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Original Investigation

Effects of sewage-water contamination on the immune response of a desert bat

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ABSTRACT

Environmental pollutants may negatively affect the immune system of animals. Yet, this phenomenon has not been studied thoroughly in terrestrial animals that use polluted water for drinking and/or foraging. We experimentally tested the hypothesis that exposure to sewage water would affect the activation of the immune response in the bat *Pipistrellus kuhlii* that drinks from bodies of open water. We selected two water sources where bats forage in the Negev desert, Israel: natural springs and a sewage-polluted man-made reservoir. We captured 13 non-reproductive female bats in the vicinity of the natural springs and offered seven of them water from the sewage-polluted source for 30 days (treatment) and the remaining six bats were offered water from the natural spring (control). Consumption of contaminated water did not alter the bactericidal ability of blood plasma or the proportions of monocytes circulating in the blood. However, our data provided evidence that the 30-day treatment can cause a decrease in the relative levels of neutrophils and an increase in the levels of lymphocytes. Our study provides a first account for the effect of sewage pollution on bat immune response which may be important in desert environments, where water sources are scarce. We suggest hypotheses for future, more focused studies.

Introduction

Anthropogenic habitat degradation or pollution can negatively affect animal health (Anderson and Maldonado-Ocampo, 2011). In particular, environmental pollutants may compromise the immune system of animals, as has been shown in a variety of taxa (e.g. Rohr and McCoy, 2010). Aquatic environments are highly sensitive to human-induced contamination (Schwarzenbach et al., 2006). Correspondingly, the effects of water contamination on vertebrate immune systems have been studied mainly in aquatic organisms (Christin et al., 2003; Milla et al., 2011). Nonetheless, the immune system of terrestrial animals that use water for drinking or bodies of open water for foraging may also be affected by pollutants (Conrad et al., 2005; Kozul et al., 2009). The risk to animal health may be exacerbated in desert environments where water is scarce and animals may be obliged to use vital but contaminated water sources. Although water pollution may have detrimental effects on desert mammals (O'Shea et al., 2001), direct evidence for the effect of drinking water quality on the immune response of mammals in desert environments is lacking.

The vertebrate immune system is composed of innate and adaptive lines of defense. The innate immune system is non-specific and includes various phagocytic cells such as neutrophils and monocytes that engulf and kill foreign cells, and are recruited in large numbers upon infection (Weiss and Wardrop, 2010). The complement component of the innate immune system involves proteins that lyse foreign cells, trigger the recruitment of inflammatory cells as well as alert other aspects of the immune system, including cells involved in memory responses (Song et al., 2000). The adaptive immune system is specific in nature and its responses are carried out by T and B lymphocytes, which are usually activated by signals received from the innate immune system (Weiss and Wardrop, 2010).

Monitoring the composition of white blood cells (WBC) is a useful tool for ecologists who aim to detect effects of external factors on vertebrate immune response (Davis et al., 2008). Sewage effluents contain a mixture of abiotic and biotic pollutants, which may be manifested by changes in the composition of WBC (Maceda-Veiga et al., 2010). An effect of sewage effluents on immune response may thus be detected by analyzing leukocyte profiles expressed as differential WBC counts (i.e. proportion of different WBC types) prior to and after treatment with polluted water. Indeed, differential

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WBC counts were previously used to assess vertebrate immune response to various factors including sewage pollution (Suorsa et al., 2004; Cottontail et al., 2009; Maceda-Veiga et al., 2010). This approach can be supplemented by using additional indices of immune function such as measuring bacterial killing ability (BKA) of complement proteins in blood plasma – a functional measurement of innate immune response (Tieleman et al., 2005).

In Israel, bats are a sensitive group with high conservation priorities. In addition, insectivorous bats can be excellent bioindicators of habitat quality, including water quality (Jones et al., 2009). Some bats are more tolerant than others to sewage-contaminated foraging environments as seen from bat activity (Park and Cristinacce, 2006; Kalcounis-Rueppell et al., 2007; Abbott et al., 2009) but no study that we know of has examined the effect of sewage water on bat immune function. Here, we tested the effect of sewage water on the immune response of the insectivorous bat *Pipistrellus kuhlii* (Kuhl, 1819). *Pipistrellus kuhlii* uses natural and man-made water sources (e.g., artificial ponds) as foraging and drinking sites (Korine and Pinshow, 2004).

Our goal was to estimate the effect of sewage-pollution on bat immune response in order to provide a basis for future studies. We hypothesized that a prolonged exposure to sewage water would alter the immune response of bats. We tested this hypothesis by offering sewage-polluted water to bats captured in a natural, unpolluted site and measuring their immune response to the treatment.

Material and methods

Study sites and water analysis

The study was done in the Negev Desert Highlands, Israel, where water sources are scarce and spatially separated. We selected two sites which were permanent water sources rich with insects. The first, clean, site was located 2–3 km from the national park Ein Avdat (30°50′N, 34°53′E), where bats forage over a complex of natural ponds (Razgour et al., 2010). The second, polluted, site was the Yeruham Reservoir (30°59′N, 34°53′E), an artificial reservoir used by bats for drinking and foraging (Korine and Pinshow, 2004). During the course of this study Yeruham reservoir was receiving untreated sewage water.

To estimate the quality of the water at the two study sites, we collected water at one representative pond in Ein Avdat (20 × 5 m) and around the shore of Yeruham Reservoir, once a month from June to August. During each sampling period, we collected the water at 3–4 locations around the Ein Avdat pool and at 7–10 locations around the shore of the reservoir (distanced ca. 100 m from each other), where bats were observed foraging and drinking. To verify a proper selection of the two sites we estimated the general differences in water quality between them. Specifically, we measured four indices of water quality: chemical oxygen demand (COD), biological oxygen demand (BOD), fecal coliforms (FC) and total suspended solids (TSS). These are commonly used indices for biotic water analysis, with well-established and standard methodology (Nollet, 2007). High values of these indices indicate sewage pollution and generally low water quality. In particular, fecal coliforms are a good proxy for the presence of pathogenic bacteria, at least for humans (Nollet, 2007). All water analyses were done at the Zuckerberg Institute for Water Research, Ben-Gurion University of the Negev.

Experimental design

We tested the immune response of bats captured at the clean site but exposed to water from the polluted site. Using mist nets, we captured 13 non-reproductive female *P. kuhlii* in the clean site during July 2010. Despite extensive efforts, very low capture success rates impeded us from collecting more bats and from collecting bats at the polluted site. During acclimation to captivity (5–14 days), bats were trained to eat mealworms (*Tenebrio molitor* larvae) independently and were offered water ad libitum from the clean site. After the end of the acclimation period (day 0 for the experiment), we randomly assigned each of the 13 bats to one of two groups: control (bats offered water from clean site; $n = 6$) and treatment (bats offered water from polluted site; $n = 7$). During acclimation, the bats were housed as a single group. For the duration of the experiment, they were housed in two separate groups, control and treatment, in the same outdoor aviaries.

To examine the association between water quality and immune function we measured (1) leukocyte profiles using differential WBC count and (2) BKA of plasma at day 0 and after 30 days of exposure to water (day 30) for all bats. We stored the water from each site at 4 °C because this is the temperature at which bacterial samples are usually kept for frequent use. We administered water at room temperature to the bats and replaced it every other day with fresh water collected from the two sites.

During the 30 days of the experiment (between day 0 and day 30), bats were fed mealworms ad libitum and their body mass was measured daily with a digital balance (PPS200, Pesola, Switzerland, ±0.01 g). We calculated a body condition index as the residuals of an ordinary least squares linear regression of body mass against forearm length (Reynolds and Korine, 2009). To monitor individual differences in water consumption, we gave the bats water (separately for each individual) on a daily basis and recorded the volume of water consumed by each bat with an accuracy of ±5 μl.

On days 0 and 30 we collected 10–20 μl of blood from each bat in a heparinized capillary (75 μl capacity, Marienfeld, Lauda-Königshofen, Germany) by puncturing the cephalic vein with a 27 G needle (Voigt and Cruz-Neto, 2009). We immediately used whole blood to prepare blood smears, centrifuged (Hettich Mikro-22R, GMI Inc., USA) the remainder for 10 min at 3000 rpm to separate plasma from formed elements, and stored plasma at −80 °C until BKA assays were performed. Samples of control and treatment groups were frozen for the same amount of time (3–7 days).

Bat captures and experimental procedures were reviewed and approved by appropriate committees and were conducted under license # 34615 given to CK by the Israel Nature and Park Authority (for animal captures) and license #BGU-R-02-2009 given to SP (for animal care protocol). We released all the animals after the experiment at the site of capture.

Immunological assays

We prepared blood smears using the wedge technique (Brown, 1993) and staining with an eosin-thiazine stain (Dip-Quick Stain Set, Product #J322, Jorgensen Laboratories, USA). We analyzed the WBC profile of the bats using the cross-sectional method of differential counting (Brown, 1993). For each bat, we examined 2–4 smears under a ×1000 magnification, until a total of 200 WBC per smear were counted. Counts were averaged across smears. We differentiated between neutrophils, lymphocytes, monocytes, eosinophils and basophils based on cell morphology (O'Connor, 1984; Brown, 1993).

Our BKA assay quantified the killing ability of bat plasma and followed Moore et al. (2011). Briefly, we made a stock of diluted *Escherichia coli* (ATCC #8739, E^{power} Microorganisms #0483E7, MicroBiologics, St. Cloud, MN, USA) that would result in the formation of 200–300 colony forming units when 50 μl aliquots were plated on agar. The killing ability of the blood plasma was compared to that of a control in the following way. Control vials were prepared by mixing 60 μl of the *E. coli* stock in 840 μl media [5 ml CO_2-independent media (product # 10010023, Invitrogen, CA, USA)

mixed with 100 μl L-glutamine (product # G7513, Sigma–Aldrich)]; test vials were prepared by mixing 20 μl of the *E. coli* stock with 280 μl of 1:100 plasma dilution (3 μl plasma in 297 μl media). For each test vial, we plated two plates at 0 min and three plates after 60 min of incubation at 25–28 °C. We plated a set of 6 control plates at time 0 and prepared 6 additional plates after 60 min of incubation. We incubated all plates at 37 °C for 8 h after which we counted the colony forming units. Bactericidal ability (% bacteria killed) was calculated as % change in control plates – % change in experimental plates (Moore et al., 2011).

Statistical analyses

We compared the water quality between the sites regardless of month or specific spatial variation within the water source itself by pooling all data across months and locations around the pool (our unit of replication was a single water sample) and using Mann–Whitney *U* tests to compare between sites for each water quality index.

To assess the effect of treatment on WBC, we used three linear mixed effects models (one for each WBC type) with experimental day (0 vs 30), group (control vs. treatment) and their interaction (day × group) as explanatory variables, bat ID as a random factor and neutrophils, lymphocytes and monocytes as response variables. These models were fit using the lme function in the nlme package in R (Pinheiro et al., 2011; R Development Core Team, 2012). This allowed us to control for individual variation within bats (Zuur et al., 2009). Each of these models had four coefficients, representing the additive values of the WBC type for the different groups and days. The reference level in these models was the control group at day 0. Note that although WBC types may act as dependent entities, we did not use a multivariate approach because a multivariate approach would not allow us to test for group × day interactions, which was our statistical goal, and because we had only three blood types, which would result in negligible power for multivariate analysis.

An effect of treatment after 30 days would be indicated by a difference between groups at day 30 but not at day 0. In statistical terms, this is expressed by the interaction term of the model. If indeed water treatment has an effect, then a model that includes a group × day interaction should provide a better fit to the data than models that do not include the interaction term. Thus, for each WBC type, we compared a set of five models using a model selection approach (Burnham and Anderson, 2002), including a global model (with an interaction term) and a null model, following Pilosof et al. (2012) (Table 1). However, because even the best model in a set of models does not necessarily provide a good fit to the data, we also calculated pseudo-R^2 values (hereafter regarded as R^2) as suggested by Nagelkerke (1991) for each model as a measure of model fit. The value of R^2 is based on maximum-likelihood estimations of the null model (i.e., intercept-only model; Table 1) and the model in question. This gave us a second measure to evaluate the importance of each model term because the ratio of the likelihoods reflects the improvement of the model in question over the intercept (i.e., null) model.

We compared models using model probabilities (w) based on corrected Akaike Information Criteria (AICc), which gives a measure of the plausibility, on a 0–1 scale, that a certain model is the best model (Burnham and Anderson, 2002). Further, we averaged model coefficients according to w across the five models and we present results for averaged coefficients. For evaluation, we use a measure of coefficient importance, calculated as the sum of w across all the models in which the coefficient appears. Coefficient importance ranges between 0 (no effect at all) and 1 (maximum importance). Model selection and multi-model inference processes were done with package MuMin in R (Barton, 2013).

Table 1
Comparison of models used to test the effect of experimental group (control vs. treatment) and day (0 vs. 30) on differential leukocyte profiles in the blood of *Pipistrellus kuhlii*. Models for neutrophils, lymphocytes and monocytes are linear mixed-effects models (bat ID as a random factor) whereas those of bacterial killing ability are two-way ANOVA (see Methods section for details). Models are ranked from the most supported (best model) to the least supported according to corrected Akaike information criteria (AICc). ΔAICc – difference in AICc between the current and best model; w_i – model probabilities. R^2 – a measure of variance explained based on model log-likelihood as suggested by Nagelkerke (1991).

Model ranks	Model structure	ΔAICc	w_i	R^2
Neutrophils				
1	~Group + day + group:day	0	0.87	0.8
2	~Day + group	3.87	0.13	0.74
3	~Day	9.04	0.01	0.64
4	~Group	28.06	0	0.26
5	Null	33.07	0	0
Lymphocytes				
1	~Group + day + group:day	0	0.89	0.79
2	~Day + group	4.29	0.1	0.72
3	~Day	8.86	0.01	0.62
4	~Group	27.11	0	0.24
5	Null	31.49	0	0
Monocytes				
1	~Group + day + group:day	0	0.84	0.66
2	~Day + group	4.34	0.1	0.55
3	~Day	5.26	0.06	0.47
4	~Group	17.72	0	0.14
5	Null	18.94	0	0
Bacteria killing ability				
1	Null	0	0.62	0
2	~Day	2.45	0.18	0.03
3	~Group	2.74	0.16	0.01
4	~Day + group	5.56	0.04	0.04
5	~Group + day + group:day	9.47	0.01	0.04

We further tested the effect of group and day on BKA with a two-way ANOVA because we resampled only 2 and 3 bats in the control and treatment groups, respectively, forbidding the use of a mixed model. The two-way ANOVA was performed on all bats. Our failure to resample bats was a result of technical difficulties in withdrawing sufficient volume of blood for BKA analysis from the bats. We performed the same model selection procedure applied to WBC on BKA.

We used repeated measures ANOVA (individual bats as subjects) to examine differences in water consumption, body mass and body condition between experimental groups across the 30 days of the experiment.

Results

Our measurements confirmed the differences in water quality between the sites: water quality in the polluted site was lower than in the clean site as the four water quality indices were significantly higher in the polluted site (Table 2).

The model selection procedure showed considerable support for the effect of water treatment on the proportion of neutrophils, lymphocytes and monocytes, as the best models included the interaction term and explained a substantial amount of variation of WBC counts (Table 1). A model with an interaction term indicates that while no apparent differences between the control and treatment groups were noted at day 0, there were differences between the groups at day 30. Specifically, our models support the notion that after 30 days of water treatment, proportion of neutrophils decreased while that of lymphocytes and monocytes increased, in the treatment group compared to the control (Fig. 1 and Table 3). This was supported by high importance of the interaction coefficient in those models (Table 3).

Table 2
Comparison between Ein Avdat and Yeruham Reservoir for four water quality indices. Samples were collected at several locations around each water source. COD – chemical oxygen demand; BOD – biological oxygen demand; FC – fecal coliforms; TSS – total suspended solids; SE – standard error of the mean.

Index	Ein-Avdat ($n=11$)			Yeruham Reservoir ($n=26$)			Mann–Whitney	
	Mean (±SE)	Median	Range	Mean (±SE)	Median	Range	U	p
COD (mg O_2/l)	65.1 ± 12	57.2	21.8–117.1	128.2 ± 7.8	124.5	83.6–199.1	43	<0.001
BOD (mg O_2/l)	2.4 ± 0.3	2.3	1–4.25	16.6 ± 0.95	15.5	7.9–25	0	0.02
FC (colonies/100 ml)	110 ± 68.2	40	0–780	635.6 ± 260.6	127.5	0–5550	76	<0.001
TSS (mg/l)	40.4 ± 1.7	52	8–58	134.2 ± 2.7	112	64–300	0	<0.001

Table 3
Effect of experimental group (control vs. treatment) and day (0 vs. 30) on differential leukocyte profiles in the blood of *Pipistrellus kuhlii*. Coefficients were obtained by a multi-model inference procedure from five linear mixed-effects models (see Methods section and Table 1). Reference level in the models was the control group at day 0. SE – adjusted standard error; I – coefficient of importance, calculated as the sum of Akaike model weights (w from Table 1) across all the models in which the coefficient appears.

	Neutrophils			Lymphocytes			Monocytes		
	Coefficient	SE	I	Coefficient	SE	I	Coefficient	SE	I
Intercept	66.1	5.33		27.92	4.79		4.59	1.87	
Treatment	−6.22	7.28	0.99	3.8	6.56	0.99	−4.45	2.51	0.94
Day 30	−20.35	5.36	1	17.76	5.37	1	4.04	2.67	1
Treatment:Day 30	−8.52	7.25	0.87	9.53	7.18	0.89	5.95	3.4	0.84

We obtained quantification of BKA only for 8 (control: $n=4$; treatment: $n=4$) and 10 (control: $n=4$; treatment: $n=6$) bats at days 0 and 30, respectively. Unlike for WBC, day and group did not explain variation in BKA, since the null model was selected as the best model. Finally, our results for WBC and BKA were not an artifact of small sample size as shown by an analysis which simulated a data set on which we executed the exact same model selection procedure as for the field data (see Supplementary material).

There were no significant differences between the two groups of bats in water consumption (mean ± SD; control: 134 ± 52.9 μl, treatment: 172.7 ± 44.1 μl; $F_{1,11}=2.07$, $p=0.18$), body mass (control: 6.05 ± 0.54 g, treatment: 6.27 ± 0.75 g; $F_{1,11}=0.45$, $p=0.52$) or body condition ($F_{1,11}=0.009$, $p=0.92$). Thus, variation in water consumption, body mass or body condition between the two bat groups did not affect any of the results.

Discussion

Our goal was to assess the effect of water contamination on bat immune responses. Our experimental data supports an effect of water pollution on differential WBC count of the bats we tested despite our small sample size (also see Electronic Supplementary

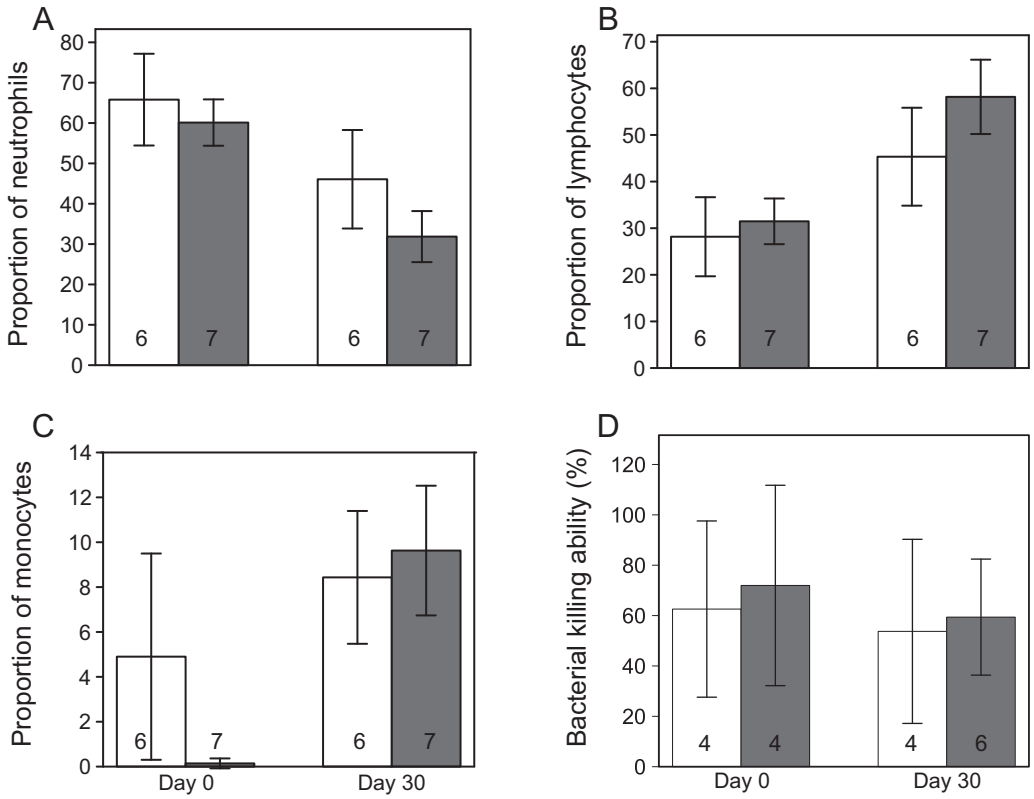

Fig. 1. Effects of experimental group and day on proportion of (A) neutrophils, (B) lymphocytes and (C) monocytes circulating in the blood, and on (D) the bacteria killing ability of *Pipistrellus kuhlii* blood plasma. Numbers inside bars are sample sizes. White bars are control group and gray bars are treatment group. Error bars are ±95% confidence intervals.

Material). Below we discuss possible mechanisms (biotic and abiotic) that could alter WBC proportions of bats.

Sewage pollution and the alteration of white blood cell counts

Sewage water often contains high levels of micro-organisms (Mitch et al., 2010). Chronic exposure to bacteria can cause an increase in levels of lymphocytes and monocytes (Beldomenico et al., 2008; Stockham and Scott, 2010) and thus in their proportion. We found a trend supporting this prediction, which was in line with the fact that the sewage water administered to our experimental group contained higher levels of coliforms compared to natural water, as shown by our water analyses (Table 2).

Another possible explanation for the observed trends is exposure of bats to abiotic pollutants. For instance, in Mediterranean barbell (*Barbus meridionalis*) at sewage-polluted sites, neutrophil and monocyte levels increased and lymphocyte proportions decreased by exposure to heavy metals (Zn, Cu, Fe and Hg) (Maceda-Veiga et al., 2010). In addition, recently collected data indicate that *M. lucifugus* bats exposed to Hg show the similar trends in neutrophils and lymphocytes as found in our study (Moore, unpublished). Recent studies have also reported accumulation of heavy metals in bats (Pikula et al., 2010), with consequences to DNA maintenance (Zocche et al., 2010).

Sewage pollution and bacterial killing ability

The effectiveness of the complement component of the innate immune system in eliminating *E. coli* was not different between treatment groups. If sewage water indeed has higher bacterial levels, this should entail an increase in complement activation, but only up to a certain point because over-activation of complement can be harmful to the host itself (Zipfel and Skerka, 2009). The 30-day experimental period might have been too long for constant activity of complement immune response. In addition, some pathogens evade or even down-regulate complement proteins (Zipfel et al., 2007; Lambris et al., 2008). For example, complement activity in fish exposed to bacteria during 35 days, a time course similar to that of our experiment, was down-regulated as adaptive immunity developed (Raida and Buchmann, 2009). Thus, our measurements may have been made after the response (if existed) was down-regulated by bacteria.

Conclusion

To the best of our knowledge, this study is the only examination so far of the effect of water contamination on bat immune response. Our results provide a starting point for future studies addressing effects of water contamination on immune response in bats and other terrestrial vertebrates. We are limited in our interpretation of the results because we could not corroborate the composition of pollutants in the water. However, the differences between the sites were maintained (clean vs. polluted), and thus our results do stimulate testable hypotheses for studies that will address immunological mechanisms more precisely.

For example, one possible and testable outcome of our results is that bats in our research sites drinking polluted water would invest resources in fighting infectious microorganisms while reducing allocation of resources to other aspects of the immune system (Cotter et al., 2011), making them more susceptible to parasitic attacks. Although experimental evidence is needed to support this hypothesis, our own field data indicate that abundance of ectoparasitic mites was higher in bats in the polluted than in the clean site (SP, unpublished data). An impaired immune response might contribute to this effect (Christe et al., 2000).

Finally, when terrestrial animals are limited in their selection of water sources, the effect of polluted water may be exacerbated (O'Shea et al., 2001). Thus, investigating the effects of water contamination on the immune response of terrestrial animals in arid environments is especially important.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mambio.2013.10.005.

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