

INTERPOPULATION DIFFERENCES IN WATER-SEEKING BEHAVIOUR IN THE GREEN TOAD *BUFO VIRIDIS*

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Many species of terrestrial amphibians can absorb water by pressing a highly vascularised region of the ventral abdominal skin against a moist surface. The degree of dehydration needed in order to evoke this response varies from species to species, suggesting that they make different use of the opportunity to use surface water for hydration. Most of the species that have been studied are each confined to a fairly narrow ecological niche, and the differences in their responses make sense in the light of their natural history. Consequently, it was of interest to compare populations of a single species living in different regions to see whether differences in the response to the presence of moisture are characteristics of the species or are related to local conditions. The steep north-south gradient of increasing aridity in Israel and the presence of populations of the toad *Bufo viridis* throughout the country provided an opportunity to make such a comparison. The frequency of this water seeking response (WR) in fully hydrated toads and the sensitivity of WR to raised plasma osmolality were determined in laboratory-acclimated samples collected from three sites in Israel, one from north-west Italy and one from Kyrgyzstan. These sites differed in altitude and climatic characteristics (mean annual temperature and total annual rainfall). A negative correlation was found between the frequency of WR and the annual rainfall at the sites where samples were collected, suggesting that WR is not a fixed characteristic of this species. The observed differences between populations are discussed with reference to evolved adaptations to aridity and the past evolutionary radiations of the species.

Key words: amphibian, adaptation to aridity, green toads, opportunistic hydration

INTRODUCTION

Most amphibians do not drink; that is, they do not swallow water. Rather, they absorb water through their skin (Bentley & Yorio, 1979). Yet, outside the breeding season, terrestrial amphibians immerse themselves in water for only relatively short periods; frequently they satisfy their water requirements by pressing a highly vascularised region of the ventral abdominal skin, the "seat patch" (Hillyard *et al.*, 1998), against a moist surface. This behaviour, described in dehydrated anurans by Stille (1958), can be observed in some species in laboratory conditions, even in the presence of standing water (Tran *et al.*, 1992).

In a long series of experiments, Hillyard and co-workers investigated factors influencing the expression of this behaviour (reviewed in Hillyard *et al.*, 1998). They found, *inter alia*, large interspecific variations in the degree of dehydration needed to evoke a consistent water absorption response, and noted that the differences between species make sense in the light of their natural history and of the environment in which they are found. The implication of their observation is that there may be evolved differences in the toads' use of opportunistic hydration. Since each of the species they studied is confined to a fairly narrow ecological niche, it became of interest to compare the responses of toads taken from different populations of a single species that lives in a variety of climatic conditions.

Such a comparison could help to determine whether the conditions required to elicit the toads' water absorption response are a fixed and constant characteristic of the species, or vary from population to population in a way that is correlated either with their geographical distribution or with their type of habitat.

The green toad, *Bufo viridis*, provides an opportunity for this type of comparison. It is widespread in Eurasia, extending from Mongolia westwards to the Rhine, and from southern Sweden to the Iberian peninsula and across to North Africa. Distant populations inhabit widely different climatic conditions. In Israel, situated between Eurasia and Africa at the eastern edge of the Mediterranean Sea, the toad approaches the southern limit of its distribution. The "crossroads" geographical position of Israel has endowed it with a diverse fauna, which includes immigrants from both north and south (Nevo, 1988). It also has a steep north-south gradient of increasing aridity, in which a mesic Mediterranean climate gives way to Irano-Turanian steppe and then to Saharo-Arabian desert in less than 400 km. Populations of *B. viridis* are widespread throughout the mesic and steppic regions, and have spread into the desert, following the line of irrigation as agriculture has been developed (Werner, 1988). There are also small isolated, possibly relict, populations at a number of oases bordering the desert. The region, therefore, provides a good opportunity to compare populations of the toad living in different habitats within a small geographical area, and to see the extent to which they differ from each other and from those from more distant regions.

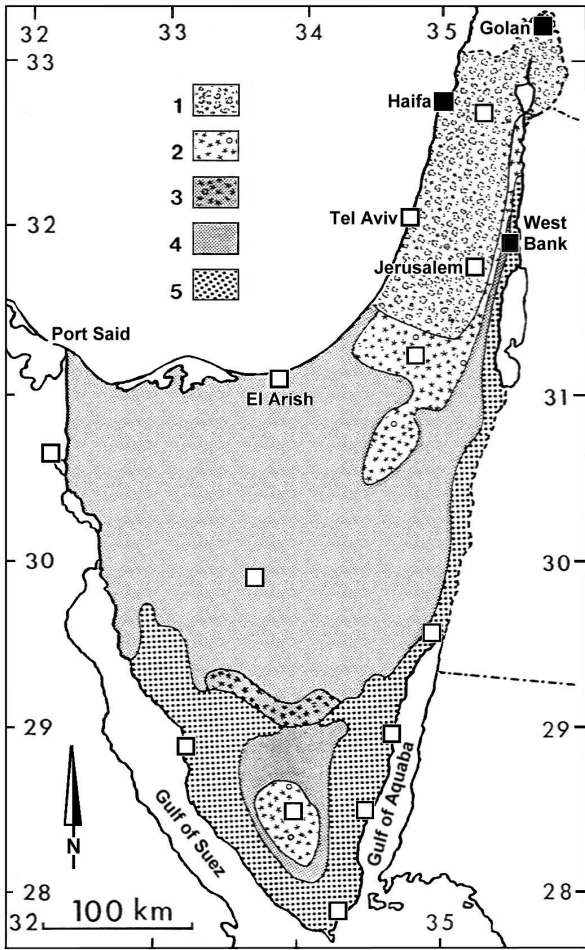


FIG. 1. Biogeographical regions of Israel and Sinai, based on Werner (1988). 1. Mediterranean; 2. Irano-Turanian; 3. Irano-Turanian/Saharo-Arabian transition; 4. Saharo-Arabian; 5. Sudanian penetration zone. Filled squares: collection sites in the present study.

In *B. viridis*, and probably in other species of terrestrial amphibians, the water absorption response consists of two steps. The first, water seeking with postural ad-

justment when moisture is detected (WR), is followed by the second, water uptake across the skin of the "seat patch" (Hoffman & Katz, 1999). In this study, I focused on the first step and compared the frequency of the response (WR) and its sensitivity to artificially raised plasma osmolality in samples of toads collected from three sites in Israel, from one in north-west Italy and one in north Kyrgyzstan. Since aldosterone, which is released under the influence of the hormone angiotensin, is known to augment the water seeking response in amphibians (Hillyard *et al.*, 1998; Hoffman & Katz, 1999), I also studied the effects of this steroid on the behaviour of toads from the different sites.

MATERIALS AND METHODS

Details of the samples of toads collected from the West Bank of the Jordan, Haifa Bay and Golan Heights areas of Israel, Vigone in north-west Italy and Kokjar in Kyrgyzstan are given in Table 1; those from the West Bank and Haifa were part of a study reported earlier by Hoffman & Katz (1999). Climatic data within Israel were obtained from meteorological stations, each within 25 km and at the same altitude as the specific collection sites. The microclimate within the West Bank wadi does not conform to the general pattern in Israel of an increase in aridity from north to south (Fig. 1), but surrounding areas at higher altitudes have a much lower annual rainfall (Meteorological Services Reports, 2000; unpublished Meteorological Services data, personal communication). Meteorological data for north-west Italy and for Kyrgyzstan are those given in Castellano & Giacoma (1998) and Castellano *et al.* (1999) respectively, and are based on the records for meteorological stations in Walter and Leith's Klimadiagramm-Weltatlas (1967).

Toads were collected at dusk, when they emerge from their shallow burrows, before they had an opportunity to reach a water source. They were acclimated to labo-

TABLE 1. Characteristics of the toads and climate conditions at the collection sites.

	West Bank	Haifa Bay	Golan	Vigone	Kokjar
Sample Size	10	10	12	9	11
Mean Standard toad weight (g)	38.3±9.97	34.9±3.80	36.5±13.33	23.3±1.71	48.6±10.44
Period of observations	Dec 1997 - May 1998	Mar 1998 - May 1998	Jan 2000 - Mar 2000	May 1998 - Jul 1998	May 1998 - Jul 1998
Altitude (m asl)	250	50	940	300	1350
Annual mean temperature (°C)	17.5	20.0	15.3	12.2	9.2
Total annual rainfall (mm)	450	399	800	680	375

ratory conditions for at least two weeks before testing. Throughout the acclimation and experimental periods they were maintained at room temperature ($20\pm 2^\circ\text{C}$) and were provided with a pool and dry area. They were fed mealworms, *ad lib*, once a week, but did not receive food during the 48 hr preceding an experimental trial.

The observations were made over a period of three years, and to test whether there were changes in the populations sampled or laboratory conditions during this period, WR frequency in untreated toads was replicated in additional samples taken from two of the sites: seven additional toads, mean standard weight (m.s.w.) 25.3 ± 4.62 g, from Vigone were the subjects in September 1999, and four from the West Bank (m.s.w. 37.7 ± 5.20 g) were observed in June 2000.

The experimental protocol was as described in Hoffman & Katz (1999), but note that that paper gives incorrect values for the quantities of NaCl injected. In brief, toads taken from the "home" dry area were catheterised in order to empty the urinary bladder, weighed, and introduced individually into observation chambers. Two filter paper discs, one dry and the other saturated with distilled water, were positioned on the floor of each container, and the animal was placed between them. Behavioural responses were monitored for up to 3 hr by observing the animals through angled mirrors beneath the chambers, the walls of which were covered to prevent the animals seeing their observers. WR was deemed to have occurred if the animal flattened itself on the wet filter paper and had gained weight after this response. Previous work has shown that neither catheterization, nor an i.p. injection *per se* (with 0.8% NaCl) affected WR frequency (Hoffman & Katz, 1999).

In each group, WR frequency was determined under three conditions: (1) untreated (control); (2) with increased plasma osmolality – the concentrating effect of dehydration to 7% weight loss was simulated by intraperitoneal (i.p.) injection of a NaCl solution (30 $\mu\text{mole}/100$ g toad); see Hoffman & Katz (1999); and (3) with increased plasma osmolality and aldosterone – 15 min after the above salt treatment, aldosterone (Sigma: Rehovot, Israel) was given by i.p. injection of 250 $\mu\text{g}/100$ g toad. In those groups that were very sensitive to the raised plasma osmolality (>80% WR response), the salt concentration was reduced by 50% to 15 $\mu\text{mole}/100$ g toad to allow a margin in which to test for any additional effect produced by aldosterone.

The toads were weighed and placed in the observation chambers immediately after completion of treatments 2 or 3. All observations were made between 0700 and 1200 hrs.

The dehydration threshold, i.e., the lowest level of dehydration at which all animals of a group exhibit WR on all occasions, was determined for toads from the Golan using the method of Hoffman & Katz (1999). Six bladder-emptied toads were subjected to rapid dehydration for 2-3 hrs in small wire cages in an airstream generated by a fan. At intervals, each was weighed, % weight loss was calculated, and WR behaviour was ob-

served until a level was reached at which all animals showed the response. The procedure was repeated three times; the least % weight loss at which all animals showed WR was taken to be the dehydration threshold. The same toads were treated with aldosterone and three further trials were carried out to establish the threshold after this treatment.

Because of the legal and ethical constraints on collecting this species in Israel and on importing them, the numbers of toads in the samples from all regions were necessarily small. Consequently, between 4 and 11 observations were made on each toad over a period of approximately three months, with a minimum of two days rest between handling. Possible systematic changes in the toads' behaviour over time were examined statistically using a sign test to compare the number of positive WRs made by each toad in the first half of the observations with that for the last half. To see whether there was heterogeneity in the responses of individual toads from the same site, the observed numbers of toads showing different proportions of positive responses were compared with the numbers predicted from a binomial distribution based on the average for the group as a whole. The *G*-test of independence was used to assess whether the proportion of positive responses differed between groups from different collection sites or the same site at different times. The effects of the three climatic factors on the response were analysed by stepwise regression (SAS; GLM procedure). A one-tailed paired *t*-test was used to compare the percentage of positive water responses of toads from the five sites before and after salt injection.

RESULTS

Levels of WR in untreated animals from populations from Vigone and the West Bank site remained stable over time (Table 2). Times of collection and study were therefore excluded from all analyses. There was also no evidence for any group of systematic changes in WR over time spent in the laboratory: when the groups were pooled, 27 of the 52 toads observed showed no difference in the number of responses in the first and last half of the observations, 8 responded more in the first half, and 17 more in the second half. The difference is not significant ($0.1 > P > 0.05$). Table 3 shows there is also no evidence of heterogeneity between toads from the same site: the observed numbers of toads showing different proportions of positive responses are very close indeed to those predicted from a binomial distribution based on the average for the group as a whole. It was therefore assumed that repeated observations on the same toad were independent of each other, and for statistical analyses observations on all toads within a group have been pooled and treated as a single set of observations.

Table 4 shows (1) the spontaneous expression of WR in fully hydrated toads differed between groups ($G_{[4]}=47.8$, $P<0.005$), i.e. it was not independent of site. It was very low (6-8%) in the groups from Golan (northern Israel) and Vigone (NW Italy) compared with those

TABLE 2. Water responses of toads collected and tested at different times.

Site	Year	Water response	No response	%WR
Vigone	1998 (May-July)	5	74	6
	1999 (Sept-Oct)	2	34	6
West Bank	1998 (Dec-May)	12	38	24
	2000 (June-July)	5	19	21

TABLE 3. Number of responses made by each toad compared with the number expected on the basis of the binomial distribution. (O is the observed number of toads making each number of responses; E is the number expected from a binomial distribution based on the mean number of responses of all toads from the site.)

Site	West Bank		Haifa Bay		Golan		Vigone		Kokjar	
Number of toads	10		10		12		9		11	
Observations per toad	5		4		8		11		6	
Number of responses	O	E	O	E	O	E	O	E	O	E
0	3	2.5	0	1.4	6	6.2	5	4.6	0	0.3
1	4	4.0	4	3.5	4	4.3	3	3.2	2	1.6
2	2	2.5	5	3.4	2	1.3	1	1.0	3	3.1
3	0	0.8	1	1.4	0	0.2	0	0.2	5	3.3
4	1	0.1	0	0.2	0	0	0	0	1	1.9
>4	0	0	-	-	0	0	0	0	0	0.6

from the other three sites. (2) Increasing the plasma osmolality by salt injection increased WR frequency in all groups ($t_{[4]} = 8.29, P < 0.005$). (3) In the two groups with low levels of WR in the untreated state, treatment with salt and aldosterone did not significantly increase WR frequency above that of salt alone, although large increases were seen in each of the other three groups.

There was a significant negative correlation ($r = -0.923, P < 0.05$) between % WR and total annual rainfall in the areas from which the groups were collected. Neither altitude, mean temperature, nor the interactions between the three climatic factors exert any further significant influence ($P > 0.05$).

The dehydration threshold of toads from the Golan was found to be marginally less than an 8% loss of their

standard weight, and treatment with aldosterone had no effect on this threshold. This compares to a threshold of 14% loss, reduced to 7% by aldosterone, in toads from the West Bank (Hoffman & Katz, 1999).

DISCUSSION

The results show that there were differences between the spontaneous water seeking response of untreated, fully hydrated *B. viridis* that had been collected from different sites and acclimated to laboratory conditions.

The phrase "fully hydrated" has not been used consistently in the literature, but here, as in a previous study (Hoffman & Katz, 1999), I use it in the sense that the toads had no apparent drive to enter water, choosing to

TABLE 4. Percentage water response in toads, *Bufo viridis* (number of individual observations in parentheses).

Treatment	West Bank	Haifa Bay	Golan	Vigone	Kokjar
none	24 (50)	39 (44)	8 (96)	6 (79)	44 (62)
NaCl	60 (20)	90 (20)	41 (64)	30 (71)	85 (20)
NaCl + aldosterone	95 (20)	-	41 (56)	39 (36)	-
NaCl (half strength)	40 (10)	70 (20)	-	-	50 (24)
NaCl (half strength) + aldosterone	-	100 (20)	-	-	93 (28)

sit in a dry area when a pool was freely available. Of course, this does not mean that all had the same total water content and urine volume, only that behaviourally they showed no signs of dehydration.

In captivity, even when housed in a group, these toads act individually regarding when they enter a pool and how long they spend in water, only entering water together after feeding or handling. So, if left undisturbed, it can be assumed that animals in the dry area of the terrarium have been out of water for different periods. In still air, in the controlled conditions of the laboratory, the amount of evaporative water loss (EWL), and hence the plasma osmolality of an individual, will depend on when it was last in water, so a group will be made up of animals with different plasma osmotic concentrations. Since raised plasma osmolality stimulates the water response (Hoffman & Katz, 1999), it follows that the level of spontaneous WR in a group probably reflects the mean period between immersions. On this basis, the results in Table 4 suggest that of any four West Bank toads taken at random from the dry area of the terrarium, one is near the level of plasma osmolality that stimulates WR; in contrast, only rarely is a toad from the Golan in this state. Stated another way, the data suggest that Golan toads enter water more frequently. The differences between the groups are not a direct outcome of weight-related differences in EWL, since toads from the Golan and from NW Italy (Vigone) have similar % WR (Table 4) even though the animals from the Golan are, on average, more than 50% heavier than those from NW Italy (Table 1). The WR is, however, correlated with the total annual rainfall in the areas from which the toads were collected: those from more arid areas showed a more frequent response.

There are several possible interpretations of the observed differences. One is that they are chance outcomes of genetic changes that occurred in the populations during periods when they were isolated. Nevo (1988) claimed that *B. viridis* has a continuous population structure in Israel, but the findings of Maxson (1981), who included populations of *B. viridis* from six sites in Israel in a wider survey of phylogenetic relationships of Eurasian *Bufo*, do not support this. Using an immunological technique to measure differences in the amino acid composition of albumin, she estimated that "a mesic population from northern Israel near the Lebanese border" (i.e. Golan) had been isolated from the others for 0.6-3 million years. The marked difference in WR and sensitivity to aldosterone found in the present study could therefore be the result of divergent genetic changes that occurred while toads in the Golan were isolated from those now found further south.

The present indigenous fauna of Israel includes immigrants from both north and south (Nevo, 1988), so today's populations of *B. viridis* could be the descendants of two independent migrations. According to Savage (1973), the species radiated from Asia during the Pleistocene (Great Ice Age) and followed two major migratory routes. One was westwards across Europe to

the Iberian peninsular, and the other went south-west, through what is now Turkey, with branches leading to the Indian subcontinent and the East Mediterranean coastal strip. Later, during the Holocene (Recent) epoch, the animals reached NW Africa via the European route. Today, populations of *B. viridis* are found along the North African Mediterranean coastal strip, including Morocco in the west and Egypt in the east. So, at least theoretically, toads could have reached Israel either via the long route, westwards across Europe and then eastwards along the North African coast, or by the shorter route from the north-east, or by both routes. However, a phylogenetic tree based on the analysis of nuclear DNA suggests they came by the shorter route (E. Balletto, personal communication). Balletto found that Asiatic toads of the *viridis* group (from 10 sites) and mid-European populations of *B. viridis* (from eight sites) formed two distinct clusters, which diverged a long time ago. On the same basis, the genetic distance between toads from the European sites and those from three sites in Israel (which included the Golan and West Bank) was estimated to be 3-4 times greater than the distance from the Asiatic populations. If, as Nei (1972) argued, genetic distance is related to the geographical distance of a migratory route, Balletto's data suggest that *B. viridis* entered the Levant only from the north. It therefore seems unlikely that the difference in WR between the Golan and the other two Israeli populations is a result of two independent migrations, one from Asia and one via Europe and N. Africa.

An alternative explanation is that the difference between populations in Israel is the result of evolutionary adaptations that occurred as *B. viridis* spread southwards into xeric habitats. As they moved into more arid regions, any behavioural and physiological adjustments that reduced the need for frequent access to water would have been an advantage. There would have been strong selection for the ability to tolerate higher plasma osmolality, which would allow longer intervals between entering water, and also, because of the favourable osmotic gradient across the skin, increase the ability to absorb soil-bound water through the water response. In the laboratory, a high WR was found only in toads from populations living in xeric habitats. Two geographically distant groups, those from Golan and Vigone, which both live in mesic conditions, showed a much lower response. However, the results following salt injection show that raised plasma osmolality, a state that toads living in mesic habitats probably rarely experience, did increase their water seeking response, indicating that they have the potential to adapt to more arid conditions.

The observation that all toads from the Golan population showed WR when they had lost only 8% of their weight through dehydration, whereas those from populations in xeric regions do not do so until they have lost 14% of their weight (Hoffman & Katz, 1999) is further evidence of the greater tolerance to raised plasma osmolality of toads from arid environments. *B. viridis* can survive more than 30% weight loss (almost entirely

due to lost water), so there is still an enormous safety margin, but the sensitivity to water loss seems to be partially suppressed in toads inhabiting the more arid regions. However, it can be reactivated by aldosterone: the present study shows that this steroid has no effect on the dehydration threshold of Golan toads, which remained at just under 8%, whereas in Hoffman & Katz's (1999) study of West Bank toads it reduced the dehydration threshold from 14% to 7%. If cues associated with the onset of the often unpredictable and irregular rainfall of arid environments are the stimulus for hormonal release, this could add flexibility to the behavioural response of toads in xeric habitats.

Travelling south from the Israel-Lebanon border today, the climate becomes increasingly arid, but there are no physical obstacles great enough to deter toads from migrating between the Golan area and regions further south. However, over the last 1.8 million years there have been great changes in paleogeography and environmental conditions in the region (Horowitz, 1988), and volcanic activity persisted on the Golan Plateau and neighbouring areas at least into the early Pleistocene (Garfunkel, 1988). Any one or more of these climatic or geological events could have temporarily cut off the northerly populations from those which had been established further south, and initiated genetic divergence through drift, the occurrence of different mutations in the populations, or selection for adaptations to dissimilar ecological conditions (Schluter, 2001). If the differences in the WR found in this study are the result of genetic divergence, they must be maintained either by strong selection, or by some degree of reproductive isolation that reduces gene flow between populations. Maxson's (1981) survey shows that a population only about 30 km south of the Golan (Dalton) has distinctly different albumin from that of the Golan toads, although DNA analyses have revealed no large differences between toads from the Golan and other Israeli locations. However, pre-zygotic isolation between populations can result from very few genetic differences (Coyne, 1992). An important pre-mating isolating mechanism in some amphibians is the advertisement call of the male, and Castellano *et al.* (2002) have shown that call patterns in *B. viridis* can differ more than the genetic distances between populations would predict. A comparative study of advertisement calls in the Golan and other regions would therefore be interesting, since selection in acoustically different environments could have led to divergence. Selection for a shift in the time of breeding and/or a change in the water temperature at which mating takes place could also lead to a mating barrier between populations. *B. viridis* in Sardinia breeds in water below the temperature at which those on the mainland are able to do so (Castellano & Giacoma, 1998), and the situation in the Golan may be similar, since at the turn of the year early rains are supplemented by the melting snows of Mt. Hermon, resulting in cool, fresh-water ponds and rivulets. At present we can only guess

at why the Golan population remains different from those in other regions in Israel, but it is reasonable to assume that any physical barrier that persisted long enough for genetic divergence in the sensitivity to water loss and use of WR would also have allowed divergence in potential isolating mechanisms.

There is a further possible explanation of the differences in WR frequency in toads from different populations: use of the response may be determined by the conditions in which the toads developed rather than by genetic differences. In the present study all toads were acclimated to laboratory conditions before testing, and their behaviour did not change over the months spent in the laboratory, but their behavioural phenotype may have been permanently set by the conditions they experienced in the pre-adult stages. If so, the differences in WR between samples from different sites reflect the behavioural plasticity of the species, rather than genetic divergence between populations. The similarity of the response of geographically distant populations inhabiting areas with similar annual rainfall is consistent with this interpretation, as rainfall determines the availability of the moist areas that are essential for the survival of vulnerable post-metamorphs, although the microclimate of the places where each individual develops will be the ultimate determining factor. If the frequency of WR is a response to the conditions experienced during ontogeny, then local variations in rainfall and other conditions would be expected to lead to differences between samples taken in different years. None were found in the present study, but the samples were small; it would require sampling over several years coupled with a detailed analysis of the microclimate at the sampling sites to determine the influence of juvenile conditions on the adult WR.

Whatever the explanation of the differences, this study of WR in populations of the same species living in very different climatic conditions has indicated a relationship between behaviour and habitat. The adaptation to aridity is apparent only in the water-seeking response, which is the first of the two steps involved in rehydration. In separate experiments (not reported here), no difference was found in the rate of water uptake in toads from the West Bank and the Golan, or in the effect of oxytocin on that rate. Reshetnikov (1998) has found that *B. viridis* is able to detect vapour pressure gradients, and judging from the observations reported here, in which the toads that exhibited WR rarely explored the observation chamber but immediately moved towards the wet disc, they can sense the presence of moisture from a distance of more than 30 cm.

Optimal exploitation of all water sources is vital to amphibians in xeric habitats, and adaptations such as an increased use of WR probably contributed to their ability to survive in these conditions. Whether or not the adaptation is a result of genetic changes or reflects developmental plasticity that is present in all populations of *B. viridis* remains to be determined. Ideally, newly-

fertilized egg strings from different populations would be reared in the laboratory in a variety of conditions, but technically this would be difficult and probably is not feasible. Sampling more toad populations from sites throughout Israel and neighbouring countries over several years, accompanied by close monitoring of the local climatic conditions, would be an alternative approach. There is also a clear need for a more detailed phylogeographic analysis of the toads in these regions using mitochondrial and satellite DNA, which might indicate whether an investigation of possible isolating mechanisms would be worthwhile. There is certainly a lot more work to be done, but the results presented here suggest that studies of *B. viridis* could yield valuable insights into how terrestrial amphibians have been able to move into arid regions.

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