

Disappearance of eggs during gestation in a viviparous snake (*Vipera aspis*) detected using non-invasive techniques

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Submitted on 2008, 19th May; revised on 2008, 22nd August; accepted on 2008, 26th August.

Abstract. The number of eggs released at ovulation may be greater than the number of offspring born, if some of these ovulated eggs and/or embryos disappear during gestation. Although this process can potentially exert significant effects on reproductive output, logistical problems have discouraged studies on the disappearance of eggs and embryos in most kinds of vertebrates. Nuclear Magnetic Resonance (NMR) imaging and ultrasound Doppler-imaging have not been applied previously to such questions. Using these techniques, we monitored changes in the female's oviduct through gestation in a viviparous snake. We documented a case of disappearance of two ovulated eggs (from a litter of four) in the aspic viper, *Vipera aspis*. The female ovulated four normal-sized eggs, two of which contained living embryos when examined by NMR and ultrasound Doppler-imaging early in gestation. Subsequent NMR imaging midway through gestation showed the same situation, but a third imaging session immediately prior to parturition revealed that the oviducts contained only the two live embryos. The two nonviable eggs had disappeared. The female gave birth to the two live offspring, with no evidence of any additional material. These data thus offer the strongest evidence so far available for egg disappearance (resorption?) during gestation in reptiles. More generally, NMR imaging offers a valuable tool for investigating processes inside the body cavity, where direct observation is otherwise difficult or impossible. The technique does not require sacrifice of the animals, and hence allows dynamic investigations over time.

Keywords. Ultrasonography, gestation, Nuclear Magnetic Resonance imaging, reproduction, reproductive output, snake, ultrasound, *Vipera*.

INTRODUCTION

Life-history theory suggests that the organisms should divide their available resources between maintenance, growth, storage and reproduction in a way that maximizes total

lifetime reproductive output (e.g., Stearns, 1989; Roff, 1992). Expenditure of resources on reproduction takes many forms in males, but in females of most animal taxa the primary form of maternal investment involves the production of eggs. Thus the control of egg production (i.e., the number and size of those eggs) is one of the most immediate targets of natural selection. Broadly, such control involves two major physiological mechanisms acting in opposition to each other: recruitment *versus* regression (follicular atresia, resorption, etc.). A large theoretical, empirical and experimental literature has accumulated on the recruitment of follicles and the maintenance of developing offspring (Lack, 1947, 1954; Jones, 1978; Skinner, 1985; Duellman and Trueb, 1986; Godfray et al., 1991; Sinervo and Licht, 1991a, b; Thibault and Levasseur, 1991; Jorgensen, 1992; Morris, 1992), but egg resorption has received less attention (Rosenheim et al., 2000). This dearth of attention partially reflects logistical problems: it is difficult to study phenomena that are hidden within the female's body. Nonetheless, follicular atresia, resorption of ovulated eggs and abortion of embryos have been documented in a wide variety of taxa (Edwards, 1954; Bell and Bohm, 1975; Byskov, 1978; Wilson, 1985; Duellman and Trueb, 1986; Gosling, 1986; Fox and Guillette, 1987; Thibault and Levasseur, 1991), and have been interpreted through both adaptive and non-adaptive explanations. For example, selective resorption of embryos enables females to manipulate sex ratio in mammals (e.g., Gosling, 1986; Forbes, 1997), and factors such as stress, poor body condition or diseases can induce females to terminate their reproductive effort (Boué and Boué, 1973; Dollander and Fenart, 1979; Hattel et al., 1998). Some species show highly specialized adaptations to reduce egg numbers subsequent to ovulation, including cases of intrauterine cannibalism (oophagy or adelphophagy) in sharks and amphibians (Springer, 1948; Vilter and Vilter, 1960; Hourdry and Beaumont, 1985). These phenomena are somewhat functionally equivalent to direct resorption, in that they notably (but not exclusively) provide mechanisms by which females can reduce their overall litter size.

Although both the ultimate selective advantages and the proximate mechanisms underlying post-ovulatory reduction in litter size are undoubtedly complex, technical difficulties preclude studies of this topic in many wild animal species. In most cases, the existence of intrauterine resorption has been inferred from autopsy. For this reason, the most extensive data sets on resorption have come from species that are killed in very large numbers for other reasons (e.g., coypu *Myocastor coypus*: Gosling, 1986). Such killing clearly cannot be justified on either ethical or ecological grounds for most species of wild animals. Furthermore, methods based on autopsy of sacrificed individuals do not allow the investigator to monitor resorption processes dynamically, by following individual females through the gestation process (Weintraub et al., 2004). In this paper we present an alternative, non-invasive, method that we have used with snakes, and that could potentially be applied to a wide range of animal species.

Although scientific interest in reptilian reproduction has increased dramatically in recent decades, a surprisingly high number of basic questions remain to be answered. One such topic is the question of embryonic resorption (Blackburn, 1998a). Despite frequent anecdotal reports, and occasional claims in the scientific literature, there has been no reliable documentation of ovulated eggs or embryo resorption in any reptile species. Intuition and logic suggest that reproducing females might often benefit by resorbing nonviable eggs or embryos. Not only may resources be recovered in this way, but females may be

able to remove dead embryos that would otherwise block the oviduct (Blackburn, 1998a, b). Such blockages can have fatal consequences for both the female and the more anteriorly-positioned offspring (pers. obs.). The ability to resorb embryos is well-documented in viviparous mammals (e.g., Westlin et al., 1995), but anatomical differences between the oviducts of reptiles and mammals may preclude resorption in the former group (Blackburn, 1998a, b).

Obviously, much of the difficulty in evaluating reports of egg/embryo resorption in reptiles (as in other groups) involves the fact that the process (if it occurs) is hidden inside the female's oviducts. Hence, most of the kinds of evidence that have been used to infer resorption are indirect, and open to other interpretations. For example, the production of smaller-than-average nonviable neonates at parturition does not necessarily mean that these offspring were normal-sized at ovulation. Ideally, we need to quantify numbers and sizes of "eggs" at the beginning of embryogenesis (i.e., immediately post-ovulation), and then monitor these variables through the period of gestation. Imaging techniques developed for medical uses are well-suited to this purpose, and in this paper we describe the application of two such techniques (Nuclear Magnetic Resonance imaging and Doppler ultrasounds) to visualizing ovulated (intra-uterine) eggs and developing embryos in a viviparous snake. This technique provides the first direct evidence for the disappearance of ovulated eggs during gestation in any squamate reptile (although we cannot claim any certitude about the mechanisms involved; resorption *versus* leakage from the oviduct *versus* expulsion into the peritoneal cavity).

MATERIALS AND METHODS

We used two techniques to examine intra-uterine embryos in gravid snakes. The first of these (Nuclear Magnetic Resonance, or NMR) relies upon the magnetic properties of atomic nuclei when they are exposed to a strong magnetic field. NMR imaging is a non-invasive technique that does not harm the subject, and hence the same animal can be examined on several occasions (Lauterbur, 1973). NMR image acquisition was carried out using a Biospec BMT 24/40 spectro-imager (Bruker) operating at 2.35 Tesla and using a 20 cm Alderman-Grant resonator. Animals were not anaesthetized, but were cooled to 15 °C. The snake was gently restrained using a purpose-built device consisting of a hollow foam-plastic tube cut lengthways and positioned on a Plexiglas rule; the animal was held in position by velcro strips. The time required to obtain an image was 2 min 34 s. For large snakes, we then moved the animal to another position and repeated the procedure (2 to 4 times depending upon SVL), to ensure that the imaging procedure covered the entire abdomen. The second technique (ultrasonography) is based upon detection of movement by red cells in the circulatory system. We performed these tests using a linear array transducer probe with electronic focusing, at a frequency of 10 MHz and connected to a color-coded Doppler echograph (ESAOTE AU4). With this equipment, it was possible not only to visualize areas of blood flow, but also to record the velocity of that flow pattern using the Doppler technique.

Data in this paper were obtained from three successive imaging sessions with a gravid female asp viper, *Vipera aspis*. The snake was collected in late April from a large wild population (Château d'Olonne, Vendée) in western central France. She measured 54.5 cm snout-vent length (62 cm total length), and weighed 113.8 g at the time she was first examined. She was maintained in captivity throughout vitellogenesis and gestation, first in an outdoor enclosure (6×3 m) during vitellogenesis and the beginning of gestation (April to late July), and then (just before the second imaging ses-

sion, until the third imaging session and parturition) in a small cage (40×40×40 cm) with the floor covered with a plastic sheet. Water was provided *ad libitum* but the female did not accept any food (mice killed by the snake by provoking the bite to induce feeding behavior), as often observed in pregnant individuals. Parturition occurred on 21st August. The cage was regularly inspected (1 to 5 times a day), and we saw no evidence of any expelled ova or other materials (e.g., yolk, membranes, blood, etc. that are very often associated with the expulsion – either of living neonates, stillborn and/or unfertilized eggs) on the floor of the cage at any stage throughout the study. Other reproductive females from the same population (n = 10) have been examined during the same sessions, but the disappearance of eggs was detected in only one. Our sample size is thus very small, and we have no precise idea about the occurrence egg disappearance in this species. However, the fact that we observed the disappearance during pregnancy of ovulated items shows that this remarkable phenomenon can indeed occur in snakes. Furthermore, the main aim of this paper is precisely to present techniques that may be useful to study and quantify such phenomenon.

RESULTS

Palpation on 14th May 1996 enabled us to detect five developing follicles. The first imaging session (19th June) took place soon after ovulation, which occurs in the first two weeks of June in western central France (Naulleau and Bidaut, 1981). NMR revealed the presence of four spherical objects in the oviduct (Fig. 1a). These four objects were very similar in size (length 44 to 47 mm; width 16 to 18 mm). Their size and location made it obvious that these were oviductal “eggs” (although the oviduct[s] that contained the eggs was not identified). This inference was confirmed by direct visual inspection of the embryos using NMR; as expected, the embryos (including embryonic membranes, etc.) were very small at this early stage of development (9 to 12 mm, within the size range we have observed in autopsied gravid females collected in mid June). Only two of the four “eggs” contained discernible embryos. Doppler imaging confirmed the presence of viable embryos in these two “eggs”, and the absence of any heartbeat in the other two “eggs”. Heartbeats were clearly discernible in the two living embryos, despite their small size. Comparisons with maternal heartbeat rates recorded either in the oviductal blood vessels or in the heart showed that the signals from the embryos were not artifacts of signals from the maternal heart; the hearts of the embryos were beating faster than the heart of their mother (75 beats m^{-1} for the embryos versus 45 beats m^{-1} for the mother). The cranial-caudal sequence of the four live and dead eggs was as follows: 1 dead egg – 2 living eggs – 1 dead egg.

The second imaging session took place 22 days later (10th July), midway through gestation. By this time the two living embryos had increased appreciably in size (19 mm), but no details of their internal anatomy could be clearly resolved (Fig. 1b). The two non-viable eggs had decreased only slightly in size (35 to 47 mm in length and 13 to 14 mm width).

The third and last imaging session occurred 41 days later, on 21st August, close to the time that we expected parturition. NMR revealed two healthy offspring fully developed, but no other objects within the mother's oviducts (Fig. 1c). Note that the resolution of imaging was 2 mm, and that even shriveled eggs would have been detected. Possibly due to handling stress, the female gave birth immediately after the conclusion of the NMR session. She produced two healthy neonates, of normal size for this study population (two

males 7.2 g, 19 cm snout-vent length and 6.2 g, 19 cm), with no evidence of any other materials from the abortive “eggs”. That is, the volume of fluids and membranes expelled with the two live offspring was typical of that usually associated with parturition in this species (pers. obs.). There was no additional yolk or degenerating tissue. Palpation confirmed that the oviducts contained no other materials. Note that we directly witnessed the birth of the two neonates, and that all material (living snakes and embryonic membranes, etc.) were carefully collected and examined.

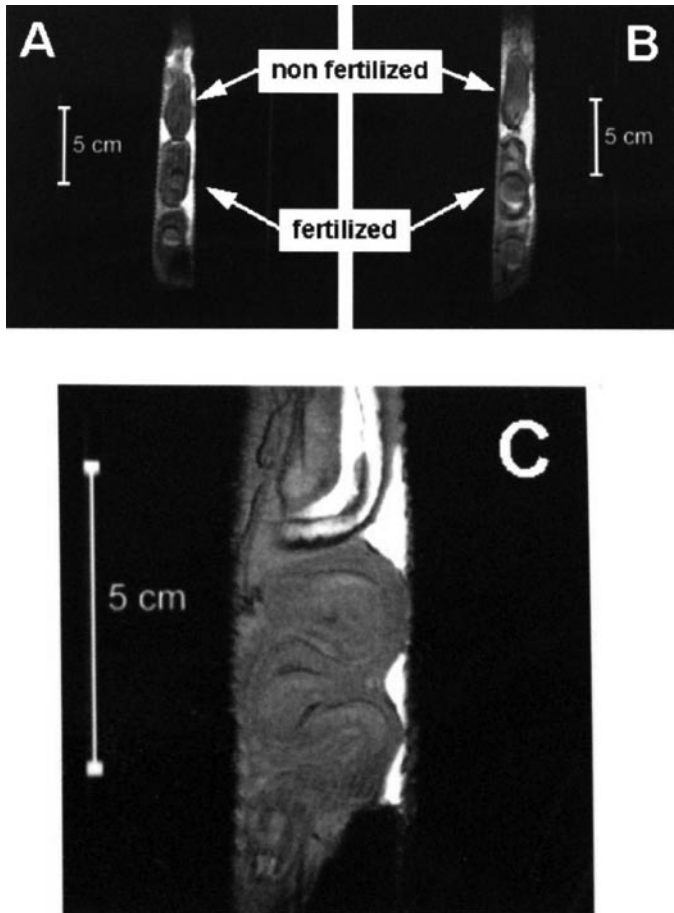


Fig. 1. (a) Gravid female asp viper soon after ovulation (first imaging session). The head of the gravid snake is to the top; only a portion of the abdomen is shown. NMR imaging shows that the oviduct contains four “eggs”, although only three are visible on this slice. Only two of these “eggs” contain visible embryos (circular structures within egg # 2 and # 3). (b) The same snake as in Figure 1a, 22 days later and one-third of the way through gestation (second imaging session). The two circular structures (containing viable embryos) have increased in size. (c) The same snake as in Figs. 1a and 1b, close to parturition (third imaging session). The two embryos in (c) are the two seen in the lower part of (a) and (b); the white area in the top right of the photograph is fat-body material in the upper embryo, and the body of the lower embryo is coiled.

DISCUSSION

Our observations clearly indicate that the female viper reduced her litter size at two stages during the reproductive cycle. First, she enlarged five follicles, but ovulated only four of them. This phenomenon (follicular atresia) has been frequently reported in other reptile (see Blackburn, 1998a, b; Blackburn et al., 1998) and vertebrate species as well (Thibault and Levasseur, 1991). Second, and more important, two of her four normalized ova failed to develop. These unviable ova somehow disappeared from the oviduct during gestation. Follicular atresia must be clearly distinguished from the disappearance of ovulated eggs, because the physiological mechanisms concerned are very different. We do not know if the two eggs that disappeared were unfertilized, or were fertilized and died very early in embryogenesis (e.g., during the first days after ovulation). Regardless, the two nonviable “eggs” disappeared from the oviduct some time after the first third of the gestation period (i.e., between our second and third imaging sessions).

Are there alternative interpretations of our data other than resorption of these eggs? Blackburn (1998a) has reviewed the kinds of evidence heretofore available on this topic, and enumerated alternative interpretations for all of them. Our data, although preliminary, are more direct and compelling than the kinds of indirect observations previously available. Two of the four ova that were ovulated disappeared prior to parturition. The only way that this could happen would be for the embryos to be resorbed via the oviductal wall, or expelled via the cloaca. Although expulsion of nonviable embryos and eggs is common in squamates (Blackburn, 1998a), we saw no sign of any such material in the female’s cage. More convincingly, one of the nonviable ova was the most anteriorly-placed in the oviduct (Fig. 1). In order for this “egg” to be expelled, it would have had to move down past at least one other “egg”. This seems unlikely (given the intimate apposition of maternal and fetal membranes during development: Stewart and Blackburn, 1988), but is not impossible because extraembryonic membranes do not fully adhere to the uterine lining in viviparous snakes (Blackburn, 1998b). Nothing remained in the female’s oviducts after parturition; extensive experience with this species indicates that palpation is effective in locating even tiny objects (< 2 g) within the digestive or reproductive tracts.

Although resorption offers a plausible explanation for our observations, we certainly do not claim certainty in this interpretation. In fact, the main limitation of our study is that the alternative mechanisms that may explain the disappearance of the eggs from oviducts cannot be teased apart. Although females of other reptile species have been recorded to consume expelled unfertilized eggs (e.g., even in snakes such as in the *Epicrates* or *Agkistrodon* genus; Lourdais et al., 2005), we have never recorded this kind of behavior in very extensive observations of female aspik vipers over several years (direct observation and monitoring of female’s behaviors for several hundreds of parturition; Bonnet et al., 2001, 2002). It remains also fully possible that the nonviable eggs degraded slowly over the latter part of gestation, with gradual expulsion of very small amounts of tissue or fluids through the cloaca over the 41 days elapsed between the second and the third session. Indeed, such amounts may have been so small as to escape our attention, or accumulation in the oviduct, or even expulsion into the peritoneal cavity (Blackburn, 1998a and references therein). A last possibility involves absorption by adjacent normal eggs (Blackburn, 1998a), although this is unlikely because the two viable eggs did not increase in size during gestation (that is, they did not incorporate additional yolk).

Overall, the most likely alternative explanations for our observations appear to be as follows: 1) leakage from the oviduct, expulsion into the peritoneal cavity, or 2) resorption by the oviducts (in the sense of Blackburn, 1998a). We cannot settle this question with the available data, because the female was not dissected after parturition to confirm the complete absence of embryonic tissue, or to examine anatomical details of the putative “resorption” areas. Hence, further investigations are still needed, such as microscopic examination of the uterine cells and tissues likely to accomplish the digestion and absorption. Such studies could be conducted on animals killed accidentally or for other purposes, or using sophisticated current techniques (Weintraub et al., 2004).

Despite these caveats, our observations have strong implications for the study of reproduction in reptiles. For example, our data indicate that estimates of reproductive output made at different stages of gestation may yield different results. Many authors have based estimates of clutch and litter sizes on the numbers of enlarging (vitellogenic) ovarian follicles, or the numbers of recently-ovulated “eggs” (Gregory et al., 1992). If egg/embryo disappearance is common, these figures will over-estimate actual fecundity levels. In the present example, the difference in litter size would be 100% (2 vs. 4 offspring). More generally, our data support the hypothesis that reproducing female reptiles may be able to manipulate their level of reproductive expenditure before, and even after ovulation (Blackburn, 1998a). In circumstances in which the “optimal” life-history tactic involves a litter size smaller than the one she currently has, a female may be able to adjust litter size by selective “resorption”.

The main result of our study is to encourage the use of the techniques described in this paper. A similar approach, using serial non-invasive magnetic resonance microscopy (but not Doppler-ultrasonography) has been successfully employed to study embryo resorption in laboratory mice (Weintraub et al., 2004). Not only do they offer powerful new opportunities to clarify the dynamics of intrauterine processes, they may also help to identify the cues that stimulate processes such as litter-size reduction. More generally, these methods allow a more direct and reliable comparison between litter sizes at ovulation and at parturition, that sometimes significantly differ in natural conditions as detected on large sample size (Bonnet et al., 2001). The techniques may also allow accurate estimation of reproductive condition under circumstances in which existing techniques such as abdominal palpation are unreliable. This may apply, for example, relatively early in gestation (pers. obs.), in large heavy-bodied species in which palpation is ineffective (e.g., NMR revealed > 30 eggs in a large gaboon viper, when palpation revealed none), or in species whose thick abdominal musculature interferes with palpation (e.g., some python species: pers. obs.). Imaging techniques also provide accurate data on the timing of events such as ovulation and various stages of embryogenesis, information that may otherwise be difficult to obtain by non-destructive techniques.

ACKNOWLEDGEMENTS

Financial support was provided by the Conseil Général des Deux Sèvres, Centre National de la Recherche Scientifique, the Faculté de Médecine de Tours (France) and the Australian Research Council. We thank Rex Cambag and J.T. Wilmslow for participation during the testing of Biospec and the Doppler apparatus.

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