

1-1-1989

The rehydration behavior of the desert toad, *Bufo punctatus*

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The rehydration behavior of the desert toad, *Bufo punctatus*

Brekke, Dona Rae, M.S.

University of Nevada, Las Vegas, 1989

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Ann Arbor, MI 48106

THE REHYDRATION BEHAVIOR
OF THE DESERT TOAD,
BUFO PUNCTATUS

By

Dona Rae Brekke

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

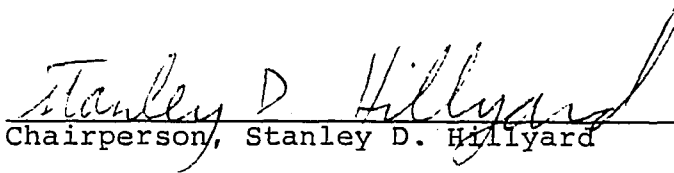
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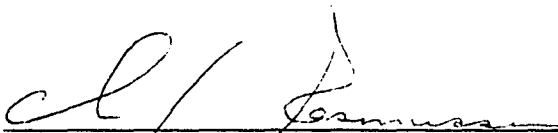
Biology Department
University of Nevada, Las Vegas
December, 1989

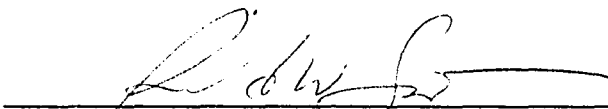
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August, 1989

ABSTRACT

The red spotted toads, (Bufo punctatus), demonstrate a "water absorption response" (WR) in which they adduct the hindlimbs and press the ventral skin to a moist surface. The "seat patch" is a region of skin located on the posterior portion of the abdomen, and is specialized for water uptake. Anterior to this is the pectoral region of skin which is not significantly involved in water absorption. The tendency of toads to demonstrate the WR was increased by subjecting toads to greater levels of dehydration, although toads dehydrated by as little as 0.6% showed the WR. When the water potential of the moist surface was reduced by using urea solutions, there was a progressive decline in the percentage of toads showing WR. No toads showed WR when the water potential of the surface was lower than that of the body fluids. Thus, toads are able to detect substrate water potential. Associated with the WR, toads showed three characteristic behaviors: a) moves, in which the toads would systematically move to adjacent regions of the moist surface to optimize water gain; b) settles, in which a toad would laterally oscillate it's body to maximize contact area when showing the WR; and c) kicks, in which toads would rub adherent particles from the skin with the hind feet. Area-specific rates of water gain declined when the substrate water potential was reduced.

It was determined that the ratio of seat patch area to total surface area was greater for the xeric species, Bufo punctatus and Bufo woodhouseii, compared to the mesic species Bufo marinus and Bufo terrestris. However, there was not an increase in this ratio with increasing body weight.

Measurements of the capacitance of the ventral skin, in vitro, indicate that the seat patch has a greater surface area than that of the pectoral region. Scanning electron micrographs of the pelvic and pectoral skin areas showed no elaboration of the external-facing membranes of cells. Therefore, the increase of surface area indicated by the capacitance values is due to the folding, evaginations, and tubercles of the skin.

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ACKNOWLEDGEMENTS

A Masters thesis is not completed by the efforts of one individual, without the contribution of time, expertise, and friendship of so many people, I could not have fulfilled this goal. I would like to thank my advising professor, Dr. Stanley D. Hillyard, for his endless assistance and enthusiasm which enabled me to complete this project. Also, I want to extend my gratitude to the members of my committee, Dr. Keith Dupre, Dr. Robert Winokur, and Dr. Charles Rasmussen, for their valuable comments and critiques. The faculty and staff of the Biology department have all, in some way, contributed to my success with my graduate studies. They are a unique group of individuals, whom I feel privileged to have been associated with.

I could not have completed my thesis without the support of my friends and family. Specifically Sonia Sutherland who has always had confidence in me and encouraged me to strive for, what seemed to me, the impossible. And most importantly my parents, William and Renate Brekke, for their guidance and instruction which instilled the qualities necessary, to have the courage to try. Obviously one does not achieve anything substantial alone.

INTRODUCTION

Although the integument of amphibians is highly permeable to water, many species are able to successfully exist in desert habitats. Desert species are able to balance high evaporative water losses by selecting microhabitats where water is available and by storing large amounts of water in their urinary bladders (Bentley, 1966). Anuran amphibians do not drink (*ibid.*, 1966). Rather, they absorb water osmotically across their integument. In bufonids, the rate of water absorption is greatest across a specialized region of skin in the posterior-ventral surface of the animal (Baldwin, 1974) which has been termed the "pelvic" region (Marrero and Hillyard, 1985) or the "seat patch" (McClanahan & Baldwin, 1969).

Physiologically, the seat patch region is known to have a greater water permeability than that of the more anterior or "pectoral" region of the ventral skin (McClanahan & Baldwin, 1969; Baldwin, 1974). A number of studies with intact animals and with isolated skin have demonstrated that the water permeability of the seat patch can be further elevated by treatment with antidiuretic hormones; both the amphibian hormone, arginine vasotocin (AVT), or the mammalian hormone, arginine vasopressin (AVP) (Baldwin 1974). In addition, blockers of β -adrenergic receptors reduce the ability of dehydrated toads to rehydrate which suggests that the sympathetic nervous system may regulate water permeability and vascular perfusion of the seat patch as well (Yokota and Hillman, 1984). The pectoral region is much less sensitive to the above agents than is the seat patch (Baldwin, 1974).

Coupled with an elevated water permeability, the seat patch region of bufonids is more highly vascularized than is the pectoral region (Roth 1973, Christiansen 1964). The degree of vascularization observed by Roth (1973) was greatest in the terrestrial anuran, Bufo alvarius. A more extensive network of capillaries in the skin will carry water which has been absorbed from a moist surface into the circulation and reduce the accumulation of water in the subcutaneous spaces under the skin. The sympathetic nervous system may affect water movement by increasing water permeability of the skin and blood flow to the skin.

A third factor which permits anurans, including bufonids, to obtain water from moist surfaces is the accumulation of solutes, especially urea, in their blood and body fluids when they become dehydrated (Katz, 1986a). This reduces the water potential of their body fluids and permits water gain from moist substrates which would not occur if the animal was fully hydrated (Katz, 1986b).

Morphologically, the seat patch is characterized by a network of elevations, thickened tubercles and channels which Lillywhite and Licht (1974) have termed "epidermal sculpturing". Epidermal sculpturing increases the surface area available for water absorption and, thus, augments the factors listed above in promoting water gain by the animal. As indicated above, desert toads utilize microhabitats where water can be obtained. In desert habitats, these microclimates may include moist soil or crevices in rocky washes where free-standing water is only available on a seasonal basis (Wright and Wright, 1949). Absorption of water from these substrates involves the transfer of water, by capillarity, from the soil particle matrix to the surface of the animal (Hillyard, 1976). The ability of epidermal sculpturing to

enhance this transfer will, therefore, increase the capacity of toads to survive in areas where free-standing water is not always available. The gracilis minor muscle makes extensive connections over the inner surface of the seat patch region. These attachments were noted by Noble (1922) who found them to be more extensive in fossorial anuran species although he suggested no function for them. More recently, Winokur and Hillyard (1985) have suggested that the attachments of the gracilis minor, and an unnamed muscle whose origin is near the cloaca, to the interior surface of the seat patch may modify the epidermal sculpturing of the seat patch in a manner which enhances water absorption from moist substrates.

Behaviorally, no function has been documented for the cutaneous attachments of the above muscles. Stille (1958) observed that several species of anurans, including bufonids, which had been dehydrated by 20% of their body weight, showed a characteristic behavior when placed on a wetted glass surface. The animals would spread their hindlimbs and wipe the ventral surface of the skin across the wetted glass in order to maximize the contact area for water absorption. This behavior was termed the "water absorption response". In an earlier study, Stille (1951) observed Bufo woodhouseii, during their daily cycle of foraging and burrowing. He found that toads would emerge from burrows at night and walk to moist sand near a lake shoreline. Examination of the footprints lead to the conclusion that the toads kept their ventral skin elevated above the ground until they encountered the moist sand. He suggested that the animals are able to detect the availability of substrate moisture with their feet.

The present study was initiated to examine the behavioral aspects of water absorption by the toad, Bufo punctatus. This species was selected because it inhabits rocky canyons in desert regions which seasonally have periods of low water availability (Wright and Wright, 1949). They are frequently found foraging in areas well away from water sources. The specific goals of the study were:

1. to determine the degree to which the hydration state of the animal affects the water absorption response.
2. to examine the effect of substrate water potential on the water absorption response; i.e. can toads detect substrate water potential and initiate or terminate behavior accordingly?
3. to examine the water absorption response of toads hydrating from a moistened surface in order to observe any cutaneous movements which could be attributed to the muscle attachments referred to above.

A second phase of the study was designed to determine whether the seat patch area, as a fraction of the total surface area, was different between Bufo punctatus and other bufonid species from xeric and mesic habitats and whether this fraction was affected by body size.

The third phase of the study was initiated to examine the degree to which epidermal sculpturing enhances the surface area for water absorption across the skin of Bufo punctatus. Bufo punctatus has a high rate of water gain across the seat patch with values of $423 \mu\text{l}/\text{cm}^2 \cdot \text{hr}$ being observed by McClanahan and Baldwin (1969). The area-specific rates of water gain, however, do not refer to the actual cell membrane area which can be increased by the extent of epidermal sculpturing. In

order to quantify the cell membrane area, the capacitance of the skin was measured, in vitro, by electrical techniques developed by Gogelein and VanDriessche (1981). Cell membranes can be modeled as equivalent electrical circuits with a resistance which corresponds to the conductive elements, i.e. ionic channels, and a parallel capacitance which corresponds to the lipid bilayer of the epithelial cell membranes. It is generally assumed that 1 cm² of membrane area has a capacitance of 1 μ F (Reeves, 1970). Thus, capacitance values in excess of 1 μ F/cm² reflect the degree to which epidermal sculpturing enhances the absorptive surface of the skin. In order to visualize the epidermal sculpturing, scanning electron micrographs were made on pieces of skin from the seat patch and pectoral region whose capacitance had been determined electrically.

METHODS

Capture and Maintenance of Animals

Red spotted toads, Bufo punctatus, were collected during June and July 1987 from First Creek and Oak Creek in the Spring Mountain range Clark County Nevada (Nevada Department of Wildlife Permit #S10615). The toads were housed in a 20 X 40 cm glass aquarium which was partially filled with soil and one end was elevated at approximately a 15 degree angle. This allowed for pooling of water at one end and relatively dry soil at the other. Rocks, 5-10cm in height, were placed in the aquarium so the animals were able to rest on rocks, moist sand or in water, ad libitum. The toads were fed crickets twice each week. The ambient temperatures ranged from 22 - 24 C. A light cycle was not systematically regulated.

Behavioral Observations

A glass-bottomed aquarium was raised above a angled mirror. The four sides of the aquarium were covered with paper so the test animals could not see the observer. This allowed the observer to see the toad from below and observe the contact of the skin with the substrate during periods when the toads were hydrating. The term, water absorption response (Stille, 1958), was chosen to describe periods when the hind limbs were adducted and the pelvic skin was pressed to a moist substrate. This term will hereafter be abbreviated, WR.

Effects of Dehydration on Hydration Behavior

For the hydration experiments, a 10 X 10cm piece of Kimwipe laboratory tissue was saturated with water and placed on the bottom of the aquarium. Toads, in

varying states of dehydration, were placed on the Kimwipe. Three treatment groups were used to vary the animal's dehydration state.

GROUP 1. Toads having free access to water were taken from the holding aquaria and their urinary bladders were emptied by inserting a polyethylene cannula into the cloaca. The standard weight, hydrated weight with an empty bladder (Ruibal, 1962), was recorded and the toads were placed in a dry aquarium for periods of one to six hours. This resulted in states of dehydration which ranged from 0.6 to 19.6% of the standard weight. For analyzing the behavioral observations, Group 1 animals were divided into two categories: Those dehydrated 4.0 - 19.6% were termed Group 1A and those dehydrated 0.6 - 3.6% were termed Group 1B.

GROUP 2. Since toads given water ad lib. tended to remain out of the water except for brief periods, a second procedure was used to assure that toads were in a fully hydrated state prior to the dehydration period. The toads were placed in an aquarium filled with 1 cm of water for 1-2 hours. The standard weight of these animals was recorded and then they were subject to dehydration as described above. The range of dehydration achieved during this procedure was between 0.5 and 3.3% of the standard weight.

GROUP 3. Toads were hydrated as in group 2 and their standard weight was recorded. Animals from this group were placed directly on the water saturated Kimwipe without experiencing a dehydration period.

Individuals from Groups 1-3 were placed on a Kimwipe and the sequence of postural adjustments and movements was recorded as a function of time. The observation period was terminated when the toad had moved completely off the

Kimwipe. Following the termination of the observation period, the toads were weighed and the change in weight was assumed to result from water gain or loss. The area of the "seat patch" was also measured, to the nearest 0.1 cm², so that water uptake could be expressed as a function of surface area and time ($\mu\text{l}/\text{cm}^2 \cdot \text{hr}$).

For a behavior to occur randomly it would be expected to be observed with frequency of 50%. Chi-squared (X^2) was used as the statistical analysis for the occurrence of the WR, with a significance level of $P < 0.05$. To test the effect of percent dehydration of toads on the occurrence of associated WR behaviors (moves, kicks, and settles), on the area-specific water flux, and on time spent on a moist substrate, a one-way analysis of variance was used to test for statistical significance. Differences between treatment means were analysed using the Duncan multiple range test. Statistical significance was accepted at a level of $P < 0.05$.

Effects of Substrate Water Potential on Hydration Behavior

A second series of experiments was conducted to determine whether toads are able to detect the water potential of a hydration surface. In order to vary water potential, urea solutions having concentrations of 100, 250, and 500 mM, as well as tap water, were used to saturate the Kimwipe on which the toads were placed. Urea was used as the solute since it is found occurring naturally in toad body fluids (Degani, 1983; Katz, 1986a). Toads having water available ad lib were dehydrated, using the methods indicated for Group 1, by 3.6 - 19.6% of their standard weight. Each toad was placed on all four substrates, and the order in which they were exposed to each substrate was randomly varied. The time course of postural changes and movements on the Kimwipes were recorded as above as was the weight change

during the observation period.

The frequency of a random behavior was expected to be 50%, therefore chi-squared (X^2) was the statistical analysis used for the occurrence of the WR on substrates of varied water potentials. The relationship between the frequency of the behaviors associated with the WR (moves, kicks, and settles) and substrate water potential, was statistically tested by linear regression analysis. A one-way analysis of variance was used to test for statistical significance of the effect of substrate water potential on the time spent on a moist substrate and the area-specific water flux. Differences between treatment means were analysed using a Duncan multiple range test. Statistical significance was accepted at a level of $P < 0.05$.

A. Effect of Covering the Feet on Hydration Behavior

The toads for this experiment had their forelimbs and hindlimbs covered by small size finger cots (ABCO Dealers, Inc., Milwaukee, WI.) placed over the limbs and secured with elastic string. The toads were then placed in an aquarium and were dehydrated to 1.2 - 13.3% of the standard weight. Following dehydration they were placed on a Kimwipe saturated with 500 mM urea and observed.

The effect of covered and uncovered feet on the time spent on a substrate saturated with 500 mM urea, was analysed with a one-way analysis of variance. Statistical significance was accepted at a level of $P < 0.05$.

Water Potential of Substrate at Termination of Behavior

In order to determine the substrate water potential at which toads terminated hydration behaviors, the water potential of the surface last in contact with the seat

patch was measured with a thermocouple psychrometer. It was found that filter paper was preferable to Kimwipes for this experiment since discs could be cut and fit in the sample chamber of the instrument (Decagon Devices SC-10A thermocouple psychrometer connected to a NT-3 Nanovoltmeter-Thermometer; Pullman, WA.) A thermocouple psychrometer compares the vapor pressure of a sample in a closed chamber with that of a film of distilled water coating a thermocouple junction in the same chamber. The cooling of the thermocouple, which is proportional to the vapor pressure difference between the sample and pure water, results in a microvolt potential difference between the leads of the thermocouple. The instrument is calibrated by recording the microvolt output of the instrument when filter paper saturated with solutions of known water potential are placed in the chambers. Distilled water and urea solutions having concentrations of 100, 150, 200, 250, and 500 mM were used to establish calibration curves (Fig. 1). The water potential of the sample was calculated by extrapolation from the linear regression fit to the calibration values. With both calibration and sample analysis, a three hour period was observed between insertion of standards and samples and the measurement of thermocouple output. A separate standard curve was determined for each experiment since the temperature varied slightly between analyses.

Morphometric Analysis of Seat Patch Area

To examine the relationship between the relative surface area of the seat patch with regards to habitat and body weight, four species were obtained locally or from commercial suppliers. Two mesic species, the marine toad (Bufo marinus) and a southern toad (Bufo terrestris), and two xeric species, the rocky mountain toad

(Bufo woodhouseii) and the red spotted toad (Bufo punctatus) were studied. Bufo punctatus were housed in a 20 x 40 cm aquarium as described in Part I. The other species were kept in the animal facility at the University of Nevada, Las Vegas in holding tanks 60 x 90cm. The light cycle was regulated on a 12:12 L:D and ambient temperature was 21° C. The animals had free access to water and were fed crickets twice per week.

The total surface area of the toad was calculated by using the formula utilizing the relation of body size and body surface area of bufonids (Hutchison et al. 1968):

$$\text{Surface Area} = 9.27W^{(0.65)} \quad (1)$$

where weight was measured in grams.

The seat patch is similar in shape to that of a trapezoid. Using calipers, the width of the anterior edge of the seat patch was measured to the nearest 0.1 millimeter. The width of the posterior edge, including the inner thigh from knee to knee, was similarly recorded. Then the length of the seat patch was measured, from the anterior edge (mid-ventral portion of abdomen) to the cloaca. Utilizing the equation for the area of a trapezoid, an estimate of the surface area of the seat patch was then calculated with the anterior width being the apex, the posterior width the base and the length as the height. The fractional contribution of the seat patch area to total surface area could thus be determined as a simple ratio: Seat Patch Area/Total Surface Area.

The statistical analysis for the relationship between the relative seat patch surface area with regards to habitat and body weight was tested with an unpaired t-test. Statistical significance was accepted at a level of $P < 0.05$.

Surface Area of Pelvic & Pectoral Skin Regions Estimated from Capacitance Measurements

Toads were anesthetized with tricaine methane sulfonate (MS 222, Crescent Research Chemicals Inc.). The concentration used was 1.0 g/L and the solution was buffered to pH 7.0 with NaHCO_3 . A piece of either pelvic or pectoral skin was dissected from the animal and mounted between the halves of an Ussing - type chamber (De Wolf and Van Driessche, 1985). The outer (apical) and inner (basolateral) surfaces of the skin were bathed with amphibian Ringer's having the following composition: NaCl , 115 mM; KHCO_3 , 2.5 mM; CaCl_2 , 1.0 mM. The chamber halves were constructed in a manner which minimized edge damage (De Wolf and Van Driessche, 1985) and which permitted continuous perfusion of solutions on both sides of the skin.

Once mounted in the chamber, the potential difference across the skin was recorded with a pair of electrodes and this potential input to a low - noise voltage clamp which passed current via a pair of current electrodes (Van Driessche and Lindemann, 1978). When the command voltage of the voltage clamp was 0 mV the current was the short-circuit current which Ussing & Zerahn (1951) have shown to correspond to the net flux of Na^+ across the skin. For capacitance measurements a computer-generated, pseudo-random, voltage signal was input to the command stage of the voltage clamp (Van Driessche, 1986). This resulted in a fluctuating current

which lagged behind the voltage according to the resistance and capacitance of the skin. This can be represented with a Nyquist plot in which the resistive and capacitive components of the skin describe a semi-circle, with each point in the semi-circle representing a specific frequency range (Fig. 2). If it is assumed that the apical membrane of the stratum corneum is the limiting barrier to water and electrolyte flux (Erlj, 1973) and has a much higher resistance than the basolateral membrane, the Nyquist plot can be fit with a computer program which calculates the resistance and capacitance (R_a and C_a) of the apical membrane (Van Driessche, 1986).

Scanning Electron Micrographs of Pelvic and Pectoral Skin Regions

Scanning electron micrographs were done on samples of the pectoral and pelvic skin which were obtained from one specimen of Bufo punctatus. Each skin sample was cut in half, one piece had capacitance measurements recorded while the remaining piece did not and served as a control. It therefore could be determined if differences in SEM of the tissue samples were due to capacitance measuring processes. The skin samples were prepared as follows:

1. A buffered glutaraldehyde solution was made by diluting a 25% stock glutaraldehyde to a 3% solution using a 0.1 M Sorenson phosphate buffer, pH 7.4-8.0.
2. Pelvic and pectoral tissue samples were fixed in the buffered glutaraldehyde solution for 1.5-3.0 hrs.
3. Following fixation, the tissues were washed in three changes of buffer of an hour duration in each.

4. The tissue samples were then dehydrated for 1 hour in each of a series of graded acetone-water solutions (30%--> 50%--> 75%--> 95%--> 100%). The tissues were stored in pure acetone (replaced daily) until further processing was done.
5. The tissues were dried using a Balzers CPD 020 chamber with acetone as the intermediate fluid and CO₂ as the drying agent.
6. The dried tissue was sputter coated in an EMScope SP5000 with a 20 nm layer of gold and palladium.
7. Finally, a coat of silver was applied and the tissues were examined with a Philips 515 scanning electron microscope at the University, California Riverside. Operating voltages ranged from 20.0 - 25.6 kV.

RESULTS

Effects of Hydration State on the Water Absorption Response

Multiple trials were made utilizing seven specimens of Bufo punctatus. Individuals were randomly selected for each trial. In 18 trials 100% of the toads in Group 1A (4.0-19.6% dehydration) demonstrated the WR. This was found to be significantly different from random behavior with the X^2 test, ($X^2=9$, $df=1$, $0.005 < P < 0.001$). Nineteen trials were made with Group 1B animals (0.6 and 2.3% dehydrated). Even at this low level of dehydration 89% of the trials showed the WR when placed on the Kimwipe ($X^2=11.84$, $df=1$, $P < < .001$). For Group 2 animals (0.5 - 3.3% dehydration) the WR was observed in 14 of 24 trials (58% $X^2=.1663$, $df=1$, $0.50 < P < 0.25$). In Group 3 only 1 out of 27 (4%) toads attempted to rehydrate ($X^2=23.14$, $df=1$, $P < < 0.001$). These data are presented in terms of percentage of trials showing the WR in Figure 3.

A. Behavior Associated with the Water Absorption Response

In addition to the WR, several other behaviors were observed and quantified. It should be noted that no consistent behavior was observed which indicated a function for the gracilis minor during WR. Toads were observed to periodically raise their pelvic skin from the substrate, move to an adjacent area of the Kimwipe and re-establish contact between the pelvic skin and the substrate. In this manner, a toad would move over the surface of the Kimwipe. This movement was detected in 100% of the trials in Group 1 and Group 2, and 92% in Group 3. Expressed in terms of the number of moves per minute of time on the Kimwipe, the frequency

was 9.24 ± 1.1 (All values reported are the mean \pm 1 SE). The mean frequency of moves declined for Groups 2 and 3 to values of 5.9 ± 0.78 and 7.0 ± 0.83 respectively, however, these values were not statistically different ($F=1.66$, $df=87$, $P=0.181$)(Fig. 4).

A second behavior termed "kicks" was also observed in all toads showing the water absorption response. Kicks were characterized by a brushing movement of one or both hind feet over the surface of the seat patch. This behavior was observed in all of the trials with Group 1A, and in 17 of 19 Group 1B trials. Group 1A had a significantly greater frequency of kicks than Group 1B (0.5 ± 0.15 and 0.1 ± 0.04 kicks/min, respectively). Kicks were only observed in 3 of 24 trials with Group 2 and the frequency of kicks was 0.05 ± 0.03 /min. No kicks were observed in Group 3. The frequency of "kicks" was significantly greater in toads which were less hydrated ($F=10.92$, $df=87$, $P=0.000$). Group 1A had a significantly different frequency of kicks than Group 1B, 2, and 3 ($P<0.05$). These data are presented in Fig. 5.

A third behavior observed during rehydration periods, was termed "settling". This behavior was characterized by lateral oscillations of the entire animal while the seat patch was in contact with the substrate. It appeared as though the toad was maximizing the amount of surface area of the seat patch in contact with the substrate. The frequency of the settling behavior decreased significantly ($F=6.16$, $df=87$, $P=0.001$) with increased states of hydration. The frequency of "settling" behavior observed for Groups 1A, 1B, and 2 were significantly different than Group 3 ($P<0.05$)(Fig. 6).

B. Time Spent on Hydration Substrate

The time spent on the substrate (including trials for the toads that did not demonstrate the WR, declined with decreasing degrees of dehydration ($F=9.58$, $df=84$, $P=0.000$)(Fig. 7). There was not a significant difference of the mean time spent on substrate between Group 1A and 1B ($P>0.05$). Group 1A had a mean value of $11.690 \pm 1.38/\text{min}$, while Group 1B was $9.607 \pm 1.86/\text{min}$. However, there was a significant difference for time spent on substrate of Group 1 (A and B combined) compared to Group 2 and Group 3 ($P<0.05$).

C. Area-Specific Water Flux

Using the time of a given trial, the weight change during the trial, and surface area measurements of the seat patch, the area-specific rate of water flux was determined (for all animals whether or not they had demonstrated the WR) and presented in Figure 8. The rates of water flux for Group 1A and 1B, (353.8 ± 73.5 and $170.5 \pm 91.6 \mu\text{l}/\text{cm}^2 * \text{hr}$, respectively), were not significantly different from each other ($P>0.05$). The mean value for Group 1 (A and B combined) was not significantly different from Group 2 ($303 \pm 196 \mu\text{l}/\text{cm}^2 * \text{hr}$; $P>0.05$). The water flux in toads of Group 3 was negative ($-150.1 \pm 198.8 \mu\text{l}/\text{cm}^2 * \text{hr}$) however, when compared to Group 1(A and B combined) the difference was not significant ($P>0.05$) due to the large variation observed between trials. There was not a significant effect between the dehydration state of the toad and the area-specific water flux ($F=1.94$, $df=83$, $P=0.130$).

Effect of Substrate Water Potential on the Water Absorption Response

There were 16 trials utilizing 7 toads. In each trial a dehydrated toad was placed randomly on tissue saturated with either water or one of the urea solutions. The range of dehydrations utilized in these trials was 0.6 to 19.6% of the standard weight. When placed on tap water, toads demonstrated the WR in 100% of the trials ($X^2=8$, $df=1$, $0.005 < P < 0.001$). On the 100 mM urea substrate the WR was observed in 93.8 % of the trials (15 out of 16 trials $X^2=12.25$, $df=1$, $P < < .001$). The percent of trials in which the WR was observed decreased to 31.3% with the 250 mM urea and was not significantly different from random ($X^2=2.25$, $df=1$, $.25 < P < .10$). None of the toads attempted to rehydrate on the 500 mM urea solution. There was an effect of substrate water potential on the occurrence of the WR ($X^2=16.0$, $df=1$, $P < < .001$) (Fig. 9).

A. Behavioral Observations on Urea Solutions

The frequency of "moves" increased significantly ($F=39.85$, $df=65$, $P=0.000$) with increasing urea concentration (i.e. decreased water potential) of the substrate (moves/min = $0.023[\text{mM urea}] + 8.44$, $r=0.62$). Although the time spent on the substrates saturated with higher concentrations of urea was short, the frequency of movement was high (Fig. 10). Specifically, toads placed on tap water showed a frequency of 9.44 ± 1.1 moves/min. The toads placed on 100 mM urea saturated substrate showed a frequency of 8.8 ± 0.7 moves/min, increasing to 14.8 ± 1.9 moves/min on 250 mM urea and 22.2 ± 2.9 moves/min on 500 mM urea.

The relationship between time on the Kimwipe and the frequency of kicks and of settles showed negative correlation (Fig. 11 & Fig. 12). The frequency of kicks

were significantly related to concentration ($F=7.96$, $df=65$, $P=0.006$), and is described by the equation; $\text{Kicks/min} = -9.17 \times 10^{-4} [\text{urea}] + 0.49$ ($r=0.33$). The relationship between the frequency of settles and urea concentration, was also significant ($F=24.22$, $df=65$, $P=0.000$), is described by the equation: $\text{Settles/min} = -1.43 \times 10^{-3} [\text{urea}] + 0.645$ ($r=0.52$).

B. Time Spent on Hydration Substrate

The total time on the Kimwipe steadily decreased ($F=27.52$, $df=63$, $P=0.000$), as indicated in Figure 13, from 11.69 ± 1.38 min on tap water to 9.61 ± 1.86 , 4.95 ± 1.18 , to finally 2.51 ± 0.83 minutes when the urea concentration was increased to 100, 250, and 500 mM, respectively. The time on the Kimwipe was significantly lower with 100, 250, and 500 mM urea-saturated substrates when compared to duration of rehydration on tap water saturated substrates ($P<0.05$).

C. Hydration Behavior with Feet Covered

The total time spent on a 500 mM urea saturated substrate was found to be significantly longer for toads with covered feet compared to toads in the above experiments, whose feet were able to directly contact the substrate ($F=12.15$, $df=35$, $P=0.001$)(Fig. 14). There were 19 trials and only one toad, having the feet covered, demonstrated WR three times. Once the seat patch made contact, however, the toad immediately left the substrate. There was no attempt to perform the WR in the 16 toads not having their feet covered.

D. Area-Specific Water Flux

The area-specific water flux across the skin of toads hydrating from tap water

($267.6 \pm 59.4 \mu\text{l}/\text{cm}^2 \cdot \text{hr}$) was not significantly different from Group 1 of the previous experiment ($t=1.56$, $df=87$, $P=0.13$). The effect of increased substrate urea concentration did not influence the area-specific water flux ($F=1.46$, $df=63$, $P=0.236$). Area-specific rates of water flux were (in $\mu\text{l}/\text{cm}^2 \cdot \text{hr}$) 30.43 ± 76.39 with 100 mM urea, 57.8 ± 110 at 250 mM urea and to -359.2 ± 434.6 at 500 mM urea (Fig. 15).

Water Potential at Termination of Hydration

The mean value for the water potential of the saturated filter paper at the termination of the WR was equivalent to a urea concentration of 64.0 ± 36.4 mM (17 trials). This value can be equated to a water potential of 1.43 ± 0.82 atmospheres (Table 1), using the Van't Hoff eq:

$$\text{Water potential (Atm)} = CRT \quad (2)$$

where C = Molar Concentration, $R = 0.0826 \text{ L} \cdot \text{Atm}/\text{mole} \cdot ^\circ\text{K}$ and $T = ^\circ\text{K}$.

Morphometric Analysis of Seat Patch Area

The fraction of seat patch of the total surface area was not significantly different between the two xeric species, Bufo punctatus and Bufo woodhouseii ($t=0.60$, $df=16$, $P=0.56$). There was no significant difference of fractional seat patch area between the mesic species, Bufo marinus and Bufo terrestris ($t=0.11$, $df=9$, $P=0.92$). However, there was a significantly greater fraction of total surface area occupied by the seat patch for the xeric species (Bufo punctatus and Bufo woodhouseii,

combined), compared to the mesic species (Bufo terrestris and Bufo marinus, combined) ($t=3.69$, $df=33$, $P=0.0008$) (Fig. 16). There was not a significant effect between seat patch surface area and body weight ($F=1.78$, $df=35$, $P=0.191$)

Surface Area of Pelvic & Pectoral Skin Regions Estimated from Capacitance Measurements

Capacitance values for pectoral skin from two toads were 1.35 and 1.07 $\mu\text{F}/\text{cm}^2$ while the capacitance values for the pelvic skin were 3.71 and 1.49 $\mu\text{F}/\text{cm}^2$ (Table 2).

Scanning Electron Micrographs of Pelvic and Pectoral Skin Regions

During the dehydration of the tissue samples for the fixation process, the tissues were pinned to styrofoam which partially dissolved in the 95% acetone solution. When the styrofoam dissolved, this decreased the tension on the tissue so that the skin sample became folded and curled. The samples attached to the styrofoam were placed in a 95% acetone bath in a larger container, so that they were more easily manipulated. The samples were then removed from the styrofoam and tension was re-established at the corners of the tissue in an attempt to flatten it out. Upon examining the SEM, it was evident that the tissue samples were excessively dried as a result of the dehydration process. The cell membranes of individual cells were pulled away from adjacent cells, while the edges were curled. However, several samples were not severely affected and were adequate for the investigation. The surface apical membranes at 600-1200X magnification, showed no obvious elaboration of apical membrane surface, in either the pelvic or pectoral skin (Fig

17a,b). At lower magnification, however, the pattern of epidermal sculpturing, which is evident upon visual examination of the skin, can be seen (Fig 18a,b).

DISCUSSION

Factors Affecting the Occurrence of the Water Absorption Response

Relative to the levels of dehydration which bufonids are able to tolerate, the WR is seen at dehydrations far below those which would be considered stressful. Bufonids are typically able to withstand dehydration levels of 30% or greater (Bentley, 1971). On the other hand, the toads are able to discriminate between substrates which contain osmotically available water from those in which water gain is not possible. It is probable that this ability can also be used to avoid attempts of the WR from substrates in the toad's natural habitat which lack sufficient water. For a desert toad, such as Bufo punctatus which occupies habitats where water is available only sporadically (Wright and Wright, 1949), the exploitation of substrate moisture when available, even at low levels of dehydration, allow toads to maintain a higher state of hydration. The greater the degree of hydration, the longer the toads will be able to subsequently forage in areas where water is not available. This is consistent with the hypothesis set forth by Bentley (1966) that terrestrial amphibians which exploit arid environments benefit by maximizing the amount of time spent away from a source of water.

Behavior Associated with the Water Absorption Response

Toads which demonstrated the WR, systematically moved to different areas over the surface of the moistened Kimwipes, extracting available water. This behavior could increase the amount of water absorbed, since water absorbed from a given area of a moist substrate will decrease its water content thus the water potential

gradient favoring water gain. Hillyard (1975) showed that water movement from moist soil, across isolated toad skin, was greatest during the period immediately following the initial contact of the skin with the soil.

When substrate moisture is not available, which was the case in the experiments when hyperosmotic urea was used to saturate the Kimwipes, the frequency of movements increased significantly which is consistent with the toads' searching for favorable substrates from which to obtain water. Putnam & Hillman (1977) have also shown that amphibians which are dehydrated show a higher degree of movement when a source of water is not available.

Kicks and settles occurred with a lower frequency in groups of animals less likely to show the WR, or when the substrate water potential was reduced. The correlation was particularly evident in the experiments with progressively more concentrated urea solutions. The observations of kicks indicated that this activity was initiated to remove adherent matter from the seat patch region. This could be sand grains which were retained from the maintenance terrarium by the sculpturing of the skin or fibers of the tissue paper which adhered to the skin during the WR. The absorption of substrate moisture requires transfer of water by capillarity to epidermal sculpturing (Lillywhite and Licht, 1974) on the skin surface. Particles from the substrate which interfere with this transfer will act as a barrier to absorption. Kicking behavior would remove adherent material and thus facilitate substrate water absorption.

The settling behavior appeared to increase the area of skin in contact with the substrate. This behavioral pattern was also observed by Seymour (1972) in juvenile

Bufo debilis which were sitting on moist sand in their natural habitat. It was suggested that this behavior was thermoregulatory but that it could also enhance substrate moisture absorption. Given the critical nature of the layer of substrate moisture immediately adjacent to the skin (Hillyard, 1975), such movement could increase water uptake. This is supported by the observation that the toads showing the greatest rates of area-specific water gain also showed the highest frequency of settling behavior.

Time Spent on Hydration Substrates

The amount of time which the toads spent on a moist substrate was greatest in toads which exhibited the highest tendency to exhibit the WR. The termination of hydration behavior, however, occurred before the toads could regain the water that had been lost during the dehydration period. Thus, the water potential of the substrate became limiting to further reabsorption. The water potential of the substrate at the termination of WR was found to be 1.433 atmospheres. This value is similar to the water potential of soils which are limiting to water gain by both anuran and urodele amphibians (Spight, 1967; Walker and Whitford, 1969). Bufo punctatus, thus appears to be capable of detecting levels of substrate water potential which become limiting to water gain from moist substrates as water is removed from those substrates.

The location of sensory neurons which are able to detect substrate water potentials are not known. That toads with their feet covered required a significantly longer amount of time to move away from an osmotically unfavorable substrate, strongly suggests that the feet are a site where substrate moisture can be detected.

Stille (1951) suggested this possibility after observing that Bufo woodhouseii, walking from burrows in drier soil to the moist sand at a lakeshore, did not contact the soil with their abdomen until they reached wet sand.

More recently Reboreda, et al. (1984) found that Bufo arenarum when placed in hyperosmotic saline solutions, show a rapid decrease in urine production before any detectable change in plasma osmolality could be observed. These authors suggested that peripheral (sensory) neurons can detect the water potential surrounding the animal. β -adrenergic antagonists prevented this response suggesting that Beta-adrenergic neurotransmitters are involved in pathways which either carry information regarding substrate water potential to the animal's central nervous system or in the efferent pathways which regulate urine production.

Area-Specific Water Flux

Physiologically, the rates of water absorption from tap water-saturated Kimwipes were within the range of area-specific water absorption values which McClanahan and Baldwin (1968) observed across the skin of Bufo punctatus which were placed in contact with free water. Packer (1963) has similarly shown that the desert anuran, Heleioporus eyrei, is able to absorb substrate moisture at rates comparable to the rates observed from standing water. Area specific rates of water uptake were comparable between toads in Groups 1 & 2 as would be expected since the osmotic gradient between the substrate and the toad was similar.

Morphometric Analysis of the Seat Patch Area

The two mesic species studied had comparably smaller seat patch/total surface area ratio despite a substantial difference in body size. As indicated previously, Bentley (1966) suggested that arid-adapted amphibians would benefit from higher rates of rehydration. However, other factors could influence rates of water uptake by the seat patch. These include the water permeability of the skin, the vascularity of the skin, and the extent to which epidermal sculpturing (Lillywhite & Licht, 1974) enhances the actual cellular surface area of a given cross sectional area of skin. This latter factor was investigated by determining the capacitance of the pelvic versus the pectoral skin regions. The pectoral skin had capacitance values near $1 \mu\text{F}/\text{cm}^2$, this value is consistent with the surface area of a planar phospholipid bilayer (Reeves 1970). The capacitance values of the pelvic skin, on the other hand, indicated a 1.5 to 3.7 fold increase in surface area. Scanning electron micrographs of the pelvic and pectoral regions show no obvious elaboration of membrane area in the outer facing cell layer of the skin. Thus, the elaboration of surface area appears to result primarily from epidermal sculpturing.

With regards to cutaneous attachments of the gracilis minor muscle, no obvious movements of the skin were observed during the WR. However, when the animals were not displaying the WR, the surface area of the seat patch skin was reduced by drawing the seat patch skin towards the posterior margin of the abdominal surface. This position of the skin would reduce the area available for evaporative water loss when water is not available and keep the skin elevated above the substrate. The orientation of muscle fibers reported by Winokur and Hillyard (1985) would produce

this observed reduction of surface area.

Bufo punctatus are opportunistic rehydraters, demonstrating WR at low levels of dehydration which are not considered stressful. The associated behaviors of WR; moves, kicks, and settles, facilitate the efficiency of water uptake from a moist substrate. Furthermore, Bufo punctatus are able to detect substrate water potential, and only demonstrate the WR on substrate where water is osmotically available to them. Although the mechanism is not known, it is postulated that sensory input may be received from the feet since this is the only part of the toad in contact with the substrate prior to showing the WR. This is supported by the fact that it takes a toad substantially longer to move away from an unfavorable substrate when it's feet are covered.

The water fluxes determined for Bufo punctatus rehydrating from a moist substrate were comparable to values obtained for rehydrating from standing water. This indicates that the red spotted toad is as efficient at obtaining water from a moist substrate as from standing water, which is a significant advantage in habitats where free water is limited.

This investigation determined that for the two xeric species, Bufo punctatus and Bufo woodhouseii, the fractional contribution of seat patch area of the total surface area was greater when compared to the mesic species Bufo marinus and Bufo terrestris. However the ratio of seat patch area to total surface area was not significantly affected by body size. Specifically for Bufo punctatus, the seat patch area was increased 1.5-3.7 times by epidermal sculpturing indicating a larger area for water uptake therefore enhancing area-specific water flux rates. The muscle

associated with the seat patch, the gracilis minor, was not found to be responsible for skin movement during the WR. However, muscle movements resulting in the bunching up of the seat patch may reduce the amount of skin surface exposed to the environment, thereby reducing evaporative water losses which are substantial in desert habitats.

Observations made in this study document a suite of behavioral and morphological characteristics that assist Bufo punctatus thriving in a hostile desert environment.

Table 1. The water potential of the substrate at termination of the water absorption response, by the desert toad Bufo punctatus, in equivalent urea concentration and atmospheres. (N = 17 trials)

Table 1

Water Potential of Substrate at Termination
of Hydration Behavior

<u>Mean</u>	<u>Standard Error</u>
63.99	36.38 mOsm/kg
1.43	0.82 -ATM

Table 2. The capacitance ($\mu\text{F}/\text{cm}^2$) of the pelvic and pectoral skin from two specimens of Bufo punctatus.

Table 2

<u>Toad #1</u>	<u>Capacitance</u>
Pelvic	3.71
Pectoral	1.35
<u>Toad #2</u>	
Pelvic	1.49
Pectoral	1.07

Figure 1. A standard curve of water potentials as mV readings, generated from data collected with the thermocouple psychrometer using samples of known urea concentrations.

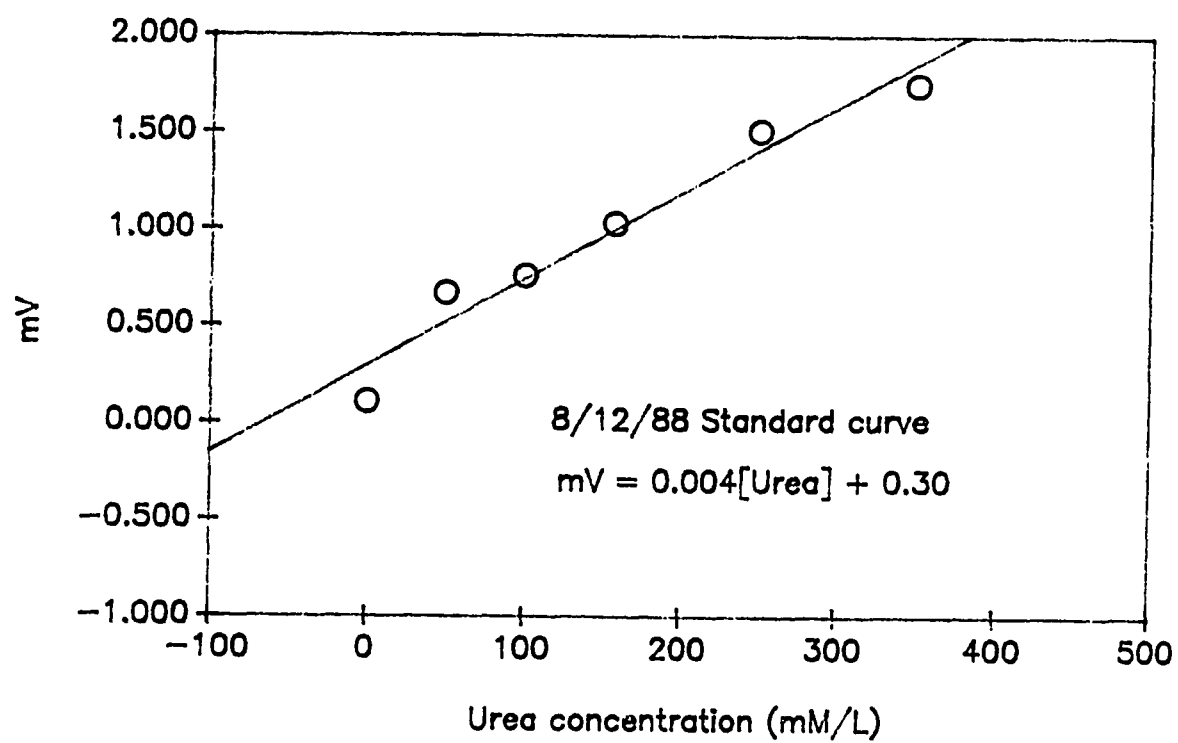


Fig. 1

Figure 2. A Nyquist plot obtained with skin from the pelvic region of Bufo punctatus. The larger semi-circle represents the apical membrane and was used to calculate capacitance. The curve to the right represents the basolateral membrane and was not fit.

Bufo punctatus

Pelvic Skin

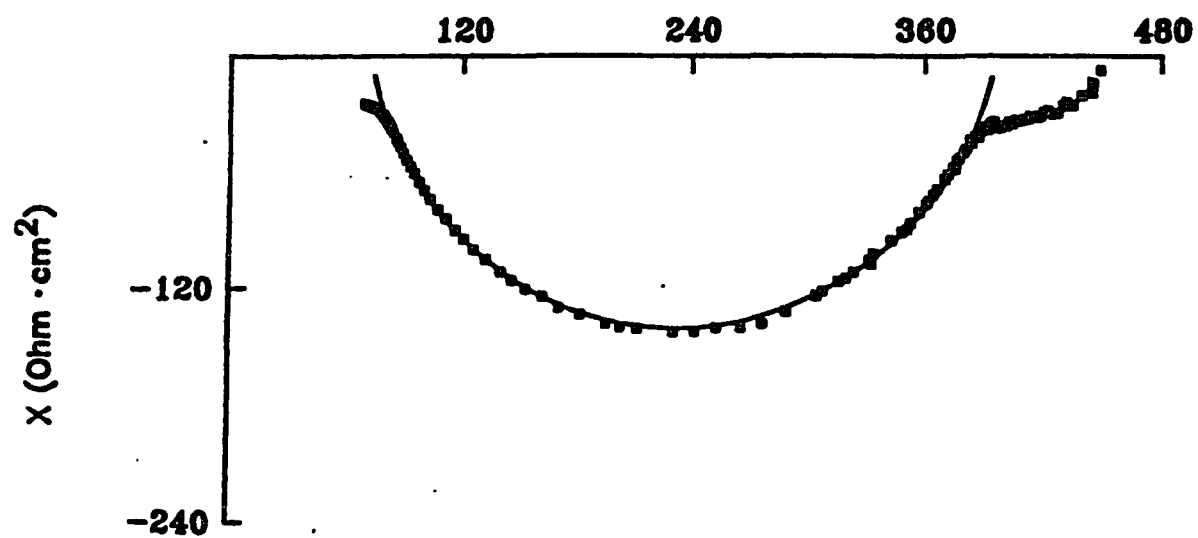
Resistance ($\text{Ohm} \cdot \text{cm}^2$)

Fig. 2

Figure 3. The percent of toads, Bufo punctatus, at different dehydration levels, showing the water absorption response. N for Group 1A (4.0-19.6%) is 18; Group 1B (0.6-3.6%), 19; Group 2 (0.5-3.3%), 24; and for Group 3, 27.

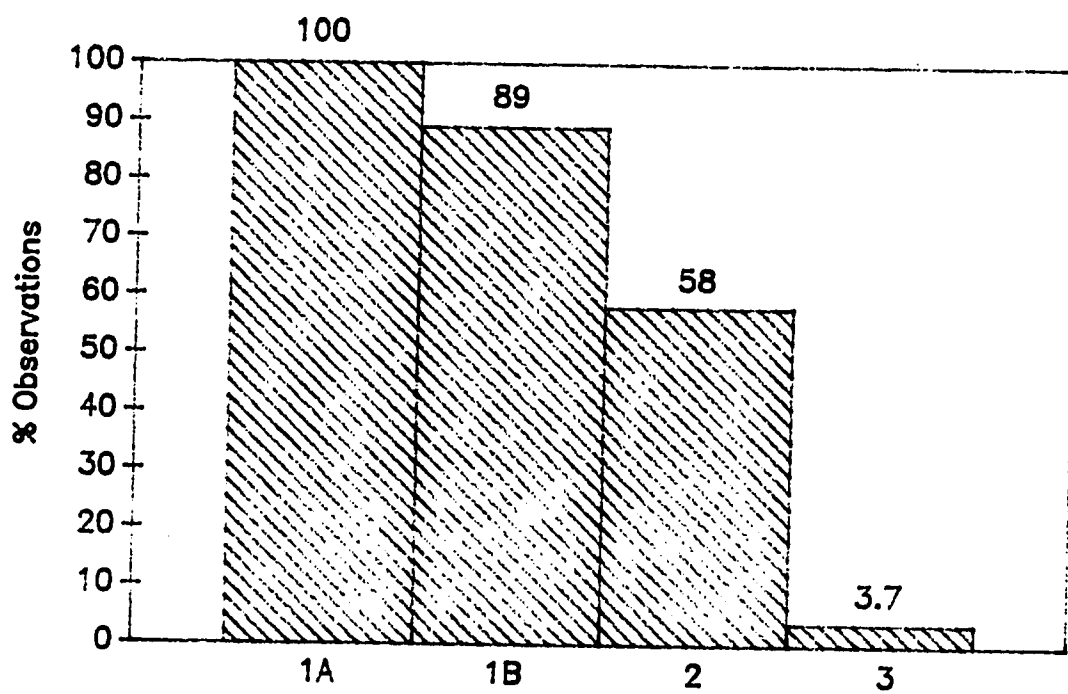


Fig. 3

Figure 4. The frequency of moves on water saturated substrates by Bufo punctatus at different dehydration levels. In all figures the histogram represents the mean and the vertical bar the standard error of the mean. N for Group 1A (4.0%-19.6%) is 18; Group 1B (0.6-3.6%), 19; Group 2 (0.5-3.3%), 24; Group 3, 27.

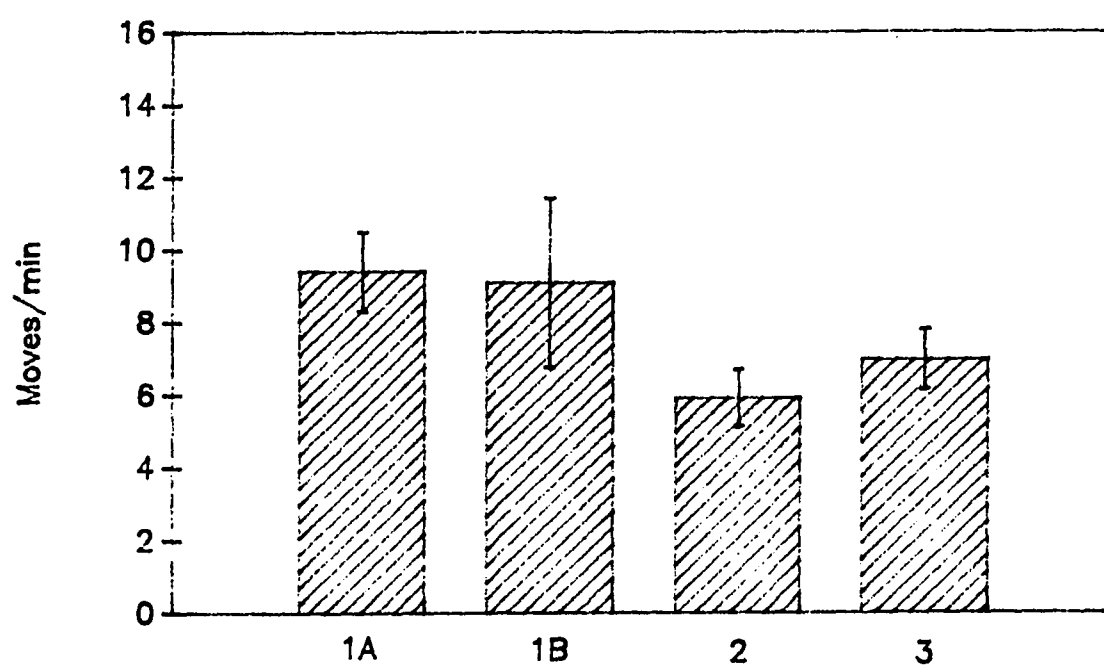


Fig. 4

Figure 5. The frequency of kicks on water saturated substrates by Bufo punctatus of different dehydration levels. The histogram represents the mean and the vertical bar the standard error of the mean. N for Group 1A (4.0-19.6%) is 18; Group 1B (0.6-3.6%), 19; Group 2 (0.5-3.3%), 24; Group 3, 27.

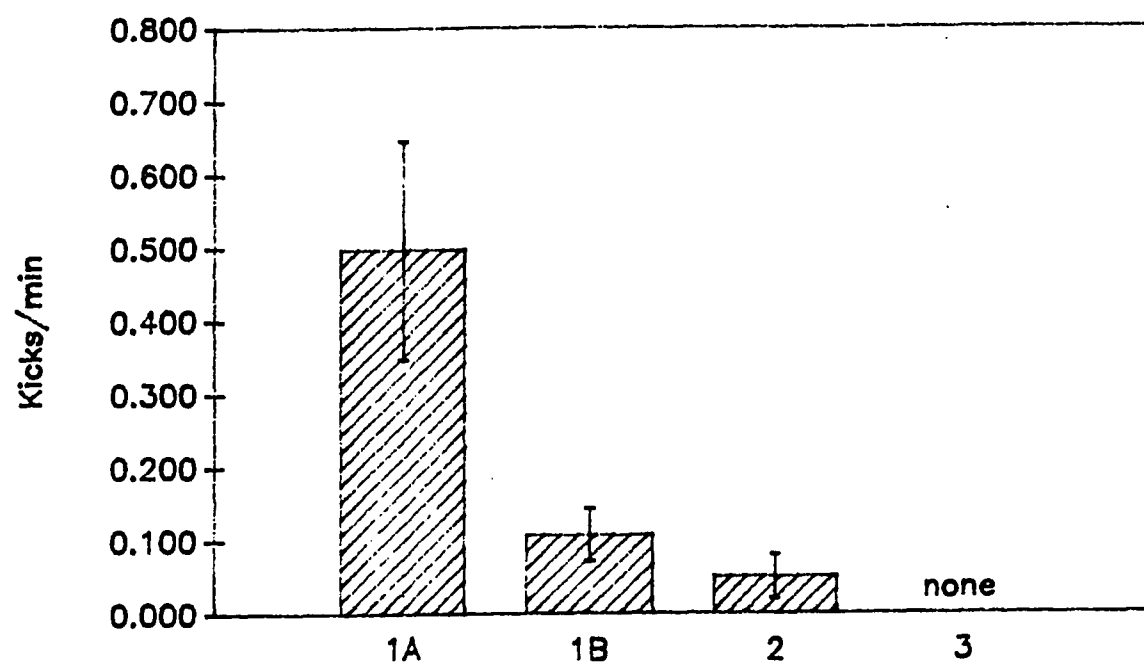


Fig. 5

Figure 6. The frequency of settles on water saturated substrates by Bufo punctatus of different dehydration levels. N for Group 1A (4.0-19.6%) is 18; Group 1B (0.6-3.6%), 19; Group 2 (0.5-3.3%), 24; Group 3, 27. Histograms represent the mean and the vertical bars the standard error of the mean.

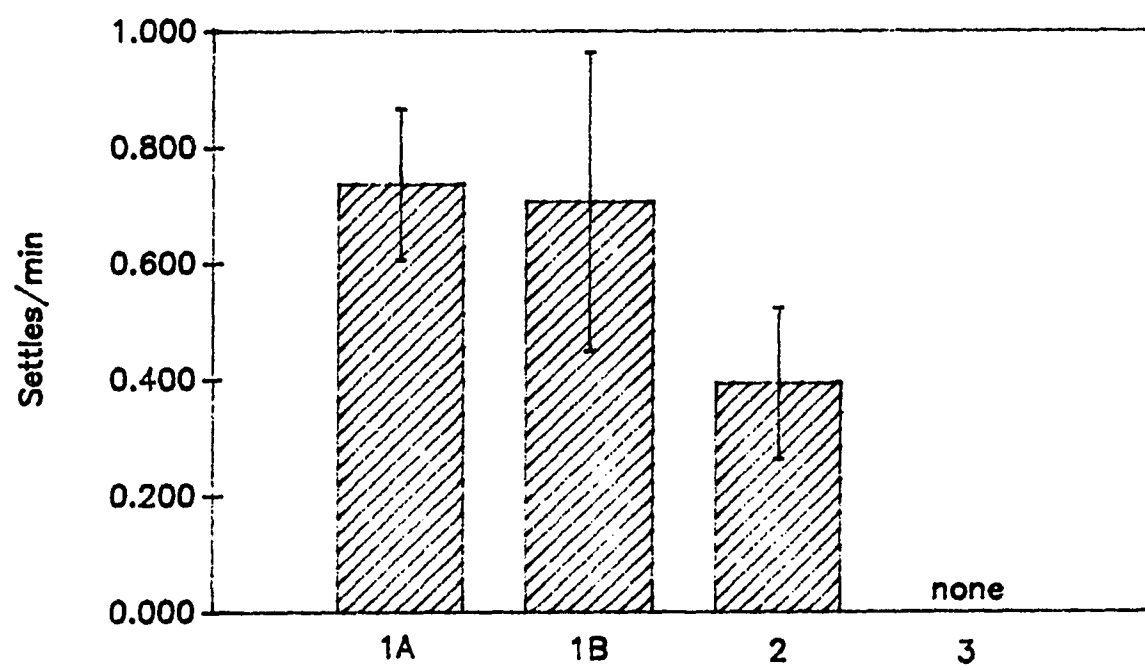


Fig. 6

Figure 7. The time spent on tap water saturated substrate by Bufo punctatus at different dehydration levels. N for Group 1A is 18; Group 1B, 19; Group 2, 24; Group 3, 27. Histograms represent the mean and the vertical bars the standard error of the mean.

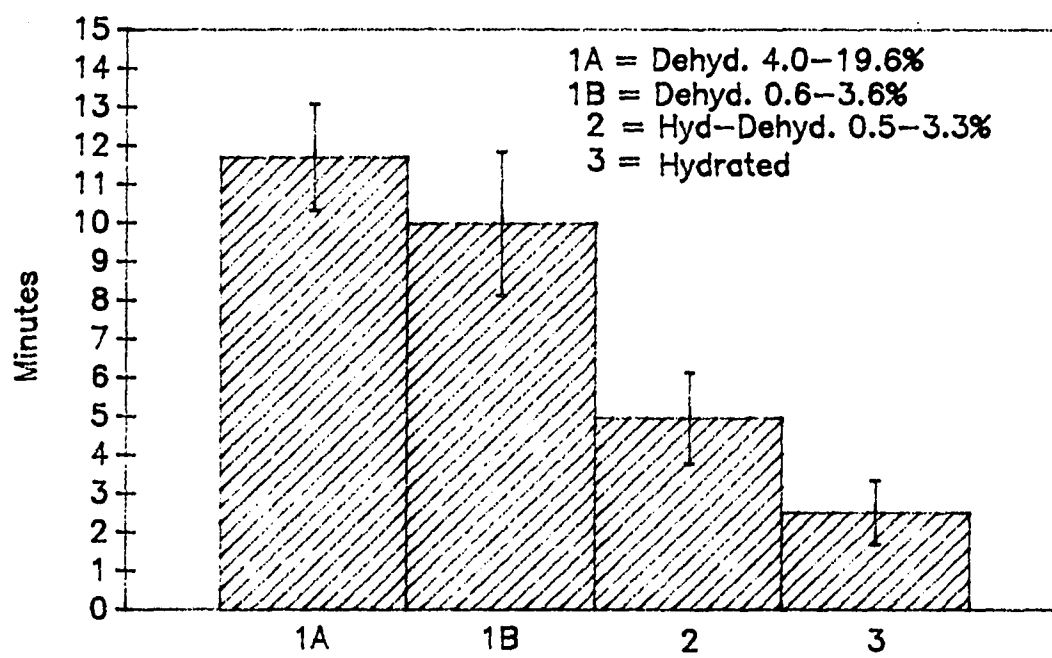


Fig. 7.

Figure 8. The area-specific water flux for Bufo punctatus at various dehydration levels rehydrating from tap water saturated substrates. N for Group 1A is 18; Group 1B, 19; Group 2, 24; Group 3, 27. Histograms represent the mean and the vertical bar the standard error of the mean.

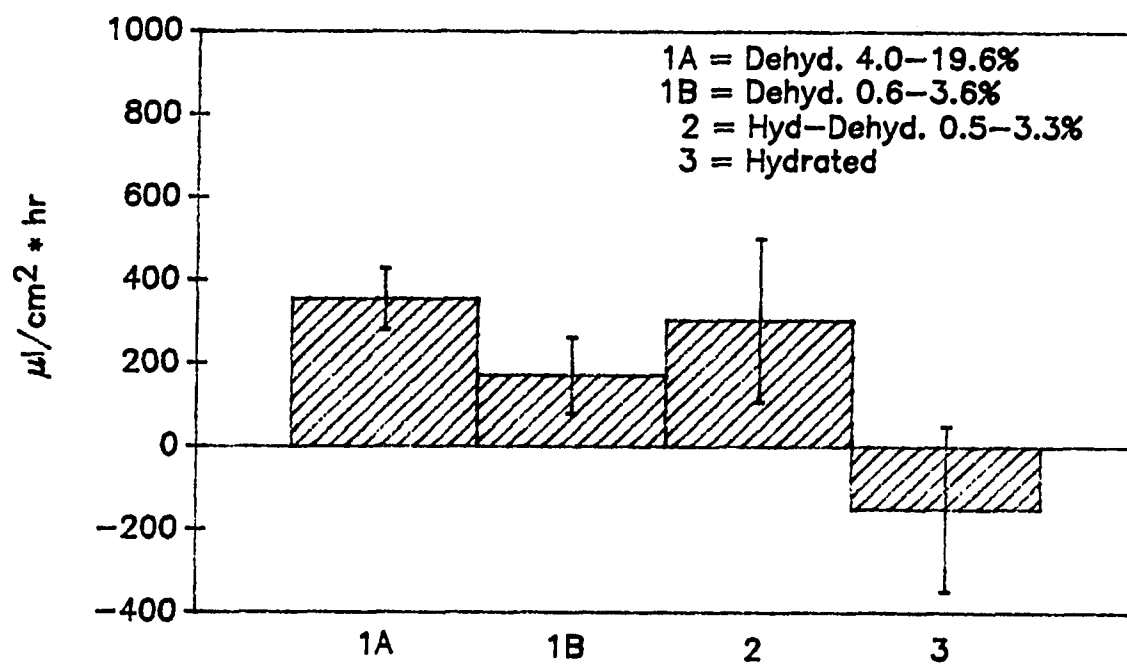


Fig. 8

Figure 9. The percent of toads, Bufo punctatus, showing the water absorption response on substrates of varied water potentials. N for tap water is 37: N for 100 mM, 250 mM, and 500 mM urea is 16.

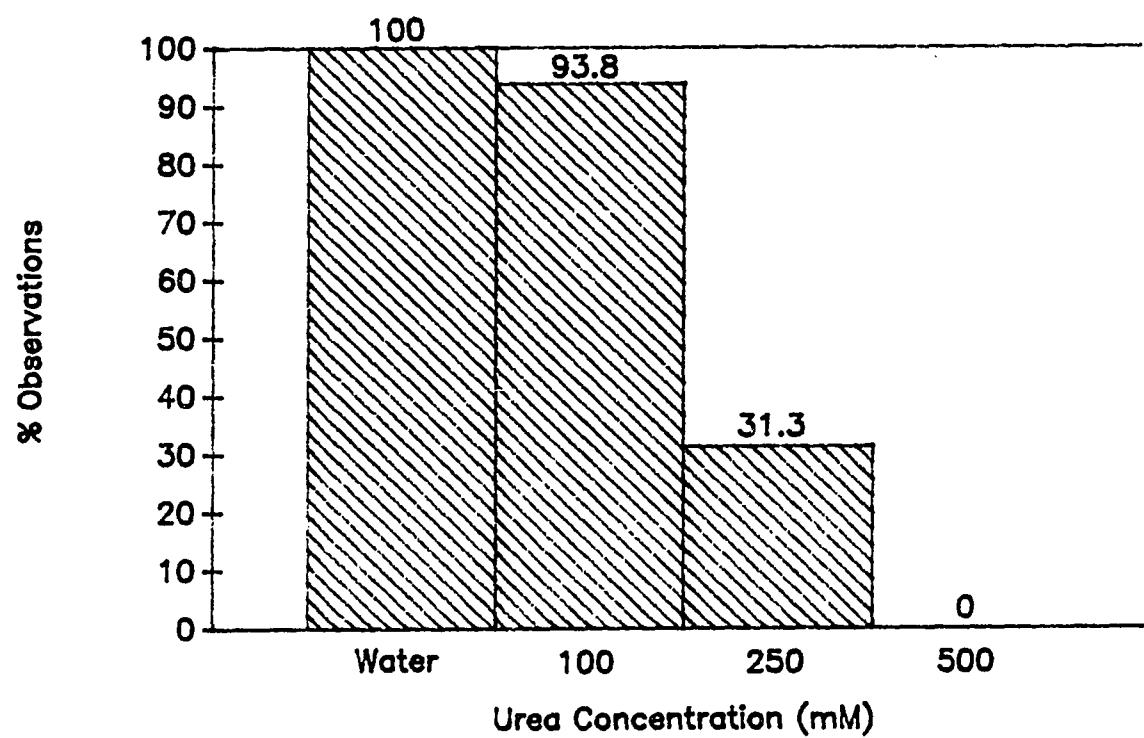


Fig. 9

Figure 10. The frequency of moves by dehydrated toads, Bufo punctatus, on substrates of varied water potentials. N for tap water is 18; N for 100 mM, 250 mM, and 500 mM urea is 16. Symbols represent the mean and the vertical bar the standard error of the mean.

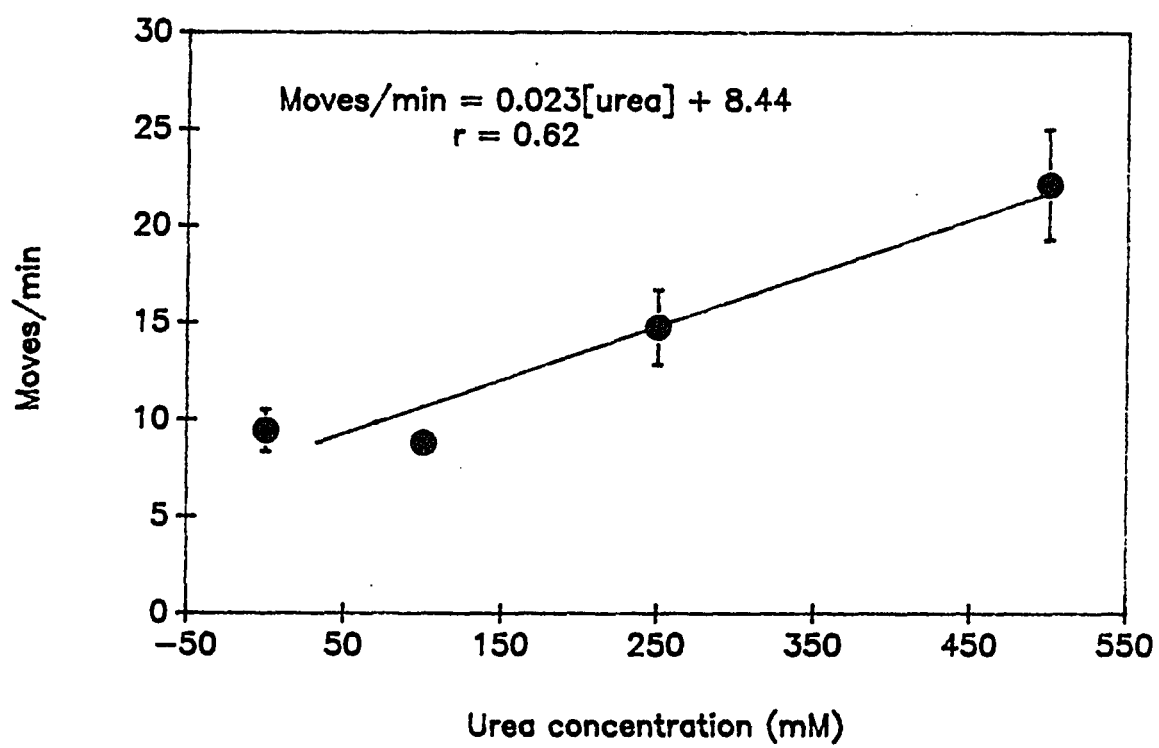


Fig. 10

Figure 11. The frequency of kicks by dehydrated toads, Bufo punctatus, on substrates of varied water potentials. N for tap water is 18; N for 100 mM, 250 mM, and 500 mM urea is 16. Symbols represent the mean and the vertical bar the standard error of the mean.

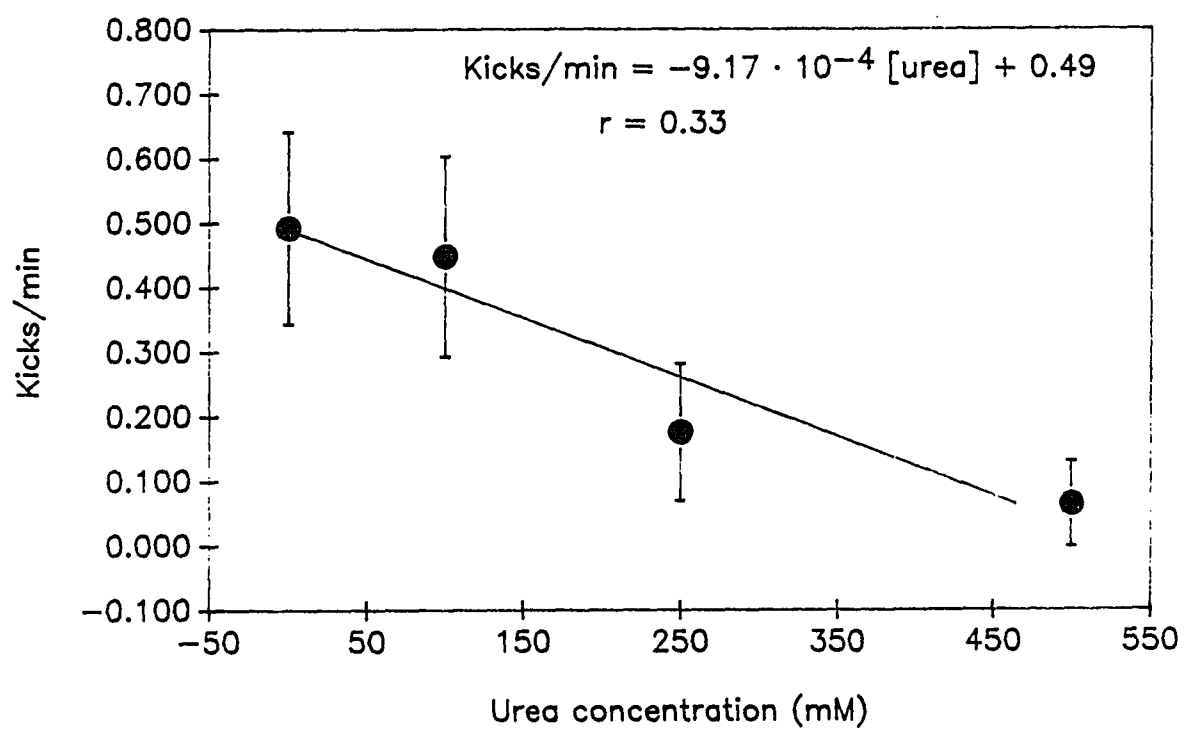


Fig. 11

Figure 12. The frequency of settles by dehydrated toads, Bufo punctatus, on substrates of varied water potentials. N for tap water is 18; N for 100 mM, 250 mM, and 500 mM urea is 16. Symbols represent the mean and the vertical bar the standard error of the mean.

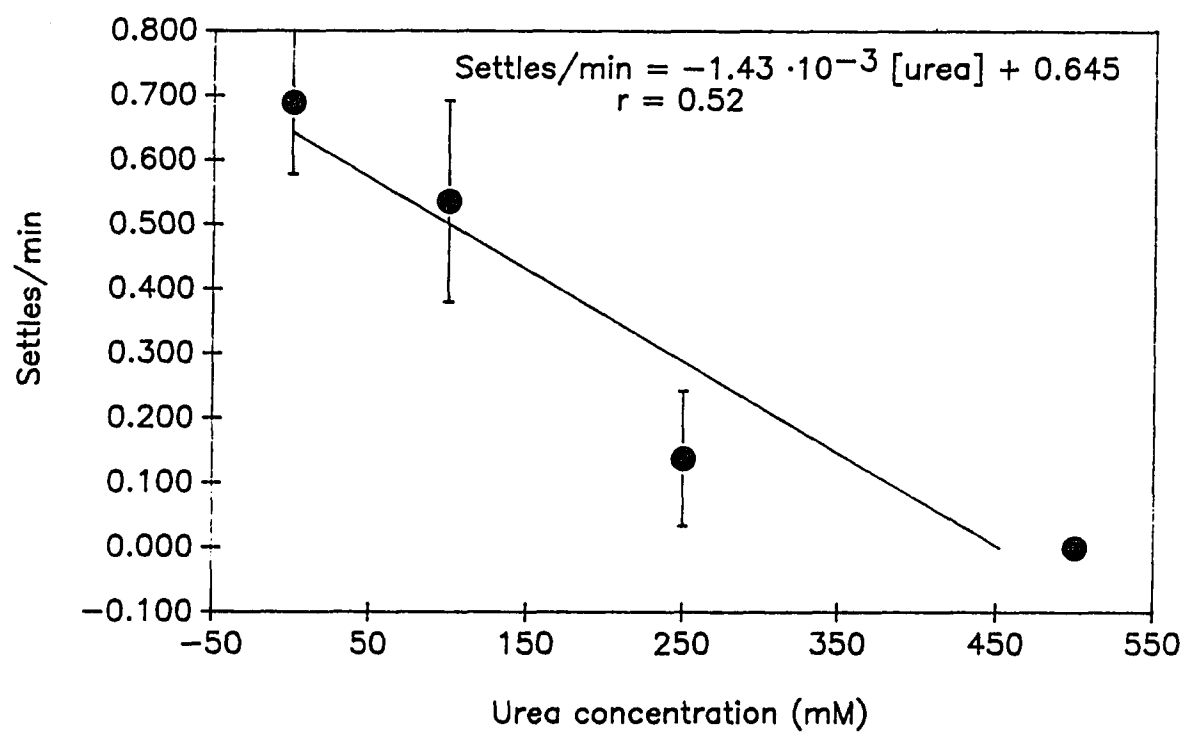


Fig. 12

Figure 13. The time spent on moist surfaces of varied water potentials by dehydrated red spotted toads. N for tap water is 37; N for 100 mM, 250 mM, and 500 mM urea is 16. Histograms represent the mean and the vertical bar the standard error of the mean.

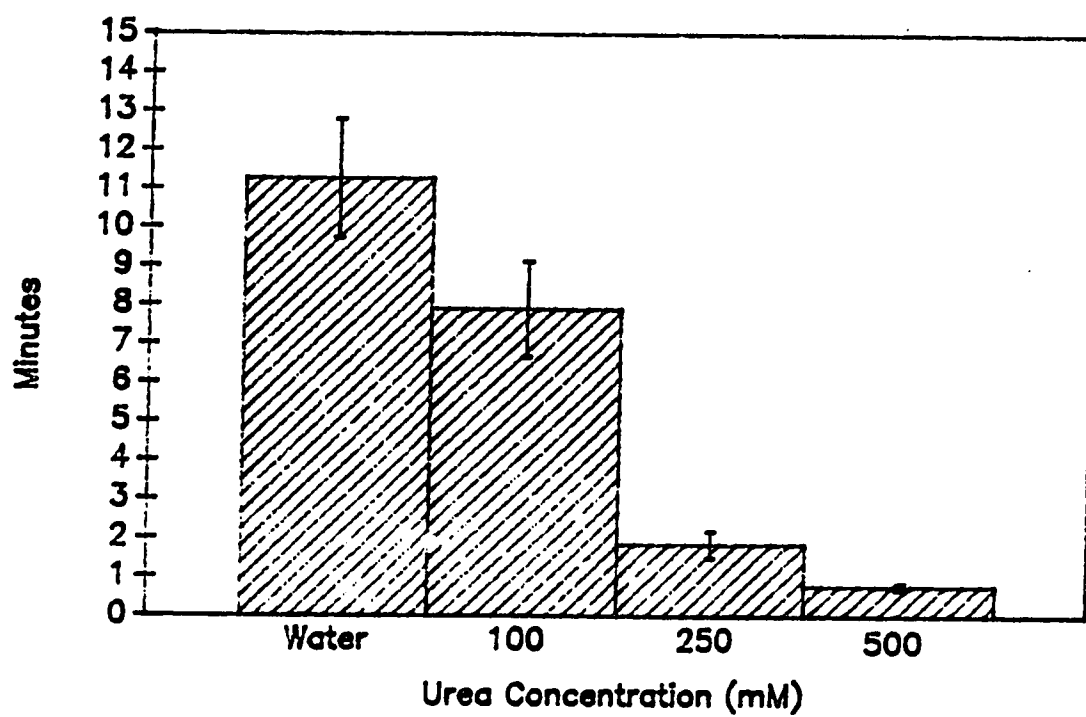


Fig. 13

Figure 14. The total time spent on moist surfaces saturated with 500 mM urea by dehydrated red spotted toads with feet uncovered and covered. N for uncovered feet is 16; for covered feet is 19. Histograms represent the mean and the vertical bar the standard error of the mean.

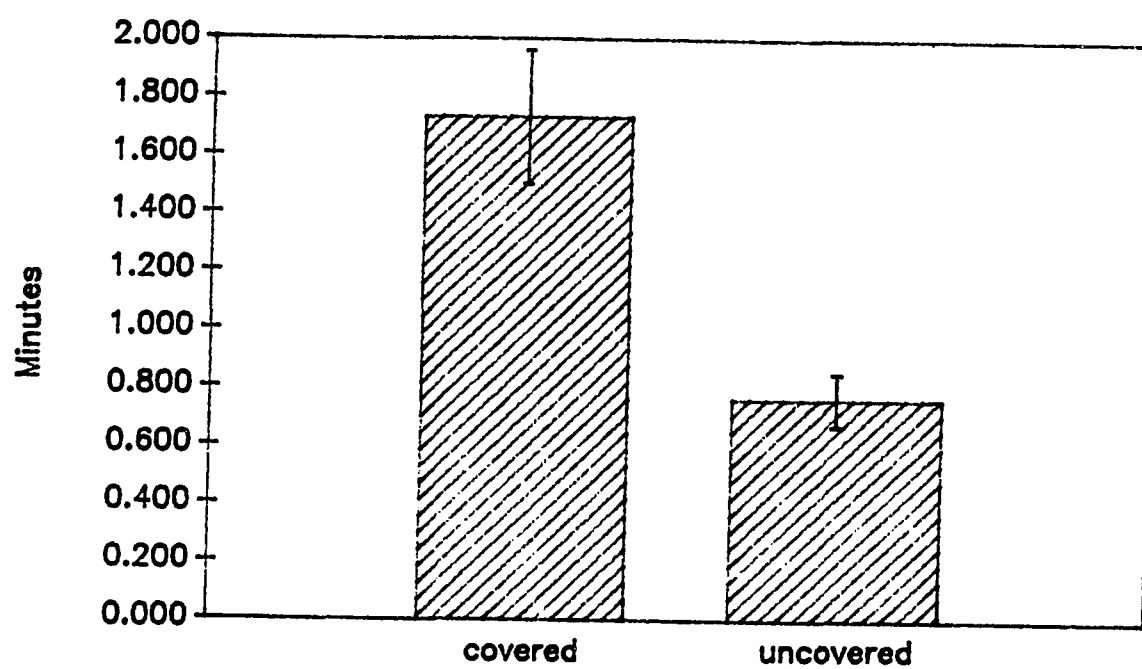


Fig. 14

Figure 15. The area-specific water flux for dehydrated red spotted toads on substrates of varied water potentials. N for tap water, 100 mM, 250 mM, and 500 mM urea is 16. Histograms represent the mean and the vertical bar the standard error of the mean.

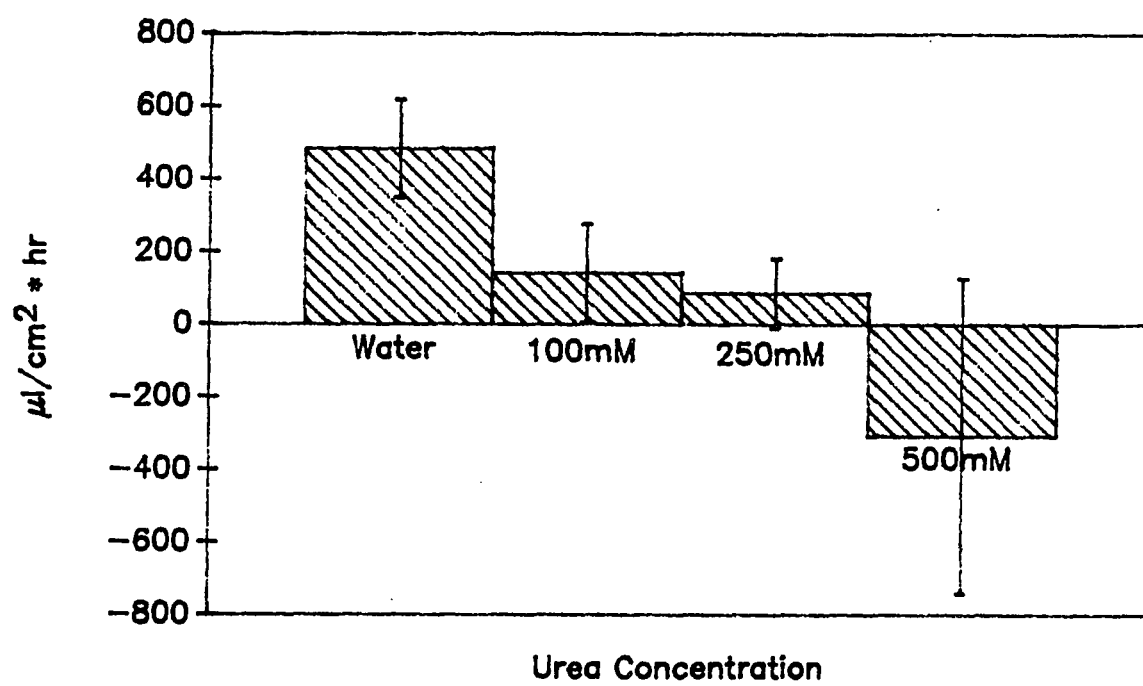


Fig. 15

Figure 16. The comparison of the ratio of seat patch area/total surface area to the body weight (g) of the toads. Symbols represent the mean and the vertical bars are the standard error of the mean.

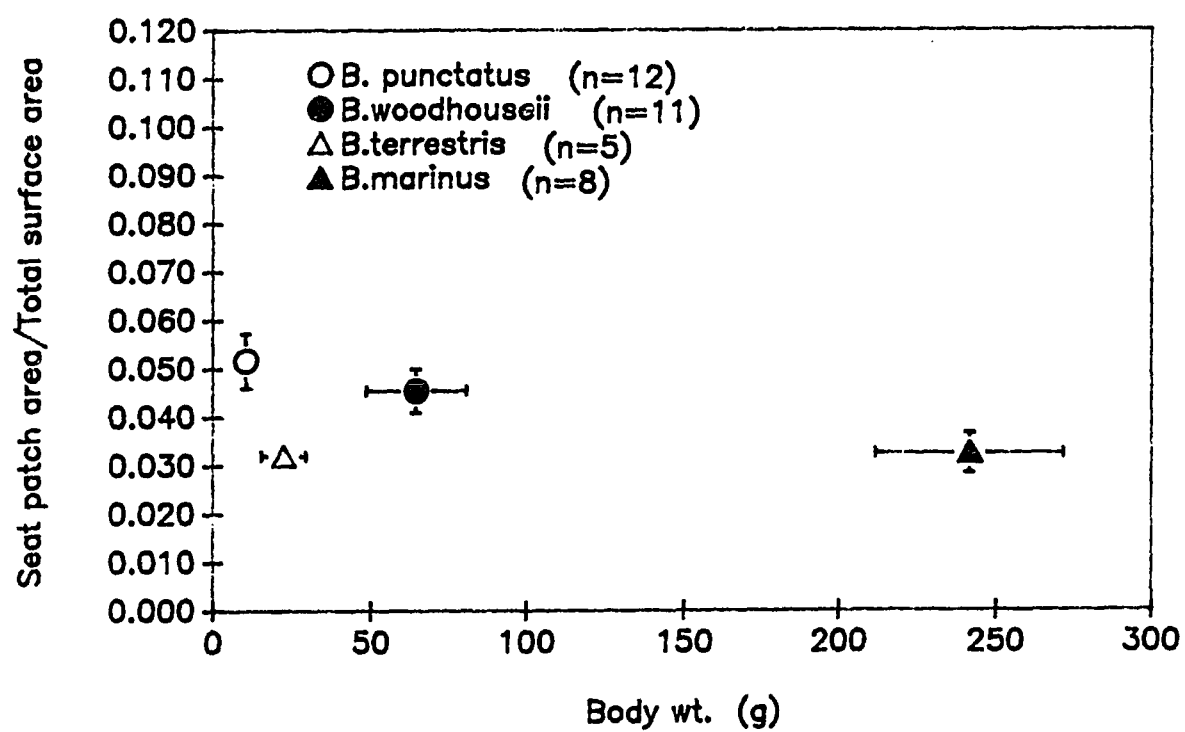
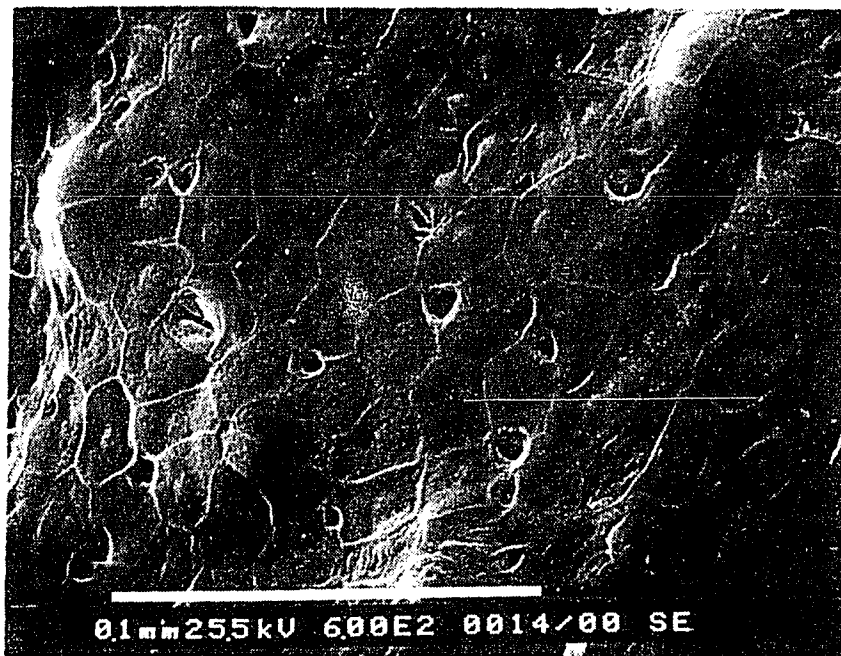
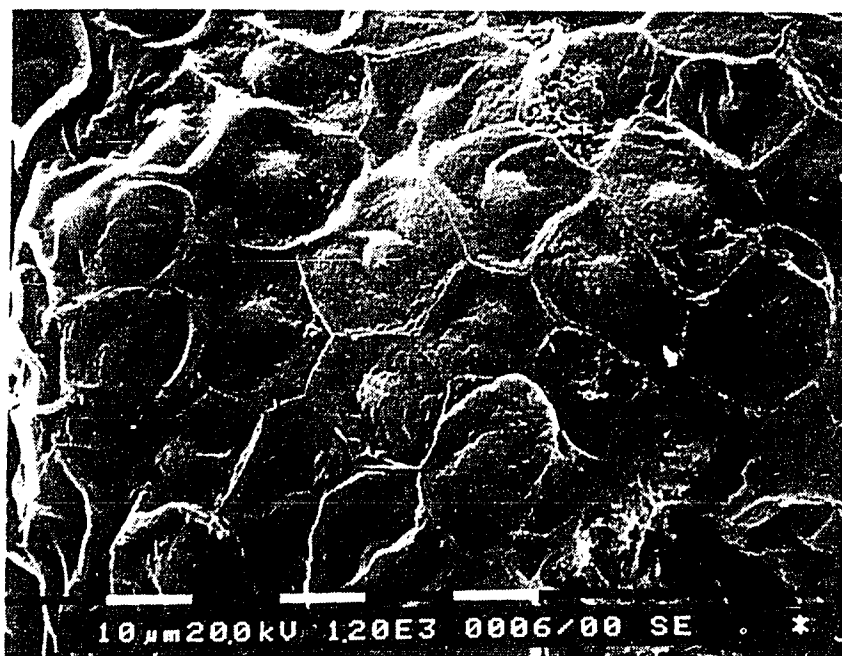


Fig. 16

Figure 17. a) The scanning electron micrograph of the pelvic skin of Bufo punctatus, scale bar is equal to 10 μm . b) The scanning electron micrograph of the pectoral skin of Bufo punctatus, scale bar is equal to 0.1 mm.



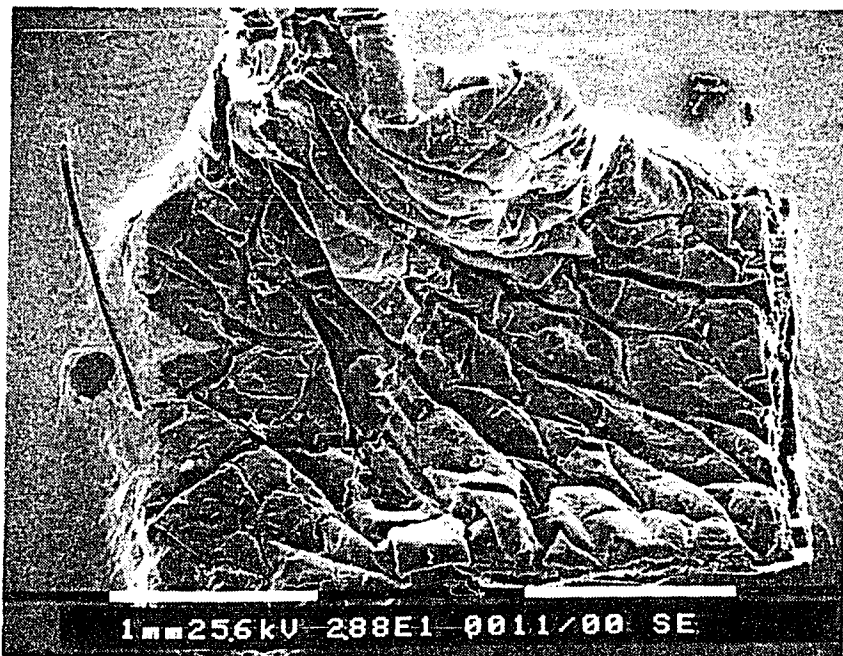
(a)



(b)

Figure 17

Figure 18. a) The scanning electron micrograph of the pelvic skin of Bufo punctatus, scale bar is equal to 1 mm. b) The scanning electron micrograph of the pectoral skin of Bufo punctatus, scale bar is equal to 1 mm.



(a)



(b)

Figure 18

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