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Anthony J. Lentz

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James R. Miller
Major professor

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AN ANALYSIS OF THE PRESENCE AND ROLE OF HUMORALLY MEDIATED, MALE-DERIVED OVIPOSITION-INDUCTION FACTORS IN INSECT MATING SYSTEMS

By

Anthony J. Lentz

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ABSTRACT

AN ANALYSIS OF THE PRESENCE AND ROLE OF HUMORALLY MEDIATED, MALE-DERIVED OVIPOSITION-INDUCTION FACTORS IN INSECT MATING SYSTEMS

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Male accessory gland factors that induce oviposition after mating (sex peptides) are not a universal feature of insects. Aqueous extracts of male reproductive tissues injected into conspecific virgin females stimulated oviposition comparable to mated controls in *Aedes aegypti* (83 to 91 eggs/♀ vs. 54 to 102 eggs/♀ for mated group) and *A. albopictus* (28 eggs/♀ vs. 37 eggs/♀ for mated group), but not in *Musca domestica, Anastrepha suspensa*, or *Lymantria dispar*. Extracts of the female seminal fluid storage organ (vaginal pouch) from mated *M. domestica* and the spermathecae from mated *L. dispar* also did not induce oviposition when injected into conspecific virgin females.

Heterospecific injection of *A. aegypti* accessory gland extract into *A. albopictus* virgin females did not cause appreciable oviposition (12 eggs/♀) but injection of *A. albopictus* accessory gland extract elicited oviposition in virgin *A. aegypti* (77 to 111 eggs/♀) comparable to mated controls. These findings suggest one physiological mechanism by which *A. albopictus* may competitively displace *A. aegypti* through asymmetrical mating interference where populations are sympatric or parapatric.

I propose three levels of intraspecific mating communication by insects within the context of sex peptide signalling: (1) Chemically-mediated mating-receptivity inhibition and oviposition induction; (2) only mating receptivity inhibition; (3) neither. Sex peptides appear to be present in both monogamous and

polyandrous species as well as in female mate choice and male-regulated mating systems.

Diverse fractionation techniques were used in extensive attempts to isolate the active factor(s) from *A. aegypti* paragonial glands: polyethylene glycol precipitation, gel filtration chromatography, DEAE ion exchange chromatography, reverse phase (C18) high performance liquid chromatography, QAE (strong anion exchange) HPLC, DEAE HPLC, capillary electrophoresis, electrodialysis, discontinuous nondenaturing polyacrylamide gel electrophoresis and gradient nondenaturing PAGE. Oviposition-induction activity, as measured by injecting females with fractionated extracts and enumerating egg output, was partially to fully recovered following PEG precipitation, capillary electrophoresis, discontinuous and gradient nondenaturing PAGE, but significant fractionation was not achieved. Activity was not recovered following the other methods. Analysis by SDS•PAGE of fractions from several experiments showed a 32.5 kDa protein common to all active fractions that was not present in nonactive fractions.

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General Introduction

Intersexual conflict. If genetic success is defined as transmitting genes from one generation to another (Thornhill & Alcock 1983), a sexually reproducing insect first has to be successful in mating. A female is genetically successful if, as a result of mating, her ova are fertilized preferably with sperm from a higher rather than lower quality male. A male attempting to transfer sperm is genetically successful if the female recipient uses his sperm. The evolution of mating systems that facilitate successful gene transfer can be examined in terms of sexual selection theory, which suggests that natural selection will favor females that evolve mechanisms maximizing mate choice (Parker 1984). In contrast, selection will favor males that employ mechanisms minimizing sperm competition (Parker 1984). In other words, males capable of inducing or forcing females to utilize their sperm when other sperm are available will be genetically successful. Therefore, the paths leading to female and male genetic success may involve conflicting reproductive strategies and the conditions that have produced these sex-specific differences likely reflect the difference in the size of parental investment by females and males.

Female insects produce relatively few ova in a lifetime, typically measured in hundreds of eggs (Ridley 1988), and provide them with nearly all of their nutrients through vitellogenesis (in sensu Thornhill & Alcock 1983). Because oogenesis and, especially, egg provisioning generally require significantly greater expenditure than spermatogenesis, females produce fewer gametes than males for propagating the next generation. Consequently, each egg has a higher intrinsic value than each sperm and

the cost of wasting an egg through combination with inferior sperm also is higher. Under these circumstances, selection favors females that mate with a male providing "superior" sperm and mating systems that increase the probability that females will mate with such males (Thornhill & Alcock 1983).

In contrast, male investment per gamete is small. Males produce thousands of sperm in a lifetime and, unless spermatophores are formed, provide few nutrients to the female that might even indirectly benefit the eggs. They rely on the female to provision the eggs. Because their investment in each sperm is minuscule, males have little to lose by mating several times and are, therefore, less selective about their mates (Thornhill & Alcock 1983). Consequently, males typically have evolved mating strategies that maximize the number of potential mates, increasing the probability of transferring their genes to the next generation. As an example, males may acquire several mates in areas where females or resources attractive to females are clumped together (Table 8.1, Thornhill & Alcock 1983). Even where females or resources are widely dispersed, males have evolved dominance-based mating systems such as leks or scramble competition polygyny that reward superior males with more than one mate.

Insect mating systems are classified by Thornhill and Alcock (1983) according to the number of copulatory partners per individual female and male. Polygynous mating systems refer to males that copulate with more than one female; polyandrous mating systems refer to females that copulate with more than one male; and monogamous systems are characterized by females or males that mate with only one partner. According to Thornhill and Alcock, most insect mating systems are characterized by monogamous females and polygynous males although dual monogamy and polyandry/polygyny also occur. The prevalence of each type of mating system as well as the mate acquisition and selection strategies that have

evolved within each mating system reflect gender-based differences in gametic investment and population-based differences in mating opportunities.

Female control. For females, many species of insects possess an internal sperm storage organ called the spermatheca in which sperm can be maintained for as long as a year or more (Gordon & Gordon 1971) and released by the female during oviposition (Davey 1985). Sperm are often stored such that the last sperm in are the first ones out and the first ones to be used to inseminate eggs descending through the oviduct (e.g., Leopold & Degrugillier 1973; Adler & Adler 1991). In fact, Walker (1980) presents a hypothesis arguing that sperm precedence has evolved not because of male intrasexual competition but because of female sperm-utilization strategies. Females that store sperm generally have more control over the paternity of their progeny (Thornhill & Alcock 1983). For polyandrous females, it is advantageous to mate with successively "better" males because the last mate before oviposition will supply most of the sperm for fertilization. Exceptions to this include queen honey bees that mate four times each nuptial flight but sperm from each are mixed evenly in the spermathecae and, presumably, disseminated equally during fertilization (Page & Metcalf 1982). If a female is monogamous it is generally to her advantage to choose the best mate available.

Male control. Male insects of many species have evolved their own means of controlling paternity, the most visible being some form of mateguarding, which I classify into three types: physical, behavioral and chemical. Each type incurs a set of costs and benefits (Table 1) and, in general, males are expected to guard females as long as the energetic and gametic costs of guarding are lower than the cost of pursuing other mating opportunities (Miller et al. 1994). Examples of physical (or barrier) mateguarding are spermatophores that seal the genital opening in female

Table 1. Costs and benefits of various forms of male mateguarding

Type of mateguarding	Definition	Characteristics
Physical	Physical barrier left in ♀ to block deposition by other ♂s	Moderately effective Relatively easy for females to overcome Moderate to high energetic expense Moderate to high loss of opportunity
Behavioral	Males remain with ♀ after mating and deter other ♂s from copulating	Highly effective Hard for females to overcome High energetic expense High loss of opportunity
Chemical	Factor delivered in semen causes ♀ to refuse additional matings	Highly effective May be overcome by selection Very low energetic expense Very low loss of opportunity

Lepidoptera, Orthoptera, some Hymenoptera and other orders (Thornhill & Alcock 1983). An unusual type of barrier mateguarding is found in honey bees in which the male detaches from his genitalia following copulation (Taber 1954). Even more dramatic are ceratopogonid males that are consumed by the female during mating, leaving their genitalia in place to block entry by other males (Downes 1978). Although spermatophores and genitalia plugs may be removed by either males or females in some cases (see Waage 1979 for description of active removal of sperm by male rivals in damselflies; Gage 1992 for description of sperm removal in *Tenebrio molitor*), they function to prevent entry of another individual's sperm.

In behavioral mateguarding, males actively defend females until they are no longer sexually receptive or have oviposited. The literature is replete with examples throughout most of the insect orders and may take the form of either precopulatory or postcopulatory mateguarding (Thornhill & Alcock 1983; Kipp et al. 1990; Alcock 1991; McMillan 1991; Sakaluk 1991). A common example of such mateguarding is

dragonflies that fly in tandem prior to or following mating. The third type, chemical mateguarding, provides the backdrop for my research on the role of humoral-based factors in insect mating systems.

Background on sex peptides. In beginning this thesis, I tentatively defined a mateguarding chemical as a factor produced by a male that when transferred to a female during copulation either inhibits further mating, induces oviposition or both. Whether such chemicals function to control paternity or simply act as a signal to the female that mating has occurred is unknown. Following Thornhill and Alcock's (1983) terminology, receptivity inhibition and oviposition induction factors, also collectively referred to as sex peptides (see below), would be classified as postcopulatory mateguarding mechanisms. Substances with sex peptide-like activity have been shown to occur in several orders of insects including Hemiptera, Orthoptera, Lepidoptera, Coleoptera, and other Diptera (reviewed by Gillott 1988). Most studies have demonstrated that this factor (or factors, if receptivity inhibition and oviposition are controlled by separate mediators) is generally secreted by the paragonial glands of the male.

In Diptera, evidence for such a substance has come primarily from transplanting male paragonial glands (also known as sex accessory glands) or ejaculatory ducts into virgin females and observing the effect on female mating receptivity. Previous transplant studies have included the mosquitoes *Aedes aegypti, A. albopictus, Culex pipiens*, several *Anopheles* species (Leahy & Craig 1965; Leahy 1967; Craig 1967), *Drosophila melanogaster* (Leahy 1967), *Musca domestica* (Riemann et al. 1967; Riemann & Thorson 1969) and the stable fly, *Stomoxys calcitrans* (Morrison et al. 1982). Other investigations have concentrated on extracting the active substance from male accessory glands for injection into females to examine receptivity inhibition in *A. aegypti* (Leahy 1967), *C. tarsalis* (Young &

Downe 1987), *D. melanogaster* (Leahy 1967), and *M. domestica* (Adams & Nelson 1968; Nelson et al. 1969). In our laboratory, Spencer et al. (1992) have conducted extensive investigations of oviposition and mating receptivity in the onion fly, *Delia antiqua*. Extracts of male paragonial glands injected into virgin females induce permanent monogamy and stimulate oviposition comparable to normally mated females. Recent experiments have demonstrated a similar factor in a closely related species, *D. platura*, the seedcorn fly (Spencer et al. 1994).

Comparatively little research has been done on neural control over female receptivity and oviposition in response to a male-produced factor. Allatectomy of *M. domestica* females less than 48 hours old inhibited mating (Adams & Hintz 1969). *M. domestica* females continued to be sexually receptive if ligated or decapitated as virgins but remained permanently unreceptive if ligated following mating, suggesting endocrine rather than neural control of mating inhibition (Leopold et al. 1971a). Using ventral nerve cord transection and ganglionectomy, Gwadz (1972) demonstrated that *A. aegypti* male paragonial factor induced mating inhibition by acting on the terminal abdominal ganglion. Decapitation of gravid crane flies induced immediate oviposition, suggesting that a neural inhibition center was removed (Chiang & Kim 1962).

In a sense, research on the isolation and characterization of male accessory gland factors began with chromatographic studies by Fox (1956), in which he demonstrated sex differences in protein content of *D. melanogaster*. Using whole fly squashes on two-dimensional paper chromatography Fox isolated a ninhydrin-positive, male-specific amino acid-containing substance, labelling it "sex peptide" (Fox 1956; Fox et al. 1959). Biological activity was not investigated and it remains unknown whether the protein comprising the spot was an oviposition-inducing or receptivity-inhibiting factor. Early fractionation studies of *A. aegypti* accessory gland extract (Fuchs et al. 1969; Fuchs & Hiss 1970; Hiss & Fuchs 1972) suggested

two factors were needed to inhibit receptivity although their character and identities were not at all clear (discussed in Chapter 4). Gel filtration chromatography of *Culex tarsalis* male accessory gland extract yielded a protein fraction of approximately 2 kDa that inhibited receptivity when injected into virgin females (Young & Downe 1987). Further characterization of *C. tarsalis* sex peptide has not appeared in the literature.

Chen et al. (1988) have established the benchmark for more recent isolation and purification studies of sex peptides. They, Chen and Balmer (1989), and Schmidt et al. (1993) have identified the primary structure of sex peptides in *D. melanogaster*, *D. sechellia* and *D. suzukii*. Peptides from the first two species consist of 36 amino acids and differ at only three positions. Sex peptide from *D. suzukii* is 41 amino acids long and has the same N-terminal and C-terminal sequences as the peptides from the other two species. All are unusually high in hydroxyproline. Purification of *D. melanogaster* sex peptide was carried out by preparing methanolic extracts of paragonial glands, fractionating by HPLC reverse-phase chromatography and testing for biological activity. A physiological dose of the active fraction (equal to ½ paragonial gland) injected into virgin females suppressed sexual receptivity and stimulated ovulation and oviposition for several days. The HPLC fraction was purified on a Vydac C_4 column and the resulting isolate was sequenced. Similar procedures were used for identifying sex peptides from the other two species (Chen & Balmer 1989; Schmidt 1993). Using the *D. melanogaster* sequence information, oligonucleotide probes were synthesized, two cDNA libraries were constructed and screened, and analysis of overlapping regions from the isolated clones provided the entire cDNA sequence of the polypeptide mRNA. Chen et al. (1988) concluded that the peptide is synthesized in a form aiding its transport across the membrane of the endoplasmic reticulum, then the

signal sequence is cleaved. They postulate that target sites for the peptide may be receptors in brain neurons and, possibly, ovarian cells.

Only a few studies have examined whether oviposition-inducing and receptivity-inhibiting factors are species specific or heterospecific. In studies of ovipositional behavior, Leahy and Craig (1965) showed that *A. albopictus* accessory gland transplants stimulated *A. aegypti* females to oviposit but the effect was not reciprocal. Studies of accessory gland transplants involving *A. aegypti, D. melanogaster,* and *C. pipiens* (Leahy 1967) also showed some heterospecific oviposition activity. Hiss and Fuchs (1972) demonstrated that injection of *D. melanogaster* accessory gland extract stimulated oviposition in *A. aegypti*. Cross-reactive mating inhibition studies by Nelson et al. (1969), demonstrated that accessory gland extracts from the screw-worm fly, *Cochliomyia hominivorax*, the black blow fly, *Phormia regina*, and *A. aegypti* prevented mating in *M. domestica* to varying degrees. In contrast, *S. calcitrans* females receiving injections of accessory gland extracts from *Sarcophaga bullata* (flesh fly), *M. domestica*, and *P. regina* were not prevented from mating (Morrison et al. 1982). Sex peptides from both *D. melanogaster* and *D. sechellia* were equally efficient in repressing sexual receptivity in each other as well as in *D. simulans* and *D. mauritiana*, but not in a more distant species, *D. funebris* (Chen & Balmer 1989). Similarly, *D. melanogaster* and *D. suzukii* sex peptides were reciprocally active with respect to mating inhibition and oviposition induction (Schmidt et al. 1993). It appears, therefore, that some species possess a heterospecific oviposition factor while others do not.

Based on my review of the literature, most of the previous research was concerned with the role of the male accessory gland material in inhibiting female sexual receptivity rather than its role in stimulating oviposition. Further, much of the work on receptivity inhibiting substances was done in only two species:

A. aegypti and *D. melanogaster*. Virtually nothing has been published on an oviposition factor in *A. albopictus* since 1965.

Objectives and hypotheses. I undertook a new set of investigations to begin addressing the many unanswered questions about the nature and extent of oviposition factors in Diptera. Important differences between most of the earlier work and my studies are that my experiments utilized gland extracts rather than gland transplants. Under the best circumstances, this approach should increase the relative concentration of the active factor as well as reduce trauma to the female resulting from surgery. Second, I focused on the role of this substance in stimulating oviposition rather than in inhibiting receptivity. Third, the investigation included the Caribbean fruit fly, *Anastrepha suspensa*, an economically important species previously unstudied. Fourth, I examined the cross-specificity of extracts shown to enhance oviposition activity. Finally, isolation and purification procedures were conducted using modern protein separation methods and equipment.

I decided on this topic because of my interest in both evolutionary and mechanistic questions about insect reproductive biology, especially insect mating systems. Second, there is a phenomenon known as asymmetric mating interference in which one species competitively displaces another because the males of one species are able to reduce the reproductive output of another species (Ribeiro & Spielman 1986; Ribeiro 1988). I wanted to investigate the possible involvement of sex peptide. Third, since virgin females activated by sex peptide readily lay unfertilized eggs, this suggests several novel forms of insect control whereby synthetic sex peptide could be delivered (e.g., in microencapsulated food or an engineered virus) to females before they become sexually active.

The majority of my research was conducted on *A. aegypti* using an oviposition bioassay. Although I chose to work with this mosquito because of its

medical importance, ease of rearing and availability, the best rationale for studying any aspect of mosquito oviposition has been concisely stated by Bentley and Day (1989):

> "Since pathogen acquisition by vector mosquitoes usually requires the taking of at least one blood meal, disease transmission usually requires the completion of at least one oviposition cycle before pathogen transfer can occur with a subsequent blood meal. Oviposition is thus an important component of most mosquito-borne diseases."

Experiments were designed to incorporate the logical components of a falsificationist test, so thoroughly described by Underwood (1990). I adhere to the terminology defined in that paper and summarize it here. *Observations* are the recognition of a pattern or departure from a pattern in space or time (aka puzzles, problems). *Models* are attempts to provide a valid explanation for the observations (aka theories or explanations). *Hypotheses* are predictions deduced from the model and are based on the model being correct. Hypotheses state an expected outcome for a defined (and as yet untested) set of circumstances consistent with the model (aka predictions, deductions). *Null hypotheses* are the logical opposites of hypotheses and are constructed "to include all possibilities except the proposition in the hypothesis. Disproof of the null hypothesis leaves the hypothesis as the only alternative, but obviates the need to attempt to prove the hypothesis." *Experiments* test the validity of a model by attempting to falsify its predictions. The results from experiments are used either to reject or retain the null hypothesis and, if appropriate, to modify the model.

In so doing, my working hypotheses for conducting this research were: (1) Sex peptides are ubiquitous across Diptera. My specific hypothesis for examining each species was that males secrete a substance in their accessory glands (or homologous structures) that, when transferred to females during copulation, induces oviposition and/or inhibits female receptivity to subsequent mating attempts.

(2) Oviposition factors function as prezygotic, postmating reproductive isolation mechanisms between closely related species. I postulated that extracts from *A. albopictus* are bispecific for *A. aegypti* but not vice-versa.

(3) Oviposition and receptivity factor(s) from *A. aegypti* can be fractionated, isolated, and identified using techniques similar to those successfully employed for *Drosophila*.

The terms accessory gland, sex accessory gland and paragonial gland are used synonymously throughout this dissertation but across all insects they do not always refer to the same structure (Gillott 1988). Similarly, sex peptide refers to a male-derived, humorally-mediated factor(s) that inhibits mating receptivity, induces oviposition or both.

Chapter 1

Effect of male accessory gland extracts on induction of oviposition in the gypsy moth, *Lymantria dispar*

Abstract

In gypsy moths, *Lymantria dispar* L. (Lepidoptera: Lymantriidae), injection of saline extracts of male reproductive glands did not cause appreciable early oviposition in virgin females. Freshly mated females ligated between the thorax and abdomen to prevent transmission of humoral factors oviposited at the same time (37 to 53 minutes) as mated, nonligated, control females (59 minutes). Transplantation of spermathecae from mated and senescent, actively ovipositing females into virgin moths did not elicit early oviposition. Females stimulated by insertion of a probe or injection of saline into the bursa copulatrix failed to oviposit. These data corroborate previous findings suggesting that initiation of oviposition in gypsy moths is mediated neurally.

Introduction

Mechanisms for induction of oviposition differ among the Lepidoptera. In most cases, the presence of eupyrene sperm and/or testicular fluids in the female reproductive tract is needed to initiate oviposition. For *Hyalophora cecropia*, the bursa copulatrix appears to secrete a bursa factor after being filled with sperm; oviposition is initiated by an undefined humoral mechanism (Riddiford & Ashenhurst 1973; Sasaki et al. 1983). A bursa factor also has been postulated for *Manduca sexta* (Sasaki & Riddiford 1984). However, in a follow-up study, Stringer

et al. (1985) found that bursa transplants from mated females, which were expected to be humorally active, did not induce oviposition in virgins. They suggested other factors, such as juvenile hormone, also may be needed and that the expanded bursa might transmit neural signals to the corpora allata to maintain the titer of JH. In the leek moth, *Acrolepiopsis assectella*, spermatozoa must migrate to the spermatheca to stimulate oviposition (Thibout 1979). Supposedly, expansion of the spermatheca generates a neural ovipositional signal; however, the data also are consistent with ovipositional stimulation by a combined neural and humoral signal. Eupyrene sperm and/or testicular fluids must be present (tissue not specified) for oviposition by spruce budworm moths, *Zeiraphera diniana*, and cabbage loopers, *Trichoplusia ni* (Benz 1969; Karpenko & North 1973).

There is ample evidence for both neural and humoral control of reproductive events in Lepidoptera as exemplified by studies of pheromone biosynthesis regulation in *Heliothis* moths (Teal et al. 1990; Christensen et al. 1991). They suggested that pheromone biosynthesis-activating neuropeptide is released by efferent nerves acting on the terminal abdominal ganglion, which then signals the pheromone gland to synthesize pheromone. They also discovered a bursa factor that suppresses pheromone production through an undefined mechanism.

Males of several insects transfer factors to females during copulation that induce oviposition and inhibit receptivity to additional mating. Such substances have been reported in Diptera, Orthoptera, Lepidoptera and Coleoptera (Leahy & Craig 1965; Leahy 1967; Nelson et al. 1969; Yamaoka & Hirao 1977; Morrison et al. 1982; reviewed by Gillott 1988). These factors are thought to be secreted by the accessory reproductive glands of the male. In Diptera, they are referred to as sex peptides based on the pioneering work by Fox (1956; 1959). Chen et al. (1988; Chen & Balmer 1989) have conducted the most extensive research on dipteran sex peptides and have been able to identify, characterize, and synthesize the sex peptides

in three species of *Drosophila*. They probably are transmitted through the bursa to the hemolymph and act on the brain to trigger oviposition (Chen 1991; Aigaki et al. 1991).

The physiological basis for induction of oviposition in gypsy moth, *Lymantria dispar*, is not fully understood. Adults are sexually mature upon eclosion and generally mate on the day of emergence (Leonard 1981). Females begin to lay an egg mass within 4-6 h after mating and complete the process within 3 days. If females have not mated within 5 days, they switch from virgin to mated behavior and spew some of their mature eggs before dying (Giebultowicz et al. 1990a). Although Giebultowicz et al. (1990b) suggest that sperm must be present in the spermathecae to initiate oviposition in gypsy moths, no studies specifically have examined whether humoral factors are involved.

Given prior research in Dr. J. Miller's laboratory on oviposition-stimulating substances (Spencer et al. 1992), the recent success in sex peptide characterization achieved by Chen et al. (1988; Chen & Balmer 1989), and the lack of comparable studies on gypsy moth, I addressed the possibility that induction of oviposition in *Lymantria dispar* might be influenced by a humoral factor from the male accessory glands. Discovery of substances promoting premature oviposition of unfertilized eggs might provide clues towards control of this economically important pest (Leonard 1981).

Methods and Materials

Rearing. Gypsy moth (*Lymantria dispar*) egg masses, obtained from the USDA-APHIS facility at the Otis ANGB in Massachusetts, were surface-sterilized by soaking in 10% formalin for 1 h followed by a cold water rinse for 1 h. Insects were maintained at 24 $\pm$ 2°C, 55 $\pm$ 5% RH, and a L:D 16:8 h cycle. Groups of 10 newly emerged larvae were placed into 60 ml clear plastic cups and reared on a high

wheat germ diet (Bell et al. 1981). During the second instar, individual larvae were transferred into cups with sufficient food for growth to pupation. Pupae were sexed according to size (females are approximately 50% larger than male pupae). Female pupae were placed into a group cage and males were placed individually into 150 ml plastic cups until adult eclosion. Since gypsy moths are sexually mature upon eclosion and because virgin females begin senescent oviposition at about 5 days old, experiments were conducted on males and females 0 to 2 days old, unless noted otherwise.

Ligation. Newly eclosed female moths (less than 18 h old) were ligated 1, 15, 30, 60 and 180 min after the onset of mating to disrupt humoral communication between the abdomen and head. A 5-0 nylon suture was looped around the thoracic-abdominal junction of female moths, then tightened sufficiently to stop hemolymph flow but not enough to sever the ventral nerve cord. In positive (normally mated) and negative (virgin) control moths, ligatures were applied but not tightened. Anesthesia was not used. The ligature was not tightened prior to copulation (at 0 min) because a preliminary experiment suggested that it reduced either a female's willingness to copulate or her attractiveness to the male. I judged it unlikely that any humoral factors would be released into the hemolymph from the bursa and be transported out of the abdomen during the first minute of copulation. All moths were injected in the abdomen with 0.5-1 ml India ink (undiluted) at the end of the experiment to identify moths with incomplete ligations, and some were injected immediately after ligation to measure failure rate of ligation. Preliminary tests showed that India ink readily circulated through all three body cavities of nonligated females.

Extract preparation and injection. Male whole reproductive tract, accessory gland/ejaculatory duct complex, vas deferens/seminal vesicle complex, and testis were dissected from freshly frozen moths and placed into a microcentrifuge vial containing Ringer's saline kept on ice. Extracts were prepared by homogenizing the tissues with a sonicating probe for 20 to 30 seconds with an ultrasonic probe, setting the tuner≈3 and the power≈35 (Blackstone Ultrasonics Inc., Sheffield, PA), then centrifuging at 6000 g for 10 minutes. The concentration of the supernatant was adjusted so that 7.0 µl of extract corresponded to 1 male equivalent of tissue. Extracts were used immediately or removed and stored for up to 5 days at -20°C before use. Female moths were anesthetized with CO_2 for 2 min, then injected with a 30 gauge needle into the ventrolateral side of the pleuron between abdominal segments A2 and A3.

Surgical procedures. Female moths were anesthetized with diethyl ether for 30 to 60 sec prior to surgery. To examine whether isolated abdomens would oviposit, female moths were allowed to mate, then their abdomens were resected at 1, 3, 5, and 7 h after the onset of mating. For tagmatal isolation studies, either the thoracic-abdominal junction or the head-thorax junction was tied off with a suture and resected anteriorly. Since the spermatheca is the target organ of sperm following dissociation of the sperm in the bursa copulatrix and oviposition is markedly reduced in females without a spermatheca but not in females without a bursa copulatrix (Giebultowicz et al. 1990b), I transplanted spermathecae to determine whether they were active humorally. One-day-old virgin females received a spermathecal transplant from one of three donor types: 1-day-old mated, ovipositing females; senescent, ovipositing virgins; 1-day-old nonovipositing virgins. For the transplantation study, spermathecae were removed from donor moths through an incision on the ventral side above the bursa opening. These were transplanted

immediately into virgins through a scalpel puncture anterior and lateral to the bursa. The sham control surgery consisted of a scalpel puncture followed by forceps insertion. All wounds were sealed with melted beeswax.

Quantification of oviposition. Since oviposition in normally mated females generally begins between 3-5 h postmating, all treatments were administered between 0 and 7 h after initiation of copulation to maximize the probability of causing a behavioral response. In the ligation and isolation experiments, ovipositional activity was monitored continuously on the first day and daily thereafter. In other experiments, females were examined daily to record the onset of oviposition. Egg masses were weighed to an accuracy of 0.1 mg.

Results

Effect of ligation on oviposition. To determine whether the presence of a ligature would affect mating and oviposition adversely, the time ligated females remained *in copulo* was compared to that of normally mated females. There were no significant differences in copulating time among ligated females compared to nonligated females, regardless of when females were ligated (Table 2). Although females ligated at 30 min tended to disengage during the ligation procedure or soon after, there was no apparent effect on other parameters measured. Overall, ligation had little effect on duration of copulation.

The effect of ligation on oviposition activity in moths was investigated by measuring elapsed time from initiation of mating to initiation of oviposition and the weight of the egg mass at three days posttreatment. Mated moths in both the ligated and nonligated groups (Table 2) had similar elapsed times to oviposition and similar egg mass weights. Virgin moths placed in oviposition cages at the same time as mated moths took significantly longer to begin ovipositing; only half of the females

Table 2. Gypsy moth mating, oviposition and egg weight at 3 days following ligation. Newly eclosed females were ligated at the times indicated after the onset of mating. In positive and negative control moths, ligatures were applied but not tightened.

Treatment	N	Mean copulation time (min)[1]	Elapsed time to oviposition (h)	Egg mass weight (mg)
Time ligated[2]				
1 min	7	51 ± 14[a]	14 ± 26[a]	522 ± 275[a]
15 min	5	53 ± 21[a]	10 ± 10[a]	579 ± 88[a]
30 min	5	37 ± 13[a]	4 ± 2[a]	568 ± 235[a]
60 min	4	46 ± 17[a]	5 ± 3[a]	663 ± 100[a]
180 min	3	53 ± 17[a]	5 ± 2[a]	548 ± 40[a]
Control				
Mated, not ligated	9	59 ± 21[a]	6 ± 3[a]	712 ± 107[a]
Virgin, not ligated	5	-------	72 ± 1[b]	15 ± 13[b]
Virgin, ligated	7	-------	57 ± 26[b]	101 ± 200[b]

[1] Results are presented as the mean ± standard deviation. Within each column, treatment means with the same letter are not significantly different ($p < 0.05$) using Student-Newman-Keuls test (Ott, 1988) for multiple comparison of means following analysis by a general linear model.

[2] After initiation of copulation

had laid any eggs at the end of three days, which is characteristic of senescent virgins. Mean egg weights were significantly lower as expected (Table 2).

Several moths with incomplete ligations were not included in the above analyses. However, in effect, they served as parallel controls revealing the effect of applying the ligature at each time period without blocking hemolymph flow. Mean latency to oviposition and mean egg weights were not different among mated moths with complete versus incomplete ligations at all time periods (Table 3).

Injection of male reproductive gland extracts. Normally mated (positive control) females oviposited sooner and with more complete formation of the egg mass than any of the treated groups (Figure 1). Virgins injected with one male tissue equivalent oviposited at approximately the same rate as virgins injected with the same volume of saline only. Untreated virgins (negative control) began egg deposition by day 4 as expected. The ovipositional response of moths receiving injections fell between the positive and the negative controls but was not appreciably different from the saline controls and was therefore judged inactive.

Transplantation of spermathecae from ovipositing females into nonovipositing females. Transplanted virgins in all groups oviposited at approximately the same rate as sham operated control virgins, which oviposited later than normally mated females (Figure 2). Both mated and transplanted females initiated oviposition sooner than untreated virgins, but this effect was due to the surgery as evidenced by egg output from sham females.

Effect of abdomen isolation and decapitation on oviposition. None of the isolated abdomens (n=3 for each time period) laid eggs during the three days following resection although they appeared to be alive. In the 5 and 7-h groups,

Table 3. Oviposition and egg weight of gypsy moths with complete and incomplete ligations[1]. Moths with incomplete ligations are shown next to those with complete ligations to illustrate the effect of ligation without blocking hemolymph exchange.

Treatment	Hours to oviposit		Egg weight (mg)	
	Complete ligation	Incomplete ligation	Complete ligation	Incomplete ligation
Time ligated[2]				
1 min	14	7	523[a]	712[a]
15 min	10	6	579[a]	667[a]
30 min	4	7	568[a]	698[a]
60 min	5	2[3]	663[a]	698[a]
180 min	5	7	548[a]	595[a]
Virgin, ligated	57	49[3]	101[b]	24[b]

[1] Treatment means with the same letter are not significantly different at $p < 0.05$ by SNK.
[2] After initiation of copulation
[3] n=1 for these ligations

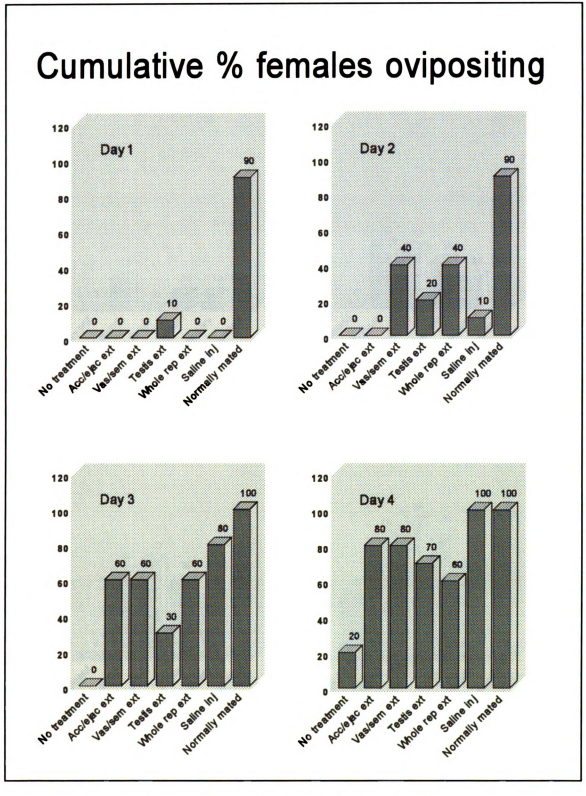


Figure 1. Gypsy moth oviposition following injection with male reproductive tissue extract.

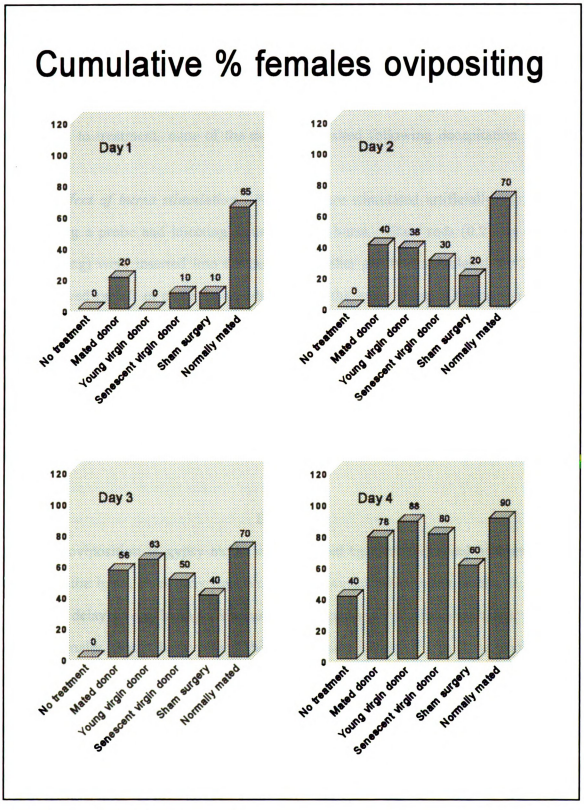


Figure 2. Oviposition by gypsy moths receiving spermathecae transplants.

2 of 3 moths had begun oviposition prior to resection of the abdomen but did not continue laying eggs after isolation. To further evaluate this phenomenon, moths were decapitated 1 h (n=2) and 3 h (n=3) after initiation of copulation so that the thorax and abdomen were intact. Although 1 moth in the 3 h group began laying eggs prior to treatment, none of the moths oviposited following decapitation.

Effect of bursa stimulation. Females were stimulated artificially by introducing a probe and injecting saline into the bursa. Glass rods (0.2 mm diam, 10 mm long) were inserted into the bursa and either gently manipulated for 2 min and removed or left in place for the duration of the experiment. Oviposition by moths at 5 days posttreatment was no greater than that by virgins handled the same way but without rod insertion. In another experiment, injection of 60-70 μl of Ringer's saline into the bursa through fine-tipped, polyethylene tubing also did not induce oviposition greater than that observed in negative controls. Oviposition was graded qualitatively for both experiments.

Discussion

If oviposition in gypsy moths were induced by a male-derived humoral factor acting on the brain, then early ligation should prevent transmission of the factor, causing a delay in egg laying. Similarly, late ligation should have no effect and treated females should behave as if they were normally mated. In these studies, ligated females oviposited at the same time as nonligated females and both groups produced comparable egg masses regardless of when a thoracic-abdominal ligature was applied (Table 2).

Injections of gypsy moth male reproductive gland extracts, including the highly secretory upper vas deferens (Riemann & Giebultowicz 1991), did not stimulate oviposition in virgin females. Studies of male paragonial gland extracts

have shown these methods to be successful for demonstrating humorally active, male-produced oviposition factors in Diptera (Leahy & Craig 1965; Riemann & Thorson 1969; Morrison et al. 1982; Chen et al. 1988). Moreover, in *Bombyx mori* injection of a saline or water extract of whole reproductive tract stimulated oviposition in virgin females (Yamaoka & Hirao 1977) whereas extract of abdomen (minus reproductive tract, alimentary canal and malpighian tubules) produced only a minor effect. In addition, whole reproductive extract added to *in vitro* preparations containing the terminal abdominal ganglion increased the spontaneous firing of associated motoneurons. Yamaoka and Hirao (1977) provided evidence in Lepidoptera for a humorally transported, oviposition-stimulating component in male reproductive tract that also appears to exert a neural effect.

My experiments involving transplantation of spermathecae from actively ovipositing females into fecund virgins elicited the same ovipositional response as transplants from nonovipositing virgins. Giebultowicz et al. (1990b) likewise reported no effect different from sham operations when transplanting spermathecae from females 1.5-3 h after mating into virgins.

The current tagmatal isolation studies suggest that oviposition is not solely controlled by the abdominal or thoracic ganglia. The head appears to be important in initiating oviposition since mated, decapitated moths did not oviposit but intact, ligated moths did. Perhaps the command to begin egg laying originates in the brain.

Several investigators have postulated that in Lepidoptera the events resulting in decreased pheromone production also may initiate oviposition (Thibout 1979; Sasaki et al. 1983; Sasaki & Riddiford 1984; Stringer et al. 1985; Giebultowicz et al. 1990a). Giebultowicz et al. (1991) showed that a 2 min stimulation of the bursa either by male genitalia or by a thin glass rod caused a temporary decline in gypsy moth pheromone production; the spermathecae needed to be present for up to 5 h after mating to result in permanent suppression of pheromone production and calling

behavior. In my studies, mechanical stimulation of the bursa failed to elicit premature oviposition by virgins.

Giebultowicz et al. (1990b; 1991) suggested that stretch receptor nerves in the spermathecae of *L. dispar* mediate an ovipositional response after the spermathecae have filled with sperm. In their studies of females mated to males kept in constant light as pharate adults, a spermatophore was formed in the bursa but few or no sperm migrated to the spermathecae and females failed to lay eggs. This is similar to the work of Klatt in 1920 (referenced in Benz 1969) who showed that castrated *L. dispar* males, which produced spermatophores without sperm, did not stimulate females to oviposit. Both of these studies concur with a 1952 study by Behrenz (referenced in Giebultowicz et al. 1990b) who showed that oviposition was reduced in *L. dispar* females in which the spermathecae had been removed or ligated to prevent sperm migration.

Given these earlier findings and my lack of evidence for humoral mediation involving the brain, I agree with Giebultowicz et al. (1990b; 1991) that only the sperm-filled spermatheca mediates the induction of oviposition in this laboratory strain of the gypsy moth. The time required for the sperm to dissociate in the bursa copulatrix and migrate to the spermatheca, approximately 3 h, would account for at least part of the elapsed time from onset of mating to initiation of oviposition. The exact mechanism remains unknown. Giebultowicz et al. (1990b; 1991) suggested that stretch receptors surrounding the spermatheca send a signal to initiate oviposition, either through a neural or neurohumoral sequence. An equally plausible explanation is that sperm receptors lining the spermathecae release a paracrine factor that acts in concert with nervous stimuli. Neither of these hypotheses explains the onset of oviposition by senescent virgins.

Chapter 2

Search for oviposition induction factor from *Musca domestica* male reproductive tract

Abstract

M. domestica ejaculatory duct extract prepared from a laboratory strain of house flies and injected into sexually mature, virgin females elicited very little oviposition (7 eggs/♀) as did male whole reproductive tract extract (7 eggs/♀), saline (10 eggs/♀) and untreated controls (34 eggs/♀). In contrast, normally mated females oviposited 179 eggs/♀ over the 6-day experiment. Experiments conducted on recent descendants of wild-caught flies yielded similar results, suggesting that use of a laboratory strain did not affect the outcome of oviposition bioassays. Ovipositional activity also was not found in SDS extracts of ejaculatory duct, saline extracts of vaginal pouches from virgin and mated females, or *D. antiqua* male accessory gland extracts injected into *M. domestica* females. These findings differ dramatically from the conclusions of house fly oviposition studies conducted by other researchers.

Introduction

Riemann et al. (1967) reported that *Musca domestica* ejaculatory duct transplants suppressed remating in 81% of recipient virgin females. Using both transplantation and ablation experiments, Riemann and Thorson (1969) provided evidence that the ejaculatory duct also was necessary for inducing female oviposition. Females mated to castrated males with ejaculatory ducts laid twice as many eggs (1153 mg) as those mated to castrated males without ducts (496 mg) but

not as much as mated control females (1543 mg). They also showed that virgin females implanted with two ejaculatory ducts oviposited five times as many eggs (523 mg) as those implanted with four testes (103 mg), but Riemann and Thorson reported no controls against which to measure the accuracy of their findings. Given that egg deposition by females receiving ejaculatory duct implants in their study was comparable to that by females mated to ductless, testesless males, the results are inconclusive. No attempts were made in later work to substantiate or expand on these findings or to isolate the active factor and conduct a detailed study of its effect on oviposition.

The reproductive tract of male *M. domestica* consists of a long ejaculatory duct that expands slightly in diameter distally, then bifurcates into the two branches of the vas deferens, each leading to a testis. Accessory glands per se are lacking in *M. domestica* but the expanded, secretory distal portion of the ejaculatory duct is considered to be their functional equivalent (Leopold 1970; Riemann 1973). The only other Dipteran investigated for male oviposition and receptivity factors that exhibits this structure also is a muscoid, the stable fly, *Stomoxys calcitrans* (Morrison et al. 1982). Other species, discussed in subsequent chapters of this dissertation, possess distinct sex accessory glands (also see review by Gillott 1988).

My objective for this series of experiments was to determine whether male accessory secretions from the ejaculatory duct stimulated oviposition in gravid females as suggested by Riemann and Thorson's experiments (1969). I intended to use the house fly as a model species for isolating and identifying a sex peptide factor, building on some of the receptivity inhibition factor studies by Adams and Nelson (1968), Nelson et al. (1969), Leopold (1970) and, later, Bird et al. (1991). Secondarily, I wanted to search for evidence of oviposition-inducing activity in other tissues dissected from males and females as well as look for cross-reactivity with

another species in the muscoid superfamily, Muscoidea (Borror et al. 1989), shown to express sex peptide.

Methods and Materials

Rearing. For the first series of experiments, *M. domestica* were obtained as pupae from Carolina Biological Supply Company (Burlington, NC). Adult males and females were sexed at less than 36 hours posteclosion by exposing them to -20°C for ~2 minutes to immobilize them for gender identification. Adults were reared on a diet of 6:6:1 by weight of sugar, dried milk, and dried egg yolk (Riemann et al. 1967) and maintained at 24±2°C, 55±5% RH, and 16:8 L:D in 12x12x12 inch aluminum and screen cages (Cat. #1450B, BioQuip Products Inc., Gardena, CA) until testing. No more than 150 flies were placed in each cage.

A colony derived from wild-caught flies captured at the Michigan State University dairy barns on College Road in East Lansing in August, 1992, was used for follow-up experiments. Captured flies were held in 12x12x12 inch aluminum and screen cages and given access to an oviposition resource as described below. Eggs were collected, placed on Purina house fly diet (Ralston-Purina, St. Louis, MO) and allowed to hatch. After 8 to 10 days, pupae were collected from the diet and placed in 12x12x12 inch cages for emergence. The cycle was repeated until flies were used for experiments, then adults were sexed as described above.

Extract preparation and injection. Reproductive tissues from 3 to 8 day-old virgin male flies (killed by brief exposure to cold) were removed into a drop of either cold, double-deionized water or onion fly saline (Spencer et al. 1992: 128 mM NaCl, 4.6 mM KCl, 1.9 mM $CaCl_2 \cdot 2H_2O$, 1mM $Na_2HPO_4 \cdot 7H_2O$, adjusted to pH 6.8) in a dissecting dish. Each tissue was immediately transferred into a saline- or water-filled microcentrifuge vial kept on ice. Tissues were processed into extracts by

homogenizing the glands for 5 to 10 seconds with an ultrasonic probe, setting the tuner≈3 and the power≈35 (Blackstone Ultrasonics Inc., Sheffield, PA). The homogenate was centrifuged at 7000 g for 10 minutes, the supernatant (extract) was removed and the concentration adjusted so that 0.5 µl was one male gland equivalent. Extracts either were used immediately for injection or stored at -20°C for up to one week.

To administer the extract, 3 to 4-day-old virgin females (except as noted) were anesthetized by exposure to -20°C or N_2 for 2 minutes, immobilized in shallow grooves (4x3x6 mm) within a block of silicone elastomer (MDX4-4210, Dow Corning Corp., Hemlock, MI), and injected into the ventrolateral region of abdominal segment 1 by inserting the needle barely past the integument. Each fly received 0.5 µl of extract (1 ♂ equivalent, except as noted). Injection needles were made from 3 mm glass tubing drawn into a very fine point (approximately 40 µm at the tip) with a pipet puller (Narishige Scientific Instrument Co., Tokyo). Treatment groups not receiving injections also were anesthetized. Treated flies were placed into 5 inch diameter x 5 inch high clear plastic cages (TriState Plastics, Dixon, KY) covered with nylon stocking and an oviposition resource was added consisting of a 1 oz. plastic cup (#P1-1, Plastics Inc., St. Paul, MN) containing 2 cotton dental wicks soaked with sucrose rich milk (condensed milk:water, 1:2).

Experimental design. All experiments were conducted as randomized block designs. Flies were randomly removed from the holding cage one block at a time. Treatments within blocks were assigned at random by drawing numbers from a container and all flies within a block were treated in the random order specified before proceeding to the next block. An experimental unit consisted of one cage containing one to three flies and one oviposition resource.

Females were allowed to oviposit for up to 10 days although results are shown only through day 6 to facilitate comparisons among experiments. Oviposition cups were replaced daily. Data collected included daily egg output per cage, measured in numbers of eggs, and the number of live females in each cage every day. Data were statistically analyzed with a SAS general linear models program and Student-Newman-Kuel's multiple range test (SAS Institute, Cary, NC).

Ovipositional activity of ejaculatory duct and whole reproductive tract extracts. All flies were from the Carolina Biological strain. Gravid, sexually mature, virgin females were injected with saline extracts of *M. domestica* ejaculatory duct or male whole reproductive tract. The five treatment groups included: a virgin control (no injection); saline injection; ejaculatory duct extract injection of 1 ♂ eq; whole reproductive tissue extract of 1 ♂ eq; and a mated control in which 2 ♂ were placed with one ♀ for 48 hours, then the males were removed. An experimental unit consisted of 1 ♀ per cage containing an oviposition resource; there were 7 to 11 experimental units per treatment.

Ovipositional activity of ejaculatory duct extract from wild-caught flies. A study using third to fourth generation flies raised from wild-caught adults was conducted to determine if laboratory selection affected expression of or sensitivity to sex peptide. Virgin females were injected with a saline extract of *M. domestica* ejaculatory duct and placed 3 ♀ per cage with one oviposition resource. The experiment consisted of three treatments with 4 experimental units per treatment: saline injection, ejaculatory duct extract injection of 1 ♂ eq, and a mated female control of 2♂/♀ for 48 hours, after which time the ♂ were removed.

Other potential sources of ovipositional activity. Following the same experimental plan as above, I examined the effect on oviposition in virgin females of *M. domestica* ejaculatory ducts extracted in 0.02% SDS, saline extract of vaginal pouches, and *Delia antiqua* (Diptera: Anthomyiidae) male accessory glands extracted in onion fly saline. The 0.02% SDS was used to extract some nonpolar and lipophilic substances in addition to polar compounds. Extracts of vaginal pouches, female organs that receive seminal fluid from males but not sperm (Leopold et al. 1971b), were prepared from virgin females and from females that had just completed mating. Since Leopold et al. (1971b) showed that transfer of seminal fluid to the pouches was completed by 40 minutes into the hour-long mating process, I reasoned that sufficient secretory material would be available for assay if I freeze-killed females immediately after decoupling, then removed the pouches into saline. The specific hypothesis for this treatment group was that the oviposition-inducing property of male seminal fluid is activated following transfer to and contact with the intima of the female pouch. *D. antiqua* accessory gland extracts, shown to be active in conspecific oviposition bioassays, were tested in case *M. domestica* females were responsive to an oviposition factor but males were not producing sex peptide. There were five females per cage containing one oviposition resource in these treatment groups. This experimental series consisted of five treatments in addition to the standard negative control (virgin ♀ or saline-injected ♀) and positive control (normally mated ♀); there were two experimental units per treatment. Treatments included injection of 0.02% SDS negative control, 0.02% SDS extract of virgin ejaculatory duct (1 ♂ eq), saline extract of vaginal pouch from mated female (0.5 ♀ eq), saline extract of vaginal pouch from virgin female (0.5 ♀ eq), and saline extract of *D. antiqua* accessory gland (0.5 ♂ eq).

Results

Ovipositional activity of ejaculatory duct and whole reproductive tract extracts. Using the Carolina Biological strain of *M. domestica*, I was unable to show any ovipositional stimulation from injections of aqueous extracts of dissected male reproductive tissues (Figure 3). Normally mated females began laying eggs 2 days after initial exposure to males, a typical oviposition pattern (Riemann et al. 1967). They continued laying through day 6, depositing a cumulative average of 179 eggs/♀. In contrast, females from all other treatment groups oviposited later and laid significantly fewer eggs ($p < 0.05$, SNK) in a manner characteristic of virgin flies (Riemann et al. 1967). Ejaculatory duct and whole reproductive tract extract injections elicited only 7 eggs/♀, a value comparable to females injected with saline (10 eggs/♀) and untreated virgins (34 eggs/♀).

Ovipositional activity of ejaculatory duct extract from wild-caught flies. Results of the follow-up study using early descendants from wild-caught flies corroborated those of the first study (Figure 4). Ejaculatory duct extract and saline-injected females laid a cumulative average of 7 and 10 eggs/♀, respectively, whereas mated females laid 41 eggs/♀. The lower egg output by mated females from the wild-caught colony compared to the Carolina Biological strain is not surprising given that mating patterns and ovipositional cues selected in the wild likely would be less suited to a laboratory environment, probably resulting in fewer successful matings and ovipositions.

Other potential sources of ovipositional activity. Extracts of vaginal pouches collected from both mated and virgin females did not stimulate appreciable oviposition compared to negative controls when injected into sexually mature females (Figure 5). Similarly, neither SDS extracts of ejaculatory duct nor saline

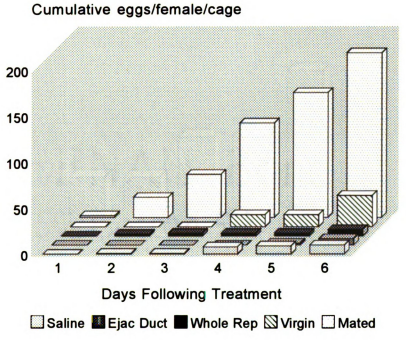


Figure 3. Virgin *M. domestica* oviposition following injection with male reproductive tissue extract. Ejaculatory duct and whole reproductive tracts were extracted in onion fly saline; females were injected with 1 ♂ equivalent. The negative controls were untreated and saline-injected virgins. For the positive control, males were placed with virgins at a density of 2 ♂/♀ for 48 h, then removed (n=7 to 11). Standard deviations on the last day ranged from 50% to 200% of the mean. Egg deposition at 6 days by mated females was significantly different than other treatments at $p < 0.05$ by SNK.

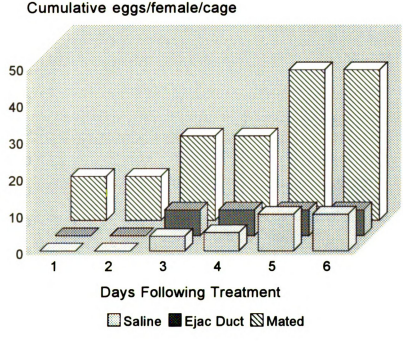


Figure 4. Oviposition by descendants of wild-caught *M. domestica* following injection with ejaculatory duct extract. Treated females were injected with 1 ♂ equivalent and the negative and positive controls were as described in Figure 3 (n=4). Standard deviations on the last day ranged from 75% to 200% of the mean. Egg deposition at 6 days by mated females was significantly different than other treatments at $p < 0.05$ by SNK.

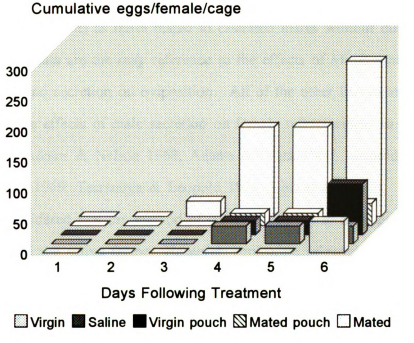


Figure 5. *M. domestica* ovipositional response to vaginal pouch extract injection. Vaginal pouches were removed from both virgin and mated females, extracted in saline and injected as 0.5 ♂ equivalent. Negative and positive controls were as described in Figure 3 (n=2).

extracts of onion fly male accessory gland, previously shown to elicit oviposition in conspecific females (Spencer et al. 1992), induced oviposition in female house flies (Figure 6).

Discussion

I was unable to demonstrate that house fly ejaculatory duct expresses a humoral factor capable of inducing oviposition in virgin females. This finding is surprising considering that Riemann and Thorson (1969) found that female house flies mated to castrated males with ejaculatory ducts laid twice as many eggs (measured by weight) as those mated to castrated males without ducts. However, it appears their data are the *only* reference to the effects of *M. domestica* male accessory gland secretion on oviposition. All of the other literature is devoted to examining the effects of male secretion on female refractoriness to mating (Riemann et al. 1967; Adams & Nelson 1968; Adams & Hintz 1969; Leopold et al. 1971a; Nelson et al. 1969; Terranova & Leopold 1971; Bird et al. 1991).

I considered that my method of using sonication to homogenize the tissues had destroyed the active factor. However, sonication of mosquito accessory glands was used routinely and activity was never lost due to the extraction process (Chapter 4). I also tested the possibility that the mass rearing conditions for the Carolina Biological strain of flies had relaxed selection for an oviposition factor. Yet, recent descendants of wild-caught flies showed no evidence of an ejaculatory duct-based oviposition factor either.

Two other species of Muscidae have been examined for oviposition factors, *Stomoxys calcitrans* and *Glossina morsitans*. The stable fly, *S. calcitrans*, does not have a separate accessory gland but, like the house fly, the bulbous tubular part of the ejaculatory duct is considered secretory and is referred to as the accessory gland (Morrison et al. 1982). Transplantation of *S. calcitrans* male accessory glands into

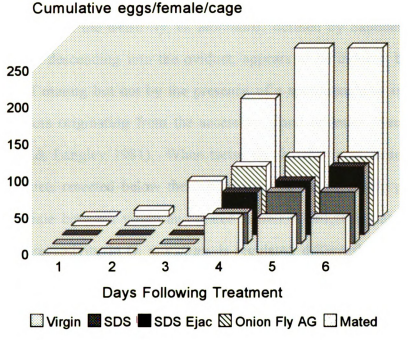


Figure 6. *M. domestica* ovipositional response to SDS extract of ejaculatory duct and *D. antiqua* accessory gland extract. Ejaculatory ducts were extracted in 0.02% SDS and injected as 1 ♂ equivalent. Accessory glands from onion flies were extracted in onion fly saline and injected as 0.5 ♂ equivalent. The injected negative control was 0.02% SDS; virgin and mated controls were as described in Figure 3 (n=2).

virgin females stimulated egg deposition of 65 to 98 eggs/female, significantly above that observed for sham-operated virgins, 9 eggs/female (Morrison et al. 1982). Accessory gland extracts were not tested for oviposition induction but were tested for receptivity inhibition; injection of 0.25 male accessory gland equivalents from blood-fed *S. calcitrans* males prevented insemination in conspecific females but had no effect on *M. domestica, Phormia regina*, and *Sarcophaga bullata* females, all of which are in the muscoid section Calyptratae (Borror et al. 1989). Apparently, both oviposition and receptivity in stable fly females is regulated by a male chemical factor(s).

Ovulation in the tsetse fly, *G. morsitans*, defined by Saunders and Dodd (1972) as eggs descending into the oviduct, appears to be induced by the physical stimulation of mating but not by the presence of a spermatophore in the uterus or by chemical factors originating from the accessory gland or testes (Saunders & Dodd 1972; Gillott & Langley 1981). When tsetse fly females were mated to males with ejaculatory ducts resected below the convergence of the accessory gland, ovulation was intermediate between virgin and mated females (Gillott & Langley 1981). Inhibition of receptivity in *G. morsitans* is regulated differently than oviposition induction, being dependent on both chemical and physical stimuli (Gillott & Langley 1981).

Given this disparity in findings among moderately related species, perhaps oviposition in *M. domestica*, like *G. morsitans*, is controlled more by some physical aspect of mating rather than a chemical factor. I did not test this hypothesis. My experiments using extracts of male ejaculatory duct did not corroborate Riemann and Thorson's (1969) data showing that accessory gland transplants stimulated oviposition. However, as discussed in the introduction to this chapter, their transplantation study lacked experimental controls, making it impossible to evaluate the veracity of their findings; they also did not provide the sample size. Because of

my results, I doubt Riemann and Thorson's (1969) interpretation that accessory gland secretions stimulate oviposition. But, of course, my inability to find a factor does not disprove its existence for all *M. domestica*. Further study of the physical effects of copulation combined with the humoral effects of accessory gland secretions is necessary to elucidate the oviposition induction mechanism in *M. domestica*. House fly is not a good model system for isolating a sex peptide influencing oviposition.

Certainly male-produced factors in addition to sperm are transferred to females during copulation. Polyacrylamide gel electrophoresis studies of ejaculatory ducts and of vaginal pouches harvested before and after mating showed ≥ 7 distinct proteins were transferred to females that did not end up in the spermathecae (Terranova et al. 1972). Further evidence that *M. domestica* mating systems involve male-derived, receptivity-inhibiting, humoral factors comes from studies examining the effect of ligation and decapitation on female mating receptivity (Leopold et al. 1971a). Both decerebrated and ligated females mated more frequently than untreated controls, suggesting that a neuroendocrine factor involving the head modulates receptivity following mating. In the same study, the authors showed that females tended to spend more time *in copulo* when mating with multiply-mated males that had depleted their accessory secretions than with virgin males, suggesting the involvement of a male chemical signal rather than a strictly physical stimulus in turning off receptivity. *M. domestica* whole body extracts fractionated on Sephadex G-10 and G-75 columns showed a mating receptivity factor in the range of 700 to over 3000 molecular weight. But mating inhibition activity from these fractionated extracts was relatively low, never exceeding 33% suppression. Follow-up cytochemical and autoradiography studies by Leopold (1970) and coworkers (Leopold et al. 1971b) indicated the factor was probably a basic protein rich in arginine and lysine. Bird et al. (1991) fractionated ejaculatory duct extract prepared

from a wild-strain of house fly using size exclusion HPLC, C18 and ion exchange Sep-Pak cartridges. Females injected with extracts were as much as 89% refractory to mating but since activity was found in all eluting fractions, not much fractionation was achieved. In addition, the effect was transient; the majority of females began to remate within 3 hours following treatment.

Perhaps *M. domestica* male accessory gland fluid has multiple functions, none of which is to regulate oviposition. For example, Leopold (1980) and Leopold and Degrugillier (1973) describe how sperm penetrance of the egg is mediated by ejaculatory duct secretions. The studies discussed above suggest some role for accessory secretions in suppressing female receptivity, but the partial response of extract-injected females versus mated females indicates something other than or in addition to accessory factors may be required to achieve full suppression. However, my data clearly indicate that accessory gland proteins acting alone do not affect oviposition patterns in *M. domestica* virgin females, leaving the mechanism for induction of oviposition unexplained for this species.

Chapter 3

Anastrepha suspensa: Effect of male accessory gland extracts on female oviposition and mating receptivity

Abstract

Anastrepha suspensa male accessory glands do not appear to possess sex peptide. Injection of accessory gland extracts from laboratory-colony males into virgin females stimulated deposition of only 4 eggs/♀/day, comparable to injections of whole reproductive tract extract (5 eggs/♀/day) and negative controls (4 to 5 eggs/♀/day). Mated females laid significantly more, 10 eggs/♀/day. Studies of wild-caught males and females yielded the same information: injection of an accessory gland/testes extract or saline both elicited 8 eggs/♀/day whereas normally mated females laid 16 eggs/♀/day. Female receptivity to mating following injection of accessory gland or whole reproductive tract extracts was comparable to the negative control group: 67% to 83% of treated females remated and 63% to 89% of control females remated. In contrast, only 43% of once-mated (positive control) females remated when placed with males. Once-mated females also took significantly longer to remate after exposure to males (359 minutes) than females from both treatment (61 to 169 minutes) and negative control groups (76 to 122 minutes). The duration of mating was similar among all groups (24 to 37 minutes). These results suggest that oviposition and receptivity inhibition in *A. suspensa* are not mediated by male-derived humoral factors.

Introduction

The Caribbean fruit fly, *Anastrepha suspensa* (Tephritidae), is a major economic pest throughout the citrus-growing regions of North and Central America, the Mediterranean, and portions of the South Pacific. Studies on the reproductive behavior and physiology of *A. suspensa* in the past several years have described the role and use of male pheromones (Nation 1972; 1990), lek formation on host plant leaves (Burk 1983), acoustic courtship signals (Sivinski et al. 1984; Webb et al. 1984), and copulation (Nation 1972; Mazomenos et al. 1977). I was interested in examining whether the Caribbean fruit fly possessed a sex peptide-signalling system capable of inducing oviposition and/or inhibiting sexual receptivity.

Oviposition by *A. suspensa* is strongly influenced by both environmental cues (Landolt & Sivinski 1992) and the quality of the oviposition site (Sivinski & Heath 1988). Mated females with access to artificial oviposition devices consisting of a rolled cloth impregnated with beeswax will lay significantly more eggs per female than those without access. In addition, the physical features of the oviposition resource such as shape and color are more important oviposition stimulants than chemical cues for polyphagous species like *A. suspensa* (Szentesi et al. 1979; Greany & Szentesi 1979).

Females will remate if given adequate time to oviposit (Sivinski & Heath 1988) or, for laboratory strain females, if males fail to inseminate them in the initial mating (Mazomenos et al. 1977). Mated females with oviposition devices are more likely to remate during weekly opportunities than those without oviposition devices (Sivinski & Heath 1988). Mated females with no opportunity to oviposit are more likely to mate only once. In another study (Mazomenos et al. 1977), none of the once-mated wild type females remated within 5 days, despite the fact that approximately 50% of the females were not fertilized after the first mating. By contrast, *Rhagoletis pomonella* females are receptive to nearby males immediately

following copulation, and *R. suavis* may alternate egg laying and copulation with several males before leaving an oviposition site (reviewed by Christenson & Foote 1960). These studies indicate there is neither significant egg dumping nor a propensity to remate during the first 5 days of sexual maturity.

Males and females of laboratory strain *A. suspensa* become sexually mature at 10 to 11 days old (Nation 1972). Males have paired testes connected to a common duct through the vasa deferentia (Dodson 1978) and the sex accessory glands originate where the vasa deferentia converge, consisting of 5 or 6 short, tubular glands. Females have three spermathecae and polytrophic ovarioles (Dodson 1978); ovaries may contain more than one flush of mature eggs at a time. When mating, flies typically remain *in copulo* for about 30 to 37 minutes (Nation 1972; Mazomenos et al. 1977).

The sterile insect technique in which sterilized males are released en masse to compete with wild males for mating opportunities is used to control *A. suspensa*. Although increasing doses of gamma irradiation have been correlated with decreased mating success of sterilized males (Calkins et al. 1988; also see Hooper 1972), the mechanism by which it occurs is not known. Given that gamma irradiation of male pupae might affect physiological mechanisms regulating female oviposition or mating receptivity, it is important to know whether *A. suspensa* uses a sex peptide. For these studies, I hypothesized that Caribbean fruit fly male accessory gland secretion induces oviposition and inhibits mating receptivity in virgin females. Tissue extraction methods designed to remove polar substances such as proteins were employed; male accessory gland-derived lipophilic compounds were not tested.

Methods and Materials

Rearing. *A. suspensa* was obtained from either the mass-rearing facilities of the USDA laboratories at Gainesville, Florida, or collected as pupae from the field in

central Florida. Females and males were sexed as newly eclosed adults, placed into separate cages of equal density and provided with an agar-based sugar, yeast, and wheat germ diet (1:1:1). They were maintained in the laboratory at ambient temperature and humidity under artificial lighting during normal working hours.

Extract preparation and injection. Reproductive tissues were removed from sexually mature (~10 to 40 day-old) virgin males (freshly killed by freezing) into a drop of cold onion fly saline in a dissecting dish. Each tissue was quickly transferred into a saline-filled microcentrifuge vial kept on ice. Tissues were processed into extracts by homogenizing them for 30 to 60 seconds in a bath sonicator (Heat Systems Ultrasonic), then centrifuging at 4500 g for 10 minutes and removing the supernatant. The concentration of the supernatant (extract) was adjusted so that 0.5 µl was one male gland equivalent. Extracts either were used immediately for injection or frozen before use.

Bioassays were conducted at the USDA facilities in Gainesville. To administer the extract, sexually mature virgin females were anesthetized by exposure to nitrogen and one male equivalent of extract was injected into the lateral metathorax through a pleuron. Injection needles consisted of 3 mm glass tubing drawn into a very fine point (~40 µm at the tip). Flies were restrained for injection on a Plexiglas® plate by covering them with Parafilm®. Treatment groups not receiving injections also were anesthetized. Treated flies were placed individually into 7.5 cm diameter by 15 cm high aluminum screen cages. An oviposition resource consisting of a 2.5 cm agar sphere surrounded with Parafilm® (Dr. C. Calkins, personal communication) was suspended from the top of the cage.

Experimental design. Experiments were conducted as randomized block designs. Flies were randomly removed from the holding cage one block at a time.

Treatments within blocks were assigned at random by drawing numbers from a container and all flies within a block were treated in the random order specified before proceeding to the next block. An experimental unit consisted of one cage containing one to three flies and one oviposition resource. Data were statistically analyzed either with a SAS general linear models program and Student-Newman-Kuel's multiple range test (SAS Institute, Cary, NC) or with Fisher's protected least squares difference test (StatView 4.0, Abacus Concepts, Inc., Berkeley, CA).

Effect of reproductive tissue extracts on oviposition and mating receptivity of lab colony flies. Flies from the mass rearing facilities were used for these experiments. An experimental unit consisted of 1 female per cage containing one oviposition resource. Experiments included up to 7 treatments with 3 to 61 experimental units per treatment. The control groups included a virgin control (no injection), females inserted with a needle only, saline injected females, and mated controls in which 3 males were placed with the female for 24 hours, then removed. Extract-injected treatment groups consisted of paragonial gland extract injection of 1 ♂ equivalent, paragonial gland extract injection of 5 ♂ equivalents, and whole reproductive tract extract injection of 1 ♂ equivalent.

Following treatment, approximately half of the females from each group were assigned to an oviposition bioassay and half to a mating-receptivity assay. For the oviposition bioassay, females were allowed to oviposit for up to 23 days on oviposition spheres replaced every few days as egg counts were performed. For the mating-receptivity assay, 3 male flies were placed with each treated female on the day following treatment and flies were observed for up to 12 hours to determine the delay until onset of mating and the duration of mating. At the end of 12 hours males were removed, females were divided according to whether they had mated, and egg output was measured as described above for the oviposition bioassay. For

each treatment, the percentage of females mating also was determined. Females not mating were included in the data set for the oviposition bioassay.

Ovipositional activity of whole reproductive tract from wild-caught flies. Since mass-rearing radically alters selection pressure and may cause changes in mating ability or mating effectiveness in some Diptera (Bush et al. 1976), wild-caught Caribbean fruit flies also were tested to determine whether they somehow were different than lab colony flies. Extracts were prepared from the accessory gland/testes complex of wild-caught *A. suspensa* males as described earlier except that they were collected into saline containing a mixture of protease inhibitors (0.0004% w/v each of antipain, leupeptin, pepstatin A, chymostatin). These were added to prevent the possibility of rapid enzymatic degradation of extracted proteins. Tissues were sonicated for ~25 seconds, centrifuged at 4500 g for 5 minutes, then the supernatant was stored in a freezer overnight. There were 25 experimental units per treatment consisting of 3 wild-caught females and one oviposition resource per experimental unit. In this test, females were given 1.5 cm diameter blue, cerasin wax domes for oviposition instead of agar balls. Females were injected with either a saline control or accessory gland/testes extract of 1 ♂ equivalent. For the mated control, 4 ♂/♀ were added to the female cage for 24 hours, after which time the males were removed. Females were allowed to oviposit for up to 10 days and oviposition spheres were replaced as counts were performed. Data collected were egg counts per live female per cage.

Results

Effect of reproductive tissue extracts on oviposition and mating receptivity of lab colony flies. Injections of male accessory gland or whole reproductive tract extracts did not increase oviposition in lab colony virgin *A. suspensa* females above

that for unmated flies (Table 4). Females from the negative control treatments and the extract treatments laid a cumulative average of 4.2 eggs/day, significantly fewer than the average 9.5 eggs/female laid by normally mated females ($p < 0.05$, SNK).

Table 4. Oviposition by lab colony *Anastrepha suspensa* injected with aqueous extract of male reproductive tissue.

Treatment	N	Eggs/♀/day
Controls		
Virgin	8	4.8 ± 4.1
Needle insertion only	10	4.3 ± 3.5
Saline	10	3.5 ± 4.6
Male extracts		
Accessory gland	13	3.8 ± 2.6
Whole tract	9	5.2 ± 2.2
Grand mean - unmated treatments	50	4.2 ± 3.4[a]
Mated females	61	9.5 ± 6.8[b]

Treatment means with the same letter are not significantly different at $p < 0.05$ by SNK.

Similarly, when virgin females receiving injections of male reproductive tissue extract were exposed to males, they mated like virgins. Treated females began mating an average of 61 to 169 minutes after males were introduced into the cage, comparable to virgin controls that began mating 76 to 122 minutes following introduction, whereas already-mated females waited 6 hours before mating again (Table 5). In addition, 78% of all treated virgin females mated during the 12-hour bioassay in contrast with only 43% of previously mated females. All groups averaged about 30 minutes mating time suggesting that once mating began, previous treatment had no effect on the duration of copulation.

Table 5. Mating by lab colony *Anastrepha suspensa* injected with aqueous extract of male reproductive tissue.

Treatment	N	Minutes		% Mating
		Delay until mating	Duration of mating	
Controls				
Virgin	7	76 ± 48[a]	25 ± 10[a]	89%
Needle insertion only	6	114 ± 102[a]	37 ± 7[a]	86%
Saline	5	122 ± 69[a]	24 ± 7[a]	63%
Male extracts				
Accessory gland	6	104 ± 66[a]	28 ± 19[a]	67%
5x♂ accessory gland	7	169 ± 100[a]	35 ± 20[a]	83%
Whole tract	9	61 ± 72[a]	27 ± 11[a]	83%
Grand mean - unmated treatments	40	104 ± 82[a]	29 ± 13[a]	78%
Mated females	3	359 ± 124[b]	30 ± 26[a]	43%

Treatment means with the same letter are not significantly different at $p < 0.05$ by SNK.

Ovipositional activity of whole reproductive tract from wild-caught flies. Oviposition by wild-caught virgin flies injected with accessory gland/testes extract was virtually identical to saline-injected virgins, 8.4 and 8.3 eggs/female after 10 days (Figure 7). Normally mated wild-caught females laid an average of 16 eggs/female over the same period. So, although egg deposition was higher among the feral population — likely due to better oviposition resources, blue wax domes — both the wild and laboratory-mated females laid twice as many eggs as unmated females regardless of the treatment.

Discussion

Inhibition of mating receptivity and stimulation of oviposition in *Anastrepha suspensa* females apparently are not mediated simply by transfer of male accessory gland factors to the female. Sexually mature laboratory-colony females injected with extracts of male reproductive tissues exhibited no observable change in egg output or mating propensity; they behaved as virgins. Protease inhibitors used to prevent or minimize digestion of extracted proteins did not change the results. Experiments using wild-caught flies yielded virtually identical results with respect to mating receptivity and oviposition induction, suggesting that laboratory selection had not removed a wild-type sex peptide.

In mating competition experiments, male *A. suspensa* rendered sterile by a low dose of gamma irradiation (3 krad) were equally competitive with normal males when placed with normal females as measured by egg output (Calkins et al. 1988). Therefore, sterile male flies are capable of inducing oviposition behavior in females without transferring viable sperm. Studies on *Rhagoletis pomonella* using normal and irradiated males showed similar results (Myers et al. 1976). Remating in the melon fly, *Bactrocera cucurbitae*, was inhibited at the same rate in females mated to either normal or sterile males as long as copulation was not terminated prematurely

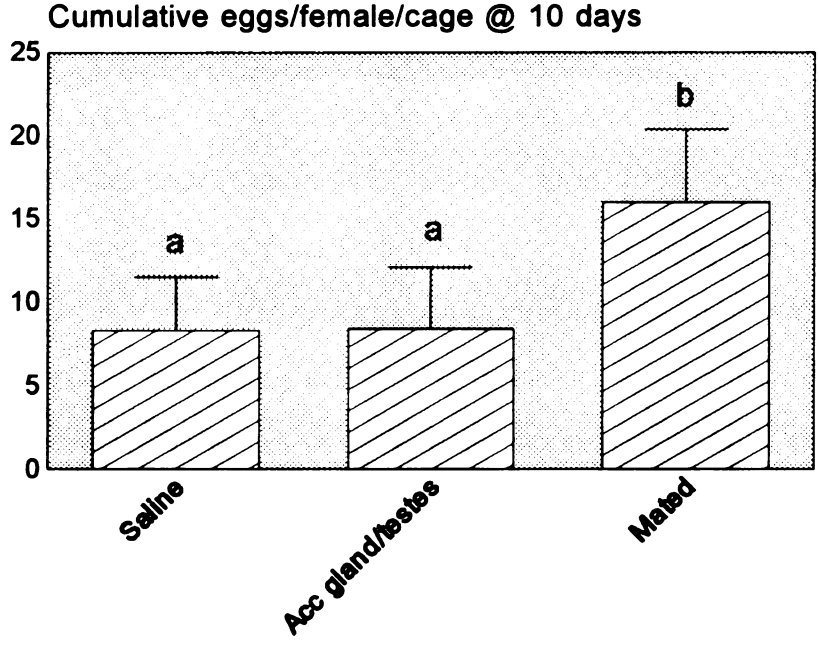


Figure 7. Oviposition by wild-caught *A. suspensa* following injection with accessory gland/testes extract. Treated females received 1 ♂ equivalent of extract and the negative control was saline-injected virgins. For the positive control, males were placed with females at a density of 4 ♂/♀ for 24 h then removed (n=25). Treatment means with the same letter were not significantly different ($p < 0.05$) using Fisher's protected least squares difference test.

(Kuba & Ito 1993). This suggests there is a factor(s) other than sperm that causes female tephritids to change from virgin to mated behavior, but I could find no evidence of a sex peptide extractable by methods amenable to protein.

Perhaps the mating system of *A. suspensa* and related species is incompatible with sex peptide communication. If the reason for sex peptide is to guarantee paternity (Miller et al. 1994), by definition, the associated mating system should favor male control. Yet *A. suspensa* mating appears to be a female mate-choice system (Nation 1972; Mazomenos et al. 1977). Males expend energy courting females, forming leks on host plant leaves in order to mate with females during the afternoon (Burk 1983). Stimuli used to attract females to the leks include pheromones deposited on the underside of leaves, acoustic signals produced by wing fanning and visual cues (Nation 1972; Sivinski et al. 1984; Webb et al. 1984). Oviposition occurs on host plant fruit the following morning (Burk 1983), suggesting that females exert greater control over mating because they approach males who are not controlling access to oviposition sites (Sivinski & Heath 1988). Females approached by males on fruit in the morning reject their attempts to copulate. Female mate choice and mating control also occur at several other levels (Nation 1972; Mazomenos et al. 1977): Females may not respond to fanning males, even at close range; they make the first contact with the motionless males by touching him with their proboscis; they extend their ovipositor up if they are receptive and down on the substrate if they are not; they can either kick males away or fly off the leaf. In essence, there are several behaviors and levels of communication in *A. suspensa* governing female acceptance and rejection of potential mates. Maybe natural selection favoring a relatively unequivocal female-choice mating system does not foster the coevolution of sex peptides that would promote male control.

Chapter 4

Aedes aegypti and *A. albopictus* male accessory gland extracts: oviposition heterospecificity and fractionation

Abstract

Virgin *A. aegypti* females injected with male accessory gland extract prepared from either *A. aegypti* or *A. albopictus* oviposited comparably to mated females, 85 eggs/♀. Saline injected *A. aegypti* females and untreated virgins typically oviposited around 12 eggs/♀. Virgin *A. albopictus* females injected with conspecific male accessory gland extract laid 28 eggs/♀, comparable to mated females that laid 37 eggs/♀. Untreated *A. albopictus* virgins and saline-injected virgins averaged 2 and 3 eggs/♀, respectively. *A. albopictus* females injected with *A. aegypti* male accessory gland extract oviposited only 12 eggs/♀. Molecular weight analysis of accessory gland extracts from both species indicates some proteins are unique to each species and may account for the difference in the asymmetrical ovipositional response.

Fractionation of *A. aegypti* glands by polyethylene glycol precipitation, capillary electrophoresis, electrodialysis, discontinuous and gradient nondenaturing PAGE yielded some recovery of activity in oviposition bioassays. Fractionation of extract by gel filtration chromatography, DEAE ion exchange chromatography, reverse phase HPLC, QAE HPLC and DEAE HPLC resulted in complete loss of oviposition activity. Significant fractionation was achieved by HPLC methods but not by methods from which activity was recovered. Analysis by SDS•PAGE of fractions from several experiments showed a 32.5 kDa protein common to all active fractions.

Introduction

In 1965, Leahy and Craig demonstrated that *Aedes aegypti* and *A. albopictus* males produce a substance that, when transferred to females during copulation, induces oviposition and inhibits receptivity to additional mating attempts; it was named matrone by Fuchs et al. (1968). Further, Leahy and Craig (1965) demonstrated that transplants of *A. aegypti* and *A. albopictus* accessory glands stimulated virgin oviposition in *A. aegypti* females but only conspecific implants stimulated *A. albopictus* oviposition. Leahy (1967) went on to characterize the ovipositional activity of *A. aegypti* accessory secretions, finding that: A full ovipositional response could be elicited with $^1/_{24}$ of a male gland equivalent; the effect persisted following a second bloodmeal; and *A. aegypti* accessory glands transplanted into *Culex pipiens* produced oviposition comparable to that observed in mated *C. pipiens*. The reciprocal transplantation experiment was not performed. She also reported on the basis of very limited data that accessory gland transplants from *D. melanogaster* elicited oviposition in *A. aegypti* (also see Hiss & Fuchs 1972) but reciprocal transplants from *A. aegypti* were only partially effective in stimulating *D. melanogaster* oviposition.

In other studies, matrone was determined to be a protein. Mating inhibition activity was destroyed by protease and by heating the extract for 5 min at 50°C (Craig 1967; Fuchs et al. 1969). Activity was nondialyzable (MW of dialysis membrane was unspecified) and the factor was precipitated by 60% $(NH_4)_2SO_4$. Treatment with $MnCl_2$ or $MnSO_4$ to precipitate nucleic acids left the active material in the supernatant. Appreciable pH sensitivity was observed below 5 and above 9 (Fuchs et al. 1969). Similarly, Leahy (1967) showed that heating *A. aegypti* accessory gland extract for 5 min at 100°C inactivated the oviposition factor.

Early efforts to isolate and characterize the active factor in *A. aegypti* met with limited success (Fuchs et al. 1969; Fuchs & Hiss 1970; Hiss & Fuchs 1972).

Active extract was prepared from whole lyophilized males by acetone homgenization and filtering, n-butanol homogenization and filtering, then acetone precipitation and resuspension of the pellet in saline. Using gel filtration chromatography to fractionate this extract, Fuchs et al. (1969) concluded that the combination of two proteins with molecular weights of approximately 60K and 30K was necessary to inhibit sexual receptivity. Young and Downe (1987) later criticized these studies because no effort was made to prevent protein aggregation during the extraction procedure, which can lead to grossly incorrect estimates of molecular weight and protein purity (see additional comments in the discussion section of this chapter). When extract prepared in the same way was separated by acetone fractionation, two fractions dubbed α and ß also were reportedly required to inhibit receptivity (Fuchs & Hiss 1970) but only the α fraction was needed to induce oviposition (Hiss & Fuchs 1972). It should be emphasized that the α and ß fractions recovered by acetone fractionation are not synonymous with the 60K and 30K MW proteins recovered by gel filtration. The purity of the α and ß fractions and the molecular weights of proteins within these fractions were not determined. Apparently, no further studies were conducted.

The mating receptivity factor in *Culex tarsalis* also was characterized using gel filtration column chromatography (Young & Downe 1987). The elution profile of the bioactive male accessory gland extract indicated the factor has a molecular weight of approximately 2K. Based on their findings, these authors suggested that the work on *A. aegypti* may have been erroneous because Fuchs and Hiss used whole bodies rather than isolated glands and that accessory gland secretions may have aggregated with other mosquito proteins.

Most of the prior work investigated and characterized receptivity inhibition of *A. aegypti* accessory gland extract. Only the studies by Leahy and Craig (1965), Leahy (1967), and Hiss and Fuchs (1972) addressed oviposition induction.

There were two main objectives of my research on mosquito oviposition factor. First, the experiments on interspecific induction of oviposition were repeated using better controls. I examined the cross-activity of *A. aegypti* and *A. albopictus* accessory gland extracts to look for species specific differences in ovipositional response. Second, I made use of more recent protein separation technology for fractionating *A. aegypti* accessory gland extract; my goal was to isolate the factor responsible for oviposition induction.

Methods and Materials

Rearing. *A. aegypti* were obtained from Michigan State University cultures (originating from the UGAL strain at the University of Georgia) and *A. albopictus* (New Orleans strain) were reared from eggs obtained from the University of Notre Dame. Larvae were fed an aqueous suspension of equal parts of ground rat chow, bakers' yeast and lactalbumin (Klowden & Lea 1978) for five days; pupation occurred 8 to 9 days after hatching (see Appendix 2 for detailed description of rearing). Pupae were transferred to mosquito breeder cages (Cat. #1425, BioQuip Products Inc., Gardena, CA) for eclosion. Individuals were sexed within 24 h of emergence and placed into separate 5 inch diameter by 5 inch high clear plastic cages (TriSate Plastics, Dixon, KY) covered with nylon stocking. No more than 75 mosquitoes were placed in each cage. They were provided with a diet of 10% sucrose and maintained at 24 $\pm$ 2°C, 75 $\pm$ 5% RH and 16:8 L:D.

Two days prior to extract injection, sexually mature females (typically 4 to 7 days old) were given a blood meal from a rat anesthetized with methoxyflurane (Pitman-Moore, Mundelein, IL). Only gravid females, as evidenced by the obvious presence of eggs in the abdomen, were used in experiments.

Extract preparation and injection. Accessory glands or male terminalia consisting of abdominal segments VII to VIII were removed from sexually mature (> 3 days old) male mosquitoes (anesthetized with anhydrous ethyl ether, Mallinckrodt Specialty Chemicals Co., Paris, KY) into a drop of cold mosquito saline (Hagedorn et al. 1977: 150 mM NaCl, 25 mM HEPES, 4 mM KCl, 1.8 $NaHCO_3$, 1.7 mM $CaCl_2 \cdot 2H_2O$, 0.6 mM $MgCl_2 \cdot 6H_2O$, adjusted to pH 7.0) on a microscope slide. Each tissue was transferred into a saline-filled, polypropylene microcentrifuge vial kept on ice. Tissues from later experiments were extracted with mosquito saline plus 0.1% n-octyl ß-D-glucopyranoside (Cat. #O-8001, Sigma Chemical Co., St. Louis, MO), a nonionic detergent intended to minimize protein aggregation (Dr. Estelle McGroarty, Department of Biochemistry, MSU, personal communication; Ersson 1989). Accessory glands were processed into extract immediately by homogenizing them with a Blackstone ultrasonic probe (tuner≈3 & power≈30), centrifuging at 8,000 g for 10 min, then removing the supernatant. Terminalia were processed identically except the supernatant was centrifuged a second time at 16,000 g for 10 min. The supernatant (extract) was divided into aliquots if needed, then either used immediately or frozen until use.

To administer the extract, blood-fed virgin females were anesthetized with ethyl ether and 0.5 µl of extract (typically 0.5 to 1.0 ♂ equivalent, except as noted) was injected into the posterior ventromedial metathorax at its junction with the abdomen. Injection needles were made from 3 mm glass tubing drawn into a very fine point measuring approximately 40 µm across the tip. For injection, mosquitoes were positioned on their side on a flat silicone sheet, the thorax was braced anteriorly with fine forceps, and the needle inserted barely past the integument. Treatment groups not receiving injections also were anesthetized. Treated mosquitoes were placed into 5 inch diameter by 5 inch high clear plastic cages covered with nylon stocking. Each cage contained an oviposition resource

consisting of a 2 oz. plastic cup (Comet Products Inc., Chelmsford, MA) lined with 2 x 4 inch Whatman No. 1 filter paper or 4 chromatography paper (Whatman International, Ltd., Maidstone, England) and filled with 25 ml of degassed, deionized water.

Experimental design. All experiments were conducted as randomized block designs. Mosquitoes were randomly removed from the holding cage one block at a time. Treatments within blocks were assigned at random by drawing numbers from a container and all mosquitoes within a block were treated in the random order specified before proceeding to the next block. An experimental unit consisted of one cage containing three mosquitoes and one oviposition resource. All three mosquitoes within a cage were treated at the same time before proceeding to the next treatment.

Females were allowed to oviposit for at least 7 days. Oviposition cups were replenished with fresh water and filter paper when eggs were present. Data collected included daily egg output per cage, measured in numbers of eggs, and the number of live females in each cage every day. Data were statistically analyzed with a SAS general linear models program and Student-Newman-Kuel's multiple range test (SAS Institute, Cary, NC).

SDS polyacrylamide gel electrophoresis. The Mini-Protean II unit (BioRad Inc., Richmond, CA) was used for analytical SDS PAGE following the procedure supplied by the manufacturer and running at constant voltage (200 V) from a Fisher Biotech power supply (Cat. #FB135, Fisher Scientific, Pittsburgh, PA). Minigels (6.5 cm x 10 cm) were cast using 0.75 mm teflon spacers, a 10-well comb, a 4% stacking gel and either a 12% or 15% resolving gel. Gel and buffer ingredients were purchased from both BioRad and Integrated Separation Systems (ISS, Natcik,

MA). Protein samples were prepared by mixing 1:1 with ISS sample buffer and heating at 55°C for 15 min. Low-range molecular weight markers were purchased from both Sigma Chemical Co. (St. Louis, MO) and ISS. Silver stain ingredients and Coomassie blue R-250 were obtained from BioRad. The upper tank buffer was 0.5 M Tris•Cl, pH 6.8, and the lower tank buffer was 1.5 M Tris•Cl, pH 8.8. All operations were performed at room temperature. Appendix 3 contains complete formulations and staining instructions.

Electroelution. Electroelution followed the the protocol of Hunkapiller et al. (1983; also see Smith 1987a) using CalTech design electroelution cells (CBS Scientific, CA), a custom-made buffer chamber, and a Fisher Biotech power supply. Electroelution cells were assembled with 3,500 MWCO Spectra/Por® 6 regenerated cellulose dialysis membranes (Spectrum, Houston, TX), both wells were filled with freshly prepared dialysis buffer and the assembled cells were allowed to equilibrate in buffer for ≥ 1 h at 3°C. Remaining operations were performed at 3°C. The electrophoresed gel was diced into 3 mm x 3 mm squares with a razor blade and loaded into the large well of the cell. The peristaltic pump for recirculating the elution buffer was set at a flow rate of 3 ml/min and elution was carried out at a constant voltage of 150 V. The elution buffer recommended by Hunkapiller et al. (1983) when running at ≤ 4°C is 10 mM NH_4HCO_3, 0.02% SDS, pH 8.2. Because I was uncertain about whether the concentration of SDS was causing mortality, buffers were prepared with and without SDS. When eluting for more than 12 h, elution buffer was replaced with fresh buffer at 8 to 12 h intervals. At the end of a run, fluid was carefully removed with a Pasteur pipet from the top of each well and discarded, then the 150 to 600 µl of fluid at the bottom of the well — overlaying the membrane and containing the protein of interest — was pipetted into microcentrifuge

vials using a pipettor fitted with polypropylene tips. Appendix 4 contains a detailed procedure.

Basic studies. I have grouped several small but relevant studies together in this section because their outcomes justify the choice of methods used in other experiments.

(1) Most of my earlier studies were conducted with extracts prepared in mosquito saline but, after performing several nondenaturing PAGE experiments, I was concerned that aggregation of accessory gland proteins was affecting my fractionation results. Dr. Estelle McGroarty suggested adding n-octyl ß-D-glucopyranoside (n-O-ß), a nonionic detergent, to the saline. A small study was conducted to determine whether mosquitoes injected with accessory glands extracted in 0.1 % n-O-ß in mosquito saline experienced any observable adverse effects or altered their oviposition response.

(2) The ability of nonparagonial gland tissue to elicit oviposition was assayed by extracting *A. aegypti* ♂ abdomens — minus paragonial glands — in mosquito saline, 0.1% n-O-ß and injecting it into virgins.

(3) Extracts during the first year of mosquito experiments were prepared solely from dissected accessory glands. Since collection of male terminalia was quicker and allowed preparation of larger quantities of extract, I conducted a comparative study of oviposition induction by accessory gland and terminalia extracts. Both extracts also were analyzed by SDS•PAGE.

(4) Although Fuchs et al. (1969; Fuchs & Hiss 1970) had previously concluded matrone was a protein because activity was lost following enzymatic digestion, I conducted a similar study using trypsin bound to agarose to verify their findings. Terminalia were extracted in mosquito saline, 0.1% n-octyl ß-D-glucopyranoside (no protease inhibitors), and the supernatant was divided into five aliquots. Aliquot 1

was placed in the freezer immediately without additional treatment; aliquot 2 was held at room temperature for 2 h; agarose beads without trypsin (exclusion limit 15 MDa, Cat.# 193453, ICN Biochemicals, Cleveland, OH) were added to aliquot 3 and the mixture was held for 2 h at 37°C; aliquot 4 was mixed with trypsin immobilized on agarose beads (1mg/ml in 4% agarose, Cat.# 191324, ICN Biochemicals) and held 2 h at 37°C; and aliquot 5 was held at 37°C for 2 h. The negative control was mosquito saline, 0.1 % n-O-ß + agarose beads held at room temperature for 2 h. All aliquots were mixed every 15 min to maintain contact between the proteins and agarose beads which tend to settle to the bottom. Following the 2 h treatment, all aliquots and the negative control were centrifuged for 1 h at 8,000 g, then placed in the freezer.

(5) Since a SpeedVac (Model SVC100, Savant Instruments Inc., Farmingdale, NY) was used at room temperature to concentrate many protein samples for injection, I examined the activity of a paragonial gland extract concentrated to low volume (not dryness, which was found to destroy activity) versus an untreated extract.

(6) Proteins also were concentrated in ultrafiltration centrifuge tubes. During the course of this research, I used two sizes of ultrafiltration tubes with a 5,000 nominal molecular weight limit (NMWL) regenerated cellulose membrane, the 0.4 ml holding capacity (Ultrafree-MC, Millipore Corp., Bedford, MA) and the 2.0 ml capacity (Ultrafree-CL). To determine whether activity of a paragonial gland extract was retained or lost following ultrafiltration, I loaded a 0.4 ml holding cup with extract, centrifuged it to 40 µl, then sonicated and removed the contents of the cup for injection into virgin females.

Heterospecificity of A. albopictus and A. aegypti. I tested the hypothesis that extracts of *A. albopictus* male accessory glands activate oviposition in females of both *A. albopictus* and *A. aegypti* while extracts of *A. aegypti* male accessory

glands activate only *A. aegypti* oviposition. In one set of assays, virgin female *A. albopictus* were injected with mosquito saline extracts of both *A. albopictus* and *A. aegypti* accessory glands, prepared as described earlier; virgin *A. aegypti* were injected with identically prepared extracts in another set of assays. To gain some sense of what was being injected, extracts from both species were analyzed by 12% SDS•PAGE to obtain a profile of accessory gland proteins within the range of 10 to 100 kDa. Matrix-assisted laser desorption/ionization time of flight (MALD/ITOF) mass spectrometry analysis, performed at the MSU Department of Biochemistry Mass Spectrometry facility, was used to profile accessory gland proteins less than 10 kDa.

Polyethylene glycol precipitation. Precipitation by PEG is considered milder than ammonium sulfate so it was selected as an initial fractionation step for *A. aegypti* accessory gland extract (Ingham 1984; Scopes 1987a; Ersson et al. 1989). A 60% aqueous solution of powdered polyethylene glycol 3350 was prepared by dissolving 3 grams in 2 ml of deionized H_2O and mixing with alternating periods of heating in a hot tap water bath to facilitate dissolution. An extract of 120 ♂ terminalia in 120 µl NH_4HCO_3 was prepared and 100 µl was set aside for the precipitation experiment. An appropriate volume of 60% PEG was added to this aliquot while vortexing to yield an extract containing 6% PEG, then the precipitating extract was placed in ice for 2.5 h; it was briefly vortexed every 15 min to maintain PEG-protein contact. The precipitated extract was centrifuged for 10 min at 16,000 g and the supernatant removed for treating with 12% PEG following the protocol just described. The pellets from each precipitation were redissolved in 50 µl of acetone to solubilize the remaining PEG, then centrifuged and the acetone supernatant removed under a microscope to help visualize the small protein pellet. Pellets were resuspended in 10 mM NH_4HCO_3 and stored at -20°C until used in the

oviposition bioassay. I previously had determined that injection of trace acetone would not affect the mosquitoes. The precipitated extracts were analyzed by SDS•PAGE.

Electrodialysis. Electrodialysis was used as a means of attempting fractionation of protein extracts and as a method for removing salts from extracts fractionated by other means. The procedure was the same as that described under electroelution except that instead of adding gel pieces to the well, I added protein extracts mixed 10:1 with 50% sucrose to help layer the protein at the bottom of the electroelution well. Three of the fractionation experiments contained 0.02% SDS in the buffer and two did not. The buffer was adjusted to pH 5.5 for one experiment in hopes that only proteins with a pI $\leq$ 5.5 would migrate to the anode. Electrodialysis times ranged from 2.25 to 3 h. Samples were removed from both the anode and the cathode sides of the cell, depending on the experiment, then concentrated if necessary.

Gel filtration chromatography. The method was based on information provided by BioRad, Hagel (1989) and Smith (1987b). A 40 µl mosquito saline extract containing 80 ♂ accessory gland equivalents was fractionated by gel filtration chromatography at 4°C using BioRad Biogel P•10 media [fractionation range 1,500-20,000 MW], an Econo-Column (10 x 100 mm), and eluting with mosquito saline. The flow rate was 66 µl/min and absorbance was set at a wavelength of 254 nm to detect peptide bonds (Model 440 Absorbance Detector, Waters Associates Inc., Milford, MA). Fractions were collected for 15 min intervals (equalling 1 ml) or less into 1 ml microcentrifuge vials kept on ice, then stored at -20°C. The run time of 126 min was terminated after the second peak had dropped to the baseline and remained stable for 5 min. A volume of 800 µl (64 ♂ Eq) was removed from each

fraction, centrifuged in a 5,000 NMWL Ultrafree-MC tube at 4,000 g, pooled with other fractions from the same absorbance peak, then centrifuged again in an ultrafiltration tube to a final volume of ≤50 µl; saline was added to the pooled tubes to bring the volume to 50 µl. Half of this amount was removed from each pool and recombined to produce "whole extract" and an equivalent amount of saline was added back to the individual pools such that the concentration of all extracts (including the unfractionated positive control extract) was 0.6 ♂/µl.

DEAE Sepharose® chromatography. The method was based on information provided by Rossomando (1990), Karlsson (1989) and Smith (1987c). An extract containing 350 ♂ accessory gland equivalents in 0.015 M mosquito saline, 0.01M Tris was fractionated by ion exchange chromatography in a 4°C room using DEAE-Sepharose (Pharmacia), a 0.7 cm x 10.0 cm Econo-Column (BioRad), and eluting with a step gradient of 1.5 column volumes each of 0.0, 0.1, 0.5, and 1.0 M NaCl in 0.01 M Tris. Flow through the column was controlled only by gravity exerting a force on the fluid above the gel bed. Absorbance was not read because the detector malfunctioned. Fractions were collected into 15 ml centrifuge tubes, then stored at -20°C for 24 h. The fractions were concentrated in 2 ml volume 5,000 MWCO Millipore ultrafiltration centrifuge tubes; typically, 2 ml filtered through in about an hour. The diluent was replaced with mosquito saline by two successive washes through the membrane, leaving about 150 to 200 µl on top of the cup. This concentrated fraction was sonicated briefly, pipetted into a polypropylene microcentrifuge vial, and stored at -20°C.

Reverse phase high performance liquid chromatography. Accessory gland extract (160 ♂ equivalents in 80 µl mosquito saline) was fractionated by microbore reverse phase HPLC (Microgradient Systems pump and controller, Brownlee Labs,

Santa Clara, CA; Spectroflow 783 detector, Kratos Analytical-Applied Biosystems Inc., Foster City, CA) through a 50 x 1.0 mm C18 column (Aquapore RP•300, Cat. #CO3-051, ABI, Foster City, CA), and eluting with a continuous gradient (100% Buffer A to 100% Buffer B) over 90 min. The flow rate was 50 µl/min and absorbance was set at a wavelength of 214 nm to detect peptide bonds. Buffer A was 5 mM phosphate buffer (0.2 g KH_2PO_4, 2.14 g $Na_2HPO_4 \cdot 7H_2O$) and buffer B was alcohol buffer (187 ml Buffer A, 75 ml isopropyl alcohol, 238 ethyl alcohol). Fractions were collected as peaks — represented by the absorbance spectra — into 2 ml microcentrifuge vials kept on ice, then stored for about 24 h. Half of the volume (80 ♂ equivalents) from each fraction was removed and pooled with other fractions and the pooled groups were processed two times through 5,000 MWCO ultrafiltration centrifuge tubes with mosquito saline to remove the salt and alcohol.

QAE (strong anion exchange) HPLC. Accessory gland extract (160 ♂ equivalents in 80 µl 0.01 M Tris) was fractionated by millibore ion exchange HPLC (Waters 600 delivery system, Waters 990 photodiode array detector, and Waters 600E controller, Waters Corp., Milford, MA) through a 30 x 2.1 mm QAE-Silica column (source unknown), and eluting with a continuous gradient of 0.01 M Tris to 1.0 M NaCl/0.01 M Tris (@ pH 8.0) over 45 min. The flow rate was 250 µl/min and absorbance was set at wavelengths of 230, 254 and 280 nm to detect peptide bonds. A guard column was not used. Fractions were collected as absorbance peaks into 1 ml microcentrifuge vials kept on ice, then stored for up to 24 h. Half of the volume (80 ♂ equivalents) from each fraction was removed and pooled with other fractions such that 4 groups of fractions were prepared for testing. The salt concentration of each pooled group was adjusted to that of mosquito saline by two successive ultrafiltrations through a 5,000 NMWL Ultrafree-MC centrifuge tube.

DEAE (weak anion exchange) HPLC. This method was developed from Regnier (1984), Karlsson et al. (1989) and Smith (1987c). Four hundred fifty terminalia were collected into 225 µl of 0.02 M Tris, 0.01 M NaCl, 0.1% n-octyl ß-D-glucopyranoside with 0.0004% protease inhibitors. Three hundred terminalia equivalents were fractionated on a Waters millibore ion exchange HPLC through a 30 x 2.1 mm ABI Polypore DEAE column (Cat. # 0711-0219, ABI, Foster City, CA) and eluted with a continuous gradient of 0.02 M Tris to 0.5 M NaCl/0.02 M Tris (@ pH 8.1) over 30 min. The flow rate was 300 µl/min and absorbance was set at wavelengths of 214, 230, 254 and 280 nm to detect peptide bonds. To increase the buffering capacity, the concentration of Tris was doubled compared to the QAE•HPLC run. A guard column was not used. Fractions were collected into 1 ml microcentrifuge vials every 2 min (= 600 µl) beginning before the gradient started and for 5 min after it ended, storing each one on ice until the completion of the run. Appendix 5 contains a detailed operating procedure for anion exchange HPLC using the Waters chromatograph. Fractions were pooled and electrodialyzed at the anode for 3 h at 3°C to remove salts. Since proteins eluting from an anion exchanger after the "void volume" should be anionic, during electrodialysis they should migrate to the anode if the two procedures are run at a similar pH, which they were. Therefore, pooled fractions were dialyzed by loading them at the anode and collecting them from the anode. Pooled fraction number 1, which eluted first from the HPLC column, also was collected at the cathode of the electrodialysis cell since it may have contained cationic proteins. The samples were concentrated in a SpeedVac at room temperature to a theoretical maximum of ~6.7♂/µl.

Capillary electrophoresis. CE separates proteins based on their net charge and movement within a buffer-filled, narrow-bore column subjected to an electric field. Accessory glands extracted in capillary electrophoresis running buffer (10 mM

sodium phosphate, 0.01% Brij-35, pH 7.0) were electrophoresed on an Isco Model 3850 CE unit (Isco, Inc., Lincoln, NE) using a 75 μm x 75 cm CE-100/C18 internally coated column from Isco (Towns & Regnier 1991). Electroendosmotic flow should be minimal to none under these operating conditions because surface charges on the inside of the C18 column should be neutralized by the nonionic Brij-35 surfactant in the phosphate buffer (Towns & Regnier 1991). Absorbance was detected at 214 nm. For a control study, one aliquot of extract was manually injected into a column then flushed out without applying any current. Another aliquot was injected into the column, 22.5 kV was applied for 7.5 min then the entire column was flushed; this was repeated fifteen times to collect enough material for assay. For the fractionation study, a sample was loaded for 15 seconds at the anode using pressure injection (pulled 53 nl or 0.1 ♂ eq into the column) and 22.5 kV was applied for 8 min so the sample was adequately subjected to a current. This was enough time to allow the fastest migrating proteins to almost reach the end of the column without passing out into the cathode buffer. The column was serially flushed with 4 x 1 μl of running buffer (column capacity = 3.5 μl) and the 1 μl fractions were collected into 4 separate sample vials. The column was cleaned with 50 μl of buffer and the procedure was repeated 50 times to yield four fractions each containing 5♂ eq in 50 μl of running buffer. Fractions were stored on ice until injection. A negative control was prepared by pipetting 50 μl of running buffer into a sample vial, storing it in an ice bucket during fractionation and in the refrigerator overnight. A positive control consisting of unfractionated extract was treated the same way. On the day of injection, the positive control, the negative control and all four fractions were concentrated in a SpeedVac at room temperature to 10 μl, yielding 0.5 ♂ eq/μl. Mosquitoes were injected with 0.25 ♂ equivalent.

Discontinuous nondenaturing PAGE. Terminalia were collected and extracted in 10 mM NH_4HCO_3 (no SDS). Electrophoresis was performed in a 3°C room at 200 V with a Mini-Protean II unit and a Fisher Biotech power supply. Minigels (6.5 cm x 10 cm) were cast using 1.0 mm teflon spacers and a single-well comb with a reference lane, a 3% stacking gel and a 5% resolving gel. Gel and buffer ingredients were purchased from ISS, and I followed the recipes and protocol described in the 1992-1993 Hoefer Scientific Instruments catalog. The remaining procedures also were carried out at 3°C. The electrophoresed gel was sliced into four rows, diced into 3 mm x 3 mm squares with a razor blade, loaded into electroelution cells and eluted at 150 V in 10 mM NH_4HCO_3. Proteins recovered from the anode were used for injection as well as analyzed by SDS•PAGE with a 12% resolving gel and a 4% stacking gel. Appendix 6 contains a detailed procedure. Variables specific to each experiment are summarized in Table 6.

Table 6. Conditions for discontinuous nondenaturing PAGE according to experiment number.

Variable	Experiment Number			
	1	2	3[1]	4
Electrophoresis time	40 min	41 min	NA[2]	2.25 hr
Electroelution time	6.1 hr	10.5 hr	NA	15 hr
SpeedVac concentration	No	No	Yes	Yes
Duration of assay	7 days	10 days	14 days	14 days

[1] Fractions from experiment 2 were concentrated in a SpeedVac to 10 ♂/µl and injected into blood-fed virgins.
[2] NA = not applicable

Gradient nondenaturing PAGE. Terminalia were collected and extracted in mosquito saline and 0.1% n-octyl ß-D-glucopyranoside (0.0004% protease inhibitors were used only in the first experiment). Electrophoresis was performed in a 3°C room with a Mini-Protean II unit using gradient minigels (6.5 cm x 10.0 cm x 1.0 mm) formed according to the protocol of Smith and Bell (1986). Typical running conditions were 50 V for 30 min, then 200 V for the remaining time. The remaining procedures also were carried out at 3°C. The electrophoresed gel was sliced into four rows, diced into 3 mm x 3 mm squares with a razor blade, loaded into electroelution cells and eluted at 150 V in 10 mM NH_4HCO_3 and 0.005% SDS (no SDS was used in experiment 1). Proteins recovered from the anode were concentrated in a SpeedVac at room temperature then used for injection and analyzed by SDS•PAGE with a 12% resolving gel and a 4% stacking gel. Appendix 7 contains a detailed procedure. Variables specific to each experiment are summarized in Table 7.

Table 7. Conditions for gradient nondenaturing PAGE according to experiment number.

Variable	Experiment Number				
	1	2	3	4	5
Gradient	5-20%	5-20%	10-25%	20-32%	20-32%
♂ equivalents loaded	400	400	200	400	400
Electrophoresis time	4 hr	16 hr	36 hr	36 hr	36 hr
Electroelution time	17 hr	6 hr	6 hr	16 hr	12 hr
Portion of gel eluted	All	All	bottom 1.6 cm	All	All

Results

Oviposition activity was easily and reproducibly obtained in *A. aegypti* male accessory gland extracts throughout all of my research on mosquitoes. Postive controls consisting of virgin females injected with unfractionated accessory gland or terminalia extract always oviposited significantly above negative controls. A detailed description of results from each series of experiments follows.

Basic studies. The use of 0.1% n-octyl ß-D-glucopyranoside in mosquito saline had no adverse effect on oviposition (Figure 8). Extract-injected virgin females oviposited the same number of eggs whether or not the saline extracting media contained nonionic detergent. Similarly, extracts prepared with *A. aegypti* male terminalia were as active as those prepared with male paragonial glands (Figure 9). In contrast, females injected with extracts of male abdomens (lacking accessory glands) deposited the same number of eggs as those injected with saline (Figure 10). Clearly, an oviposition factor resides within the male accessory glands.

When terminalia extracts treated with agarose-immobilized trypsin were injected into females, oviposition was significantly reduced compared to females injected with untreated extract, agarose only and the other control treatments (Figure 11). Although egg output in the trypsin group was not as low as saline-injected females, the marked effect of trypsin on oviposition activity suggests that oviposition factor either is a protein or is part of a protein complex, e.g., involving a carrier protein.

Injection of paragonial gland extract concentrated by SpeedVac evaporation elicited virtually the same egg output as did mating by the end of the experiment (Figure 12). The difference in egg output during the early part of the experiment either may have been due to random variation or to the atypically low dosage (0.15 ♂ equivalents) injected into the females. Later studies written up in this chapter,

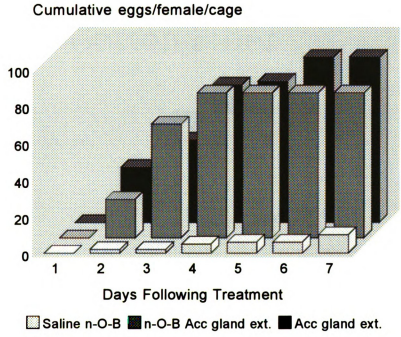


Figure 8. Oviposition by *A. aegypti* virgins injected with mosquito saline, 0.1% n-octyl ß-D-glucopyranoside accessory gland extract. The negative control was saline, 0.1% n-O-ß and the positive control was glands extracted in mosquito saline only (n=5 blocks). Egg deposition at 7 days by saline-injected females was significantly different than other treatments at $p < 0.05$ by SNK.

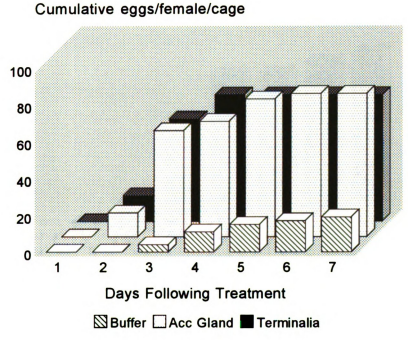


Figure 9. Comparison of oviposition by *A. aegypti* virgins injected with accessory gland extract and male terminalia extract. Terminalia contain the accessory glands, fat body, distal gut, integument and the associated genital structures. The negative control was elution buffer, 10 mM NH_4HCO_3 (n=5). Egg deposition at 7 days by buffer-injected females was significantly different than other treatments at $p < 0.05$ by SNK.

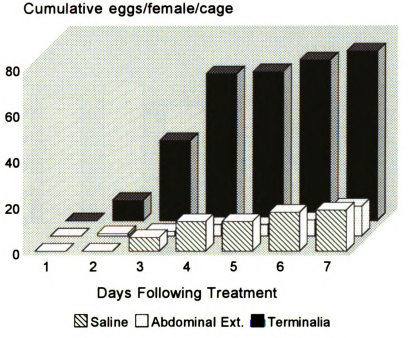


Figure 10. Effect of abdominal extract on oviposition by *A. aegypti* virgins. The extracted abdomen consisted of the entire tagma minus the terminalia. Saline, 0.1% n-O-ß was the negative control and terminalia extract was the positive control (n=5). Egg deposition at 7 days by terminalia-injected females was significantly different than other treatments at $p < 0.05$ by SNK.

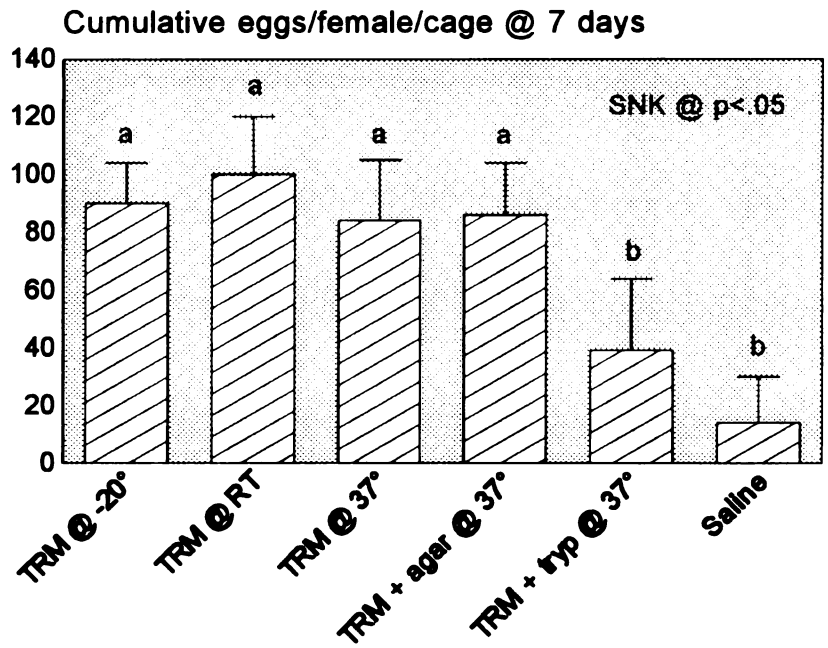


Figure 11. Injection of trypsin-treated terminalia extract into virgin *A. aegypti*. Treatments included terminalia extracted in mosquito saline, 0.1% n-o-ß (TRM @ -20°); mosquito saline + agarose; terminalia held @ room temperature for 2 h; terminalia + agarose @ 37°C for 2 h; terminalia + trypsin immobilized on agarose @ 37°C for 2 h; and terminalia @ 37°C for 2 h. All females received an injection of 0.5 ♂ equivalents (n=5).

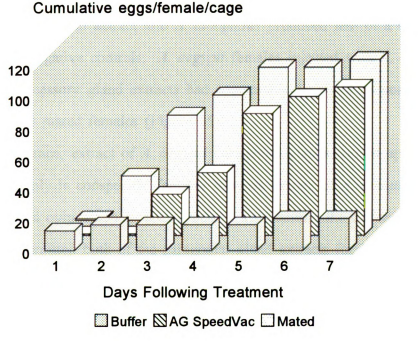


Figure 12. Effect of SpeedVac concentration on ovipositional activity of *A. aegypti* male accessory gland extract. A 60 µl extract of accessory glands in 10 mM sodium phosphate, 0.01% Brij-35 was concentrated to 10 µl, diluted and injected. The negative control was sodium phosphate buffer only and the positive control consisted of normally mated females (n=5). Egg deposition at 7 days by buffer-injected females was significantly different than other treatments at $p < 0.05$ by SNK.

in which SpeedVac concentration was used as part of the treatment process, corroborated these results and showed no adverse effect on extract activity. Similarly, mosquitoes injected with ultrafiltrated accessory gland extract oviposited comparably to untreated extract injected females (Figure 13); a negative control was not performed.

Heterospecificity of A. albopictus and A. aegypti. Mosquito saline extracts prepared from *A. albopictus* male accessory glands elicited oviposition in conspecific females comparable to that of mated females (Figure 14). Injection of *A. aegypti* male accessory gland extract into *A. albopictus* produced less of a response albeit greater than negative controls. *A. aegypti* females injected with *A. aegypti* and *A. albopictus* accessory gland extracts had comparable levels of oviposition, greater than normally mated females (Figure 15).

In essence, extract of *A. aegypti* paragonial glands caused appreciable oviposition only in conspecific females whereas extract of *A. albopictus* paragonial glands elicited oviposition in both species. To examine the difference in protein content, I submitted both of the paragonial gland extracts for MALD/ITOF mass spectrometry and analyzed each of them by SDS•PAGE. MALD/ITOF-MS analysis showed at least two proteins under 10 kDa for *A. aegypti* and at least five proteins under 10 kDa for *A. albopictus* (Figure 16), two of which were in the same range as the *A. aegypti* proteins. SDS•PAGE revealed numerous protein bands, a few which were unique to each species (Figure 17). These results, combined with the oviposition bioassays, suggest the *A. aegypti* and *A. albopictus* oviposition factors are not identical; they produced an asymmetric response in conspecific vs. heterospecific females.

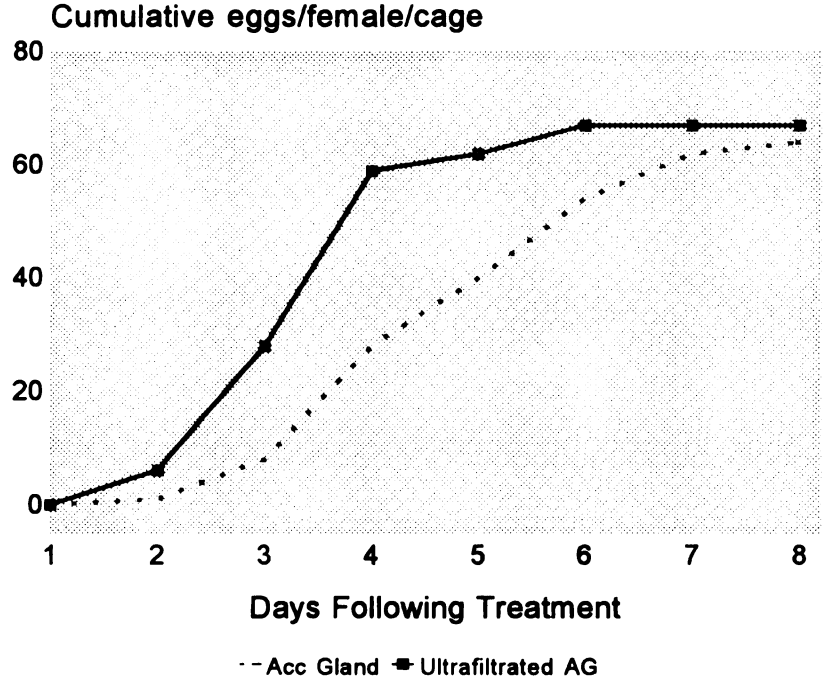


Figure 13. Oviposition by virgin *A. aegypti* injected with accessory gland extract concentrated in ultrafiltration centrifuge tubes. The positive control was a group of females injected with 1 ♂ equivalent of untreated accessory gland extract (n=5).

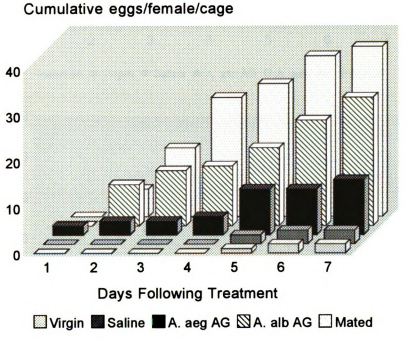


Figure 14. Oviposition by *A. albopictus* females injected with 1 ♂ equivalent of *A. albopictus* and *A. aegypti* accessory gland extracts. The negative controls consisted of untreated virgins and saline-injected females. For the positive control, male *A. albopictus* were placed with virgins at a density of 3♂/♀ for 48 h, then removed (n=9 to 19).

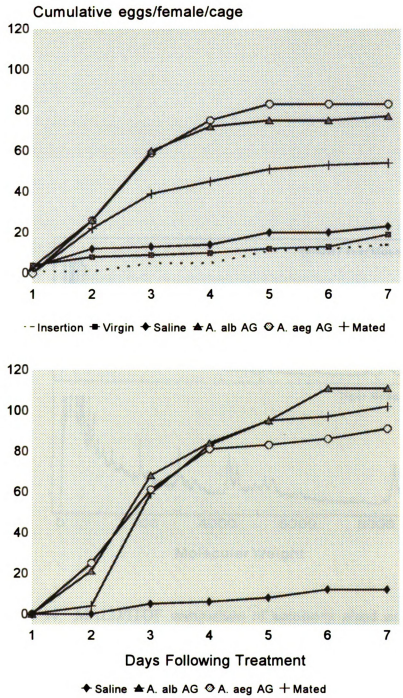


Figure 15. Oviposition by *A. aegypti* females injected with 1 ♂ equivalent of *A. aegypti* and *A. albopictus* accessory gland extract. The treatments are the same as in Figure 14 plus one group received a sham injection (insertion). The top graph are data from 1992 (n=10 to 19) and the bottom graph are data from 1994 (n=10).

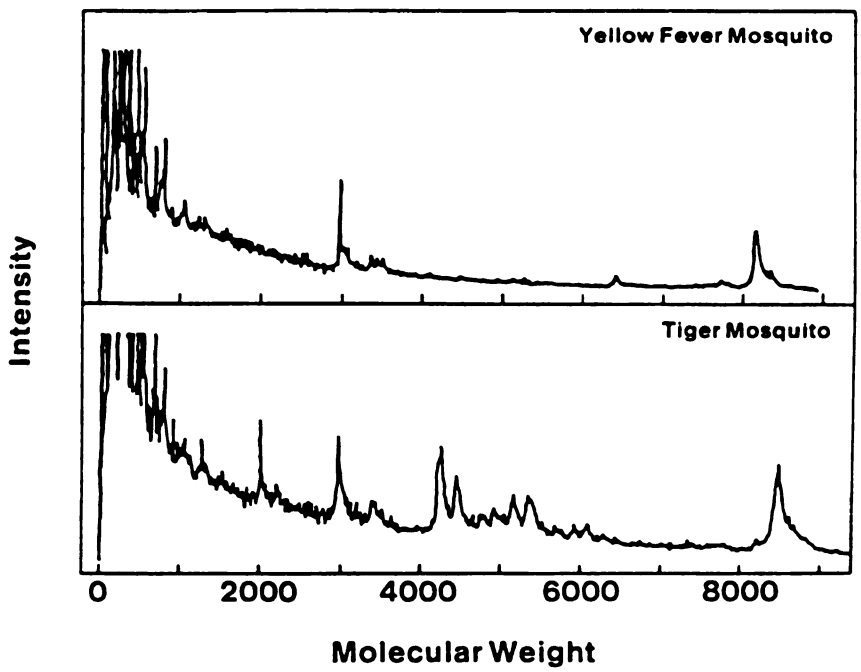


Figure 16. MALD/ITOF comparison of accessory gland extracts from *A. aegypti* and *A. albopictus*. The top tracing shows the mass spec analysis for *A. aegypti*, the yellow fever mosquito, and the bottom tracing is for *A. albopictus*, the Asian tiger mosquito. The detection range was set for < 10 kDa.

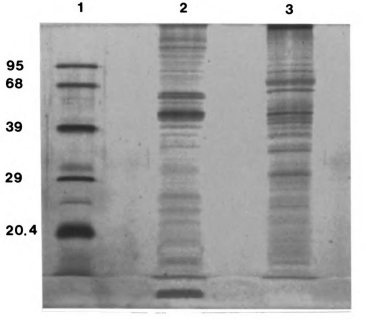


Figure 17. 12% SDS•PAGE of *A. albopictus* and *A. aegypti* accessory gland extracts. Lanes: (1) molecular weight markers of 95, 68, 39, 29 and 20.4 kDa; (2) *A. albopictus* extract; (3) *A. aegypti* extract.

Polyethylene glycol precipitation. Oviposition by females injected with 6% and 12% PEG precipitated terminalia extracts was greater than negative control injected females over the course of the experiment (Figure 18) but lower than females receiving the positive control untreated extract. Both precipitates appeared to have nearly identical banding profiles and intensities when analyzed by SDS•PAGE (gel not shown). Most of the bands present in the untreated extract were also in the precipitates but in much lower concentration as evidenced by staining intensity. It is possible that precipitate-injected females laid fewer eggs simply because they received less protein. Alternatively, perhaps a concentration of 12% PEG was not enough to induce significant aggregation and precipitation of the active factor. It is also possible that exposure of the precipitated pellet to acetone caused some denaturation. In any case, I did not pursue this approach because the fractionation achieved with precipitation (as determined by SDS•PAGE) was marginal and more promising results were unfolding with nondenaturing PAGE.

Electrodialysis. Initially, I investigated whether extract activity could be maintained following electrodialysis because it was a necessary step (in the form of electroelution) in recovering proteins from nondenaturing gels after electrophoresis. Three variables were examined: sample loading location (cathode vs. anode), the presence or absence of SDS in the dialysis buffer, and pH. The results, summarized in a composite graph (Figure 19), conclusively show that activity was not lost due to electrodialysis and that more activity was generally recovered from the anode than the cathode, regardless of which side contained the starting sample. Although analysis by SDS•PAGE of electrodialyzed extracts indicated that fractionation did not occur under these operating conditions, these findings encouraged use of electrodialysis in combination with other techniques when warranted. Because

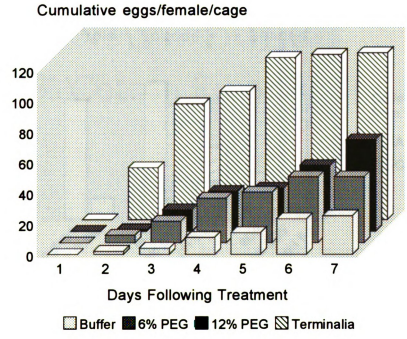


Figure 18. Oviposition following injection of accessory gland extract precipitated with polyethylene glycol 3350. Pellets precipitated by 6% and 12% PEG were resuspended in 10 mM NH_4HCO_3. Females were injected with 2 ♂ equivalents. Buffer was the negative control and terminalia extract was the positive control (n=5).

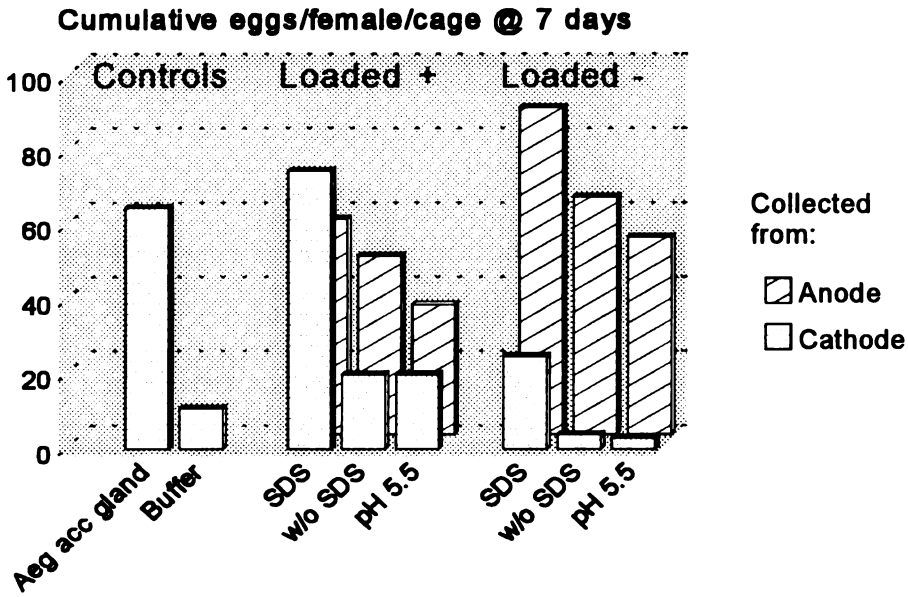


Figure 19. Ovipositional activity of electrodialyzed *A. aegypti* accessory gland extract. Extract was loaded at the cathode or anode well, then collected from both wells for bioassay. Electrodialysis was done in the presence and absence of SDS in 10 mM NH_4HCO_3 at pH 8.2 except one run was performed at pH 5.5. Females were injected with from 0.3 ♂ to 1 ♂ equivalent (n=5).

activity was generally higher at the anode, it also reinforced my CE results suggesting that active factor is anionic (acidic).

Another electrodialysis experiment was set up in which one cell was fitted with a 3,500 MWCO membrane and another was fitted with a 50,000 MWCO membrane to see if I could remove lower molecular weight proteins from the extract. Terminalia extract containing 150 ♂ equivalents was loaded into the anode well of each cell and dialyzed for 12 h in 10 mM NH_4HCO_3, 0.02% SDS. Samples removed from both wells were analyzed by SDS•PAGE. Protein bands were equally represented at all molecular weights (~15 to >100 kDa) indicating I had achieved no fractionation, so an oviposition bioassay was not conducted.

Gel filtration chromatography. Paragonial gland extract eluted as two broad peaks, presumably greater than and lesser than 20,000 MW, respectively, the exclusion limit of the Biogel P•10 column (Figure 20). The fractions encompassing each of these peaks were pooled, then ultrafiltrated to concentrate the extracts into a usable volume and dosage. In addition, the two fractions were recombined, yielding a total of three different treatments. Females injected with these treatments deposited only a fraction of the eggs deposited by postive control females injected with untreated accessory gland extract (Figure 21). The results were surprising since the chromatographic separation occurs at low pressure conditions that are gentle on proteins and because favorable results, measured by receptivity inhibition, had been obtained previously with this method in fractionating *A. aegypti* (Fuchs et al. 1969) and *Culex tarsalis* (Young & Downe 1987) extracts. Both groups used a Sephadex G-50 column (exclusion limit of 30,000 MW), which is made from dextran, as opposed to Biogel, which is polyacrylamide. Pohl (1990) recommends Sephadex over Biogel because it is "more gentle", but he doesn't explain why. Deutscher (1990) and others (Pohl 1990; Scopes 1987b) recommend using a protein

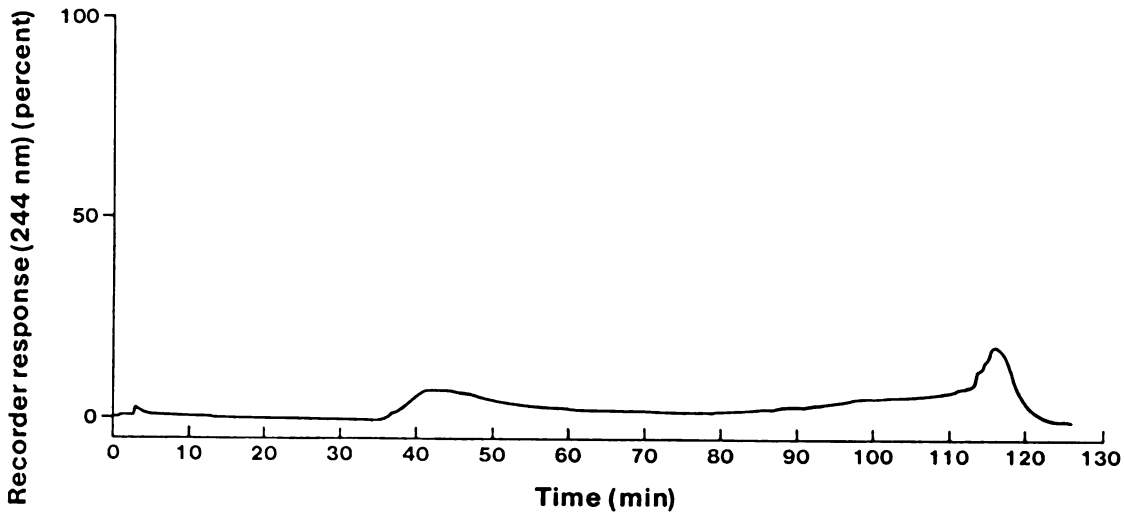


Figure 20. Gel filtration chromatography of *A. aegypti* accessory gland extract. The flow rate was 66 µl/min and absorbance was set at a wavelength of 254 nm to detect peptide bonds. Fractions were collected for 15 min intervals (equalling 1 ml) or less into 1 ml microcentrifuge vials kept on ice, then stored at -20°C. The run time was 126 min.

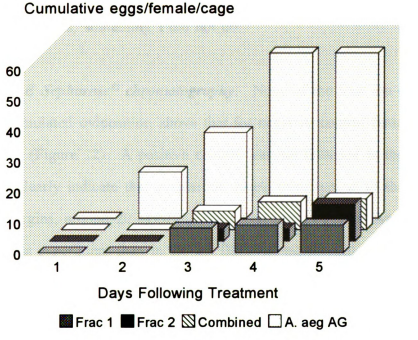


Figure 21. Oviposition by *A. aegypti* injected with accessory gland extract fractionated by gel filtration chromatography. Two fractions were collected from the Biogel P10 column (exclusion > 20 kDa), concentrated to 0.3 ♂ equivalents and injected. A recombined treatment also was injected. The positive control was accessory gland extract (n=5).

concentration of greater than 1 mg/ml to maintain stability, an important precaution in chromatographic procedures like gel filtration that tend to dilute rather than concentrate the starting protein sample. A mosquito accessory gland extract containing 2 ♂ equivalents/µl has roughly 1 mg of total protein per ml (not shown) so although the sample applied to the column (80 ♂ in 40 µl) had a minimally acceptable concentration, it was quickly diluted when elution was started. Perhaps the amount of active protein passing through the column was too low and was retained by nonspecific interaction with the 000gel media. Stellwagen (1990) suggests including a nonionic detergent in the eluting buffer to mimimize protein-column interactions, something I did not do.

DEAE Sepharose® chromatography. None of the four ion exchange fractions stimulated oviposition above that for negative control females injected with 0.01 M Tris (Figure 22). A positive control was not included in the experiment but the results clearly indicate that oviposition levels were typical of that observed for untreated virgins, around 20 eggs/♀. The protein loading level was four times higher in this experiment than in the gel filtration experiment and the column was smaller, factors that should increase the probability that some protein would elute from the column. Given that Fuchs et al. (1969) recovered receptivity inhibition activity from a DEAE-cellulose column loaded with *A. aegypti* whole body extracts, I expected to recover oviposition activity. Perhaps activity would have been restored by combining two or more fractions. It is also possible that methods shown to be successful for studying receptivity inhibition factors are not applicable to oviposition factors. However, Fuchs et al.'s (1969) ion exchange column was eluted with 0.0 M to 1.0 M NaCl in 0.01 M Tris, and their protein extract sample was in physiological saline, not the starting buffer of 0.01 M Tris; in that respect, it is not surprising that the active fraction in their experiment eluted first because the salt solution likely

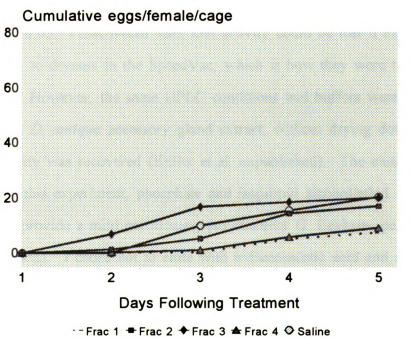


Figure 22. Oviposition by *A. aegypti* injected with accessory gland extract fractionated by DEAE Sepharose chromatography. Four fractions were collected, one from each step gradient of 0 M, 0.1 M, 0.5 M and 1 M NaCl in 0.01 M Tris, and injected as 1 ♂ equivalent along with the negative control, mosquito saline (n=5).

would carry it right through the column. Ion exchange samples should be loaded onto the column in the starting buffer (Karlsson et al. 1989).

Reverse phase HPLC. Many peaks were resolved by microbore reverse phase HPLC of *A. aegypti* accessory gland extract (Figure 23). However, there was no significant oviposition activity among any of the four treatment groups representing all possible fractions eluted from the C18 column (Figure 24). Egg output at the end of the experiment ranged from 18 to 29 eggs/♀ for the fractionated extracts versus 81 eggs/♀ for the unfractionated extract, a significant difference by SNK at $p \leq 0.05$. I discovered later that activity could be lost if extracts were concentrated to dryness in the SpeedVac, which is how they were treated in this experiment. However, the same HPLC conditions and buffers were employed for fractionating *D. antiqua* accessory gland extract, without drying down the fractions, and no activity was recovered (Keller et al. unpublished). The elution buffers selected for this experiment, phosphate and isopropyl alcohol/ethyl alcohol, were intended to provide a mild partitioning environment (J. Leykam, personal communication). I chose not to elute with trifluoroacetic acid and acetonitrile, buffers that were successful in fractionating *D. melanogaster* extracts (Chen et al. 1988), because reverse phase HPLC of *D. antiqua* accessory gland extract in TFA/acetonitrile had resulted in a loss of activity (Miller et al. unpublished). Voet and Voet (1990) suggest that proteins are precipitated by alcohols at low temperatures and denatured by them at higher temperatures, so in retrospect, the alcohol gradient buffer may not have been the best choice either, especially if substances responsible for activity were to elute late in the run when the alcohol concentation was highest.

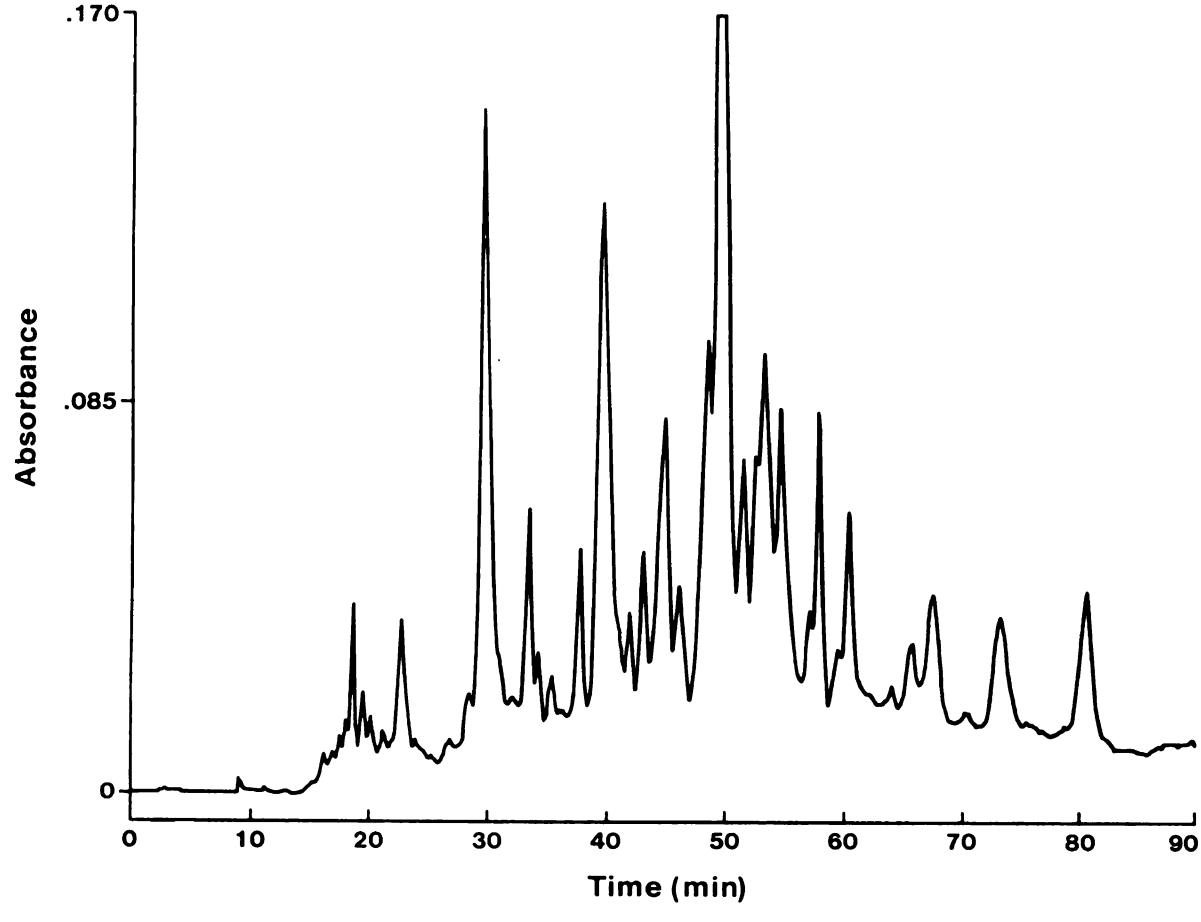


Figure 23. Reverse phase HPLC of *A. aegypti* accessory gland extract. 160 ♂ equivalents were loaded onto a 50 x 1.0 mm C18 column and eluted with a continuous gradient (100% Buffer A to 100% Buffer B) over 90 min. Buffer A was 5 mM phosphate and buffer B was phosphate in alcohol. The flow rate was 50 µl/min and absorbance was read at a wavelength of 214 nm.

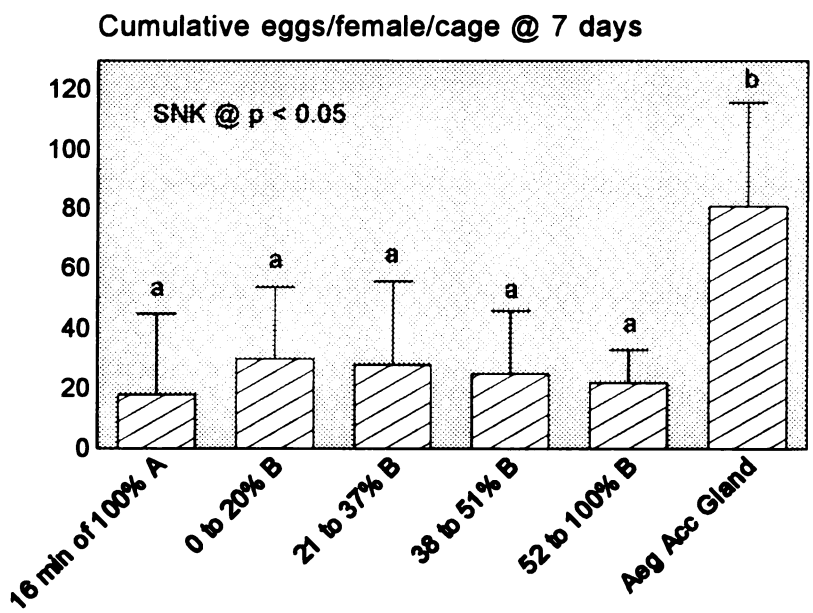


Figure 24. Oviposition following injection with reverse phase HPLC fractions of *A. aegypti* extract. Fractions were collected as peaks then pooled, concentrated and injected. Females received 1 ♂ equivalent injection (n=5).

QAE HPLC. There were numerous peaks detected during the QAE HPLC ion exchange run (Figure 25) but none of the fractions was active in the oviposition bioassay (Figure 26). The untreated accessory gland extract-injected group averaged 64 eggs/♀ whereas output by the pooled fraction-injected groups ranged from 4 to 27 eggs/♀. Even though pool 1 had seven times more egg laying than pool 2, the difference was not significant. Based on a review of recent biochemical literature, preparative ion exchange was used in 37% of protein purification procedures, presumably because it frequently works (Karlsson et al. 1989). Yet, as I found in the other chromatography experiments, I was unable to recover activity with ion exchange. Reasoning that the stronger binding potential of the QAE column might be causing problems, I conducted an experiment using a weak anion exchanger, hoping to expose accessory gland proteins to less stringent conditions.

DEAE HPLC. The chromatogram from the DEAE HPLC run is shown in Figure 27. The concentrated sample from pooled fraction number 1 (removed from the anode) was too viscous to take up into the glass injection needle and when diluted and injected into 3 mosquitoes, resulted in their deaths. Either the salt or the sucrose overlay solution was too concentrated, so I decided to electrodialyze again. This time, pooled fractions were dialyzed from the cathode to the anode to facilitate separation of protein from the sugar or salt, then fluid at the anode was removed, concentrated on a SpeedVac and injected. The results clearly show that no activity was recovered in any of the fractions (Figure 28).

I feel that this experiment had addressed all of the issues that may have plagued the previous chromatographic studies: Terminalia were extracted in a starting buffer of low molarity but acceptable buffering capacity (Karlsson et al. 1989; Stoll & Blanchard 1990; Voet & Voet 1990) with a pH of 8.1, the pK_a (and maximal buffering capacity) of Tris; protease inhibitors were included in the extract;

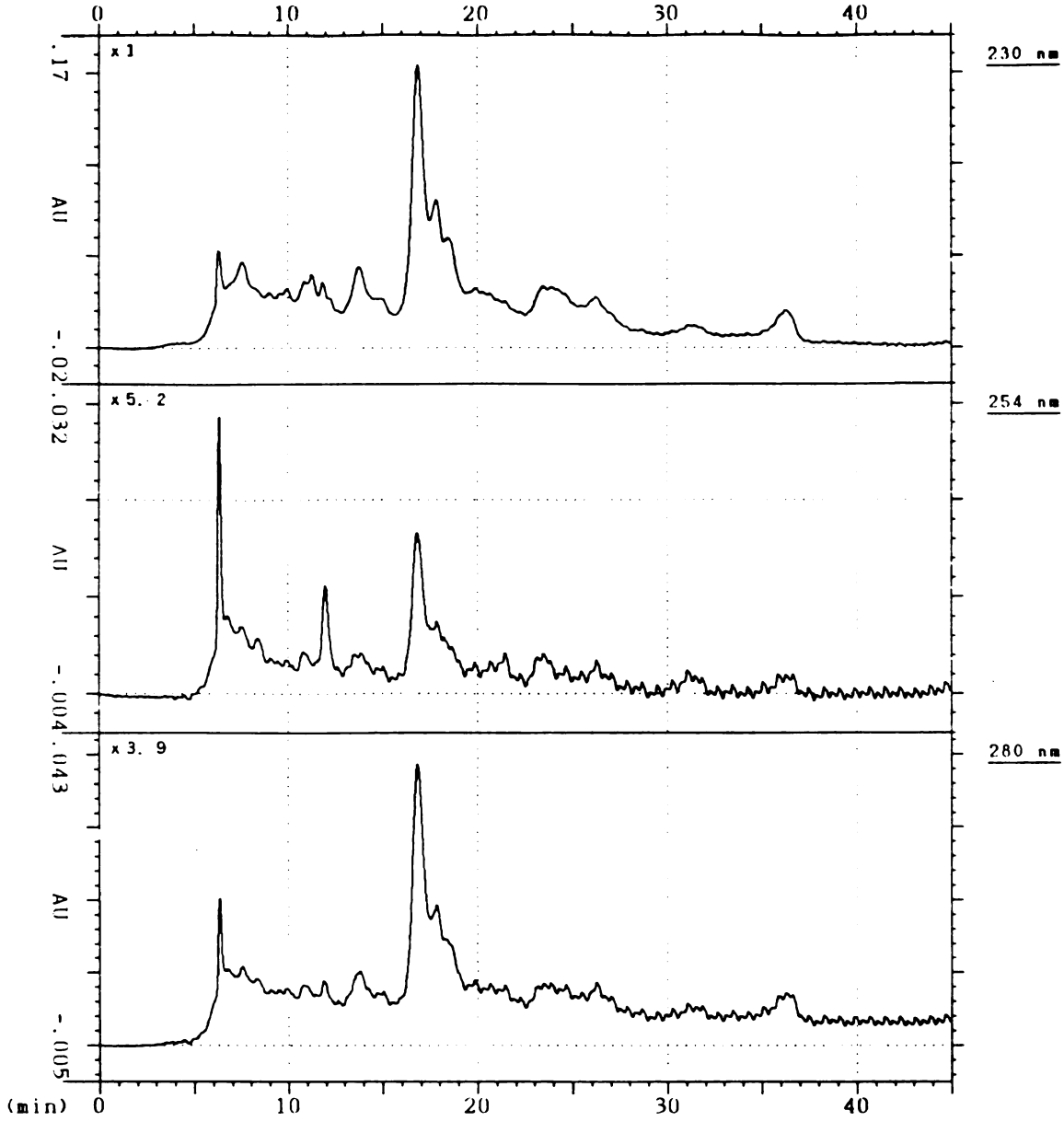


Figure 25. QAE ion exchange HPLC of *A. aegypti* accessory gland extract. 160 ♂ equivalents were loaded onto a 30 x 2.1 mm QAE-Silica column and eluted with a gradient of 0 to 1 M NaCl in 0.01 M Tris (@ pH 8.0) over 45 min. The flow rate was 250 µl/min and absorbance was read at wavelengths of 230, 254 and 280 nm.

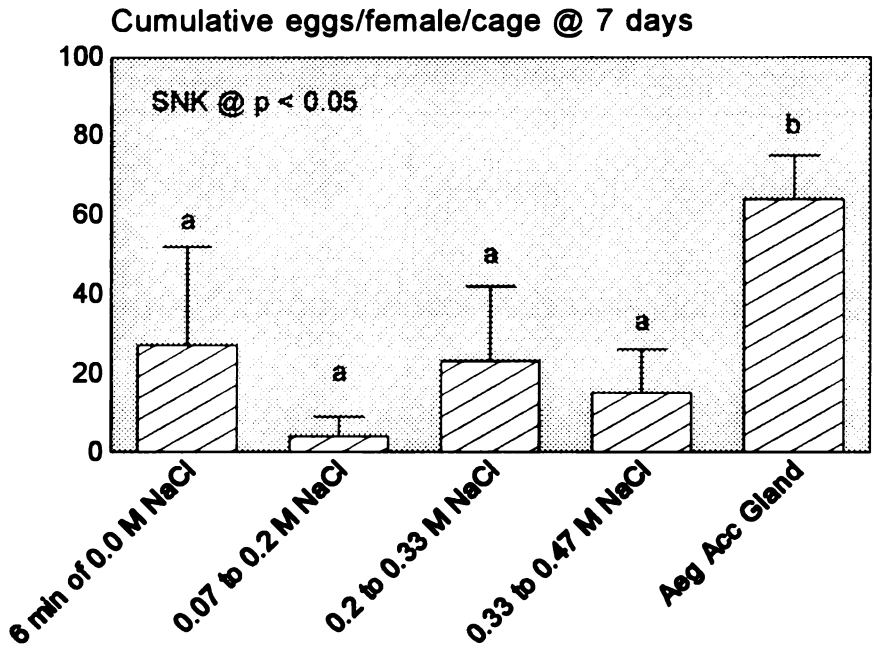
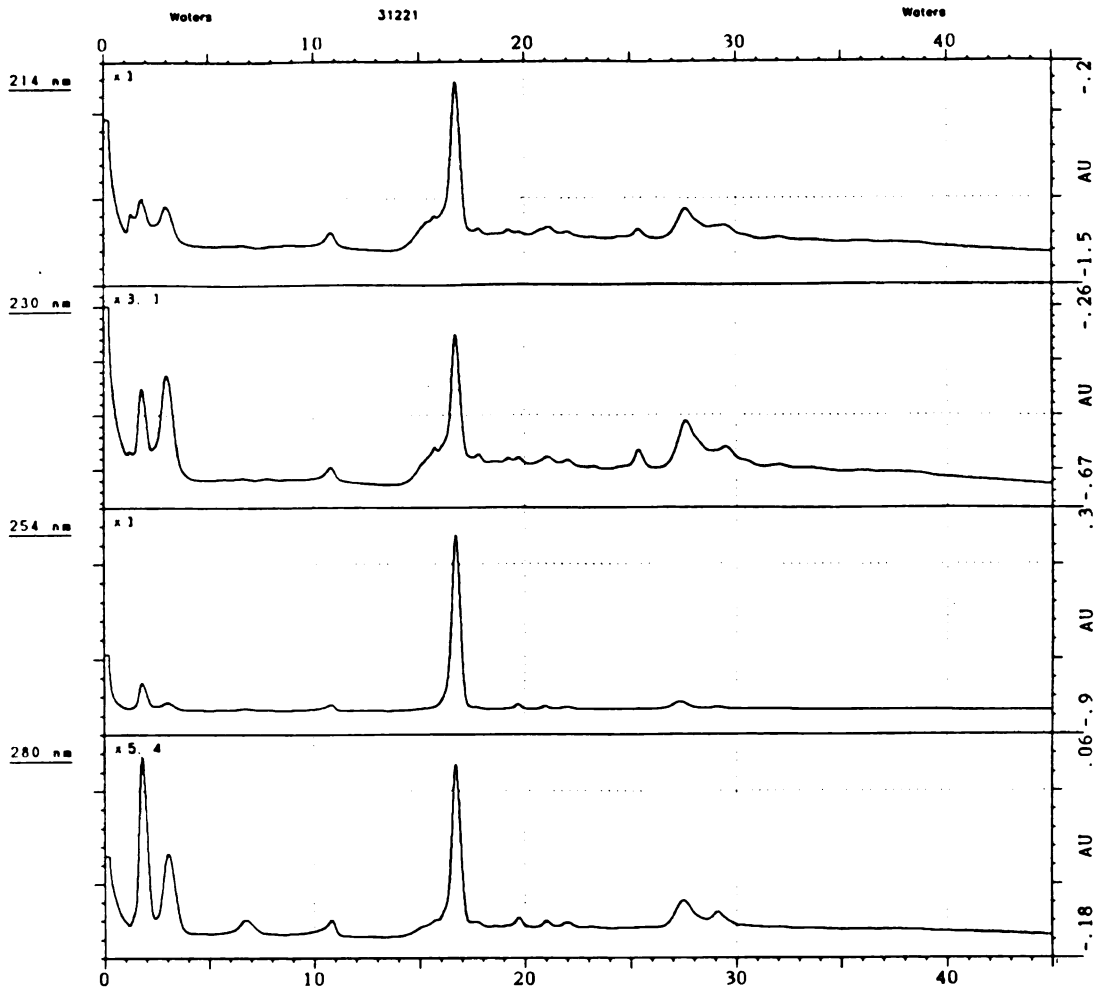


Figure 26. *A. aegypti* oviposition following injection with QAE HPLC fractionated accessory gland extract. Fractions containing peaks were pooled, desalted and injected as 1 ♂ equivalent (n=5).



Time in minutes

Figure 27. DEAE ion exchange HPLC of *A. aegypti* accessory gland extract. 300 ♂ equivalents were loaded onto a 30 x 2.1 mm Polypore DEAE column and eluted with a continuous gradient of 0.02 M Tris to 0.5 M NaCl/0.02 M Tris (@ pH 8.1) over 30 min. The flow rate was 300 µl/min and absorbance was 214, 230, 254 and 280.

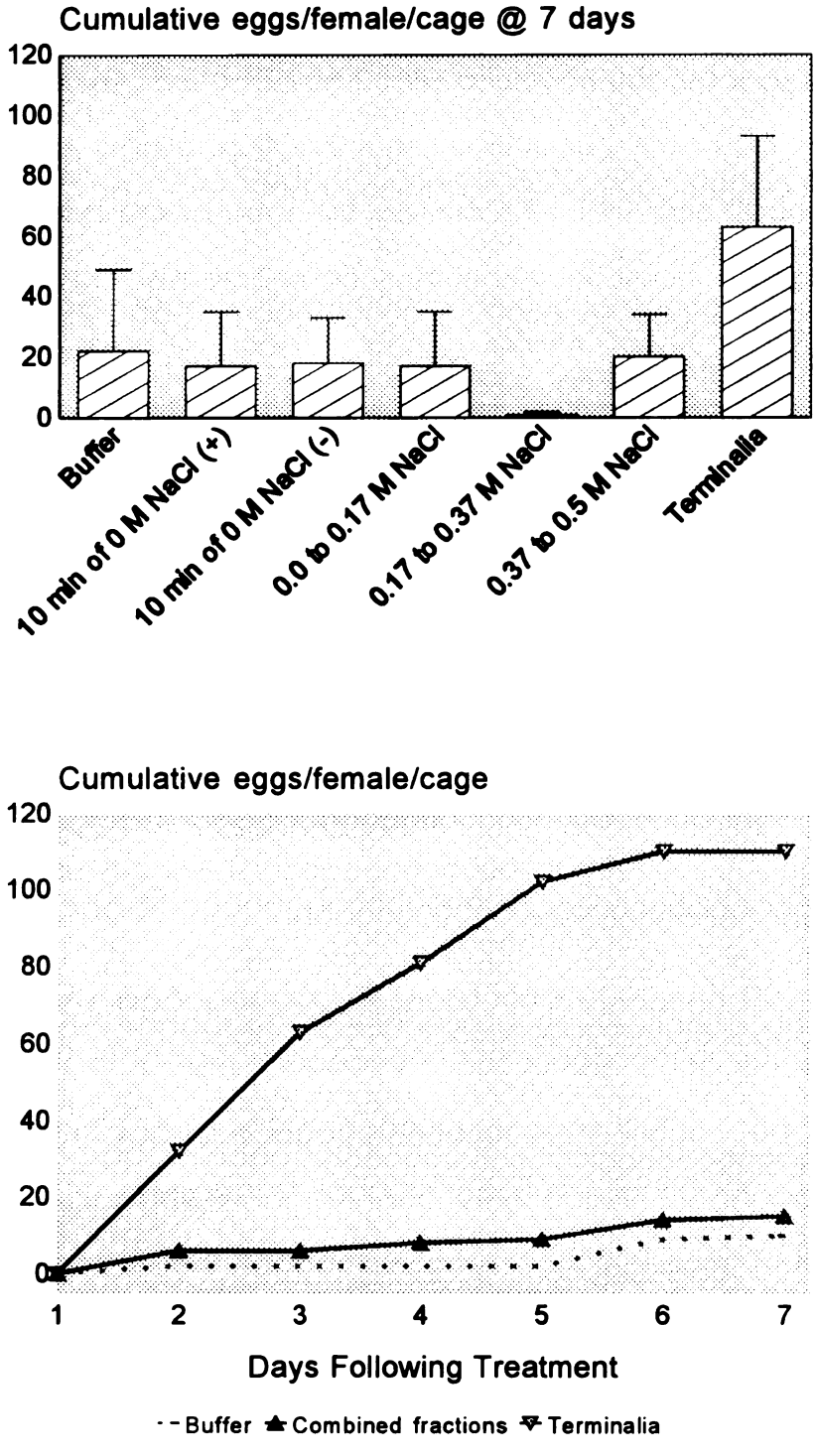


Figure 28. Oviposition by *A. aegypti* injected with DEAE HPLC fractionated accessory gland extract. Fractions in the top graph were desalted and injected as ≤ 5 ♂ equivalents (n=5). The bottom graph shows oviposition following injection of recombined fractions (n=5).

the protein concentration of the starting sample was reasonable (1 mg per ml) and would be concentrated further during the run; the amount of protein loaded onto the column (~ 200 μg) was sufficient for the column, which had a capacity of 150 to 200 μg; proper column equilibration and conditioning procedures were followed to remove potential contaminants and establish a level baseline (Smith 1987c); a nonionic detergent was used to minimize or prevent protein aggregation in the extract and nonspecific interaction with the column [Hutchens (1989) indicates that the low ionic strength of buffers and columns in chromatofocusing sometimes induces protein aggregation. He states that nonionic detergent additives may prevent aggregation until proteins adsorb to the column, when aggregation should no longer be a problem.]; samples were processed via electrodialysis and SpeedVac concentration, methods previously shown to be hospitable to mosquito accessory gland extracts; extracts were injected at a relatively high dosage (≤ 5 ♂ equivalents); and all fractions were recombined to test the hypothesis that two or more components are required for oviposition induction.

Capillary electrophoresis. A typical electropherogram from capillary electrophoresis of *A. aegypti* accessory glands reveals several low absorbance peaks on either side of a group of higher absorbance peaks that probably reflect compounds with nearly neutral net ionic charge (Figure 29). A control oviposition study (results not shown) demonstrated that activity was maintained following a 7.5 min exposure of extract to the CE column in the presence and absence of a typical operating voltage of 22.5 kV. Results from the two fractionation studies were equivocal. In the first study, mosquitoes receiving the most anionic fraction (proteins migrating slowly through the column) laid the most eggs whereas those receiving the most cationic fraction (proteins migrating quickly) laid the least eggs, comparable to negative control mosquitoes (Figure 30). The two other fractions

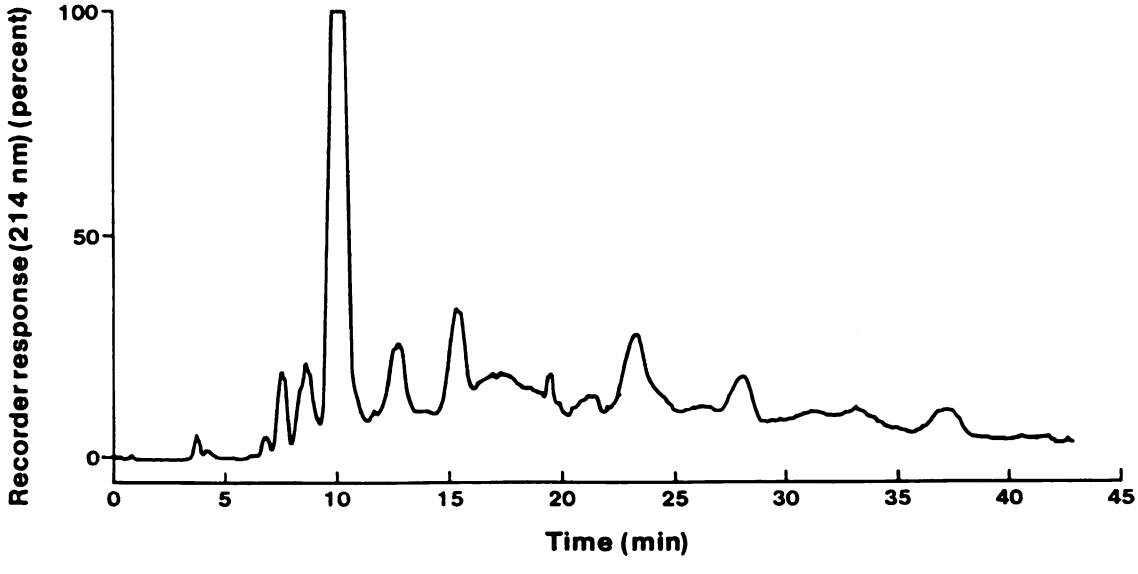


Figure 29. Capillary electrophoresis of *A. aegypti* accessory gland extract in a coated C18 column. A voltage of 22.5 kV was applied for 8 min through a 75 μm column containing a buffer of 10 mM sodium phosphate, 0.01% Brij-35, pH 7.0. Absorbance was read at 214 nm.

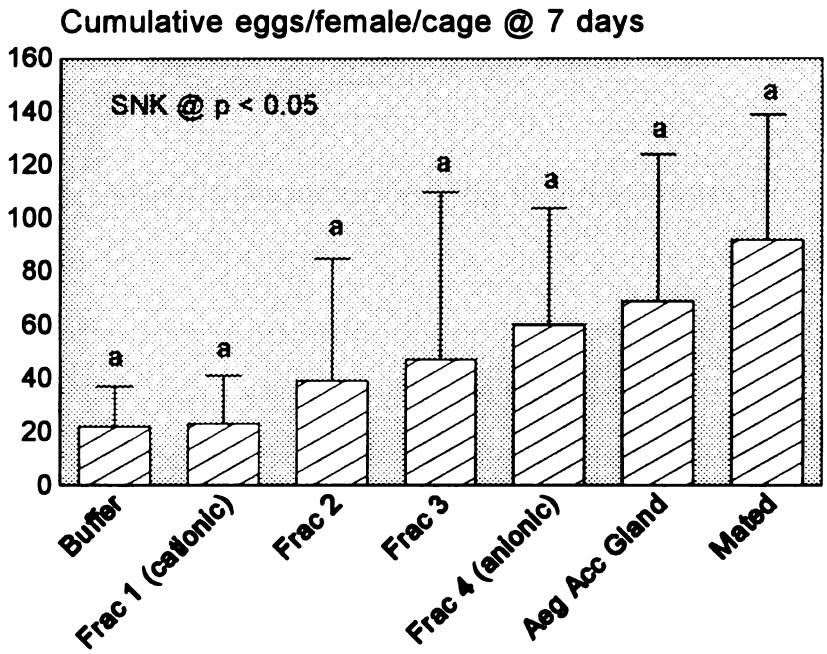


Figure 30. Ovipositional activity of *A. aegypti* accessory gland extract electrophoresed in a coated C18 capillary column. Fifty runs were made to collect sufficient material for injecting 0.25 ♂ equivalents in each female (n=5).

gave intermediate results. Some activity may have been present in up to three of the fractions, perhaps because of random mixing during the flushing procedure or incomplete separation of proteins during the electrophoresis run. They also suggest that extract-induced mosquito oviposition is dosage-dependent. Unfortunately, attempts to use CE to analyze each fraction were confounded by the increased concentration of analyte following SpeedVac evaporation, causing all proteins to elute together as two peaks, so I was unsure how much fractionation was actually achieved. In the second study, the collected fractions were unintentionally evaporated to dryness in the SpeedVac, immediately resuspended in mosquito saline, but failed to show any activity above negative control values (results not shown).

I also tried using a PACE 2000 unit (Beckman Instruments Inc., Fullerton, CA) with an automatic collector and cooling chamber but encountered problems including rapid depletion of cathodic buffer and mortality among mosquitoes injected with concentrated fractions. Probably the dosage of either Brij-35 or sodium phosphate buffer was too high to be tolerated.

It is interesting that the profiles of the absorbance spectra from the QAE HPLC run (Figure 25) and the CE run (Figure 29) were similar. Both methods separate proteins according to charge differences, retarding the elution of anions and hastening the elution of cations.

Discontinuous nondenaturing PAGE. In the first discontinuous native gel experiment I assumed that all of the proteins in the extract would migrate out of the 3% stacking gel and into the 5% resolving gel. The resolving gel was divided into four fractions, electroeluted for 6.1 h and the resulting eluted samples were injected, theoretically at a concentration of 0.67 ♂ equivalents. No activity was recovered from any of the four fractions (Figure 31). The second experiment was run essentially under the same conditions except that I eluted both the stacking gel and

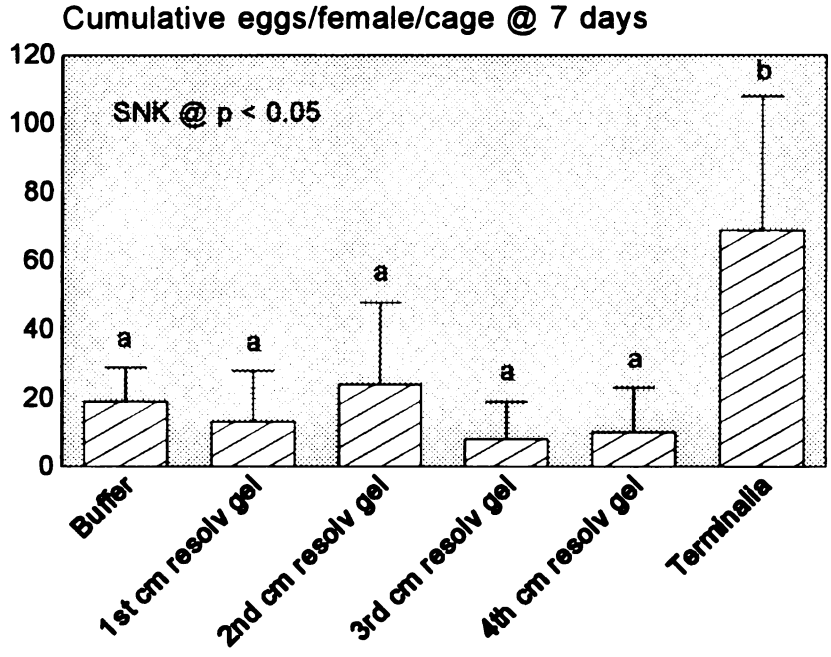


Figure 31. *A. aegypti* ovipositional activity of terminalia extract electrophoresed 40 min in 5% discontinuous nondenaturing gel and eluted. The gel slices described above were electroeluted for 6 h in 10 mM NH_4HCO_3, then extracts were injected at 0.7 ♂ equivalent. The positive control was terminalia in elution buffer (0.5 ♂ equivalents) and the negative control was buffer (n=5).

the resolving gel for 10.5 h and injected a theoretical dosage of 1 ♂ equivalent. Since there was no significant oviposition activity after 10 days (see Figure 32, black bars) the leftover eluted fractions were concentrated 2.5 to 5-fold and injected into another group of mosquitoes. This time significant activity appeared in the stacking gel fraction, peaking at 74 eggs per female versus 14 eggs/female in the negative control (buffer) injected group (Figure 32, shaded bars). However, none of the activity had moved from the stacking gel into the resolving gel so I decided to electrophorese for a longer time in hopes of effecting some fractionation of the extract. When terminalia extract was electrophoresed for 2¼ h, electroeluted for 15 h, then concentrated to a theoretical maximum of 5 ♂ equivalents, I recovered full oviposition activity comparable to the positive control but it remained in the stacking gel (Figure 33).

Analysis of the eluted fractions from the last experiment by SDS•PAGE showed that a wide range of proteins had migrated into the nondenaturing gel along with the front, indicating that most sizes of proteins were not being retained in the stacking gel (Figure 34). Yet activity continued to be recovered only from the stacking gel, suggesting several possible explanations: Perhaps the oviposition factor consisted of two or more components being separated once they entered the resolving gel but enough material was being held back in its native form in the stacking gel to produce reasonable activity. Alternatively, maybe the active factor was not entering the resolving gel because it was aggregating with other proteins preventing their migration out of the lower percentage gel; intuitively, however, it is difficult to believe that the slightly smaller pore size of the resolving gel would retard migration of any sized protein complex. A third possibility is that the pI of the active factor is intermediate between the pH of the resolving gel and stacking gel, causing it to precipitate at the interface. A fourth possibility is the active factor

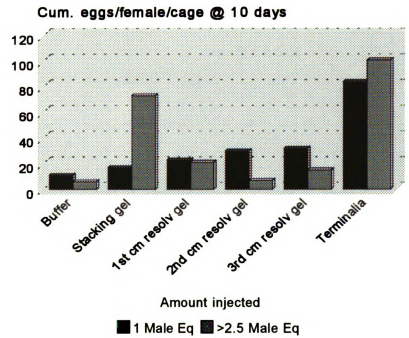


Figure 32. *A. aegypti* ovipositional activity of terminalia extract electrophoresed 41 min in 5% discontinuous nondenaturing gel, eluted and concentrated. The gel slices described above were electroeluted 10½ h in 10 mM NH_4HCO_3, then extracts were injected immediately (1 ♂ equivalent) or concentrated and injected (2.5 to 5 ♂ equivalents). Buffers, positive and negative controls were the same as the previous experiment (n=5).

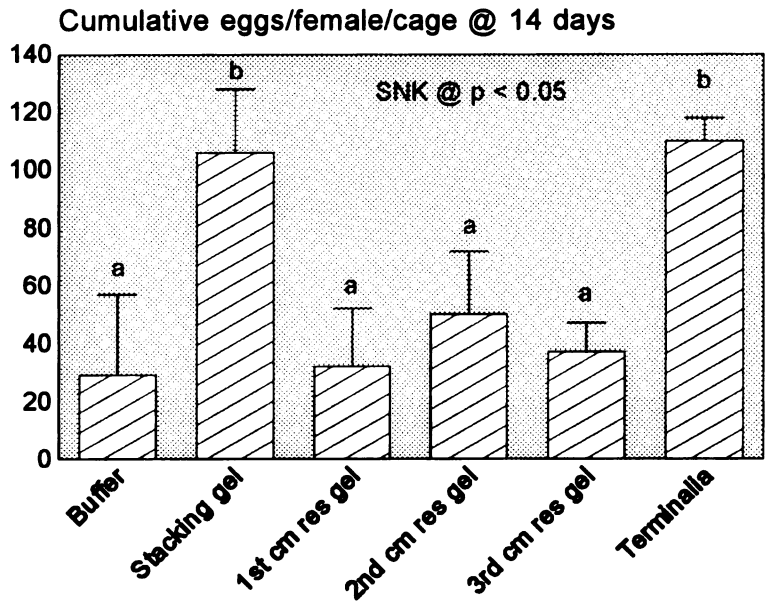


Figure 33. *A. aegypti* ovipositional activity of terminalia extract electrophoresed 2¼ h in 5% discontinuous nondenaturing gel, eluted and concentrated. The gel slices described above were electroeluted for 15 h in 10 mM NH_4HCO_3, then extracts were concentrated to ≤ 5 ♂ equivalents and injected. Buffers, positive and negative controls were the same as before (n=5).

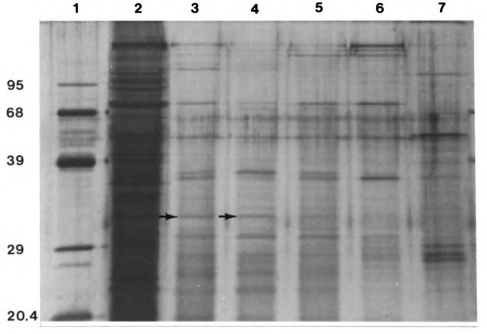


Figure 34. 12% SDS•PAGE of *A. aegypti* terminalia extract separated by nondenaturing discontinuous PAGE. Lanes: (1) molecular weight markers of 95, 68, 39, 29 and 20.4 kDa; (2) unfractionated terminalia extract; (3) concentrated stacking gel fraction from Figure 33; (4) stacking gel fraction from Figure 34; (5) through (7) the 1st, 2nd and 3rd cm of the resolving gel from Figure 34. The arrows in lanes 3 and 4 indicate a 32.5 kDa protein unique to these active fractions.

is almost neutral at the stacking gel pH of ~ 8.9, causing it to move very slowly; but it still behaved as an anion at pH 5.5 during electrodialysis, so this is unlikely.

Despite being able to recover activity, however, the results of the SDS analytical gels suggested not much fractionation of the extract had been achieved. Many protein bands were found in each fraction, most of them common to all fractions. In addition, based on the differences in staining intensity between the fractions and the untreated extract, it was apparent there was significant loss of material during the electrophoresis and/or electroelution steps.

In reviewing SDS•PAGE results from the electrodialysis study, I found little difference in staining intensity of SDS gels between dialyzed and nondialyzed samples at comparable protein loading levels, indicating that protein loss from the electrolelution process was minimal. The operating voltage for electroelution was 150 V whereas electrophoresis ran at 200 V, a potentially more severe environment for protein. Based on a qualitative evaluation of staining intensity, I also determined that the presence of SDS in the buffer was critical for protein recovery when electroeluting gels but seemed to have little effect when dialyzing protein samples (results not shown). Perhaps some aspect of the electroelution conditions (insufficient or excessive elution time, wrong buffer) was preventing good recovery of proteins from the gel. Alternatively, proteins were possibly precipitating within the gel, making them difficult to extract. I did not think that further refinement of the discontinuous gel technique would be fruitful and switched to using gradient gels at the suggestion of Dr. Tom Mowry (personal communication). One potential advantage is that gradient gels have the same pH throughout, eliminating potential problems due to pH discontinuities.

Gradient nondenaturing PAGE. Dr. Estelle McGroarty (Department of Biochemistry) related that if a protein of interest is remaining in the stacking gel

during nondenaturing PAGE, it is probably aggregating with other proteins. She suggested using a nonionic detergent such as n-octyl ß-D-glucopyranoside to break up the aggregation. Although this series of electrophoresis experiments did not involve a stacking gel, n-octyl ß-D-glucopyranoside was used in all extracts as a precaution.

Marginal activity was recovered from the middle of a 5% to 20% gradient gel loaded with 400 ♂ equivalents following 4 h of electrophoresis. Egg output averaged 37 eggs/female at the end of 14 days versus 13 eggs/female for the buffer-injected group and 92 eggs/female for mosquitoes injected with the untreated extract (Figure 35). The experiment was repeated except that electrophoresis was run for 16 h instead of 4 to try to achieve more separation and narrower focusing of protein bands. Electroelution was reduced from 17 h to 6 h because longer elution times seemed to be associated with some protein loss (based on my analysis of protein recovery from the discontinuous native gel experiments). Slightly greater activity, 51 eggs/female, was recovered by the end of 10 days, this time in the bottom fraction, which makes sense since the gel ran longer (Figure 36). The 15% SDS analytical gel for this experiment illustrates the degree of fractionation achieved (Figure 37).

Since protein band resolution on the native gel was still not satisfactory, a third experiment was set up to run for 36 h, using a gradient of 10% to 25% and loading on 200 ♂ equivalents. Excellent band resolution was obtained, as evidenced by a silver-stained slice of native gel, and there was some activity recovered from one fraction (all fractions were from the bottom of the gel) but the signal was not significantly different than the negative control: 38 eggs/female versus 15 eggs/female for the saline group and 90 eggs/female for the positive control.

The last two experiments were run on 20% to 32% gels for 36 h, reasoning that activity recovered from the bottom of the 10% to 25% gel in the previous

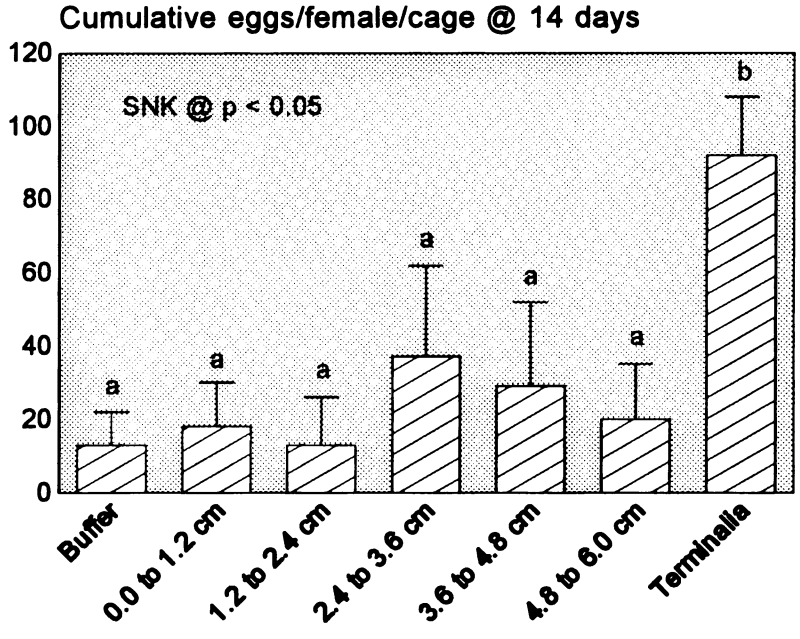


Figure 35. Ovipositional activity of *A. aegypti* terminalia extract electrophoresed 4 h by 5-20% gradient nondenaturing PAGE. An extract containing 400 ♂ equivalents in mosquito saline, 0.1% n-O-ß and 0.0004% protease inhibitors was electrophoresed for 4 h, the gel slices described above were eluted for 17 h, then the eluted samples were concentrated and injected at ≤ 5 ♂ equivalents. Electroelution buffer was 10 mM NH_4HCO_3, the positive control was terminalia extract and the negative control was elution buffer (n=5).

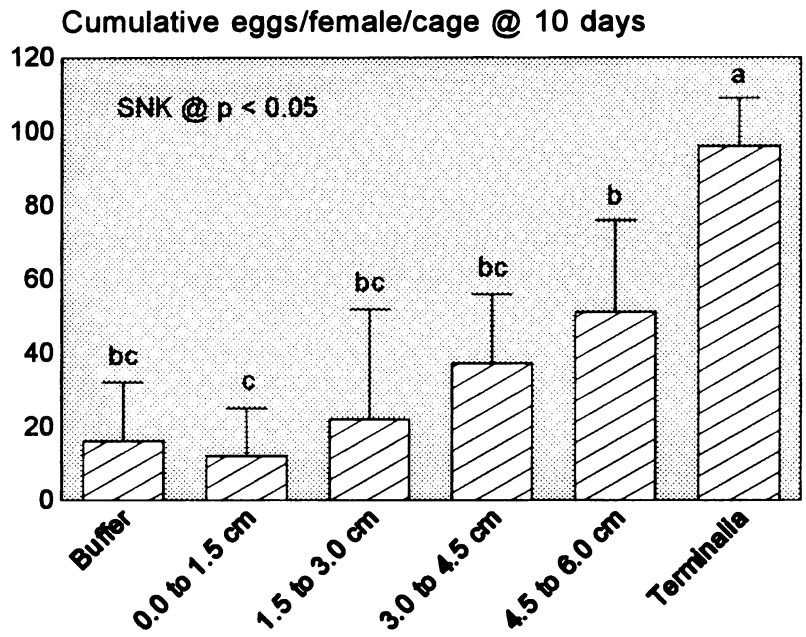


Figure 36. Ovipositional activity of *A. aegypti* terminalia extract electrophoresed 16 h by 5-20% gradient nondenaturing PAGE. An extract containing 400 ♂ equivalents in mosquito saline, 0.1% n-O-ß was electrophoresed for 16 h, the gel slices described above were eluted for 6 h, then the eluted samples were concentrated and injected at ≤ 5 ♂ equivalents. Electroelution buffer was 10 mM NH_4HCO_3 in 0.005% SDS, the positive control was terminalia extract and the negative control was mosquito saline, 0.1% n-O-ß (n=5).

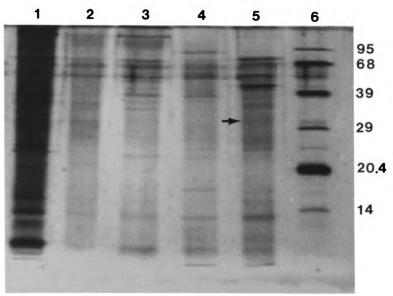


Figure 37. 15% SDS•PAGE of *A. aegypti* terminalia fractions generated by nondenaturing 5-20% gradient PAGE. Lanes: (1) unfractionated terminalia extract; (2) through (5) 1.5 cm horizontal slices of nondenaturing gel — going from top to bottom — as described in Figure 37; (6) molecular weight markers of 95, 68, 39, 29, 20.4 and 14 kDa. The arrow in lane 5 indicates a 32.7 kDa protein unique to this active fraction.

experiment would show up here as well. Mosquitoes injected with fractions from both experiments suffered significant mortality so the data are not shown. It appears that even though the procedures were identical to the ones used earlier, something about the higher percentage gel was lethal to the mosquitoes. (These experiments were conducted sequentially. I ran the second experiment thinking the first was somehow flawed but they corroborated each other.)

SDS•PAGE analysis. If activity for a given treatment is defined as egg deposition equalling ≥ 50% of the positive control, a total of five fractions showed activity following polyethylene glycol precipitation, discontinuous native PAGE and gradient native PAGE. In all five of these fractions, as well as their respective positive controls, there was a protein band at 32.5 ± 0.3 kDa on SDS•PAGE not present in any of the nonactive treatments from these fractionation experiments (Table 8; also see arrows in Figures 35 and 38). Certainly, this does not prove that

Table 8. Molecular weights of unique protein bands from active fractions

Experiment Date	Fraction	Eggs in fraction/ eggs in pos. ctrl.[1]	Molecular weight of unique band
10/15/93	12% PEG precipitation	60/109	32.2 kDa
10/15/93[2]	Disc. stacking gel[3]	102/109	32.2 kDa
10/22/93	Disc. stacking gel[3]	74/101	32.5 kDa
10/29/93	Disc. stacking gel	106/110	32.5 kDa
01/30/94	Bottom 1.5 cm of 5-20% gradient gel	51/96	32.7 kDa

[1] Positive control = terminalia extract-injected virgin females
[2] The results presented for this experiment are not shown elsewhere in this disseration.
[3] These fractions were from the same native gel but were bioassayed and analyzed by SDS•PAGE twice (in separate experiments), suggesting good reproducibility.

this protein is the oviposition induction factor since many other bands also were represented on the SDS gels, but it is worthwhile to note that all of the other bands were prominent in one or more nonactive fractions. The ~32.5 kDa protein was the only protein unique to and common to all active treatments and, notably, is in the molecular weight range of an active fraction found by Fuchs et al. (1969) to be important in receptivity inhibition.

Discussion

Heterospecificity. It is significant that *A. aegypti* male accessory gland extract stimulates oviposition only in conspecific females whereas *A. albopictus* extract activates females of both species. These data corroborate the accessory gland transplant experiments between *A. aegypti* anbd *A. albopictus* performed 30 years ago (Leahy & Craig 1965). At least two interpretations are tenable: either the male-produced *A. albopictus* factor has broader specifity or the female *A. albopictus* receptor has narrower specificity than *A. aegypti* males and females, respectively. The scant biochemical data support the former interpretation. If mosquito oviposition factor is in the molecular weight range of 4 kDa — notwithstanding the findings above — as has been found for *D. melanogaster* (Chen et al. 1988), *D. sechellia* (Chen & Balmer 1989) and *D. suzukii* (Schmidt et al. 1993), then perhaps the species differences in paragonial gland content observed with MALD/ITOF account for differences in oviposition factor specificity (Figure 16). Qualitatively, the protein banding patterns on SDS gels also indicate more kinds of proteins are present in *A. albopictus* accessory glands than in *A. aegypti* (Figure 17). My interpretation is that if accessory glands from *A. albopictus* contain the same proteins as those from *A. aegypti*, as suggested by comparable molecular weights, but also possess some unique proteins, the unique proteins probably confer species specificity on *A. albopictus* with respect to oviposition induction. In the absence of these

proteins, *A. aegypti* accessory gland extract is unable to effect a significant ovipositional response when injected into *A. albopictus* virgins.

A recently published study contradicts the notion of asymmetric ovpositional activation. Yeh & Huang (1991) found that artificial insemination of *A. aegypti* with conspecific male accessory gland extract induced preoviposition behavior but insemination with *A. albopictus* accessory gland extract did not. Preoviposition was measured by placing female mosquitoes in an olfactometer and observing whether they oriented by anemotaxis to an experimental oviposition site (Klowden & Blackmer 1987). Yeh and Huang concluded that accessory gland extracts are species specific. However, oviposition per se was not measured; perhaps either oviposition is regulated differently than preoviposition or the preoviposition assay is not an accurate indicator of oviposition behavior.

It would be greatly beneficial to know the chemistry of the oviposition factor because it may help explain the basis for the difference in ovipositional activation by *A. aegypti* and *A. albopictus* accessory secretions. Second, it may shed more light on their evolutionary and phylogenetic relationships. Third, it might elucidate an underlying mechanism for asymmetric mating interference by which one species effectively reduces the reproductive output of a closely-related species, either through removal of receptive, monogamous females from the mating population or through production of sterile, hybrid offspring (Ribeiro 1988). Such knowledge might be used as a biological control method by introducing a nonpest species into a population to displace a closely related pest or vector species, a strategy recently proposed for the malaria vector, *Anopheles gambiae* (in sensu Collins & Besansky 1994). Knowledge of the chemistry of these factors also may make it possible to develop specific "fertilicides", synthetic oviposition factors that induce females of pest species to lay unfertilized eggs without affecting nonpest species.

According to Mattingly (1957), *Aedes aegypti* originated in Ethiopia and *A. albopictus* has Australasian and Oriental origins (Leahy & Craig, 1967). Both species are widely distributed over the tropics and subtropics. Although they occupy the same subgenus, *Stegomyia*, given their reputed origins I would qualitatively state they are distantly related.

Oviposition factors in *A. aegypti* and *A. albopictus* appear to function as prezygotic, postmating reproductive isolation mechanisms (Mettler et al. 1988). However, several other parameters besides sex peptide differences regulate their ability to interbreed. In an extensive study of mating compatibility, Leahy & Craig (1967) demonstrated that interspecific mating did not readily occur and identified five barriers to gene flow between laboratory populations of these two species: mating behavior, structural incompatibility, sperm inactivation, reduced oviposition, and genetic incompatibility.

Differences among species in mating behavior function as prezygotic, ethological reproductive isolation mechanisms but apparently the barrier between *A. aegypti* and *A. albopictus* mating is sufficiently plastic to allow some interaction. However, studies of the degree of mating heterospecificity have produced contradictory results. Nasci et al. (1989) found that *A. albopictus* males inseminated ~80% of both *A. albopictus* and *A. aegypti* females in free-choice experiments whereas *A. aegypti* males rarely inseminated *A. albopictus* females; whether this was by female choice or male ability was not stated. In contrast, Harper and Paulson (1994) showed that heterospecific insemination occurred less than 11% of the time for both species combinations. Yet another cross-mating study suggested these two species freely interbreed and produce viable progeny but the experiments may have been contaminated by impure cultures of each species (Downs and Baker, 1949). Leahy and Craig (1967; also Black et al. 1989) observed that female *A. aegypti* were

more receptive to males of both species than *A. albopictus*, which were apt to retract their claspers and terminate mating by flying away.

Structural incompatibility between male genitalia represent another prezygotic barrier to hybridization. Males of both *Aedes* species struggled to disengage from females following heterologous matings (Leahy & Craig 1967). In addition, sperm masses normally deposited inside the genital chamber in intraspecific matings were often found outside following interspecific matings. The inability to successfully transfer sperm would constitute a mechanical barrier to fertilization.

There is contradictory evidence on whether *A. albopictus* x *A. aegypti* hybridization is preempted via prezygotic, gametic incompatibility barriers. Dissection of spermathecae retrieved from cross-insemination experiments involving *A. albopictus* ♀ x *A. aegypti* ♂ revealed that sperm were either always alive (Nasci et al. 1989) or partially inactivated (Leahy & Craig 1967). Pairings of *A. aegypti* ♀ x *A. albopictus* ♂ showed that sperm were either always dead (Nasci et al. 1989) or partially inactivated (Leahy & Craig 1967). Studies on spermathecal filling in mosquitoes (Jones & Wheeler 1965; review by Linley & Simmons 1981) suggest the female plays an active role in determining whether sperm migrate to the spermathecae from the bursa, where they are initially deposited by the male. Given the above data, perhaps *A. aegypti* females do not possess the requisite factors to maintain sperm viability or they actively destroy sperm from heterospecific males. Following the same logic, *A. albopictus* females may be less able to differentiate and inactivate sperm from *A. aegypti*.

Finally, none of the eggs laid by cross-inseminated females hatched (Harper & Paulson 1994), although some of the sperm transfers were presumed to be viable (Leahy and Craig, 1967). Taken together, these five reproductive isolating mechanisms constitute a formidable barrier to hybridization between *A. aegypti* and *A. albopictus*.

If these species can be said to be evolving away from one another, then male sex peptide or its female receptor has not sufficiently differentiated to preclude all interspecific communication. Given that many barriers already exist to prevent mating, this suggests sex peptide differentiation may be arising as a byproduct of speciation but is not an integral driving force of reproductive isolation.

Perhaps adding other species of *Aedes* to this study might have provided valuable, if not better, clues on the evolution of sex peptides in this genus of mosquitoes. It is relevant to an evolutionary study of sex peptides as a mate recognition system that at least two species be examined, that both species possess an oviposition factor, and that the factors exhibit asymmetrical, heterospecific activity. Although my studies and those conducted previously used *A. aegypti* and *A. albopictus* for investigating sex peptides, their postulated separate origins and the barriers to gene flow suggest that more closely related species might be better subjects for future evolutionary study as *A. albopictus* and *A. aegypti* appear to be rather distant cousins.

Differences in sex peptide probably would be less between closely related species than between distantly related species. Specifically, I would investigate sex peptide activity (and sequence data) in known sympatric species as well as in allopatric species, expecting that differences would be greater in sympatry than allopatry. The *Scutellaris* group may be appropriate because it contains species that show true unidirectional hybrid compatibility (*A. polynesiensis & A. scutellaris*), bidirectional hybrid compatibility (*A. polynesiensis & A. pseudoscutellaris*), and noncompatibility as exemplified by *A. albopictus* mating with any other species (Gubler 1970; Ali and Rozeboom 1971; Dev & Rai 1985; Meek 1988). There also are three species of the *Albopictus* subgroup that might elucidate the specificity of sex peptides. An *A. albopictus* strain from Malaysia is sympatric with but

ethologically isolated from *A. pseudalbopictus* but is not reproductively isolated (other than geographically) from the allopatric *A. seatoi* (McLain & Rai 1986).

A study of ethological isolation of species in the *Scutellaris* subgroup found asymmetrical isolation among older and new species. Females of older species, *A. pseudoscutellaris* and *A. polynesiensis*, actively discriminated against the newer species males, *A. cooki* and *A. kesseli*, but the reciprocal did not occur (McLain et al. 1985). Thus descendants, which diverged about 2 million years ago (mya) according to allozyme data, exhibited more heterospecificity than ancestors, which diverged about 5 mya.

In light of the asymmetry in sex peptide activity as well as the number of major barriers to hybridization between *A. aegypti* and *A. albopictus*, it is provocative to recall the oviposition studies by Leahy (1967) and Hiss and Fuchs (1972). Leahy showed that *D. melanogaster* accessory gland implants activate *A. aegypti* oviposition. Hiss and Fuchs demonstrated that injection of *D. melanogaster* accessory gland extract into *A. aegypti* elicited the same response as mosquito α factor: It stimulated oviposition when injected alone and suppressed receptivity when injected along with ß factor. Yet when trying to use the methanol extraction methods published by Chen et al. (1988) for *D. melanogaster* as a prelude to fractionating by reverse phase HPLC, activity was diminished (results not shown). This suggests that oviposition factors for *A. aegypti* and *D. melanogaster* are not biochemically similar. In addition, it is difficult to reconcile how *A. aegypti* and *D. melanogaster* sex peptides could be reciprocally active when seemingly more closely related species exhibited sex peptide asymmetry. Perhaps this means that sex peptide receptor for *A. aegypti* females is quite nonspecific.

Fractionation. None of the techniques used in separating accessory gland extract yielded significant fractionation. Although activity was recoverable at many

stages in the overall process, when activity was lost it usually occurred during a fractionation step. The fractionation procedures from which activity was recovered include capillary electrophoresis, polyethylene glycol precipitation, electrodialysis, nondenaturing discontinuous polyacrylamide electrophoresis and nondenaturing gradient polyacrylamide electrophoresis. Activity also was retained under the following conditions or through the indicated processing steps: whole extract stored for 2 months at -20°C or ≥ 48 h at 3°C; extract held at or electrodialyzed at pH 5.5 to pH 8.3; extraction in mosquito saline, 10 mM NH_4HCO_3, ≤ 0.02% SDS, 0.1% n-octyl ß-D-glucopyranoside, and 10 mM sodium phosphate and 0.01% Brij-35; SpeedVac concentration to low volumes.

Loss of activity occurred as a result of fractionation by reverse phase high performance liquid chromatography, strong and weak anion exchange HPLC, gel filtration chromatography, conventional ion exchange chromatography, extraction in 80% methanol, and SpeedVac concentration to dryness. In reviewing all of the fractionation techniques and experimental conditions, it is my impression that significant or total loss occurred during any sieving process whether it was through a column or a gel. High pressure also may have caused permanent loss. Other possible explanations are that the protein concentration was too low either at the beginning or the end of an experiment, the carrying capacity (molarity) of the buffer was not optimal, or that the active factor consists of two or more components that do not spontaneously reassemble when recombined.

Because of difficulty retaining activity following standard protein fractionation protocols, I wondered whether the oviposition factor was a protein. Given the decline in activity following treatment with trypsin (Figure 11) and the previously published data (Fuchs et al. 1969), the active factor certainly appears to be a protein. Yet these studies do not exclude the possibility that protein could be functioning as a carrier molecule for a nonproteinaceous oviposition factor.

Ideally, fractionation methods should employ as few steps as possible between preparation of an extract and injection into virgin females. With each additional processing step, I likely was losing active material either as a result of the process or during transfers, a potential problem when exposing the protein to so many surfaces (Felgner & Wilson 1976). Table 9 outlines the sequence of transfers involved in fractionating an extract by gel filtration chromatography. This was not an atypical number of steps for any of my fractionation methods. Given the amount of starting material used by other groups in isolating a protein (e.g., Cho et al. 1991 started with a crude protein extract of 793 mg and isolated 13 μg of pure aspartic protease, a 14-fold reduction), I should have started each fractionation with a much greater amount of protein and, possibly, at a higher concentration.

The incorporation of a nonionic detergent, n-octyl ß-D-glucopyranoside, was intended to address at least part of this problem. Besides trying to minimize protein adsorption to foreign surfaces, it also minimized protein aggregation. Young and Downe (1987) found that the factor inhibiting insemination in *Culex tarsalis* eluted at different places on a gel filtration column depending on what tissues were extracted. Activity derived from extracts of accessory glands only eluted at around 3 kDa. Activity of extracts prepared from combining accessory glands with whole males eluted at greater than 30 kDa. They concluded that the difference in elution profiles occurred because the small molecular weight receptivity-inhibition factor from accessory glands aggregated with other proteins when extracted in a complex protein mixture, falsely indicating a higher molecular weight. Young and Downe suggested that the molecular weights of the *A. aegypti* receptivity-inhibition and oviposition-induction factors characterized by Fuchs et al. (1969) were incorrect, also because of aggregation. Fuchs et al. (1969) estimated the molecular weights at approximately 60 and 30 kDa using gel filtration chromatography but extracts were prepared from whole bodies, not isolated accessory glands.

Table 9. Protein handling steps in gel filtration chromatography

Process	Exposure to other surfaces & transfers
Extract preparation	Original collection vial Sonicating probe Pipet tip for removing supernatant Vial for storing supernatant (extract)
Chromatography	Pipet tip for applying extract to gel Gel media and column walls Collection tube under column
Concentration	Pipet tip to transfer fraction Ultrafiltration centrifuge tube Pipet tip to remove & pool fractions Second ultrafiltration tube Pipet tip to remove pooled fractions Vial for storing pooled fractions
Injection	Aspiration of extract into glass needle

An alternative explanation for the large difference in molecular weight estimates may be found by considering the accessory gland ultrastructure of an *Aedes* and a *Culex* species. *A. aegypti* accessory glands are characterized by two types of columnar cells, each producing a different type of secretory granule (Dapples et al. 1974). In contrast, *C. pipiens* accessory glands contain four types of cells and three types of secretory granules (see Dapples et al. 1974). Perhaps the nature and character of the accessory secretion is, in fact, different. However, *A. aegypti* accessory glands transplanted into *C. pipiens* stimulated oviposition comparable to mated controls (Leahy 1967), which implies that, despite their morphological differences, *A. aegypti* glands express a protein(s) similar to *C. pipiens*.

Follow-up recommendations. I have several recommendations regarding future efforts at fractionation. First, it would be more efficient and might be worth the effort to consider developing an alternative assay to oviposition such as a behavioral assay that clearly distinguishes the sequences of preovipositional behaviors between the mated and virgin mosquitoes. Second, the starting extract for a fractionation procedure should consist of several hundred to a thousand milligrams of protein (~ 2000 ♂) rather than 200 µg. Third, I would investigate either ammonium sulfate or polyethylene glycol precipitation in more detail as an initial fractionation procedure to remove bulk proteins from the crude extract. Fourth, I would continue to use a nonionic detergent as it does not appear to cause any problem and may facilitate protein recovery. Fifth, I would maintain the protein in a buffer of at least 200 mM whenever possible (Voet & Voet 1990; Stellwagen 1990; Pohl 1990). Sixth, there should be as few steps as possible from start to finish. Seventh, consider using bovine serum albumin in the initial mixture to provide excess protein for adsorption (Pohl 1990).

One final note: I made three attempts to sequence the N-terminus of the darkest staining proteins (and, therefore, the ones in greatest quantity) from *A. aegypti* accessory glands separated by SDS•PAGE. In one case a ~30 kDa protein was recovered by electroelution using an established procedure (Hunkapiller et al. 1983; Smith 1987a) and submitted to the Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility. In the other two instances, a ~40 kDa protein and a ~25 kDa protein were isolated on SDS gels and electroblotted to PVDF membranes using the MSSSF-recommended procedure of Matsudaira (1987; also see the BioRad publication #LIT-240 and BioRad Mini Trans-Blot® Electrophoretic Cell instruction manual). None of the three protein bands yielded sequence information even though there was enough material present (J. Leykam, personal communication). It is likely that the proteins I selected were blocked at the NH_2 termini, a characteristic of most eukaryotic proteins (Stone et al. 1989). An appropriate strategy for future sequencing attempts would be to digest the protein with trypsin and sequence each of the fragments.

General Summary and Conclusions

Why do some species of Diptera possess an oviposition factor while others do not? I propose there are at least three levels of intraspecific mating communication by insects within the context of sex peptide signalling: (1) Male-derived, chemically-mediated mating-receptivity inhibition and oviposition induction; (2) only mating-receptivity inhibition; and (3) neither. Perhaps certain types of mating systems are associated with each level. If so, what is the adaptive significance of each within the mating system?

Aedes aegypti and *A. albopictus* (as well as *Delia antiqua, D. platura, Drosophila melanogaster*, and *D. sechellia*) fall into the first category as present and past experiments have demonstrated both mating inhibition and oviposition induction in these species. In *A. aegypti*, previous work (Fuchs et al. 1969; Fuchs & Hiss 1970; Hiss & Fuchs 1972) found that two fractions were necessary to inhibit receptivity but only one was required to elicit oviposition. Because Young and Downe's (1987) experiments with *Culex tarsalis* cast doubt on Fuchs et al.'s isolation studies (see discussion in chapter 4), the question remains whether two factors really are necessary. Nonetheless, it is clear that *Aedes* male accessory glands secrete an active sex peptide. In *Drosophila*, it appears that one factor is responsible for both behaviors although the receptivity inhibition effect produced by synthetic sex peptide is of considerably shorter duration, 2 to 3 days, than that produced by normal mating, 7 to 9 days (Hihara 1981; Chen et al. 1988; Aigaki et al. 1991).

Female *A. aegypti* are considered monogamous. They retain semen in the copulatory bursa after a first mating but expel semen following second matings even

though they appear to mate (Spielman et al. 1967; Jones & Madhukar 1976). In contrast, *D. melanogaster* females mate two or three times if given adequate opportunities for oviposition between matings (Pyle & Gromko 1978). Apparently sex peptide has evolved under two different mating systems in these genera: one monogamous and the other polyandrous. What would be the adaptive advantage in each case?

Mating in *Aedes* mosquitoes appears to be initiated by males, which are attracted to female wingbeat frequency (Leahy & Craig 1967; Jones 1968; Williams et al. 1978; Black et al. 1989). Upon contact a male immediately clasps the female's genitalia, inserts his copulatory organ, discharges the semen then disengages, all in 6 to 20 seconds (Jones 1968; Spielman et al. 1967). I roughly classify this as a male mate-control system because the male does not appear to discriminate among potential partners — indicating it is not a male mate-choice system — and the mating encounter seemingly gives the female no choice. Based on these data for *Aedes* mosquitoes, natural selection apparently has favored both males and females that employ mechanisms minimizing sperm competition. For males, sperm precedence is achieved through sex peptide-induced behavioral change. Females minimize sperm competition through monogamy.

Polyandrous female *D. melanogaster* preferentially mate with virgin males rather than recently mated males (Markow et al. 1978), qualifying their behavior as a female mate choice system. In evolutionary terms, female *Drosophila* have evolved mechanisms maximizing mate choice as a means of protecting their gametic investment. They use behavioral cues and humoral cues (i.e., sex peptide) provided by the male to assess male quality. Therefore, sex peptide use and female mate choice systems need not be mutually exclusive, reinforcing the notion that sex peptides are not simply "paternity proteins" existing only within male mate-control mating systems.

In monogamous females, once sexual receptivity is permanently inhibited either by sex peptide or other means, the need for a male-derived, oviposition-inducing chemical factor is moot, provided ovipositional activation can occur otherwise. Paternity is guaranteed by rendering females unreceptive to other males and it is essentially unimportant to a male's genetic success when a female actually oviposits as long as she does so under appropriate conditions. In fact it may be evolutionarily advantageous for the female to exert control over the timing of oviposition since she, rather than the male, is more likely to assess the quality of the oviposition site. Perhaps *Musca domestica*, which is mostly monogamous (Riemann et al. 1967), fits this paradigm, placing it into the second category of sex peptide communication (see page 123). As discussed in Chapter 2, I did not disprove the existence of a house fly oviposition factor but, using techniques shown to be successful with other Diptera, was unable to document one. It may be that selection pressure has not favored evolution of an oviposition factor in the house fly mating system when mating receptivity already is regulated.

Insects in which any form of sex peptide signalling might be considered a disadvantage, particularly to females, fall into the third category. *Anastrepha suspensa* and other Tephritidae have evolved lek mating systems in which males congregate to attract and court females, affording the female an opportunity to select a mate. By definition (Thornhill & Alcock 1983), mate and resource monopolization potential is low in lek mating systems because females are widely dispersed so males cannot realistically defend either females or resources without significant investment. Lekking systems are characterized by long-lived insects with prolonged breeding seasons and high mobility, making male territoriality the default strategy for males. Typically, tephritid females mate several times during their adult life (Christenson & Foote 1960), suggesting polyandry has greater benefits than monogamy or those mechanisms such as sex peptides that might induce monogamy.

Clearly, if this is the case, there are advantages to being polyandrous even if it is the exception rather than the rule among insects. It may be that genetic variation in a population is maintained by multiple matings during multiple cycles of oviposition. If variation is selected because it confers a fitness advantage, natural selection might not favor mechanisms like sex peptide that could decrease the frequency of multiple mating and lower genetic variation in a population. Whether sex peptide is primitive or derived is unknown. Since it occurs in the Nematocera, perhaps sex peptide signalling is the primitive condition for Diptera but lekking, polyandrous species like Tephritidae have lost it as they have evolved.

Thornhill and Alcock (1983) cite other benefits according to the type of polyandry (described in their Table 14.4): *sperm replenishment* — females add to inadequate supplies; *material benefit* — males provide nutrients or protection from unwanted mating attempts (mateguarding is a material benefit); *genetic benefit* — females acquire superior sperm; *convenience* — energy is not expended repelling unwanted suitors. *Ceratitis capitata* (the Mediterranean fruit fly, a Tephritidae) females remate as sperm are depleted from the spermathecae, suggesting receptivity is affected by sperm volume (Cunningham et al. 1971; Nakagawa et al. 1971). Thornhill and Alcock (1983) classify this mating as sperm replenishment polyandry and I would place *A. suspensa* in this category as well.

What is the role of sex peptide? Does it signal matedness (inhibit receptivity), guarantee paternity (elicit oviposition), provide a means for assessing male quality, or some combination of all three? I think it differs according to the type of mating system. Sex peptide signalling viewed from the male perspective is classified within sexual selection theory as a form of postcopulatory intrasexual selection (Thornhill & Alcock 1983). In other words, the message conveyed by sex peptide is read by the female during or following coupling. In *D. antiqua*, 100% of

virgin females oviposit when injected with greater than $^{1}/_{20}$ of a male equivalent of male reproductive tract extract (Spencer et al. 1994). Concentrations less than this elicit a correspondingly lower ovipositional response. Classified this way, sex peptide signalling can be viewed as being part of a female mate choice system because females accept or reject the signal according to its strength. Sex peptide can signal matedness but guarantee paternity only if present in sufficient quantity. By this argument, sex peptides have not simply evolved as paternity proteins nor are they found exclusively within male mate-control systems. However, if present in male mate-control systems, sex peptide probably functions to guarantee paternity either in concert with or independent of other physiological or behavioral mechanisms operating to assure male mating success.

What favors evolution of sex peptide signalling? Perhaps natural selection acting on monogamous mating systems fosters the evolution of sex peptides because sex peptides are efficient signallers of matedness and reduce the risks inherent in time-consuming mating strategies. Clearly, my evidence combined with that from the literature suggests that some monogamous species have a sex peptide signalling system (*A. aegypti*) while others do not (*M. domestica, L. dispar*), just as some polyandrous species express sex peptides (*D. melanogaster*) while others do not (*A. suspensa*). Certainly, sex peptides need not always induce permanent monogamy but simply can function to ensure paternity for the current round of fertilization in species with multiple cycles of oogenesis. Such a mating system would not require sex peptides and polyandry to be mutually exclusive. Such is the case with *D. melanogaster*, which mate two to three times over their adult life (Pyle & Gromko 1978). Yet sex peptides exist in monogamous species. Most culicids are considered primarily monogamous (Thornhill & Alcock, Table 14.1, 1983) and there is ample evidence across this family for sex peptides (Craig 1967). If monogamy reduces

variation in a population, there must be something conferring a greater benefit to individuals than the potential loss from reduced diversity. Thornhill and Alcock (1983) suggest the costs of multiple mating relative to monogamy are: (1) the time allocated to courtship and copulation, and (2) the time required to repel unwanted suitors. The risk due to increased predation is thought to be a minor component, occurring only in cryptically colored insects (Thornhill & Alcock 1983). In short-lived insects, time is a precious resource not to be squandered on acquisition of unnecessary gametes, especially if spermathecae are capable of holding large numbers of sperm (Davey 1985).

Do sex peptides play a primary role in speciation? Sex peptides do not appear to play a dominant role in the evolution of geographic and habitat races. Since sympatric speciation of animals involves the development of intrinsic barriers to gene flow (Diehl & Bush 1989), sex peptides would have to exhibit high species specificity to be considered as a driving force for speciation, but the data show they are not. Also, in host-race formation, premating reproductive isolating mechanisms evolve early in sympatric speciation (Bush 1975; Diehl & Bush 1989; Mettler et al. 1988). However, I would classify sex peptides as postmating, prezygotic isolation mechanisms.

Other evidence arguing against a primary role for sex peptides in speciation draws from mosquito genetics studies. Comparison of populations of *A. aegypti* mosquitoes from a sylvan habitat and a domestic habitat in East Africa showed significant differences in allelic frequency at several polymorphic loci (Tabachnick et al. 1979). Further, the two populations exhibited heterozygosity at different loci and there were significant differences in genetic distances between forms. The authors concluded that gene flow was restricted and the populations could be considered sympatric because each form readily travelled to the other's habitat;

outdoor forms were occasionally found inside and vice versa. Following the classification of Bush (1975), Tabachnick et al. (1979) suggested that positive assortative mating may exist due to differences in host preferences: The domestic form feeds on humans and the sylvan form feeds on nonhuman animals. The authors also stated that this conforms to the Maynard-Smith speciation model in which initial genetic differentiation may be due to different habitat preferences. Although no mechanism was discussed, I assume that differences in host preference likely arose due to mutations at a single or a few alleles (Bush 1975). Additional studies by Tabachnick and Powell (1979) and Wallis et al. (1983) demonstrated that the worldwide variation among *A. aegypti* was the same as shown for Kenya and that there were seven distinct gene pools. They found a strong correlation of allozyme variation among populations with similar behavioral, morphological and ecological differentiation; that is, allozyme studies showed that East African domestic forms were more closely related to New World domestic forms than to their siblings in the forest less than 1 kilometer away.

Since trait differentiation is more crucial in sympatry than in allopatry (Otte 1989), I would expect to find a preponderance of differentiated sex peptides in sympatric populations compared to allopatric populations. Yet given the lack of premating or postmating isolation mechanisms in the sylvan and domestic forms of *A. aegypti* described above (Tabachnick et al. 1979), it appears that competition for host resources in sympatric populations is under significantly stronger selection pressure than mate signalling. Certainly there is more evidence to support the change in host preference as a primary speciating event. Further, because these populations of *A. aegypti* can still interbreed, although they do not (Tabachnick et al. 1979), it is clear that sex peptide differentiation has not preceded a niche shift. Therefore, I think that differential host recognition has provided a sufficient isolating barrier for speciation to occur and there is insufficient selection pressure to evolve a

novel or species-specific signalling system such as sex peptide. According to Bush (1981) reproductive isolation may arise as a byproduct of adaptation or develop as an integral part of the adaptive process. In sympatry, perhaps sex peptide specificity would be useful and necessary only as the niche space becomes full and other habitat-specific isolating mechanisms are exhausted.

With respect to *A. aegypti* and *A. albopictus*, they exist sympatrically or parapatrically throughout tropical and subtropical Asia (Hawley 1988). *A. aegypti* typically occupy domestic habitats and *A. albopictus* occupy the surrounding rural areas, not unlike the situation described for sympatric forms of *A. aegypti* in Kenya (Tabachnick & Powell 1979). In both cases, host and habitat specificity seem to be well established yet sex peptide specificity is only partially fixed based on my findings in the laboratory using colonized forms of *A. aegypti* and *A. albopictus*.

Similarly, there is little support for differentiation of sex peptides in allopatry. Given their island distribution, it appears that many *Aedes* groups have evolved allopatrically (Rai et al. 1982). Yet *A. albopictus* from Japan, Hawaii, Indonesia, India, Malaysia and the United States are bidirectionally compatible; they readily mate and produce viable progeny (reviewed by Hawley 1988) and appear to constitute a single species (McLain & Rai 1986). Powell (1978; 1982) states that reproductive isolation results as a byproduct of founder-flush-crash cycles and that mate recognition systems may be the first isolating mechanism to evolve as a result of the founder event. Given the allopatric distribution of *A. albopictus* on island populations, it is conceivable that they were founded by small numbers of individuals and that random genetic drift could have led to evolution of a mate recognition system such as sex peptide. But, in founder-flush populations, heterozygosity is decreased substantially and if sex peptides were to evolve under these conditions (i.e., during the flush), they probably would be highly specific and not show cross-specificity with other island populations. Yet strains of *A. albopictus*

from several geographic regions freely interbreed just as strains of *A. aegypti* from similar habitats around the world show allozyme similarities. I would state, therefore, that sex peptides have not evolved in allopatry but were present prior to establishment of island populations. This also suggests they may be a primitive rather than a derived trait. Further, I speculate that either sufficient time has not elapsed to result in differentiation of sex peptide or that sex peptide has been around a long time but there is insufficient selection pressure to produce significant differentiation. This would explain why so much heterospecificity of paragonial gland extracts has been observed in laboratory studies.

If *A. aegypti* and *A. albopictus* are not derived from a common ancestral stock, as discussed earlier, then how would convergent evolution explain the development of a heterospecific signalling system? Perhaps there is another way to examine the question. If sex peptide simply functions as a signal to the female that insemination has occurred, then sex peptides might occur universally across insects, similar to the sesquiterpenoid juvenile hormones or the ecdysteroid hormones. In this case, sex peptide probably would be highly conserved. Male-produced oviposition and receptivity factors have been found in several orders of insects (reviewed in Gillott 1988), but until the structures are known for several species, such hypotheses cannot be critically examined.

Practical applications. Knowledge of the biology and chemistry of sex peptides leads naturally to developing it for biorational insect control. If sex peptides are found to have reasonably high species specificity, it would afford the ability to selectively reduce the fertility of target populations by minimizing mating or inducing precopulatory egg dumping, an especially attractive strategy for species with monogamous females or single vitellogenic cycles. Control systems might include (1) feeding sex peptide in a microencapsulated form, (2) delivering sex

peptide through recombinant viruses such as Baculoviruses, or (3) capitalizing on the potential for closely related species to interbreed asymmetrically and replacing a pest species with a nonpest species (Miller et al. 1994).

Future inquiry. Several questions I wrote into my lab notebook on October 3, 1990, less than three weeks after starting my Ph. D. program, are still relevant now and suggest investigations yet to come. I have paraphrased them here:

(1) Are sex peptides a universal characteristic of insects?

(2) Does the degree of specificity of sex peptides change across orders?

(3) What adaptive significance is represented by sex peptides?

(4) What is the similarity of sex peptide primary structure across orders?

(5) What is the time delay between transmission and acknowledgment of sex peptide?

(6) At what age is sex peptide produced by the male?

(7) What is the target site of sex peptide in the female?

(8) Are there circumstances under which the effects of sex peptide are preempted or superseded?

A random assortment of specific questions have occurred to me since: Is the male oviposition factor located on an autosome or a sex chromosome? If it is autosomal, is expression controlled by regulatory genes with sex-specific differences in RNA splicing (similar to *D. melanogaster* sex differentiation) that lead to translation of a functional oviposition factor in males and a nonfunctional factor in females? Alternatively, could the male oviposition factor actually be a *trans* factor that binds with an enhancer in females to promote transcription of an endogenous, egg-releasing factor RNA? If so, does male oviposition factor resemble a DNA binding protein?

The study of oviposition factors is really the study of a male activator and a female receptor complex. Although research on the female receptor was not addressed in these studies, if both the activator and the female receptor can be identified for a single species, the physiology of the oviposition induction sequence can be more fully understood. In addition, if the structure of the receptor can be characterized, similarities and differences between the alleles coding for male factor and female receptor can be identified, which would provide the basis for understanding evolution of the activator-receptor complex. Such investigation would enhance our knowledge of insect reproductive biology and help elucidate evolutionary and phylogenetic relationships among taxa.

Appendices

APPENDIX 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 1994-1

Title of thesis or dissertation (or other research projects):

An analysis of the presence and role of humorally mediated, male-derived oviposition-induction factors in insect mating systems

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name (s) (typed)
Anthony J. Lentz

Date 16 May 1994

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24:141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.
Copies: Included as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Musca domestica Linnaeus	Carolina Biological, Burlington NC 4 March 1994					10	10		MSU
Aedes aegypti (L.)	UGAL Strain, U. Georgia, Athens, GA 6 April 1994					10	10		MSU
Aedes albopictus (Skuse)	New Orleans strain, U Notre Dame, South Bend, IN 6 April 1994					10	10		MSU
Lymantria dispar (L.)	USDA-APHIS, Otis ANGB, MA 31 March 1994					3	3		MSU
Anastrepha suspensa (Loew)	USDA-ARS, Gainesville, FL 13 April 1994					5	5		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Anthony J. Lentz

Date 16 May 1994

Voucher No. 1994-1

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator 15 June 1994 Date

Appendix 2

Mosquito-rearing procedure

(1) Set up ~200 eggs per 500 ml degassed water (yields over 150 larvae). For *A. albopictus* only, moisten egg paper several hours before adding water.

(2) Add 1 ml of mosquito larval food to each dish.

(3) Label each dish with the following information and note the date that each life stage appears.

	Brood:
Species	Hatch:
(set-up date)	Pupae:
	Adult:

(4) Continue larval feeding schedule as directed in record-keeping sheet (next page).

(5) Remove and discard egg paper on second day.

(6) On second day after pupae appear, transfer them into an adult emergence cage.

(7) As adults begin to emerge, sex them into virgin cages every 24 hours, allowing no more than ~75 adults per 5 inch high x 5 inch diameter plastic cage. Provision each cage with 10% sucrose-soaked cotton in a 1 oz. cup. Indicate dates of emergence on each cage - use new cages every 2 days or less so adults are close in age. Discard remaining pupae after about 3 days.

Aedes aegypti & *A. albopictus* Larval Diet

Larval Food Solution (store in the refrigerator for 2-3 weeks)

ground rat chow (40 mesh sieve) . . . . . 3.3 g
fresh baker's yeast . . . . . 3.3 g
lactalbumin . . . . . 3.3 g

Mix dry ingredients together, then add with stirring:

dd•H_2O . . . . . 200 ml

Larval Feeding Schedule & Record

Write in start date (egg paper is placed in water) and each date of feeding.

Day	Amt.	Date egg paper placed in water					
1	1 ml						
2	2 ml						
3	5 ml						
4	9 ml						
5	10 ml						

Appendix 3

SDS•PAGE formulations and staining procedure

Sample (Reducing) Buffer

dd•H_2O	360 µl
0.5 M Tris•Cl, pH 6.8	100 µl
Glycerol	120 µl
10% SDS	160 µl
ß-mercaptoethanol	40 µl
0.1% bromphenol blue	20 µl
	800 µl

Store sample buffer @ room temperature. To prepare protein for electrophoresis, dilute 1 part protein with 3 parts sample buffer and heat at 60°C for 15 min.

1.5 M Tris•Cl pH 8.8

Tris base $_{MW\ 121.1}$	18.15 g
dd•H_2O	60 ml

Bring to pH 8.8 with 1N HCl. Add dd•H_2O to 100 ml and store @ 4°C.

0.5 M Tris•Cl pH 6.8

Tris base $_{MW\ 121.1}$	6.0 g
1N HCl	45.0 ml

Bring to pH 6.8 with 1N HCl. Add dd•H_2O to 100 ml and store @ 4°C.

5X Electrode Buffer pH 8.3

Tris base $_{MW\ 121.1}$	15.0 g
Glycine	72.0 g
SDS	5.0 g
dd•H_2O	to 1000 ml

Store @ 4°C. Dilute 80 ml buffer with 320 ml dd•H_2O for one run.

Acrylamide/bis Solution (30%T, 2.6%C w/v)

Acrylamide	29.2 g
N'N'-bis-methylene-acrylamide	0.8 g
dd•H_2O	to 100 ml

Filter and store @ 4°C in the dark (30 days maximum).

SDS•PAGE Resolving Gel
0.375 M Tris pH 8.8

	7.5%	12%	15%
dd•H_2O	4.85 ml	3.35 ml	2.30 ml
1.5 M Tris•Cl, pH 8.8	2.50 ml	2.50 ml	2.50 ml
10% SDS	100 µl	100 µl	100 µl
Acrylamide/bis (30%)	2.50 ml	4.00 ml	5.00 ml
Mix above ingredients, degas for 15 min, then add:			
10% ammonium persulfate	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl

SDS•PAGE 4% Stacking Gel
0.125 M Tris pH 6.8

dd•H_2O	3.05 ml
0.5 M Tris•Cl, pH 6.8	1.25 ml
10% SDS	50 µl
Acrylamide/bis (30%)	650 µl
Mix above ingredients, degas for 15 min, then add:	
10% ammonium persulfate	25 µl
TEMED	5 µl

Silver Staining
(for one 0.75 mm minigel)

---

1. Fixing Step

Combine the following ingredients in a glass tray, add gel and fix for 20 min with gentle shaking.

MeOH	50.0 ml
Acetic acid	10.0 ml
dd•H_2O	35.0 ml
Fixative enhancer concentrate	5.0 ml

2. Rinse Step

Decant the fixing solution and rinse the gel 2 x 10 minutes with 100 ml dd•H_2O

3. Stain & Develop Step

While stirring, mix the following ingredients in order:

dd•H_2O	17.5 ml
Silver complex solution	2.5 ml
Reduction moderator solution	2.5 ml
Image development reagent	2.5 ml

then add, just prior to staining the gel:

5% development accelerator reagent	25.0 ml

4. Stop Bath Step

When gel has developed to the desired intensity, place it in the stop bath solution for at least 10 minutes:

dd•H_2O	190.0 ml
Acetic acid	10.0 ml

Coomassie Blue R-250 Staining

1. Staining (0.5% Coomassie Blue)

Pour 50 ml of the following stain solution into a glass tray and stain the gel for 20 min with gentle shaking.

dd•H_2O	120 ml
2-propanol	60 ml
Acetic acid	20 ml
Coomassie blue R-250	1 g

2. Destaining

Line a glass tray with paper towelling and add 200 ml of:

dd•H_2O	785 ml
MeOH	165 ml
Acetic acid	50 ml

Destain with gentle shaking, replacing the destaining solution as needed until the gels have a clear background (~2 hr).

Appendix 4

Electroelution procedure

Electroelution is run in a 3°C room using CalTech electroelution cells, a custom-made buffer chamber, and a Fisher power supply.

1. Assemble electroelution cells with 3500 MWCO Spectrum cellulose ester dialysis membranes and fill both wells of each cell with *freshly prepared* 10 mM NH_4HCO_3 buffer halfway up to the transverse channel. Examine and correct for leaks as needed.

2. Following electrophoresis, disassemble the gel plate.

3. Place the gel, still on the plate, on top of a grid marked with horizontal lines spaced as wide apart as the desired slice width.

4. Slice the first fraction (A) away from the rest of the gel. Cut it into 3 mm wide strips of gel, then dice each strip into 3 x 3 mm squares, keeping them slightly moist with buffer.

5. Pick up the gel pieces with a spatula and place them into the large well of an electroelution cell. Fill the channels with buffer, avoiding air bubbles, and place the cell into the electroelution tank.

6. Repeat the process for each of the other fractions.

7. Remove air bubbles from the bottom of the dialysis membranes of all cells.

8. Start the peristaltic pump @ a flow rate of 3 ml/min, then connect the electrodes and turn on the power supply to 150 V constant voltage, yielding ~ 6 mA for four cells.

9. Electroelute for the desired number of hours, then remove the sample from the small well on the anode side, comprising a volume of ~ 150 µl. Note the ending current.

10. Concentrate the sample in a SpeedVac @ room temperature to a volume yielding 10♂/µl or the desired concentration. Store in the freezer.

Appendix 5

Anion exchange HPLC procedure

1. Sparge buffers and dd•H_2O with Helium at 100 ml/min for 20 min. Continue sparging but decrease rate to 20 ml/min for remainder of protocol.

2. Wet the media by flushing the column with 50 volumes of 100% MeOH @ the maximum flow rate for the column:

   (column volume x 50)/(flow rate) = flushing time

   (_____ µl x 50)/(_____ µl/min) = _____ min

3. Remove the MeOH by flushing the column with 50 volumes of dd•H_2O @ the maximum flow rate for the column.

4. Flush the column with buffer B for 20 min @ the flow rate to be used in the chromatography.

5. Equilibrate the column with buffer A for 20 min @ the flow rate to be used in the chromatography.

6. Make a blank run using a linear gradient of 0 to 100% buffer B over 30 min, hold at 100% buffer B for 5 min to allow complete flushing of column at maximum gradient, return to 0% buffer B over 10 min, and hold at 0% buffer B for 15 min.

   Presence of peaks greater than 5% full scale indicates contaminants in the column or the buffers. Repeat the blank run until a satisfactory baseline is obtained.

7. While pumping 100% buffer A through the column @ the chromatography flow rate,
   - switch the injection loop to LOAD
   - load the protein sample into the loop
   - switch the injection loop to INJECT and start the chromatogram
   - begin collecting fractions immediately into 1.5 ml polypropylene vials

8. Hold the gradient @ 100% buffer A for 6 min to allow the proteins to adsorb to the column and flush out any nonadsorbing proteins, **switch the injection loop to LOAD**, then
   - run a gradient of 0% to 100% buffer B over 30 min
   - hold at 100% buffer B for 5 min ... then stop collecting fractions
   - return to 0% buffer B over 10 min
   - hold at 0% buffer B for 15 min

HPLC Specifications

Operator	Date
Delivery system Detector System controller	
Column type	
Column size	Injection loop size
Flow rate	Absorbance
Buffer A	
Buffer B	

Do not use a guard column!

Sample information	
Description	
Buffer	
Volume	
Concentration	
Collection strategy	
Peaks	Frequency: every __ min

Fraction Collection Record

Date:

Vial #	Time collected	Vial #	Time collected
1		16	
2		17	
3		18	
4		19	
5		20	
6		21	
7		22	
8		23	
9		24	
10		25	
11		26	
12		27	
13		28	
14		29	
15		30	

Notes:

Waters Model HPLC Operating Procedure

Notes

After wetting the column with methanol, pull dd•H_2O into the pump heads through line A using a 5cc syringe (works faster than pumping directly) to replace the methanol. Set *pump diverter* to

- vertical to pull in new liquid (e.g. dd•H_2O)
- diagonal to prime the pumps (if they haven't been used recently)
- horizontal to operate normally, pumping fluid through lines

When changing flow rate, *line diverter* (a metal thumb lever) is pointed to right so fluid exits immediately from tubing and does not go through column. After resetting flow, divert line back through column by pointing thumb lever to left.

Flow through the column should never exceed the maximum recommended rate.

Pull some buffer A into the syringe before loading protein, then pull protein in gently (to minimize mixing); this eliminates most of the material that otherwise would remain in the syringe barrel following injection.

Changing buffers (e.g. B > A) and flushing the column

1. ***Stop flow*** by setting flow rate to 0 ml/min.
2. Rinse line filters and move both sparge line and inlet line into buffer A bottle.
3. Divert flow away from the column directly to the exit by turning the *line diverter* (thumb lever) to the right.
4. Set controller to 100% A @ 5 ml/min and run until valve clicks. (Establishes pumping through line A instead of previous line.) Stop flow by entering 0 ml/min.
5. Set *pump diverter* to vertical and pull 3 x 5 cc syringe volumes of buffer A through line A to fill pumps with buffer A.
6. Set *pump diverter* to horizontal, start flow @ 5 ml/min and run until valve clicks.
7. Reset flow to 0.3 ml/min (or desired running flow rate) and run until valve clicks.
8. Divert flow back through the column by setting the *line diverter* (thumb lever) to the left.

Programming the system controller

- Set up the program under **PROG TABLE**. For example,

Time	Flow	Buffer	Gradient
INIT	0.3	100% A	6 (linear)
30	0.3	100% B	6
35	0.3	100% B	6
45	0.3	100% A	6
60	0.3	100% A	11 (stop)

- Save this method under **PROG METHOD** ensuring that the correct table number assigned in PROG TABLE is entered into the last column on the right.
- Activate the method by pressing **OPER METH** then pressing it again (to go to the new conditions).
- The **DIRECT** screen is used for all flushing operations.

Setting computer-generated chromatogram parameters

- Set AUFS to -0.1 to 0.25
- Set wavelength to 214, 230, 254, 280 nm.
- Set time to 45 min.
- Save all conditions.
- Enter into startup mode by pressing **(3) Measure**, which asks for file-saving information.
- Switch chromatogram **ON** by pressing (-).

Appendix 6

Discontinuous nondenaturing PAGE procedure & formulations

(for BioRad Mini-Protean II unit)

1. Make catalyst solution fresh and store on ice in dark

2. Mix buffer, monomer, water and catalyst for resolving gel. Degas 5 min in dark (wrap foil around flask).

3. Add TEMED and swirl, then pipet into glass sandwich. Overlayer with 50% MeOH.

4. ***Photopolymerize*** 30 min @ room temperature, ~ 3 inches from fluorescent light.

5. Make the stacking gel following the same degassing procedure as above.

6. Decant the buffer from the resolving gel. Add TEMED to stacking gel mixture, swirl and pipet onto resolving gel up to top of glass. Insert Teflon comb.

7. ***Photopolymerize*** for 30 min @ room temperature, ~ 3 inches from fluorescent light.

   Overlay with 25% resolving gel buffer following polymerization and refrigerate for 4 hours to eliminate free radicals that can contribute to protein degradation.

8. Assemble electrophoresis unit, add lower tank and upper tank buffers, and place in 3°C room to equilibrate for 20 min.

9. Add 10% v/v of 50% sucrose/0.1% bromphenol blue solution to protein extract, vortex and layer onto stacking gel.

10. Electrophorese @ 200 V.

Catalyst

dd•H_2O	9.0 ml
0.02% riboflavin phosphate	1.0 ml
Ammonium persulfate	6.0 mg

NATIVE•PAGE Separating Gel

	5%	10%	15%
dd•H_2O	5.00 ml	3.75 ml	2.50 ml
Separating gel monomer	1.25 ml	2.50 ml	3.75 ml
4X separating gel buffer	2.50 ml	2.50 ml	2.50 ml
Catalyst	1.25 ml	1.25 ml	1.25 ml
Mix above ingredients, wrap flask in foil, degas for 5 min, then add:			
TEMED	20 µl	20 µl	20 µl

NATIVE•PAGE 3.125% Stacking Gel

Stacking gel monomer	5.0 ml
4X stacking gel buffer	2.5 ml
Catalyst	2.5 ml

Mix above ingredients, wrap flask in foil, degas for 5 min, then add:

TEMED	10 µl

Coomassie Blue G-250 Staining

1. Fixing

Combine the following ingredients in a glass tray, add gel and fix for 60 min with gentle shaking.

Trichloroacetic acid	6.25 g
dd•H_2O	to 50 ml

2. Staining (0.25% Coomassie Blue)

While stirring, mix the following ingredients in order:

dd•H_2O	50 ml
Coomassie blue G-250	125 mg

Add 2.0 ml of above solution to fixing solution and gel; stain for 2 hours.

3. Destaining

Decant the staining solution and add 200 ml of:

dd•H_2O	880 ml
Acetic acid	70 ml
MeOH	50 ml

Replace the destaining solution as needed until the gels have a clear background.

Upper Tank Buffer

37.6 mM Tris, 40 mM glycine pH 8.89

Tris base $_{MW\ 121.1}$	2.28 g
Glycine	1.50 g
dd•H_2O	to 500 ml

Lower Tank Buffer

63 mM Tris, 0.5N HCl pH 7.47

Tris base $_{MW\ 121.1}$	7.63 g
1N HCl	50 ml
dd•H_2O	to 1000 ml

Sample Buffer

50% sucrose, 0.1% bromphenol blue

Sucrose	500 mg
1% bromphenol blue	100 µl
dd•H_2O	to 1.0 ml

Combine 1 volume of protein sample with 0.1 volume of sample buffer and vortex.

Separating Gel Monomer
(40%T, 5%C w/v)

Acrylamide	38.0 g
Bis	2.0 g
dd•H_2O	to 100 ml

Stacking Gel Monomer
(6.25%T, 20%C w/v)

Acrylamide	5.0 g
Bis	1.25 g
dd•H_2O	to 100 ml

4X Separating Gel Buffer
947 mM Tris, 0.289N HCl pH 8.48

Tris base	11.47 g
1N HCl	28.92 ml
dd•H_2O	to 100 ml

4X Stacking Gel Buffer
158 mM Tris, 0.256N H_3PO_4 pH 6.90

Tris base	1.91 g
1N phosphoric acid	25.6 ml
dd•H_2O	to 100 ml

Appendix 7

Gradient nondenaturing PAGE procedure & formulations

Catalyst for gradient gel:

dd•H_2O	5.88 ml
0.02% riboflavin phosphate	2.00 ml
10% ammonium persulfate	120 µl

1% gel (for top of gradient mixer)

dd•H_2O	6.75 ml
1.5 M Tris•Cl	2.50 ml
acrylamide monomer	250 µl
glycerol	None
catalyst	500 µl
TEMED	20 µl

15% gel (for bottom of gradient mixer)

dd•H_2O	2.55 ml
1.5 M Tris•Cl	2.50 ml
acrylamide monomer	3.75 ml
glycerol	700 µl
catalyst	500 µl
TEMED	20 µl

20% gel (for bottom of gradient mixer)

dd•H_2O	1.30 ml
1.5 M Tris•Cl	2.50 ml
acrylamide monomer	5.00 ml
glycerol	700 µl
catalyst	500 µl
TEMED	20 µl

25% gel (for bottom of gradient mixer)

dd•H_2O	None ml
1.5 M Tris•Cl	2.50 ml
acrylamide monomer	6.50 ml
glycerol	700 µl
catalyst	500 µl
TEMED	20 µl

Gradient Nondenaturing PAGE

BioRad Mini-Protean II electrophoresis unit
1 mm spacers and single well comb with reference lane
Custom gradient mixer (Keller design)
Fisher Biotech power supply

1. Assemble a gel electrophoresis sandwich using clean glass plates and 1 mm spacers. Mark the position on the outside of the glass plates indicating the bottom of the comb.

2. If necessary, prepare catalyst solution; store in the refrigerator.

3. Mix water, buffer, monomer and catalyst for 1% gel. Degas 5 min in dark (wrap foil around flask). Repeat the process for the $_{higher}$% gel, but including the glycerol.

4. Place a 1" stir bar in the bottom tube of the gradient mixer, secure it to a ring stand and set it on a stir plate. Place the electrophoresis sandwich below the mixer with the outlet tube of the mixer hanging over the middle of the glass plate assembly.

5. Turn off the laboratory lights, add TEMED to the $_{higher}$% gel ingredients, swirl and pour ___ ***ml*** into bottom of gradient mixer. *Immediately* begin stirring. Add TEMED to the 1% gel ingredients, swirl and pour ***6 ml*** in the top of the gradient mixer.

6. Immediately open up the stopcock of the outlet tube to allow the mixing gel to flow into the glass sandwich. Fill to a point **2 mm** above the where the bottom edge of the comb will be.

7. Place the comb on top of the gel carefully by lowering it in between the glass plates at an angle to prevent trapping air bubbles.

8. *Photopolymerize* **30** min @ room temperature, ~ 3 inches from fluorescent light.

9. Assemble the electrophoresis unit, add lower tank and upper tank buffers, and place the unit in a 3°C room to equilibrate for ___ **hr**.

10. Add 10% v/v of [50% sucrose, 0.1% bromphenol blue solution] to the protein extract, vortex slightly, and layer it onto the top of the gradient gel.

11. Electrophorese @ **50 V for** ___ **hrs**, then @ **200 V for** ___ **hrs** (=___ hrs total).

Gradient Nondenaturing PAGE Profiles

To create a gradient from:	4-15%	5-20%	6-25%
High acrylamide concentration is	15%	20%	25%
& volume in bottom tube is	3 ml	3 ml	3 ml
Low acrylamide concentration is	1%	1%	1%
& volume in top tube is	6 ml	6 ml	6 ml
Thickness of gel is	1 mm	1 mm	1 mm

To create a gradient from:	4-15%	10-25%	10-25%
High acrylamide concentration is	15%	20%	25%
& volume in bottom tube is	3 ml	3.75 ml	5.0 ml
Low acrylamide concentration is	1%	1%	1%
& volume in top tube is	6 ml	6 ml	6 ml
Thickness of gel is	1 mm	0.75 mm	1 mm

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