

Does testosterone influence activity budget in the male Greek tortoise (*Testudo graeca graeca*)?

Matthieu Sereau^a, Frédéric Lagarde^a, Xavier Bonnet^{a,*}, El Hassan El Mouden^b, Tahar Slimani^b, Laurent Dubroca^a, Colette Trouvé^a, Stéphanie Dano^a, André Lacroix^a

^a Centre d'Études Biologiques de Chizé, CNRS, 79360 Villiers en Bois, France

^b Biodiversité et Dynamique des Écosystèmes, Université Cadi Ayyad, Faculté des Sciences Semlalia, BP 2390, 40000 Marrakech, Morocco

ARTICLE INFO

Article history:

Received 25 August 2009

Revised 3 March 2010

Accepted 4 March 2010

Available online 10 March 2010

Keywords:

Dissociated pattern

Hormonal implant

Reptiles

Sexual behavior

Testosterone

Tortoise

ABSTRACT

The stimulatory effect of testosterone on male sexual activity is one of the clearest examples linking hormones and behaviors. However, this relationship is complex in Chelonians. We experimentally studied the influence of testosterone levels on the activity budget and space use in male Greek tortoises (*Testudo graeca graeca*) during the spring mating season. We first described the annual pattern of changes in plasma testosterone levels in free-ranging animals in Morocco. Two peaks, one in winter and one in summer, corresponded to periods of inactivity; whereas mating periods in spring and to a lesser extent in autumn were associated with low plasma testosterone levels. Second, we experimentally manipulated plasma testosterone levels in free-ranging males, and analyzed the behavioral consequences. The strong contrasts in plasma hormone levels induced by the experimental treatments did not result in changes in activity budget or space use, both in the short-term or more than one month after the beginning of the hormonal treatment. Our results suggest that testosterone levels did not influence directly behavioral activity in this species, either immediately or after a time delay of one month.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

Since Berthold's experiments (Berthold, 1849), a profusion of studies demonstrated that hormones play a central role in regulating animal behaviors (Moore and Lindzey, 1992; Becker et al., 2002). For historical and practical reasons, the positive influence of testosterone on sexual behaviors has been deeply investigated in behavioral endocrinology, essentially in males and to a lesser extent in females (Davidson et al., 1982; O'Carroll et al., 1985; Licht et al., 1992; Nelson, 2005). Notably, it has been shown that an elevation of plasma testosterone stimulates mate searching effort, territoriality, aggressiveness and more generally promotes the expression of various sexual behaviors and general activity.

Intensive sexual activity requires abundant energetic resources, is time consuming and compromises other activities (e.g. foraging); this demand often triggers the mobilization of body reserves which is regulated via the variation of hormone levels (e.g. corticosteroids, androgens; Marler and Moore, 1988; Wingfield et al., 1990; Folstad and Karter, 1992; Nolan et al., 1992; Wilson and Wingfield, 1994; Moore et al., 2000; Moore and Mason, 2001; Aubret et al., 2002; Cease et al., 2007). The increase of sexual activity entails important costs and is sometimes associated with signifi-

cant mortality (Bradley et al., 1980). Therefore, the influence of testosterone levels on the overall behavioral activity of animals plays an important role in evolutionary ecology (Nelson, 2005).

Some reptiles however, display complex relationships between changes in steroid levels and behaviors. The positive influence of testosterone levels on general activity, sexual or other behaviors has been validated for many species (Crews and Silver, 1985), but not all (King, 2002). In other species, an important time-delay (1–2 weeks) has been observed between the occurrence of hormonal peaks and subsequent behavioral effects (Naulleau et al., 1987; Bonnet and Naulleau, 1996; Krohmer et al., 2002). A particularly interesting system called 'dissociated reproductive pattern' was discovered in the garter snake (*Thamnophis sirtalis parietalis*) and subsequently in other taxa (Hosken et al., 1998; Canosa and Ceballos, 2002; Sarkar et al. 2002; Woolley et al., 2004; but see Benner and Woodley, 2007). Comprehensive experimental studies have shown that sexual behaviors do not depend on increased levels of gonadal steroid hormones during the mating season but on environmental cues (e.g. ambient temperatures) (Camazine et al., 1980; Crews, 1984; Crews et al., 1984; Moore and Lindzey, 1992).

Among reptiles, chelonians represent the most problematic group. Although an associated pattern was described for one Galapagos tortoise (Schramm et al., 1999), the far more widespread strong temporal disconnection between gametogenesis, fecundation, sexual behaviors, general activity, and hormone levels gener-

* Corresponding author.

E-mail address: bonnet@cebc.cnrs.fr (X. Bonnet).

ates multifaceted pictures of reproductive cycles without clear patterns emerging from life style (e.g. terrestrial versus aquatic) or phylogenetic comparisons (Kuchling, 1999). The roles of marked annual variations of testosterone levels observed in many chelonians remain therefore obscure, especially to understand seasonal changes of general and sexual activity.

Clarification requires long-term sampling of large numbers of free-ranging individuals to encompass the strong inter-individual variations typically documented in reptiles, in combination with experiments where hormone levels are manipulated. Unfortunately, such data are almost totally lacking. To date, the only experiment exploring this issue has been carried out on *Kinosternon odoratum* (Mendonça, 1987), but the results remained difficult to interpret, perhaps due to the disruptive action of captivity on the sexual behaviors of the tested animals (Kuchling, 1999). The scarcity of data does not reflect researcher's neglect but rather the notorious difficulties to record the highly sporadic sexual behaviors of wild tortoises (Lagarde et al., 2003a,b, 2008).

We benefited from a long-term mark-recapture study of the Greek tortoise (*Testudo graeca graeca*) to perform a field experiment on the relationships between testosterone levels, activity and behaviors. The strength of this study relies on the use of a technique recently developed that allows continuous monitoring of the main behaviors of free-ranging tortoises (Lagarde et al., 2008), combined with hormonal manipulation of testosterone levels. We addressed the following questions:

- (1) Do seasonal changes of testosterone levels correlate with male activity and sexual behaviors?
- (2) Does an artificial elevation of testosterone level increase sexual behaviors with a concomitant decrease of competing activities (e.g. foraging) and/or generate a significant increase of total activity time?
- (3) If so, does this effect occur rapidly (days) or after a marked time decoupling (weeks)?
- (4) Do testosterone antagonists artificially diminish general activity and sexual behaviors?

In order to frame the experiments into a realistic context, the study was based on the description of annual changes of plasma testosterone using a large sample size. Therefore the structure of the paper is as follows: (1) a descriptive phase to obtain the general profile of testosterone variations in free-ranging males; (2) the experimental phase combining hormonal manipulation and field monitoring.

2. Material and methods

2.1. Study area

We studied free-ranging Greek tortoises in a 33 ha area localized in the Central Jbilet-Mountains (25 km north of Marrakech, southern Morocco, 31°37'N, 8°02'W, 580 m above sea level). The region is arid, with mean annual rainfall averaging 240 mm, most precipitations occurring between September and February (El Mouden et al., 1999; Znari et al., 2002). Ambient temperatures vary greatly over time. The mean average air temperatures recorded under shelter in the hottest month (July, mid-summer) exceed 39 °C, while the minimal air temperatures drop below 0 °C in winter (Emberger, 1933; Le Houérou, 1989). The sclerophylic vegetation consists mainly in scattered bushes of Jujube (*Ziziphus lotus*) in association with Acacias (*Acacia gummifera*) and Retams (*Retama monosperma*). An ephemeral herbaceous stratum grows in late winter–early spring following the main rainfalls (El Mouden et al., 2005).

2.2. Study species

The Greek tortoise (*Testudo graeca graeca*) is a medium sized herbivorous testudinae, widely distributed in the Mediterranean region (Iverson, 1992; Ernst and Barbour, 1989). The activity pattern of the Greek tortoise, shaped by local climatic conditions, is bi-modal: individuals are active from February to June and then from September to November (Andreu et al., 2000; Slimani et al., 2003). Both winter (cold) and summer (hot and dry) periods are characterized by an almost total cessation of activity (Lambert, 1981). In our study area, foraging, displacements, sexual behaviors and egg-laying occur essentially in late winter and early spring from February to April. From late May to mid-September (dry and hot months) and in January to mid-February (cold months) the tortoises remain virtually immobile buried in the soil (El Mouden et al., 2001; Slimani et al., 2001). A second, albeit markedly attenuated, activity period occurs in autumn after the aestivation phase, in late September–early December (Ernst and Barbour, 1989; Andreu et al., 2000; El Mouden et al., 2001; Slimani et al., 2001).

This study focuses on the activity budget and behaviors of adult males. Each animal was permanently marked with shell notches. The tortoises were sexed using the classical criterion used for the *Testudo* genus: males notably exhibit a markedly longer tail than females and a concave plastron (Andreu et al., 2000; Bonnet et al., 2001b; El Mouden et al., 2001; Ben Kaddour et al., 2008). Males larger than 100 mm (shell length) and presenting marked contracting of the last annual growth rings were considered as adults: these two factors indicate sexual maturity (El Mouden et al., 2001; Lagarde et al., 2001; Slimani et al., 2001; Ben Kaddour et al., 2005, 2008). Morphological studies of the ultrastructure of the testes have shown a spring recrudescence of spermatogenesis, with complete germinal series in autumn and testicular regression in winter (Ibargüengoytia et al., 1999). Spring and autumn increases of testes activity involved both Sertoli and Leydig cells, suggesting a synchrony between spermatogenesis and steroidogenesis (Ibargüengoytia et al., 1999).

Importantly, the density in our study population was six adult individuals per hectare, a value that ranks high for terrestrial chelonians (Ben Kaddour et al., 2006). Moreover, both male and female Greek tortoises aggregate under bushes (essentially Jujube bushes) that act as meeting points. Each male had abundant opportunities to find and court a mature female within the course of the study. Consequently, it was possible to discard a putative low population density effect on sexual behaviors.

2.3. Description of annual changes of plasma testosterone in free-ranging males

Blood samples were collected every month (except October) from February 2003 to April 2004, immediately after capture on 140 different free-ranging males. The sampling effort was more intense during the spring activity period (see Fig. 1 for sample size per month). A total volume of 250–1000 µl of blood (adjusted to the mass of the tortoise) was taken. Each male was sampled only once; our analyses on annual changes of plasma testosterone did not include pseudo-replicates. We collected blood by jugular puncture (Jacobson et al., 1992), a method we used intensively on a closely related species (*Testudo horsfieldii*; Lagarde et al., 2003a). The blood was collected within 5 min after capture, immediately transferred into dry heparinized Vacutainers, and stored on ice pending centrifugation in the laboratory within 3–4 h after collection. The plasma was then separated into Sarstedt tubes and stored below –25 °C until assays.

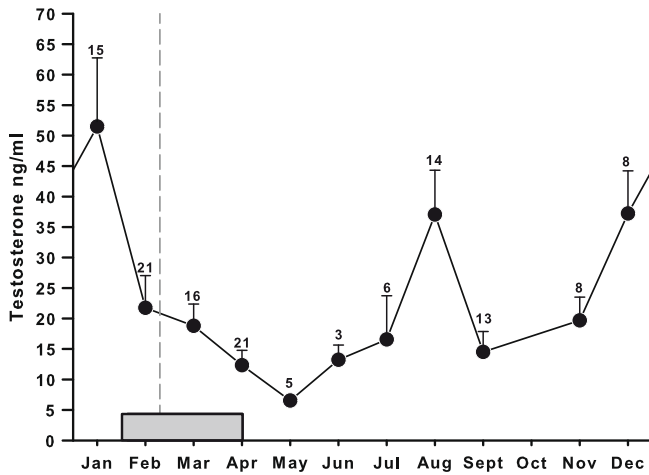


Fig. 1. Annual changes in plasma testosterone levels in free-ranging male Greek tortoises in the Marrakech region. The open circles provide the mean values obtained each month, the error bars represent standard deviations, and sample sizes are indicated above. The gray box indicates both mating time and the dashed vertical line corresponds to the date of implantation (see text and Fig. 2).

2.4. Hormonal manipulation in the field

In early spring 2004 (late February–early April), twenty-four adult male Greek tortoises were individually monitored during more than two months to encompass the main annual peak of activity, notably the major period during which sexual behaviors are exhibited. Individuals were randomly selected in the population and randomly allocated into four experimental batches. There was no significant difference in mass, body size or body condition (mass scaled by size, Bonnet et al., 2001b) between the four groups (all $P > 0.25$). The four batches were composed as follows:

- Eight males were fitted with testosterone implants in order to artificially increase their plasma testosterone level (henceforth named batch T).
- Eight males received flutamide implants (batch F). The flutamide is a powerful non-steroidic anti-androgen that occupies the testosterone receptors, and hence, blocks the action of testosterone (Liao et al., 1974; Neumann et al., 1977; Alexandre and Balthazart, 1987; Ayub and Levell, 1987).
- Four sham-operated males were fitted with empty implants, exactly under the same technical conditions as for the two first groups (batch S).
- Four control males were not fitted with any implant, but were manipulated in the same way as the tortoises from the first three groups to equally distribute handling stress (batch C).

The implants consisted of an empty segment of a flexible silicone tube (microporous silicone: Silastic Cat N°601–335) cut to an average length of 5 ± 1 mm. One extremity was sealed with silicone glue; the implant was then filled with crystallized steroid, flutamide (Sigma Laboratory), or left empty, and the second extremity was sealed (see Bonnet, 1996 for details). The mass of the active substance within each implant was measured using a precision balance to the tenth of a microgram. The implants were kept in sterile airtight plastic bags and stored at 5°C until use. The implants were slipped under the skin of the tortoises, through a small incision (< 2 mm), near the insertion of the posterior legs, on the level of the inguinal fold (two per side) using a sterilized nozzle fitted with a pushrod. Each individual from batches T and F received four implants containing, respectively, $16 \pm 0.3 \mu\text{g}$ of testosterone, or $20 \pm 0.6 \mu\text{g}$ of flutamide. The tortoises from batch

S were fitted with four empty implants. All the implants were removed at the end of the experiment, and no sign of infection was observed.

2.5. Monitoring of the experimental males

Each male was equipped with a radio-transmitter (AVM or Telonics; 5–20 g), and was located twice a day (morning and evening) using a receiver (LA12Q AVM) and a Yagi three element antenna. The transmitters were fixed horizontally using synthetic resin on the front face of the carapace. Each tracked individual was sighted and its location noted on each tracking occasion. Locations were recorded using a GPS (Garmin 3+; accuracy 8 ± 4 m [mean \pm standard deviation] tested with 34 different records on fixed points in the study site).

Each male was also regularly sampled for hormonal assays. The first sample of blood was collected 16 days before the hormonal manipulations; the second one immediately after (< 1 min); the next three samplings occurred three days; 22 days; and finally 38 days after the insertion of the implants. None of the tortoises exhibited any visible distress (e.g. from blood sampling).

The activity budget was automatically recorded using acceleration data loggers (ActiTrac© 1998 IM SYSTEMS). Lagarde et al. (2008) recently developed an inductive model to discriminate the five main behaviors of free-ranging Greek tortoises: immobility, walking, foraging, digging and sexual behaviors. The procedure was able to identify and to quantify correctly 92% of the behaviors (79–98%; Lagarde et al. (2008)). The data loggers were fixed to the top of the shell using synthetic resin. The overall equipment (transmitter, resin and acceleration data logger) represented $16 \pm 4\%$ of individual body mass, a value relatively low in Chelonians, without detectable effect on the behavior or body condition of free-ranging tortoises (see Lagarde et al., 2002 for details on this issue). Unfortunately, only six acceleration data loggers were available; it was thus impossible to record the activity of the 24 males simultaneously. Therefore, we used a short-term alternation system. We split the 24 males in four groups of six males (2 T, 2 F, 1 S and 1 C). Each group was monitored during one day; the data loggers were then removed and glued on six other tortoises, etc. One day of preparation was also required because transferring the loggers was time-consuming and to minimize the possible disturbing effect of handling on behaviors, the acceleration data loggers were fixed at least 11 h before the start of recording; thus, each animal had ample time to resume normal activity. Greek tortoises are diurnal, at least in spring (Lagarde et al., 2008), as such, the onset of the automatic recording was initiated at 6:00 AM and lasted 18 h, thereby encompassing the entire daily activity period. Each individual was thus fitted with the data logger for 2 days (1 day of preparation + 1 day of recording). Consequently, 8 days (2 days \times 4 groups) were required to monitor the 24 males once. Each 8-day time period was subsequently referred as a session. Over the 68 days of the study period (8 of February to mid-April 2004) we were able to run 8 consecutive sessions during which the activity of each male was automatically monitored for an identical amount of time (144 h, 8 sessions) spread over the entire duration of the study. Overall, we performed 2 sessions 1–2 weeks before hormonal implantations, 1 session shortly after implantation, and 5 sessions during the following 5–6 weeks. The main advantage of the rotation system compared to alternative options (e.g. successive monitoring of 6 groups) was to distribute the environmental effects over time. Indeed, both environmental conditions (i.e. food and water availability, ambient temperatures), and physiological status (i.e. body reserves, reproductive stage) influence behaviors markedly, and these factors vary significantly over time, generating major complications. Note that blood samples were taken on all

tortoises regularly and that radio-tracking was continuous for all individuals.

2.6. Testosterone assays

Hormonal assays were performed in the Centre d'Etudes Biologiques de Chizé where plasma steroid levels are routinely measured on a wide variety of vertebrates. Testosterone level was measured by Radio-Immuno-Assay (RIA) on 50 μ l of plasma after an extraction using 500 μ l of di-ethyl-ether (the extraction efficiency was 0.93 ± 0.1 , mean \pm standard deviation). The cross reactivity of the specific antibody (Sigma Laboratory) with other steroids, including androgens, was low (percentages of cross reactivity for B/BO on antibodies anti-testosterone: 5 α -dihydrotestosterone: 17.8%; 5 β -androstene-3 β , 17 β -diol: 1.4%; 5 α -androstene-3 β , 17 β -diol: 1.2%; androstenedione: 1.4%; epitestosterone: 0.7%; progesterone: 0.07%). The assay's sensitivity was 7.8 pg by tube, i.e. 156 pg/ml. The variations intra- and inter-assay were low, respectively, 6% and 12%. Dehydrotestosterone (DHT) was not assayed. However, changes of DHT and testosterone plasma levels are parallel in reptiles; testosterone assays thus provide an indicator of possible seasonal variations of DHT (Saint Girons et al., 1993; Whittier et al., 1997). DHT concentrations are almost systematically lower than testosterone concentrations. Androgens are aromatized in the brain to induce behavioral effects; testosterone and DHT are therefore only slightly different in terms of male behavior regulation (Callard et al., 1979; Naftolin, 1994; Becker et al., 2002; Santen et al., 2009). For these different reasons, although different hormones interact, most studies on androgen/behavior relationships focus on testosterone.

2.7. Analyses and precautions

Body condition index was calculated as the residual value of the regression between body mass (g, log) against carapace length (mm, log). Individuals were sampled only once to describe the patterns of plasma testosterone levels in free-ranging animals, hence no pseudo-replicates were included. The distribution pattern of the hormonal data deviated markedly from normality thus we performed non-parametric Kruskal–Wallis ANOVAs to compare the average values calculated each month. For the 24 tortoises monitored in the field during three months, behavioral and hormonal data were gathered several times on the same individual. Therefore, we compared the four experimental groups using ANOVAs for repeated measures over time (O'Brien and Kaiser, 1985). Post hoc tests were performed to better examine the differences in behaviors and/or plasma testosterone level between the groups at certain time periods (LSD tests; Winer, 1962; Milliken and Johnson, 1984; Hays, 1988). Given the abundance of statistical results owing to the multiplicity of possible combinations when performing post hoc tests, only relevant (usually significant) differences are presented in Section 3. All these tests were run using the software Statistica 7.1 (Statsoft, 2005).

Displacements and home range (minimum convex polygons) were estimated using one fix per day on the 24 radio-tracked animals (total, 68 fixes per individual). The estimates were performed using Arcview (ESRI, Animal movement extension and Geographical Information System). In order to analyze the possible effects of the hormonal manipulation on displacement and home range (two indexes of the intensity of mate searching in males) we estimated a mean daily-displacement value and calculated the home range regularly over time (before, during and after the onset of hormonal manipulation). Observed home range size increases automatically over time as more fixes are collected, and a minimum number of fixes are required. Therefore, we divided the total study period into six equal time periods (to include at least six fixes per individual

and per unit of time). This procedure entailed a slight difference in the time scale used between several analyses (i.e. 8 sessions versus 6 time periods). However, this did not hamper our ability to compare the four groups of tortoises and thus to address the main biological questions: does hormonal manipulation influence activity in male Greek tortoises?

We also examined a possible negative effect of the experiment and of the monitoring on the tortoises. Change of body condition is an integrative measurement of energy budget and foraging success. During the two months of the study, variations of mean body condition were not different between the 24 experimental males compared to 24 other randomly sampled males (repeated measure ANOVA with body condition index as the dependent variable and the experimental status [involved or not in the experiment] as the factor, $F_{1,46} = 1.14$, $P = 0.28$). We found no significant effect of the extra-load associated with the activity data loggers on the displacements exhibited by the radio-tracked males (Friedman ANOVA for repeated measures over time with the distance traveled by each radio-tracked tortoise as the dependent variable and the presence of the acceleration data logger as a factor: $\chi^2 = 2.43$, $df = 2$, $P = 0.30$). Overall, the experimental procedures had no detectable impact on the tortoises (Lagarde et al., 2008).

As expected, we found no significant difference between the sham-operated (S) and the control (C) batches ($P > 0.05$ for all tests). Consequently, these two groups were pooled together for the subsequent analyses, and were referred as the control group ($C = C + S$).

3. Results

3.1. Description of annual changes of plasma testosterone in free-ranging males

Mean plasma testosterone levels varied greatly over months, from 5 ng/ml to 50 ng/ml (Kruskal–Wallis: $df = 10$, $H = 36$, $P < 0.001$; Fig. 1). Two peaks were visible, in winter (December–January) and in late summer (August). Post hoc tests suggested the existence of only two groups of values: high values in December and January and in August (not statistically distinguishable from each other but significantly different from all the other months), versus low values for the rest of the year. The highest mean value was observed in January (culminating at approximately 50 ng/ml) preceded by an increase in December. The second peak occurred in August (35 ng/ml). During the rest of the year, the mean values remained low, with the minimal mean value recorded in May (5 ng/ml). The period of intensive general and sexual activity (February–April) was not clearly associated with high values of plasma testosterone, although the onset of this active period was preceded by a peak one month before, in January (Fig. 1).

3.2. Hormonal manipulation in the field

In the testosterone-implanted group (T), plasma levels of testosterone increased drastically, demonstrating that the technique was efficient (Fig. 2). Indeed, shortly after fitting the animals with the implants, the mean plasma value peaked to approximately 90 ng/ml. The circulating levels of testosterone decreased later, but nonetheless remained high during several weeks, close to 40 ng/ml (a value significantly higher compared to those recorded in the other batches; all post hoc tests: $P < 0.015$; Fig. 2). Importantly, the artificially increased level of plasma testosterone ranged within the values recorded in January on free-ranging tortoises (extreme values ranging from 0.8 to 166.0 ng/ml in the un-manipulated tortoises), and enabled us to examine the results within a

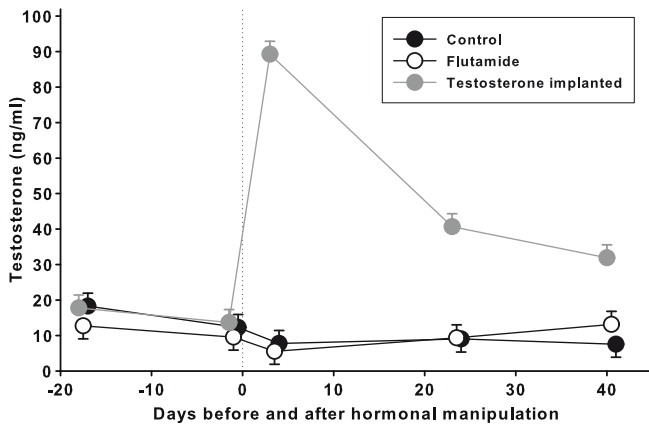


Fig. 2. Effects of the silastic implants fitted with hormones on plasma testosterone concentration in male Greek tortoises (means \pm SE). Gray circles indicate males fitted with testosterone implants ($N = 8$), open circles indicate flutamide implanted males ($N = 8$), and black circles represent control animals (C + S [see text], $N = 8$). The dashed vertical line corresponds to the date of implantation (see Fig. 1). For ease of reading, the symbols have been slightly shifted on the “x” axis.

realistic hormonal context. As expected, the plasma testosterone levels remained low in all the other experimental groups and followed the same general testosterone levels observed in the free-ranging un-manipulated animals (all $P < 0.05$; Fig. 2). We detected a significant effect of the experimental treatment on testosterone levels; repeated measures ANOVA with plasma testosterone level assayed during the four successive samplings as the dependent variable, and the three experimental batches (T, F, S + C) as the factor, with a significant effect of the experimental group and time ($F_{2,21} = 77.8$, $P < 0.001$ and $F_{3,63} = 21.8$, $P < 0.001$, respectively); and a strong interaction between time and the treatment ($F_{6,63} = 32.8$, $P < 0.001$; Fig. 2). There was no significant effect of the flutamide on testosterone level (i.e. compared to batch C, all post hoc tests: $P > 0.05$).

3.3. Effect of the hormonal manipulation on the activity budget

We failed to detect any significant effect of the hormonal manipulation on any aspects of the behavioral activity (Table 1). Similarly, we found no interaction between the experimental treatment and the time period (i.e. no interaction was detected using the experimental batches and the eight successive sessions as the factors; see Table 2). By contrast, we found significant variations over time (Table 1). For instance, climatic conditions during the

3rd session were unfavorable (cold) and associated with low activity; but they became favorable and were associated with important activity during the 5th session (Table 2). These results clearly show that tortoise activity responded to climatic conditions, that our technique enabled the observation of such variations, and thus that the short-term alternation system was the appropriate procedure to take into account such significant time variability. Most of the activity variations were due to marked differences of the time allocated to foraging (Tables 1 and 2): indeed the significant effects were limited to the parameters that involve, directly (time spent foraging) or indirectly (total activity, time spent walking, proportion of time walking), the amount of time devoted to finding and ingesting food resources. Sexual behaviors were clearly detected, but they represented a modest proportion of the activity budget, confirming previous findings (Lagarde et al., 2008). As was observed for the general activity, the intensity of sexual behaviors varied accordingly to climatic condition, with a peak mid course of the study (Table 1).

3.4. Effect of the hormonal manipulation on space occupation

The results obtained with continuous radio-tracking on space use, notably home range and displacements, mirror the results above (Tables 3 and 4). We found no significant effect of the experimental batch, and no interaction between the experimental batches and the time period. Interestingly, we observed again significant effects of time (Tables 3 and 4). Such parallel findings between behavioral activity, mobility and thus home range, obtained using completely different methods (automatic acceleration data loggers versus classical radio-tracking), were expected and considerably strengthened our results.

4. Discussion

Our results provide another example of the atypical and complex patterns exhibited by different chelonian species, characterized by the absence of a clear link between testosterone levels, behaviors, and male activity during the mating season (Kuchling, 1999). Explanations broadly fall into three categories: methodological caveats, simple time-disconnection between changes in plasma testosterone levels and the effects on behaviors (e.g. hormone peak precedes the expression of behaviors), and more complex time-dissociated reproductive tactics (interplay between environmental cues and long-term decoupling between hormone peak and their effects on behaviors).

Table 1

Total daily activity (minutes per day, mean \pm SD) and main behaviors (% of total activity) of free-ranging male Greek tortoises: controls (sham-operated or un-manipulated), implanted with flutamide, or implanted with testosterone. The measures were repeated 8 times: before (columns 1–2) and after hormonal manipulation (columns 3–8).

Behavior	Group	1	2	3	4	5	6	7	8
Total (min)	Control	53.3 \pm 60.6	52.3 \pm 49.7	22.0 \pm 21.7	64.1 \pm 56.4	93.7 \pm 62.6	73.8 \pm 39.7	81.0 \pm 71.6	48.1 \pm 50.0
	Flutamide	95.9 \pm 115	55.8 \pm 52.0	15.4 \pm 24.7	45.2 \pm 68.1	85.6 \pm 67.9	17.6 \pm 30.4	95.5 \pm 66.4	56.7 \pm 91.0
	Testosterone	64.2 \pm 58.4	25.2 \pm 38.7	22.4 \pm 31.5	63.3 \pm 58.2	165 \pm 152	61.0 \pm 54.8	102 \pm 108	49.3 \pm 63.7
Walking	Control	14.5 \pm 12.4	19.7 \pm 19.2	21.0 \pm 17.9	23.9 \pm 16.7	27.1 \pm 15.5	25.9 \pm 11.2	19.6 \pm 19.6	19.4 \pm 17.4
	Flutamide	15.4 \pm 13.4	17.6 \pm 16.9	8.20 \pm 13.9	14.2 \pm 18.8	30.8 \pm 13.2	15.0 \pm 22.3	22.1 \pm 14.8	17.1 \pm 18.2
	Testosterone	18.0 \pm 15.4	9.10 \pm 12.6	9.80 \pm 14.4	18.4 \pm 19.5	25.0 \pm 11.1	20.9 \pm 16.2	23.2 \pm 13.3	18.3 \pm 19.7
Foraging	Control	10.2 \pm 15.9	8.70 \pm 9.90	19.8 \pm 20.3	13.0 \pm 15.7	12.5 \pm 13.1	23.2 \pm 26.9	8.30 \pm 9.80	23.4 \pm 25.5
	Flutamide	11.6 \pm 19.9	12.9 \pm 15.4	18.1 \pm 28.2	22.3 \pm 31.4	30.7 \pm 21.2	11.5 \pm 21.6	10.4 \pm 9.70	23.1 \pm 28.6
	Testosterone	15.2 \pm 13.1	24.0 \pm 31.5	20.4 \pm 31.9	10.9 \pm 10.6	23.4 \pm 26.9	22.5 \pm 21.1	21.1 \pm 21.4	17.3 \pm 22.5
Hidden	Control	27.6 \pm 23.6	25.2 \pm 22.8	18.9 \pm 18.0	29.1 \pm 21.7	34.6 \pm 17.5	37.8 \pm 13.3	35.6 \pm 25.6	45.1 \pm 41.3
	Flutamide	24.9 \pm 23.5	23.1 \pm 21.7	21.0 \pm 31.8	12.7 \pm 16.3	32.6 \pm 12.8	10.2 \pm 14.3	30.2 \pm 19.3	22.7 \pm 21.8
	Testosterone	30.4 \pm 20.9	15.3 \pm 19.6	5.60 \pm 11.1	24.0 \pm 22.8	35.1 \pm 12.4	25.3 \pm 16.4	34.0 \pm 20.2	22.6 \pm 19.7
Sexual	Control	0.76 \pm 1.34	0.25 \pm 0.48	0.01 \pm 0.02	0.13 \pm 0.30	0.85 \pm 1.19	0.22 \pm 0.48	0.30 \pm 0.49	0.34 \pm 0.73
	Flutamide	0.84 \pm 1.21	0.33 \pm 0.58	0.83 \pm 2.13	0.12 \pm 0.23	0.15 \pm 0.24	0.02 \pm 0.06	0.51 \pm 0.51	0.10 \pm 0.18
	Testosterone	0.30 \pm 0.48	0.16 \pm 0.31	0.00 \pm 0.00	0.33 \pm 0.58	1.40 \pm 2.42	0.44 \pm 0.81	0.69 \pm 1.09	0.37 \pm 0.69

Table 2
Effects of hormonal treatment and time elapsed since the beginning of the experiment on the activity budget of free-ranging male Greek tortoises (ANOVA for repeated measures with behavior as the dependent variable, the experimental group and time as independent variables). Significant effects are indicated in bold.

Activity	Group			Time			Group × time		
	df	F	P	df	F	P	df	F	P
Total activity	2	0.231	0.795	7	5.01	<0.001	14	0.967	0.49
Walking	2	0.143	0.867	7	4.597	<0.001	14	0.777	0.693
Foraging	2	0.019	0.981	7	4.779	<0.001	14	0.993	0.464
Hidden	2	2.01	0.159	7	1.301	0.254	14	0.648	0.821
Sexual behaviors	2	1.383	0.273	7	1.685	0.117	14	1.115	0.35

Table 3
Comparison of the displacements and home range (mean ± standard deviation) between free-ranging Greek tortoises.

Space utilization	Group	Experimental period (time)					
		1	2	3	4	5	6
Home range (ha)	Control	0.86 ± 1.05	0.21 ± 0.38	1.10 ± 1.33	0.84 ± 1.00	0.43 ± 0.58	0.93 ± 0.98
	Flutamide	0.63 ± 1.06	0.09 ± 0.14	0.40 ± 0.48	0.98 ± 1.80	0.55 ± 0.94	0.48 ± 1.02
	Testosterone	1.46 ± 1.46	0.18 ± 0.24	1.20 ± 1.37	1.57 ± 1.66	0.73 ± 0.65	1.21 ± 1.21
Average distance travelled (m)	Control	50.1 ± 68.3	24.8 ± 43.3	42.7 ± 54.4	50.7 ± 64.6	37.9 ± 60.1	62.4 ± 79.5
	Flutamide	39.1 ± 55.7	33.7 ± 72.2	32.6 ± 63.2	47.9 ± 73.9	33.7 ± 56.2	36.4 ± 53.1
	Testosterone	50.7 ± 67.9	24.0 ± 31.7	42.8 ± 62.6	79.4 ± 108	42.3 ± 66.2	63.2 ± 86.8

Table 4
Effects of hormonal treatment and time on the displacements and home range in free-ranging Greek tortoises with different hormonal treatments (ANOVA for repeated measures with displacements or home range as dependent variables, experimental batch and time as independent variables). Significant effects are indicated in bold.

Space utilization	Experimental batch			Time			Batch × time		
	df	F	P	df	F	P	df	F	P
Average displacements	2	1.367	0.278	5	3.621	0.003	10	0.718	0.742
Home range	2	2.066	0.152	5	2.851	0.019	10	0.385	0.951

4.1. Methodological caveats

Although rarely acknowledged, sampling limitations should be examined. Notably, there is no surrogate to small sample size, especially in reptiles where inter-individual variations are generally extremely pronounced. For instance, in snakes the controversial roles of oestradiol and progesterone levels during vitellogenesis and gestation (e.g. Saint Girons et al., 1993) have been eventually clarified using large samples (and experiments) of both reproductive and non-reproductive females during the entire annual cycle (Bonnet et al., 1994, 2001a).

Regarding chelonians, large data sets collected over long-term periods (at least during the active season) on free-ranging tortoises are extremely scarce (Lagarde et al., 2003c), impeding inter-specific comparisons. Experiments performed under field conditions are lacking. The current study combined long-term sampling on a reasonably large number of tortoises in combination with an experiment on free-ranging individuals. Importantly, our method involved an automatic procedure to monitor accurately and regularly (>100 h per individual) the main behaviors and displacements (every day over 2 months), providing a mean to assess time variations, the impact of hormonal manipulation, and their interactions. Our results clearly revealed marked seasonal changes of plasma testosterone (Fig. 1). Interestingly, histological investigations of the ultrastructural characteristics of the testes in *Testudo graeca* suggested a pattern of steroidogenic activity that matches nicely our hormonal data with little steroidogenesis in winter, but marked signs of androgen synthesis after winter emergence and autumn (Ibargüengoytia et al., 1999). However, such changes were not associated with the intensity of individual activity and the expression of sexual behaviors. Peaks of testosterone were dissociated from the period during which maximal activity and sexual behaviors were observed; and more convincingly, an artificial in-

crease in plasma testosterone, and the use of an antagonist, failed to induce any effect. Artificial increase of testosterone successfully prolonged the winter peak. However, no behavioral consequence was observed, suggesting that a simple delayed effect (1 month) between testosterone peak and activity was unlikely. Furthermore, the implantation of flutamide did not have any effect on the testosterone levels. Several studies have shown that flutamide can cause an increase in testosterone levels, likely because it tempers the negative testosterone feedback on the hypothalamo-hypophysis axis on its own secretion (Deputte et al., 1994; Shin et al., 2002); although such assumption might not hold because the spermatogenic cycle might have been quiescent at the time of experimentation. Overall, the stimulatory effects of testosterone, despite the use of realistic experimental doses, and the expected feedbacks were not observed in the Greek tortoise. However, because both hormonal and histological information showed that maximal gonadal and steroidogenesis activities occur before and during the mating season, and not after, our study does not clearly support a typical dissociated reproductive tactic (Kuchling, 1999; Crews and Moore, 2005).

4.2. Dissociated reproductive patterns

Several studies suggest a close temporal association between peaks of plasma testosterone and sexual activity in chelonians (Callard et al., 1976; Lance and Callard, 1980; Licht et al., 1985; Ernst and Barbour, 1989; Messner et al., 1993; Mahmoud and Licht, 1997; Schramm et al., 1999; Andreu et al., 2000; El Mouden et al., 2001; Lagarde et al., 2003c). Other studies, have shown that high testosterone levels are observed in summer, long after or long before the mating season; suggesting a possible time delay between testosterone peaks and behaviors (Kuchling et al., 1981; Kuchling, 1982; Licht et al., 1985; Mendonça, 1987; Mendonça and Licht,

1986; Rostal et al., 1994; Silva et al., 1984; Wibbels et al., 1992). In the current study, the main peak of testosterone occurred one month before (January) the period of intensive activity during which most sexual behaviors were observed (February–March); perhaps that several weeks were necessary to induce behaviors? However, our experiment suggested an absence of testosterone stimulation on activity (including sexual behaviors). Therefore, a more complicated dissociated pattern where long-term changes of testosterone levels (e.g. before or during hibernation) combined with current (spring) environmental cues influence male activity, remains the most parsimonious alternative (Crews et al., 1984; Moore et al., 2000; Krohmer et al., 2002; Crews and Moore, 2005).

A complex dissociated reproductive pattern could be an adaptation to extreme environments, enabling the limitation of costly sexual behaviors to the periods when environmental conditions are favorable (Crews, 1998). The production of spermatozooids, under the control of testosterone, would be time-dissociated from sexual behaviors: different components of reproductive effort being distributed over time (Callard et al., 1976; Kuchling et al., 1981; Licht, 1982; McPherson et al., 1982; Kuchling, 1999). This would alleviate the costs of reproduction that would otherwise be a very intensive reproductive episode if both gametogenesis and mate searching occurred simultaneously. The Greek tortoise indeed combines a dissociated reproductive pattern with a long mating period while the steppe tortoise (*Testudo horsfieldi*), a species that faces extreme environmental conditions, exhibits an associated reproductive pattern and a very short mating period (Lagarde et al., 2003c). Such contrasts suggest that environmental conditions likely play a major role. Climatic conditions, especially temperature and precipitations, constrain the activity of tortoises (Ataev, 1975; Pough, 1980; Lambert, 1981; Hailey et al., 1984; Willemssen, 1991; Panagiota and Valakos, 1992; Pough et al., 1999). The activity (general and sexual) of the free-ranging Greek tortoises dropped during the cool and dry periods of our study; and increased during favorable mild and wet periods. During the main mating season (spring) sexual behaviors were sporadic, representing on average less than 1% of the total activity budget (Lagarde et al., 2008). By contrast, sexual behaviors reach 40% in the steppe tortoise during the spring mating period (Lagarde et al., 2002). This latter species inhabits deserts characterized by hyper-continental climatic conditions (Lagarde et al., 2001), and the activity period is concentrated over three months only. In such a context, sexual behaviors can be expressed only over a two-week time period (Lagarde et al., 2002, 2003c). Less harsh climatic conditions prevail in the Central Jbilet (arid Mediterranean climate), allowing an annual activity period of more than seven months (Ernst and Barbour, 1989; Andreu et al., 2000; El Mouden et al., 2001; Slimani et al., 2001). Our study indeed revealed that sexual behaviors represented a modest proportion of the activity budget, but they were displayed over long time periods (at least two months [current study], but often much more [unpublished]). The necessity for species such as the steppe tortoise to exhibit intense sexual behaviors at a precise time period may favor a strong underlying endocrine regulation linking tightly spring emergence with sexual activity (Lagarde et al., 2003c). Similarly, for the desert tortoise (*Gopherus agassizii*), the active period is short, sexual behaviors are concentrated at that time and preceded by peaks of testosterone (Nagy and Medica, 1986; Schramm et al., 1999; Averill-Murray et al., 2002). Such time-links between environmental constraints and reproduction represent the classical pattern in the animal kingdom (Becker et al., 2002). By contrast, male Greek tortoises may benefit from any opportunity to court females over a long time period (any time in spring and in autumn when the weather is favorable), without committing themselves to very intense mate searching activity. In such a case, sexual behaviors would be mostly regulated by the current climatic conditions rather than by current

changes in testosterone levels. Testosterone peak could be synchronized with the production of spermatozooids (Kuchling et al., 1981; Licht, 1982; McPherson et al., 1982; Kuchling, 1999). This hypothesis is supported by the fact that many temperate tortoises can express sexual behaviors over the whole duration of their activity cycle, but only do so when environmental conditions are favorable for general activity (Kuchling, 1999). Our hormonal manipulation provides a support to this hypothesis.

4.3. Alternative explanations

It remains possible that male activity and sexual behaviors are regulated through hormonal mechanisms independent from testosterone. Progesterone plays important roles in the sexual behaviors of male lizards, and this steroid has been qualified as a “male” hormone (Phelps et al., 1998; Weiss and Moore, 2004; Crews and Moore, 2005); but evidence indicating that this hormone could replace, or dominate testosterone to trigger and maintain sexual behaviors in males is still lacking. In addition, progesterone tends to inhibit sexual behaviors in different species of vertebrates (Bottoni et al., 1985).

5. Conclusions

Currently a complex dissociated reproductive tactic is the most likely pattern for male Greek tortoises. The physiological role of seasonal variation of testosterone levels remains to be explained however. Recent studies in the male red-sided garter snakes showed that androgen-sensitive regions of the brain that are critical for courtship behaviors are hypertrophied shortly before the onset of the mating season, at the end of hibernation precisely when circulating testosterone is elevated (Krohmer, 2009). These results suggest a more important role for testosterone in the control of reproductive behavior for dissociated reproductive patterns than previously considered, and they also provide a plausible underlying mechanism for the indirect influence of sex-steroid hormones (Krohmer, 2009). Both the annual changes of plasma testosterone in free-ranging male Greek tortoises and our experimental results fit well with this scenario for a complex time-dissociated reproductive pattern where sex steroids interplay with seasonal variations of ambient temperatures.

Although the current study poses more questions than it provides answers, the methodology we successfully employed, combining classical hormonal manipulations, radio-tracking and automatic recording of the behaviors provides a solid basis for further tests in natural conditions. The unexpected patterns between testosterone levels and male sexual behaviors exhibited by several chelonians reinforce the image of the diversity and perhaps physiological plasticity displayed by ectothermic vertebrates (Merilä et al., 2000). It also emphasizes the complications associated with unexpected and non-significant effects despite their scientific value (Kotze et al., 2004). Finally, the current study illustrates the interest of using complementary methods on various study models (Bonnet et al., 2002) if we wish to describe and better understand the general patterns between hormones and behaviors (Bartholomew, 1982; Crews, 1998).

Acknowledgments

Financial support was provided by the Integrated Action number MA/02/54 (International Program for scientific Collaboration between Morocco and France, Comité Mixte Inter Universitaire Franco-Marocain) and the PICS 3949. The Conseil Général des Deux Sèvres provided logistical support. We warmly thank Hussein Boujoudi and his family for his help in the field. Rex Cambag carried

the implants and encouraged all the members of the research team.

References

- Alexandre, C., Balthazart, J., 1987. Inhibition of testosterone metabolism in the brain and cloacal gland of the quail by specific inhibitors and antihormones. *Journal of Endocrinology* 112, 189–195.
- Andreu, A.C., Diaz-Paniagua, C., Keller, C., 2000. La tortuga mora (*Testudo graeca* L.) en Doñana. *Asociación Herpetológica Española. Monografías de Herpetología* 5, 70.
- Ataev, C.A., 1975. On winter activity of reptiles in the Kopet-Dag and Badkhyz. *Izvestiya Akademiyu, Nauk. TSSR Biologica, Nauk* 4, 63–67.
- Aubret, F., Bonnet, X., Shine, R., Lourdaux, O., 2002. Fat is sexy for females but not males: the influence of body reserves on reproduction in snakes (*Vipera aspis*). *Hormones and Behavior* 42, 135–147.
- Averill-Murray, R.C., Martin, B.E., Bailey, S.J., Wirt, E.B., 2002. Activity and behaviour of the Sonoran Desert Tortoise in Arizona. In: Van Devender, T.R. (Ed.), *The Sonoran Desert Tortoise: Natural History, Biology, and Conservation*, Tucson, Arizona. The University of Arizona Press, pp. 135–158.
- Ayub, M., Levell, M.J., 1987. Inhibition of rat testicular 17- α -hydroxylase and 17, 20-lyase activities by anti-androgens (flutamide, hydroxyflutamide, RU23908, cyproterone acetate) in vitro. *Journal of Steroid Biochemistry* 28, 43–47.
- Bartholomew, G.A., 1982. Scientific innovation and creativity: a zoologist point of view. *American Zoologist* 22, 227–235.
- Becker, J.B., Breedlove, S.M., Crews, D., McCarthy, M.M., 2002. *Behavioral Endocrinology*, second ed. MIT Press, Cambridge, MA.
- Ben Kaddour, K., El Mouden, E.H., Slimani, T., Lagarde, F., Bonnet, X., 2005. Dimorphisme sexuel et cinétique de croissance et de maturation chez *Testudo graeca graeca* dans les Jbillets Centrales, Maroc. *Revue d'Ecologie Terre et Vie* 60, 265–278.
- Ben Kaddour, K., Slimani, T., El Mouden, E.H., Lagarde, F., Bonnet, X., 2006. Population structure, population density and individual catchability of *Testudo graeca* in the Central Jbillets (Morocco). *Vie et Milieu* 56, 49–54.
- Ben Kaddour, K., El Mouden, E.H., Slimani, T., Bonnet, X., Lagarde, F., 2008. Sexual dimorphism in the Greek Tortoise: a test of the body shape hypothesis. *Chelonian Conservation and Biology* 7, 21–27.
- Benner, S.L., Woodley, S.K., 2007. The reproductive pattern of male dusky salamanders (genus *Desmognathus*) is neither associated nor dissociated. *Hormones and Behavior* 51, 542–547.
- Berthold, A.A., 1849. Transplantation der hoden. *Archiven der Anatomie und Physiologie Wissenschaft der Medizin* 249, 455–461.
- Bonnet, X., Naulleau, G., Mauget, R., 1994. The influence of body condition on 17- β Estradiol levels in relation to vitellogenesis in female *Vipera aspis* (Reptilia viperidae). *General and Comparative Endocrinology* 93, 424–437.
- Bonnet, X., 1996. Gestion des réserves corporelles et stratégie de reproduction chez *Vipera aspis*. Unpublished Thesis. Université de Lyon 1, France.
- Bonnet, X., Naulleau, G., 1996. Are body reserves important for reproduction in male dark-green snakes? *Herpetologica* 52, 137–146.
- Bonnet, X., Naulleau, G., Bradshaw, D., Shine, R., 2001a. Changes in plasma progesterone in relation to vitellogenesis and gestation in the viviparous snake, *Vipera aspis*. *General and Comparative Endocrinology* 121, 84–94.
- Bonnet, X., Lagarde, F., Henen, B.T., Corbin, J., Nagy, K.A., Naulleau, G., Balhoul, K., Chastel, O., Legrand, A., Cambag, R., 2001b. Sexual dimorphism in steppe tortoises (*Testudo horsfieldi*): influence of the environment and sexual selection on body shape and mobility. *Biological Journal of the Linnean Society* 72, 357–372.
- Bonnet, X., Shine, R., Lourdaux, O., 2002. Taxonomic chauvinism. *Trends in Ecology and Evolution* 17, 1–3.
- Bottoni, L., Lucini, V., Massa, R., 1985. Effect of progesterone on the sexual behavior of the male Japanese quail. *General and Comparative Endocrinology* 57, 345–351.
- Bradley, A.J., McDonald, I.R., Lee, A.K., 1980. Stress and mortality in a small marsupial (*Antechinus stuartii*, Macleay). *General and Comparative Endocrinology* 40, 188–200.
- Callard, I.P., Callard, G.V., Lance, V., Eccles, S., 1976. Seasonal changes in testicular structure and function and the effects of gonadotropins in the freshwater turtles, *Chrysemys picta*. *General and Comparative Endocrinology* 30, 347–356.
- Callard, G.V., Hoffman, R.A., Petro, Z., Ryan, K.J., 1979. In vitro aromatization and other androgen transformations in the brain of the hamster (*Mesocricetus auratus*). *Biology of Reproduction* 21, 33–38.
- Camazine, B., Gartska, W., Tokarz, R., Crews, D., 1980. Effect of castration and androgen replacement on male courtship behaviour in the red-sided garter snake (*Thamnophis sirtalis parietalis*). *Australian Journal of Zoology* 51, 307–316.
- Canosa, L.F., Ceballos, N.R., 2002. Seasonal changes in testicular steroidogenesis in the toad *Bufo arenarum* H. *General and Comparative Endocrinology* 125, 426–434.
- Cease, A.J., Lutterschmidt, D.L., Mason, R.T., 2007. Corticosterone and the transition from courtship behavior to dispersal in male red-sided garter snakes (*Thamnophis sirtalis parietalis*). *General and Comparative Endocrinology* 1, 124–131.
- Crews, D., 1984. Gamete production, sex hormone secretion and mating behaviour uncoupled. *Hormones and Behavior* 18, 22–28.
- Crews, D., 1998. The evolutionary antecedents to love. *Psychoneuroendocrinology* 23, 751–764.
- Crews, D., Camazine, M., Diamond, R., Mason, R.T., Tokarz, R.R., Gartska, W.R., 1984. Hormonal independence of courtship behaviour in the male garter snake. *Hormones and Behavior* 18, 29–41.
- Crews, D., Silver, R., 1985. Reproductive physiology and behavior interactions in nonmammalian vertebrates. In: Adler, N.T., Pfaff, D., Goy, R.W. (Eds.), *Handbook of Behavioural Neurobiology, Reproduction*, vol. 7. Plenum Press, New York, New York, pp. 101–182.
- Crews, D., Moore, M.C., 2005. Historical contributions of research on reptiles to behavioral neuroendocrinology. *Hormones and Behavior* 48, 384–394.
- Davidson, J.M., Kwan, M., Greenleaf, W.J., 1982. Hormonal replacement and sexuality in men. *Journal of Clinical Endocrinology and Metabolism* 11, 599–623.
- Deputte, B.L., Johnson, J., Hempel, M., Scheffler, G., 1994. Behavioral effects of an antiandrogen in adult male rhesus macaques (*Macaca mulatta*). *Hormones and Behavior* 28, 155–164.
- El Mouden, E.H., Znari, M., Brown, R., 1999. Skeletochronology and mark-recapture assessments of growth in the North African agamid lizard (*Agama impalearis*). *Journal of Zoology*, London 249, 455–461.
- El Mouden, E.H., Slimani, T., Ben Kaddour, K., 2001. Croissance et dimorphisme sexuel chez la Tortue mauresque (*Testudo graeca graeca* L. 1758). *Chelonii* 3, 7–10.
- El Mouden, E.H., Slimani, T., Ben Kaddour, K., Lagarde, F., Ouhammou, A., Bonnet, X., 2005. *Testudo graeca* feeding ecology in an arid and overgrazed zone in Morocco. *Journal of the Arid Environment* 64, 422–443.
- Emberger, L., 1933. Nouvelle contribution à l'étude de la classification des groupements végétaux. *Revue Générale de Botanique* 45, 473–486.
- Ernst, C.H., Barbour, R.W., 1989. *Turtles of the World*. Smithsonian Institution Press, Washington, Washington.
- Folstad, I., Karter, A., 1992. Parasites, bright males and the immunocompetence handicap. *American Naturalist* 139, 603–622.
- Hailey, A., Pulford, E., Stubbs, D., 1984. Summer activity pattern of *Testudo Hermanni* Gmelin in Greece and France. *Amphibia Reptilia* 5, 69–78.
- Hays, W.L., 1988. *Statistics*, Fourth ed. CBS College Publishing, New York, New York.
- Hosken, D.J., Blackberry, M.A., Stewart, T.B., Stucki, A.F., 1998. The male reproductive cycle of three species of Australian vespertilionid bat. *Journal of Zoology* 245, 261–270.
- Ibargüengoytia, N.R., Pastor, L.M., Pallares, J., 1999. A light microscopy and ultrastructural study of the testes of tortoise *Testudo graeca* (Testudinidae). *Journal of Submicroscopic Cytology and Pathology* 31, 221–230.
- Iverson, J.B., 1992. A revised checklist with distribution maps of the turtles of the world. Richmond, IN, Privately published, 363 p.
- Jacobson, E.R., Schumacher, J., Green, M., 1992. Field and clinical techniques for sampling and handling blood for hematologic and selected biochemical determinations in the desert tortoise, *Xerobates agassizii*. *Copeia* 237, 241.
- King, R.B., 2002. Family, sex and testosterone effects on garter snake behaviour. *Animal Behaviour* 64, 345–359.
- Kotze, D.J., Johnson, C.A., O'Hara, R.B., Vepsäläinen, K., Fowler, M.S., 2004. Editorial: The Journal of Negative Results in Ecology and Evolutionary Biology. *Journal of Negative Results* 1, 1–5.
- Krohmer, R.W., Bieganski, G., Balekaitis, D.D., Balthazart, J., 2002. Distribution of aromatase immunoreactivity in the forebrain of red-sided garter snakes at the beginning of the winter dormancy. *Journal of Chemical Neuroanatomy* 23, 59–71.
- Krohmer, R.W., (2009). Neuroendocrine and environmental regulation of courtship and mating in the male red-sided garter snake. *Snake Reproduction Symposium. Joint Meeting of Ichthyologists and Herpetologists*, Portland, July 2009.
- Kuchling, G., 1982. Effect of temperature and photoperiod on spermatogenesis in the tortoise, *Testudo hermanni hermanni* Gmelin. *Amphibia Reptilia* 2, 32–34.
- Kuchling, G., 1999. *The Reproductive Biology of the Chelonia*. Berlin, Germany, Springer.
- Kuchling, G., Skolek-Winnisch, R., Bamberg, E., 1981. Histochemical and biochemical investigation on the annual cycle of testis, epididymis, and plasma testosterone of the tortoise, *Testudo hermanni hermanni* Gmelin. *General and Comparative Endocrinology* 44, 194–201.
- Lagarde, F., Bonnet, X., Henen, B., Corbin, J., Nagy, K.A., Naulleau, G., 2001. Sexual size dimorphism in steppe tortoises (*Testudo horsfieldi*): growth, maturity and individual variation. *Canadian Journal of Zoology* 79, 1433–1441.
- Lagarde, F., Bonnet, X., Henen, B., Corbin, J., Nagy, K.A., Naulleau, G., 2002. A short spring before a long jump: the ecological challenge to the steppe tortoises (*Testudo horsfieldi*). *Canadian Journal of Zoology* 80, 493–502.
- Lagarde, F., Bonnet, X., Henen, B., Corbin, J., Nagy, K.A., Mardonov, B., Naulleau, G., 2003a. Foraging behaviour and diet of an ectothermic herbivore: *Testudo horsfieldi*. *Ecography* 26, 236–242.
- Lagarde, F., Bonnet, X., Henen, B., Legrand, A., Corbin, J., Nagy, K., Naulleau, G., 2003b. Sex divergence in space utilisation in the steppe tortoise (*Testudo horsfieldi*). *Canadian Journal of Zoology* 81, 380–387.
- Lagarde, F., Bonnet, X., Henen, B., Nagy, K.A., Corbin, J., Lacroix, A., Trouvé, C., 2003c. Plasma steroid and nutrient levels during the active season in wild *Testudo horsfieldi*. *General and Comparative Endocrinology* 134, 139–146.
- Lagarde, F., Guillon, M., Dubroca, L., Bonnet, X., Ben Kaddour, K., Slimani, T., El Mouden, H.E., 2008. Slowness and acceleration: a new method to quantify the activity budget of chelonians. *Animal Behaviour* 75, 319–329.
- Lambert, M.R.K., 1981. Temperatures, activity and field sighting in the Mediterranean spur-thighed or common garden tortoise *Testudo graeca* L. *Biology of Conservation* 21, 39–54.

- Lance, V.A., Callard, I.P., 1980. Phylogenetic trends in hormonal control of gonadal steroidogenesis. In: Pang, P.K.T., Epplé, A. (Eds.), *Evolution of Vertebrate Endocrine Systems*. Texas Tech. Press, Lubbock, Texas, pp. 167–231.
- Le Houérou, H., 1989. Classification écoclimatique des zones arides (s.l.) de l'Afrique du Nord. *Ecologia Mediterranea* 249, 455–461.
- Liao, S., Howell, D.K., Chang, T.M., 1974. Action of a non-steroidal antiandrogen, flutamide, on the receptor binding and nuclear retention of 5-dihydrotestosterone in rat ventral prostate. *Endocrinology* 94, 1205–1209.
- Licht, P., 1982. Endocrine patterns in the reproductive cycle of turtles. *Herpetologica* 38, 51–61.
- Licht, P., Wood, J.F., Wood, F.E., 1985. Annual and diurnal cycles in plasma testosterone and thyroxine in the male green sea turtle, *Chelonia mydas*. *General and Comparative Endocrinology* 57, 335–344.
- Licht, P., Frank, L.G., Pavgi, S., Yalcinkaya, T.M., Siiteri, P.K., Glickman, S.E., 1992. Hormonal correlates of 'masculinization' in female spotted hyaenas (*Crocuta crocuta*). 2. Maternal and fetal steroids. *Journal of Reproduction and Fertility* 95, 463–474.
- Mahmoud, I.Y., Licht, P., 1997. Seasonal changes in gonadal activity and the effects of stress on reproductive hormones in the common snapping turtle, *Chelydra serpentina*. *General and Comparative Endocrinology* 107, 359–372.
- Marler, C.A., Moore, M.C., 1988. Evolutionary costs of aggression revealed by testosterone manipulations in free-living male lizards. *Behavioural Ecology and Sociobiology* 23, 21–26.
- McPherson, R.J., Boots, L.R., McGregor III, R., Marion, K.R., 1982. Plasma steroids associated with seasonal reproductive changes in a multi-clutched freshwater turtle, *Sternotherus odoratus*. *General and Comparative Endocrinology* 48, 440–451.
- Mendonça, M.T., 1987. Timing of reproductive behaviour in male musk turtles, *Sternotherus odoratus*: effects of photoperiod, temperature and testosterone. *Animal Behaviour* 35, 1002–1014.
- Mendonça, M.T., Licht, P., 1986. Seasonal cycles in gonadal activity and plasma gonadotropin in the musk turtle, *Sternotherus odoratus*. *General and Comparative Endocrinology* 62, 459–469.
- Merilä, J., Laurila, A., Timenes Laugen, A., Räsänen, K., Pahlka, M., 2000. Plasticity in age and size at metamorphosis in *Rana temporaria* – comparison of high and low latitude populations. *Ecography* 23, 457–465.
- Messner, P.W., Mahmoud, I.Y., Cyrus, R.V., 1993. Seasonal testosterone levels in Leydig and Sertoli cells of the snapping turtle (*Chelydra serpentina*) in natural populations. *Journal of Experimental Zoology* 266, 266–276.
- Milliken, G., Johnson, D.E., 1984. *Analysis of messy data. Designed Experiments*, vol. 1. Van Nostrand Reinhold Company, New York, New York.
- Moore, M.C., Lindzey, J., 1992. The physiological basis of sexual behaviour in male reptiles. In: Gans, C., Crews, D. (Eds.), *Hormones, Brain and Behavior, Biology of the Reptilia*, vol. 18, New York, pp. 70–113.
- Moore, I.T., Lerner, J.P., Lerner, D.T., Mason, R.T., 2000. Relationships between annual cycles of testosterone, corticosterone, and body condition in male red-spotted garter snakes, *Thamnophis sirtalis concinnus*. *Physiological and Biochemical Zoology* 73, 307–312.
- Moore, I.T., Mason, R.T., 2001. Behavioral and hormonal responses to corticosterone in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. *Physiology and Behavior* 72, 669–674.
- Naftolin, F., 1994. Brain aromatization of androgens. *Journal of Reproductive Medicine* 39, 257–261.
- Nagy, K.A., Medica, P.A., 1986. Physiological ecology of desert tortoises in Southern Nevada. *Herpetologica* 42, 73–92.
- Naulleau, G., Fleury, F., Boissin, J., 1987. Annual cycles in plasma testosterone and thyroxin for the male asp viper, *Vipera aspis* L. (Reptilia, Viperidae), in relation to the sexual cycle and hibernation. *General and Comparative Endocrinology* 65, 254–263.
- Nelson, R.J., 2005. *An Introduction to Behavioural Endocrinology*. Sinauer Associates, Inc., Massachusetts.
- Neumann, F., Graf, K.J., Hasan, S.H., Schenck, B., Steinbeck, H., 1977. Central actions of antiandrogen. In: Martini, L., Motta, M. (Eds.), *Androgens and Antiandrogens*. New York, Raven Press, pp. 163–177.
- Nolan, V.J., Ketterson, E.D., Ziegenfuss, C., Cullen, D.P., 1992. Testosterone and avian life histories: effect of experimentally elevated testosterone on prebasic molt and survival in male dark-eye juncos. *Condor* 94, 364–370.
- O'Brien, R.G., Kaiser, M.K., 1985. MANOVA method for analysing repeated measures design: an extensive primer. *Psychological Bulletin* 97, 316–333.
- O'Carroll, R., Shapiro, C., Bancroft, J., 1985. Androgens, behaviour and nocturnal erection in hypogonadal men: the effects of varying the replacement doses. *Clinical Endocrinology* 23, 527–538.
- Panagiota, M., Valakos, E.D., 1992. Contribution to the thermal ecology of *Testudo marginata* and *Testudo hermanni* (Chelonia: Testudinidae) in semi-captivity. *Herpetological Journal* 2, 48–50.
- Phelps, S.M., Lydon, J., O'Malley, B.W., Crews, D., 1998. Regulation of male sexual behavior by progesterone receptor, sexual experience and androgen. *Hormones and Behavior* 34, 294–302.
- Pough, F.H., 1980. The advantages for ectothermy for tetrapods. *American Naturalist* 115, 92–112.
- Pough, F.H., Janis, C.M., Heiser, J.B., 1999. *Vertebrate Life*. Upper Saddle River, New Jersey, Prentice Hall.
- Rostal, D.C., Lance, V.A., Grumbles, J.S., Alberts, A.C., 1994. Seasonal reproductive cycles of desert tortoise (*Gopherus agassizii*) in the eastern Mojave Desert. *Herpetological Monographs* 8, 72–82.
- Saint Girons, H., Bradshaw, S.D., Bradshaw, F.J., 1993. Sexual activity and plasma levels of sex steroids in the Asp viper *Vipera aspis* L. (Reptilia, Viperidae). *General and Comparative Endocrinology* 91, 287–297.
- Santen, R.J., Brodie, H., Simpson, E.R., Siiteri, P.K., Brodie, A., 2009. History of aromatase: saga of an important biological mediator and therapeutic target. *Endocrine Reviews* 30, 343–375.
- Sarkar, S., Sarkar, N.K., Mait, B.R., 2002. Oviductal sperm storage structure and their changes during the seasonal (dissociated) reproductive cycle in the soft-shelled turtle *Lissemys punctata punctata*. *Journal of Experimental Zoology Part A: Comparative Experimental Biology* 295A, 83–91.
- Shin, J.H., Kim, H.S., Moon, H.J., Kim, T.S., Seok, J.H., Kim, I.Y., Park, K.L., Han, S.Y., Nam, S.Y., 2002. Effects of flutamide on puberty in male rats: an evaluation of the protocol for the assessment of pubertal development and thyroid function. *Journal of Toxicology and Environmental Health A* 65, 433–445.
- Schramm, B.G., Casares, M., Lance, V.A., 1999. Steroid levels and reproductive cycle of the Galapagos tortoise, *Geochelone nigra*, living under seminatural conditions on Santa Cruz Island (Galapagos). *General and Comparative Endocrinology* 114, 108–120.
- Silva, A.M.R., Morales, G.S., Wassermann, G.F., 1984. Seasonal variations of testicular morphology and plasma levels of testosterone in the turtle *Chrysemys dorsignii*. *Comparative Biochemical Physiology A* 78, 153–157.
- Slimani, T., El Mouden, E.H., Ben Kaddour, K., 2001. Structure et dynamique d'une population de *Testudo graeca graeca* L. 1758 dans les Jbillets centrales. *Chelonii* 3, 7–10.
- Slimani, T., El Mouden, E.H., Ben Kaddour, K., Boumezzough, A., Lagarde, F., Bonnet, X., 2003. Ecological characteristics and habitat status of the spur-thighed *Testudo graeca graeca* in an arid area of Morocco: implications for conservation. In: *Second International Congress on Chelonian Conservation*, Saly, Senegal, June 18–22.
- Statsoft (2005). StatSoft France, STATISTICA, version 7.1. Available from: <www.statsoft.fr>.
- Weiss, S.L., Moore, M.C., 2004. Activation of aggressive behavior by progesterone and testosterone in male tree lizards, *Urosaurus ornatus*. *General and Comparative Endocrinology* 136, 282–288.
- Whittier, J.M., Corrie, F., Limpus, C., 1997. Plasma steroid profiles in nesting Loggerhead Turtles (*Caretta caretta*) in Queensland, Australia: relationship to nesting episode and season. *General and Comparative Endocrinology* 106, 39–47.
- Wibbels, T., Owens, D.W., Licht, P., Limpus, C., Reed, P., Amoss, M., 1992. Serum gonadotropins and gonadal steroid concentrations associated with migration, mating, and nesting in loggerhead sea turtles. *General and Comparative Endocrinology* 79, 154–164.
- Willemsen, R.E., 1991. Differences in thermoregulation between *Testudo hermanni* and *Testudo marginata* and their ecological significance. *Herpetological Journal* 1, 559–567.
- Wilson, B.S., Wingfield, J.C., 1994. Seasonal and interpopulational variation in plasma levels of corticosterone in the side-blotched lizard (*Uta stansburiana*). *Physiological Zoology* 67, 1025–1049.
- Winer, B.J., 1962. *Statistical Principles in Experimental Design*. New York, McGraw Hill.
- Wingfield, J.C., Hegner, R.E., Dufty Jr., A.M., Ball, G.F., 1990. The challenge hypothesis: theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *American Naturalist* 136, 829–846.
- Woolley, S.C., Sakata, J.T., Crews, D., 2004. Evolutionary insights into the regulation of courtship behavior in male amphibians and reptiles. *Physiology & Behavior* 83, 347–360.
- Znari, M., El Mouden, E.H., Francillon-Vieillot, H., 2002. Long-term variation in reproductive traits of Bibron's agama, *Agama impalearis*, in Western Morocco. *African Journal of Herpetology* 51, 57–68.