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Sperm Storage in the Oviduct of the American Alligator

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ABSTRACT Oviducts of the American alligator (*Alligator mississippiensis*) were examined histologically for the presence of stored sperm. Two regions containing sperm were identified, one at the junction of the posterior uterus and the vagina (UVJ) and the other at the junction of the tube and isthmus (TIJ). In these areas, sperm were found in the lumina of oviductal glands. The glands in these areas of the oviduct are diffuse and shallow and appear to allow better access to sperm than glands located elsewhere. Histochemically, the glands of the UVJ reacted weakly for carbohydrates and proteins, whereas those of the TIJ reacted strongly for these same two components, secretions of which are associated with sperm storage structures in other reptiles. Sperm were not in contact with the glandular epithelium, and glands at the UVJ contained more sperm than those at the TIJ. Oviductal sperm storage was observed not only in recently mated females but in all females possessing uterine eggs as well as all females known to be associated with a nest. We conclude that female alligators are capable of storing sperm in their oviductal glands, but not from one year to the next. *J. Exp. Zool. 309A:581–587, 2008.* © 2008 Wiley-Liss, Inc.

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The reproductive cycles of female American alligators from various populations have been examined including Louisiana (Lance, '89), Florida (Guillette et al., '97) and South Carolina (Wilkinson, '83). Reproduction is an annual event, with a single clutch of eggs produced in the summer; however, a variable percentage of females fail to become reproductively active in any given year. Mating occurs in late spring (c.a. May) followed by ovulation and oviposition some 3.0–3.5 weeks later (Joanen and McNease, '80). The size of the annual egg clutch is variable, generally ranging around 40 eggs.

Considerably less is known regarding the details of folliculogenesis, ovulation, fertilization, and the formation of egg investments. Vitellogenic growth of follicles commences the previous autumn in Florida populations and in the spring in more temperate locations (Guillette et al., '97). Follicles grow from 1 to 4 mm in diameter to a preovulatory size of 45 mm, resulting in a megalecithal egg. The trigger for ovulation remains unknown although it is speculated to be neuroendocrine in nature (Lance, '89). Alligators are believed to possess an autochronic form of ovulation (Smith et al., '72), in which mature follicles from both ovaries are ovulated to the oviduct within a short period of time. Whether eggs are released sequentially or simultaneously remains unknown. The alligator oviduct resembles that of the fowl (Johnson, '86) and possesses separate regions for the production of egg white proteins

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(magnum: alligator = tube) and egg shell investments (uterus). These are separated by a short, aglandular region, the isthmus (Palmer and Guillette, '92). Similar to the fowl, ova are believed to descend the tuba rapidly, gaining egg white proteins, and are held in the uterus for a much longer time, gaining shell investments, until oviposition (Jacob and Bakst, 2007).

In birds, freshly inseminated sperm enter specialized tubules located at the uterovaginal junction (UVJ) of the oviduct (see Birkhead and Moller, '92; Stepinska and Bakst, 2007). Sperm are released from the specialized tubules in a continual stream to fertilize recently ovulated eggs in the infundibulum (Bakst, '94). Sperm storage in oviductal glands is well known in the Squamata and Chelonia, although the location of the glands and their specialization for sperm storage is quite variable (Sever and Hamlett, 2002). Oviductal sperm storage has not been reported from the Rhynchocephalia (Gabe and Saint Girons, '64) or the Crocodylidae (Gist and Jones, '87; Sever and Hamlett, 2002). The apparent absence of sperm storing structures from the alligator's oviduct (Palmer and Guillette, '92) is surprising in that both mating behaviors (Garrick and Lang, '77) and genetic markers (Davis et al., 2001) suggest that multiple paternity exists in this species. Multiple paternity could result from either a mixing of sperm from different males within the oviduct and/or a storage of gametes from different males within the oviduct itself. Indirect evidence of sperm storage in crocodilians is provided by Davenport ('95). If female alligators do store sperm, one would expect sperm densities to be greatest at the time of ovulation. This study examines oviducts of periovulatory alligators and confirms the existence of sperm storage structures.

MATERIALS AND METHODS

Adult alligators examined in this study were collected from the Rockefeller Wildlife Refuge, Grand Chenier, Louisiana, a total of 13 animals were used. All animals were over 1.8 m in length. They were collected by Rockefeller staff biologists and humanely sacrificed. Paired oviducts were removed together with the ovaries and placed in 10% neutral buffered formalin; where possible, oviducts were perfused with fixative at the time of oviduct removal. After a minimum of 24 hr in neutral buffered formalin, portions of the oviducts were cleansed of adhering connective tissue, dehydrated in a graded series of ethanol and embedded in Paraplast. Sections (6 and 10 μm) were placed on albuminized slides, stained with Harris hematoxylin and eosin, and examined using an Olympus BX40 microscope. Digital micrographs were taken using a Spot camera (Diagnostic Instruments) and manipulated using Adobe Photoshop software. Histochemical analysis was performed using periodic acid-Schiff's (PAS) with an alcian blue counterstain for neutral carbohydrates and glycosaminoglycans, respectively. Bromphenol blue (BB) was used to stain proteins. The slides were viewed with a Leica DM2000 microscope and images acquired with a Leica DFC420 mounted camera.

RESULTS

The majority of alligators examined in this study were associated with nests at the time of capture and were estimated to be within 30–45 days of ovulation. The reproductive status of alligators was assessed at necropsy (Table 1). The three nonreproductive alligators examined were captured in February and April. Pre-ovulatory alligators were collected in May. Two general areas of sperm storage were observed histologically, one at the uterovaginal junction (UVJ) and the other at the junction of the tube and isthmus (TIJ). Except for recently mated alligators, sperm were not observed in other areas of the oviduct.

Uterovaginal junction

The vagina of the alligator oviduct is a short (c.a.1 cm) segment connecting the uterus to the cloaca. This segment is absent from the diagram of the alligator oviduct contained in Palmer and Guillette ('92). The vagina is surrounded by thick, smooth muscle layers, and the mucosa is thrown up into highly branched folds that generally run

TABLE 1. Criteria used to assess reproductive condition of alligators

N	Reproductive status	Assessment criteria
3	Pre-ovulatory	Large ovarian follicles, no oviductal eggs
3	Postovulatory, pre-oviposition	Eggs in utero, corpora lutea, nesting behavior
4	Postovulatory, postoviposition	Eggs in nest, nesting behavior, small ovarian follicles
3	Nonreproductive	Small ovarian follicles, regressed oviduct

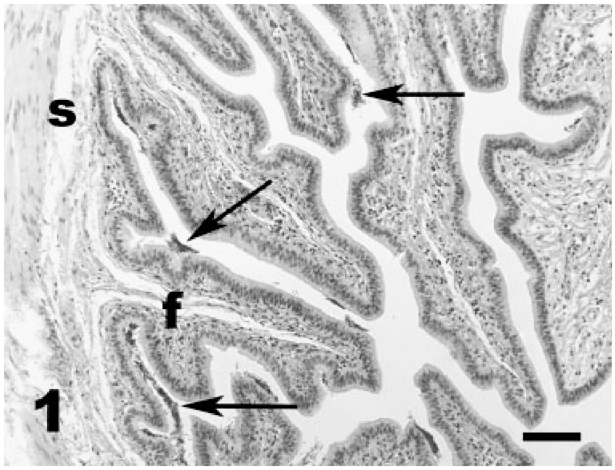

Fig. 1. Transverse section through a portion of the vagina of a recently mated alligator. Note longitudinal mucosal folds (f) and sperm aggregates (arrows) in the lumen. Bar = 100 µm; s, smooth muscle.

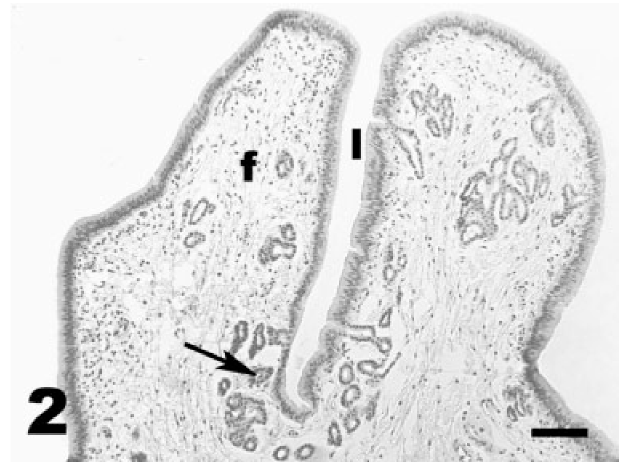

Fig. 2. Transverse section through a portion of the uterovaginal junction showing the beginning of the uterine glands. Note the prominent openings to the lumen and the gland containing sperm (arrow). Bar = 100 µm; f, mucosal fold; l, oviduct lumen.

longitudinally. The mucosa is devoid of glands, and the overlying simple epithelium consists of ciliated cells and mucous cells (Fig. 1). In recently mated alligators, aggregations of sperm are observed in between the folds. The transition from vagina to uterus (UVJ) is indicated by the appearance of compound tubular glands extending from the luminal epithelium into the connective tissue of the folds (Fig. 2). Here, the number of glands is small, and the openings of these glands to the oviduct lumen are prominent compared with the same glands located more anteriorly in the body of the posterior uterus (Fig. 3). The glands in this region stain slightly positive for both neutral carbohydrates (PAS+) and proteins (BB+). However, the luminal epithelium of the posterior uterus stains highly PAS positive and alcian blue positive at the apical portion of the cells.

The sperm storing region of the UVJ is a section of the oviduct rostral to the vagina approximately 1 cm in length. In this region, uterine glands begin to appear among the vaginal folds, particularly in the furrows in between the folds. Here, individual glandular systems are easily recognized and are separate from adjacent systems. When present, sperm are observed, singly or in aggregates, within the lumen of these glands (Figs. 1 and 4). Sperm entering the glands appear to do so arranged in a corkscrew manner, but once within the glands, their orientation becomes less ordered. Typically, they are found with their heads towards the terminus of the uterine glands (Fig. 5). The epithelium of the glands consists of a simple layer

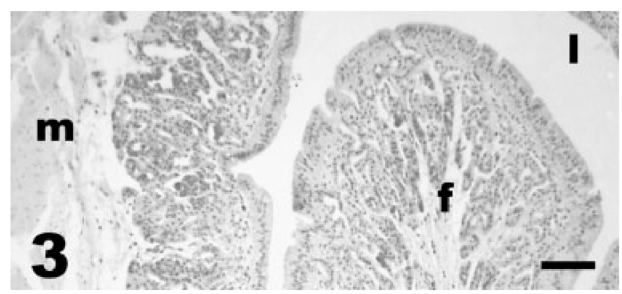

Fig. 3. Section through the posterior uterus showing the distribution of uterine glands and their openings to the oviduct lumen. Bar = 100 µm. f, mucosal fold; l, oviduct lumen; m, smooth muscle.

of cuboidal cells (Fig. 5); in some alligators these cells may contain a secretory material. Sperm do not appear to be in contact with the underlying glandular epithelium and are easily displaced during microtomy.

Tubal-isthmus junction

The tube of the oviduct lies rostral to the isthmus and contains glands that secrete the egg white proteins. These tubal glands occupy the mucosal folds, are compound tubular in structure, and communicate with the lumen via nonsecretory ducts that project deep into the mucosa (Fig. 6). Cells forming the tubal glands are generally columnar with basal nuclei, and the cytoplasm is filled with secretory granules. Histochemically the glands of the tube stain for high amounts of

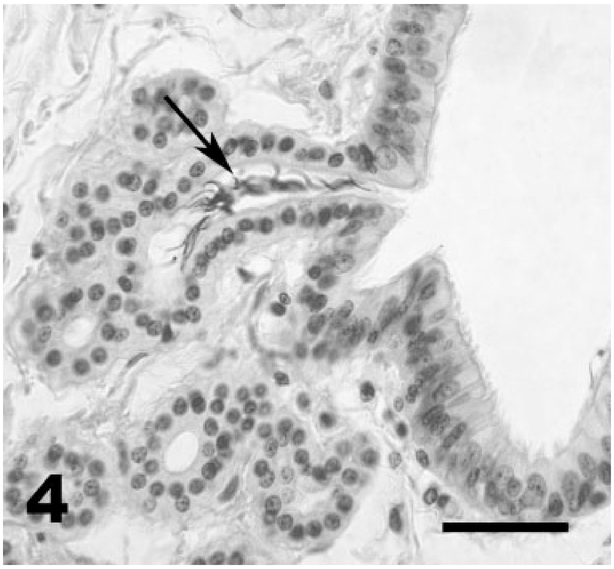

Fig. 4. Section through the uterovaginal junction of the oviduct of a recently mated alligator showing sperm (arrow) occupying the lumen of a uterine gland. Bar = 50 μm.

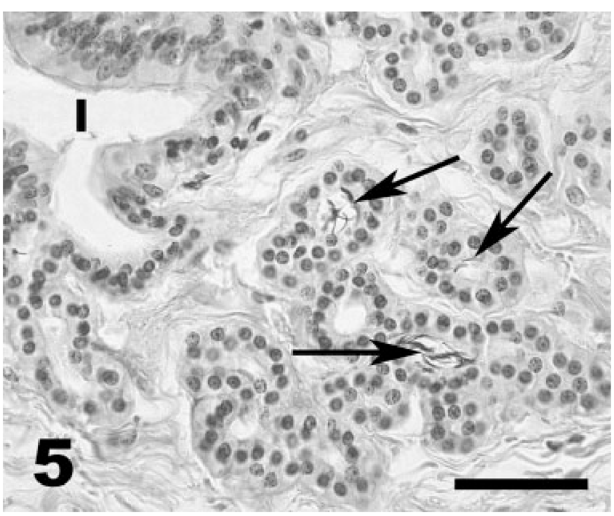

Fig. 5. Section of the uterovaginal junction of the oviduct of a recently mated alligator showing sperm residing in the tubules of uterine glands. Bar = 100 μm. l, oviduct lumen.

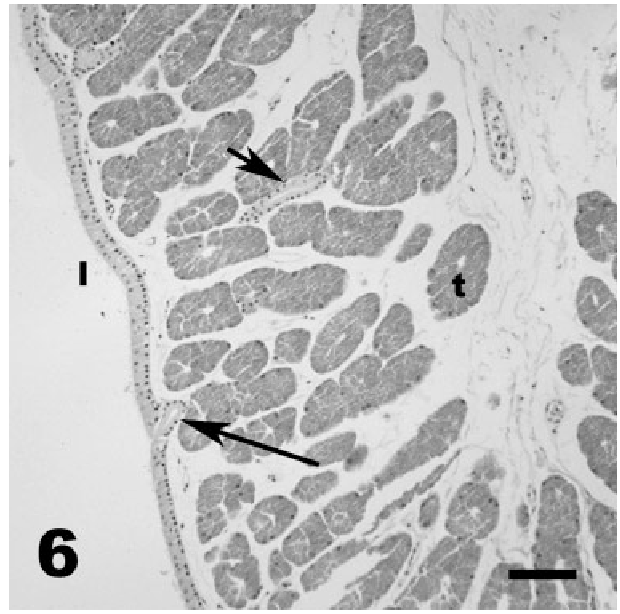

Fig. 6. Section through a portion of a mucosal fold in the tube of the alligator oviduct showing glandular organization. Note ducts within the mucosa (short arrow) and opening (long arrow) to oviduct lumen (l). t, secretory tubule. Bar = 50 μ.

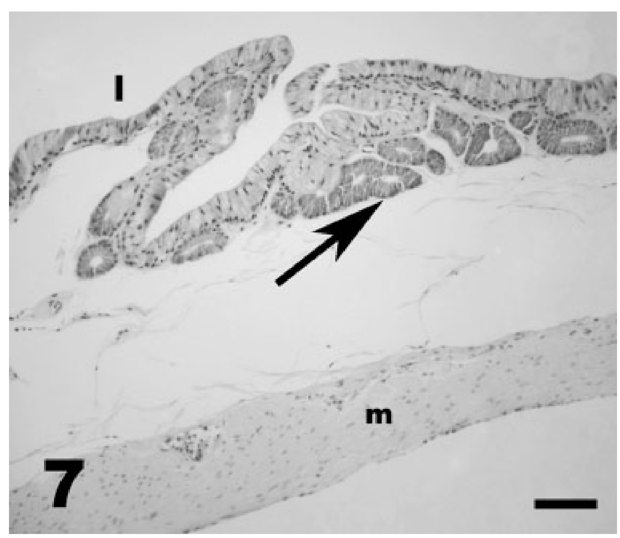

Fig. 7. Longitudinal section through the tubal-isthmus junction of the alligator oviduct showing the caudal tubal glands (arrow). Bar = 50 μm. l, oviduct lumen; m, smooth muscle layer.

neutral carbohydrates (PAS+) and proteins (BB+). The luminal epithelium of the tube does not secrete proteins but reacts highly for neutral carbohydrates. The sperm storage portion of the tube is approximately 1 cm in length and lies just rostral to the isthmus where the glands of the tube are beginning to form (Fig. 7). Here the tubal glands are small and poorly developed compared with those in the body of the tube. Ducts connecting the secretory tubules to the lumen in this area are short. Isolated sperm or small groups of spermatozoa are found in the lumen of tubal glands (Fig. 8). As in the UVJ, the spermatozoa stored in the tubal glands do not appear to be attached to the glandular epithelium.

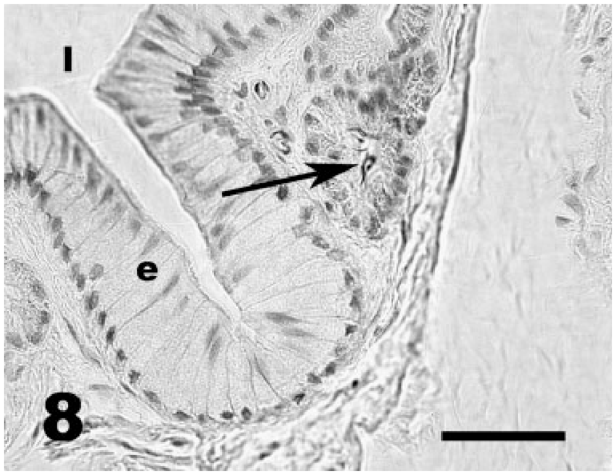

Fig. 8. Section through the tubal-isthmus junction of the alligator oviduct showing sperm (arrow) stored within the lumen of tubal glands. Bar = 50 μm. l, oviduct lumen; e, epithelium of tube.

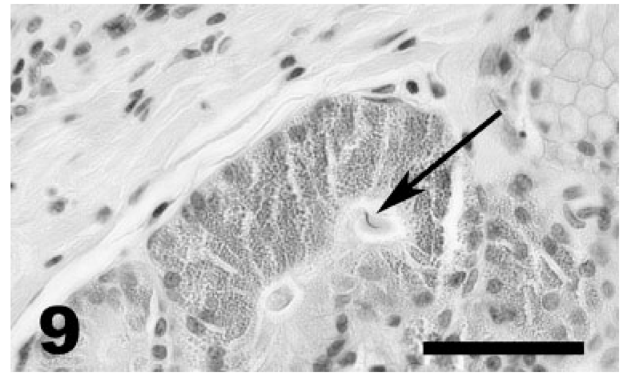

Fig. 9. Section through the tubal-isthmus junction of the alligator oviduct showing a single spermatozoon (arrow) within the lumen of a tubal gland. Bar = 50 μm.

Quantity and duration of sperm storage

Although sperm counts were not made, the greatest amounts of stored sperm were seen in the uterovaginal (UV) area. In recently mated alligators, large masses of sperm were observed in the lumen of the vagina (Fig. 1) and UVJ, outside the uterine glands, and in the ducts leading to them. Considerably fewer sperm were observed in these same areas in postovulatory animals. Glands at the TIJ, on the other hand, typically contained large whorls of sperm in the recently mated individual; however, in postovulatory animals less than 2–3 gametes were seen in any given section (Fig. 9). Oviductal sperm storage was observed not only in recently mated females but in all females possessing uterine eggs as well as all females known to be associated with a nest. In contrast, no sperm were observed in unmated pre-ovulatory females or in females captured outside the reproductive period (May, June). Thus, sperm appear to be stored for a minimum of 2 months and, as samples were not taken between June and the following February, possibly as long as 7 months.

DISCUSSION

Sperm storage in crocodilians was suggested by Davenport ('95), who reported that a captive female caiman laid 16 eggs, in one of which an embryo developed 488 days after isolation from a male, and by the findings of Davis et al. (2001), who demonstrated multiple paternity in annual egg clutches of alligators. However, the site(s) of potential storage remained unknown until this study. This is most likely owing to the short duration of sperm retention in the alligator and the small portion of the oviduct in which it occurs. On the basis of the absence of sperm from the oviduct during the nonreproductive periods, it is unlikely that alligator sperm are retained in the oviduct from one year to the next but are used to ensure paternal diversity in the egg clutch oviposited each year. The ovulatory pattern of the alligator provides an additional rationale for oviductal sperm storage. At ovulation, each ovary in the alligator sheds approximately 20 megalecithal eggs into the oviduct within a short period of time (Lance, '89). These eggs begin their descent down the oviduct in tandem, each distending the oviduct and receiving oviductal secretions (Palmer and Guillette, '92). Sperm residing in the lumen of the oviduct would be displaced by the descending ova, leaving greatly reduced quantities of sperm to fertilize the last of the ovulated eggs to descend the oviduct. By holding sperm in the lumina of oviductal glands, a continuing supply of sperm remains available to fertilize eggs at the end of a clutch. This mechanism for sperm conservation may be the ancestral reason for sperm storage. Vertebrate groups that use sperm storage extensively include the birds and reptiles, groups that possess megalecithal eggs.

The locations of the sperm storage sites in the alligator are the same as those found in the turtle (Gist and Jones, '89; Gist and Congdon, '98) at the caudal terminations of the large glandular areas of the oviduct. In these locations, the gland systems are diffuse and the openings to the lumen

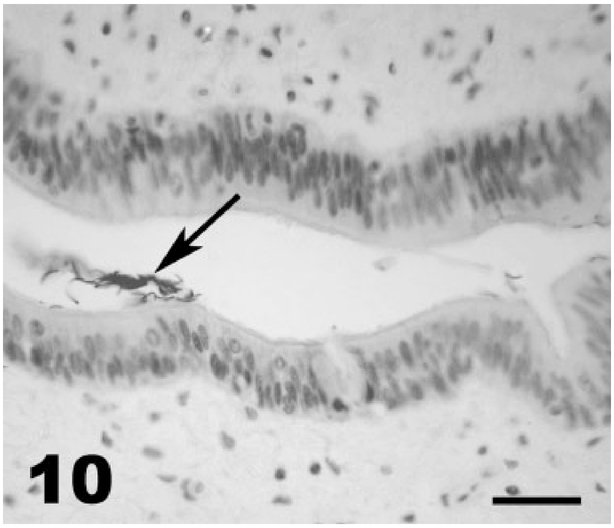

Fig. 10. Micrograph of a portion of the vagina of a postovulatory alligator showing sperm masses (arrow) in the lumen. Bar = 100 μm.

prominent compared with the body of the glandular regions. In micrographs of freshly inseminated animals (Fig. 2), sperm appear to be attracted to or move directly into the glands and reside there within the lumina of the glands with no intimate contact with the underlying cells as in the turtle (Fischer and Gist, '93), suggesting that the glandular epithelium plays a minimal role in sperm support and maintenance. The association of neutral carbohydrate and protein secretions with sperm storage structures has also been seen in other reptiles (Siegel, personal communication; Sever and Ryan, '99). The protein secretions that occur in the glands of the TI region are mainly egg white secretions but may serve as a medium in which sperm can be maintained. However, the small amount of neutral carbohydrates and proteins in the UV glands suggests that the secretions may not be a main source of nutrition for the stored sperm. It is possible that sperm longevity in the female may be a property of the male gamete. Turtle spermatozoa are remarkably hardy compared with those of other vertebrates (Gist et al., 2000), and the observation of (presumably residual) sperm masses in the vagina of postovulatory females suggests that the same may hold true for alligators (Fig. 10). Further studies are being conducted to look at the ultrastructure of the sperm storage glands and whether sperm degradation is occurring in the latter weeks of storage.

Freshly inseminated sperm undergo a massive selection as they move up the female reproductive tract (Drobnis and Overstreet, '92). Much of this selection occurs at the level of the vagina, but additional selection and capacitation can occur at the level of the fallopian tube and above. The observation that the TI glands contain fewer sperm than the UVJ glands in both pre-ovulatory and postovulatory females was not unanticipated and suggests that sperm selection is occurring in the alligator oviduct as well. Whether occupancy of both the UVJ and TI storage areas is a requirement for fertilization is possible but cannot be confirmed by this study.

How stored sperm fertilize eggs is not known. In birds, sperm residing in the UV sperm storage tubules leave them in a steady stream and move towards the infundibular site of fertilization (Bakst, '94). In the alligator, distances are considerably greater. In this study, the TI sperm storage glands were 100–125 cm away from the infundibulum, and the UV storage glands 200–225 cm; thus, sperm movement and/or transport would have to be rapid in order to deliver sperm to the infundibulum to achieve fertilization for all the eggs in a clutch. Given these distances, one has to raise the possibility that fertilization might occur at a site other than the infundibulum.

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