimulation of the outer surface of the skin. A flow rate of approximately 0.1 ml/s was found to minimize the level of mechanosensory stimulation due to perfusion and solution changes were made by switching solution reservoirs so that flow was continuous and flow rates were consistent within each experiment.

The skin was perfused with a dilute NaCl solution (0.5 or 1.0 mM) in the control condition. Once a stable background of neural activity had been attained, a test solution (de-ionized water, 50 mM, 100 mM, 250 mM or 500 mM NaCl) was perfused for 25-30 seconds. This was followed by a perfusion of the control NaCl solution for 145-150 seconds followed by perfusion with the next test solution. This pattern was followed until all of the test solutions had been evaluated.

Results

Hydration Behavior Experiments

In typical trials with de-ionized water as the substrate, the toads adopted the SPU posture first. Following a short period (20-25 seconds) of SPU behavior, if the toads remained on the tissue, they usually spent a brief period in SPD posture, allowing limited contact of the specialized pelvic skin to the substrate. The toads then initiated WR posture which allowed maximal contact of the specialized pelvic skin to the hydration surface and rapid water uptake through the exposed epithelium. The toads varied the duration of the behaviors with different substrates.

The average time during a 30 minute observation period that dehydrated toads spent in each of the four discrete behaviors, OFF, SPU, SPD and WR, is shown in Figure 2 (page 39). In general, the duration of time OFF increases and the duration of time spent in WR decreases as the NaCl concentration increases above 50 mM. Surprisingly, WR time on 50 mM NaCl is greater than that on deionized water. Results for each behavior are described below.

OFF:

The time that toads spent off the tissue was significant ($p < .05$) between the 50 mM NaCl (567s $\pm$ 137s) ($\overline{X} \pm$ SE) and the three highest NaCl concentrations of 100 mM (1073s $\pm$ 134s), 250 mM (1388s $\pm$ 97s) and 500 mM (1421s $\pm$ 68s). Off time was significantly different also between de-ionized water (805s $\pm$ 83s) and both 250 mM and 500 mM NaCl. There was no significant difference between DI and 50 mM NaCl or 100 mM NaCl.

SPU:

Although the time spent in SPU posture was greatest on the 500 mM NaCl (374s $\pm$ 68s) and shortest on 50 mM NaCl (153s $\pm$ 46s), the only significant difference between substrates for SPU was between 500 mM and 50 mM NaCl and 500 mM NaCl

and de-ionized water (202s $\pm$ 38s) (both p < .05). The values for 250 mM NaCl (277s $\pm$ 61s) and 100 mM NaCl (212s $\pm$ 52s) were not significantly different from any other substrate.

SPD:

Time spent displaying SPD behavior was low and variable for all substrates and the only significant difference was between 250 mM NaCl (124s $\pm$ 77s) and 500 mM NaCl (3s $\pm$ 2) (p < 0.05). The values for de-ionized water (78s $\pm$ 16s), 50 mM NaCl (62s $\pm$ 22s) and 10 mM NaCl (53s $\pm$ 19s) were not significantly different from any other substrate.

WR:

The duration of the WR behavior is shown in Figure 2 (page 39) and separately in Figure 3 (page 40). De-ionized water, 50 mM and 100 mM NaCl were similarly selected as suitable hydration substrates for dehydrated *B. punctatus* whereas the 250 mM and 500 mM NaCl concentrations were found unsuitable.

The mean value of time spent in WR was high for de-ionized water (714s $\pm$ 81s), 50 mM NaCl (1017s $\pm$ 145s) and on 100 mM NaCl (461s $\pm$ 137s). There was virtually no time spent in WR on 250 mM (10s $\pm$ 4s) and 500 mM NaCl (1s $\pm$ 1s). Except for de-ionized water versus 100 mM NaCl and 250 mM versus 500 mM NaCl the duration of WR varied significantly among substrates (p value <.05). As noted above, toads spent more time in WR posture on 50 mM NaCl than on the more osmotically favorable de-ionized water and this difference was significant (p < 0.05).

<u>Rehydration Experiments</u>

The results of weight change for dehydrated toads immersed in de-ionized water with or without various NaCl concentrations is shown in Figure 4 (page 41). To account for differences in toad size and amount of dehydration, each measurement was normalized to the toad standard weight. The highest average weight gain of standard

weight was for toads placed for 20 minutes in 50 mM NaCl (7.5% $\pm$ 3.1%) ($\bar{X} \pm$ SE) substrate. The average weight gain amounts for de-ionized water (4.2% $\pm$ 3.5%) and 100 mM NaCl (4.9% $\pm$ 2.6%) are significantly less than 50 mM NaCl ($p < .05$). There was a small weight loss of approximately 1% for the toads in 250 mM (-1.1% $\pm$ 2.3%) and 500 mM NaCl (-0.5% $\pm$ 2.3%) substrate, and both are significantly different from the all other substrates ($p < .05$).

The results of the second rehydration experiment are shown in Figure 5. Again, the highest average weight gain measured as percent of standard weight was for toads placed in 50 mM NaCl (10.2% $\pm$ 1.0%) substrate, however, weight gain for toads in 50 mM NaCl with 10 μM amiloride was significantly reduced (7.2% $\pm$ 2.6%). The average weight gain amounts for de-ionized water (5.0% $\pm$ 2.4%) and de-ionized water with amiloride (6.7% $\pm$ 3.2%) were significantly different but were significantly less than those with 50 mM NaCl ($p < .05$). There was a small weight gain of approximately 1% for the toads in 250 mM NaCl (0.8% $\pm$ 3.5%) and a small weight loss of approximately 1% for the toads in 250 mM NaCl with amiloride (-1.1% $\pm$ 0.7%) substrate. Both values are significantly different from the all other substrates ($p < .05$) but the 250 mM NaCl treatments with and without amiloride did not differ significantly from each other.

Discussion

Hydration Behavior Experiments

As shown in figure 3, the hydration behaviors indicate that de-ionized water, 100 mM NaCl and especially 50 mM NaCl are suitable hydration sources for dehydrated *B. punctatus* while the 250 mM and 500 mM NaCl concentrations are unsuitable. The results of the behavioral assays demonstrate that toads have the ability to detect and react to differing salt concentrations in potential hydration sources. Toads also show a clear

preference for some salt over no salt. The duration of WR on hypertonic NaCl solutions is drastically lower compared to the duration of WR on hypotonic NaCl concentrations (Figure 3) and reveals a threshold of tolerance. A preference by toads for some salt but not very much salt in their hydration substrate is shown by the significant preference of 50 mM NaCl over de-ionized water and 100 mM NaCl.

WR allows maximal contact of the pelvic patch skin to the hydration surface and rapid water uptake through the exposed epithelium. The skin of the pelvic patch has a network of channeling and skin thickenings (epidermal sculpturing) which increases the area of skin that comes into contact with the hydration surface and increases potential for water uptake (Lillywhite and Licht 1974). The epidermal sculpturing of *Bufo punctatus* is especially pronounced and allows expansion of the contact area of the seat patch by 1.5 to 3.7 times (Brekke et al., 1991).

SPD posture allows limited contact of the specialized pelvic patch skin to the substrate. The posture allows some water uptake as well as a more thorough chemosensory evaluation of the substrate than SPU posture. The SPD is consistently of short duration on all substrates except 500 mM NaCl, where it is not displayed at all. This suggests that a suitable surface is readily detected by the toads. The SPD behavior may be an information gathering posture that indicates some acceptance of the substrate.

When toads evaluate the suitability of a hydration source they first assume SPU posture. This posture allows the toad to make a tentative evaluation of the substrate, possibly directly through the feet (Stille, 1952; Brekke *et al..*, 1991). As shown in Figure 2, SPU durations are comparable for all substrates except 500 mM NaCl where sustained SPU behavior is noted.

A similar method was used in a study of salt taste discrimination behavior that measured salt preference of the axolotl salamander, *Ambystroma mexicanum,* using a simple behavioral assay that equated acceptance with swallowing and rejection with

spitting out food pellets containing various salt concentrations (Takeuchi , Masuda and Nagai, 1994). The present study emulates that assay. Acceptance or rejection of salt is evaluated using distinctive behaviors: the time toads spent in WR posture is acceptance and the time spent in SPU or OFF the tissue is rejection.

<div align="center">Coupling of Water and Na$^+$ Transport</div>

The ability of toads to discriminate among water sources is apparent, but the relationship between the ability to discriminate and the ability to transport water has, here-to-for, not been explored. Overall, the amount of weight change of toads immersed in various concentrations of NaCl (Figure 4, page 41) corresponds to the relative duration of the WR posture displayed for the corresponding NaCl concentrations in Figure 3 (page 40). As one would expect, there is a reasonable amount of water uptake in hypotonic salt concentrations. Within the hypotonic concentrations there is a significantly greater rate of water uptake by toads placed in 50 mM NaCl over toads placed in de-ionized water or 100 mM NaCl. The hypertonic salt concentrations of 250 and 500 mM NaCl showed no weight gain and some water loss.

Rehydration for toads, as for most amphibians, occurs by transcellular transport of water across the skin in a favorable osmotic gradient (Rick et al 1981) as illustrated in Figure 6 (page 43). The extracellular NaCl concentration is approximately 115 mM and the intercellular NaCl concentration is approximately 5 mM. Water transport across the toad epithelium is thought to be *via* water channels inserted across the apical membrane of the outermost cell layer, the stratum granulosum, but presence of water channels has yet to be verified in toad seat patch skin.

Among vertebrates, amphibians alone are able to offset Na$^+$ loss in dilute media by its reabsorption across the skin against a sizable concentration gradient (Krogh, 1939). As shown in the model of Koefoed-Johnsen and Ussing (1958), Na$^+$ diffuses across the apical membrane of the stratum granulosum through specific Na$^+$ channels.

The Na$^+$ is then quickly transported across the basolateral membrane *via* the Na$^+$/K$^+$ pump which actively transports 3 Na$^+$ ions out of the cell and 2 K$^+$ ions in by use of ATP (Bonting and Canaday, 1964). The K$^+$ passively leaks out of the cell, establishing a negative gradient which allows continuous Na$^+$ diffusion across the apical membrane (Koefoed-Johnson and Ussing, 1958).

The ability of amphibian skin to actively transport Na$^+$ may facilitate the uptake of water. As Na$^+$ enters the outer cell layer of the skin it creates a favorable osmotic gradient that allows water to diffuse across also. The epithelial cell layers work in syncytium. As Na$^+$ is pumped out across the basolateral side, by the Na$^+$/K$^+$ pump active transport mechanism, water follows into the deeper, neighboring cell layer.

Early studies of water transport across amphibian skin coupled the water transport to the sodium induced electrical potential of the skin, called electro-osmosis. The source of the electrical potential was shown to result from the flux of sodium ions across the skin (Ussing and Zerahn, 1951). The transport was thought to be through pores in the skin until Shier (1985) demonstrated separate channels for water transport. This study demonstrates in vivo the coupling of sodium and water transport across the toad skin and how presence and concentration of NaCl can effect water uptake rate across the toad skin.

As shown in these experiments, NaCl concentrations significantly in excess (250 and 500 mM) of the extracellular concentration of 115 mM NaCl are not favorable for water uptake. Substrate concentrations below (50 and 100 mM) the extracellular concentration are favorable for water uptake. Water uptake rate studies conducted on leg and thigh skin of *Rana pipiens* showed that half concentration Ringers solution allowed faster water transport across the skin than full strength ringers solution when applied to the mucosal side of the skin (Steinbach, 1967). These results are similar to the water uptake rate results for *B. punctatus* presented in this paper.

The second set of rehydration experiments (Figure 5, page 42) examine the effects of the sodium channel blocker amiloride on the rate of water transport across the toad skin. In those experiments, there was no difference in water transport rate between toads immersed in DI and DI with amiloride. However, there is a significant difference between 50 mM NaCl and 50 mM NaCl with amiloride, demonstrating that the increased water transport rate at low salt concentrations is facilitated by Na^+ transport through amiloride blockable sodium ion channels.

Separate Na^+ Pathways for Water Transport and Chemosensation

The results from this study, from the study of Rick, *et al.* (1981) and previous work in this lab, suggest that the two functions of toad seat patch skin, water transport and salt taste transduction, may be separate. The water transport is through specialized epithelial cell layers as a syncytium as previously described (Figure 6, page 43). The number of active Na^+ channels and thus the amount of Na^+ transport through amiloride blockable Na^+ channels into these cells varies with ADH level (Baker and Hillyard, 1991) and, thus, depends on the toad's hydration state. However, the chemosensory mechanism must continuously monitor the salt concentration in the environment and may transduce salt concentration information imprecisely to the CNS if that mechanism is affected by ADH.

Toad discrimination among salt concentrations is displayed during substrate contact with the pelvic skin and during substrate contact with the feet only (SPU). When amiloride is added to a hyperosmotic sodium solution, the toads display SPU for a longer time but show no difference in the duration of seat patch contact with or without amiloride (Hoff and Hillyard 1993). This suggests that amiloride blockable Na^+ channels in the apical membrane may function in the Na^+ sensory transduction mechanism in skin regions other than the seat patch. The Na^+ sensory transduction mechanism of the seat patch may be located not in the apical membrane, but several cell

layers beneath the apical membrane and the route of sodium ion transport for chemosensation may be paracellular. Furthermore, when the sodium ion channel blocker, amiloride, is applied to the skin of the toad *Bufo marinus* a several minute wash of the blocker on the skin is required before there is any inhibition of neural response from 250 mM NaCl (Maleek *et al.*, 1998). If the sensory cells are in a sub-epithelial location the prolonged wash time may be needed to allow the large impermeant amiloride molecule to diffuse into the epithelial cell layers beneath the apical membrane.

A Possible Neural Transduction Pathway for Salt Discrimination

Preliminary electrophysiolgy experiments conducted on *B. punctatus* in this lab measured neural response of spinal nerve #6, which innervates the lower pelvic area skin, to the same NaCl concentrations used in the hydration behavior experiments (Figure 7, page 44). Results of the neural recording experiment correlate with the results of the hydration behavior experiment. There is a distinct increase of neural response to the application of hypertonic NaCl as compared with the neural response to application of hypotonic NaCl solutions to the lower pelvic skin area. Since there is also a slight increase of neural response to 50 mM NaCl compared to de-ionized water, the threshold seemed to be 50 mM, indicating that Na^+ flows into the skin at this concentration.

To determine if the source of neural stimuli was a taste mechanism or a measure of osmolality, another short set of experiments were conducted to look at the effects of 0.5 mM and 1.0 mM sucrose on neural response of spinal nerve #6. Preliminary results showed very little neural response when sucrose was applied as compared to the substantial response of 250 mM NaCl applied both before and after the sucrose (data not shown). Similar preliminary results of sucrose application were found for *Bufo marinus* as well.

Overall there is a trend showing that as the salt concentration increases, the amount of neural response also increases. Although inconclusive as to the specific transduction mechanism, these results support the hypothesis that the transduction pathway for salt discrimination in toads is at least in part, *via* the 6th spinal nerve.

Conclusion

Red Spotted toads, *Bufo punctatus* dwell in arid habitats of the southwestern United States. They forage at night as much as 100 meters away from their canyon springs habitat. Although they can withstand dehydration of up to 40% of body weight (McClanahan and Baldwin, 1969; McClanahan, Ruibal and Shoemaker, 1994), when they are faced with the mostly dry habitat of the desert, the ability to rehydrate quickly is crucial. Their need to rehydrate is likely to be a limiting factor for forays away from hydration sources. Therefore the ability for these toads to evaluate favorable hydration sources is critical to their survival.

Bufo punctatus are able to detect varying concentrations of salt in a potential hydration substrate. The display of a distinct acceptance or rejection behavior by the toad to the various NaCl concentrations suggests a threshold exists for salt tolerance. Results of the rehydration experiments also suggests a similar threshold for favorable water uptake in salt solution that may correspond with the toad interstitial fluid concentration of about 115 mM NaCl. In the Red-spotted toad, the preference or avoidance of the various NaCl concentrations correlates with the water uptake or water loss rate. All hyposmotic salt concentrations were found to be acceptable hydration substrates in the behavioral experiment as well as favorable hydration sources for the toads. Conversely, the hyperosmotic salt concentrations were avoided and were shown to deplete much needed water from the toads. This distinct behavioral difference between high and low salt concentrations suggests a threshold exists for salt tolerance

in *Bufo punctatus* and indicates that the toads can evaluate whether a surface is acceptable or not and act accordingly by either adopting WR on the substrate or avoiding the substrate.

The uptake of NaCl through amiloride blockable Na^+ ion channels facilitates the uptake of water and this mechanism for water uptake appears to be present in toad pelvic skin and within the concentrations studied, is highest at the 50 mM NaCl concentration. Within the hyposmotic substrates tested in the hydration behavior experiments, some salt (the 50 mM concentration), was found to be preferred over deionized water as a hydration source. This suggests that the toad has a mechanism to discriminate between different salt concentrations and that there is a physiological reason for such a mechanism to exist in desert dwelling anurans.

Further studies investigating salt uptake through paracellular pathways may help to clarify the possible functional distinction between transcellular and paracellular Na^+ transport. Behavioral and neural studies manipulating the cellular junctions on the apical membrane of toad seat patch may reveal the nature of salt chemosensory and water uptake functions of toad pelvic skin.

CHAPTER 3

EFFECTS OF SODIUM GLUCONATE CONCENTRATION ON WATER TRANSPORT AND HYDRATION BEHAVIOR IN RED-SPOTTED TOADS, *Bufo punctatus*.

Introduction

Amphibians rehydrate by transporting water across the skin down a favorable osmotic gradient (Bentley and Yorio, 1979). Many amphibian species have specialized areas of skin that enhance water uptake. Toads in the genus *Bufo* have a highly permeable region in the lower ventral skin that is specially adapted for water uptake termed the seat patch or pelvic patch (McClanahan and Baldwin, 1969; Marrero and Hillyard, 1985). Rehydrating toads press their pelvic patch onto the moist surface with feet parallel to the body axis with just the central patch region touching the surface. This is called seat patch down (SPD) behavior. In the same posture but with hindlimbs splayed to allow maximal patch contact the behavior is called water absorption response (WR). These behaviors together are called hydration behavior posture (Maleek *et al*.., 1998).

Brekke, Hillyard and Winokur (1991) showed that when dehydrated *Bufo punctatus* were placed on a tissue saturated with hyperosmotic urea solutions they would not initiate WR. Studies by Hoff and Hillyard (1993b) further showed that dehydrated toads avoided surfaces saturated with hyperosmotic NaCl and KCl. This

24

study also found that amiloride, a blocker of epithelial Na^+ channels, increased the frequency of initiation of WR behavior on NaCl but not on KCl or urea solutions. The duration of the episodes of WR behavior was very brief so that the total time showing this behavior was not increased during a five-minute observation period. These results suggest that toads initially evaluate an hydration surface with a sensory mechanism that includes an amiloride-sensitive transport pathway that is selective for Na^+ but that a commitment to maintain skin contact involves their ability to discern the osmolality of an hydration source by an amiloride-insensitive mechanism.

Further studies in this lab demonstrated that in Red-Spotted toads, *Bufo punctatus*, water uptake is more rapid when the animals are immersed in 50 mM than in water containing no salt (de-ionized water) or in 100 mM NaCl. As demonstrated in chapter 2, toads fail to gain weight and often lose weight when immersed in hyperosmotic NaCl solutions of 250 and 500 mM NaCl. It was also demonstrated that Red-spotted toads show preference for an hydration source that contains 50 mM NaCl over de-ionized water or 100 mM NaCl and hyperosmotic salt concentrations of 250 mM and 500 mM NaCl. Preliminary electrophysiology studies suggest that the transduction of sodium taste is at least in part through the spinal nerves that innervate the lower abdominal skin (including the seat patch) of the toad *Bufo punctatus*.

The electropositive gradient established by active transport of Na^+ is neutralized by the cotransport of Cl^- across the toad skin (Koefoed-Johnsen and Ussing, 1958). Although much is known about the transport of chloride across epithelium, but the relative amount of paracellular *versus* transcellular Cl^- transport remains controversial. Ye, Heck and DeSimone (1991) noted that in canine lingual epithelium there is a paracellular shunt for chloride associated with the taste receptor cells. They showed that blockage of the movement across the tissue reduced the neural response to sodium suggesting that the transport of Na^+ ions across the lingual epithelium is dependent, in

part, on the cotransport of the chloride anion. Jensen, Sorensen, Larsen, and Willumsen (1997) showed that in dilute media the active transport of chloride is driven by a Cl^- - HCO_3^- exchange mechanism and is coupled to an active transport of Na^+ through the Na^+/K^+ pump. Both mechanisms are located on the apical membrane of mitochondrial rich cells in toad epithelium. Previous studies by Larsen (1991) on principle cells of toad skin demonstrated that there was no transcellular Cl^- transport across these cells. As discussed previously (see chapter 2), the chloride ion may diffuse across the toad skin by way of an electrogradient driven paracellular route or, alternatively, the chloride ion may transport actively across the apical membrane of the mitchondrial rich cells in the toad skin.

Little is known about the coupling of Na^+ and Cl^- transport across the toad seat patch skin. In this study we look at the impact of the Cl^- on epithelial sodium transport by comparing the effects of the Cl^- and gluconate anions on the transport of Na^+ across the toad skin. Weight change of dehydrated toads placed in de-ionized water and 50 and 250 mM concentrations of both NaCl and NaGluconate was measured to determine if the presence of the Cl^-, together with Na^+, effects the rate of water uptake in Red-spotted toads.

The gluconate anion is much larger than the chloride anion and , unlike chloride, it is impermeant to passive diffusion across the epithelium. There are no known gluconate channels that may accommodate transcellular transport of the large anion across the skin. The rate of gluconate penetration into the skin might thus relate to the size of the anion and the tightness of the junctions between the epithelial cells.

An hydration behavior assay was used to investigate the ability of dehydrated toads, *Bufo punctatus*, to detect salt in hydration substrates with or without the presence of the Cl^-. Time spent in hydration behavior is compared among deionized water, 50

mM, and 250 mM concentrations of both NaCl and NaGluconate and related to water weight gain by toads immersed in these same solutions.

Methods

Animals

Bufo punctatus were collected from several sites in the Spring Mountains in Clark County Nevada (Nevada Department of Wildlife Scientific Collection Permit # S14965). Experiments were conducted from May through November of 1996 and 1997. Toads were kept in 75 x 30 cm terraria containing local desert sand, large stacked rocks and pooled tap water that simulated the hydric conditions of their natural habitat. The toads were kept on a 12:12 L:D cycle at room temperature (21-24 °C) and were fed crickets twice or three times a week. Toad mass ranged from 7 to 20 grams.

For all experiments the urinary bladder contents were emptied with a polyethylene cannula and the standard weight, the weight of a hydrated toad with empty urinary bladder (Ruibal 1962), was recorded. The toads were placed in a dry 40 x 21 x 27 cm glass tank for two to four hours, until dehydrated by approximately 10% of the standard weight. The actual dehydration of the toads used in the experiment ranged from 7% to 14%.

Toads were selected at random but without repetition for each experiment. Once a toad was used in an experiment it was not used again until all other toads in the group were used. In the behavioral experiments, the substrate was randomly selected.

Rehydration Experiments

When in contact with a hydration surface, *Bufo punctatus* can take up water rapidly. The purpose of this experiment was to measure the amount of water weight gain when hydration behaviors do not affect contact with the substrate. Each toad was placed in a straight sided widemouth glass jar 17 cm in height x 9 cm in diameter filled with 150 ml of water or aqueous solutions of NaCl or NaGluconate to a depth of

approximately 4 cm. This depth gave constant submersion of the lower abdominal area of the toad while allowing the toad to breath air when in a comfortable sitting posture. For the smaller toads the water level was reduced.

For this experiment the substrates used were de-ionized water, and aqueous solutions of 50 mM, and 250 mM concentrations of both NaCl and NaGluconate. Each toad was immersed in the substrate for 20 minutes. The difference between the standard pre-experiment weight and the post experiment weight was the rehydration weight change. Before each weighing, the toads were dipped in de-ionized water so that the mass of water adhering to the skin would not bias the observed weight gain or loss.

<u>Hydration Behavior Experiments</u>

Each experiment was conducted in a 40 x 21 x 27 cm glass aquaria with sides covered with black plastic or paper. Two observational cubicles were made with a cardboard wall dividing down the middle to allow experimental and control observations to be run in the same place and at the same time. Clear observation of the rehydration posture displays by the toads was made through the underside of the glass tank by use of an angled 30 cm square mirror placed beneath the tank. Presentation of the substrates to the toads was by saturation on a 10 x 10 cm piece of laboratory tissue centered within each observational cubicle. Three ml of substrate was used for each trial. The control substrate was deionized water from the building tap supply.

Behavioral assays were designed to see what choices were made by dehydrated toads when presented with 50 mM and 250 mM solutions of NaCl and NaGluconate. Previous studies (discussed in chapter 2) have shown that *Bufo punctatus* shows preference for 50 mM NaCl but avoids 250 mM NaCl. In the same study several discrete behavioral states used in this experiment were defined and discussed in detail (see chapter 2 for illustrations). In summary they are:

1. OFF the toad is completely off the substrate saturated tissue.

2. Seat patch up (SPU) the toad has one or more feet pressed against the tissue.

3. Seat patch down (SPD) the toad has a portion of the lower abdominal skin , the pelvic patch area, pressed against the tissue while placing hindlimbs parallel to the body.

4. Water absorption response (WR) the toad has a portion of the lower abdominal skin, the pelvic patch area, pressed against the tissue while placing hindlimbs at an outward angle. This posture allows more pelvic patch skin contact with the moist surface than in SPD posture.

To evaluate the impact of chloride on the decision making process as well as the decision of whether a substrate is a favorable hydration source this study combined the time spent in SPD and WR postures into what is termed the hydration behavior.

In typical control experiments with de-ionized water as the substrate, the toads adopted the SPU posture first. Following a short period (20-25 seconds) of SPU behavior, if the toads remained on the tissue, they usually spent a brief period in SPD posture allowing limited contact of the specialized pelvic skin to the substrate. The toads then initiated WR posture which allowed maximal contact of the specialized pelvic skin to the hydration surface and rapid water uptake through the exposed epithelium. The toads varied the duration of the behaviors with different substrates.

Barometric pressure and relative humidity were recorded at the time of bladder cannulization and before and after each experimental trial. Changing barometric pressure (Hoff and Hillyard 1993a) and relative humidity (Hoff and Orgeron, in prep.) are known to affect hydration behaviors. The time of onset of each discrete behavioral state was observed visually. For each experimental trial the duration in seconds for each behavioral state was tallied. Mean values for duration of hydration behaviors and weight change were compared by ANOVA using the Statview statistical software package (Abacus Concepts).

Results

Rehydration Experiments

Results of the NaCl and NaGluconate rehydration experiment are shown as percent weight change for toads immersed in the experimental substrates (Fig. 8, page 45). To account for differences in toad size and amount of dehydration, each measurement of water gain was expressed as a percentage of the toad's standard weight. Experiments for the two salts were done over a two year time period and are normalized to the control group (de-ionized water) done during the same time period. Figure 8 shows the weight change of toads immersed in solutions of 50 mM NaCl (2.52 $\pm$ 0.40) ($\bar{X} \pm$ SE), 50 mM NaGluconate (0.96 $\pm$ 0.29), 250 mM NaCl (-0.64 $\pm$ 0.31) and 250 mM NaGluconate. (-0.26 $\pm$ 0.24). All substrates are significantly different from each other ($p < 0.05$) except between 250 mM NaCl and 250 mM NaGluconate.

Toads regained water significantly faster from 50 mM NaCl than from 50 mM NaGluconate ($p < 0.05$). Differences between water loss in 250 mM were not significant between NaCl and NaGluconate; however, both were significantly different from the 50 mM solutions.

Hydration Behavior Experiments

Duration of hydration behavior did not differ between the 50 mM salts but was significantly longer with gluconate in the 250 mM concentrations. Figure 9 (page 46) shows the results of hydration behavior (measured as a fraction of the associated deionized water control group) of toads on 50 mM NaCl (1.26 $\pm$ 0.20) ($\bar{X} \pm$ SE), 50 mM NaGluconate (1.28 $\pm$ 0.01), 250 mM NaCl (0.17 $\pm$ 0.08) and 250 mM NaGluconate. (0.638 $\pm$ 0.17).

The duration of hydration behavior for toads on 250 mM NaGluconate is significantly longer than the duration of hydration behavior on 250 mM NaCl ($p < 0.05$)

demonstrating that the toads have a higher chemosensory tolerance for 250 mM sodium when coupled with the larger, less permeable anion, gluconate.

Discussion

Anion Transport Across the Epithelium

Past studies indicate that the Na^+ transport across the chemosensory epithelium may be enhanced when coupled with chloride, a small ion of opposite charge (Ye *et al.*, 1991). Studies presented in Chapter 2 suggest two separate modes of sodium ion transport across the skin: transcellular through amiloride blockable sodium ion channels located on the apical membrane for water transport function, and an amiloride insensitive component that is possibly a paracellular route for the chemosensory function of toad seat patch skin. It is likely that an anion cotransport would be involved in both means of Na^+ transport.

The effects of the anion coupling on Na^+ transport has been suggested to impact the transcellular water uptake mechanism in toad epithelium (Larsen, 1991). Early studies by Ussing and Zerahn (1951) showed that Na^+ is actively transported across the toad skin by a basolaterally located Na^+/K^+ pump that maintains a favorable osmotic gradient across the apical membrane for Na^+ diffusion. Rick *et al.* (1981) demonstrated that the epithelial cell layers were a syncytial Na^+ transport compartment. Previous studies in this lab on the water uptake rate in toads (chapter 2) showed that some NaCl (50 mM) increases water uptake rate over no NaCl (deionized water) in the hydration source, demonstrating that the active transport of Na^+ across the toad skin facilitates water transport. Studies for this chapter replaced the Na^+ coupled anion chloride with gluconate. Results show that when Na^+ solutions are made with the impermeant anion gluconate, the rate of water transport is reduced. This suggests that the chloride anion is coupled to the transport of sodium across the apical membrane in toad seat patch skin.

There are two probable pathways for the epithelial transport of chloride, passive paracellular diffusion or active transport across the membrane of mitochondrial-rich cells found in the toad skin.

The same water uptake experiment was done with hyperosmotic sodium solution and results showed no difference between the two anions, chloride and gluconate, on water uptake rate at the scale of measurement allowed by the methodology. This indicates that osmotic water movement is the primary mechanism of water loss into solutions having a higher external osmolality.

Anion Transport and Chemosensation

The presence of chloride may facilitate the transport of Na^+ across the epithelium. If so, it could enhance the detection of salt by the toad. Previous studies in this lab showed that toads display different and reproducible behaviors in response to high and low NaCl concentrations. Conversely, the reduced Na^+ transport across the toad epithelium may reduce the amount sodium detected by the toad chemosensory mechanism the nature of which is unknown.

As in the studies of Ye *et al.*, (1991) that demonstrated that in canine lingual epithelium, the presence of chloride in the microenvironment of the taste cell impacts the neural response to NaCl, the impact of chloride on the behavior of the toads may be due to the passive diffusion of chloride into the microenvironment of the chemosensory mechanism embedded several cell layers into the toad skin.

In this study, the higher concentration of NaGluconate shows a significant increase in tolerance to 250 mM sodium. This may be caused by a decreased influx of sodium across the epithelium and subsequent reduction of salt detection by chemosensory mechanisms in the toad. The increase of seat patch exposure to the 250 mM NaGluconate is about equal between the SPD and WR postures (SPD 107s $\pm$ 53s, WR 133s $\pm$ 57s). This may indicate that lack of chloride to facilitate Na^+ across

the skin makes the higher 250 mM concentration appear to be marginally potable. Further analysis of duration of hydration behavior posture as a function of the number of times that the behaviors are initiated (NaCl 13.2s $\pm$ 5s, NaGluconate 61s $\pm$ 19s, p < 0.05)) shows more clearly that the toads are actually allowing a longer duration of seat patch contact on hyperosmotic sodium concentrations when the sodium ion is presented with gluconate. In other words, to a thirsty toad the substrate may not seem salty enough to be avoided, but the salt is not low enough to warrant lengthy exposure of the specialized seat patch skin when in WR posture.

Conclusion

Toads are able to detect varying concentrations of salt in a potential hydration substrate and the acceptance or avoidance of given sodium chloride concentrations parallells the benefit or detriment of the substrate to water uptake rate in *B. punctatus*.

When the sodium ion is coupled to gluconate instead of chloride in an hyperosmotic (250 mM) sodium solution, the toads show less avoidance of the substrate and display hydration behavior for significantly longer. This suggests that the transport of sodium ions across the skin is coupled to the transport of the chloride anion. An impermeant anion will slow down the rate of sodium transport across the skin thus reducing the amount of salt detected by the toads after adopting the hydration behavior.

In the immersion studies, the water uptake rate for 50 mM NaCl is much higher than the rate for 50 mM NaGluconate. Past studies have suggested a facilitation of water uptake by the transport of Na^+ across the skin. The epithelial water transport rate difference between the two substrates may in part result from the presence of chloride.

The mechanism of Cl^- transport, whether through chloride channels associated with the syncytial Na^+ transport compartment, *via* a paracellular route or through chloride channels in mitochondrial rich cells, is unknown. Future studies of water

uptake measurement that incorporate methods to block Cl^- channels, may shed light on the route(s) of sodium coupled chloride transport for the chemosensory and water uptake mechanisms in toad seat patch skin.

REFERENCES

Baker C.A. and S.D. Hillyard. 1992 Capacitance, short-circuit current and osmotic water flow across different regions of the isolated toad skin. J. Comp. Physiol. 162:707-713.

Barker Jorgensen, C. 1997. 200 Years of Amphibian Water Economy: From Robert Townson to the Present. Biol. Rev. 72:153-237.

Bentley P.J. and T. Yorio. 1979. Do frogs drink? J. Exp. Biol. 79:41-46

Bonting, S.L. and M.R. Canady 1964. Na-K activated adenosine triphosphate and sodium transport in toad bladder. Am. J. Physiol. 207:1005-1009.

Brekke, D.R., S.D. Hillyard and R.M. Winokur. 1991. Behavior associated with the water absorption response by the toad, *Bufo punctatus*. Copeia 1991:393-401

Christoffersen B.C., L.J. Jensen, L.J., E.H. Larsen, J.B. Sorensen and N.J. Willumsen. 1997. Proton pump activity of Mitochondria-rich cells. J. Exp. Physiol. 109:73-84.

DeSimone, J.A., G.L. Heck, S. Miersen and S.K. DeSimone 1984. The active ion transport properties of canine lingual epithelium in vitro. J. Gen. Physiol. 83:633-656.

Hillyard, S.D. 1988. Comparison of the surface area of pelvic and pectoral regions of toad skin using impedance analysis. Am. Zool. 28:17A.

Hillyard, S.D., K. vS. Hoff and C. Propper. 1998. The water absorption response: a behavioral assay for physiological processes in terrestrial amphibians. Phys. Zoo. 09:43:48 1-12.

Hillyard, S.D., K. Hoff and P. Sullivan. 1997. Species differences in the sensory function of epithelial Na^+ channels (ENaCs) in toad skin. APS Conference: The physiology and functional diversity of amiloride-sensitive Na^+ channels: a new gene superfamily. Park City Utah, October 29-November 1, 1997. The Physiologist 40:A-18.

Hoff K. vS. and S.D. Hillyard. 1991. Angiotension II stimulates cutaneous drinking in the toad, *Bufo punctatus*. Physiol. Zool. 64:1165-1172.

Hoff K.vS. and S.D. Hillyard. 1993a. Inhibition of cutaneous water absorption in dehydrated toads by saralasin is associated with changes in barometric pressure. Physiol. Zool. 66:89-98.

Hoff, K. vS. and S.D. Hillyard 1993b. Toads taste sodium with their skin: sensory function in a transporting epithelium. J. Exp. Biol. 183:347-351.

Hoff, K vS. and K. Orgeron. Effects of environmental parameters on hydration behavior of the toad, *Bufo marinus*. in prep.

Jensen L.J., J.N. Sorensen, E.H. Larsen and N.J. Willumsen. 1997. Proton pump activity of mitochondria-rich cells: The interpretation of external proton-concentration gradients. J. Gen. Physiol. 109:73-91.

Jorgensen, C.B. 1997. 200 years of amphibian water economy: from Robert Townson to the present. Biol. Rev. 72:153-237

Koefoed-Johnsen , V., H. Levi and H.H. Ussing 1952. The mode of passage of chloride ions through the isolated frog skin. Acta Physiol. Scand. 25:150-163.

Koefoed-Johnsen, V. and H.H. Ussing. 1958. The nature of frog skin potential. Acta Physiol. Scand. 42:298-308.

Krogh, A. 1939. Osmotic Regulation in Aquatic Animals. Cambridge: Cambridge University Press.

Larsen, E.H. 1991. Chloride transport by high-resistance heterocellular epithelia. Am. Physiol. Soc. 71:235-283

Lilliywhite, H.B. and P. Licht .1974. The movement of water over toad skin: the role of epidermal sculpturing. Copeia 1974:165-171.

Lindemann, B. and W. Van Driessche. 1977. Sodium specific membrane channels of frog skin are pores: current fluctuations reveal high turnover. Science 195:292-294.

Maleek, R., P. Sullivan, K. vS. Hoff and S.D. Hillyard. 1998. Salt sensitivity and hydration behavior of the toad *Bufo marinus*. submitted to Physiol. Beh.

Marrero, M.B., and S.D. Hillyard. 1985. Differences in cyclic AMP levels in epithelial cells from pelvic and pectoral regions of toad skin. Comp. Biochem. Physiol. 82C:69-73.

McClanahan, L.L. Jr. and R. Baldwin. 1969. Rate of water uptake through the integument of the desert toad, *Bufo punctatus*. Comp. Biochem. Physiol. 28:381-389.

McClanahan L.L., Jr., R. Ruibal, and V.H. Shoemaker. 1995. Frogs and toads in deserts. Sci. Am. 274:64-70.

Nouwen, E.J. and E.R. Kuhn. 1984. Radioimmunoassay of arginine vasotocin and mesotocin in serum of the frog *Rana ridibunda*. Gen. Comp. Endochrinol. 50:242-251.

Phillips M.I. 1987. Functions of angiotension in the central nervous system. Annu. Rev. Physiol. 49:413-435.

Rick, R., A. Dorge, E. von Arnim, M. Weigal and K. Thurau. 1981. Properties of the outer and inner barriers to transepithelial Na transport: an electron microprobe analysis. in. Macknight, A.D.C. and J.P. Leader eds. Epithelial Ion and Water Transport. Raven Press New York.

Ruibal, R. 1962. The adaptive value of bladder water in the toad, *Bufo cognatus*. Physiol. Zool. 35:218-223.

Schrier, R.W. (ed.) 1985. Vasopressin. New York: Raven Press.

Steinbach H.B.. 1967. On the ability of isolated frog skin to manufacture Ringer's fluid. J. of Anat. Physiol. 50:2377-2389.

Stewart, R.E., J.A. DeSimone and D.L. Hill. 1997. New perspectives in gustatory physiology: transduction, development, plasticity. Am.J. Physiol. (Cell Physiol.) 41: C-1-C26.

Stille, W.T. 1952. The nocturnal amphibian fauna of the southern Lake Michigan beach. Ecology 33:149-162.

Stille, W.T. 1958. The water absorption response of an anuran. Copeia 1958:217-218.

Takeuchi, H.A., T. Masuda and T. Nagai. 1994. Electrophysiological and behavioral studies of taste discrimination in the Axolotl, *Ambystoma mexicanum*. Physiol. Beh. 56:121-127.

Townson, R. 1799. Tracts and Observations in Natural History and Physiology. London: printed for the Author by J. White.

Ussing H.H. and K. Zerahn. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol. Scand. 23:110-127.

Winokur, R.M. and S.D. Hillyard .1992. Pelvic cutaneous musculature in toads of the genus *Bufo*. Copeia 1992:760-769.

Ye, Q., G.L. Heck and J.A. DeSimone 1991. The anion paradox in sodium taste reception: resolution by voltage clamp studies. Science 254:724-726.
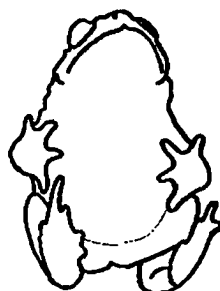
SPU:
Only the feet are
in contact with
the substrate

SPD:
Minimal seat patch
is in contact with
the substrate and
the hindfeet are
parallel to the body
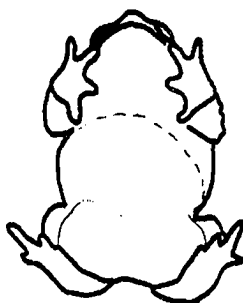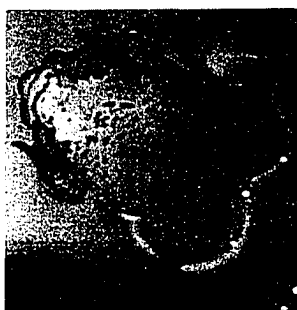
WR:
Maximal seat patch
 is in contact with
the substrate and
hindfeet are at an
outward angle to
the body

Figure 1: Hydration Behavior Postures of the Toad *Bufo punctatus*.
Stages of hydration behavior of *Bufo punctatus* used in the
hydration behavior assays. Postures are shown in photographs and
the critical identifying features of each are emphasized in line drawings

Time-activity Budget for 30 Minute NaCl
Hydration Behavior Experiments
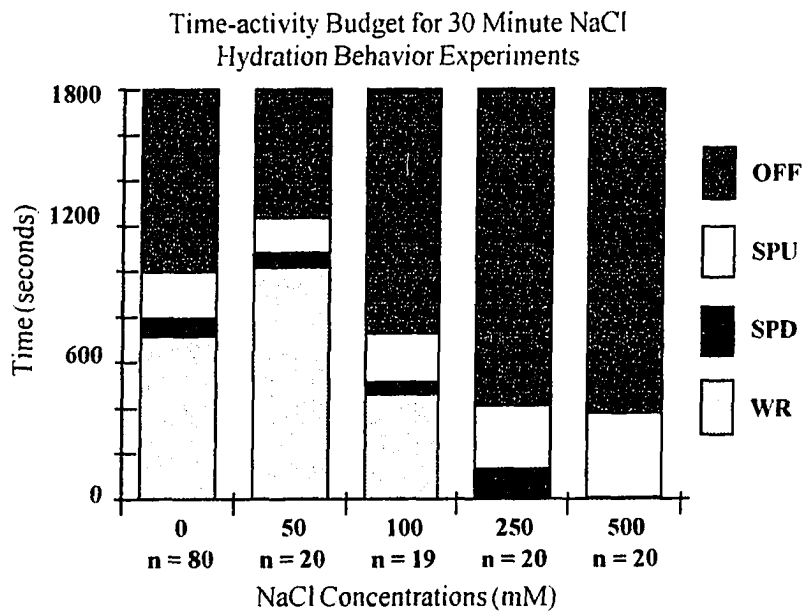
Figure 2: Time activity budget for hydration behavior of *Bufo
punctatus*. The stacked bar charts show that the mean duration of
hydration behaviors varies with the concentration of NaCl. Note that
WR behavior varies with salt concentration and duration is greater on
hypo-osmotic concentrations than on hyperosmotic concentrations of
NaCl. Numbers indicate sample size.

Duration of WR Posture for 30 Minute NaCl
Hydration Behavior Experiments
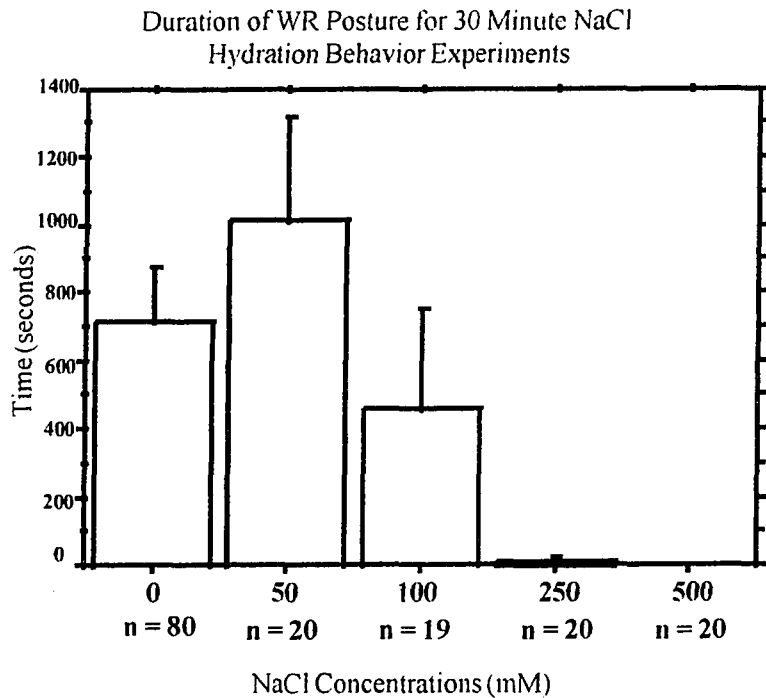
Figure 3: Mean duration of WR behavior for *Bufo punctatus* on several
concentrations of NaCl. Note that WR behavior is significantly greater
on 50 mM NaCl than on any other solution. Numbers indicate sample
sizes. Bars indicate 95% confidence intervals.

Weight Change for NaCl Rehydration Experiments
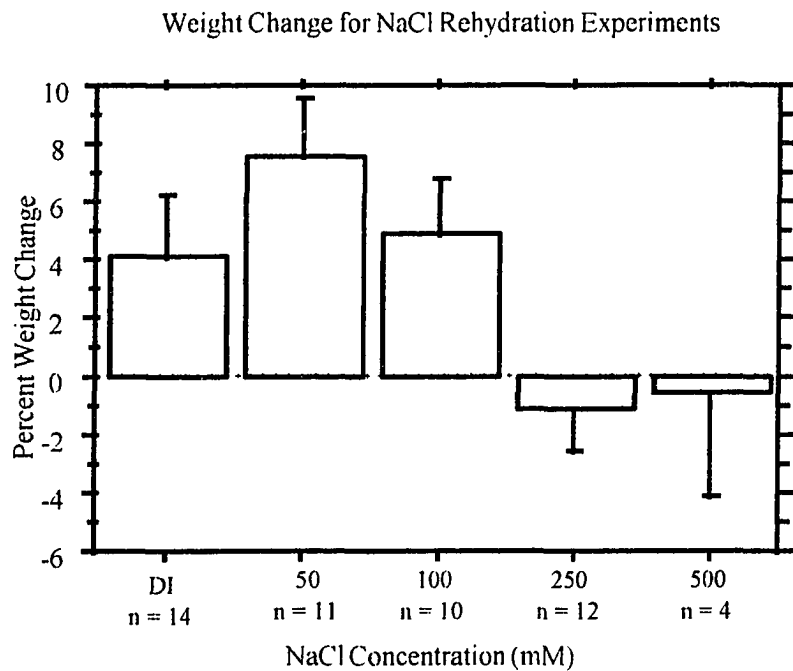
Figure 4: Mean weight change in *Bufo punctatus* rehydration experiments after 20 minute immersion in varying concentrations of NaCl. Numbers indicate sample sizes. Note that weight change is greater in 50 mM NaCl than in any other solution. Bars indicate 95% confidence interval. Negative values indicate weight loss.
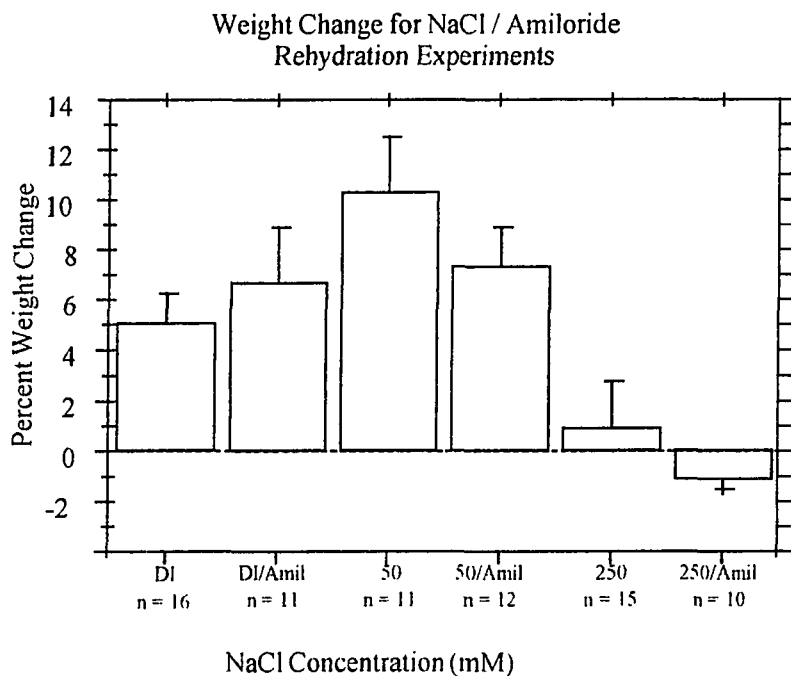
Figure 5: Mean weight change in Bufo punctatus rehydration experiments after 20 minute immersion in varying concentrations of NaCl in the presence of 10 uM amiloride. Note that toads in 50 mM NaCl with amiloride gained significantly less weight than toads in 50 mM NaCl without amiloride. Numbers indicate sample sizes. Barsindicate 95% confidence interval. Negative values indicate weight loss.
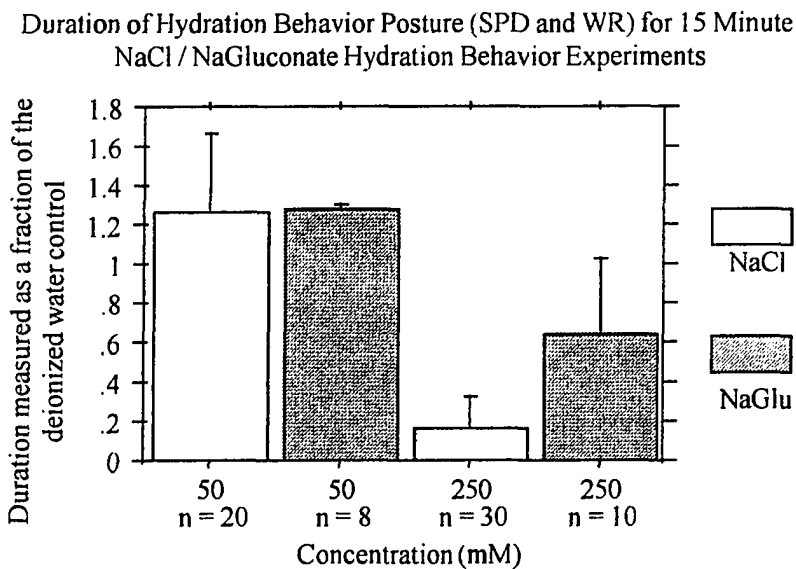
The Apical Cell Layer of Toad Seat Patch Skin
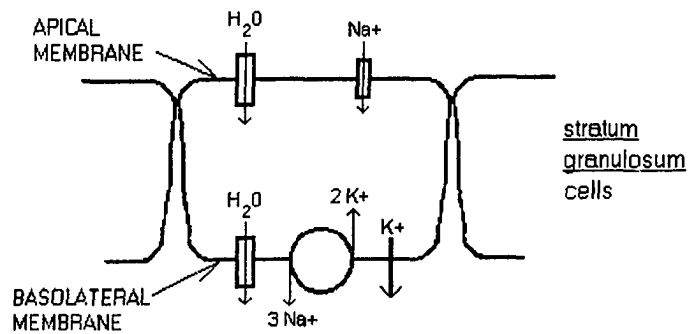
Figure 6: Illustration of sodium active transport and water transport across the apical membrane of toad seat patch epithilium.

Integrated Neural Response of Toad Spinal Nerve #6
(normalized to the 500 mM NaCl response)
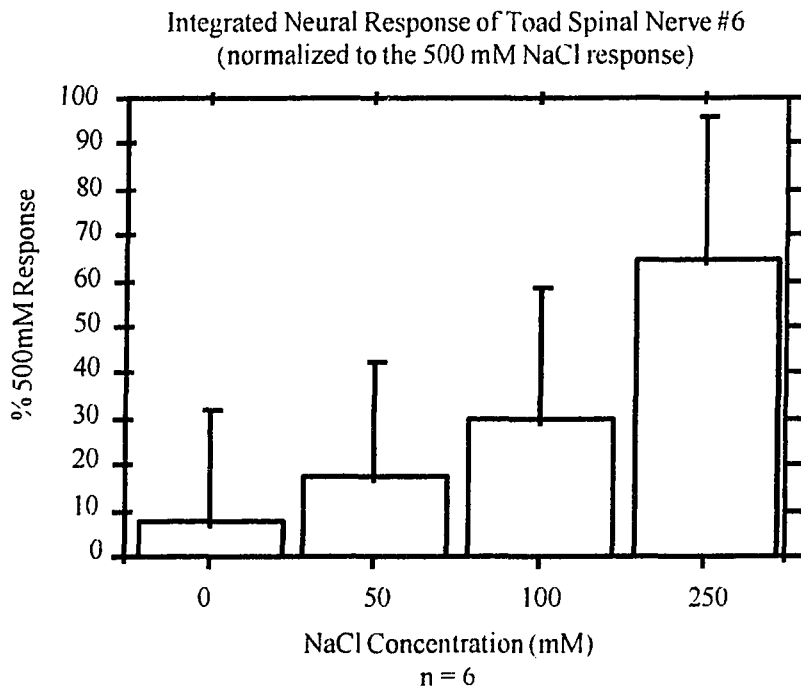
Figure 7: Mean integrated neural response from *Bufo punctatus* spinal nerve #6 to skin exposure of varying concentrations of NaCl. Note that neural response is significantly greater to 250 mM NaCl than to any other solution. Sample size is 6. Bars indicate 95% confidence interval.

Weight Change for NaCl/ NaGluconate
Rehydration Experiments
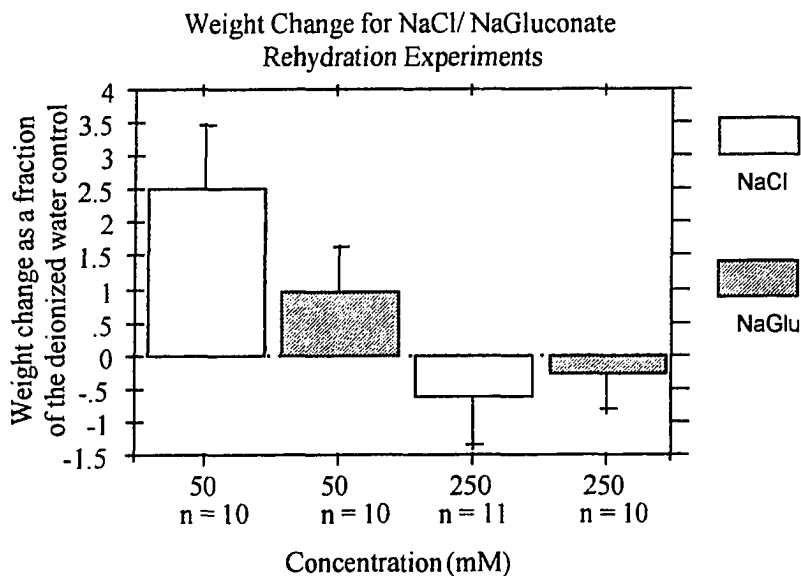
Figure 8: Mean weight change in *Bufo punctatus* rehydration experiments
after 20 minute immersion in aqueous solutions of 50 mM and 250 mM
concentrations of both NaCl and NaGluconate. Subtrates are measured
as a fraction of the control substrate, deionized water. Note that toads in
50 mM NaGluconate gained significantly less weight than toads in
50 mM NaCl solution. Numbers indicate sample sizes. Bars indicate
95% confidence interval. Negative values indicate weight loss.

Duration of Hydration Behavior Posture (SPD and WR) for 15 Minute
NaCl / NaGluconate Hydration Behavior Experiments

Figure 9: Mean duration of hydration behavior posture (SPD and WR) of
Bufo punctatus on the solutions of 50mM and 250 mM concentrations of
NaCl and NaGluconate expressed as a fraction of the control. Note that
toads allowed significantly longer duration of seat patch contact on 250
mM NaGluconate than on 250 mM NaCl. Numbers indicate sample
size. Bars indicate 95% confidence interval.

VITA

Graduate College
University of Nevada, Las Vegas

Polly Ann Sullivan

Local Address:
  3896 Commanche Avenue
  Las Vegas Nevada 89121

Degrees:
  Bachelor of Arts, Anthropology, 1993
  University of Nevada Las Vegas

  Master of Arts, Anthropology, 1995
  University of Nevada Las Vegas,

Special Honors and Awards:
  NSF EPSCOR Women in Science and Engineering Research Grant;
  June - Aug. 1996, Sept.1996 - May 1997, Sept. - Dec. 1997
  UNLV Graduate College Research Scholarship, Spring 1996
  UNLV Alumni Award for Academic Excellence in the Department of Anthropology
  1992-1993
  UNLV Anthropological Society Scholarship Award 1992-1993 school year
  UNLV Pen Pals Journal essay "Desert Treasures" 1992

Publications and Papers Presented:
  Maleek, R., P. A. Sullivan, K. von Seckendorff Hoff and S. D. Hillyard. 'Salt
  sensitivity and hydration behavior of the toad, *Bufo marinus*'. (submitted to
  Physiology and Behavior)

  Sullivan, P.A.. 'Effects of salt concentration on hydration behavior in Red-spotted
  toads, *Bufo punctatus*' In preparation. (plan to submit to Experimental Biology)

  Hillyard,S. D., K. Hoff and P. A. Sullivan. 'The effect of impermeant anions on Na+
  chemoreception and water transport by the toad skin. (Submitted poster to The
  AChemS XX meeting. April 1998

  Sullivan, P.A., G. Spaulding. 'A method of paleoenvironmental reconsruction using
  plant macrofossil assemblages from packrat middens'. In preparation

  Hillyard,S. D., K. Hoff, K. Mace, P. A. Sullivan and K. Martin. 1997. 'Salt appetite
  and avoidance in an amphibian *Bufo punctatus*'. The ISOT XII/AChemS XIX
  meeting. Park City Utah. October 1997

  Hillyard, S.D., K. Hoff and P.A. Sullivan. 1997. 'Species differences in the sensory
  function of epithilial Na+ channels (ENaCs) in toad skin' (poster). The American
  Physiological Society Conference, San Diego, California. 5-12 July

47

Sullivan, P.A., K.S. Hoff, S.D. Hillyard, C. Orgeron and K. Martin. 1997. 'Effects of NaCl concentration on rehydration of the Red-Spotted Toad, *Bufo punctatus*' The Arizona Nevada Academy of Sciences Conference, Las Vegas, Nevada. April 19th 1997.

Hoff, K.S., S.D. Hillyard, P.A. Sullivan and K. Martin. 1996. 'Salt appetite in a desert anuran, *Bufo punctatus*'. The Society for Integrative and Comparative Biology meeting, Albuquerque, New Mexico. 26-30 Dec. 1996.

Sullivan, P.A. 1992. ' Tactile interaction among Bottlenose dolphins (Tursiops truncatus) in captivity'. UNLV Journal of Anthropology 2 (1): 1-8

Thesis Title: Packrat middens: a tool for environmental reconstruction and human adaptation analysis

Thesis Examination Committee:
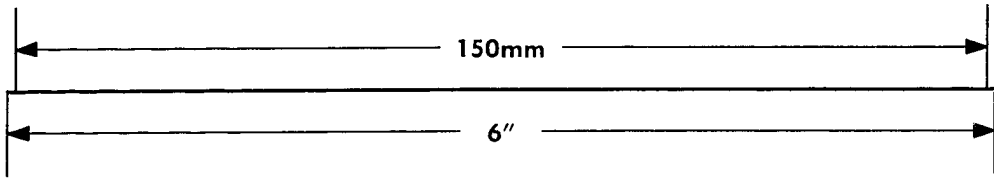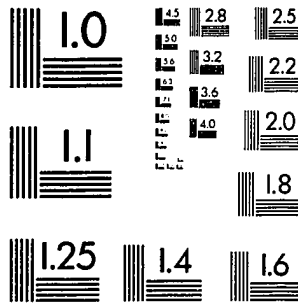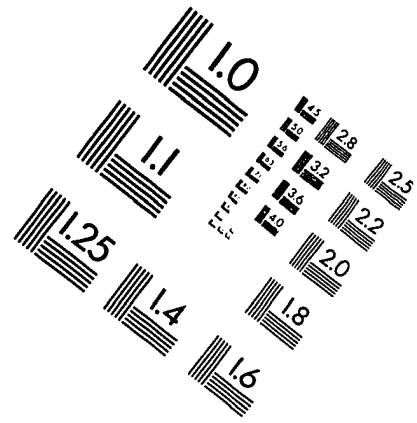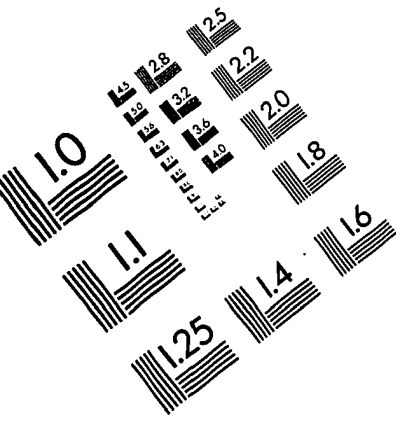 Chairperson, Dr. Claude Warren, Ph.D.
  Committee Member. Dr. John Swetnam, Full Professor, Ph.D.
  Committee Member, Dr. Bernardo Arriaza, Assistant Professor, Ph.D.
  Committee Member, Dr. Geoffry Spaulding, Adjunct Faculty, Ph.D.
  Graduate Faculty Member, Dr. Charles Douglas, Full Professor, Ph.D.

IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"