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Changes in Plasma Progesterone in Relation to Vitellogenesis and Gestation in the Viviparous Snake *Vipera aspis*

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Viviparity has arisen from oviparity on more than 100 independent occasions in squamate reptiles, providing an unique opportunity to investigate the ways in which endocrine control of gestation length has been modified by natural selection during this major transition in reproductive modes. Intuitively, the evolution of viviparity might be expected to involve an increasingly important role for the steroid hormone progesterone, rather than estradiol. Unfortunately, published data on this topic in snakes are scarce and often contradictory. Females of the viviparous snake *Vipera aspis* reproduce with a lower than annual frequency, providing the opportunity to examine steroid profiles simultaneously in vitellogenic, pregnant, and postparturient versus nonreproductive females. From 1990 to 1994, more than 500 blood samples were collected from more than 100 females. Progesterone, estradiol-17β, and several plasma metabolites were assayed by radioimmunoassay and spectrophotometry. In contrast to earlier studies, we found significant differences between plasma progesterone levels in reproducing (10.5 ± 9.1 ng ml^{-1}, $N = 168$) and nonreproducing (5.1 ± 4.2 ng ml^{-1}, $N = 121$) females. Estradiol reached high levels during vitellogenesis (4.8 ± 4.0 ng ml^{-1}, $N = 16$), and progesterone levels increased during gestation (from 3.7 ng ml^{-1} before gestation to 18.7 ng ml^{-1} at midgestation). However, experimental elevation of plasma progesterone levels with implants (up to 44.4 ng ml^{-1}) did not modify plasma metabolite levels or delay parturition.

Key Words: viviparity; gestation; progesterone; snakes; vitellogenesis; *Vipera*.

INTRODUCTION

Major transitions in reproductive modes offer an exceptional opportunity for scientists to unravel complex systems (Ridley, 1993; Gerhart and Kischner, 1997). The transition from oviparity (egg laying) to viviparity (live bearing) is perhaps one of the best examples of such a shift in reproductive mode. Among the vertebrates, squamate reptiles (lizards and snakes) represent the lineage with by far the highest frequency of such transitions (Weekes, 1935; Blackburn, 1982, 1985; Shine, 1985; Guillette, 1993). This situation has been exploited by a comprehensive series of investigations focusing on the evolutionary and ecological factors associated with the shift (Mell, 1929; Weekes, 1935; Tinkle and Gibbons, 1977; Shine and Bull, 1979; Shine, 1983a,b, 1995; Guillette, 1982, 1993; Xavier and Gavaud, 1986; Stewart, 1989; Heulin *et al.*, 1992, 1993; Faibairn *et al.*, 1998; Lee and Shine, 1998).

The situation with physiological and (especially) endocrinological studies is, however, quite different. Despite an abundant literature (Highfill and Mead, 1975a,b; Callard *et al.*, 1972; Yaron, 1972; Chan *et al.*, 1973; Guillette *et al.*, 1981; Mead *et al.*, 1981; Kleis-San

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Francisco and Callard, 1986; Callard *et al.*, 1992; Gobbetti *et al.*, 1994), we still have no clear idea of how the evolution of viviparity has been achieved at a mechanistic level. For example, what hormones have been modified (either in levels of production or in their effects) and what tissues (both maternal and foetal) have been changed to allow long-term retention of the offspring *in utero?* At least for snakes, published studies in this field are often complex and the results sometimes contradictory. For example, is estradiol involved in vitellogenesis? Although this steroid has proven to be the primary stimulus for vitellogenesis in most invertebrates and vertebrates that have been examined to date (Ho *et al.*, 1982; Callard *et al.*, 1990; Wada *et al.*, 1990), snakes are more problematic. Several studies suggest that changes in estradiol levels are independent of vitellogenesis in squamate reptiles (see Whittier *et al.*, 1987; Becker *et al.*, 1992; Crews, 1992; Crews and Gans, 1992; Alcock, 1993; Saint Girons *et al.*, 1993; Eberhard, 1996; but see Licht, 1984; Bona-Gallo *et al.*, 1980; Bonnet *et al.*, 1994). The situation with progesterone is even more confusing. Although levels of this hormone are typically elevated during pregnancy in viviparous vertebrates, the role of this steroid is not always clear, even in intensively studied taxa, such as mammals (Cake *et al.*, 1980; Thibault and Levasseur, 1991). In the case of reptiles, are progesterone levels higher in reproducing females than in nonreproducing females (Chan *et al.*, 1973; Mead *et al.*, 1981; Naulleau and Fleury, 1990; Saint Girons *et al.*, 1993)? Are progesterone levels in the plasma as high in males as in females, as reported by Saint Girons *et al.* (1993)?

Some of these apparent contradictions, however, may reflect real diversity. Those mechanisms that have evolved to extend gestation may differ substantially from one lineage to another. Alternatively, some of these contradictions may result from methodological shortcomings. For example, most studies have relied upon killing snakes to obtain blood, rather than following individuals over an entire reproductive cycle. The first step to resolve such issues is to establish the basic patterns of hormone levels in both viviparous and oviparous snakes and document how these are modified during reproduction. The next step should involve manipulative experiments to investigate the functional role of these hormones. This paper provides such baseline data for a viviparous snake, the Aspic viper (*Vipera aspis*), focusing essentially on progesterone, which has proved to be a problematic steroid in terms of plasma levels, origins, and functions in snakes (Saint Girons *et al.*, 1993). Herein, we provide the first empirical data on the changes in plasma levels of progesterone, covering the whole course of the complex reproductive cycle (>2 years) in a viviparous snake.

MATERIALS AND METHODS

Animals

The Aspic viper is a medium-sized (on average 50 cm snout–vent length (SVL); 100 g body mass) viviparous venomous snake widely distributed in western Europe. In northern parts of its distribution, females typically reproduce with a lower than annual frequency, depending on fat stores (Saint Girons, 1957; Bonnet and Naulleau, 1996; Naulleau and Bonnet, 1996). Females emerge form hibernation in March and vitellogenesis starts immediately (Bonnet *et al.*, 1994). Ovulation occurs during the first 2 weeks of June (Naulleau and Bidaut, 1981), and parturition is generally observed form late August until late September. After birth, females are very emaciated and cannot replenish their body reserves before hibernation. Thus, all postparturient females are nonreproductive the following spring. They usually need 1 to 3 years to regain sufficient body condition for breeding (Bonnet and Naulleau, 1996; Naulleau and Bonnet, 1996; Bonnet *et al.*, 2000a,b). Overall, populations contain large numbers of females at various stages of their life cycle, and nonreproductive individuals are often more numerous than reproductive individuals (Bonnet and Naulleau, 1996). Females mature at approximately 41.5 cm SVL (47 cm total length; the smallest size at which parturition has been observed).

Experimental Groups and Housing

Three main groups were composed as follows.

(1). Females maintained under natural climatic conditions: reproductive and nonreproductive females monitored in the course of their "normal" cycle of reproductive and nonreproductive years. The comparison between these two categories of "outdoor" snakes is direct; one may expect to observe high levels of sex steroids only in reproductive females and principally during reproduction (vitellogenesis and gestation).

(2). Females maintained under artificial and stable conditions and not allowed to reproduce (e.g., absence of reproductive stimuli such as artificial hibernation). These "indoor" females were not expected to display the complex reproductive cycle exhibited by outdoor females. They should exhibit low, and probably stable, plasma levels of sex steroids and hence may provide a basal plasma level for the species.

(3). Males maintained under natural climatic conditions: our aim was to compare the plasma concentrations of males with those of reproductive females, nonreproductive females, and indoor females. Intuition suggests that progesterone levels should be low in males (perhaps similar to indoor females?), but Saint Girons *et al.* (1993) did not find any significant difference between average progesterone levels in male and female (reproductive and nonreproductive) snakes kept in outdoor enclosures. Although their sample size was small, the high plasma concentrations that they reported for some males are intriguing and deserve further study.

Below, we provide more details on these three groups. Note that they have been studied during the same period (1990–1996) and that no females were exchanged between groups.

Outdoor females. Eighty-eight adult females were caught by hand in the field in central west France (Vendée, Loire Atlantique, and Deux-Sèvres) between March 1990 and October 1994. Forty-three of these animals were caught soon after hibernation in March and April, and the other 45 were caught later during the active season from May to late October. Immediately after capture, the snakes were weighed to the nearest 0.1 g, measured to the nearest 0.5 cm (snout–vent-length and total body length), marked by scale-clipping, and placed in outdoor enclosures under natural climatic conditions (Forêt de Chizé, Deux-Sèvres, 46°07′ N, 00°25′ W). The terraria had surface areas of 8 m^2 and included three hibernating dens (50 cm underground). Three to 10 vipers were placed in each terrarium. Vipers were fed *ad libitum* with laboratory mice, and water was available. Snakes were weighed at least monthly. Several snakes were kept in the outdoor enclosures for 3 years, whereas others were released after much shorter periods (1 week to 1 month after capture). Reproductive status was determined by palpation of embryos and was confirmed by parturition in each case. These 88 females are referred to as the outdoor vipers. Of these, 17 were sampled both during reproduction and during the following (nonreproductive) year.

Indoor females. Thirteen other females had been captured at least 3 years before the beginning of this study. These snakes were kept under artificial conditions in the laboratory (12:12 L:D h; constant ambient temperature of 20°) in individual cages (40 × 40 × 40 cm with a 100-W light bulb to provide a thermal gradient during the day). Food was provided weekly (laboratory mice weighing 15 to 40 g) and water *ad libitum*. These females were in good condition but were not allowed to mate or reproduce. Under artificial conditions such as these, adult female Aspic vipers rarely initiate vitellogenesis (one observation, not included in this data set), whereas females kept in outdoor enclosures (under natural conditions) may initiate vitellogenesis even in the absence of males (although this phenomenon is rare: four observations in total).

Males. Forty-four adult males (total body length greater than 45 cm) caught in the wild and housed in outdoor enclosures (same characteristics as those used for females) were also bled.

Determination of Vitellogenesis

Vitellogenesis was determined by abdominal palpation and confirmed by the large increase in the level of plasma metabolites and calcium which occurs at this time.

Plasma assays

Five hundred and twenty-nine blood samples (100 to 500 μl into 1-ml heparinized syringes, needle 27G × 3/4 inch 0.4 × 20 mm) were taken by cardiac puncture (Naulleau and Fleury, 1990). This technique does not cause any detectable problems for the animals and

snakes were bled either immediately (<5 min) or more than 1 h after capture, depending upon the numbers being processed. High levels of plasma progesterone, which may have been due to the stress of handling, were recorded in several individuals. However, such putative stress responses were equally distributed among the different groups and unrelated to the time of bleeding. Furthermore, at certain periods (e.g., March or October in outdoor females, see Results) plasma progesterone levels were very low in all individuals, discounting any stress-induced elevation of this hormone. Samples were taken around the 20th of each month. During hibernation (broadly 4 months, from November to February), mortality (which was very low during the active season) increased in outdoor individuals that had been manipulated. Thus, blood samples were limited and were subsequently collected in a cold room (adjusted to the temperature of the hibernation dens, between 5 and 10°) to minimize thermal disturbance during this critical period. Blood samples were immediately centrifuged and plasma was stored at −25° until assayed. Plasma progesterone was measured by radioimmunoassay on 30 to 60 μl plasma after extraction with 0.5 ml of diethyl ether (mean extraction efficiency was 0.98 ± 0.10). Cross-reactivity of the Sigma antibody with other steroids was low (percentage of cross-reactivity at B/B_o: deoxycorticosterone <6.6%, 5α-dihydroprogesterone <3.9%, 5β-dihydroprogesterone <2.6%, 6β-dihydroprogesterone <2.1%). The sensitivity of the assay was 7.8 pg/tube. Inter- and intraassay variations were, respectively, 12 and 6%. Plasma levels of estradiol-17β and metabolites (phospholipids, triglycerides, cholesterol, total protein, albumin, free and total calcium, total phosphorus) were determined, respectively, by radioimmunoassays and spectrophotometry (see Bonnet *et al.*, 1994).

Steroid Implants

Six pregnant females kept in outdoor enclosures were given subcutaneous implants of progesterone 2 weeks before the expected date of parturition, shortly before the August blood sample was taken (see Results). Silastic tubes (Silastic Catalog No. 601-335; i.d. of 3.13 mm, o.d. of 4.16 mm) were cut into 1.5-cm-long pieces. Each piece was filled with 21.43 ± 0.90 mg of crystalline progesterone (4-pregnene-3,20-dione; Sigma). Implants were placed subcutaneously in a dorso–lateral position in the posterior one-third of the snake. The implants were removed shortly after partutition, soon after the September blood sample was taken.

Statistics

All hormone values were ln-transformed to meet the assumption of normality. Comparisons among groups, treatments, and time periods were performed using ANOVAs. Correlations were performed using a simple linear model (Pearson correlation rank) because the source of error variation should be relatively equivalent in the X and Y values (see Results). Some of the 88 outdoor females were bled several times, and most of the 13 indoor females were bled more than five times. Because individual variations in hormonal plasma concentrations are substantial in this species (Naulleau and Fleury, 1990; Saint Girons *et al.*, 1993), this resampling may introduce a pseudo-replication effect in these females. However, because the influence of reproductive status was much greater than interindividual differences (see Results), none of our conclusions was altered if we randomly used individuals only once (also reported under Results), except for the loss in power for some analyses due to smaller sample sizes. Similarly, the changes over time in plasma steroids in outdoor females were great enough to escape being obscured by any interindividual effect (see Results). In this paper we report results from analyses based on the complete data set (henceforth called "full data set"), along with the analysis where individual replicates have been excluded. Analyses were performed using Statistica 5.1.

RESULTS

Comparisons between Reproductive and Nonreproductive Outdoor Females

In contrast to previous studies, we found a significant difference in plasma progesterone concentrations in reproductive and nonreproductive females (ANOVA with reproductive status as the factor and progesterone (ln) levels as the dependent variable: $F_{1,287} = 47.5, P < 0.0001$ with the full data set; $F_{1,109} =$

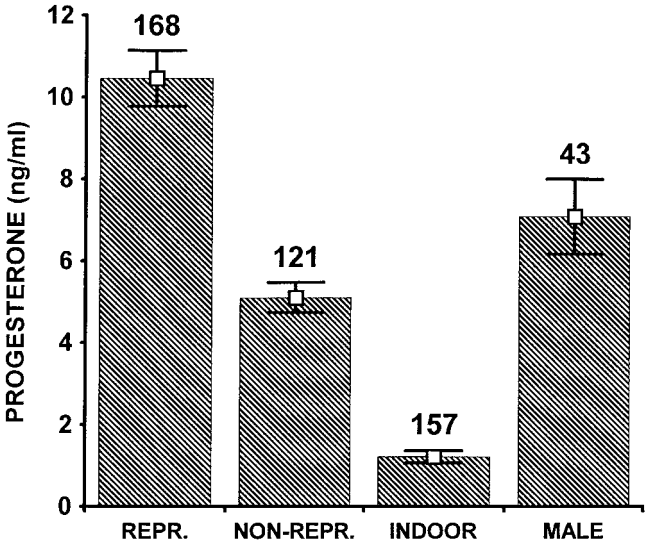

FIG. 1. Comparison of average plasma progesterone among sex categories in Aspic vipers (means are presented with their standard errors). Repr., reproductive females caught in the wild; non-repr., nonreproductive females caught in the wild (both first categories were kept in outdoor enclosures after capture); indoor, females kept under artificial conditions in the laboratory for several years and not allowed to reproduce; male, males caught in the wild and kept in outdoor enclosures. See text for statistics.

32.37, $P < 0.0001$ with each female represented only once in the analysis). On average, reproductive females exhibited plasma progesterone levels double those of nonreproductive individuals (Fig. 1).

Comparisons between Outdoor and Indoor Females

Indoor females showed very low levels of plasma progesterone (same design ANOVA as above: $F_{2,443} = 242.3$, $P < 0.0001$ with the full data set; $F_{2,113} = 42.30$, $P < 0.0001$ with each female represented only once; Fig. 1). *Post hoc* tests showed that these levels were lower than those in any other group (i.e., versus outdoor females, reproductive and nonreproductive; all P values <0.0001, either with the full data set or with each female represented only once). Thus, adult female Aspic vipers which were not reproducing (alternate phases of vitellogenesis–pregnancy versus body-reserve recovery periods) secreted very little progesterone. Although nonreproductive females had lower levels of plasma progesterone than reproductive females, they still had higher circulating blood levels than indoor females.

Comparisons between Males and Females

We first compared males and females kept in outdoor enclosures. One-factor ANOVA with reproductive/sex categories (1, males; 2, reproductive females; and 3, nonreproductive females) as the factor and progesterone (ln) levels as the dependent variable revealed significant differences among the three groups ($F_{2,329} = 23.72$, $P < 0.0001$ with the full data set; $F_{2,151} = 15.54$, $P < 0.0001$ with each female represented only once). However, the plasma values observed in males were intermediate between those observed in reproductive and those observed in nonreproductive females (Fig. 1). *Post hoc* tests showed a significant difference between males and reproductive females ($P = 0.018$ and $P = 0.041$ with the full data set and with females represented only once), but not a clear difference between males and nonreproductive females ($P = 0.188$ and $P = 0.031$ with the full and the limited data sets). Thus, males exhibited significant levels of plasma progesterone and hence showed higher levels than indoor females ($F_{1,198} = 85.45$, $P < 0.0001$ with the full data set; $F_{1,52} = 50.45$, $P < 0.0001$ with each female represented only once). Few males were sampled in April and June and none in October and November. To determine whether this temporal bias may have influenced our conclusions, we repeated the analyses excluding these 4 months (two-way ANOVA with months and sex/categories as the factors). None of our results was altered.

Monthly Changes in Plasma Progesterone in Relation to the Female Reproductive Cycle

For this analysis, we compared the monthly changes in plasma progesterone observed in reproductive versus nonreproductive outdoor females. A two-way ANOVA with reproductive status and months as the factors and progesterone level as the dependent variable showed significant differences among months ($F_{10,264} = 8.88$, $P < 0.00001$ with the full data set; $F_{10,89} = 6.97$, $P < 0.00001$ with each female represented only once) and a significant interaction between the two factors ($F_{10,264} = 2.78$, $P = 0.028$ with the full data set; $F_{10,89} = 1.99$, $P < 0.04$ with each female represented only once; Fig. 2). A closer inspection of the data from the onset of vitellogenesis to parturition, restricting analyses to the activity period

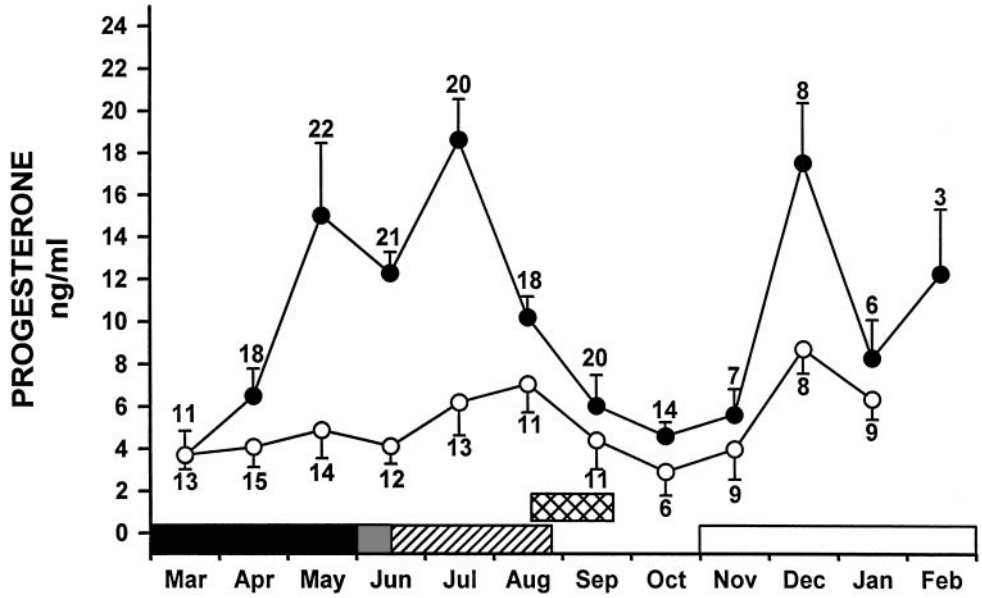

FIG. 2. Changes in plasma progesterone (mean ± SE) during the active period (March to November) and hibernation (November to February) in reproductive (black dots) and nonreproductive (open dots) female Aspic vipers. Sample sizes are indicated. Black box, vitellogenesis period; light gray box, ovulation period; hatched box, gestation; crosses box, parturition; open box, hibernation period. Values are expressed as means (in ng/ml) for clarity, but statistics were performed using Ln transformed values. See text for statistics.

(March to October), revealed that plasma progesterone was more variable in reproductive females (one-way ANOVA with month as the factor: $F_{7,136} = 12.91$, $P < 0.00001$ with the full data set; $F_{7,102} = 6.87$, $P < 0.00001$ with each female represented only once) than in nonreproductive females (same design ANOVA: $F_{7,87} = 1.80$, $P = 0.097$ with the full data set; $F_{7,41} = 2.50$; $P = 0.031$ with each female represented only once).

Figure 2 and *post hoc* tests show that plasma progesterone levels were low in both reproductive and nonreproductive females shortly after hibernation in March (no difference between the two groups; $P = 1.00$). Progesterone levels increased in reproductive females in May (with a significant difference between March and May; $P = 0.003$) to reach a plateau during the first two-thirds of pregnancy (all $P > 0.72$ in all comparisons among May, June, and July; all $P < 0.01$ in comparisons with all the other months). Progesterone levels decreased sharply after parturition in September to again reach low levels (no significant difference from values recorded in March, April, or October (all $P > 0.98$). Overall, progesterone levels of reproductive versus nonreproductive females differed only during late vitellogenesis in May and during pregnancy (all $P < 0.0001$).

Plasma progesterone showed a peak during hibernation in both reproductive and nonreproductive females (Fig. 2). Restricting analyses to the period from October to March to encompass the periods before and after hibernation, a two-way ANOVA (with months and reproductive status as the factors) revealed significant monthly variation ($F_{4,81} = 10.66$, $P < 0.0001$ with the full data set; $F_{4,31} = 14.26$, $P < 0.0001$ with each female represented only once). This analysis also showed a significant difference between reproductive and nonreproductive females ($F_{1,81} = 7.52$, $P = 0.007$ with the full data set; $F_{4,31} = 6.99$, $P = 0.013$ with each female represented only once), but no interaction between months and reproductive status ($F_{4,81} = 0.25$, $P = 0.91$ with the full data set; $F_{4,31} = 0.70$, $P = 0.60$ with each female represented only once). The plasma progesterone concentration in December also differed from the level recorded soon after hibernation in March ($P = 0.0001$; Fig. 2).

Balance between Progesterone and Estradiol Plasma Concentration during Vitellogenesis

We also tested the possibility that progesterone and estradiol plasma levels may be negatively correlated

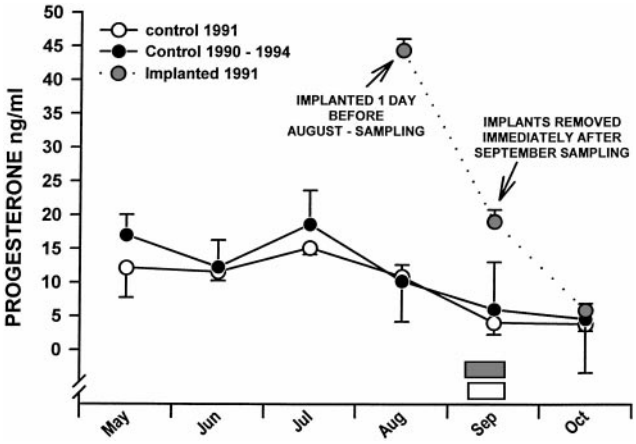

FIG. 3. Influence of exogenous progesterone (mean ± SE) on parturition in reproductive female Aspic vipers. In August 1991, six pregnant females were fitted with a silastic implant of progesterone 1 month before the estimated date of parturition. These females exhibited very high plasma progesterone values (gray dots), but the parturition was not delayed. Gray box, range of parturition for the 1991 implanted females; white box, range of parturition for the 1991 control reproductive females. Because yearly variations may have influenced the patterns, the 1991 control group (open dots) is shown separately from the 1990, 1992–1994 control groups (black dots). Note that after removing the silastic implants, the plasma progesterone values dropped to the "normal" levels observed in the control groups. Values are expressed as means (in ng/ml) for clarity, but statistics were performed using Ln transformed values (see text).

during vitellogenesis (Callard *et al.*, 1992). To encompass the period of vitellogenesis and early pregnancy, we restricted analyses to the period from March (onset of vitellogenesis) to July (postovulation). We found a significant negative correlation between progesterone and plasma estradiol levels ($r = -0.26$, $N = 81$, $P = 0.017$ with the full data set; $r = -0.56$, $N = 23$, $P = 0.005$ with each female represented only once). During vitellogenesis, estradiol levels are initially very high (see Bonnet *et al.*, 1994) and then fall progressively as progesterone levels increase.

Effect of Exogenous Progesterone on Parturition

Silastic implants with exogenous progesterone significantly elevated plasma progesterone levels in the six manipulated reproductive females in comparison with the other reproductive females in late August (ANOVA with group treatment as the factor: $F_{1,21} = 61.02$, $P < 0.0001$; Fig. 3). Plasma progesterone levels remained elevated in the implanted females during parturition in September (same design ANOVA: $F_{1,14} = 17.35$, $P < 0.001$), and these levels were even higher than those in nonmanipulated pregnant females (ANOVA; $F_{1,39} = 12.20$, $P < 0.002$). Because parturition dates are influenced by climatic conditions (Naulleau, 1986) and hence show significant yearly variations (unpublished), we restricted the following analyses to the year in which we conducted the experiment. Exogenous progesterone did not delay parturition in the six implanted females relative to the seven nonmanipulated females (ANOVA with treatment group as the factor and parturition date as the factor: $F_{1,12} = 1.29$, $P > 0.05$; Fig. 3).

Effect of Exogenous Progesterone on Plasma Metabolite Levels

We found no significant effect of exogenous progesterone on plasma metabolites (Table 1). Because a certain delay may be necessary for the mobilization of any metabolites, we repeated the above analyses 1 month later, in September. At that time, progesterone levels were still very high in the manipulated females, but only one marginally significant effect was found (decrease in phospholipids from 3.09 ± 1.42 g/L ($N = 18$) to 1.72 ± 0.37 g/L ($N = 4$); $P = 0.046$). Thus, exogenous progesterone seems to have little (if any) effect on plasma metabolites (and hence on the mobilization of maternal lipids, proteins, or calcium) either in pregnant or postparturient females.

DISCUSSION

Our results provide the first direct evidence that the progesterone profile shifts significantly during vitellogenesis and gestation, and more generally over the course of the long (>2 years) and complex reproductive cycle, in this viviparous snake. Previous studies have been contradictory and/or carried out during a limited period of the reproductive cycle (e.g., gestation only). Previous studies with *V. aspis* noted lower levels of progesterone in "seasonal anestrous" individuals, but found no significant variation with changes in reproductive condition (i.e., pregnant or mating; see Saint Girons *et al.*, 1993). The null result from this

TABLE 1
Effects on Exogenous Progesterone in Plasma Estradiol and Plasma Metabolites during Late Pregnancy in Reproductive Female Aspic Vipers

	Intact	Manipulated	*F*	*df*	*P*
Progesterone	10.222 ± 4.137 (18)	44.417 ± 8.282 (5)	61.016	1,21	0.00001
Estradiol	0.091 ± 0.068 (19)	0.168 ± 0.018 (6)	3.434	1,23	0.077
Calcium	95.1 ± 15.0 (19)	87.0 ± 13.0 (4)	0.893	1,21	0.355
Phospholipids	3.23 ± 1.29 (16)	2.30 ± 0.50 (5)	1.831	1,19	0.192
Glucose	0.40 ± 0.16 (19)	0.35 ± 0.11 (5)	0.554	1,22	0.464
Tryglycerids	0.87 ± 0.78 (18)	0.60 ± 0.29 (5)	0.405	1,21	0.531
Cholesterol	4.42 ± 2.07 (19)	3.20 ± 0.82 (5)	1.01	1,21	0.326
Total proteins	39.2 ± 8.7 (19)	37.3 ± 10.0 (5)	0.219	1,22	0.644
Albumin	14.0 ± 2.8 (19)	14.5 ± 3.3 (5)	0.952	1,22	0.760

Note. Intact group refers to nonmanipulated females; manipulated individuals were fitted with silastic implant of progesterone shortly before blood sampling. Values are expressed as means with SD and sample size in parentheses; steroids in ng/ml; plasma metabolites in g/ml or mg/ml for calcium. Note that ANOVAs were performed with ln-transformed values.

earlier study probably reflects the great individual variability in plasma progesterone levels of the Aspic viper combined with small sample sizes. With the benefit of a much larger data set, we see that progesterone levels do increase significantly during gestation in the Aspic viper and are also higher in reproductive than in nonreproductive females. Progesterone levels then fall at the time of parturition, as in other viviparous reptiles studied to date (Highfill and Mead, 1975a,b; Arslan *et al.*, 1978; Callard *et al.*, 1992; Fergusson and Bradshaw, 1991). The relationship between pregnancy and high levels of progesterone suggests that progesterone plays an important role in reproduction in this viviparous species, particularly during the 3-month pregnancy (i.e., stimulating the vascularity of the oviducts—Mead *et al.*, 1981; Masson and Guillette, 1987). On the other hand, progesterone does not appear to be directly implicated in the onset of parturition in this species. Other studies with viviparous reptiles have shown a similar precipitous fall in plasma progesterone levels close to parturition (e.g., Highfill and Mead, 1975a). Fergusson and Bradshaw (1991) showed that this fall was preceded by a significant increase in levels of arginine vasotocin. Our preliminary implant experiment suggests that doubling progesterone levels does not prolong parturition in Aspic vipers and that, although plasma progesterone levels fall naturally at the end of pregnancy, this decline does not appear to initiate parturition. Instead, the fetus may play a significant role in the timing of its birth, as occurs in mammals (Yaron, 1972; Thibault and Levasseur, 1991).

Callard *et al.* (1990, 1992) and Guillette (1993) have suggested that the transition from oviparity to viviparity involves an alteration in the balance between estradiol and progesterone, the hormones believed to control vitellogenesis and gestation, respectively. Estradiol-17β has been described as a vitellogenic hormone (stimulating the production of vitellogenin by the liver), which also promotes follicular development and the formation of the protective layers of the egg. Increases in plasma levels of estradiol which are observed exclusively in vitellogenic females are directly responsible for the mobilization of maternal reserves during vitellogenesis in the Aspic viper (Bonnet *et al.*, 1994). Progesterone, on the other hand, has been viewed as a hormone antagonistic to estradiol, slowing the rate of vitellogenesis and enhancing feto–maternal exchanges at the level of the oviduct. This scenario is supported by *in vitro* and *in vivo* experiments (Ho *et al.*, 1981, 1982; Riley *et al.*, 1987, 1988; Riley and Callard, 1988; Perez and Callard, 1989). However, almost all the evidence of such antagonism has been inferred from studies with oviparous lizards rather than viviparous species, including snakes (Ho *et al.*, 1981, 1982; Riley *et al.*, 1988; see Callard *et al.*, 1992). Our results do not provide a rigorous test of the hypothesis that progesterone acts to inhibit vitellogenesis, but the rapid increase in progesterone levels at the end of vitellogenesis is certainly consistent with this hypothesis. It seems possible that, soon after ovulation, progesterone reorients the hepatocytes toward functions other than the production of vitellogenin. In experiments outlined in Bonnet *et al.* (1994), the effects

of estradiol implants did not cease after their removal. In contrast, virtually all of the plasma metabolites of normal females display a return to basal values as levels of estradiol fall after vitellogenesis. This observation suggests that, under normal circumstances, some inhibitory mechanism terminates vitellogenesis at the appropriate time. Progesterone may well be the active component in this negative feedback process, as suggested by the negative correlation found between progesterone and estradiol levels in this study. A hibernation peak in progesterone levels in *V. aspis,* already noted by Naulleau and Fleury (1990), was observed in females, and it was very marked in postparturient snakes (greater absolute value). This progesterone peak may function to block vitellogenesis in these emaciated animals, which could ill afford to engage in another reproductive bout.

Only our reproducing females had higher progesterone levels than males. This result mirrors the earlier report by Saint Girons *et al.* (1993) of no sex difference in progesterone levels in males versus females. This puzzling result suggests that progesterone plays some additional role in male vipers and nonreproductive females, as well as supporting gestation in the female. In addition, the very low values observed in indoor females suggest that, under natural conditions, both nonreproductive females and males maintain relatively high levels of plasma progesterone. What possible functions does progesterone subtend? Plasma progesterone levels measured in males were very similar to those reported in the study by Saint Girons *et al.* (1993), which involved chromatographic separation of the steroid on Lipidex columns. This raises the question of the origin of the progesterone in males, which may be from adrenal or from other steroidogenic tissue. Highfill and Mead (1975a,b) have shown that corpora lutea are the major source of progesterone during pregnancy, but the adrenals are the major source of this steroid in nonpregnant female *Thamnophis;* this may equally apply to nonreproductive female and male *V. aspis.* Interestingly, Belvedere and Colombo (1992) have reported autonomous sites of extraglandular steroidogenesis and progesterone secretion in rats and frogs, and Schwann cells in the central nervous system have also been shown to be capable of progesterone synthesis (Koenig *et al.*, 1995).

The results of this study provide greater insight into the progestational hormone of a viviparous snake and its modulation throughout the period of reproductive activity. More data are required, however, from other species in the context of the oviparity–viviparity transition. Experiments on the influence of progesterone levels on gestation length for example (the primary difference between oviparous and viviparous populations) are contradictory (see Mason and Guillette, 1987; Guillette *et al.*, 1984; Shine and Guillette, 1988; Dunbrack and Ramsay, 1989; Braña *et al.*, 1991; Jones and Guillette, 1992; Stewart and Thompson, 1993). In one viviparous lizard species (*Sceloporus jarrovi*) injection of progesterone delays parturition (Guillette *et al.*, 1991). However, the same procedure had no effect on gestation length in the Aspic vipers that were the subject of our study and probably in other viviparous snakes (Bradgon, 1951). Although such contradictory results are frustrating, they hint that the interspecific diversity of endocrine mechanisms in reptiles has enormous potential for comparative study.

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