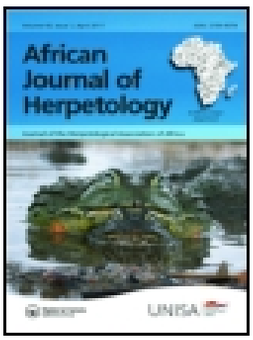

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Tolerance to salinity and dehydration in the Sahara Desert blue-eyed turtle, *Mauremys leprosa saharica* (Testudines: Geoemydidae) from a brackish pond in the Lower Draa basin, southern Morocco

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ABSTRACT

The marginal populations of the Sahara blue-eyed pond turtle, *Mauremys leprosa saharica*, in the southern-most species distribution range in the pre-Saharan areas of north-west Africa, are faced with extreme environmental conditions of arid climate and anthropogenic and climate change mediated water and land salinisation. In the current study, we investigated a small and isolated population of *M. l. saharica* at Sidi El Mehdaoui oasis in the Lower Draa River, southern Morocco, in order to assess its osmo- and iono-regulatory abilities and tolerance to salinity and dehydration. Upon capture, turtles were weighed and measured for shell dimensions and blood and voided urine were taken. Tests of exposure to different levels of water salinity (0%, 35%, and 50% seawater) and maintenance out of water (estivation simulation) were carried out. Osmolalities and Na^+, Cl^-, K^+, and urea concentrations were determined in plasma and voided urine, and glycaemia was measured in blood, before and after tests. Turtles were able to survive in brackish waters with a salinity as high as 24% seawater (8.4 ppt). Their voided urine was hypotonic to plasma, which indicated that they could use their bladder water reserves for osmo- and iono-regulation until the iso-osmocity level beyond which osmotic and ionic anhomeostasy can occur. Experimental tests showed that the osmo- and iono-regulatory capacities of these turtles are relatively limited, and not enough effective to allow them to survive for long-term periods in brackish/saline waters or out of water, because of dehydration indicated by progressive weight loss to a critical threshold. The increased drought, water salinisation and habitat fragmentation related to anthropogenic activities and climate change, represent great threats that can create habitats exceeding the species' threshold for a long-term persistence of the vulnerable small marginal populations of the Saharan pond turtle. So, conservation measures of these populations and their habitats are urgently needed.

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Introduction

The constraining environments of arid regions of north-west Africa are home to various animal species, including turtles: two terrestrial species: *Astrochelys sulcata* and *Testudo graeca* (Testudinidae), and one freshwater species: *Mauremys leprosa* (Geoemydidae) (Turtle Taxonomy Working Group 2017). If the physiological and/or behavioural characteristics that permit different New World freshwater turtle species to occupy some of the driest areas of North America and Australia are well documented (e.g. Dunson 1981; Seidel 1975; Grigg et al. 1986; Dunson and Seidel 1986; Peterson and Stone 2000; Roe et al. 2008; Bower et al. 2016; see also Agha et al. 2018), there is very little information on the physiological effects of osmotic stress in the Old World freshwater turtles (Gilles-Baillien and Schoffeniels 1965; Schoffeniels and Tercafs 1965), with no studies under natural conditions.

A turtle faced with the drying or highly salinised home pond has two options: migrating in search of more permanent water with lower salinity, or digging down to await more favourable conditions by estivating. Estivation is operationally defined as a terrestrial inactivity during dry seasons (Gregory 1982). Both strategies have been reported for arid-land freshwater turtles in US, especially in mud turtles, the Family Kinosternidae (Skorepa and Ozment 1968; Gibbons et al. 1983). These species inhabit areas where water is generally scarce and rainfall highly seasonal, which are quite similar to those of the Sahara blue-eyed pond turtle in north-west Africa. However, other behavioural strategies allow some freshwater turtles to reduce the physiological effects of brackish/saline conditions, such as moving along salinity gradients, drinking fresh water from surface sources, and reducing feeding (Bower et al. 2016; Agha et al. 2018). Many coastal (estuarine) species have evolved different morphological (e.g. functional lachrymal salt glands in the diamondback terrapin, *Malaclemys terrapin*) and physiological adaptations (e.g. regulation of blood, urea, and intercellular fluids) that allow them to tolerate, rather than avoid, saline conditions (Agha et al. 2018). In contrast, arid inland freshwater turtles have developed similar, but apparently limited morphological (i.e. cloacal urine bladder; see Jørgensen (1998)) and physiological adaptations to enable them to tolerate permanently relatively high-water salinity (e.g. Dunson 1981; 1983; Bower et al. 2016).

Maran (2010) reported several dead Sahara Desert blue-eyed turtles, *M. l. saharica*, on different occasions (1993 and 2009) in an isolated brackish water pond (~20% seawater) at the Sidi El Mehdaoui oasis, lower Draa, southern Morocco, during the hot and dry season, but the true cause of their death was not established. *Mauremys leprosa* kept during two weeks in 100% seawater, revealed to have non-efficient osmoregulatory abilities, but were able to tolerate, at least temporarily, high blood osmotic and ionic variations (Schoffeniels and Tercafs 1965). These authors suggested that the occurrence of this species in exceptionally saline habitats, would be rather incidental and in all cases temporary. Beside the problem of high salinity, water scarcity is the major constraint that forces freshwater turtles to use upland terrestrial habitats for estivation, with no access to food and water. They would then be exposed, for relatively long periods, to starvation and dehydration resulting in body mass loss.

The increase in both water scarcity and salinisation, under current climate change and anthropogenic impacts in many continental arid basins, places freshwater turtle species at risk of habitat drying up, fragmentation, and salinisation. Given that freshwater turtles are

more threatened than other taxa, understanding the role of water scarcity and salinity in determining their current distribution and evolution is of a high research priority. This would be also of a critical value to conservation managers in arid basins where increasing aridity and water and soil salinisation are predicted to have the greatest effects on species' ranges.

In the current study, we assessed the tolerance and physiological responses of the Sahara blue-eyed turtles to exposure to brackish water hyperosmotic to their body fluids and that was naturally avoided by turtles, and to total water and food deprivation (simulated estivation out of water). We investigated the osmotic and ionic turtles' responses from either their natural habitat at Sidi EL Mehdaoui (salinity of 24% seawater) or from experimental exposures to different water salinities (i.e. 35% and 50%). We hypothesised that the studied turtles would not be able to maintain homeostasis in water with salinity higher than 24% seawater and out of water and this would be reflected through reduced feeding in the former case (indicated by a decrease in glycaemia), resulting in a loss of body mass and increased concentrations of plasma osmolytes (sodium, chloride, potassium and urea) over time in both cases.

All animal procedures used in the current study were in accordance with guidelines of the European Council Directive (EU2010/63). The study received the approval of the Council Committee of research laboratories of the Faculty of Science-Semlalia, Cadi Ayyad University of Marrakech.

Materials and methods

Study site

The Draa River (Oued Draa), in southeast and southwest Morocco, the longest one in the country (1 100 km), is formed by the confluence of the Dadès and Imini Rivers in the High Atlas Mountains and ends in the Atlantic Ocean north of Tan-Tan, in south-west Morocco. The Draa Valley consists of several oases with palm groves of *Phoenix dactylifera*. The current study was carried out in mid-spring 2016 at Sidi El Mehdaoui, a Saharan oasis located at 27 km south to Tata in the lower Draa (29°29'03.66' N, 07°59'08.29' W; 453 m asl). Tata province extends from the southern slopes of the Anti-Atlas range in the Draa River that marks the border with Algeria and the Moroccan Saharan region (Figure 1). The oasis contains several discontinuous brackish ponds and saline puddles (Figure 2). The largest pond is 50 m long, 20 m wide and 2 m deep (Maran 2010). The riparian vegetation is dense and dominated by *Juncus maritimus*, *Tamarix aphylla*, *Nerium oleander*, *Phragmites australis*, *Acacia raddiana* and *P. dactylifera*. The aquatic animal community included turtles along with Lepinay's barbels (*Luciobarbus lepinayi*) and different species of insects, but no amphibians or watersnakes.

Analysis of physical and chemical characteristics of water

Water samples were collected in three main ponds and three isolated puddles. They were immediately measured for the following parameters: temperature, pH, electric conductivity, and dissolved oxygen, using a multiprobe analyser (EUTECH; ThermoFisher Scienfitific, CyberScan, PCD 650, Singapore). Water was taken at the same period

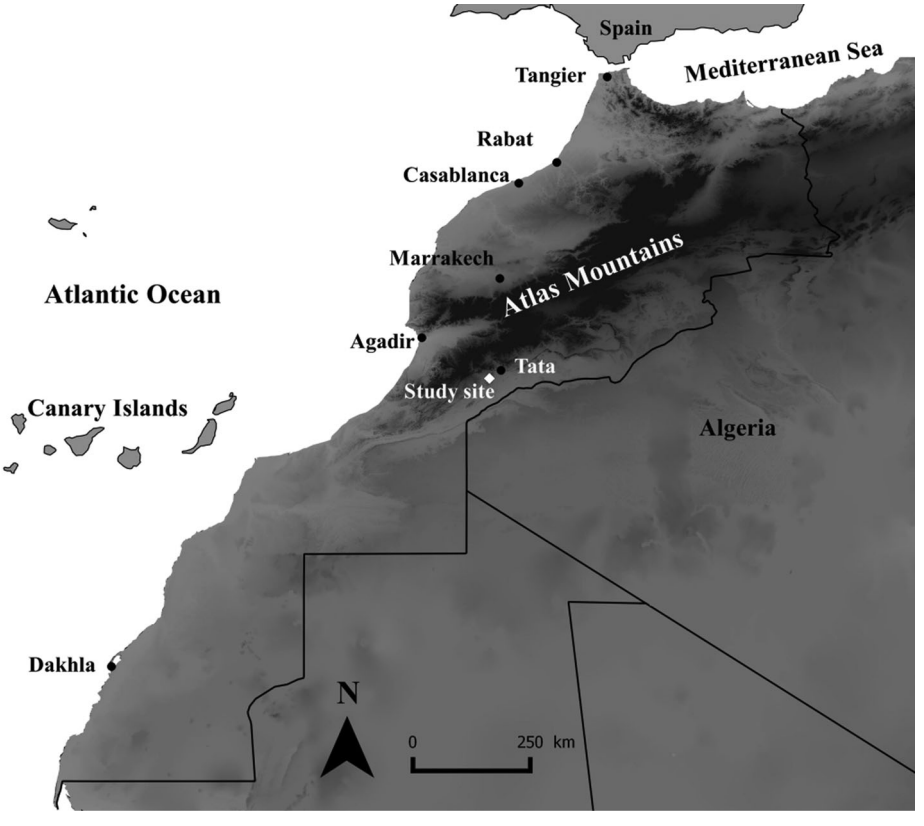

Figure 1. Map of Morocco representing the study site 'Sidi El Mehdaoui', Lower Draa, southern Morocco.

when setting traps for catching turtles. Water was sampled once in the middle of each habitat at mean depths of 10 cm and 40 cm (lowest depth of set traps), respectively for puddles and ponds.

Capture, weighing and measurement of animals

Given the small size of the studied population, only seventeen adult turtles (10 males and 7 females) were caught (under permit) using hoop traps baited with canned sardines. Upon capture, each specimen was weighed (to the nearest 0.1 g) then measured using a vernier calliper (to the nearest 0.1 mm) for the following shell dimensions: CL: carapace length, ACW: anterior carapace width, PCW: posterior carapace width, and H: maximal height of the carapace. Three small groups of 5 to 6 individuals were kept in aquaterraria (70 x 40 x 40 cm) filled to their one-third height with tap water and equipped with 75-Watt infra-red lamps and basking supports (Suber barks and bricks). They were fed three times a week with dried commercial trout pellets and rehydrated cat food or fresh sardines provided *ad libitum*. The average temperature in the room was 25 ± 2 °C and the photoperiod was 14 h light:10 h dark. The aquaterraria were cleaned three times a week.

Figure 2. Brackish ponds and puddle and mud with salt from Sidi El Mehdaoui, Lower Draa, southern Morocco. 1: Large pond, 2: Medium pond, 3: Puddle, 4: An adult *Mauremys leprosa saharica* with salt crust on the carapace (Photo: J Maran).

Body condition index

We calculated the volumetric body condition index (BCI), which allowed estimating body density as the ratio of the live body weight of the animal to its estimated volume in cm^3. This index expresses the weight status of the individual in relation to its size in terms of weight loss (wasting), overweight (accumulation of energy reserves) or normal weight (no loss or gain of weight). It was calculated using the following formula:

$$\mathrm{BCI} = \frac{\text{weight (g)}}{\text{volume } (cm^3)}$$

Where V = volume calculated by equating the shape of the turtle to an ellipsoid using the equation:

$$\mathrm{V} = \pi \times \mathrm{H} \times \frac{(\mathrm{ACW} + \mathrm{PCW})}{2} \times \mathrm{CL}$$

Collection of blood and voided urine

We collected samples of blood and voided urine in turtles upon their capture and in turtles kept in the laboratory, before (in controls, after 2 months) and at the end of the different experimental tests. Blood was taken from the caudal vein using a 2.5 ml heparinised syringe (lithium heparin). A blood droplet was used for measuring glycaemia

(see below) and then all the remaining blood was transferred into heparinised vacuum containers and centrifuged at 3 000 rpm (1 500 g). The plasma extracted is preserved in Eppendorf tubes, and stored at −20 °C for further analyses. Voided urine samples were eventually obtained during measurements otherwise they were removed by inserting a soft plastic pipette in the cloaca. All contaminated urine samples with faecal materials were excluded. The urine samples were stored at −20 °C for subsequent analyses.

Experimental tests

Turtles previously acclimatised in fresh water (three groups of 5 to 6 individuals per container) for a minimum of 60 days (controls), were divided into three experimental groups of 5 to 7 individuals each. Turtles of the first and second batches ($n = 5$ for each group) were tested for their tolerance to salinity, respectively at 35% and 50% seawater prepared with synthetic sea salt (Treasure Blue; Qingdao Sea-Salt Aquarium Technology Co., China). The experiments consisted of a forced immersion of turtles by placing them in aquaria (70 × 40 × 40 cm) filled to their half depth with salted water for a period of 15 days according to the protocol previously used by Schoffeniels and Tercafs (1965) for the same species. Turtles kept in salted water were also fed *ad libitum* with a mixture of commercial trout, cat pellets and fresh minced sardine balls; pieces of each kind of food were counted after all turtles stopped eating. The third group was tested for tolerance of turtles to water and food deprivation ($n = 7$). This group was kept out of water in a single large (90 × 50 × 25 cm) wood storage box with small holes for breathing and filled to a depth of 15 cm with dry vermiculite. The size of the box allowed enough space for all turtles to bury themselves without interference from others. The box was placed on a quiet air-conditioned room: the photoperiod was 14 h light:10 h dark, the ambient room temperature ranged from 25 to 30°C, measured using a thermocouple recorder (Omega, RD255, USA), and the relative humidity varied from 39 to 55%, measured using a digital hygrometer (Cole-Parmer, EXTECH, EW-39759-08, USA). Humidity inside the box was not recorded but would have been very likely higher and less variable. Turtles were left in the box until the loss of a maximal critical percentage range of 30–35% of their initial hydrated body mass was reached according to the values reported for other arid-land freshwater turtles (e.g. Ligon and Peterson 2002). During this experiment, every week (3 to 11 times depending on the individual), we gently excavated each turtle and quickly weighed it before being replaced where found in the box. Turtles were completely deprived of food and water (simulating estivation) for a period of a maximal critical body weight loss of 30% of initial body weight. At the end of the experiments, each dehydrated turtle from the three experimental groups was weighed (to the nearest 0.1 g) and placed in a separated plastic tubs filled to 1 cm of depth with tap water. After a turtle stopped drinking (for a maximum of 15 min), it was removed, dried with a paper towel, and then reweighed. The drinking rate was estimated as the change in body weight expressed as the percent of initial body weight.

At the end of the experiments, all (15 among 17) surviving turtles were taken back to their natural habitat in Sidi El Mehdaoui oasis in the following autumn. The two dead turtles were preserved in the collections of the Natural History Museum of Marrakech.

Physical and chemical analysis of plasma and urine

The osmotic pressure, concentrations of different osmolytes in the blood/plasma and urine were measured. We selected the ionic (Na^+, K^+ and Cl^-), and non-ionic solutes (urea and glucose), because these constitute the major contributors to the osmolality (see Dorwart and Chalmers 1975). The osmotic pressure was measured using a micro-osmometer (Kryometer, Löser, MODE200Plus, Germany). Na^+ and K^+ concentrations in the plasma and urine were performed using a flame photometer (AFP 100, FisherScientific, France). Chloride (Cl^-) concentrations were determined using the MOHR method (see Skoog et al. 1996). This latter determines the chloride ion concentration of a solution sample (plasma or urine in our case) by titration with silver nitrate. As the silver nitrate solution is slowly added, a precipitate of silver chloride forms. The endpoint of the titration occurs when all the chloride ions are precipitated. Then additional silver ions react with the chromate ions of the indicator, potassium chromate, to form a red-brown precipitate of silver chromate. The determination of urea was assayed using Urea Kits (BioSystems, REF 11536, Barcelona, Spain) according to the Berthelot's method (Berthelot 1859). This latter is based on the following principle: the urease hydrolyses urea to ammonia (NH_3) and CO_2. The ammonia formed further reacts with a phenolic chromogen and hypochlorite to form a green coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample. Glycaemia was measured in blood using a pre-calibrated Glucometer (BIONIME, GM110, Taiwan) based on electrochemical test strips to perform measurements. All measurements were made in duplicates and when the average values of measurements differed by more than 1%, samples were then re-measured after re-calibration of the corresponding used device or method.

Statistical analyses

Mean values are assigned to their standard deviations. Statistical differences among the different groups (for BCI and plasma/blood and urine variables) and between plasma and urine for a given group were tested using Duncan multi-comparisons (one-way ANOVAs) and Student's *t*-tests, respectively. The statistical significance was set at 5%. All statistical analyses were performed using the software SPSS v22.0.

Results

Characteristics of the aquatic environments and turtle population

The measured physical and chemical parameters of water in the investigated ponds and puddles (Figure 2) are shown in the Table 1. In all the pools, the pH was almost neutral with a value around 7. The salinity varied from 8.35 ppt (~24% seawater) in the large pond to 93.30 ppt (~267% seawater) in the smallest isolated puddle. The dissolved oxygen concentration was between 2 and 3 mg l^{-1} (32.2 to 36.06% saturation; sub-hypoxia) in the large, medium and little ponds, but lower (≈hypoxia) in the small isolated puddles varying from 0.69 to 1.75 mg l^{-1} (15.71 to 26.03% saturation).

Turtles were present only in the large pond. In previous visits [1993, 1997 and 2009 (Maran 2010) and 2012 and 2014 (personal observations)] to Sidi El Mehdaoui oasis, several turtles were found dead in the intermediate pond, which has a much higher salinity

Table 1. Physical and chemical characteristics of the different ponds and puddles at Sidi El Mehdaoui Oasis, Lower Draa basin, southern Morocco.

		pH	Temperature (°C)	Dissolved oxygen (mg l^{-1}) (% saturation)	Conductivity (µS cm^{-1})	Salinity (ppt) (% seawater)
Large pond		6.76	22.6	2.97 (36.06)	13 720	8.35 (23.86)
Medium pond		6.98	24.8	2.7 (35.09)	21 850	13.18 (37.66)
Little pond		7.03	27.2	2.32 (32.2)	29 480	17.38 (49.66)
Small isolated puddles	A	7.04	29.4	1.75 (26.03)	39 770	23.09 (65.70)
	B	7.01	29.2	1.45 (22.61)	53 460	32.25 (92.43)
	C	7.06	32.2	0.69 (15.71)	140 300	93.3 (266.57)

than the large pond and many turtles were coated with a pale mineralised layer on their carapaces (Figure 2). The captured turtles were small-sized with nearly 60% (19/32) of the sample comprising individuals of a shell size (CL) lower than 80 mm. In adult individuals, the mean carapace lengths were 102.94 ± 20.99 mm (range: 65.2 to 132 mm) and 104.38 ± 34.85 mm (range: 81.6 to 120 mm), respectively in males and females. The corresponding mean body weights were 172.68 ± 92.39 g (range: 49.1 to 323.9 g) and 134.56 ± 51.16 g (range: 86.2 to 248.8 g). There were no significant differences between sexes (Student's t-test = 0.107; df = 15; $p = 0.917$, and Student's t-test = 0.937; df = 15; $p = 0.364$, respectively).

Tolerance to salinity and dehydration

Weight loss and body condition index

Turtles maintained in fresh water (controls) ate voraciously and showed a net increase in their body weight of 1.70 ± 0.66% of initial body weight d^{-1}. However, when placed in 35% and 50% seawater, they fed much less (barely a few pellets/balls or not at all) than controls and lose an average of −0.57 ± 0.29% and −0.69 ± 0.22% body weight d^{-1}, respectively, with no significant difference between the two salinity tests (one-way ANOVA: $F_{2,24} = 54.75$; $p < 0.001$).

Turtles kept out of water lost a maximal critical percentage of up to 36% of their initial hydrated body mass over an average period of 58.5 ± 16.80 days of simulated drought (range 19–78 days). The mean rate of mass change (% initial body weight per day) during this period was −1.23 ± 0.43%. This average value is significantly lower than those of controls, 35%, and 50% seawater groups, but with no significant difference between these two latter groups (one-way ANOVA: $F_{3,30} = 67.34$; $p < 0.001$).

When provided with fresh water, turtles drank an average amount of water of 8.8 ± 3.21% of their final body weight. The corresponding values for turtles maintained in 35% and 50% seawater were similar (1.68 ± 1.12% and 1.75 ± 1.08 and %, respectively), but significantly much lower than those in out of water (one-way ANOVA: $F_{2,14} = 20.24$; $p < 0.001$) (Table 2).

BCI (g cm^{-3}) varied significantly among groups (one-way ANOVA: $F_{4,46} = 4.14$; $p = 0.006$). The mean BCI of turtles from the natural habitat was significantly lower than that of controls (0.174 ± 0.0285 g cm^{-3} vs 0.223 ± 0.0423 g cm^{-3}), but not significantly different

Table 2. Body weight change, body mass condition index (BCI) and amount of drunk fresh water in *Mauremys leprosa saharica*: Mean values ± standard deviation. (Number of individuals: 5–17). Different letters (a, b, c) indicate significant differences at $p < 0.05$.

Conditions	Body weight change (% initial body mass d^{-1})	BCI (g cm^{-3})	Water drunk (% body mass)
Natural conditions (24% seawater) ($n = 17$)	–	[a]0.174 ± 0.029	–
Experimental conditions			
Control (Fresh water) ($n = 17$)	[a]1.70 ± 0.66	[b]0.223 ± 0.042	–
35% seawater ($n = 5$)	[b]−0.57 ± 0.29	[a]0.189 ± 0.029	[a]1.68 ± 1.12
50% seawater ($n = 5$)	[b]−0.69 ± 0.22	[a]0.182 ± 0.038	[a]1.75 ± 1.08
Out of water ($n = 7$)	[c]−1.23 ± 0.43	[c]0.169 ± 0.06	[b]8.80 ± 3.21

to those in 35% and 50% seawater (0.174 ± 0.0285 g cm^{-3} vs 0.189 ± 0.029 g cm^{-3} and 0.182 ± 0.038 g cm^{-3}). These values are not significantly different from that obtained in turtles maintained out of water (0.169 ± 0.060 g cm^{-3}), but significantly lower than in controls (Table 2).

Osmolality and osmolyte concentrations of plasma and urine

Plasma osmolality (mOsm l^{-1}) was highly significantly different among groups (one-way ANOVA: $F_{4,46} = 7.08$; $p < 0.001$) (Figure 3). Turtles from the natural environment had a mean plasma osmolality significantly higher than that of controls (291.36 ± 31.41 mOsm l^{-1} vs 261 ± 38.64 mOsm l^{-1}). The turtles placed in 35% and 50% seawater and those kept out of water had plasma osmolalities not significantly different of those from the natural environment (322.20 ± 27.14 mOsm l^{-1}, 344.3 ± 45.33 mOsm l^{-1} and 316.38 ± 44.86 mOsm l^{-1} vs 291.36 ± 31.41 mOsm l^{-1}). On the other hand, urine osmolalities differed very significantly among the five groups (one-way ANOVA: $F_{4,46} = 98.34$; $p < 0.001$) in the following ascending order: controls < natural environment < out of water < 35% seawater < 50% seawater (41.25 ± 16.78 < 181 ± 41.90 < 258.67 ± 59.53 < 289.17 ± 29.3 < 332.17 ± 42.80 mOsm l^{-1}). The urine (U) to plasma (P) osmolality ratio (U/P) was low (16%) (hypo-tonicity) in controls; this difference was highly significant (Student's t-test = 21.51; df = 32; $p < 0.001$). For turtles maintained in 35% and 50% seawater and those out of water, this ratio was higher and close to isotonicity (89.75% and 96.47% and 81.76%, respectively), with no significant differences for both groups (Student's t-test = 1.85; df = 8; $p = 0.102$; Student's t-test = 431.61; df = 8; $p = 0.674$, and Student's t-test = 2.05; df = 12; $p = 0.631$). As for the turtles from the natural environment, this ratio was intermediate of 62%, indicating a state of moderate hypotonia; the difference was highly significant (Student's t-test = 8.69; df = 32; $p < 0.001$).

The mean values of plasma Na^+ concentration (mM) varied very significantly among groups (one-way ANOVA: $F_{4,46} = 28.30$; $p < 0.001$). In turtles from the natural brackish pond, mean plasma Na^+ concentration was higher, but not significantly different from those in controls (143.19 ± 6.73 mM vs 131.52 ± 4.64 mM). These values are significantly lower than those in turtles maintained in 35% and 50% seawater or out of water, which are not significantly different from each other (159.16 ± 15.87, 178.26 ± 18.84 and 158.62 ± 11.87 mM, respectively).

The Na^+ concentration (mM) in urine varied also very significantly among groups (one-way ANOVA: $F_{4,46} = 27.20$; $p < 0.001$). Turtles from natural brackish pond, those kept in 35%

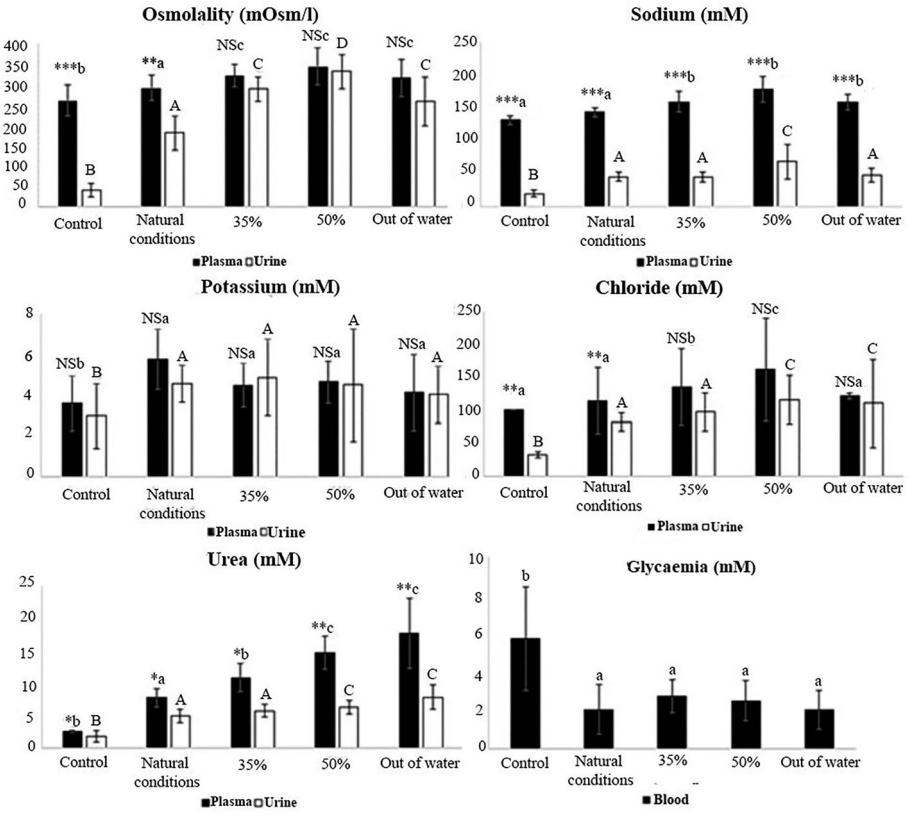

Figure 3. Osmolality, Na^+, K^+, Cl^- and urea concentrations in plasma and voided urine and glycaemia in blood of *Mauremys leprosa saharica* adapted to different hydration and salinity conditions: Mean values ± standard deviation. (Number of individuals: 5–17). Different letters (Lowercase for plasma and capital letter for urine) indicate significant differences at $p < 0.05$. Significant differences between means in plasma and urine for a given parameter are indicated as follows: * < 0.05 significant, ** < 0.01 very significant, *** < 0.001 highly significant, NS >0.05 non-significant.

seawater and those out of water were not significantly different and were twice as high as those of controls (45.32 ± 7.11 mM, 47.39 ± 10.77 mM and 45.16 ± 17.2 mM vs 20.1 ± 5.85 mM). For turtles kept in 50% seawater, concentrations were three times higher than those in the control group (68.44 ± 26.04 mM vs 20.1 ± 5.85 mM). These values are significantly higher than those in turtles maintained in the natural brackish pond, in 35% seawater and out of water (68.44 ± 26.04 mM vs 45.32 ± 7.11 mM, 45.2 ± 7.16 mM and 47.39 ± 10.77 mM, respectively). The difference of the concentration values between plasma and urine was highly significant for all groups (Student's *t*-test range = 7.64–41.22; df range = 8–32; $p <$ 0.001 in all cases) (Figure 3).

Plasma K^+ concentration (mM) showed very significant differences among groups (one-way ANOVA: $F_{4,46} = 5.43$; $p = 0.001$), with no significant variation among the four groups (natural conditions, 35% and 50% seawater and out of water) that were significantly different of controls (5.77 ± 1.49 mM, 4.47 ± 1.07 mM, 4.64 ± 1.03 mM, 4.13 ± 1.86 mM vs 3.53 ± 1.34 mM, respectively). The values of urine K^+ concentration varied very significantly

among groups (one-way ANOVA: $F_{4,46} = 2.96$; $p = 0.029$). The mean value does not differ significantly among turtles from the natural pond, 35% and 50% seawater, out of water, but are much higher than those in controls (4.55 ± 0.89 mM, 4.85 ± 1.89 mM, 4.5 ± 2.76 mM, 4.02 ± 1.38 mM and 2.97 ± 1.58 mM, respectively). Except for controls (Student's *t*-test range = 2.89; df = 32; $p = 0.007$), the differences in K^+ between plasma and urine were not significant in the other groups (Student's *t*-test range = 0.106–1.11; df range = 8–32; $p = 0.706$–0.918).

The concentrations of Cl^- (mM) in plasma varied significantly among groups (one-way ANOVA: $F_{4,46} = 2.41$; $p = 0.047$) and were in the same order of magnitude as for Na^+ with values of 114.03 ± 50.50 mM, 100.15 ± 0.53 mM and 121.06 ± 4.03 mM for turtles in the natural pond, controls and those out of the water, but significantly lower than those in turtles placed in 35% and 50% seawater that had a relatively high plasma Cl^- concentration (135 ± 57.3 mM and 161.15 ± 77.9 mM, respectively) . The Cl^- concentrations in urine changed very significantly among groups (one-way ANOVA: $F_{4,46} = 15.13$; $p < 0.001$). They were not significantly different among turtles from the natural brackish pond, those in 35% and 50% seawater and those out of water, but are much higher than controls (82.33 ± 13.32 mM, 97.21 ± 28.9 mM, 115.53 ± 36.81 mM, and 109.69 ± 67.39 mM vs 31.89 ± 4.57 mM, respectively). The differences in Cl^- concentration between plasma and urine were significant in the natural group and the controls (Student's *t*-test = 2.50; df = 32; $p = 0.018$; and Student's *t*-test = 66; df = 8–32; $p < 0.001$; respectively). On the other hand, the differences in all remaining groups were not significant (Student's *t*-test range = 0.45–1.32; df range = 8–12; $p = 0.224$–0.663) (Figure 3).

The plasma urea concentration (mM) varied significantly among groups (one-way ANOVA: $F_{4,46} = 64.33$; $p < 0.001$). The values were significantly higher in turtles from the natural environment, those kept at 35 and 50% seawater and out of water, compared to the values obtained in controls (7.69 ± 1.45 mM, 10.8 ± 2.12 mM, 14.65 ± 2.59 mM and 17.6 ± 5.39 mM vs 2.5 ± 0.17 mM, respectively). The concentrations of urea in urine were highly significantly different among groups (one-way ANOVA: $F_{4,46} = 43.42$; $p < 0.001$); they were not significantly different between turtles from the natural pond and those maintained in 35%, but significantly different in 50% seawater and those out of the water and significantly higher than those in controls (4.85 ± 1.01 mM, 5.67 ± 0.98 mM vs 1.78 ± 0.92 mM and 6.16 ± 1.05 mM, 7.77 ± 1.87 mM vs 1.78 ± 0.92 mM) . The difference of the concentration values between plasma and urine was significant for all groups (Student's *t*-test range = 3.17–6.79; df range = 8–32; $p < 0.001$ to = 0.0033) (Figure 3).

The mean value of glycaemia (mM) showed high significant differences among groups (one-way ANOVA: $F_{4,46} = 10.51$; $p < 0.001$). It was significantly higher in controls and was twice as high as the values obtained in the turtles from the natural brackish pond, 35% and 50% seawater and out of water (5.75 ± 2.69 mM vs 2.06 ± 1.3 mM, 2.52 ± 1.04 mM, 2.76 ± 0.86 mM and 2.07 ± 1.01 mM, respectively) (Figure 3).

Discussion

Based on the results of the current study, *M. l. saharica* from northern margin of the Sahara Desert, exhibits adaptive physiological mechanisms related to salinity previously reported in the same species kept in 100% seawater for two weeks (Schoffeniels and Tercafs 1965). Such physiological adaptations and/or behavioural ones were also documented in various

New World freshwater turtle species from North America and Australia (see introduction and Agha et al. 2018 for a review).

Effects of salinity and dehydration on physical appearance and body condition

Elevated levels of salinity and turbidity in the Sidi El Mehdaoui ponds/puddles, combined with extreme exposure to the sun, have resulted in a salt crust on the turtles' shell. More recently, Lovich et al. (2017) documented a significant mortality event that affected a southwestern pond turtle (*Actinemys pallida*) population living in Lake Elizabeth, Southern California, US. A large wildland fire affected the area around the lake that occurred during a protracted drought with the water salinity reaching 46 ppt (130% seawater). Many turtles, covered with a pale mineralised layer on their shells and skin, were severely emaciated. The water salinity level in the large pond at Sidi El Mehdaoui was lower than in the small and medium ponds and in the Californian one, and apparently did not affect the productivity of the ecosystem and turtles there had an apparently good body condition. Moreover, Silva and Davies (1999) reported a sharp decrease in oxygen consumption in aquatic invertebrates beyond salinity of 8.2 ppt (23.4% seawater), as a result of the organism's collapse. This could explain the absence of turtles in ponds with salinity levels higher than 24% resulting in a lack of food for turtles and fish. Blue-eyed turtles from the large brackish pond exhibited a body condition index lower than controls. When kept at higher salinity levels for just two weeks, captive turtles reduced their food consumption and exhibited a significant decrease of their body condition comparatively to controls. The reduced food consumption in *M. l. saharica* exposed to high salinity is also a behavioural adaptation reported for other species to minimise salt intake (Davenport and Macedo 1990). The lower values of glycaemia in *M. l. saharica* kept in brackish/saltwater compared to those in controls are indicative of reduced food consumption in the formers.

The relatively small body size of turtles from Sidi El Mehdaoui oasis could be attributed to a low growth rate at high salinity levels presumably imputed to reduced food consumption. In this regard, hatchlings of *M. terrapin* showed no growth at 100% seawater (Dunson 1985). The rate of sodium efflux in fed terrapins was correlated with feeding rate. However, the main source of sodium uptake at higher salinities seems to be incidental swallowing of water during ingestion of food not the salt content of the food itself. In order to restrict salt ingestion, *M. l. saharica* in its natural brackish environment, could also feed out of water on terrestrial prey with high water, but low salt contents. This is still to be confirmed from the diet composition.

Blue-eyed turtles exhibited a low body mass loss during the two-week immersion in saline waters, indicating that the increased plasma osmolality was sufficient to offset osmotic water loss through the integuments, as in other turtle species (Bentley et al. 1967; Bower et al. 2016). The elevated osmolality is obviously attributable to the increased concentrations of Na^+, Cl^-, and urea in such conditions (see also Schoffeniels and Tercafs 1965). The reduced salt intake is an important mechanism to limit dehydration that would inevitably occur in saline environments, which would occur in conditions requiring the elimination of excess of Na^+ and Cl^-. The fact that these two mechanisms of generation and tolerance of elevated osmolality and reducing salt intake occurred together in turtles, with no evidence of apparent pathological effects, indicate a high level of

tolerance, at least temporarily, to elevated saline conditions, as had previously been reported for the same species (Schoffeniels and Tercafs 1965).

As a reptilian species with a reduced capacity of producing hyperosmotic urine compared to plasma, *M. leprosa,* with no salt glands, is limited in eliminating excess salt. *Mauremys l. saharica* was found to tolerate a salinity of up to 24% seawater (this study) under natural conditions. Schoffeniels and Tercafs (1965) showed that this species kept in 100% seawater, has no effective osmo- and iono-regulatory mechanisms; osmolyte concentrations (Na^+, K^+, Cl^- and urea) increased with the plasma osmolality, which resulted in a state of adaptive 'anhomeostasis'. These authors suggested that the occurrence of this species in saline waters should be, in all cases, coincidental and temporary.

The rate of weight loss in freshwater turtles placed at different salinities or out of water is commonly assumed to be an indicator of net water loss (Dunson and Dunson 1973) and provides a good index of their ability to resist dehydration, whereas the BCI is a useful indicator of nutritional and energy reserves status. The dehydration limits for turtles, the point at which dehydration can cause death, are 30–35% of hydrated body mass with most of body mass loss being probably evaporative water loss (Ernst 1968; Minnich 1979; Mautz 1982; Peterson and Stone 2000; Ligon and Peterson 2002). Considering this range of maximal critical body weight loss and given that the Saharan pond turtle in saline waters lost 0.63% weight (g) per day, these turtles are expected to survive up to 7 to 8 weeks in saline waters. Under similar conditions, Dunson (1979) predicted a lower survival time at 30% body weight loss of less than two weeks in the soft-shell turtles *Trionyx ferox, Kinosternon leucostomum, K. baurii palmarum,* which showed respectively a weight loss four times higher (2.3–2.8 vs 0.63% d^{-1}) with a very short mean survival time of only 3 to 5.5 days (Dunson 1981). On the other hand, unfed *Pseudemys nelsoni* (730–1 240 g) had especially low rates of body mass loss in 100% seawater of about 0.4% initial mass per day. Smaller *T. decussata* (200–240 g) exhibited higher values (about 0.8% per day), yet these rates were lower than four typical freshwater species: *Chrysemys picta* (1.8%), *Clemmys guttata* (2.4%), *K. b. palamarum* (2.8%) and *Sternotherus odoratus* (7.6%) held in 100% seawater (Robinson and Dunson 1976).

Dehydrated turtles in the current study showed an average weight loss of 1.23% per day after a mean period of about two months. Two turtles that died lost respectively 32% and 35.4% of their maximal hydrated body mass, but one turtle survived at a higher body mass loss (36%). During experimental three-month long dormancy (at 28 °C), Yellow mud turtles, *K. flavecens*, lost 27.3% of their initial body weights; two turtles that did not burrow during acclimation to long dormancy died in the third month after losing 31% and 34% of their original weights (Seidel 1975). Peterson and Stone (2000) reported in the Sonora Mud turtle, *K. sonoriense,* a mean body mass loss of 26.4% in a longer average time of 76 days with one individual dead at 32% of its maximum hydrated body mass, but 2 individuals survived at a higher body mass loss. One turtle (a female) from our study survived to a comparable value of 77.5 days with a weight loss of only 20.5% of initial hydrated body weight. Ligon and Peterson (2002) reported in three other kinosternid species average weight loss values of 0.38%/d over 55 days and 0.49%/d over 30 days, respectively, in desiccation resistant and sensitive groups. These authors also reported two turtles (*K. hirtipes*) that died at 25 and 51 days with respective loss of 32 and 29%. The difference between the average values could be related to the

high variability among turtles and to the small sample size. Roe et al. (2008) reported an extreme case of an estivating eastern long-necked turtle *Chelodina longicollis* from south-eastern Australia, which lost less than 5% of its body mass after 54 days and could survive 455 days in estivation before reaching vital dehydration limits.

Drinking rate has not previously been reported in dehydrated turtles. In our study, turtles kept out of water drunk around 9% of their final dehydrated body mass, which was five times higher than the corresponding percentage (less than 2%) in turtles kept in saline waters. This large difference might be attributed to a probably higher evaporative water loss in dry conditions and starvation (loss of energy reserves) in the formers. After rehydration, a marked decrease in plasma and urine osmolalities, indicative of body fluid and bladder urine dilution are expected as had previously been reported for some arid-land kinosternid turtles (Ligon and Peterson 2002).

Osmotic responses to salinity and dehydration effects

The plasma osmolality in *M. l. saharica* increased with the salinity level. In *M. leprosa* kept in 100% seawater, plasma osmolality increased to more than 200% compared to fresh water (Schoffeniels and Tercafs 1965). The increase of plasma osmolality with salinity has also been reported in different other freshwater species (e.g. Gilles-Baillien 1970; Seidel 1975; Hong et al. 2014).

Mauremys l. saharica from the natural environment and those kept in 35% and 50% seawater and out of water, had relatively concentrated urine comparatively to controls. This agrees with data reported for other freshwater turtle species, (e.g. Bentley et al. 1967; Gilles-Baillien 1970; Seidel 1975; see also Agha et al. 2018 for a review).

The voided urine to plasma osmolality ratio (U/P) could constitute a good indicator of the salt stress and hydration status. *Mauremys l. saharica* from the natural habitat showed a U/P ratio much higher than the controls, but lower than those maintained in saline water. This had also been the case for *M. terrapin* in 50–100% seawater (Gilles-Baillien 1970) and the soft-shell turtles from Texas, kept at 20% seawater (Seidel 1975), which exhibited high U/P ratios in brackish water compared to freshwater. This suggests that Blue-eyed turtles from the natural brackish habitat at a relatively lower salinity level had lost less water and could have drunk freshwater (e.g. rain, underwater springs) and/or had eaten some food that provided them with an excess of water stored as diluted urine in their bladders. With an increasing salinity and/or prolonged drought period, isotonicity between urine and plasma would be reached and then the body condition of turtles will decrease, becoming progressively dehydrated and could die in the long-term if they do not drink freshwater. However, and even though they are able to move to surrounding land habitats for estivation, they cannot survive for a long period because their urine is concentrated and then would no longer be a source of water.

The dehydrated Saharan pond turtles kept out of water exhibited an anhomeostatic increase in osmolality and plasma osmolyte concentrations compared to controls. This is in accordance with findings reported for other turtle species (e.g. Dantzler and Schmidt-Nielson 1966; Ligon and Peterson 2002). In natural conditions, Snake-necked turtles, *C. rugosa*, from northern tropical Australia, have a mean plasma osmolality 20% higher in estivating compared to non-estivating animals (Grigg et al. 1986). However, this process depends on the initial state of hydration and the upper limit of the osmolality

range of the extracellular fluid of turtles (Chilian 1976; Ligon and Peterson 2002). In dehydrated *M. l. saharica*, the mean urine osmolality was 82% of plasma osmolality after an average period of 59 days (30 to 36% body mass loss).

Impacts of salinity and dehydration on osmolyte concentrations

The plasma sodium concentrations in *M. l. saharica* kept in fresh and salt waters are in the ranges of the values reported for other freshwater turtle species (e.g. Schoffeniels and Tercafs 1965; Bower et al. 2012; Gilles-Baillien 1970). The difference between the values among species, including *M. leprosa*, kept in fresh or saline waters could be related to the maintenance conditions, namely water quality and type of food.

The sodium concentrations in urine of *M. l. saharica* (expressed as % plasma sodium concentration) from the natural habitat, in controls and saline waters were in the corresponding ranges of other turtle species in similar conditions (Gilles-Baillien 1970; Seidel 1975). Except in *M. terrapin*, with the lowest concentrations related to the presence of salt glands, an excess of excreted sodium in the other species, including *M. l. saharica*, is probably adsorbed on the mono-sodium urates.

The plasma chloride concentration found in *M. l. saharica* kept in freshwater was relatively higher than that reported for *M. leprosa* (Schoffeniels and Tercafs 1965) (100.15 vs 88 mM), and slightly out of range in other turtle species (81.4 to 92.3 mM) (Bower et al. 2012). This was probably because of a relatively higher chlorinity of tap water used in our experiments. The values in blue-eyed turtles maintained in brackish or salt waters were in the range of other turtles species, but higher than what had been reported for the same species by Schoffeniels and Tercafs (1965).

In contrast to plasma, and except in *Trachemys scripta elegans* (Hong et al. 2014), there are no data on urine chloride concentration in other studied freshwater turtle species. In *M. l. saharica*, the percentages of excreted chloride in urine were 30 and 72%, respectively in freshwater and brackish/salt waters. These values in *M. l. saharica* are comparable to those reported for *T. sripta* kept in water salinities varying from 14% to 72% seawater for 30 to 90 days. These percentages in these two species are higher than concentrations of sodium, because unlike this mono-cation, chloride is not adsorbed onto urates.

Concerning the potassium, the plasma concentration of this mainly intracellular mono-cation, the mean value in controls was well in the range reported for the same species (Schoffeniels and Tercafs 1965). In brackish/saline waters, the corresponding values were at the upper limit of the range in other species (4.5 mM). As for the urine, potassium concentrations in *M. l. saharica* kept in brackish/saline waters were lower than those for other species (Gilles-Baillien 1970; Hong et al. 2014); this might be because of the fact that this mono-cation, just like sodium, can also usually be adsorbed onto urates.

The plasma urea concentration in *M. l. saharica* kept in fresh water was approximately half of that reported for the same species (4.7 mM) by Schoffeniels and Tercafs (1965). A very low value (0.4 mM) had been reported for the Australian species *Emydura macquarii* (Bower et al. 2012) and a much higher (21.5 mM) one for *M. terrapin* (Gilles-Baillien 1970). The obtained value in *M. l. saharica* kept in saline waters were lower than the 25.7 mM that had been reported for the same species maintained in 100% seawater (Schoffeniels and Tercafs,1965). An extremely high concentration of 115.2 mM had been reported in *M. terrapin* (Gilles-Baillien 1970). All these variations could be attributed to the nutritional

quality of food offered, namely protein content in turtles kept in fresh water or mobilisation of body proteins in non-feeding turtles maintained in salt water.

In turtles kept out of water, plasma sodium, potassium and chloride concentrations were higher than in hydrated turtles. Similarly, in water-deprived Sonora mud turtles, plasma osmolality, BUN, sodium and potassium concentrations were strongly and positively correlated (Peterson and Stone 2000). Conversely, estivating snake-necked turtles showed just 7% higher plasma sodium than non-estivating turtles, but chloride and potassium values were similar in all groups (Grigg et al. 1986). This increase in urine osmolality was also accompanied by an increase in Na^+, K^+, Cl^-, and urea concentrations comparatively to hydrated turtles. This is in accordance with the data obtained for the yellow mud turtle, *K. flavescens* (Ligon and Peterson 2002).

Implications for conservation

Despite the precarious conservation status of many turtles, (e.g. Todd et al. 2010), the extent to which they can occur in brackish or saline environments and thus, our ability to predict the future impact of water salinisation is still not well known (Neill 1958; Rasmussen et al. 2011; Agha et al. 2018). Salinisation can indeed affect the organisms in various ways, from increasing stress to causing a high risk of mortality, determining the population viability. The future predictions clearly indicate that river salinisation will globally increase (see Cañedo-Argüelles et al. 2013). Although the current study demonstrates that the Saharan Blue-eyed pond turtles have mechanisms to survive temporarily in brackish to saline waters, it is likely that sustained salinisation of streams or isolated ponds will exceed their short- to medium-term capacity to survive increased salt levels, making salinisation a potentially key threatening process for these marginal freshwater turtles. Fragmentation and habitat loss, because of a potential increase of drought and water salinisation, might co-act with many other processes that are simultaneously threatening freshwater turtles in the Draa basin (e.g. resource exploitation, habitat destruction and deterioration, human development, water pollution, and water diversion). Accordingly, future research directed at life-history, behavioural, and physiological responses of freshwater turtles to salinisation that might be amplified by climate change could be of critical value to conservation managers in arid areas where salinisation is expected to have the highest impacts on species' ranges. Freshwater turtles are among the semi-aquatic vertebrates that can tolerate temporarily living out of water for their terrestrial activities or for estivation under acute dry conditions, especially in arid areas. Drought and habitat fragmentation along with salinisation are predicted to increase under different climate changes scenarios. Therefore, understanding and evaluating the ability of turtles and the extent to which they can survive in terrestrial arid environments, would be also useful in conservation and management programs.

Conservation biologists and managers have traditionally paid attention to the characteristics (e.g. abundance, structure, trends) of populations, species, communities, and ecosystems, and simple indicators of the responses to environmental disturbances and other anthropic activities. However, an understanding of the specific mechanisms underlying conservation problems is becoming more and more important for decision-making, partly because physiological knowledge and tools are particularly helpful for developing cause-and-effect relationships, and for identifying the optimal

range of habitats and stressor thresholds for different organisms. When physiological data are integrated into ecological models, they can improve predictions of organism responses to environmental changes and provide tools to reinforce management decisions. Conservation physiology (see Cooke et al. 2012 for more details) could then be utilised as an integral part of monitoring programs and active conservation action plans (Cooke and Suski 2008), and moreover, to assist with identification of actions likely to be most successful.

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