This is to certify that the

dissertation entitled

Onion Fly Reproductive Behaviors
as Influenced by
Mating and Paragonial Gland Extracts

presented by

Joseph Lee Spencer

has been accepted towards fulfillment
of the requirements for

the Ph.D. degree in Entomology

James R. Miller
Major professor

Date 11-11-94

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ONION FLY REPRODUCTIVE BEHAVIORS AS INFLUENCED BY MATING AND PARAGONIAL GLAND EXTRACTS

By

Joseph Lee Spencer

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Entomology

1994

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ABSTRACT

ONION FLY REPRODUCTIVE BEHAVIORS AS INFLUENCED BY MATING AND PARAGONIAL GLAND EXTRACTS

By

Joseph Lee Spencer

Onion fly, *Delia antiqua* (Meigen) (Diptera: Anthomyiidae) males produce sex peptide in their paragonial (accessory) sex glands. When delivered via the ejaculate to virgin females, paragonial secretions caused permanent mating inhibition and oviposition at *ca.* 14 eggs/female/d. Dosages of aqueous paragonial extract at ≥1/20th male equiv/female caused mated behavior, and those <1/20th male equiv/female stimulated oviposition but did not always preclude mating.

Significant oviposition (8.0 ± 2.3 eggs/virgin female/d) occured in virgin females beginning at 24.7 ± 1.5 d (mean ± SE); mated female oviposition begins at 6.4 ± 0.2 d. Ovipositional lifespans of mated (34.4 ± 1.8 d) and virgin females (30.9 ± 3.6 d) were not significantly different (P =0.30). Significantly longer overall lifespans for virgins (59.0 vs 47.2 d) could be partially explained by delayed ovipositional onset. Lifetime cumulative ovipositional patterns, if corrected for differences in rate and age at oviposition, were similar for mated and virgin females. Duration of egg depositional behaviors were not different between mated and virgin females.

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Delia platura (seedcorn fly) and *D. antiqua* sex peptides were symmetrically cross-active. *Delia radicum* (cabbage fly) sex peptide was fully active in *D. platura,* but not vice versa. *D. antiqua* sex peptide stimulated mating inhibition and partial ovipositional activation in *D. radicum ; D. radicum* peptide was fully active in *D. antiqua.* Sex peptide cross-activity patterns were consistent with published morphologically-based phylogenies and genetic distance determinations. Though not thought to be initiators of reproductive isolation, sex peptide cross-activity asymmetries may intensify the consequences of occasional reproductive interactions between related sympatric species.

D. antiqua sex peptide is a highly polar proteinaceous material. Biological activity is lost if exposed to: lipophillic solvents, temperatures > 50°F, or pH <3.9. Biologically active material was recovered from microcentrifuge sizing filters and electroeluted fractions of native-PAGE gels. No activity was recovered using C4, C8, or C18 RP-HPLC, gel filtration or anion exhange chromatography. Conventional protein separation techniques were not successful in purifying onion fly sex peptide.

To my par

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DEDICATION

To my parents, grandparents and long-suffering siblings; without your loving support and enthusiastic approval this work would not have been possible.

I dedicate this dissertation to my grandmother, the late Mildred Engemann, whose unconditonal love, strength of will and unshakable faith in better tomorrows inspires me to push ever onward.

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ACKNOWLEDGMENTS

In recognition of invaluable help, inspiration, advice, and support during my work and the preparation of this dissertation, I thank my major professor James R. Miller, an excellent mentor and friend. I also thank the members of my guidance committee: Guy L. Bush, Fred C. Dyer, Joseph L. Leykam, and J. Mark Scriber, as well as my "ad hoc" committee members: Bob Lederhouse and Ned Walker, for their comments, suggestions and advice.I extend my gratitude to Jim Keller, a good friend and technician of extraordinary skill and resourcefulness; without your assistance this dissertation would not have been possible nor would my tenure in the Miller lab have been nearly so much fun. I also extend my thanks to my lab mates over the last few years Marco Candolfi, Tony Lentz, and Adam Peters; it has been a pleasure to do science with you. Many thanks are also due to Trista Mowry, Holly McCartney, Kurtis Markely, and all the other student workers who faithfully collected fly pupae from pans of rotted onions so I could do experiments. I acknowledge Jim Miller's aid and significant contribution in preparing the historical timeline in the general introduction. Portions of the Research in this disseration was supported by a Pesticide Research Center Enhancement Grants and a USDA Competitive Grant # 90-37153-5437 to J.R.M. and J.F. Leykam, a grant from the Rackham Foundation, and a Swiss NSF Grant to M.P. Candolfi.

I acknowledge my bugs, thanks for just being.

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KEY TO ABBREVIATIONS

AcN	Acetonitrile
ANOVA	Analysis of Variance
BR_{50}; BR_{100}	Behavioral Response 50; 100
BT_{50}; BT_{100}	Behavioral Threshold 50; 100
buf	buffer
d	day
diam	diameter
Drm-SP-I	*Drosophila melanogaster* Sex Peptide
Drs-SP-I	*Drosophila sechellia* Sex Peptide
Drsuz-Sp-I	*Drosophila suzukii* Sex Peptide
ELH	Egglaying Hormone
equiv	equivalent(s)
Fmoc	9-fluorenylmethyloxycarbonyl
g	gram
h	hour
HPLC	High performance liquid chromatography
HPHIC	Hydrophobic interaction chromatography
JH	Juvenile Hormone
k	kilodalton
MEOH	Methanol
min	minute
MWCO	Molecular Weight Cut Off
n	number
OSS	Oviposition-stimulating substance
PAGE	Polyacrylamide Gel Electrophoresis
PBAN	Pheromone biosynthesis activating neuropeptide
pmol	picomole
PO_4	Phosphate
Pr_2S_2	n-Dipropyl Disulfide
RH	Relative Humidity
RIS	Receptivity inhibiting substance
RTF	Receptivity terminating factor
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SE	Standard Error
SP	Sex Peptide
SV	Speed Vac
TFA	Trifluoroacetic Acid
xg	times the force of gravity
µl	microliter

"I have come to suspect that this long descent down the ladder of life, beautiful and instructive as it may be, will not lead us to the final secret...it is only that somewhere among these seeds and beetle shells and abandoned grasshopper legs I find something that is not accounted for very clearly in the dissections to the ultimate virus or crystal or protein particle."

Loren Eiseley, Biologist
From The Immense Journey

GENERAL INTRODUCTION AND OVERVIEW

This thesis, emphasizing onion fly, *Delia antiqua* (Meigen), oviposition as affected by mating, is part of a model-system study of factors and mechanisms governing insect colonization of host plants. Before offering an historical synopsis of major developments in this field of insect-plant behavioral interactions, the basic biology and behavior of *D. antiqua* are presented to facilitate interpretation of onion fly results in a larger context.

Onion Fly Biology and Behavior. *D. antiqua* is a muscid fly in the family Anthomyiidae. Adults are similar in size and appearance to the common house fly. As do a number of its congeners, *D. antiqua* females probe and lay eggs in soil cracks and cervices around the base of host plants, where the larvae feed on below-ground plant tissues. As the common name suggests, onion fly specializes on the onion; *Allium cepa*, and a few closely related species like shallot (*A. ascolonicum*), leek (*A. porrum*), and chive (*A. schoenoprasam*) (Loosjes, 1976). All highly suitable hosts of *D. antiqua* produce mainly propyl sulfides like n-dipropyl disulfide (Pr_2S_2) and propanethiol (PrS_2H) rather than allyl sulfides (Whitaker, 1976), and have a similar onion-like anatomy (Loosjes, 1976). In the temperate regions of the northern hemisphere, *D. antiqua* is a pest of commercially grown onions and

those in backyard gardens, and in many locations is thought to have no sustainable non-domesticated hosts (Loosjes, 1976).

In Michigan, adults of the first of 3 yearly generations emerge around the time of peak dandelion bloom from puparia having over-wintered in the soil. Given current commercial production methods where crops are infrequently rotated, encounters between emergin adult insects and host plants are likely (Miller and Cowles, 1990). Adult flies are thought to feed on pollen as well as to scavenge organic residues in and around the onion fields. An exogenous source of protein is necessary for the maturation of eggs (Niemczyk, 1964), which may take 7-15 days under field conditions. Each of a female's two ovaries contains *ca.* 25 polytrophic ovarioles, that can produce a flush of mature eggs every 2-3 days at room temperature (Weston *et al.*, 1992). On the complete diet of Ticheler (1971), mated females reared in the laboratory can produce several hundred eggs over a lifespan ranging up to eight weeks; in the field, lifetime fecundity is thought to be about 50-100 eggs over a three to four week lifespan (Loosjes, 1976).

Males mature sexually within several days of emergence and for several-hour periods in the afternoon perch atop onion foliage and defend *ca.* 2 m^2 diam territories in onion fields (J.R. Miller, personal observation). The polygynous males fly out from their perches to intercept flying fly-sized objects. A male and a prospective mate usually fall to the ground (or floor of their cage in the lab) after contact. Unreceptive females reject males by buzzing their wings and flying away; receptive females remain quiet and permit copulation which takes only 2-4 min. Because females are monocoitic (Martin and McEwen, 1982) and copulation is so brief, matings are rarely observed even in laboratory cultures (Loosjes, 1976). Following mating, the

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female becomes unreceptive to subsequent mating attempts and will begin to engage in host-oriented behaviors before oviposition.

In the field, the larvae of first generation adults feed on seedling onions and may destroy 10 or more seedlings during their development (Workman, 1958). Later generations feed on the more mature bulbs which can support numerous larvae. First and second-generation larvae pupate in the soil and emerge as adults *ca.* 2 weeks later. Following onion harvest in September, mature 3rd generation larvae having fed on the leftover unharvested onions pupate and overwinter in the upper layers of the soil. Current controls for onion maggot infestation includes row application of Lorsban™ (fonofos) during seeding and field sanitation which includes burying piles of highly stimulatory and suitable culls (unmarketably small or sprouting onions) (Cowles and Miller, 1992) and plowing under field waste.

Historical Synopsis of Major Developments in Insect-Plant Behavioral Interactions. Due to their successful competition for human crops, the value of their critical pollination services, and because they are a predominant life form on earth, insect herbivores have long been considered important objects of research. Mechanisms of insect host-finding, examining, and consuming have been studied since at least 1888, and interest in the subject continues (Table 1).

From the onset, thinking about host-plant acceptance by insects focused on distinctive plant chemistry as the critical mediator of insect-plant interactions (Table 1: Stahl, 1888; Verschaffelt, 1910). The early suggestion by Brues (1920) that insects might exploit multiple plant attributes during their interactions was soon supplanted by a chemocentric view following the discovery of gustatory receptors specific to salts, sugars, and acids (Dethier,

Table 1. Temporal comparison of developments in conceptualization of factors and mechanisms governing host-plant acceptance by onion fly relative to insects generally

Table 1. Temporal comparison of developments in conceptualization of factors and mechanisms governing host-plant acceptance by onion fly relative to insects generally

	Models/Concepts Developed for:		
Author; Date	Herbivorous Insects Generally	Onion fly	Author; Date
Stahl; 1888	Suggested that botanical compounds protect many plants against enemies, including insects.		
		First published record on onion fly as a pest in the new world.	Swaine; 1909
Verschaffelt; 1910	Single and distinctive plant secondary compounds can stimulate insect feeding.		
		Documented onion fly as a significant agricultural pest and outline its basic biology, including information on host range.	Severin and Severin;1915 Smith; 1922 Armstrong; 1924 Gray; 1924 Hammond; 1924
Brues; 1920	Insects identify suitable plants via odors, tastes, and other attributes; "Botanical Instinct" governs host-plant range.		
von Frisch; 1921	Definitive identification of the antennal receptors responsible for detection of plant odors by honey bees.		

Table 1. continued

Table 1. continued

Author; Date	Models/Concepts Developed for: Herbivorous Insects Generally	Onion fly	Author; Date
		Cull onions first tested as a trap crop for onion fly.	Dudley; 1925
Ilse; 1937	Butterflies foraging for ovipositional sites respond to green cards as surrogate leaves.		
Dethier; 1937 Dethier;1939	Caterpillar mouthparts possess gustatory receptors sensitive to sugars, salts, and acids.		
Dethier, 1941	The cues primarily responsible for host-plant selection by caterpillars are olfactory and gustatory.		

Table 1. continued

Table 1. continued

	Models/Concepts Developed for:		
Author; Date	Herbivorous Insects Generally	Onion fly	Author; Date
Fraenkel; 1953 Fraenkel; 1959	Secondary plant substances evolved as botanical defense mechanisms, then secondarily were exploited as "token stimuli" or "sign stimuli"[1] for plant recognition by insects. Some of these allelochemicals may be stimulatory to feeding or oviposition while others may inhibit consumptive behaviors.		
Dethier; 1954	Host selection occurs as a series of progressive steps each triggered by a particular cue (usually chemical).		
Hodgson *et al.*; 1955	Successfully recorded action potentials from insect gustatory receptors.		
Moericke; 1957 (as cited in Thorsteinson; 1960)	Aphids are strongly attracted to yellow.		

[1] As defined by Dethier (1975), a sign stimulus was viewed as "a certain specific compound among all those present in the plant [that] may be sufficient to initiate one or more of the steps in the ovipositional, or feeding sequence, similarily, that a single compound acting as a deterrent might block such behavior."

Table 1. continued

Table 1. continued

Author; Date	Models/Concepts Developed for: Herbivorous Insects Generally	Onion fly	Author; Date
Kennedy; 1958 Thorsteinson; 1960	Both nutrients and allelochemicals recognized as important mediators of host acceptance--"Dual Discrimination Hypothesis."	[Onion maggot is one of the first documented cases (1953) of insecticide resistance to organochlorides in the United States.]	[Howitt; 1958]
Gupta and Thorsteinson; 1960	Moth oviposition on crucifers is strongly influenced by allyl isothiocyanate but also by ovipositional substrate texture.		
Thorsteinson; 1960	Plant size, shape, and color viewed as "being too variable and lacking the identifiable uniqueness required to explain the obvious discriminating power of insects." "The decisive act of host selection is regulated predominantly by the chemical sense." Several stimuli might simultaneously converge to move an herbivore through a particular step in a host-selection behavioral chain.		
Kennedy; 1958 Kennedy *et al.*; 1959 Kennedy and Booth; 1963	Appreciation that herbivore (aphid) behavioral states vary substantially over time and physiological history.		

Table 1. continued

Table 1. continued

Models/Concepts Developed for:			
Author; Date	Herbivorous Insects Generally	Onion fly	Author; Date
Ehrlich and Raven; 1964	Argued that a majority of host shifts are to plants that share similiar chemistry with the ancestral host. The insects that make shifts are then "preadapted" to their new hosts via their special chemical sensitivity.		
Garcia-Bellido;1964 Leahy; 1966	Transplantation of male accessory reproductive glands into virgin female *Drosophila melanogaster* stimulated oviposition and inhibited remating.		
Janzen; 1968	Host plants hypothesized to function as chemically bounded islands during the evolution of insect-plant interactions.		
Schoonhoven and Dethier; 1966	Proved sensilla basiconica on herbivore antennae are olfactory receptors.		
Schoonhoven; 1968	Host selection is determined largely by responses to tastes generated by sugar, salt, water, and specialized deterrent/stimulant receptors. Suggestion that if this "sensory code" can be "cracked" insect behavior can be manipulated.	Organic sulfur compounds like dipropyl disulfide (Pr_2S_2), the major volatile emanating from onions, release oviposition by *Delia antiqua.*	Matsumoto and Thorsteinson; 1968a

Table 1. continued

Table 1. continued

Models/Concepts Developed for:			
Author; Date	Herbivorous Insects Generally	Onion fly	Author; Date
Prokopy; 1967 Prokopy; 1968 Prokopy and Boller;1970 Prokopy*et al.*; 1973	True fruit fly (*Rhagoletis pomonella*) oviposition is strongly influenced by shape and color of artificial fruits. Odor enhances visitation of fruit.	*D. antiqua* larvae orient strongly to organic sulfur compounds like (Pr_2S_2).	Matsumoto and Thorsteinson; 1968b
Benz; 1969	Fecundity of mated female larch bud moths is *ca.* 10x that of virgin females. Presence of ovipositional site (larch twig) also increases oviposition by 10x over resource-deprived mated females.	Onion fly reported to have a preference for brightness but not for color; plant shape and brightness are effective short distance attractants for oviposition?	Müller; 1969
Dethier; 1971; 1973	Host recognition involves the pattern of neural firing across multiple units of the differentially tuned chemoreceptors--"Across-fiber patterning" begins to replace the concept that host plant acceptance is mainly driven by labeled-line processes.	The onion volatiles Pr_2S_2 and propanethiol are reported to be good attractants of onion flies in the field.	Matsumoto; 1970
Whittaker and Feeny; 1971	Allelochemical interactions may be essential aspects of niche differentiation and contribute to the adaptation of species, including plants and insects, to one another.	First report of sex peptide effects in *Delia*. Injection of male *Delia radicum* reproductive tract extracts caused ovipositional activation and mating inhibition in virgin females.	Swailes; 1971

Table 1. continued

Table 1. continued

Author; Date	Models/Concepts Developed for: Herbivorous Insects Generally	Onion fly	Author; Date
Rausher; 1978	*Battus philenor* butterflies form visual search images and selectively seek either narrow- or broad-leaved larval host plants (*Aristolochia* sp.). Butterflies with the strongest preferences for one leaf type or the other discovered larval food plants at a higher rate.	Plant foliar parts discounted as important in onion fly oviposition.	Vernon *et al.*; 1977
		Behavioral responses of *D. antiqua* to host chemicals requires a single sulfur atom with two unshared electron pairs bonded to a saturated hydrocarbon chain 3-5 carbons long.	Ishikawa *et al.*; 1978 Vernon *et al.*; 1978
Rausher; 1981	Predation, nutrition and plant chemistry influence host selection in *Battus philenor* butterflies.	Confirmed that onion flies are attracted to dipropyl disulfide, but even more strongly to microorganisms from freshly decomposing onions. Trap catch in onion fields requires higher than normal release rates of Pr_2S_2.	Dindonis and Miller; 1980a; 1980b; 1981 Miller *et al.*; 1984
Dethier; 1982	Recognized that host acceptance is dynamic and involves balance between external excitatory and inhibitory inputs as well as internal excitatory and inhibitory inputs.	Chemical and foliar visual stimuli act synergistically to release oviposition. Very poor response to only Pr_2S_2 with sand.	Harris and Miller; 1982

Table 1. continued

Models/Concepts Developed for:

Table 1. continued

Author; Date	Models/Concepts Developed for: Herbivorous Insects Generally	Onion fly	Author; Date
Singer; 1982	Quantified individual *Euphydryas editha* host specificity and rank order of preference based on time intervals between initial rejection of potential hosts and eventual host use. When oviposition was not permitted, acceptance thresholds dropped steadily, and insects became increasingly accepting of hosts initially rejected.	Color strongly affects both alighting and postalighting preovipositional behaviors. Quantification of cue effects on acceptance behaviors suggests multiple cues are involved in each step and hints that there may be parity among modalities.	Harris and Miller; 1983
Strausfeld *et al.*; 1984	Neuroanatomical studies of dipteran brains reveal highly multimodal giant descending neurons suggested to be regulators of major behaviors.	Foliar form and spatial orientation strongly affect ovipositional behavior.	Harris and Miller; 1984

Table 1. continued

Table 1. continued

Author; Date	Models/Concepts Developed for: Herbivorous Insects Generally	Onion fly	Author; Date
Miller and Strickler; 1984	Emphasizes the advantages of broad sensitivity to host cues via all possible modalities and behavioral responsiveness to particular multimodal cue combinations correlated with high host-plant suitability. Where chemicals are involved, blends are emphasized over "key" chemicals, especially for adult insects. The Rolling Fulcrum Model of host acceptance formalized the idea that host acceptance results from a dynamic balancing of multimodal external excitatory *vs.* inhibitory inputs against waxing and waning internal excitatory *vs.* inhibitory inputs. This model suggests that decision-making units are likely to be multimodal, complex, and highly integrative.	Concluded that early research on onion fly ovipositional behavior was erroneously constrained by the "key chemical paradigm" which obscured the strongly multimodal nature of this phenomenon. A suggested replacement model is across-modality stimulus summation where hypothetical command neurons with rolling fulcrum properties trigger an ovipositional motor program when stimulated repetitively by a contemporaneous and synergistic convergence of descending outputs from visual, olfactory, gustatory, and tactile centers during a female's predepositional examining of an onion. Attention is drawn to the possible relevance of Meredith and Stein's (1983) cat study showing synergistic increases on firing rates of integrative neural units in the superior colliculus receiving multimodal stimulation.	Miller and Harris; 1985

Table 1. continued

Table 1. continued

Models/Concepts Developed for:			
Author; Date	Herbivorous Insects Generally	Onion fly	Author; Date
[1987]	[Vincent G. Dethier retires]	A surrogate onion plant is designed that embodies the near-optimal suite of multimodal onion cues and evokes behaviors identical to those on authentic onions. (Availability of this defined and reproducible surrogate host becomes an important tool for deeper, mechanistic studies).	Harris *et al.*; 1987 Harris and Miller; 1991
		Ovipositional behaviors and egg deposition occur predominantly in the late afternoon and appear to be cued by a diel rhythm.	Havukkala and Miller; 1987

Table 1. continued

Models (Concepts Developed for:	

Table 1. continued

Author; Date	Models/Concepts Developed for: Herbivorous Insects Generally	Onion fly	Author; Date
		External inhibitory inputs (repellents/deterrents) are evaluated for onion fly oviposition. A wide range of cinnamyl derivatives and monoterpenoids are deterrent when released in the range of a few ng/ml of air around the base of onions. Ovipositional deterrents alone do not effect control of this pest due to a waning of deterrency over time of constant exposure.	Javer *et al.*; 1987 Cowles *et al.*; 1989; 1990
Chen *et al.*; 1988	*Drosophila melanogaster* sex peptide is completely sequenced and synthesized: found to be a 36mer. Upon injection into virgin females, oviposition stimulating and receptivity inhibiting effects lasted 24-72 h, mated females are normally are unreceptive for 7-9 days.	Optimal substrate (soil) parameters are defined for *D. antiqua* oviposition, females prefer pores or cracks slightly wider and deeper than their *ca.* 1 mm diameter, 4mm long ovipositors. The surrogate onion is further perfected.	Mowry *et al.*; 1989a

Table 1. continued

Models/Concepts Developed for:

Table 1. continued

Author; Date	Models/Concepts Developed for: Herbivorous Insects Generally	Onion fly	Author; Date
Courtney *et al.*; 1989	The Hierarchy-Threshold model of host selection incorporates the Rolling -Fulcrum model of Miller and Strickler (1984) and its implicit multimodal balancing of positive and negative factors from each prospective host against a shifting internal physiological state. However Courtney *et al.*'s model spells out the behavioral consequences for encounters of a rolling fulcrum mechanism with a range of hosts varying in stimulant/deterrent ratios. The Hierarchy-Threshold model proposes that insects have a hard-wired rank ordering (hierarchy) for the net positive stimulation from prospective hosts. Interaction of a deprived insect with a given less positive host can result in acceptance (lowered apparent specificity); however, an insect in this behavioral state will always be most accepting of the higher ranked host if different plants are encountered simultaneously.	While examining behaviors were shown to be a highly repetitive behavioral web (probabilistic), actual movement and deposition of eggs appear to be a fixed action pattern (highly deterministic). The motor program driving egg deposition is envisioned to reside in the fused thoracic ganglion while its controlling command neuron (activated by across-modality stimulus summation) resides in the brain. Egg movement is synonymous with ovipositional host acceptance.	Mowry *et al.*; 1989b Harris and Miller; 1991

Table 1. continued

Models/Concepts Developed for:

Table 1. continued

Models/Concepts Developed for:			
Author; Date	Herbivorous Insects Generally	Onion fly	Author; Date
		Onion fly oviposition is highly dynamic: ovipositional resources receiving few eggs in a choice test may receive considerably more in a no-choice test. Mated females confined to poor ovipositional resources (stimulus deprived) for a lifetime lay *ca*. 70% as many eggs as those given optimal hosts.	Harris and Miller; 1988 Weston *et al.*; 1992
		Development of the Stimulo-Deterrent Diversion strategy of pest control which for onion fly recommends simultaneous deployment of: 1) deterrents on seedling onions (value crop), and 2) deeply planted cull onions (trap crop) acting as a behavioral releaser envisioned to preclude shifting response thresholds leading to deterrent ineffectiveness.	Miller; 1986 Cowles *et al.*; 1989; 1990 Miller and Cowles; 1990 Cowles and Miller; 1992
		Deterrents operating across several modalities are more effective than several within one modality.	Cowles and Miller; (in manuscript)
		Inception of studies of internal excitatory inputs of *D. antiqua* oviposition.	**This thesis:**

Table 1. continued

Table 1. continued

Models/Concepts Developed for:			
Author; Date	Herbivorous Insects Generally	Onion fly	Author; Date
Schmidt *et al.*; 1993	*D. melanogaster* ovulation and mating inhibition occur at the same concentration of purified and synthetic sex peptide (0.6 and 3.1 pmol/female respectively). Structure activity studies indicate that an intrachain disulfide bridge is necessary for function, though N-terminal amino acids and post-translationally modified acids are not needed.	**Quantification of the effect of mating on release of onion fly oviposition. Demonstration that all of this stimulatory effect can be reproduced by injecting virgin females with an aqueous extract of male paragonial (accessory reproductive) glands.**	**Chapter 1 (Spencer *et al.*; 1992)**

Table 1. continued

Table 1. continued

Author; Date	Models/Concepts Developed for: Herbivorous Insects Generally	Onion fly	Author; Date
Miller *et al.*; (in preparation)	A synthetic analysis of the accumulated behavioral data on onion fly oviposition (including data generated by this thesis) has lead to the major hypothesis that oviposition by onion flies can be accurately described by an exponential expression. This Neural Math Model of host acceptance combines the attributes of the Rolling Fulcrum and the Across-Modality Stimulus Summation models. J.R. Miller postulates that this integration is accomplished by egg movement command neurons equivalent or similar to the giant descending neurons whose anatomy (see Figure 1 for a schematic representation) is being elucidated by Strausfeld and coworkers. The credibility of this bold hypothesis has been increased by the recent findings of Carandini and Heeger (1994) that integrative neurons (or simple neural circuits) seem indeed capable of arithmetical functions like division.		

Table 1, continued

Table 1. continued

Models/Concepts Developed for:			
Author; Date	Herbivorous Insects Generally	Onion fly	Author; Date
		Evaluation of sex peptide dosage effects on ovipositional stimulation.	**Chapter 2 (Spencer *et al.*; 1994-in press)**
		Characterization of lifetime oviposition by *D. antiqua* virgins.	**Chapter 3**
		Cross-reactivity of *Delia* sp. paragonial extracts.	**Chapter 4**
		Chemical characterization of onion fly "sex peptide".	**Chapter 5**

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1937; Dethier, 1939; Dethier, 1941), and evidence that such receptors were involved in host detection (von Frisch, 1921).

The onion fly played no role in influencing concepts of host-plant acceptance in the early period. As for herbivorous pests, studies were limited to basic biology (Table 1, p. 4-5) and attempts at management by cultural and behavioral manipulations, e.g., trap cropping (Dudley, 1925). In the 1940s, insect control was revolutionized as a consequence of the discovery and development of potent synthetic organic insecticides (e.g., DDT) which came to dominate control of pests like the onion fly.

In spite of scattered evidence that non-chemical modalities like plant form, color and tactile cues also affected host finding by insect adults (e.g., Ilse, 1937; Moericke, 1957(as cited in Thorsteinson, 1960)), plant secondary substances acting as excitatory or inhibitory "token stimuli" were given primacy as mediators of host-plant recognition (Fraenkel, 1953; 1959). By assigning the influence of individual cues to progressive steps of host selection, Dethier (1954) departed from the single key-chemical model but remained faithful to the idea of chemical primacy. The flood of recordings of action potentials from insect chemoreceptors following Hodgson *et al.*'s (1955) technological breakthrough seemingly increased the level of resolution and sophistication in host-acceptance studies. At the same time, identification of cells responding specifically to certain compounds strengthened the chemocentric view and labeled-line thinking.

In an attempt to integrate the belief of some that insects used nutritive chemicals to identify host plants and the belief of other researchers who held that only plant secondary compounds mediated host acceptance, Kennedy (1958) offered the "Dual Discrimination Hypothesis" which incorporated both ideas. In the following years, evidence supporting the involvement of non-

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chemical modalities continued to appear, and the dynamic nature of host acceptance as influenced by physiological state came to be appreciated (Kennedy, 1958; Kennedy *et al.*, 1959; Kennedy and Booth, 1963). The importance of host chemistry in the coevolution of insects and plants and as bridges for host shifts as emphasized by Ehrlich and Raven (1964) promoted evolutionary dimensions to studies of host acceptance.

A strong stimulatory effect of male accessory secretions (later known as "sex peptides") on female *Drosophila melanogaster* mating refusal (Garcia-Bellido, 1964; Leahy, 1966) and oviposition (Leahy, 1966) set off a flurry of interest in these behavioral modulators and their possible application as sterilization agents. Interest in alternative pest control was by necessity growing during this period as insecticide resistance was becoming a pervasive problem. *D. antiqua* was one of the first species resistant to organochlorides (Howitt, 1958).

On the heels of the identification of the first olfactory receptors (Schoonhoven and Dethier, 1966) and electrophysiological recordings therefrom, it seemed that the insect chemosensory code might soon be broken, and insect behavior would be vulnerable to manipulation by appropriate chemicals. Work on *D. antiqua* host colonization began in this era (Table 1, p. 8) and focused on chemicals mediating behaviors of both larvae and adults; foliar influences were largely discounted (Matsumoto and Thorsteinson, 1968a; Matsumoto and Thorsteinson, 1968b; Müller, 1969; Matsumoto, 1970). *D. antiqua* host colonization was thought to be chemically driven. Meanwhile, among true fruit flies of the genus *Rhagoletis* (Tephritidae) visual cues and odor were found to be very important in host-finding and oviposition (Prokopy, 1967; Prokopy, 1968; Prokopy and Boller, 1970; Prokopy *et al.*, 1973). Near this time, the effect of mating on oviposition

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was quantified relative to host quality in the larch bud moth (Benz, 1969); it was suggested that the presence or absence of host or mating stimuli have predictable effects on total fecundity. Benz made a crude attempt at developing a mathematical expression for predicting moth egg output.

Dethier's (1971; 1973) Across-Fiber Patterning hypothesis presented a new picture of insect chemoreception in which cross-comparison and integration of primary inputs accounted for broad discrimination abilities from few receptor types. Nevertheless, among onion fly researchers of the time, labeled-line concepts of host recognition prevailed. Perhaps the pinnacle of this chemocentric reductionism was reached with the declaration (Ishikawa *et al.*, 1978; Vernon *et al.*, 1978) that *D. antiqua* behavioral responses were explained by the presence of alkyl sulfides with one pair of unshared electrons.

The entry of Miller and coworkers onto the onion fly scene marked a change from the chemical paradigm to an openness to the potential influence of other types of stimuli. Quickly, the importance of once-discounted non-chemical stimuli was re-established, and synergy between them recognized (Dindonis and Miller, 1980; Harris and Miller, 1982; Harris and Miller, 1983; Miller *et al.*, 1984). Onion fly host acceptance behavior was found to be mediated by interacting multimodal cues affecting progressive steps during examining. Tested factorially, the contribution of modal combinations toward oviposition suggested parity between interacting modalities.

Consistent with observations of onion fly ovipositional behavior, the dynamic nature of interacting external and internal excitatory and inhibitory inputs proposed by Dethier (1982) became the foundation for Miller and Stickler's (1984) Rolling Fulcrum Model of host acceptance (Figure 1). Emphasizing broad peripheral sensitivity, integration of cues from all

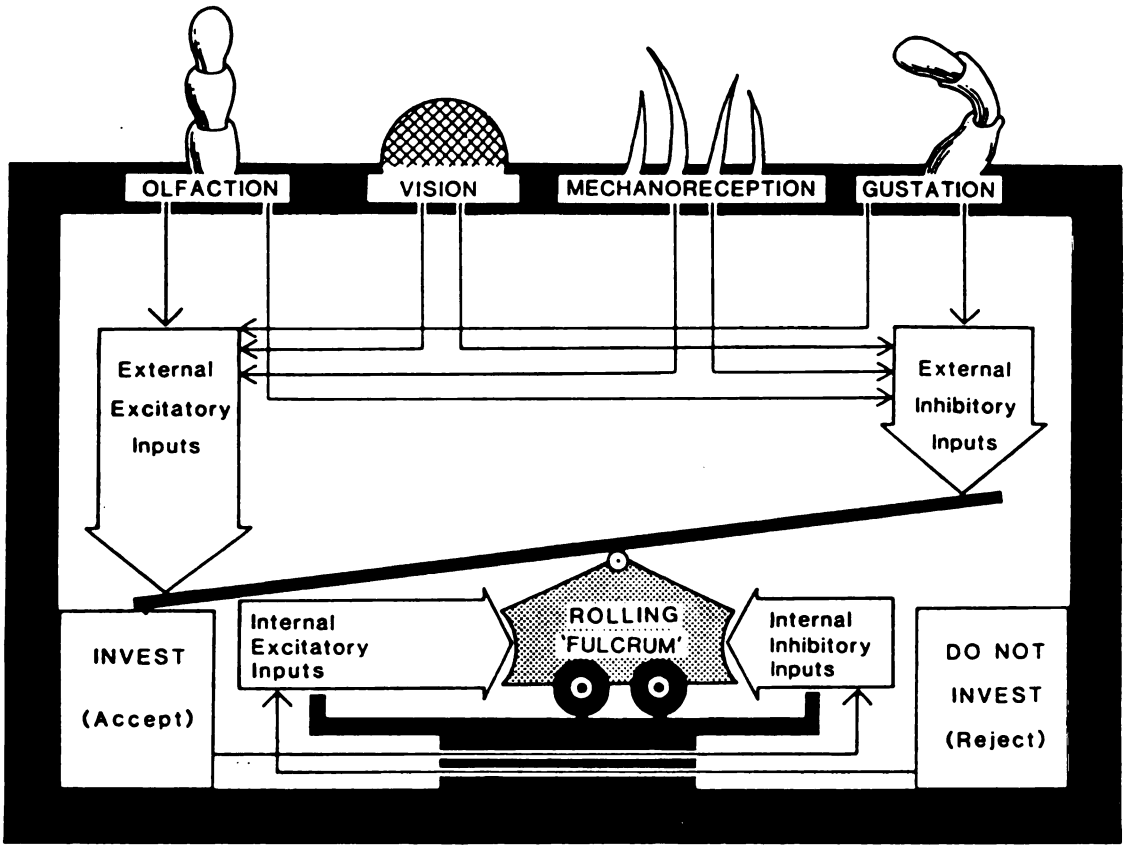

Figure 1. Miller and Strickler's (1984) rolling-fulcrum model of host-acceptance by insects.

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modalities, and the dynamic balance of internal and external inputs, this model suggested insect nervous systems possess complex decision-making units.

The kind of multimodal integration necessitated by rolling-fulcrum concepts is, in fact, increasingly supported by Strausfeld *et al.*'s (1984) neuroanatomical studies of giant descending neurons (GDNs) in dipteran brains (Figure 2). These unusually large interneurons have multiple synaptic connections throughout the protocerebrum and receive inputs from: most or all sensory modalities, ascending afferent trunks bearing sensory information about states of internal organs, neuropils, and peptidergic sites. Because they are highly multimodal and situated between brain sensory regions and motor units, GDNs are thought to be decision making units and activators of major behaviors via connectives to thoracic motor interneurons. The discovery of neuroanatomical structures and connectives situated so as to receive and integrate inputs from multiple sensory modalities and influenced by peptidergic sites provided strong support for reconfiguring long-held chemocentric views about sensory ecology.

The concept of Across-Modality Stimulus Summation developed for onion fly (Miller and Harris, 1985) extended Dethier's (1982) across-fiber patterning concept by incorporating simultaneous interplay among different sensory modalities. Extension of the model effectively brought host acceptance thinking into register with neuroanatomical reality.

Development of a standardized, highly reproducible surrogate onion (Harris *et al.*, 1987; Harris and Miller, 1991) made possible measurement of the effects of changing internal states of *D. antiqua* against a controlled set of optimized external inputs. Observation of ovipositional periodicity provided evidence for the influence of diel rhythms on host acceptance (Havukkala

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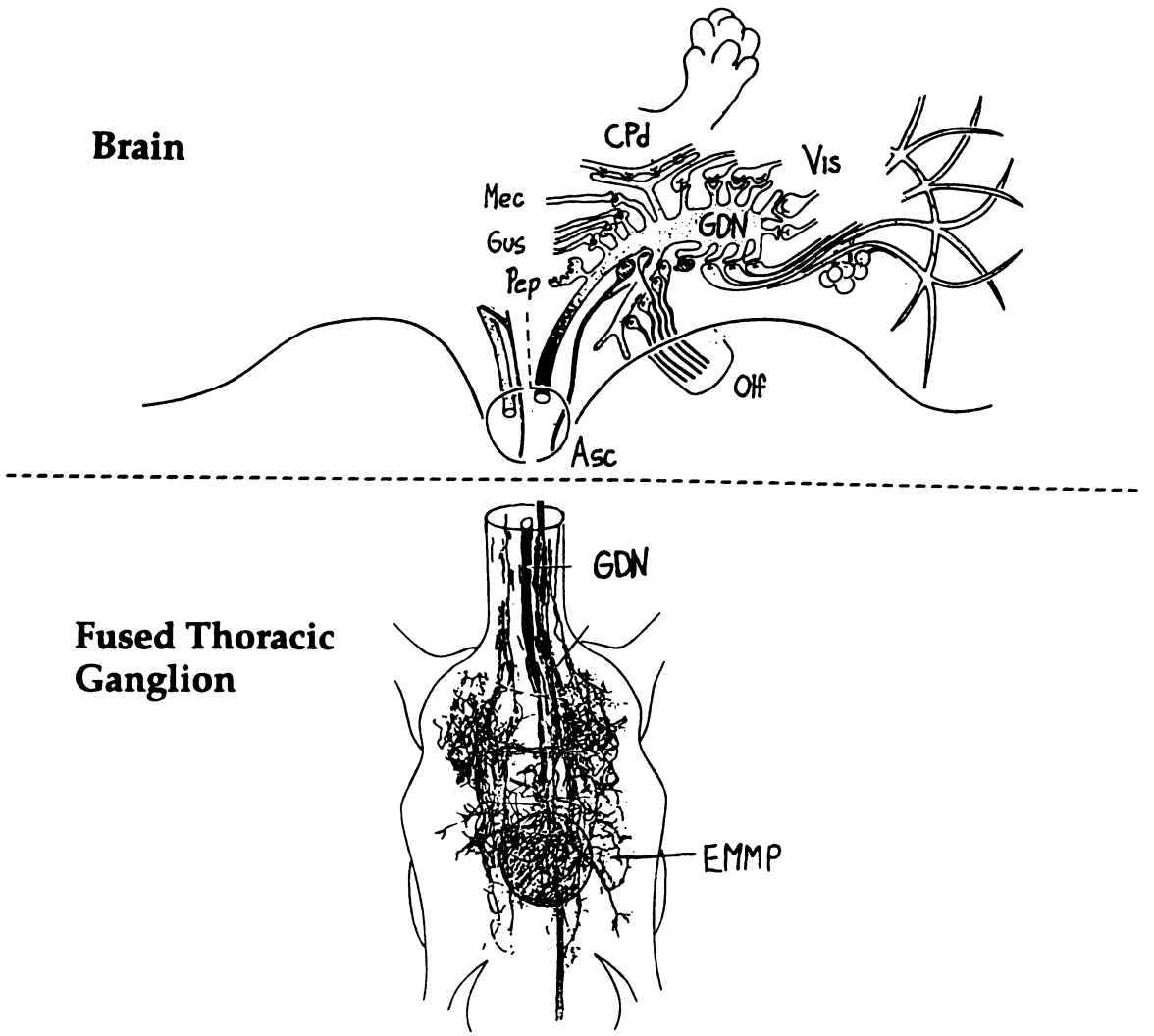

Figure 2. Dipteran giant decending neuron (GDN), its sensory inputs and thoracic connectives (modified from Strausfeld *et al.*, 1984). Multimodal sensory inputs from visual (Vis), gustatory (Gus), olfactory (Olf), and mechanoreceptory (Mec) receptors are indicated. Additional inputs from higher brain centers (Corpora pedunculata; CPd), ascending fibers (Asc) and peptidergic centers (Pep) are also noted. In *D. antiqua*, egg deposition is thought to be triggered following accumulation and processing of appropriate multimodal stimulation in the brain which activates an egg movement motor program (EMMP) residing in the fused thoracic ganglion. In an intact insect, GDSs would be found as *ca.* bilaterally symmetric pairs.

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and Miller, 1987). Quantification of substrate parameters (Mowry *et al.*, 1989a) led to further refinement of the surrogate onion, and to detailed analysis of predepositional and depositional behaviors (Mowry *et al.*, 1989b). The transition from probabilistic predepositional behaviors to deterministic depositional sequences suggested that actual egg movement was under control of a fixed action pattern residing in the thoracic ganglion. Decapitation of females before they entered depositional behavior never resulted in movement of eggs, while females decapitated during deposition could continue to lay additional eggs.

The dynamic nature of *D. antiqua* ovipositional behavior was evident in no-choice tests (Harris and Miller, 1988) and studies of lifetime oviposition by mated flies (Weston *et al.*, 1992). A few days of deprival from an optimal ovipositional resource resulted in acceptance of sub-optimal resources. Surprisingly, lifetime oviposition by onion flies confined to poor ovipositional resources was *ca.* 70% that of females receiving the best resources (Weston *et al.*, 1992), a result emphasizing the malleability of host acceptance mechanisms for this insect.

Courtney *et al*'s. (1989) Hierarchy-Threshold model, used a rolling fulcrum mechanism to predict outcomes of encounters with a range of hosts under conditions thought to be realistic in the field. This model explicitly states that insects have a hard-wired rank ordering of hosts, an unstated but implicit assumption of the Rolling Fulcrum model. Courtney linked proposed proximate behavioral mechanisms with anticipated ultimate outcomes of insect-plant interaction.

External inhibitory inputs in the form of cinnamyl derivatives and monoterpenoids were found to be onion fly ovipositional deterrents over short distances and at high release rates around optimally configured hosts

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(Cowles *et al.*, 1989; 1990); however, their effects waned with a few days of deprival. An understanding of stimulatory as well as deterrent factors for *D. antiqua* oviposition, along with rolling fulcrum properties of the host-acceptance mechanism led to proposal of Stimulo-Deterrent Diversion (SDD) (Miller and Cowles, 1990), which, for onion fly, involves simultaneous deployment of: 1) deterrents on onion seedlings (valued crop), and 2) highly stimulatory cull onions as an egg sink so as to avoid states of high deprival. Successful use of SDD depends on generating a sufficiently high preference differential between the trap crop and the valued crop. Application of deterrents alone creates essentially a "no choice" condition in the field, which is eventually ineffective in deterring oviposition. Likewise, insecticide treatment alone leaves no ovipositional alternative for surviving resistant members of the population, who oviposit on treated plants contributing to rapid selection for physiological resistance. By applying SDD, deprival effects should be avoided in the value crop and the trap crop would become the focus of oviposition and a refugia for susceptible genes.

Importantly, the most effective deterrents or combinations of deterrents seemed to be those which operated across modalities (Cowles and Miller; in manuscript). The effectiveness of two chemical deterrents was less than that of a chemical deterrent and a visual deterrent, suggesting that integration between modalities may involve multiplicative (non-linear) processes.

Over the last 5 years, studies of *D. antiqua* oviposition have expanded to and focused upon internal excitatory inputs (Figure 1). Sex peptide effects, like those first found in *Drosophila*, had been noted for cabbage fly, *Delia radicum* (Swailes, 1971), but never explored further. Rediscovery of the potent ovipositional-stimulating effects of male reproductive tract secretions

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transferred to females during mating (Spencer *et al.*, 1992) opened an avenue for manipulating the internal state of female *Delia* flies in defined ways.

A growing appreciation for the complexity of sensory interactions and an ever increasing capability to manipulate external and now internal inputs affecting the outcome of host encounters, lead to a recent reevaluation by J.R. Miller of the lab's earlier studies. When egg output in choice tests with treatments varying from sand only to the optimal surrogate onion was plotted on the y-axis against the number of optimally stimulated modalities on the x-axis, a very smooth exponential curve of the general form $f(x) = a^x$ was discovered, where: **x**= the number of modalities optimally stimulated and **a**= a base of magnitude *ca.* 4.

Drawing on wealth of *D. antiqua* data, Dr. Miller recently formulated the Neural Math Model. His synthetic analysis of the accumulated behavioral data on onion fly oviposition (including data generated by this thesis) has lead to the major hypothesis that oviposition by onion flies can be described by the expression:

$$f(x) = a^x \cdot e/(b \cdot c \cdot d)$$

where from empirical data across various experiments:

f(x)= number of eggs laid/female/day in a choice test deploying various combinations of cues optimized for each modality.

x= the number of modalities optimally stimulated.

a= a base of *ca.* 4 (irrespective of modality type) (Harris and Miller; 1982).

b= an inhibitory constant (of ca. magnitude 20) that suppresses oviposition to well below 1 egg/female/day under null stimulation.

c= a mating factor of *ca.* 10 for virgin flies (this dissertation, Chapter 3); this value diminishes to 1 with mating.

d= effects of deterrents; for two deterrents operating within one modality d= ($d_1 + d_2$); for two deterrents operating in different modalities d= ($d_1 \cdot d_2$) (Cowles and Miller; in manuscript).

e= a hypothetical internal excitatory input arising from, e.g. accumulating eggs which, with ovipositional resource deprivation, increases to *ca.* 60.

This Neural Math Model of host acceptance combines the attributes of the Rolling Fulcrum and the Across-Modality Stimulus Summation models. J.R. Miller postulates that this integration is accomplished by egg movement command neurons equivalent or similar to the giant descending neurons (GDNs) whose anatomy (see Figure 2 for a schematic representation) is being elucidated by Strausfeld and coworkers. Furthermore, the model postulates modality parity and integrative properties consistent with the neuroanatomy of GDNs. This mathematical model generates precise predictions that are eminently testable and the focus of recently initiated studies as well as future research. The credibility of this bold hypothesis has been increased by the recent findings of Carandini and Heeger (1994) that integrative neurons (or simple neural circuits) seem indeed capable of arithmetical functions like division.

The five chapters of this dissertation reveal how onion fly oviposition is influenced by changing internal excitatory inputs. Chapter 1 identifies the existence and behavioral effects of sex peptide (a presumed internal excitatory input). Chapter 2 measures the dosage effects of sex peptide and addresses the literature presumption that the effect is all-or-none. Chapter 3 quantifies patterns of egg output for populations of mated and virgin females held individually for their lifetimes. In the absence of mating and sex peptide, virgin females are considered to be in a state of mating deprival, and they

experience the presumed internal excitatory input of accumulating mature eggs. Chapter 4 addresses species-specificity of sex peptides among three *Delia* flies. Chapter 5 departs from the examination of the decision-making system's properties, and reports on attempts to isolate and chemically-characterize the onion fly sex peptide.

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CHAPTER 1

Modification of Female Onion Fly, *Delia antiqua* (Meigen), Reproductive Behavior by Male Paragonial Gland Extracts

ABSTRACT

Egg depositional rates of virgin onion flies, *Delia antiqua* (Meigen), injected thoracically with extracts of male paragonial glands were statistically identical (14.5 eggs/female/d) to those of normally mated females. Moreover, when continuously exposed to males, extract-injected virgin females refused to mate and produced unfertilized eggs for the duration of the > 15 d experiment. For this normally monocoitic dipteran, < 1 male equiv of paragonial secretion completely reproduced the ovipositional response characteristic of a normal mating, and, this effect required no involvement of the genitalia or genital chamber. We suggest that the receptor for the active chemical(s) (sex peptide?) would be an excellent target for biorational insect control by sterilization. The role of these primer sex pheromones in insect reproductive isolation and evolution is not clear.

INTRODUCTION

Female insects of many species become refractory to mating and commence egg laying following copulation. Products of the male paragonial glands, transferred in the ejaculate, are often responsible for the observed changes in female behavior (Leopold, 1976). In *D. melanogaster*, materials thought to be associated with sperm or the ejaculate were long suspected of

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stimulating oviposition and suppressing female mating receptivity (Gottschewski, 1937; Kummer, 1960; Manning, 1962). Transplantation and injection experiments localized the active component in the male paragonial glands (Garcia-Bellido, 1964; Leahy, 1966; Leahy and Lowe, 1967). Chen and Diem (1961) had previously determined that a peptide they found exclusively in the paragonia of adult male *D. melanogaster* was in fact the sex-specific peptide that Fox (1956) had detected in whole-body extracts of *D. melanogaster* males. Leahy and Lowe (1967) found that the behaviorally active component they extracted from male paragonia was chromatographically similar to Fox's (1956) "sex peptide"; however, they never demonstrated that the two materials were identical. Nevertheless, other substances with similar origins and effects are commonly referred to as sex peptides. A paragonial substance from the mosquito *Aedes aegypti* ("matrone", Fuchs *et al.*, 1968) also caused females to become refractory to further mating and increased oviposition (Leahy and Craig, 1965; Craig, 1967; Hiss and Fuchs, 1972).

Loss of mating receptivity and/or activation of oviposition in females has since been demonstrated for reproductive gland extracts or transplants from a number of Diptera, including: *Musca domestica* (house fly) (Riemann *et al.*, 1967; Riemann and Thorson, 1969; Leopold *et al.*, 1971b), *Lucilia cuprina* (sheep blow fly) (Smith *et al.*, 1989; Smith *et al.*, 1990), *D. radicum* (Swailes, 1971), and *Stomoxys calcitrans* (stable fly) (Morrison *et al.*, 1982).

Though male-derived factors with similar or related functions are known in several insect orders (Gillott, 1988), there has been little progress on structural elucidation of sex peptides, except within *Drosophila*. Chen *et al.* (1988) isolated and sequenced the 36 amino acid *D. melanogaster* sex peptide, which according to Raina and Gäde (1988) would be abbreviated Drm-SP-I. This sex peptide is thought to be rapidly released into and transported by the

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hemolymph to receptors in the brain (Kubli, 1992). Interestingly, Drm-SP-I was equally effective in suppressing mating receptivity and inducing oviposition in virgin females of *Drosophila simulans*, *Drosophila mauritania*, and *Drosophila* sechellia, all sibling species from the *D. melanogaster* species complex (Chen *et al.*, 1988).

The amino acid sequence of *D. sechellia* sex peptide; Drs-SP-I, has also been determined (Chen and Balmer, 1989); this peptide, which is active in *D. melanogaster*, differs from Drm-SP-I by 3 amino acid substitutions. Neither Drm-SP-I or Drs-SP-I had any effect on sexual receptivity or oviposition when administered to *Drosophila funebris*, a *Drosophila* only distantly related to the *D. melanogaster* species complex. Sex peptides may be involved in reproductive isolation (Chen, 1976; Fuyama, 1983); however, the appreciable cross-reactivity within the *Drosophila* requires that other isolating mechanisms are also involved.

Here we report that an extract of male *Delia antiqua* paragonial glands (*sensu* Gillott, (1988)) contains a substance that terminates sexual receptivity for weeks and activates oviposition in virgin female onion flies.

MATERIALS AND METHODS

Insects. A *D. antiqua* culture was initiated from late-instar larvae collected in autumn 1986 from Grant, MI., and maintained as described by Havukkala and Miller (1987). Virgin insects were obtained by collecting flies within 24 h following emergence and segregating males and females in separate cages with food, water, and an artificial onion ovipositional resource modified from Harris *et al.* (1987) by the addition of optimal ovipositional holes (Mowry *et al.*, 1989a) around the base of each stem.

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Extract Preparation. Paragonial glands were dissected from 6-10 d old *D. antiqua* males in physiological saline (128 mM NaCl; 4.6 mM KCl; 1.9 mM $CaCl_2 \cdot 2H_2O$; 1 mM $Na_2HPO_4 \cdot 7H_2O$; adjusted to pH 6.8), modified from Mowry *et al.* (1989a). Reproductive tracts were obtained by pulling on the terminalia of freshly freeze-killed males until the gut and reproductive tract were exposed. The testes/paragonial gland complex was then separated from the gut with forceps and the testes excised. To minimize carry over of saline solution, paragonial glands were transferred via suction, using a hydrophobic polyethylene micropipet pulled over heat to yield a tip diammeter of 0.25mm, to a 0.5 ml DOT™ polyethylene microcentrifuge tube containing a measured volume of ice-cold onion fly saline. Collected glands were homogenized using a a Blackstone™ Probe Sonicator (Model SS 2) with a 90 mm long x 3 mm diam probe tuned for maximum disruption at 90% of full power and centrifuged for 10 min at 12000xg and 4°C. The supernatant was transferred to another microcentrifuge tube and stored at -16°C. The final extract concentration was 1 male equivalent (two paragonial glands) per µl of supernatant.

Injection. Injections were carried out using heat-pulled glass needles made from Fisher™ 5 µl disposable micropipets. Needles were slipped into a 10 cm length of PE 60 polyethylene tubing glued with cyanoacrylate adhesive to a 5 cm section of Intramedic™ PE 10 tubing fit snugly over the needle of a 10 µl Hamilton™ syringe loaded with onion fly saline and held in a micromanipulator. A tiny reference air bubble was introduced into the tip of the needle before 5 µl of paragonial gland extract was drawn inside. The volume of extract dispensed was monitored by observing the bubble passing volumetric calibration marks on the needle as the syringe plunger was depressed.

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Treatments. Seven-day-old virgin females were randomly selected from the female cage and assigned to nine treatment groups: 1) untreated with no exposure to males, 2) untreated and exposed to males from day 1 onward, 3) untreated and exposed to males from day 5 onward, 4) saline injected with no exposure to males, 5) saline injected and exposed to males from day 1 onward, 6) saline injected and exposed to males from day 5 onward, 7) injected only once with *D. antiqua* paragonial gland extract and no exposure to males, 8) injected only once with *D. antiqua* paragonial gland extract and exposed to males from day 1 onward, and 9) injected only once with *D. antiqua* paragonial gland extract and exposed to males from day 5 onward. Treatments were administered in accordance with a completely randomized design, replicated ten times. Injected females were anesthetized with CO_2 and restrained on a small glass plate under a thinly stretched piece of laboratory Parafilm™ prior to receiving a dorso-lateral thoracic injection of either 0.25 µl of paragonial gland extract (0.25 male equivalents) or physiological saline. The untreated controls were not anesthetized.

Following treatment, females were placed singly into 20 cm x 6 cm cylindrical screen cages with food, water, and an ovipositional resource. On days 1 and 5 following the initial treatments, two 7-10 day old sexually mature and previously virgin males were added to the cages of females to be exposed to males beginning on day 1 or day 5 respectively, to assess mating receptivity. Males dying during the experiment were replaced with similarly aged virgin males so that these females were continuously exposed to males. Resources were changed daily throughout the experiment and the eggs were collected by flotation, counted, and held in small Parafilm™-covered petri dishes on moistened sand to assay hatching. Females producing viable eggs subsequent to addition of males were considered sexually receptive at the time males

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were added. Mean daily egg production for each female was the measure of ovipositional response. The onset of oviposition or an increase in the rate of oviposition after the addition of males was taken as evidence of mating. By day 15, the population of females had declined by 1/2 in a majority of the treatments and the experiment was terminated. The mating status of all remaining live females, and those that had died during the experiment, was determined by postmortem dissection of the spermathecae. Total egg counts were analyzed by ANOVA following $(y+0.375)^{1/2}$ transformation. Mean separation was by Fisher's Protected LSD.

RESULTS AND DISCUSSION

During the first 4 days of the experiment, the daily rate of oviposition by females injected with paragonial gland extract was not significantly different from that of saline-injected or untreated females exposed to males on day 1 (Table 2, Figure 3). These groups did oviposit at a significantly greater rate than did saline-injected or untreated females not exposed to males ($P<0.01$). During this period, fertile eggs were produced only by untreated or saline-injected females exposed to males. Extract-injected and previously untreated or saline-injected females receiving males at the beginning of day 5 laid eggs at a rate not significantly different from that for the same treatments receiving males on day 1 ($P<0.01$). During the course of the experiment, there was no significant change in the rate of oviposition or viability of eggs produced by unmated females of either the saline-injected or untreated groups. The timing of male exposure had no significant effect on oviposition or the probability of mating, as non-extract injected females exposed to males on days 1 or 5 laid similar numbers of eggs and mated with comparable frequency.

Table 2. *Egg production and mating of onion fly females exposed to males after injection with male paragonial gland extract, along with respective controls.*

Table 2. Egg production and mating of onion fly females exposed to males after injection with male paragonial gland extract, along with respective controls.

Pretreatment			Days 1–4		Treatment		Days 5–15	
Injection	Exposure to males, day 1	*n*	$\bar{X}$ eggs/♀/d (± SE)*	% ♀♀ mated	Males added at end of day 4	*n*	$\bar{X}$ eggs/♀/d (± SE)	% ♀♀ mated
None	No	10	1.4 ± 1.1 d	0	No	9	1.3 ± 1.1 d	0
Saline	No	10	1.1 ± 0.5 d	0	No	8	2.0 ± 1.0 cd	0
Extract	No	10	14.7 ± 2.7 ab	0	No	7	10.1 ± 2.0 ab	0
None	No	10	0.2 ± 0.2 d	0	Yes	10	9.3 ± 2.3 bc	80
Saline	No	10	1.6 ± 0.8 d	0	Yes	6	8.7 ± 2.3 abc	50
Extract	No	10	15.3 ± 2.2 ab	0	Yes	5	9.8 ± 1.9 ab	0
None	Yes	10	8.9 ± 2.3 ab	80	No"	10	13.2 ± 1.6 ab	100
Saline	Yes	10	10.2 ± 2.7 ab	70	No	7	10.2 ± 1.6 ab	100
Extract	Yes	9	16.9 ± 2.0 a	0	No	6	10.7 ± 0.7 ab	0

"Females receiving males on day 1 were continuously in the presence of males thereafter.

*Means followed by a common letter are not significantly different (Fisher's protected LSD on $\sqrt{y + 0.375}$ transfomed data; $P < 0.01$).

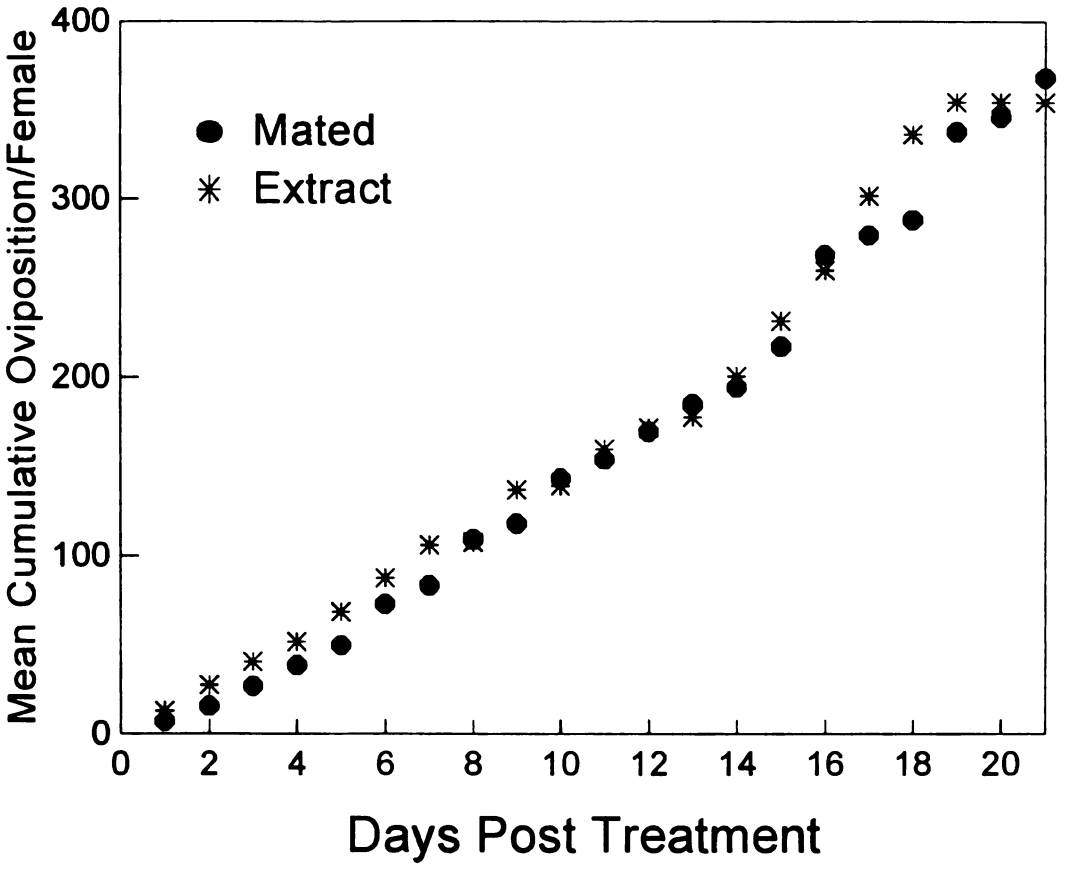

Figure 3. Mean cumulative egg production for mated (n = 106) and extract-injected (n = 178) onion fly females. Slopes of the best fit lines through the virgin and mated data were 14.7 and 14.4 eggs/female/d. Beyond day 16, increased variation among the points for mean daily cumulative oviposition/female is probably due to reduced sample sizes caused by death of the least robust individuals.

Notably, the ovipositional rate of the extract-injected females did not change following addition of males, nor did any extract-injected female mate during this experiment. Extract injection had a rapid and long-lasting effect; extract-injected females were unreceptive to mating and most had begun ovipositing by the morning following injection. All of these females had empty spermathecae and failed to produce any fertile eggs during the experiment, indicating that they remained sexually unreceptive even when given 15 days of continuous access to males. Moreover, additional replicates from other experiments (methods identical to those above) demonstrated that cumulative egg production of extract-injected females was identical to that of untreated females held with males (normally mated) for more than 20 days (Figure 3), further supporting the hypothesis that male paragonial gland extracts contain the factor(s) responsible for ovipositional behavior of mated females. We also observed that some untreated, unmated females may begin to oviposit in a manner indistinguishable from mated or extract-injected females. Of the 341 eggs laid by 20 saline-injected and untreated females not exposed to males, 88% were contributed by three laying virgins. Thereafter, one of these laying virgins, given constant access to males for more than 2 weeks, continued to oviposit at a mated-like rate, (17.1 eggs/d), but none of her eggs hatched and her spermathecae were empty upon her death. That some virgin females may become as ovipositionally-activated and unreceptive to males as do extract-injected or normally mated females is interesting and potentially important mechanistically.

In *Drosophila;* extract-injected females regain their mating receptivity and egg production wanes after 24 to 72 h vs. 7 to 10 days for mated females (Burnet *et al.*, 1973; Chen *et al.*, 1988). Apparently, some factor or stimulation (e.g. sperm effect (Gromko *et al.*, 1984)), in addition to Drm-SP-I is necessary

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for the maintenance of the complete response associated with normal mating in *D. melanogaster*. However, in the monocoitic onion fly (Martin and McEwen, 1982), injected chemical cues from the paragonial glands duplicate completely the effects of normal mating. It is not necessary for a female to receive the physical stimulation associated with copulation or to experience the normal pathway of sex peptide release via the genital chamber.

The finding that *D. antiqua* reproduction is so dramatically affected by paragonial factors emphasizes the potential value of chemically identifying the active factor(s) and their receptors (unpublished data of J.L. Spencer, J.R. Miller, and J.F. Leykam implicate a moderately sized, polar peptide(s), this dissertation, Chapter 5). Artificially stimulating receptors for sex peptides early in the life of a female onion fly would represent a novel, apparently irreversible control by sterilization conforming to most of the ideals laid down by Knipling (1979). For example, the apparent reactivity across a species group within *Drosophila*, would suggest usable breadth for a given chemical, with low probability of sterilizing non-target insects.

Knowledge of sex peptide cross-reactivity is also likely to add significantly to understanding of mate recognition systems. Where the ranges of related species overlap, it is possible that the consequences of interspecific pairings may not be symmetric. Males of one species might be capable of delivering a cross-reactive sex peptide with genetically incompatible sperm to females of another species. Due to a less specific sex peptide receptor, females of the second species might be susceptible to the sex peptides of aggressively mating heterospecific males. These females would waste their reproductive efforts by fertilizing eggs with incompatible heterospecific sperm, while rejecting the sexual advances of conspecific males. Such interspecific sexual interference competition has been termed

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"satyrism" by Ribeiro and Spielman (1986), and Ribeiro (1988) but no explanation of a mechanism has accompanied the description of this phenomenon. The extent to which genetically incompatible insect populations become reproductively isolated by mating site, sex attractant pheromones, or sex peptide pheromones is an open and intriguing question that can be addressed when research on sex peptides catches up with these sister research areas. However, broad specificity within genera suggests that sex peptides are not primary reproductive isolating mechanisms (this dissertation, Chapter 4).

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CHAPTER 2

Onion fly, *Delia antiqua*, Oviposition and Mating as Influenced by Insect Age and Dosage of Male Reproductive Tract Extract: Comparisons with Other Diptera.

ABSTRACT

One hundred percent of virgin female onion flies, *Delia antiqua*, receiving ≥1/20th of a male equivalent of mature male reproductive tract extract refused to mate and began laying unfertilized eggs at a normally mated rate. The 50% behavioral response (BR_{50}) fell between 1/40th and 1/20th of a male equivalent. Sex peptide responses are not always all-or-none. Some females receiving extract at ≤ 1/40th male equivalent oviposited at an intermediate rate. Moreover, in some cases mating inhibition and ovipositional activation became decoupled at intermediate dosage.

A low level of sex peptide was present in 1-day-old males. Sex peptide titer rose with age until plateauing by 6 days post-eclosion. Males began mating at 3 days, when they first had ample mature sperm; 50% of 6-day-old males mated. The mean number of females inseminated per male exposed to an excess of virgin females over a 24 h period was 4.3 ± 0.6 (mean ± SE).

Presence of mature eggs was not always a prerequisite for mating, although probability of insemination was correlated with egg maturation. One-day-old pre-ovipositional females receiving 1/20th of a male equivalent of extract began ovipositing when they had mature eggs at 5-6 days old. Therefore, sex peptide may act early and permanently or have a long half-life and affect behaviors once females reach sexual maturity. Male insects

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provide females with an excess of sex peptide in many cases. *D. antiqua* males transfer *ca.* 5-10 times more pheromone than necessary to fully activate females. We suggest this excess is related to the speed of female response. It is yet unclear whether sex peptide potency or titer in Diptera have been influenced by an evolutionary arms race between the sexes, whose reproductive interests may conflict.

INTRODUCTION

Sex peptides are components of male paragonial gland secretions, transferred in the ejaculate to the female during copulation. Once deposited in the genital chamber, paragonial proteins travel to the brain via the hemolymph where they act as neuropeptides (Kubli, 1992). Found in several insect orders and widely in the Diptera (reviewed by Gillott, 1988), sex peptides are thought to function as chemical mate-guarding substances (Miller *et al.*, 1994), insuring a male's paternity by *both* terminating female sexual receptivity *and* stimulating sperm use by releasing oviposition . Loss of mating receptivity and/or activation of oviposition in females have been demonstrated for reproductive tract extracts or gland transplants from more than 20 species of Diptera (Craig, 1967; Spencer *et al.*, 1992). Sex peptide-like effects have recently been documented for several additional Dipterans: *Phormia regina* (black blowfly) (Merritt *et al.*, 1994), *Mayetiola destructor* (Hessian fly) (Bergh *et al.*, 1992), and *Delia platura* (seed fly) (Spencer, this dissertation Chapter 4).

The duration of sexual receptivity loss following mating or injection of glandular extracts is variable; it may be temporary as is seen in *Drosophila melanogaster* (Chen *et al.*, 1988; Aigaki *et al.*, 1991) or be permanent, as is the case with *D. antiqua* (Spencer *et al.*, 1992). In *D. antiqua*, normal mating or

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injection of paragonial gland extracts results in a *ca.* 10 fold increase in the rate of oviposition over virgin egg laying (Spencer *et al.*, 1992).

D. melanogaster sex peptide (Drm SP-I) has been isolated, identified, and synthesized; it is a 36mer (i.e., it is composed of 36 amino acids)(Chen *et al.*, 1988). The paragonial glands of male *D. melanogaster* contain *ca.* 6.2 pmol of Drm-SP-1 (Kubli, 1992). As little as 3 pmol of purified peptide or 0.6 pmol of synthetic Drm SP-I affects mating, a quantity equivalent to the transfer of *ca.* 1/2 to 1/10th of one male equivalent (male equiv) from a 7-day-old sexually mature virgin male. Dosages for sex peptide action are reported for few other species. The threshold for mating inhibition and activation of oviposition of *Aedes aegypti* sex peptide (matrone) has been measured as ≤1/64th of a male equiv (Craig, 1967; Fuchs *et al.*, 1968; Fuchs *et al.*, 1969; Fuchs and Hiss, 1970). Extrapolating from serial mating experiments with *Lucilia cuprina* (sheep blow fly) (Smith *et al.*, 1990), *ca.* 1/25th of a male equiv was sufficient to cause loss of receptivity in 50% of females for 8 days. Numerous other studies report mating effectiveness declines when males are serially mated (Leopold *et al.*, 1971a; Hihara, 1981; Bergh *et al.*, 1992; Cook, 1992); such studies can provide valuable information about male copulation capacity where dose-response data are lacking.

In the field, male *D. antiqua* appear to use a form of resource guarding polygyny. For periods of several hours in the afternoon, males perch on the top of onion foliage and intercept objects flying within *ca.* 1.5 meters (J.R. Miller, personal observation). Female receptivity is likely signaled by contact pheromones, as is the case with other higher Diptera (Nelson *et al.*, 1981; Scott and Jackson, 1990; Trabalon *et al.*, 1992). In our laboratory setting, males can mate when >2 days old, but females do not generally mate before days 5-6, an age when most females possess vitellogenic eggs. The onset of female

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receptivity in female Diptera is variously associated with active corpora allata/juvenile hormone (Manning, 1967; Adams and Hintz, 1969; Ringo and Pratt, 1978), protein feeding (Barton Browne *et al.*, 1976), the presence of vitellogenic ovaries (Chaudhury and Ball, 1973; Kambysellis and Craddock, 1991) or combinations of these factors (Pappas and Fraenkel, 1978; Raabe, 1986b and references therein).

Chen *et al.* (1988) reported that, with each copulation, *D. melanogaster* males deliver *ca.* 1/3 to 1/2 of their paragonial secretion to a female. Hihara (1981) found that females copulating with serially mated males who had previously mated ≥4 times, remated more often and laid fewer eggs than females who mated earlier in the mating series. The lack of an activating effect in late-mated females was apparently due to the depletion of paragonial secretion, as ample sperm were transferred to even 5th mated females (Hihara, 1981).

Males of some sex peptide-using species have a high capacity to mate. Some *M. destructor* males may successfully mate 15 or more females in 10 h (Bergh *et al.* 1992). *L. cuprina* males can effectively copulate with ten or more females in 24 h (Smith *et al.*, 1990). Schmidt *et al.* (1985) note that replenishment of paragonial secretion in *Drosophila* begins within 30 min to 6 h after copulation. In experiments with *L. cuprina*, significant replenishment occurred in 30 min after mating (Smith *et al.*, 1990).

In this paper we quantify effects of reproductive tract extract dosage on onion fly oviposition and mating and establish BR_{100} (Behavioral Response) and BR_{50} values for sex peptide activity. We also address: whether the response to sex peptides is always all-or-nothing, how sex peptide titer and male mating propensity is influenced by age, and whether male extract injected into newly eclosed females is active and when?

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MATERIALS AND METHODS

Rearing. A *D. antiqua* culture originally from University of Guelph, Canada was used in these experiments. Flies were reared at 22 ± 2°C , 35 ± 10% RH, under a photoperiod of 16:8 (L:D), in 1 m x 1 m x 0.75 m screened cages. Adults were given free access to water and a dry diet of 10 parts powdered milk, 10 parts powdered sugar, 1 part brewer's yeast, 1 part soy flour, and 20 parts yeast hydrolosate. Every second day eggs were collected by water floatation from an artificial onion ovipositional resource modified from Harris *et al.* (1987) by the addition of optimal ovipositional holes (Mowry *et al.*, 1989) around the base of each stem. Eggs were then placed into plastic rearing boxes (30 cm x 25 cm x 10 cm) containing a layer of moistened gravel and longitudinally halved onions. A plastic lid with 1 mm mesh screening covered each rearing container. After 14 days, a fresh layer of sliced onions was added, and covered with moistened gravel. Pupae were collected by floatation 7-10 days later, and stored in moist gravel at 4°C.

Adult virgin insects were obtained by collecting flies within 24 h following emergence and segregating the sexes in separate cages with *ad libitum* food and water (Spencer *et al.*, 1992).

Extract Preparation. For Experiments 1, 2 and 3, extracts were prepared from whole male reproductive tracts consisting of testes, paragonial glands, ducta ejaculatoria and the seminal vesicle as per this dissertation, Chapter 2. Male reproductive tracts from 200 individuals (unless otherwise noted) were collected into 100 µl of ice-cold physiological saline (this dissertation, Chapter 1) and homogenized for 10 s with a probe sonicator. Previous experiments (this disseration, Chapters 2 and 4) demonstrated that paragonial gland extract alone elicited the behavioral responses associated with mating, and that

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injected testes extracts had no effect on ovipositional activation and mating inhibition in onion fly; thus, to save time, testes were not removed and the entire reproductive tract was used. Following sonication, reproductive tract homogenates were immediately centrifuged for 30 min at 16,000xg and 4°C. The supernatant, at a concentration of 1, 1/20th, or 1/10th of a male equivalent (male equiv) per 0.5 µl (1 male equiv is 1 reproductive tract), was collected and used as starting extract. Concentrations <1 male equiv/0.5 µl were obtained by collecting fewer reproductive tracts into 100 µl of saline as above.

Injection Insects selected for treatment were randomly drawn from their holding cages and assigned to treatment groups. Females were anesthetized with CO_2 before receiving 0.5 µl of extract or saline via a thoracic injection, using a glass needle pulled from a calibrated 5 µl micropipette attached to a 10 µl syringe as described in this dissertation, Chapter 1. The injection needle was hand-held, and insects were unrestrained throughout the process. Controls included saline-injected as well as untreated virgins and virgin females held with two sexually mature virgin males for mating.

Treated flies were placed singly into 20 cm x 6 cm cylindrical screen cages with food, water and an ovipositional resource modified from Harris *et al.* (1987) by the addition of optimal ovipositional holes (Mowry *et al.*, 1989a). Following treatment, eggs were collected by floatation and counted every other day for 8 days to establish ovipositional rates for each female, unless otherwise noted. Mean eggs/female/d was computed by dividing total eggs laid by the number of days since the onset of oviposition. After the initial 8-day egg-counting period, two 7- to 10-day-old males were added to each cage to test for mating inhibition unless otherwise indicated. Thereafter eggs were collected and counted every other day for an additional 4 to 6 days and held in

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Parafilm™-covered 35 x 10 mm petri dishes on moistened sand. Larvae, hatching from the eggs of mated females, wander and become trapped in droplets of condensed water on the Parafilm™ where they were easily observed. Females whose eggs hatched subsequently to addition of males were considered sexually receptive at the time males were added. The onset of oviposition or an increase in the rate of oviposition after the addition of males was taken as further evidence of mating.

When adequate numbers of eggs were not available to test for hatching or when hatching data were inconsistent with other observations, spermathecae were dissected, crushed and evaluated for the presence of sperm. The 100 µm sperm of *D. antiqua* were readily visible at 100x. Males dying during an experiment were replaced with virgin males of similar age. Only females surviving the entire experiment were included in the analyses. Mortality averaged 14% and never exceeded 23% in any treatment.

Experiment 1: Dosage Response. Extracts from a dilution series prepared from reproductive tracts of sexually mature males were tested for activation of oviposition and mating inhibition. Starting extract was diluted from 1 male equiv per 0.5 µl to 1/10th, 1/20th, 1/40th, 1/80th and 1/160th male equiv per 0.5 µl with physiological saline and injected into 6-day-old virgin females along with three control treatments: untreated, saline injection or normal mating. At the completion of the experiment all spermathecae were dissected to verify female mating status. Each treatment was replicated 35 times (1 fly = 1 replicate).

Experiment 2: Male Age and Extract Activity. This effect was evaluated using extracts prepared as above from males aged 1, 3 or 6 days, at concentrations of 1 and 1/10th male equiv/0.5 µl of extract (prepared from 20 male reproductive tracts collected into 100 µl of onion fly saline) along with

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untreated, saline injected and mated controls. At the completion of the experiment all females were dissected to verify mating status. Treatments were replicated 15 times. Mean daily oviposition per female in Experiments 1 and 2 were analyzed by a Kruskal-Wallis nonparametric test (Zar, 1984).

Experiment 3: Female Age. To assess the effect of female age at injection on ovipositional response, extracts of mature males at 1 and 1/20th male equiv/0.5 µl were injected into 1-, 3-, and 6-day-old virgin females. Eggs were collected daily beginning on the day following injection until flies were 9-days-old. Females were not tested for mating receptivity in this experiment. Treatments were replicated ten times. Data were analyzed by ANOVA and SNK following $(X + 0.5)^{1/2}$ transformation.

Experiment 4: Male Age and Mating Probability. The probability of successful mating by males of various ages was evaluated so that realized matings could be correlated with the age-related mating potential determined in Experiment 2. Single males, aged 1, 2, 3, 4, 5 or 6 days, were enclosed with two 6-day-old females in a screened cage for 24 h to establish probability of mating for males of different ages. After 24 h, the male was removed and the females were left to oviposit for 24 h, whereupon eggs were collected and observed for hatching. If fewer than 20 eggs were laid, a fresh ovipositional cup was returned to the cage and the flies were allowed two more days to oviposit before a second egg count was made. All spermathecae were assessed for sperm presence. There were 20 replicates for each male age treatment. Percent mating as influenced by age was analyzed by linear regression.

Experiment 5: Male Mating Capacity. Dipteran male potency is known to decline with serial mating (Leopold *et al.*, 1971a; Hihara, 1981; Bergh *et al.*, 1992; Cook, 1992). We evaluated the capacity of 6-day-old males to inseminate multiple females by holding 15 males singly with 10 six-day-old virgin

females in 20 cm x 6 cm cylindrical screened cages for 24 h. Thereafter, females were distributed to individual cages with food, water and an ovipositional resource. Resources were changed every other day and any eggs laid were collected and monitored for hatching. After four days, the spermathecae of all females whose eggs had not hatched were examined for sperm. Realized male mating capacity could then be compared to the theoretical mating potential based on the response to dosage of male extract as measured in Experiment 1.

Experiment 6: Ovarian development and female mating receptivity. In an attempt to separate the relative contributions of age and ovarian maturity upon mating receptivity, 120 virgin females were maintained on an *ad libitum* sucrose and water diet for seven days following adult emergence. Removing protein from the diet generated females lacking mature eggs; yet, these females were at an age when normally maintained virgins would be receptive to mating. Four groups of 30 virgin females were transferred into screened cages (30 cm x 30 cm x 37 cm) and held with 30 mature males. After 24 h, the 8-day-old females were removed and dissected to score the developmental state of eggs in their ovaries and the presence of sperm. Eggs in ovaries were classified according to the scale of (Kozhanova and Bogoslovskaya, 1983) as simplified by Kostál (1993) and, where possible, the number of eggs in each ovary was recorded.

RESULTS AND DISCUSSION

Experiment 1: Dosage Response. Analyzed at the treatment level, dosages of 1/20th of a male reproductive tract equivalent and higher caused *D. antiqua* females *both* to increase their rate of egg laying significantly above the negative controls (15 or more eggs/female/d is typical for mated flies

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(Spencer *et al.*, 1992)) *and* to refuse mating even when given continuous access to sexually mature males (Figure 4). Females receiving ≤ 1/40th of a male reproductive tract equivalent had ovipositional rates and mating inhibition patterns not significantly different from negative controls. Thus onion fly males appear to be using chemicals as mate guarding agents (*sensu* Miller *et al.*, 1994). We agree with the interpretation of Schmidt *et al.* (1993a) that simultaneous expression of both mating inhibition and ovipositional activation at a common threshold is suggestive of a single receptor type.

For *D. antiqua,* the rich variation in individual rates of oviposition is better appreciated when the distribution of mean eggs/female/d for each treatment is presented as a dose response surface (Figure 5). This presentation reveals two major ovipositional response classes in the population of extract-injected females (Figure 5). Individuals behaving as virgins (receptive to mating and not ovipositionally active) have low oviposition rates (< 3 eggs/female/d), and occupy the steep-sided peak rising along the top of the response landscape. Mated females or extract-injected females, unreceptive to mating with oviposition rates of ≥ 15 eggs/d and are represented by the diffuse peak at the right center of the surface.

The proportion of females in a treatment laying >15 eggs/d increased with dosage up to 1/20th of a male equiv and then plateaued (Figure 5). The presence of some females laying > 15 eggs/d even at the lowest dosage suggests there is considerable variation among females in the threshold for activation of the mated-like ovipositional response (Figure 5). Ovipositionally activated and unreceptive individuals at 1/160th to 1/40th male equiv are evidence for females with response thresholds below 1/20th male equiv. Of females laying ≥ 15 eggs/d (exclusive of females in the mated treatment), only 2 out of 127 were receptive to mating; both had received

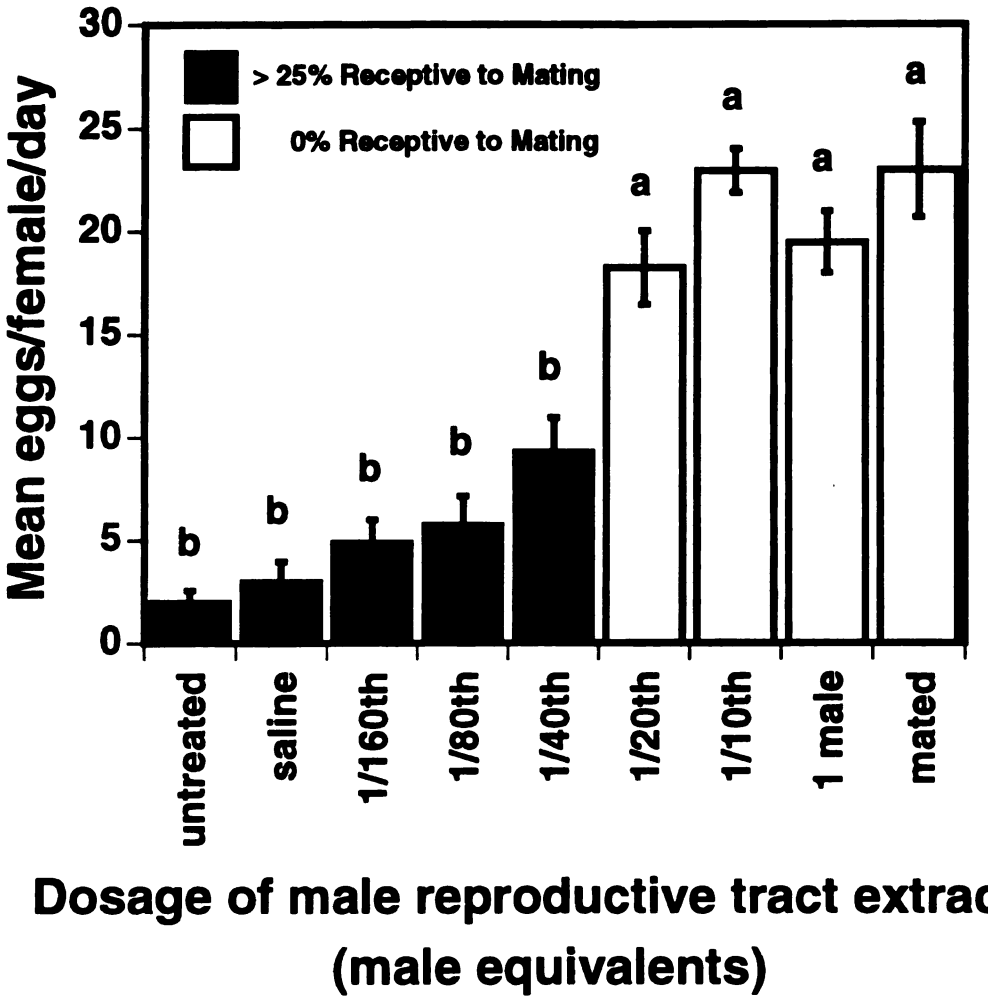

Figure 4. Onion fly mean egg production/female/d ± 1 SE and receptivity status following injection with dilutions of male reproductive tract extract or controls. Number of individuals/treatment: untreated=33; saline=28; 1/160th=32; 1/80th=27; 1/40th=29; 1/20th=29; 1/10th=31; 1 male=32; mated=22. Means with different letters are statistically different (p=0.05, Kruskal-Wallis test, nonparametric multiple comparison test as described by Zar (1984)).

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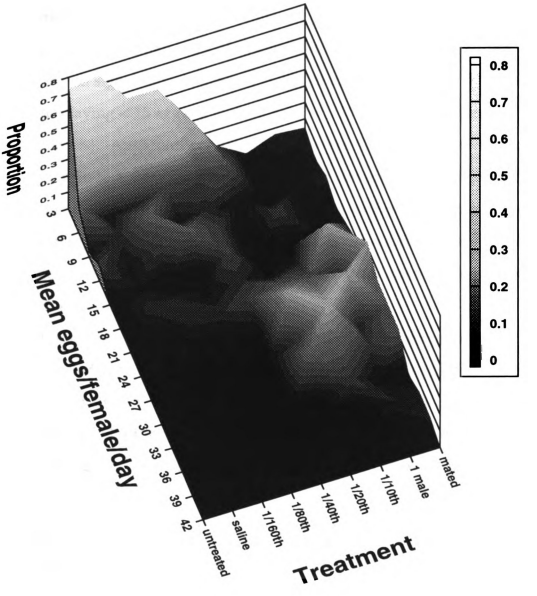

Figure 5. Response surface for onion fly females receiving various dosages of male reproductive tract extract. At any treatment x ovipositional response interval combination, surface elevation represents the proportion of females within a treatment laying eggs at a given daily rate. The mean eggs/female/d axis is presented in three egg increments.

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1/160th of a male equiv injections. All females refused mating at dosages ≥ 1/20th male equiv regardless of how many eggs a female laid; 83% (76/92) of these flies had mated-like rates of oviposition before males were added. There were no significant differences in the percent of receptive females among flies laying < 15 eggs/d at dosages of <1/20th of a male equiv (range 23.1% to 53.6%). Apparently certain females were only partially switched from the virgin to the mated state at low extract dosages. At dosages of ≤1/40th male equiv, females exhibited a range of ovipositional rates, with the majority laying few eggs (≤10 eggs/female/d); however, there were females at all dosages with elevated oviposition (Figure 5), the majority of which were unreceptive to mating.

At dosages ≥ 1/20th of a male equiv, and in mated females, 81.5% (93/114) of individuals had high rates of oviposition (≥15 eggs/female/d) and all were unreceptive to mating. At 1/40th male equiv, 27.5% laid ≥ 15 eggs/female/d, all of these were unreceptive to mating. However, among 1/40th male equiv flies laying < 15 eggs/female/d, 28.6% were receptive to mating.

Before they were tested for mating inhibition, the ovipositional rates of females who were later receptive to mating (3.3 ± 2.9 eggs/female/d (±SE)) was significantly lower than that of unreceptive females (22.2 ± 2.3 eggs/female/d; mean ± SE; $P < 0.0001$); *ca.* 95% of receptive females and half of unreceptive females laid fewer than 10 eggs/d. There were no significant differences in rates of oviposition between treatments for receptive females ($P = 0.68$), indicating that a low rate of oviposition is correlated with receptivity. However, among unreceptive females, there were significant treatment differences in ovipositional rate ($P<0.0001$) mirroring the magnitude and shape of the overall extract dosage response as seen in Figure 4.

That some virgin females lay eggs independent of mating or extract treatment complicates interpretation of the surface plot. The measured distribution of egg production by the combined negative controls could be subtracted from that of extract treatments to correct for virgin oviposition. After that calculation, a ridge, representing *ca.* 5-10% of females at 1/160th, 1/80th, and 1/40th male equiv with intermediate ovipositional rates (3-15 eggs/d) remained between the virgin and mated ranges on the response surface. The existence of unreceptive females whose ovipositional response is intermediate between virgin and mated suggests that the sex peptide-mediated behavioral changes are not always all-or-none, but may be intermediate, with respect to oviposition, at dosages of 1/80th and 1/40th male equiv. There were also females at dosages of 1/160th, 1/80th, and 1/40th male equiv with egglying rates ≥ 15 eggs/female/d and low mating receptivity: only 2/14 of such females mated. These females probably had low thresholds for response to injected extract. Based on the distributions of mean eggs/female/d and the patterns of receptivity for each treatment, the threshold for full ovipositional activation and 100% mating refusal (BR_{100}) is 1/20th of one male reproductive tract equivalent injected into a virgin female. Likewise, we estimate that the BR_{50} for onion fly ovipositional activation lies between 1/20th and 1/40th of one male equiv.

According to our data, expression of sex peptide-mediated behavioral changes in *D. antiqua* can be decoupled at intermediate dosage. In *D. melanogaster*, the linkage has been thought to be tight (Schmidt *et al.*, 1993a), however, quantification of the female response based on the binary variables of mated vs. not mated and ovulated vs. not ovulated, may conceal some graded effects. Existence of decoupled responses suggests that the signal

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processing mechanism for onion fly sex peptide is capable of more complex integration than would be predicted by a simple switch-like module.

When the substantial individual variability (Figure 5) was damped by plotting mean ovipositional rate against extract dosage from zero male equiv (saline injection) to 1/20th male equiv (non-plateauing dosages) a striking linearity was evident (Figure 6). The steep linear response by females to increases in sex peptide titer quantifies average relative rewards males can realize due to increments of paragonial secretion delivered. With respect to full activation, the cost of risk avoidance is only 1/20th of a male equiv. The linearity of Figure 6 indicates that the ovipositional effect of *D. antiqua* sex peptide is decidedly not switch-like at a population level. The now oft-referenced all-or-none switching effect of sex peptides may largely be attributable to an overwhelming effect at high dosages, like those normally delivered by males.

Experiment 2: Male Age. Extracts made from 1-, 3- or 6-day-old males all were capable of eliciting mating inhibition and ovipositional activation, however, the full female response to 1 day-old-male extract was achieved only upon injection of 1 male equiv/female rather than 1/10th male equiv/female (Figure 7). All females receiving extracts from 3 or 6-day-old males refused mating when tested. Injections of 1 male equiv from 1-day-old males also caused mating inhibition in females, however, among females receiving 1/10th male equiv from a 1-day-old male, 14% were receptive to mating when tested. The reduced response to 1 day-old-male extract at 1/10th male equiv suggests that reproductive tracts of 1-day-old males may not be fully charged. Morphometric data (K. Grimnes and J. Miller, In manuscript)

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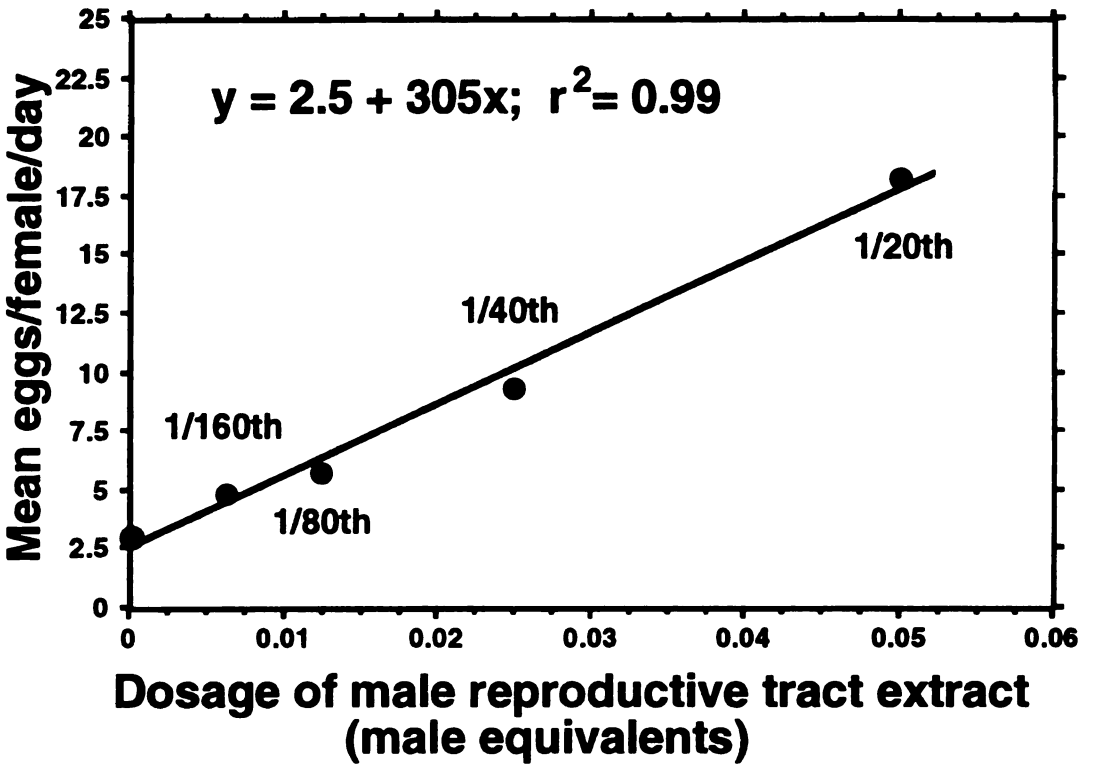

Figure 6. Linear regression on onion fly mean eggs/female/d for treatments 1/160th to 1/20th male equiv. Beyond 1/20th male equiv the ovipositional response plateaued.

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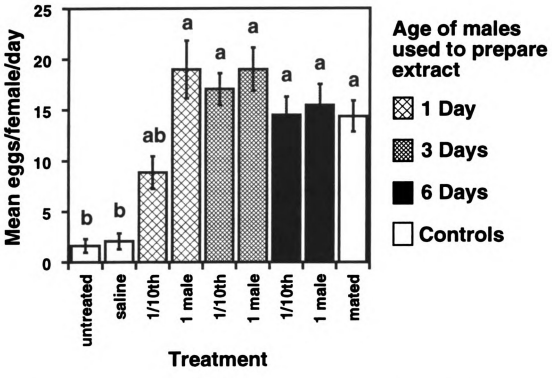

Figure 7. Onion fly mean egg production/female/d following injection with male reproductive tract extracts prepared from 1-, 3-, or 6-day-old virgin male onion flies. Number of individuals/treatment: untreated=28; saline=21; 1-day-old male extracts: 1/10th=14, 1 equiv=13; 3-day-old male extracts: 1/10th=13, 1 equiv=13; 6-day-old male extracts: 1/10th=13, 1 equiv=15. Means with different letters are statistically different. p=0.05, Kruskal-Wallis test, nonparametric multiple comparison test as described by Zar (1984)).

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support this interpretation; they show secretion volume doubling during the first few days of adult life and plateauing by *ca.* 7 days.

Experiment 3: Female Age. When held with males for 24 h, only 6-day-old flies mated and laid eggs, indicating that the onset of sexual maturity occurred between days 3 and 6. In our laboratory culture, the usual time of mating for onion fly females coincides with maturation of the first flush of eggs at age 5-6 days; oviposition commences the photophase following mating. 1-, 3- & 6-day-old female *D. antiqua* injected with 1/20th or 1 male equiv of male reproductive tract extract all expressed ovipositional activation when they were *ca.* 6-days-old (Figure 8). Expression of mated-like behavior at normal times by mature flies following early injection of male extracts, suggests that the active factor(s) is stable in the hemolymph, or is able to stably bind neural receptor(s) after entering the hemolymph.

In *D. melanogaster* experiments using heat shock and yolk protein promoters to express Drm-SP-I, sex peptide effects on receptivity and oviposition can occur within 1-1.5 h after gene expression in transformed females. Moreover, the waning of response seen within 8 h following a single 30 min pulse of exposure to Drm-SP-1 can be eliminated when the sex peptide gene is continuously expressed (Aigaki *et al.*, 1991). We speculate that Drm-SP-I has a rather short half-life in *Drosophila* females and is degraded or removed from the hemolymph rapidly, thus the transient response. Under normal conditions, the sustained response in *Drosophila* (lasting 7-9 days (Chen *et al.*, 1988)) due to the sperm effect would take over before the sex peptide influence waned.

Given that *D. antiqua* sex peptide effects may be manifest even 6 days after injection, the dynamics of the interaction between the *D. antiqua* sex

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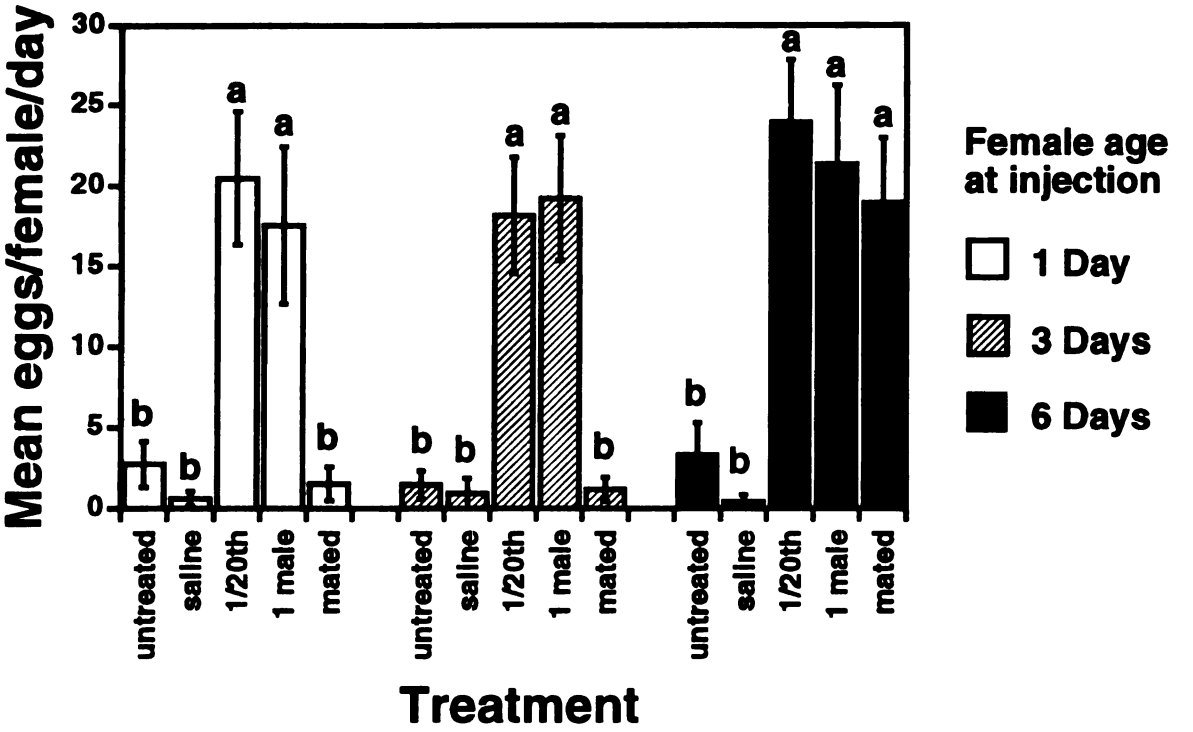

Figure 8. Onion fly mean egg production/female/d for females injected at age 1, 3 or 6 days with male reproductive tract extract dilutions or controls. Data analysis performed by ANOVA with mean separation by SNK following $(X + 0.5)^{1/2}$ transformation.

peptide and its receptors must be quite different from that in *D. melanogaster.* There is no evidence for any role of sperm or testes secretions in maintenance of the effect in *D. antiqua,* as paragonial gland extracts alone sustained ovipositional activation and mating inhibition for a lifetime (this dissertation, Chapter 1). Pursuit of the biochemical bases of such differences in sex peptide dynamics between *Drosophila* and *Delia* might lead to an understanding of monogamy vs. polygamy in Diptera.

Experiment 4: Male Age and Mating Probability. Over the first 6 days of adult life, there was a significant increase in the probability of male mating with age ($r^2 = 0.86$, $F=24.7$, $P<0.01$)(Figure 9). Among males who mated, the mean number of females mated per cage also increased significantly with age: 3-, 4-, 5-, and 6-day-old males averaged 1.0, 1.3, and 1.9 matings per cage, respectively, (slope significant at $P<0.01$) suggesting male mating ability improved with age. The percent of males mating successfully was 50% by age 6 days. In previous experiments, 50% male mating success was typical under these conditions and did not rule out female refusal as a contributing factor. To achieve ≥ 80% female mating success, we included two males per female in mating controls. The data of Experiment 2 indicated that males possess some sex peptide in their paragonia *ca.* 2 days before they first mate, though few mature sperm are present in the testes of 1 or 2-day-old males (K. Grimnes, personal communication). It appears that a lack of sufficient numbers of mature sperm limits mating until age 3 days.

Experiment 5: Male mating capacity. If at mating, an onion fly male transfers *ca.* 1/3 to 1/2 of its paragonial secretion, like *D. melanogaster* (Kubli, 1992), and that product has a threshold of effectiveness of *ca.* 1/20th male equiv, a male would transfer above-threshold doses for 5 consecutive

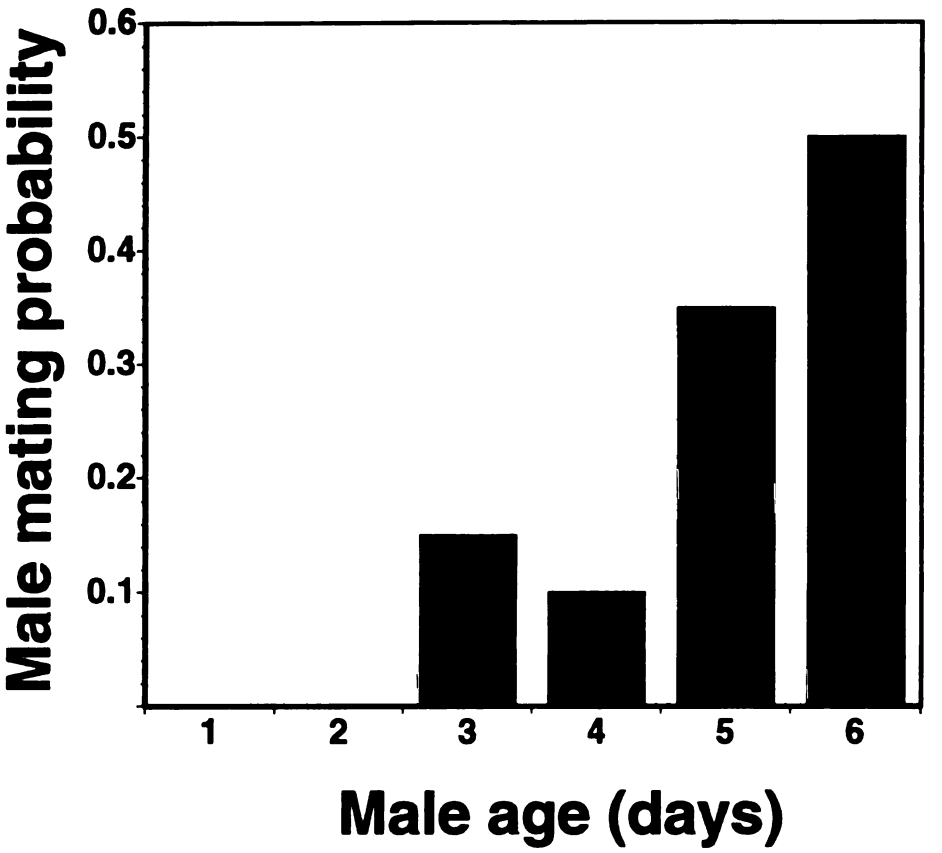

Figure 9. Probability of virgin male onion flies, between age 1 and 6 days, mating when held with two 6-day-old females for 24 h. There were 20 replicates for each male age treatment.

matings. Of the 12 males mating, the mean number of matings per mature male was 4.3 ± 0.6 (SE), (range: 1-9 matings/24 h); this number falls very close to the estimated capacity of 5 matings/male *D. antiqua.* In these and other experiments (this dissertation, Chapter 1), we have never observed males to transfer sperm without switching female behavior to the mated state. The behaviors of male onion flies appear to be reproductively efficient.

Experiment 6: Ovarian development and female mating receptivity. Autogenous development of the first batch of eggs had not been reported for *D. antiqua* until now; however autogeny was known in the close relative *D. radicum* (Kostal, 1993). Egg developmental state was bimodally distributed among 8-day-old sugar-fed females (Figure 10); 59% had previtellogenic or early vitellogenic eggs, 10% had eggs well into vitellogenesis but lacking chorions, and 31% of females had at least some mature chorionated eggs. The mean number of mature eggs for sugar-fed females with some stage 8 eggs was only 23.0 ± 1.3 (mean ± SE, n=33). A wide variety of egg developmental stages was common for ovaries of sugar-fed females. Virgin females, fed the complete diet of Ticheler (1971), have a full complement of *ca.* 50-60 uniformly mature eggs by age 8 days. Sugar-fed females could use only larval-derived proteins to provision eggs. It appeared that certain eggs garnered a majority of the limited protein resources and matured during vitellogenesis at the expense of the other eggs.

Females with complements of eggs in different developmental stages mated in this experiment. No significant differences in probability of mating ($P = 0.25$) were found across females with egg complements at different stages of development. However, flies with mature eggs were more likely to be mated than females with immature eggs at any stage ($P = 0.02$). Among sugar

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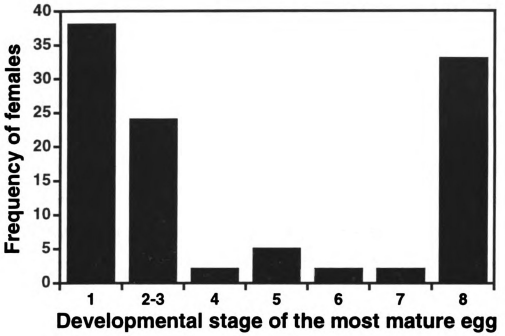

Figure 10. Frequency of 8-day-old sugar-fed females with their most developed eggs in each developmental category (after Kostál, 1993)(n=106). Stage 1 eggs were completely undifferentiated previtellogenic eggs. Stages 2-3 and Stage 4 were progressively larger previtellogenic eggs. Stage 5 were early vitellogenic eggs, stage 6 and 7 were late vitellogenic eggs. Mature chorionated eggs were stage 8 (after Kostál, 1993) .

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fed females with only previtellogenic eggs (n=64), 35% mated. Given the mating data from Experiment 3, these results falsify the notion that mature eggs are required for mating, though mating is more likely with advancing ovarian development. This suggests that the onset of receptivity is associated with an ontogenic change rather than egg maturation *per se.*

Other unevaluated factors are likely to impact the onset of receptivity and ovarian development in onion fly females. In many Diptera and other insects, changing titers of juvenile hormone play a critical role in determining the onset of female receptivity and the timing of vitellogenesis. Application of exogenous JH or analogs can induce the onset of precocious sexual activity and pheromone production (Adams and Hintz, 1969; Truman and Riddiford, 1974; Dillwith *et al.*, 1983; Adams *et al.*, 1984 and references therein). Application of juvenile hormone or its analogs can effect precocious sexual receptivity in *Drosophila grimaldi* (Ringo and Pratt, 1978) and will restore receptivity to allatectomized *Musca domestica* (Adams and Hintz, 1969). Implantation of active ring glands into *D. melanogaster* pupae resulted in precocious copulation by females (Manning, 1967). Perhaps a JH increase explains the discrepancy in mating probability among differently fed females with eggs at similar stages of development. If an increase in the titer of JH is developmentally timed, this would explain why older previtellogenic females in our experiment were receptive to matings. An association between maturing eggs and the onset of mating receptivity could free a female from the attention of males until after the normally anautogenous *D. antiqua* female has completed foraging necessary to provision her eggs.

GENERAL DISCUSSION

Sex peptide, produced in male reproductive tract of *D. antiqua* and other insects, is a potent modifier of female behavior. Purified *D. melanogaster* sex peptide elicited mating refusal and oviposition in conspecific females at 3.0 pmol/female (Kubli, 1992). The concentrations of synthetic (0.6 pmol/female) or purified (3.0 pmol/female) Drm-SP-I necessary to elicit mating refusal and oviposition in female *D. melanogaster* fall within the dosage range of other insect peptide hormones, suggesting that its effect may be hormone-like: oxidized and reduced forms of *Helicoverpa zea* Pheromone Biosynthesis-Activating Neuropeptide (PBAN) have pheromonotropic activity at 0.5 and 2.0 pmol/female respectively (Raina *et al.*, 1991); *Manduca sexta* diuretic hormone is active at 20 pmol/larvae (Kelly *et al.*, 1994), and *Ae. aegypti* oostatic hormone shows activity at 240 pmol/female (Borovsky *et al.*, 1991).

Sperm competition by males may have selected for potent sex peptide delivery systems that are effective even after repeated matings over short periods deplete paragonial products. Among Diptera using sex peptides, thresholds for response to glandular extracts are reported to be < 1/64 of a male equivalent for *Ae. aegypti* (Craig, 1967), though the average male only has the capacity to induce unreceptivity and completely inseminate 4-6 females and to partially inseminate an additional 2-6 during his lifetime (Hausermann and Nijhout, 1975). In radiolabelling studies of *L. cuprina* receptivity-inhibiting substance (RIS) (Smith *et al.*, 1990), virgin males transferred 23% of their initial quantity of accessory gland secretion during the first mating of a series. This quantity was sufficient to keep 50% of females unreceptive for 8-12 days. By the 6th mating (males average 10

matings in 10 h of access to virgin females), males transferred labeled material equal to 1/30th male equiv; 50% of 6th-mated-females were still unreceptive 8 days after mating (Smith *et al.*, 1990). Beyond the 6th mating, the RIS effect and sperm transferred declined rapidly and a marked loss of effectiveness was evident.

In many insects using sex peptides, the number of matings per male is not limited by a male's potential capacity to effect behavioral change in females (calculated from the sex peptide threshold). Moreover, there appears to be a disparity between the sex peptide dosages for full activity (BR_{100}) or threshold activity (BR_{50}) and the quantity males actually transfer to females. Based on mean frequency of male *D. antiqua* mating (4.3 times/d) and the dosage of male reproductive tract extract needed to reach the BR_{100} or BR_{50} (*ca.* 1/20th and 1/40th male equiv respectively), we calculate that *D. antiqua* males are providing *ca.* 5-10 times the amount of sex peptide necessary to activate females. In *Ae. aegypti,* males normally pass *ca.* 1/5th of the contents of their seminal vesicles and accessory glands to females at mating (Jones and Wheeler, 1965), *ca.* 12 times what is necessary (based on a threshold (BR_{50}) of *ca.* 1/64th male equiv (Craig, 1965)). Male *L. cuprina* transfer 23% of a male equiv resulting in *ca.* 100% loss of receptivity for >12 days; however, as little as 1/30th (3.3%) of a male equiv causes 50% of females to remain unreceptive for 8 days (Smith *et al.*, 1990); the safety factor here is *ca.* 7x. In *M. destructor,* a 0.4 male equiv extract of male abdomens caused mated behavior in all females recovering from injection (Bergh *et al.*, 1992), suggesting that this concentration is well above the threshold for activity. The fact that *M. destructor* males effectively induced mated behavior in 10-15 serially mated females (Bergh *et al.*, 1992) indicates sex peptide is active at fractions of a male equivalent.

Oviposition and prolonged remating inhibition follow copulation with a fully charged male or injection of male extracts in each of the above species. *D. melanogaster* represents an interesting exception; both the threshold and the amount of sex peptide transferred are the same; 3.0 pmol (for purified sex peptide (Kubli, 1992)), there is no apparent safety factor. *D. melanogaster* is unique in having a sex peptide causing only a 24-72 h loss of receptivity and stimulation of oviposition. However, if the sex peptide is acquired along with sperm during mating, the female will not remate until 7-9 days after mating. Injection of sex peptide-containing extracts alone is sufficient to elicit mated-like behavior in *Ae. aegypti* (Fuchs *et al.*, 1969), *L. cuprina* (Smith *et al.*, 1989), *M. destructor* (Bergh *et al.*, 1992), or *D. antiqua* (Spencer *et al.*, 1992); there is no "sperm effect" (Manning, 1967) for these species. The association of excess sex peptide delivery with long periods of mating inhibition suggests that monogamy may be a consequence of sex peptide overdoses.

In a treatise on the physiological manifestation of the maternal-fetal conflict during human pregnancy, Haig (1993) notes that genetic conflicts tend to escalate and may be quantifiable by their inefficient outcomes, e.g., extraordinarily high titers of critical hormones. He suggests that the interchanges during an escalating interaction could contain information about the quality and robustness of the interactants. Accordingly, sex peptide chemistry and titer might offer a means for a female insect to evaluate mate quality via the "chemical abstract" (Sivinski, 1984) associated with sperm.

High doses of sex peptide may be critical if a male is to avoid sperm competition by quickly rendering a female refractory. Males transferring too little sex peptide to preclude remating usually fertilize few eggs because of the strong tendency for the last sperm into the spermathecae to be the first dispensed (sperm precedence, Parker, 1984). Though for onion fly we did not

measure the time course of mating inhibition vs. dosage, the onset of oviposition varied with dosage. Of ovipositing onion flies who received 1/160th, 1/80th or 1/40th male equiv, 40% delayed oviposition by *ca.* 2 days, while only 11% of flies receiving ≥ 1/20th male equiv had similar delays.

Sex peptide dosage is likely to be influenced by mating duration. When females control cessation of copulation and sperm are transferred before the accessory secretions are passed, males would be selected to rapidly transfer a potent dose of sex peptide. From the male standpoint, the transfer schedule of ejaculate components may represent a compromise between maximizing potential offspring and insuring rapid induction of mating refusal within an female-controlled interval. When males control the termination of mating, the probability of passing an adequate dose of sex peptide could be increased by extending the duration of secretion transfer. In *M. domestica,* the duration of copulation increased in serially mated males (Leopold *et al.,* 1971a), suggesting that males may be compensating for depleted secretion stores. In both *L. cuprina* and *M. destructor* the mating duration of serially mated males did not change, even as males became increasingly depleted of accessory secretion (and gametes) late in the mating series (Smith *et al.,* 1990; Bergh *et al.,* 1992). Perhaps in these species, the female determines when copulation terminates. In that capacity, she may be able to "force" males to rapidly transfer a potent dose of sex-peptide. If they cannot, impotence is revealed. Female *M. destructor,* mating late in the mating series, ignored inadequate matings and began calling again after soon after uncoupling with depleted males (Bergh *et al.,* 1992).

It is unclear whether sex peptides represent an interesting intersexual conflict. Perhaps monogamy in some Diptera is the favored state for females who might benefit from an uninterrupted life of oviposition free from the

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distracting advances of males. Extended exposure to multiple males is known to reduce female *D. melanogaster* longevity and fecundity (Partridge *et al.*, 1986). However, monogamous females would experience a trade off in the reduced diversity of their progeny, and perhaps reduced fertility with time.

CHAPTER 3

Lifetime Ovipositional Patterns of Mated and Virgin Onion Flies, *Delia antiqua*

ABSTRACT

Over their 47.2 ± 1.9 (S.E.) day lifetime, mated onion flies, *Delia antiqua*, held individually and provided with optimal diet and ovipositional resources, oviposited much more uniformly than did virgins. Mated females began ovipositing at 6 to 7 days old and regularly deposited 14.0 ± 0.6 (S.E.) eggs/d for *ca.* 3-4 weeks. Thereafter, oviposition slowed and stopped *ca.* 1 week before death. By contrast, virgin flies began ovipositing at 24.7 ± 1.5 (S.E.) days into their 59.0 ± 3.8 (S.E.) day lifespan, and deposited eggs at an increasing rate over the next *ca.* 3-4 weeks, so as to generate a mean overall ovipositional rate of 7.3 ± 2.1 (S.E.) eggs/female/d. An 11.8 day disparity in the mean lifespan of mated and virgin females was largely accounted for by the later onset of oviposition by virgins and not a longer ovipositional period. Mean lifetime egg production of mated females was 475 ± 27 eggs versus 179 ± 30 eggs for virgins. Ovipositional and post-ovipositional periods (34.4 ± 1.8 days and 7.2 ± 1.0 days, respectively) for mated females were not significantly different from those of virgin females (28.2 ± 3.5 days and 6.7 ± 1.2 days; P =0.30 and P =0.74 respectively). Over 90% of virgin females laid some eggs before death. The durations of egg depositional behaviors (initial subsurface probing, stationary phase, and terminal subsurface probing) did not differ with mating status. Thus, virgins deposit eggs via an identically

running motor program as that for mated females; but virgins activate this program less frequently on average. This study revealed substantial individual variation in virgin oviposition.

INTRODUCTION

The change from the virgin to the mated state in most insects is marked by a temporary to lifelong loss of mating receptivity (Barth and Lester, 1973; Leopold, 1976; Raabe, 1986a). Mated behaviors are affected by stimuli associated with copulation and transfer of spermatophores, sperm, or male reproductive secretions. Interaction of spermatophore contents with the genital tract of virgin female *Hyalophora cecropia* initiates release of an hormonal signal from the bursa copulatrix, resulting in mated behavior (Riddiford and Ashenhurst, 1973). In *Lymantria dispar*, mechanical stimuli and sperm transfer were required for permanent suppression of virgin calling behavior (Giebultowicz *et al.*, 1991). In *Pieris rapae crucivora*, physical presence of a spermatophore stimulates bursa copulatrix stretch receptors to trigger a mate refusal posture (Obara *et al.*, 1975; Sugawara, 1979). Where mechanical stimulation is initially responsible for inhibiting mating in Lepidoptera, chitin within the walls of spermatophores may keep the structure inflated until sperm are stored and humoral factors assume control of nonreceptivity (Drummond, 1984). In Orthoptera, contributions from the male accessory reproductive glands and mechanical stimulation from the spermatophore or insertion of male genitalia are all involved in mediating calling behavior and oviposition (Tantawy and El-Helw, 1965; Liang and Schal, 1994 and references therein).

Sex peptides produced in male paragonial glands, and transferred in the ejaculate during mating, are responsible for the onset of mated behavior

in a number of Diptera, e.g., *Aedes* (Leahy and Craig, 1965; Craig, 1967; Hiss and Fuchs, 1972; Adlakha and Pillai, 1975; 1976), *Drosophila* (Manning, 1967; Hihara, 1981; Chen, 1984; Chen and Balmer, 1989; Ohashi *et al.*, 1991; Schmidt *et al.*, 1993b), *Delia* (Swailes, 1971; Spencer *et al.*, 1992), and others (Adams and Nelson, 1968; Riemann and Thorson, 1969; Leopold, 1976; Smith *et al.*, 1989; Bergh *et al.*, 1992). Both sex peptide and sperm presence are necessary for longterm suppression of female *Drosophila melanogaster* mating receptivity and ovipositional stimulation (Kubli, 1992). In *D. antiqua*, mating or injection of as little as 1/20th male equivalent of paragonial gland extract terminates receptivity and activates oviposition for >2 weeks (this dissertation, Chapter 2). Rapid termination of receptivity immediately following copulation in sex peptide-using flies may be due to mechanical stimulation (Leopold, 1976). The response may bridge the interval between uncoupling and the time required for humorally mediated (sex peptide) mating suppression to take effect.

Reduced mating receptivity and activation of oviposition are not exclusive traits of mated females. Where the probability of mating is normally 100%, just the passage of time may cause virgin females to become unreceptive to mating, as is the case for the chalcidoid wasp *Megastigmus bipunctatus* (Thornhill and Alcock, 1983 p. 453). Virgin females of many species will eventually lay some eggs, e.g., *Rhodnius prolixus* (Davey, 1967), *Teleogryllus commodus* (Loher and Edson, 1973), *Hyalophora cecropia* (Truman and Riddiford, 1971), *Bombyx mori* (Yamaoka and Hirao, 1973), *L. dispar* (Barbosa and Martinat, 1987; Giebultowicz *et al.*, 1990), and *Helicoverpa zea* (Teal *et al.*, 1990), though virgin oviposition is usually delayed. With time, some virgins may become unreceptive to mating and lose their sexual attractiveness. For example, gypsy moths, *L. dispar*, cease pheromone

production and begin oviposition by age 5-6-days if not mated (Giebultowicz *et al.*, 1990). Reduced pheromone titer and increased oviposition in aging *H. zea* virgins are related to a bursa copulatrix-produced factor responsible for suppression of pheromone production in both mated and older virgin females (Teal *et al.*, 1990).

In the Diptera, virgin oviposition has been quantified for: *Drosophila melanogaster* (Partridge *et al.*, 1986; Bouletreau-Merle, 1990), *Drosophila viriis* (Aigaki and Ohba, 1984), *Mayetiola destructor* (Harris and Rose, 1991), *Rhagoletis pomonella* (Prokopy and Bush, 1973), *Anastrepha suspensa* (Lentz, 1994), *Aedes aegypti* (Fuchs and Kang, 1978; Vandehey *et al.*, 1979), and *Musca domestica* (Riemann and Thorson, 1969). Bouletreau-Merle (1990) selected *D. melanogaster* for the ability to retain eggs for long or short periods, a trait thought to be mediated by JH (Hudak and Gromko, 1989). It has been suggested that virgin oviposition in *Aedes* mosquitoes may be due to loss of an ovipositional inhibitor (Fuchs and Kang, 1978). Virgin oviposition was not selectable in *Ae. aegypti* (Vandehey *et al.*, 1979) or in *D. antiqua* (A. Peters, unpublished).

Oviposition has been suggested to be under constant inhibition in *Tipula* and *Nephrotoma* crane flies; decapitation of gravid females leads to the rapid release of all of a female's eggs (Chiang and Kim, 1962). Headless *D. antiqua* do not initiate oviposition, however if carefully decapitated after a bout of deposition has commenced, several eggs can be deposited in a normal fashion (J. Spencer, personal observation). This observation is consistant with a brain-activated egg-movement motor program residing in the fused thoracic ganglion, that once activated, runs independently of the higher centers (Mowry *et al.*, 1989b).

In the present study, I quantify the phenomenon of virgin oviposition for *D. antiqua* individuals whose egg output was monitored for their lifetime and compared to the ovipositional patterns of mated individuals. Also, video recordings were used to compare mated and virgin egg depositional behaviors. This study was undertaken as part of an in-depth investigation into the mechanisms governing host-colonization behaviors by *D. antiqua* with the goal of interrelating the effects of external and internal inputs acting over time (Miller and Strickler, 1984), and to gather baseline ovipositional data for mated and virgin females.

MATERIALS AND METHODS

Rearing. A. *D. antiqua* culture ("Field-Grant") originally collected in 1986 (and supplimented with additional field flies in 1989) from onions left in harvested fields in Grant, Michigan was used for all experiments. Flies were reared at 22 ± 2°C , 35 ± 10% RH, under a photoperiod of 16:8 (L:D), in 1 m x 1 m x 0.75 m wooden-framed screen cages as described in this dissertation, Chapter 1. Adult virgin insects were obtained by collecting flies within 24 h following emergence and segregating the sexes in separate 30 cm x 30 cm x 42 cm screened wooden cages with *ad libitum* food and water. Experiments were conducted in an environmental chamber under conditions reported in Chapter 2.

Ovipositional resources for bioassays: The *D. antiqua* ovipositional resource consisted of a 1 oz. jelly cup filled with moist silica sand into which an artifical onion stem (Harris *et al.*, 1987) was inserted vertically and 8 optimally sized ovipositional holes (Mowry *et al.*, 1989a) were punched into the sand around the base.

Lifetime Ovipositional Experiment. Cohorts of 3-day-old virgin female *D. antiqua* were randomly drawn from their holding cages and individually placed into 20 cm x 6 cm cylindrical screen cages with food, water, and an ovipositional resource. Food was provided *ad libitum* in 1 cm diam plastic vial caps replenished every three days. Water was supplied in 1.5 cm diam x 1.5 cm plastic cup inverted on a filter paper-lined 3 cm diam petri dish and was renewed every other day.

There were two treatments: virgin and mated. Virgin females were held individually without access to males for their entire lives. Females to be mated were confined with two sexually mature 7-day-old males for five days beginning at age 3 days. Females failing to deposit fertilized eggs within the seven days were provided with an additional 7-day-old male, for three more days. All males were removed, regardless of female mating status on day 10, and females were held alone for the remainder of their lives. Most of the data on the longevity and ovipositional response of virgin females were collected in 1991, while most of those for mated females were recorded early in 1992. Where runs for a given treatment were initiated at different dates, there was no appreciable effect of starting date. The lifetime ovipositional data generated here for individually held mated flies were virtually identical to those collected in 1985 and 1988 as reported by Weston *et al.*(1992) who also drew from this *D. antiqua* culture. Our results agree well with those of Vernon and Borden (1979) who used a culture maintained at Simon Frasier University, British Columbia, Canada.

For the first 30 days of the experiment, ovipositional resources for virgins were changed every other day, while those for mated flies who laid more eggs were changed daily. After day 30, the resources in the virgin and mated cages were changed every third and every other day respectively until

all the flies were dead. Eggs were collected from the ovipositional resources by flotation, counted, and held in Parafilm™-covered 35 mm x 10 mm petri dishes on moistened sand. Larvae hatching from fertilized eggs wander and become entrapped in droplets of water condensed on the Parafilm™ where they are easily counted. Eggs from inseminated vs. uninseminated females can thus be readily distinguished. The eggs of females verified to be mated were reassayed periodically over the whole experiment.

All virgin and mated ovipositional data were converted to mean eggs/female/d for rate analysis. Data were analyzed by ANOVA (StatView 4.0, Abacus Concepts).

Video Records of Mated and Virgin Oviposition; Egg depositional behaviors of 10-day-old mated and 20-day-old virgin females (aging increased the probability of observing virgin oviposition) were videorecorded using the methods of Mowry et al. (1989b). Groups of five, virgin or mated female *D. antiqua* were color-coded with a dot of tempera paint applied to the thorax and introduced into a 15 cm x 15 cm x 24 cm video arena in the late morning. In early afternoon an optimal ovipositional resource configured to allow observation of subsurface depositional events (Mowry *et al.*, 1989b) was added to each video cage. A JVC BY110 U video camera with C-mount adapter and a Nikon 50mm Nikkor lens was used to record behavior on a Panasonic AG 1950 VCR. Videotaping began when predepositional examining behaviors (Harris and Miller, 1991), such as frequent ovipositor probing of the substrate, indicated deposition was imminent; and, it continued until a bout of deposition yielding several eggs was completed. Twenty mated and virgin females were observed for several hours during the afternoon and evening for two days. Ovipositional records were obtained for five virgins and nine mated females. Depositional records were replayed in slow motion and the

duration of the elements as identified by Mowry *et al.* (1989) was quantified and analyzed by ANOVA (StatView 4.0, Abacus Concepts).

RESULTS

Lifetime Ovipositional Experiment. Mated female *D. antiqua* began ovipositing earlier, had higher ovipositional rates, laid more total eggs, and died sooner than virgins (Table 3). Nevertheless, mated and virgin ovipositional lifespans, defined as the interval between first and last oviposition, were not significantly different. Post-ovipositional lifespans were also not significantly different.

Patterns of oviposition for mated and virgin females (Figure 11) differ in both the onset and rate of oviposition. There was little variation in the age at first oviposition among mated females (Table 3, Figure 12); and once beginning to oviposit at age 6.4 ± 0.2 days (mean ± SE) most continued at a high and steady rate for *ca.* 3-4 weeks (Figures 11 & 13A). The linearity of the early ovipositional response is characteristic of mated females during the first weeks of their ovipositional lifespan (also see Figure 3, this dissertation Chapter 1). The BT_{50s} (age by which females had deposited 50% of their lifetime total egg output) of the virgin and mated populations were offset by 31 days.

Virgin *D. antiqua* have a very different ovipositional pattern dominated by the relatively slow recruitment of laying virgins from the ranks of the non-layers (Figures 12 & 13B). Compared to mated females, virgins began laying eggs much later at *ca.* 24.7 ± 1.5 days (mean ± SE)(Table 3); 89% of mated females had begun to oviposit before any virgin females had laid eggs (Figure 12). Combining ovipositional results from many individual virgins whose age at first oviposition varied (Figure 12 & 13), yields a cumulative

Table 3. Lifetime ovipositional measures for mated and virgin onion flies

Variable	Mated Females (mean ± SE)	n	Virgin Females (mean ± SE)	n	P value[b]	
Lifespan (days)	47.2 ± 1.9	69	59.0 ± 3.8	70	0.009	**
Age at first oviposition (days)	6.4 ± 0.2	69	24.7 ± 1.5	64[a]	<0.0001	**
Ovipositional lifespan (days)	34.4 ± 1.8	69	30.9 ± 3.6	64	0.3025	ns
Lifetime egg production (eggs)	475.0 ± 26.8	69	179.0 ± 30.0	70	<0.0001	**
Ovipositional rate (eggs/female/day):						
over lifespan	10.1 ± 0.4	69	2.7 ± 0.4	64	<0.0001	**
over ovipositional lifespan	14.2 ± 0.6	69	8.0 ± 2.3	64	0.0089	**
Post-ovipositional lifespan (days)	7.2 ± 1.0	69	6.7 ± 1.2	64	0.74	ns

[a]Six virgin females did not oviposit.
[b]Data analyzed by ANOVA

Cumulative Mean

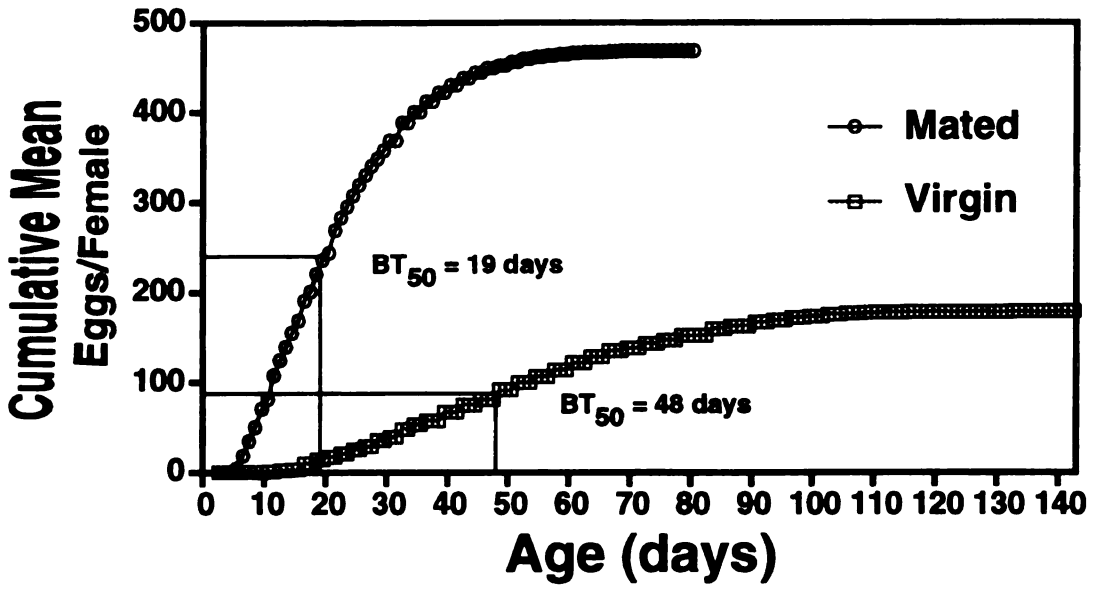

Figure 11. Cumulative mean eggs per female for mated and virgin *D. antiqua*. Mated individuals were maintained with two males for ten days and then held singly for the remainder of their lives. Virgin females were held singly throughout their lifetime. There were initially 70 females in each treatment.

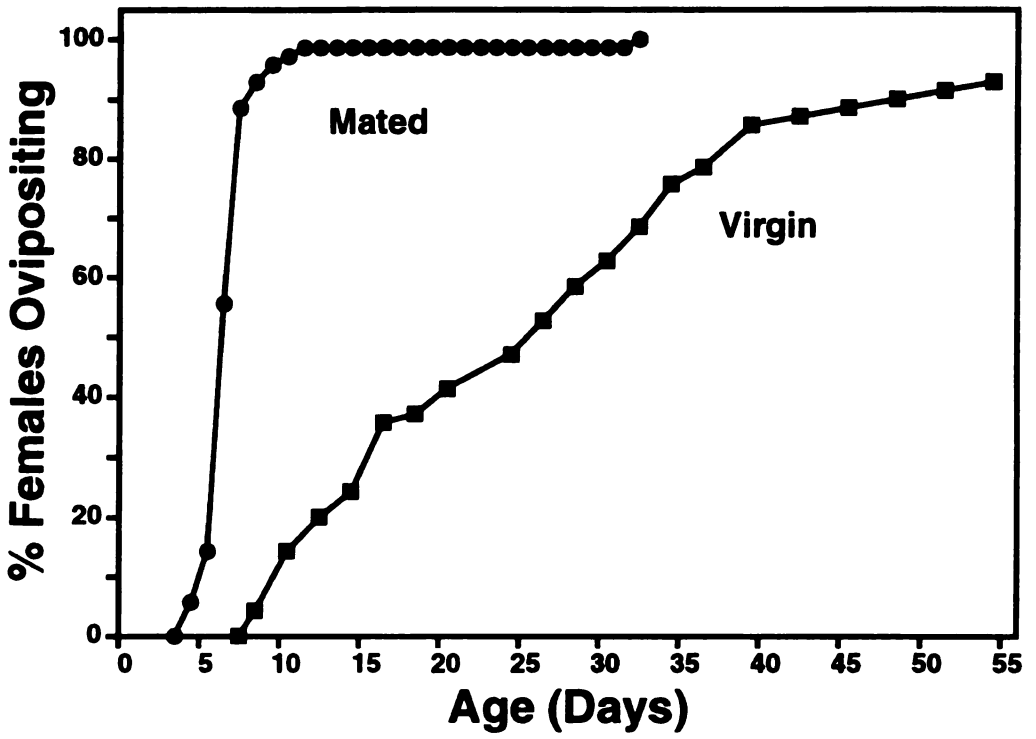

Figure 12. Cumulative percent of mated and virgin female *D. antiqua* having oviposited as a function of time.

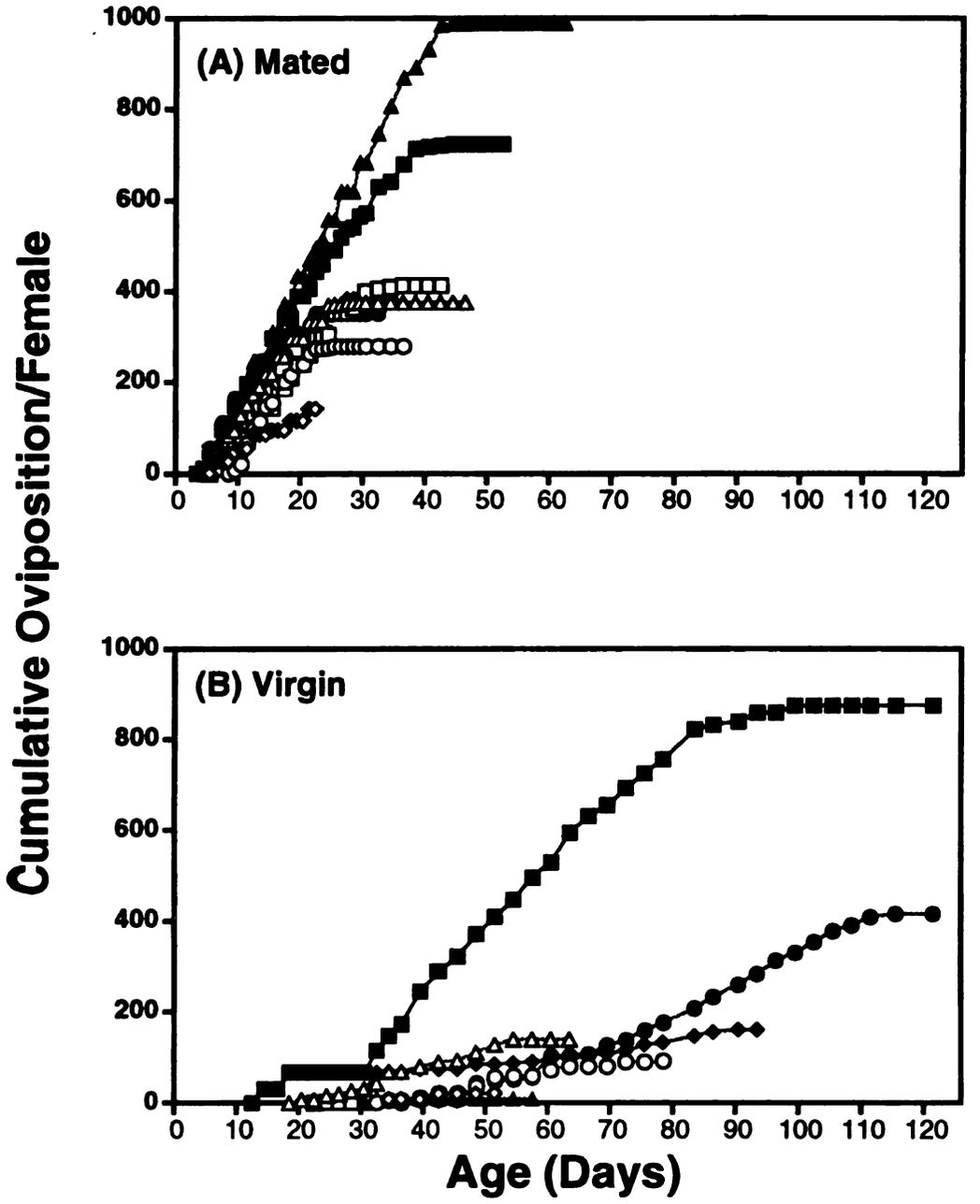

Figure 13. Cumulative lifetime ovipositional records for eight mated (A) and eight virgin (B) female *D. antiqua* randomly selected from the pool of 70 records for each.

oviposition curve having a gradual slope (Figure 11) which conceals considerable variation for individuals (Figure 13B).

When cumulative oviposition was normalized with respect to maximum number of eggs laid and synchronized according to the day of first oviposition for both mated and virgin flies, cumulative ovipositional curves and BT_{50}s of Figure 11 became quite similar (Figure 14). The slope of cumulative oviposition was still highest for mated females.

The ovipositional landscape of Figure 15 presents a three dimensional frequency histogram of mean eggs/female x age for mated (A) and virgin (B) females. Nearly all mated females rapidly sustained oviposition at a rate of 10–18 eggs/female/d, and then gradually slowed and ceased egg-laying before dying (also see Figure 13A). The steady pace of mated female oviposition is reflected in the ridge of ovipositional peaks at *ca.* 10-14 eggs/d in Figure 15A. Some mated individuals were capable of even higher rates of oviposition, laying eggs at twice the typical mated rate for short periods (Figure 15A). Very few mated females averaged less than 10 eggs/female/d early in the experiment, though late in their lives, rates of oviposition declined and fell into the former valley between the virgin and young-and-mated states (Figure 15A). A similar void was found in this zone in a landscape plot of young-and-mated female oviposition from a *D. antiqua* reproductive tract dose response study (this dissertation, Chapter 2, Figure 5).

Once ovipositing, few virgins sustained rates as high as those for mated females, and the frequency of egg-laying was more irregular (Figure 15B). However, just as in the mated population, exceptional individuals are evidenced by the scattered peaks of oviposition around 16 eggs/d (Figure 15B). These females seem to behave indistinguishably from mated females except for elapsed time until oviposition begins. When synchronized with respect

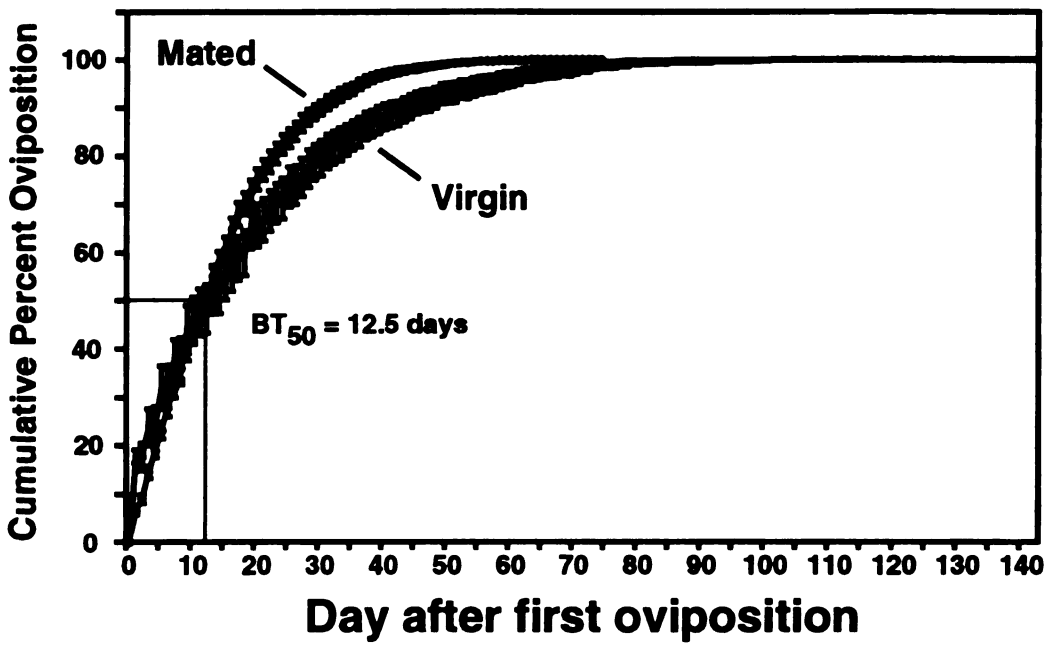

Figure 14. Cumulative oviposition for mated and virgin *D. antiqua* normalized as a proportion of total eggs (± SE), and synchronized to the day of first oviposition for both mated and virgin females.

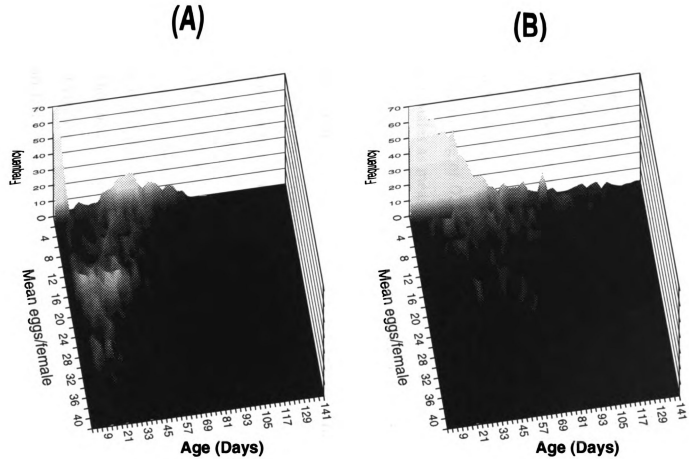

Figure 15. Ovipositional landscape for mated (A) and virgin (B) *D. antiqua*. At any mean eggs/female x age combination, surface elevation represents the number of females laying eggs at that rate. Frequencies are presented in increments of 2 flies, with frequencies of less than 2 per interval not shown.

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to age at first oviposition (Figure 15B & 16A) the scattered distribution of virgin oviposition contracts somewhat and (Figure 16B) becomes a bit more similar to the mated landscape (Figure 15A).

Nearly half of *D. antiqua* virgins laid 50 or fewer eggs (6 laid zero eggs, 12 between 1 and 10 eggs, an 16 between 11 and 50 eggs), whereas only 2/70 mated females laid less than 50 eggs (they laid 1 and 46 eggs, respectively) (Figure 17). Surprisingly, the greatest number of eggs laid in this experiment came from a virgin female who laid 1297 eggs over 87 days, averaging 14.9 eggs/d.

Video Records of Mated and Virgin Oviposition; The behavioral elements and timing of *D. antiqua* oviposition were very similar for mated and virgin females as measured from video records (Table 4). Time spent during the initial subsurface examining phases before deposition and the interval between deposition of successive eggs within a bout were not significantly different between mated and virgin flies. The difference between duration of subsurface probing following the deposition of the last egg in a depositional bout(terminal subsurface probing) by mated or virgin females was just significantly different. Both virgins and mated females engaged in "stationary phase" (*sensu* Mowry *et al.*,(1989)), a period of complete arrestment during deposition when an egg rests in the bursa copulatrix for *ca.* 16 s before egg expulsion; fertilization occurs during this time in mated females. Duration of stationary phase was not significantly different between mated and virgin flies. The number of eggs laid/bout by mated (2.6 ± 0.52 eggs; mean ± SE, n=9) and virgin (3.8 ± 0.97; mean ± SE, n=5) females during video observation was also not significantly different and was similar to egg numbers reported by Mowry *et al.* (1989).

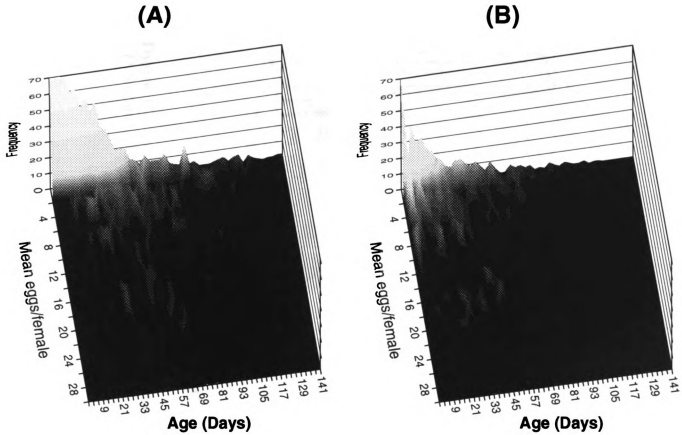

Figure 16. (A) ovipositional landscape for virgin *Delia antiqua*. At any mean eggs/female x age combination, surface elevation represents the number of females laying eggs at a that rate. (B) virgin *D. antiqua* oviposition transformed to synchronize the day of first oviposition for all flies to day 1.

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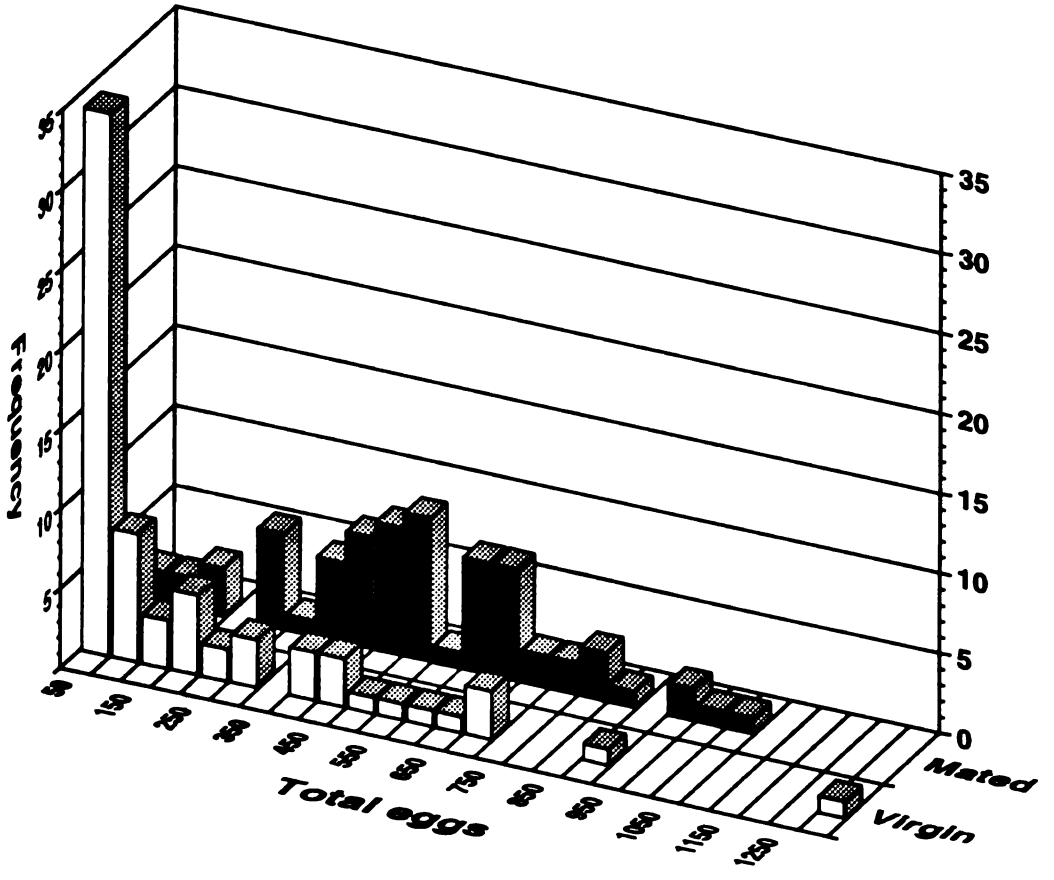

Figure 17. Frequency histogram of total oviposition for mated and virgin *D. antiqua*. Mated and virgin n=70.

Table 4. *Mean duration of egg depositional behaviors for mated and virgin female*

Table 4. Mean duration of egg depositional behaviors for mated and virgin female *D. antiqua*.

Treatment	n	Duration (seconds) (mean ± SE) Initial Subsurface Probing[a]	Stationary Phase	Interegg Subsurface Probing	Terminal Subsurface Probing	Mean eggs/ depositional bout (±SE)
Mated	9	25.7 ± 4.4	14.7 ± 0.3	14.4 ± 1.2	8.1 ± 1.0	2.6 ± 0.5
P value[b]		0.3480 ns	0.0928 ns	0.1582 ns	0.0482 *	0.2700 ns
Virgin	5	36.2 ± 13.2	16.6 ± 1.4	27.9 ± 12.3	4.3 ±1.5	3.8 ± 1.0

[a]Terminology of Mowry *et al.* (1989)
[b]Data analyzed by ANOVA

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DISCUSSION

Oviposition by virgins is probably uncommon in commercial field populations of *D. antiqua*. In the lab, where densities are high, females are likely mated soon after they become sexually receptive, making prolonged virginity atypical for most *D. antiqua*. In the field, farming practices and restriction of onion culture to scattered patches of rich organic soil, concentrate field populations of *D. antiqua* and put receptive females in proximity with nutritive resources, ovipositional sites and potential mates. Under the resource defense polygyny mating system of *D. antiqua* (J.R. Miller, unpublished), aggressive mate searching by males make it unlikely that a female would remain unmated for long after becoming sexually receptive. Nevertheless, under conditions of lifetime mating deprival, most virgin *D. antiqua* will eventually lay eggs (that do not hatch) at about half the rate of mated females.

Virgin oviposition may become important during rare selection events. Under conditions of low population density and thus reduced likelihood of timely mating upon reaching sexual maturity, females capable of retaining their eggs until they were eventually mated may have a selective advantage over those who commence virgin oviposition. If the onset of oviposition initiates a fixed reproductive period, females retaining eggs until mated will enjoy a fecundity advantage over laying virgins who may eventually mate having expended a portion of their limited reproductive period in the laying of unfertilized eggs.

How does virgin oviposition differ from mated? ***Lifespan.*** Virgins lived on average 11.4 days longer than mated females (Table 3). Longer virgin lifespans are known for various insect species (Lockshin and Zimmerman,

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1983); the phenomenon is particularily well documented in *Drosophila* (Partridge and Andrews, 1985; Partridge *et al.*, 1986; 1987), where exposure to males and increased egg-laying both shorten female *D. melanogaster* lifespan (Partridge *et al.*, 1987). However, these factors are not independent, since increased egg-laying and the attendant depletion of sperm stores, leads to re-mating (Gromko and Gerhart, 1984; Gromko *et al.*, 1984). The significant life-shortening effects of male *D. melanogaster* exposure on females were reversible if males were removed (Partridge *et al.*, 1986).

A lifetime study of the effect of mating status on individual *Drosophila virilis* (Aigaki and Ohba, 1984) yielded results similar to ours for onion fly. Virgin females lived significantly longer (87.6 vs 72.5 days), reached peak oviposition at age 30 days; 20 days later than mated flies, and had total egg production equal to 85% of mated females. Roitberg, (1989) has shown that in the egg- and time-limited fly, *Rhagoletis basiola*, opportunities to oviposit and not a female's mated state were key factors in determining lifespan.

Age at first oviposition. High variability in the age at first oviposition for virgins and low variation in this measure for mated females suggests mating synchronizes the inception of oviposition. By the age of 8 days, 93% of mated females had laid fertilized eggs (Figure 13). Correcting for the lack of virgin oviposition synchrony yielded an ovipositional pattern somewhat resembling that for mated females in its compact form (Figures 16B & 15A). The difference in age at first oviposition (18 days) may partially account for the difference between virgin and mated lifespan. Harris and Rose (1991) found similar ovipositional patterns for virgin and mated *M. destructor;* longer virgin lifespan could be accounted for by the *ca.* 2 day delay until oviposition in this short-lived species.

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For both mated and virgin females, the age at first oviposition was independent of ovipositional and post-ovipositional period duration (see below: How are virgin and mated oviposition similar?). Earlier age at first oviposition was correlated with a 0.92 eggs/d increase in the rate of oviposition ($r^2 = 0.06$, $P = 0.04$) for mated females, though it was not correlated with the rate of virgin oviposition ($r^2 = 0.005$, $P = 0.56$). Age at first oviposition was independent of total eggs laid for both mated ($r^2 = 0.042$, $P = 0.09$) and virgin ($r^2 = 0.0002$, $P = 0.91$) females. A delayed onset of oviposition was correlated with longer lifespan in virgins ($r^2 = 0.22$, $P < 0.0001$); lack of correlation between ovipositional onset and lifespan ($r^2 = 0.006$, $P = 0.59$) is not unexpected among mated females, especially since there was little variation in age at first oviposition in this group.

Rate and pattern of oviposition. Kostal (1993) reported "great variability" in the ovipositional rates of unmated *D. radicum* females, individual rates of oviposition varied from normal mated-like rate (*ca.* 8 eggs/d) to near complete inhibition of oviposition. For *D. antiqua* during the ovipositional period, mated onion flies laid eggs at twice the rate of virgins (Table 3); mean eggs/female/d during the ovipositional lifespan was independent of lifespan for both mated ($r^2 = 0.027$, $P = 0.18$) and virgin females ($r^2 = 0.013$, $P = 0.17$) indicating that there was no longevity cost for producing more eggs. During shorter-term ovipositional bioassays which are usually completed well before significant virgin oviposition begins, mated and virgin ovipositional rates typically differ by a factor of *ca.* 10 (this dissertation, Chapters 1 & 2).

Compared with oviposition by mated females, virgin oviposition in insects is often variable. In *H. zea* and *Spodoptera ornithogalli*, erratic temporal oviposition patterns of virgins relative to mated females suggest

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that mating is important in establishing the temporal pattern of the ovipositional response (Adler *et al.*, 1991). *Choristoneura fumiferana* do not exhibit a preference when given a choice between oviposition sites of a different suitability unless they are mated (Rivet and Albert, 1990).

Conversely, some virgin females display very regular ovipositional patterns. Virgins of the cricket *Telogryllus commodus* initially lay very few eggs (11/week), but by 3 weeks post-emergence average a steady *ca.* 160 eggs/week, compared to *ca.* 260 eggs/week for mated females (Tantawy and El-Helw, 1965). Ovipositional behavior of mated and virgin females of the arrhenotokous (males develop from unfertilized eggs, and females develop from fertilized ones) sawfly *Dineura virididorsata,* is also very similar, even down to the time spent in egg deposition (Walter *et al.*, 1994).

When normalized with respect to maximum number of eggs laid and synchronized according to the day of first oviposition, the two cumulative oviposition curves of Figure 11 became very similar but not identical (Figure 14). The slope of cumulative oviposition for virgin *D. antiqua* was still lower than that for mated females. However, similarity in the overall pattern of oviposition following transformation of mated and virgin cumulative oviposition curves (Figure 14) suggests that there does not appear to be a fundamental difference in the process of egg-laying for mated or virgin females once the onset and rate of oviposition are factored out. The similarity in depositional behavior durations (Table 4) also supports this hypothesis. Unpublished results of A.S. Peters indicate that laying virgin onion flies have the same ovipositional host preferences as mated females.

Total oviposition. Mated *D. antiqua* females laid significantly more eggs (2.6 x) than virgins (Table 3; Figure 11). Mated females reached 50% of their total egg production (BT_{50}) in only 19 days; the average virgin needed 48

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days. That offset in the BT_{50} vanished when both curves are normalized and synchronized to the day of first oviposition for each individual (Figure 14). Some individual virgins exceed mated output (Figure 17), however, mated-like egg production was the exception. Nearly half of *D. antiqua* virgins laid 50 or fewer eggs (6 laid zero eggs, 12 between 1 and 10 eggs, and 16 between 11 and 50 eggs), whereas only 2/ 70 mated females laid less than 50 eggs (they laid 1 and 46 eggs respectively) (Figure 17).

How are virgin and mated oviposition similar? ***Ovipositional and post-ovipositional periods.*** It is remarkable that ovipositional lifespans, elapsed time between deposition of the first and last eggs, were not significantly different for mated and virgin females (Table 3). Working only with mated flies Weston and Miller (1987) and Weston *et al.* (1992) also found no difference in the ovipositional lifespans of host-deprived or undeprived *Delia* flies even though there were significant differences in total oviposition. The ovipositional lifespan is not determined by the number of eggs laid. The same conclusion was reached in this experiment. However, because the ovipositional rate was independent of ovipositional lifespan (see below), flies with longer ovipositional lifespans had more opportunity to lay eggs.

Post-ovipositional lifespans, the interval between the day of last oviposition and death, were also not significantly different for mated and virgin females (Table 3). Given the similarity in ovipositional and post-ovipositional periods it is not surprising that lifespan and ovipositional lifespan were correlated for mated ($r^2 = 0.77$, $P < 0.0001$) and virgin females ($r^2 = 0.76$, $P < 0.0001$).

Ovipositional and post-ovipositional lifespan durations were independent of the age at first oviposition for mated ($r^2 < 0.001$, $P = 0.79$; $r^2 = 0.056$, $P > 0.05$) and virgin females ($r^2 = 0.007$, $P = 0.47$; $r^2 = 0.006$, $P = 0.53$)

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respectively, even though the mean age at first oviposition was different. Similarities in duration of ovipositional lifespans for both mated and virgin females suggest that mechanisms responsible for generating these patterns of oviposition are independent of how or at what age oviposition is initiated. Ovipositional lifespan may be a genetically determined interval. Perhaps ovipositional activation entails programmed gene expression which once activated is irreversible and independent of the number of eggs produced and deposited. It is interesting to note that by the time an average mated female reached the end of her ovipositional period at *ca.* 40-days-old, the first of her offspring would be nearing sexual maturity and might begin competing for the same resources.

Depositional behavior. Similarity in the duration of depositional behaviors and the fact that virgins engage in a stationary (fertilization) phase suggests that the normal egg depositional program can run independently of mating status. Apparently *D. antiqua* females do not sense the absence of sperm via reproductive tract feedbacks. These data lend further support to the suggestion of Mowry *et al.,* (1989b) that *D. antiqua* egg deposition is under the control of a deterministic fixed action pattern.

Why do virgins oviposit?

Dispersal. In *D. antiqua,* virgin oviposition seems to be maladaptive, especially if the female is capable of mating and laying viable eggs. Though its onset is delayed relative to mated females, eggs laid by some 66-day-old mated females were viable implying that ovipositing virgins might not be too old to produce offspring if mated. However, the mating receptivity of old laying virgins is not known. In commercial onion fields, male contact is very highly probable.

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Perhaps virgin oviposition plays a role in dispersal. In *D. antiqua,* where provisioning of the first eggs occurs before mating, females unable to find mates (or held without access to males) may reduce the mass of eggs they are carrying in order to facilitate dispersal to a different patch where a mate may be encountered. If virgin oviposition is not accompanied by a loss of mating receptivity and serves to jettison eggs to relieve build-up, laying females may not be severely penalized if a mate can later be secured. The similarity between mated and virgin depositional behavior and ovipositional patterns might suggest that virgins are not "throwing away" eggs. Or, perhaps "normal" oviposition is the only way eggs can be expelled by this animal.

Senescence. Lockshin and Zimmerman (1983) suggest that an "unequivocal decline in physiological function long before death of the insect" is the best criterion to identify senescence. Under that definition, declining oviposition during the later portion of the ovipositional period (Figures 11, 15(B) & 16(B)) and the occurence of post-ovipositional periods (Table 3) may qualify *D. antiqua* virgin oviposition as a senescence phenomenon. In that light, the tendency for oviposition and other changes typical of the virgin-to-mated transition (like reduced pheromone titer and mating inhibition in Lepidoptera (Giebultowicz *et al.,* 1990)) to begin at an age well beyond when mating and oviposition normally occur may be evidence for senescence.

Eggload. *D. antiqua* produces mature eggs in flushes of *ca.* 50 every 2.8 days (one mature egg in each ovariole) (Weston *et al.,* 1992). When deprived of an optimal host, mated females overcome host deprivation in *ca.* 2 days (*ca.* one ovarian cycle) and begin sustained oviposition (Weston *et al.,* 1992). Eventual oviposition following deprival may be a function of accumulating mature eggs which promote oviposition to make room for developing eggs.

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Assuming that females in the Weston *et al.*, (1992) experiment began with a full compliment of 50 mature eggs, after 2 days of deprival each could have fully provisioned an additional 40 eggs so as to have 90 or more mature eggs stored in her ovaries at the time oviposition began. It is not uncommon for some *D. antiqua* to lay 90 or more eggs on occasion (6 times the average of *ca.* 15 eggs/female/d), suggesting that normal mated females have the capacity to hold at least *ca.* 90 eggs (2 eggs/ovariole) for a short time.

In a sample of 50-day-old virgin females who had never oviposited (A.S. Peters, unpublished), a few exceptional individuals were found to be retaining nearly 200 eggs, (an average of >3 eggs per ovariole, range 2-7 eggs/ovariole). Many of the eggs in the ovarioles of these retaining females were dented, evidently due to compression from eggs surrounding them. Egg accumulation in the ovaries of most 50-day-old females was not so extreme; typically they had ovarioles with at most two or three mature eggs in each, suggesting that under conditions of mating deprival the upper limit for egg retention may be *ca.* 125 eggs. Extreme overaccumulation of eggs may explain dented and deformed eggs and empty chorions recovered in this experiment from ovipositional resources and the ovaries of some aging virgin and mated females.

In this experiment, virgin females retained eggs an average of 18.7 days beyond sexual maturity (Table 3). If egg production were not attenuated and eggs were accumulating in virgins, those ovipositing later would have a larger stock of eggs and be capable of depositing larger clutches than earlier laying virgins. In fact, there was no correlation between age at first oviposition and the number of eggs laid by virgins ($r^2 = 0.03$; $P = 0.19$) or mated females ($r^2=0.014$; $P =0.33$). There was also no evidence that first-time ovipositing females laid more eggs on average; laying virgins averaged only

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6.8 ± 0.9 eggs/d (mean ± SE) eggs on their first day of oviposition, a value close to the 7.8 eggs/d average (Table 1) during their ovipositional period. On their first day of oviposition, mated females averaged 25.1 ± 1.9 (mean ± SE) eggs, nearly double their average daily rate (Table 1). Mature eggs do not appear to be very effective at initiating virgin oviposition.

In the cabbage fly, *D. radicum*, vitellogenesis of the second flush of eggs occurs only after the first has been deposited at *ca.* 4.7 days (Kostal, 1993). Development of the second flush of eggs was arrested (Kostal, 1993) and the mature eggs were retained when mated females had no suitable ovipositional site. Among mated *D. radicum* females deprived of ovipositional sites, development of oocytes in the penultimate follicles resumed (in spite of the presence of mature eggs) and some females began to oviposit by age 8 days. It appears that for mated female *D. radicum*, mature eggs have a limited capacity to inhibit subsequent flushes even in the absence of optimal stimuli. On the other hand, virgin females, held with an ovipositional resource, had arrested egg development; they required *ca.* 16 days to oviposit their first eggs (Kostal, 1993).

A similar mechanism for regulating egg maturation may be functioning in *D. antiqua*. Weston *et al.* (1992) interpretted similar numbers of eggs remaining in the ovaries of females deprived of various ovipositional stimuli throughout their lifetimes as evidence that the rate of egg deposition and not egg manufacture determined the length of an ovarian cycle. They went on to hypothesize that host-deprived flies spent more time examining before they laid eggs. If unable to lay all their mature eggs, the next cycle of vitellogenesis would be delayed, which would disrupt the expression of ovipositional periodicity on subsequent days (Weston *et al.*, 1992). If egg

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The mechanism behind the gradual onset of virgin oviposition was not revealed by the present study, though qualitative similarities between laying virgin and mated female ovipositional patterns suggest that whatever initiates egg laying in virgins acts like mating or injection of sex peptide. Hall (1994) notes that in *D. melanogaster*, pleiotropy makes it is difficult to identify specific genes responsible for most mating behaviors or reproductive abilities. A notable exception is the finding that only a few genes are responsible for the pheromonal differences contributing to reproductive isolation between *Drosophila* species (Coyne *et al.*, 1994). The onset of virgin oviposition is likely the result of interactions among various internal excitatory and inhibitory inputs associated with sexual maturity and as well as insect age and host availability.

In several-day no-choice tests, Harris and Miller (1991) found mated female *D. antiqua* females on wax plus Pr_2S_2-treated artifical onion stems laid eggs at twice the rate of those on wax alone stems (13.6 vs 6.5 eggs/ovipositional bout respectively). On a per egg laid basis, females on the less stimulatory, wax-only surrogate spent twice as much time examining as females on the wax plus Pr_2S_2 surrogate. In addition, the most frequent behaviors of females on the less stimulatory host had long latencies to behaviors (surface and subsurface probing) more directly associated with eventual oviposition. Females on the wax plus Pr_2S_2 surrogates spent twice as much time surface probing or subsurface probing; the latter is strongly associated with deposition of eggs (Harris and Miller, 1983). Thus, flies that laid more eggs had quicker transitions to behaviors associated with oviposition, progressed faster through examining behaviors, and on average

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spent less time in predepositional examining for each egg laid. In this example, flies that progressed through examining twice as fast laid twice as many eggs. Dissections later revealed that flies from both groups had begun with similar numbers of mature eggs (Harris and Miller, 1991), indicating that differences in ovipositional rate were not related to eggload.

I postulate that internal conditions (e.g., mating status, eggload, and recent ovipositional experience) determine female responsiveness to host cues, and that, once engaged, the pace of predepositional examining is a function of that responsiveness. Mated females or females not having recently oviposited would be more responsive and progress through predepositional examining to repeated bouts of egg deposition quicker than lesser stimulated females. Because predeposition precedes deposition (Harris and Miller, 1983; 1991) and there is constancy in eggs deposited/bout (Table 4), differences in ovipositional rate could be a reflection of individual differences in propensity to engage in predepositional examining.

If earlier age at first virgin oviposition were a reflection of greater propensity to oviposit, then the first *D. antiqua* virgins to oviposit should have had higher rates of oviposition than later ovipositing virgins. Lack of correlation between age at first virgin oviposition and ovipositional rate (r^2 = 0.005, P = 0.56) suggests that the onset of virgin oviposition is not solely a function of a female's propensity to oviposit. No reliable predictor of virgin oviposition was uncovered in this study.

If propensity to oviposit is an important factor determining how many eggs a female lays on a host, I propose that inputs which make oviposition more probable, like mating/sex peptide transfer and, in time, mating deprival, affect oviposition by first acting on the predepositional examining phase of onion fly host colonization. The duration of predepositional

examining is envisioned to be a function of a female's baseline level of excitation measured against the requisite level of stimulation needed for engagment of the depositional program. Females at a higher initial level of stimulation, would require less examining before oviposition is released, and engage in more bouts per unit time. These data suggest that mating increases the likelihood of host acceptance and plays a defining role in tuning the rate and onset of oviposition.

Even though oviposition is not stimulated to mated-like levels in most virgins, virgin oviposition provides a unique opportunity to study endogenous factors leading to the induction of oviposition, in a species where exogenous factors affecting oviposition are relatively well understood (Miller and Strickler, 1984; Harris *et al.*, 1987; Havukkala and Miller, 1987; Mowry *et al.*, 1989a; Keller and Miller, 1990). Deciphering how specific stimulatory and inhibitory inputs interact to affect behavior is crucial to developing realistic models of insect host acceptance behavior. Characterization of mating effects and other stimuli which bias the outcome of the probabilistic stages of host acceptance behavior is an important step in the identification of major decision-making units and the discernment of their properties.

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CHAPTER 4

Specificity of Onion (*Delia antiqua*), Seedcorn (*D. platura*), and Cabbage Fly (*D. radicum*) Sex Peptide Communication

ABSTRACT

Reproductive gland extracts of onion (*Delia antiqua*), seedcorn (*Delia platura*), and cabbage fly (*Delia radicum*) males suppressed mating and stimulated normal oviposition when injected into conspecific virgin females. The extracts of *D. antiqua* and *D. platura* were completely cross-active. *D. radicum* extract fully activated *D. antiqua*, and stimulated full mating inhibition and partial ovipositional activation in *D. platura*. *D. antiqua* extract was only partially active in *D. radicum* and that of *D. platura* had no effect on either ovipositional activation or mating inhibition in *D. radicum*. These results suggest that *D. antiqua* and *D. platura* are more closely related to one another than either is to *D. radicum* and agree with published anatomically-based phylogenies and a genetic distance calculation based on 8 enzyme loci. The occurrence of sex peptide cross-activity, though asymmetrical, between *D. radicum* vs. *D. antiqua* and *D. platura* indicates that, functionally, sex peptides have changed little during the evolution of this genus. Paragonial gland extracts of *Aedes aegypti* (mosquito) and *Drosophila melanogaster* did not activate *D. antiqua*, nor did synthetic *D. melanogaster* sex peptide or *Aplysia californica* (sea slug) egg-laying hormone. An emerging pattern of broad cross-activity within genera suggests that sex peptides are not an initiator of reproductive isolation. However, depending on the direction of sex peptide asymmetry relative to the direction of

intermating asymmetry, sex peptides might influence the intensity and outcome of interspecific reproductive conflicts between genetically incompatible populations; possibly by promoting satyrization (Ribeiro, 1988).

INTRODUCTION

The paragonial glands of various male Diptera (e.g., *Aedes* (Leahy and Craig, 1965; Fuchs *et al.*, 1968), *Lucilia* (Smith *et al.*, 1990), *Musca* (Adams and Nelson, 1968; Riemann and Thorson, 1969), *Drosophila* (Chen *et al.*, 1988; Schmidt *et al.*, 1993b) produce potent pheromones that, when transferred to virgin females via the semen, inhibit mating and stimulate oviposition. The active factor(s) are so far proving to be proteinaceous and are called "sex peptides". The possibility that sex peptides or their chemical mimics might be exploited as agents of insect birth control (Miller *et al.*, 1994), has stimulated research into both behavioral effects and chemistry of these molecules. From the applied perspective, it will be important to understand sex peptide specificity both to assess potential product breadth as well as likelihood for affecting non-target species. Sex peptide specificity might be an important attribute of insect biology, e.g., if involved in mate recognition.

There is already evidence that some sex peptides are cross-specific even though their chemical structures may vary slightly among close relatives. For example, the cross-active sex peptides of *Drosophila melanogaster* and *Drosophila sechellia*, Drm-SP-I and Drs-SP-I (according to the nomenclature of Raina and Gäde (1988)) are both 36mers, but they differ by three amino acids at positions 8, 11, and 12 (Chen and Balmer, 1989). *Drosophila suzukii* sex peptide, Drsuz-SP-I, is a 41mer with homology to the N- and C-terminal portions of both Drm-SP-I and Drs-SP-I (Schmidt *et al.*, 1993a). Though *D. suzukii* and *D. melanogaster* belong to different subgroups of the

melanogaster group (Schmidt *et al.*, 1993b), Drsuz-SP-I and Drm-SP-I are completely cross-active for both mating and ovipositional effects. Drm-SP-I is also active in *Drosophila simulans* and *Drosophila mauritiana*, other members of the *melanogaster* subgroup (Chen and Balmer, 1989).

A second sex peptide from *D. suzukii*, initially identified by its ovulational stimulating activity, also suppressed mating, and was cross-active in *D. melanogaster* (Ohashi *et al.*, 1991). Though incompletely characterized, it is known to be > a 35mer, with a molecular weight of 3990 (Ohashi *et al.*, 1991). Its amino acid composition is different from Drsuz-SP- I (Ohashi *et al.*, 1991), Drs-SP-I (Chen and Balmer, 1989), and Drm-SP-I (Schmidt *et al.*, 1993b), No evidence for a second sex peptide has been found in *D. melanogaster* (Schmidt *et al.*, 1993b).

Considerable sex peptide cross-activity also exists among mosquito species. Gland implants or injection of *Ae. aeqypti* accessory gland extracts stimulated mated-like behavior in a number of *Aedes*, *Anopheles* and *Culex* mosquitoes (Craig, 1967).

But, heterospecificity is not always reciprocal. In *Aedes* mosquitoes, *Aedes albopictus* paragonial extracts injected into *Ae. aegypti* females suppress receptivity and fully activate oviposition, but reciprocally there is minimal effect (Leahy and Craig, 1965; Lentz, 1994). A similar asymmetry was found between *Aedes atropalpus* and *Ae. aegypti* (Ramalingam and Craig, 1976). Between *D. suzukii* and *Drosophila pulchrella*, members of the *suzukii* subgroup of the *melanogaster* species group, 0.25 male equiv of *D. suzukii* male paragonial extract had little stimulatory effect on females of *D. pulchrella* (Fuyama, 1983). However, reciprocal injections of *D. pulchrella* extract into *D. suzukii* caused ovulation (an ovulated egg present in the

uterus could be expelled and thus detected by squeezing the tip of the abdomen with forceps), at even 1/64th male equiv.

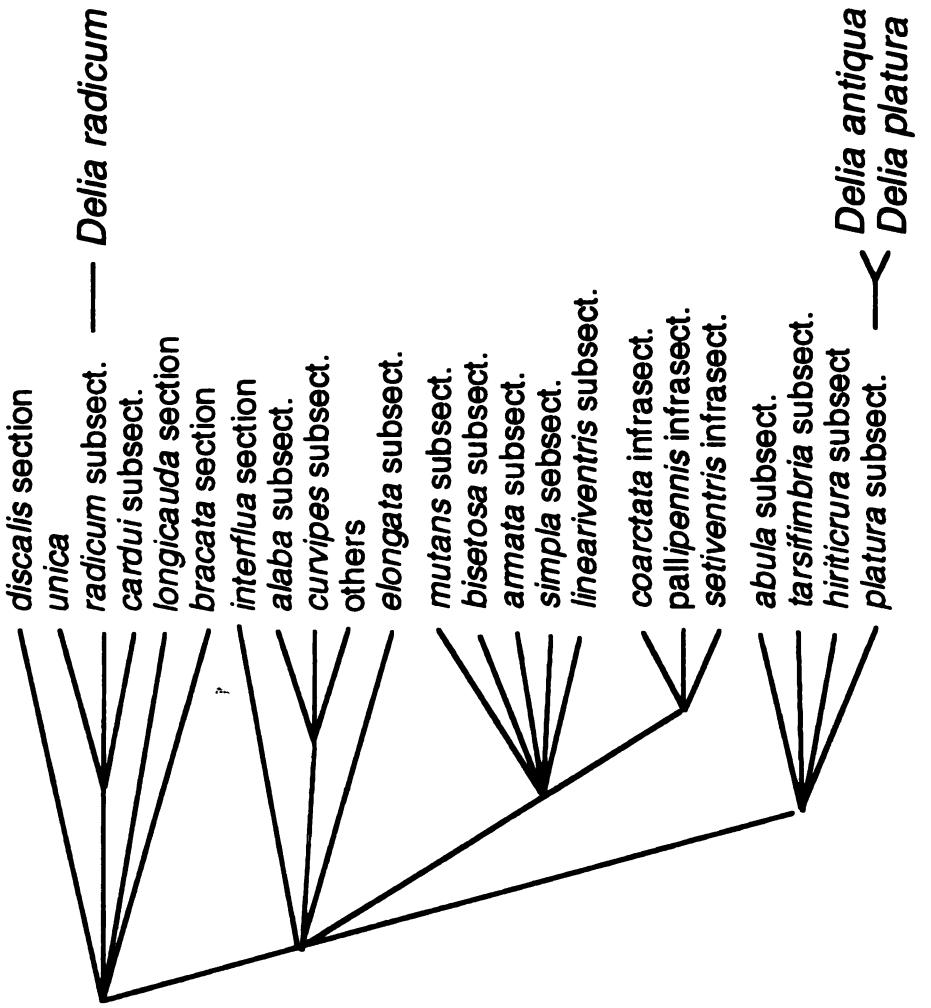
The apparent lack of cross-activity from *D. suzukii* to *D. pulchrella* was overcome by injection of 4 pmol of synthetic Drsuz-SP-I. The activity threshold of the synthetic form of Drm-SP-I is lower (0.6 pmol) than that of purified sex peptide (3 pmol) (Kubli, 1992; Schmidt *et al.*, 1993a), making the apparent insensitivity of *D. pulchrella* all the more remarkable given its sibling status with *D. suzukii.*

The current study documents the profile of cross activity of paragonial gland extracts for three readily available and economically important vegetable flies in the family Anthomyiidae, genus *Delia* (8 sections; 162 Nearctic species (Griffiths, 1991)), and tribe Anthomyiini (Figure 18). The onion fly, *D. antiqua,* (larvae feed on *Allium cepa* and close relatives), and seed corn fly, *D. platura,* (larvae feed on and around germinating seeds of diverse plant families including Alliaceae and Cruciferae (Harris *et al.*, 1986)) are close relatives in the *platura* subsection (Figure 18). The more distantly related cabbage fly (larvae feed on and around Cruciferae), *D. radicum,* falls into the *radicum* subsection of the *radicum* section of the Anthomyiini (Figure 18). Although populations of these three species overlap in time and space, and sometimes on plant hosts, each is considered a distinct species (Griffiths, 1991) thought not to hybridize with the relatives examined here.

MATERIALS AND METHODS

Insects and culturing. All *Delia* flies were reared at 22 ± 2°C, 35 ± 10% RH, under a photoperiod of 16:8 (L:D), in 1 m x 1 m x 0.75 m screened cages.

D. antiqua were originally from the University of Guelph, Canada, and cultured as described by Havukkala and Miller (1987). Adults were given free



Genus *Delia*, tribe Anthomyiini

Figure 18. Morphologically based phylogeny of the *Delia* flies, subdivided according to Griffiths (1991, pg. 955). *D. antiqua* and *D. platura* are both found in the *platura* subsection of the *albula* section. *D. radicum* is placed in the *radicum* subsection of the *radicum* section.

access to water and a dry diet of 10 parts powdered milk, 10 parts powdered sugar, 1 part brewers' yeast, 1 part soy flour, and 20 parts yeast hydrolosate. Every second day eggs were collected by water floatation from an artificial onion ovipositional resource modified from Harris *et al.* (1987) by the addition of optimal ovipositional holes (Mowry *et al.*, 1989a) around the base of each stem. Eggs were then placed into 30 cm x 25 cm x 10 cm plastic rearing boxes containing a layer of moistened gravel and longitudinally sliced onion bulbs. A plastic lid with 1 mm mesh screening covered each rearing container. After 14 days, a fresh layer of sliced onions was added, and covered with moistened gravel. Pupae were collected by floatation 7-10 days later, and stored in moist gravel at 4°C.

D. radicum was obtained from a culture maintained at the London Research Centre, London, Ontario. Adults were given free access to water and dry *D. antiqua* diet (see above) following emergence. Every second day eggs were collected by water floatation from ovipositional resources consisting of a three to four, 2-3 cm square chunks of rutabaga on dry silica sand in a 9 cm diam petri dish. Eggs were then placed into 30 cm x 25 cm x 10 cm plastic rearing boxes containing ten 2 cm thick slices of whole rutabaga upright in moistened gravel. A plastic lid with 1 mm mesh screening covered each rearing container. After 14 days, fresh rutabaga slices were added, and covered with moistened gravel. Pupae were collected by floatation 14 days later, and stored in moist gravel at 4°C.

D. platura was not cultured in our lab; all adults used in experiments came from pupae obtained from a laboratory culture maintained at the London Research Centre, London, Ontario. Adults were given free access to water and dry *D. antiqua* diet (see above) following emergence.

Drosophila melanogaster (Oregon-R strain) were obtained from the Department of Zoology, Michigan State University. Insects were reared on Instant *Drosophila* Medium (Carolina Biological Supply Company, 2700 York Rd, Burlington, North Carolina) in 20 dram vials. Upon emergence, adults were anesthetized with moist CO_2, sexed and held in single sex vials on media for six days at 22 ± 2°C under a photoperiod of 16:8 (L:D) until dissected for extract preparation.

Aedes aegypti, (originating from the UGAL strain at the University of Georgia) were reared on equal parts of yeast, lactalbumin, and ground rat chow as larvae. Following emergence, adults were separated by sex, held in 1 m^3 screened aluminum cages with *ad libitum* access to 10% sucrose solution on cotton balls in 1 oz. plastic cups and held at 25 ± 2°C, 70% RH under a photoperiod of 16:8 (L:D).

Extract Preparation and Injection. For each species, reproductive tracts were dissected under saline from sexually mature (6 to 8-day-old) virgin males by pulling on the terminalia of freshly freeze-killed males until the gut and reproductive tract were exposed (*Ae. aegypti* males were ether-anesthetized before dissection). The paragonia were separated from the gut with forceps and a scalpel blade and transferred via a polyethylene suction pipette to microcentrifuge tubes containing physiological saline (128 mM NaCl; 4.6 mM KCl; 1.9 mM $CaCl_2 \cdot 2H_2O$; 1 mM $Na_2HPO_4 \cdot 7H_2O$; adjusted to pH 6.8; modified from Mowry *et al.* (1987)). Collected material was sonicated for 15-30 sec (*Delia* fly paragonia were not sonicated), centrifuged for *ca.* 30 min at 16000xg and 4°C, and the supernatants used for injection. All extracts were stored at -20°C until use.

In addition to paragonia, *Delia* fly testes were also collected (as above); both were prepared at a concentration of 0.5 male equivalent/µl. *Ae. aegypti,*

and *D. melanogaster* paragonial extracts were prepared at two male equiv/µl. Whole reproductive tract extracts (testes and paragonia) were also prepared for each *Delia* species. They were processed as above, starting with testes/paragonia complexes separated from the gut and transferred into saline via a bent minutin pin inserted in wooden applicator stick.

Adult virgin insects of all *Delia* species were obtained by collecting flies within 24 h following adult emergence and segregating the sexes in separate cages with *ad libitum* food and water (Spencer *et al.*, 1992).

Extract injections were carried out using the device and techniques of this dissertation, Chapter 1. All injections were made into the dorsolateral thorax of CO_2 anesthetized virgin females. 0.5 µl of extract (1/4 male equiv) was injected unless otherwise noted.

Bioassay materials and procedures. In addition to treatments injected, all experiments included mated controls, consisting of a single virgin female, unless otherwise noted, held continuously with two sexually mature conspecific males for the duration of the experiment. Saline-injected controls received a volume of physiological saline equal to that for extract-treated flies. Untreated controls were transferred directly from single sex cages to the 20 cm x 9 cm cylindrical screen cages capped with 9 cm diam petri dishes. An experimental unit consisted of a single female held in a screen cage, unless otherwise noted. Water dishes consisted of a water-filled 2.5 cm x 2.7 cm plastic cup inverted on a filter paper lined 4 cm petri dish. Each *Delia* species was provided with their respective food(s) (see culturing methods above) in a 1.5 cm plastic shell-vial cap.

The *D. antiqua* ovipositional resource consisted of a 1 oz. jelly cup filled with moist silica sand into which a surrogate onion stem (Harris *et al.*, 1987) was inserted vertically and provided with optimally sized ovipositional holes

around the base (Mowry *et al.*, 1989a). The ovipositional resource for *D. radicum* was a 4 cm petri dish bottom filled with dry silica sand with a 2 cm diam x 2 cm cylinder of freshly cut rutabaga upright in the center. The *D. platura* ovipositional resource was a 1 oz. jelly cup filled with moistened dark gravel having a water-soaked lima bean protruding from the surface and 4 circular groupings of eight, 1 mm diam x 8 mm deep ovipositional holes evenly spaced around the bean.

To establish ovipositional rates (mean eggs/female/d) following treatment, eggs were collected by floatation and counted daily for 7 days. On day 7, two sexually mature virgin males were added to each cage to test female mating receptivity. Eggs were collected for five additional days, and held on moistened silica sand in Parafilm™-covered 4 cm diam petri dishes to monitor hatching as evidence of mating (this dissertation, Chapter 1).

Experiment 1. **Testes vs Paragonial Extracts** To demonstrate that the sex peptide effect is attributable only to paragonia and to justify the time-saving use of whole reproductive tract extracts in the cross-activity experiment (see Experiment 2), 7-day-old virgin female *D. antiqua*, *D. radicum* and *D. platura* (n=30 for each species) were bioassayed for response to injection of conspecific male extract of testes or paragonia

Experiment 2. **Cross-activity of *Delia* Sex Peptides.** Seven-day-old virgin females of each species were bioassayed for response to injection of conspecific (n=30) or heterospecific (n=30) male reproductive tract extracts.

Experiment 3. **Sex peptides and reproductive extracts of other species** Paragonial gland extracts of *D. melanogaster* and *D. antiqua* males were injected into 15, nine-day-old virgin female *D. antiqua*. *D. melanogaster* extract was injected into *D. antiqua* at 1 *Drosophila* male equiv/0.5 µl. *D. antiqua* control extract was injected at 0.25 µl/individual from extracts

prepared at 0.5 male equiv/µl. There were three replicates. Following treatment, females were held in groups of five per bioassay cage containing one ovipositional resource. Eggs were collected and counted daily for 10 days. Mating propensity was not evaluated.

Paragonial gland extracts from *Ae. aegypti* (2 male equiv/µl) or *D. antiqua* (1 male equiv/µl) were injected into five, 6-day-old *D. antiqua* virgin females at 0.5 male equiv/female. Only oviposition was monitored daily for 10 days.

Synthetic *D. melanogaster* sex peptide and *Aplysia* egg laying hormone were prepared by J.F. Leykam, Director of the Macromolecular Structure and Synthesis Facility in the Department of Biochemistry, Michigan State University. Both peptides were synthesized on a Model 270 Applied Biosystems Peptide Synthesizer using Fmoc (9-fluorenylmethyloxycarbonyl) amino group protection (Roberts and Vellaccio, 1983) and obtained in phosphate buffered saline (pH 7.0) at 1 µg protein/µl and frozen until use. Synthetic peptides were injected at 0.5 µl/individual into ten, 7-day-old virgin female *D. antiqua*. Oviposition was monitored daily for five days.

A second sample of synthetic *D. melanogaster* sex peptide, was obtained from Dr. Eric Kubli, Zurich, Switzerland. A 16 µg sample of lyophilized Drm-SP-I was suspended in 60 µl of physiological saline to make an extract at 1 male equiv/0.05 µl, an aliquot was diluted to 2 male equiv/µl and frozen at -20°C until use. Synthetic *D. melanogaster* sex peptide was injected into 25 (5 flies/cage and 5 replicates), 12-day-old virgin female *D. antiqua* at 0.5 µl/individual (0.5 male equiv). Following treatment, females were held in groups of five per screen cages (as described above) with one ovipositional resource/cage. Oviposition was monitored daily for eight days.

Statistical Analysis. All experiments were conducted in a completely randomized design. Before analysis, data were tested for fit to a normal distribution. Analysis began with 1-way ANOVA; mean separations were performed using Sheffe's multiple comparison test, unless otherwise noted, on mean eggs/female/d before addition of males. Data from females dying during a bioassay (mean mortality/treatment ± SD = 22.5 ± 14.6%) were not included in statistical analyses.

RESULTS

***Experiment 1.* Testes vs Paragonial Extracts** Only paragonial gland extracts activated oviposition and suppressed mating (Figure 19) in conspecific tests for the three *Delia* sp. Conspecific sex peptide activity from male reproductive tracts and paragonia had been reported previously for *D. radicum* (Swailes, 1971) and *D. antiqua* (Spencer *et al.*, 1992), but not from *D. platura*. Whether extract from testes had any effect on remating propensity and oviposition had never been carefully evaluated in *Delia* flies. The finding that testes extracts contributed nothing to the measured reproductive behaviors justified collection of whole reproductive tract extracts as a time-saving measure.

***Experiment 2.* Cross-activity of *Delia* reproductive tract extracts** Following injection with *D. platura* and *D. radicum* paragonial extracts, *D. antiqua* females refused mating and oviposited indistinguishably from those normally mated (Figure 20). *D. antiqua* and *D. radicum* extracts injected into *D. platura* stimulated mated-like levels of oviposition (Figure 20). However *D. radicum* extract caused only partial mating refusal in *D. platura* females, while that from *D. antiqua* was fully active. Extract of *D. antiqua* paragonia increased *D. radicum* oviposition but not to the level of the positive controls

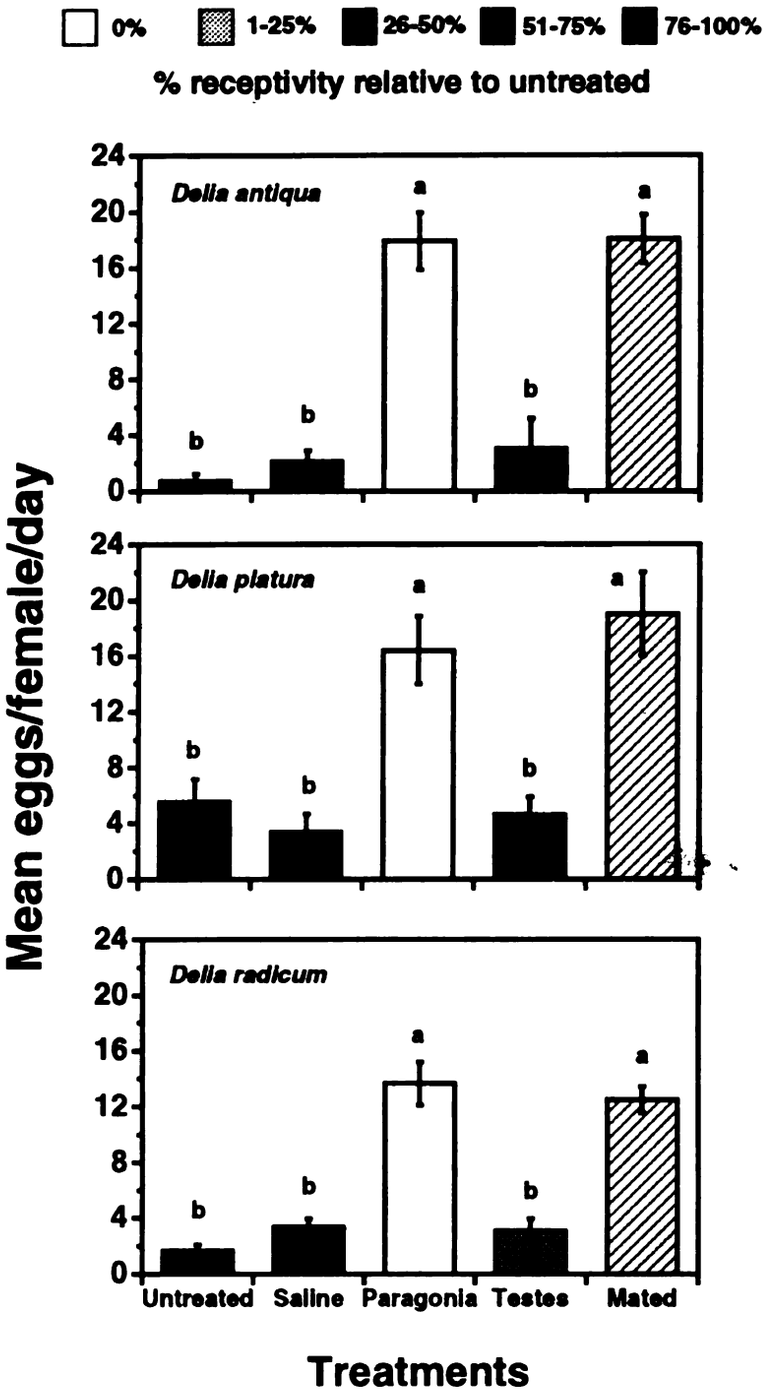


Figure 19. Influence of conspecific male paragonial gland and testes extracts on oviposition (mean eggs/d ± SE) of the onion fly (*Delia antiqua*), the cabbage fly (*Delia radicum*), and the seed corn fly (*Delia platura*). Oviposition by each species was analyzed separately. Mated female bars are striped to indicate that they were not tested for mating receptivity.

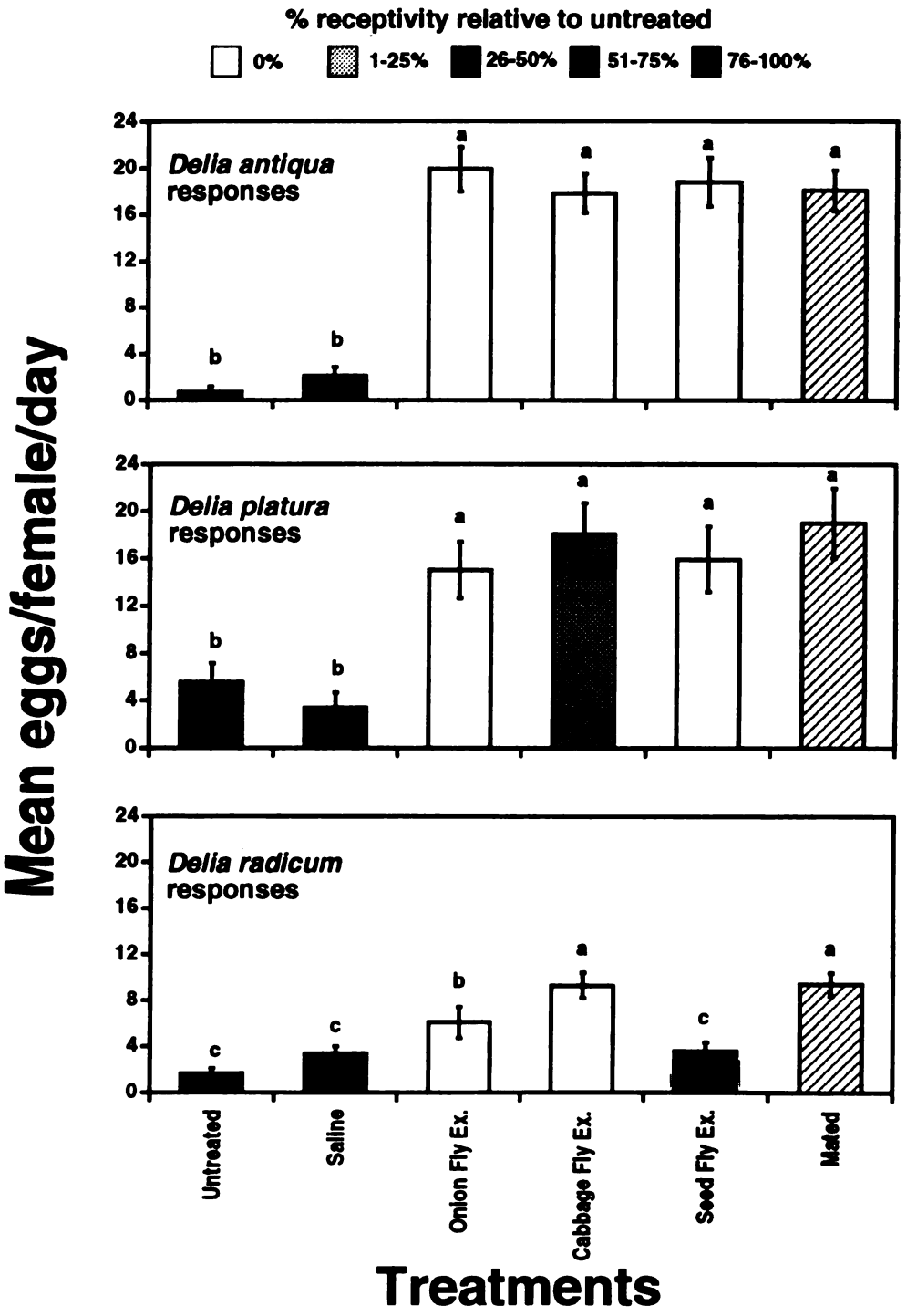


Figure 20. Influence of onion (*Delia antiqua*), seed corn (*Delia platura*), and cabbage fly (*Delia radicum*) male reproductive tract extracts on the oviposition and mating receptivity of each species. Mated female bars are striped to indicate that they were not tested for mating receptivity.

(Figure 20). *D. platura* extract had no effect on oviposition or mating inhibition in *D. radicum*.

***Experiment 3.* Sex peptides and reproductive extracts of other species** Neither *D. antiqua* oviposition nor mating inhibition was affected by injection of: *Ae. aegypti* reproductive tract extracts (Fig 21), *D. melanogaster* paragonial gland extracts (Figure 22A), or the synthetic forms of Drm-SP-I and *Aplysia* ELH (Figure 22B & C).

DISCUSSION

Breadth of Insect Sex Peptide Cross-Activity. The sex peptide activity profile across *D. antiqua, D. platura,* and *D. radicum* is schematically summarized in Figure 23. Sex peptides of *D. antiqua* and *D. platura* were cross-active with respect to both induction of oviposition and mating inhibition. Full mating inhibition, but only partial ovipositional induction, occurred when *D. antiqua* sex peptide was injected into *D. radicum*. The sex peptide of *D. radicum* was completely cross-active when injected into *D. antiqua*. The *D. radicum* secretion stimulated full ovipositional activation and partial mating inhibition in *D. platura,* however, *D. platura* sex peptide was inactive in *D. radicum*. Though sex peptide cross-activity was not always complete, the pattern suggests broad sex peptide heterospecificity in this genus.

Full sex peptide cross-activity between *D. antiqua* and *D. platura* and limited activity between them and *D. radicum* is congruent with a phylogeny based on morphological characters which indicate *D. antiqua* and *D. platura* are more closely related to one another than each is to *D. radicum* (Figure 18). An electrophoretic study using 8 enzyme loci (Harris *et al.*, 1986) supports this

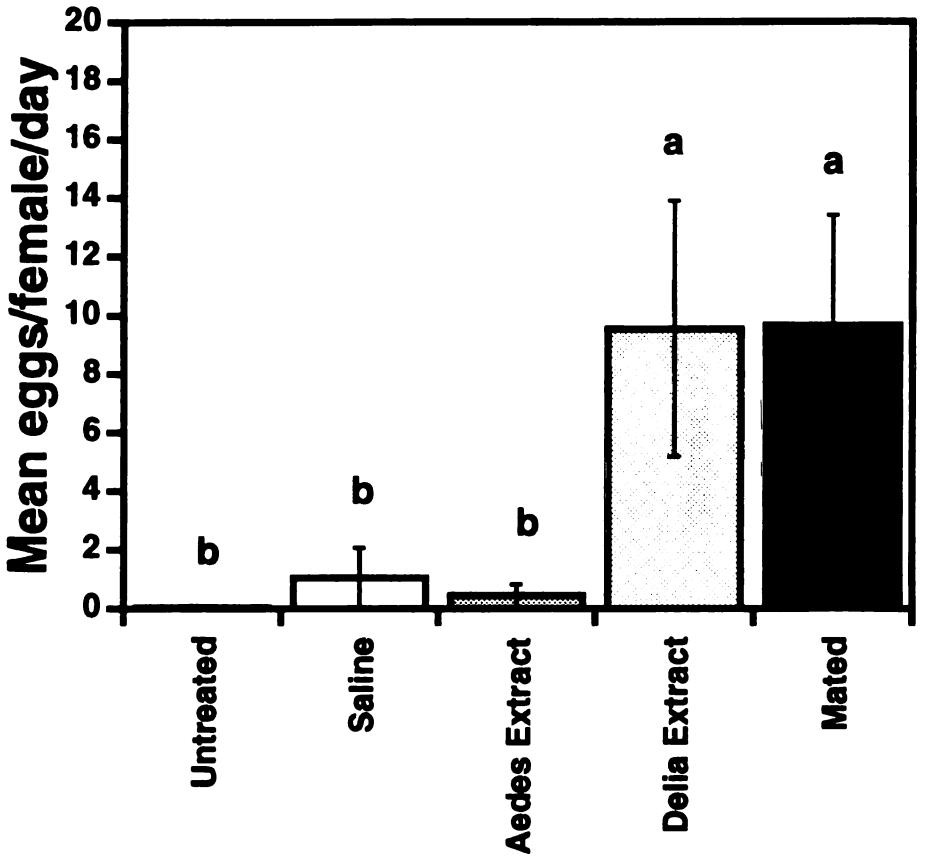


Figure 21. Influence of *Aedes aegypti* paragonial gland extracts on the ovipositional response of onion fly (*Delia antiqua*).

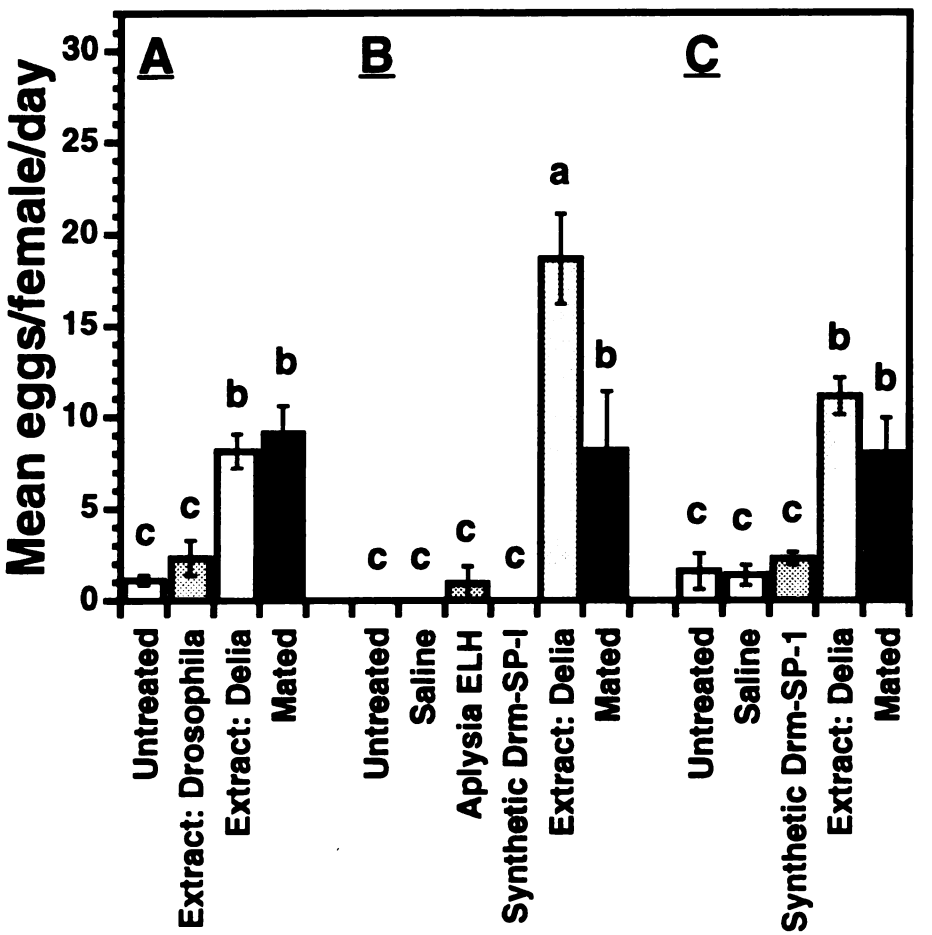


Figure 22. Onion fly (*D. antiqua*) oviposition as influenced by *Drosophila melanogaster* paragonial gland extract, synthetic *Aplysia* egg laying hormone (ELH), and synthetic *D. melanogaster* sex peptide. (A) *D. melanogaster* paragonial gland extract injection (n= 5). (B) Synthetic *Aplysia* ELH and Synthetic *D. melanogaster* sex peptide (from J.F. Leykam) (n= 10). (C) Synthetic *D. melanogaster* sex peptide (from E. Kubli) (n= 5). A-C were analyzed separately; means followed by the same letter were not significantly different at $P \leq 0.05$ (Student-Newman-Keuls).

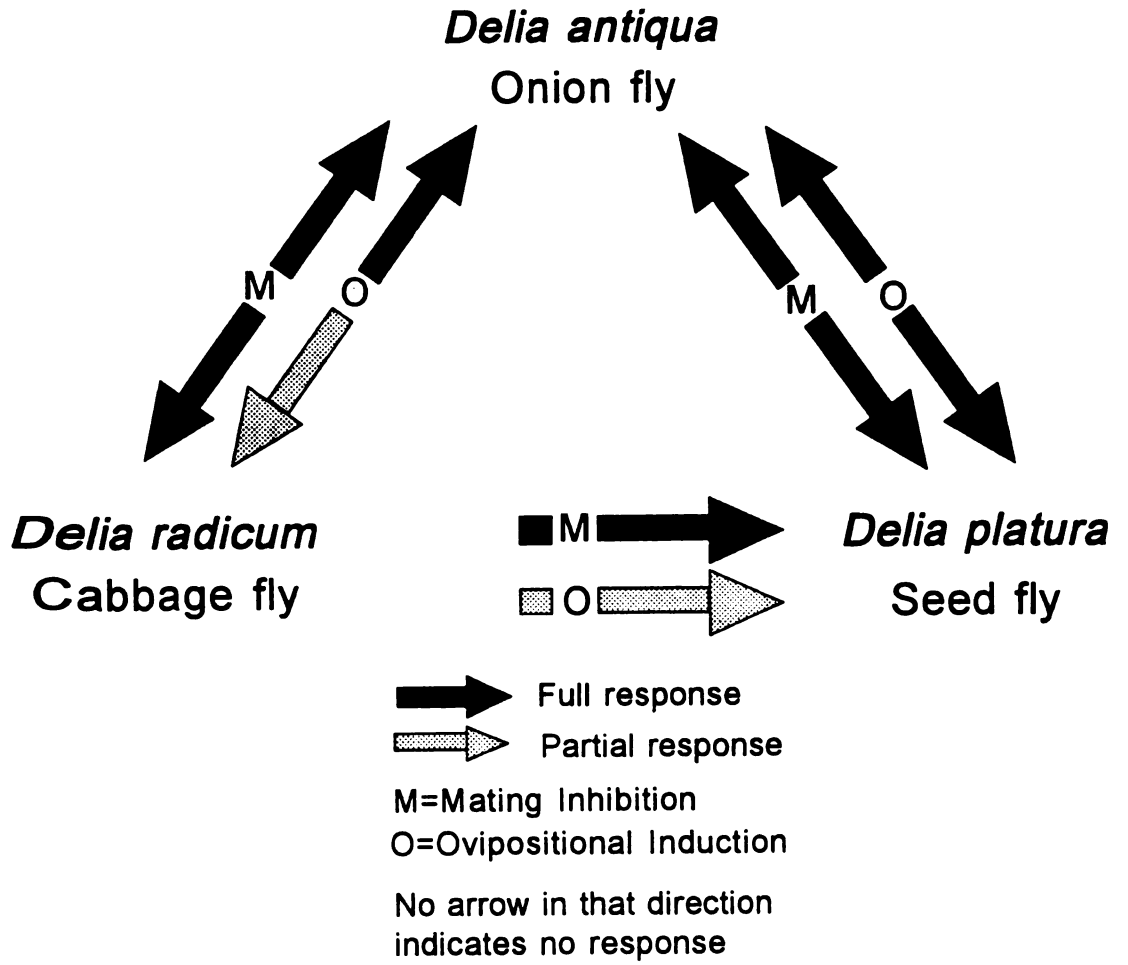


Figure 23. Summary of sex peptide cross-activity between *D. antiqua*, *D. platura*, and *D. radicum*.

interpretation. The calculated Nei genetic distances between *D. antiqua* vs. *D. platura* or *D. radicum* are 0.398 and 1.369, respectively. Between *D. platura* and *D. radicum* the Nei genetic distance was 0.9 (Harris *et al.*, 1986).

Sex peptide cross-activity within *Aedes* mosquitoes (Craig, 1967), and amongst related *Drosophila* (Fuyama, 1983; Stumm-Zollinger and Chen, 1988; Chen, 1991; Schmidt *et al.*, 1993b) suggests that sex peptide cross-activity within genera may be common. Between *D. melanogaster* and *D. simulans* or *D. mauritiana*, the Nei genetic distances are 0.4 and 0.6, respectively, and that for *D. simulans* and *D. mauritiana* is 0.2 (Gonzalez *et al.*, 1982). *D. sechellia* is thought to be more closely related to *D. simulans* and *D. mauritiana* than to *D. melanogaster*, though no genetic distance was reported (Lachaise *et al.*, 1986). Sex peptides of these four species are mutually cross-active with respect to mating inhibition and oviposition induction (Stumm-Zollinger and Chen, 1988; Chen and Balmer, 1989). Thus in both *Delia* and *Drosophila*, it appears that sex peptide cross-activity is not always highly correlated with genetic distance.

Reports of sex peptide cross-activity between different insect families (Craig, 1967; Leahy, 1967), should be viewed with skepticism, as there is only scant evidence to support interfamilial sex peptide cross-activity. Reported sex peptide cross-activity following implantation of *D. melanogaster* paragonial glands into *Ae. aegypti* females (Leahy, 1967) has never been corroborated. Interfamilial cross-injection of male accessory gland extracts of *Stomoxys calcitrans* (family Muscidae) had little effect on mating refusal when injected at 0.5 male equiv into *Musca domestica* (Muscidae), *Phormia regina* (Calliphoridae), and *Sarcophaga bullata* (Sarcophagidae) (Morrison *et al.*, 1982). *D. antiqua* oviposition was unaffected by injection of *Ae. aegypti, D.*

melanogaster, or *Aplysia* peptides. Heterospecificity at the generic level, but not at the family level or above appears to be the general pattern.

Mechanistic interpretation of sex peptide cross-activity. The pattern of sex peptide cross-activity between *D. radicum*, *D. platura*, and *D. radicum* is more complex than comparisons across two of these species would have suggested. An assumption of sex peptide structural identity based on complete cross-activity between *D. antiqua* and *D. platura* is falsified by their divergent effect on *D. radicum*. The decidedly asymmetric relationship arising from cross-injection of *D. platura* and *D. radicum* sex peptides (Figure 23) suggests the sex peptides for these two species are not structurally identical, yet both extracts produced an identical full effect when injected into *D. antiqua*.

This pattern is unlikely to be explained by only one cause. Differences due to sex peptide dosage effects, amino acid sequence divergence, receptor affinity/specifity changes, or combinations thereof may be involved. Sex peptide sequence data, were it available, would only solve part of the puzzle. Because the outcome of a sex peptide transfer is an interaction dependent on which species donates or receives the secretion, as shown in the present experiments, full understanding of sex peptide interactions and specificity will require characterization of both receptors and sex peptide sequences.

Sex peptides and reproductive isolation. Though they share sex peptide cross-activity and are closely related, species within the *D. melanogaster* species complex do not normally interbreed because of considerable pre-mating mate recognition barriers and post mating genetic incompatibility (Lachaise *et al.*, 1986). Crosses never lead to fertile males, and fertile females are obtained only from some crosses between *D. simulans*, *D. sechellia* and *D. mauritiana*. In addition, the intermating propensity is highly

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asymmetrical between members of the *D. melanogaster* species group (Coyne *et al.*, 1994).

Although there is no literature documenting the occurrence of intermating or hybridization, the *Delia* flies studied here are thought to be genetically incompatible (Harris *et al.*, 1986). In addition to their use of different host plants, strong premating isolation between *D. radicum* and *D. antiqua* seems likely because *D. radicum* exhibits distinct courtship behaviors including wing-buzzing, whereas *D. antiqua* males do not seem to engage in any courtship before copulatory strikes (J. L. Spencer, personal observation).

Given that divergences in sex peptide communicational channels seem to develop subsequently to or late in speciation events and that such divergences are rather modest among even widely separated species like *D. radicum* and *D. antiqua*, sex peptide shifts are likely the product not the initiator of reproductive isolation. They may merely represent drift in a once common interspecific communicational channel that is now only intraspecifically tuned and thus need not be entirely conserved, or an adaptive shift in reponse to divergent mate recognition systems.

However, before sex peptides are discounted as playing any significant role in the reproductive interactions of genetically diverging populations, their unique properties as possible postmating, prezygotic isolating mechanisms need careful consideration. Sperm of *D. antiqua* are wrapped in a tight ball within the female's spermatheca (see Miller *et al.*, 1994), like other higher Diptera, they are expected to get dispensed with a strong bias of last-in-are-first-out (sperm precedence; Parker, 1984). Females of such an animal also using sex peptides to turn off female mating receptivity and to turn on oviposition and thus fertilization have the unique potential to correct mating mistakes, e.g., a female mating with a genetically incompatible male donating

a divergent sex peptide is unlikely to turn off remating and to turn on oviposition; after a proper mating, this female could be normally fecund, as the incompatible sperm would be buried beneath a sufficient supply of good sperm.

Some unusual possibilities for reproductive interference arise if sex peptide asymmetries were to develop before genetically diverging populations became completely reproductively isolated or if males of respective species were to accomplish interspecific matings. Monogamous females receiving genetically incompatible sperm and fully active sex peptides would be unwittingly sterilized, those sensitive to the sex peptide of only genetically compatible males could correct interspecific mating mistakes.

The possibilities for pronounced disruptive selection due to asymmetries in interspecific mating frequency has been described and theoretically quantified by the pioneering work of Ribeiro (1988) and Ribeiro and Spielman (1986), who used the term "satyrization" to describe the situation of having females of one type be sterilized by behaviorally satisfying matings with males of a genetically incompatible type (satyr males). Asymmetries in frequency of intertype/interspecific matings were seen as critical in setting up conditions where the satyrized type could be driven to parapatry or extinction. As each mating by male insects like Diptera is not energetically or reproductively expensive, it was argued that the benefits of satyristic matings could outweigh the costs, particularly if satyr matings cleared a once overlapping ecological niche for the satyr males' offspring.

Although the satyrization hypothesis is yet largely untested (see Ribeiro (1988) and Ribeiro and Spielman (1986) for some support), we suggest it should be given serious attention. This idea, previously conceptualized with no consideration of sex peptides, gains additional credibility with the

realization that sex peptide asymmetries could facilitate intermating frequency asymmetries and also asymmetrically offer the satryizing species a mistake-correction mechanism in a dangerous and unstable intermating game.

Presented in Table 5 are some tentative predictions for theoretical outcomes of reproductive interactions by genetically incompatible insects expressing sperm precedence and using sex peptides. Based upon the emerging data for sex peptide cross-activity across the Diptera, Table 5 supports the idea that premating barriers are more likely to resolve reproductive conflicts than are alterations in sex peptide communicational channels. Though sex peptides are not likely to initiate reproductive isolation, it remains to be determined whether they enhance mate recognition, help drive species apart once divergence is well underway or whether they might favor brief and forceful periods of satyrization.

Table 5. Suggested consequences of reproductive interactions mediated through symmetries and asymmetries of intermating frequency and sex peptide cross-activity. Genetic incompatibility, use of sex peptides, and sperm precedence are assumed for all interactants.

Do they Intermate?	Reason for no intermating	Intermating Frequency[1]	Type of Sex Peptide Cross-activity	Table cell designation	Condition	Likely Adjustments (if not extinction or parapatry)-- Interactants move to:
Yes	—	Symmetrical	Symmetrical	1	Major, balanced mating interference	4, 2, 3[2]
			Asymmetrical	2	Satyrization	14, 3
			None	3	Minor, balanced mating interference[3]	6
		Asymmetrical	Symmetrical	4	Satyrization	13, 5, 6
			Asymmetrical	5	Hypersatyrization **or** Balanced mating interference[4]	14, 15 6
			None	6	Minor, unbalanced mating interference	15
No	No opportunity (e.g., allopatric)	Potentially symmetrical	Symmetrical	7	No interaction, but potentially 1	
			Asymmetrical	8	No interaction, but potentially 2	
			None	9	No interaction, but potentially 3	
		Potentially asymmetrical	Symmetrical	10	No interaction, but potentially 4	
			Asymmetrical	11	No interaction, but potentially 5	
			None	12	No interaction, but potentially 6	
	Won't (behavioral or anatomical block)	—	Symmetrical	13	Adjustment end point, but by chance mutations likely to move to 14	14
			Asymmetrical	14	Adjustment end point, but by chance mutations likely to move to 15	15
			None	15	Stable end point	

[1] Intermating frequency would be highly influenced by density of the respective populations as well as propensity to intermate.

[2] In order of likelihood.

[3] Each type would lay eggs only after insemination by a genetically compatible male.

[4] One of these two conditions would be determined by whether the sex peptide asymmetry was in the same or opposite direction as the intermating asymmetry.

CHAPTER 5

Chemical Characterization of Onion Fly Sex Peptide

ABSTRACT

Delia antiqua sex peptide activity from paragonial glands or whole reproductive tracts was extractable in: distilled water, physiological saline, concentrated and unconcentrated phosphate-buffered saline, and 0.01% or 0.1% sodium dodecyl sulfate. Activity was recovered from resuspended centrifuge pellets of precipitated extracts, nitrogen or speed-vac-dried extracts resuspended in distilled water or physiological saline, the tops of microcentrifuge filters with molecular weight cutoffs (MWCO) of 300k, 30k, 10k, and 5k, and from electroeluted fractions of 5 & 10% native-PAGE gels. Activity was lost following: exposure to temperatures above 50°C for 10 min, pH <3.9, lipophilic or organic solvents (>0.01% trifluroacetic acid, ≥10% acetonitrile, 50mM acetic acid, and 20% isopropanol), gel filtration chromatography, weak anion exchange chromatography, polyacrylamide gel electrophoresis (SDS-PAGE), RP- and NP-HPLC.

Loss of activity following tryptic digestion of extracts demonstrated involvement of a protein in sex peptide activity. Low protein recovery from extracts exposed to an agarose-only control, underscores the importance of a qualitative and quantitative assessment of biological activity. SDS-PAGE of male reproductive tract extract on 12% gels revealed a complex mixture of >40 major proteins with molecular weights ranging from *ca.* 300k to <14.4k. Crude size fractionation microcentrifuge filters along with 2000 and 1000

MWCO dialysis membranes suggested *D. antiqua* sex peptide activity is associated with molecules of ≥ 1-5k, however activity was also recovered from the top of a 300k MWCO filter. *D. antiqua* sex peptide activity may have a high capacity to bind surfaces or form aggregates.

It is yet unclear whether sex peptide activity is the result of a single or multicomponent protein or polypeptide. Biologically active material was recovered following 5x concentration of fractions electroeluted from the uppermost portion of native-PAGE resolving gels; on SDS-PAGE these fractions contained some of all proteins present in the starting extract. Fractionation was evident in fractions excised from the same 10% native-PAGE gels that had migrated the length of the gel, but no activity was recovered. The approach to isolating onion fly sex peptide may need to be shifted to e.g., affinity chromatography as convential chromatographic and electrophoretic techniques were ineffective.

INTRODUCTION

Background Information on Previous Characterizations of dipteran Paragonial Secretions and Sex Peptides. The secretory products of *Drosophila melanogaster* paragonia are complex, containing in addition to protein: amino acids and their derivatives, carbohydrates, and lipids (Chen, 1984). Though the precise functions of few paragonial proteins are known, their involvement in precluding mating and activating oviposition is well established (Chen, 1984).

The paragonial glands of *D. melanogaster*, contain *ca.* 1200 proteins resolvable with 2 dimensional gel electrophoresis (Ingham-Baker and Candido, 1980). The molecular weights of 41 major bands separated by Von Wyl (1976) on 10% SDS-PAGE ranged from 12k-122k. At least 85 of these were

judged to be exported secretory products (Stumm-Zollinger and Chen, 1985). Following copulation, secretory proteins rapidly re-accumulate in the gland lumen resulting from accelerated translation of existing stable mRNAs and *de novo* synthesis of ribosomes rather than enhanced synthesis of mRNA (Schmidt *et al.*, 1985).

Some characterized *Drosophila* proteins share homology with proteins of known reproductive function. The mst355a protein shows high sequence homology (11 in a stretch of 17 amino acids are in identical sequence) with *Aplysia californa* egg-laying hormone (ELH, a 36mer), however there is yet no evidence for an analogous function in *Drosophila* (Monsma and Wolfner, 1988; Chen, 1991). Esterase-6, an enzyme produced in the male ejaculatory duct epithelium and transferred to female *D. melanogaster* early in copulation, metabolizes *cis*-vaccenyl acetate into cis-vaccenyl alcohol which acts as a short-lived antiaphrodisiac pheromone involved in changing female receptivity behavior (Mane *et al.*, 1983; Scott, 1986) and may play a role in sperm motility (Gilbert, 1981).

The isolation and identification of the *D. melanogaster* sex peptide by Chen *et al.* (1988) culminated more than two decades of work and made a major contribution to this field. Chen *et al.* (1988) began their successful preparative purification by extracting several hundred pairs of male accessory glands in 0.15 ml of Bennett's solution (Chen *et al.*, 1988). After centrifugation and collection of the supernatant, the pellet was re-extracted twice and the pooled supernatants were loaded onto a Vydac C_4 HPLC column and eluted with a linear gradient of 0%-50% buffer of 30mM orthophosphoric acid and 95% methanol. Active fractions were further purified on the same column by eluting with a gradient of 0%-15% buffer B

(0.04%TFA in 95% acetonitrile) for 10 min and 15%-30% buffer B for 80 min (Chen *et al.*, 1988).

For injection purposes, *D. melanogaster* male paragonial glands were collected into 80% methanol, sonicated, and centrifuged to yield a supernatant which was lyophilized with a Speed Vac. The purified peptide was taken up in Ringer's solution to achieve a final concentration of 10 paragonial gland equivalents (or 5 male equivalents)/μl. The extract was injected into 4-6 day-old virgin female *D. melanogaster* (Chen *et al.*, 1988). Purified material, recovered following both protocols, suppressed mating and induced ovulation (presence of an egg in the uterus) and oviposition in virgin females at a physiological dose of 3 pmol. Amino acid analysis of purified active material and cDNA cloning confirmed that the *D. melanogaster* sex peptide, Drm-SP-I according to the nomenclature of Raina and Gäde (1988), was a 36mer (Figure 24). Synthetic sex peptide was prepared to confirm the activity of the sequence (Chen, 1991; Kubli, 1992).

Induction of sex peptide expression using heat shock promotor or yolk protein enhancer linked to sex peptide cDNAs activated oviposition and caused mating inhibition, confirming the functionality of the cDNA derived-peptide (Aigaki *et al.*, 1991). The method of sex peptide expression affected the duration of ovipositional stimulation and mating inhibition. Thirty min of heat induction at 37°C, altered receptivity and ovipositional behavior for *ca.* 1 day. Continuous expression of the sex peptide gene under control of the yolk protein enhancer caused sustained loss of receptivity and ovipositional activation for more than four days (Aigaki *et al.*, 1991). However, Aigaki *et al.*, (1991) caution that the lifetime of the ectopically expressed protein may not be as long as that of the natural sex peptide. They hypothesize that sustained sex peptide effects may be due to a second factor acting as a

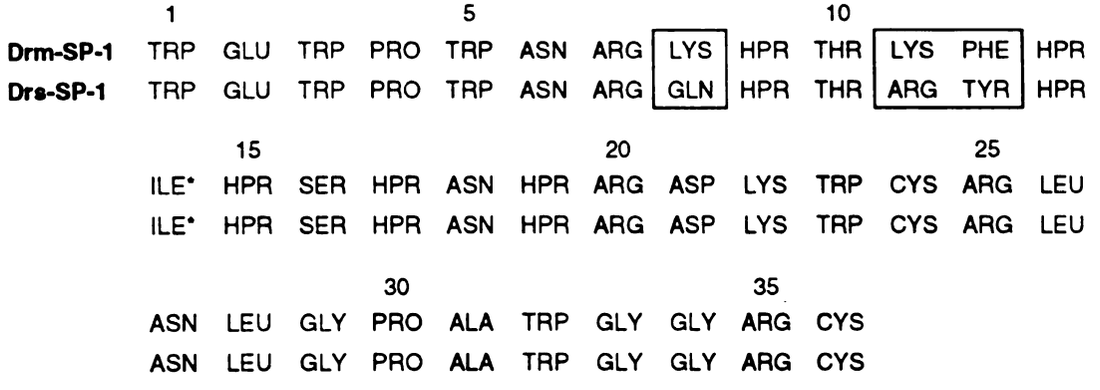

Figure 24. Amino acid sequence of the sex peptides of *Drosophila melanogaster* (Drm-SP-I) and *Drosophila sechellia* (Drs-SP-I) (adapted from Chen (1991)). The isoleucines marked an * are tentative assignments (Chen *et al.*, 1988; Chen and Balmer, 1989).

stabilizing molecule, or that a sperm factor (Manning, 1962), independent of sex peptide, maintains mated-like behaviors in this species (Aigaki *et al.*, 1991).

Sex peptides from three other related *Drosophila*: *Drosophila sechellia*, *Drosophila suzukii*, and *Drosophila funebris*, have been identified and at least partially characterized (Federer and Chen, 1982; Chen and Balmer, 1989; Schmidt *et al.*, 1993b) since the structure of Drm-SP-I was reported. The amino acid sequence of *D. sechellia* sex peptide, isolated using the procedure of Chen *et al.* (1988), is very similar to that of *D. melanogaster* (Figure 24); it differs by only three amino acids at positions 8, 11, and 12. Cysteines at positions 24 and 36 suggest both have an intrachain disulfide bridge to the C-terminus. The high hydroxyproline content is reminiscent of vertebrate connective tissues (J.F. Leykam, personal communication). In collagen, hydroxyprolines are thought to confer temperature stability via hydrogen-bonding between the residues of adjacent strands (Voet and Voet, 1990). Compositional similarities to structural protein suggests that sex peptides may be derived from accessory gland structural products perhaps originally associated with spermatophores. Spermatophores are thought to be a symplesiomorphic characteristic of arthropods (Schaller, 1988); occurence of sex peptides or sex peptide-like effects across diverse insect groups is consistent with such a spermatophore origin for sex peptides.

An oviposition-stimulating substance (OSS) of molecular weight *ca.* 4000 was purfied from *D. suzukii*, using methanol extraction and HPLC gel filtration and anion exchange for final cleanup (Ohashi *et al.*, 1991). Amino acid and sequence analyses revealed a peptide of >35 amino acids; a masked N-terminal prevented complete sequencing.

Primary structures of two *D. funebris* paragonial proteins, PS-1 and PS-2, with somewhat obscure reproductive effects have been partially determined (Baumann, 1974a; 1974b). A third male paragonial gland peptide from *D. funebris*, was found to be a 63mer, with protease inhibiting activity (Schmidt *et al.*, 1989). Though not technically a sex peptide, this serine protease inhibitor may play a role in sperm motility.

Though work on non-*Drosophila* sex peptides began shortly after they were recognized in *D. melanogaster*, progress toward chemical characterization has been slow. In the mosquitoes, *Aedes aegypti* and *Ae. albopictus*, quantification of sex peptide effects began in earnest with the work of Craig and Leahy (Leahy and Craig, 1965; Craig, 1967; Leahy, 1967; Leahy and Lowe, 1967).

Ae. aegypti sex peptide ("matrone") (Fuchs *et al.*, 1968) was nondialysable, readily precipitated in 60% ammonium sulfate, and was deactivated when exposed to 50°C for 5 min or following exposure to 5% TFA. When extracts were treated with $MnCl_2$ or $MnSO_4$ to precipitate nucleic acids, active principle remained in the supernatant. Treatment with 5% TFA destroyed behavioral activity. The protein nature of the active material was suggested by protease digestion (Fuchs *et al.*, 1969). *Ae. aegypti* sex peptide was pH sensitive below 5 and above 9, and activity was lost following exposure to lipophilic solvents.

Loss of activity during dialysis against distilled water was attributed to aggregation or precipitation; the interpretation being that the sex peptide was a globular protein, perhaps composed of 2 fractions (Fuchs and Hiss, 1970). Matrone's α and β fractions, with molecular weights between 30k and 60k, were reported to be required for mating inhibition; the α fraction alone could stimulate oviposition (Fuchs and Hiss, 1970). It should be noted that in

mosquitoes the paragonial secretion may have effects beyond mating suppression and activation of oviposition including egg development (Klowden and Chambers, 1991) and preovipositional behavior (Yeh and Klowden, 1990).

Using gel filtration, Young and Downe (1987) fractionated *Culex tarsalis* paragonial peptide and demonstrated that it was absorbed by the brains of females injected with radiolabelled paragonial secretions. They also demonstrated that whole body extracts could cause a small receptivity inhibiting substance to aggregate with larger proteins, casting some doubt on the accuracy of the Fuchs and Hiss (1970) earlier molecular weight determination.

Characterization of *Musca domestica* sex peptide of has progressed little since the late 1960s. Isolated from the male ejaculatory ducts (*M. domestica* does not have paragonial glands), activity nearly equivalent to mated was reported to be associated with a heat stable, dialysable protein with a molecular weight reported to be between 0.8k and 3k (Adams and Nelson, 1968). Recent investigations (Bird *et al.*, 1991) using field-collected strains have found stronger mating suppression than previously reported (Adams and Nelson, 1968), yet the effects on mating suppression were not detected beyond 3 h post-treatment. The potency of house fly extracts was significantly affected by the route of administration. Three to six male equiv were required for female monogamy if injected abdominally, however, only 0.4 male equiv generated the same effect if injected directly into the vaginal pouches (Terranova and Leopold, 1971). Strain effects are likely to be important. Using a highly laboratory inbred Carolina Biological strain of *M. domestica*, Lentz and Miller (Lentz, 1994) found no mating or oviposition effects from extracts of male ejaculatory ducts.

Recently recognized sex peptides or sex peptide-like factors from *Mayetiola destructor* (Diptera: Cecidomyiidae)(Bergh *et al.*, 1992), *Prokelisa* (Homoptera: Delphacidae) (Heady, 1993), *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) (Sherwood and Levine, 1993), *Phormia regina* (Diptera: Calliphoridae) (Merritt *et al.*, 1994), and *Delia platura* (Diptera: Anthomyiidae) (this dissertation, Chapter 4) await chemical characterization.

The pheromone biosynthesis activating neuropeptide (PBAN) system of *Heliocoverpa zea* (Lepidoptera: Noctuidae) and other Lepidoptera is affected by male paragonial gland secretions. Notably, injection of paragonial gland extracts of *H. zea* reduced female pheromone titers and stimulated oviposition (Bird *et al.*, 1991). Purification is underway, employing C-18 SEP-PAKs, ion exchange, and HPLC. Activity from the receptivity terminating factor (RTF) has been recovered using each technique. High variability in female response to injected PBAN and RTF, combined with low numbers of replicates (typically 3 per treatment), is reflected in highly variable pheromone responses. Because chemical analyses have not been completed for sex peptide-using species outside the *Drosophila*, it is unclear if the *Drosophila* chemistry is typical.

Objective of this Report. A program of chemical characterization and behavioral quantification of *D. antiqua* sex peptide and its activity has been underway in the laboratory of J.R. Miller since mid-1989. Here I offer a progress report on attempts to chemically characterize the *D. antiqua* sex peptide.

Extracts were prepared in a variety of HPLC solvents over a range of concentrations, combinations and exposure protocols to evaluate solvent and procedural tolerances. Thermal sensitivity and the protein nature of *D. antiqua* male reproductive tract extracts were also tested. Our experiments

have focused on peptide isolation (using RP-HPLC, microbore RP-HPLC, anion exchange chromatography, electroelution from SDS- and native-PAGE and capillary electrophoresis) and molecular weight determination (using gel filtration, dialysis, SDS-PAGE and native gel electrophoresis, and centrifuge microconcentrating spinfilters). The order of presentation does not necessarily reflect the order in which experiments were conducted.

MATERIALS AND METHODS

Extract preparation and injection. Extracts were prepared by collecting whole reproductive tracts or paragonial glands of male *D. antiqua* into known volumes of ice-cold physiological saline (see Chapter 1, p. 32). Physiological saline needed to be prepared every two to three weeks , as changes occurred during storage and sometimes lead to high mortality among injected females. Reagent contamination during preparation is also a problem, for this reason chemicals should be replaced periodically; a small expense compared to the cost of time and energy wasted when bad saline causes unacceptable high mortality (e.g., see p. 192). Following collection, tissues were sonicated for 15-20 s using a Blackstone™ Probe Sonicator and centrifuged to pellet particulates (this dissertation, chapter 1). The supernatant was collected and used as starting extract. Extracts prepared from whole reproductive tracts or paragonia were as effective as normal mating at initiating oviposition and suppressing mating (this dissertation, Chapter 4). Fraction or extract injections were carried out as described in Chapter 1 of this dissertation.

Insects and bioassay. Within an experiment, *D. antiqua* were of the same laboratory strain and age. Sexually mature six to twelve-day-old virgin females were selected at random from 30 cm x 30 cm x 42 cm single sex

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holding cages (this dissertation, Chapter 3) and assigned to treatment groups. One to five females were used per replicate.

Negative controls for all experiments included: 1)untreated females transferred directly from their holding cages to treatment cage without any manipulation; and 2) saline-injected flies anesthetized with CO_2 and injected with a volume of physiological saline identical to the volume of injected into treated flies. Positive controls consisted of "n" virgin female(s) held with n+1 sexually mature (>6-day-old) males.

Following treatment, females were placed singly, unless otherwise noted, into 20 cm x 9 cm cylindrical screen cages with food, water, and an ovipositional resource (this dissertation, Chapter 1). Ovipositional resources were changed daily throughout experiments and the eggs were collected by flotation and counted. Periodically, samples of eggs from laying females were held in small Parafilm™-covered petri dishes on moistened sand to assay hatching and verify mating status (as per Chapter 1). Mean daily egg production per individual for each female or group of females was the measure of ovipositional response.

Statistics. Experiments were executed in accordance with a completely randomized design. All ovipositional data were computed and analyzed on a per female basis. Following ANOVA, mean separations were accomplished via Student-Newman-Keuls procedure at a=0.05, unless otherwise noted.

Experiments.

Methanol Extraction. Following the 1988 procedure of Chen *et al.* we attempted to extract *D. antiqua* sex peptide from 200 male accessory glands in

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200 µl of 20% aqueous methanol (MeOH), but recovered no activity in the supernatant. After brief sonication using a Blackstone™ Probe Sonicator (Model SS 2) (this dissertation, Chapter 2), extract was centrifuged for 14 min at 16,000xg and 4°C, the supernatant was collected and dried in a chilled vacuum apparatus to remove the MeOH. Once dry, the sample was resuspended in distilled H_2O and stored frozen, the pelleted material was also frozen. The supernatant was injected, at 0.25 µl/female, into 10-day-old virgin females Each treatment was replicated three times, there were three flies per cage.

A second experiment used the pellet from the first experiment. The pellet was thawed and dried under chilled vacuum and resuspended in 200 µl of physiological saline. After vigorous vortexing for *ca.* 30 seconds, the pellet extract was centrifuged for 5 min at 4°C, and the supernatant collected. This saline resuspension of the methanol pellet was injected at 0.25 µl/female into five groups of three virgin females/cage, along with a sample of the original MeOH supernatant as a separate treatment.

Tryptic digestion of male reproductive tract extract. Enzymatic digestion of the putative *D. antiqua* sex peptide was performed to determine if proteins were involved in sex peptide activity as was reported for other Diptera. Temperature sensitivity of *D. antiqua* sex peptide activity (see Thermal sensitivity, below) precluded methods of digestion involving enzyme deactivation by heating. Trypsin immobilized on agarose beads at a concentration of 1mg/ml of 4% agarose bead suspension in 10mM acetic acid (Trypsin-Agarose, cat. no. 191324) was purchased from ICN Biomedicals (1263 South Chillicothe Road, Aurora, Ohio 44202). Immobilization of the trypsin on agarose allowed separation of the enzyme from the protein solution by centrifugation.

A 200 male equiv extract of reproductive tracts in 200 µl of physiological saline was collected and divided into 10 - 20 µl aliquots treated as follows: Aliquot 1 was frozen at -16° until used. Aliquots 2 and 3 were held at room temperature (21.7°C) for 2 h or 4 h with vortexing every 15 min. Aliquots 4 and 5 were mixed with 1 µl of 4% agarose solution, incubated at 37.0°C for 2 h or 4 h (with an additional 1 µl of 4% agarose added after 2 h) in a water bath with vortexing every 15 min. Aliquots 6 and 7 were mixed with 1 µl of trypsin-4% agarose bead solution, incubated at 37.0°C for 2 h, or 4 h (with an additional 1 µl of trypsin/agarose added after 2 h) in a water bath with vortexing every 15 min. The amount of 2% trypsin-agarose solution added to the extracts was determined based on an estimated *ca.* 0.7ng of total protein/male accessory gland pair, as measured by a Bio-Rad™ DC Protein Assay, a colorimetric assay based on the Lowry *et al.* (1951) assay. Given a 20 male equiv/extract sample with *ca.* 13 µg of protein/sample (20 males x 0.7 ng/male), 1 µl of enzyme-agarose solution was added to extracts to be digested. One µl of enzyme solution in 20 µl of extract solution yielded a 7% enzyme concentration in solution, a >3x excess relative to what was recommended by the manufacturer. Aliquots 8 and 9 were incubated at 37°C for 2 h or 4 h in a water bath with vortexing every 15 min. After treatment, extracts were centrifuged for 30 min at 8,000xg and 4°C, and then stored frozen at -16°C.

The differently treated aliquots were injected at 0.5 µl/individual. Twenty four, 6-day-old virgin females per treatment were used in the bioassay. Eggs were counted daily for 6 days. On day 5, two sexually mature males were added to the cages of each surviving female. After 3 days, eggs were collected, counted, and set up in Parafilm™-covered petri dishes to assay for evidence of mating. The intent of the mating test was to establish

the receptivity status each surviving female in order to determine if the digestion treatment would also eliminate the receptivity-inhibiting effect of male extract.

HPLC Solvent Bioassay. During isolation attempts using *Drosophila* methodology, we came to suspect that *D. antiqua* sex peptide was sensitive to some chromatographic solvents. Therefore, onion fly sex peptide activity was assessed for extracts exposed to: trifluoroacetic acid (TFA), acetonitrile (AcN), formic acid, isopropyl alcohol, distilled water (dH_2O), phosphate-buffered saline (137 mM NaCl; 8 mM $Na_2HPO_4 \cdot 7H_2O$; 2.7 mM KCl; 1.5 mM KH_2PO_4; pH 7.2) , phosphate buffered saline concentrated 10x, onion fly physiological saline (this dissertation, Chapter 1), sodium dodecylsulfate (SDS) at 0.01%, 0.1% and 0.5%. Tests were also run on the effect of concentration techniques: nitrogen or speed-vac drying, on sample activity, as well as on combinations of solvents and concentration techniques. In most cases, paragonial glands, were collected into the respective solvent, concentrated or dried, and resuspended in distilled water or solvent. These experiments were conducted over a 4-year period, often in response to equivocal bioassays. In this work, both single and multiple females per cage were used; however, accumulated data from all bioassays were analyzed as mean eggs/female/cage. The number of replicates/treatment ranged from 3 to 51.

Thermal stability.

Experiment 1. 10 µl aliquots of a 76 male equiv/50 µl extract prepared in onion fly physiological saline were held for 10 min at 40°, 60°, 80°, or 100°C in a Brinkman circulating water bath. These extracts, along with an unheated

extract stored at 4°C were injected at 0.25 µl/individual. There were two replicates/treatment, with three flies injected per replicate.

Experiment 2. Pellets and supernatants from a onion fly physiological saline extract (80 male reproductive tracts in 75 ul of onion fly physiological saline; sonicated and centrifuged) were tested for ovipositional activity after 10 min of exposure at 40°, 50°, or 60°C in a Brinkman circulating water-bath. Extracts of pelleted material were prepared by sonicating and centrifuging the pellet remaining once the original supernatant was collected. This second supernatant was called the pellet extract. A sonicated, but uncentrifuged, extract (39 male reproductive tracts in 60 µl of onion fly physiological saline) was also treated as above to see if the presumed greater complexity of crude extracts made them less sensitive to elevated temperature. All extracts were held at 4°C before and after heat treatment. Injected flies received *ca.* 0.75 µl of extract. There were four replicates/treatment, with one female/replicate.

Molecular weight estimation.

Microcentrifuge filter sizing. Microcentrifuge filters with defined molecular weight cutoffs (MWCOs) were used to estimate the molecular weight of *D. antiqua* sex peptide. Ultrafree-MC Microcentrifuge filters were obtained from Millipore™ with MWCOs of 300k, 100k, 30k, and 5k (according to the manufacturer 90% of materials at or above the stated MWCO are retained). Filter units were preconditioned by rinsing them with *ca.* 100 µl of distilled H_2O and centrifuged at 3000xg. Three experiments were conducted to estimate *D. antiqua* molecular weight range with these filters.

Experiment 1. 100 µl of 98 male equiv/125 µl extract, sonicated and centrifuged, was placed in a 300k MWCO microcentrifuge filter and centrifuged at 5000xg until the solution had passed through the filter (<5

min). The top of the filter unit was washed with two-50 µl aliquots of physiological saline to recover retained protein and the combined wash volume stored frozen (estimated concentration 1 male equiv/µl). The filtrate (90ul) was recovered from the unit and added to the top of a 100k MWCO microcentrifuge filter and centrifuged as described above. Approximately 20 min were required for all of the extract to pass through the 100k filter. The top of this filter was washed as described above, and the filtrate (80 µl) was added to a 30k MWCO microcentrifuge filter and centrifuged as above. The top of the filter was washed to recover the retentate which, along with the the filtrate was stored at 4°C. This process created 5 fractions: a ≥ 300k fraction, a <300k and ≥ 100k fraction, a <100k and ≥30k fraction, a < 30k fraction, and a 25 µl sample of unfiltered starting extract.

Each fraction was injected at *ca.* 0.25 µl/individual into 7-day-old virgin females. There were three replicates/treatment with three females/replicate cage. Eggs were counted for six days.

Experiment 2. An extract of 138 male reproductive tracts collected into 160 µl of physiological saline, sonicated and centrifuged was the starting material for this experiment. 40 ul of extract (supernatant) were retained for use as a positive control. To the remainder (*ca.* 120 ul) containing *ca.* 112 male equiv was added 280 µl of physiological saline to bring the volume to 400 µl. By diluting the extract, I hoped to reduce the probability that the filter would be quickly plugged so as to promote aggregation and restriction of flow through the filter. To further reduce that likelihood, extract was passed through the filters in 100 µl aliquots. Two microcentrifuge filters with MWCOs of 300k and 5k were used sequentially as described in Experiment 1. Three fractions were generated; ≥ 300k, <300k and ≥5k, and <5k. All were concentrated to 50 µl under a stream of nitrogen before being injected at *ca.*

0.33 µl/individual into twelve 6-day-old virgins/treatment. In addition to the fractions and starting material, untreated, sham injected, saline injected, and mated controls were administered. There were four replicates per treatment and three females per replicate cage. Eggs were counted for five days.

Experiment 3. An extract of 100 male paragonial gland pairs collected into 100 µl of physiological saline, sonicated and centrifuged was divided into two 50 µl aliquots. The extracts were passed through either a 30k or 5k MWCO microcentrifuge filter by centrifugation at 3000xg. After 17 min of centrifugation, 50 ul of distilled water was added to each filter and centrifugation continued for another 17 min. The filtrate was collected from both filters and the tops of the filters washed by vortexing each filter unit after two-50 µl aliquots of distilled water were added to the top of the filter. Both the filtrate and the collected retentate were concentrated to 50 µl under nitrogen. Filtered fractions along with starting extract, untreated, and mated controls were administered to 8-day-old virgin females. There were three replicates per treatment and two females per replicate cage. Eggs were collected daily for seven days.

Dialysis

Experiment 1. Three 50 male equivalent reproductive tract extracts were collected into 45 µl of distilled water, 45 µl of phosphate buffer (see p. 137) or 118 µl of 10x concentrated phosphate buffer. The extracts were dialyzed for 2.5 h at 4°C against 250 µl of distilled water across a Spectra/Por Cellulose Ester membrane (Spectrum Medical Industries, Los Angeles, CA) with a MWCO of 1000 . Retained material was injected at 0.5 µl/individual into ten 13-day-old virgin females per treatment. There were five replicates

with two females/replicate for each treatment. Eggs were counted for three days. Experiment 1 was twice repeated using 40-50 male equiv extracts prepared as above and injected into 7 to 8-day-old virgins. Total number of replicates was 11 for all treatments except the phosphate buffer extract (PO_4) which was replicated five times.

Experiment 2. 98 and 70 male equiv extracts collected in 50 µl of phosphate buffer. The 98 male extract was dialyzed for 4 h at 4°C against distilled water across 1000 MWCO Spectra/Por membrane tubing (dia. 7.5 mm; length *ca.* 2.5 cm after closing the ends of the tubing with dialysis tubing closures (Spectra/Por)). The 70 male equiv extract was dialyzed for 20 h at 4°C against distilled water across 500 MWCO Spectra/Por membrane tubing (dia. 7.5 mm; length *ca.* 2.5 cm). Both extracts doubled in volume during dialysis. Samples of starting extract were injected at 0.25 µl/individual. Retentate was collected from the dialysis tubing and injected at 0.5 µl/individual into six, 11-day-old virgin females per treatment. To compensate for the dilution during dialysis, injection volume was doubled. There were three replicates and two females/replicate for each treatment. Eggs were counted for four days.

Experiment 3 20 µl samples of active extract from Experiment 2 were used to test the effect of exposure to dialysis membrane on extract activity. A 2.5 cm length of well-rinsed 1000 MWCO Spectra/Por membrane tubing was cut into seven pieces and added to a microcentrifuge tube containing a sample of the 98 male equiv extract from Experiment 5 and held at 55°C in a water bath for 3 h. An equivalent sample from the 70 male equiv extract (from Experiment 2) was treated identically, except it was not exposed to the membrane. Samples were injected at 0.25 µl/individual into six, 6-day-old virgin females. There were three replicates and two females/replicate for each treatment. Eggs were counted for three days.

Protein separations.

Gel filtration chromatography. *Gel Filtration of male reproductive tract extract with Sephadex G-50.* An extract of 100 male reproductive tracts collected in 100 µl of physiological saline was sonicated and centrifuged for 30 min at 12,000xg. A 75 µl sample of the supernatant was loaded onto a 0.7 mm diam x 8 cm long gel filtration column packed with Sephadex™ G-50 (exclusion limit: 30k). The column was equilibrated in physiological saline (*ca.* 150mM) with a flow rate of 0.061 ml/min, maintained by a peristaltic pump; a Waters Model 440 UV detector was set at 254nm. Seven fractions spanning a broad void volume and elution peaks were collected during the 92 min run and immediately frozen. Fractions were concentrated using Millipore™ 5000 MWCO microcentrifuge filters spun at 4500xg to yield a retentate which was recovered in *ca.* 50 µl.

Two fractions, corresponding to the centers of the void volume and elution peaks were bioassayed along with a sample of starting extract and mated and untreated controls. Flies injected with elution fractions received *ca.* 1 µl/individual; the starting extract was injected at 0.5 µl/individual. There were eight replicates/treatment and one female/replicate.

HPLC gel filtration of male paragonial gland extract with polyhydroxyethyl A. An extract, prepared from 150 male paragonial gland pairs collected into 50 ul of distilled water, sonicated, and centrifuged was applied to a PolyLC™ polyhydroxyethyl A 250 mm x 9.4 mm HPLC Gel Filtration column with a pore size of 20nm. Running isocratically in a pH 5.5 phosphate buffer (see pg. 136), 17 fractions were collected into 1 ml polyethylene microcentrifuge tubes during the 30 min run. Each fractions was concentrated under nitrogen to *ca.* 100 µl, and injected at 0.4 µl/individual (equivalent to 0.6 male equiv) into ten, 6-day-old virgin

females. Samples of each concentrated fraction were recombined, further concentrated and injected as pooled extracts. Fractions 1-4, 5-10, and 11-17 were combined into Fractions P1, P2, and P3 respectively and injected as above. There were two replicates/treatment and five females/replicate cage. Eggs were counted for four days.

OTHER HPLC

Vydac C4 RP-HPLC. 300 male equivalents of *D. antiqua* male paragonial gland extract collected and processed in physiological saline were injected onto a Vydac C_4 analytical column and eluted at 1.5 ml/min with a 50 min linear gradient 0-25% buffer B (95:5 AcN:H_2O) in 0.04% TFA (buffer A). A Milton Roy Spectro Monitor 3100 detector was set at 220nm and attenuation of 0.05 AUFS. Five fractions labeled A-E were collected, lyophilized and resuspended in a diluted (10%) physiological saline solution, lyophilized again and resuspended in 300 µl of distilled water. Fractions were injected at 0.25 µl/individual (*ca.* 1/8th male equiv) into twelve, 7-day-old virgin female *D. antiqua*. There were three replicates/treatment with four females/replicate cage. Eggs were collected and counted for four days.

C8 Microbore HPLC, Microbore HPLC was used repeatedly in attempts to separate active material. A 212 male-equivalent extract collected in 200 ul of physiological saline, sonicated and centrifuged, was fractionated on a 250 mm x 1 mm C_8 column. A Brownlee Microgradient Systems pump supplied a flow rate of 50 µl/min. Solvent A was 90% AcN in 0.1% TFA, solvent B was 0.1% TFA in H_2O. The gradient was 0-100% B in 90 min and began 8 min after injection; the Spectroflow 783 detector was set at 214 nm. Fractions were collected every two min until a peak appeared and then peaks were collected as individual fractions. 63 fractions were collected during the first 45 min of

the run and brought to near dryness in a speed vac to eliminate the solvents (Fractions 1-53 went briefly to dryness), resuspended in 100 µl of distilled water, brought to near dryness again and resuspended in physiological saline. Fractions corresponding to peaks were bioassayed first, and then 25 µl of each remaining non-peak fraction were pooled in sequential groups of five, reduced to 31 µl to achieve a concentration of *ca.* 1 male/µl and injected at 0.25 µl/individual. There were three replicates/treatment with five females/replicate for each peak fraction or pooled fraction. Eggs were counted for five days.

C18 microbore RP-HPLC Run 1. For C_{18} reverse-phase HPLC fractionation we used a microbore column (1 mm x 40 mm) and a linear gradient of 0-100% buffer B (475 mM ethanol, 150 mM isopropyl alcohol in 5 mM phosphate buffer) in 90 min (buffer A was 5 mM phosphate buffer @ pH 6.73). An extract of 190 pairs of paragonial glands in 70 µl of distilled water was injected onto the column. Flow rate was 50 µl/min with UV detector set at 214 nm. Total run length was 110 min; the gradient began 17 min into the run (9 min later than intended). Fractions were collected into methanol-washed polyethylene microcentrifuge tubes and frozen immediately. Fractions were later concentrated to a final volume of 100 µl using a speed vac.

Concentrated Fractions 2, 6, 7, 8, 9, 10, 11, 12, & 14, corresponding to peaks on the chromatogram (Figure 41), were injected into 7-day-old virgin females at 0.75 µl/female (*ca.* 1.5 male equiv). There were five replicates/treatment and one female/replicate. Eggs were counted for one week.

Runs 2, 3, & 4. Three additional microbore HPLC runs were made using the same column and solvent system. A 90 min gradient was also used,

but it was started at 8 min into the run. A 509 male equiv extract of paragonial glands in H_2O divided into three *ca.* 30 µl aliquots was used. Fractions were collected as above; those corresponding to two apparently active fractions: 2 (2.5-10.3 min) and 6 (35.5-38.3 min), from the original run were concentrated to *ca.* 50 µl. 0.5-0.75 µl (1.7-2.6 male equiv) injections of each fraction were administered to 6-day-old virgin females. There were five replicates/treatment and one female/replicate cage. Eggs were counted for one week.

Run 5. 128 male equivalents were fractionated using a new C_{18} microbore column, and a protocol identical to the original C_{18} run including the 17 minute delay to start the gradient in an attempt to replicate exactly the previous running conditions. Fractions containing peaks from the early portion of the run, where activity had been found before, were collected on ice and injected directly into 6-day-old virgin females within 2 h of elution. Each of five females/treatment received a 0.75 µl injection. Remaining portions of each fraction were concentrated with a speed vac, frozen, and injected the following morning. Eggs were counted for one week.

C_{18} buffer bioassay. The effect of exposure to solvent conditions like those occurring during a microbore run was examined by adding 9 µl of buffer B (475mM ethanol, 150mM isopropyl alcohol in 5 mM phosphate buffer) to *ca.* 24 µl of active extract (0.75 male equiv/µl) to duplicate the approximate concentration of buffer B (*ca.* 28%) at the time Fraction 6 eluted if the gradient began at 8 min. Three tubes of extract were mixed with buffer B. Two were quickly (within <30 min) taken to near dryness, one with a speed vac and the other under a stream of nitrogen, and then brought up to 25 µl with distilled water. The third was not dried.

Three additional tubes of extract were also prepared, but were not treated with buffer B; instead, 9 µl of distilled water was added to each. Two were taken to near dryness with a speed-vac or under a stream of nitrogen and brought up to 25 µl with distilled water as above. After resuspension, all extracts were frozen before bioassay. An untreated, undried extract was also prepared at this time and frozen. After 1 h, 0.5 -0.75 µl of each extract was injected into 7-day-old virgin females. There were five replicates/treatment with one female/replicate cage.

Weak Anion Exchange Chromatography. An anion exchange column was prepared in a 4°C coldroom by loading 5 ml of DEAE Sepharose™ CL-6B weak anion exchange resin (equilibrated in 50 mM NaCl-20 mM TRIS/HCl solution) into a 1 cc Tuberculin™ syringe with a stopcock and a small amount of glass wool to retain the resin. After the slurry settled, several ml of 50 mM NaCl-20 mM TRIS/HCl solution were allowed to flow through the column and when the solution level dropped to just above the top of the resin-bed the stopcock was closed.

An 85 µl sample of a 300 male equiv paragonial gland extract, collected in 100 µl of 20 mM TRIS/HCl; 50 mM NaCl solution (pH 7.5), sonicated and centrifuged, was then loaded on the top of the column along with an additional 115 µl of 20 mM TRIS/HCl; 50 mM NaCl solution and the stopcock opened. 200 µl of fluid was collected, bringing the solution level to just above the top of the bed. A 150 mM NaCl-20 mM TRIS/HCl solution was then added to the top of the column and the stopcock again opened. Eight, 400 µl fractions were collected before the stopcock was closed and a 250 mM NaCl-20 mM TRIS/HCl solution was added to the top of the column. The stopcock was opened and 8 more 400 µl fractions were collected before a 500 mM NaCl-

20mM TRIS/HCl solution was added to the top. Nine 400 µl fractions were collected while the final solution flowed through the column.

All 25 fractions were immediately stored at 4°C and then frozen after the run was completed. Fractions were concentrated and desalted using Millipore™ Ultrafree-MC microcentrifuge filters (5000 MWCO) spun at 4000xg in a Eppendorf 5414 C Microcentrifuge at 4°C for 3 h. After centrfugation, the filters were twice washed with 50 µl of dH_2O. The final volumes of the recovered fractions were between 110-120 µl.

Fractions were injected at 0.5 µl/individual into 7-day-old virgins. Given the initial loading rate, the final concentration in any one tube could be no more than 2.5 male equiv/µl. The injection volume was equivalent to >1 male equivalent/female. There were four replicates/treatment and two females/replicate cage. Eggs were counted everyday for five days.

Capillary Electrophoresis. Capillary electrophoresis was used in an attempt to separate and visualize components of *D. antiqua* male paragonial gland, testes and whole reproductive tract extracts. We used a model 3850 ISCO capillary electrophoresis system with a CE-100 (moderately hydrophobic) 75 cm x 75 µm column. The running buffer was 10mM sodium phosphate/0.01% Brij 35 at pH 7.0. Column rinsing buffer was 0.5% Brij.

When a potential difference is applied across the capillary during an electrophoretic run, molecules of varying charge and mass move through the column at different rates as a consequence of electrical force. The running conditions set up an electroosmotic flow moving from the anode to the cathode. Positively charged species are carried toward the cathode in the electroosmotic flow and are further attracted by their charge (electromigration). Anionic species move more slowly in the electroosmotic

flow because electromigration draws then toward the anode. Neutral species move by force of the electroosmotic flow only. Positively charged species elute first, followed by neutrals and then negatively charged species.

Extracts of whole male reproductive tracts, paragonial glands, and testes were collected from 8-day-old male *D. antiqua* at 0.5 male equiv/ul. Extracts were individually loaded onto the capillary column during two second injections and run at 22.5kV and 27 μA, with detector set at 214nm.

Electrophoresis and electroelution of proteins from denaturing (SDS-PAGE) and non-denaturing (native-PAGE) gels.

Electrophoresis and electroelution procedures: Elution of proteins from SDS- and native-PAGE was investigated as a method to separate and recover sex peptide activity. Recipes for preparing denaturing (SDS-PAGE) and non-denaturing (native-PAGE) electrophoretic gels and their respective buffers are found in Appendix 2, along with silver and Coomassie blue staining protocols. All electrophoretic gels were 8 cm x 10 cm, 0.75 mm thick mini gels unless otherwise noted. Native-PAGE and electroelution were conducted at 4°C.

The electroelution chamber was constructed, and fractions were eluted from gels according to the method of Hunkapillar *et al.*, (1983). Elution cells were purchased from CBS Scientific (Del Mar, California). Accessory gland extracts were electrophoresed on nondenaturing discontinuous mini gels using a mini Protean II unit (BioRad).

While the mini gel was running, the electroelution unit was preconditioned by adding elution buffer (0.05 M NH_4HCO_3) to cover the buffer intakes (*ca.* 400 ml) in the return chamber so that circulation was maintained . Elution buffer was added to both wells of each elution cell to

check for leaks around the dialysis membranes (3500 MWCO Spectra/Por 6) capping the gel holding and protein recovery wells. Completed cells were placed in the electroelution chamber.

After electrophoresis, the stacking gel was removed and placed in a microcentrifuge tube and stored at 4°C. Strips of gel were sliced from each edge of the resolving gel and stained with colloidal Coomassie blue. Bands identified from the side strips were used as locators to guide alignment and horizontal slicing of the gel based on typical banding patterns observed on native-PAGE reference gels. Figure 25, is a photograph of a 10% native-PAGE gel used as a reference to plan the slicing of gels for the native-PAGE electroelution experiments that follow.

After removing the locator slices, the remainding gel was stored frozen until the band locator lanes had been stained and compared to the pattern of bands known from the reference gel. Based on the locater-reference comparison, the gel was aligned and cut into slices perpendicular to the direction of protein migration. Each slice was then diced into *ca.* 1-2 mm cubes and added to the gel well of an elution cell, and overlaid with soaking buffer (0.4 M NH_4HCO_3). After 5 h, the buffer electroelution unit (0.05 M NH_4HCO_3) was exchanged with fresh elution buffer. The peristaltic pump was turned on and the elution cells were filled with enough elution buffer to fill the cross passage between the cathodic and anodic cells. Elution began at 50 V DC and continued for 18 h, whereupon the elution buffer was changed to dialysis buffer (0.01 M NH_4HCO_3) and eluted for 24 h. When completed, the now-concentrated eluants (*ca.* 150 µl) were collected from the protein recovery well (anode) and bioassayed.

Analytical SDS-PAGE using 12% or 15% gels was often used to assess the quality of fractionation in the electroeluted native-PAGE fractions. The

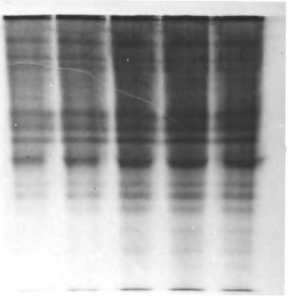

Figure 25 *Delia antiqua* male reproductive tract dilution series on an 8 cm x 10 cm, 10% native gel. Lanes from left to right contain 10, 20, 30, 40, and 50 male equiv. The protein banding pattern from this gel was used to locate the major bands and to plan the slicing of gels for the native gel electroelution experiments.

recipes for the gels, buffers, and staining procedures are presented in Appendix 2. Analytical SDS-PAGE of eluted fractions was run at 200V constant current on the same apparatus used for native-PAGE. ISS™ Mid Range Molecular Weight Standards (Integrated Separation Systems, Natick, MA) 95k, 68k, 39k, 29k, 20.4k, and 14k were also run on each gel. Analytical gels were silver stained using a BioRad™ silver staining kit (BioRad™, Hercules, Ca).

Non-denaturing SDS-PAGE. Recovery of activity was attempted using analytical SDS-PAGE of unreduced extract, i.e. they were processed for electrophoresis without addition of the reducing agent β-mercaptoethanol to the sample buffer or heating intended to disrupt disulfide bonds and secondary protein structure. Using this system, protein banding patterns were very sharp and distinct. If activity were recovered, fractionation potential was great.

An Extract of 400 male reproductive tracts was collected into *ca.* 400 µl of cold physiological saline, sonicated for *ca.* 10 s, and centrifuged at 16,000xg for 20 min. The supernatant was collected and stored at -16°C until it was concentrated under nitrogen to *ca.* 200 µl from an original volume of 400 µl.

Four µl of sample buffer (2.89 ml of H_2O, 0.5 ml of 0.5 M Tris-HCl, 0.4 ml of glycerol, 0.16 ml of 10% SDS, and 0.05 ml of 0.05% bromphenol blue; total volume = 4.0 ml) was added to the 190 µl extract containing *ca.* 380 male equiv. The extract plus sample buffer was loaded onto the 4% stacking gel of a 12% SDS-PAGE gel (see appendix 2). The gel had been chilled for 1 h at 4°C before the sample was applied and electrophoresis begun. A small sample, 10 ul, of the extract was saved for use as a positive control.

Due to a leak in the upper buffer chamber, current was applied for only 16 min. This was long enough for the bromphenol blue front to just enter

the resolving gel. This gel was divided into only two fractions, the stacking gel and the top most portion (1 cm) of the resolving gel.

The gel slices were processed and electroeluted for 6 h, as decribed for native-PAGE electroelution (see above), yielding two - 100 µl concentrated fractions. These were injected at 0.5 µl (1 male equiv)/individual into 8-day-old virgin females. There were ten replicates/treatment and one female/replicate. Eggs were counted for five days.

5% Native-PAGE. A 5% native gel, with a 3% stacking gel was prepared by adjusting the 8% native-PAGE recipe (Appendix 2). 240 µl of a 250 µl sample of a 400 male reproductive tract extract collected into 250 µl of physiological saline, sonicated and centrifuged, was used. 24 µl of sample buffer (50% sucrose; 0.1% bromphenol blue) was added to the *ca.* 384 male equiv sample before electrophoresis. After the preparative comb was removed, the upper buffer unit (cathode) was assembled and placed in the lower buffer (anode) tank. Upper tank buffer (37.6 mM Tris, 40 mM glycine; pH 8.9) was added to the upper buffer chamber to a level 3 mm above the stacking gel surface. Lower tank buffer (63 mM Tris, 0.5 mM HCL; pH 7.5) was added. Any air bubbles along the bottom of the gel, which could interfere with the electrical conductivity, were removed with a glass rod, and the sample (extract + sample buffer) was pipetted into the preparative well. Prestained mid-range molecular weight standards (Integrated Separation Systems, Natick, MA) were also run to monitor the progress of the electrophoresis. The gel was run at 200 V constant voltage for 60 min.

This gel was run only until the bromphenol front was approximately 1 cm into the resolving gel. It was then cut into three pieces: 1) the stacking gel, 2) the top 1 cm of the resolving gel, and 3) the region of the resolving gel ahead of the bromphenol front (position of the front was determined by

examining the bands from stained edge slices of the gel). The three fractions were electroeluted for 6 h, aliquoits were collected after 3 h. Protein from gel Fractions 2 and 3 was also collected by diffusion (10 h at 4°C) into fresh electroelution buffer.

Fractions were injected at 0.5 µl/individual into 6-day-old virgin females. There were ten replicates/treatment, and one female/replicate cage. Eggs were counted for seven days.

8% Native -PAGE An 8% native-PAGE resolving gel (thickness = 1 mm) was allowed to polymerize overnight in a refrigerator at 4°C (Appendix 2). The following morning, a 4% stacking gel, with preparative comb in place, was poured and polymerized for 30 min. The gel was then moved to a 4°C coldroom to equilibrate for 30 min. 300 µl of sample (320 male equiv in 100 ul of physiological saline + 200 µl of sample buffer (50% sucrose, 0.1% bromphenol blue) was added to the prep well. Electrophoresis proceded as described above. When complete, the gel was processed for electroelution as described above. The resolving gel was divided into ten equal sized slices *ca.* 0.5 cm wide. 7-day-old virgin females were injected with 0.5 µl of fractions. There were five replicates/treatment and one female/replicate cage. Eggs were counted for seven days.

10% native-PAGE. *Experiments 1 & 2.* Recovery of activity from electrophoretic gels was a step forward in attempts to isolate the *D. antiqua* sex peptide. However for gels to be useful for fractionating extracts, activity had to be recoverable from gels run longer than a few minutes.

An extract of 300 male reproductive tracts was collected into 100 µl of physiological saline, sonicated and centrifuged. The 105 µl supernatant was used in a full-length fractionation. A 10% resolving gel (Appendix 2), 1 mm thick was poured, overlaid with gel buffer and allowed to polymerize

overnight in at 4°C. A 1.5% stacking gel was poured atop the resolving gel and polymerized under UV light. The gel was assembled in the BioRad™ Mini Protean II Electrophoresis Unit and chilled to 4°C, 10.5 µl of sample buffer was added to 104 µl of starting extract and the sample was added to the sample well. The gel was run at 200V. After 50 min, the run was stopped 1 cm wide locator strips were cut from the sides and stained. The present banding pattern was compared to the reference gel and cut into five fractions. Fraction 1 was the stacking gel. Fraction 2 was the first 1 cm of the resolving gel. Fraction 3 consisted of the next 1.5 cm of resolving gel. Fraction 4 was the 1 cm of gel immediately below Fraction 3, and Fraction 5 was the bottom 1 cm of the gel. Each slice of gel was immediately diced into *ca.* $1mm^2$ pieces and loaded into one of five elution cells. Taking into account the material used at the sides of the gel, *ca.* 240 male equiv were available to be electroeluted.

Fractions were electroeluted at 150 V. After 7.5 h, the five eluted fractions, each *ca.* 150 µl, were collected and stored frozen. Analytical SDS-PAGE verified that protein was present in the eluted gel slice fractions.

Each of the 5 fractions was injected at 0.5 µl/individual into 5-day-old virgin females. There were ten replicates/treatment and one female/replicate. Eggs were collected for 8 d. After 2 d, few eggs had been laid. A sample (50 µl) from Fraction 2, which was showing borderline activity was concentrated 5x under nitrogen to increase the concentration of any active material, and injected into 7-day-old virgins. 50 µl samples from all five fractions (1-5) were also combined and concentrated 5x under nitrogen and injected into additional females.

Experiment 2 followed the first protocol exactly. However, all five electroeluted gel fractions were individually concentrated 5x under nitrogen before injection, no unconcentrated fractions were bioassayed.

Experiment 3. A third native-PAGE electroelution experiment was intended to verify the results of Experiments 1 & 2 and to discern the precise location of activity within Fraction 2. All running and electroelution conditions were identical to Experiments 1 & 2. Following electrophoresis, the slice of gel corresponding to Fraction 2 was subdivided into four-2.5 mm wide slices (from the top of the fraction downward they were identified as 2.1, 2.2, 2.3, and 2.4), electroeluted and concentrated 5x before injection. Four 25 ul aliquoits of each electroeluted component of Fraction 2 were combined and concentrated under nitrogen to 25 µl in order to reconstitute Fraction 2 in concentrated form. Fractions 1, 3, 4, and 5 were processed as in Experiments 1 & 2 and concentrated 5x. As before, all concentration was done under a stream of nitrogen at room temperature. Injection an bioassays were carried out as per Experiments 1 & 2.

RESULTS AND DISCUSSION

Attempted methanol extraction of *D. antiqua* sex peptide Unlike the work on *Drosophila* (Chen *et al.*, 1988), *D. antiqua* sex peptide activity was not recovered from the supernatant of an male reproductive tract extract collected and centrifuged in 80% MeOH (Figure 26A); but activity was recovered from the extract pellet resuspended in physiological saline (Figure 26B). This suggests onion fly sex peptide is more polar than that of the *D. melanogaster*.

Consistently high activity was obtained from extracts prepared in onion fly physiological saline (also see Table 6, below), and thus it was adopted for preparation of most extracts (this dissertation, Chapters 1, 2, & 4). Active material may also be reliably extracted with distilled water or phosphate buffers. Extraction of reproductive glands in concentrated phosphate buffer resulted in very active extracts (e.g., see dialysis p. 170). Perhaps the ability of

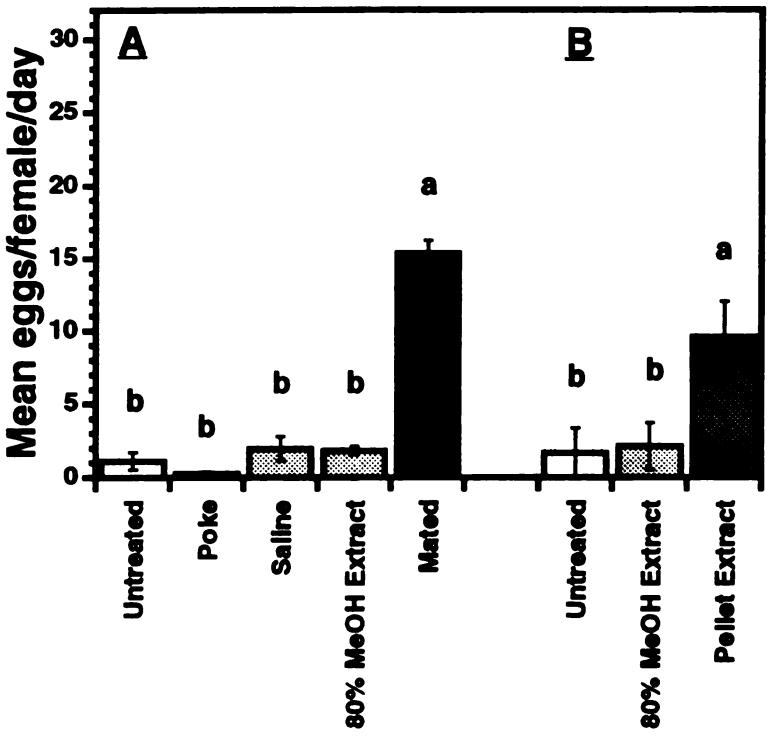

Figure 26. Ovipositional response of female *D. antiqua* injected with extracts prepared in 80% aqueous methanol (MEOH). (A) Bioassay results for the supernatant of a 200 male equiv extract of male paragonial glands extracts in 80% MEOH. (B) Bioassay results for the pellet from a MEOH extract resuspended in physiological saline. (A) and (B) were replicated 3 and 5 times respectively and analyzed separately. There were three females/replicate. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

the -$H_2PO_4^-$ ion to stabilize protein structure (Voet and Voet, 1990) is partially responsible for the high activity.

Drying under nitrogen or in a Speed-vac did not inactivate extracts; however, the low threshold for activity of the bioassay (this dissertation, Chapter 2) could conceal considerable losses with no apparent diminution of behavioral effects. The stability of sex peptide activity under drying conditions suggests that in spite of its solvent sensitivity it can withstand conditions of increasing salt concentration.

Tryptic digestion of male reproductive tract extract. Extracts exposed to agarose-immobilized trypsin were inactivated, those not subjected to trypsin digestion retained high activity (Figure 27). There was no difference between extracts treated for 2 or 4 h, thus the data were pooled for analysis. Both mating inhibition and ovipositional activity of females injected with trypsin-digested extract resembled the negative controls. Behavioral action of *D. antiqua* paragonial glands appears to be proteinaceous or at least requires the presence of a protein component.

Since digestion abolished both mating inhibition and the ovipositonal activating effects of extract, I speculate that the same protein(s), destroyed by digestion, is intimately involved in both effects. In *D. melanogaster*, mating receptivity and ovipositional activation both occur at 0.6 pmol/female, suggesting the effects are caused by the same molecule (Schmidt *et al.*, 1993a). In dose response studies of *D. antiqua* sex peptide activity (this dissertation, Chapter 2), the linkage between receptivity and ovipositional effects was strong. Only at dosages below the BR_{50} (*ca.* 1/40th male equiv/female), was there evidence for decoupling of receptivity and ovipositional effects. Sex peptide may not be just a protein. A nonproteinaceous material associated

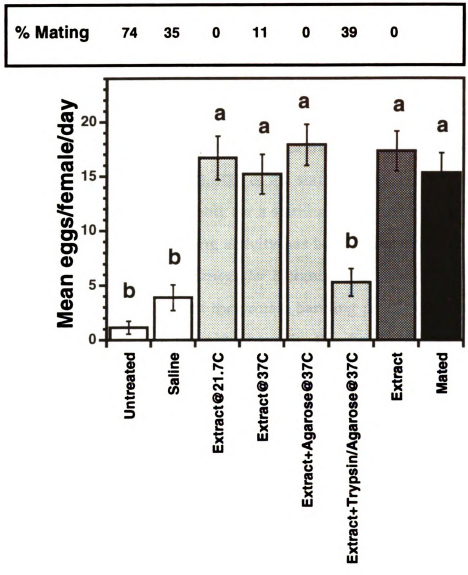

Figure 27. Oviposition and receptivity status of female *D. antiqua* injected with trypsin-digested male reproductive tract extracts. Extracts were injected at 0.5 µl/individual (0.5 male equiv). There were 24 replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

with a carrier or action by a requisite chaperone protein could yield a similar activity profile following the above treatments.

D. antiqua paragonial extracts are apparently devoid of significant protease activity, as extracts are active after many months in cold storage and could be left out at room temperature (21.7°C) for several h (overnight on one occassion), or incubated at 37°C for 4 h without measurable loss of activity. However, it should be pointed out that because of the high sensitivity of *D. antiqua* females to extract (Chapter 2), 90% of the starting activity could be lost before response would decline noticably for a starting dosage of 1 male equiv *per* 0.5 µl injected. Protease-inhibiting activity has been documented in accessory gland secretions of *D. melanogaster* (Schmidt *et al.*, 1989).

Electrophoretic data show that the protein banding profile of extract following digestion is changed (Figure 28, Lane 5). Few bands of large molecular weight species remain unchanged in the digested extract, and shifted bands are seen when compared with untreated extract (Lane 2). Though still highly active, significant protein loss was evident for various components of the extract incubated with only agarose beads (Figure 28, Lane 4). Clearly, the qualitative and quantitative composition of solutions can vary substantially but yet yield similar activity in the bioassay.

Solvent Bioassay. The combined results of solvent bioassay are presented in Table 6. Ovipositional stimulating activity was lost upon even brief exposure to 0.04% TFA, 20% AcN, 20% isopropanol or 50 mM formic acid. Only at 0.01% TFA (pH 3.9) was marginal activity retained. Sensitivity to solvents (TFA and AcN) favored in high resolution RP-HPLC systems may be one reason chemical characterization of these molecules is difficult. Use of the surfactant SDS at 0.01% in extracts offered no protection from organic

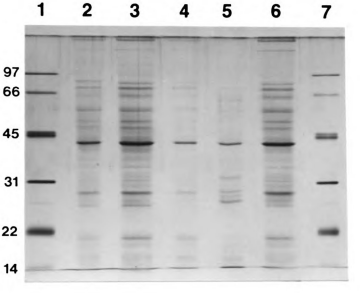

Figure 28. Silver stained 12% SDS-PAGE of male reproductive tract extracts from the tryptic digest experiment. Lanes 1 and 7 are molecular weight markers. Lane 2 is untreated extract of male reproductive tracts. Lane 3 is male extract heated to 21.7°C. Lane 4 is male extract exposed to 4% agarose solution. Lane 5 is male extract exposed to agarose bound trypsin. Lane 6 is male extract held at 37°C. Treatments were for 4 h. 10 male equiv of extract were used in each lane 2-6.

Table 6. Effect of extraction medium and exposure to HPLC solvent systems on oviposition stimulating activity of male paragonial gland extracts. Treated extracts were injected into 6-10 day old virgin female onion flies at 0.25 to 0.5 male equiv/individual. All extracts were resuspended in distilled water to restore original concentration before injection.

Extraction medium	n	Solvent exposure treatment	Final pH	Drying conditions	mean eggs/ female/d (mean±SE)
H_2O	9		7.0	N_2	17.4 ± 5.7
Saline (see p. 32)	5		6.8	--	18.3 ± 3.6
Saline	9		6.8	SV	17.8 ± 2.8
Saline	42		6.8	N_2	13.4 ± 1.5
0.01% SDS	21		6.8	N_2	16.9 ± 2.6
0.1% SDS	9		6.8	--	13.1 ± 3.7
0.5% SDS	5		6.8	--	2.0 ± 1.5
PO4 Buf. (see p. 136)	5		3.0	--	0.6 ± 0.6
PO_4 Buf.	13		7.4	N_2	14.8 ± 4.3
10x Conc. PO_4 Buf.	3		7.4	N_2	23.5 ± 2.8
PO_4 Buf.;5%AcN	5		7.4	N_2	1.1 ± 0.4
PO_4 Buf.;10%AcN	5		7.4	N_2	2.0 ± 1.4
20% AcN	10		7.0	N_2	1.8 ± 1.1
20% Isopropanol	5		6.4	N_2	3.0 ± 1.7
50mM Formic acid	5		2.5	N_2	0.0 ± 0.0
0.01% TFA	5		3.9	N_2	8.8 ± 5.0
0.04%TFA;10%AcN	6		3.0	N_2	2.5 ± 2.0
0.04%TFA;15%AcN	6		3.0	N_2	1.1 ± 0.9
0.04%TFA;20%AcN	6		2.9	N_2	0.0 ± 0.0
0.1%TFA	5		2.5	N_2	0.5 ± 0.4
H_2O	10	50mM Formic acid; 0.01%SDS	2.5	N_2	0.4 ± 0.4
Saline	6	0.04%TFA;10%AcN	3.0	N_2	0.0 ± 0.0
Saline	6	0.04%TFA;15%AcN	3.0	N_2	0.1 ± 0.1
Saline	6	0.04%TFA;20%AcN	2.9	N_2	0.1 ± 0.1
Saline	10	0.1%TFA;20%AcN	2.5	N_2	0.0 ± 0.0
Saline	10	0.04%TFA;10%AcN;0.01%SDS	3.2	N_2	0.2 ± 0.2
0.01% SDS	6	0.04%TFA;10%AcN	2.5	N_2	3.1 ± 2.1
0.01% SDS	10	0.1%TFA;20%AcN	2.0	N_2	1.7 ± 1.7
0.01% SDS	10	50mM Formic acid	2.4	N_2	0.0 ± 0.0
Untreated	46				1.0 ± 0.4
Mated	51				10.3 ± 1.7

solvents and low pH, though high activity was obtained when it was used alone at 0.01% and 0.1%; 0.5% SDS destroyed activity. The effect of alkaline conditions was not formally tested, however, activity was recovered after exposure to the pH 8.9 native-PAGE upper tank buffer (Appendix 2).

Exposure to pH variations, detergents, high salt concentration, and water soluble organic solvents all can denature protein (Voet and Voet, 1990). Intolerance for low pH and exposure to lipophillic solvents are characteristics *D. antiqua* extracts share with mosquito sex peptides (Lentz, 1994). The order or the brevity of exposure to low pH and/or lipophillic solvent did not alter the outcome.

Thermal Stability. The temperature sensitivity of male extract as measured by ovipositional response is shown in Figure 29. No activity was recovered from any extract heated to 60°, 80°, or 100°C (Figure 29A). Some activity was retained in all extracts subjected to 50°C or lower for 10 min regardless of whether they came from supernatants, pellets, or crude extracts (Figure 29B). During the trypsin digestion experiment, longer exposure to elevated temperature did not noticably decrease activity; an extract subjected to 37°C for 4 h was as active as starting material (Figure 27).

A trend (though nonsignificant) toward diminished activity for the separated pellet and supernatant extracts relative to the unseparated starting extract suggests that the presence of suspended particulates may have had a stabilizing effect on activity. However, low thresholds for activity in the bioassay could have concealed considerable losses, leaving open the possibility that some activity is lost at ≤ 50°C. This experiment also demonstrated that not all activity is harvested upon first extraction; above-threshold concentrations of active material were recoverable from once

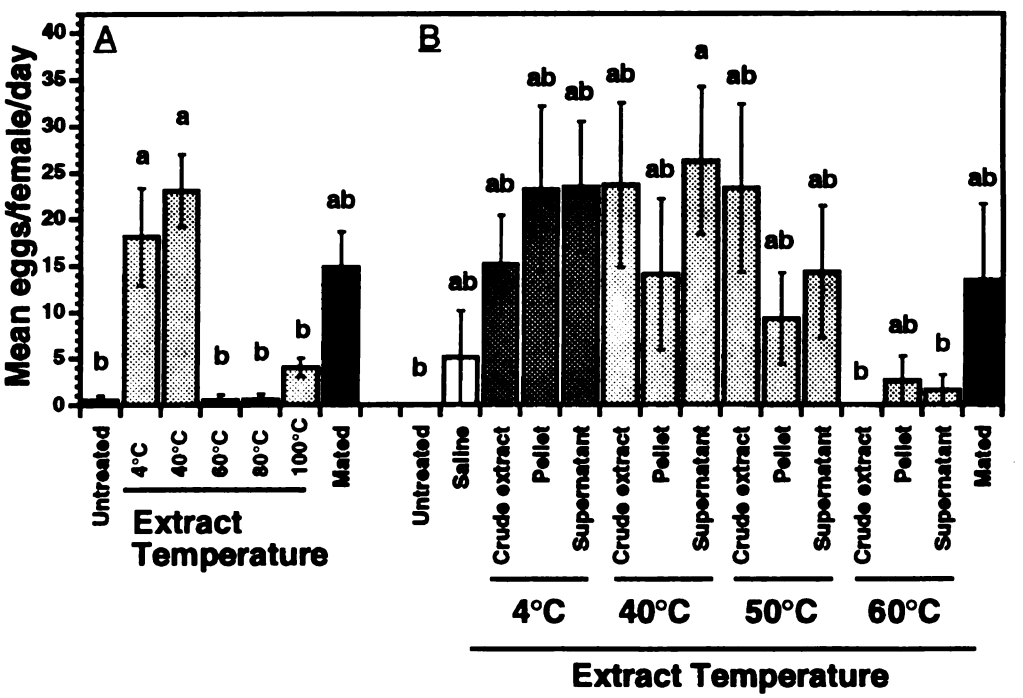

Figure 29. Thermal stability of *D. antiqua* sex peptide activity. (A) 10 µl samples of extract exposed to 40-100°C (n=2). (B) Thermal stability of crude uncentrifuged extracts, plus pellet and supernatant derived extracts exposed to 40°, 50°, or 60°C for 10 min. Extract-crude, pellet, and supernatant treatments were samples of untreated starting extracts (n=4). (A) and (B), were analyzed separately. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

extracted pellets of whole male reproductive tracts resuspended in physiological saline and resonicated.

Molecular weight estimation.

Centrifuge microfilter sizing. Active material was recovered using microcentrifuge filters (Figure 30). Analytical SDS-PAGE of fractions from microcentrifuge filter experiments (Figures 31, 32, and 33) clearly showed that protein was recoverable from the tops of filters of widely varying MWCO (300k, 10k and 5k) suggesting that the active material(s) are "sticky" or may form aggregates. Unfortunately, proteins from the entire range of molecular weights above and below stated MWCOs were recovered on most filters. Taking into account this lack of precision, molecular weight estimations using this method are at best crude and the filters of limited use for determining molecular weight. They are perhaps best used as an initial clean up device or for desalting extracts (see electrophoresis and electroelution of proteins from SDS- and native-PAGE).

Experiment 1. The ≥300k fraction was more active than either the <300k and ≥100k, or <100k and ≥30k fractions (Figure 30A). It was not significantly different from the positive controls or the <30k fraction. Recovery of high activity from only the top of the 300k MWCO filter in the series is suggestive of a fairly large active material. However, analytical 12% SDS-PAGE of the retentate from each filter (Figure 31) showed that little protein was recovered from filters other than the 300k MWCO even though much of the secretion was composed of proteins with molecular weights well below the 300k MWCO. The 300k MWCO filter may have become overloaded and plugged. Alternatively, components of the extract may have inherent affinities that favor formation of aggregates too large to move through the

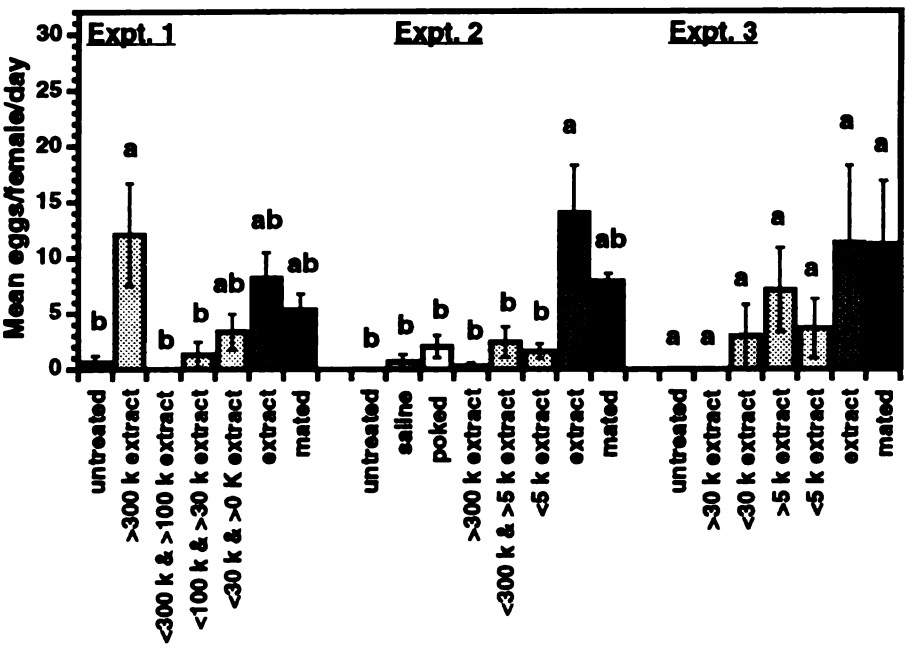

Figure 30. Ovipositional response of female *D. antiqua* injected with male reproductive tract extracts filtered through Millipore™ Ultrafree-MC microcentrifuge filters. (Expt. 1) 98 male equiv sequentially filtered through 300k, 100k and 30k microcentrifuge filters to produce 4 fractions injected at 0.25 µl/individual. There were three replicates. (Expt. 2) 138 male equiv sequentially filtered through 300k and 5k microcentrifuge filters to produce 3 fractions which were injected at 0.33 ul/individual. There were four replicates. (Expt. 3) 50 µl aliquots of a 100 male equiv extract of paragonial glands filtered through 30k or 5k microcentrifuge filters. 4 fractions, corresponding to the retentate and filtrate from each microfilter, were collected and concentrated under nitrogen to 50 µl and injected at 0.5 µl/female. There were three replicates. Experiments were analyzed separately; treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

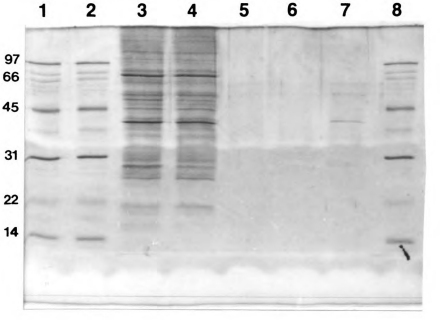

Figure 31. Silver stained 12% SDS-PAGE of fractions collected by passing reproductive tract extract sequentially through 300k, 100k, and 30k MWCO microcentrifuge filters. Lanes 1, 2, and 8 are molecular weight standards. Lane 3 is a saline extract of male reproductive tracts. Lane 4 is material recovered from the top of the 300k MWCO microcentrifuge filter. Lane 5 is material recovered from the top of the 100k MWCO filter. Lane 6 is material recovered from the top of a 30k MWCO filter. Lane 7 is the filtrate from the 30k MWCO filter. Seven male equiv of extract were used in each lane 3-7.

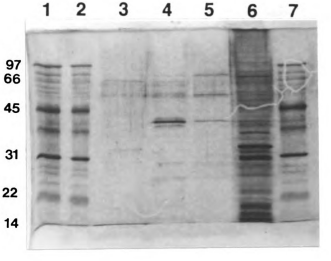

Figure 32. Silver stained 12% SDS-PAGE of fractions collected by passing reproductive tract extract sequentially through 300k and 5k MWCO microcentrifuge filters. Lanes 1, 2, and 7 are molecular weight standards. Lane 3 is material recovered from the top of the 300k MWCO microcentrifuge filter. Lane 4 is material recovered from the top of the 5k MWCO microcentrifuge filter. Lane 5 is the filtrate of the 5k MWCO microcentrifuge filter. Lane 6 is a saline extract of male reproductive tracts. 10 male equiv of extract were used in each lane 3-6.

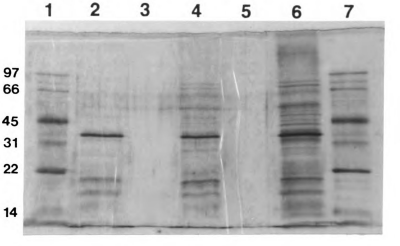

Figure 33. Silver stained 12% SDS-PAGE of retentates and filtrates collected from 30k and 5k MWCO microcentrifuge filters. Lanes 1 and 7 are molecular weight standards. Lane 2 is the filtrate collected from a 30k MWCO microcentrifuge filter. Lane 3 is the retentate material from the top of a 30k MWCO microcentrifuge filter. Lane 4 is a saline extract of male reproductive tracts. Lane 5 is the filtrate collected from a 5k MWCO microcentrifuge filter. Lane 6 is the retentate material from the top of a 5k MWCO microcentrifuge filter. 7 male equiv were used in each lane 2-6.

filter. The fact that nearly every protein associated with the starting extract was found atop the 300k MWCO filter was greatly at variance the with the expected performance of microcentrifuge filters as described by the manufacturer.

Experiment 2. Results of Experiment 2 were also unexpected (Figure 30B); none of the fractions was active. 12% SDS-PAGE of the molecular weight range fractions revealed little size fractionation of the extract (Figure 32) and confirmed that protein recovery had been very poor. Dilution of the extract and filtration in stages may have increased unwanted binding of active material to surfaces. Proteins with molecular weights well above the 5k MWCO were recovered in the filtrate from the 5k MWCO microcentrifuge filter, again calling into question the utility of these filter units for this project.

Experiment 3. As in Experiment 2, the fractions were not significantly different from the negative controls (Figure 30C), however there was a trend toward ovipositional activity for materials between 30k and 5k. Unlike Experiment 1, the aliquots were passed through only one microcentrifuge filter and a nitrogen concentration step was used to reduce the volume for bioassay, however there is no evidence that nitrogen concentration causes qualitative loss of activity (Table 5). 12% SDS-PAGE of the retentate and filtrate from each microcentrifuge filter (Figure 31) illustrated that protein moved through the 30k MWCO filter well but was retained atop the 5k MWCO filter (Figure 33). Extracts are also known to be stable under a variety of drying regimes (see Table 6; p. 160, and C_{18} buffer bioassay; p. 187). Loss of activity may be attributed to partial fractionation of extracts, which separated the active material from a carrier or protecting protein resulting in loss of activity.

In a subsequent experiment (data not shown) using a 10k MWCO filter to fractionate 119 male equivalents of extract in 100 µl of physiological saline, no activity was recovered from the filtrate. Highly active material was collected directly from the top of the 10k filter (top: mean eggs/female/d = 23.8 ± 4.8 (mean ± S.E.); flow-through: mean eggs/female/d = 0.9 ± 0.9; n=4). These data suggest that the active principle is of molecular weight >10k, however, because the starting extract was not divided among multiple filters, twice as many male equiv were potentially recoverable.

Discounting the results of Experiment 1 where the filter may have been overloaded, sex peptide activity seems easily lost when passed through microcentrifuge filters. The loss of activity was likely due to physical factors rather than any chemical sensitivity since extracts had been prepared in physiological saline, and were not exposed to any materials known to cause loss of activity. Under these conditions the loss of activity is consistent with physical separation of multiple component sex peptide or perhaps surface losses to the filter walls or the filter itself during passage. In retrospect, it would have been instructive to combine the inactive retentates and the filtrate from Experiment 2, to see if activity could be reconstituted.

Dialysis. *Experiment 1.* Extract dialysis through Membranes. The extract collected in phosphate buffer was significantly more active than the other extracts and controls (Figure 34). Though high treatment variability and limited sample sizes prevented statistical separation of the other treatments and controls, ovipositional rates from the positive controls and the dialyzed H_2O extract are within the range expected for fully activated females.

Experiment 2. Extract dialysis through dialysis Tubing. There was no activity recovered from either extract after dialysis (Figure 34), however, the

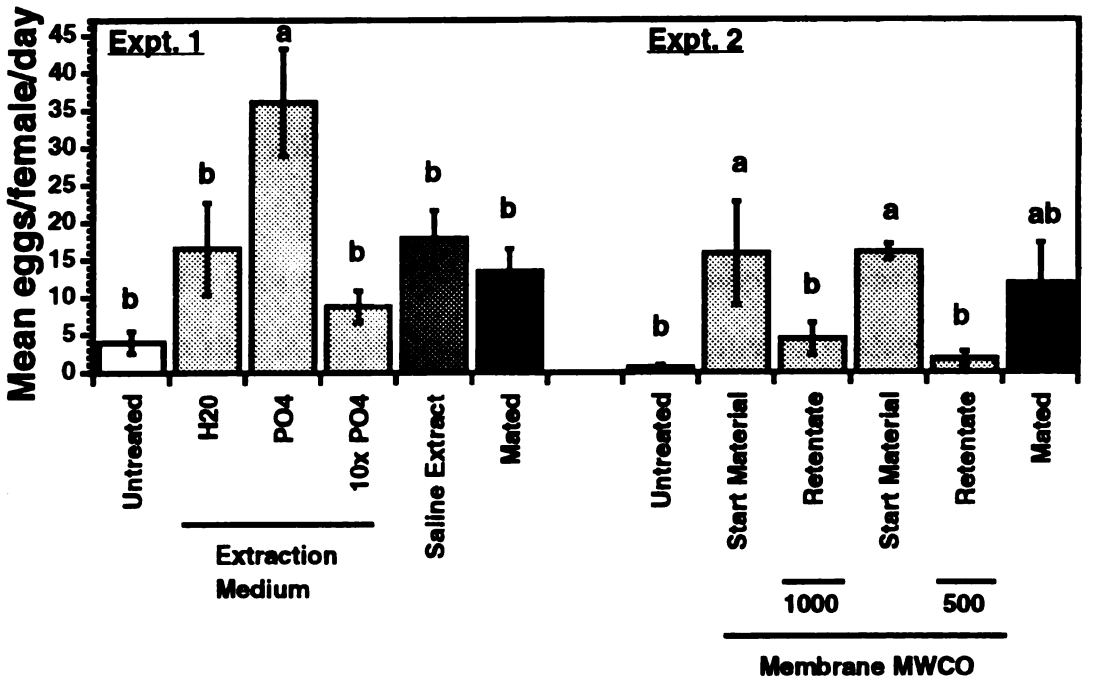

Figure 34. Ovipositional response of female *D. antiqua* injected with dialyzed male reproductive tract extracts. (Expt. 1) Three 50 male equiv extracts collected into 45 ul of distilled water, phosphate buffer, or 10x concentrated phosphate buffer were dialyzed against distilled water across a 1000 MWCO dialysis membrane for 2.5 h. The retentate was bioassayed. There were 5 replicates. (Expt. 2) Two male reproductive tract extracts, containing 100 and 70 male equivalents at a concentration of 1 male/µl of phosphate buffer were dialyzed for 4 h against against distilled water across a 1000 or 500 MWCO dialysis membrane respectively. There were three replicates. Experiments were analyzed separately, treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

starting extracts used for each dialysis tubing test were active. Not unexpectedly, the volume of the dialysis bags doubled during dialysis against distilled water. To compensate for the increase, the dialyzed extracts were injected at twice the volume of the starting extract. The male equiv/injection were the same.

The surface area to which the extracts were exposed was greater in this experiment than that in Experiment 1. The total interior surface area of the *ca.* 2.5 cm long segments of dialysis tubing was *ca* 600 mm^2 vs. *ca.* 80 mm^2 for Experiment 1 where dialysis occurred across a 1 cm diam window of the same membrane. Both types of dialysis membrane were produced by Spectra/Por and were throughly rinsed and conditioned according to instructions accompanying the product.

Loss of activity in this situation is mystifying, given that other extracts prepared in phosphate buffer for Experiment 1 were highly active. Given that the exposed surface areas for dialysis bags was greater than that for strips of dialysis membrane, losses via increased surface interactions may be one explanation.

Experiment 3. Measure of Surface Interaction with dialysis Membranes. Extracts, whether exposed and unexposed to dialysis membrane, averaged 13.2 and 16.6 eggs/female/d respectively (Figure 35). Exposed extracts interacted with more than twice the surface area available in Experiment 2 (the same length of tubing was used, but extracts were in contact with **both** sides of the chopped tubing). These data suggest that membrane binding alone cannot explain activity losses in the previous experiments. In fact, the Spectra/Por Cellulose Ester membranes used in these experiments have a low affinity for protein as they are designed for high protein recovery.

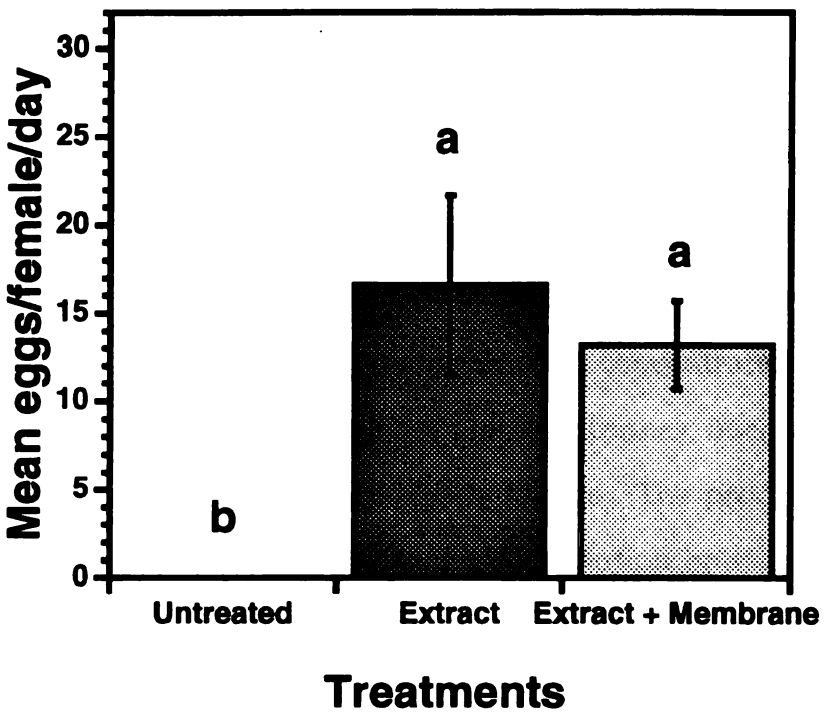

Figure 35. Ovipositional response of female *D. antiqua* injected with male reproductive tract extracts exposed to dialysis membrane. A 20 µl sample of male extract was held at 55°C in a microcentrifuge tube with 1" of chopped 1000k MWCO Spectra/Por CE dialysis membrane. The extract was injected at 0.25 µl/individual (0.5 male equiv). There were three replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

The results from dialysis were contradictory, procedures yielding activity on one ocassion, failed when repeated a second time. Retention of activity following dialysis with a 1000 MWCO membrane provides evidence that the active principle has a molecular weight of >1k, or that it was bound to the dialysis membrane and failed to move. Separation of protein components by dialysis was a poor method for dealing with crude extracts; dialysis is better suited to concentrating protein. 3.5k MWCO Spectra/Por dialysis membranes are an important component of our electroelution system (see protein separations section, p. 141). We reliably recover concentrated biologically active protein by 3.5k MWCO Spectra/Por membranes, even after long exposure to membrane surfaces and high voltage. The procedures used in the successful dialysis of Experiment 1 and electroelution both employ small disks of membrane held tightly in place. In my view, the weakness of the method used in unsuccessful Experiment 2, was the tubing closure method which uses clips to seal both ends, which if improperly done creates two routes for loss of material. Small extract volumes also make it difficult to distribute and recover proteins from within the tubing. For the *D. antiqua* project, dialysis provided little information and should be used to concentrate proteins, but not as a primary separation tool.

Protein separation.

Gel filtration chromatography. With Sephadex G-50. Neither of the two gel filtration fractions was more active than the negative control (Figure 36). Higher rates of oviposition from Fraction 1 are suggestive of some activity associated with a material of >30k in the void volume (the exclusion limit of Sephadex G-50 is 30k), however the result was not significantly different from the negative controls. Mated data are not shown because the ovipositional rate was atypically low (only *ca.* < 3 eggs/female/d) due to poor

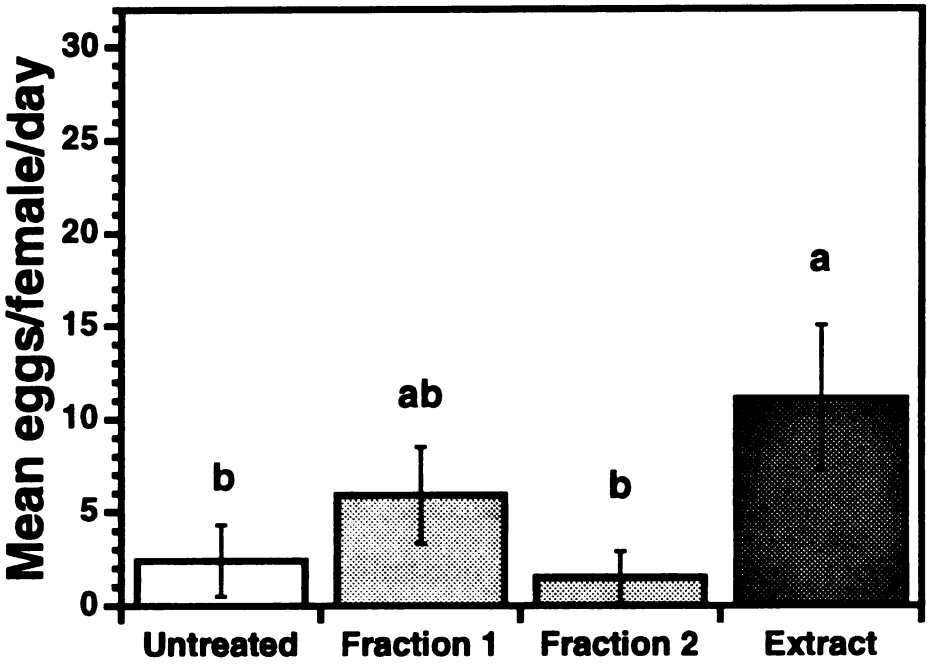

Figure 36. Oviposition by female *D. antiqua* injected with male reproductive extract fractionated by Sephadex G-50 Gel Filtration. Two fractions from a 100 male equiv starting extract, containing the void volume and the elution peak were desalted and concentrated to 50 µl, and injected at 0.5 µl/individual (equivalent to 0.5 male equiv) into eight, 6-day-old virgin females along with starting extract. There were eight replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Duncan New Multiple Range Test).

mating and not ability of this group of flies to lay eggs as evidenced by the performance of extract-injected flies.

Within 3 days of injection 15/16 females injected with fractions from this column were dead. Salts concentrated during the nitrogen drying step may have been responsible, however, >50% mortality among the starting extract-injected flies, and 0% mortality in the positive and negative controls (uninjected controls) suggests that the starting material may have had some toxicity apart from any concentration effect. It was later determined that contaminated reagents had caused the mortality, but the experiment was not repeated.

HPLC gel filtration of male paragonial gland extract with polyhydroxyethyl A. HPLC gel filtration resolved the extract into multiple peaks (figure 37). None of the individual or pooled fractions retained activity equivalent to the positive controls which averaged an unusually low 6.8 eggs/female/d (Figure 38), typical rates of ovipostion for mated or extracted injected females are *ca.* 14.5 eggs/female/d (this dissertation, Chapter 1). All 10 flies injected with Pooled Fraction 3 (P3) were dead the morning after treatment. P3 contained the last 8 min of the run, including the flow-through material which likely contained a high concentration of salts. Given that the phosphate buffer solvent system used here had a demonstrated compatability with sex peptide activity (Table 6) though it was never tested at pH 5.5, it is surprising that activity was not recovered somewhere among the fractions. Certainly the potential exists for significant surface losses during the run or the subsequent fraction concentration from 1.5 ml to 100 ul.

Separation of multiple components may also explain the loss of activity. After individual fractions were tested, limited pooling of fractions from different regions of the run occurred, however, all fractions were not

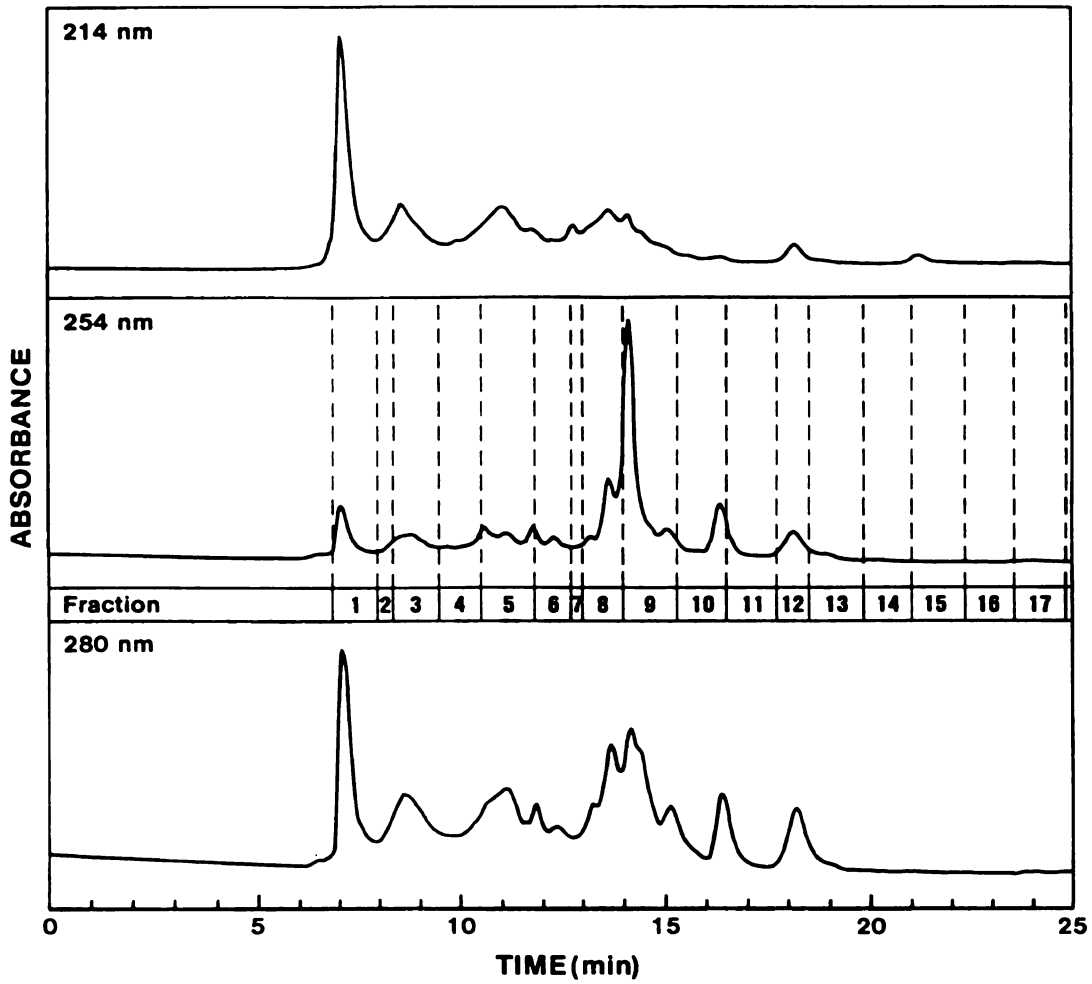

Figure 37. Chromatograms generated by HPLC gel filtration of a 150 male equiv extract of male paragonial glands on a PolyLC polyhydroxyethyl A 250 X 9.4 mm column viewed at 214, 254, and 280 nm. The run was made *isocratically* in pH 5.5 phosphate buffer. 17 fractions were collected, concentrated to *ca.* 100 µl under nitrogen. Samples of each fraction were combined, further concentrated and injected as pooled extracts. No activity was recovered from any region of these chromatograms.

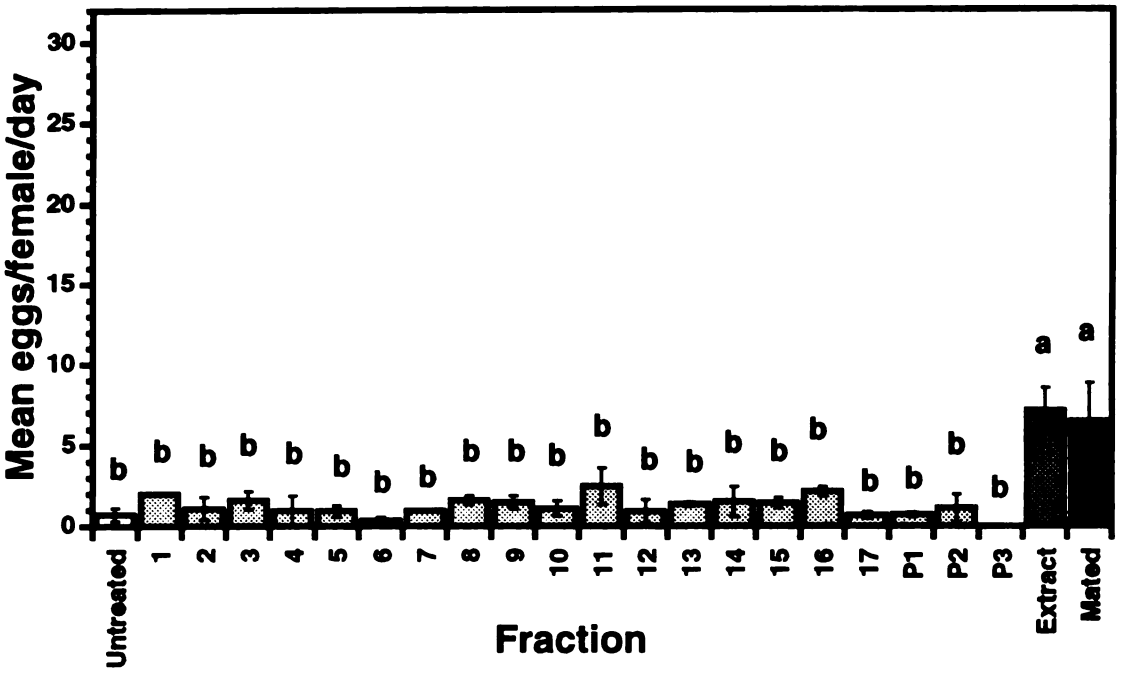

Figure 38. Oviposition by virgin female *D. antiqua* injected with male reproductive tract extract fractionated by HPLC Gel Filtration. 17 fractions and 3 pooled fractions were collected and concentrated under nitrogen to 100 µl and injected at 0.4 µl/individual (0.6 male equiv) into two replicates of five females. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Fishers PLSD).

combined to reconstitute whole extract. In Experiment 1, there was no baseline separation between the two peaks corresponding to the void volume and elution peak. The peaks likely shared many components, nonetheless they were tested individually but never formally pooled. The bioassay protocol used here did not specifically address the multiple component hypothesis for loss of activity in either experiment. Given that gel filtration had been used to recover sex peptide activity from *Musca* (Bird *et al.*, 1991), *Culex* (Young and Downe, 1987), and even the chemically sensitive *Aedes* extracts (Fuchs *et al.*, 1969) further efforts using compatiable buffers with provision to evaluate a fully reconstituted extract seem warranted.

Other HPLC. HPLC chromatograms reveal that *D. antiqua* male paragonial gland extracts are composed of a complex assortment of components varying widely in their relative abundance and chemical properties (as judged by their broad distribution of elution times under different running conditions). Secretion complexity is a feature of glands from *Ae. aegypti* (Lentz, 1994) and various *Drosophila* (Chen *et al.*, 1985; Stumm-Zollinger and Chen, 1988).

Protein components from the array of male accessory secretions are likely involved in aspects of reproductive biology. Nevertheless, even among the well studied *Drosophila*, only a few easily assayed secretion components with have been identified and characterized.

<u>*Vydac C4 RP-HPLC.*</u> No biological activity was recovered from any of the fractions collected in this or subsequent runs using the C4 column and solvent system (Figure 39 & 40). Loss of activity was likely due to the harsh chemical environment of the separation and losses during lyophilization of the fractions. As we later learned, exposure to the starting concentration of

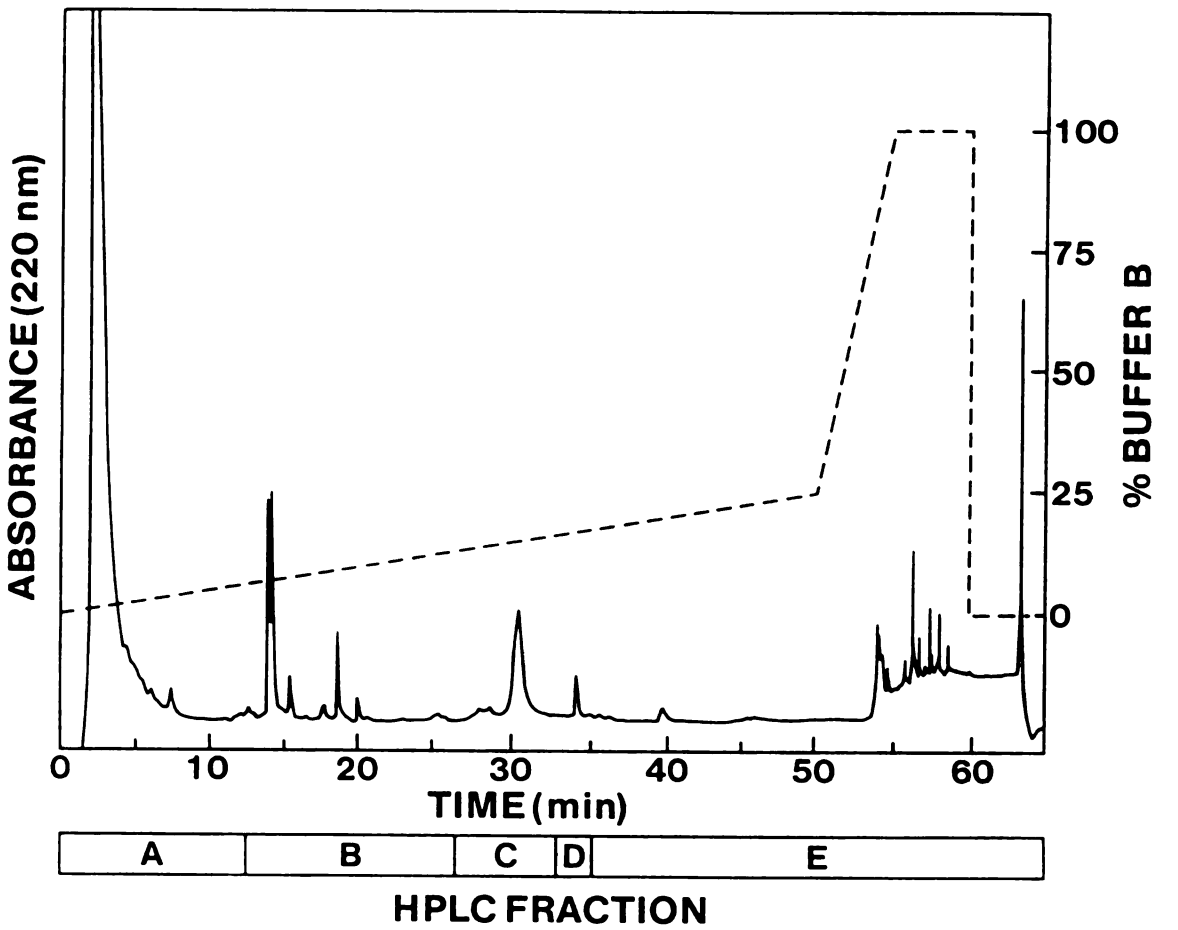

Figure 39. C_4 RP-HPLC fractionation of *D. antiqua* paragonial gland extract. 300 equiv of male paragonial gland extract were injected onto a Vydac C_4 analytical column and eluted at 1.5 ml/min with a 50 min linear gradient 0-25% buffer B (95:5 AcN:H_2O) in 0.04% TFA. A Milton Roy Spectro Monitor 3100 detector was set at 220nm and attenuation of 0.05 AUFS. Five Fractions A-E were collected, lyophilized, resuspended and bioassayed for ovipositional stimulating effects.

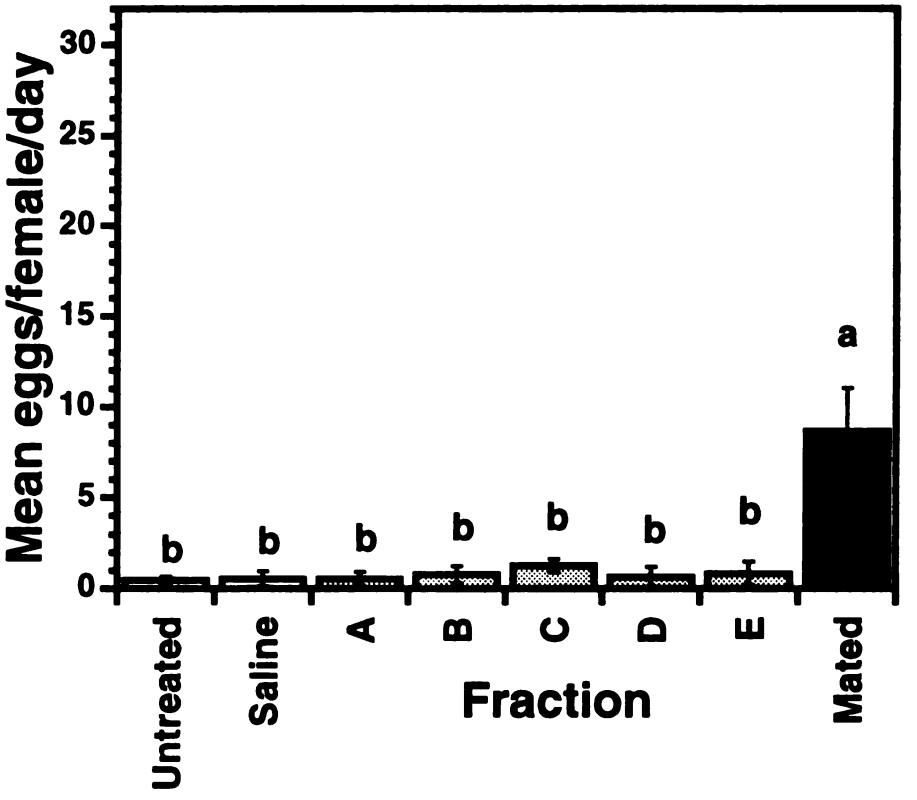

Figure 40. Results of RP-HPLC fractionation of a 300 male equiv extract of male *D. antiqua* paragonial glands separated on a Vydac C_4 under conditions given in the caption of Figure 5.1. Each treatment was replicated three times, with four females/replicate. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

buffer A alone; 0.04%TFA, would destroy sex peptide activity, the combination of TFA with and increasing concentration of AcN also have a demonstrated capacity to destroy sex peptide activity based on solvent test results (Table 6).

The post-elution processing introduced a significant potential for surface loses; fraction volumes were large (3 to 30 ml) and they were twice concentrated by lyophilization in glass round bottom flasks; substantial surface losses were likely.

C8 Microbore HPLC. There were no active peaks or active combined fractions identified from the C8 microbore column (Figure 41). In light of solvent stability data (Table 6), the running conditions alone, which included a gradient beginning at 90% AcN in 0.1% TFA, can explain the loss of activity.

C18 Microbore RP-HPLC. Run 1. On the first of three attempts two microbore fractions from the C_{18} column (Fractions 2: 2.5-10.3 min, corresponding to Peak 1 in Figure 42 and Fraction 6: 35.5-38.3 min, corresponding to Peak 2 in Figure 42), had activity suspiciously higher than the negative controls and other fractions (Figure 42 & 43). Fraction 2 eluted very early and was likely unbound material eluting in the void volume. Fraction 6 was composed primarily of the first peak seen to elute at 214 nm after the gradient began.

The earliest eluting peaks from RP-HPLC are the most polar, suggesting that, if activity were verified in subsequent runs, material was likely to be polar; polarity is consistent with the methanol extraction result (see above). Subsequent RP-HPLC of Fraction 6 on an Aquapore RP-300 50 mm x 1 mm column, with a 40 minute gradient from 0.1% TFA to 90:10AcN:H_2O revealed one major peak (Fraction 2 was found to be composed of many materials and was not further analyzed). Dr. J. Leykam (MSU Macromolecular Structure

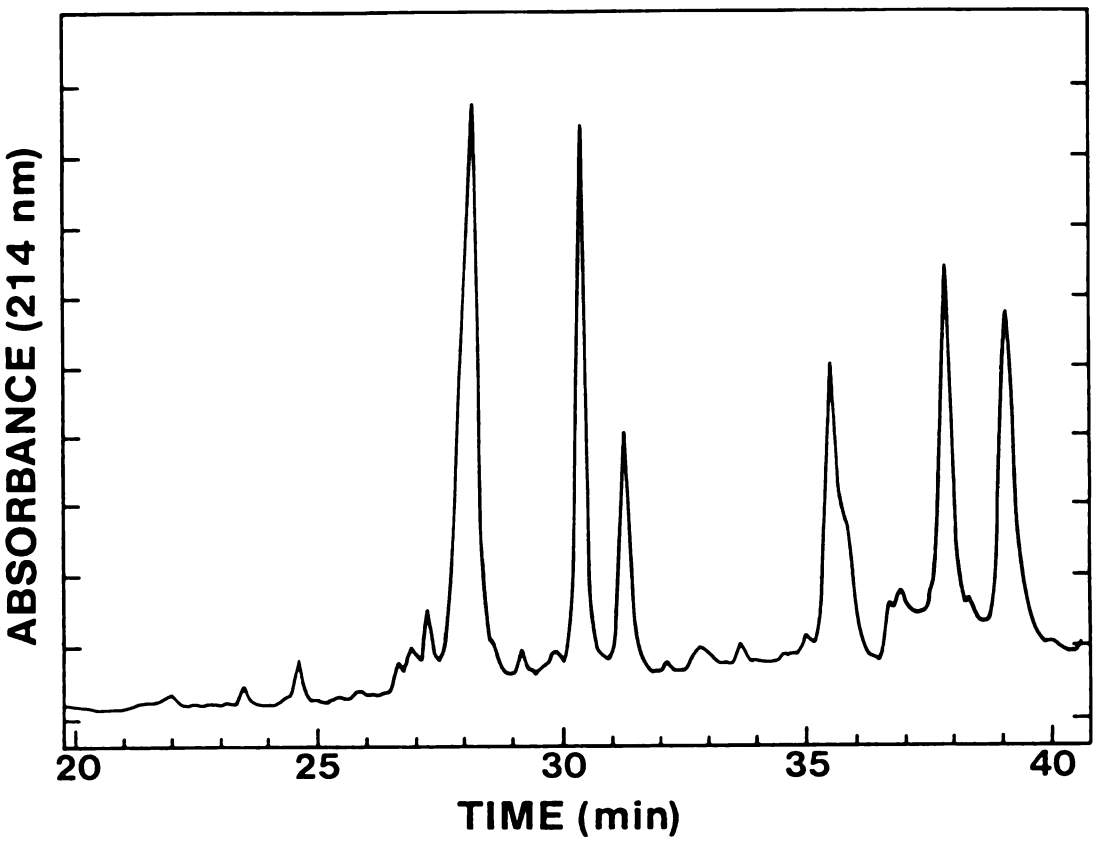

Figure 41. Chromatogram generated by RP microbore HPLC fractionation of a 212 male equiv extract of *D. antiqua* male paragonial glands on a 250 mm x 1 mm C_8 column. A Brownlee Micrograd ient Systems pump supplied a flow rate of 50 ul/min. Solvent A was 90% AcN in 0.1% TFA, solvent B was 0.1% TFA in H_2O. Gradient was 0-100% B in 90 min and began 8 min after injection, with Spectroflow 783 detector set at 214nm. 63 fractions were collected, twice lyophilized, and resuspended on physiological saline at a concentration of 1 male equiv/µl. No activity was recovered from any region of this chromatogram.

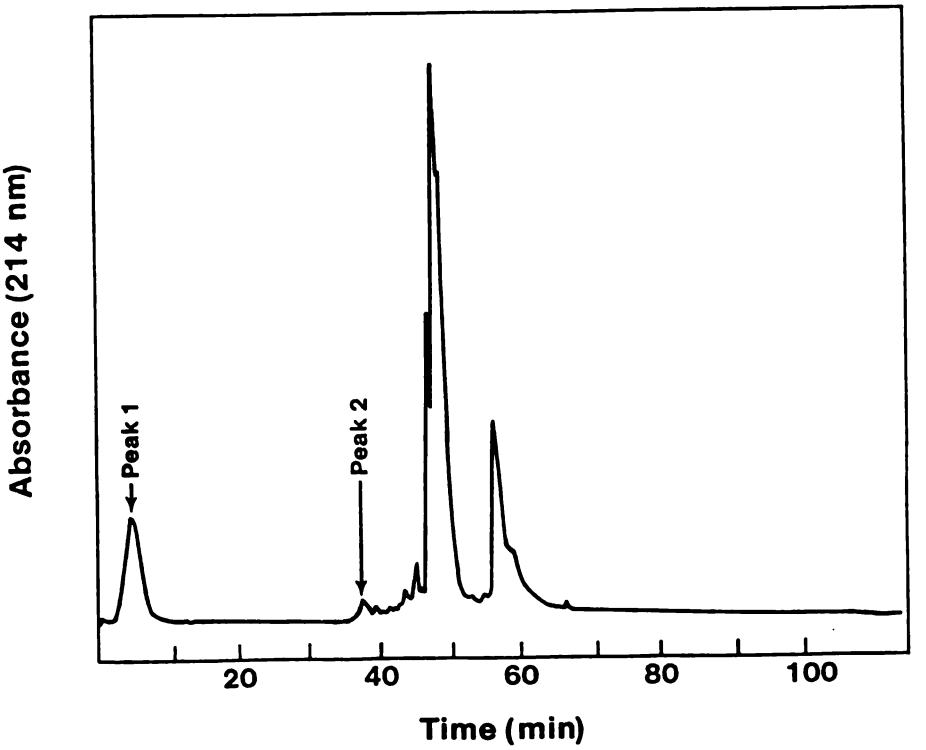

Figure 42. Chromatogram generated by C_{18} RP microbore HPLC fractionation of a 190 male equiv extract of *D. antiqua* male paragonial glands collected in distilled water. A linear gradient of 0-100% buffer B (buffer A was 5 mM phosphate buffer @ pH 6.73, buffer B was 475 mM ethanol, 150 mM isopropyl alcohol in 5 mM phosphate buffer) in 90 min was used. Flow rate was 50ul/min with UV detector set at 214 nm. Total run time was 110 min; the gradient began 17 min into the run. Fractions 2 (2.5-10.3 min), 6 (35.5-38.3 min), 7 (38.3-40.1 min), 8 (40.1-42.2 min), 9 (42.2-44.3 min), 10 (44.3-45.8 min), 11 (45.8-48.3 min), 12 (48.3-52.7 min) and 14 (54.4-62.2 min) were tested for activity. Activity was associated with Fractions 2 and 6 corresponding to labelled peaks 1 and 2 respectively.

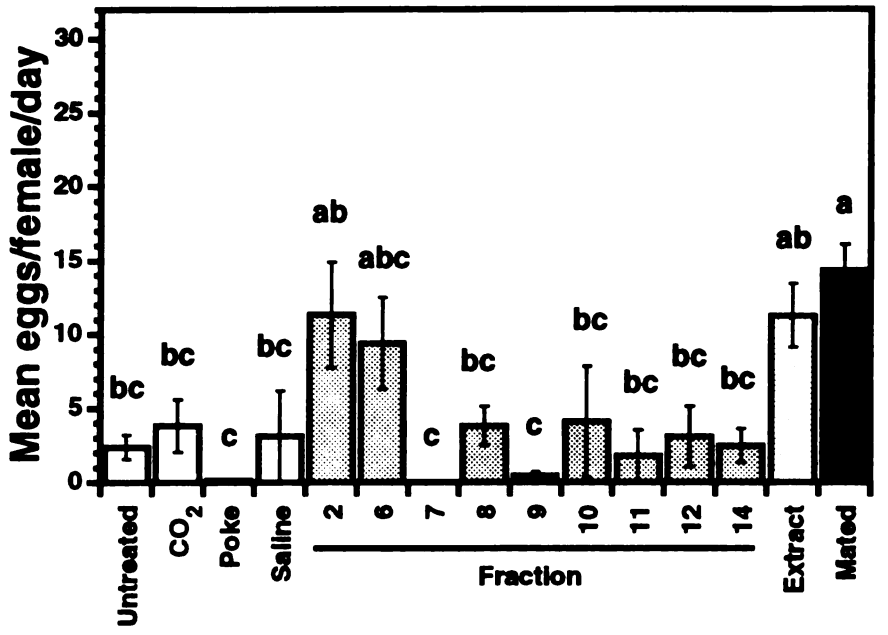

Figure 43. Ovipositional response of female *D. antiqua* injected with fractions from C_{18} RP microbore HPLC fractionation. A linear gradient of 0-100% buffer B (buffer A was 5 mM phosphate buffer @ pH 6.73, buffer B was 475 mM ethanol, 150 mM isopropyl alcohol in 5 mM phosphate buffer) in 90 min was used to fractionate a 140 male equiv of paragonial glands collected in distilled water. Virgin females were injected with *ca.* 0.5 male equiv in 0.5-0.75 µl. Treatments were replicated 5 times, with 1 female/replicate. Fractions 2 and 6 correspond to peaks 1 and 2 respectively in Figure 28. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

and Synthesis Facility) began sequencing the major component of 6 on an Applied Biosytems Sequenator. Four residues were determined before sequencing halted for unknown reasons: Residue 1: Lys or Glu or Gly or Val (could be any of these), Residue 2: Gly, Residue 3: Pro, Residue 4: Pro.

Runs 2, 3, & 4. Bioassay of fractions from three subsequent microbore runs recovered no activity and did not corroborate the results of Run 1 (Figure 44). In the first run, the gradient started at 17 min. Thus the sample was not in contact with solvent B (475 mM ethanol, 150 mM isopropyl alcohol in 5 mM phosphate buffer) as long as the other runs whose gradients began at 8 min. Isopropyl alcohol was susbequently found to destroy activity at 20% (v/v) (Table 6); sex peptide activity was never tested for sensitivity to ethanol. For the fractions equivalent to Fractions 2 and 6 from Run 1, both ethanol and isopropyl alcohol (2-propanol) were at very low concentrations (0.9% v/v and 2% v/v, respectively), well below that of isopropyl alcohol as tested in the solvent bioassay (Table 6).

Run 5. A duplication of the original microbore run including a 17 min delay to starting the gradient, with prompt processing and injection of fractions did not yield behavioral activity from the region of suspected activity or anywhere else in the chromatogram. The Run 1 positive result is surprising as a "false positive" result from our bioassay was otherwise unknown.

C_{18} buffer bioassay. When tested, the conditions of exposure to buffer B in Runs 1-5 did not destroy sex peptide activity (Figure 45). All fractions, regardless of how they were handled with respect to buffer B exposure and drying conditions were as active as "non-dried saline extract" (Figure 45). However, the combination of lower than average ovposition from the extract-treated and positive control insects and significant egg-laying by one

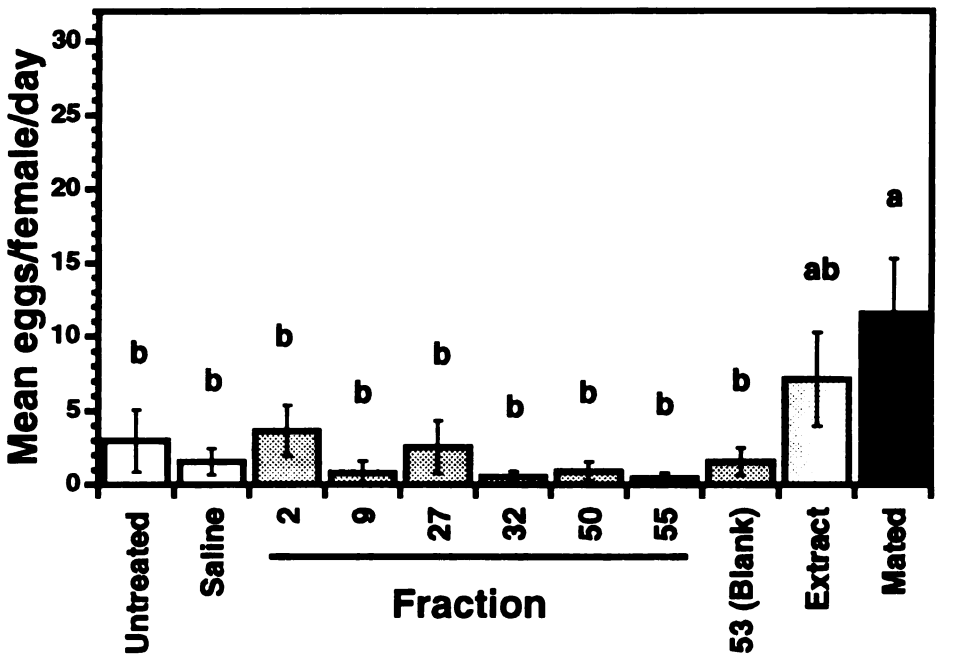

Figure 44. Ovipositional response of female *D. antiqua* injected with fractions from three C_{18} RP microbore HPLC fractionations. A linear gradient of 0-100% buffer B (buffer A was 5 mM phosphate buffer @ pH 6.73, buffer B was 475 mM ethanol, 150 mM isopropyl alcohol in 5 mM phosphate buffer) in 90 min was used to fractionate three 170 male equiv extracts of paragonial glands collected in distilled water. Fractions 2, 27, and 50 correspond to the active Fraction 2 from the original microbore fractionation (Figure 5.3), and Fractions 9, 32, and 55 correspond to active Fraction 6. Fraction 53 was a solvent blank. Treatments were replicated five times, with one female/replicate. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

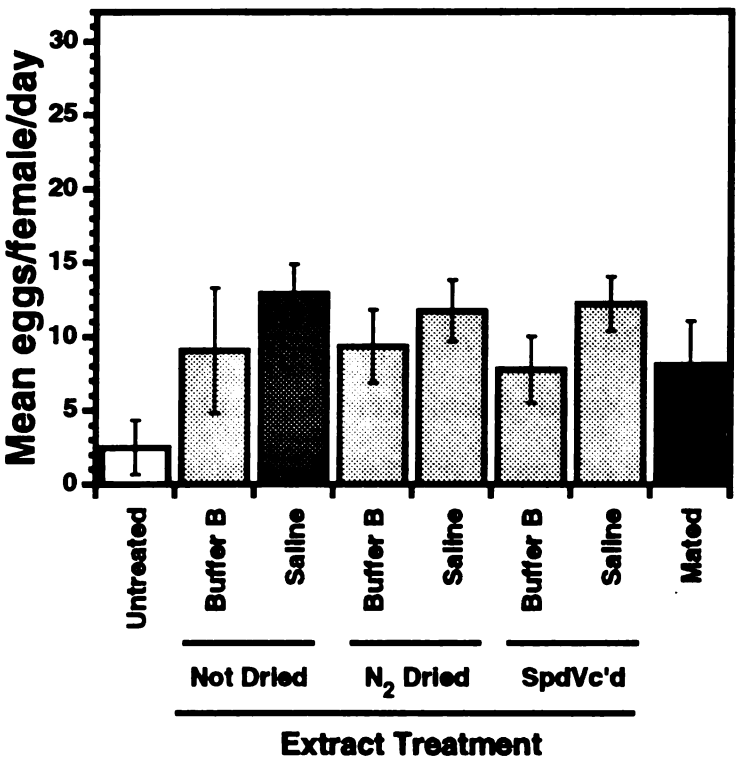

Figure 45. Ovipositional response of female *D. antiqua* injected with fractions exposed to components of a RP microbore HPLC solvent system. Three 24 ul samples of active male paragonial gland extract (concentration = 1 male equiv/µl) were mixed with 9 µl of microbore buffer B (475 mM ethanol, 150 mM isopropyl alcohol in 5 mM phosphate buffer) and taken to near dryness in a speed vac or with a stream of nitrogen or left untreated. Three additional 24 µl samples were not exposed to solvent B, but, after 9 µl of distilled water was added, were taken to near dryness as above. Dried extracts were resuspended in 24 ul of distilled water. Treatments were not significantly different at $P<0.05$ (Student-Newman-Keuls).

out of five virgins (laying virgin mean eggs/female/d = 16.8; mean of the other four females = 0.75 eggs/female/d) in the negative control yielded low statistical power. In light of data on virgin oviposition (this dissertation, chapter 3), the occassional appearance of a virgin female with exceptionally high ovipositional output is to be expected. Using the liberal Fisher's Protected LSD test, the saline extracts were significantly different from untreated, but the buffer B-exposed extracts were not.

Parity between all of the injection treatments suggests that the buffer system and processing was not the culprit in the loss of activity. However, these extracts were exposed to buffer only briefly before drying and resuspension or injection into flies. Extracts from C_{18} microbore Runs 1-5 may have experienced losses not accounted for in this check procedure; the actual microbore fractions were stored frozen for several days in buffer before they were processed. In the run identical to the original (Run 5), including the 17 minute gradient delay, fractions were kept on ice and injected into females within 2 h of their elution; nevertheless, activity was not recovered. If exposure to the buffer solution were destroying activity, loss occured between 30 and 120 min after exposure. We know that significant losses can be tolerated before loss of biological activity would be seen (this dissertation, Chapter 2); perhaps losses are continuously occurring once exposed to buffer B, and the cumulative losses reduce activity below the 1/20th male equiv threshold between 30 and 120 min after elution. Immediate bioassay of microbore fractions, within 30 min of elution, might increase the likelihood of recovering sex peptide activity. However, this methodology seems at best only marginally suited to this isolation problem.

Weak Anion Exchange Chromatography.; No activity was recovered from any fraction from anion exchange (Fig 46). The method of concentration with 5000 MWCO filters, which is reliable with concentrated extracts, may not work with more dilute fractions (see Centrifuge Microfilter Sizing; Experiment 2; when dilute samples were processed, no activity was recovered). In concert with suspected surface binding losses during the chromatography, dilution and the increased time to process the extract may have reduced activity below the detection threshold of the bioassay. Alternatively, activity may be "smeared out" over the run and diluted to below the threshold for activity. Regions of higher average oviposition (though not statistically significant) near the end of the run (Fractions 19-24), may be indicative of smeared activity. However, extracts were amply concentrated to reveal even a low level of activity; the high dosage used (2.5 male equiv) is 50 times greater than the threshold dose (this dissertation, Chapter 2).

Capillary Electrophoresis. Three capillary electrophorograms of male *D. antiqua* extracts of reproductive tissues are presented in Figure 47. Capillary electrophoresis revealed two main clusters of compounds in whole male extracts (Figure 47A) eluting between *ca.* 12.5-17 min and 25.5-28 min. Paragonial gland extract (Figure 47B) had peaks in both of these regions. Testes extracts (Fig 47C) had peaks in the second cluster only. As expected, testes and paragonial gland extracts together account for most of the peaks seen in the whole reproductive tract extract. The small sample volumes used in CE (<1 µl) precluded bioassaying of fractionated materials. It might have been possible to pool samples from multiple runs, however non-autoinjection CE seemed better suited to visualize the components of an

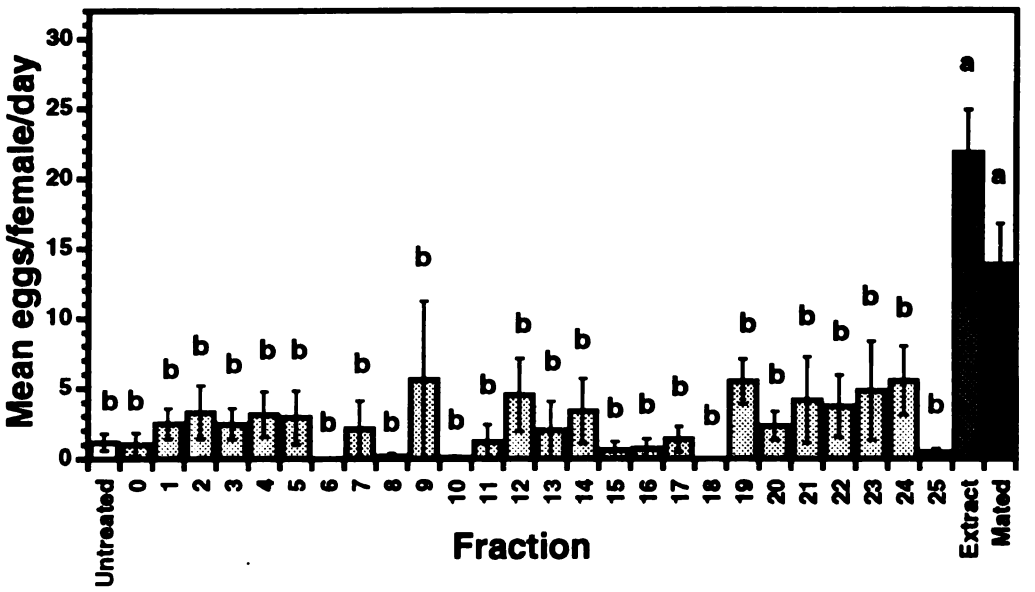

Figure 46. Ovipositional response of female *D. antiqua* to injection of anion exchange fractions. A 260 male equiv extract of *D. antiqua* male paragonial glands collected in 20 mM TRIS/HCl was fractionated by anion exchange using Sepharose™ CL-6B weak anion exchange resin (equilibrated in 50 mM NaCl-20 mM TRIS/HCl solution) and eluted in a 3-step gradient from 150 mM NaCl-20 mM TRIS/HCl to 500 mM NaCl-20 mM TRIS/HCl. Fractions were concentrated and desalted using Millipore™ 5k MWCO Ultrafree-MC filters. Virgin females were injected with *ca.* 2.5 male equiv in 0.5 µl. There were four replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

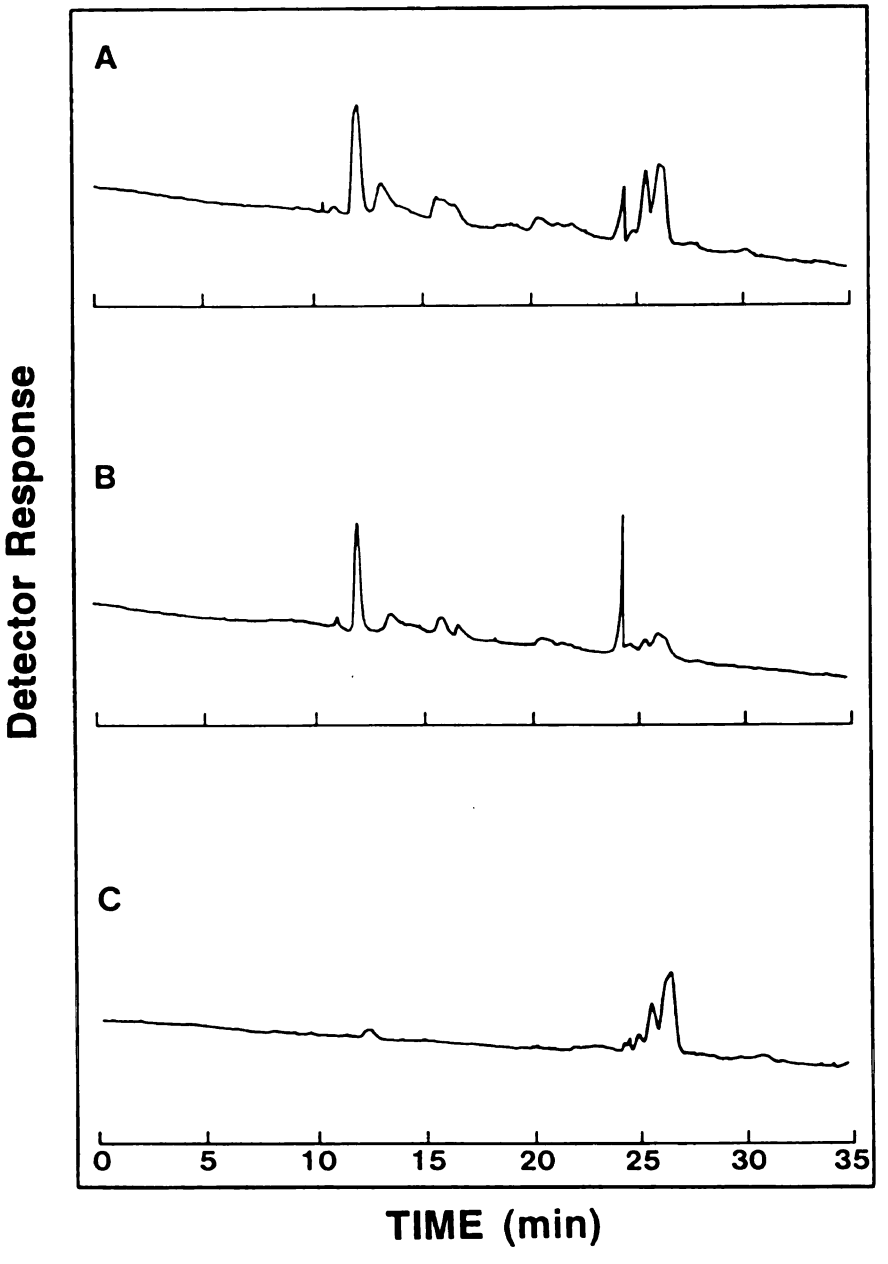

Figure 47. Capillary electrophoresis of *D. antiqua* extract of whole reproductive tracts (A), paragonial glands only (B) and testes only (C). An ISCO capillary electrophoresis system model 3850 with a CE-100 moderately hydrophobic 75 cm x 75 µm column was used. The running buffer was 10 mM sodium phosphate/0.01% Brij 35 at pH 7.0. Column rinsing buffer was 0.5% Brij. 2 second injections of 0.5 male equiv/µl extracts were run at 22.5kV with a current of 27 µA, and detector set at 214 nm. Numbers indicate elution time.

extract than to fractionate them, at least until improved preparative CE equipment becomes available. The speed and sensitivity of CE, along with the small sample volume requirements, could be exploited in the future to analyze variation in secretion composition or concentration between individuals.

Electroelution from SDS-PAGE and Native PAGE

Electroelution of Unreduced Male Reproductive Tract Extract From Non-Denaturing SDS-PAGE. No activity significantly above the level of the negative controls was recovered from the unreduced extract fractions (Figure 48). There was high variability in ovipositional rates among females receiving Fraction 1 injections (range = 0-43 eggs/female/d; Fraction 2 range = 0-10.6 eggs/female/d) reminiscent of a near threshold effect (this dissertation, chapter 2).

8% Native-PAGE None of the 8% native-PAGE fractions was significantly different from the untreated control (Fig 49). However, a trend toward increased activity in the later fractions suggested that repeating part of the assay may be worthwhile. The filtrate and retentate from Fractions 9 and 10 were later reassayed, along with a concentrated "reconstituted" extracts made by combining all the extracts and concentrating the extract with a 5k MWCO microcentrifuge filter. Unfortunately, more than half of the fraction-injected flies were dead in 2 days, and there was not enough sample left to inject other flies. The mortality was later determined to be due to contamination of the $CaCl_2 \cdot 2H_2O$ used in preparation of the physiological saline used for this experiment.

5% Native-PAGE electroelution. 5% Native-PAGE with 6 h electroelution yielded mated-like ovipositional activity from both the

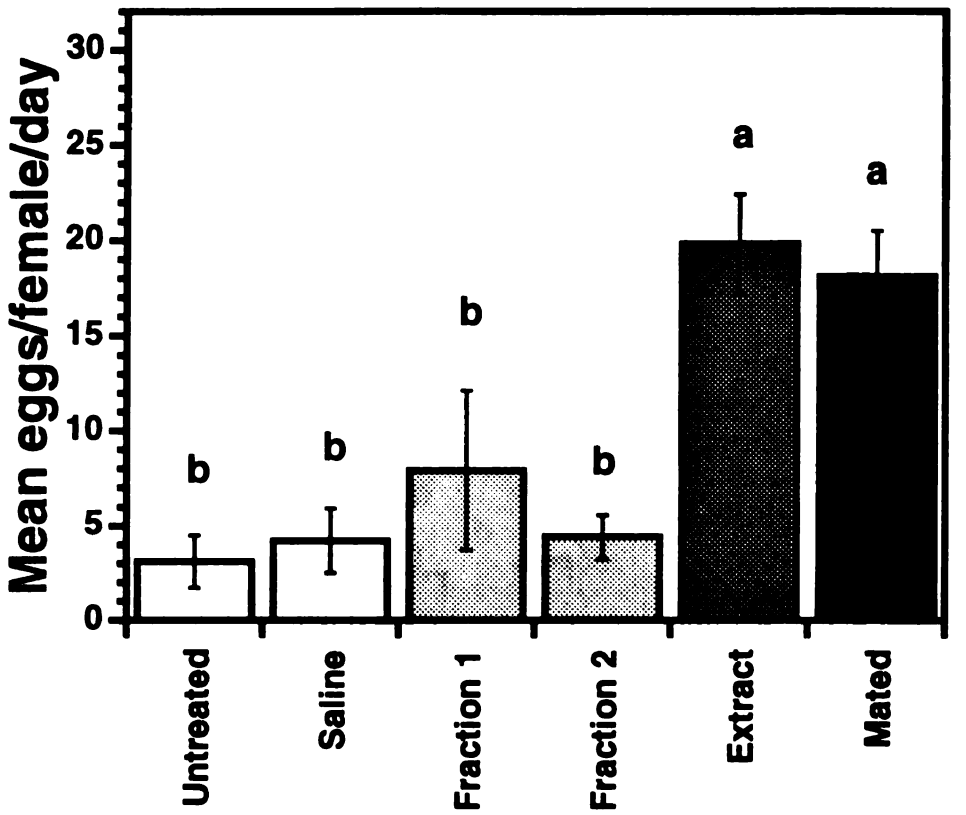

Figure 48. Ovipositional response of female *D. antiqua* injected with electroeluted fractions from 12% SDS-PAGE under non-denaturing conditions. Two fractions corresponding to the 4% stacking gel, and the first 1 cm of the resolving gel were injected at 0.5 µl/individual (1 male equiv). There were ten replicates/treatment. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

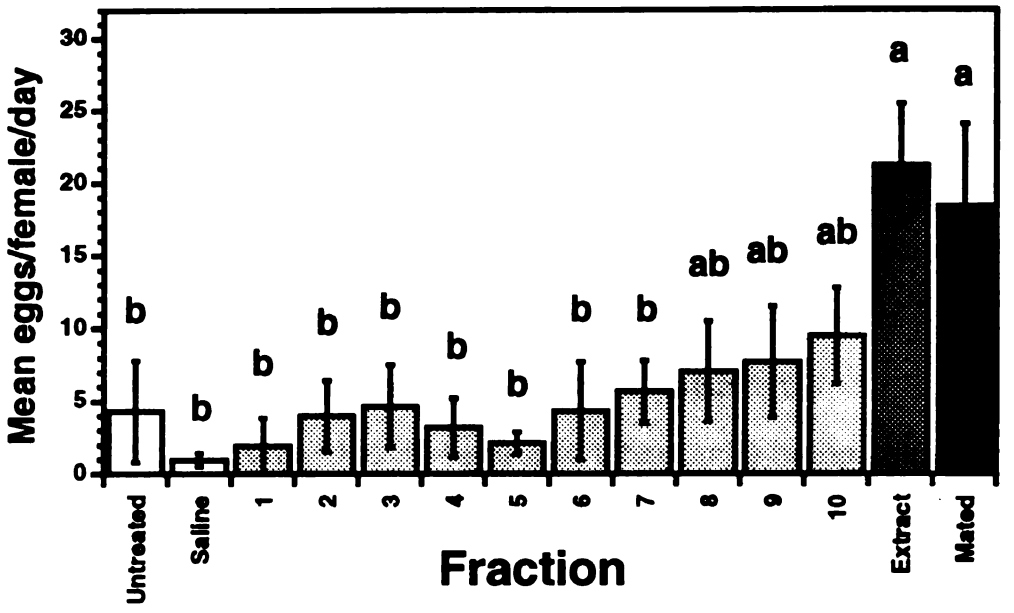

Figure 49. Ovipositional response of virgin female *D. antiqua* injected with 10 electroeluted fractions from an 8% native gel. A 320 male equiv extract of male reproductive tracts fractionated on an 8% native gel and protein in the gel slices electroeluted. There were five replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

stacking gel and the top 1 cm of the resolving gel (Fig 50). Electroeluting from 6 rather than 3 h increased recovery; 10 h of diffusion alone did result in recovery of activity. In later experiments, gels were run full-length, allowing protein to completely clear the stacking gel. The presence of activity in the stacking gel was likely a consequence of the shortness of the run.

Electroelution from Native-PAGE. Experiments 1 & 2. Highly active material was recovered from concentrated Fraction 2 (the top 1 cm of the resolving gel), and the pooled concentrate of all 5 fractions in both experiments (Figures 51 & 52). Though the mean activity of Fraction 2 in Experiment 1 was only marginal, a bimodal distribution of individual ovipositional rates among females in that fraction suggested some females were clearly activated and others were unaffected (4/10 averaged >5.5 eggs/female/d) . This distribution of activity is consistent with near threshold levels of activity (this dissertation, Chapter 2) being present only in the first 1.0 cm of the resolving gel. Since pooled fractions contained all lanes including active Fraction 2, it is reassuring, though not particularily suprising, that activity was recovered in the pooled concentrate of all five. In retrospect, an attempt to falsify the unique activity supposed for Fraction 2 by pooling Fractions 3-5 should also have been tried. Injection of pooled inactive fractions would also have revealed multiple component effects.

Experiment 3. Subdivision of Fraction 2 indicated that the active material was present in only the upper-most layer of the resolving gel (Figure 53). However, on 12% SDS-PAGE, the first 1 mm of the resolving gel, Fraction 2.1, (Figure 54, Lane 4) contained nearly all the bands found in the starting extract (Figure 54, Lane 2) though somewhat reduced in intensity. Very little protein was left in the stacking gel, unfortunately inactive fractions shared many bands with the active fraction.

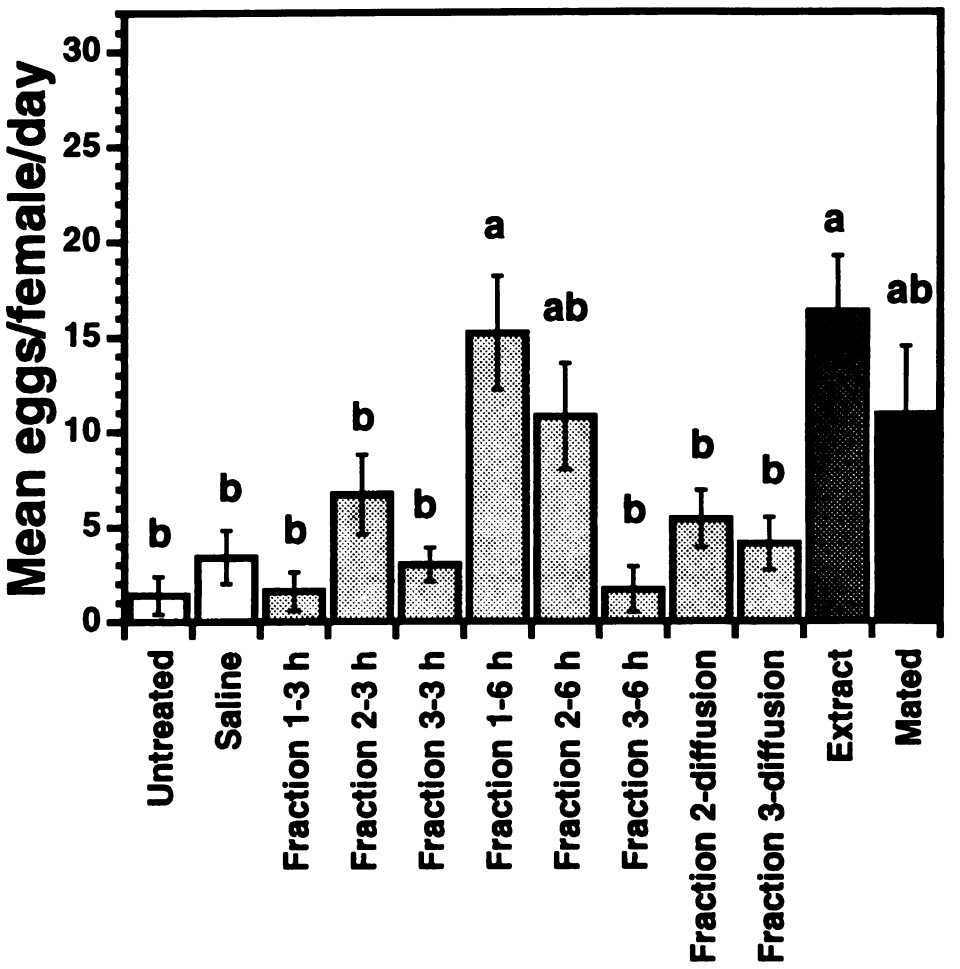

Figure 50. Ovipositional response of female *D. antiqua* injected with fractions which were electroeluted or allowed to diffuse from an 8% native gel. Protein was eluted from Fractions 1, 2, and 3, corresponding to the stacking gel, the top 1 cm of the resolving gel, and the resolving gel ahead of the bromphenol front. Fractions were electroeluted for 6 h, with a sample removed at 3 h. Protein was also collected by a 10 h diffusion from gel slices 2 and 3. Fractions were injected at 0.5ul/individual (*ca.* 1 male equiv), there were ten replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

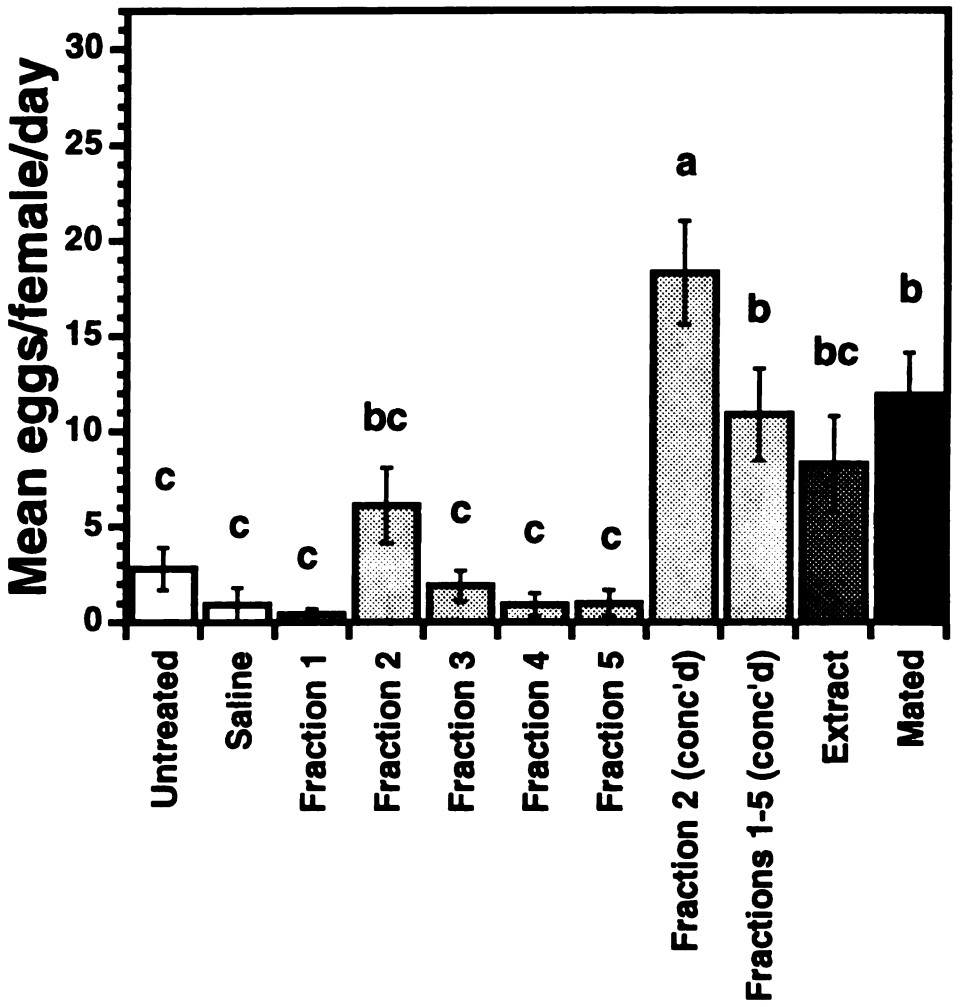

Figure 51. Ovipositional response of female *D. antiqua* injected with electroeluted fractions from a 10% native-PAGE gel. A 300 male reproductive tract extract was fractionated on a 10% native-PAGE gel. Fraction 1 was the 1.5% stacking gel, Fractions 2 through 5 corresponded to 1-1.5 cm horizontal slices of the resolving gel. Gel slices were electroeluted before injection into virgin females at 0.5 µl/individual. 5x nitrogen concentrated (conc'd) samples of Fraction 2 and a pooled sample of all 5 fractions were also prepared. There were ten replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

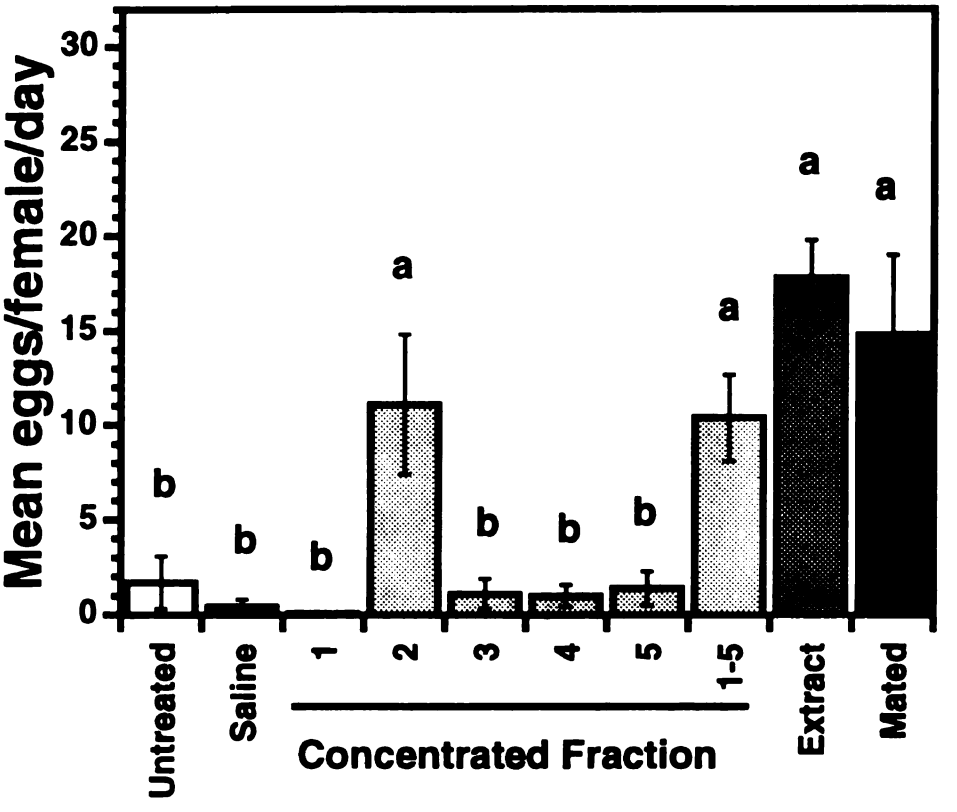

Figure 52. Ovipositional response of female *D. antiqua* injected with 5x concentrated electroelution fractions from a 10% native-PAGE gel. A 300 male reproductive tract extract was fractionated on a 10% native-page gel. Fractions 1 was the 1.5% stacking gel, Fractions 2 through 5 corresponded to 1-1.5 cm horizontal slices of the resolving gel. All electroeluted fractions were concentrated 5x under nitrogen before injection into virgin females at 0.5 µl/individual. There were ten replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

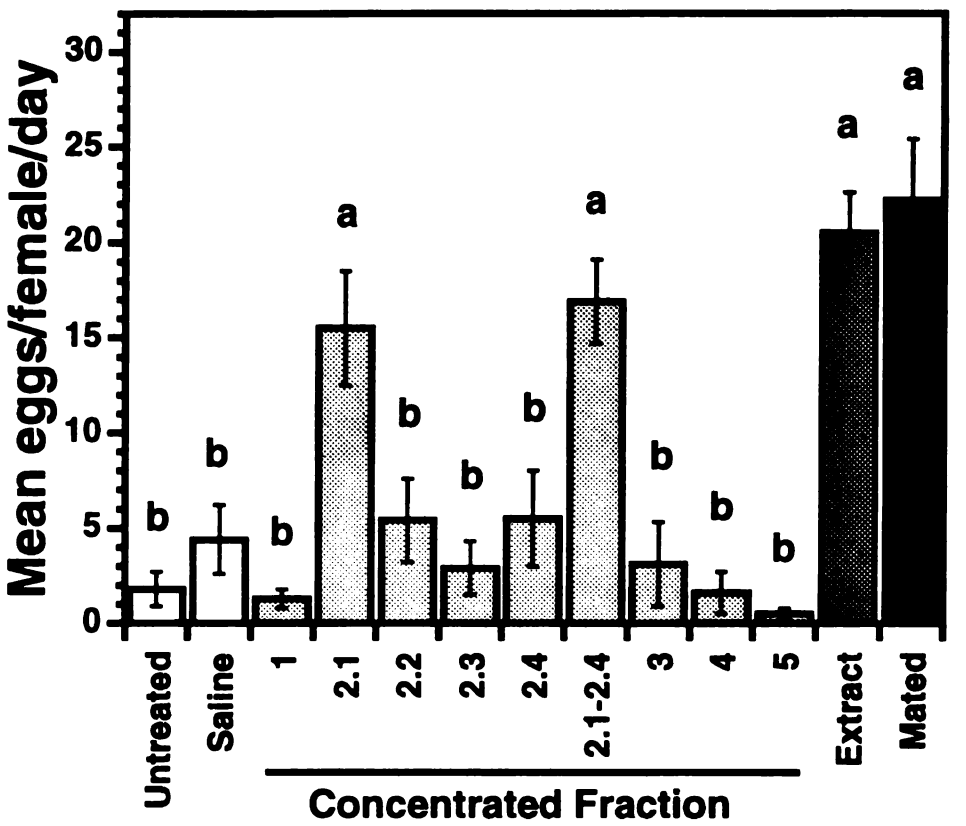

Figure 53. Ovipositional response of female *D. antiqua* injected with electroeluted fractions from a 10% native-page gel. Fractions 1 was the 1.5% stacking gel, Fractions 2 through 5 corresponded to 1-1.5 cm horizontal slices of the resolving gel. Fraction 2 was subdivided into 4, 2.5mm wide slices; 2.1-2.4. Fractions were electroeluted and concentrated 5x under nitrogen before injection into virgin females at 0.5ul/individual. There were ten replicates. Treatments accompanied by the same letter were not significantly different at $P<0.05$ (Student-Newman-Keuls).

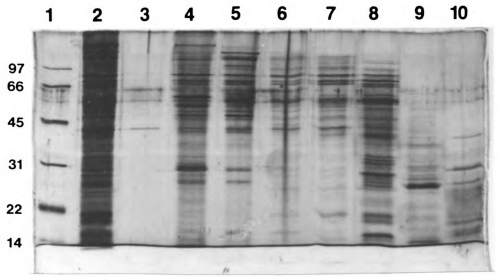

Figure 54. 12% SDS-PAGE of fractions derived from 10% native-PAGE, electroeluted and concentrated 5x before injection. Lane 1 is molecular weight standard. Lane 2 is a saline extract of male reproductive tracts. Lanes 3-10 are 5x concentrated Fractions 1, 2.1, 2.2, 2.3, 2.4, 3, 4, and 5 from native-PAGE. 7 male equiv were used in each lane 2-10.

A subtractive process comparing band patterns from inactive fractions could narrow the field of candidate sex peptide bands. It is evident from the number of shared bands across fractions that many were widely spread several centimeters down from the top of the resolving gel. Nevertheless, there is evidence of fractionation, particularily in lanes 8, 9, and 10, which correspond to the inactive Fractions 3, 4, and 5. The lack of activity from anywhere but the very top of the gel may indicate that fractionation separates two or more components necessary for activity. The stacking gel (Figure 54, Lane 3) was nearly devoid of banding, save for some contaminant present in all lanes. There was an abrupt increase in protein at lane 4 (Fraction 2.1). Overall protein content appears to decline from Fraction 2.1 to 2.4, suggesting that conditions at the interface between the stacking and resolving gels may offer some resistance to free movement of protein. In retrospect, pooling of Fractions 3, 4, and 5 into an extract for bioassay would have been appropriate.

Recombination of inactive fractions from this run was carried out 6 months after the original bioassay. 15 µl samples of each concentrated inactive fraction 2.2, 2.3, 2.4, 3, 4, & 5 were combined and injected at 0.75 µl/female into 10 virgins, held singly in screen cages. Four of the ten females laid eggs after 3 d; the mean egg production of the whole group was just 4.5 ± 1.9 eggs/female/d compared to 10.1 and 8.2 eggs/female/d for the active extract and mated female controls respectively. However, among the ovipositing females, the average ovipositional rate was 11.2 eggs/female/d (not different from the positive controls). The pattern of response to recombined concentrated extracts was not unlike that to low dosages of sex peptide (1/160th and 1/80th male equiv), where a percentage (5-10%) of females exhibited intermediate ovipositional rates (this dissertation, Chapter 2). This result, though by itself inconclusive, suggests that *D. antiqua* sex

peptide may be a multicomponent material and begs for more definitive testing.

Activity from the combination of a known active fraction with inactive fractions is not surprising (see Experiments 1, 2, & 3). Knowing whether or not activity resulted from recombining and/or concentrating all inactive fractions would be very important. If activity appeared only after recombination **and** concentration, then a dispersed but active unitary sex peptide would be suspected. If strong activity came from simply combining but not concentrating inactive fractions, then a multi-component sex peptide would be suggested. A quantitative measure of activity, perhaps densitometry readings to compare SDS-PAGE lanes bearing electroeluted fractions with others having samples of active material at known concentrations, would also shed light on the likelihood of one or the other hypothesis.

From the perspective of further purification, a multiple component sex peptide at a reasonable concentration could be handled using native-PAGE and electroelution. A single component sex peptide sustaining significant losses is much less amenable to this methodology.

If *D. antiqua* sex peptide is a single entity, it is sobering to contemplate the quantity of material likely lost during electrophoresis and electroelution so as to generate the observed results. If sex peptide is localized to one region of a lane, then in a horizontal fraction containing that region, all of the male equivalents of sex peptide originally loaded on the gel (except for those lost to the locator lanes) are found there. In Experiment 3, 240 male equiv were available after removal of the locator bands. The electroeluted fraction, with a volume of *ca.* 150 µl before concentration, contains 1.6 male equiv/µl (240 male equiv/150 µl) and 0.8 equiv/0.5 µl injection volume. Obtaining BR_{50}-

like responses (this dissertation, Chapter 2) from such an extract suggests that *ca.* 97% of the activity was lost along the way.

If sex peptide were distributed broadly (smeared) down a lane, then some may be in all 5 fractions and be at a concentration of *ca.* 0.33 male equiv/µl (48 male equiv/150 µl) and 0.167 male equiv/0.5 µl injection volume. Obtaining BR_{50}-like responses from this extract would mean that *ca.* 85% of activity was lost.

In either case, significant losses are occurring during electrophoresis and electroelution. This technique, though appropriate and adequate to recover activity, is not well suited for isolating sex peptide in quantity. Given that active fractions contain many proteins and sex peptide in low concentration, the probability of avoiding additional losses during subsequent purfication is low.

DISCUSSION

The characterization of *D. antiqua* sex peptide chemistry is very incomplete. Biologically active material was not recovered following most separation procedures; however, analysis of where activity was lost or recovered may offer some clues about the chemical nature of the *D. antiqua* sex peptide.

Loss of biological activity from a single component *D. antiqua* sex peptide might occur as a consequence of: (1) surface binding, (2) insolubility, (3) solvent incompatibility, (4) denaturation (by temperature or detergent exposure), or (5) proteolysis. Effects of (6) low dosage or (7) isolation techniques which are inappropriate for the molecule in question (as a result of combinations of mechanisms 1-5) could also be responsible for lost biological activity. In addition, activity of a multiple component sex peptide

could be lost due to 1-7 and because (8) necessary components were separated (e.g. a polypeptide broken into peptide subunits, enzyme separated from its substrate, or separation of protein from a associated nonprotein-molecule or carrier).

Evidence for loss of material due to probable surface binding is seen on SDS-PAGE gels of extracts from the trypsin digestion experiment (Figure 28). The amount of protein recovered from extracts exposed to solutions of 4% agarose beads (with or without bound trypsin) is much less than unexposed extracts. The porous agarose beads present a large surface area for protein interaction; in chromatography that character is reversibly exploited to effect separations. Though activity was recovered following this procedure, diminished protein recovery identifies a proclivity for substantial loss upon exposure to matrices of high surface area. That losses from extracts of relatively high specific activity (1 male equiv/μl) can be tolerated is clear from this experiment, however, similar losses from extracts of lower activity might cause active extracts to be judged inactive.

Microcentrifuge filters also present large surface areas to extracts during passage through the filter frit. No behavioral activity and little protein was ever recovered from the filtrate of even the highest MWCO microcentrifuge filter (Figures 30-33). Proteins may bind the internal structure of the filter unit. Activity was recoverable from the tops of these filters; SDS-PAGE of this material recovered from the tops of filters usually showed banding patterns like starting extract, suggesting little fractionation had occurred. Alternatively, recovery of *D. antiqua* sex peptide activity from the tops of many different microcentrifuge filters may also suggest that the material is prone to aggregation.

Activity was lost during tube dialysis. When the potential for losses onto/into dialysis tubing was tested in a separate experiment, that possibility was judged to be insignificant (Figure 35). However the short duration of that test (3 h) was not appropriate given that the original experimental conditions to be simulated were maintained for 20 h. Though incompletely evaluated in this particular instance, exposure to high surface area and potential binding sites are known to reduce protein recovery.

Unlike *D. melanogaster* sex peptide, *D. antiqua* sex peptide activity was insoluble in 80% aqueous methanol, an indication that it is likely more polar than Drm-SP-I. *D. antiqua* sex peptide activity was extractable in other polar solvents: distilled water and several saline buffer solutions. Starting material and positive control extracts for all experiments were prepared in solutions (saline, phosphate buffer, or distilled water) in which activity was reliably extracted. Activity was lost following extract exposure to various solvents (Table 3); unfortunately extract pellets from inactive extracts were never resuspended in saline to see if active material had only been precipitated as happened in methanol.

Low solvent pH was incompatible with recovery of sex peptide activity. Extraction in an acceptable solvent (e.g. phosphate buffer) at low pH (3.0) destroyed activity, suggesting that pH should be a major concern when planning separations. In Table 3, all the extracts exposed to acidic conditions were resuspended in neutral solutions before bioassay, but were still inactive; the effects of extreme pH apparently have permanent effects on sex peptide activity, or special steps (not evaluated here) may be required to renature then. Marginal activity (8.8 ± 5.5 eggs/female/d) was recovered from an extract prepared in 0.01% TFA at pH 3.9; no extract prepared in or exposed to a

lower pH was ever active (Table 6). Most HPLC solvent systems used in these experiments were more acidic than pH 3.9.

Though not specifically evaluated, there is indirect evidence for tolerance of elevated solvent pH. In native-PAGE procedures samples experience a range of neutral and higher pH's (pH 8.9 in upper tank buffer, pH 6.9 in the stacking gel, and pH 8.5 in resolving gel) during electrophoresis and electroelution. Recovery of activity from extracts fractionated by native-PAGE, implies some tolerance of alkaline pH. Solvents like isopropyl alcohol and AcN used in HPLC, were incompatible with sex peptide activity even when tested at near neutral pH, indicating that some aspect of their interaction other than pH alone destroyed activity. Taken as a whole, these results suggest that attributing loss of activity during HPLC runs and other unsuccessful separations to pH and other solvent effects is not unreasonable.

High temperature could also have denatured sex peptide. Activity was abruptly lost in a variety of extracts following brief exposure to temperatures above 50°C, yet, activity was stable in extracts from the trypsin digestion experiment when held at 21.7°C or 37°C for 4 h. Knowing this, sex peptide activity could be expected to withstand the conditions of handling and processing without loss of activity over a range of temperatures. Nevertheless, temperatures during separations were kept as low as possible. Native-PAGE and electroelutions were conducted at 4°C, as was anion exchange. Extracts processed in microcentrifuge filters were always spun at 4°C. HPLC was run at room temperature, but the fractions were collected on ice. With the exception of the first 53 fractions from C_8 microbore HPLC, (which may have briefly experienced temperatures above 50°C when they inadvertantly went to dryness while being speed vacced), extracts were shielded from temperatures that could destroy activity.

The pattern of abrupt activity loss associated with increasing temperature or acidic pH is compatible with a globular protein undergoing a loss of secondary structure, or dissociation of the subunits of a multicomponent protein. For single domain proteins, denaturation is often very abrupt, as certain internal residues ionize or growing electrostatic repulsion between charged groups on the protein surface open up the protein as the pH drops (Creighton, 1993).

Spontaneous renaturation of proteins is possible for many small proteins, however some post-synthetically modifed proteins, like those with disulfide bridges, do not renature. Insulin is an example; disulfide bridges hold the proprotein in an energetically unfavorable conformation which permits the enzymatic cleavage of an internal peptide generating active insulin (Voet and Voet, 1990; Creighton, 1993). If denatured in the active form, the two insulin chains do not easily reassemble, as proinsulin's proper conformation is not readily reassumed (Creighton, 1993).

Sodium dodecylsulfate (SDS) is a detergent used, among other things, to impart a uniform negative charge to proteins in SDS-PAGE. When the SDS concentration is at least 8×10^{-4} M, SDS disrupts protein structure and dissociates polypeptides into component peptide chains, binding to them at 1.4 grams of SDS per gram of protein in solution (Creighton, 1993). Based on 700ng of protein per male equiv, and extracts at 1 male equiv/ul, SDS at a concentration of 0.5% would be predicted to denature onion fly sex peptide. *D. antiqua* sex peptide activity at 1 male/µl was lost after extraction in 0.5% SDS (extraction in 0.01% or 0.1% SDS did not affect activity, see Table 1). Insects receiving 0.5 µl injections of 0.5% SDS extract were initially incapacitated for 12-24 h indicating that this concentration of SDS presented a serious physiological challenge. But 4/5 of the females recovered from the

malaise and survived for the duration of the experiment. Loss of activity upon exposure to this critical concentration of SDS provides additional evidence for the protein nature of *D. antiqua* sex peptide, though the details of the protein involvement are unknown.

It is likely that SDS concentrations used may have denatured extracts during nondenaturing SDS-PAGE. The final SDS concentration in the mixture of nondenaturing SDS-PAGE sample buffer (0.4% SDS in 650 µl, see appendix 2) plus extract sample (150 µl) was 0.3% when the sample was added to the gel. This concentration lies within the (0.1-0.5% SDS) range where *D. antiqua* sex peptide activity was lost. Exposure to 0.3% SDS during nondenaturing SDS-PAGE likely caused the loss of activity.

The dosage-response relationship of *D. antiqua* sex peptide and the all-or-none behavioral response of "activated" females favors the positive outcomes. Activity is detectable at concentrations of *ca.* 1/40th male equiv or more (this dissertation, Chapter 2). It was standard practice after a separation procedure, to bring extracts to a concentration (based on starting concentration and recovered volume) where if they were at all active, elevated egg counts would have been evident. Most were prepared so that females were injected with *ca.* 0.5 male equiv, 10 times the threshold for 100% behavioral response (BR_{100}) in females and 20 times the BR_{50} (this dissertation, Chapter 2). The need to concentrate extracts after native-PAGE and electroelution in order to get above-threshold responses is an indication of a significant loss of activity during electroelution.

There is an interesting inconsistency between protein recovery and recovery of sex peptide activity. In Figure 54, Lane 4 contains material from the active Fraction 2.1, Lane 2 contains material from an untreated active extract loaded on the gel at the same concentration. Lane 2 is at least twice as

dark as Lane 4 (more than twice as much protein is present). To attain mated-like levels of activity from Lane 4 (and homologous fractions from other gels), that extract had to be concentrated 5x. The level of activity from Fraction 2.1 before concentration was typical of an extract at or near the activity threshold of *ca.* 1/40th -1/20th male equiv/0.5 µl. If activity were strictly related to protein recovery, Lane 4 should have been 1/20th as dark as Lane 2. In combination with the bioassay data, perhaps SDS-PAGE, which presents a picture of all the protein in a denatured state, is telling us that degredation is actually occurring, but we aren't seeing it because of the low threshold for activity. Comparisons of this type are important because they relate protein loss to activity recovered. This also indicates that activity cannot be inferred from measures of total protein. If SDS-PAGE had been run on fractions collected from HPLC and other chromatographic techniques, measurement of the magnitude of protein loss could have been compared with controls to determine if activity losses were due to low protein recovery or denaturation of active materials.

In *D. antiqua,* there is no evidence of sex peptide activity loss due to degredation by proteases or microbial attack. There was no measurable loss of activity when extracts were held at room temperature or higher for 4 h (Figure 27), exposured to elevated temperature (37°C) (Figure 27), or when stored for weeks or months at -16°C between experiments. Male-specific antibacterial peptides which may be transferred to the female at mating are known from the ejaculatory ducts of *D. melanogaster* (Samakovlis *et al.*, 1991) and the accessory glands of *D. funebris* (Schmidt *et al.*, 1989). Two antibacterial proteins have recently been identified in the female accessory glands of Mediterranean fruit fly, *Ceratitis capitata* (Marchini *et al.*, 1991; Marchini *et al.*, 1993). Extracts of *D. antiqua* reproductive tracts or paragonia

may also contain antimicrobial peptides, which protect the female reproductive tract, the eggs and stored sperm from pathogens and may contribute to the stability of *D. antiqua* sex peptide activity after long storage.

If sex peptide activity were associated with a complex, many of the separation procedures used here (e.g. Electrophoresis, HPLC, anion exchange, and gel filtration) could have disrupted the association, be it a protein and its carrier, polypeptide subunits, or an enzyme-substrate relationship. There is a precedent for enzyme-substrate relationships between products of male reproductive tracts. Esterase 6 from the ejaculatory duct of male *D. melanogaster,* catalyzes the conversion of cis-vaccenyl acetate, produced in the ejaculatory bulb, to cis-vaccenyl alcohol (thought to act as an antiaphrodisiac) once transferred to the female (Mane *et al.,* 1983).

The pattern of activity from electroeluted native-PAGE fractions bears on the question of sex peptide structure. The only activity recovered from a fractionated extract came from electroelution of native-PAGE fractions, however, that activity was recovered only from the very top of resolving gels. The transition from the 1.5% acrylamide stacking to the 10% acrylamide resolving gel in these discontinous gels is marked by a decrease in gel pore size and an increase in pH from 6.75 to 8.48, conditions at this boundry may precipitate sex peptide (or one of its components) if its pI is between these values or physically halt the migration of a large molecule.

If electrophoresis is incompletely separating two or more components, it is possible they may be still be in association near the stacking-resolving gel boundry. In fact, SDS-PAGE of eluted fractions showed that protein was concentrated at this boundry even after full length runs; materials of all molecular weights were represented. Some evidence of fractionation is seen in fractions sliced from below the top of the resolving gel.

Perhaps a portion of all protein precipitated near the gel interface and never moved beyond that point, while the remainder passes into the resolving gel and is fractionated. If some precipitation occurred along with fractionation, a logical step would be to recombine and concentrate "inactive" fractions from beyond the initial active fraction to see if activity could be reconstituted. A sensible step may be switch to continuous gradient gels and thus avoid the stacking gel altogether.

If separation of protein occurring during electrophoresis inactivates extracts, then other chromatographic procedures like HPLC, anion exchange, and gel filtration might be expected to inactivate extracts as well. Demonstrated sensitivity to the running conditions, suggest that recombination of HPLC fractions would have been a futile effort. However, combining inactive fractions from procedures like anion exchange, where solvent conditions are more compatible with activity, may have been telling.

Could incomplete recovery of protein account for all loss of activity? Changing the duration of electroelution was an important factor in initially recovering biological activity from native-PAGE fractions. Six hour electroelution increased the amount of activity recovered, as measured by oviposition. Subsequent electroelutions were carried out for at least 6 h, concentration of eluted fractions increased the specific activity and increased the probability of finding even low level activity. Some procedures where no activity was recovered, but the solvent conditions seemed compatible (e.g. anion exchange) may have benefited from further concentration of fractions; however, it is difficult to justify additional expenditure of time and energy in the hope of recovering only remant activity.

Lastly, were the techniques used inappropriate for the molecule of interest? Demonstration of protein involvment in *D. antiqua* sex peptide

activity (Figures 27 & 28) suggests that the protein-oriented techniques we used (HPLC, electrophoresis, gel filtration, and anion exchange) had the potential to recover biological activity. It is unfortunate that the chemical environments of some separation procedures were harsh enough to destroy activity outright, and that this fact was recognized only after significant time and material were wasted. The strategy of assuming that onion fly sex peptide would be chemically similar to that for *Drosophila* has been thoroughly falsified!

In summary, though this work has not isolated an active material, a composite picture can be drawn of what *D. antiqua* sex peptide may be like. *D. antiqua* sex peptide is a protein or has a protein component. The active material is quite polar and may be extracted in distilled water and saline solutions. It is sensitive to acidic pH, lipophilic solvents and temperatures above 50°C. It's molecular weight is probably greater than 10k, though it seems to be prone to forming aggregates of apparently higher molecular weight, and is easily lost to surfaces. Loss of activity during separation procedures may be interpreted as evidence that two or more components are involved, unfortunately many of the separations used solvents that probably destroyed activity independently of any hypothesized component disassociation.

Recovery of active material from only one zone of native-PAGE gels suggests that sex peptide may be a single molecule, however, when run out on SDS-PAGE, nearly all componets of starting extract were present in the active fraction. Such a concentration of all activity near the boundry between the stacking and resolving gels may indicate that precipitation is occurring as proteins encounter the higher pH of the resolving gel. Later fractions from the same native-PAGE gel do show evidence of fractionation, but were never

active. Are two distinct phenomena responsible for the distribution of protein among native-PAGE fractions, e.g., precipitation of some of all the protein and fractionation of the remainder. Inactive fractions were recombined from native-PAGE gels, however the active fraction was also included in the mix, recombination of **only** inactive fractions was not tried. Knowing whether combined inactive fractions stimulate mated-like behavior would help in the interpretation of this important set of experiments. Until a definitive activity determination is made from only inactive fractions, an important piece of the single vs. multiple component sex peptide puzzle will be missing.

If the activity recovered from native-PAGE was associated with a specific band which could be identified on SDS-PAGE, it would be possible to determine if sex peptide was a polypeptide by comparing native and denatured protein banding.

The pool of potential separation techniques is far from exhausted. Our approach had been to bring very clean, concentrated extracts to experiments, in the hope that identifying the active material in our separations would be aided by having minimized extraneous protein. The lack of clarification evident from the protein profile of active native-PAGE fractions indicates that we have made little progress toward extracting active sex peptide from the pool of other proteins in paragonial gland extracts. The potential exists for comparing active and inactive lanes and using subtractive processes to rule out inactive bands.

Given the poor progress in isolating material from extracts, I would now propose changing the isolation strategy. A method is needed to selectively retain only a portion of the protein diversity of extracts, making the complexity more manageable and allowing more active material to be

recovered per unit of effort. Male reproductive tract extracts are very complex. Up to this point, we have tried and failed to reduce the complexity of active fractions using a variety of separatation techniques. When active material was recovered (using native-PAGE), significant concentration was needed to generate a mated-like behavioral response. I believe affinity chromatography with group specific adsorbents may be a better way to isolate active material or at the very least exclude very specific types of molecules.

Drosophila accessory reproductive glands produce a variety of products including proteins, glycoproteins, lipoproteins, carbohydrates, and other post-translationally modified proteins that may number near 1200. In cells, modified amino acid residues in protein are often exploited as signals to orchestrate specific uptake and transport of molecules from sites of synthesis to where they are used or stored.

The identified *Drosophila* sex peptides have an abundance of post-translationally modified amino acids, principally hydroxyproline (7/36 amino acids in *D. melanogaster* sex peptide). Structure activity studies of the *D. melanogaster* sex peptide reveal that the modified amino acids are not required for biological activity (Schmidt *et al.*, 1993a). It is postulated that the hydroxylated amino acids, which are clustered near each other in *D. melanogaster* sex peptide, may be neccessary transport signals for moving sex peptide into the hemolymph following mating (Schmidt *et al.*, 1993a). Clusters of hydroxyprolines are also favored sites for glycosylation in proteins. The carbohydrate moieties of glycoproteins are also important recognition markers involved in specific recognition and uptake into cells (Voet and Voet, 1990).

Similarity between some characteristics of sex peptides and secreted proteins, and the occurence of glycoprotein in male accessory glands suggest

that exploiting glycoprotein-specific affinity columns may be a way to rapidly test activity from a specific subset of male secretion. Using a Con A Sepharose affinity column (Con A Sepharose has group specific affinity for α-D-mannosyl and α-D-glycosyl residues which are common on glycosylated proteins), it would be possible to prepare extracts, and to selectively collect glycosylated molecules. Even if activity remained in the unbound fraction after affinity chromatography, narrowing the potential pool of candidate molecules by exluding a very specific class of molecule would be progress.

The elution conditions of affinity chromatography do not require the incompatible solvents typical of HPLC separation, but instead use salt solutions at near neutral pH. Because of the binding specifity, extracts could be prepared from whole abdomens or terminalia, saving time and allowing more material to be collected. If successful, selective retention also would produce active extracts of high specific activity, which could be used in subsequent analytical procedures where losses are likely.

Another technique which should be attempted is hydrophobic interaction chromatography (HPHIC), which separates on the basis of protein surface characteristics. Hydrophobic interactions are important forces stabilizing the tertiary and quaternary structures of proteins. High concentrations of organic solvents (which tend to denature proteins) characteristic of RP-HPLC separations are not employed; instead, HPHIC uses aqueous solvent systems and changing salt concentrations to bind and elute protein. Proteins often have some hydrophobic amino acids on their surfaces. These exposed hydrophobic amino acids impart some hydrophobic character. In a polar environment, hydrophobic interactions are forced on nonpolar molecules (Kennedy, 1990). In energetic terms, it is favorable for water to be less structured (Voet and Voet, 1990). Aggregation of nonpolar

regions into hydrophobic pockets reduces the structure of water by reducing the nonpolar surface exposed to the polar environment. The hydrophobic character of the column offers a favorable site for hydrophobic interaction with proteins under highly ionic solvent conditions.

Elution of proteins is based on the strength of interaction with the hydrophobic matrix. Protein samples are added in high salt to increase the structure of water and enhance hydrophobic interactions between the protein and the alkyl groups bonded to the solid support. As the solvent strength decreases, or as the structure of water is reduced by the addition of chaotrophic salts (salts which disrupt the structure of water) like $(NH_4)_2SO_4$, the strength of the hydrophobic interactions declines and it becomes more energetically favorable for the proteins to increase their interactions with the aqueous mobile phase; thus, they begin to reenter the aqueous phase and elute. With a decreasing salt gradient, elution is in order of increasing hydrophobicity.

Recent data on the dose-response profile of sex peptide activity (this dissertation, Chapter 2) should be a prime consideration in the planning and execution of future purification steps. Because "full" activity can result from as little as *ca.* 0.05 male equiv, it is imperative to know at what concentration activity is found. Increasing the concentration of starting extracts to 500 or more male equivalents is one justified method if analytical SDS-PAGE indicates protein losses are significant.

In the native gel electroelution experiment, activity was recovered only after 5x concentration. Ovipositional responses from the unconcentrated fractions were suggestive of activity at the threshold. Recovering activity after such a concentration suggests that as much as 95% of all activity was lost during handling and processing. A more quantitative approach to judging

the quality of activity is needed. Regular total protein determination at each step should be done to identify where loses are occurring and then take action to minimize them. "Active" fractions should be judged on the basis of both qualitative and quantitative measures of activity. Because of the potency of male extracts, caution should be used in the interpretation of "activity" identified from a given fraction. I suggest that SDS-PAGE and densitometry be used to test fractions and control extracts so that a quantitiative measure of protein content/recovery in extracts can be made.

The search to find a compatible solvent system for use with microbore HPLC should continue to be a priority. Identification of an active fraction is critical to the short-term viability of this project, and would open the door for powerful analytical and perhaps molecular techniques to be applied once a peptide can be isolated.

Development of a successful separation protocol for a non-drosophilid sex peptide would be very informative for those who have tried and failed to successfully recover activity using *Drosophila* techniques. Until such a time, it will be unclear whether *Drosophila* sex peptide chemistry is typical for Diptera.

THESIS GENERAL DISCUSSION

The contributions of onion stimuli to host acceptance are well-characterized for the onion fly, *D. antiqua*. Ovipositional acceptance is determined by the multiplicative interaction of onion-like physical, chemical and visual stimuli also interacting with a variable physiological state. The internal excitatory and inhibitory inputs affecting physiological status are now known to be as important in regulating egg output as are external stimuli. Holding external host stimuli constant via an optimized and constant ovipositional resource enabled inroads in the characterization and quantification of internal inputs on *D. antiqua* ovipositional acceptance.

This dissertation has established that mating provides one major type of internal excitatory input in onion fly. Sex peptide produced in male paragonial glands and transferred in the ejaculate, effects dramatic behavioral changes in virgin females. Mated *D. antiqua* or virgin females injected with as little as 1/20th male equiv of sex peptide display mating inhibition and *ca.* a 10x increase in oviposition compared to untreated virgins (this dissertation, Chapter 2).

Because sex peptide is delivered directly into the female genital chamber, this dissertation has treated sex peptide as an internal excitatory input to ovipositional host acceptance (*sensu*. Miller and Strickler, 1984). Whether the effect of sex peptide is removal of inhibition or excitation in the face of counteracting internal inhibition is not known. Once in the hemolymph, sex peptide is apparently carried to neural receptors in the posterior mid brain (Kubli, 1992). In that integrative region are the giant descending neurons (GDNs) (Figure 2) thought to act as ovipositional decision-making units for *D. antiqua*. Once bound to a peptidergic receptor

on a GDN, sex peptide is envisioned to affect signal transduction after processing of multimodal external inputs from the periphery but before signals reach the spike initiation zone.

I speculate that a neural center, like a GDN (Figure 2), responsive to sex peptide, activates finding and examining behaviors before the GDN controlling egg laying is engaged. In the presence of onion stimuli, a mated or sex peptide-injected female apparently has a greater propensity to engage in host-oriented examination of her surroundings than a virgin, a phenomenon not studied here but worthy of research.

Once a potential host is found, repeated movements over and around the plant and nearby substrate, interspersed with mouthparts tasting and ovipositor dragging on and near the foliage provide a female with information about the chemical, visual and physical characteristics of the host (Harris and Miller, 1983; 1984). Different assessment behaviors are highly interspersed during examining, their order of execution is best described as probabilistic. Throughout the examining process, information arriving via the peripheral nervous system, through respective modal integration centers, is impinging upon the GDN controlling egg deposition (Figure 2). As examining progresses, the rate of stem runs increases, probing of foliage and substrate crevices near the plant becomes more intense until, with her ovipositor inserted, a highly aroused female settles over a substrate hole to deposit some eggs (Harris and Miller, 1991). I propose that repetitive exposure to multimodal stimulation leads to a growing depolarization within the GDN controlling egg deposition.

In addition to the multimodal external inputs, I envision that other information regarding internal status also impinges upon the GDN. Ascending afferents arising in the abdomen and carrying information about

e.g., egg load (abdominal stretch) or substrate hole quality (as sensed by ovipositor mechanoreceptors) are likely to influence the probability of egg deposition. Information regarding internal status could also come from the highly multimodal corpora pedunculata, where learning and the state of circadian functions may further bias the decision-making unit.

Together the effects of sex peptide and internal inputs are envisioned to act near the point where the summated peripheral sensory signal (a graded, nonpropagated potential) is transduced into propagated action potentials. The internal inputs may affect a greater frequency of signal transduction by lowering the axon's threshold for generating action potentials or perhaps by changing the neuron's cable properties (e.g., membrane resistance or capacitance) and attenuating the signal before transduction. Thus, at the synapses with the egg movement motor program (a command interneuron controlling the activation of multiple motoneurons each responsible for coordinating elements of deposition) (EMMP, Figure 2), a higher probability of signal propagation from the GDN of mated females would translate into more frequent expression of the stereotypical series of behaviors we recognize as egg deposition.

When the ovipositional fixed action pattern is activated by the EMMP, most other behaviors are inhibited (Mowry *et al.*, 1989b). An ovipositing female remains nearly motionless after moving an egg from her ovaries to the bursa copulatrix for fertilization. Contractions of the reproductive tract expel the fertilized egg, whereupon she probes briefly with her ovipositor before moving another egg or withdrawing from the hole. Following deposition of a clutch of 3-4 eggs, females usually withdraw from the hole and cycle back into another period of intense predepositional examining before laying additional clutches (Mowry *et al.*, 1989b; Harris and Miller, 1991). After

a few more cycles of examining and deposition, the intensity of examining wanes, and the female eventually leaves the host, perhaps as a consequence of depleted mature eggs reserves or reduction in a some other internal input contributing to diminished ovipositional propensity.

Mounting intensity of host interaction leading to ovipositional investment can occur in the absence of any mating or sex peptide stimulation. The course of egg deposition by virgin is similar in both form and duration to that of mated femals (this dissertation, Chapter 3). Its eventual, but delayed, onset in virgins is congruent with a waning inhibition of oviposition. Because virgin oviposition occurs at a reduced rate compared to mated females, I postulate that virgin oviposition is not due to the peptidergic "switch" having been thrown endogenously. Rather, I suspect that in the absence of mating stimuli, virgin oviposition is activated by mounting deprival which eventually overcomes inhibition, yet for mated females the acquisition of sex peptide acts dramatically increasing egg deposition and production. If mating inhibition were found to be concomitant with virgin oviposition, involvement at the receptor for peptidergic inputs would become the favored explanation.

The circumstances of virgin oviposition suggest that the GDN which serves as the ovipositional decision-making unit is one where sex peptide removes ovipositional inhibition. However, the rapid ability of mated females to overcome host deprival and oviposit (Weston *et al.*, 1992) suggest that there is a precedent for mounting endogenous stimuli to overcome missing external stimulatory inputs. Although Weston *et al.*, (1992) in lifetime no-choice tests and Harris and Miller (1984) in short-term no-choice tests found rapid adjustment to deprival of external excitatory inputs, I did

not find rapid adjustments to be the case for deprival of mating (this dissertation, Chapter 3).

Intermediate rates of oviposition, with or without mating inhibition occurred at sex peptide dosages less than 1/20th male equiv (this dissertation, Chapter 2). Threshold effects with decoupling of mating inhibition and oviposition could mean that these functions are controlled by separate components of male secretion or that they are regulated by a single compound acting at different sites. I favor the interpretation that sex peptide binds at different sites (perhaps on different neurons) which have slightly different thresholds for response; the mating inhibition threshold may be higher than that for ovipositional activation. Offset thresholds would assure that mating inhibition was always accompanied by oviposition. However, this decoupling probably occurs only at dosages far below those actually delivered by a male.

The multimodal integrative capabilities of insect GDNs envisioned to act as ovipositional decision-making units are qualitatively and quantitatively represented in the Neural Math Model (p. 26). The potent modulatory effect of sex peptide on reproductive behavior (Neural Math Model term **c**) is only one of several inputs determining the likelihood and magnitude of ovipositional host acceptance. The 10x increase in oviposition following mating or sex peptide injection is very robust, remaining at 10x regardless of host quality (Peters and Miller, unpublished).

In the course of defining a model for an observed phenomenon, the relationships between model variables become testable predictions. As formulated (Table 1), the Neural Math Model predicts that **c** (sex peptide input) is independent of $\mathbf{a}^{x}$ (the term representing the contribution of multimodal integrated external inputs). If the **a** term is really a characteristic

of a species' ovipositional decision-making unit, stimulation with a cross-active sex peptide from a heterospecific should not change the slope of the ovipositional response predicted by the Neural Math Model.

Changes in the ovipositional response slope following sex peptide cross-injection would indicate that the sex peptide affects the processing of sensory information at the periphery. This seems highly unlikely given that the slopes of mated and virgin female *D. antiqua* ovipositional responses are identical (their y-intercepts differ by 10x)(A.S. Peters, unpublished). An even more unlikely occurrence would be ovipositional patterns consistent with an **a** term characteristic of the heterospecific sex peptide donor, suggesting that species-specific sex peptide effects on peripheral processing are transferable.

Intraspecifically, sex peptide and other inputs to the Neural Math Model are likely to have some effect on predepositional host examining. Whether manifestations of Neural Math Model parameters would have the same magnitude with respect to the more probabilistic elements of host examining expressed in a separate predepositional function is an intriguing but untested idea. I hypothesize that a parameter, like **e** (representing internal inputs to the GDN, and expected to vary over the short term as eggs are laid and internal conditions change), would dominate predepositional finding and examining and thereby affect the propensity for oviposition.

Perhaps male choice of a perching site during mate finding involves integration of complex multimodal information within a GDN similar to the one(s) affecting oviposition in females. If females form associations with potential hosts before mating, shared acceptability parameters may be important for bringing the sexes together near appropriate ovipositional sites.

Outlook for the future. Insights into the interplay of multimodal inputs, sex peptides and other internal inputs position the onion-onion fly

system at the cutting edge of theory development in insect-plant behavioral interactions. The onion fly system is very amenable to continued study to understand gating of oviposition. Productive directions would be: identification of sex peptide, tracing labelled sex peptide to receptors in the brain via fluorescent binding studies, identification and neuro-anatomical characterization of egg deposition decision making units and insertion of electrodes into these cells in an attempt to stimulate *in vivo* oviposition.

APPENDICES

APPENDIX 1

Voucher specimen depository forms

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.: 1994-3

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

Onion fly reproductive behaviors as influenced by mating and paragonial gland extracts.

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

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name (s) (typed)
Joseph L. Spencer

Date 16 August 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
Delia antiqua (Meigen)	Lab culture E. Lansing, MI 15 June 1994					5	5		MSU
	Field-Grant culture E. Lansing, MI 15 June 1994					5	5		MSU
	DI-90 culture E. Lansing, MI 15 June 1994					5	5		MSU
Delia platura Linnaeus	Whistlecraft culture London, Ontario Canada 15 June 1993					10	10		MSU
Delia radicum Linnaeus	Whistlecraft culture London, Ontario Canada 15 June 1993					10	10		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Joseph L. Spencer

Date 16 August 1994

Voucher No. 1994-3

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

Curator 12 Sept 1994

Date

APPENDIX 2

Native- and SDS-PAGE gel, buffer and staining recipes

NATIVE•PAGE
SEPARATING GEL
(Stock sol. is 40%T,5%C_{bis})

	5%T(5%C_{bis})	10%T(5%C_{bis})	15%T(5%C_{bis})
SEP. GEL MONOMER	1.25 ml	2.50 ml	3.75 ml
SEP. GEL BUFFER	2.50 ml	2.50 ml	2.50 ml
2D•H_2O	5.00 ml	3.75 ml	2.50 ml
CATALYST	1.25 ml	1.25 ml	1.25 ml
TEMED	20 µl	20 µl	20 µl

NATIVE•PAGE
STACKING GEL
(3.125%T, 20%C_{bis})

STACK. GEL MONOMER	5.0 ml
STACK. GEL BUFFER	2.5 ml
CATALYST	2.5 ml
TEMED	10 µl

NATIVE•PAGE
SEPARATING GEL MONOMER
(40%T, 5%C_{bis})

ACRYLAMIDE .....38.0 g
(Acrylamide is a neurotoxin and should be handled with care)
BIS .....2.0 g
2D•H_2O .....to 100 ml

NATIVE•PAGE
STACKING GEL MONOMER
(6.25%T, 20%C_{bis})

ACRYLAMIDE .....5.0 g
(Acrylamide is a neurotoxin and shoud be handled with care)
bis .....1.25 g
2D•H_2O .....to 100 ml

NATIVE•PAGE
SEPARATING GEL BUFFER(4X)
(947mM Tris, 0.289N HCl, pH 8.48)

TRIS .....11.47 g
HCl ..... 28.92 ml (1N)
2D•H_2O .....to 100.0 ml

NATIVE•PAGE
STACKING GEL BUFFER(4X)
(158mM Tris, 0.256N H_3PO_4, pH 6.90)

TRIS .....1.92 g
PHOSPHORIC ACID..... 25.6 ml(1N)
2D•H_2O .....to 100 ml

NATIVE•PAGE
LOWER TANK BUFFER
(63mM Tris,0.5N HCl,pH 7.47)

TRIS ..... 7.63 g
1N HCl ..... 50 ml
2D•H_2O ..... to 1000 ml

NATIVE•PAGE
UPPER TANK BUFFER
(37.6mM Tris,40mM glycine,pH8.89)

TRIS ..... 2.28 g
GLYCINE ..... 1.50 g
2D•H_2O ..... to 500 ml

NATIVE•PAGE
SAMPLE BUFFER
(50% sucrose, 0.1% bromphenol blue)

SUCROSE ..... 500 mg
BROMPHENOL BLUE ..... 100 µl(1% solution)
2D•H_2O ..... to 1.0 ml

-Combine 1 volumn sample to 0.1 volumn sample buffer solution.
-e.g. 50 µl sample and 5 µl sample buffer solution.

NATIVE•PAGE
CATALYST
(0.06%ammonium persulfate,
0.002% riboflavin phosphate)

AMMONIUM PERSULPHATE ..... 60 µl (10% solution)
RIBOFLAVIN PHOSPHATE ..... 1.0 ml (0.02% solution)
2D•H_2O ..... to 10 ml

NATIVE•GEL

COOMASSIE BLUE STAINING

---

1. FIXING STEP (12.5% trichloroacetic acid)

TCA ..... 6.25 g
2D•H_2O ..... to 50.0 ml.

-combine ingredients in staining tray, add gel and fix for 60 min.

2. STAINING STEP (0.01% coomassie blue G-250)

COOMASSIE BLUE G-250 ..... 125 mg
2D•H_2O ..... to 50 ml

-add 2.0 ml of above solution to fixing solution and gel.
-stain for 2 hours.

3. DESTAINING STEP (7% acetic acid, 5% methanol)

ACETIC ACID .. .. 70 ml
MeOH ..... 50 ml
2D•H_2O ..... to 1000 ml

-Decant the stain solution.
-Add 200ml of destaining solution.
-Replace the destaining solution until the gels have a clear background.

SDS•PAGE SEPARATING GEL

0.375 M TRIS, pH 8.8

	7.5%	12%	15%
2D•H_2O	4.85 ml	3.35 ml	2.30 ml
1.5M TRIS/HCl, pH 8.8	2.50 ml	2.50 ml	2.50 ml
10% SDS	100 µl	100µl	100 µl
Acrylamide/Bis (30% stock)	2.50 ml	4.00 ml	5.00 ml
10% Ammonium Persulfate	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5µl

SDS•PAGE STACKING GEL

4.0% GEL,0.125M TRIS, pH6.8

2D•H_2O	3.05 ml
0.5M TRIS/HCl	1.25 ml
10% SDS	50 µl
ACRYLAMIDE/BIS (30% stock)	650 µl
10% AMMONIUM PERSULFATE	25 µl
TEMED	5 µl

.SAMPLE BUFFER

SDS•PAGE

2D•H_2O ..... 180 µl

STACKING GEL BUFFER ..... 50 µl

GLYCEROL ..... 60 µl

10% SDS ..... 80 µl

β-MERCAPTOETHANOL ..... 20 µl

BROMPHENOL BLUE ..... 10 µl

SEPARATING GEL BUFFER

1.5M TRIS/HCl, pH 8.8

TRIS BASE ..... 18.15 g

2D•H_2O ..... 35.0 ml

Bring to pH 8.8 with 1N HCl. Add 2D•H_2O to 100 ml.

STACKING GEL BUFFER

0.5M TRIS/HCl, pH 6.8

TRIS BASE ..... 6.0 g

1N HCl ..... 45.0 ml

Bring to pH 6.8 with 1N HCl. Add 2D•H_2O to 100 ml.

ACRYLAMIDE/BIS STOCK SOLUTION

30% T, 2.6% C

ACRYLAMIDE ..... 29.2 g

N'N'-BIS-METHYLENE-ACRYLAMIDE ..... 0.8 g

$2D•H_2O$ ..... 68.5 ml

5X ELECTRODE BUFFER

(RUNNING BUFFER)

TRIS BASE ..... 15.0 g

GLYCINE ..... 72.0 g

SDS ..... 5.0 g

$2D•H_2O$ ..... TO 1000 ml

Dilute 80 ml with 320 ml $2D•H_2O$ for one run.

SILVER STAINING

For One 0.75 mm Mini Gel

1. FIXING STEP

MeOH	50.0 ml
ACETIC ACID	10.0 ml
2D•H_2O	35.0 ml
FIXATIVE ENHANCER SOLUTION	5.0 ml

-Combine above ingredients in glass tray, add gel and fix for 20 min.

2. RINSE STEP

-Decant the fixing solution
-Rinse the gel 2 times for 10 minutes with 100 ml 2D•H_2O.

3.STAIN & DEVELOP STEP

-In a 100 ml beaker add, with stirring and in order:
(While the gel is rinsing)

2D•H_2O	17.5 ml
SILVER COMPLEX SOLUTION	2.5 ml
REDUCTION MODERATION SOLUTION	2.5 ml
IMAGE DEVELOPMENT REAGENT	2.5 ml
*DEVELOPING ACCELERATOR REAGENT	25.0 ml

*Add this reagent to the others just prior to adding solution to the mini gel.

-Decant the rinse bath.
- Add Stain and Develop solution.
-Develop until the desired intensity is reached. (Approx. 10 min.).

4. STOP BATH STEP

2D•H_2O	190.0 ml
ACETIC ACID	10.0 ml

When gel is developed to the desired intensity, add it to the above stop bath solution for at least 10 minutes.

SAMPLE BUFFER

Modified for SDS•PAGE without
Mercaptoethanol & Heating
(Non-denaturing SDS-PAGE)

2D•H20 ..... 2.89 ml

0.5 M TRIS/HCl ..... 500 µl

GLYCEROL ..... 400 µl

10% SDS ..... 160 µl

0.1% BROMPHENOL BLUE ..... 50 µl

-Combine 650 µl Sample Buffer with 150 µl of sample before loading onto gel wells

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