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**Functional long-term storage of spermatozoa in oviducts of the
common house gecko, *Hemidactylus frenatus***

Murphy-Walker, Susan Gene, Ph.D.

University of Hawaii, 1994

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FUNCTIONAL LONG-TERM STORAGE OF SPERMATOZOA IN
OVIDUCTS OF THE COMMON HOUSE GECKO,
HEMIDACTYLUS FRENATUS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

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ABSTRACT

The functional life span of mature gametes is generally quite short and, as a result, mating occurs at or near the time of ovulation in order to maximize fertilization potential. Prolonged sperm survival (and delayed fertilization) within the oviduct has been reported for almost every vertebrate class, being particularly common among reptiles. Most reports involve temperate species. Fall matings are followed by an overwintering period during which females do not ovulate. Oviductal sperm storage, therefore, promotes reproductive success by allowing fertilization to occur in the spring when females resume ovulation. Oviductal sperm storage in tropical reptiles is less commonly reported, perhaps because year-round reproduction is generally observed. This dissertation verifies oviductal sperm storage in the tropical gekkonid lizard, *Hemidactylus frenatus*, and proposes several advantages associated with this capacity.

Hemidactylus frenatus produces clutches consisting of a maximum of two calcareous-shelled eggs at variable intervals throughout the year. Histology revealed the presence of sperm sequestered within crypts located in a particular region of the oviduct. This was observed in virtually every adult female examined, regardless of her ovarian condition. Isolated from males, captive females each produced several viable clutches for up to nine months, demonstrating that sequestered sperm remained viable over several ovarian

cycles. Scanning electron microscopy revealed that distension of the oviduct released sperm from storage crypts as a result of egg passage through the oviduct following ovulation. Transmission electron microscopy revealed cyclical secretory activity within the oviduct which correlated with ovulation, and may prove to be beneficial to sperm, either while in storage or after release.

That oviductal sperm storage exists in *Hemidactylus frenatus* is certain. Although the mechanism is yet unclear, there appear to be both mechanical and physiological contributing factors. The advantages of oviductal sperm storage to this tropical reptile may derive from reproductive (small, irregularly produced clutches), behavioral (temporal separation of males and females), and distributional characteristics (dispersal events) of the species; advantages which provide almost guaranteed reproductive success.

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INTRODUCTION

Many vertebrates exhibit synchronized reproductive cycles such that male and female gametes mature during the same time of the year or season. The functional life span of mature gametes, however, is often quite short, and within hours or days, the capacity for fertilization may be lost. This rarely presents a problem as mating generally occurs at or around the time of ovulation with fertilization following shortly thereafter. Almost every vertebrate class, however, contains examples of reported delayed fertilization via prolonged sperm survival within the oviduct (see review by Howarth, 1974).

Sperm are energetically inexpensive to produce; eggs are not. Because egg production requires a great deal of energy utilization, females must generally reach a particular body size or mass before egg production can begin. In addition, energy consumption must be at a level that maintains body mass in order to produce fully mature eggs. Sperm storage within the female reproductive tract may have evolved as a means of ensuring fertilization during egg production intervals.

External fertilization occurs mainly in aquatic organisms. The aquatic medium both activates gametes and prevents desiccation prior to actual fertilization. Terrestrial organisms must also release gametes into a medium that is both suitable for motility and fusion and which protects against

desiccation. A great many vertebrates, however, utilize habitats with little or no water readily accessible. Internal fertilization allows for the direct transfer of gametes from male to female within a suitable internal environment. Additionally, internal fertilization allows for the exploitation of a wider variety of terrestrial habitats. One potential hazard of greater mobility on land is that males and females may become separated. Females with mature ova may not encounter males and eggs may go unfertilized. Males also suffer decreased reproductive success if females are not available. Oviductal sperm storage ensures optimal reproductive success for both males and females. Mature sperm, capable of fertilizing an egg, can be transferred to females regardless of the female's reproductive condition. Females, in turn, can utilize the received sperm when ovulation actually occurs. As a result, the sexes need not be reproductively synchronized, allowing for greater independence from each other.

How sperm are maintained and later released from storage sites is unclear. Maintenance and release may be due to the sperm themselves, the oviduct, or both. Basic sperm structure is generally conserved from one vertebrate group to the next. Sperm consist of a head (nucleus and acrosome), neck or mid-piece (mitochondria), and flagellum (axoneme and fibrous structural components). Interspecific modifications in sperm morphology may reflect differences in modes of fertilization, environmental constraints, and

egg-related species-specific recognition (Baccetti and Afzelius, 1976).

Regardless of apparent differences, vertebrate sperm in general appear to have similar metabolic requirements. During sperm maturation, the epididymis often secretes materials that may become associated with the sperm themselves. It is quite possible that the female reproductive tract secretes similar materials, enhancing the oviduct's ability to keep sperm viable during storage.

In all sperm-storing vertebrates examined, sperm storage occurs only in specialized regions of the oviduct. In order to understand the process of sperm storage, one would need to examine these specialized regions (and enclosed sperm) in detail and compare them to similar regions within the male tract. Unfortunately, some of the organisms involved are either difficult to obtain and study, or have reproductive cycles that are strongly seasonal and difficult to examine with regard to the dynamics involved in sperm storage. In addition, many of the organisms involved utilize more than one region of the oviduct as storage sites, belaboring the examination process.

Sperm storage: Reptiles

Reports of prolonged sperm survival within the oviduct are particularly common among reptiles (Saint Girons, 1975; Gist and Jones, 1987). Most reports, however, involve temperate species. Fall matings are

followed by an overwintering period during which females do not ovulate. Oviductal sperm storage guarantees reproductive success in the event that males are not available in the spring when females resume ovulation. Oviductal sperm storage is less commonly reported in tropical reptiles, perhaps because year-round reproduction is generally observed in both sexes. In this situation, males and females are not seasonally separated and no obvious pause in reproduction exists, making the “need” for and advantage to be gained by oviductal sperm storage less clear. This manuscript verifies oviductal sperm storage in a tropical reptile and draws conclusions regarding the possible advantages gained as a result.

How sperm storage reports are verified varies. Most are based on one or more of the following observations: 1) the production of fertile clutches by females after isolation from males, 2) noted asynchrony between copulation and ovulation and, 3) histological observations of sperm within the female reproductive tract. Inherent in each of these methods are issues which may contradict or negate oviductal sperm storage within the animal observed.

The production of fertile clutches by females after isolation from males certainly suggests that oviductal sperm storage is a possibility. Several questions arise, however. Is the observer assuming that egg production only occurs as a result of prior mating activities? How is “fertility” assessed? Are the eggs truly fertilized (produced by sperm-egg fusion)? Parthenogenesis

(another phenomenon known to occur in reptiles) must be contraindicated. In addition, and more importantly, are the eggs "viable" (resulting in offspring)?

Fertile eggs that result in abnormal embryonic development may not produce viable offspring. In the absence of offspring sperm storage becomes irrelevant.

Noted asynchrony between copulation and ovulation also suggests sperm storage. Again, questions arise. Has ejaculation occurred? Rarely are females examined for the presence of sperm within the female reproductive tract. What are the lengths of the various phases of the ovarian cycle for the females involved? Sperm can certainly survive for hours or days within the female reproductive tract. If the time of observed (or presumed) mating is very close to that of actual ovulation, the delay seen between mating and egg deposition may, in fact, be due to alterations of the egg during transit (post-ovulation) and not at all to sperm storage.

Histological evidence of sperm within the female reproductive tract also suggests the possibility of oviductal sperm storage, but needs to be verified in terms of the functional activity of those sperm. Because histological examination requires the termination of involved females, these individuals cannot be observed for subsequent egg production and offspring.

As reviewed above, there are many areas that must be addressed when attempting to verify oviductal sperm storage in any animal. A large number

of animals must be available and, in addition, must often be maintained in captivity. These considerations make verifying oviductal sperm storage particularly difficult in reptiles. Reptiles are rarely maintained in captivity in the numbers required for such observations due to spatial requirements involved and lack of adequate information regarding appropriate captive environments. That oviductal sperm storage exists in certain reptiles is certain, but how common it is among reptiles requires additional, carefully addressed study.

Sperm storage: The Family Gekkonidae

The family Gekkonidae is comprised of at least 800 species of lizards and is, perhaps, the largest reptilian family. Geckos typically produce multiple clutches of one or two calcareous eggs throughout a year (Mattison, 1987; Dunham, *et al.*, 1988). Ovaries exhibit follicular growth, vitellogenesis, follicular atresia, and corpus luteum formation (Fox, 1976; Jones and Summers, 1984). The oviduct is typically reptilian, consisting of vaginal, uterine, tubal (some species), and infundibular regions. Geckos appear to store sperm only within the infundibular region of the oviduct (Cuellar, 1966a).

Sperm storage: *Hemidactylus frenatus*

Church (1962) postulated the occurrence of oviductal sperm storage in *H. frenatus* based on an observation of asynchronous mating between a single pair of animals, a histological examination of the testes ("found to be full of mature spermatozoa"), and a gross examination of the female's reproductive condition ("the largest eggs were in an early stage of development"). Functional oviductal sperm storage, however, was never verified.

H. frenatus is a tropical lizard abundant on Oahu, Hawaii, and are fairly easily captured. Females exhibit short, regular ovarian cycles throughout the year (pers. obs.). Asynchrony between ovulation and copulation has been observed within this species (Church, 1962; pers. obs.). These animals exhibit monoautochronic ovulation and both oviducts are histologically similar (pers. obs.). Preliminary histological observations identified the presence of only one oviductal storage site. The large number of animals obtainable, the need to examine only one region of the oviduct, and that two similar storage regions could be examined per animal, made *H. frenatus* an acceptable model for the study of oviductal sperm storage in a tropical vertebrate.

Objectives

1) In order to verify the possibility of oviductal sperm storage in *Hemidactylus frenatus*, I first examined ovaries and testes of adult animals (>40 mm snout-vent length) for evidence of gametogenesis. Because sperm are ultimately released from the epididymis into the oviduct, I then examined oviducts for sperm presence, orientation, and location. Specific location and orientation within the oviduct, regardless of ovarian condition, would certainly suggest the possibility of oviductal sperm storage.

2) In order to verify the viability of “stored” sperm, I maintained adult animals in captivity. Following an initial assessment of captive requirements, I analyzed the reproductive activity of captive male-female pairs. Once this analysis was completed, females were isolated from males. The reproductive capability of isolated females was compared to initial observations in the event that isolation affected egg production. In addition, egg fertility, viability, sex of offspring, and functional sperm storage duration were assessed.

The aforementioned experiments and results are included in Chapter One, “Functional Sperm Storage Duration in Female *Hemidactylus frenatus* (Family Gekkonidae)” as submitted to *Herpetologica*.

3) In order to assess characteristics of sperm maintenance within and release from storage sites, I examined both epididymal and oviductal sperm

via transmission and scanning electron microscopy for evidence of morphological alterations that might contribute to sperm maintenance during storage. I also examined the oviductal epithelium via histochemical and ultrastructural methods (light and transmission electron microscopy) throughout an ovarian cycle (follicular, vitellogenic, and luteal phases) for evidence of cyclic activity that might contribute to sperm maintenance during storage. Finally, I tested a prevailing hypothesis that sperm release occurs as a result of distension during egg passage through the oviduct by artificially distending oviducts and viewing the lumen via scanning electron microscopy for the presence of sperm. This information is addressed in Chapter Two, “Histochemical and Ultrastructural Characteristics of Oviductal Sperm Storage in *Hemidactylus frenatus* (Family Gekkonidae): With A Proposed Sperm Recycling Hypothesis.

4) In order to assess sperm metabolic requirements, I performed *in vitro* pilot experiments: a) analyzing sperm motility and survival within defined media, b) analyzing oviductal tissues for the presence of D-glucose, using high performance liquid chromatography (HPLC) analysis, and c) analyzing potential D-glucose uptake by sperm, using a modified membrane-uptake technique. This information is addressed in Chapter Three, “Physiology of *Hemidactylus frenatus* Sperm: Results of Pilot Experiments.”

The final chapter attempts to draw conclusions regarding the possible advantages of oviductal sperm storage to *H. frenatus*, using information obtained within Chapters 1-3.

CHAPTER 1

Functional Sperm Storage Duration in Female *Hemidactylus frenatus* (Family Gekkonidae)

ABSTRACT

Oviductal sperm storage has been demonstrated in *Hemidactylus frenatus*, a tropical gekkonid lizard that produces clutches of two calcareous-shelled eggs throughout the year on the island of Oahu, Hawaii. Histological examination of females revealed that sperm are stored within an area between the uterine and infundibular regions of the oviduct regardless of the ovarian condition of the female. In order to assess functional oviductal sperm storage, captive animals were maintained at ambient temperatures and natural lighting for a period of one year. Male/female pairs were maintained throughout November and December, 1991, and in January, 1992, females were individually isolated from males. Eggs were collected from all females and monitored for fertility and viability through November, 1992. A range of 3-5 wk separated egg production intervals during March through August, 1992. An average of seven viable clutches was produced per isolated female throughout this period, resulting in a sperm storage duration of up to 36 wk. Hatchlings (64 % of all fertile eggs) included males, contraindicating

parthenogenesis. Females ceased egg production in October, 1992, possibly due to cooler temperatures and the inability to thermoregulate beyond the confines of the cages. Oviductal sperm storage is of possible benefit to *H. frenatus* because of the small clutch size produced and variable egg production intervals.

INTRODUCTION

Most vertebrate classes contain examples of delayed fertilization via prolonged sperm survival within the oviduct (Howarth, 1974). When ovulation and mating are not synchronized, sperm storage may serve to increase female fecundity and offspring survival (Smyth and Smith, 1968). Sperm storage may also increase the reproductive success of females when colonizing new habitats or when males are scarce (Conner and Crews, 1980).

Reports of oviductal sperm storage are particularly common for reptiles (Gist and Jones, 1987; Saint-Girons, 1975) and have been based primarily on: (1) the production of fertile clutches by females after isolation from males (Atsatt, 1953; Cuellar, 1966b; Ewing, 1943; Lin, 1982; Woodward, 1933), (2) noted asynchrony between copulation and ovulation (Church, 1962; Smyth and Smith, 1968), and (3) histological observations of sperm within the oviduct (Adams and Cooper, 1988; Aldridge, 1992; Bou-Resli, *et al.*, 1981; Conner and Crews, 1980; Cuellar, 1966a,b; Gist and Jones, 1989; Halpert, *et*

al., 1982; Hattan and Gist, 1975; Hoffman and Wimsatt, 1972; King, 1977; Kumari, *et al.*, 1990; Palmer and Guillette, 1988; Schaefer and Roeding, 1973; Uribe, *et al.*, 1988). There is also evidence that stored sperm contribute to subsequent offspring in the garter snake, *Thamnophis sirtalis* (Gibson and Falls, 1975; Schwartz, *et al.*, 1989) and in the copperhead snake, *Agkistrodon contortrix* (Schuett and Gillingham, 1986).

Members of the family Gekkonidae typically have multiple clutches of one or two calcareous eggs throughout a breeding period (Dunham, *et al.*, 1988; Mattison, 1987). *Hemidactylus frenatus* Dumeril and Bibron, the Asian House Gecko, is a gekkonid lizard widely distributed throughout India, the IndoPacific, New Guinea, Australia, Oceania, Mexico, Africa, and Madagascar (Seufer, 1991). Reproductive studies in Java and China suggest variable reproductive patterns based on geographic distribution (Cheng and Lin, 1977; Church, 1962; Lin and Cheng, 1984). On the islands of Java, and Oahu, Hawaii, females do not exhibit seasonal reproduction, but are capable of egg production throughout the year (Church, 1962; this study).

Asynchrony between ovulation and copulation has been observed (Church 1962; personal observation). The potential for long term oviductal sperm storage and sperm viability in these animals has never been verified.

The objectives of this study were to: (a) determine if and where sperm are stored within the oviducts of *H. frenatus*, (b) maintain captive individuals

in order to establish captive reproductive patterns, and (c) establish the potential duration of functional sperm storage within the oviducts of captive females.

MATERIALS AND METHODS

Animal Collection and Measurements

Adult male and female *Hemidactylus frenatus* with snout-vent lengths (SVL) >40 mm were hand or net collected from buildings in the Waianae and Honolulu areas of Oahu, Hawaii.

Light Microscopy

Twenty-five females were sacrificed within 15 h of capture. Reproductive structures (ovaries and oviducts) were immediately immersed in Steive fixative (Humason, 1979) for 24 h at room temperature. Samples were washed in 50% ethanol (EtOH) for 30 min, and post-treated with iodine-alcohol for 5-8 h. Samples were dehydrated in a graded series of EtOH, embedded in Paraplast-Plus, and sectioned at 7 μ m. Every fourth slide was stained with either Harris' or Delafield's Hematoxylin and Eosin Y (H&E, regressive method: Humason, 1979) for general histology as well as for the presence/absence of sperm. Ovaries were categorized into follicular, vitellogenic, and luteal phases as described by Jones and Summers (1984).

Five males were sacrificed as controls for characterizing sperm morphology. Reproductive structures (testes and epididymes) were processed as above for examination. The reproductive condition of each male was recorded.

Ex Situ Environment

All captive animals (as pairs or individuals) were maintained in standard acrylic aquaria (7.56 l) on a semi-enclosed platform on Oahu, Hawaii. Ambient temperature (ranging from a daily average of 22.2 °C to 27.7 °C), direct sunlight (2 h/morning), and daylight (10-12 h/day) were present. The location additionally allowed for the presence of local sensory stimuli (sights, sounds, and smells). Each cage was lined with gravel and sphagnum moss to assist animal shedding. Hiding places in the form of electrical junction boxes (a common egg laying site in the field), egg cartons, and cardboard tissue rolls were present in each cage. Mediterranean fruit flies (*Ceratitidis capitata*) were used as the primary food source. Additional foods included *Drosophila melanogaster* cultures and insect sweepings from local areas. Calcium was provided daily in the form of crushed chicken egg shell. Reptile vitamins (Reptovite) were mixed with a sucrose-water solution for consumption by both insects and lizards. A moistened sponge in a petri

dish added humidity and served as water for insects. Water for lizard consumption was supplied by the daily misting of aquarium walls.

Captive assessment

Observations included: months in which eggs were produced, number of eggs produced/female/month, and egg fertility and viability. Eggs were collected bi-weekly from cages and placed in covered tissue culture dishes (Falcon) for individual observation. Eggs were maintained at ambient temperatures throughout incubation. Observations were made through the clear plastic of the culture dishes. Egg fertility was defined by observable color changes (from pink to lavender to gray) associated with embryonic development (Brown, personal communication). Eggs remaining creamy white (condition at laying) or becoming yellow-white several months after deposition, were presumed infertile or a result of early embryonic mortality. Egg viability was defined by hatching success. Eggs remaining unhatched after a minimum of four months post-deposition were opened and examined for evidence of embryonic development.

The first year's analysis included data for eight females. Five females were paired with males from August, 1990, through August, 1991. The remaining three females were isolated from males in August, 1990, and maintained separately throughout the study in order to determine possible

effects of isolation on reproduction. All animals were returned to the original collection site in August, 1991.

The second year's analysis included data for 13 females. In November, 1991, 26 adult animals (13 males, 13 females) were collected and maintained as male/female pairs for a period of two months. Then, 11 females were isolated from males for the remainder of the study in order to assess functional sperm storage potential. Removed males were sacrificed and examined for the presence of epididymal sperm. For this study, analysis of the reproductive patterns for all females (both paired with males and isolated from males) continued through November, 1992. A Spearman's Rank Correlation Coefficient was utilized to correlate average daily temperature and photoperiod to egg production and number of females producing eggs in this study.

RESULTS

The oviduct can be separated histologically into vaginal, uterine, and infundibular regions (Fig. 1). The mucosa of the posterior vagina, continuous with the cloaca, is characterized by thin longitudinal folds of ciliated columnar cells surrounded by smooth muscle. The mucosa of the anterior vagina is characterized by thin, ciliated transverse folds of columnar cells with little surrounding muscle. The uterine mucosa consists of thick

longitudinal folds of possibly pseudostratified columnar cells. Uterine gland cells are present and only scattered patches of cilia exist here. The mucosa of the posterior infundibulum consists of shallow folds of ciliated pseudostratified columnar cells. The thinner mucosa of the anterior infundibulum consists of both ciliated and non-ciliated pseudostratified columnar cells, is vascular, and contains no folds.

Sperm are regularly located in a “transition zone” that separates uterine and infundibular regions of the oviduct (Fig. 1.). The mucosa of this “transition zone” consists of deep folds (crypts) lined by both ciliated and non-ciliated pseudostratified columnar cells that extend into the neck of the crypts. Three females contained sperm within both the vagina and the transitional zone regions. Sperm within the posterior vagina were seen as large, unlocalized and unoriented masses (Fig. 2A). Within the anterior vagina, sperm were clustered in parallel manner with heads oriented toward the oviductal epithelium, but randomly clustered along it (Fig. 2B). Sperm within the transitional region were localized deep within crypts, oriented parallel to each other with heads toward the oviductal epithelium and were present during anovulatory phases of the ovarian cycle (Fig. 2C). No sperm were seen within the oviduct connecting these regions.

All males examined were reproductively capable as evidenced by mature sperm within both the testes and epididymes. Sperm were randomly

oriented throughout the epididymal lumen (Fig 2D) and, upon release from the epididymis, exhibited vigorous movement.

During the first year, eggs were produced in every month except January and February, 1991. (Fig. 3A). Females paired with males always produced fertile clutches, whereas isolated females produced no more than two fertile clutches. Egg production continued in a pattern similar to that of females paired with males, indicating that males were not required for egg production. One female, isolated immediately after mating, produced two fertile, viable clutches before producing only infertile eggs, establishing a sperm storage potential of approximately 4-6 weeks.

I collected 132 eggs during the first year. Eighty percent of these eggs belonged to two-egg clutches; 20% to one-egg clutches. Of the total number collected, 88 were assessed as fertile, and among these, 70% hatched. No egg assessed as infertile hatched. Each female produced an average of 11 clutches during this period.

During the second year, the pattern of egg production was similar, although cessation of egg production occurred during different months (Fig. 3B). Functional sperm storage potential differed considerably. Females produced fertile, viable eggs up to 36 wk after isolation from males (Fig. 4), but not thereafter. Males were found among hatchlings produced, contra-

Fig. 1. Schematic of left oviduct from *Hemidactylus frenatus*. This configuration is observed in females with ovaries in the follicular phase of development. Abbreviations (length in mm): i= infundibulum (3.0), t= transitional region (0.5; site of sperm storage), u= uterine region (5.0), av= anterior vagina (0.5), pv= posterior vagina (1.5), c= cloaca.



Fig. 2. Sperm orientation within the oviduct (A-C) and epididymis (D) of *Hemidactylus frenatus*. A. posterior vagina, B. anterior vagina, C. transitional region (site of sperm storage), D. epididymis. Abbreviations: s= sperm, sh= sperm heads, oe= oviductal epithelium, ee= epididymal epithelium.

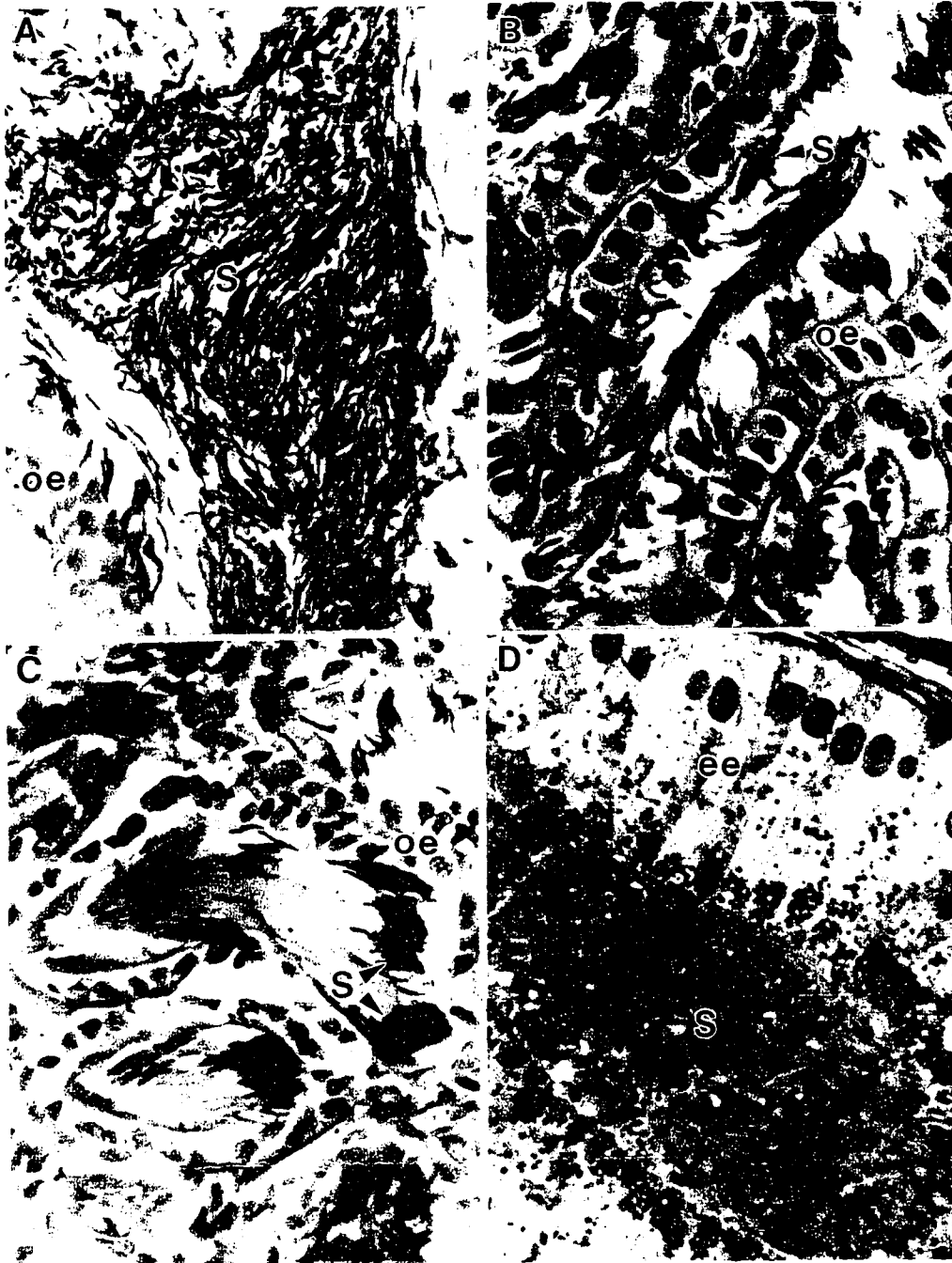
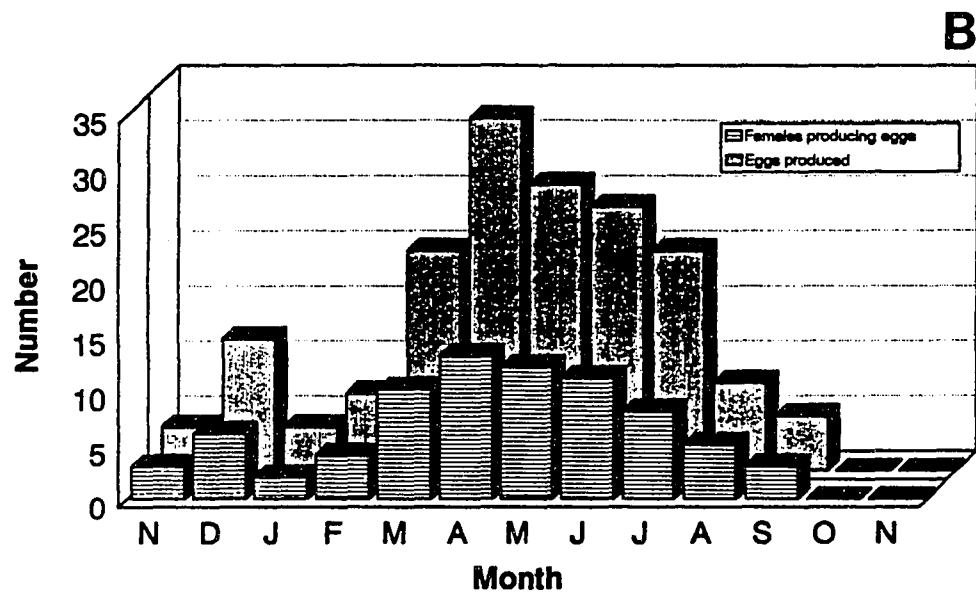
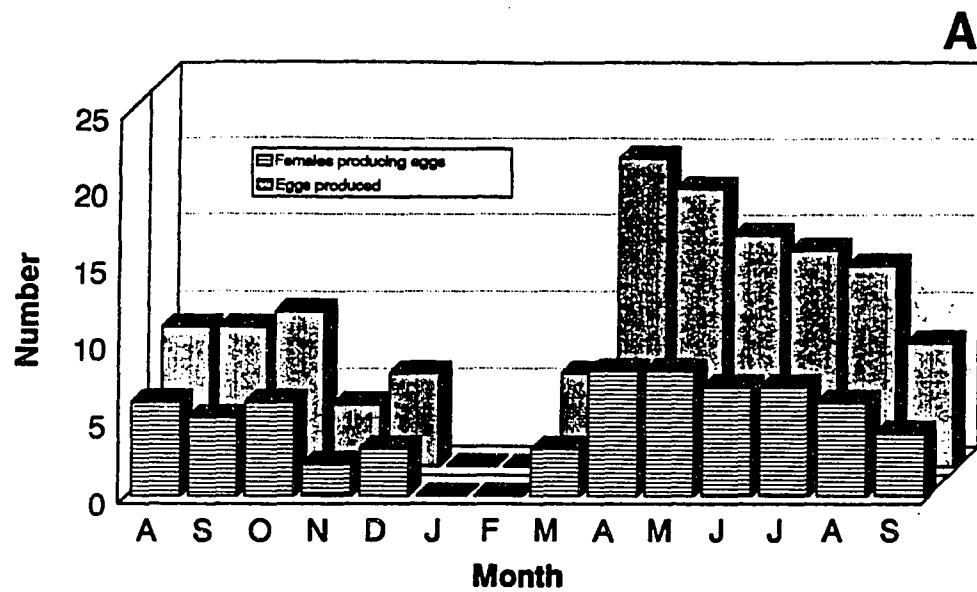


Fig. 3. Monthly egg production by captive *Hemidactylus frenatus*. A. August, 1990, through September, 1991 ($n=8$). B. November, 1991, through November, 1992 ($n=13$).

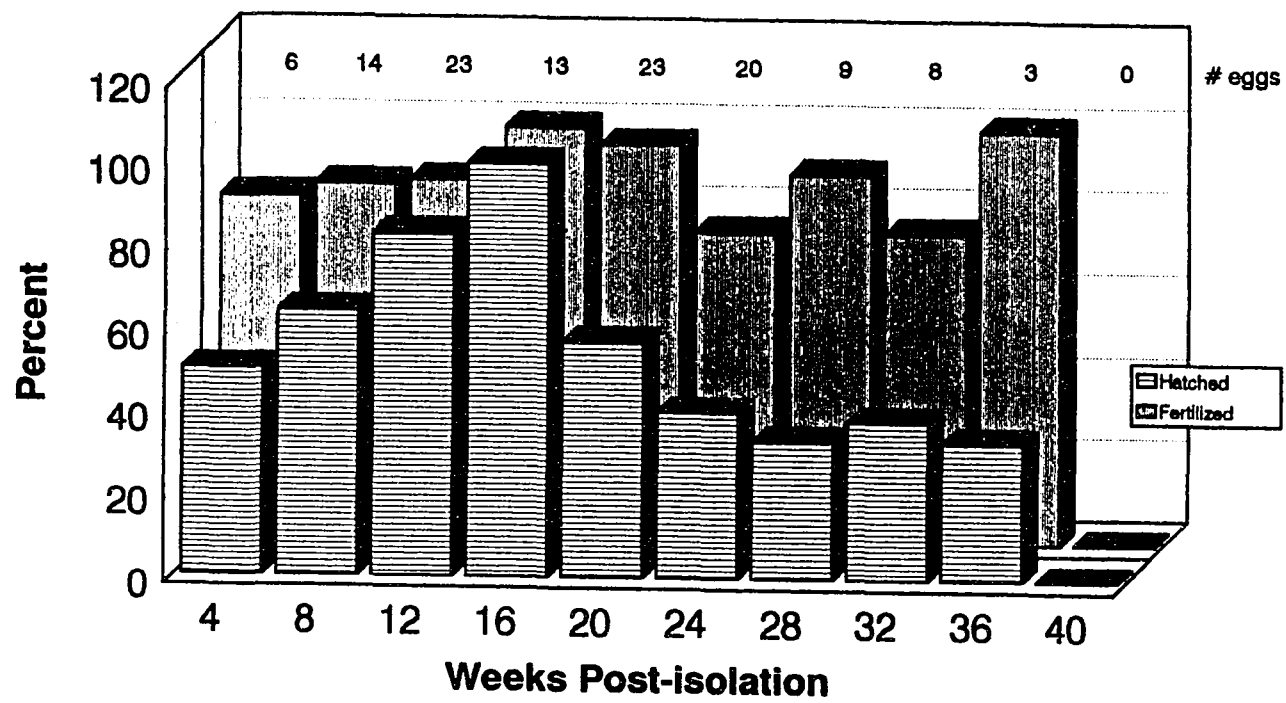


indicating parthenogenesis. Forty-five unhatched eggs were opened at least 4 mo post-deposition and were examined for evidence of embryonic development. Eggs originally defined as fertile contained evidence of embryonic material, whereas eggs defined as infertile contained only yolk. Only one egg originally defined as infertile contained a darkened area that may have represented aborted early embryonic development. If fertile, this embryo failed to demonstrate visible color changes.

I collected 162 eggs during the second year. Eighty two percent of all clutches were two-egg clutches; 18 % were one-egg clutches. A total of 140 eggs were assessed as fertile, and 64 % of them hatched. No egg assessed as infertile hatched. An average of seven clutches per female was produced during this period. Hatching success rates were twice as high during March through August (61%) when compared to September through February (27%). In addition, ovulatory cycles were shorter during March through August (ranging 3-5 wk) when compared to September through February (7-16 wk).

Temperature was not significantly correlated with either egg production or number of females producing eggs in this study ($r=0.325$, $P=0.36$; and $r=0.317$, $P=0.37$, respectively). Photoperiod was significantly correlated with both variables ($r=0.725$, $P=0.04$; and $r=0.7$, $P=0.048$, respectively).

Fig. 4. Fertility and hatching success of eggs produced by isolated female *Hemidactylus frenatus* during January, 1992, through October, 1992 ($n=11$).



DISCUSSION

Members of the family Gekkonidae apparently retain sperm only within a region separating the uterine and infundibular portions of each oviduct (Cuellar, 1966a; this study). The anterior vagina in *Hemidactylus frenatus* does not appear to be a site for long term sperm storage as sperm are infrequently located here and probably cannot be sequestered due to the region's shallow mucosal folds. Virtually every adult female collected and examined histologically contained sperm within the "transitional" and/or vaginal region of the oviduct although no sperm were seen within the oviduct connecting these regions. This could suggest that multiple mating occurs in *H. frenatus* or that sperm are transferred in groups between these regions.

All captive females had reduced egg production primarily during the months of September through February which may have been related to changes in metabolic activity. Thermoregulatory behavior occurs in *H. frenatus* (Werner, 1990) and captive females (this study), subject to ambient temperatures, could not regulate internal body temperatures beyond the confines of their cages. Although apparently healthy, captive females were not as active and did not feed as frequently during fall and winter months, perhaps unable to meet the energy requirements needed for egg production.

A similar pattern was found by Lin and Cheng (1984) for *Hemidactylus frenatus* in Taiwan and *Anolis* species in Puerto Rico (Gorman,

1974). Lin and Cheng associated this reduction in egg production primarily with food scarcity, perhaps as it related to temperature and photoperiod. Temperature and photoperiod explain seasonal ovarian cycles in Puerto Rican *Anolis* lizards (Gorman, 1974), although only photoperiod was significantly correlated with egg production in this study. I was unable to distinguish between photoperiod and temperature, however, as the proximate factor in reduced egg production during winter months, and clearly not for the variable egg production rates seen. Precipitation has been positively correlated with egg-laying in the gecko, *Lepidodactylus lugubris* (Brown and Duffy, 1992), but was not calculated here.

The sperm storage duration of 4-6 wk seen during the first year's analysis may have been due to the initial caging situation. Animals in the first data set were initially housed as groups of 2-3 females per male. It is possible that, under those conditions, some females mated more frequently than others and that sperm depletion occurred during ovulations preceding isolation. Additionally, the sample size of isolated females was quite small, consisting of only three females. The second data set included a larger sample size of 11 females who were individually paired with males prior to isolation and may have mated several times, ensuring a large oviductal sperm population.

For most reptiles with prolonged sperm storage, the actual number of ovulations has been low, producing a finite number of fertile clutches in the absence of males (Cuellar, 1966b; Ewing, 1943; Woodward, 1933). This may be due to a depletion of sperm from storage sites as each ovulated egg passes through the oviduct (Saint-Girons, 1975). Sperm depletion is defined as decreased numbers of sperm within the oviduct over time, and has been shown to occur in both the domestic hen and the lizard, *Holbrookia propinqua* (Adams and Cooper, 1988; Bushman, *et al.*, 1985). If cessation of fertile egg production is due to sperm depletion from storage sites, the more frequently a female ovulates, the more rapidly stored sperm should be depleted.

Temperate reptiles that "over-winter" cease ovulation until the spring, when stored sperm may then be used to produce subsequent offspring (Aldridge, 1992; Halpert, *et al.*, 1982). These animals, therefore, show prolonged sperm storage, perhaps as a result of infrequent ovulations. Tropical and subtropical species should show shorter storage durations because these animals rarely experience any lengthy pause in ovulation. Sperm stores, therefore, should be depleted more rapidly than in temperate species unless multiple or frequent matings occur, ensuring a constant sperm supply. Although females isolated from males in this study could not replenish sperm stores, egg production intervals varied throughout the study

and may have contributed to the prolonged sperm storage duration shown by captive females.

In addition to ovulation rates, clutch size may be important. Larger clutches would deplete sperm more rapidly as more eggs pass through the oviduct per cycle. *Hemidactylus frenatus* is a monoautochronic ovulator, releasing a single egg from each ovary per cycle. The small clutch size produced during each cycle may have maintained larger sperm stores over time and thus contributed to the relatively large number of fertile clutches produced by isolated females.

That *H. frenatus* sperm are certainly capable of prolonged viability within storage sites is evidenced by the successful hatching of eggs produced by females up to 36 wk of isolation from males. Overall hatching success rates were lower for eggs produced by these females, perhaps due to sperm aging or suboptimal incubation temperatures. Abnormal fertilization due to sperm age may have prevented embryonic development and subsequent hatching, as in birds, for which a decrease in hatching success has been correlated (Howarth, 1974). Eggs were not incubated at controlled temperatures, making it difficult to separate sperm aging events from environmental conditions affecting embryonic development and hatching success. Depletion from sperm stores may have contributed to the decreased

hatching success over time by reducing the number of functionally viable sperm present within the oviduct.

The adaptive value of oviductal sperm storage in *H. frenatus* may be related to its year-round reproduction, lack of gregariousness, and dispersal. How sperm are maintained for prolonged periods during storage and subsequently released at ovulation is the focus of our current research.

CHAPTER 2

Histochemical and Ultrastructural Characteristics of Oviductal Sperm Storage in *Hemidactylus frenatus* (Family Gekkonidae), With A Proposed Sperm Recycling Hypothesis

ABSTRACT

Oviductal sperm storage in *Hemidactylus frenatus* may be accomplished by both physical and chemical properties of the oviduct. Sperm appear to move actively through the oviduct but are not seen within the infundibular region. Infundibular mucosubstances, secreted throughout the ovarian cycle, may physically or chemically prohibit further anterior sperm movement. Sperm become localized within deep epithelial infoldings, or crypts, located posterior to the infundibulum. Narrow entrances and frequently branched blind ends in these crypts may serve physically to immobilize sperm during storage. Secretory cells lining the crypt entrances and oviductal lumen secrete carbohydrate-rich material into the oviductal lumen, but not into the crypts themselves. Oviductal distension as a result of egg passage may explain the mechanical exposure of sperm within storage crypts, allowing for chemical activation of sperm as they directly contact potential energy substrates. Once the egg has passed posteriorly, the oviductal epithelium collapses to its

original folded condition, allowing for “re-storage” of sperm until the next ovulation.

INTRODUCTION

Although oviductal sperm storage appears to be common among all classes of vertebrates, it is especially prevalent among reptiles (Howarth, 1974; Saint-Girons, 1975; Gist and Jones, 1987). The location of sperm storage sites within the reptilian oviduct varies from species to species, with many reptiles possessing two storage sites per oviduct: one vaginal and one infundibular. Members of the Family Gekkonidae apparently utilize only the infundibular site for long-term sperm storage (Cuellar, 1966a). Storage sites may consist of epithelial folds or furrows, and crypts or tubules (Gist and Jones, 1987). In all cases examined, sperm in these storage sites characteristically orient their acrosomes toward the storage epithelium (Fox, 1963; Cuellar, 1966a; Hoffman and Wimsatt, 1972; Adams and Cooper, 1988; Gist and Jones, 1989). Using light microscopy, Cuellar (1966a) concluded that the acrosomal regions of sperm appeared to penetrate oviductal epithelial cells. Absence of cellular penetration has been noted in the oviducts of the snake, *Thamnophis sirtalis*, and in bats when examined by transmission electron microscopy (Hoffman and Wimsatt, 1972; Racey, *et al*, 1973; Son, *et al*, 1987). Scanning electron microscopy has also demonstrated a lack of direct contact

between sperm and oviductal cells in the turkey, *Meleagris gallopavo* (Bakst, 1987).

Following insemination, sperm may or may not undergo morphological changes as they travel through the female reproductive tract or during extended oviductal storage. No morphological differences have been noted between oviductal and epididymal sperm in the snake, *Thamnophis sirtalis*, or the bat, *Myotis lucifugus* (Hoffman and Wimsatt, 1972; Wimsatt, *et al*, 1966). Similarly, Wimsatt, *et al*, (1966), were also unable to detect morphological differences between oviductal sperm obtained from female bats isolated from males for 14 months and oviductal sperm from freshly inseminated females. Newton and Trauth (1992), however, did observe differences between oviductal and epididymal sperm in the non-sperm-storing lizard, *Cnemidophorus sexlineatus*. Acrosomes of sperm obtained from the ductus deferens possessed spatulate anterior tips, while sperm flushed from the oviduct possessed pointed acrosomes with exposed acrosomal filaments. In addition, sperm obtained from the male reproductive tract were characteristically bent at the midpiece, such that sperm heads were reflected posteriorly. The heads of oviductal sperm did not demonstrate such a bend, but instead projected forward. This led Newton and Trauth (1992), to suggest that sperm structural maturation events were completed while in the oviduct of *C. sexlineatus*.

Sperm appear to swim actively through the oviduct of the lizard, *Anolis carolinensis* (Conner and Crews, 1980), but are passively transported with carrier matrices by ciliary and contractile actions of the oviductal epithelium in the snake, *Thamnophis sirtalis parietalis* (Halpert, *et al*, 1982). How sperm are released from storage sites remains unclear. Events related to ovulation and/or oviposition may play a role in sperm release from storage sites within domestic fowl (Bobr, *et al.*, 1964; Bushman, *et al*, 1985). While oviductal distention has not been demonstrated as a primary factor initiating sperm release from the uterovaginal region in birds (Van Krey, *et al*, 1967), it has been hypothesized as a means for release from infundibular regions (Grigg, 1957).

Whether or not morphological changes occur, requirements for sperm maintenance and activity during storage remain unknown at the present time. Prolonged sperm viability may be due to characteristics of the sperm themselves, the oviductal epithelium, or both. With respect to the oviductal epithelium, there is evidence that supports hormonal control as several investigators (Hoffman and Wimsatt, 1972; Halpert, *et al.*, 1982; Kumari, *et al.*, 1990; Jones and Summers, 1984) have demonstrated a correlation between changes within the oviductal epithelium and the ovarian cycle. For example, non-ciliated secretory cells appear to dominate the oviductal epithelium in the snakes, *Thamnophis sirtalis* and *Thamnophis sirtalis*

parietalis, during the breeding season while ciliated non-secretory cells dominate during the non-breeding state (Hoffman and Wimsatt, 1972; Halpert, *et al.*, 1982). Ovarian and oviductal mass increase to a maximum during late vitellogenesis in the tortoise, *Gopherus polyphemus* (Palmer and Guillette, 1990) and secretory granules and blebbing of microvilli also increase in amount as vitellogenesis proceeds. Increased secretory activity has also been demonstrated in oestradiol-treated oviductal glands of the lizard, *Uromastix hardwicki* (Akhtar, 1988).

Although research on reproductive cycles in reptiles has been extensive, few analyses have examined tropical or subtropical reptiles whose breeding patterns and cycles may be quite different (Fitch, 1982). While Jones and Summers (1984) characterized the gekkonid ovarian cycle and found that oviductal weights were correlated with the ovarian cycle, examinations were not made of the oviductal epithelium in order to describe the histological dynamics underlying this correlation.

Hemidactylus frenatus is a tropical, oviparous, gekkonid lizard abundant on Oahu, Hawaii, and oviductal sperm storage has been documented in isolated females for up to nine months (pers. obs.). The objectives of this study were (1) to describe the histochemistry and ultrastructure of the oviductal epithelium in *H. frenatus* with reference to sperm storage during follicular and vitellogenic phases of the ovarian cycle,

and (2) to propose a possible mechanism for sperm release and “recycling” over many ovarian cycles.

MATERIALS AND METHODS

Fixation procedures

Adult *Hemidactylus frenatus* greater than 40 mm snout-vent length were collected during peak evening hours from buildings on Oahu, Hawaii. One oviduct and ovary (or testis and epididymis) from each animal were immersed in Steive's fixative (Humason, 1979) for 24 h at room temperature. These samples were processed for light microscopy. The opposite ovary and oviduct (or testis and epididymis) were immersed in 2% glutaraldehyde fixative in 0.1 M Na-phosphate buffer for 3-24 h at room temperature. Oviducts and epididymes fixed in glutaraldehyde were processed for electron microscopy. Ovaries and testes fixed in glutaraldehyde were processed for light microscopy and served as controls for differences in fixation procedures.

Light microscopy

Reproductive organs placed in Steive's fixative were washed in 50% ethanol for 30 min, and post-treated with iodine-alcohol for 5-8 h. Samples were dehydrated in a graded series of ethanol and embedded in Paraplast-Plus. Ovaries and testes placed in glutaraldehyde were washed in 0.1 M Na-

phosphate buffer for three hours. No post-treatment was necessary for these samples which were immediately dehydrated and embedded as described for samples fixed in Steive's fluid. All blocks were sectioned at 7 μm on either an American Optical 820 Microtome or Reichert Jung Microtome for general histology and to determine if sperm were present. Every fourth slide was stained with either Harris' or Delafield's hematoxylin and eosin Y (H&E). Additional slides containing sperm, as indicated from H&E analysis, were stained for the presence of carbohydrates (Periodic acid-Schiff technique) and for mucosubstances (Alcian Blue technique; Humason, 1979) possibly contributing to sperm survival during storage. Ovaries were categorized into follicular, vitellogenic, and luteal phases as described by Jones and Summers (1984).

Transmission Electron Microscopy (TEM)

Epididymes were separated into anterior and posterior regions, cut into 1 mm³ pieces, and processed for examination by transmission electron microscopy (TEM). Samples were washed in 0.1 M Na-phosphate buffer for 1 h, post-fixed in 1% osmium tetroxide for 1-2 h, washed again in fresh buffer and dehydrated in a graded series of ethanol. After dehydration, samples were embedded in Spurr's epoxy resin. Semi-thin sections were made at 1-2 μm for block orientation and stained with toluidine blue. Thin sections were

made at 90 nm with glass knives and placed on copper grids. Grids were stained for 20 seconds with 0.3% lead citrate at room temperature, washed once in 10N NaOH followed by distilled water, stained again with warm 2% uranyl acetate for 5-8 minutes, washed twice in distilled water, and stained a final time for 20 seconds with lead citrate at room temperature. Grids were washed again in 10N NaOH followed by distilled water and stored in a desiccator until viewing. Observations were made using a Zeiss EM 10/A TEM (Zeiss Instruments, FDR, Germany) operated at 80 kV. The sperm storing region of each oviduct was treated similarly and observed. Additional semi-thin sections were also stained using Periodic Acid-Schiff and Alcian Blue techniques for comparison with paraffin sections (Lane and Europa, 1965).

Scanning Electron Microscopy (SEM)

Epididymal sperm were obtained by stripping one complete epididymis of all contents with forceps. The contents (sperm and supportive fluids) were diluted in 500 µl of amphibian Ringer's solution and suspended thoroughly. A sperm smear was made by drawing 10 µl of sperm suspension across the surface of a round glass coverslip. Freshly smeared samples were immediately exposed to fumes of 1% osmium tetroxide for 15 minutes at room temperature and air-dried. The coverslips were mounted on viewing stubs,

coated with gold (Hummer II sputter coater), and viewed at 10 kV with a Hitachi S-800 scanning electron microscope.

The sperm storage region of the left oviduct was removed, pinned open on Sylgard (Dow Corning) and immersed in 2% glutaraldehyde/1M phosphate buffer for 3 hr at room temperature. The right oviduct was injected with 1 cc of amphibian Ringer's solution in order to distend the sperm storage region to a size approximating that of an egg passing through the region. The sperm storage region was then removed, pinned open, and fixed as before. Both samples were then washed in fresh buffer for 1 hr and post-treated in 1% osmium tetroxide for 1 hr at room temperature. Samples were washed again in fresh buffer for 1 hr and dehydrated in a graded ethanol series prior to critical point drying. Once dried, samples were mounted on stubs, coated with gold and viewed.

Epididymal and oviductal morphology

Observations were made with reference to sperm presence, morphology, and orientation. Ultrastructural characteristics of the associated epithelium were also noted. Oviductal samples were categorized according to the ovarian condition at the time of processing.

RESULTS

Sperm were randomly distributed throughout the epididymal lumen of *H. frenatus*. Cytoplasmic droplets were frequently associated with the midpiece of sperm found within the anterior epididymis when viewed by transmission electron microscopy. Few cytoplasmic droplets were seen among sperm from the posterior epididymis. All epididymal sperm consisted of discernible head (nucleus and acrosome), midpiece (mitochondria), and tail regions when viewed by scanning electron microscopy (Fig. 5A, B), although many sperm heads were folded over onto the tail region. There were no discernible differences in morphology between epididymal and oviductal sperm. Sperm could not be identified in uninjected oviducts prepared for SEM, but were readily observed in Ringer's injected, distended oviducts (Fig. 5C). Again, many sperm heads were folded over onto the tail region.

Oviductal sperm storage regions were characterized by simple epithelial infoldings (crypts). These crypts frequently branched, communicating with the oviductal lumen via a common channel. All sperm observed were localized within the blind ends of the crypts and were oriented parallel to each other with heads toward the crypt epithelium.

Transmission electron microscopy clarified that sperm storage crypts were lined by two distinct types of pseudostratified columnar epithelial cells. Non-secretory ciliated cells dominated the entrance to each crypt (Fig. 6A),

whereas non-ciliated secretory cells occupied primarily the blind ends of the crypts (Fig. 6B). In addition, non-ciliated cells frequently protruded into and narrowed the crypt lumen (Fig. 6C). Sperm heads were not embedded in the crypt epithelium as suggested by Cuellar (1966a), but interdigitated with membrane extensions located within the blind ends of the crypts (Fig. 6D). Sperm were present throughout all phases of the ovarian cycle, including anovulatory phases. No gross ultrastructural differences were noted between sperm found in male and female reproductive tracts.

Transmission electron microscopy of oviducts from follicular-phase females also revealed the presence of electron-dense granules within non-ciliated secretory cells. These granules appeared structurally homogenous and were most numerous in the apical cytoplasm (Fig. 7A). Correlated with ovulation, however, the granules vacuolated and secreted their contents into the oviductal lumen (Fig. 7B). While Periodic Acid-Schiff treatment (light microscopy) demonstrated that the granules and secretions were carbohydrate in nature, the contents were not digested by amylase. Development of granules and their subsequent secretion was not affected by the presence or absence of sperm.

At pH 2.5 and 1.0, secretory cells at the entrance to and presumptive secretory cells at the blind ends of the storage crypts did not stain positively for Alcian Blue when viewed by light microscopy. This indicated the absence

of mucosubstances in the area of sperm storage. In contrast, infundibular cells demonstrated prominent non-cyclical secretion of mucosubstances throughout the ovarian cycle. Infundibular mucosubstances stained positively at both pH 2.5 and 1.0, indicating the presence of sulfate (and perhaps carboxyl) groups.

Fig. 5. Sperm morphology (SEM). A, B. epididymal sperm; C. oviductal sperm after release from storage crypt. Abbreviations: a= acrosome, h= head, m= midpiece, n= nucleus, t= tail.



Fig. 6. Oviductal crypt ultrastructure (TEM). A. crypt entrance, B, D. blind end of crypt, C. crypt lining. Abbreviations: c= cilia, cc= ciliated cell, cl= crypt lumen, m= epithelial membrane, nc= non-ciliated cell, ol= oviductal lumen, s= sperm.



Fig. 7. Secretory cell activity (TEM). A. follicular phase, B. vitellogenic phase.

Abbreviations: c= cilia, cc= ciliated cell, sg= secretory granule(s), ol= oviductal lumen, vg= vacuolated granule(s).



DISCUSSION

Materials positive for the Periodic Acid-Schiff reagent (PAS) have been associated with oviductal sperm masses in the snake, *Thamnophis sirtalis* and *Calotes versicolor* (Halpert *et al*, 1982; Hoffman and Wimsatt, 1972; Kumari *et al*, 1990). In *T. sirtalis*, epithelial cells within vaginal storage sites stained strongly with PAS and, in caudal areas, sloughed off and surrounded sperm. PAS-positive sperm masses were also found in infundibular storage sites in these reptiles. Because of this, transportation and nutritional roles were suggested for the sperm/oviductal cell association (Halpert and Crews, 1981; Halpert, *et al.*, 1982). Hoffman and Wimsatt (1972), noting PAS-positive material throughout the oviduct of *T. sirtalis*, suggested that secretory activity was not confined to the sperm storage sites and was possibly of nutritional value, being present at the time of sperm deposition. Stored sperm masses were not PAS-positive in this study. The secretions seen in our study may have been a byproduct of oviductal hypertrophy, contributing to sperm activity after release as this material was seen about the time of ovulation at the entrances to sperm storage regions, and not seen deep within the crypts themselves. Whether PAS-positive material contributes to sperm motility upon release from storage sites, or is utilized by newly ovulated eggs in *H. frenatus* remains unknown.

Attempts to determine the nature of oviductal secretions have shown the presence of glucose within sperm storage sites of the domestic hen, *Gallus domesticus*; the garter snake, *T. sirtalis*; and the bat, *Pipistrellus pipistrellus* (Gilbert, *et al*, 1968; Hoffman and Wimsatt, 1972; Racey and Potts, 1970). In addition, free glucose and fructose have been identified in infundibular homogenates from *T. sirtalis* (Hoffman and Wimsatt, 1972). Preliminary carbohydrate analyses, via high performance liquid chromatography, demonstrated the presence of glucose throughout the oviduct of *H. frenatus*, and *in vitro* analysis suggested potential uptake of H³-glucose by *H. frenatus* epididymal sperm (unpublished data). Whether or not oviductal sperm would demonstrate similar uptake capabilities has not been resolved. While glucose is indicated as a component of the secretory material seen in the present study, the source is not glycogen, as the material was not removed with amylase treatment.

It has been suggested that secretory cells, rich in acid mucopolysaccharides, may serve a nutritional function for sperm while in storage (Hoffman and Wimsatt, 1972). We did not observe mucopolysaccharides within the storage crypts of *H. frenatus*. Rather than serving a nutritional role, observed mucosubstances may serve to lubricate the oviduct in preparation for passage of the relatively large egg. The mucosubstances may also serve as a barrier to sperm movement, as these materials stained at very

acidic pH values and sperm were found only posterior to oviductal regions with these secretions.

The structure of epididymal sperm has been documented for several reptilian species (Furieri, 1970; Butler and Gabri, 1984; Al-Haji, *et al.*, 1987). These observations, using transmission electron microscopy, concurred with those made by Furieri, (1970). The absence of cytoplasmic droplets in *H. frenatus* sperm from both the posterior epididymis and oviduct may be due to sperm maturation within the epididymis (Bedford, 1979). There were no observable differences between posterior epididymal sperm and oviductal sperm examined in the present study by both scanning and transmission electron microscopy. The morphological and ultrastructural integrity seen in this study may be due to the “sperm-storing” capacity within this species. Newton and Trauth (1992), observed visible differences between epididymal and oviductal sperm in the “non-sperm-storing” lizard, *Cnemidophorus sexlineatus*. Perhaps sperm integrity is maintained in sperm-storing species as a result of the capacity for prolonged storage.

That sperm were identifiable only in Ringer’s injected, distended oviducts suggests that sperm are sequestered deep within storage crypts and that distension aids in their release from these sites. In non-distended oviducts, sperm remained sequestered within the storage crypts and were, therefore, not readily visible when viewed by scanning electron microscopy.

In addition, sperm identified within injected, distended oviducts were randomly oriented, suggesting that these sperm may not have been metabolically active at the time of release.

That the histochemical and ultrastructural characteristics described for the oviduct of *H. frenatus* were not altered by the absence of sperm from storage crypts suggests that the secretory activity of epithelial cells is “pre-set” and perhaps under the hormonal control of the reproductive cycle. This is supported by the fact that both Hoffman and Wimsatt (1972), and Halpert, *et al* (1982), have demonstrated that sperm transport and storage is integrated with the reproductive cycle in *T. sirtalis* and *T. sirtalis parietalis*.

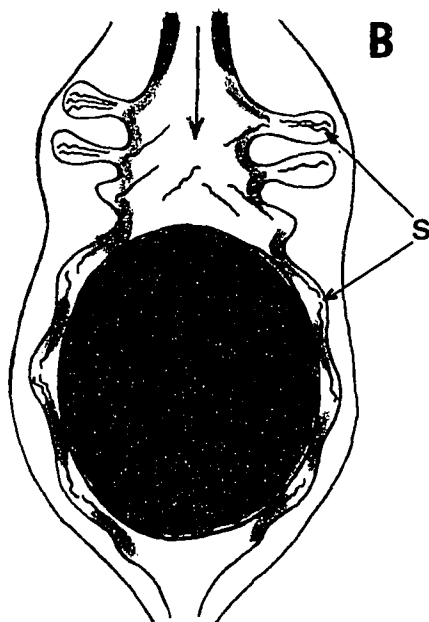
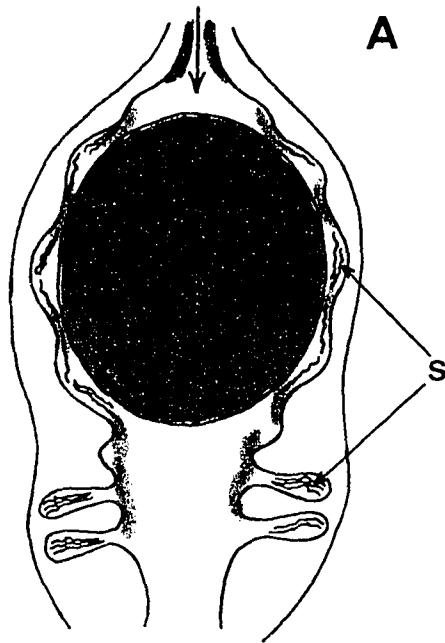
As in the present study, Hoffman and Wimsatt (1972) also observed both increased secretory activity within the sperm storage epithelium and increased cytoplasmic vacuolation within storage cells as ovulation neared. The same investigators were also able to demonstrate a correlation between cytoplasmic vacuolation and sperm release from storage sites, but this could not be substantiated in *H. frenatus*. One additional observation that supports the role of hormonal control in preparing the oviduct of *H. frenatus* for egg passage is that similar changes in the thickness of the oviductal epithelium, which are directly related to the ovarian cycle, have also been noted in the lizard, *Calotes versicolor* (Kumari, *et al.*, 1990).

The problem of sperm maintenance during storage would, in the present study, appear to implicate both physical and chemical mechanisms. Sperm density, head association with membranous extensions at the blind end of storage crypts, and a narrow luminal passageway may contribute mechanically to sperm maintenance during storage. The protrusion of crypt epithelial cells into the crypt lumen (Fig. 6C) may be equivalent to the “blebbing” observed by Palmer and Guillette (1990) in the tortoise, *Gopherus polyphemus*.

A sperm recycling hypothesis is proposed based upon the following series of events: Oviductal distension during egg passage, as a means for sperm release from storage, is supported by scanning electron microscopy in this study. In conjunction with this, microvilli and other membrane extensions presumably allow for increased distension of the oviduct during egg passage, resulting in the mechanical release of sperm by “loosening” the physical restraints on the sperm heads within storage crypts. Once released from storage, sperm may directly contact potential metabolites (PAS-positive material), becoming activated and capable of fertilizing the passing egg (Fig. 8A). As the egg continues posteriorly, the sperm are again restricted to the storage region by infundibular mucosubstances anteriorly. As the epithelium collapses back on itself, sperm are resequenced within the epithelial foldings, away from luminal secretions (Fig. 8B). In this way, the sperm are

again mechanically (and perhaps metabolically) inactivated and stored until the next ovarian cycle.

Fig. 8A, B. Schematic of sperm recycling hypothesis (see pages 56-57 for description of events). A. Egg entrance into oviduct following ovulation. B. Continued posterior egg passage. E = egg, S = sperm, blue = infundibular mucosubstances, pink = PAS-positive material.



CHAPTER THREE

Physiology of *Hemidactylus frenatus* Sperm: Results of Pilot Experiments

INTRODUCTION

Both the reptilian oviduct and epididymis exhibit cyclic secretory activity related to the reproductive cycle (Hoffman and Wimsatt, 1972; Conner and Crews, 1980; Halpert, *et al.*, 1982; Depeiges, *et al.*, 1985; Jadhav, *et al.*, 1986; Haider and Rai, 1987; Akhtar, 1988; and Kumari, *et al.*, 1990). In general, the secretions are carbohydrate or mucoglycoprotein in nature, as determined by Periodic Acid-Schiff (PAS) technique (Hoffman and Wimsatt, 1972; Halpert, *et al.*, 1982; Kumari, *et al.*, 1990) or by lectin probes (Depeiges, *et al.*, 1985). PAS-positive material is commonly associated with sperm masses as they are transported through the female reproductive tract (Conner and Crews, 1980; Halpert, *et al.*, 1982; Kumari, *et al.*, 1990). It is presumed that such material is of nutritive or protective value to sperm (Hoffman and Wimsatt, 1972). If oviductal secretions (PAS-positive) are for sperm, sperm should demonstrate the ability to transfer these materials across the plasma membrane or demonstrate changes in motility or survival with regard to the presence or absence of such materials.

Glucose is one of the carbohydrates that react positively to PAS treatment. D-glucose is present within epididymal secretions of the lizards, *Lacerta vivipara* and *Hemidactylus flaviviridis* (Depeiges, *et al.*, 1985; Jadhav, *et al.*, 1986). However, the functional significance of glucose to reptilian sperm in both male and female reproductive tracts is not clear. D-glucose has been shown to be essential for maintaining motility and subsequent fertilizing ability of certain mammalian sperm (Frenkel, *et al.*, 1975; Niwa and Iritani, 1978; Brooks, 1979). Murphy *et al.* (1986) found that D-glucose somewhat decreased the overall motility of guinea pig sperm. When D-glucose and Na-pyruvate were combined, however, good motility and the ability of spermatozoa to undergo the acrosome reaction was maintained.

Sperm are stored in the oviducts of *Hemidactylus frenatus*. Secretory granules within the oviductal epithelium are PAS-positive throughout the ovarian cycle. Their contents are secreted into the oviductal lumen just prior to ovulation. Sperm appear to be immotile (or at least not vigorously motile) while in storage sites (see Chapter 2) but they must be motile at or near ovulation in order to fertilize passing eggs.

The objectives of the following series of pilot experiments were to determine: 1) the presence and location of glucose within the oviduct, 2) the potential metabolic use of glucose by epididymal sperm, and 3) the effect of energy substrates on the motility of epididymal sperm *in vitro*.

MATERIALS AND METHODS

I. Animal collection and general tissue preparation

Hemidactylus frenatus greater than 40mm (females) and 50mm (males) in snout-vent length (SVL) were collected during peak activity hours from residential buildings in Oahu, Hawaii. Following decapitation, each body was placed immediately into ice-cold saline (0.7% NaCl) for 30-60 seconds (to slow reflexive movements that make surgery difficult) and then dissected in fresh cold saline. Reproductive organs were removed and placed in fresh saline or amphibian Ringer's solution for viewing and experimentation.

II. High performance liquid chromatography (HPLC) analysis of glucose

A total of ten oviducts from six females were analyzed. Each oviduct was cut grossly into vaginal, uterine, infundibular, and transitional (sperm storage) regions. Four were cut immediately upon removal from the body; the remaining six were flushed with 1cc 0.7% NaCl prior to cutting. Each sample was frozen, lyophilized, and weighed. Solvents and standards used, as well as sample extraction and HPLC analysis followed the procedure of Higa and Womersley (1993). High performance liquid chromatography analysis for this study differed only in eluant concentration (50 mM NaOH) and flow rate (a constant 0.8 ml/min). Glucose was calculated as percent dry weight values.

III. Membrane transport analysis

1. General procedure: Sperm were collected from four males, for a total of four experimental sperm samples. Each sample was obtained by gently stripping both epididymes and associated vas deferens using forceps, releasing free sperm into 0.5ml of amphibian Ringer's solution. The sperm were gently mixed to create a homogenous sperm suspension. Each suspension was held at room temperature for the duration of all experiments. Prior to experimentation, 20 μ l of the sperm suspension to be tested was removed and viewed with a light microscope at both low and high dry objectives (10X and 40X). Motility and general morphology were assessed. Only samples with greater than 80% motile intact sperm (visual estimate within the field of view for evidence of active progressive swimming motion) were used for the following experiments. Upon completion of all experiments, a second 20 μ l sperm sample from the initial suspension was again analyzed.

2. Protein assay: A Shimadzu UV-160 was used to determine mg/protein in 20 μ l of sperm suspension. A working curve display was obtained using Sigma #540-10 protein standards and compared to a 10-20 μ l sample of sperm solution. Only sperm samples with protein concentrations within the working curve were used.

3. Transport experiments: Two treatments were tested: D-glucose alone and D-glucose plus phloridzin (an inhibitor of Na⁺-dependent glucose transport). Three repetitions per treatment were performed.

a. D-glucose uptake: Two μ l of 0.1mM ³H-D-glucose (New England Nuclear, Boston, Mass.) were oven-dried in 3 clean glass test tubes. Each tube represented a treatment repetition. One hundred seventy μ l of amphibian Ringer's solution containing 0.1mM D-glucose was added to each tube and vortexed. This produced three stock treatment solutions. A 10 μ l sample was removed from each tube, placed into separate scintillation vials, and combined with 3 ml scintillation cocktail for counting. These samples were designated I₀ samples and identified the amount of radioactivity present initially.

Each repetition was carried out at time intervals of 30 sec, 2 min, 5 min, 10 min, 20 min, and 60 min. At time 0, 20 μ l of sperm solution was added to the first stock solution. At each designated time interval, a 20 μ l aliquot was removed and placed into 2 ml of iced Ringer's solution. This was then poured through a 0.22 μ filter (on vacuum) and washed with 4 ml of iced Ringer's solution. The filter was then removed and placed inside a scintillation vial with 3 ml of scintillation cocktail for subsequent counting. The second and third stock solutions were handled similarly. All counts were obtained with a Beckman scintillation counter.

b. D-glucose with phloridzin: Two μl of 0.1mM ^3H -D-glucose was dried into 3 clean test tubes as before. One hundred seventy μl of amphibian Ringer's solution containing 0.1mM D-glucose and 0.5mM phloridzin was added. I_0 and sperm samples were collected as before.

c. Uptake activity was calculated by the equation:

$$\begin{aligned} &\text{CPM (counts/minute) + specific activity (} I_0 \text{ samples)} \\ &+ \text{mg protein/20}\mu\text{l sample} = \text{nmoles activity/mg protein} \end{aligned}$$

Once calculated, uptake was plotted against time.

IV. In situ analysis of sperm motility

Testes, epididymes, and oviductal storage regions were placed separately in cold amphibian Ringer's solution and gently pressed between two clean glass slides. Observations were made using a dark phase-contrast light microscope. Moving objects within samples were judged to be ciliated cells if attached or moving perpendicularly to the epithelium and sperm if free or moving parallel to the epithelium, as suggested by earlier histological information (Chapters 1 and 2).

V. In vitro analysis of anterior and posterior epididymal sperm

Epididymes were cut into anterior and posterior regions. Sperm were stripped from each region, placed into approximately 0.5 ml of amphibian

Ringer's solution and mixed. Each sample was assessed for percent motile, quality of motility, and general morphology by light microscopy.

VI. In vitro analysis of sperm motility over time in various media

Sperm were collected from the posterior regions of both epididymes into 250µl amphibian Ringer's solution and placed in a capped microcentrifuge (Eppendorf) vial for ease of handling and to prevent evaporation. Initial sperm concentrations were approximately $2.0 \times 10^6/\text{ml}$. An initial test sample (20µl) was assessed for overall motility and morphology. Only samples containing 80% or greater motile, intact sperm were used for motility analysis. An additional 250 µl of Ringer's solution was added to samples meeting the criteria and mixed thoroughly. The vial was vortexed briefly to break up clumps and then centrifuged at 1100 rpm for 4 minutes. The supernatant was discarded and the pellet was resuspended in 500 µl Ringer's solution. The sample was vortexed and centrifuged again at 1100rpm for 4 minutes. The supernatant was discarded and the pellet was resuspended in 220µl Ringer's solution and vortexed. Twenty µl final sperm suspension was added to 600µl of test medium (amphibian Ringer's solution, Tyrode's medium, a modified Tyrode's medium (reg mT), or a modified TALP4 medium (mTALP4); Tables (1a-d)). After incubation for 15-20 min at room temperature, aliquots (25-30 µl each) were examined with the light microscope and visual estimates were made regarding the overall percentage of

motile sperm, quality of motility, morphology, and the acrosome reaction. The quality of motility was defined by the following characteristics: grade 1, twitching or only weakly motile (no forward progression); grade 2, moderate “eel-like” movement (forward, non-spiraling progression within a single plane); and grade 3, rapid movement (forward, spiraling progression within one or more planes). Only motile sperm with clearly absent acrosomes were recorded as acrosome-reacted.

VIII. In vitro assessment of the acrosome reaction using acridine orange

Sperm from the posterior epididymis were collected, examined, and mixed with mTALP4 medium as previously described. At 2, 5, and 24h, 0.001% acridine orange was added to sperm subsamples (1:1) which were then examined by fluorescence light microscopy. Acrosomes, if present, fluoresced bright red. In a series of experiments, sperm were exposed to lysophosphatidyl choline (LC; final concentration 85µg/ml) at the end of 24h incubation. Thirty minutes or 2.5h later, sperm were examined for the acrosome reaction using acridine orange.

Table 1a. Composition of Amphibian Ringer's solution.

Component	g/l
NaCl	6.5
KCl	0.14
NaHCO ₃	0.2
CaCl ₂	0.12

mOsm = 240

pH = 7.0

mM NaCl = 110

Table 1b. Composition of Tyrode's medium.

Component	g/l	mM	mOsmol
NaCl	8.0	136.98	273.9
KCl	0.2	2.68	5.4
CaCl ₂	0.2	1.8	5.45
MgCl ₂ · (6H ₂ O)	0.1	0.49	1.47
NaH ₂ PO ₄ · (H ₂ O)	0.05	0.36	0.72
NaHCO ₃	1.0	11.9	23.8
D-glucose	1.0	5.56	5.56
antibiotics, BSA			

mOsmol = 316

pH = 7.4-7.8

Table 1c. Composition of modified TALP4 medium.

Component	mg/100ml	mM
NaCl	729.0	124.8
KCl	20.0	2.68
CaCl ₂	20.0	1.8
MgCl ₂ · (6H ₂ O)	10.0	0.49
NaH ₂ PO ₄ · (H ₂ O)	5.0	0.36
NaHCO ₃	100.0	11.9
D-glucose	81.0	4.5
Na-lactate (60% syrup)	0.15 ml	(9.0)
Na-pyruvate	11.0	1.0
hypotaurine	5.5	(0.5)
L-epinephrine	0.9	(0.05)
BSA	15. mg/ml	
Penicillin G/ streptomycin	100. IU/ml/50µg/ml	

mOsmol = 310

pH = 7.5

Table 1d. Composition of modified Tyrode's medium (reg mT).

Component	mg/100ml	mM
NaCl	635.2	108.76
KCl	20.0	2.7
CaCl ₂	22.2	2.0
MgCl ₂ · (6H ₂ O)	10.0	0.49
NaHCO ₃	210.0	25.07
D-glucose	100.0	5.56
Na-lactate (60% syrup)	0.15 ml	(9.0)
Na-pyruvate	11.0	1.0
BSA	3. mg/ml	
Penicillin G	100. IU/ml	

mOsmol = 308

pH = 7.5

IX. In vitro assessment of energy source-variable mT on sperm motility, survival, and the acrosome reaction

Energy source-variable media were prepared by omitting one or more of the energy sources (D-glucose, Na-pyruvate, and Na-lactate) available in reg mT such that a total of eight combinations (Table 3) were prepared. Constant osmolarity (about 308 mOsmol) was maintained by adjusting the concentration of NaCl. Sperm were prepared and added to each test medium as previously described. Aliquots (25-30 μ l each) were examined as before for overall percentage of motile sperm, quality of motility, morphology, and the acrosome reaction.

Table 2. Glucose content (HPLC analysis) within various regions of the oviduct (*H. frenatus*, n = 6).

Sample SVL (mm)	Ovarian Condition	Flushed +/-	Left/ Right	Glucose Values (% dry weight) Oviductal Region*			
				I	SS	U	V
S9206	Follicular	-	L	0.131	0.168	0.04	N/A
(45)		-	R	0.106	0.063	0.02	0.012
S9207	Follicular	-	L	0.114	N/A	0.09	N/A
(42)		-	R	N/A	0.14	0.08	N/A
S9211	Follicular	+	L	0.31	0.30	0.06	0.06
(43)		+	R	0.50	0.058	0.13	0.17
S9209	Vitellogenic	+	L	0.345	0.10	0.08	0.65
(46)		-	R	N/A	N/A	N/A	N/A
S9210	Vitellogenic	-	L	N/A	N/A	N/A	N/A
(48)		+	R	0.127	0.075	0.06	0.023
S9208	Luteal	+	L	N/A	0.085	N/A	0.101
(44)		+	R	0.14	0.20	0.08	0.04

* I = infundibular; SS = sperm storage; U = uterine; V = vaginal

Table 3. Quality of sperm at 8 hr after transfer to test medium
(summary of 3 experiments using 3 different males).

Test Media			Mean % Motile among Total	Quality of Motility Range (mean)	Acrosome Reaction among Live Sperm
Energy Sources Present					
Glucose	Pyruvate	Lactate			
+	+	+	55	2-3 (2.3)	0
-	-	-	63	1-3 (1.7)	0
-	-	+	62	1-2 (1.8)	0
+	-	-	54	1-3 (1.9)	0
-	+	-	67	1-3 (2.1)	0
+	+	-	64	1-2 (1.8)	0
-	+	+	67	1-2 (1.4)	0
+	-	+	59	2-3 (2.2)	0

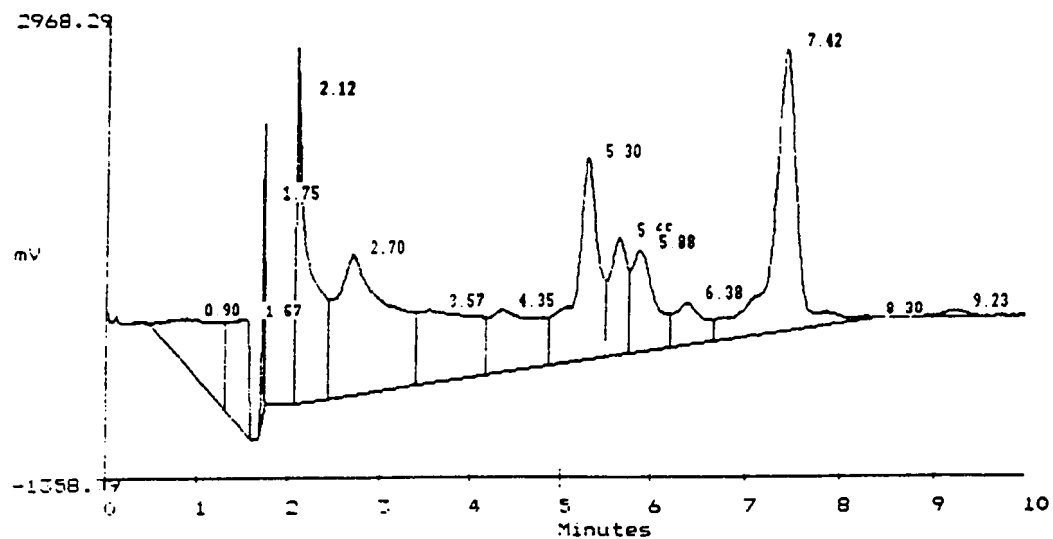
RESULTS

Glucose was present throughout the oviduct, along with other unidentifiable putative carbohydrates, regardless of ovarian condition. Glucose values ranged from 0.012 - 0.345 percent dry weight (Table 2). Representative graphic analysis from the oviduct of one female is depicted by the Figure 9.

Enough sperm to complete three uptake repetitions were obtained from two of the four males used. Figures 10A and 10B demonstrate glucose uptake by sperm from the two males. For each repetition, both glucose and glucose-phloridzin are graphed together. With few exceptions, sperm samples presented with D-glucose alone demonstrated greater glucose uptake than those presented with D-glucose plus phloridzin. An initial "overshoot" of glucose uptake within 2-5 minutes frequently appeared in sperm presented with glucose alone. Sperm in the original suspension were still motile at the end of all experiments.

Sperm released from the testes between glass slides were primarily immotile with a few weakly motile. Sperm released from the epididymis, on the other hand, quickly exhibited vigorous motion. Oviductal sperm viewed between glass slides did not exhibit motility although many epithelial cells showed active ciliary movement.

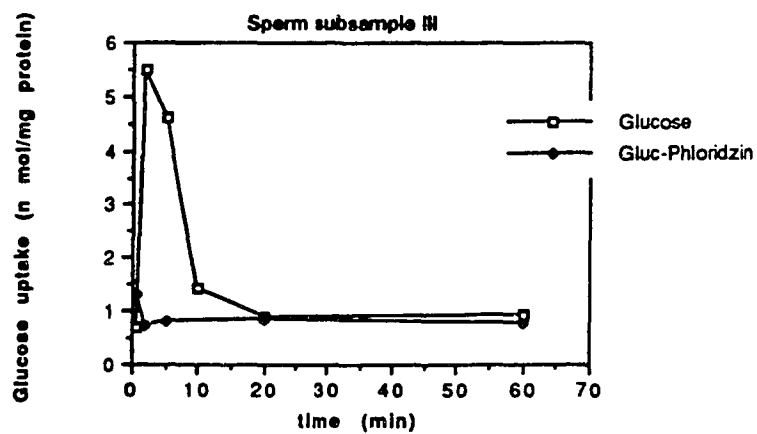
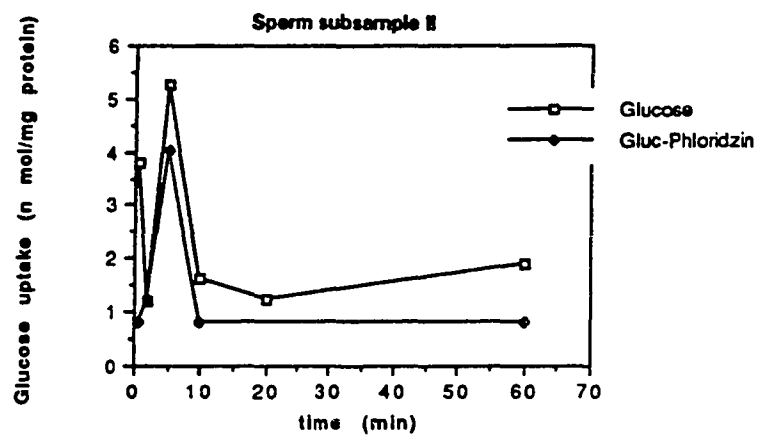
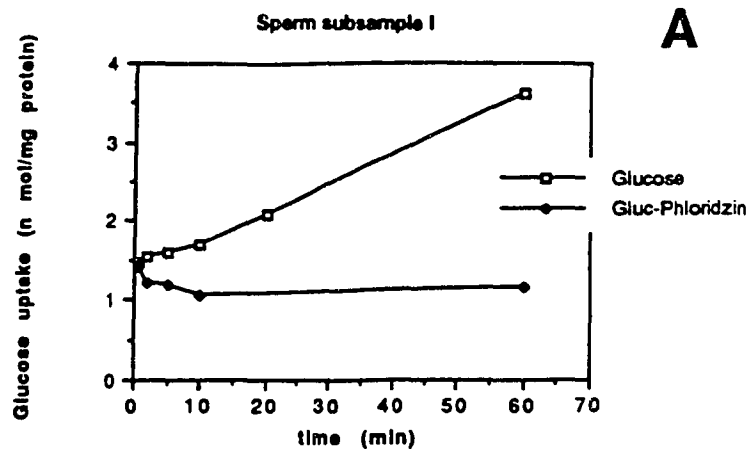
Fig. 9. Oviductal glucose content from the left sperm storage region of one female *Hemidactylus frenatus*.



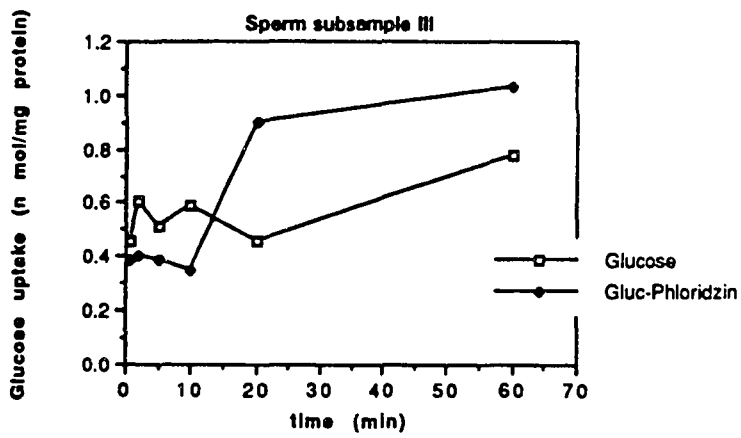
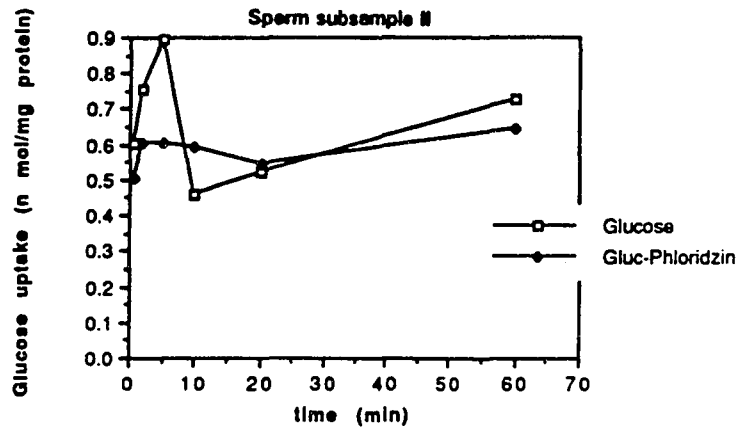
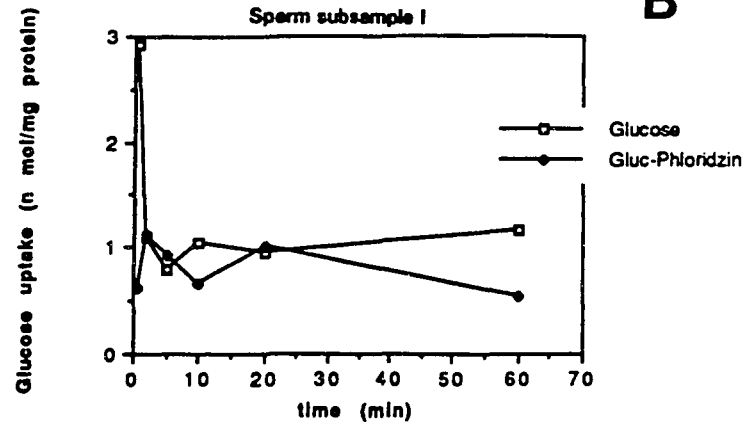
Peak No.	Ret Time	Peak Name	Conc. $\mu\text{g}/\text{ml}$	Area	Height	BL	Ref. Peak	% Delta Ret Time
1	0.90		0.000e+000	2.108e+007	429111	2		
2	1.57		0.000e+000	1.245e+007	1050434	2		
3	1.75		0.000e+000	3.876e+006	2042521	1		
4	2.12		0.000e+000	2.914e+007	2874046	2		
5	2.70		0.000e+000	5.332e+007	1315345	2		
6	3.57		0.000e+000	2.797e+007	690739	2		
7	4.35		0.000e+000	2.097e+007	602899	2		
8	5.30	glucose	2.793e-004	3.354e+007	1861327	2	1	-0.56%
9	5.65		0.000e+000	1.308e+007	1092672	2		
10	5.88		0.000e+000	1.634e+007	951846	2		
11	6.38		0.000e+000	8.199e+006	408901	2		
12	7.42		0.000e+000	4.767e+007	2629677	3		
13	9.23		0.000e+000	8.166e+005	53198	1		

**Fig. 10A. Glucose uptake by epididymal sperm (*Hemidactylus frenatus*).
Male #1**

A



**Fig. 10B. Glucose uptake by epididymal sperm (*Hemidactylus frenatus*).
Male #2**

B

The anterior epididymis contained more cellular “debris” than the posterior epididymis. The incidence of cytoplasmic droplets and “clumping” of sperm were more common in the anterior epididymis than in the posterior epididymis.

Regardless of the combination of energy sources, epididymal sperm exhibited vigorous progressive movement upon dispersal into the medium. In amphibian Ringer’s solution the percent motile and quality of motility were low after 8 h of incubation. Many sperm appeared dead (non-motile with degenerating acrosomes as indicated by a needle-like appearance at the tip of the nucleus) while others were only weakly motile. In Tyrode’s, reg mT, and mTALP4, on the other hand, the majority of sperm were motile and exhibited vigorous movement even up to 24 hours after the start of incubation. Subjectively, sperm survival and motility were greatest in reg mT and mTALP4, media containing three energy substrates (glucose, Na-pyruvate, and Na-lactate) as well as bovine serum albumin.

When stained with acridine orange and viewed by fluorescent light microscopy, heads of live sperm fluoresced blue-green in color; those of dead sperm appeared red-orange. Acrosomes also appeared red-orange in both live and dead sperm. The heads of non-motile sperm incubated in Tyrode’s solution for 24 hours all appeared red. The heads of 50-100% of the sperm incubated for the same time period in mTALP4 fluoresced blue-green; those of remaining

sperm fluoresced red-orange. Because the acrosome always fluoresced red, a distinction could be made between acrosome-reacted and non-reacted live sperm. Lysophosphatidyl choline, added to the sperm suspension after 24h of incubation, could not induce the acrosome reaction in living sperm.

The effects of the energy source-variable media on sperm motility and survival are summarized in Table 3. All samples demonstrated great variability in response to each test medium. Under no conditions were live acrosome-reacted sperm seen.

DISCUSSION

The percent dry weight of glucose within oviducts varied from one region of the oviduct to another. Variations were also evident among females and even between the left and right sides of the same region of the oviduct of one individual. Several possibilities for this are apparent. First, only six females were used in this study; larger samples may be needed to demonstrate significant differences. In addition, ovarian conditions were not the same for all females studied. Ultrastructural analyses (Chapter Two) revealed that secretory cells reacting positively to Periodic Acid-Schiff treatment secrete their contents into the oviductal lumen just prior to ovulation. Some material secreted into the oviductal lumen may have been lost during tissue processing. Only half of the oviducts were “flushed” prior to regional dissection, resulting in further potential

variation in glucose content. Secondly, while glucose is a possible component of the material reacting positively with PAS treatment, it is probably not the major component of the PAS-positive material in the oviduct. Other unidentifiable potential carbohydrates present may be contributing to the material indicated. The change of an electron-dense material to “vacuolated” material within the oviductal secretory cells (Chapter Two) seems to reflect a change in overall composition of the material within secretory cells.

Results of sperm glucose uptake experiments were also quite variable from male to male and within the same sperm suspension. Again, the sample size was small and, under my experimental conditions, sperm tended to clump together rather than remain as isolated single cells. Sperm were still motile at the end of all experiments indicating that amphibian Ringer’s solution was not deleterious to lizard sperm and that the time frame required to complete all experiments was not detrimental to the sperm.

Glucose transport into cells is enhanced by a Na^+ -dependent carrier transport mechanism. A primary active transport mechanism (Na^+K^+ -ATPase) sets up a Na^+ gradient such that Na^+ continually crosses the plasma membrane or enters the cell. Glucose entry is coupled to Na^+ entry, using the Na^+ gradient as a driving force (Kinne and Murer, 1976). The initial overshoot (Fig. 9a,b), represents the driving force for glucose uptake based on an actively maintained Na^+ gradient. In the absence of this driving force, a more gradual slope would

have appeared, representing simple or facilitated diffusion not coupled to an energy source. As carrier proteins are satiated with glucose, the slope is reduced, reflecting the finite number of carrier proteins available for transport.

Phloridzin, an inhibitor of Na⁺-dependent glucose transport, works by effectively blocking glucose carrier proteins. This may explain why sperm exposed to glucose-phloridzin-containing solutions demonstrated lower glucose uptake values.

While the data from the present study suggest that *H. frenatus* sperm may be capable of utilizing glucose via a Na⁺-dependent carrier transport mechanism, the results are inconclusive. Several factors must be taken into consideration when reviewing this initial data set. First, the uptake values for sperm collected from each male greatly differ. This may represent individual variation from one male to another. A much larger sample size would therefore be required. Secondly, epididymal fluid was unavoidably collected with sperm. The chemical composition of such fluid is unknown. If glucose is already present within the epididymal fluid, sperm may not be properly “challenged” by the presence of glucose in experimental solutions. In addition, glucose may not be the only carbohydrate source present or primarily used by sperm. Thirdly, the “age” of the sperm must be considered. Sperm samples used several hours after collection may be “starved” of epididymal components and therefore, transport labeled materials more rapidly. Initial stock populations of sperm were

maintained at laboratory room temperature throughout the experimental period; a condition allowing for continued cellular metabolism, even if at slower than normal rates.

Sperm are immotile within the testis and perhaps within the epididymis as well. Sperm must be motile after being deposited in the female reproductive tract, but in the sperm storage region of the oviduct, motility may be suppressed. High sperm density within the epididymis and the oviductal sperm storage crypts may temporarily immobilize sperm.

That sperm from the posterior epididymis had fewer cytoplasmic droplets and maintained progressive motility longer in higher numbers than those of the anterior epididymis suggests that sperm maturation occurs within the epididymis of *H. frenatus* as in a variety of other reptiles (Bedford, 1979; Depeiges and Dacheux, 1984). It has been reported that the percent motility-competent sperm increases from proximal to distal regions of the epididymis in the lizard, *Lacerta vivipara* (Depeiges and Dacheux, 1984) and the water snake, *Natrix spenodon* (Bedford, 1979).

Exogenous energy sources seem to be necessary after sperm release from storage, as sperm must certainly be mobilized to fertilize the passing egg. What energy substrates are utilized by or available to reptilian sperm during this period is unknown. The *in vitro* effects, if any, of D-glucose, Na-pyruvate, or Na-lactate on sperm motility and survival were not clear cut in the present study. In

no case was an acrosome reaction detected in living sperm. More tests would have to be performed before knowing whether or not the response (or lack of) seen in this study was due to the procedure or to characteristics of reptilian sperm.

CONCLUSION

The capacity for long-term sperm storage and survival within the oviduct of *Hemidactylus frenatus* has been verified by this study (Chapter One). Certain reproductive, behavioral, and distributional characteristics of *H. frenatus* seem to make oviductal sperm storage of advantage to the long-term survival of these reptiles.

Males contain sperm throughout the year (pers. obs., Church, 1962). Females exhibit variable egg production intervals, produce at most only two eggs per clutch, and are not synchronized cyclically (Chapter One). During collection periods contributing to this study, I noticed spatial separation of males and females. On some occasions, only males were located at a majority of feeding sites, while at a later time period on the same evening, females and juveniles, or a mix could be seen at the same sites. While a 1:1 ratio of adult males and females could be captured on any given evening, additional time (or a search of different sites) was often necessary in order to obtain the required number of both sexes. Males may spend a great deal of time patrolling or defending sites from other males, essentially ignoring females except for brief copulation periods (Petren, 1994). Such temporal and spatial separation of the sexes could be reproductively "dangerous" in that females ready to ovulate might not encounter a male. Thus, oviductal sperm storage would be advantageous.

Little is known about the mating behavior of these animals. Histological evidence obtained during the present study (Chapter One) suggests that multiple mating may occur in *H. frenatus*. Because stored sperm could not be traced back to the male(s) involved, it was not possible to determine whether the sperm seen in histological preparations came from one or more males (or mating periods). Mating does appear to be somewhat opportunistic, however, with no evidence supporting courtship behavior (pers. obs.). Whereas Petren (1994) observed copulations in his study, his animals were essentially “captive”, being restricted to a particular area (even if a relatively large area). This may have increased the frequency of male-female encounters. Opportunistic (perhaps multiple) mating, in conjunction with oviductal sperm storage, would allow for the acquisition of mature sperm regardless of the immediate ovarian condition of the female and offset temporal and spatial distribution differences between the sexes.

Approximately 800 species within the Family Gekkonidae exhibit worldwide distribution, with at least 70 species within the genus *Hemidactylus* alone (Seufer, 1991). It has been suggested that *H. frenatus* arrived in Hawaii with humans around the time of World War II (McKeown, 1978). These reptiles possess several characteristics that enhance this type of dispersal. The lizards are small, primarily nocturnal, and readily reside within and around human habitations, making them prime for dispersal as a

result of human activities. Oviductal sperm storage enhances colonization by offering newly-arrived females the opportunity to reproduce in the absence of immediately available mates. In addition, eggs produced by *H. frenatus* are calcareous-shelled, preventing desiccation. A female storing sperm could, in mid-voyage, produce a clutch of eggs that would survive transportation.

Oviductal sperm storage also offers an opportunity to delay reproduction until suitable environmental conditions are met. Appropriate environmental conditions in Hawaii (relatively stable temperatures, ample food and water supply, and minimal predation), in conjunction with oviductal sperm storage, have allowed these animals to survive and populate the islands in large numbers.

The advantages offered by oviductal sperm storage to *H. frenatus* seem to result from the morphological and physiological design of the oviduct itself. The morphology of the crypts, as described in Chapter Two (blind-ended, narrow entrances, frequently branched, etc.), seems designed for mechanical restraint of sperm. Distension experiments (Chapter Two) have demonstrated that sperm may be released from the crypts as eggs pass through the oviduct. The location of the crypts allows for fertilization of the passing egg by released sperm before the egg contacts the shell glands of the uterus. While the composition of secretory granules within the oviductal epithelium is unclear at present, the contents of the granules are secreted

just prior to ovulation and may be utilized by sperm prior to fertilization (Chapter Three). These morphological and physiological characteristics of the oviduct provide for the possibility of sperm storage; a situation allowing *H. frenatus* almost guaranteed reproductive success.

Despite the verification of oviductal sperm storage in *H. frenatus*, there are many questions left unanswered that could further describe the derived benefits. Do multiple copulations occur? Do copulations involve more than one male? Does mating occur prior to a female's first ovulation? With regard to the mechanism involved in oviductal sperm storage, each of the pilot experiments described in Chapter Three offers an opportunity for further characterization of the chemical nature of the oviduct, as well as sperm physiology.

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