Sex-specific ecophysiological responses to environmental fluctuations of free-ranging Hermann’s tortoises: implication for conservation

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Physiological parameters provide indicators to evaluate how organisms respond to conservation actions. For example, individuals translocated during reinforcement programmes may not adapt to their novel host environment and may exhibit elevated chronic levels of stress hormones and/or decreasing body condition. Conversely, successful conservation actions should be associated with a lack of detrimental physiological perturbation. However, physiological references fluctuate over time and are influenced by various factors (e.g. sex, age, reproductive status). It is therefore necessary to determine the range of natural variations of the selected physiological metrics to establish useful baselines. This study focuses on endangered free-ranging Hermann’s tortoises (\textit{Testudo hermanni hermanni}), where conservation actions have been preconized to prevent extinction of French mainland populations. The influence of sex and of environmental factors (site, year and season) on eight physiological parameters (e.g. body condition, corticosterone concentrations) was assessed in 82 individuals from two populations living in different habitats. Daily displacements were monitored by radio-tracking. Most parameters varied between years and seasons and exhibited contrasting sex patterns but with no or limited effect of site. By combining behavioural and physiological traits, this study provides sex-specific seasonal baselines that can be used to monitor the health status of Hermann’s tortoises facing environmental threats (e.g. habitat changes) or during conservation actions (e.g. translocation). These results might also assist in selection of the appropriate season for translocation.

Key words: Body condition, corticosterone, population managment, reptile conservation, translocation methodology

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Introduction

Understanding the physiological responses of organisms to environmental changes can improve conservation strategies (Wikelski and Cooke, 2006). Indeed, even closely related species exhibit different triggering factors and different physiological limits to external fluctuations, and these divergences determine their respective adaptability to changing

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conditions (Helmuth et al., 2005). Relevant physiological mechanisms should be scrutinized in each species to forecast population responses to global changes (Kearney and Porter, 2009). Furthermore, the intensity, appropriateness or failure of the responses to external conditions depends on the physiological status of each individual at a given time. For example, in female reptiles, body condition at the onset of the breeding season influences the decision to reproduce, the mobilization of maternal reserves and post-reproduction survival, and all these traits are impacted by environmental conditions (Nauleau and Bonnet, 1996; Shine and Madsen, 1997; Bonnet et al., 1999, 2001a; Warner et al., 2008).

Survival rate, fecundity and offspring quality, hence population viability, are thus determined by the sum of the physiological responses of individuals to environmental factors. Besides these idiosyncratic traits, most populations can be divided into major categories, notably sex and age. These categories exhibit physiological peculiarities that should also be considered to derive useful metrics. Overall, implementing selected ecophysiological measurements into population surveys is an asset to improve conservation actions (Fairbairn et al., 2007; Cooke and Suski, 2008; Dantzer et al., 2014).

Stress regulation is one of the major ecophysiological systems that allow individuals to adjust their behaviour, energy expenditure and reproductive effort to environmental constraints (Teixeira et al., 2007; Dickens et al., 2010). Glucocorticoid (GC) hormones are important effectors of the stress response; environmental stressors trigger an increase of GC concentrations that stimulates metabolism, vigilance and the mobilization of energetic resources. Thus, changes of GC blood concentrations have been widely used as key metrics of the adaptive capacities or health status in vertebrates (Wingfield and Romero, 2000; Romero and Wikelski, 2001; Kahn, 2006; Breuner et al., 2008). However, high GC concentrations can perturb sex steroid regulation and may negatively affect other functions (e.g. immunity; Charmandari et al., 2005; Breuner et al., 2008; Dantzer et al., 2014; Michel and Bonnet, 2014). Extreme and chronic stress responses can be detrimental to reproduction, survival and population viability, thereby revealing trade-offs between physiological functions (Sherill et al., 2009; Zanette et al., 2011).

Nonetheless, many studies have failed to find clear relationships between GC concentrations, sex steroid concentrations and demography (Cree et al., 2009; Crespi et al., 2013; Dickens and Romero, 2013; Dantzer et al., 2014). Simple general patterns where elevated GC concentrations systematically correlate with population threats may not exist (Bonier et al., 2009; Boonstra, 2013). For example, although high chronic stress GC concentrations are interpreted as warning signs, a significant chronic/acute stress response may instead correlate positively with physiological stamina and, ultimately, with elevated reproductive performances (Dantzer et al., 2014). A flat stress response may indicate that the organism is exhausted and incapable of reacting to environmental factors rather than being unstressed (Dickens et al., 2010). In contrast, individuals exhibiting an extreme stress response would face difficulties in responding appropriately to environmental fluctuations. Either a lack of response (e.g. very low GC concentrations) or saturated responses (e.g. plateauing maximal GC concentrations or very high chronic concentrations relative to baseline values) suggest that physiological limits have been reached, with possible negative consequences. Therefore, determining the range of natural variation of physiological metrics (e.g. GC plasma concentrations) is a prerequisite to establish baselines in order to seize pertinent deviations that can be useful to field managers.

We measured the basal GC plasma concentrations of free-ranging Hermann’s tortoises (Testudo hermanni hermanni) monitored by radio-tracking during 3 years. This endangered subspecies (IUCN, 2016) has faced a drastic reduction of its distribution range during recent decades, especially in continental areas (Livoreil, 2009; Bertolero, 2011). Many remaining populations occur at low densities, are fragile and are threatened by illegal harvesting, urbanization, closing of habitats and forest fires (Celse et al., 2014). Reintroduction (Bertolero et al., 2007; Livoreil, 2007; Bertolero, 2014) and, more recently, reinforcement programmes have been launched to bolster populations decimated by fire (Lepeigneul et al., 2014). Assessing the success of these translocations is important, but for comparative objectives, assessing the health status of resident individuals living in the remaining populations is needed. Although tortoises are robust animals that can afford harsh environmental conditions during prolonged periods, their adaptive capacities are not unlimited (Henen et al., 1998; Christopher et al., 1999). Consequently, an evaluation of the health status of tortoises sampled in contrasting and thus possibly challenging habitats (e.g. burned, closed) is important; therefore, we studied two populations in open and closed habitats, respectively.

Monitoring GC concentrations of individuals in different habitats represents a means to identify environmental stressors (Drake et al., 2012). Nonetheless, gathering CG concentrations in isolation is poorly informative because contrasted GC concentration profiles do not necessarily translate into contrasting demographic response. Taking into account a panel of traits is required to provide a better evaluation of threats to populations (Christopher et al., 1999; Cooke et al., 2013; Dantzer et al., 2014). In tortoises, behaviours, body condition and several other physiological metrics (e.g. haematocrit) are notably useful to evaluate the health and reproductive status of individuals (Henen, 1997; Christopher et al., 1999; Marin et al., 2002; Nagy et al., 2002; Lagarde et al., 2003a, 2008; Loehr et al., 2007; Lecq et al., 2014). It is also important to consider seasons and genders. In chelonians, each sex exhibits specific timing of reproductive effort (e.g. vitellogenesis, egg laying, spermatogenesis) reflected by sex seasonal patterns of blood chemistry in free-ranging (Henen et al., 1998; Christopher et al., 1999; Ott et al., 2000; Longepierre, 2001; Huot-Daubremont et al., 2003;
Lagarde et al., 2003a; Currylow et al., 2013; Bonnet et al., 2016a) and captive tortoises (Kölle et al., 2001; Scope et al., 2013; Andreani et al., 2014).

The objective of this study was to establish dynamic eco-physiological references in Hermann’s tortoises, taking into account possible sex and time effects. Therefore, in addition to CG concentrations, we monitored movements, body condition and several haematological traits involved in various functions (e.g. plasma concentrations of glucose as an index for energy budget; triglycerides for vitellogenesis; osmolality for water balance; uric acid for excretion). Given that habitats, seasons and years are likely to influence physiology, we sampled both sexes during the main activity periods, during 2 or 3 years consecutively, in both open mosaic and dense closed habitats.

Materials and methods

Study species and field sites

This study is part of a Life program (2010–2014; LIFE08NAT/F/000475) that aims to set up practical actions for the conservation of the Hermann’s tortoise (Celse et al., 2014). Previously abundant in south-eastern France, this sub-species has markedly declined during recent decades; relict continental populations persist in the Massif des Maures and adjacent plains (Livoreil, 2009; Bertolero et al., 2011). This tortoise exhibits typical life-history traits of terrestrial chelonians, including delayed maturity, low fecundity and low population turnover (Bertolero et al., 2011). Females are on average 12% larger than males (Bertolero et al., 2011). Emergence from hibernation usually occurs from mid-March to the beginning of April, and the active season ends in November. Hermann’s tortoises are found in various habitats, notably mosaic landscapes that comprise small cultivated fields, meadows, bushy zones and closed forest areas. They exhibit a generalist diet (mostly herbivorous) and are philopatric (Calzolai and Chelazzi, 1991).

We studied two populations, Flasans and Callas, separated by ~38 km and seven roads (including a highway) and thus without any possibility for exchanges between them (Fig. 1). The respective habitats of these two populations are very different; they reflect variations across the current distribution range caused by human activities.

Figure 1: Location of the two study sites. The black dots indicate the initial position of the radio-tracked tortoises (all years pooled).
**Flassans population**

The Flassans population site (~50 hectares) is situated in the west region of the distribution range. Adult population density is estimated to be 4.2 tortoises/hectare J-M. B. and S. C. (unpublished data). This value is categorized as moderately high for French continental areas (Celse et al., 2014) but is low compared with less impacted areas (Bonnet et al., 2016b). This study site is relatively flat, with a mean elevation of 270 m (maximum 295 m). The geological substrate is calcareous. The habitat is open, characterized by small grassy meadows (29%), diverse and patchy shrub formations (29%, *Paliurus spina-christi*, *Cistus monspeliensis* and *Cistus albidus*), small woods (34%, *Quercus pubescens* and *Quercus ilex*), several small vineyards (7%), a temporary lake (1%) and numerous hedgerows. This study site is typical of traditionally managed agricultural areas and is considered to be favourable for reptiles, including tortoises.

**Callas population**

The Callas population (~225 hectares) is situated near the north-eastern limit of the distribution range. The adult population density is low, estimated to be 1.4 tortoises/hectare J-M. B. and S. C. (unpublished data). The study site is characterized by a hilly landscape, where elevation varies between 178 and 300 m. The geological substrate is siliceous. The sclerophyllous vegetation is mainly represented by a dense and close mixed forest with large trees (76%, *Quercus suber*, *Quercus pubescens* and *Pinus halepensis*), alternating with dense thick scrub vegetation (16%, mainly *Erica arborea*) and few partly open areas (8%, open patches populated by *Cistus albidus* and *Cistus monspeliensis*). This study site is typical of unmanaged areas where the habitat is progressively closing and is considered to be unfavourable for reptiles owing to a lack of basking and laying sites, shrub shelters and herbaceous layer (Todd and Andrews, 2008; Sirami et al., 2010).

Considering a broad geographical scale (100 km²) during the study period (2010–2012), mean temperatures in spring and summer were 17.7 and 21.5°C, respectively; mean precipitations were 86.2 and 28.6 mm, respectively (GES DISC, NASA, MERRA Monthly analyses). Climatic conditions varied among years (Table 1); probably without major differences between the two study sites considering the relatively short distance (<40 km) in terms of climatology [there is no major barrier (e.g. mountain) between sites].

### Population monitoring and radio-tracking

Free-ranging individuals were visually searched and captured after hibernation in April and early May. They were sexed (Djordjević et al., 2013), weighed [body mass (BM) ±1 g using a portable scale CL-Series; OHAUS], measured [shell length (SL) ±1 mm using callipers] and marked with a metallic clip (Livoreil, 2009). We selected tortoises with a shell length >120 mm; they were supposedly adult (Bertolero et al., 2011). Tortoises were fitted with an AVM-K16 transmitter glued onto the shell in early May. The tortoises were then immediately released. The transmitter plus resin represented <10% of individual body mass, a value assumed to be well tolerated in free-ranging tortoises (Lagarde et al., 2008). Each individual was located once a day, alternatively in the morning, around midday and in the afternoon. Coordinates were recorded using a Garmin GPS. Tortoises were weighed every 2 weeks, measured for SL, and blood sampled on two occasions during each active season, in spring and in summer. We selected these two periods because vitellogenesis occurs in spring, whereas intensive reproductive sexual behaviours are displayed by males in summer (Lagarde et al., 2003a).

In total, 82 different adult tortoises were monitored between 2010 and 2012. Eleven were monitored during 2 years and one was monitored during 3 years (total = 94 annual tortoise monitoring). Fifty tortoises were captured and radio-tracked at Flassans (23 in 2010, 22 in 2011 and 14 in 2012; eight monitored during more than 1 year). Thirty-two tortoises were studied at Callas (14 in 2010 and 21 in 2011; three monitored for more than 1 year). The overall sex ratio was balanced (42 females and 40 males). Daily displacements of radio-tracked tortoises were calculated as the mean distance travelled per day. There were measured during the spring season (before 21 June) to include vitellogenesis and the laying period and summer season (from the 21 June to 20 September), corresponding to the post-laying and main mating period.

### Body condition index

Body condition index (BCI) is an integrative metric that can involve various elements: body reserves, gut content, urinary

| Mean temperature (°C) | Precipitation (mm) |
|-----------------------|--------------------|
| **Spring** | **Summer** | **Spring** | **Summer** |
| 2010 | 17.55 | 23.30 | 452.2 | 72.4 |
| 2011 | 18.84 | 23.04 | 31.8 | 85.5 |
| 2012 | 18.23 | 24.18 | 221.3 | 55.8 |
Blood sampling

To limit the possible stress effect during device fitting on blood parameters, we waited 15 days from initial capture before taking the first blood sample. Radio-tracked tortoises were sampled in spring (from 7 May to 5 June) and in summer (from 6 August to 21 September). We increased our sample size by incorporating tortoises opportunistically captured (OC) during radio-tracking sessions (n = 18 in Flassans and n = 6 in Callas). Some individuals were OC in a given year and radio-tracked another year. A total of 208 blood samples were collected from 96 individuals: 50 females and 46 males (two radio-tracked males from Callas were not blood sampled). Punctures were performed in the field before 12.00 h to limit the influence of daily variation. Samples were collected within 5 min to limit the impact of handling stress (Jessop et al., 2003; Drake et al., 2012; Bonnet et al., 2016a). Between 0.5 and 1.0 ml of blood was retrieved from the dorsal subcarapacial cervical plexus with a 25 or 26 gauge needle connected to a 1 ml syringe and transferred in a lithium heparinized tube. Blood samples were immediately placed on an ice bed in an icebox; they were transferred to the laboratory within 3–4 h after collection. We first measured the haematocrit (HCT; the percentage packed blood cell volume per unit volume of blood) by centrifuging blood in two capillaries (37 800 g, 3 min; Sigma 112 microcentrifuge). Haemodiluted samples detected by visual inspection during puncture (i.e. streaks of red liquid mixed up into a clear liquid; Bonnet et al., 2016a) contained substantial amounts of lymph (HCT <12%); they were discarded from statistical analysis. Samples were then centrifuged (10 000 rpm for 5 min), and the plasma was collected in small tubes and stored at −25°C until analysis.

Hormonal assays

All assays could not be performed on several samples owing to limited amounts of blood retrieved, generating slight variations in sample sizes. The main GC in reptiles is corticosterone (CORT; concentrations usually expressed as nanograms per millilitre); plasma concentrations were assayed in the Centre d’Études Biologiques de Chizé (CEBC France) using radio-immunoassay (Bonnet et al., 2013, 2016a). The steroids were extracted from 40 μl of plasma using diethyl ether (mean extraction rate was of 97.3 ± 5.2%); the sensitivity of the assay was 1.9 pg/tube. Cross-reactions with other steroids were low (<0.1% for 11-deoxycorticosterone, cortisol, testosterone and androstenedione; 7% for compound S and progesterone). Intra- and interassay coefficients of variation remained <4%.

Plasma metabolites and ion assays

The glycaemia (in milligrams per decilitre) was assayed directly in the field using a portable device, an Accu-Chek® Performa blood glucose meter. Plasma concentrations of three circulating metabolites, triglycerides (in grams per litre), cholesterol (in grams per litre) and uric acid (in milligrams per litre), and of two ions, Na⁺ (in milliequivalents per litre) and K⁺ (in milliequivalents per litre), were measured at the BIO CONVERGENCE laboratory for medical analyses (Le Luc, France) using MODULAR de Roche automaton (ADVIA 2400 Siemens, Colorimetry, potentiometry). Osmolarity (in millimoles per litre) was calculated using the following formula: 2(Na⁺ + K⁺) (Dallwig et al. 2010; Guzman et al. 2011).

Statistical analysis

Morphological data were logarithmically transformed to meet normality and homogeneity of variance assumptions. However, after transformation some variables were not normally distributed (corticosterone, uric acid and cholesterol concentrations); other variables (glycaemia, osmolality, haematocrit, triglyceride concentration, BCI and daily movements) were normally distributed. In addition, blood samples were repeated on some but not all individuals, resulting in a complex data set with several pseudo-replicates. Consequently, generalized linear mixed models with Gaussian or γ error and penalized quasi-likelihood estimation were used to assess the influence of sex and environmental variables on physiological markers (Bolker et al., 2009). Individual identity was added as a random factor. Each model included sex, year, season, site, BCI and SL as fixed variables. Initial models contained each variable and the first-order interaction between session and sex, session and year, and the interaction between sex and BCI; the interaction between sex and site was also integrated for the daily displacement analysis. Model selection was performed by backward elimination. Results with P < 0.05 were considered statistically significant, and tests were bidirectional. All analyses were performed with R version 2.13.1 (R version 2.13.1, 2011-07-08, © 2011, the R Foundation for Statistical Computing).

Many variables were measured, generating a possible risk of inflation in the presentation of the results. For conciseness, we retained a synthetic selection of the statistics, providing the final output from backward elimination summarized in Table 1. In addition, we provide more detailed results when appropriate in order to provide better focus on important issues (e.g. sex, season). Mean values ± SEM are indicated.

Permits and ethical note

All procedures were performed in accordance with international regulations. Permits for population monitoring were issued by prefectural authorities on 13 January 2011. Ethical procedures were approved by the ethical committee COMETCH (permit no. CE2013-6). No tortoise was injured during handling and blood sampling.
Results

Daily displacements

We found significant effects of sex, study site, season, body size and body condition on mean daily displacements, with several significant interactions among these factors but without an effect of year (Table 2). Greater spring movements in females compared with males (39.9 ± 2.7 vs. 31.4 ± 1.7 m/day, respectively; Table 2) and females exhibited lower HCT compared with males (19.98 ± 0.6 vs. 24.05 ± 0.7). Other effects were either very weak (correlation between HCT and body size: $r^2 = 0.02$) or inconsistent regarding explanatory variables.

Mean values of CORT were influenced by most of the factors tested, with significant interactions among them (Table 2), resulting in complex effects. However, a close inspection of the data revealed a consistent broad pattern. Females systematically exhibited lower CORT compared with males, with almost no overlapping of the values between the sexes in summer (Fig. 5). In females, CORT decreased significantly from spring to summer (years pooled: spring CORT = 1.34 ± 0.2 ng/ml; summer CORT = 0.54 ± 0.04 ng/ml), whereas on average the opposite pattern was observed in males (spring CORT = 2.28 ± 0.2 ng/ml; summer CORT = 3.22 ± 0.2 ng/ml). Pooling sexes and seasons, CORT was significantly higher in 2010 (2.05 ± 0.2 ng/ml) and 2012 (2.65 ± 0.3 ng/ml) than in 2011 (1.47 ± 0.2 ng/ml).

Mean plasma concentrations of triglycerides varied significantly between sexes and among years (Table 2). Triglycerides were significantly higher in females (3.84 ± 0.2 g/l) than in males (0.64 ± 0.1 g/l). They were significantly lower in 2010 than in 2011 and 2012 (1.35 ± 0.2, 2.32 ± 0.2 and 2.52 ± 0.4 g/l, respectively).

Mean plasma concentrations of cholesterol varied significantly between sexes and among years (Table 2). Cholesterol concentrations were significantly higher in females (1.65 ± 0.08 g/l) than in males (0.89 ± 0.07 g/l). They were higher in 2010 than in 2011 and 2012 (1.53 ± 0.06, 1.24 ± 0.1 and 1.43 ± 0.10 g/l, respectively).

Mean concentrations of uric acid were mainly impacted by season and year (Table 2). Mean values were almost 2-fold higher in spring than in summer (19.3 ± 1.4 vs. 12.0 ± 0.9 mg/l). Uric acid concentrations differed among years (in 2010, 2011 and 2012: 18.5 ± 2.0 mg/dl, respectively; Fig. 6); the opposite pattern was observed in males (57.07 ± 2.4 and 75.70 ± 3.1 mg/dl, respectively). Glyceremia was higher in 2012 (78.45 ± 3.5 mg/dl) than in 2011 (64.52 ± 2.0 mg/dl).

The situation was partly reversed in females; glyceremia decreased from spring to summer (2010: 75.70 ± 2.4 and 57.07 ± 3.1 mg/dl, respectively; it varied between seasons in females (274.70 ± 8.6 mmol/l, respectively). It was significantly higher in 2010 than in 2011 and 2012 (18.5 ± 2.0 mg/dl, respectively; Fig. 6); the opposite pattern was observed in males (57.07 ± 2.4 and 75.70 ± 3.1 mg/dl, respectively). Glyceremia was higher in 2012 (78.45 ± 3.5 mg/dl) than in 2011 (64.52 ± 2.0 mg/dl).

Mean values of HCT exhibited a complex pattern. They were significantly influenced by sex, size, year and season, with an interaction between year and season (Table 2). The females exhibited lower HCT compared with males (19.98 ± 0.6 vs. 24.05 ± 0.7). Other effects were either very weak (correlation between HCT and body size: $r^2 = 0.02$) or inconsistent regarding explanatory variables.
Table 2: Effect of temporal (season, year), spatial (site) and individual parameters (sex, body condition index, shell length) on displacements, body condition and a set of seven physiological markers

| Dependent variable                  | Effect       | d.f. | t-value | P-value |
|-------------------------------------|--------------|------|---------|---------|
| Daily displacements (n = 157)       | Intercept    | 85   | -0.03   | 0.975   |
|                                     | Sex          | 65   | 2.28    | 0.026   |
|                                     | Site         | 65   | -2.54   | 0.014   |
|                                     | Season       | 85   | 2.00    | 0.049   |
|                                     | SL           | 65   | 2.24    | 0.028   |
|                                     | BCI          | 65   | 2.35    | 0.022   |
|                                     | Sex*BCI      | 65   | -2.01   | 0.048   |
|                                     | Sex*Season   | 85   | -2.73   | 0.008   |
| Body condition index (n = 209)      | Intercept    | 114  | 3.22    | 0.002   |
|                                     | Sex          | 91   | -2.94   | 0.004   |
|                                     | Season       | 114  | -0.96   | 0.338   |
|                                     | Sex*Season   | 114  | -2.94   | 0.004   |
| Haematocrit (n = 157)               | Intercept    | 82   | 1.16    | 0.248   |
|                                     | Sex          | 82   | 5.08    | <0.001  |
|                                     | Year 2010–11 | 67   | -4.41   | <0.001  |
|                                     | Year 2010–12 | 67   | -0.56   | 0.579   |
|                                     | Year 2011–12 | 67   | 3.46    | 0.001   |
|                                     | Season       | 67   | -2.88   | 0.005   |
|                                     | SL           | 67   | 2.93    | 0.005   |
|                                     | Year 2010–11*Season | 67   | 5.79    | <0.001  |
|                                     | Year 2010–12*Season | 67   | 1.79    | 0.077   |
|                                     | Year 2011–12*Season | 67   | -2.95   | 0.004   |
| Corticosterone (n = 196)            | Intercept    | 96   | 7.68    | <0.001  |
|                                     | Sex          | 92   | -3.23   | 0.002   |
|                                     | Year 2010–11 | 96   | 4.48    | <0.001  |
|                                     | Year 2010–12 | 96   | 0.13    | 0.898   |
|                                     | Year 2011–12 | 96   | -3.89   | <0.001  |
|                                     | Season       | 96   | 6.70    | <0.001  |
|                                     | Sex*Season   | 96   | -6.65   | <0.001  |
|                                     | Year 2010–11*Season | 96   | 3.77    | <0.001  |
|                                     | Year 2010–12*Season | 96   | -1.42   | 0.157   |
|                                     | Year 2011–12*Season | 96   | 2.39    | 0.019   |
| Glycaemia (n = 128)                 | Intercept    | 65   | 19.78   | <0.001  |
|                                     | Sex          | 65   | -3.12   | 0.003   |
|                                     | Year 2011–12 | 61   | 3.66    | <0.001  |
|                                     | Season       | 61   | -1.63   | 0.107   |
|                                     | Sex*Season   | 61   | 4.22    | <0.001   |

(Continued)
Table 2: continued

| Dependent variable | Effect  | d.f. | t-value | P-value |
|--------------------|---------|------|---------|---------|
| Triglyceride (n = 122) | Intercept | 78 | 16.11 | <0.001 |
|                     | Sex | 78 | -12.65 | <0.001 |
|                     | Year 2010–11 | 40 | 2.90 | 0.006 |
|                     | Year 2010–12 | 40 | 2.94 | 0.005 |
|                     | Year 2011–12 | 40 | 0.53 | 0.601 |
| Cholesterol (n = 120) | Intercept | 77 | 9.25 | <0.001 |
|                     | Sex | 77 | 6.60 | <0.001 |
|                     | Year 2010–11 | 39 | -3.04 | 0.004 |
|                     | Year 2010–12 | 39 | -3.30 | 0.002 |
|                     | Year 2011–12 | 39 | -0.75 | 0.459 |
| Uric acid (n = 177) | Intercept | 92 | 9.00 | <0.001 |
|                     | Year 2010–11 | 79 | 3.31 | 0.001 |
|                     | Year 2010–12 | 79 | 1.92 | 0.058 |
|                     | Year 2011–12 | 79 | -0.50 | 0.619 |
|                     | Season | 79 | 4.56 | <0.001 |
|                     | Year 2010–11*Season | 79 | -2.12 | 0.037 |
|                     | Year 2010–12*Season | 79 | -1.99 | 0.050 |
|                     | Year 2011–12*Season | 79 | -0.38 | 0.703 |
| Osmolarity (n = 144) | Intercept | 86 | 67.98 | <0.001 |
|                     | Sex | 86 | 3.03 | 0.003 |
|                     | Year 2010–11 | 52 | 0.74 | 0.463 |
|                     | Year 2010–12 | 52 | 5.11 | <0.001 |
|                     | Year 2011–12 | 52 | 5.83 | <0.001 |
|                     | Season | 52 | 4.46 | <0.001 |
|                     | Sex*Season | 52 | -2.64 | 0.011 |

BCI, body condition index; SL, shell length. Significant P-values are indicated in bold.

Fig. 7) but not in males (spring 274.65 ± 3.8 mmol/l; summer 274.73 ± 2.4 mmol/l).

**Discussion**

Setting up simple field technique(s) to determine sex-specific baselines along with the range of variations of several major ecophysiological metrics may assist field managers to monitor the health status of tortoises. Chelonia are robust organisms that can survive harsh conditions, but they may not reproduce at a sufficient rate for population viability if, for example, vitellogenesis and thus egg production is perturbed (Turner et al., 1986). Detection of underlying physiological disorders requires reference values gathered in normally functioning individuals in various conditions.

Obtaining these values from free-ranging tortoises is relatively simple. Tortoises are slow-moving animals tolerant to handling, to the electronic device glued onto their back and to repeated blood sampling (Lagarde et al., 2008; X.B., unpublished observations). Thus, individuals can be monitored on a regular basis and their global status (BCI, behaviours and blood parameters) can be measured accurately. Possible physiological disorders (e.g. decreasing BCI, chronic high CORT) caused by translocation and/or degraded habitats can be detected early, possibly prompting interventions (e.g. removal of individuals from unsuitable habitats). More subtle effects, such as a lack of elevation of plasma concentrations of cholesterol and triglycerides in spring in females (i.e. an index of vitellogenesis), would reveal reproductive disorders, motivating further investigations.
In practice, however, interpretation of individual and mean values can be tricky because of strong variations in most behavioural and physiological traits of chelonians. For example, a very low glycaemia (<0.4 g/l) can be lethal in endotherm vertebrates (Chajek-Shaul et al., 1990), whereas it may simply reflect non-pathological natural fluctuations in ectothermic reptiles, where variations are driven by ambient temperatures and reproductive effort (Bonnet and Naulleau, 1993). Consequently, although a very low glycaemia may well suggest serious health problems in an active female during vitellogenesis, it should be considered as normal in a resting and cold female sampled in late summer. Considering a set of metrics and environmental factors is thus important to assist the diagnosis of health status in organisms that display a very flexible physiology, such as reptiles (in less flexible organisms, such as birds, for instance, any deviation of natraemia or body temperature may represent a warning sign). For instance, a counterintuitive strong reduction of albuminuria concomitant with an increase in total plasma proteins can be explained by a shift of liver function during vitellogenesis in reptiles (Bonnet et al., 1994), but this does not indicate a pathological state, as would be the case if...
Table 3: Reference levels for haematological metrics of Testudo hermanni hermanni

| Parameter               | Mean  | n   | SD   | Minimum | Maximum | 95% Confidence interval | Outlier |
|-------------------------|-------|-----|------|---------|---------|--------------------------|---------|
| Haematocrit (%)         | 22.05 | 161 | 6.31 | 12      | 38      | 21.08–23.03              | No      |
| Female                  | 19.98 | 79  | 5.51 | 12      | 32      | 18.77–21.20              |         |
| Male                    | 24.05 | 82  | 6.41 | 12      | 38      | 22.66–25.43              |         |
| Corticosterone (ng/ml)  | 1.85  | 196 | 1.60 | 0.15    | 8.64    | 1.63–2.08                |         |
| Female                  | 0.96  | 100 | 0.90 | 0.15    | 5.25    | 0.78–1.13                |         |
| Male                    | 2.79  | 96  | 1.64 | 0.38    | 8.64    | 2.47–3.13                |         |
| Glycaemia (mg/dl)       | 68.03 | 131 | 20.8 | 14      | 129     | 64.46–71.59              |         |
| Female                  | 68.04 | 68  | 22.78| 14      | 129     | 62.63–73.46              |         |
| Male                    | 68.01 | 63  | 18.62| 16      | 108     | 63.42–72.61              |         |
| Triglyceride (g/l)      | 2.24  | 122 | 2.05 | 0.00    | 7.52    | 1.88–2.60                |         |
| Female                  | 3.84  | 61  | 1.74 | 0.50    | 7.52    | 3.40–4.28                |         |
| Male                    | 0.64  | 61  | 0.50 | 0       | 2.55    | 0.52–0.77                |         |
| Cholesterol (g/l)       | 1.28  | 120 | 0.70 | 0.11    | 3.83    | 1.16–1.41                |         |
| Female                  | 1.65  | 61  | 0.65 | 0.49    | 3.34    | 1.49–1.82                |         |
| Male                    | 0.89  | 59  | 0.51 | 0.11    | 3.83    | 0.76–1.02                |         |
| Uric acid (mg/l)        | 15.90 | 177 | 12.25| 0.2     | 68      | 14.10–17.71              |         |
| Female                  | 15.89 | 93  | 13.09| 1       | 68      | 13.23–18.55              |         |
| Male                    | 15.91 | 84  | 11.34| 0.2     | 60      | 13.49–17.71              |         |
| Osmolarity (mmol/l)     | 272.25| 144 | 15.67| 241.2   | 328.4   | 269.69–274.81            |         |
| Female                  | 270.12| 77  | 14.35| 251.2   | 328.4   | 266.91–273.32            |         |
| Male                    | 274.70| 67  | 16.84| 241.2   | 326     | 269.69–274.81            |         |

Removing outliers from the data set markedly affected reference value that might be used as ecophysiological references for the species.

Figure 4: Effect of sex and season (a) and sex, season and year (b) on the mean variations of body condition index (±SEM) in radio-tracked Hermann’s tortoises. The numbers above or below the bars indicate sample size. We found significant effects of sex, season and their interactions (see Table 2).
endothermic references were used (Hill et al., 1977; Lumeij, 1987; Cerón et al., 2005; Roche et al., 2008). It is thus important to describe the range of physiological variations in free-ranging and healthy individuals in the course of their normal activity in order to derive useful reference values.

This study combined various metrics recorded in adult female and male tortoises monitored during several years. None of the individuals presented any sign of disorder, exhibited a marked decrease of BCI or displayed unusual behaviours. Many matings were observed (n = 82). Several females were observed while laying their eggs (n = 4 individuals; one female laid two clutches), and we found 31 nests in the field. Considering that witnessing laying females and finding nests represent rare events in the field for this species, we crudely estimate that reproductive rate was normal. On average, tortoises travelled 30–40 m/day, covering greater distances in the closed habitat (Callas) compared with open mosaic habitat (Flasseens) that is considered to be more favourable for Hermann’s tortoises (Couturier et al., 2014). Perhaps the closed habitat forced tortoises to move more often between shelters, foraging and basking spots. Whatever the case, these values fall within the range of variations for Testudo species living in relatively similar habitats [15–30 m/day for T. h. hermanni in Italy, Chelazzi and Francisci (1979); 32 m/day for T. h. boetgerri in Romania, Rozylowicz and Popescu (2013); 20–120 m/day for T. graeca in Spain, Díaz-Paniagua et al. (1995)]

Figure 5: Annual and seasonal variations of plasma corticosterone concentration (shown as mean values + SEM) in Hermann’s tortoises. We found significant effects of year and season, and significant interactions between sex and season and between year and season (see Table 2). The numbers above the bars indicate sample size.

Figure 6: Effect of sex and season on glycaemia (shown as mean values + SEM) of radio-tracked Hermann’s tortoises. Two periods were considered; spring corresponds to vitellogenesis and laying periods, whereas summer corresponds to intensive male sexual activity. The numbers above the bars indicate sample size.

Figure 7: Effect of sex and season on Osmolarity (shown as mean values + SEM) of radio-tracked Hermann’s tortoises. Two periods were considered; spring corresponds to vitellogenesis and laying periods, whereas summer corresponds to intensive male sexual activity. The numbers above the bars indicate sample size.
daily displacements for males during the mating period 
(160 ± 40 vs. 67 ± 28 m/day during the post-mating period),
with lower mean daily displacements and a larger home
range in females have been recorded in the steppe tortoise,
Testudo horsfieldii (Lagarde et al., 2002, 2003b). Focusing
on Mediterranean habitats, the daily displacements we
recorded are representative of tortoises in the course of their
usual daily activity.

As expected for this species, females were larger com-
pared with males (Willemsen and Hailey, 2003; Djordjević
et al., 2013). Body condition increased in spring and
decreased in summer, probably because feeding activity cul-
minates in spring (Calzolai and Chelazzi, 1991; Rugiero and
Luiselli, 2006; Christopher et al., 1999; Nagy et al., 2002).
Yet, dehydration may participate in the summer decrease of
BCI. In addition, males tend to feed less during the mating
season in summer. These results are typical for tortoises
(Hen, 1997; Loehr et al., 2007) and they confirm that we
monitored ‘normal’ individuals exposed to natural environ-
mental fluctuations. Overall, we believe that our blood sam-
ple did not include sick or abnormal individuals and that
the range of variations of the parameters measured reflect
sex-specific responses to annual fluctuations of normally
breeding, free-ranging Hermann’s tortoises living in contrast-
ing habitats. Thus, Table 3 provides reference values that
can be used to gauge to what extent any individual may devi-
ate from the expected range of variations. However, in or-
der to interpret these values correctly, several factors should be
considered. We generally found strong effects of sex, season
and year, and interactions among them.

The most consistent effects involved sex and season. Corti-
costerone concentrations were notably higher in males
than in females; a result in accordance with previous studies
(Schramm et al., 1999; Lance et al., 2001; Drake et al.,
2012; Selman et al., 2012). Corticosterone decreased during
the active season in females but increased in males; a similar
pattern was observed for glycaemia. Thus, the present study
corroborates the notion that CORT concentration is
involved in the mobilization of energy stores, such as glucose
(Sapolsky et al., 2000; Moore and Jessop, 2003; Crespi
et al., 2013). The sex–season interaction may mirror the sex-
ual seasonal difference of reproductive effort; vitellogenesis
in spring vs. mate searching in summer. Plasma concentra-
tions of the main sex steroids also vary between sexes and
seasons in chelonians (Schramm et al., 1999; Ott et al.,
2000; Lance et al., 2001; Huot-Daubremont et al., 2003;
Sereau et al., 2010; Currylow et al., 2013; Wack et al.,
2008). If CORT (basal concentrations) supports the mobili-
zation of resources for reproduction, then parallel seasonal
fluctuations of plasma concentrations of estradiol and testos-
erone (in females and males, respectively) and CORT should
occur (Crespi et al., 2013). As expected, females exhibited
higher concentrations of plasma lipids (triglycerides and
cholesterol) than males. Indeed, lipids are typical markers of
vitellogenesis (Bonnet et al., 1994; Duggan et al., 2001;
Lagarde et al., 2003a). Moreover, it has been shown that
CORT enhances food intake and daily activity (Cote et al.,
2006). We observed similar pattern of variations between
sex and season in CORT, glycaemia and daily movement,
with an overall decrease for females between spring and
summer and an overall increase for males. Uric acid concen-
trations were higher in spring than summer. The opposite
pattern was found for the osmolality, a trait mainly influ-
enced by a low osmolality of the females in spring (Fig. 7),
followed by an elevation in summer, perhaps owing to low
precipitation. Osmolality and HCT were higher in males
than in females, possibly because of their higher surface/
body mass ratio that promotes dehydration. High HCT
might be related to male velocity (Bonnet et al., 2001b).
Substantial water lost during egg laying may also impact
female hydration level (Osmolarity and HCT) because eggs
contain important amounts of water at deposition (Tracy
et al., 1978; Turner et al., 1986). Further studies are required
to clarify these issues in tortoises.

Annual variations in interaction with seasons represent
another important source of fluctuations of the blood para-
meters measured; notably, considering uric acid, CORT and
HCT. Annual changes in food and water availability associated
with fluctuating ambient temperatures influence almost all life-
history traits in ectotherms, including metabolism and haem-
atological parameters (Packard, 1991). Other factors (e.g. food
availability, diet, drinking sites, shelter abundance, predators)
and interactions among individuals may also influence physio-
logical traits. Most of these factors were present in our two
study sites and applied in a peculiar way to each individual (e.g.
predators killed several tortoises in our two study sites and
probably threatened others, but not all), and thus our metrics
already incorporate these sources of variation. However, moni-
toring individuals during extreme climatic events (e.g. pro-
longed drought) would be helpful to calibrate ecophysiological
references better. Our 3 years of study, with the alternation of
springs and summers, nonetheless offered substantial varia-
tions. But we probably missed extreme and thus very inform-
ative events; opportunistic assessments would be helpful to
examine whether strong droughts are detrimental or not (e.g.
individuals may simply aestivate; Lagarde et al., 2002).
Extreme events (e.g. repeated fires, prolonged droughts) are
likely to be very important in terms of physiological response
and population viability.

Conclusions

Our results suggest that, in order to be exploitable by field
researchers, ranges of fluctuations of ecophysiological
metrics should be considered for each sex and season
(Table 3). The set of parameters measured, encompassing
behaviours, body condition and various haematological
traits, suggest that spring is a crucial period for females,
whereas summer is the most demanding season for males.
This sex difference may guide the selection of distinct and
supposedly appropriate periods to set up field actions, such as translocations. Importantly, the reference levels for haematological metrics provided in Table 3 should allow rapid and simple monitoring of the health status of tortoises in the future, both during field experiments and to survey remaining populations. The development of portable devices facilitates this type of investigation (Stoot et al., 2014), but interpretations rely on accurate baselines.

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