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Does Less-than-annual Production of Offspring by Female Vipers (*Vipera aspis*) Mean Less-than-annual Mating?

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Copulation does not necessarily result in the production of offspring. Copulatory behavior unrelated to reproductive output has been recorded in a wide variety of species (e.g., Hrdy, 1986; De Ruiter et al., 1994; Poldmaa et al., 1995). These non-reproductive matings have been interpreted as nonadaptive by some workers (e.g., as consequences of high sex-steroid levels: e.g., Symons, 1979) but as adaptations by others. For example, females that mate more often than is necessary for reproduction may enhance their nutritional status through nuptial gifts or sperm acquisition (Ward and Landolt, 1995), or may be able to manipulate the subsequent behavior of their male partners (Hrdy, 1986; De Ruiter et al., 1994). Because studies on reptiles have generally interpreted copulations with females strictly in terms of sperm transfer, most herpetologists have tacitly assumed that mating only occurs in circumstances where the sperm that are transferred are likely to contribute to paternity of a female's offspring. The inference is logical and plausible, but definitive evidence is weak.

Snakes with less-than-annual frequencies of female reproduction offer an exceptional opportunity to examine this question. In many species of snakes, especially viperids from temperate zone habitats, adult females do not produce offspring every year. Instead, females delay the production of their next litter until they have accumulated sufficient body reserves, a process that may take from one to several years (e.g., Saint Girons, 1957; Fitch, 1960a; Brown, 1991). In consequence, the population each year contains both reproductive and non-reproductive females. We define as "reproductive" those females which contained large (>10 mm diameter) vitellogenic follicles or embryos (detected by palpation or observed at parturition). Females were classified as "Non-reproductive" if they were caught without enlarged ova or embryos during the period of vitellogenesis and/or gestation (Bonnet and Naulleau, 1996a). This definition is strictly based on physiological events (Bonnet et al., 1994), and thus is somewhat different from a purely behavioral one (e.g., "sexually active" versus "non sexually active" females). This clarification is important because the concepts of "reproductive" versus "sexually active" although biologically overlapping and often used as synonyms, are not strictly identical.

One obvious question is thus: do females mate as usual during their "non-reproductive" years (i.e., while they are accumulating energy stores), or do they mate only in years when they will undergo vitellogenesis and produce a litter? Surprisingly, there appears to be little reliable information with which to answer this simple question (Schuett et al., 1993). Some authors have inferred that females do not mate except in reproductive years (e.g., Macartney and Gregory, 1988), whereas others have concluded that females mate every year despite a less-than-annual schedule of offspring production (Saint Girons, 1949, 1952, 1957, 1996; Saint Girons and Duguy, 1982; Luiselli, 1993; Capula et al., 1995). In most cases, these inferences have been based on relatively limited data.

The reason for the lack of information on mating frequency is straightforward; it is easier to determine a female's reproductive status (by body shape, etc.: Fitch, 1960a, 1987) than it is to detect whether or not she has mated. Snakes are often secretive at this time, and matings may be difficult to observe under field conditions. Nonetheless, there is a simple and long-established technique by which prior mating can be determined, by sampling fluids from the female's reproductive tract and examining them for the presence of spermatozoa (Fukada, 1959; Fitch, 1960b, 1987; Naulleau 1992; Bonnet and Naulleau 1996b). We have gathered an extensive data set on female aspic vipers (*Vipera aspis*) using this method, to test Saint Girons' (1949, 1952, 1957, 1996) hypothesis that females of this species continue to mate even during years in which they do not produce offspring. If such non-reproductive matings occur in this species, it would offer an exciting opportunity to test ideas on the factors responsible for such behavior.

Snakes were captured in the field in western central France (Loire Atlantique, Vendée, Charente Maritime, and Deux Sèvres) as part of extensive mark-recapture studies on reproductive biology of this species (Naulleau and Bonnet, 1996; Bonnet and Naulleau, 1996a; Bonnet, 1997). Since 1990, more than 340 adult females (>41 cm snout-vent length, 47 cm total length: Naulleau and Bonnet, 1996) have been tested for the presence of oviductal sperm following the methods of Fukada (1959) and Fitch (1960b). The posterior part of the abdomen was softly massaged to expel fluids from both oviducts and cloaca. The fluids were then spread across a glass microscope slide and allowed to dry. They were later examined microscopically (60× magnification). Dried spermatozoa are easily identified and no specific preservation method is needed. With this method, the presence of spermatozoa can only be detected for few days after mating. We base this conclusion on hundreds of examinations of cloacal swabs from female snakes of several species (e.g., *Vipera aspis, Coluber viridiflavus, Elaphe longissima, Natrix natrix*): sperm were never seen in swabs from this posterior region >10 d after the date of mating (pers. obs. by GN, XB, MV, OL). Hence, the technique can be used to estimate the period of mating.

These measurements revealed a strong link between reproductive status and mating (Fig. 1). Restricting analysis to the two populations intensively studied (Loire Atlantique and Vendée) and to years when systematic "sperm" examinations were carried out, 81 of

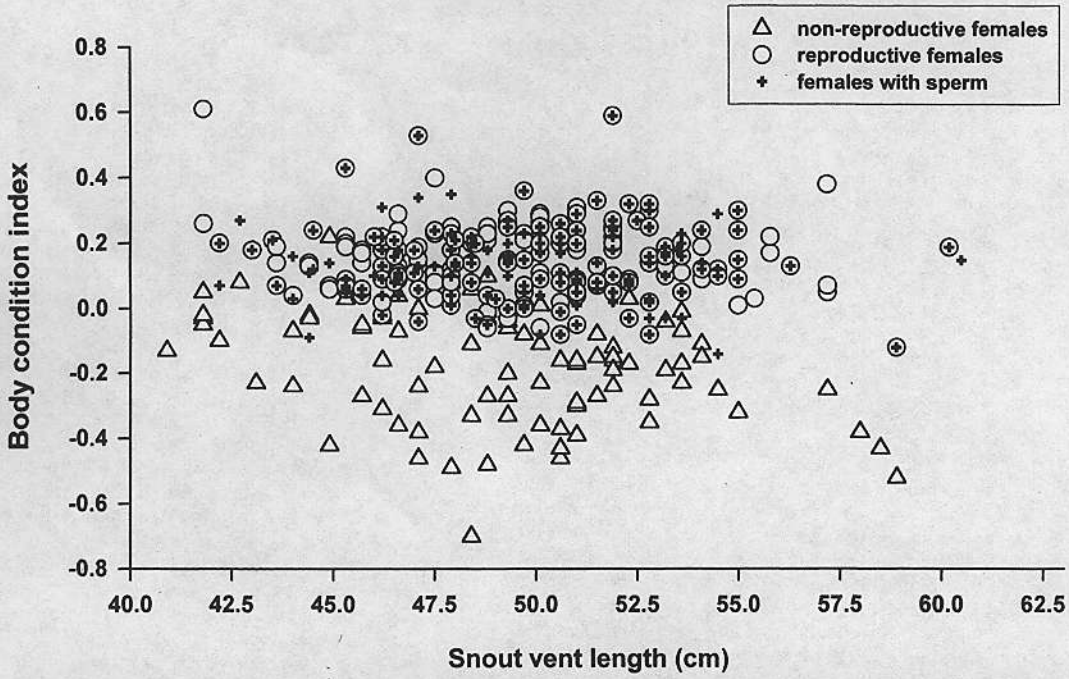

FIG. 1. A comparison of body length and body condition (mass relative to length) for three groups of female aspic vipers collected during the mating season. Body condition (as assessed by the residual score from the general linear regression between ln-transformed values of mass versus snout-vent length) was much higher for females captured in years when they were reproducing (i.e., producing offspring) than in females captured in their non-reproductive years (i.e., not producing offspring). Cloacal samples showed that sperm were present in many of the reproductive females and in other females that were in similarly high body condition, but for which we could not reliably determine reproductive status because they were not recaptured later in the season. However, females in poor body condition, that would not produce offspring in that year, never contained sperm.

144 reproductive females were found to contain sperm (56%), compared to 0 of 86 non-reproductive individuals ($\chi^2 = 41.7$, 1 df, $P < 0.0001$).

We also examined cloacal swabs from many other females for which we had no direct information on reproductive condition. Fortunately reproductive status in females of this species can be confidently assessed from the animal's body condition. Female aspic vipers do not initiate vitellogenesis unless they exceed a very consistent, precise threshold in body condition (reflecting the magnitude of their energy reserves: Naulleau and Bonnet 1996). Thus, if females mate only during years when they undergo vitellogenesis, we predict that the only animals to contain sperm will be those with a relatively high body condition. Examination of the data provides strong support for this hypothesis (Fig. 1).

The clear result from these data is that female aspic vipers mate only in years in which they also undergo vitellogenesis and produce offspring (Naulleau and Bonnet, 1996). There are two plausible mechanisms by which a non-reproductive female could forego mating: either she is not attractive to males (and thus, is not courted), or she is attractive but unreceptive. Our observations both in the field and in captivity support the former hypothesis: non-reproductive females attract little interest from males.

Why do female vipers copulate only in years when they undergo vitellogenesis and produce offspring? Several answers are plausible, from the viewpoint of both sexes. A male that courts and mates non-reproductive females may thereby waste considerable time and energy, as well as potentially costly semen (Olsson et al., 1997). Courtship and mating may also expose the male to a higher vulnerability to predation (Magnhagen, 1991; Madsen and Shine, 1993), and possibly sexually-transmitted disease (Williams, 1975). The same factors may apply to females, with the added constraint that active courtship by a male is likely to compromise the female's ability to thermoregulate, feed, and escape detection by predators (e.g., Slip and Shine, 1988). The only advantage to either partner from mating in a female's non-reproductive year would be in situations where long-term sperm storage is feasible and opportunities for mating during the female's reproductive year are limited (Schuett, 1992). Neither condition seems likely to apply to the aspic viper population that we studied. First, long-term sperm storage may well occur in this species, but is unlikely to be important: we have recorded autumn mating only very infrequently, and data on the closely related *Vipera berus* suggest that long-term sperm storage does not play a major role in the mating system (Stille et al., 1986; Luiselli 1993; Höggren and Tegelström, 1996). Second, population densities of the snakes in our study area are high enough for frequent

male-female encounters during the mating season (pers. obs. by GN, XB, MV, OL based on observations of radio-tracked snakes).

An alternative approach would be to focus on proximate rather than ultimate mechanisms. For example, the act of copulation may directly stimulate vitellogenesis, as it is believed to do in female garter snakes (Gartska et al. 1985). Our data on aspic vipers do not accord with this scenario. The female viper's "decision", as to whether or not to reproduce is determined almost entirely by the relative magnitude of her energy reserves (Bonnet et al., 1994; Naulleau and Bonnet, 1996). Reproductive females display characteristic steroid profiles (high levels of circulating oestrogen) soon after emerging from hibernation, before mating (Bonnet et al., 1994). Thus, reproductive status is determined prior to, and thus independently of, copulatory behaviour.

In summary, our extensive data set strongly challenges previous reports (Saint Girons, 1996 and earlier papers) that female aspic vipers mate even during non-reproductive years. Additional data on other species are needed before we can assess the generality of this result, and we encourage other workers to apply the simple technique of oviduct-flushing to obtain reliable information for this «missing link» in snake reproductive biology.

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Literature Cited

Bonnet, X. 1997. Influence of size on survival in newborn asp vipers (*Vipera aspis*), preliminary results. Amphibia-Reptilia 18:107–112.

———, and G. Naulleau 1996a. Catchability in snakes: consequences on breeding frequency estimates. Can. J. Zool. 74:233–239.

———, and ———. 1996b. Are body reserves important for reproduction in male dark green snakes (Colubridae: *Coluber viridiflavus*)? Herpetologica 52: 137–146.

———, ———, and R. Mauget 1994. The influence of body condition on 17-β estradiol levels in relation to vitellogenesis in female *Vipera aspis* (Reptilia, Viperidae). Gen. Comp. Endocrinol. 93:424–437.

Brown, W. S. 1991. Female reproductive ecology in a northern population of the timber rattlesnake, *Crotalus horridus*. Herpetologica 47:101–115.

Capula, M., E. Phillippi, and L. Luiselli. 1995. Annual mating in female colubrid snakes with irregular reproductive frequency. Herpetozoa 8:11–15.

De Ruiter, J. R., J. A. R. A. M. Van Hoof, and W. Scheffrahn. 1994. Social and genetic aspects of paternity in wild long-tailed macaques (*Macaca fascicularis*). Behaviour 129:203–224.

Fitch, H. S. 1960a. Autecology of the copperhead. Univ. Kansas Pub. Mus. Nat. Hist. 13:85–288.

———. 1960b. Criteria for determining sex and breeding condition in snakes. Herpetologica 16:49–51.

———. 1987. Collecting and life-history techniques. *In* R. A. Seigel, J. T. Collins, and S. S. Novak (eds.), Snakes Ecology and Evolutionary Biology, pp. 143–164. Macmillan, New York.

Fukada, H. 1959. A method for detecting copulated female snakes. Herpetologica 15:181–182.

Gartska, W. R., R. R. Tokarz, M. Diamond, A. Halpert, and D. Crews. 1985. Behavioral and physiological control of yolk synthesis and deposition in the female red-sided garter snake (*Thamnophis sirtalis parietalis*). Horm. Behav. 19:137–153.

Höggren, M., and Tegelström, H. 1996. Does long-term storage of spermatozoa occur in the adder (*Vipera berus*)? J. Zool. (London) 240:501–510.

Hrdy, S. B. 1986. Empathy, polyandry, and the myth of the coy female. *In* R. Bleier (eds.), Feminist Approaches to Science, pp. 119–146. Pergamon Press, Oxford.

Luiselli, L. 1993. Are sperm storage and within-season multiple mating important components of the adder reproductive biology? Acta Oecologia 14: 705–710.

Macartney, J. M., and P. T. Gregory. 1988. Reproductive biology of female rattlesnakes (*Crotalus viridis*) in British Columbia. Copeia 1988:47–57.

Madsen, T., and R. Shine. 1993. Costs of reproduction in a population of European adders. Oecologia 94: 488–495.

Magnhagen, C. 1991. Predation risk as a cost of reproduction. TREE 6:183–186.

Naulleau, G. 1992. Reproduction de la couleuvre d'Esculape *Elaphe longissima* Laurenti (Reptilia, Colubridae) dans le centre ouest de la France. Bull. Soc. Herp. France 62:9–17.

———, and X. Bonnet. 1996. Body condition threshold for breeding in a viviparous snake. Oecologia 107:301–306.

Olsson, M., T. Madsen, and R. Shine. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. Proc. R. Soc. Ser. B 264:455–459.

Poldmaa, T., R. Montgomerie, and P. Boag. 1995. Mating system of the cooperatively breeding noisy miner *Manorina melanocephala*, as revealed by DNA profiling. Behav. Ecol. Sociobiol. 37:137–143.

Saint Girons, H. 1949. Les moeurs nuptiales de la Vipère aspic. Terre Vie 96:110–113.

———. 1952. Ecologie et éthologie des vipères de France. Ann. Sc. Nat., Zool. 14:263–343.

———. 1957. Le cycle sexuel chez *Vipera aspis* (L.) dans l'ouest de la France. Bull. Biologique. 91:284–350.

———. 1996. Structure et évolution d'une petite population de Vipera aspis (L.) dans une région de Bocage de l'ouest de la France. Terre Vie 51:223–241.

———, and R. Duguy. 1982. Données histologiques sur les variations de l'hypophyse au cours du cycle annuel chez *Vipera aspis*. Zeit. Zellforsch 56:819–856.

Schuett, G. W. 1992. Is long-term sperm storage an important component of the reproductive biology of temperate pitvipers? *In* J. A. Campbell and E. D. Brodie, Jr. (eds.), Biology of the Pitvipers, pp. 169–184. Selva, Texas.

———, BUTTENHOFF, P. A., AND DUVALL, D. 1993. Corroborative evidence for the lack of spring-mating in certain populations of prairie rattlesnakes, *Crotalus viridis*. Herpetol. Natur. Hist. 1:101–106.

SLIP, D. J., AND R. SHINE. 1988. The reproductive biology and mating system of diamond pythons, *Morelia spilota* (Serpentes, Boidae). Herpetologica 44:396–404.

STILLE, B., MADSEN, T., AND NIKLASSON, M. 1986. Multiple paternity in the adder, *Vipera berus*. Oikos 47: 173–175.

SYMONS, D. 1979. The Evolution of Human Sexuality. Oxford Univ. Press, Oxford.

WARD, K. E., AND P. J. LANDOLT. 1995. Influence of multiple matings on fecundity and longevity of female cabbage looper moths (Lepidoptera: Noctuidae). Annals Entomol. Soc. Am. 88:768–772.

WILLIAMS, G. C. 1975. Sex and Evolution. Princeton Univ. Press, Princeton, New Jersey.

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Seasonal Comparison of Hemoglobins in Three Species of Turtles

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Freshwater turtles are renowned for their capacity to tolerate prolonged periods of apnea while submerged. This ability stems from three physiological traits; metabolic depression coupled with (1) high tolerance for anaerobic respiration and/or (2) non-pulmonary O_2 uptake (Ultsch, 1989). Both terrestrial and aquatic turtles have some capacity for extra-pulmonary O_2 uptake (King, 1995). The relative importance of extra-pulmonary uptake, however, differs interspecifically. It is considered more important for turtles during winter than summer, and some species have been reported to maintain aerobic metabolism during simulated underwater hibernation (Ultsch, 1989; Ultsch and Jackson, 1995).

Some turtle species have remarkable morphological adaptations that enhance their extra-pulmonary uptake in water. These include the use of the buccopharyngeal cavity, cloacal bursae, and skin (King and Heatwole, 1994). Absorption of oxygen from water poses problems not encountered in air where oxygen is 20–40 times more plentiful, depending on temperature and salinity (J. Graham, 1990). This shift in oxygen availability in different media raises the question of whether there are corresponding alterations in the hemoglobins that transfer oxygen to body tissues.

Most ectothermic vertebrates, including turtles, possess multiple hemoglobins (Ingermann, 1992). Adaptations of hemoglobin in response to changing environmental conditions have been identified in fishes (Hattingh, 1976; Tun and Houston, 1986) but less is known about such changes in turtles.

The present study examines seasonal variation in the hemoglobins of three species of turtles with lifestyles ranging from terrestrial to highly aquatic. The musk turtle, *Sternotherus odoratus* (Kinosternidae), is a highly aquatic species (Ernst, 1986) that rarely comes out of the water and relies on extra-pulmonary O_2 uptake to maintain aerobic respiration during hibernation (Ultsch, 1988). The eastern box turtle, *Terrapene carolina carolina*, is a terrestrial species in the family Emydidae, the other genera of which are aquatic (Carr, 1952). The spotted turtle, *Clemmys guttata* (Emydidae), is intermediate between *S. odoratus* and *T. c. carolina* in its degree of aquatic habitat use. It has annual periods of terrestrial migration and terrestrial estivation and hibernates underwater (T. Graham, 1995). The degree of aquatic habitat use of these turtles correlates with the relative importance of extra-pulmonary O_2 uptake (King, 1995).

Three adults of each species were collected in spring and summer, 1994; *S. odoratus* and *T. c. carolina* from Wake County, North Carolina, and *C. guttata* from Pamlico County, North Carolina. They were housed in outdoor compounds with free access to both water and dry land throughout the summer. The turtles were allowed to hibernate in winter. Hibernating turtles were removed from outdoor hibernacula in late January 1995 and taken to the laboratory to obtain winter samples of blood. In the spring and summer of 1995, five adults of each species were collected from the same areas and kept for a short period as described above for summer blood-sampling.

Blood samples (approx. 2 ml) were taken by heparinized syringe from a blood sinus dorsal to the 7th cervical vertebra. Samples were immediately transferred to sterilized Vacutainer™ tubes containing 0.048 ml 7.5% EDTA and chilled with ice before separation of erythrocytes from the plasma.

At the time of blood collection, part of each sample was transferred to a capillary tube and hematocrits were determined. Differences between seasons for each species were tested using Student's t-test and differences among species were tested using ANOVA and Fisher's protected least square difference (PLSD).

Erythrocytes were separated, washed, and lysed. Hemolysate was treated with aprotinin (24 µl/ml) to prevent protein breakdown, 2-mercaptoethanol (1.8% v/v) to prevent polymerization, and 0.1M KCN (5% v/v) and 0.1M $K_3Fe(CN)_6$ (5% v/v) to convert hemoglobins to cyanmethemoglobin (Braman et al., 1977; Maginniss et al., 1980; Sullivan and Riggs, 1967). Hemolysate was either stored at 4°C for up to 24 h until isoelectric focusing was carried out, or stored at −70°C.

Hemolysate was subjected to isoelectric focusing (IEF) using Phastsystem™ electrophoresis equipment and Phastgel™ IEF 5–8 media. Proteins were stained with coomassie blue and with benzidine/peroxidase (200 mg tetramethylbenzidine, 15 ml glacial acetic acid, in 100 ml deionized water; 0.2% v/v peroxidase was added immediately before staining) (adapted from Gordon, 1969).

Proteins stained with coomassie blue were scanned using an Apparatus Corp. EC-910 densitometer. The relative abundance of each hemoglobin was calculated