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Cardiovascular and behavioural changes during water absorption in toads, *Bufo alvarius* and *Bufo marinus*

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Summary

Blood cell flux (BCF) in the pelvic skin of *Bufo marinus* was lower than *Bufo alvarius* when toads rehydrated from deionised water (DI) or 50 mmol l^{-1} NaCl (NaCl). Despite the lower BCF in *B. marinus*, water absorption was not different between the species when toads rehydrated from DI or NaCl. When fluid contact was limited to the pelvic skin, water uptake from NaCl was lower than from DI, but became greater than uptake from DI as the immersion level increased. Hydrophobic beeswax coating the lateral sides reduced absorption from NaCl but not from DI. Toads settled into water absorption response posture well after maximal BCF was attained in both DI and NaCl, indicating that the behavioural response requires neural integration beyond the increase in BCF. Water exposure increased BCF in hydrated *B. alvarius* with empty bladders but not in those with stored bladder water. Hydrated *B. marinus* with an empty bladder did not increase BCF when given water. Handling stress depressed BCF but increased central arterial flow (CAF), measured using a flow probe around the dorsal aorta. In undisturbed toads, CAF increased with the same time course as BCF while heart rate remained relatively constant, suggesting redistribution of blood flow.

Key words: toad, *Bufo marinus*, *Bufo alvarius*, water absorption, blood flow.

Introduction

Toads in the genus *Bufo* have a highly vascularized area of skin on the ventral surface, termed the seat patch, which is specialized for water absorption (McClanahan and Baldwin, 1969; Christensen, 1974). Using non-invasive Laser Doppler Flowcytometry, a rapid rise in seat patch blood cell flux (BCF) associated with increased water absorption was demonstrated (Viborg and Rosenkilde, 2004) when previously dehydrated toads (*Bufo bufo*) were given access to water. In contrast, hydrated toads injected with arginine vasotocin absorbed water faster without increasing BCF. BCF and water absorption by dehydrated *Bufo woodhouseii* and *Bufo punctatus* were higher than in hydrated toads, but the two were not correlated (Viborg and Hillyard, 2005). Thus, the relationship between BCF and water absorption remains unclear. *B. punctatus* and *B. woodhouseii* inhabit dryer environments than *B. bufo*, and some of the differences in water absorption may relate to the more extensive vascularization in the ventral skin of arid-adapted toads (Roth, 1973). Specifically, *B. alvarius* that inhabit deserts of the south western USA and northern Mexico have a denser array of blood vessels than the more mesic *B. marinus*. This provided the opportunity to determine whether BCF is correspondingly larger in *B. alvarius vs B. marinus* and if it results in a greater rate of water absorption by dehydrated toads.

Despite the reduced osmotic gradient, dehydrated toads, *B. punctatus* and *B. marinus* rehydrate faster when immersed in 50 mmol l^{-1} NaCl than in deionised water (Sullivan et al., 2000; Hillyard and Larsen, 2001). Hillyard and Larsen suggested that increased blood flow to the seat patch is responsible for the elevated water uptake during immersion in 50 mmol l^{-1} NaCl (Hillyard and Larsen, 2001). We tested this hypothesis using a chamber in which BCF and water absorption can be measured simultaneously (Viborg and Hillyard, 2005). This chamber also allowed us to evaluate the effect of different immersion levels on BCF and water absorption from deionised water (DI) and salt solutions. Because the toads were conscious and unrestrained, we were able to observe whether the time course for behaviours associated with water absorption (Hillyard et al., 1998) corresponded to the physiological response to water contact (increase in BCF).

The increase in BCF was characterized as a reflex stimulated by water potential receptors in the skin (Viborg and Rosenkilde, 2004). The degree to which internal factors might affect this reflex was investigated (Viborg and Hillyard, 2005),

and it was shown that hydrated toads (*B. punctatus*) whose bladders had been emptied had a significantly greater BCF than toads that were allowed to retain bladder water. However, *B. punctatus* is a small toad and the process of emptying the bladder resulted in mild dehydration, so the effect of bladder content alone was not established. Bladders of larger toads used in the present study could be emptied without appreciable dehydration. This permitted a more conclusive demonstration that the reflexive increase in BCF is sensitive to the presence or absence of stored bladder water.

A rise in BCF requires diversion of blood from other organs or a rise in cardiac output. Earlier studies with anesthetized anurans have used measurement of central arterial blood flow (CAF) to infer levels of BCF (Parsons and Schwartz, 1991). In some cases, the heart was stopped by direct injection of MS222, so BCF was abruptly stopped. Here we measure the relationship between CAF and BCF in conscious *B. marinus* that were outfitted with flow probes placed around the dorsal aorta to determine whether changes in CAF and heart rate (f_H) correlate with the increase in BCF as dehydrated toads were presented with a hydration surface.

Materials and methods

Capture and maintenance of animals

Bufo alvarius Girard were collected at the Buenos Aires National Wildlife Refuge, Sasabe Arizona, USA. The toads were kept in terraria (80 cm×40 cm×40 cm) with access to pooled tapwater and shelters made of plastic tubing. They were maintained on a 12 h:12 h L:D cycle at room temperature (21–24°C) and were fed crickets 2–3 times a week. The five toads used for experiments ranged in body mass from 200 to 390 g. *Bufo marinus* L. were obtained from commercial suppliers and kept in a 2 m×5 m room having water troughs along the sides and a dry ledge in the middle. The temperature of the room was maintained at 22–24°C and a heat lamp was provided at one end of the room. The toads were fed mealworms *ad libitum*. The five toads used for experiments ranged in body mass from 230 to 371 g. Toads kept with free access to water can be assumed to maintain a hydrated state (Jorgensen, 1991; Jorgensen, 1994). Hydration status was evaluated relative to the standard weight (Ruibal, 1962), which is the weight of a fully hydrated toad with an empty urinary bladder.

Measurements of skin blood flow

BCF was measured using the technique described (Viborg and Hillyard, 2005), with a Periflux PF 2B 2 mW He-Ne laser (Perimed, Sweden) connected to a PF 313 Integrating Probe (Perimed AB, Sweden) that is designed for measurement of skin blood flow (Salerud and Nilsson, 1986). BCF is linearly related to the product of the number of blood cells and their average velocity in the explored volume of tissue. The measurements can not be calibrated to absolute values, but can be expressed in relative terms as voltage (V), and have frequently been used to assess microvascular flow, including skin blood flow (Perimed Literature Reference list; http://www.lisca.se/). During recording of seat patch skin BCF, toads were placed individually in a Lucite chamber measuring 12.5 cm×16 cm×6.5 cm containing a water reservoir at the bottom to allow the seat patch region of the skin to have direct contact with water. A port in the centre of the water reservoir held the laser Doppler probe, so BCF could be recorded prior to (dry) and after (wet) water was added to the reservoir. The diagrams showing the increase in BCF in dehydrated toads (Figs 1B, 2B) were constructed by averaging BCF over 20 s (2000 points) intervals every 1 or 2 min. Data points 10 s prior to and after the minute were averaged to give mean BCF values for each interval.

Measurements of seat patch water uptake

A silicon tube connected the reservoir to a 0.50 ml glass pipette calibrated in 0.01 ml divisions that was positioned horizontally and adjusted a few millimetres above the floor of the chamber, so the water surface in the reservoir was immediately above the nylon mesh that covered the openings to the water reservoir. Water absorbed from the reservoir was read directly from the pipette at various times. Multiple measurements of time taken to absorb the 0.5 ml water contained in the pipette were made during a given trial. The large size of the toads ensured that measurements of BCF and water uptake were made in the centre of the seat patch region.

Water absorption behaviour

When given access to a wet surface, dehydrated toads will abduct the hind limbs and press the seat patch towards the surface. This behaviour is termed the 'water absorption response' (WR) (Stille, 1958; Hillyard et al., 1998). In addition to WR, toads will display a series of moves and body oscillations to reposition the seat patch on the hydration surface (Brekke et al., 1991). The experimental chamber allowed observation of the WR and related behaviours that could be quantified with respect to the increase in BCF.

Experimental protocol

BCF, water absorption and WR behaviour on deionised water vs 50 mmol l^{-1} NaCl

The urinary bladders of toads obtained from the maintenance terraria were emptied by inserting a polyethylene cannula into the cloaca combined with gentle abdominal pressure. The resulting standard weights were recorded and the toads placed overnight in a dry terrarium to obtain a level of dehydration (12–20% relative to the standard weight) that is sufficient to consistently stimulate the toads to initiate water absorption behaviour (Maleek et al., 1999; Nagai et al., 1999). Following dehydration, BCF and water uptake read from the pipette were measured for 40 min with the reservoir containing either deionised water (DI) or 50 mmol l^{-1} NaCl (NaCl). At the end of the trials the toads were blotted on paper tissue and weighed so that rates of water uptake measured by the pipette could be compared with the gravimetrically measured increase.

In this and subsequent experiments, we observed that differing levels of immersion affect rehydration rates from

water *vs* dilute NaCl solutions. Because factors such as barometric pressure (Hoff and Hillyard, 1993) affect hydration behaviour, we elected to serially evaluate different levels of immersion on a given day to keep conditions as similar as possible. To do this, toads that had completed a rehydration period at one level of immersion were placed for 2 h in a dry terrarium with a fan to circulate air over the animal. This procedure, which we term a 'dehydration interval', produced a level of dehydration that was comparable to that following the overnight dehydration used for the initial measurements of BCF and water absorption, i.e. toads lost the water that had been absorbed during the previous trial.

After the initial dehydration interval, the toads were placed in a 2 l glass beaker holding 200 ml of either DI or NaCl. This produced a level of immersion that increased the skin area available for water absorption relative to that of toads absorbing water from the chamber reservoir, across the seat patch. Water gain was determined gravimetrically after immersion for 20 min. We termed this 'full immersion', for comparison with toads placed in the chamber with increased water levels but not enough to allow the toads to float freely.

Except where noted, three trials were performed with each of the five toads on DI and with NaCl, giving a total of 15 trials for each group. For repetitive trials the toads were allowed to recover for a week between dehydrations.

Effects of immersion level on BCF, WR behaviour and rates of water uptake

Toads were again dehydrated overnight and SW recorded. For these experiments, the chamber was initially filled to a depth of 1.5 mm with either DI or 50 mmol l^{-1} NaCl. WR behaviour and BCF were monitored in the chamber for 20 min. Then the toads were carefully blotted in paper tissue and body weight were recorded for gravimetric determination of water gain. After a dehydration interval, body weights were recorded and the toads were again placed individually in the chamber that was now filled to a depth of 6 mm with either DI or NaCl. BCF and WR behaviour were monitored for 20 min, the toads were blotted in paper tissue and body weights were again recorded for gravimetric measurement of water gain. Finally, a second dehydration interval was observed and the toads were fully immersed for 20 min in 200 ml DI or NaCl in a 2 l glass beaker, for comparison with the first set of experiments. As before, water absorption was measured gravimetrically.

Regional water absorption from DI vs NaCl

Only *B. marinus* were available for the third set of experiments. Toads were dehydrated overnight as previously described and water absorption was measured gravimetrically following immersion for 15 min in either 200 ml DI or NaCl in a 2 l glass beaker. After a dehydration interval this procedure was repeated to establish a baseline level for water absorption from DI and NaCl. A second dehydration interval was then observed and one of two separate procedures were conducted in which water absorption was measured in toads having defined areas of the skin covered with a mixture of beeswax dissolved in vegetable oil (0.14 g ml^{-1}). In the first procedure, the ventral and lateral skin was covered from a point 2 cm caudal from the forelimbs to the posterior margins of the thighs at a level just below the cloaca. This area includes the seat patch, where most water absorption is believed to occur (Christensen, 1974). Rehydrating toads are also known to draw water from the ventral to the dorsal surface of the skin by way of capillarity in grooves and channels that Lillywhite and Licht referred to as epidermal sculpturing (Lillywhite and Licht, 1974). In the second procedure, the skin on the lateral sides, including the anterior part of the thigh, was covered by the mixture to prevent this transfer but keep the ventral skin available for absorption. After applying the mixture, toads from both treatment groups were immersed for third and fourth 15 min periods separated by a dehydration interval. The toads were allowed to moult at least once between trials to ensure that all beeswax and vegetable oil had disappeared.

Effects of bladder water on BCF

Body weights of *B. alvarius* obtained from their maintenance terraria were recorded, prior to removing bladder urine. The volume of bladder urine voluntarily retained by toads has previously been termed '*ad libitum* bladder urine' (Tran et al., 1992). The toads were placed individually in the chamber and BCF was recorded for 6 min with no water in the reservoir (dry), and then for 6 min after the addition of deionised water to the reservoir (wet; hydrated toads, *ad libitum* bladder). The urinary bladders were emptied, standard weights (SW) were recorded and the toads were left in dry terraria (40 cm×40 cm×40 cm) for 10 min. This provided a standardized interval following the stress of handling so that toads could be positioned in the chamber. The bladder volumes for each toad were assumed to be the difference between the initial weight and the SW. Because *B. marinus* spontaneously voided their bladders when handled, the initial weighing was the SW. BCF for both species (hydrated, empty bladder toads) was measured for 6 min before and after the addition of water to the reservoir. The toads were then transferred to the dry terraria for 3–4 h with a ventilating fan, to induce a state of mild dehydration that may correspond to brief activity periods in the field (Stille, 1952). Body weights were recorded and BCF was again measured for 6 min before and after the addition of deionised water to the reservoir (mild dehydration). The toads were then transferred to the dry terraria and left without access to water overnight to produce a greater degree of dehydration. The following day, body weights were recorded and the percent dehydration calculated. Then BCF was measured for 6 min with empty reservoir, and for 40 min with deionised water added to the reservoir. For repetitive experiments, the toads were allowed to recover for 1 week between dehydrations.

Central arterial flow and BCF

Central arterial blood flow (CAF) and BCF were measured simultaneously in five *B. marinus* (body mass 253–371 g). One or two trials were performed for each toad, resulting in a total of 9 measurements. Prior to surgery, the toads were

anesthetized by immersion in a 2‰ solution of benzocaine, until the corneal reflex disappeared. During surgery, the toads were placed on water-saturated paper tissue. A 3 cm incision was made on the dorsal side approximately 1 cm lateral to the vertebrae in regio lumbalis. The muscles were gently separated to access the abdominal cavity and expose aorta abdominalis. A Transonic blood flow probe (Transomic Systems Inc., Ithaca, NY, USA) was placed around the aorta abdominalis and two holding sutures in the muscles secured the probe. The incision was closed in two layers (muscles and skin) by nylon sutures. The toads were allowed to recover for 2 full days in a plastic cage (50 cm×35 cm×25 cm) containing 2 cm of tapwater (Andersen and Wang, 2002). One day before the experiment, the urinary bladder was emptied, SW was recorded and the toad was placed in a dry plastic cage for dehydration. The next day, body weight was recorded and toads were transferred individually to the chamber after filling the reservoir with deionised water. CAF and BCF were measured for 40 min with water in the reservoir.

In a separate experiment handling was avoided and only CAF was measured in four *B. marinus* (210–434 g). After emptying the urinary bladder and overnight dehydration, water was added gently to the dehydration chamber through a silicon tube in order to disturb the toads as little as possible, and CAF was measured over a 40 min period.

Analysis of data

A serial protocol with paired trials was used for the experiments. The serial design was less time consuming and allowed trials for an individual toad to be completed within a day. This served to reduce effects of environmental factors like barometric pressure, which have been shown to affect rehydration behaviour (Hoff and Hillyard, 1993). Paired trials were chosen to minimize effects of individual variation, as both rates of water absorption and BCF may vary considerably between individual animals.

Two-way analysis of variance (ANOVA) applied to the randomized block design was used to compare treatment groups where toads served as a blocking factor with 3 replications for each toad. In order to check the model assumptions of normally distributed residuals and subsequently apply ANOVA, the Kolmogorov–Smirnov test was performed on the model residuals. An ANOVA was used to compare BCF between species and the χ^2-test was used to compare the occurrence of oscillations in *B. alvarius* on DI or NaCl. The linear regressions take account for variation due to differences between individual toads.

Averages were calculated for each individual toad, an average representing all toads was then calculated and s.e.m. determined from this average with *N*=number of toads.

Results

Species comparisons

Fig. 1A shows a representative trace for the increase in BCF in a dehydrated *B. alvarius* placed in contact with deionised

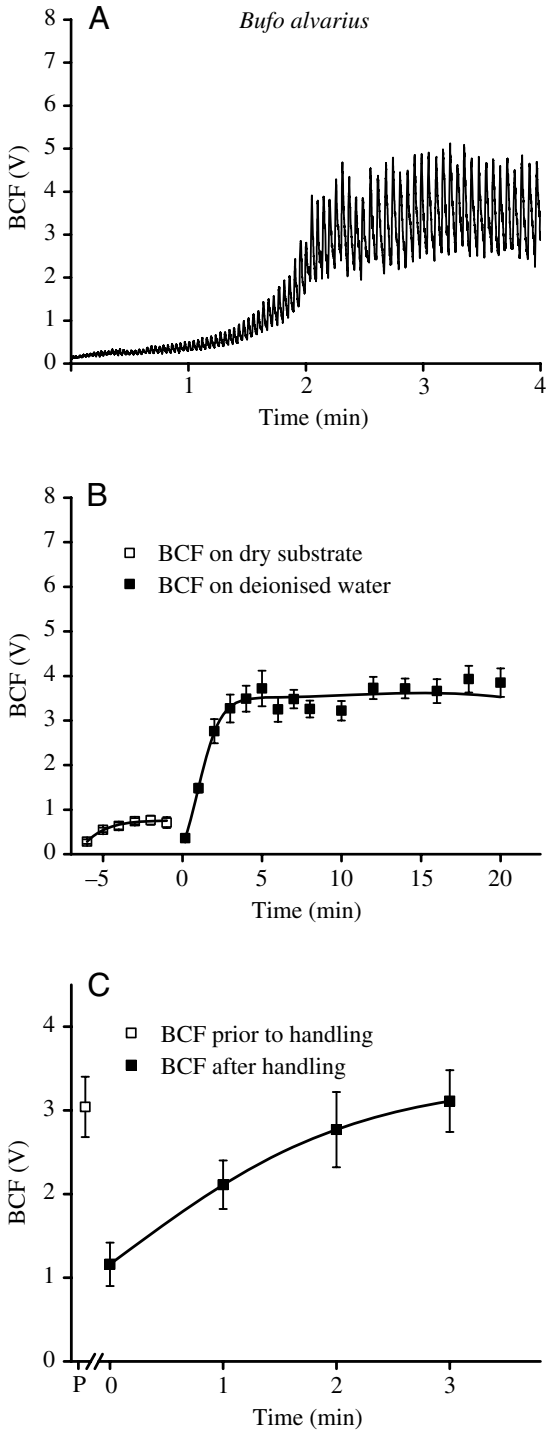

Fig. 1 (A) Trace from the flow cytometer showing the increase in seat patch BCF of a *B. alvarius* dehydrated by 9.2% and placed on DI at time 0. (B) Mean seat patch BCF values ± s.e.m. from 15 trials (three replicates in five toads) with dehydrated *B. alvarius*; open symbols, BCF in the dry chamber; filled symbols, BCF on DI. (C) Effect of handling on BCF. Open symbol, BCF prior to handling; repositioning the toads on the probe caused a rapid decline in BCF, when the toads were left undisturbed BCF increased to the prehandling level within 3 min (filled symbols). The increase in BCF by dehydrated toads with 50 mmol l^{-1} NaCl in the reservoir was not different from that in A and B. P, pre-handling level.

water (DI). The mean response is shown in Fig. 1B. After a lag time of approximately 1 min, BCF rose gradually over the next minute and reached a stable level within the third minute. In 8 of the 15 trials, toads moved off the flow probe after maximal BCF values had been attained and had to be repositioned. This handling caused a rapid reduction in BCF, but BCF returned to the pre-handling level within 3 min (Fig. 1C). The time course for increased BCF by *B. marinus* was similar to *B. alvarius*, but the magnitude of the response was significantly smaller (Fig. 2) despite the greater degree of dehydration in *B. marinus* (16.4±1.1 *vs* 14.5±0.7% of SW). *B. marinus* also exhibited a decrease in BCF upon handling (data not shown).

BCF, WR behaviour and water absorption on NaCl vs DI

The temporal change of BCF and its magnitude was not affected by 50 mmol l^{-1} NaCl compared to DI for either species; however, BCF remained higher for *B. alvarius* compared to *B. marinus*. Although *B. marinus* gradually adopted the WR posture rather than abducting the hind limbs in a distinct movement, full abduction of the hindlimbs generally occurred after maximal BCF had been achieved. *B. alvarius* also settled into the WR posture well after maximal BCF had been attained (mean settling time on DI was 10.5 min and on NaCl it was 10.6 min. The time and the BCF when WR was initiated were not affected by NaCl. However, the number of moves during the first 40 min was greater on NaCl than on DI (Table 1). Further, oscillations of the body following a move, when skin contact with the reservoir was re-established, were observed in 9 of 12 trials on NaCl but in only 2 of 15 trials on DI (P<0.01, N=27, χ^2-test). The lower number of trials on NaCl was due to one toad, which consistently rejected water uptake from NaCl by leaving the chamber.

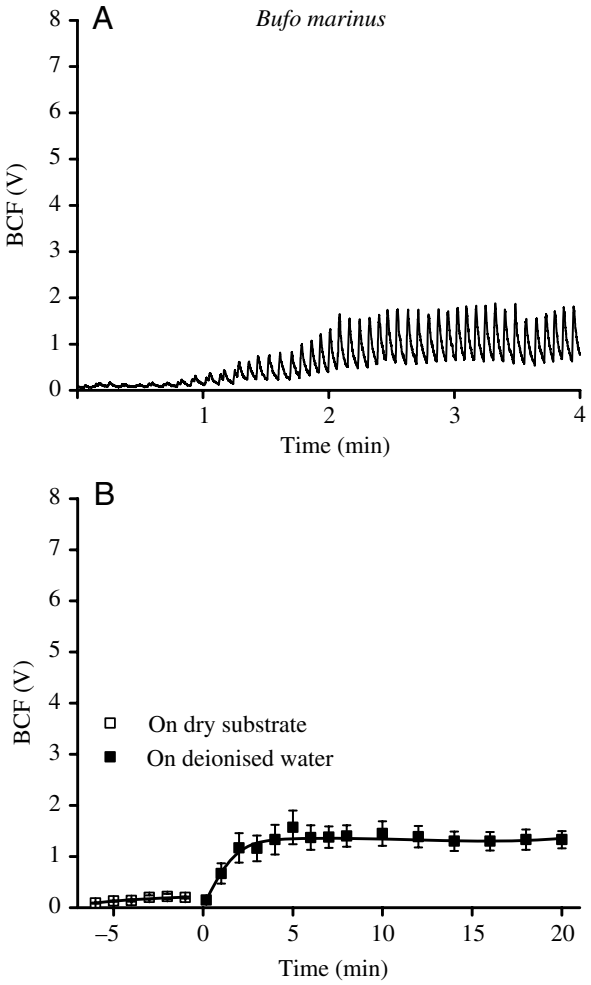

Fig. 2. (A) A trace from the flow cytometer showing the increase in seat patch BCF of a dehydrated (14.1%) *B. marinus* placed on water at time 0. (B) Mean seat patch BCF values ± 1 s.e.m. from 15 trials (three replicates in five toads) in dehydrated *B. marinus*; open symbols, BCF in the dry chamber; filled symbols, BCF on water. On water, BCF was significantly lower in *B. marinus* compared to *B. alvarius* (P<0.001, Fig. 1B *vs* Fig. 2B). Values for toads with 50 mmol l^{-1} NaCl were not different from DI.

For both species, the rate of water uptake from the reservoir was significantly lower from 50 mmol l^{-1} NaCl compared to DI (Fig. 3). In contrast, water uptake was significantly greater when the toads were fully immersed in 50 mmol l^{-1} NaCl compared to DI by toads immersed in the beakers (Fig. 3). The two species absorbed water at similar rates in any of the experimental conditions and the values obtained from the pipette were not different from weight change.

Water absorption by *B. alvarius*, obtained from successive pipette readings, did not correlate with BCF values averaged during the same intervals, on either DI or NaCl (Fig. 4A,B). Each point represents 8–20 simultaneous measurements of water uptake and mean BCF on DI (15 trials) and NaCl (10 trials). The smaller sample size resulted from two toads on NaCl not remaining in the chamber for the entire trial period. Similar results were obtained with *B. marinus* (data not shown).

Effects of immersion level on BCF, WR behaviour and rates of water uptake

When the fluid level in the chamber was raised to 1.5 mm or 6 mm, all toads readily engaged in WR behaviour and remained in the chamber for the full experimental period in both DI and NaCl. The increase in BCF observed in both species was not different between the solutions at either immersion level, nor was there a difference between

Table 1. *The number of times during a trial that B. alvarius moved and resettled into WR posture*

Immersion level	Time (min)	No. of movements DI	No. of movements NaCl	P
Reservoir	40	6.4±1.2	14.5±1.8	<0.05
1.5 mm	20	6.3±1.2	19.5±1.9	<0.001
6.0 mm	20	8.1±1.2	13.1±1.8	<0.02

Values are means ± s.e.m. of 15 trials, except for Reservoir NaCl (12 trials).

WR, water absorption response; DI, deionised water; NaCl, 50 mmol l^{-1} NaCl solution.

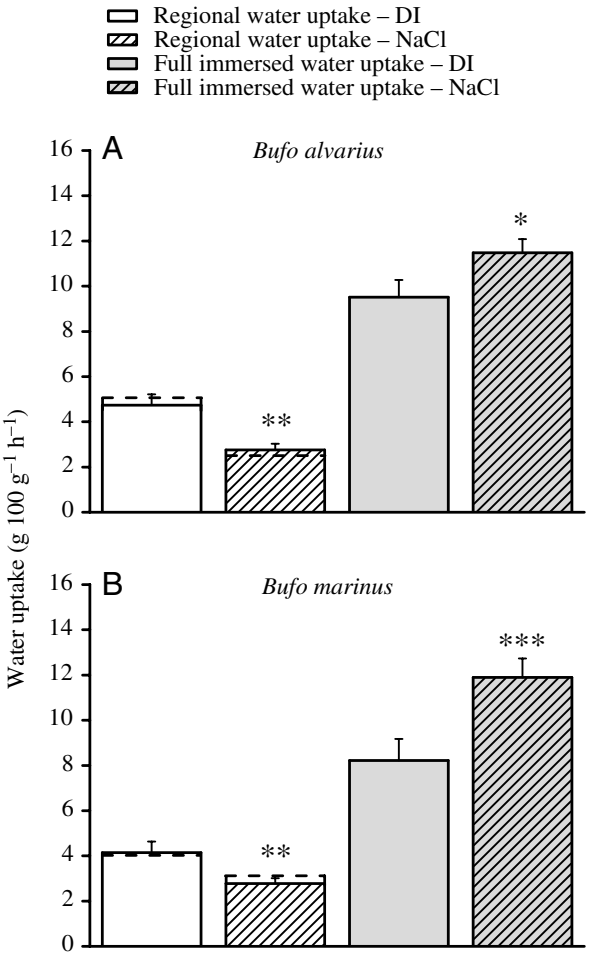

Fig. 3. Seat patch and fully immersed water uptake from DI water or NaCl (50 mol l^{-1}) in *B. alvarius* (A) and *B. marinus* (B). Each column represents mean ± 1 s.e.m. for 15 trials, except seat patch water uptake from NaCl (12 trials). Regional water uptake read from the pipette or determined gravimetrically (broken lines) were not different. Regional seat patch water uptake was significantly lower from NaCl (**P<0.01). In contrast, full immersion resulted in significantly higher water uptake from NaCl (*B. alvarius*, *P<0.05; *B. marinus*, ***P<0.001). There were no differences between *B. marinus* and *B. alvarius* in water absorption from a given rehydration source.

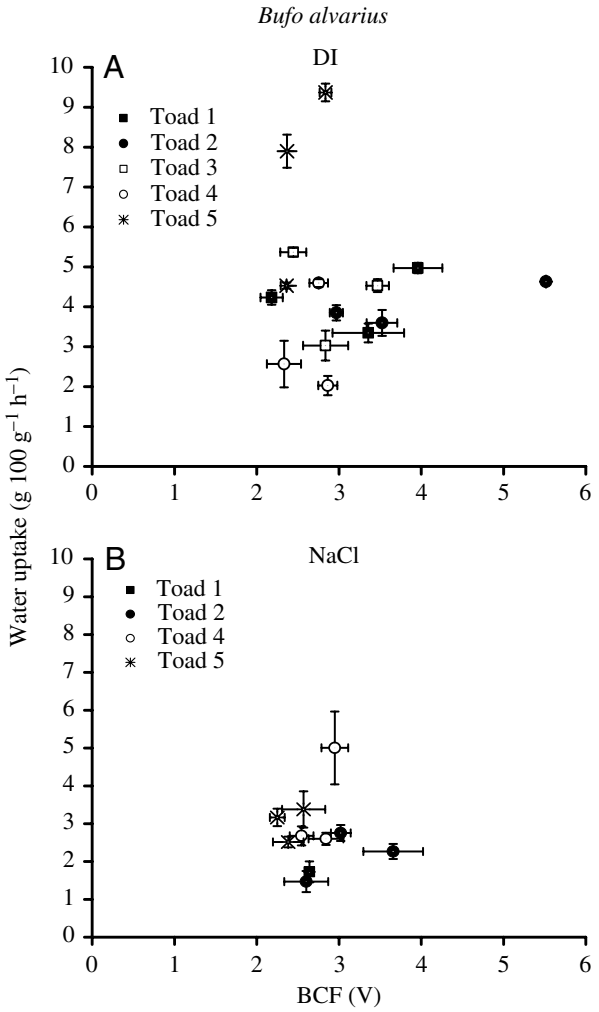

Fig. 4. Seat patch water uptake read from the pipette in *B. alvarius* placed on DI water (A) or NaCl (50 mol l^{-1}) (B) plotted as a function of seat patch BCF during the same period. In A, 15 trials were performed and in B, only 10 trials as two of the toads often left the chamber within 10–15 min. Each point represents from 8–20 simultaneous measurements ± s.e.m. There was no correlation between BCF and water uptake from either DI or NaCl. Similar results were obtained with *B. marinus*.

immersion level and the pattern observed in Fig. 1 for a fluid level confined to the seat patch area (data not shown). With both immersion levels, *B. alvarius* settled into WR posture after maximal BCF had been attained. (Immersed in 1.5 mm of solution, mean settling times were 7.7±1.6 min in DI and 4.1±0.4 min in NaCl; immersed in 6 mm of solution, mean settling times were 6.5±1.4 min in DI and 4.8±0.6 min in NaCl). As with absorption from the reservoir, toads moved and resettled more often on NaCl than on DI (Table 1). Again, it was not possible to detect discrete times when *B. marinus* initiated WR behaviour, but these toads also moved more often during 1.5 mm immersion in NaCl (10.5±1.6) compared to DI water (4.7±0.9), (P<0.003). With 6 mm immersion, no difference in moves was observed between the two solutions.

Rates of water uptake by *B. alvarius* immersed in 1.5 mm, 6 mm or fully immersed in DI were not significantly different from each other (Fig. 5A). In contrast, water absorption from NaCl increased significantly when the immersion level was increased from 1.5 mm to 6 mm, and a further significant increase was observed with full immersion. A similar pattern was observed with *B. marinus* (Fig. 5B): rates of water uptake from DI were not different among immersion levels while the rate of water uptake from NaCl increased significantly with 6 mm *vs* 1.5 mm immersion, and with full immersion *vs* 6 mm.

Regional water absorption from DI vs NaCl

Covering the seat patch and the lateral sides significantly reduced water uptake of *B. marinus* immersed in either DI or

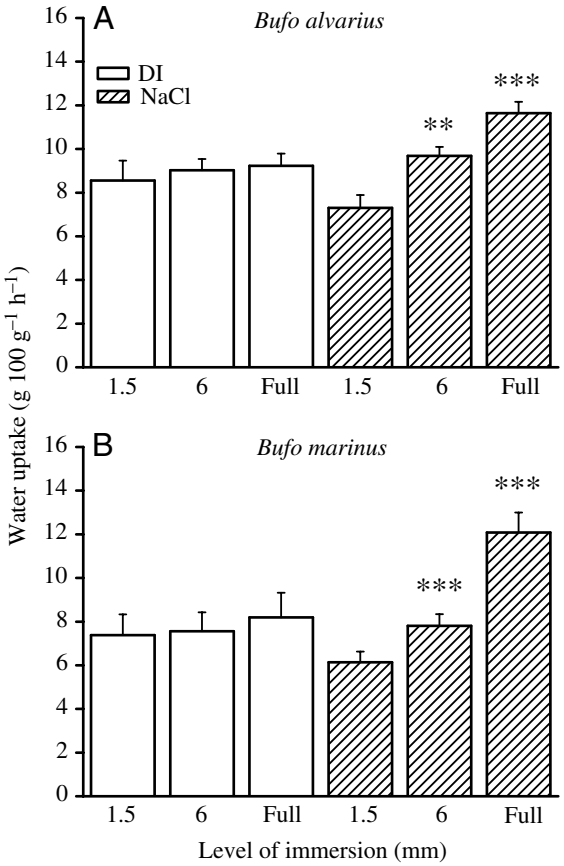

Fig. 5. Rates of water uptake in *B. alvarius* (A) or *B. marinus* (B) immersed in 1.5 mm, 6 mm or fully immersed in DI water or NaCl (50 mol l^{-1}). Each column represents mean ± 1 s.e.m. for 15 trials. Water uptake from DI was not different irrespective of immersion level. In NaCl, increased immersion resulted in significantly increased rates of water uptake. ***P*<0.01; ****P*<0.001.

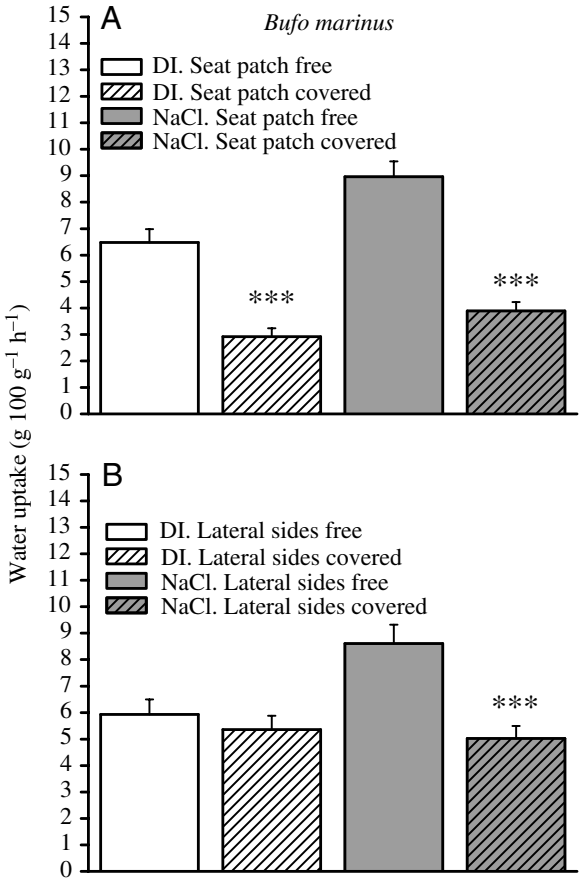

Fig. 6. (A) Water uptake in *B. marinus* fully immersed in DI water or NaCl (50 mol l^{-1}), with or without the seat patch and the skin on the lateral sides covered by a mixture of beeswax and vegetable oil. Each column represents mean ± 1 s.e.m. of 15 trials. Water uptake from both DI and NaCl was significantly reduced when the skin was covered by the hydrophobic mixture. (B) Water uptake from DI or NaCl, with or without the skin on the lateral sides covered by the hydrophobic mixture. Water uptake from DI was not affected, but water uptake from NaCl was significantly reduced when the lateral skin was covered by the hydrophobic mixture. ****P*<0.001.

NaCl (Fig. 6A). If only the skin on the lateral sides between forelimbs and hindlimbs was covered with the hydrophobic mixture, water uptake by toads immersed in DI was not significantly reduced (Fig. 6B) while water uptake from NaCl was significantly reduced (Fig. 6B).

Effects of bladder content and dehydration on seat patch BCF

For hydrated *B. alvarius* with *ad libitum* bladder water, BCF did not increase following water contact (Fig. 7A, group A). In contrast, removal of bladder water resulted in a highly significant stimulation of BCF upon water contact (Fig. 7A, group B). Mild dehydration (mean 3.6±0.2% of SW) resulted in a similar stimulation of BCF following water contact (Fig. 7A, group C) that was not different from empty bladder toads. Overnight dehydration (mean 15.0±0.44% of SW) caused a further significant increase in BCF following water contact compared to mild dehydration (Fig. 7A, groups C and D). The gradual increase in BCF values on the dry surface as the level of dehydration increased was not significant.

As noted earlier, *B. marinus* spontaneously voided their bladders when handled, so experiments on toads with *ad libitum* bladders could not be performed. Empty bladder toads did not show an increase in BCF following water exposure (Fig. 7B, group B). Mild dehydration (mean 3.3±0.3% of SW) did result in a small but significant stimulation of BCF following water contact (Fig. 7B, group C). Overnight dehydration (16.9±0.7% of SW) produced a significant increase in BCF following water contact (Fig. 7B, group D). BCF values on the dry chamber were not significantly different between treatments.

B. alvarius maintained in terraria with access to water retained amounts of bladder water ranging from less than 5% to nearly 25% of the body mass (group A in Fig. 7). A linear regression of BCF *vs* retained bladder volumes for the individual trials showed a significant correlation (R^2=0.7403; *P*<0.001).

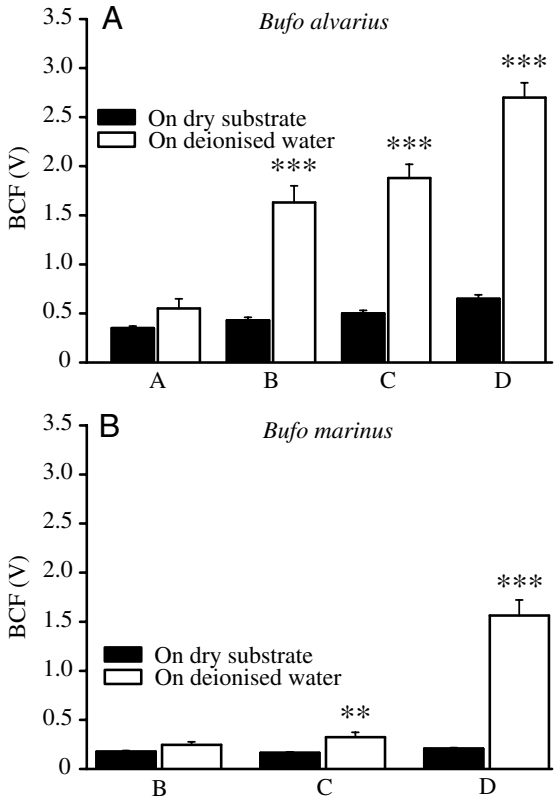

Fig. 7. BCF in toads in the dry chamber (filled columns) and in the wet chamber (open columns). Each column represents mean ± 1 s.e.m. of 15 trials. (A) In *B. alvarius* BCF did not increase when toads with *ad libitum* bladder water were placed on water (group A). Removing the bladder depot resulted in a significant stimulation of BCF in the wet chamber (group B). Mild dehydration resulted in a similar increase in BCF following water contact (group C). Dehydration (mean 15%) caused a further increase in BCF (group D). (B) *B. marinus* voided their bladders so *ad libitum* bladder measurements could not be obtained. Empty bladder *B. marinus* did not increase BCF when placed in on water (group B). Mild dehydration produced a small but significant increase in BCF (group C). Dehydration (16.9%) caused a further significant increase in BCF on water (group D). On dry substrate BCF did not increase significantly with dehydration in any of the species. $^{**}P<0.01$; $^{***}P<0.001$.

Central arterial blood flow and BCF

Typical traces of CAF and BCF are shown in Fig. 8A for a dehydrated toad transferred to the wet chamber. Heart rate (f_H) was also obtained from these records. The increase in BCF (Fig. 8B) was similar to that of dehydrated toads placed on water (Fig. 2B). In contrast, CAF was elevated after transfer and declined from 15.2±2.0 to 9.4±1.0 ml min^{-1} kg^{-1} during the first 3 min followed by a steady rise (to 12.5±1.0 ml min^{-1} kg^{-1} at 10 min) that was maintained throughout the following 40 min. Heart rate followed a similar pattern.

In two toads, CAF was measured prior to handling (four recordings), while the toads remained in the dehydration chamber. Average CAF in these toads was 6.7±2.2 ml min^{-1} kg^{-1} (Fig. 8B). When these two toads were handled by being transferred to the wet chamber the initial CAF was 14.9±4.1 ml min^{-1} kg^{-1} and declined over the initial 3 min, during which BCF increased, as noted for the entire group shown in Fig. 8B. This indicates that the observed initial decline in CAF was due to recovery from handling stress.

The addition of water to the dehydration chamber without handling the toads elicited a steady rise in CAF (Fig. 8C) that followed a time course similar to that seen for BCF in Fig. 8B. CAF and f_H were normalized as the fractional changes relative to the values recorded before water exposure. CAF increased by approximately 80% over a 10 min period and remained at this elevated level for the next 30 min. Heart rate was initially elevated but remained almost unchanged over most of the 40 min period.

Discussion

Species comparison

Species inhabiting arid habitats, such as *B. woodhouseii* and *B. punctatus* (Viborg and Hillyard, 2005), as well as *B. alvarius* (present study), had BCF in the range 3.5–4.0 V, while BCF ranged between 1.5 and 2.0 V in *B. bufo* (Viborg and Rosenkilde, 2004) and *B. marinus* (present study) that inhabit more mesic environments. The lower BCF of dehydrated *B. marinus* compared to *B. alvarius* is consistent with the much lower vascularisation of the pelvic skin of *B. marinus*, and the suggestion that differences in seat patch skin vascularization reflect adaptations to different habitats (Roth, 1973).

Despite the much higher BCF in *B. alvarius*, rates of water uptake, whether from DI or NaCl in the reservoir or during full immersion, were similar between the two species. Furthermore, within each of the two species, there was no correlation between BCF and rates of water uptake. These inter- and intraspecific observations support the hypothesis (Viborg and Hillyard, 2005) that the increase in BCF is facultative once a favourable osmotic gradient has been detected although internal factors, such as handling stress and bladder reserve, are able to affect the magnitude of BCF. Nevertheless, a weak correlation between BCF and water uptake in *B. bufo* was reported (Viborg and Rosenkilde, 2004), but this correlation was based on a rehydration period of 120 min during which the toads were immersed in water between BCF and weight measurements and were returning to a fully hydrated condition. This contrasts with the present study where efforts were made to maintain a uniform dehydration state at the beginning of each trial. As evidence that serial treatments reflect reproducible physiological effects, BCF values obtained initially and after a dehydration interval were not significantly different. Similarly, water absorption by *B. alvarius* immersed in DI was similar when the experiment was run before or after a dehydration interval (Figs 3 and 5).

Because the seat patch is highly vascularized (Roth, 1973; Christensen, 1974) and the rate of capillary ultrafiltration of plasma proteins in the amphibian skin is very high (Conklin,

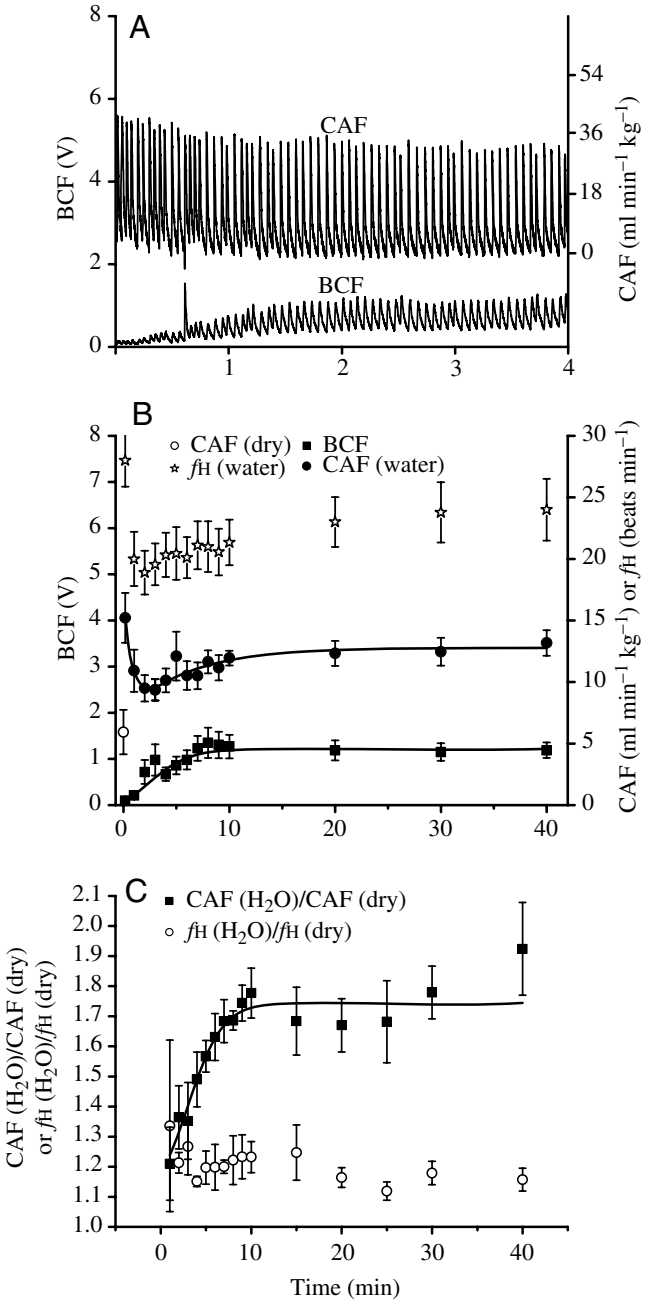

Fig. 8. (A) Representative traces from simultaneous recordings of central arterial flow (CAF, upper trace) and blood cell flux (BCF, lower trace) in a *B. marinus* dehydrated by 14.4%. (B) Time course for changes in CAF (filled circles) and seat patch BCF (filled squares). Each point represents mean ± 1 s.e.m. of nine trials in five toads. Recordings were started immediately after the toads had been transferred from the dehydration chamber to the wet chamber. CAF and heart rate (stars) declined during the first 3 min of recording, followed by an increase to a stable level, which was reached in 10 min. Initial CAF was much lower in two toads (four recordings, open circle), which remained unhandled in the dehydration chamber prior to water contact. (C) Time course for CAF and f_H when dehydrated toads were presented with water but not handled. Because of the small sample size, data points are normalized as the ratio between the control values and those recorded sequentially after water exposure was initiated.

1930), the circulation continuously adds solutes to the interstitial fluid that maintains an osmotic gradient favouring water gain (Christensen, 1975; Parsons et al., 1993). A high rate of transcutaneous water gain also depends on the water permeability of the skin, which may limit water movement to the cutaneous capillaries. Recently, AQP-2 and 3 were demonstrated in the stratum granulosum of the ventral pelvic skin of amphibians (Tanii et al., 2002; Hasegawa et al., 2003; Willumsen et al., 2003). In response to AVT stimulation, both AQP-2 and 3 are translocated to the apical membrane of the stratum granulosum (Hasegawa et al., 2003). While AQP-2 and AQP-3 are only present in the ventral pelvic skin, AQP-1 is associated with the vascular system both inside and outside the seat patch (Willumsen et al., 2003). Aquaporins 3, 2 and 1 thus form a serial pathway from the exterior through the skin into the capillaries. The results of the present study could be due to a lower capillary density in *B. marinus* that is compensated for by a greater expression of AQP-1 in the capillary endothelial cells or AQPs 2 and 3 in the stratum granulosum. Viborg and Rosenkilde also proposed this hypothesis (Viborg and Rosenkilde, 2004), noting that the increase in water absorption by *B. bufo* was stimulated by AVT without an increase in BCF.

Behavioural correlations

The initiation of WR behaviour by *B. alvarius* consistently occurred after maximal BCF values were attained, regardless of the fluid level or salinity of rehydration medium in the chamber. *B. marinus* appeared to behave similarly even though WR posture was adopted more gradually. The rise in BCF appears to be a sympathetic reflex that is mediated by water potential receptors in the skin (Viborg and Rosenkilde, 2004). The delay in WR initiation indicates further integration of the sensory information before a large area of skin is committed to a rehydration source and that the lower water potential of the NaCl solution (*vide supra*) did not affect the time course for WR initiation despite the lower rehydration rate. Both *B. alvarius* and *B. marinus* moved and resettled more often when absorbing water from the NaCl solution. However, the number of moves decreased, significantly so in *B. marinus*, when the immersion level was increased from 1.5 to 6 mm, where the latter condition enhanced water uptake. Toads appear to be able to evaluate not only the osmotic content of a hydration source but also the efficacy of water absorption.

The time course for changes in BCF and the initiation of WR appear to depend on body size. In the large toads of both species (300–400 g), BCF reached maximal values within 3–4 min and WR was initiated well after maximal BCF was attained. For *B. woodhouseii* weighing about 75 g, WR was initiated within 1 min, before BCF was maximal, while 15–20 g *B. punctatus* initiated WR within 20–30 s when BCF had already attained a maximal value (Viborg and Hillyard, 2005). The differences as to whether WR is initiated during or after the increase in BCF may relate to accumulation of solutes required for water absorption, so WR behaviour is not initiated until a favourable osmotic gradient can be sustained. The larger toads have a greater volume to surface ratio so the additional

contact area presented by WR behaviour will be less beneficial for water absorption, compared to small toads.

BCF and water absorption from NaCl vs DI

Rehydration rates of both species from NaCl were 30–40% lower than from DI when fluid contact was limited to the seat patch and became significantly greater only when the immersion level was raised to 6 mm or in a beaker. Lymph osmolality of *B. marinus* dehydrated by 10–15% is approximately 260 mOsm kg^{-1} (Hillyard and Larsen, 2001). Assuming ideal osmotic behaviour for NaCl, the osmotic gradient should be reduced by a factor of 0.62 (160/260) relative to DI, which corresponds well to the observed ratios (0.58 for *B. alvarius* and 0.67 for *B. marinus*).

Coating the lateral skin with the oil/wax mixture abolished the rise in water uptake upon full immersion in NaCl, but there was no effect when toads were immersed in DI. As a control, coating the entire ventral and lateral skin reduced rehydration from both DI and NaCl, which is consistent with numerous studies showing that the seat patch accounts for most water absorption (McClanahan and Baldwin, 1969; Christensen, 1974; Marrero and Hillyard, 1985). The oil/wax mixture will interfere with water transfer to the lateral and dorsal skin *via* epidermal sculpturing, which suggests that these regions of the skin contribute to the greater rehydration rates from dilute NaCl solutions. The mechanism whereby the lateral and dorsal skin might couple salt and water absorption is not known. There was no effect of amiloride on the stimulation of water absorption by 10 and 50 mmol l^{-1} NaCl (Hillyard and Larsen, 2001), but rehydration from 50 mmol l^{-1} sodium gluconate was reduced, as predicted from the osmotic gradient.

Effect of stored bladder water

Arid-adapted toads appear to be more sensitive to hydration status. An empty bladder stimulated BCF in hydrated *B. alvarius* (present study). The empty bladder condition, in combination with mild dehydration (2.7% of SW), elicited a large increase in seat patch BCF in *B. punctatus* (Viborg and Hillyard, 2005). In contrast, a greater level of dehydration (5–10%) was necessary to elicit a pronounced increase in BCF of *B. bufo* (Viborg and Rosenkilde, 2004), and in *B. marinus* neither the empty bladder condition nor mild dehydration elicited a substantial increase in seat patch BCF when the toads were exposed to deionised water (present study). Marked differences were also observed in the behaviour displayed by the two species in the holding terraria. *B. alvarius* remained in their shelters and were never encountered in the hydration tray during the day, while *B. marinus* were usually taken from the water trough. *B. marinus* that remain in water are known to have lymph osmolality that is more dilute than the plasma (Hillyard and Larsen, 2001). *B. marinus* had to be kept dry for 2 h to obtain equilibration between lymph and plasma (Hillyard and Larsen, 2001). The surplus of water temporarily stored in the lymph spaces of *B. marinus* could delay dehydration when toads are transferred to a dry environment and thus the minimal response of BCF to mild dehydration observed in the present study. However, overnight dehydration greatly exceeds the small volume contained in the diluted lymph and we observed no appreciable difference in BCF values for toads dehydrated over a range of 12–25% dehydration.

The negative correlation between BCF and the *ad libitum* bladder volume retained by *B. alvarius* indicates that the amount of stored water reserve contributes to the regulation of pelvic skin BCF in this species. The pathway for cutaneous water absorption remains controversial. It has been proposed that water moves first to the lymphatic pathway and is then returned to the circulation (Carter, 1979; Toews and Wentzell, 1995). More recently, Word and Hillman provide evidence that water movement is primarily *via* the blood capillaries (Word and Hillman, 2005). If this is the case, the generally observed increase in BCF is sufficient to transport all of the water molecules absorbed. Alternatively, the lack of correlation between BCF and water absorption could result from variable utilization of either pathway in response to the ensemble of sensory cues that conscious animals receive. With an empty bladder but no osmotic stress, the primary need is to fill the bladder, which requires water absorption by the circulation and subsequent filtration by the kidneys.

The present study allowed us to quantify the relationship between CAF and BCF in conscious, unrestrained animals. The high initial values seem to be caused by handling stress, as indicated by the low CAF values recorded in toads that remained unhandled in the dehydration chamber. When handling was avoided CAF increased by approximately 80% over a 10 min period following water contact. Heart rate was initially elevated by about 30% but declined as CAF rose to stable elevated values, in contrast with the six- to sevenfold increase in BCF following water exposure in the rehydration chamber. It appears that the increase in BCF is not just the result of increased cardiac output but is due to a redistribution of blood to arteries supplying the seat patch skin, and further may involve local opening of capillaries in the skin (Krogh, 1919). Increased CAF and decreased BCF in response to stressful stimuli can be explained in terms of a fight or flight response. Turning off seat patch perfusion corresponds to a decrease in peripheral circulation, while increased CAF combined with shunting of blood to the muscles will meet increased oxygen demands in response to muscle activity. These observations support the hypothesis that regulation of seat patch perfusion is mediated by the autonomic nervous system, as suggested by Viborg and Rosenkilde (2004).

List of abbreviations

BCF	blood cell flux
CAF	central arterial flow
DI	deionised water
f_H	heart rate
SW	standard weight
WR	water absorption response

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