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Plasma steroid and nutrient levels during the active season in wild *Testudo horsfieldi*

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Abstract

Plasma concentrations of sex steroids (testosterone and progesterone), proteins (total protein and albumin), lipids (phospholipids, cholesterol, and triglycerides), and minerals (calcium and phosphorus) were measured in wild *Testudo horsfieldi* in Uzbekistan, during the short, 3-month activity period (March–May, 1998). Testosterone concentration in males was highest (52 ng/ml) when they had just emerged from brumation (hibernation) in mid-March, which was also the beginning of the mating period, and fell in April. In females, progesterone peaked in mid-April (at 10 ng/ml), just before ovulation of the first clutches at the end of April and beginning of May. Testosterone levels in females and progesterone levels in males were low (<3 ng/ml) throughout the activity period. In general, the plasma concentrations of proteins, lipids, and phosphorus increased slowly in males, but more rapidly in females, during the activity season. These increases were particularly strong in females in the second half of April, coinciding with the peaks in female hormone levels. The changes in plasma hormones and nutrients reflected the timing of the different behaviours. The four first weeks of above-ground activity (mid-March to mid-April) by males, when they had high testosterone levels, were primarily allocated to fighting other males, courtship, and mating, while females spent much of that time feeding. Thereafter, both sexes concentrated on feeding. Females were probably preparing to ovulate in late-April, when their progesterone levels were highest and when plasma nutrient levels increased considerably.
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1. Introduction

The steppe tortoise (*Testudo horsfieldi*) is probably one of the most widespread and the most abundant among terrestrial tortoises, living in the south east of the former USSR, the north east of Iran, Afghanistan, north west of Pakistan, and northern and western Baluchistan (Ernst and Barbour, 1989; Iverson, 1992). Unfortunately, wild populations have been declining rapidly due to massive harvesting for the pet trade and extensive disruption of their habitat by intensive agriculture. Despite its prevalence, several basic aspects of the biology of this species still remain unknown, so we initiated a long-term field study of the Steppe tortoise in Uzbekistan to document its ecology (Lagarde et al., 1999, 2002, 2003a,b), morphology (Bonnet et al., 2001), growth and maturation processes (Lagarde et al., 2001), behaviour (Lagarde et al., 2002, 2003a), reproductive success (Henen et al., 2002), and physiology. Consequently, we now have a better understanding of this chelonian, especially the strong environmental constraints shaping its biological cycle.

In our study area, climatic conditions are typical from extreme continental regions. The average air

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temperature is −1 °C in January and more than 33 °C in July (Lagarde et al., 1999, 2002). Annual rainfall is less than 250 mm a year, with almost of which falling between October and mid-May. As a result, summer is extremely dry and hot and the annual vegetation (the *T. horsfieldi* feeding resources) is available for only 3 months, from the beginning of March to the end of May. Then, the steppe tortoise is characterised by a short 3-month cycle of activity, between mid-March and the mid-June, probably due to climatic and trophic constraints (Lagarde et al., 1999, 2002). During this short activity period, males and females behaved differently from each other. From the time of emergence from brumation (winter dormancy or hibernation) in mid-March until mid-April, males showed intensive sexual behaviours (fighting and mating, 30% of active time), whereas females had cryptic sexual behaviour during that period (2% of active time), were not very mobile and allocated most of their daily activity time to feeding (20% of active time) (Lagarde et al., 2002, 2003a,b). After mid-April, sexual activities ceased, both sexes fed frequently, and females produced an average of 5.8 eggs in two or three consecutive clutches between the beginning of May and mid-June (Henen et al., 2002). Females presumably had to eat more than males to obtain the nutrients needed for egg production (Lagarde et al., 2003a).

Males began their long (9-month) inactivity period at the end of May, 15 days earlier than did females (Lagarde et al., 2002). The males carry out sperm maturation while underground, in July and August (Brushko, 1981). The exact chronology of vitellogenesis in female *T. horsfieldi* remains unknown.

The goal of this study was to describe and interpret, for the first time, the circulating levels of steroids (testosterone and progesterone) and selected nutrients in a wild population of *T. horsfieldi* during its activity season. We hypothesised that these parameters reflect the strong behavioural differences we observed between males and females, as well as female nutrient demands before ovulation and oviposition.

2. Methods

2.1. Study area

The study population was situated in the Republic of Uzbekistan, at the Djeiron Ecocenter of Bukhara (40°N, 65°E). This region neighbours the Kyzyl Kum desert and receives less than 250 mm of rain annually (Lagarde et al., 2002; Pereladova et al., 1998). The sandy soil supports xerophytic vegetation in spring (see Lagarde et al., 2002; Pereladova et al., 1998 for details).

2.2. Blood samples

We sampled 0.5–1.0 ml of blood in the field by jugular puncture (Jacobson et al., 1992) from the middle of March to the middle of May 1998. Each animal was identified with shell notches using a code, and with a number painted on the shell (Lagarde et al., 2002) and sampled only once in the season. The samples were collected within 5 min of capturing a tortoise, transferred to dry, heparinised Vacutainers, and stored on ice pending centrifugation in the laboratory within 3–4 h of collection. Portions of plasma were pipetted into Sarsteadt tubes, and stored frozen (at −25 °C) until analysed.

2.3. Steroid analysis

Testosterone and progesterone were measured by radio-immunoassay on 50 μl plasma after extraction using 0.5 ml of diethyl ether (mean extraction efficiency was 0.93 ± 0.10 for testosterone and 0.98 ± 0.10 for progesterone). Cross-reactivity of the Sigma specific antibodies with other steroids was generally low (percentage of cross-reactivity at B/B0 for progesterone antibody: deoxycorticosterone <6.6%, 5α-dihydroprogesterone <3.9%, 5β-dihydroprogesterone <2.6%, 6β-dihydroprogesterone <2.1% and percentage of cross-reactivity at B/B0 for testosterone antibody: 5α-dihydrotestosterone 17.8%, 5β-androstene-3β,17β-diol 1.4%, 5α-androstene-3β,17β-diol 1.2%, androstenedione 1.4%, epitestosterone 0.7%, progesterone 0.07%). The only hormone that could potentially complicate some of our results is DHT. Changes in plasma levels of DHT are parallel to testosterone variations (Saint Girons et al., 1993; Whittier et al., 1997) and hence will not modify our major conclusions. The sensitivity of the assay were 7.8 pg/tube (0.156 ng/ml) for both testosterone and progesterone. Intra- and inter-assay variations were, respectively, 12 and 6% for testosterone and 12 and 7% for progesterone.

2.4. Nutrient assays

Plasma nutrient levels were determined by automatic spectrophotometer (TECHNICON RA 1000) and bioMérieux reagents (enzyme kits). Total protein present in the plasma was determined using the Biuret method. Plasma albumin was estimated by the colorimetric enzymatic reaction with bromocresol green (Albumin-Kit). Total calcium was determined by the colorimetric method without deproteinisation, using *O*-cresolphthalein complexone (Ca-OCP) and interference due to Mg^{2+} ions was eliminated by 8-hydroxyquinoline. Total plasma phosphorus was determined with ammonium heptamolybdate. Plasma triglycerides were measured by the full enzymatic method involving

lipase, glycerokinase, glycerol-3 phosphate oxidase, and peroxidase. Plasma phospholipids were hydrolysed by phospholipase D and the liberated choline was measured by the Trinder reaction using choline oxidase and peroxidase. Total cholesterol was assayed using a coupled enzymatic system involving esterase, cholesterol oxidase, and peroxidase. Because within-assay variations (range between 4.2 and 9.8%) were lower than inter-assay variations (range 8.5–19.2%), all the samples were assayed in one batch for each metabolite. Such nutrient assays were validated by numerous studies on reptiles physiology (e.g., Bonnet et al., 1994) and more particularly on chelonian eco-physiology (Callard et al., 1978; Pagés et al., 1992; Seidel and Reynolds, 1980).

2.5. Statistics

The data were grouped in four different periods: period 1, 16–30 March; period 2, 1–15 April; period 3, 16–30 April; and period 4, 1–17 May. The first two periods covered the entire mating season (Lagarde et al., 1999, 2002). Periods 3 and 4 covered most of the second half of the activity season for males and the first half of the egg-laying season for females (Lagarde et al., 1999, 2002; Henen et al., 2002).

Mean plasma concentrations of steroids and nutrients are given ±standard deviation.

In order to standardise the data and respect the assumption of variance homogeneity, data were log transformed and then tested again for the homogeneity of variance. Our data on steroid levels in the plasma typically fell into two major categories: virtually zero, or very high. Even after log transformation, the distribution of steroid levels was not normal, precluding the use of parametric tests. We used the non-parametric Median test to compare plasma values obtained in males and females and for each sex, between the subsequent periods. Using this method, we simply count the number of cases in each sample that fall above or below the common median, and compute the χ^2 value for the resulting $2 \times k$ contingency table. The major disadvantage inherent in such a crude version of non-parametric tests to compare two samples is that the power of the analysis is lowered. The compensating advantage, however, is that the analysis is less sensitive to outliers. The median test is particularly useful when many case fall at either extreme of the scale (undetectable versus high plasma values of steroids, see Section 3). In such a situation, the Median test is the most appropriate method for comparing samples (Statistica user's manual, 1995).

The variance homogeneity was respected for nutrient levels and then the results were compared using a two way ANOVA (sex and period as factors, nutrient concentrations as dependent variables). We carried out post hoc comparisons (tests LSD; Hays, 1988; Milliken and Johnson, 1984; Winer, 1962) to determine which periods showed significant temporal variations in nutrient concentrations, and when these temporal variations lead to significant differences between the sexes. Relationships between plasma constituents were analysed using least square linear regression analysis.

All tests were performed using STATISTICA 5.1 software.

3. Results

3.1. Steroids

Analysis of testosterone levels showed significant effects of sex and period. Testosterone in males was higher than in females throughout the cycle (Median test, $\chi^2 \geqslant 10$, df $= 1$, $P \leqslant 0.001$ for each measurement period; Fig. 1, Table 1) and was significantly affected by the period (Median test, $\chi^2 = 14.20$, df $= 3$, $P = .0026$). Testosterone concentration in males was highest at the beginning of the activity season (51.8 ± 35.2 ng/ml), and fell to significantly lower values in April (Median test, $\chi^2 = 4.05$, df $= 1$, $P = 0.04$). The high standard deviations indicate large variations in testosterone levels between individuals (for example from 0.47 to 146.3 ng/ml at mid-March). Despite lower testosterone levels in females (maximum 9.4 ng/ml), we detect significant temporal variation (Median test, $\chi^2 = 10.6$, df $= 3$, $P = .013$) and the maximum levels were reached in late-April (2.17 ± 1.53 ng/ml).

The majority of the samples were characterised by very low progesterone levels (<3 ng/ml) except for 7 females in late-April on 27 sampled (7–107 ng/ml, Fig. 1, period 3). We then detect a significant sex effect on progesterone levels in late-April (Table 1) and a period effect in females (Median test, $\chi^2 = 17.54$, df $= 3$, $P < 0.001$).

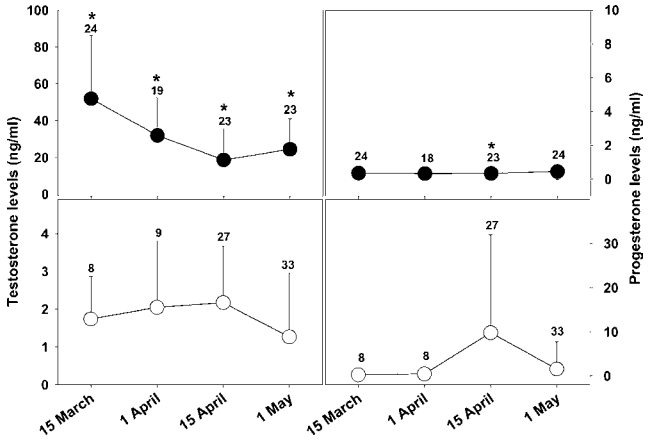

Fig. 1. Mean (±SD) plasma testosterone and progesterone in male (filled circles) and female (open circles) *T. horsfieldi*. Sample sizes are indicated near each point. An asterisk indicates that significant differences existed between the sexes during that period.

Table 1
Statistical analyses of sex differences in plasma steroid concentrations in *T. horsfieldi*

Periods	Testosterone			Progesterone		
	df	χ^2	*P*	df	χ^2	*P*
1	1	10.66	**0.001**	1	5.06	**0.024**
2	1	13.26	**<0.001**	1	0.29	0.58
3	1	29.06	**<0.001**	1	13.60	**<0.001**
4	1	40.99	**<0.001**	1	0.92	0.33

Median tests were performed with plasma concentration as the dependent variable and sex as independent variables (see Section 2) for the different periods of the activity cycle (period 1, 16–30 March; period 2, 1–15 April; period 3, 16–30 April; and period 4, 1–17 May). Significant effects are indicated in boldface type. df: degree of freedom.

3.2. Nutrients

The plasma concentrations of all nutrients varied among the four periods, increasing significantly between the periods 2 and 3 (transition between the mating and the post-mating periods; Fig. 2, Table 1) ($P < 0.02$ for post hoc tests). Most nutrient levels were significantly affected by an interaction between sex and measurement period, but calcium was influenced only by measurement period (Table 2). However, when we performed the analysis for each sex separately, we detected no significant period effect on the calcium levels in males ($F_{3,67} = 1.90$, $P = 0.13$) but a significant one in females ($F_{3,78} = 5.90$, $P = 0.001$). Cholesterol levels (Fig. 2) were affected by period and sex, with no interaction between these factors; females had higher cholesterol concentrations than did males. Protein and albumin levels (Fig. 2) were characterised by higher concentrations in males than in females during the mating season (periods 1 and 2) whereas phosphorus, cholesterol, phospholipid, and triglyceride levels were higher in females than in males during the post-mating season (Fig. 2).

All nutrients we measured were positively correlated with each other (for sexes combined, all correlations had $r^2 > 0.20$ and $P < 0.001$).

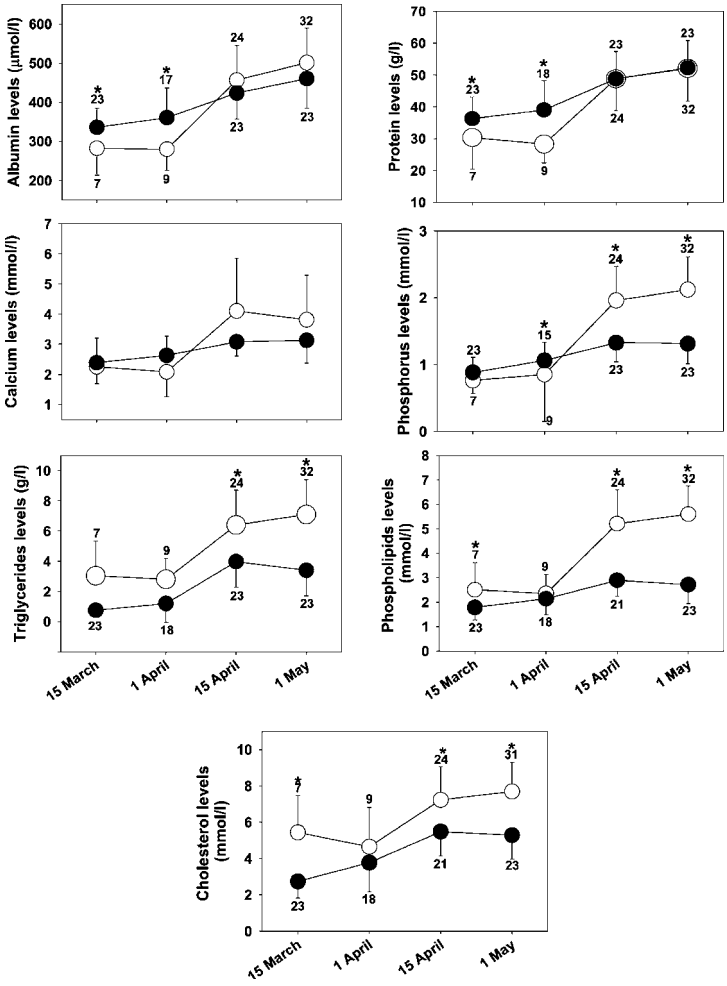

Fig. 2. Mean (±SD) proteins (total protein and albumin), lipids (phospholipids, cholesterol, and triglycerides) and minerals (calcium and phosphorus) levels in male (filled circles) and female (open circles) *T. horsfieldi*. Sample sizes are indicated near each point. An asterisk indicates that significant differences existed between the sexes during that period.

Table 2
Statistical analyses of plasma nutrient concentrations in *T. horsfieldi*

Nutrient	Sex			Period			Sex × period		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Protein	1,151	11.04	**0.001**	3,151	38.80	**<0.001**	3,151	3.75	**0.012**
Albumin	1,150	4.51	**0.035**	3,150	48.27	**<0.001**	3,150	6.64	**<0.001**
Calcium	1,145	0.07	0.78	3,145	4.57	**0.004**	3,145	0.73	0.53
Phosphorus	1,148	0.58	0.44	3,148	49.61	**<0.001**	3,148	16.53	**<0.001**
Triglycerid	1,151	11.04	**0.001**	3,151	38.80	**<0.001**	3,151	3.75	**0.012**
Phospholipid	1,151	69.03	**<0.001**	3,151	46.20	**<0.001**	3,151	8.06	**<0.001**
Cholesterol	1,148	27.51	**<0.001**	3,148	19.67	**<0.001**	3,148	1.98	0.11

ANOVAs were performed with plasma concentration as the dependent variable and sex and time period as independent variables (see Section 2). Significant effects are indicated in boldface type. df: degree of freedom.

4. Discussion

4.1. Males

4.1.1. Hormones

In some chelonians, high testosterone levels in cold periods may result from low turnover or renal clearance rates caused by low body temperatures and metabolism (Callard et al., 1976; Kuchling, 1999). The testosterone levels in male *T. horsfieldi* were highest during the mating season, and fell quickly thereafter. However, these high testosterone concentrations may organise the intense sexual behaviour in *T. horsfieldi*. A link between sexual behaviour and testosterone apparently exists in males of other chelonians (Callard and Ho, 1980; Callard et al., 1976; Lance and Callard, 1980; Licht, 1984; Licht et al., 1985; Messner et al., 1993; Mahmoud and Licht, 1997), but this association is less clear or non-existent in other species (Kuchling, 1982; Kuchling et al., 1981; Licht et al., 1979; Mendonça, 1987; Mendonça and Licht, 1986; Rostal et al., 1994; Silva et al., 1984; Wibbels et al., 1992). For many, but not all species of tortoises (see review by Kuchling, 1999), plasma testosterone levels peak in association with peak sperm production and maturation (Kuchling, 1999; Kuchling et al., 1981; Licht, 1982; McPherson et al., 1982). This includes *Testudo hermanni,* a close relative to *T. horsfieldi* (Kuchling, 1999; Kuchling et al., 1981). Brushko (1981) indicated that sperm maturation in *T. horsfieldi* occurs during July and August while they are buried underground in aestivation. Then, to understand better the male reproductive cycle, it would be necessary to measure male testosterone levels and spermatogenic activity throughout the entire year.

4.1.2. Nutrients

All nutrient levels, except calcium, increased in males during the mating and post-mating period. The intense male sexual activity, coupled with infrequent and sporadic feeding until mid-April (Lagarde et al., 1999, 2003a) may explain the low blood concentrations of some nutrients during the mating period. From mid-April until their retreat underground in late-May, males had short daily active periods, more time was spent feeding (Lagarde et al., 2002, 2003a), and concentrations of all measured nutrients rose. Increased ambient temperatures following the mating season may have facilitated the increase in feeding, and probably increased the digestion and metabolic rates of males and females. The increased feeding and plasma nutrient concentrations, especially plasma proteins and lipids, is consistent with accentuated demands to prepare, in less than 3 months, for the subsequent 9 months of aestivation and brumation. They also suggest that males may develop lipid and protein stores for use in dormancy as *Gopherus agassizii* (Henen, 1997, 2002).

4.2. Females

4.2.1. Hormones

We failed to detect 17β-estradiol in our female blood samples and it could be considered as a serious lack in our study because estrogen is classically the primary stimulus to vitellogenesis in amphibians, reptiles, and birds (Bonnet et al., 1994; Callard et al., 1978; Callard et al., 1990; Carnevalli et al., 1991; Ho et al., 1981, 1982). Nevertheless, testosterone and progesterone level fluctuations corresponded to the temporal organisation of steppe tortoise major physiological events. Testosterone and progesterone concentrations in female blood peaked in mid-April, just prior to the ovulation of first clutches, which appeared synchronised among females (Henen et al., 2002). Testosterone in female chelonians is generally associated with regulation of vitellogenesis (Callard and Ho, 1980; Ho et al., 1981) with follicles becoming sensitised to LH action just before ovulation (Wibbels et al., 1992). Testosterone levels generally increase during vitellogenesis, and reach maximum levels at the time of ovulation (Lance and Callard, 1980; Schramm et al., 1999). Thus part of vitellogenesis may occur just prior to ovulation in *T. horsfieldi*.

Progesterone appears to be related to oocyte maturation (Nagahama, 1987) and to oviduct motility control (Callard and Hirsch, 1976; Giannoukos and

Callard, 1996; Mahmoud et al., 1988 as cited in Kuchling, 1999). Progesterone generally peaks near ovulation time also, being produced by pre- and post-ovulation follicles or yellow body secretion activities (Guillette et al., 1991; Lance and Callard, 1980; Licht, 1984; Schramm et al., 1999). Progesterone peaks are usually short in duration (Callard et al., 1978; Licht et al., 1982; Rostal et al., 1994; Schramm et al., 1999; Wibbels et al., 1992) and tend to decline as egg shelling progresses (Kuchling, 1999). This probably explains the high peak in late-April as females appeared to ovulate first clutches during the last week of April or the first week of May (Henen et al., 2002). Unfortunately, we did not have plasma samples that corresponded to the ovulation of second clutches, which occurred primarily after 15 May, 1998; we may have missed a second peak in plasma progesterone concentrations. However, the short clutch retention times and interclutch intervals (ca. 11 and 6 days, respectively; Henen et al., 2002) would make it difficult to document progesterone fluctuations within and among clutches without undue manipulation and stress to the tortoises.

4.2.2. Nutrients

As with males, all nutrient levels in females increased between the mating and post-mating period. Most nutrient levels were higher in females than in males during the ovulation–oviposition period (sample periods 3 and 4). Nutrient concentrations in females increased dramatically just after the mating season, suggesting mobilisation of resources for vitellogenesis and egg production. Vitellogenesis can be a long process in chelonians (Callard et al., 1978; Christiansen and Dunham, 1972; Christiansen and Moll, 1973; Combescot, 1955; Kuchling, 1999; White and Murphy, 1973). It can occur during inactivity and torpor periods using body reserves (e.g., in *Kinosternon flavescens*, Long, 1985 and in *Pseudemydura umbrina*, King et al., 1998; see also Kuchling and Bradshaw, 1993; Kuchling, 1999) or it can occur during activity periods using resources acquired from food (e.g., in *Chrysemys picta*, Congdon and Tinkel, 1982 and *G. agassizii*, Henen and Oftedal, 1998). Elevated plasma calcium is characteristic of active vitellogenesis and egg production in female tortoises (Callard et al., 1978) and in female reptiles in general (Bonnet et al., 1994; Lance, 1976). Several results indicate that female *T. horsfieldi* devoted themselves strongly to vitellogenesis and/or egg production after the mating period. Ovulation of the first clutches appeared synchronised in late-April and early-May, with two-thirds of females being gravid by 7 May and over 90% were gravid by 17 May (Henen et al., 2002). Females also quickly produced second or third clutches before they entered aestivation in early-June (Henen et al., 2002). Female feeding activity increased in association with sharp increases in plasma proteins, particularly albumin which may be used to complete vitellogenesis. The elevation of plasma, lipid, protein, and phosphorus concentrations by both sexes in April and May was probably associated with preparing body reserves for the 9 months of dormancy. However, plasma lipid and phosphorus lipids were often higher for females than for males, and compared to males, females showed greater increases in plasma proteins and minerals between the first and second halves of the study. This sexual divergence is most likely linked to females meeting the production costs for eggs, and perhaps vitellogenesis and production of shell, at the same time they were preparing for estivation and brumation. Female *G. agassizii* also have higher plasma levels of cholesterol, triglycerides, calcium, and phosphorus compared to their male counterparts (Christopher et al., 1999), with vitellogenesis requiring at least 3 or 4 months prior to brumation (Henen and Oftedal, 1998; Rostal et al., 1994) and possibly additional time in spring. The large investment by females in April and May does not preclude them from carrying out vitellogenesis at other times of the year (i.e., mating or dormancy periods) or over several years; as has been documented for other chelonians (see review by Kuchling, 1999).

4.3. Conclusions

Testudo horsfieldi accomplish all of its tasks (eating, mating, producing eggs, nesting, and preparing for dormancy) within its short 3-month activity period. Our profiles of the plasma steroids and nutrients clearly reflect the temporal partitioning of biological functions in *T. horsfieldi*, and help us to evaluate the chronology of physiological events. The study of follicular development by ultrasonography could determine the chronology of this costly process for females, helping reveal how much of the elevated plasma concentrations result from vitellogenesis and other egg formation requirements. It will be important to measure plasma parameters during the time of inactivity in order to understand the physiological significance of this underground way of life. Is the long 9-month "inactivity period" a dormancy period or a crucial time for several physiological events to occur (e.g., spermatogenesis, vitellogenesis, and mobilisation of body reserves)?

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