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MALE-INDUCED STIMULATION OF EGG LAYING IN THE HOUSE CRICKET

The Ohio State University

Ph.D. 1980

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MALE-INDUCED STIMULATION OF EGG LAYING

IN THE HOUSE CRICKET

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

by

Michael P. Murtaugh, B.S.

* * * *

The Ohio State University

1980

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INTRODUCTION

House crickets (*Acheta domesticus* (Linnaeus)) are in the Family Gryllidae, an ancient group which arose in the Jurassic Period or earlier (Zeuner 1939, in Alexander 1968). They originated in North Africa or Western Asia and now exist throughout Europe, in Africa, Asia, and the United States in association with human habitations (Chopard 1938). Since this early beginning, house crickets have developed an elaborate panoply of acoustical signals and tactile cues into which courtship and mating have been integrated, and have complemented this with physiological machinery highly specialized in both male and female for a large and long-term reproductive effort. Thus the process of reproduction in the house cricket combines behavioral interactions and physiological phenomena to a degree that is unique among insects.

A. Courtship

Male crickets stridulate using their tegmina to communicate acoustically, and utilize this mechanism in their intraspecific interactions, including sexual behavior (Alexander 1961). Females will respond and move toward a stationary male emitting its shrill, high pitched calling song with the tegmina arched to an angle of about 45° over the abdomen. When the female locates the calling male, they antennate mutually, the female becomes immobile, and the male switches to the mating song, a distinctive, soft, noise-like chatter. The wings are lowered roof-like over the tegmina and scraped together with much less pressure (Alexander
1961, Khalifa 1950). A receptive female (Stout et al. 1976, Crankshaw 1979) mounts the male or the male will ease backwards under the female, she lowers the tip of her abdomen, and the male seizes the bursa copulatrix (copulatory papilla in the terminology of Alexander and Otte 1967) with his epiphallic hook, extrudes a spermatophore and attaches it to the female. The capillary tube is directed into the spermathecal duct by the male's guiding rod and the spermatophore handle is held to the base of the ovipositor by the female's subgenital plate (Alexander and Otte 1967). When this process is completed in 3-5 minutes, the male releases the female and they disengage. The male then turns to guard the female for an indeterminate length of time with a characteristic "watching" behavior (Khalifa 1950), presumably to protect the spermatophore from being dislodged or eaten until sperm have been evacuated.

B. Sperm transfer

Sperm transfer in crickets is uniquely difficult, owing to the external attachment of the spermatophore to the female. The spermatophore must provide its own propulsive force and supply a viable, sealed environment for sperm, yet be rigid enough to resist desiccation and abrasion while being carried by the female. Sperm and seminal fluid are incorporated into the ampulla of the spermatophore and surrounded with a thick, hard proteinaceous coat (Fig. 1). At its anterior end the ampulla opens into an extremely thin capillary tube. The posterior end contains a pressure body consisting of two symmetrical translucent masses enclosed in separate membranes. Beyond that, between the inner and outer layer of the ampullar wall, is the evacuating fluid.
Figure 1

A. External appearance of the spermatophore of Acheta domesticus (L.).
B. Horizontal section through the ampulla. Redrawn from Khalifa (1949).
When a spermatophore is attached to the female, the evacuating fluid diffuses across the inner layer to the pressure body. The difference in pressure between these two fluids is great (480 mOsm for evacuating fluid and 1646 mOsm for pressure body material, Khalifa 1949) and the resultant influx causes the pressure body membranes to rupture. The pressure body swells, forcing the sperm and seminal fluid through the capillary tube into the female. The sperm then migrate from the bursa copulatrix, through a long, convoluted spermathecal duct to the spermatheca, where they are stored.

C. Male reproductive physiology

The developmental and physiological events in the male that culminate in mating start early in nymphal life. Spermatogenesis begins in about the seventh instar (Tessier and Pallotta 1973) and mature sperm can be found in the last (tenth) instar nymph. Spermatocyte development persists throughout adult life. Regulation of sperm development in the house cricket has not been studied, but evidence from many other insects suggests that ecdysterone accelerates the rate of spermatocyte division and differentiation, while juvenile hormone retards testicular growth (Dumser 1980).

At three to four days after the adult molt, mature sperm begin to migrate from the testes to the seminal vesicles and at this point they are competent for delivery to the female. Nuclear organization and biochemical differentiation during sperm maturation have been thoroughly characterized (Kaye 1962, Tessier and Pallotta 1973, Pallotta and Tessier 1976, Tres and Kierszenbaum 1976, Kierszenbaum and Tres 1978, McMaster-Kaye and Kaye 1976, Kaye et al. 1978).
The plasma that accompanies sperm from the testes to the seminal vesicles also includes prostaglandin synthesis enzymes and various prostaglandins (Destephano et al. 1974, 1976, Destephano and Brady 1977). Prostaglandins modulate numerous hormonal and pharmacological actions and are especially active in reproductive processes (Samuelsson et al. 1978, Karim 1975). The discovery of prostaglandins in insect reproductive tissue, particularly in the house cricket, is provocative and opens up much of the older literature relating to male-induced stimulation of ovulation and oviposition in insects to new interpretations.

In the last nymphal instar, small buds appear on the surface of the accessory gland body. Following adult eclosion, the gland initiates massive differentiation culminating in a large, polytubular structure highly specialized for protein export (Kaulenas et al. 1975, Kaulenas 1976, Kaulenas et al. 1979, Pustell 1979). At six days the gland is sufficiently differentiated to discharge protein for spermatophore formation. By this time, remarkable amounts of cyclic guanosine-3',5'-monophosphate (cyclic GMP) have also accumulated in the accessory gland (Fallon and Wyatt 1975a,b, 1977a,b, Pustell 1979).

Cyclic GMP appears to be ubiquitous in animal tissues and functions in the regulation of many biological processes (Goldberg et al. 1973, 1975, Goldberg and Haddox 1977). In insects it participates in several aspects of pupal and adult development (Filburn and Wyatt 1976, Bodnaryk 1975, Delinger and Wingard 1978). Fallon and Wyatt (1975b, 1977a) propose in the house cricket a role in sperm maturation or motility. The occurrence of prostaglandins and cyclic GMP in adjoining tissues of
the male reproductive system is tantalizing in view of their interactions in other organ systems (Kuehl 1974, Kuehl et al. 1976).

The male house cricket matures sexually at about six days after adult eclosion: the seminal vesicles are filled with sperm and the accessory gland is synthesizing the full complement of proteins required for spermatophore formation. The nature of the hormonal and neural mechanisms regulating the development of the male reproductive system is not known. The control of spermatophore formation is likewise poorly understood. This process follows a circadian pattern (McFarlane 1968, Loher 1974), and thus may be regulated by release of 5-hydroxytryptamine from the brain, as Cymborowski (1973), Cymborowski and Muszynska (1974) and Muszynska-Pytel and Cymborowski (1978a,b) have hypothesized for circadian locomotory activity.

D. Female reproductive physiology

Unlike males, female house crickets begin reproductive development only after the imaginal molt. Belyaeva (1966, 1967) demonstrated by extirpation and reimplantation experiments that ovarian development and egg production require juvenile hormone. Eggs fail to mature following allatectomy, while implantation of corpora allata into an intact animal accelerates and intensifies oviposition. Removal of the ovaries resulted in an enlarged corpora allata. Juvenile hormone presumably acts by inducing synthesis of yolk proteins, as it does in many insects (see reviews by Wyatt 1972, Hagedorn and Kunkel 1979, and Engelmann 1979). The ovaries may also play a modulatory role since the net rate of vitellogenin synthesis is reduced in the absence of ovarian tissue (Bradley and Edwards 1978). Endocrine control of oogenesis also
appears to involve secretions from the subesophageal ganglion (Huignard 1964, Thomas 1964): implantation of the ganglion augments the onset of egg laying.

Precise knowledge of many other aspects of house cricket sexual maturation are lacking. We know little of the processes leading to competence of the spermatheca for storing sperm or the acquisition of mating competence. It has been noted, however, in females that are sexually mature, responsiveness to courting males varies and is mediated by the corpora allata (Stout et al. 1976). Crankshaw (1979) also found that females exercise choice in responding to calling males. Control of sexual receptivity by the corpora allata has been demonstrated in other species of insects (Manning 1967, Engelmann and Barth 1968).

E. Oviposition stimuli in insects

The variety of stimuli that evoke oviposition by female insects is manifold. In a few of the species that have been studied, oviposition is not dependent on an association with a male. Schistocerca gregaria (Hamilton 1955), Gomphocerus rufus (Loher and Huber 1964) and Pyrrhocoris apterus (Slama 1964, Zdarek 1970) lay as many eggs in the virgin state as in the mated state. Likewise virgin Cochliomyia hominivorax lay nearly the same number of eggs as mated females (Crystal and Meyners 1965). Porthetria dispers (Schedl 1936) and Anthonomis grandis (Mayer and Brazzel 1963) lay eggs as virgins, but the ovipositional behavior is highly aberrant.

In certain cockroaches, the mechanical stimulation of spermatophore deposition triggers the mating syndrome (Engelmann 1959, Roth and Stay 1964) while in the tsetse fly, Glossina morsitans, the copulatory act
itself constitutes the signal (Saunders and Dodd 1972, Chaudhury and Dhadialla 1976).

In the great majority of species, however, some factor transmitted from the male to the female induces or markedly increases egg deposition by the female. Frequently, no attempt is made to deduce the nature of the stimulus (Mundall 1978, and many others in Engelmann 1970, p. 195). In those cases in which it has been examined, however, the source of the oviposition stimulus is found in the testes or accessory gland.

Frequently, females mated to castrated males retain the virgin condition, failing to accelerate the deposition of eggs (Regen 1910 with Gryllus campestris, Davey 1965 with Rhodnius prolixus, Truman and Riddiford 1971 with Hyalophora cecropia, Loher and Edson 1973 with Teleogryllus commodus, Karpenko and North 1973 with Trichoplusia ni). Some authors (e.g., Truman and Riddiford 1971) have inferred from this type of experiment that sperm are the oviposition stimulant. Irradiation experiments with various Lepidoptera (Acrolepiopsis assectella, Thibout 1969, 1979; Heliothis virescens, Flint and Kressin 1969; Pectinophora gossypiella, LaChance et al. 1978) corroborate a putative role for sperm in triggering oviposition.

In many other insects, male accessory gland secretions promote egg laying (Leopold 1976). In Schistocerca gregaria (Leahy 1973) and Melanoplus sanguinipes (Pickford et al. 1969, Friedel and Gillott 1976), implantation of select accessory gland tubules into virgins evokes egg release. In two Lepidoptera, Zeiraphera diniana (Benz 1969) and Trichoplusia ni (Karpenko and North 1973), accessory secretions facilitate maximum egg deposition. Accessory gland enhancement of
oviposition is perhaps most prevalent among the Diptera. A "sex peptide" from male paragonia has been identified as the oviposition stimulant in *Drosophila melanogaster* (Kummer 1960, Garcia-Bellido 1964, Leahy 1966, Chen and Buhler 1970). A similar accessory gland product has been found in the house fly (Riemann and Thorson 1969), *Drosophila funebris* (Baumann 1974a,b), the cabbage maggot (Swailes 1971) and several mosquitoes (Leahy and Craig, 1965, Leahy 1970, Ramalingam and Craig 1976).

In the house cricket, prostaglandins are reported to trigger oviposition (Destephano and Brady 1977). Prostaglandins participate in regulating numerous events in vertebrate reproduction, including parturition (Karim 1975, Thorburn and Challis 1979); hence, this first report of a physiological role for prostaglandins in an arthropod is extremely interesting and requires validation. Oviposition in most insects depends on products from the accessory gland or testes and in the cricket, cyclic GMP is abundant in the accessory gland and prostaglandins occur in high levels in the testes. The putative interactions of cyclic nucleotides and prostaglandins (Kuehl 1974, Kuehl et al. 1976), their close proximity in the male reproductive tract, their regulatory functions in a wide variety of physiological processes, and the possible role of prostaglandins in oviposition endow the events of cricket reproduction, including oviposition, with great potential for elucidating specific functional roles for cyclic GMP, prostaglandin E₂ and prostaglandin F₂α in invertebrate reproduction.
In this study, I determine the basic parameters that characterize the egg-laying response of a female house cricket to mating, describe the nature of the oviposition stimulus contributed by the male, including its origin and the importance of sperm and prostaglandins, examine the movement and destination of cyclic GMP, and identify the target tissue in the female which receives and transmits the egg laying signal. The mechanism that functions in the house cricket is compared to strategies governing ovipositional regulation in other insects, and the consequences of this mechanism are considered.
A. Food

Development of a reliable rearing technique was essential to insure a ready supply of physiologically uniform and healthy adults for experimentation. Crickets are highly sensitive to nutritional deficiencies, and much research has been devoted to selecting diets for maximizing growth and development (McFarlane et al. 1959b, McFarlane 1972, 1978c, Patton 1967, 1978, Clifford et al. 1977). In the present procedure, all life stages were provided a diet consisting of 95% rodent lab chow (Ralston Purina, St. Louis) and 5% liver powder (United States Biochemicals, Cleveland). Since food particle size exerts selection pressure on house crickets, especially affecting growth rate and total reproduction (Tennis et al. 1979), the lab chow pellets were initially ground in a Wiley mill to pass through a 1 mm mesh screen (Clifford et al. 1979); later it was purchased in a finely ground meal form that provided particle sizes suitable for all ages. Liver powder was then mixed in and the diet stored at -20°C until use. The basic ingredients of this diet, the crude levels of protein, fat and carbohydrate, and the elemental analysis were comparable to those available in the literature cited above.

Free-standing water was made available to nymphs and adults at all times. Details of the method of supply to nymphs or adults are given below. Free-standing water was preferred since even slight water
deprivation due to the use of cotton wicks causes marked changes in hemolymph composition (Clifford et al. 1977).

B. Conditions for rearing nymphs

Nymphs were placed in plastic boxes 31 x 25 x 10 cm with plastic lids soon after hatching. The standard diet was supplied in a 10 x 100 mm petri dish lid ringed with masking tape and water was provided by inverting a notched plastic vial (3.5 x 8 cm) filled with distilled water in a 15 x 100 mm plastic petri dish filled with washed pea gravel. The dish was ringed with masking tape to enable small hoppers to climb up the sides or jump to the water without drowning. At first, the density was maintained at about 0.4 nymphs/cm² of box floor (Patton 1978). Later, density was monitored by experience, and box populations were reduced when signs of overcrowding (e.g., rapid food depletion, excessive fouling of the water dish, obvious accumulation of feces) appeared.

The rate of growth of house cricket nymphs appears to be enhanced by group rearing (Federhen 1955, Chauvin 1958, McFarlane 1962, 1978b, Kieruzel 1976, Johnston and McFarlane 1973, Patton 1978), although Bate (1971) and Ghouri and McFarlane (1958) found no such positive effect. The point at which nymphal density becomes inimical for optimum growth is not clear (viz., compare Chauvin 1958, Tennis et al. 1977 and Patton 1978); nevertheless, under my conditions of temperature and humidity the principal factors limiting rate and extent of development were availability of food and water.

Shelter for nymphs was provided with the bottom half of a cardboard egg carton. The presence of a shelter, such as an egg carton or
folded corrugated cardboard, improves survival (Ghouri and McFarlane 1958) and satisfies innate photo- and thigmokineses (Federhen 1955, Kieruzel 1976). In daylight hours, nymphs were always hidden in the interior surfaces of the carton away from the light.

The nymphs were maintained in an incubator at 30-32°C and 12 hr light, 12 hr dark. The sealed lids provided young nymphs with high humidity. By the third or fourth instar these were exchanged for lids with screening to lower humidity (Clifford et al. 1977). Food and water were usually replaced three times each week. Under these conditions nymphal development lasted 40-50 days. The number of molts was never determined, but is presumed to be 8-10 (Patton 1978, Clifford et al. 1977) though it can range from 7 (Ghouri and McFarlane 1958, Clifford et al. 1977) to 14 (Bate 1971).

C. Conditions for maintaining adults

When nymphs reached the last instar or when the first adults appeared, the crickets were transferred to a large plastic rodent cage (45 x 23 x 21 cm, Fisher Scientific), covered with aluminum window screen, in an environmental chamber at 25±1°C and 12 hrs light, 12 hrs dark. Humidity varied from 30-90% in concert with prevailing weather conditions. Food was supplied in a plastic petri dish 10 x 100 mm and water was available in a pint-size chick watering device (Brower Manufacturing, Quincy, Illinois) whose trough was filled with washed pea gravel. Surface area in the cage was again increased by adding egg cartons or corrugated cardboard. Adults were removed every 1-3 days, isolated by sex, and retained for experiments, or added to the breeding colony.
The breeding colony was maintained in a glass terrarium 61 x 33 x 30 cm with egg carton shelters, food, and water in the chick watering device.

D. Egg collection

Crickets in laboratory culture classically have been provided with moistened sand as an oviposition substrate (Browning 1953, McFarlane et al. 1959a, Hogan 1960, Thompson 1977, Clifford et al. 1977, Loher 1979). Recently, Patton (1978) suggested a peat moss-vermiculite mixture. In the present study, it was crucially important to present females with a desirable oviposition source, then to recover the eggs quantitatively and reproducibly. The techniques for separation from sand rely on egg flotation in an osmotically dense solution such as saturated ducrose, but this type of recovery was unpredictable and the procedure was extremely messy. Peat moss-vermiculite was also unsuitable since all attempts to separate the mixture from the eggs left the eggs in a brown soup.

I developed a method using perlite (Terra-lite, W. R. Grace, Cambridge, Massachusetts) as an oviposition substrate. Females readily deposit their eggs in perlite and they can readily be recovered without loss or damage using only tap water. Before using, perlite was placed in a large vessel of water, agitated and all the material that sank to the bottom was discarded. The clean perlite was then dried and stored. For oviposition, dry perlite was placed in a 15 x 100 mm petri dish (experiments), or a 190 ml plastic cup (breeding colony) and moistened with water. More water had to be added if the dish was not processed within 3 days. To collect eggs, the cup contents were washed into a
polyethylene basket lined with aluminum window screen. The basket was in a plastic box 19.5 x 14.5 x 10 cm. The box was filled with tap water and the basket with perlite was raised slightly, gently agitated back and forth, then dunked several times. The screen retained the granular perlite while the eggs passed through and sank to the bottom. After the eggs were separated the basket was lifted out, excess water poured off and the eggs were collected and counted. Used perlite was washed with hot water in a sieve and dried, thus eliminating carryover contamination from the occasional egg that was not separated out.

When it was desirable to incubate the eggs for hatching or to determine fertility, eggs were concentrated in a 30 ml plastic cup and washed onto filter paper in a 10 x 100 or 15 x 60 mm petri dish. Excess water was removed, the dish covered and incubated at 30-32°C in a desiccator containing a saturated solution of KCl to maintain a constant relative humidity of 75% (Winston and Bates 1960). Fertility was confirmed by noting the appearance of eyespots about 10 days after egg deposition. Hatching occurs within 13-14 days.

E. Handling the animals

Crickets are hardy and easy to handle. Certain precautions are necessary, however. Carbon dioxide causes severe physiological distress (Woodring et al. 1977), so nymphs were never anaesthetized. Carbon dioxide was never used, while chilling was used only to relax adults for surgical procedures.
F. Maintenance of experimental animals

Using these rearing conditions, I can obtain virgin adults of well-defined ages. For most experiments, individual age was known to within one or two days. Adults were placed in cylindrical, plastic-coated pint cardboard containers (82 x 80 mm) whose ends had been removed and replaced with aluminum window screen. The bottom screen was sealed on with melted paraffin and the top screen was fitted tightly into the lid. Up to 20 crickets were placed in each container, and food and free-standing water were supplied in glass shell vials 21 mm in diameter x 16 mm high. These containers were used throughout to store adults and for holding experimental animals. When they were placed on moist perlite, females deposited eggs directly through the screen without difficulty, and the eggs could be removed without disturbing the crickets. Unless otherwise noted, all adults were kept at 25±1°C, 12 hr light, 12 hr dark, both for experiments and maintenance.

G. Cleanliness

Stringent precautions to insure a healthy, vigorous colony were observed throughout the study. Following each use, rearing containers, watering dishes and vials, food dishes, shell vials, and pea gravel were soaked in dishwashing detergent, washed and thoroughly rinsed. Rinsing the gravel was especially critical to remove all traces of detergent. Egg cartons were discarded when they became fouled with feces or fungal growth.

Pint cardboard containers were soaked in water to remove feces and spilled food from the bottom screen, and the inside was scrubbed lightly
with water after each use. Using these measures, I have maintained the colony for nearly three years without any serious rearing problem.
A. Introduction

Details of egg production and deposition throughout the adult life of house crickets are reported for the first time. In particular, I describe the age at which females become receptive to courting males, the onset of egg laying, the long-term pattern of oviposition, the complications of senescence, and the requirement for a suitable substrate.

I use this information, which has not been available to earlier investigators, to design experiments to probe the regulation of egg release in the house cricket, and to expand our understanding of its reproductive biology. The methods used for these observations are described in Chapter 2. The environmental conditions were maintained at 25±1°C and 12 hr light, 12 hr dark. As in all experiments, adults were aged by considering animals that had eclosed in the previous 24 hr to be one day old.

B. Results

1. Age of first mating and onset of egg laying

Female house crickets become receptive to courting males and mate within two days of adult eclosion (Fig. 2), well in advance of egg maturation. Vitellogenin uptake is not noticeable until day four and only at seven days of age does a female possess a full complement of mature eggs. Curiously, even when mated, the female does not lay eggs until approximately day 13-14 (Fig. 2).
Figure 2

Ability to mate and oviposition in relation to mating in the first 20 days after adult eclosion of female house crickets. Each graph represents 5-7 females that were allowed to mate with mature males on the day indicated by the arrow on the X-axis. For days 1-8, groups of 3-4 females were given 3-4 males and after 24 hr, females were isolated in cylindrical cups with food and water on moist perlite. For days 10-16, females were paired individually with one mate then treated as described. Each fraction represents the proportion of females that mated at a given age. Unmated females laid no eggs and are not included in the data. Mating was confirmed by the presence of sperm in the spermatheca at the end of the experiment.
Days after Eclosion

Figure 2
2. Long-term oviposition

Once oviposition has begun it will continue for many weeks. Under my conditions females lay an average of 25-30 eggs/day until they are about 60 days old, after which egg deposition falls off markedly (Fig. 3). Oviposition can be stimulated temporarily at this point by another mating (Fig. 4), indicating that egg production has not ceased.

To determine if the decline in egg laying were due to exhaustion of the sperm supply, females were examined at death for the presence of sperm in the spermatheca. Of 10 females that mated twice, all contained sperm. And although ovipositional activity was low, five had a large supply of eggs and three others had a small number (10-50) of eggs. Twelve of 14 females that were mated once contained sperm in the spermatheca at death, while 11 had a large number of eggs.

Egg release in crickets is not steady and predictable. It seems to follow a loose pattern of high activity for a day followed by two or three days of relative inactivity. Exceptions to this rule are numerous so that individual patterns vary widely.

3. Mating in old age and oviposition

Females that are not permitted to mate until they are very old still respond by laying a large number of eggs, most of which are infertile (Table 1). Crickets will resorb eggs if not mated or provided an oviposition site and by this time, although the ovaries remain replete, a large proportion of eggs are discolored. Their capacity to renew oogenesis and produce more eggs is reduced with age, as indicated by the low ovipositional rate following the initial response to mating.
Figure 3

Onset and duration of oviposition in female house crickets. (A) Virgin females provided with moist perlite, (B) females mated on day 7 (n=8), and (C) females mated on day 16 (n=16) or day 42 (n=8). Arrows indicate adult age at mating.
Stimulatory effect of a second mating on oviposition. Twenty-five females age 4-11 days were mated to mature males and transferred in groups of 1-3 to oviposition cups. 58 days after mating (arrow) a portion (33%) of the females from this experiment and those in Fig. 5 were allowed to mate again; the subsequent rise in oviposition is indicated by the dashed line.
Oviposition and egg fertility in virgin and mated females 65-80 days old. After mating, eggs were collected daily and incubated as described in Chapter 2 to determine fertility.

<table>
<thead>
<tr>
<th>Status</th>
<th>Sample size</th>
<th>Eggs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mated</td>
<td>18</td>
<td>laid</td>
<td>92±16</td>
<td>202±26</td>
<td>16±4</td>
<td>7±3</td>
<td>9±4</td>
<td>6±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5±2</td>
<td>22±15</td>
<td>1±1</td>
<td>3±1</td>
<td>4±2</td>
<td>4±2</td>
</tr>
<tr>
<td>Virgin</td>
<td>10</td>
<td>laid</td>
<td>2±1</td>
<td>31±25</td>
<td>6±4</td>
<td>8±7</td>
<td>2±1</td>
<td>3±2</td>
</tr>
<tr>
<td>Virgin</td>
<td>9</td>
<td>laid</td>
<td>2±1</td>
<td>6±4</td>
<td>0.4±0.2</td>
<td>1±0.2</td>
<td>1±1</td>
<td>0.7±0.3</td>
</tr>
</tbody>
</table>

* These data exclude a single outlier in the virgin females which laid 79% of all the eggs. These adjusted values more accurately represent the egg laying pattern of old virgin females.
4. Requirement for mating and an oviposition site

Females are extremely reluctant to lay eggs without having mated or finding an oviposition substrate. Despite possessing several hundred eggs, a virgin female will deposit virtually no eggs even when offered a suitable oviposition substrate (Figs. 2 and 3). Mated females likewise retain all of their eggs until an oviposition site is found; then oviposition begins immediately (Fig. 5).

C. Discussion

The house cricket, like many insects, attains mating competence independently of oviposition competence. It appears to be unique, though, in the long delay between the acquisition of mature eggs and their deposition. Mating is a prerequisite for oviposition, and in animals older than 15 days it provokes an ovulatory/ovipositional response—the rapid release of mature eggs stored in the ovary, and a synthetic response—the renewed manufacture of more eggs. In young animals the same processes are activated by mating, but only after a considerable period of delay. This age-dependent delay may be related to a need for sperm maturation in the spermatheca. Changes in sperm after mating have been reported in *Acheta domesticus* (McMaster-Kaye and Kaye 1976) and other insects (Das *et al.* 1964, Makielski 1966); these maturational steps may require factors from the female which do not appear until two weeks after eclosion. The delay in oviposition may also be related to the strain or temperature used. Destephano and Brady (1977), using rearing conditions of 28°C and constant light, routinely obtained eggs from 8 day old females.
Figure 5

Oviposition pattern of females mated at 4-11 days and held without an oviposition cup until (A) 17 days after mating, n=16 or (B) 34 days after mating, n=14.
The oviposition signal is retained by the female for long periods of time. Females denied an oviposition site for more than 30 days still lay eggs readily when a site is provided. Thus the signal appears sustained: females can respond to it at any point in their life including senescence.

The apparent inability of a single mating to sustain a high rate of oviposition until the death of the female appears to be some function or complication of senescence. Females continue to produce eggs since a second mating evokes a dramatic but transient egg release and females usually contain many eggs at death, but even recently donated sperm from a second mating fail to prolong significant oviposition. The effects of aging are certainly accumulating in females by this time. Burns and Kaulenas (1979) report that most of their animals died by 60 days, life-table studies by Nowosielski and Patton (1965) showed adults lived 30-40 days, and Bate (1971) found adult longevity to average less than 30 days. Thus the onset of senescence adds a confounding factor that makes an analysis of the regulation of oviposition excessively difficult during late stages of adult life.

Oviposition is regulated at two levels in the house cricket. First the female attains the competence to lay eggs by mating. A trigger is released that permits egg deposition when other sensory cues are present. The second level, a suitable oviposition site, which typically is some type of moist soil, must then be present for eggs to be laid. This substrate is detected by sensory receptors on the ovipositor (deWilde and deLoof 1973).
These results provide a useful framework for studying the control of cricket oviposition. Previous investigations (Destephano and Brady 1977, Loher 1979, Sakaluk and Cade 1980) were carried out without an appreciation of the full response to mating, and these results are now subject to alternative interpretations. Using this information, I will reinvestigate the male-induced stimulation of egg laying in the house cricket in the following chapter.
NATURE OF THE OVIPOSITION STIMULUS

A. Introduction

Mating is a prerequisite for egg laying in the house cricket. This is especially interesting because, unlike other insects, the house cricket has large stores of cyclic GMP in the accessory reproductive gland (Fallon and Wyatt 1975b) and prostaglandins in the testes (Destephano and Brady 1977). The prostaglandins in particular modulate numerous events in vertebrate reproduction (e.g., see Karim 1975), and their occurrence may be widespread in insects (Yamaja Setty and Ramaiah 1979).

A role for prostaglandins in house cricket oviposition has been postulated based on the work of Destephano et al. (1974, 1976), Destephano and Brady (1977), and Loher (1979). A short-term stimulation of egg laying is induced by administering prostaglandin E₂ (PGE₂) directly to virgin females, while an initial suppression of oviposition results from feeding prostaglandin synthesis inhibitors to mated females.

The putative role of prostaglandins in cricket reproduction remains equivocal, however. Males fed high levels of acetaminophen, a potent inhibitor of PG synthesis, were still capable of inducing egg deposition in females (Destephano and Brady 1977). Furthermore, the long-term stimulation of oviposition that mating provokes in crickets is inconsistent with the highly unstable and evanescent nature of prostaglandins.
To determine the nature of the oviposition stimulus in the house cricket, I located its tissue of origin in the male, determined when it is transferred during mating, examined the effects of prostaglandin inhibition and injection, and determined the importance of sperm in eliciting oviposition.

B. Methods

1. Surgery

To remove the testes, adult males less than three days old were chilled on ice and restrained on a paraffin dissecting dish with plasticine. The abdomen was sponged with 70% alcohol and the testes were removed with No. 5 jeweler's forceps through lateral incisions in the intersegmental membrane between abdominal segments four and five. The animals were released and replaced on ice for a few minutes before being returned to a rearing container. Survival averaged about 80%. Sperm do not descend from the testes to the seminal vesicles until day three or four, so operated animals were deprived of all testicular products.

2. Prostaglandin injection

Prostaglandins E₂ and F₂α-tromethamine salt were generous gifts of Dr. John E. Pike, the Upjohn Co., Kalamazoo, Michigan. Samples were dissolved in 0.2M phosphate buffer, pH 7.4, and 100 μg PGE₂ or PGF₂α-tromethamine salt in 5 μl solution was injected into chilled females through an intersegmental membrane on the abdominal venter using a 10 μl Hamilton syringe. Controls received 5 μl of 0.2M phosphate buffer.
3. Prostaglandin inhibition

To inhibit prostaglandin formation in reproductive tissues, crickets were fed diet supplemented with 5% powdered acetaminophen or aspirin and given water saturated with acetaminophen. Water was changed every other day. Acetaminophen and aspirin were from Sigma Chemical Co., St. Louis.

4. Prostaglandin radioimmunoassay (RIA)

Animals were chilled on ice and tissues quickly removed in the absence of aqueous medium to a Beckman Microfuge tube containing 1 ml absolute ethanol and placed in a dry ice-ethanol bath. Tritiated PGE\(_1\) (4500 cpm) or PGF\(_2\alpha\) (1300 cpm) was added to selected samples to determine rate of recovery. Samples were then stored at -20\(^\circ\)C and processed within three weeks.

To extract prostaglandins from the tissue, the sample was added to a 10 ml all-glass grinding tube (Kontes, Vineland, N.J.) containing 2 ml distilled water, 2 ml ethyl acetate and 100 \(\mu\)l of 2N formic acid. The pH of the grinding solution was 2.5-3.0. The homogenate was transferred to a 12 ml centrifuge tube and the grinder was washed with 1 ml water and 2 ml ethyl acetate which was added to the homogenate. After vigorous vortexing, samples were centrifuged 5 min at 10,000 g and 4\(^\circ\)C. The ethyl acetate layer was removed to a 20 ml centrifuge tube and the aqueous fraction was reextracted twice with 4 ml ethyl acetate. The ethyl acetate fractions containing the prostaglandins were combined, vortexed with Na\(_2\)SO\(_4\) to remove water, and centrifuged 5 min at 10,000 g. The supernatant was transferred to a 12 ml conical test tube and dried under a stream of N\(_2\) at 30\(^\circ\)C or less. Tubes were then sealed
with Parafilm (American Can Co., Greenwich, Connecticut) and refrigerated at 4°C until separation.

After silicic acid (100 mesh) was activated at 160°C, columns were prepared according to Jaffe and Behrman (1974). Dried samples were dissolved, applied to the column, and the E and F prostaglandins eluted following Jaffe and Behrman (1974) except that 4 ml of solvent 2 was used to elute the F prostaglandins. Elution was assisted by applying \( \text{N}_2 \) to the column to achieve a flow rate of 1-2 ml/min. Eluted samples were dried under \( \text{N}_2 \) at 30°C or less, sealed with Parafilm and refrigerated until RIA (5-7 days). Recovery of PGE\(_1\) and PGF\(_{2\alpha}\) after extraction and separation was about 40%.

Radioimmunoassay for PGE\(_1\) and PGE\(_2\) or PGF\(_{2\alpha}\) was carried out using kits purchased from Clinical Assays, Cambridge, Massachusetts. In the only adjustment to their protocol, following the final incubation at 4°C, tubes were centrifuged at 4°C for 15 min at 4340 g rather than 30 min at 1600 g. Redissolved samples were placed in polyethylene scintillation vials with 10 ml scintillation cocktail (2 parts PCS scintillation cocktail (Amersham), 1 part spectro-grade xylene; v/v) and counted for 5-10 min in a Hewlett-Packard scintillation counter.

The antiserum for PGF\(_{2\alpha}\) was highly specific. In the assay for PGE\(_1\) and PGE\(_2\), these PGs are converted to PGB\(_1\) and PGB\(_2\) before radioimmunoassay. The antiserum was prepared against PGB\(_1\) and has an approximate 18% cross-reactivity against PGB\(_2\); thus, it detects both PGE\(_1\) and PGE\(_2\) in the sample. These results are presented as PGE since the two were not differentiated.
The extraction and separation procedures were adapted from Caldwell et al. (1971), Auletta et al. (1974), Jaffe and Behrman (1974), Salmon and Karim (1976), Eklund et al. (1979) and James Greenwald (Ohio State University, personal communication). All solvents were spectro-grade or were redistilled in glass.

5. Protein determination

Protein measurements were needed in order to compensate for large differences in tissue mass among the samples. Thus after the prostaglandins had been extracted, the aqueous fraction was agitated with 2 ml chloroform and centrifuged about 2 min at 2000 g to remove lipids. Samples were then frozen, lyophilized, and redissolved in 0.5 ml of 1N NaOH, after which 0.5 ml of 0.05M Tris buffer, pH 7.5, containing 4 mM EDTA, was added. Protein was then determined (Bradford 1976) in appropriately diluted samples using ovalbumin subjected to the same procedures as a standard.

6. X-irradiation

Adult males age 1-4 days were exposed to 250-8000 rads of X-irradiation delivered from a Norelco MG 300 X-ray tube operated at 250 KV and 10 mamps. Cricket juveniles of mixed sex, and approximately at the stage when external genitalia appear, were given 1200 rads from the X-ray tube operated at 150 KV and 10 mamps.

7. Extract injection

Testes, seminal vesicles, and the accessory gland from 12 30- or 50-day old males were homogenized in 0.4 ml cricket saline (Fallon and Wyatt 1975b), centrifuged 4 min in a Beckman Microfuge, and 5 μl of
supernatant was injected into 16-22 day old females through the abdominal venter using a 10 μl Hamilton syringe. Spermatophore extracts were prepared by homogenizing 10 spermatophores from 20 day old males in 100 μl cricket saline. Samples were centrifuged at 2100 g for 10 min and 5 μl of supernatant was administered as described above.

C. Results

1. Source of the oviposition stimulus

Castrated males form a spermatophore, court and engage females, and pass a spermatophore. Their mates, however, fail to lay eggs, retaining the virgin condition (Table 2). This effect is independent of age and previous mating experience of the male.

2. Transfer kinetics of the oviposition stimulus

Although a spermatophore remains attached to a female for up to an hour or longer and evacuation takes about 45 min (Khalifa 1950, Pustell 1979), the oviposition signal is transferred in less than three minutes, frequently in the first minute after the capillary tube has been inserted into the female bursa copulatrix (Fig. 6).

3. Role of prostaglandins

a. Prostaglandin injections

To examine if the stimulus were a prostaglandin, I first injected PGE$_2$ and PGF$_{2α}$-tromethamine into 5-, 10-, or 20-day old females and maintained equal numbers in each group at 25°C, 28°C, and 31°C for 7 days. Oviposition cups were collected and eggs counted daily. It is clear from Table 3 that the injections elicited only a small and erratic response on the first day and nothing at all on succeeding days.
Table 2

Effect of male age, mating experience, and castration on cricket oviposition. Females 15-17 days old were mated and eggs collected for 7 days. Data represent mean±standard error; sample number is in parentheses.

<table>
<thead>
<tr>
<th>Male status</th>
<th>Eggs laid/female/day</th>
<th>sham-operated ♀</th>
<th>castrated ♀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 day old</td>
<td>32.4±6.4 (12)</td>
<td>0.6±0.3 (14)</td>
<td></td>
</tr>
<tr>
<td>30 day old</td>
<td>31.6±5.3 (14)</td>
<td>0.6±0.3 (12)</td>
<td></td>
</tr>
<tr>
<td>Non-virgin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 day old</td>
<td>35.1±4.0 (10)</td>
<td>0.5±0.2 (15)</td>
<td></td>
</tr>
</tbody>
</table>
Egg laying pattern in females following removal of the spermatophore after one or three minutes. Data for zero minutes include three females who received no sperm in one minute. Ten of 13 females had sperm after one minute; by three minutes all females had sperm. N=4-7 for each graph.
Table 3

Effect of prostaglandin injections on oviposition by virgins. Females received PGE\(_2\) or PGF\(_{2\alpha}\)-tromethamine in 5 µL phosphate buffer and were maintained at 25, 28 or 31°C for 7 days. Temperature replicates were pooled since no differences were found. Data represent means obtained from 10-18 individuals. Standard errors are included where valid.

<table>
<thead>
<tr>
<th>Female age at time of injection</th>
<th>Egg laying interval</th>
<th>Eggs laid/female/day</th>
<th>PO(_4) buffer (5 µL)</th>
<th>PGF(_{2\alpha}) (100 µg)</th>
<th>PGE(_2) (100 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 days</td>
<td>day 1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>days 2-7</td>
<td>0</td>
<td>0.9</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>10 days</td>
<td>day 1</td>
<td>0.4</td>
<td>0.3</td>
<td>2.9±0.6 ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>days 2-7</td>
<td>0.3</td>
<td>0.7</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>20 days</td>
<td>day 1</td>
<td>1.3</td>
<td>9.5±2.3 ns</td>
<td>11.9±1.1*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>days 2-7</td>
<td>0.4</td>
<td>0.1</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

ns = not significant at α = 0.05, one-sided Wilcoxon rank sum test.

* = significant at α = 0.05, one-sided Wilcoxon rank sum test.
Unlike the normal mating response in which all mated females lay eggs, 15% of the 10- and 20-day old females given PGE\(_2\) or PGF\(_{2\alpha}\) laid 81% of the eggs.

Since young crickets will mate but not oviposit for several days, young females were given an injection of PGE\(_2\) and then egg release over the following 15 days was compared to females that were mated or injected with phosphate buffer (Fig. 7). Again, prostaglandin E\(_2\) fails to mimic the mating syndrome.

b. Injection of tissue extracts

To examine the possibility that an additional molecular factor from the testes was missing from these injections, I homogenized and injected 5 µl supernatants from testes, seminal vesicles, spermatophore or the accessory gland (as a nonspecific control) into virgin females. As Table 4 shows, none of the extracts elicited egg laying.

c. Inhibition of prostaglandin synthesis

If prostaglandins stimulate egg laying then inhibition of prostaglandin synthesis in males prior to mating should abolish the deposition of eggs that follows mating. Nevertheless, even with high levels of aspirin and acetaminophen present in the diet of males from eclosion to the time of mating, no significant impairment of oviposition was observed (Table 5, Fig. 8). When adult females were reared on this diet and returned to a normal diet after mating, oviposition was strongly suppressed at first, but in six to eight days the effect had regressed and egg laying resumed at the normal rate (Table 5, Fig. 8).
Figure 7

Egg laying response when 4-5 day old females were mated to 15-21 day old males (n=18), given 100 µg PGE\(_2\) in 5 µl phosphate buffer through an intersegmental membrane of the ventral abdomen (n=20), or given 5 µl 0.2M phosphate buffer in the same location (n=20). Data represent the mean±standard error.
Table 4

Oviposition by virgin female house crickets in response to injection of male reproductive tissues. Sample preparation is described in Methods. Data represent mean±standard error.

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Number of females</th>
<th>Eggs laid in 7 days/female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cricket saline</td>
<td>5</td>
<td>19.2±9.9</td>
</tr>
<tr>
<td>Testes</td>
<td>5</td>
<td>13.4±9.1</td>
</tr>
<tr>
<td>Seminal vesicles</td>
<td>5</td>
<td>3.4±1.7</td>
</tr>
<tr>
<td>Spermatophore</td>
<td>9</td>
<td>7.1±3.2</td>
</tr>
<tr>
<td>Accessory gland</td>
<td>5</td>
<td>2.0±0.4</td>
</tr>
</tbody>
</table>
Table 5

Effect of prostaglandin synthesis inhibitors on oviposition. Males and females were provided with normal (N) diet or inhibitor (-PG) diet (food includes 5% aspirin, water saturated with acetaminophen) when collected as adults. Sixteen-17 days after adult eclosion the following crosses were made and eggs collected at two day intervals. All females received normal food and water after mating. Data represent mean±standard error.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Sample size</th>
<th>Eggs laid/female on days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-2</td>
<td>3-4</td>
</tr>
<tr>
<td>N ♀ x N ♂</td>
<td>18</td>
<td>170±33</td>
</tr>
<tr>
<td>N ♀ x -PG ♂</td>
<td>15</td>
<td>92±27</td>
</tr>
<tr>
<td>-PG ♀ x N ♂</td>
<td>12</td>
<td>9±4</td>
</tr>
</tbody>
</table>
Effect of prostaglandin synthesis inhibitors on oviposition in mated crickets. One day old adults were placed on normal or inhibitor (-PG) diet as described in Methods and mated 12-14 days later. All females received normal food and water after mating. N=12-13 for each cross except normal female x normal male (n=5).
No overt sperm dysfunction was detected when either sex was fed prostaglandin synthesis inhibitors after eclosion. In data taken from the experiments of Fig. 8, 86% of 908 eggs from the cross normal female x -PG male were fertile, 87% of 296 eggs from normal female x normal male, 76% of 312 eggs from -PG female x normal male, and 78% of 77 eggs from -PG female x -PG male.

The suppression of egg laying caused by feeding PG synthesis inhibitors to the female are readily inducible and reversible. Return to the normal diet is followed in 6-8 days by the resumption of normal egg deposition, while presenting normal females with inhibitor after mating and initial egg laying results in immediate constraint of oviposition (Fig. 9).

Perhaps prostaglandins are required during sperm development or the early stages of spermatogenesis. Hence inhibition during adulthood would not affect the large number of spermatozoa that have already differentiated. Thus a group of young nymphs that had not yet elaborated external genitalia were placed on the inhibitor diet. Males which were 23-40 days old and had been on inhibitor diet as juveniles and adults for 61 days at the time of mating induced oviposition just as proficiently as normal males and their sperm fertilized eggs with great success (Table 6).

Twenty- to 35-day old females given the inhibitor diet for 58 days as juveniles and adults prior to mating respond to mating as before: egg release is suppressed as long as they remain on the inhibitor diet (Fig. 10). The intensity of suppression lessens, although it is still evident, as mated females remain on inhibitor diet. The rise in
Figure 9

Effect of dietary prostaglandin synthesis inhibitors on oviposition in mated female house crickets. Females were mated to mature males when 16 days old. Eggs were collected at four day intervals after mating. N=5-6 for each group.
Normal diet throughout

Normal diet until day 10, then inhibitor diet.

Inhibitor diet until mating, then normal diet.

Days after mating

Figure 9
Table 6

Fecundity and fertility of females mated to males reared on inhibitor diet. Nymphs were given inhibitor diet as described in Methods and maintained on it as adults (-PG males). Adult males 23-40 days old that had been on inhibitor diet a total of 61 days were mated to 16-18 day old females. Eggs were collected and fertility determined as described in Methods. Control males were reared on normal diet and were 16 days old when mated.

<table>
<thead>
<tr>
<th>Male</th>
<th>Sample size</th>
<th>Eggs obtained on days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-2</td>
</tr>
<tr>
<td>Fecundity</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>-PG</td>
<td>18</td>
<td>30±11</td>
</tr>
<tr>
<td>Fertility</td>
<td>eggs examined</td>
<td>27</td>
</tr>
<tr>
<td>normal</td>
<td>fertile eggs</td>
<td>12</td>
</tr>
<tr>
<td>-PG</td>
<td>% fertility</td>
<td>44</td>
</tr>
<tr>
<td>eggs examined</td>
<td>452</td>
<td>1130</td>
</tr>
<tr>
<td>fertile eggs</td>
<td>366</td>
<td>999</td>
</tr>
<tr>
<td>% fertility</td>
<td>81</td>
<td>88</td>
</tr>
</tbody>
</table>

a mean±standard error of eggs/female/two days
Figure 10

Oviposition in mated female crickets reared on a diet containing prostaglandin synthesis inhibitors as described in Methods. Females 20-35 days old which had been on inhibitor diet a total of 58 days were mated to 11-18 day old normal males and returned to a normal diet 0 (n=10), 8 (n=11), or 16 (n=6) days after mating. Data represent mean±standard error of the number of eggs laid/female/two days.
Figure 10
oviposition rate is not gradual on an individual basis. As is indicated by the large fluctuations in standard error, the majority of eggs in groups of animals under the suppressive influence of PG synthesis inhibitors are laid by a small proportion of individuals. A trend emerges in which prostaglandin inhibition blocks implementation of the oviposition signal, but when the inhibition diminishes, a normal rate of oviposition rapidly ensues. Variation in female sensitivity thus accounts for the variability of the "average" response to removal of inhibition.

The presumed inhibition of prostaglandins in mated females appears to exert no effect on sperm function regardless of the time at which inhibition begins. From the females of Fig. 10 the fertility of 972 eggs collected while inhibitor was present in the diet was 80% (range of 14 samples was 57-100%).

d. Prostaglandin levels in reproductive tissues

Prostaglandins E and F₂₅ were measured to determine the effectiveness of prostaglandin removal by inhibition of its synthesis, and to monitor prostaglandin levels in key tissues involved in transfer and maintenance of the oviposition stimulus.

Availability of aspirin and acetaminophen throughout adult life strongly suppresses PGE accumulation in the testes, reducing its level about 90% (Table 7). Prostaglandin F₂₅ is less abundant, and inhibition causes an approximate 60% reduction in the testes. Indomethacin effected less of a suppression when administered for a long duration or at high concentration (5% of the food). If given a short time at 1% of the food, it was very effective and virtually abolished both PGE and PGF₂₅.
Table 7

Effect of prostaglandin synthesis inhibitors on PGE and PGF$_{2\alpha}$ levels in cricket testes and spermatophore. Adult males were fed aspirin and acetaminophen (asp + acet) or indomethacin and assayed 10-20 days after eclosion.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatments</th>
<th>Number of individuals per sample</th>
<th>pg PGE/individual</th>
<th>pg PGE/mg protein</th>
<th>pg PGF$_{2\alpha}$/individual</th>
<th>pg PGF$_{2\alpha}$/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A  B  C</td>
<td>A  B  C</td>
<td>A  B  C</td>
<td>A  B  C</td>
<td>A  B  C</td>
</tr>
<tr>
<td>Testes</td>
<td>untreated</td>
<td>9.3  203 72 341</td>
<td>35 14 68</td>
<td>48 45 56</td>
<td>8 10 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>asp + acet</td>
<td>10  26 12 18</td>
<td>6 4 2</td>
<td>26 18 10</td>
<td>6 4 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indomethacin$^a$</td>
<td>10  3 - -</td>
<td>1 - -</td>
<td>0 - -</td>
<td>0 - -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indomethacin$^b$</td>
<td>9.5  143 128</td>
<td>29 25 -</td>
<td>30 0 -</td>
<td>6 0 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indomethacin$^c$</td>
<td>12  71 41 -</td>
<td>11 7 -</td>
<td>8 0 -</td>
<td>1 0 -</td>
<td></td>
</tr>
<tr>
<td>Spermatophore</td>
<td>untreated</td>
<td>8.3  17 12 15</td>
<td>266 90 197</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>asp + acet</td>
<td>8.5  11 0 -</td>
<td>154 0 -</td>
<td>9 0 -</td>
<td>129 0 -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indomethacin$^c$</td>
<td>21  16 - -</td>
<td>157 - -</td>
<td>21 - -</td>
<td>267 - -</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ 1% of food for 5 days before assay

$^b$ 5% of food for 5 days before assay

$^c$ 1% of food since adult eclosion
in the testes. Although inhibition greatly reduces prostaglandin levels in male testes, its effect on spermatophore PG levels is only slight.

Egg laying in females mated by these PG-inhibited males is not affected and prostaglandins are still found in the females' spermathecae (Table 8). When females receive the inhibitor, however high prostaglandin levels are found in the spermatheca after mating, yet egg laying is retarded for several days. Likewise prostaglandin levels in young mated females are very high (Table 8), even though eggs will not be laid for many days.

4. Irradiation

An alternative to removing prostaglandins from the mixture of seminal components that enter the female is to remove the sperm. Treatment of juveniles with 1200 rads X-irradiation completely blocks reproductive development if oogenesis or spermatogenesis has not begun. Treated animals continue to develop, molt, and reach adulthood as rapidly as their untreated siblings. Adult longevity seems unimpaired. Yet even if treated only 10 days before adult eclosion, the development of reproductive tissue is severely retarded. At 12-13 days after eclosion, median ovarian wet weight of irradiated females is 4.5 mg (n=10) while 8 of 10 contained no mature eggs. Untreated siblings had ovaries filled with eggs; the median weight was 156 mg (n=10).

Males irradiated 11-12 days before eclosion produce spermatophores with sperm as 10-11 day old adults, but testicular growth is greatly retarded: median wet weight of these testes is 18.5 mg (n=10) while in a group of siblings reared under identical conditions it is 43.5 mg (n=10).
Table 8

Effect of prostaglandin synthesis inhibitors on levels of PGE and PGF$_{2\alpha}$ in the spermathecae of mated females. Adult males or females were fed aspirin and acetaminophen (-PG, A) or 5% indomethacin (-PG, I). Adult females 8-18 days old were mated and PGs assayed two days later. Young females 4-6 days old were mated to normal (N) males in the last cross.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Number of individuals per sample</th>
<th>pg PGE/individual</th>
<th>pg PGF$_{2\alpha}$/individual</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>N</td>
<td>-PG, A</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>-PG, A</td>
<td>N</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>-PG, I</td>
<td>N</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>N</td>
<td>N</td>
<td>12.3</td>
<td>90</td>
</tr>
</tbody>
</table>
The amount of sperm in adults is an approximate function of the age at which juveniles are irradiated. Thus irradiated males can transfer sperm to females, who then lay eggs in the normal pattern (Fig. 11). Juveniles irradiated at an earlier age contain virtually no sperm as mature adults, and when these males mate, females fail to lay eggs (Fig. 12). The dose of X-rays received by the male does not control the egg laying stimulus, but it may modulate the resultant intensity of egg laying (Fig. 13).

The oviposition stimulus that young females receive becomes expressed later when they become competent to release eggs. Mating to X-irradiated males that lack sperm fails to evoke this latent response and the females remain functional virgins, whereas those females which receive sperm demonstrate delayed oviposition as expected (Fig. 14).

Application of higher X-ray doses to recently emerged adults impairs sperm function but does not disturb sperm production. Treated males mate and transfer sperm readily, but are less able to fertilize eggs. Females continue to lay eggs at the normal rate even when none are fertile (Fig. 15).

In order to confirm that irradiation did not reduce prostaglandin production, PGE and PGF\textsubscript{2\alpha} levels were measured in the testes and spermatophores of treated animals. In X-irradiated males, PGE and PGF\textsubscript{2\alpha} levels in the testes are both greatly elevated (Fig. 16). The reduced size of irradiated testes which is reflected in their smaller protein content (Fig. 16) makes these differences even more striking. PGE levels in spermatophores of irradiated males were about the same (10-30 pg/spermatophore) as in unirradiated males. PGF\textsubscript{2\alpha}, which was not
Figure 11

Effect of X-irradiation on sperm transfer and oviposition in house crickets. Males 13-18 days old were given 1200 rads of X-irradiation 28 days before mating. Two days after they had mated with 3-5 females, males were mated to 17-20 day old females. Of 37 such females, 34 received sperm (stippled bars), three did not (open bars). Virgin females were used as controls (cross-hatched bars, n=3). Data represent mean and standard error.
Figure 11

Eggs/female/two days

Days after mating

Figure 11
Figure 12

Effect of X-irradiation on sperm transfer and oviposition in house crickets. Males 13-16 days old received 1200 rads X-irradiation 48 days before the test females were mated. Of 25 females 15-18 days old that accepted a spermatophore, 4 had sperm in the spermatheca at the end of the experiment (open bars) and 21 did not contain sperm (cross-hatched bars). Five females were mated to 15-16 day old untreated siblings (stippled bars); all received sperm. Females were maintained and eggs collected as described in Methods. Data represent mean and standard error.
Figure 12
Effect of various doses of X-rays on sperm transfer and oviposition in the house cricket. Last instar male nymphs were irradiated and 34 days later (as 23-31 day old adults), surviving males were each given five females. Females mated and received sperm from males given 400 or 800 rads X-irradiation. 41 days after irradiation, 25 males (400 rads, n=9; 800 rads, n=10; 1600 rads, n=6) were allowed to mate with up to 5 females (median=2). Eggs were collected for 7 days as described in Methods. Sperm transfer was determined by examining spermathecae at the end of the experiment and by incubating eggs. Data represent the mean.
Figure 14

Oviposition by female house crickets after mating at an early age with X-irradiated males. 2-4 day old females were mated to X-irradiated or untreated males 10-13 days old or were held as virgins. Closed circles represent the means of 7 mated females that received sperm from 5 normal and 2 irradiated males; open circles represent the means of 22 mated females that did not receive sperm; and closed squares represent the means of 7 unmated females.
Figure 15

Effect of X-irradiation of males on oviposition and fertility in females to which they are mated. Males were irradiated at 1-4 days of age, then given 15-23 day old females seven days later. Each point represents the mean of 6-8 females.
Figure 16

Association of age and PG levels in testes of X-irradiated males. Males were irradiated as nymphs 30-41 days before assay. PG levels in untreated testes are shown for comparison. Each sample consisted of testes from 8-18 individuals.

Inset: protein content of the testes in irradiated (●) and untreated (○) males.
Figure 16
detected in spermatophores of normal males, was abundant in irradiated males (10-100 pg/spermatophore).

D. Discussion
1. Identity of the oviposition stimulus

The results of these experiments show that the composition of the insemination mixture which males transfer to females can be altered by castration, irradiation and administration of prostaglandin synthesis inhibitors. When manipulation of the male resulted in removal of sperm from the insemination mixture, a female he mated would not lay eggs. But if sperm remained in the mixture which was transferred to a female, then she laid eggs. Thus it appears that sperm are the oviposition stimulus in house crickets.

Lack of egg laying following removal of sperm from the insemination mixture is often used to implicate sperm as the oviposition signal. Hence, sperm participation in egg release has been postulated in Hyalophora cecropia based on castration experiments (Truman and Riddiford 1971), in Pectinophora gossypiella based on irradiation and repeated mating studies (LaChance et al. 1978), in Zeiraphera diniana based on irradiation studies (Benz 1969), in Acrolepiopsis assectella based on high temperature rearing (Thibout 1979), and in an acarid, Ornithodoros tholozani, using irradiation (Galun and Warburg 1967).

In Rhodnius prolixus (Davey 1965) and Acrolepiopsis assectella (Thibout 1979), enhancement of oviposition following implantation of tissues containing sperm also implicates sperm in the induction of oviposition. In all of these studies, however, the interpretation of the results is confounded by the failure to consider, or the inability
to exclude, other testicular, seminal or accessory gland products. Consequently, the evidence that sperm stimulate egg laying in many insects remains equivocal.

Only in *Cimex lectularius* has an absolute requirement for sperm been established: virgin females lay no eggs but injection of washed, active sperm into the spermalege induces a normal, long-term ovipositional response (Davis 1965a). Since all accessory and seminal secretions were eliminated, the sperm, itself, appears to be the egg laying stimulus.

In the house cricket, though, the anatomy and physiology of male reproduction allow a more careful discrimination of the oviposition stimulus. Castration experiments exclude a stimulatory role for accessory gland products; in this respect the house cricket is similar to *T. commodus* (Loher and Edson 1973). I used irradiation supplemented by repeated mating to delete sperm from the insemination mixture and generated males totally lacking or with very low levels of sperm. Those males that mated but transferred no sperm consistently failed to provoke egg release in the female; those males that did transfer sperm induced egg deposition.

Removal of the spermatophore soon after it is attached to the female shows that the egg laying signal is transmitted to the female early in the process of spermatophore evacuation. Again, oviposition was strongly correlated with the transfer of sperm. Spermatophore removal one minute after attachment sometimes prevented egg laying and in every case sperm were absent from the spermatheca. In the remaining cases egg deposition occurred and sperm were found.

Prostaglandins, also found in the testes, are unlikely candidates for stimulating oviposition. Prostaglandin injections are completely
ineffective in promoting long-term oviposition, inhibition of prostaglandin synthesis in males does not alter the female response to mating, and irradiated males lacking sperm have excessively high levels of prostaglandins yet are unable to induce oviposition.

This conclusion contradicts the findings of Destephano and Brady (1977) and Loher (1979), who reported that prostaglandins or prostaglandin synthesis enzymes stimulated crickets to lay eggs. These results were derived from injections of large doses of \( \text{PGE}_2 \) or administration of PG synthesis inhibitors to females, and in contrast to my results, represent only one or two days of observation. No long-term enhancement of oviposition was reported.

"Prostaglandin synthetase," in particular, was thought to be the stimulatory agent transferred from the male (Destephano and Brady 1977), but three features of cricket oviposition make it an unlikely candidate for the trigger. First, young females two days old will mate but do not begin to lay eggs until 12-14 days later. Second, mature, mated females that are denied an oviposition site will not lay eggs, but when one is provided they oviposit readily. Finally, females lay eggs throughout their adult life so that the stimulus must be active for several months.

In these situations, the delay between reception of the stimulus and the initiation of egg laying can be extremely long. Yet male-contributed "prostaglandin synthetase" probably persists in the female only for a short time. (But longer than its products: prostaglandins are notoriously labile.) The average half-life of more than 50 silkmoth galeal proteins is 21 hr and the greatest is 89 hr, or less than four days (Kafatos 1972). Thus the likelihood that proteins entering the
female with sperm from the male would persist in an active form for up to two months is not high. A similar argument suffices to show that prostaglandins are also poorly suited to be the oviposition stimulus.

E and F₆ prostaglandins occur in the reproductive tract of male Bombyx mori (Yamaja Setty and Ramaiah 1979) but they do not participate in stimulating oviposition (Yamaoka and Hirao 1977). In many species of Lepidoptera, in fact, sperm appear to stimulate oviposition (Flint and Kressin 1969, LaChance et al. 1978, Thibout 1979).

2. Role of prostaglandins in reproduction

Despite my failure to confirm a role for prostaglandins in regulating oviposition, a function in reproduction remains likely based on their occurrence in both male and female reproductive tissues. Prostaglandin E is much more abundant than PGF₂α in these tissues, although the levels of PGE that I obtained were considerably lower than those reported by Destephano and Brady (1977). These differences may be due to strain variation or methodology. Our extraction procedures were similar. My rate of recovery (40%) was typical for tissue samples (Salmon and Karim 1976). Differences in PGE determination may also result from variation in the RIA. The accuracy of this technique when applied to many biological tissues is low and often leads to erroneously high measurements (Samuelsson et al. 1978).

Nevertheless, by using RIA, I was able to test the ability of various treatments to alter prostaglandin levels in vivo. Irradiation does not suppress prostaglandin synthesis in testes. In fact it appears to augment its production. Ionizing radiation is associated with increased PG synthesis (Mennie et al. 1975), an effect that may be due to
the inhibition of thromboxane A₂ biosynthesis with resultant overproduction of other prostaglandins (Horrobin et al. 1978).

Irradiation of young nymphs also abolishes sperm production. Thus the rise in PG levels in adults after irradiation is correlated with the absence of sperm. This suggests that sperm may require PGs for development and regulate their levels through a feedback mechanism. Absence of sperm would result in continuous production of PGs. Such a model accounts for the elevated testicular levels of PG which were noted to increase with adult age.

Inhibition of PG synthesis in cricket tissues by aspirin and acetaminophen was effective in suppressing PG levels. It is assumed that the PG synthesis enzymes which a female acquires at mating are also inactive since these inhibitory agents are irreversible (Smith and Lands 1971, Raz et al. 1973, Abdel-Halim et al. 1978, Demers et al. 1980). The effectiveness of these inhibitors may be further enhanced by their action in preventing PG binding to serum proteins, thus increasing exposure to catabolic enzymes (Attallah and Lee 1980).

In vivo inhibition by indomethacin is especially noteworthy since it appears to be ineffective in vitro (Destephano et al. 1976). It apparently is metabolized to an active form in the tissue (Flower and Vane 1974). Indomethacin was more effective in crickets than aspirin and acetaminophen combined; such a difference most likely represents tissue-specific sensitivity of PG synthetase to various inhibitors (Raz et al. 1973, Abdel-Halim et al. 1978). Surprisingly, indomethacin is most effective in suppressing PG synthesis at the lower concentration tested for the shortest duration (5 days). High dietary concentrations
of indomethacin may provoke an antifeeding effect (which was noticed with aspirin) so that relatively little inhibitor is ingested. Long-term exposure may induce an alternate synthetic pathway that is refractory to inhibition.

Measurement of prostaglandins in female tissues shows that PG synthesis inhibitors have little effect on PGE and PGF$_{2\alpha}$ levels in the spermathecae. High PG levels are found in the spermathecae of young mated females long before they begin to lay eggs. Hence, the presence of prostaglandins in the spermatheca does not cause egg laying.

The function of prostaglandins in insects is unknown, in part because so little is known; their occurrence has been documented in only two species. Participation of prostaglandins in house cricket spermatogenesis has been indirectly suggested based on the deleterious effects of dietary lipid deficiencies (Richot and McFarlane 1962, Meikle and McFarlane 1965, McFarlane 1978a), even though tocopherol and linoleic acid are not PG precursors. Similarly, a deficiency of arachidonic acid, the direct substrate of PG synthetase, causes loss of flight in Culex pipiens (Dadd and Kleinjan 1979). A connection with PGE or PGF$_{2\alpha}$ is tenuous in this case also.

The role of prostaglandins in vertebrate reproduction has been studied intensively in recent years and some of these findings may shed light on the function of PGs in cricket reproduction.

In males of many animals, prostaglandins are abundant in seminal fluid but their physiological significance is obscure (Karim and Hillier 1979). Kelly (1977) and Conte et al. (1979) found no relationship between E and F$_{\alpha}$ prostaglandins and metabolism or fructose levels in
human sperm. By contrast, Cohen et al. (1977) reported that PGF$_2$α inhibits sperm motility. In rats, PGE$_2$ increases testicular weight, sperm number and RNA content, while PGF$_2$α lowers DNA content (Didolkar and Roychowdhury 1980). Low levels of prostaglandins have been associated with high sperm concentrations in human semen (Kelly et al. 1979) and high levels in testes and epididymides disrupt spermatogenesis and render males sterile (Abbatello et al. 1975, Tso 1976, Saksena et al. 1978a,b, Tso and Lacy 1979). Conversely, the sperm content of the porcine ejaculate is increased by treatment with PGF$_2$α (Hemsworth et al. 1977).

The regulation of reproductive tract prostaglandin levels is complex. Prostaglandin synthesis enzymes are found in sperm tails, interstitial cells of the testes and epididymis, adipose cells and others in the rat (Johnson and Ellis 1977) while endogenous inhibitors are found in human semen (Dineen et al. 1974), rat testis (Johnson and Ellis 1977) and cricket accessory gland (Destephano and Brady 1977). Further regulation may occur at the sperm membrane surface, where differential binding of PGE and PGF$_2$α has been reported (Mercado et al. 1978).

The variety of reports suggesting that prostaglandins in some way regulate or perturbate sperm development and function and the acknowledged occurrence of PGE and PGF$_2$α in cricket testis and spermatophore indicate that a role in the male cricket cannot be disclaimed, in spite of the experimental results presented here. Endogenous prostaglandins were not entirely abolished by the presence of inhibitors, and only the obvious overt physiological effects of healthy sperm were observed.
Though not the primary stimulus, prostaglandins appear to be involved in ovulation or oviposition by female crickets. The temporary boost in oviposition following PG injection and the suppression caused by feeding PG synthesis inhibitors to mated females indicated that PGs participate in regulating the actual ovulatory/ovipositional process. In hens, E and F prostaglandins occur in the follicles and uterus, and treatment of the hen with indomethacin or PGE₁ antiserum delays oviposition for up to 48 hr (Day and Nalbandov 1977, Hertelendy and Biellier 1978a,b).
A. Introduction

Uniquely high levels of cyclic GMP are found in the house cricket male accessory reproductive gland (Fallon and Wyatt 1975b). As the tissue differentiates during the first two weeks of adult life (Kaulenas et al. 1979), cyclic GMP increases from nearly undetectable amounts to more than 4000 pmol/gland. Changes in the activity of guanylate cyclase and cyclic GMP phosphodiesterase during this period are not significant and fail to explain the dramatic rise in cyclic GMP (Fallon and Wyatt 1977a,b).

This striking accumulation of reproductive tract cyclic GMP appears to be limited to Acheta domesticus. Accessory gland (ARG) levels of cyclic GMP are 10-fold higher than in other representative Orthoptera with accessory glands and mating strategies similar to those of the house cricket (Fallon and Wyatt 1975b).

Similar notable levels of cyclic GMP have been described in vertebrate rod outer segments: in darkness, a light-dependent phosphodiesterase remains inactive and levels of cyclic GMP reach 600 pmol/mg protein (Farber and Lolley 1974, Krishna et al. 1976). Even higher levels, about 2500 pmol/mg protein, in mutant C3H mouse outer rod segments result from developmental lesions resulting in the failure to produce high $K_m$-cGMP phosphodiesterase (Farber and Lolley 1974). In other systems utilizing cyclic GMP as a physiological regulator, the
concentration of cyclic GMP does not nearly approach levels typically encountered in the house cricket ARG.

Cyclic GMP production is localized in a small group of ARG tubules near the seminal vesicles (Pustell 1979) and it is incorporated into the spermatophore (Fallon and Wyatt 1975b). Fallon and Wyatt (1977a) and Pustell (1979) have suggested that cyclic GMP plays a role in sperm function. The physiological significance of the remarkable accumulation of ARG cyclic GMP is unclear but it may involve regulation or modulation of reproductive processes in the female, including oviposition. Such a role would be especially likely if cyclic GMP is a component of the insemination mixture and if it is associated with sperm. Thus I have examined the sequestration and distribution of cyclic GMP in the ARG, its incorporation into the spermatophore and its eventual fate in mating to ascertain its role in cricket reproduction.

Unlike cyclic GMP, the level of cyclic AMP in the ARG is not exceptional, but in several organisms cyclic AMP does appear to be involved in the metabolism and function of sperm (Casillas and Hoskins 1971, Morton and Albagli 1973, Rosado et al. 1974, Lee and Iverson 1976). Thus I have also documented by immunocytochemical localization the presence and cellular distribution of cyclic AMP and other components of the cyclic nucleotide system.

B. Methods
1. Surgery

After chilling on ice, adult crickets less than two days old were castrated by withdrawing the testes through a pair of lateral incisions between the fourth and fifth abdominal segments. Sperm had not yet
migrated to the seminal vesicles. Survival was about 80%.

2. Determination of cyclic GMP

Male tissues were homogenized in 0.05M Tris buffer, pH 7.5, containing 4mM EDTA and approximately 1000 cpm of tritiated cyclic GMP to monitor recovery. Samples were then placed in boiling water for three minutes and centrifuged at 6000-10,000 g for 10 minutes to remove protein. The precipitate was dissolved in 1N NaOH and a portion was used for determination of protein (Bradford 1976) using ovalbumin as a standard. Cyclic GMP was measured in a portion of supernatant by the radioimmunoassay method of Steiner et al. (1972) using a commercially available kit (Amersham Searle, Arlington Heights, Illinois). Recovery from extraction averaged 88%.

Female tissues and spermatophores were homogenized in 10% trichloroacetic acid containing about 1000 cpm tritiated cyclic GMP. The extract was centrifuged at 10,000 g for 10 minutes. The precipitate was treated as described above. Trichloroacetic acid was removed from the supernatant by extraction four times with ether; ether was removed by incubating the samples at 50°C. Samples were then lyophilized and redisolved in Tris buffer, or a portion was taken directly for radioimmunoassay as described above. Recovery of cyclic GMP by this method was 90-95%.

3. Immunocytochemistry of cyclic nucleotides

In order to determine the immunocytochemical localization of cyclic nucleotides, tissues were dissected from ice-immobilized crickets, embedded in OCT compound (Fisher Scientific) and quickly frozen in a
Section (4-6 µm) were cut on a cryostat at -20°C, transferred to clean glass slides and air-dried.

The sections were washed with phosphate-buffered saline (PBS) and stained with appropriately diluted antisera of cyclic GMP or cyclic AMP for 30 minutes. Specific antisera were raised in rabbits as described by Steiner et al. (1972). The sections on the slides were rinsed 3 times for a total of 10 minutes with PBS and then covered with appropriately diluted goat antisera to rabbit IgG labelled with fluorescein isothiocyanate (Miles Laboratories, Elkhart, Indiana) for 30 minutes. The sections again were rinsed 3 times for a total of 10 minutes with PBS. Coverslips were mounted using PBS:glycerine (1:1). Specimens were examined on a Leitz fluorescence microscope equipped with episcopic illumination. The specificity of the fluorescence was determined as described previously (Steiner et al. 1976).

4. Analysis for calcium in the spermatophore

Insemination fluid was collected under mineral oil or was discharged into distilled water on an aluminum stub for X-ray microanalysis of calcium. Whole spermatophores were crushed directly onto the stub. Samples were dried thoroughly in a desiccator, coated with 30-40 Å of carbon and examined by energy dispersive X-ray analysis in an Ortec 6230 micro-analyzer. This method will detect calcium when it constitutes 0.5% of a homogeneous sample. Sensitivity is greater if the distribution of calcium is heterogeneous.
C. Results

1. Cyclic GMP in the accessory gland

The ARG accumulates cyclic GMP to impressive levels and maintains them throughout adult life (Fig. 17). Accumulation of cyclic GMP peaks at about two weeks after eclosion, remains relatively constant at about 4000 pmol/gland until 8-9 weeks, and finally declines in very old animals. The amount of cyclic GMP in the ARG of males older than two weeks averages 3790±220 pmol (X±S.E., n=37), or 985 pmol/mg protein.

Castration early in life, while not affecting these values shortly thereafter (Fallon and Wyatt 1975b), reduces the level of cyclic GMP significantly in older males (p<0.01, Wilcoxon rank-sum test) (Fig. 17). At all ages tested, these levels are similar to those found in very old non-castrated crickets.

Feeding prostaglandin synthesis inhibitors to adult males does not alter glandular accumulation of cyclic GMP; however protein levels are reduced 37%. Thus the relative concentration of cyclic GMP is significantly higher in males fed aspirin and acetaminophen (Table 9).

In general the ARG is bilaterally symmetric. When bissected in the dorso-ventral plane, each half contains equivalent amounts of protein (0.91 mg in the right half and 1.14 mg in the left half, averages from 5 glands). Yet cyclic GMP is entirely restricted to the left half of the ARG. The ARGs from 5 males 21-22 days old were bissected in a dissecting dish with a razor blade and assayed as described in Methods and materials. No cyclic GMP was detected from the right side of the ARG, whereas the left side contained 1822±163 pmol.
Changes in cyclic GMP content of normal (o) and castrated (o) male accessory reproductive glands in the adult. Levels plateau at about 15 days but decline in very old animals. Castration early in life reduces the level of cyclic GMP, especially at 40-50 days. Data represent X±S.E. of 3-6 observations.
Table 9

Effect of aspirin and acetaminophen ingestion on cyclic GMP levels in the cricket male ARG. Adult males were placed on PG synthesis inhibitor diet soon after eclosion as described in the Methods of Chapter 4. Cyclic GMP and protein levels in the ARG were determined in treated 15-20 day old males as described.

<table>
<thead>
<tr>
<th>Male</th>
<th>Sample number</th>
<th>Cyclic GMP&lt;sup&gt;a&lt;/sup&gt; pmol/ARG</th>
<th>Protein&lt;sup&gt;b&lt;/sup&gt; mg/ARG</th>
<th>pmol cyclic GMP&lt;sup&gt;c&lt;/sup&gt; mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>1802±142</td>
<td>2.03±0.06</td>
<td>889±68</td>
</tr>
<tr>
<td>-PG</td>
<td>9</td>
<td>1573±130</td>
<td>1.28±0.07</td>
<td>1247±107</td>
</tr>
</tbody>
</table>

<sup>a</sup> X±S.E. Difference is not significant at α= 0.05 (one-sided Wilcoxon rank-sum test).

<sup>b</sup> X±S.E. Difference is highly significant, p<0.005 (two-sided Wilcoxon rank-sum test).

<sup>c</sup> X±S.E. Difference is highly significant, p=0.005 (two-sided Wilcoxon rank-sum test).
Synthesis and sequestration of cyclic GMP is limited to an unexpectedly select number of tubules. Immunocytochemical staining distinguishes an array of 2-4 tubule types that contain cyclic GMP. Cyclic GMP appears to dominate the secretory product of about 20 tubules in the mature cricket ARG. It completely fills the lumen and is usually most dense at the apical margin of the luminal epithelium (Plate I). It is present in only modest amounts in the cell cytoplasm and is absent from the nuclei. In other tubules it is located in the matrix surrounding the protein aggregates of several types (Plate I,B). In these tubules it is most evident at the perimeter of the epithelium and only faintly evident in the cytosol. Cyclic GMP is completely absent from many tubules, although all tubules of the ARG export protein for spermatoaphore construction.

Cyclic GMP accumulation in the ARG commences with the onset of glandular differentiation at adult eclosion. On day two, many tubules stain intensely (Plate I,C), and differences can already be noted in the intensity of staining of cyclic GMP, including its absence from many tubules.

Cyclic AMP is not secreted nor is it evident in the cytoplasm of ARG tubule epithelia. It is however, occasionally observed in the tubule nuclei, apparently in the chromatin region, and in the circular muscle sheath surrounding each tubule (Plate II).

The receptor subunit of Type I cyclic AMP dependent protein kinase (receptor subunit I) that binds cyclic AMP was localized immunocytochemically in nucleoli of the ARG tubule epithelium (Plate III). The analogous binding protein of cyclic GMP, the cyclic GMP dependent
Plate I

Cyclic GMP in the accessory reproductive gland (ARG) of the male house cricket.

A Cyclic GMP immunofluorescence in two types of cyclic GMP-containing tubules from a 15 day old cricket ARG. Cyclic GMP antiserum was diluted 1:10 with PBS, second antibody dilution was 1:20. Note the more intense fluorescence at the luminal border and its general absence from the epithelium (arrows). 400x.

B Similar to A. Stain dilutions were 1:20 and 1:20. Fluorescence is most evident at the apical border of the epithelium and fills the interstices surrounding protein aggregates of different types in the three tubules shown. 400x.

C Cyclic GMP staining in the ARG of a 2 day old male. Selective cyclic GMP secretion into the lumen is already evident, but many tubules are lacking it. Stain dilutions were 1:30 and 1:30. 160x.

D Normal rabbit serum diluted 1:20. Second antibody was also diluted 1:20. No fluorescence is observed. 400x.
Plate II

A  Cyclic AMP immunofluorescence in nuclei and on the muscle sheath surrounding tubules from a mature 15 day old male ARG. Cyclic AMP staining is absent from the lumen. Staining dilutions for primary and secondary antibodies were 1:20 and 1:20. 400x.

B  Normal rabbit serum control diluted 1:15. Second antibody was diluted 1:20. 400x.
Plate III

A Immunofluorescent staining of the Type I receptor subunit of cyclic AMP dependent protein kinase. Staining is restricted to the nuclei of tubule epithelial cells in the ARG from a 15 day old male. Stain dilutions were 1:10 and 1:20. 160x.

B Immunofluorescent localization of Type I receptor subunit in the nucleoli of the epithelial nuclei. Stain dilutions were 1:10 and 1:20. 400x.

C Normal rabbit serum control diluted 1:10. Second antibody was diluted 1:20. 160x.
protein kinase, was not found in tissues of the ARG. Likewise, calmodulin, the calcium-binding protein that regulates cyclic nucleotide phosphodiesterase (Cheung 1980), was not found in the ARG by immunocytochemistry.

2. Localization of cyclic nucleotides in the spermatophore

Cyclic GMP, along with the seminal products and many structural proteins, is incorporated into the spermatophore, but its distribution is not uniform. It is found in all areas of the spermatophore (see Fig. 1 in Chapter 1), but the vast majority, about 80%, is concentrated in the handle and capillary tube (Table 10). By contrast, this part constitutes only 5% of the spermatophore dry weight.

To determine if the insemination mixture in the ampulla contains cyclic GMP, the handle-capillary tube complex was removed from spermatophores and the insemination fluid collected in Tris buffer until evacuation stopped. The average amount of cyclic GMP in the fluid from 11 day old males (n=3) was 19 pmol. Insemination fluid from old castrated males (n=3) contained 67 pmol cyclic GMP.

Sperm in the spermatophore do not bind cyclic GMP (Plate IV,C). Immunocytochemical staining for cyclic AMP, however, is intense on the long flagellum (Plate IV,A). Inhibition of prostaglandin synthesis with aspirin and acetaminophen for 35-40 days as an adult and about 30 days as a nymph had little effect on this binding (Plate IV,B).

3. Presence of calcium in the spermatophore

Calcium was not detected in either the spermatophore or in the insemination fluid.
Table 10

Cyclic GMP content and dry weight of various regions of the spermatophore. Cyclic GMP was measured in 20 spermatophores (10 samples of 2) as described. Spermatophore weights were determined in a different sample of 10 individuals. Each value represents the mean ± the standard error.

<table>
<thead>
<tr>
<th>Region of spermatophore</th>
<th>pmol cyclic GMP</th>
<th>mg dry wt.</th>
<th>pmol cGMP/ mg dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papilla</td>
<td>6.6±1.4</td>
<td>0.143±.025</td>
<td>46</td>
</tr>
<tr>
<td>Ampulla</td>
<td>19.7±2.9</td>
<td>0.855±0.023</td>
<td>23</td>
</tr>
<tr>
<td>Handle-capillary tube</td>
<td>99.6±18.6</td>
<td>0.064±0.0001</td>
<td>1556</td>
</tr>
<tr>
<td>Total</td>
<td>125.7±17.4</td>
<td>1.048±0.021</td>
<td>120</td>
</tr>
</tbody>
</table>
Plate IV

Cyclic AMP and cyclic GMP distributions in the spermatophore insemination mixture.

A Cyclic AMP immunofluorescence on sperm from the spermatophore secretion. Intense staining occurs on the sperm flagellae. Stain dilutions were 1:15 and 1:20. 400x.

B Similar to A. The spermatophore was taken from a male about 40 days old which had been fed a diet containing aspirin in the food (5%) and acetaminophen in the water (saturated solution) for 70 days. Stain dilutions were the same as in A. 1250x.

C Cyclic GMP fluorescence on the secretion of the spermatophore. Specific staining on sperm is absent. The irregular blotches that stain intensely indicate isolated protein aggregates that are binding cyclic GMP. Stain dilutions are 1:10 and 1:20. 400x.

D Normal rabbit serum control treated in the same manner as B. 400x.
4. Fate of cyclic nucleotides in mating

Results presented in Table 11 show that a small portion of cyclic GMP enters the female at mating. Within one minute after the spermatophore is attached to the female, the cyclic GMP content increases 10- to 20-fold in both the bursa copulatrix and the spermatheca. After 30 minutes these levels have begun to decline although spermatophore evacuation is not completed until after 40-60 minutes. Cyclic GMP levels eventually return to the premated level.

Immunocytochemical evidence suggests that these transient increases are derived from the spermatophore. Cyclic GMP appears in the lumen of the spermatheca but not in the epithelial lining (Plate V,A) but the sperm show no significant immunofluorescence of cyclic GMP (Plate V,B). The total contribution of cyclic GMP to the female is small; levels in spermatophores do not change markedly during mating (Table 11).

Cyclic AMP remains on the sperm flagellar membrane after sperm have been deposited in the spermatheca (Plate V,C). It is also localized in the nuclei of the spermatheca after mating but not in the virgin spermatheca (Plate VI). Its receptor protein is found in the nuclei of spermathecae from mated females at the same location in which it is found in the ARG (Plate VI).
Table 11

Changes in cyclic GMP content of spermatophore, bursa copulatrix, and spermatheca during mating. Cyclic GMP was determined as described. Number of individuals used for the determination is given in parentheses. Virgin females were 35-50 days old and males were greater than 20 days old.

<table>
<thead>
<tr>
<th>Time after spermatophore attachment</th>
<th>pmol cyclic GMP/individual</th>
<th>Spermatophore</th>
<th>Bursa copulatrix</th>
<th>Spermatheca</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes</td>
<td>---</td>
<td>0.09 (11)</td>
<td>0.11 (11)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>60 (3)</td>
<td>0.95 (3)</td>
<td>1.11 (3)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>207 (3)</td>
<td>2.13 (3)</td>
<td>1.17 (3)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>166 (4)</td>
<td>0.77 (6)</td>
<td>0.74 (6)</td>
<td></td>
</tr>
<tr>
<td>&gt; 24 hours</td>
<td>---</td>
<td>0.12 (7)</td>
<td>0.12 (7)</td>
<td></td>
</tr>
</tbody>
</table>
Cyclic GMP and cyclic AMP in the spermatheca of a mated female house cricket.

A Cyclic GMP staining on the luminal contents of the spermatheca. No fluorescence is detected in the epithelial lining. Stain dilutions were 1:20 and 1:20. 400x.

B One minute after the spermatophore is attached, cyclic GMP immunofluorescence is evident in the insemination mixture but is not observed on the sperm. Stain dilutions were 1:20 and 1:20. 400x.

C Sperm in the spermatheca of a mated female that are stained for cyclic AMP. Cyclic AMP is not evident elsewhere. Stain dilutions were 1:15 and 1:20. 160x.

D Normal rabbit serum control. Section is from the luminal contents of the spermatheca of a mated female. Treated the same as the sample in C. 400x.
Plate VI

Cyclic AMP and its receptor subunit I in the spermathecal epithelium.

A Cyclic AMP staining in the nuclei and cytoplasm of epithelial cells from the spermatheca of a mated female. Arrows indicate typical nuclei. Stain dilutions were 1:15 and 1:20. 400x.

B Cyclic AMP staining in the spermathecal epithelium of a virgin female. Note the lack of nuclear staining and the greatly reduced intensity of the cytoplasmic staining. Stain dilutions were 1:15 and 1:20. 400x.

C Receptor subunit I in the nuclei of epithelial cells from the spermatheca of a mated female. Stain dilutions were 1:10 and 1:20. 400x.

D Lack of receptor subunit I immunofluorescence in the epithelium of the spermatheca of a virgin female. The intima of the epithelium which borders the lumen shows nonspecific staining (arrow). Stain dilutions are 1:20 and 1:20. 400x.
D. Discussion

1. Significance of cyclic nucleotides in the ARG

The accumulation of cyclic GMP in the male ARG has been thoroughly documented since Ishikawa et al. (1969) first suggested that the cricket abdomen contained high levels (Fallon and Wyatt 1975a,b, Pustell 1979). The results of Pustell (1979) and of those presented here unexpectedly describe an exceedingly restricted localization of this large reservoir of cyclic GMP. These findings that extremely large amounts of cyclic GMP are limited to a select group of tubules on one side only of a bilaterally symmetrical gland are novel.

The histological localization of cyclic GMP in the ARG differs in some respects from its distribution in tissues previously examined. In the ARG tubules, cyclic GMP is found along the apical border of the cellular epithelium and in the tubule lumen, but no association with nuclear elements is evident. This distribution contrasts that found in rat liver, intestine, and testis, where cyclic GMP is located in the nuclear elements (Ong et al. 1975, Spruil and Steiner 1976). In addition it is found along the apical borders of secretory tissues in the rat.

Biological regulation involving cyclic nucleotides in many cases appears to be mediated through opposing influences of cyclic AMP and cyclic GMP (Goldberg et al. 1974a,b, 1975) acting via their specific protein kinases (Weinryb 1979). Calmodulin and calcium also play pivotal roles in cellular regulation, in part by modulating cyclic nucleotide levels (Schultz et al. 1970, Berridge 1975, Cheung 1980). The distributions of cyclic AMP, receptor subunit I of cyclic AMP, cyclic GMP
dependent protein kinase, and calmodulin in the ARG, however, are strikingly dissimilar to patterns found in vertebrate tissues.

Cyclic AMP has been reported previously from cell cytoplasm and from the lateral and basal margins of tubule epithelium (Ong et al. 1975, Spruill and Steiner 1976). By contrast, in the ARG it is located only in the nucleus on chromatin and in the muscle sheath surrounding the tubules. The receptor subunit I localization in epithelial nucleoli corresponds to previous reports that it occurs in cell nuclei (Steiner et al. 1978, Steiner et al. 1979). By contrast cyclic GMP dependent protein kinase, which was found previously to have the same distribution as cyclic GMP (Steiner et al. 1979), was not demonstrated in the ARG.

The functional significance of these findings (which will be discussed later) depends in part on the reliability of the immunohistochemical procedure that I utilized. This technique for cyclic GMP and cyclic AMP localization has been thoroughly tested in numerous systems and consistently shows a high degree of specificity. The procedure is assumed to result in the loss of unbound, soluble cyclic nucleotide, however, which can obscure hormone-stimulated changes (Cumming et al. 1979). Thus, the fluorescence that is observed represents cyclic nucleotide that is associated with its protein kinase or some other receptor. It is thus a reflection of the physiologically active cyclic nucleotides.

The interpretation of staining patterns for receptor subunit I, cyclic GMP dependent protein kinase, and calmodulin is more tentative because these procedures have only recently been described. Receptor subunit I was purified from bovine skeletal muscle, cyclic GMP dependent
protein kinase from bovine lung, and calmodulin from bovine brain; thus the antibodies I used are immunospecific for these molecular species. In order to be detected in other tissues, these proteins must be sufficiently similar to possess the same antigenic determinants. In contrast, cyclic AMP and cyclic GMP retain their chemical identities regardless of their biological origin.

In spite of the vast evolutionary difference between the cricket and the cow, evidence is available which suggests that my immunohistochemical results represent authentic distributions of these proteins. Antibodies to receptor subunit I from rabbit skeletal muscle recognize the antigen in rat liver and localize it primarily in nuclei (Steiner et al. 1978). In the cricket ARG it is found exclusively in nuclei (apparently in the nucleoli).

Antibodies to soluble cyclic GMP dependent protein kinase from bovine lung recognize a particulate form of the antigen in several rat tissues. Thus it can cross-react with a different form of the protein in a different species. Although the cricket ARG may possess a cyclic GMP dependent protein kinase sufficiently modified that it lacks appropriate antigenic determinants, it is more likely that it lacks the kinase altogether. The apparent extrusion and accumulation of cyclic GMP into the lumen suggests that it is not physiologically active in this organ. It may be bound to another protein, however. A novel cyclic GMP binding protein in which the active site is neither a kinase nor a phosphodiesterase has recently been described (Francis et al. 1980).

Calmodulin occurs throughout the animal and plant kingdoms in a highly conserved structure. An antibody prepared from rat testis
calmodulin cross-reacts with calmodulin from *Tetrahymena pyriformis*, a protist (Jamieson et al. 1979). Calmodulin has been localized in rat liver, muscle and adrenal cortex using an antiserum prepared against bovine brain immunogen (Harper et al. 1980). Hence cricket calmodulin should be recognizable immunologically by my procedure; the negative results from staining indicate its absence from the ARG. In view of the many ways in which calmodulin affects cells, though, these results must be regarded as tentative.

a. Regulation of cyclic GMP levels in the ARG

The steady rise in cyclic GMP levels in the ARG during the first two weeks after adult eclosion are paralleled by a similar increase in protein content of the gland (Fallon and Wyatt 1975b). The increase does not result from a change in the activity of guanylate cyclase or cyclic GMP phosphodiesterase (Fallon and Wyatt 1977a,b). During this period the gland is characteristic of a terminally differentiating system (Kafatos 1972, Kaulenas et al. 1979) whose development is presumably triggered by events associated with adult eclosion.

After the gland matures, a steady level of cyclic GMP is maintained in the ARG. Cyclic GMP synthesis and secretion is thought to continue at approximately the same rate while cyclic GMP is removed by the periodic production of spermatophores (McFarlane 1968, Loher 1974). Cyclic GMP levels begin to decline about 60 days after eclosion in conjunction with a gradual decline in protein synthesizing capacity (Burns and Kaulenas 1979). The fall in cyclic GMP levels characteristic of senescent males is mimicked by castration shortly after adult eclosion. Thus a product released from the testes or transported to the seminal vesicles and
released there may be involved in sustaining a high level of cyclic GMP in the ARG. The seminal vesicles are buried in the ARG near the opening where the cyclic GMP-containing tubules empty into the main gland body (Pustell 1979).

Prostaglandin E₂ (or E₁) or PGF₂₀ are obvious candidates for this putative regulatory role since they are found in the testes and seminal fluid. Interactions between prostaglandins and cyclic nucleotides are common (Kuehl 1974, Kuehl et al. 1976) and several reports describe PGE₁ or PGE₂ regulation of cyclic AMP levels (Tomasi 1976, Dalton 1977, Penit et al. 1977, Zor et al. 1978, Gemsa et al. 1979, Goodwin et al. 1979). But, such a role in the house cricket is improbable since the addition of PG synthesis inhibitors to the diet does not affect cyclic GMP levels in the ARG although protein levels are significantly reduced. The reduction in protein probably represents an antifeeding effect of the diet. It also shows that protein synthesis and cyclic GMP accumulation can be dissociated; thus their regulatory mechanisms are not linked.

b. Extrusion of cyclic GMP into the lumen of ARG tubules

The concentration of cyclic GMP in the cyclic GMP-containing tubules is three orders of magnitude higher than in the most active vertebrate system (Pustell 1979) and the immunocytochemical evidence shows that almost all of it is in the lumina. Cyclic GMP may leave the epithelial cells during the export of secretory proteins (Kapoor and Krishna 1977), or extrusion may be a separate, energy-dependent process. Cyclic GMP was originally isolated from rat urine (Ashman et al. 1963), in which it is a secretory/excretory product. Since then, ATP-dependent efflux systems for cyclic AMP have been described in several animal
cells (Davoren and Sutherland 1963, Doore et al. 1975, Rindler et al. 1978, Brunton and Mayer 1979). Curiously, Doore et al. (1975) reported that prostaglandins (especially in the A and B series) inhibit efflux.

c. Cyclic nucleotide functions in the ARG

The localization of cyclic GMP along the apical plasmalemma and in tubule lumina is similar to the distribution of cyclic GMP in guinea pig pancreatic lobules that are actively secreting protein (Kapoor and Krishna 1977). The ARG is highly specialized for export protein synthesis and discharge (Kaulenas et al. 1975, Kaulenas 1976, Kaulenas et al. 1979), a process in which cyclic GMP has been implicated in vertebrate tissues secreting digestive enzymes (Schulze 1975, Wojcik et al. 1975, Haymovits and Scheele 1976, Albano et al. 1976, Singh 1979, Spearman and Pritchard 1979). A direct role for cyclic GMP in protein discharge remains unproven, however, as whole tissue increases in cyclic GMP levels have also been dissociated from the induction of protein secretion (Heisler and Lambert 1978, Gunther and Jamieson 1979).

The tubule distribution of cyclic GMP is also consistent with a role in membrane function. In rod photoreceptors, cyclic GMP regulates membrane permeability and membrane potential (Miller and Nicol 1979, Caretta et al. 1979).

The regulation of nuclear-directed events by cyclic GMP has been proposed (Ong et al. 1975, Steiner et al. 1978) based on the localization of cyclic GMP in nuclei of cells from diverse tissues. In addition, cyclic GMP modifies DNA-dependent RNA polymerase activity in vitro (Johnson and Hadden 1975, 1977), induces mRNA release from nuclei (Schumm and Webb 1978), and is found on an active puff site in
Drosophila salivary gland chromosomes (Spruill et al. 1978). Neither cyclic GMP nor its protein kinase were found on nuclei of ARG tubule epithelial cells. Thus, a role in nuclear regulation seems unlikely, even by cyclic GMP activation of cyclic AMP dependent protein kinase (Lincoln and Corbin 1977, 1978).

Cyclic AMP appears to be involved in the function of the muscle sheaths that surround the ARG tubules and contract to eject the tubule contents at the time of spermatophore construction. Cyclic AMP and its receptor protein appear to participate in the regulation of nuclear- and nucleolar-directed events in epithelial cells of the ARG tubules. Recently, cyclic AMP involvement in nuclear regulation was demonstrated by Cho-Chung et al. (1979).

2. Cyclic nucleotides in the spermatophore
a. Distribution and function of cyclic GMP

During spermatophore formation cyclic GMP is highly localized in the handle-capillary tube (see Table 10). Yet due to its location, bound in a rigid protein matrix and segregated from the rest of the spermatophore, a significant physiological function seems improbable. Relatively high levels occur in other sections of the spermatophore, however, including the seminal fluid. As a component of the seminal fluid, cyclic GMP is in a position to directly participate in sperm development or function. Evidence for such a role exists in other invertebrates (Garbers et al. 1975, Gray et al. 1976, Gray and Drummond 1976, Kopf et al. 1979).

Although cyclic GMP could affect sperm in the ampulla or as they pass through the capillary tube during insemination, I was unable to
induce motility with \textit{in vitro} preparations of sperm using exogenous cyclic GMP. (The significance of this result is tempered by the fact that I was unable to develop a system in which active sperm were observed.) Based on its distribution, the possibility that the spermatoaphore serves as a sink for excess cyclic GMP from the ARG cannot be excluded.

b. Cyclic AMP localization on sperm

Although much literature is available that points to cyclic AMP involvement in sperm function, the immunohistochemical results presented here are the first evidence for a direct association. Although this finding was unexpected in the house cricket since cyclic GMP had been proposed to participate in sperm function (Fallon and Wyatt 1975b, 1977a, Pustell 1979), it agrees with many reports which show that sperm possess all the components of a cyclic AMP second messenger system.

Adenylate cyclase has been characterized and localized on plasma membrane in bull and sea urchin sperm (Braun and Dods 1975, Herman et al. 1976, Garbers 1977), is activated by egg factors in sea urchins (Watkins et al. 1978), and is stimulated by \textit{in vitro} capacitation in hamsters (Morton and Albagli 1973). Cyclic AMP phosphodiesterase is reported from the sea urchin (Wells and Garbers 1976) and cyclic AMP dependent protein kinase has been isolated in bull (Garbers et al. 1973a) and sea urchin (Lee and Iverson 1976) sperm.

The addition of exogenous cyclic AMP or phosphodiesterase inhibitors to sperm preparations from several sources induces motility, stimulates respiration, and results in capacitation or the acrosome reaction (Casillas and Hoskins 1971, Garbers et al. 1971a,b, 1973b,c, Frenkel et
al. 1973, Rosado et al. 1974, Hyne and Garbers 1979). Nevertheless, to assume that cyclic AMP increases generally mediate the sperm functions associated with mating would be premature; cyclic AMP and agents that elevate cyclic AMP levels are also reported to inhibit the acrosome reaction and fertilization in guinea pig and hamster sperm (Rogers and Garcia 1979). In addition, exogenous cyclic AMP does not induce motility in sperm of the saturniid silkmoth Antheraea pernyi (Shepherd 1974).

Although many effects of prostaglandins on sperm are reported (see Chapter 4), no change in the immunohistochemical staining for cyclic AMP from males fed PG synthesis inhibitors was noticed in crickets. No agents tested in this study were found to alter membrane-bound cyclic AMP on the sperm.

The absence of Ca$^{2+}$ from the spermatophore was unexpected since calcium appears to be involved in sperm activities in other organisms, either alone or in concert with cyclic AMP (Morton et al. 1974, Babcock et al. 1979, Peterson et al. 1979, Hyne and Garbers 1979, Gibbons and Gibbons 1980). Also, calmodulin from sea urchin eggs may be involved in activating sperm adenylate cyclase (Head et al. 1978). Calcium may have an active role in cricket sperm function, though. Babcock et al. (1979) suggest that Ca$^{2+}$ is present at a very low concentration in seminal fluid. It may have been present in my preparations but at a level below the limit of detection.

In these studies I was unsuccessful in identifying the cricket sperm activator. A variety of cyclic nucleotide derivatives, phosphodiesterase inhibitors and pharmacological agents were tested and found to have no
effect. Sperm in the spermatophore are nonmotile even though they are both in a cyclic GMP-containing fluid and are coated with cyclic AMP. A cyclic nucleotide requirement may not be necessary until much later (e.g., at fertilization). In certain marine invertebrates, the cyclic AMP system in sperm is not activated until it is stimulated by factors released from eggs (Garbers and Kopf 1978, Kopf and Garbers 1979, Tubb et al. 1979).

3. Fate of cyclic GMP and cyclic AMP at mating

Cyclic GMP appears in the spermatheca early in the process of spermatophore evacuation but is already absent by the end of mating, which lasts 45-60 minutes. The immunohistochemical evidence and physiological evidence (from matings with castrated males) fail to suggest an interaction of cyclic GMP with the tissues of the spermatheca.

The large quantities of cyclic GMP and protein remaining in the depleted spermatophore represent a potential nutrient and nitrogen source for a mated female. Some butterfly males package nutrients in the spermatophore that are transferred to the female and incorporated into developing oocytes (Boggs and Gilbert 1979), and urates in the cockroach spermatophore provide nitrogen for the female and for developing embryos (Mullins and Keil 1980).

After cyclic GMP has disappeared from the sperm mass in the spermatheca, cyclic AMP remains on the flagellar membrane. Thus cyclic AMP may have a function in maintaining the sperm during storage in the spermatheca (up to three months) or at capacitation and egg fertilization. The localization of cyclic AMP and its receptor subunit I in the nuclei
of cells in the mated spermatheca also suggests that it is involved in regulating nuclear-directed events that are initiated by, or after, mating.
ROLE OF THE SPERMATHECA IN CRICKET REPRODUCTION

A. Introduction

The spermatheca, the sperm storage organ of the female, is at the crossroads of several key reproductive events. At the time of mating, the spermatheca of the house cricket receives the oviposition stimulus (sperm) as well as prostaglandins, cyclic GMP, and other components of the insemination mixture. In a mature female the onset of ovulation and oviposition occurs at mating and the spermatheca must precisely regulate discharge of the sperm needed to fertilize the eggs as they are periodically deposited. The spermatheca also plays a vital role in maintaining sperm for long periods. In crickets, sperm can retain their fertilizing capacity for at least three months.

I have explored the functional role of the spermatheca in the receiving and processing of the oviposition signal and have examined its structural organization as a basis for its role in sperm storage.

B. Methods

1. Surgical procedures

After females were immobilized on ice, the abdomen was swabbed with 70% ethanol and the animals were restrained ventral side up in a dissecting dish with plasticine. Using a syringe needle an incision was made across the middle of the seventh sternite just anterior to the bursa copulatrix which was visible beneath the cuticle. In extirpation...
experiments, the spermatheca was grasped with forceps, drawn out and removed. In "loose spermatheca" experiments the spermatheca was drawn out, neural connectives were severed, and the spermatheca was reinserted without damaging the spermathecal duct. The incision was closed, blood blotted off, and females were returned to the rearing container.

For implantation studies, 20-21 day old females were immobilized on ice, immersed for 3 min in 0.5% sodium hypochlorite containing 0.5% Triton X-100 and rinsed in 3 changes of sterile distilled water. Surface-sterilized females were then restrained ventral side up and the spermathecae were removed into sterile cricket saline (Fallon and Wyatt 1975b). After 3 changes of cricket saline, spermathecae were stored in Grace's insect tissue culture medium (Grand Island Biological Co., Grand Island, NY) for not more than two hours prior to implantation. Virgin recipients 15-17 days old were treated as described above but, instead of removing the spermatheca, a donor virgin or mated (up to 15 hr previously) spermatheca was placed in the incision.

To sever the ventral nerve cord, a small incision was made between the sixth and seventh sternites of chilled, restrained, 15 day old females. The nerve cord was withdrawn and a small segment, sometimes including the sixth abdominal ganglion, was removed. A piece of fat body was removed from sham-operated individuals.

2. Electron microscopy

Female crickets were immobilized on ice and the spermatheca was fixed in situ with 2% glutaraldehyde in 0.1M cacodylate HCl buffer, pH 7.4, then dissected into ice-cold 2% glutaraldehyde and fixed for about 2 hr. The tissue was rinsed in 3 changes of cold buffer containing
0.2M sucrose for 30 minutes and post-fixed with 2% osmium tetroxide in buffer containing 0.1M sucrose for 1 hr.

After samples were rinsed in 3 changes of buffer for 30 min, they were dehydrated through a graded series of ethanol in water to 100% ethanol. Samples were transferred through two changes of propylene oxide and infiltrated with Epon/Araldite (Mollenhauer 1964) in propylene oxide (1 hr at 1:1, then 12 hr at 2:1 resin to propylene oxide). Final embedding in BEEM capsules was carried out slowly. The block was degassed in a vacuum oven for 12 hr at room temperature, transferred to 45°C for 12 hr and to 60°C for 24 hr.

Thin sections were cut with glass knives on a Porter-Blum MT-2 ultramicrotome (Sorvall, Norwalk, Conn.), placed on copper grids, stained in 2% aqeous uranyl acetate and Reynold's lead citrate (Reynolds 1963) and then examined with a Philips EM 200 electron microscope.

3. Light microscopy

Thick sections cut with glass knives at 1-2 μm for light microscopy were stained with methylene blue and basic fuschin (Jha 1976), mounted in Permount (Fisher Scientific) and examined on an Olympus compound light microscope. Additional specimens for light microscopy were fixed in Bouin's fluid, processed according to Humason (1967) and stained with Harris' hematoxylin and eosin.

To test for presence of glycogen, spermathecae from 15 day old mated or 23-30 day old virgin females were dissected into alcoholic formalin, processed and stained by the periodic acid-Schiff (PAS) procedure (Humason 1967) using saliva as the control.
C. Results

1. Reception of the oviposition stimulus

   The normal response of a mature female is abolished if the spermatheca has been removed prior to mating (Fig. 18). Rather than prolonged ovipositional activity, a temporary release of eggs in the first 24 hours is followed by a virtual cessation of egg laying.

2. Transmission of the stimulus

   Following reception of the oviposition stimulus by the spermatheca, it is possible to relay the message to other organs neurally or by hormones. In the cricket, denervating the spermatheca as described in Methods abolishes egg deposition in response to mating while sham-operated animals lay many eggs (Fig. 19). Implantation of a filled spermatheca was also ineffective in eliciting egg deposition in mature females. Of ten 15-17 day old virgins that received a filled spermatheca from a mated female, the median number of eggs laid/female in each of the following 6 days was between 1 and 3.5. This is typical for virgins. Four females that received empty spermathecae from virgin donors showed the same response.

   An intact ventral nerve cord appears to be required not only for oviposition, but also for mating. Of 9 virgin females that survived the operation only one was able to mate as judged by the presence of sperm in the spermatheca and laid only a few eggs (32 in 9 days). Sham-operated females mated and laid eggs readily. When the nerve cord was severed in these animals, egg laying ceased. Six days after mating 7 sham-operated females each had laid between 119 and 398 eggs. Nerve cord severance at this point reduced egg deposition in the following
Figure 18

Effect of removal of the spermatheca on egg deposition by female house crickets. Spermathecae were extirpated from 12-13 day old females as described in Methods, and mated to 15-16 day old males 15 days later. A portion were not mated. Females were then individually placed in oviposition cups and eggs collected for six days. Cross-hatched bars represent the egg production by 11 sham-operated virgins (mean and standard error); stippled bars represent oviposition in 12 mated females that lacked spermathecae; and the open bars represent oviposition in 12 sham-operated mated females.
Figure 18

Days after mating

Eggs/female/day
Days after mating

Figure 19

The effect of spermathecal isolation on egg deposition by female house crickets. Spermathecae were denervated from 2-5 day old females and mated to 28-38 day old males when they were 15-16 days old. Eggs were collected at 2 day intervals from 9 females with a denervated spermatheca (stippled bar with standard error) and 14 sham-operated females (open bars with standard error).
3 days to 1-4 eggs/female.

3. Structural organization of the spermatheca

a. Histology

The spermatheca is organized into four regions that are structurally and functionally distinct. At the outermost margin the gland is invested with a sheath of muscle containing nerves and tracheae (Plate VII). A thick basement membrane separates the enveloping muscle sheath from the underlying gland epithelium. The epithelium consists of two regions: an outer layer of secretory cells containing at the basal margin extremely large nuclei with prominent nucleoli, and an inner layer of duct cells which secrete a cuticular intima lining the lumen. Cells in the duct layer are poorly defined in thick sections but contain numerous dense granules. The lumen, the fourth region, is filled with a faintly staining flocculent material in virgins, but becomes filled with sperm during mating.

b. The muscle sheath

(Plates VIII-X) Muscle cells dominate this region. The contractile system of actin and myosin fills most of the cell volume and is organized into A bands and I bands in irregular fibrils. Transverse tubules occur frequently where the plasma membrane is invaginated into the fiber and it commonly forms dyads with sarcoplasmic reticulum. The cells are richly supplied with mitochondria and ribosomes and contain a large irregularly shaped nucleus. Aggregations of microtubules are observed in various regions. Axons of nerve cells that innervate the muscle sheath contain neurosecretory granules. Vesicles presumably filled with transmitter substances can be located, apparently near a neuromuscular junction.
Plate VII

Thick transverse sections of spermathecae from female house crickets.

A Section from the spermatheca of a mated female showing the thin muscle sheath (MS), the secretory cell layer (SC), the duct cell layer (DC) and the lumen which is filled with sperm. A large trachea (TR) is visible in muscle tissue that is not part of the sheath. Large nuclei with prominent nucleoli are arranged along the basal margin of the secretory cell layer. 1500x.

B Section from the spermatheca of a virgin female. Note the thick muscle sheath (MS) and folding in the epithelium, which can be seen by following the trail of large secretory cell nuclei to the area where they intermingle with the duct cell layer containing a zone of densely-staining granules. The lumen is greatly reduced but contains a faintly staining material. The dark spots in the lumen bordering the cuticular intima are protrusions of intima which can be seen at another angle at the arrow. 600x.
Tranverse section through a portion of the muscle sheath and epithelium of a spermatheca from a virgin female house cricket. Muscle cells (MC) contain large bundles of fibers, numerous mitochondria and irregularly shaped nuclei (N). Several tracheal cells (TR) are visible in this micrograph; in addition to the large tracheal canal, a few mitochondria and free ribosomes are scattered throughout the cytoplasm. The matrix of basement membrane (BM) holding the muscle cells and separating the sheath from the epithelium is greatly elaborated. A portion of the epithelium consisting of secretory cells is in the lower right of the micrograph. These cells are bounded by sinuous, contorted plasma membranes (PM) and contain mitochondria (M), and swollen rough endoplasmic reticulum (RER). 9,000x.
Plate VIII
Plate IX

Transverse section through the spermathecal muscle sheath of a virgin house cricket. The muscle fibrils (F) consist of A bands (A) containing thick filaments of myosin surrounded by thin filaments of actin, and I bands (I) composed of actin only. The plasma membrane is frequently invaginated into the fiber to form transverse tubules (T) which form dyads with the sarcoplasmic reticulum (arrows). Mitochondria are scattered throughout the cell (M). The cells are enclosed by basement membrane (BM). A tracheal cell contains a large tracheal trunk (TR). 30,000x.
Plate X

Nerve cells innervating the spermathecal muscle sheath of a virgin house cricket.

A Nerve cell containing several axons (A). The large axon on the left is filled with neurosecretory granules. 84,000x.

B Section near a neuromuscular junction. The axon contains many vesicles (V) which are concentrated near the muscle fiber (MF). Mitochondria (M) are distributed throughout the axoplasm. A mesaxon invagination arises from the glial cell membrane (arrow), while the mesaxon (Ma) describes an irregular course around the axon. 56,000x.
Many large tracheae are evident in the muscle sheath. The tracheal cells contain a large nucleus, scattered mitochondria and numerous free ribosomes.

c. The secretory cells

(Plates XI-XIII) When the spermatheca is empty, a great excess of plasma membrane obliterates cell boundaries but the nature of the cell organelles remains clear. Large nuclei with prominent nucleoli are situated basally and rough endoplasmic reticulum, Golgi bodies, free ribosomes, mitochondria and tracheoles are scattered densely throughout the cytoplasm.

Rough endoplasmic reticulum (rough ER) is widely distributed and has a characteristic appearance consisting of small lobes or short strands with swollen cisternae. Golgi bodies are numerous and occasionally appear to contain an electron-dense product. Large bundles of microtubules also occur in the cytoplasm.

At least some, and possibly all, secretory cells contain a large reservoir invaded by an extensive network of microvilli. In the virgin female the reservoir contains a fine, flocculent material. ER and Golgi bodies are not found in the vicinity of the reservoir, but the cytoplasm of the immediate cell and of the adjoining cells is extensively dotted with intracellular spaces and large vesicles. Some of the spaces resemble tracheoles but others are filled with secretions of varying electron opacity.
Survey micrograph of the secretory cell region of the epithelium and muscle sheath in the spermatheca of a virgin house cricket. This region is characterized by cells with large basal nuclei (N) and profuse, sinuous cell membranes (PM). The cytoplasm contains tracheoles (Tr), rough endoplasmic reticulum (RER), Golgi bodies (G), intracellular spaces (arrows), and an abundance of round or oblong mitochondria (M). A thick basement membrane (BM) separates this region from the muscle sheath. 9,000x.
Plate XI
Plate XII

A secretory cell in the spermatheca of a virgin house cricket. Rough endoplasmic reticulum (RER), Golgi bodies (G), mitochondria (M), tracheoles (Tr) and intracellular spaces (arrows) surround the elongate portion of the nucleus (N). A bundle of microtubules (Mt) is seen in longitudinal profile. Scattered strands of microtubules are visible in other areas of the cell. The remainder of the cytoplasm is filled with free ribosomes. 30,000x.
Plate XII
Plate XIII

Transverse section through the reservoir of an inactive secretory cell in a virgin female spermatheca. Microvilli project into the cavity from all angles. Mitochondria are also scattered in the cavity lodged in the microvilli (arrow). Mitochondria are the dominant cytoplasmic organelle in the vicinity of the reservoir; tracheoles (Tr) are also numerous. The extensive layering of cytoplasm to the right of the reservoir probably represents storage of excess plasma membrane prior to its utilization during spermathecal filling when the gland expands enormously. Two nuclei (N) in the lower portion of the picture bracket the reservoir. 9,000x.
Plate XIII
d. The duct cells

(Plates XIV-XVI) The cells of the duct region are packed with round, ovoid, or irregularly-shaped mitochondria. Nuclei are not apparent in this region, but the sparse cytoplasm contains scattered ribosomes and occasional segments of rough ER. Bundles of microtubules are interspersed throughout the cells, as are intracellular spaces of uncertain derivation. Certain spaces appear as voids in the cytoplasm. Others, possibly tracheoles or ducts, are bordered by a membrane and occasionally contain circular membranes within. Large secretory vesicles containing electron dense material are prominent in some areas.

The apical margin bordering the cuticular intima of the lumen is completely occupied by microvilli. Dense aggregations of microvilli in this area are found in spermathecae of both virgin and mated females. The intima is constructed of laminated chitin and protein that is extensively folded in virgins.

e. The lumen

In virgin females the lumen of the spermatheca contains fine granular material of uniform consistency. In the mated spermatheca, sperm are the only structural element observed in the lumen. Glycogen deposits were not observed in the lumen but positively PAS-staining material was located in the lumen and in the duct cell region of the epithelium.

In the virgin female these regions are each many cells thick and the gland as a whole is collapsed on the lumen. The secretory and duct cell layers are deformed by extensive folding and overlapping. At mating,
Plate XIV

Duct cell region of the spermathecal epithelium of a virgin house cricket. The cells are almost completely filled with mitochondria (M), while microvilli (MV) proliferate along the apical margin. The cuticular intima (I) secreted by these cells consists of lamellae of chitin and protein (asterisk). The lumen (L) contains a fine granular substance. 21,000x.
Plate XV

Duct cell region of the spermathecal epithelium of an old mated house cricket. In this field, the cytoplasm is packed with mitochondria (M). Several bundles of microtubules (Mt) can be seen in cross-section. Intracellular spaces (arrows) and a large lysosome (Ly) are also present. The even plasma membrane delimiting the cell is interrupted at intervals by aggregations of microvilli (MV). 30,000x.
Duct cell region near the lumen of the spermatheca of a virgin female. Large vacuoles (V) containing electron-dense material are interspersed in the cytoplasm which is packed with mitochondria. Free ribosomes (R) fill the interstices around the mitochondria. A large network of microvilli (MV) occupy the lower portion of the field. A bundle of microtubules (Mt) is also present. 30,000x.
Plate XVI
the spermatheca expands greatly: the muscle layer is stretched to a thin sheath of cells and the secretory and duct cell regions become distinct and highly organized. The lumen is filled with sperm and the gland volume increases many-fold.

D. Discussion

1. Role of the spermatheca in mating

The results of these experiments indicate that in the house cricket the spermatheca is the immediate target of the ovipositional signal. The information contained in the signal is transmitted through nerves, presumably to the brain which then coordinates the female response. Hormones do not appear to be involved: ventral nerve cord transection alone abolishes oviposition in mated females and prevents successful sperm transfer to virgin females.

Reception and initial processing of the oviposition stimulus are poorly understood for most species but studies have been completed for several species. Like the cricket, the signal is received by the spermatheca in *Rhodnius prolixus* (Davey 1965), and in the Cimicidae the receptor site is the conceptacula seminis, a sperm storage organ in the upper reproductive tract (Davis 1965b). However the bursa copulatrix serves as the target organ in *Diploptera* (Roth and Stay 1961) and *Hyalophora* (Riddiford and Ashenhurst 1973).

Because the neuroendocrine centers in the insect head direct and regulate many aspects of female reproductive physiology (Engelmann 1970, Highnam and Hill 1977), it is likely that the cricket stimulus is transmitted to the brain which then organizes an egg laying response.
The oviposition signal is transmitted to the brain by hormones in *Hyalophora* (Riddiford and Ashenhurst 1973) and *Rhodnius* (Davey 1965) or through the nervous system as in *Triatoma* (Mundall 1978), *Cimex* (Davis 1965b) and *Acheta*. The final signal that causes oviposition appears in several insects to be a myotropic factor released from the corpora cardiaca (Davey 1967, Truman and Riddiford 1971, Highnam and Hill 1977), though in both *Triatoma* (Mundall 1978) and the house cricket an intact ventral nerve cord is required.

The mechanism for translating a mating event into an oviposition event varies widely among the insects, but the lack of evidence for hormonal involvement seems to be unusual. Oviposition in the house cricket, however, is independent of egg development and occurs at irregular intervals during the long reproductive life of the female. Egg laying episodes also require certain environmental stimuli (viz., substrate moisture) that are perceived by sensory neurons. Thus, in the cricket at least, neural mediation appears to be the more efficient means of regulating unpredictable ovipositional events.

2. Role of the spermatheca in sperm maintenance

The spermatheca is well-adapted for storing sperm over long periods. In the muscular sheath investing the gland, the muscle fibers contain A bands with large amounts of actin relative to myosin, and I bands composed entirely of actin are numerous. Such a pattern is typical of slowly contracting visceral fibers (Smith 1968). Axons of the nerve cells contain neurotransmitter vesicles appropriate for muscle excitation and neurosecretory granules consistent with a role in regulating glandular secretion. The presence of numerous tracheae
indicates a large requirement for oxygen and gas exchange.

The epithelium, divided into two layers clearly distinguishable at the histological level, possesses a glandular region modified for synthesis and secretion of products for the sperm and a duct region which secretes the cuticular intima, guides the glandular secretion into the lumen and is adapted for ion and small molecule exchange. The packed mitochondria and extensive microvilli along the apical border resemble similar structures in the rectum and Malphigian tubules of *Calliphora* (Gupta and Berridge 1966, Berridge and Gupta 1967, 1968, Smith 1968). These tissues are intensively active in ion and water uptake.

The massive change in volume that accompanies spermathecal filling is achieved by rearranging cellular components, especially plasma membranes, that had been present in large excess. The intracellular spaces disappear and the glandular regions are reduced in thickness to single cells. Intercellular boundaries are maintained intact during these changes by the well-developed system of microtubules.

The structural organization of the house cricket spermatheca is similar to that of other insects that must maintain sperm for long periods and which lack an accessory gland to the spermatheca (Dallai and Melis 1966, Dallai 1966, Gupta and Smith 1968). If a spermathecal gland is present, the spermatheca itself is usually less complex (Jones and Fischman 1970, Dallai 1975).

The profusion of mitochondria in the house cricket spermatheca is notable and suggests a physiological demand for energy that is not present in other insects. It could be related to the mechanism of sperm storage. Sperm survival in *Apis* appears to depend on an ionic
mechanism (Verma 1973) which would require a large energy expenditure for its maintenance. The nature of the secretion that bathes sperm in the cricket spermathecal lumen is not clear, but may be a polysaccharide based on the PAS-staining test. Davey and Webster (1967) reported a non-glycogenic polysaccharide from the spermatheca of Rhodnius. Unlike the house cricket, Gryllus campestris and Gryllotalpa gryllotalpa utilize glycogen as an energy substrate for sperm survival (Dallai and Melis 1966, Melis and Dallai 1966).

The general features of the cricket spermatheca are schematically illustrated in Fig. 20. Actual ducts leading from the glandular reservoir and intersecting the cuticular intima were not observed, but are proposed based on their occurrence in spermathecae from other species (Dallai and Melis 1966, Dallai 1966, Gupta and Smith 1968) and other integumentary glands (Beams et al. 1962, Eisner et al. 1964, Happ et al. 1966, Dallai 1967).
Diagrammatic representation of the spermathecal wall showing the muscle sheath, epithelium, and cuticular lining of the lumen.
SUMMARY

1. Except for the first day after eclosion, adult female house crickets will mate at any time. A mated female begins to lay eggs when she is 12-14 days old under my conditions (25°C, 12 hr light, 12 hr dark). Beyond this age, females begin laying eggs shortly after mating.

   Females require a moist substrate for egg laying, and if one is provided, they lay about 25-30 eggs/day for two months. The rate of egg laying declines in senescent individuals but can be temporarily stimulated by a second mating.

2. Sperm constitute the egg laying stimulus transmitted by the male at mating. Females which mate but are denied sperm by removal of the spermatophore or by male castration or irradiation fail to lay eggs. Females which receive sperm despite these treatments lay eggs. While the presence of sperm in the female is required for egg laying, the number of eggs laid is not related to the quantity of sperm received.

3. Prostaglandins E and F2α were found in testes and spermatophores of males and in the spermathecae of mated females. Prostaglandins do not evoke and regulate oviposition, although a temporary suppression of egg laying was observed in females fed large amounts of aspirin and acetaminophen.

   Evidence from vertebrates suggests that the PGs are involved in sperm development or function. Irradiation studies and the occurrence
of PGs in young females' spermathecae containing sperm indicate that such a role is plausible in crickets.

4. Exceedingly high levels (about 4000 pmol) of cyclic GMP are found in the ARG, but its distribution is extraordinarily restricted to a small number of tubules on one side of the gland. Cyclic GMP is secreted into tubule lumina where it is protected from phosphodiesterases, and is incorporated into spermatophores.

In the spermatophore it is highly concentrated in the handle and capillary tube although a small portion is found in the insemination mixture. During mating the level of cyclic GMP in the bursa copulatrix and spermatheca rises briefly. This increase is abolished by the end of spermatophore evacuation and cyclic GMP levels remain high in the empty spermatophore.

Although its localization and movement are known, a physiological role in cricket reproduction has not yet been ascribed to cyclic GMP.

5. Cyclic AMP was found on spermatozoa in the seminal vesicles, spermatophore and spermatheca. This represents the first direct association of a cyclic nucleotide with sperm. These findings in conjunction with the striking pattern of cyclic GMP distribution and with evidence for prostaglandin participation in sperm development or function make this a system with obvious potential for elucidating cyclic nucleotide and prostaglandin interactions in biological regulation and in reproductive processes.
6. In the female the target organ receiving the oviposition stimulus is the spermatheca. Sperm in the spermatheca cause the release of a signal transmitted via the nervous system, allowing eggs to be laid. It presumably acts on the brain which then coordinates ovulation, oviposition and other reproductive events.

In addition to transponding the oviposition signal the spermatheca stores sperm so that eggs can be laid and fertilized throughout adult life after a single mating. Structural modifications that enable the spermatheca to accept and store large amounts of sperm are similar to those found in other insects.

7. House crickets have developed a relatively direct and simple method for insuring that females will be competent to lay fertile eggs throughout their reproductively active lives. The presence of sperm in the spermatheca is the only requirement needed for egg laying, and thus assures that the eggs can be fertilized.

The significance of the biochemical findings cannot be assessed yet because too little information is available. Nevertheless, this area holds great promise as an experimental model for determining physiological roles in reproduction for cyclic GMP, cyclic AMP and the E and F prostaglandins.
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