Saint Girons, H. (1975). Observations preliminaires sur la thermoregulation des Vipères d'Europe. *Vie et Milieu* **25**, Fasc. I, Sèrie C, 137-168.

Saint Girons, H. (1978). Thermorègulation comparèe des Vipères d'Europe. Etude biotèlèmetrique. *Terre et Vie* **32**, 417-440.

Saint Girons, H. (1979). Les cycles alimentaires des Vipères europèennes dans des conditions semi(naturelles. *Ann. Biol. Anim. Bioc. Biophys.* **19**, 125-134.

Saint Girons, H. and Naulleau, G. (1981). Poids des nouveau-nès et stratègies reproductives des Vipères europeenes. *Rev. Ecol (Terre et Vie).* **35**, 597-616.

Simpson, E. H. (1949). Measurement of diversity. *Nature* **163**, 688.

Smith, M. (1951). *British Amphibians and Reptiles*. London, Collins.

Stjernberg, M. (1978). Huggorm som holkfagelplundrare (A Viper *Vipera berus* plundering nest-box with nestlings of *Parus major*). *Memoranda Soc. Fauna Flora Fenn.* **54**, 1-58.

Toschi, A. (1965). Fauna d'Italia, III. *Mammalia* (*Lagomorpha, Rodentia, Carnivora, Artiodactyla, Cetacea*). Bologna, Calderini.

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PHOSPHATE AND CALCIUM LEVEL VARIATIONS IN THE PLASMA OF THE SNAKE *VIPERA ASPIS* DURING THE ANNUAL CYCLE AND THE REPRODUCTIVE PERIOD

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ABSTRACT

The measurement of phosphate and calcium levels in the plasma of *Vipera aspis* during two consecutive year cycles shows significant seasonal variations. For males and non-breeding females, phosphate levels are higher during the active period of the year than during hibernation. Conversely, calcium levels appear generally more elevated for the hibernating period than for the active period. Breeding females present an important increase of phosphate and calcium levels with a peak near ovulation. Clearly, this phenomenon is related to vitellogenesis. The relationship between plasma phosphate and calcium levels and bone tissue mineralization is discussed, the latter being the only reservoir of these mineral salts available to the snake.

INTRODUCTION

Literature on annual variations of plasma calcium and phosphate levels in non-mammalian vertebrates is scarce (see Dacke, 1979; Clark, 1983). Variations of calcium level have been studied more than those of phosphate. It appears that fish (Booke, 1964; Meunier, 1978) and amphibians (Robertson, 1978; 1985) present significant annual variations of calcium level. For reptiles, we are not aware of any publications dealing with calcium and phosphate variations during the entire annual cycle. During the breeding period, however, female fish (Lopez and Martelly-Bagot, 1971; Martelly, Milet, Legrand, Girard and Fontaine, 1979), amphibians (Dacke, 1979), reptiles (Dessauer and Fox, 1959; Dacke, 1979) and birds (Dacke, 1979) are all known to exhibit an increase of plasma calcium and phosphate levels. These data suggest that the regulation of plasma calcium and phosphate levels in these groups is quite different from that of mammals, because they show such high and significant natural annual variations. Nevertheless, for reptiles and especially for snakes, the available data are so scarce that they prevent any general conclusions concerning phosphocalcic regulations.

This work, part of a more general study dealing with the phosphocalcic metabolism of *Vipera aspis* (Alcobendas and Castanet, 1985; Alcobendas and Baud, 1988; Alcobendas, 1988; Alcobendas, Baud and Castanet, 1991), presents data concerning plasma calcium and phosphate level variations in this species during the annual cycle and breeding period.

MATERIALS AND METHODS

MATERIAL

Some of the vipers used in this study originated from a wild population living in the center of France (Loir et Cher), (capture permission delivered by the french Ministry of Environment). Before the beginning of the experiment these vipers remained at least six months in captivity. A second sample was born at the laboratory, from parents which also originated from the same locality. Finally some females caught in nature in June were immediately punctured.

The breeding conditions used at the laboratory correspond to an artificial seasonal cycle defined by an active period of 8 months with high temperature and a light cycle similar to that encountered in nature (from March to October) during which vipers feed and reproduce, and a hibernating period of 4 months (November to February) with low temperature, darkness and no food. These breeding conditions provide vipers in good physiological condition, all year round. All the vipers used in this study had reached sexual maturity.

Intracardiac punctures were conducted monthly, using a standstill system fitted to the snakes (Naulleau, Fleury and Boissin, 1987). 400 to 500 µl of blood are taken monthly from each viper. This technique was used for two years in some vipers and did not cause any trouble to the animals which went on eating and reproducing normally.

Method of Measurement

The blood samples were centrifuged (Beckman centrifuge) for three minutes at 15,000 rpm. The plasma obtained was divided into two fractions. The first, stored at 4°C, was used, within 24 to 48 hours of the intracardiac puncture, for the measurement of phosphorus by direct colorimetry, without deproteinization (Phosphore Seratest Eurobio). The second fraction of plasma was stored at -18°C (no more than two months) before the measurement of calcium level by spectrophotometry of atomic absorption (Perkin Elmer Model 401 spectrophotometer).

Statistical Analysis

The individual values of plasma calcium and phosphate levels were recorded. Monthly average values were then calculated, according to the sex of the animals. The number of individuals punctured each month is indicated on the curves (Figs. 1-4).

The importance of calcium and phosphate level variations during the annual cycle, was estimated using analysis of variance. When the calcium and phosphate levels were compared between two periods of the year (e.g. active period compared with hibernation), the individual values were pooled for each period and the two periods compared as two groups of values using Student's *t*-test.

The two consecutive annual cycles of calcium and phosphate levels were compared using the Student *t*-test for paired series derived, in this case, from an analysis of variance (ANOVA).

When the calcium level was compared between sexes (very small samples), in January and February 1985, we used the non-parametric Mann-Whitney U-test.

RESULTS

Males and Non-breeding Females

Phosphate level. During the two annual cycles, 1984 and 1985, plasma phosphate levels did not show significant differences between males and non- breeding females, regardless of the month (Fig. 1).

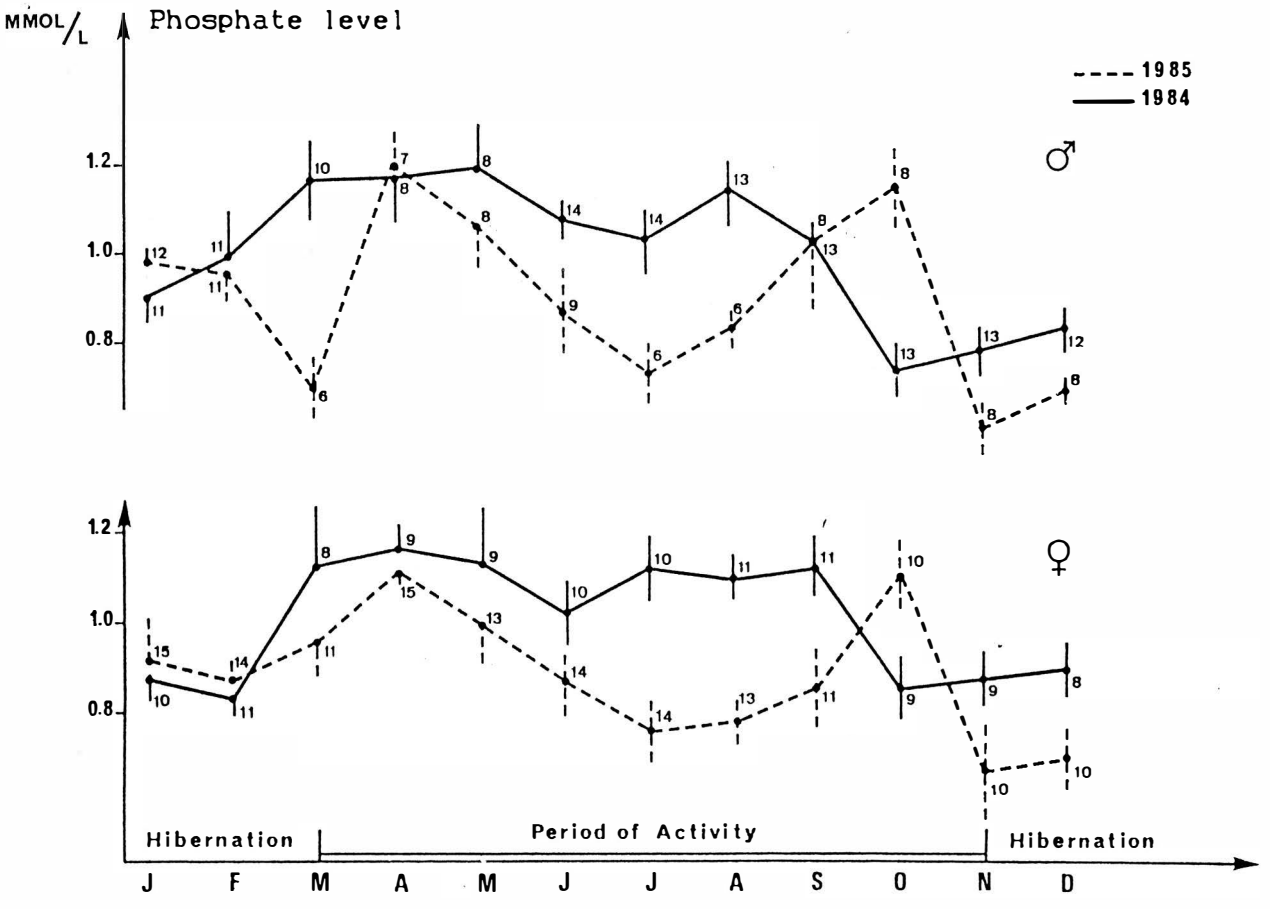

Fig. 1. Comparison of the annual variations of plasma phosphate level for the *Vipera aspis* males and non breeding females during two consecutive annual cycles, 1984 and 1985 (numbers = *n*; bars = ± SD).

For each annual cycle, we observed in both sexes significant monthly variations of phosphate level (*1984*, males: $F_{10,118} = 2.47$, females: $F_{10,95} = 2.54$, $P < 0.01$; *1985*, males: $F_{11,86} = 2.50$, females: $F_{11,122} = 2.25$, $P < 0.01$). The values appeared to be more elevated for the active period than for the hibernating one. A shift of one month could be observed between the two annual cycles: the phosphate level increased in March for 1984 but in April, for 1985, and decreased in September for 1984 but in October for 1985.

A comparison of the two consecutive annual cycles in females, with the test for paired series, showed differences ($t = 2.20$, df = 11, $P > 0.05$). For males, the differences between the two annual cycles were not significant (t-test, df = 11, $P > 0.05$).

Calcium level. In 1984, the monthly values of calcium level were not equivalent between sexes. For the non-breeding females, the calcium level showed significant variations during the annual cycle ($F_{11,116} = 2.40$, $P < 0.01$): i.e. lower from April to August than from September to March ($t = 2.57$, df = 91, $P < 0.05$). For the males, the calcium level also showed significant variations during the annual cycle ($F_{11,116} = 1.87$, $P < 0.05$). But these variations were not as well marked as for females. The calcium level increased slowly from June to November. It decreased significantly in December and remained low until February. The calcium level increased again in March (at the end of the hibernation) but decreased in April-May (Fig. 2).

In 1985, the differences of the monthly calcium levels between sexes were not so marked. They were significant only in January ($n_1 = 9$, n_2-$n_1 = 8$, $U<39$, $P < 0.05$) and February ($n_1 = 10$, n_1-$n_2 = 4$, $U<36$, $P < 0.05$) where the calcium level was higher in females. For both sexes, the calcium level values showed significant variations during the annual cycle (males: $F_{11,75} = 2.58$, females: $F_{11,125} = 2.40$, $P < 0.01$). Two periods could be identified. The first one — January to July — was characterized by higher values while the second period — August to December — was characterized by lower values (males: df = 85, females: df = 135; both $t = 2.57$, $P < 0.01$).

Breeding Females

The breeding female group had a heterogeneous origin. Differences between the laboratory born females and those that had been recently caught in nature were noticeable.

Annual variations of phosphate level. The values of phosphate level of the breeding females born in the laboratory present significant variations during the annual cycle ($F_{10,22} = 2.30$, $P < 0.05$). The phosphate level appeared significantly higher in March or April (according to each individual), than during the rest of the year. The maximal values obtained were 2 or 3 times higher than the average measured during the rest of the year (Fig. 3).

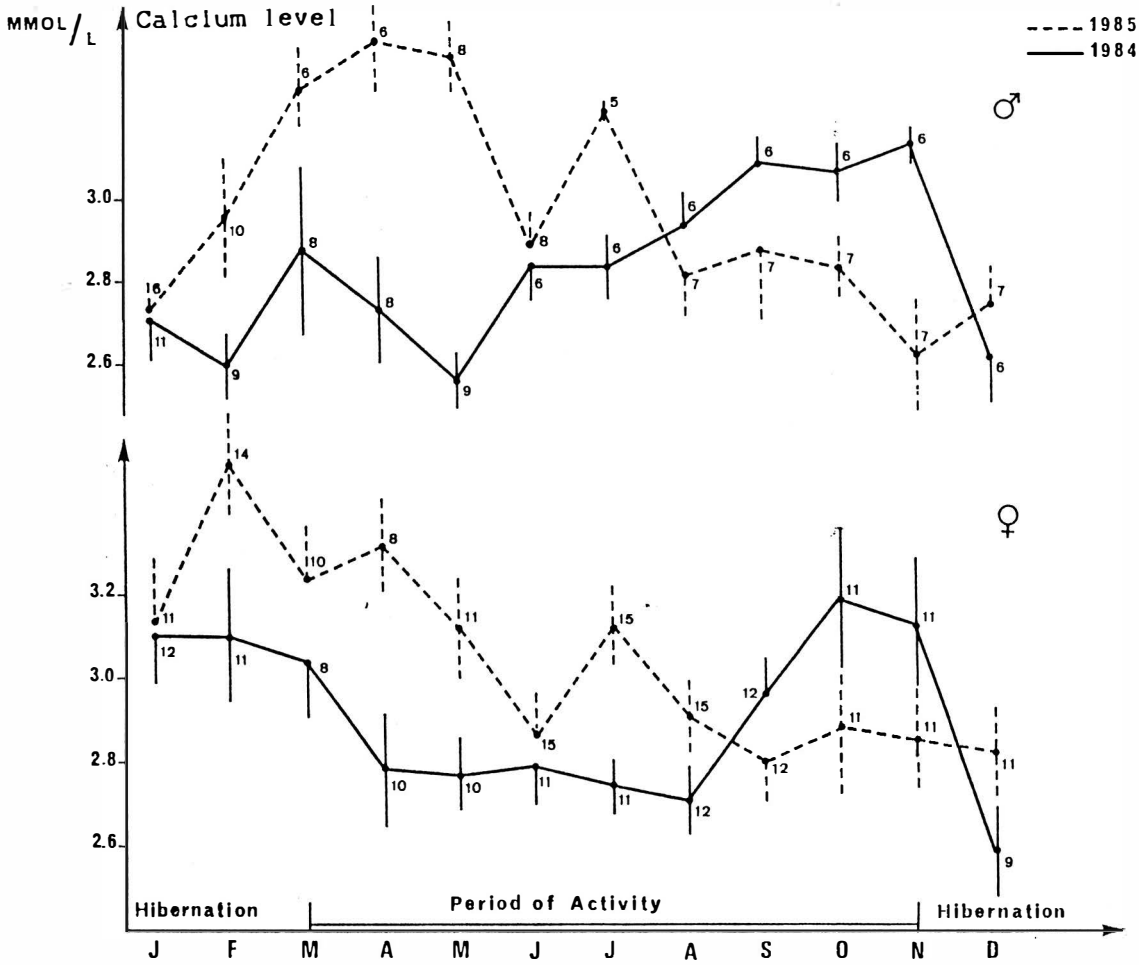

Fig. 2. Comparison of the annual variations of plasma calcium level for the *Vipera aspis* males and non breeding females during two consecutive annual cycles, 1984 and 1985 (numbers = n; bars = $\pm$ SD).

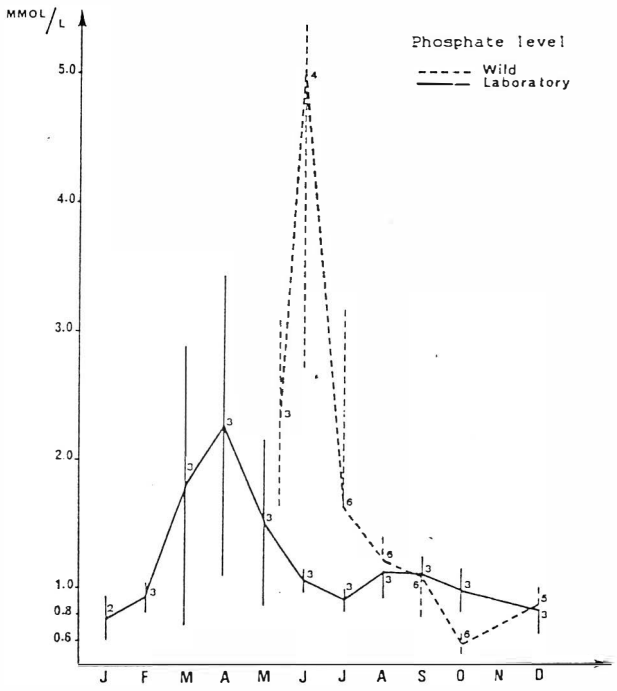

Fig. 3. Annual variation of plasma phosphate level for *Vipera aspis* breeding females. For the wild vipers, we do not possess values of phosphate level from January to April.

For the breeding females caught in nature, the phosphate level showed significant variations during the annual cycle ($F_{7.33} = 3.23$, $P < 0.01$). High values appeared in June or July, according to each individual. The maximal values obtained appeared five times higher than the average, measured for the rest of the year. For these females, we did not have the phosphate level values at the beginning of the breeding cycle (March to April).

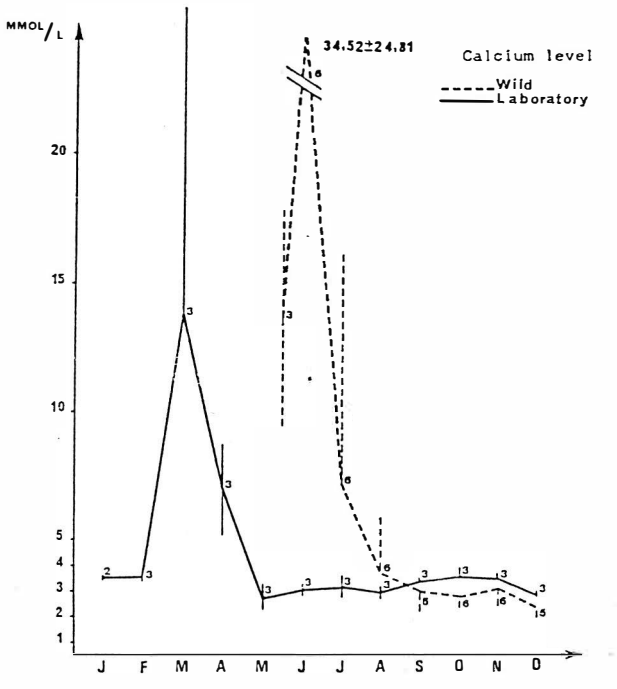

Fig. 4. Annual variation of plasma calcium level for *Vipera aspis* breeding females. For the wild vipers, we do not possess values of calcium level from January to April.

The annual variations of calcium level. The calcium level of the females born in the laboratory showed, in March or April depending on the individual, values significantly higher than the rest of the year ($F_{11.23} = 2.23$, $P < 0.05$). The maximal values were 5 or 10 times higher than the values measured for the rest of the year (Fig. 4). For the females caught in nature, the calcium level values were significantly higher in June or July than for the rest of the year ($F_{7.36} = 3.21$, $P < 0.01$). The maximal values measured were 10 or 15 times higher than the average ones obtained for the rest of the year. For these wild vipers, we did not have data for the beginning of the breeding cycle (March-April).

Thus, for plasma calcium and phosphate levels, the maximal values obtained for the females caught in nature were more elevated than those obtained for the females born in the laboratory. For each category the increase of the two parameters was synchronous each year, but we note that these peaks appeared earlier in the vipers born in the laboratory.

DISCUSSION

The results presented here, reveal important variations of plasma phosphate and calcium levels during the annual cycle, for non-breeding vipers between two consecutive years and for breeding females during the reproductive period.

Calcium and Phosphate Levels in Males and Non-breeding Females

Although the variations of plasma phosphate and calcium levels of *Vipera aspis* between the two consecutive annual cycles do not appear similar, these differences are not significant. An equivalent result has been observed in the eel (Fontaine *et al.*, 1969). Such variations appear characteristic of heterothermic vertebrates, taking into account the peculiarities of their physiology, more dependent on environmental conditions than homeothermic animals. These variations suggest that the regulation of phosphate and calcium levels in heterotherms is different than that is observed in mammals.

Although the phosphate level of plasma appears to be directly related to diet, since it increases when the vipers begin to eat during early spring, we have seen that the increase in phosphate level can be postponed, e.g. by one month in 1985. Furthermore, the yearly 7 months duration of high phosphate level values is invariable. Thus, this duration seems to be independent of variations in external conditions. Clark (1972) suggests the possibility of endocrine regulation of phosphate level in reptiles. The data concerning *Vipera aspis* tends to support this hypothesis. Pending the availability of more data, we consider that the variations of phosphate levels for the non-breeding vipers seem to be correlated with the yearly activity of the animals, the lower values always being observed during hibernation, the higher during the active season.

The variations of plasma calcium level reported here in the males of *Vipera aspis* appear to be similar to those in the frog *Rana pipiens* as reported by Robertson (1977). In *Vipera aspis*, the calcium level presents complex annual variations which are apparently difficult to link to environmental conditions or to a particular

physiological condition. The regulation of the calcium level also appears to be partially independent of the feeding season, but is probably related to endogenous factors. Such a hypothesis is supported by the fact that the calcium level for females is already high in February 1985, when the vipers are still in hibernation, and not feeding. Later, in autumn 1985, when the vipers are still actively feeding, the calcium level drops.

During the two annual cycles, calcium levels appear higher for females than for males in February and March. This could be related to a preparatory phase of vitellogenesis in the adult females. Because females do not eat during that time, the calcium needed has to be released from a mineral salt reservoir (see below).

Calcium and Phosphate Levels in Breeding Females

We will define a breeding female as a female which, after mating, realizes a complete vitellogenesis followed by oviductal embryonic development. A non-breeding female is a female which, after hibernation, does not mate and does not carry out a complete vitellogenesis.

In our laboratory, we noted that only the females which have mated realize vitellogenesis. Considering the small size of our sample, we cannot maintain that mating is a causal factor of vitellogenesis but our experiment gives some credibility to this hypothesis. The only differences in the monthly values of calcium and phosphate levels between breeding and non-breeding females are peaks of calcium and phosphate for the breeding females. This observation is consistent with the rough data obtained by Izard, Detrait and Bocquet (1961) showing an increase of calcium and phosphate in spring, but for a sample of pooled male and female blood.

The temporal localization and the intensity of the peaks show important individual variations, independent of the origin of the vipers (laboratory or nature). This variability is demonstrated by the importance of the standard deviation observed only for the peaks. As it is not always easy to capture wild females in the beginning of the active season, we cannot establish if the increase of calcium and phosphate levels begins in May or earlier. Dessauer and Fox (1959) in a study of another snake, *Thamnophis sauritus*, showed that the increase of calcium and phosphate in the vitellus occurs "near ovulation" and not all along the period of vitellogenesis. We have shown that for females born in the laboratory, for which the timing of ovulation has not yet been determined but probably appears earlier than in wild vipers, as observed by Naulleau and Bidaud (1981), calcium and phosphate levels peak for one month, and do not last as long as the duration of vitellogenesis. Females of *Vipera aspis* in the wild ovulate in June (Saint Girons, 1957; Naulleau and Bidaud, 1981), when the peaks of plasma calcium and phosphate appear. These data suggest that the increase of calcium and phosphate levels does not last more than one month for the wild asp vipers, as for *Thamnophis sauritus*. Our results support the idea that the peaks of plasma calcium and phosphate level which only appeared in the breeding females are almost certainly linked to vitellogenesis. This phenomenon exists in all non mammalian vertebrates (see Dacke, 1979) and corresponds to storage in the ovules of the elements needed for embryonic development (Packard, Tracy and Roth, 1977; Packard and Packard, 1984; Packard, Packard and Gutzke, 1984). The delay of two months which exists between the wild and laboratory born females is probably caused by the different living conditions of the two groups. Standardized laboratory breeding conditions in laboratory are known to influence the timing of biological events (e.g. mating, birth) (Naulleau, 1975; Naulleau and Bidaud, 1981; Castanet and Naulleau, 1985). Our laboratory conditions, conducive to an active metabolism from the beginning of March to the end of October, may explain why these biological events come sooner in the yearly cycle of laboratory specimens.

Origin of Mineral Salts

The mobilization of mineral ions, essentially calcium and phosphate, results from different processes, depending on the group of vertebrates considered: uptake of mineral salts from their aquatic environment for cyclostomes, chondrichthyans and amphibians (Dacke, 1979), and from scales and bone tissue in bony fishes (Fontaine, Bertrand, Lopez and Callamand, 1964; Lopez and Martelly-Bagot, 1971). Martelly et al. (1979) noticed in *Anguilla anguilla*, a direct relationship between higher serum values of calcium and phosphate and the decreasing mineralization of bone, during experimental maturity. Birds obtain mineral ions by dissolution of medullary bone (Benoit and Clavert, 1947; Meister, 1951; Simkiss, 1975; Dacke, 1979). Reptiles extract mineral salts from bone tissue and/or from endolymphatic sacs, depending on the species (Edgren, 1960; Suzuki, 1963; Dacke, 1979; Wink and Elsey, 1986).

What is the origin of the important quantity of mineral salts needed during the vitellogenesis in *Vipera aspis*? The increase of calcium and phosphate in plasma appears just at the end of the hibernating period for the females caught in nature. As most of these vipers had not eaten since the beginning of the hibernating period, four or five months before, the mineral salts in their blood must have an origin other than dietary. It is known that vipers, like most other snakes, do not possess salt reservoirs other than bone tissue (Alcobendas, 1989). Moreover, during vitellogenesis, a significant increase of bone porosity is observed in the vertebral bone of the breeding females, and in this part of the skeleton only (Alcobendas, 1989). Consequently, a close relationship can be hypothesized between vertebral bone tissue resorption and the providing of mineral salts to the vitellus in *Vipera aspis* via increased plasma calcium and phosphate levels. This process of bone resorption, in addition to a strong decrease in the degree of the vertebral bone mineralization observed during the hibernating period for all the vipers (Alcobendas and Castanet, 1985), offers a strong case that bone tissue, and especially the vertebrae, acts as the only calcium and phosphate releasing reservoir when those ions are needed by the organism of the Asp viper.

REFERENCES

Alcobendas, M. (1988). Données sur le métabolisme phosphocalcique chez la femelle de *Vipera aspis* (L.) (Ophidia, Viperidae) en reproduction. *Bulletin de la Société Hepétologique de France* **46,** 22-30.

Alcobendas, M. (1989). Recherches sur le métabolisme phosphocalcique au cours du cycle annuel et du cycle reproducteur chez un reptile, *Vipera aspis* (L.)(Ophidia, Viperidae). *Thèse Dr. Université Paris* 7. 235 pages.

Alcobendas, M. and Baud, C.A. (1988). Halos périlacunaires de déminéralisation de l'os cortical chez *Vipera aspis* (L.) (Reptilia, Ophidia) dans diverses conditions physiologiques. *Comptes rendus de l'Académie des Sciences (Paris)* **307**, 177-181.

Alcobendas, M. and Castanet, J. (1985). Variations du degré de minéralisation osseux au cours du cycle annuel chez *Vipera aspis (L.)* Ophidia, Viperidae. *Comptes rendus de l'Académie des Sciences (Paris)* **301**, 187-190.

Alcobendas, M., Baud, C.A. and Castanet, J. (1991). Structural changes of the periosteocytic area in *Vipera aspis* (L.) (Ophidia, Viperidae) bone tissue in various physiological conditions. *Calcified Tissue International* **49**, 53-57.

Benoit, J. and Clavert, J. (1947). Action de la folliculine sur le métabolisme du calcium et l'ostéogénèse chez les oiseaux. *Problèmes Actuels d'Endocrinologie et de Nutrition* **15**, 313-320.

Booke, H. E. (1964). Blood serum proteine and calcium levels in yearling brooktrout. *The Progressive Fish Culturist* **26**, 107-110.

Castanet, J. and Naulleau, G. (1985). La squelettochronologie chez les reptiles: Résultats expérimentaux sur la signification des marques de croissance squelettiques chez les Serpents. Remarques sur la croissance et la longévité de la Vipère Aspic. *Annales de Sciences Naturelles Zoologie* 7, 41-62.

Clark, N. B. (1972). Calcium regulation in reptiles. *General and Comparative Endocrinology suppl.* **3**, 430-440.

Clark, N. B. (1983). Evolution of calcium regulation in lower vertebrates. *American Zoologist* **23**, 719-727.

Dacke, C. G. (1979). *Calcium regulation in sub-mammalian vertebrates*. London: Academic Press.

Dessauer, H. C. and Fox, W. (1959). Change in ovarian follicule composition with plasma levels of snakes during oestrus. *American Journal of Physiology* **197**, 360-366.

Edgren, R. A. (1960). A seasonal change in bone density in female musk turtles, *Sternothaerus odoratus* (Latreille). *Comparative Biochemistry and Physiology* **1**, 213-217.

Fontaine, M.; Bertrand, E.; Lopez, E. and Callamand, O. (1964). Sur la maturation des organes génitaux de l'anguille femelle (*Anguilla anguilla* L.) et l'émission spontanée des oeufs en aquarium. *Comptes rendus de l'Académie des sciences* (Paris) **259**, 2907-2910.

Fontaine, M.; Chartier-Baraduc, M. M.; Deville, J.; Lopez, E. and Poncet, M. (1969). Sur les variations de la calcémie, observées chez *Salmo salar* L. à diverses étapes de son cycle vital. Leur déterminisme endocrinien et leur intervention éventuelle sur le comportement. *Comptes rendus de l'Académie des Sciences* (Paris) **268**, 1958-1961.

Izard, Y.; Detrait, J. and Bocquet, P. (1961). Variations saisonnières de la composition du sang de *Vipera aspis*. *Annales de l'Institut Pasteur* **100**, 539-545.

Lopez, E. and Martelly-Bagot, E. (1971). L'os cellulaire d'un poisson téléostéen, *Anguilla anguilla* L. III: Etude histologique et histophysique au cours de la maturation provoquée par injection d'extraits hypophysaires de carpe. *Zeitschift für Zellforschung* **117**, 176-190.

Martelly, E.; Milet, C.; Legrand, A.; Girard, M. L. and Fontaine, M. (1979). Modifications métaboliques observées au cours de la maturation provoquée *d'Anguilla anguilla* L. *Rapport P. v. Réunion du Conseil International des Explorations en Mer*, 84-90.

Meister, J. (1951). Changes in histological structure of the long bones of birds during the molt. *Anatomical Record* **3**, 1-21.

Meunier, F. J. (1978). Variations saisonnières de la calcémie de la carpe. *Cahiers du laboratoire de Montereau* **6**, 45-52.

Naulleau, G. (1975). Cycle d'activité de *Vipera aspis* (L.) et choix des conditions climatiques naturelles et artificielles. *Vie et Milieu* **25**, 119-136.

Naulleau, G. and Bidaud, Cl. (1981). Intervalle entre accouplement, ovulation et parturition chez *Vipera aspis* (L.) (reptiles, ophidiens, vipéridés), dans différentes conditions expérimentales, étudié par radiographie. *Bulletin de la Société Zoologique de France* **106**, 137-143.

Naulleau, G.; Fleury, F. and Boissin, J. (1987). Annual cycles in plasma testostérone and thyroxine for the male aspic viper *Vipera aspis* (L.), (reptilia, viperidae), in relation to the sexual cycle and hibernation. *General and Comparative Endocrinology* **65**, 254-263.

Packard, G. C.; Tracy, C. R. and Roth, J. J. (1977). The physiological ecology of reptilian eggs and embryos and the evolution of viviparity within the class Reptilia. *Biological Reviews* **52**, 71-105.

Packard, M. J. and Packard, G. C. (1984). Comparative aspects of calcium metabolism in embryonic reptiles and birds. In *Respiration and metabolism of embryonic vertebrates*, 155-179. Seymour R. S. (ed). Dordrecht: Dr. W. Junk Publisher.

Packard, M. J.; Packard, G. C. and Gutzke, W. H. N. (1984). Calcium metabolism in embryos of the oviparous snake *Coluber constrictor*. *Journal of Experimental Biology* **110**, 99-112.

Robertson, D. R. (1977). The annual pattern of plasma calcium in the frog and the seasonal effects of ultimobranchialectomy and parathyroidectomy. *General and Comparative Endocrinology* **33**, 335-343.

Robertson, D. R. (1978). Seasonal changes in plasma and urinary sodium potassium and calcium in the frog *Rana pipiens*. *Comparative Biochemistry and Physiology* **60**, 387-390.

Robertson, D. R. (1985). Annual and diurnal patterns of plasma Ca fractions in anuran *Rana pipiens*. *American Journal of Physiology* **249**, 290-296.

Saint Girons, H. (1957). Le cycle sexuel chez *Vipera aspis* (L.) dans l'ouest de la France. *Bulletin Biologique de France et de Belgique* **91**, 284-350.

Simkiss, K. (1975). Calcium and avian reproduction. *Symposium of the Zoological Society of London* **35**, 307-337.

Suzuki, H. K. (1963). Studies on the osseous system of the slider turtle. *Annals of the New York Academy of Science* **109**, 351-410.

Wink, C. S. and Elsey, R. M. (1986). Changes in femoral morphology during egg-laying in *Alligator mississipiensis*. *Journal of Morphology* **189**, 183-188.