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Physiological and behavioral factors affecting feeding and satiation in Tabanus nigrovittatus and Phormia regina.

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PHYSIOLOGICAL AND BEHAVIORAL FACTORS AFFECTING FEEDING AND SATIATION IN *TABANUS NIGROVITTATUS* AND *PHORMIA REGINA*

A Thesis Presented

by

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DEDICATION

To the men and women who pioneered the science of entomology, paving the way for future entomophiliacs, and especially to my mentors who not only fostered the scientific life in me, but also evoked passion for learning. They continue to be a tremendous source of encouragement. They are the giants whose shoulders' I stand on.

"Our treasure lies in the beehive of our knowledge. We are perpetually on the way thither, being by nature winged insects and honey gatherers of the mind."

Friedrich Nietzsche

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ABSTRACT

PHYSIOLOGICAL AND BEHAVIORAL FACTORS AFFECTING FEEDING AND SATIATION IN *TABANUS NIGROVITTATUS* AND *PHORMIA REGINA*

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For most organisms, feeding is an absolute necessity. Yet, there remain many unknown aspects about the feeding behavior of insects that beg to be discovered. Specifically, little is known about the chemical short-term and long-term satiety factors that are involved in feeding. Sulfakinin is an invertebrate neuropeptide that has recently been shown to be homologous to vertebrate cholecystokinin.

In this study, sulfakinin has been used to investigate the role it has in blood engorgement of the salt marsh horse fly, *Tabanus nigrovittatus*, and in carbohydrate and protein ingestion of both sexes of the black blow fly, *Phormia regina*. Horse flies injected with 1 nmol dose of sulfakinin (perisulfakinin) were inhibited from feeding by 45-58%, when blood fed using an artificial membrane. However, no feeding inhibition was observed when blood fed using blood-soaked Kimwipes. In the blow fly, feeding was significantly inhibited by 34% in the males and 44% in the females when injected with sulfakinin (drosulfakinin I). Sulfakinin had no significant inhibitory effect on protein feeding in either sex.

Several other factors affecting feeding in the horse fly were also examined. The effect of odors on blood engorgement has not previously been looked at in the Tabanidae. Octenol, which is a known odor attractant, was found to significantly stimulate engorgement in the horse fly. The effect of blood temperature on engorgement has never been examined in this species, and the results of this study demonstrate that temperature is a significant stimulus for successful engorgement of a blood meal. Also, the percentage of engorgement throughout the season was recorded and found to fluctuate depending on whether it was the beginning, middle, or end of the season.

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CHAPTER I

LITERATURE REVIEW

Introduction

The idea of food is an all-consuming thought in and of itself. Food consumption infiltrates practically every area of human life, the least of which includes dieting, disorders, and survival. Feeding has been argued as one of the most important behaviors in all living organisms. Not only does feeding behavior affect the individual or even the species, but it has implications for other organisms as well. The feeding habits of other organisms, specifically insects, greatly affect the quality of human life, more so than what we often give credit for. Food consumption by insects is responsible for much of the major global economic and health related problems that plague humans. The feeding behaviors of insects have a fascinating breadth of diversity, and the Diptera prove no different. The two species of flies, *Tabanus nigrovittatus* and *Phormia regina*, used in the following studies have quite different feeding habits and mechanisms for feeding regulation. There are a series of 'events' that must take place in order for both species to feed.

Feeding Behavior. For *T. nigrovittatus*, only blood feeding will be examined. In order to successfully engorge (i.e., feed to repletion on blood) a blood meal several things must occur. Once the female horse fly emerges as an adult on the marsh and has mated she is immediately able to lay a batch of eggs without a blood meal (autogenous). The egg production and laying process takes about 7-10 days (Magnarelli and Stoffolano, 1980). After she has completed her first gonotrophic cycle, the female voraciously seeks out her first blood meal from a vertebrate host.

One of the first events that needs to take place in order to find a blood meal is for the female to visually orient herself to a host, more specifically, to detect and recognize appropriate background contrast (Allen and Stoffolano, 1986a), as well as hue and intensity (Allen and Stoffolano, 1986b). In fact, visual attraction is so central to *T. nigrovittatus* orientation that much of the control effort for this fly is based on color (i.e., the blue and black box traps) and shape (i.e., compact, solid box traps) (Allen and Stoffolano, 1986c). The second sequential and complementary event that leads to the female finding a blood meal is the odor stimulus. Research with odorants for tsetse flies in the 1970's proved very successful in setting the stage for isolating specific compounds involved in attraction to a vertebrate host (Vale, 1977). Certain compounds, such as octenol, carbon dioxide and acetone, have been identified as important factors in the host seeking behavior of tsetse flies (*Glossina* spp.: Vale, 1979; 1980; Vale and Hall, 1985), stable flies (*Stomoxys calcitrans* Linnaeus: Warnes and Finlayson, 1985), black flies (*Simulium* spp.: Thompson, 1976) and horse flies as well (Hayes et al., 1993). Other stimuli that ensure hematophagous Diptera will find a blood meal is host movement (O'Meara, 1987). Once the female has located the host, appropriate cues must be received while on the host before probing is initiated, such as an appropriate heat and humidity stimulus (Friend and Smith, 1977). According to Dethier (1957) the most important factor to induce probing is heat. Friend and Smith (1977) explained in their review of factors affecting blood feeding that after landing on the host the female tabanid uses her '…foretarsi to palpate a wide area' in order to find an appropriate stimulus which would elicit the fly to lower and spread her labellum and begin probing. After she has pierced the skin and located the blood from the hemorrhage, ingestion is initiated by

the taste of blood via the chemoreceptors on the labellum (Dethier, 1957) and the blood meal is directed to the midgut (Stoffolano, 1983). The behavioral and physiological factors that affect blood feeding and satiation in *T. nigrovittatus* will be discussed below and in the coming chapters.

The blow fly, *P. regina*, actively searches for its two essential nutrients, carbohydrates and proteins. In the wild, sources of these nutrients include nectar, sucrose, fructose, decaying matter, and feces. When an acceptable food source is encountered, the blow fly stops the searching behavior and orients toward the source. If the chemoreceptors on the tarsi are appropriately stimulated, the fly extends its proboscis and spreads the labellar lobes. Ingestion will occur if there is further stimulation by the chemoreceptors of the mouthparts and cibarium. The nutrient source enters the alimentary canal and can either be stored in the crop or diverted to the midgut for digestion. The extent of time that feeding takes determines the amount of food ingested (Gelperin, 1971). The known mechanisms of feeding control in non-hematophagous insects are discussed below and in chapter V. After feeding has been terminated, if food is stored in the crop, it will be moved up the crop duct and re-shunted to the midgut. According to Gelperin (1971), the mechanism regulating feeding in *P. regina* does not function to maintain a constant caloric intake, but rather meal size. In addition to carbohydrates for energy, the blow fly requires a protein meal before the onset of oogenesis.

Feeding Control in Vertebrates. Feeding regulation in vertebrates has been well studied and well established in the literature. Current research suggests that the endocrine system plays a significant role in feeding regulation (Geary, 2004). Long-term feeding

regulation, the homeostasis of a stable body weight, is controlled through insulin and leptin signals that are involved in indicating the body fat stores (Geary, 2004; Strader and Woods, 2005). Short-term feeding regulation is controlled by meal-related signals and function to maintain the meal size. According to Strader and Woods (2005), satiety signals are referred to as gastrointestinal signals that stop an ongoing meal. There have been several hormones identified and known to be secreted in response to an ingested meal, but one of the most studied and most important regulatory hormones is cholecystokinin (CCK). Most satiety signals, including CCK, are released from endocrine cells that line the lumen of the gastrointestinal tract (Strader and Woods, 2005). These specialized endocrine cells have projections that have chemoreceptors sensitive to the food that passes through the gut. The cells secrete peptides that act as hormones and enter the bloodstream or act on other cells. CCK is secreted from endocrine cells in the duodenum in response to proteins and fats (Beglinger and Degen, 2004), and interacts with specific CCK receptors on the vagus nerve. The vagus nerve then transmits signals to the brain, causing the termination of the ongoing meal. Other biological actions in response to the release of CCK include myostimulatory effects on the gut and gall bladder, enzyme secretion from the pancreas, and gastric emptying (Strader and Wood, 2005). There are also stretch receptors on the afferent fibers of the vagus nerve. These fibers line the gut and are sensitive to volume and lumen pressure from the resulting meal. They act additively to shut down feeding (Strader and Woods, 2005). CCK also acts additively with other hormones (i.e., bombesin, glucagon) and other absorbed nutrients from the meal, as well as the biogenic amine, serotonin (Hayes et al., 2006). Exogenous injections of CCK have reduced meal size in animals and humans while CCK

antagonists (blocking CCK receptors) have increased meal size. CCK is thought to also interact with long-term feeding regulation signals (Strader and Woods, 2005).

Feeding Control in Insects. For hematophagous and non-hematophagous insects alike, little research has been done examining the long-term feeding regulation mechanisms. For hematophagous insects, it is well known that stretch receptors play a significant role in short-term control of blood feeding (Gwadz, 1969; Friend and Smith, 1977). Stretch receptors are activated once a blood meal enters the midgut and distend the abdomen. Exogenous applications of juvenile hormone have been shown to terminate blood feeding in *Culex* mosquitoes and consequently inhibit further host seeking behavior (Meola and Petralia, 1980). The role of neuropeptides in satiation has not been investigated.

For non-hematophagous insects, several neural mechanisms of short-term satiety have been outlined by Bernays and Simpson (1982) and include chemosensory adaptation, the decay of the excitatory state, stretch receptors, and negative feedback from hemolymph factors following the meal. Biogenic amines, specifically serotonin, have been shown to inhibit carbohydrate food intake in the flesh fly (Dacks et al., 2003) and protein food intake in the blow fly (Haselton, 2005; Stoffolano, *unpublished data*). More recently, neuropeptides have been shown to have a role in feeding satiety (Maestro et al., 2001; Wei et al., 2000).

The role of sulfakinin, an invertebrate homologue to vertebrate cholecystokinin, has been identified in many insects; thus far, its effects on feeding have only been studied in the German cockroach (Maestro et al., 2001) and desert locust (Wei et al., 2000). Sulfakinins are a family of invertebrate neuropeptides that are physiologically and

structurally homologous to vertebrate cholecystokinin (Schoofs and Nachman, *in press*). The first invertebrate sulfakinin to be reported was leucosulfakinin (LSK), isolated from the cockroach, *Leucophaea maderae,* and shown to increase hindgut contractions (Nachman et al., 1986). As reported in Nichols (2003), sulfakinins have also been identified in other insects such as the cockroach, *Periplaneta americana* (Veenstra, 1989), the locust, *Locusta migratoria* (Schoofs et al., 1990), the flesh fly, *Neobellieria bullata* (Fonágy et al., 1992), the fruit fly, *Drosophila melanogaster* (Nichols, 1992) and the blow fly, *Calliphora vomitoria* (Duve et al., 1994). Sulfakinin immunoreactive cells have been found in the blow fly, *P. regina* (Haselton, 2005), in the midgut of *Aedes aegypti* (Veenstra et al., 1995), as well as in the brain, abdominal ganglion and in the endocrine cells of the gut where the foregut and midgut merge in *T. nigrovittatus* (Haselton, 2005).

Sulfakinins have been shown to have myostimulatory effects on the gut of both the cockroach and locust, but have not been shown to have myotropic effects in any of the Diptera examined to this point (Duve et al., 1994; Haselton et al., 2006). Research by Aguilar et al. (2004) identified a myosuppressin, leucomyosuppressin (LMS), in the German cockroach as another inhibitor of food intake. The authors hypothesized that myoinhibitory factors produce accumulations of food in the gut, further inhibiting food intake due to constant signals from the appropriate stretch receptors. The authors found that, in response to LMS, food accumulated in alimentary compartments (specifically the foregut) in a dose-dependent manner. While sulfakinin also had anti-alimentary effects, it did not cause an accumulation of food in the foregut.

The authors proposed that sulfakinins were inhibiting food intake though a different mechanism than that of LMS, suggesting the satiety factor acts on the CNS. The role of sulfakinin on feeding inhibition in the Diptera will be discussed in chapters III, IV, and V.

CHAPTER II

THE EFFECT OF OCTENOL ON ENGORGEMENT BY THE SALT MARSH HORSE FLY, *TABANUS NIGROVITTATUS*.

Abstract

Adult female *Tabanus nigrovittatus* were field collected from a salt marsh in Essex Co., MA. The horse flies were transported back to and tested in the laboratory to determine the effects of octenol (1-octen-3-ol) on engorgement. Flies exposed to octenol strips had a significantly higher engorgement response compared to control flies. This is the first study to demonstrate an important link between an odor stimulus and the feeding response in Tabanidae. Research examining the link between odor attractants and repellents on the engorgement response is lacking or limited in most hematophagous Diptera. Understanding the role odors have on ingestion is essential to knowing how to interrupt feeding behavior of blood feeding arthropods, especially for important vectors.

Introduction

Tabanus nigrovittatus Macquart is found in the salt marshes of the Atlantic coast. This fly is a notorious nuisance to tourists, locals, and livestock alike, especially as its three to four week presence on the marsh coincides with the peak tourist season of the summer. *T. nigrovittatus* is an excellent blood-feeding insect to study because the flies can be collected in extremely high numbers on the marsh during the season, they have been shown to readily feed through a parafilm membrane (Stoffolano, 1979), and considerable information already exists on phagostimulants (Friend and Stoffolano, 1983; 1984; 1991), food diversion (Stoffolano, 1983), oogenesis and oviposition (Magnarelli and Stoffolano, 1980; Graham and Stoffolano, 1983). Research examining the effects of odor on probing and ingestion is lacking for most hematophagous Diptera. Most, if not all, research involving host-seeking stimuli and odor attractants is concerned with trap effectiveness and control measures (Takken and Kline, 1989; Jaenson et al., 1991). Those studies that do examine the role of odors only investigate probing and not ingestion (Hopkins, 1964; Gatehouse, 1970). There is a general absence in the literature of studies that look at the function of odors in the next behavioral step of obtaining a blood meal (i.e., engorgement). I used octenol to study how it affects engorgement. Octenol (1-octen-3-ol), identified from ox breath and originally used in tsetse fly research (Vale and Hall, 1985), is an odorous compound that has been identified as an olfactory stimulant for several hematophagous insects, including tabanids (Hayes et al., 1993; Foil and Hribar, 1995). Octenol is used in box traps and has proven to be an effective attractant for increasing trap collection of *T. nigrovittatus* (Hayes et al., 1993; Foil and Hribar, 1995).

Materials and Methods

Collecting and Maintaining Flies. Female host-seeking *T. nigrovittatus* were collected from box traps on the salt marsh in Essex Co., Massachusetts, during July 2005. Flies were moved from black box traps on the marsh to metal screened cages (24 x 24 x 45 cm) and given access to granulated sugar and water during transportation back to and while housed in the laboratory. Females were maintained at 25-27^{0}C and 50-60% relative humidity. Prior to experimentation, all flies were deprived of granulated sugar for 16 h and were tested one day after being collected in the field. The exact chronological ages of all flies used are unknown. However, the first collection date for the 2005 experiments occurred on 4 July 2005 and field collections were made every other day consecutively throughout the season. Therefore, all flies used in experimentation were assumed to have only been in the field traps for at most two days.

Feeding Assay. Following the starvation period, flies were cold immobilized in the freezer. Each experimental group (control and octenol) consisted of 20 flies and a total of 8 replicates were performed. Citrated beef blood was warmed on a hot plate to 37^{0}C and continuously stirred. The bottoms of 500 ml plastic deli cups were cut off; parafilm was fitted over the opening and secured to the cup with a rubber band. A single cup was placed on top of each cage with the parafilm positioned on the bottom to act as a membrane for the flies to probe.

The warmed blood was poured into each cup and a lamp with a 60-watt bulb was positioned over it to provide adequate light and to keep the blood warmed. The flies were placed in the cages and allowed to feed *ad libitum* for one hour. Octenol strips (BioSensory Inc., Willimantic CT.) were placed on top of the cage next to the cups of

blood for the octenol-exposed treatments. The octenol release rate at room temperature in an open area is 0.0075 g/h (Biosensory Inc.). Octenol feeding assays were performed in a different laboratory from that containing the control cages to ensure no odor stimulus affected any other feeding assays, and only octenol-exposed cages were used for octenol-exposed flies for the same reason. The two laboratories were not tested beforehand using a non-treatment to ensure equivalency; however, optimal lighting and temperature was ensured before each experimental replication.

Dissection Technique and Analysis. After the feeding assays, flies were put into a deep freezer for approximately an hour. Once the flies were dead they were submerged in 70% ethanol and each one was then held up to a light bulb to check for the presence of a blood meal. Flies appeared deep red if there was a blood meal in the midgut and appeared yellow if lacking one. Any questionable individuals were dissected and the midgut was checked for the presence of blood. The data was analyzed using a *t*-test to compare the means of the non-treatment and the treatment group.

Results

A total of 320 flies were used in this study. A *t*-test was used to compare the means of the controls (2.375 ± 1.4497) and the octenol-exposed flies (7.250 ± 1.4497) (JMP, SAS Institute Inc. 2005). I found that 36.3% of the 160 flies exposed to octenol took a blood meal while only 10.4% of the 160 control flies took a meal. Thus, octenol significantly stimulates blood feeding in *T. nigrovittatus* ($F = 5.65$; $df = 14$; $P < 0.05$). While not quantified, with the flies exposed to octenol, there appeared to be a greater number of individuals spending more time moving about on the top of the cage near the octenol strips and blood.

Discussion

There is abundant literature describing how hematophagous insects locate their host and all the factors involved. There is also a wealth of knowledge available on how hematophagous insects ingest their blood meals, as well as destination of meals, and the factors affecting feeding physiology. The results of this study demonstrate that there is also an important link between an odor stimulus and the feeding response (i.e., probing and ingestion) in this hematophagous insect.

It is not intuitively surprising that an odor attractant stimulates biting in a hematophagous fly. However, it is an important missing link in the literature when investigating the sequence of events a fly must respond to in order to obtain a blood meal. Since I did not make any observations of individual flies, it is impossible to make a direct correlation in this study between an individual fly probing and its engorgement. However, what is clear is that the number of individuals probing was increased based on the increased engorgement rate that has been demonstrated.

The observation that octenol-exposed flies spend more time moving about on the underside of the top of the cage near the octenol strips and that they appeared to be in a more excited state compared to flies not exposed to the odorant suggests that the octenol is positively affecting the central excitatory state (CES) as related to probing and gorging. The effect of one stimulus on a specific, often unrelated, behavior was termed central excitatory state if the stimulus caused an increased expression of the behavior being examined (Dethier et al., 1965). Later, Tully and Hirsch (1983) evaluated the previous studies examining the central excitatory state with respect to feeding in *Drosophila* and *Phormia* and noted, "It is conceivable that other sensory modalities may induce CES

too." I propose in this study that octenol, which is a non-feeding modality perceived by the antenna, alters the central excitatory state of the female tabanids such that it increases their CES as it relates to probing and ingestion behavior. Few if any studies using hematophagous insects have looked at the CES as it relates to obtaining a blood meal.

This study only looked at the effect of octenol on membrane feeding. Future studies that should provide insight concerning *T. nigrovittatus* might include time trials to observe how fast blood meals are taken once probing has been initiated, and whether an odor stimulus stimulates a faster ingestion response. Another study could examine how Kimwipe feeding (described by Stoffolano, 1979) differs and whether one could observe a 100% engorgement response from flies treated with octenol. Knowing how to increase the engorgement response of hematophagous insects in the laboratory would be beneficial and have practical applications for physiological and behavioral studies investigating the consequences of engorgement or for research examining parasite transmission and blood feeding.

Other experiments that would prove insightful would be to test the effects of insect repellents on membrane feeding. A repellent is defined as 'any stimulus which elicits an avoiding reaction' and therefore, according to Vincent Dethier, there should be a distinction made between those repellents that act on the contact chemoreceptors after the insect lands on the host and those repellents that act on the olfactory system and deter the insect from finding the host (Dethier, 1957). Most past and current literature examining the effects of repellents on probing are performed in the field with mosquitoes (Barnard et al., 2002) and biting midges (Perich et al., 1995) and most studies tend to generalize any fly 'settling' on a human volunteer as one that would eventually bite

(Dethier, 1957). While performing behavior studies in a laboratory environment has certain limitations, it may provide more accurate information when evaluating the engorgement response and what is actually happening with the flies physiologically. Field tests and even some laboratory tests using mosquitoes (Klun et al., 2004; Konan et al., 2003) with repellents have examined the repellency duration and biting rate but have not necessarily examined in detail how the repellent interrupts the physiological feeding sequence of the fly.

Understanding how to interrupt feeding, especially when trying to control pests like *T. nigrovittatus*, requires knowing not only what attracts and repels the flies to their hosts but also recognizing each physiological and behavioral step that stimulates the fly to successfully feed. Thus, understanding the role of attractants on engorging is essential.

CHAPTER III

THE EFFECT OF PERISULFAKININ AND SEASONALITY ON ENGORGEMENT BY THE SALT MARSH HORSE FLY, *TABANUS NIGROVITTATUS*.

Abstract

Insect sulfakinins are homologues to cholecystokinin, which in vertebrates functions as a satiety factor. Recently, non-hematophagous insect studies demonstrated that sulfakinins function in feeding inhibition. Using a hematophagous insect (i.e., *Tabanus nigrovittatus* Macquart), flies injected with 1 nmol of perisulfakinin were inhibited from blood feeding by 45-58%. This percentage of inhibition is comparable to previous research on non-hematophagous species. When flies were injected with 10 nmol of perisulfakinin, engorgement was increased relative to the sham-injected flies. The stimulation of feeding may be due to the fact that the endogenous levels of sulfakinins in insects remain unknown. This study is the first to examine sulfakinin in a hematophagous insect and suggests that sulfakinins act additively with other mechanisms to regulate blood feeding. Also, the percentages of flies engorging throughout the season was recorded and revealed that the percentage of flies gorging fluctuates, leading to a decrease in engorgement as the season comes to an end. Understanding what controls blood feeding will allow researchers to interrupt engorgement more successfully for control efforts.

Introduction

Tabanus nigrovittatus Macquart is an ideal hematophagous model to study feeding behavior because flies can be collected in extremely large numbers on the marsh and considerable information already exists on phagostimulants (Friend and Stoffolano, 1983; 1984; 1991), food diversion, and feeding methods (Stoffolano, 1979; 1983). To date, the least understood aspects of its biology are the factors affecting satiety. In addition, little is known about the natural engorgement pattern throughout the fly's three to four week season, especially in regards to aging.

Sulfakinins (SKs) are a family of invertebrate neuropeptides that are physiologically and structurally homologous to vertebrate cholecystokinin (Schoofs and Nachman, 2006), and thus have a role in vertebrate satiety. Recent studies reported that sulfakinins significantly inhibited food intake by 55% (at 1 nmol, Wei et al., 2000) in the desert locust, *Schistocerca gregaria* Förskal, and by 60% (at 10 μg, Maestro et al., 2001) in the German cockroach, *Blatella germanica* Linnaeus. In *T. nigrovittatus*, sulfakinin immunoreactive cells have been found in the brain, abdominal ganglion, and in the endocrine cells of the gut where the foregut and midgut merge (Haselton, 2005). The goal of the present study is to investigate the role of sulfakinins on blood feeding and to determine what the natural pattern of engorgement is over the season.

Materials and Methods

Collecting and Maintaining Flies. Female host-seeking *T. nigrovittatus* were collected from box traps, as previously described in chapter II, during July of 2004 and 2005. Prior to experimentation, all flies were deprived of granulated sucrose for 16-20 h. All flies used in experimentation were tested the day after being collected in the field.

The exact chronological ages of all flies used are unknown. However, during the 2005 field season the first observation of *T. nigrovittatus* in surveillance traps (3 flies) occurred on 22 June 2005. The first collection date for the 2005 experiments occurred on 4 July 2005 and field collections were made every other day consecutively throughout the season until the flies were no longer on the marsh. Flies were only collected once a week during 2004.

Injection Technique for Perisulfakinin Experiments. All flies were cold immobilized in the freezer and only flies of approximately the same size were used. Flies were then placed in a Petri dish on ice to prevent them from moving prior to injections. Sham-injected and treatment flies were injected with 1 µl in the second to last intersegmental membrane on the right ventral side of the abdomen. All flies were injected using a 30-gauge needle attached to a 25 µl glass gastight Hamilton #1750 syringe (Hamilton Co., Reno, Nevada). Sham-injected flies were injected with *Phormia* saline (Chen and Friedman 1975) and treatment flies were injected 1 nmol and 10 nmol perisulfakinin (PSK) (Bachem, PA, USA) dissolved in *Phormia* saline. The sulfakinin was prepared in a stock solution of 80% acetonitrile and 20% water, made up to 0.01% trifluoroacetic acid. The nanomolar doses were chosen based on the doses used in the previous insect studies with sulfakinins (Masestro et al., 2001; Wei et al., 2000).

Control flies (used in the seasonality experiments described below) were cold immobilized and set on ice for the same duration as the sham-injected and treatment flies but were not injected with saline. After an individual fly was injected, it was placed back on ice until the entire experimental (control, sham, or treatment) group was completed. 40 flies were used for each experimental group in 2004 and 20 flies were used for each

experimental group in 2005. The entire injection process for each experimental group took less than 10 minutes. Each experimental group of flies was placed in a 23 cm^3 metal-screened cage and the feeding assay was started. There was zero mortality with all injections. All flies recovered from injections and resumed normal 'fly behavior' of walking, grooming, etc. The recovery period usually took less than 5 minutes.

A total of 1,200 flies were used in experimentation for the sulfakinin study and a total of 15 replicates were performed throughout the study (5 replicates in 2004 and 10 replicates in 2005). Here and throughout the rest of this thesis, one replicate consists of a single run of all of the experimental cages (sham-injected and sulfakinin-injected) simultaneously. All statistical analyses were performed using ANOVA to compare the percentage engorged by treatment and Tukey-Kramer HSD test (JMP, SAS Institute Inc. 2005). The percentage of difference between the sham-injected group and the treatment group was calculated by:

$$\frac{\textit{\% engorged by treatment} - \textit{\% engorged by sham}}{\textit{\% engorged by sham}} \times 100 = \textit{\% Difference}$$

Feeding Assay. Friend and Stoffolano (1983) found that tabanids only successfully blood fed in a group setting of more than 5 flies, so all flies were group-fed. Citrated beef blood was warmed on a hot plate to 37^0C and stirred with a magnetic stirrer. Horse flies have been shown to blood feed in the laboratory using two different methods (Stoffolano, 1979), using an artificial membrane or blood-soaked Kimwipes. Both of those feeding techniques were examined for the seasonality experiments. For the parafilm membrane feeding technique, plastic deli cups were fitted with a parafilm membrane and

prepared as previously described in chapter II. Warmed blood was poured into each cup and a lamp with a 60-watt bulb was positioned over it to provide adequate light and keep the blood warmed. For the blood-soaked Kimwipe feeding technique (used in the seasonality experiments described below, not in PSK experiments), Kimwipes were placed on top of the cages and warmed blood was pipetted onto the Kimwipes until thoroughly soaked. Only the parafilm membrane feeding technique was used in the PSK experiments. The flies were then allowed to feed *ad libitum* for one hour. The flies used in the seasonal engorgement experiments were prepared and treated the same, with the exception of no injections. After the feeding assays were completed, flies were killed and their midguts checked for the presence of a blood meal.

Seasonality and Engorgement. Stoffolano (1979) demonstrated that horse flies could be blood-fed in the laboratory using two methods. The parafilm membrane feeding technique employs the probing mechanism from the fly, while the Kimwipe feeding mechanism does not require probing through a membrane and has been shown to be a more successful method for blood feeding in the laboratory. For this reason, the percentage of flies engorging throughout the 2005 season was recorded for both feeding techniques. Based on dissections of the ovarioles of females throughout the 2005 season, as well as previous work conducted by Magnarelli and Stoffolano (1980), there is enough information to make plausible predictions about how seasonality, and ultimately aging, affects engorgement patterns in this species.

A total of 1,315 flies were used for the seasonal engorgement studies with 34 replicates performed for the parafilm membrane feeding technique throughout both field seasons (2004 and 2005) and 23 replicates performed for the Kimwipe feeding technique

in 2005. All dates (total of 11) in 2005 had a minimum of two replicates per date and a maximum of four replicates per date for each feeding technique. Here and throughout the rest of this thesis, figures included, 'n' refers to the total number of flies used in the entire assay from all of the replicates. All statistical comparisons were performed using ANOVA to compare the percentage of engorgement by date and Tukey-Kramer HSD test (JMP, SAS Institute Inc., 2005). A t-test was used to compare the mean (±SEM) percentage of engorgement for 2004 and 2005 for flies fed through parafilm membranes (JMP, SAS Institute Inc., 2005).

Results

Perisulfakinin and Satiety. Perisulfakinin (PSK) had a marginally significant effect on blood feeding during the 2004 field season ($F_{2, 10} = 3.94$; $P = 0.054$). However, only the 10 nmol and 1 nmol doses were significantly different from one another. Flies that were injected with a high dose of PSK (10 nmol) showed an increase in engorgement (62.5% of flies engorged), while flies injected with a low dose (1 nmol) showed a decrease in engorgement (15.1% of flies engorged), compared to 36.6% of the sham-injected flies that engorged (Fig. 1). The significant difference observed here between the 10 nmol and 1 nmol doses may be a physiological effect of PSK stimulating and/or inhibiting the probing mechanism, which is examined in chapter IV.

During the 2005 field season, the effect of PSK on engorgement was also marginally significant ($F_{2, 27} = 3.17$; $P = 0.058$; Fig. 2). The 2005 data were obtained under more controlled conditions (i.e., flies were collected every other day from the traps). The pattern of engorgement remained the same when examining the sham-injected and treatment groups for both the 2004 and 2005 field season.

Flies that were injected with a high dose of PSK (10 nmol) engorged at higher percentages (15.7%), while flies injected with 1 nmol doses of PSK engorged at lower percentages (6%), compared to the 11% of the sham-injected flies that engorged.

Previous research (Maestro et al., 2001; Wei et al., 2000) reported data as the percentage of feeding inhibition. When the results are calculated as the inhibition of engorgement (compared to the percentage engorged), 1 nmol of PSK inhibited feeding by 58.7% and 10 nmol of PSK stimulated feeding by 70.8% in 2004 (Fig. 3). For the 2005 experiments, 1 nmol of PSK inhibited feeding by 45.5% and 10 nmol of PSK stimulated feeding by 42.7% (Fig. 3).

Seasonality and Engorgement. Initially, the experiments with sulfakinins led me to question what the normal percentage of engorgement was for the flies throughout the season. During the 2004 field season, I noted increases and decreases in the percentage of females engorging using the parafilm membranes from the beginning of the season and to the end of the season. I measured the percentage of females engorging during the following season as well. A lower percentage of flies engorged during the beginning and end of the season and a higher percentage of flies engorged during the 'peak' season, when fed through parafilm membranes (especially for 2005). In 2005, there were two peaks (13 July and 19 July) in the engorgement behavior during the middle of the season (Fig. 4). Towards the end of the season, *T. nigrovittatus* engorged less than 'peak' season flies. The mean percentage of flies that engorged for the 2004 (27.95 ± 8.62) and 2005 (18.54 ± 6.37) field seasons did not differ statistically when exposed to warmed blood through parafilm membranes ($F_{1, 14} = 0.77$; $P = 0.39$) (Fig. 4). There was a significant statistical difference in the percentage of flies engorging using parafilm membranes

throughout the 2005 season ($F_{10, 17} = 2.58$; $P = 0.04$). Data for 2004 were not statistically analyzed because there were not enough replicates for each date. However, at similar times during the month, the percentage of engorgement was similar in both years.

For the Kimwipe feeding technique, there was a highly statistical significant difference in the percentage of females engorging throughout the 2005 season ($F_{10, 12} = 5.07$; $P = 0.005$) (Fig. 5). It is interesting to note that the percentage of females engorging spiked up (from 25.7% to 78%) on 30 July. The peaks and valleys over the season for flies exposed to blood-soaked Kimwipes were not as pronounced throughout the season as they were for flies exposed to parafilm membranes (Fig. 5). However, engorgement increased and decreased at similar times during the month for the two feeding techniques.

Discussion

Perisulfakinin and Satiety. This is the first study to examine sulfakinin in a hematophagous insect, as well as the first study using sulfakinin as a possible satiety factor to examine its effect on a natural insect population. Using a field-collected population has certain limitations, particularly in that the physiological and chronological age remains unknown, both of which can cause variability in the data. The lack of highly significant statistical effect on feeding inhibition could be a product of the uncontrolled variables that are present when examining the effect of sulfakinin in a natural population.

Perisulfakinin has dose-dependent effect on the gorging behavior of *T. nigrovittatus* with the 10 nmol stimulating and 1 nmol inhibiting engorgement. The inhibition pattern of this species (58.7% in 2004 and 45.5% in 2005) at the 1 nmol dose of PSK is comparable to the previous research, which demonstrated a 60% inhibition of

feeding by *B. germanica* (Maestro et al. 2001) and a 55% inhibition of feeding by *S. gregaria* (Wei et al. 2000).

The stimulation of engorgement is unexpected and has not been previously demonstrated in another insect with sulfakinin. It is possible that 10 nmol injections of PSK increase engorgement behaviorally by affecting probing through either a neurological pathway or physiologically through a water deficit pathway. On the other hand, increased engorgement could be a pharmacological effect of the drug since the endogenous amounts of SK that are released in tabanids, as other insects, are unknown. It is also possible that a highly significant effect was not observed in this species because the sulfakinin used (perisulfakinin from the cockroach, *Periplaneta* spp.) is not native to Tabanidae.

The cockroach and locust are continual feeders that consume differing amounts of food. There is no evidence in any other rsearch (Maestro et al., 2001; Wei et al., 2000) to indicate that the authors included or removed individuals that did not feed in their analysis. Wei et al. (2000) and Maestro et al. (2001) reported insects taking meals but in smaller meal sizes. *T. nigrovittatus* normally feeds until repletion in the wild and only takes one blood meal in between each gonadotrophic cycle. The amount of blood engorged was not measured for this reason, and instead I measured the percentage of females engorging. Thus, it is interesting that low doses of sulfakinin deterred individual flies from taking a meal, as opposed to regulating the size of the meal, as it was shown by Wei et al. (2000) and Maestro et al (2001). The deterrence of individual flies from taking a meal also raises the question of whether the SK is affecting the probing mechanism differently from the engorgement mechanism, and is examined in chapter IV.

Horse flies, as most hematophagous flies, divert carbohydrates to the crop and blood meals to the midgut. Stoffolano (1983) showed that when horse flies engorge on blood they still take a sugar meal, or vice versa. A full blood meal does not satiate all nutritive feeding (i.e., carbohydrate) in this species; thus, feeding to repletion on a previous meal does not inhibit the feeding response to another nutrient. It is very likely then, as previously suggested by Stoffolano (1983), that there are different mechanisms regulating feeding for both carbohydrates and proteins in female *T. nigrovittatus*. Sulfakinins may be more important in regulating the meal size of sucrose feeding and may play a more significant role in the males, which do not blood feed and lack the distendable midgut. The effect of sulfakinin on sucrose feeding in this species has not been tested.

The differences in the percentages of engorgement between the two field seasons in this study are most likely due to how long the flies were in the traps on the marsh. In 2004, flies were collected once a week, whereas in 2005 they were collected every other day. In 2004, flies could have been in the traps anywhere from a week to just a couple hours. The difference between the shams (36.6% in 2004 versus 11% in 2005) suggests that starvation and/or thirst could account for the higher engorgement in 2004 because flies would have been in the traps longer. It has been shown in tsetse flies that starvation changes the feeding thresholds so that a more starved fly will elicit a greater probing response (Brady 1973). Thus, the 2005 data may be more indicative of the fly's response to PSK since their physiological condition was more controlled.

Most of the female hematophagous insects studied to date appear to rely on the abdominal stretch receptors as the primary feedback mechanism for terminating blood

feeding and host seeking behavior (Adams 1999, Friend and Smith 1977, Gwadz 1969, Hocking 1971, Rice 1972). Why then would there be a need for a chemical satiety factor (e.g., sulfakinins) to regulate blood feeding when there appears to already be an effective mechanism in place? It may be that female horse flies rely on stretch receptors to relay messages via the ventral nerve cord as the primary mechanism inducing satiety following blood feeding and/or use chemical satiety factors (e.g., sulfakinins) to regulate carbohydrate feeding in both sexes. Satiation in insects, as in vertebrates, is surely not the result of one peptide, one hormone or one neural mechanism. There are probably many different mechanisms acting additively to produce satiety in insects.

Seasonality and Engorgement. When the 2004 data were initially analyzed, the high and low engorgement pattern from the effect of the PSK led to the question of what the normal percentage of engorgement was throughout the season. A careful review of the literature revealed no data describing seasonal probing or engorgement (and ultimately aging) patterns in *T. nigrovittatus*. Nor is there a wealth of published information detailing whether this species emerge all at once in the beginning of the season or whether they are continually emerging on the marsh all season long. Freeman and Hansens (1972) published on collection methods of *T. nigrovittatus* in New Jersey and report larval collections from June 2 to July 6; however, the authors state that, "…larvae were still abundant in the marsh sod even during the summer peak of adult abundance." The authors didn't report when larval abundance on the marsh decreases (if at all) and when the peak adult abundance occurs. Notes on emergence for this species would be helpful in understanding whether the same flies that emerged in the beginning of the season are on the marsh all 3-4 weeks of the season, whether they have a peak

probing activity, and whether aging contributes to a decrease in their probing because of physiological and/or endocrinological degradation.

The results from this study, specifically using the parafilm feeding technique, demonstrate an increase in engorgement in *T. nigrovattus* as the season peaks and a decrease in engorgement as the season ends. Presumably, all flies that enter the box traps in the field are in the blood-feeding mode. Why then are the engorgement rates different throughout the season? Why does the highest percentage of successful engorgement in the laboratory coincide with the peak abundance of *T. nigrovittatus* during the season? Senescence in *T. nigrovittatus* may influence host seeking behavior differently from engorgement behavior, possibly explaining the lower engorgement rates in the laboratory towards the end of the season, despite flies still being caught in the box traps. As the season progresses and flies begin to age on the marsh, they probably experience a physiological degradation of chemoreceptors, become less responsive to stimuli and less able to execute probing and ingestion. In addition, *T. nigrovittatus* probably also experiences a change in hormone levels, which could also account for the lack of engorgement response later in the season since hormones have been shown to affect probing in *Culex* mosquitoes (Meola and Petralia, 1980).

The low engorgement in the beginning and end of the season makes biological sense when thinking about what the normal aging patterns probably are in the horse fly. The first collection of *T. nigrovittatus* in the marsh traps was on 4 July 2005. Most likely, those flies emerged sometime in the end of June, mated and laid eggs [oogenesis taking 7-10 days, Magnarelli and Stoffolano (1980)] and were seeking their first blood meal. The host-seeking behavior resulted in getting the flies caught in the box traps. Based on

my observations, made through random dissections, none of the flies used in the beginning of the season had previously engorged. On 27 July 2005, I noted the first observations of flies that had produced at least two batches of eggs prior to being caught in the traps. This was based on conditions of the ovarioles, the distended midguts, and in several instances, the leftover remnants of blood meals in the midgut. Counting backwards 10 days (for oogenesis) means that the flies would have had a blood meal and mated somewhere around 17 July 2005. The females probably first mated another 10 days before that (7 July 2005) without a blood meal, because they are autogenous, putting their emergence somewhere in the first week of July. Further, survivorship curves performed in the laboratory by Stoffolano and Majer (1997) showed that *T. nigrovittatus* could possibly live for about 20 days after engorgement, but the mean survivorship was about 9 days for blood-fed females. Thompson and Krauter (1978) showed similar results with less than 10% of the population surviving past 15 days in the laboratory, and zero percent survivorship after about 25 days in the laboratory. Therefore, it is entirely possible that the flies that emerged at the end of June and the first week of July are the same flies on the marsh at the end of July (i.e., towards the end of their season). It is likely then that aging, expressed here as the percentage of engorgement throughout the season, takes a toll on blood feeding in *T. nigrovittatus.* This concept has also been demonstrated by Mather and DeFoliart (1984) where the authors showed that older mosquitoes had reduced feeding success on squirrels and chipmunks. Fully understanding engorgement behavior, especially in light of seasonality, is important to uncovering all of the factors that affect *T. nigrovittatus* feeding behavior.

Figure 1. The effect of perisulfakinin (PSK) on engorgement by *Tabanus nigrovittatus* in 2004. PSK had a borderline significant effect on blood 000000-engorgement ($F_{2,10} = 3.94$; $P = 0.054$). The percentage of flies engorged was 62.5% when injected with 10 nmol of PSK. Only 15.1% of flies engorged when injected with 1 nmol. The percentage of flies engorged for the sham treatment was 36.6%. A total of 600 flies were used, with 120 flies per replicate and 5 replicates performed. Small bars represent SEM.

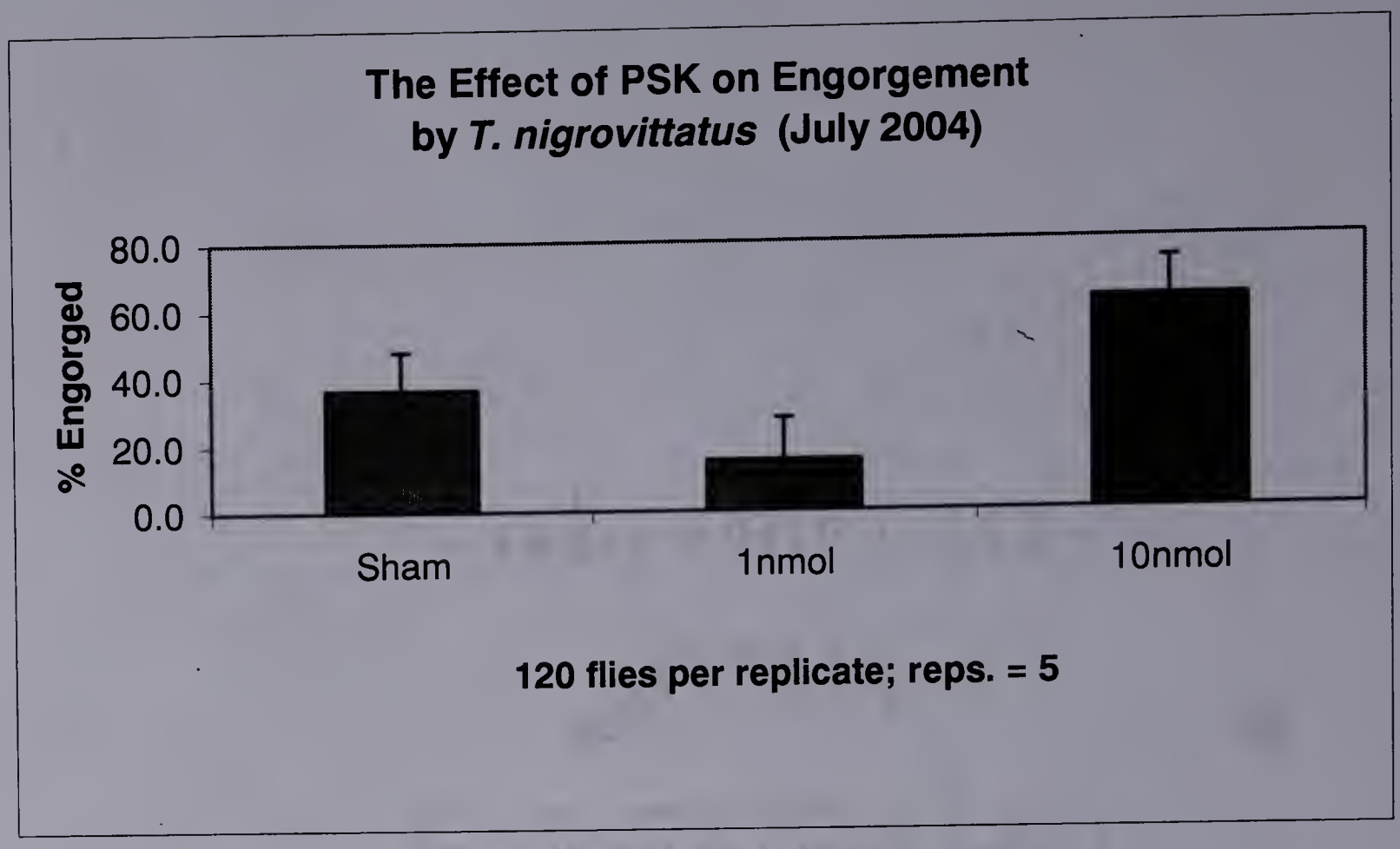

The Effect of PSK on Engorgement
by T. nigrovittatus (July 2004)
% Engorged
80.0
60.0
40.0
20.0
0.0
Sham
1nmol
10nmol
120 flies per replicate; reps. = 5

Figure 2. The effect of perisulfakinin (PSK) on engorgement by *Tabanus nigrovittatus* in 2005. PSK had a borderline significant effect on blood engorgement ($F_{2, 27} = 3.17$; $P = 0.058$). The percentage of flies engorged was 15.7% when injected with 10 nmol, while only 6% of flies engorged when injected with 1 nmol. The percentage of flies engorged for the sham treatment was 11%. A total of 600 flies were used, 60 flies per replicate and 10 replicates performed. Small bars represent SEM.

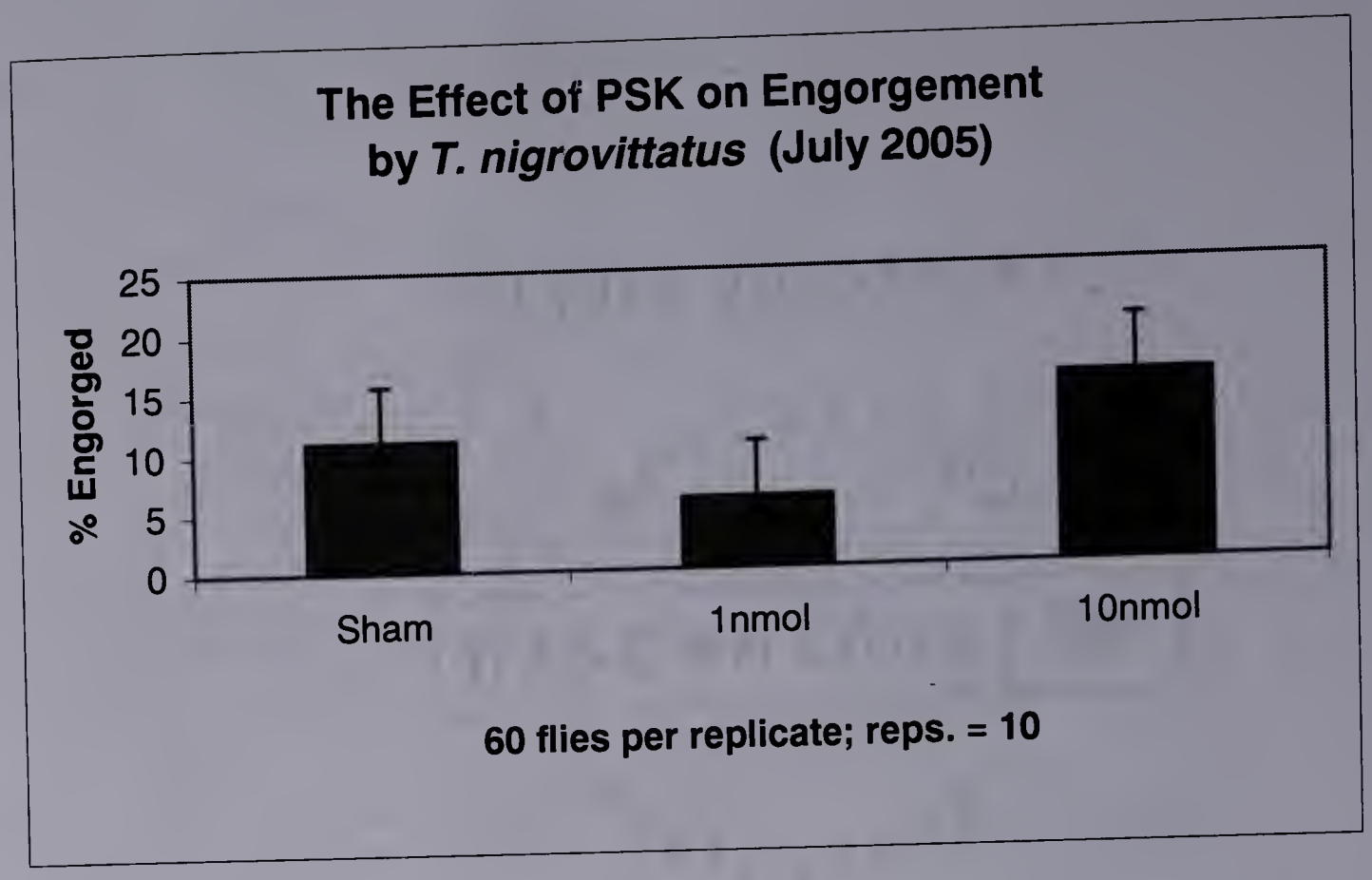

The Effect of PSK on Engorgement
by T. nigrovittatus (July 2005)
% Engorged
25
20
15
10
5
0
Sham
1nmol
10nmol
60 flies per replicate; reps. = 10

Figure 3. The effect, expressed here as inhibition or stimulation, of PSK on engorgement by *T. nigrovittatus*. The peptide was injected at 1 nmol/fly and 10 nmol/fly. In 2004, 1 nmol injections of PSK inhibited engorgement by 58.7%, while 10 nmol injections stimulated engorgement by 70.8%, compared to the sham-injected group. In 2005, injections of 1 nmol PSK reduced the percentage of flies gorging by approximately 45.5%, whereas injections of 10 nmol PSK increased the percentage of flies gorging through an artificial membrane by approximately 42.7%, compared to the sham-injected flies.

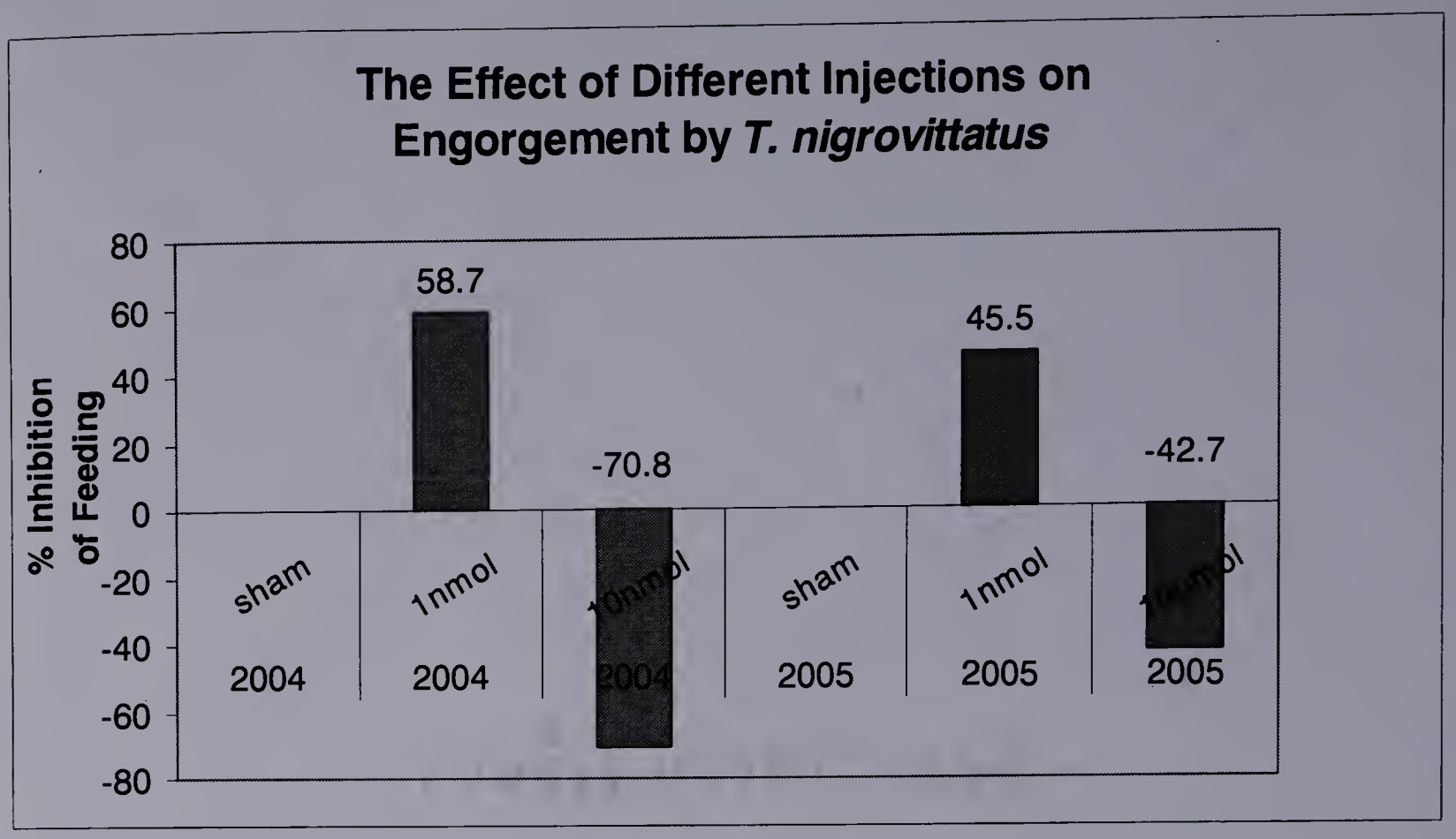

The Effect of Different Injections on Engorgement by T. nigrovittatus
% Inhibition of Feeding
80
60
40
20
0
-20
-40
-60
-80
58.7
-70.8
45.5
-42.7
sham
1nmol
10nmol
sham
1nmol
1nmol
2004
2004
2004
2005
2005
2005

Figure 4. The seasonal engorgement response by *T. nigrovittatus* when fed through a parafilm membrane (July 2004 & 2005). Data points are similar for 2004 and 2005, suggesting that females have a consistent pattern of engorgement in which there is a higher percentage of blood feeding during peak abundance in the season.

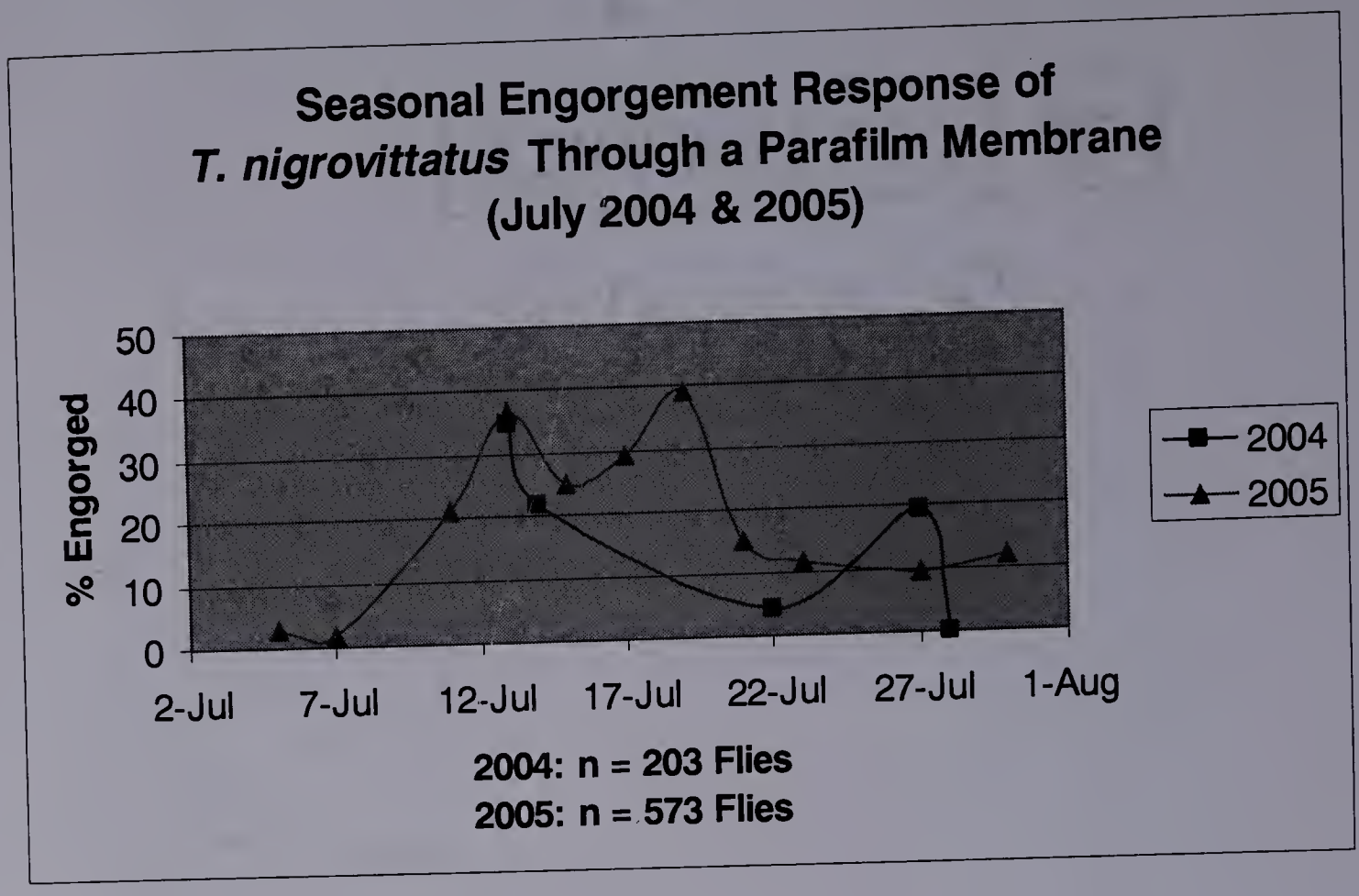
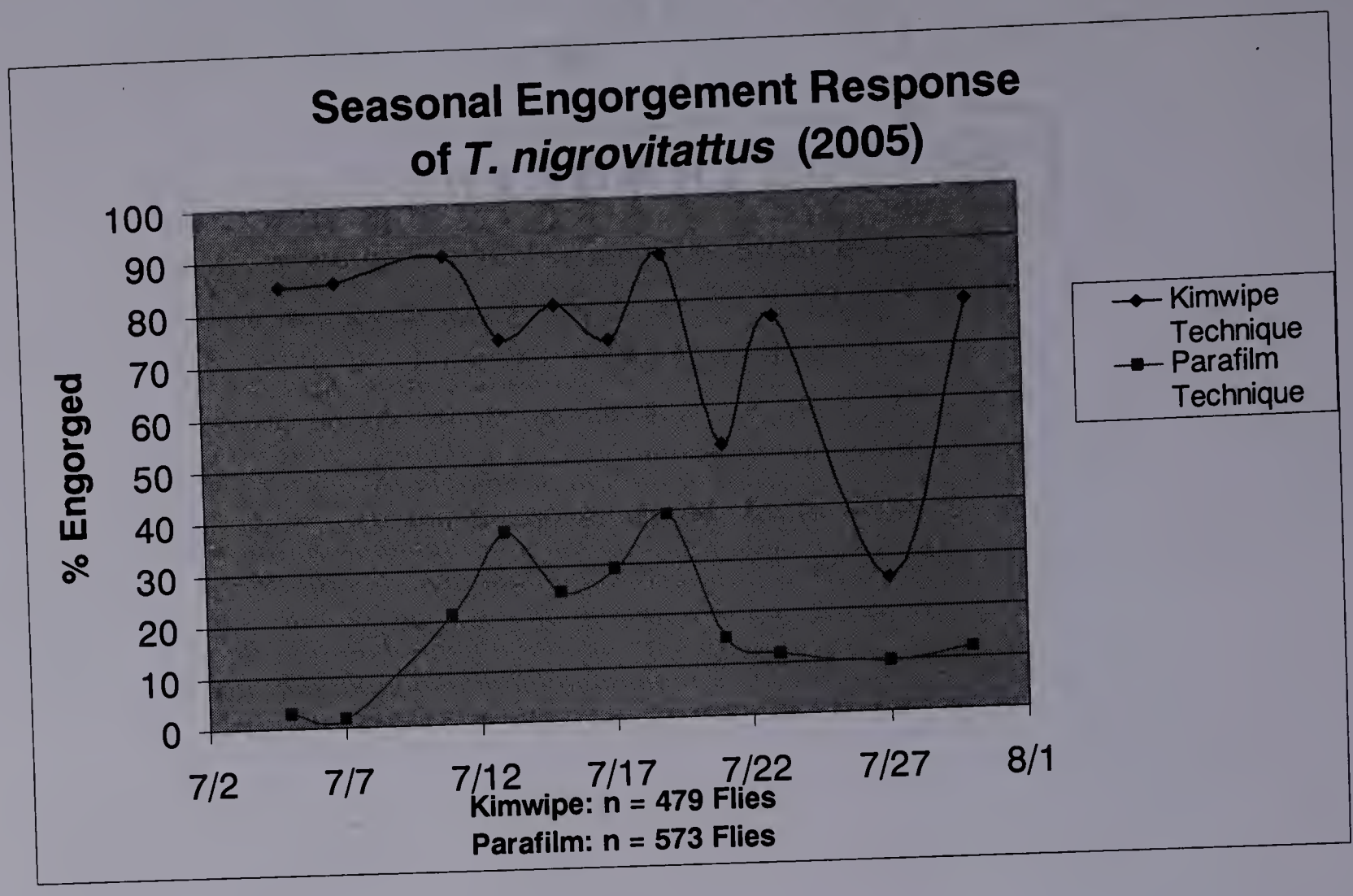

Seasonal Engorgement Response of
T. nigrovittatus Through a Parafilm Membrane
(July 2004 & 2005)
% Engorged
50
40
30
20
10
0
2-Jul
7-Jul
12-Jul
17-Jul
22-Jul
27-Jul
1-Aug
2004
2005
2004: n = 203 Flies
2005: n = 573 Flies

Figure 5. The seasonal engorgement response by *T. nigrovittatus*, comparing Kimwipe feeding technique to parafilm feeding technique (July 2005). The engorgement patterns between the two methods of blood feeding express similar peaks and valleys throughout the season; however, the flies exposed to blood through the Kimwipe feeding technique engorge at higher percentages. In addition, the flies exposed to blood through the Kimwipe feeding technique do not express lower percentages of engorgement in the beginning of the season as do the flies exposed to blood through the parafilm membrane feeding technique.


Seasonal Engorgement Response
of T. nigrovitattus (2005)
% Engorged
100
90
80
70
60
50
40
30
20
10
0
7/2
7/7
7/12
7/17
7/22
7/27
8/1
Kimwipe Technique
Parafilm Technique
Kimwipe: n = 479 Flies
Parafilm: n = 573 Flies

CHAPTER IV

FACTORS AFFECTING ENGORGEMENT BY THE SALT MARSH HORSE FLY, *TABANUS NIGROVITTATUS*.

Abstract

Feeding is one of the most important behaviors of an organism, yet the mechanisms that control blood feeding in the salt marsh horse fly remain largely unknown. Female *Tabanus nigrovittatus* were field collected and used in laboratory experimentation to explore physiological and behavioral factors that affect engorgement. Previous studies showed sulfakinins act as feeding satiety factors in insects. This study demonstrates that sulfakinins have differing effects on engorgement that is dependent on the feeding technique used in the laboratory. The satiety effect of sulfakinin on flies engorging using the blood-soaked Kimwipe feeding technique is not in agreement with previous experiments (see chapter III) using an alternate feeding technique with artificial membranes. This study is the first to demonstrate that the temperature of blood is a significant factor for engorgement in this species. Also, the percentage of flies engorging is significantly different depending on the feeding technique used. It is clear from these results that there are many factors that affect the successful engorgement of a blood meal in the salt marsh horse fly.

Introduction

Tabanus nigrovittatus, commonly referred to as the greenhead horse fly, inhabits the salt marshes along the Atlantic coast of Massachusetts. This fly is only present on the marsh for 3-4 weeks, but can be collected in extremely high numbers. While research on its biology has come a long way over the years (see Stoffolano, 1979; Magnarelli and Stoffolano, 1980; Friend and Stoffolano, 1983; Stoffolano, 1983; Graham and Stoffolano, 1983), a great deal of information remains unknown about what controls blood feeding. Understanding the feeding behavior of insects continues to be one of the most vital aspects of entomology, especially when trying to control hematophagous insects.

Perisulfakinin and Engorgement. Very little is generally known about short-term feeding satiety regulation in insects (see Bernays and Simpson, 1982), and even less is known about long-term satiety regulation. Recent research with the neuropeptide sulfakinin has demonstrated short-term feeding inhibition in two non-hematophagous insects (Maestro et al., 2001; Wei et al., 2000). In the previous studies (chapter III) where I investigated the role of sulfakinin in the regulation of blood feeding in *T. nigrovittatus,* I showed an inhibiting effect of 45.5% when insects were injected with 1 nmol of perisulfakinin (PSK) and blood-fed using artificial membranes. PSK at the 1 nmol dose inhibited individuals from engorging a blood meal and raised the question of whether PSK was affecting the probing mechanism or the ingestion process as a whole (Chapter III). The following experiments were conducted in order to determine whether sulfakinin has the same effect on engorgement when exposed to blood through an alternate laboratory feeding technique. The blood-soaked Kimwipes provide an alternative method of feeding that may not require the probing mechanism to be employed.

Temperature and Engorgement. Thermal stimuli are well known to be important factors in the successful engorgement of a blood meal in tsetse flies, mosquitoes, and *Rhodnius* spp. (Dethier, 1954, 1957; Friend and Smith, 1977). However, to my knowledge, the effect of blood temperature on engorgement has not previously been shown for this species or other Tabanidae. Thus, experiments were conducted with blood-soaked Kimwipes in order to elucidate the effect of thermal stimuli on engorgement.

Comparison of Two Feeding Techniques. Stoffolano (1979) found that the best method for feeding *T. nigrovittatus* was by providing grouped females with blood-soaked Kimwipes. He found that Kimwipe-soaked, group-fed females ingested significantly more blood (51.4 μl) than did parafilm, group-fed females (40 μl). However, the author did not look at the percentage of engorged flies per trial. In fact, an overwhelming number of papers on hematophagous arthropods and their feeding habits do not report the percentage of engorgement. Reporting percentages is important for understanding feeding success rates of blood feeders. Since the horse fly is thought to be an 'all-or-none' blood feeder, recording the success rate (percentage engorged) of feeding, especially in response to certain factors, will allow researchers to gain better insight into all of the physiological and behavioral events that lead to a blood meal and provide greater knowledge in how to interrupt blood feeding.

Materials and Methods

Collecting and Maintaining Flies. Female host-seeking *Tabanus nigrovittatus* were field collected from box traps on the salt marshes of Essex Co., Massachusetts, during July 2005. Flies were laboratory maintained as previously described in Chapter II.

Prior to experimentation, all flies were deprived of granulated sucrose for 16-20 h. All flies used in experimentation were tested the day after being collected in the field. The exact chronological ages of all flies used are unknown. The first collection date for the 2005 experiments was 4 July 2005 and field collections were made every other day consecutively throughout the season until the flies were no longer present on the marsh. .

Perisulfakinin and Engorgement. Prior to experimentation, flies of approximately the same size were cold immobilized in the freezer. Sham-injected and treatment flies were injected with a 1 µl of solution in the second to last intersegmental membrane on the right ventral side of the abdomen. All flies were injected using a 30-gauge needle attached to a 25 µl glass gastight Hamilton #1750 syringe (Hamilton Co., Reno, Nevada). Sham-injected flies were injected with *Phormia* saline (Chen and Friedman, 1975) and treatment flies were injected with perisulfakinin (Bachem, PA, USA) dissolved in *Phormia* saline. Treatment flies were injected with 1 nmol and 10 nmol doses of perisulfakinin. The sulfakinin was prepared in a stock solution of 80% acetonitrile and 20% water, made up to 0.01% trifluoroacetic acid. Control flies (used in the experiments described below) were cold immobilized and set on ice for the same duration as the sham-injected and treatment flies, but were not injected with any solution. After an individual fly was injected it was placed back on ice until the entire experimental group (20 flies per treatment) was completed. An experimental group consists of the control, sham, or treatment. The entire injection process for each group took less than 10 minutes. Each experimental group of flies was placed in a 23 cm^3 metal-screened cage and the feeding assay was started.

There was zero mortality with all injections. All flies recovered from injections and resumed normal behavior in less than 5 minutes.

For the feeding assay, citrated beef blood was warmed on a hot plate to 37° C and stirred with a magnetic stirrer. Kimwipes were placed on top of each 23 cm^3 cage and the warmed blood was pipetted onto the Kimwipes until they had been completely soaked. A lamp with a 60-watt bulb was positioned over the cage to provide adequate light and keep the blood warmed to approximately 37° C. The flies were then allowed to feed *ad libitum* for one hour.

After the feeding assays were completed, flies were killed in the freezer. Once dead, they were submerged in 70% ethanol and each one was held up to a light bulb to check for the presence of a blood meal. The abdomen of the flies appeared red if a blood meal was taken and appeared yellow if lacking one. Any questionable individuals were dissected to check for the presence of blood in the midgut.

A total of 587 flies were used during experimentation with 10 replicates performed. A replicate is a single simultaneous run of all of the sham and treatment groups. All statistical comparisons were performed using ANOVA to compare the percentage of engorgement by treatment and post-hoc comparison using a Tukey-Kramer HSD test (JMP, SAS Institute Inc. 2005). The percentage of difference between the sham-injected group and the treatment group was calculated by using the following formula:

$$\frac{\%\ engorged\ by\ treatment\ -\ \%\ engorged\ by\ sham}{\%\ engorged\ by\ sham} \times 100 = \%\ Difference$$

Temperature and Engorgement. The experiments testing the effect of blood temperature were run simultaneously with the perisulfakinin experiments. The procedures remained the same, but there were no injections made and 'cold blood' was kept at room temperature (no heating involved) for the feeding assay. In addition, a lamp was not positioned directly over the Kimwipes in the 'cold blood' experimental group, but the cage was placed next to the warmed, blood-soaked Kimwipe cage in order to still have access to adequate light. Also, control flies exposed to warmed blood using parafilm membrane were also run simultaneously in order to compare the normal percentage of engorgement to those exposed to blood-soaked Kimwipes. Approximately 20 flies were used for each treatment. Ten replicates were performed with 648 flies used in these experiments. An ANOVA was used to compare the percentage of engorgement by treatment and post-hoc comparison using a Tukey-Kramer HSD test was used (JMP, SAS Institute Inc. 2005).

Comparison of Two Feeding Techniques. I compared the percentage of flies that engorged on an artificial membrane to the percentage of flies engorged on blood-soaked kimwipes. I also compared the two methods in alternate and successive feeding assays. A group of flies (20 flies for each experimental treatment) were allowed to blood feed *ad libitum* for 1 h using a parafilm membrane, by the method previously described in chapter II. After the time trial was finished, flies were cold immobilized and the number of flies that had taken a blood meal was counted. Those flies that blood fed were discarded and the flies that had not taken a blood meal were put back into another metal-screened cage and allowed to feed *ad libitum* for 30 minutes, using the Kimwipe feeding technique. At the end of the feeding assay the flies were killed and then recounted to

determine how many had engorged during the subsequent feeding method. The opposite feeding assay (i.e., Kimwipe-fed first and then parafilm-fed) was also tested to see if the order of the feeding technique had any effect. A total of 429 flies were used and 10 replicates performed for each feeding technique. A *t*-test was used to compare the mean percentage (± SEM) of flies that had engorged in the first part of the feeding trial to those that had fed in the second part of the feeding trial (JMP, SAS Institute Inc. 2005). However, the data is presented in the form of the number of flies that fed out of the total flies exposed to blood using the two different techniques in order to amplify the differences.

Results

Perisulfakinin and Engorgement. Perisulfakinin (PSK) had no statistically significant effect on engorgement by *T. nigrovittatus* when fed using the warmed, blood-soaked Kimwipe technique ($F_{2, 27} = 3.1$; $P = 0.06$). The percentage of sham-injected flies engorged was 58.7%, while the percentage engorged for the 10 nmol and 1 nmol treatments was 72.6% and 70.5%, respectively (Fig. 6). While not statistically significant, 10 nmol PSK stimulated engorgement by 23.7% and 1 nmol stimulated engorgement by 20.1%, compared to the sham-injected experimental group.

Temperature and Engorgement. The percentage of *T. nigrovittatus* engorging when offered warmed blood-soaked Kimwipes was 82.1%, while only 12% of the flies offered room temperature blood-soaked Kimwipes engorged. Only 19.2% of *T. nigrovittatus* engorged when fed using the parafilm membrane, compared to the 82.1% offered blood-soaked Kimwipes. The effect of feeding technique and the temperature of blood significantly effects engorgement ($F_{2, 27} = 91.9$; $P < 0.0001$) (Fig. 7). According to

the Tukey-Kramer HSD test, the parafilm control and the Kimwipes soaked in room temperature blood are not significantly different. There is a significant interaction between warmed blood versus room temperature blood and parafilm versus Kimwipe feeding techniques.

Comparison of Two Feeding Techniques. There is no significant difference in the mean percentage of females engorged when exposed to parafilm membranes first (15.8 ± 7.5) or second (32.0 ± 7.5) in the two separate assays ($F_{1, 20} = 2.3$; $P = 0.14$). Nor was there was a statistically significant difference in the mean percentage of females engorged when Kimwipe-exposed first (74.7 ± 5.5) compared to flies Kimwipe-exposed second (59.5 ± 5.5) in the two assays ($F_{1, 18} = 3.9$; $P = 0.07$). Thus, the order of the feeding technique made no difference in the percentage of females engorging.

When flies were exposed first to the membranes and second to the Kimwipes, the mean percentage of flies that fed using the parafilm (15.8 ± 4.9) was significantly lower than the mean percentage of flies that fed using Kimwipes (59.5 ± 4.9) ($F_{1, 18} = 39.1$; $P < 0.0001$). In other words, of the total number of flies (211) exposed to blood using a parafilm membrane first, only 34 flies engorged. Of the remaining 174 flies, those that did not engorge by probing the parafilm membrane, 101 flies did successfully engorge blood using the warmed, blood-soaked Kimwipes (Fig. 8).

For the assay where the flies were exposed first to warmed, blood-soaked Kimwipes and second to the membranes, the mean percentage of flies that fed using the Kimwipes (74.7 ± 7.8) was significantly different from the mean percentage of flies that fed using the membranes (32.0 ± 7.8) ($F_{1, 20} = 14.9$; $P < 0.0012$). Therefore, of the 218 flies allowed to feed on warmed, blood-soaked Kimwipes first, 163 flies successfully

engorged. Out of the remaining 52 flies that failed to engorge a blood meal, 22 flies did successfully engorge blood through the parafilm membrane (Fig. 8) (*Note*: Some flies were lost during the transferring phase, accounting for the difference in numbers between feeding assays).

Discussion

Perisulfakinin and Engorgement. Previous research (chapter III) examining the effect of PSK on engorgement through a parafilm membrane in *T. nigrovittatus* showed that 10 nmol of PSK stimulated engorgement by 42.7%, while 1 nmol inhibited engorgement by 45.5% relative to the sham-injected group (2005 data). This study demonstrates the same trend of increased engorgement by the 10 nmol dose of PSK. However, the percentage of increased engorgement is lower when fed using blood-soaked Kimwipes (23.7%) compared to using parafilm membranes (42.7%) from previous experiments (chapter III). Interestingly, the 1 nmol dose of PSK does not demonstrate the same inhibition as previously observed in the parafilm membrane study (e.g., increased engorgement of 20.1% when fed using blood-soaked Kimwipes compared to inhibition of engorgement by 45.5% when fed using parafilm membranes). These experiments were designed to determine what effect sulfakinin has on engorgement in hematophagous insects and to determine if the type of laboratory feeding technique plays any role in the expression of the drug effect. The difference between the contrasting effects at the 1 nmol dose for the parafilm feeding technique (inhibition of engorgement by 45.5%) and the Kimwipe feeding technique (stimulation of engorgement by 20.1%) suggests that PSK may influence the probing mechanism since the parafilm technique requires the flies to puncture the membrane in order to blood feed. Other research found that sulfakinins, at

all doses, inhibited feeding in non-hematophagous insects (German cockroach, *Blattodea germanica*: Maestro et al., 2001; Desert locust, *Schistocerca gregaria*: Wei et al., 2000). The endogenous amounts of sulfakinins that naturally occur in the tabanid, as other insects, are unknown at this time, thus adding to the difficulty of determining whether the observed effects are a physiological or a pharmacological effect. It is possible that the lack of statistical significance is due to experimental testing being conducted on a wild population of flies (i.e., flies not raised in a laboratory). The chronological and physiological ages of the flies were unknown when testing and those factors could influence the fly's response to PSK.

If sulfakinin affected short-term satiety, it would be expected that meal size would be regulated in blood feeding (as was shown in the other non-hematophagous insects). The role of stretch receptors in regulating the blood meal size (i.e., short-term feeding satiety) in hematophagous insects has been well documented (Gwadz, 1969; Friend and Smith, 1977). Further, horse flies only require one blood meal between each gonadotrophic cycle. The implication of sulfakinin (specifically 1 nmol PSK) inhibiting probing suggests that it may act as a long-term satiety regulation mechanism, terminating probing and theoretically blood feeding until after oogenesis. It may be that sulfakinins act additively with the stretch receptors to produce short-term satiety and/or act on long-term blood-feeding regulation in this species.

Temperature and Engorgement. Most studies examining engorgement in hematophagous insects do not report the percentage of insects that engorge, but rather report the amount of blood engorged. In the few studies that do report the percentages of hematophagous insects that successfully engorge (either through artificial membranes or

live hosts), those percentages are relatively low (30% by *Aedes* spp. on live hosts: Mather and DeFoliart, 1984; 27.3% by *T. nigrovittatus* fed by artificial membrane in the field: Thompson and Krauter, 1978). The percentage of engorgement by *T. nigrovittatus* using the parafilm membrane feeding technique (19.2%) is in agreement with the few studies that have reported percentages of engorgement. Reporting the percentage of engorgement is beneficial for those studies looking at feeding success rates and consequences of blood engorgement.

There is a 62.9% difference in engorgement between the two feeding techniques (19.2% for parafilm membrane and 83.1% for Kimwipes). It is interesting how much of a significant difference there is in the engorgement response between the two different feeding techniques, especially when the method thought to be employed normally in the wild (probing through a membrane) by the fly is so much lower than the uncharacteristic way (open pools of blood) of encountering a blood meal. It is highly unlikely that the reason for increased engorgement on Kimwipes is because of tarsal stimulation from contacting the blood. Stoffolano et al. (1990) tested the chemosensilla from the tarsi, tibia, terminal end of the antenna, labrum and the labral groove to various substances. The authors found that all of the chemosensilla responded to salts and sugars; however, the only chemosensilla that responded to sera and plasma were those at the tip of the labrum and lining the labral groove. Chirov and Alekseyev (1970) were also able to show that tabanids would feed from free liquids (blood, water, and sugar water) when a pipette was inserted over the mouthparts with the labium moved aside. Friend and Stoffolano (1984) suggest, "...Mouthpart deployment may not play as significant a role in establishing the blood-feeding mode in pool feeders as it does in vessel feeders."

However, it is unlikely that the flies would often encounter open pools of blood in the wild. The parafilm feeding assays are probably not as successful in eliciting engorgement because of the lack of other natural stimuli, which are missing in the laboratory.

Thermal stimuli have proven to be an important factor in probing by other hematophagous insects (Dethier, 1954; Friend and Smith, 1977). However, this is the first study to examine the effect of blood temperature on the engorgement response in Tabanidae, especially using the Kimwipe feeding technique. *T. nigrovittatus* engorged significantly more on warmed blood than on blood that was kept at room temperature. The results of this study indicate that the flies are thermally stimulated by the warmed blood on the Kimwipes and once the chemosensilla on the labrum and labral groove contact the blood they are stimulated to engorge. The flies exposed to Kimwipes soaked in cold blood lacked the thermal stimulation and failed to engorge.

Comparison of Two Feeding Techniques. Stoffolano (1979) showed that there are two types of techniques that can be used to feed *T. nigrovittatus* in the laboratory. The alternate and successive feeding assays were designed in order to have a better understanding of why there were low percentages of flies engorging using the parafilm feeding technique. I wanted to determine if the flies were not engorging because they were not in the blood-feeding mode or if the failure to engorge was because of missing stimuli. In both techniques, parafilm membrane and Kimwipes, there were always some flies that failed to engorge. When I took the flies that failed to feed through parafilm membranes and presented them with blood-soaked Kimwipes, I always observed a higher percentage of engorgement than in the original parafilm assay. The same pattern was observed when the Kimwipe assay was performed first in that more flies engorged by the

Kimwipe technique. While there was no statistical difference in the percentage of engorgement when examining the order of exposure using parafilm membranes, engorgement was higher for those flies previously exposed to blood-soaked Kimwipes. This may be due to the central excitatory state being elevated after having more contact with open pools of blood. It is interesting that 100% engorgement is never observed, especially because all of the flies should be in the blood-feeding mode since they were engaging in host-seeking behavior (i.e., collected from the field traps). The results suggest that there are cues missing from the parafilm feeding technique essential to stimulate the flies to successfully probe and ingest a blood meal in the laboratory.

Figure 6. The effect of perisulfakinin (PSK) on engorgement using warmed, blood-soaked Kimwipes by *T. nigrovittatus* (July 2005). Engorgement was increased by 23.7% and 20.1% when injected with 10 nmol and 1 nmol of PSK (respectively), compared to the sham-injected group. However, the effect of PSK on engorgement using this feeding technique was not significant ($F_{2, 27} = 3.1$; $P = 0.06$). Small bars represent SEM.

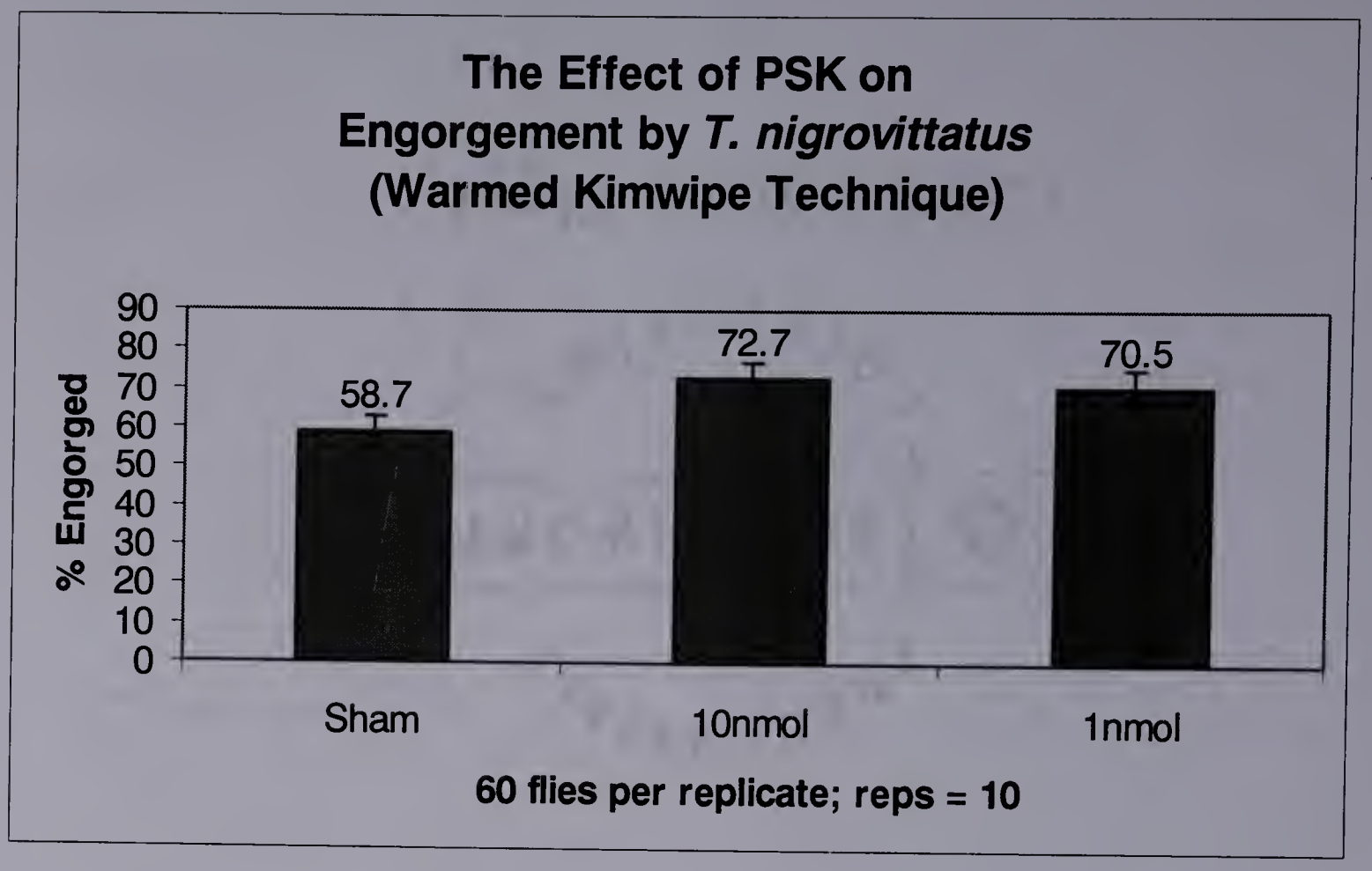

The Effect of PSK on
Engorgement by T. nigrovittatus
(Warmed Kimwipe Technique)
% Engorged
90
80
70
60
50
40
30
20
10
0
58.7
72.7
70.5
Sham
10nmol
1nmol
60 flies per replicate; reps = 10

Figure 7. The effect of blood temperature and feeding technique on the percentage of engorged female *T. nigrovittatus*. The percentage of flies engorged when offered warmed, blood-soaked Kimwipes was 82.1%. Flies offered room temperature ('cold blood'), blood-soaked Kimwipes engorged less (12%), while 19.2% of the flies offered warmed blood through artificial membranes (parafilm) engorged. The temperature of the blood meal and the feeding technique has a significant effect on engorgement ($F_{2, 27} = 91.9$; $P < 0.0001$). Small bars represent SEM.

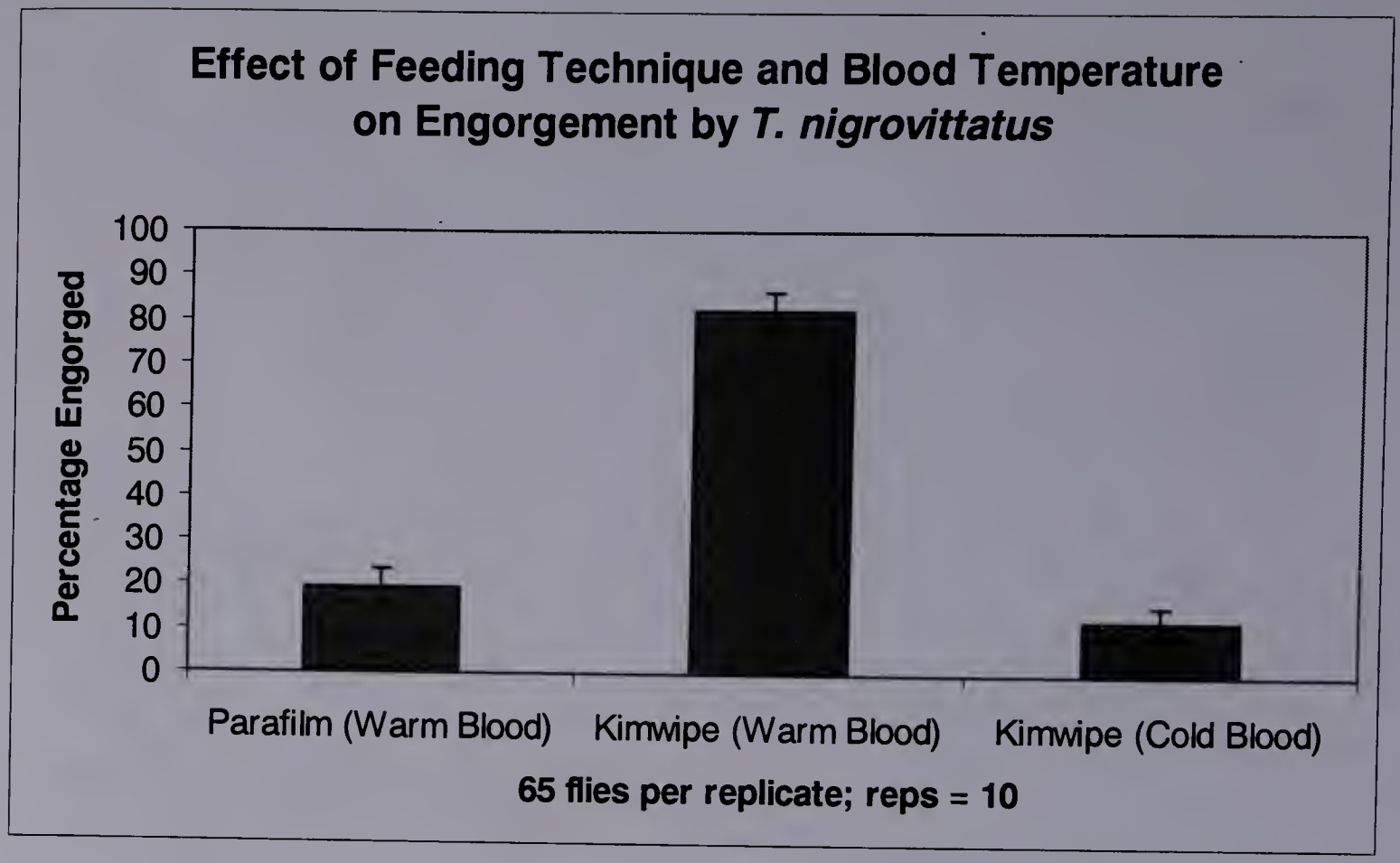
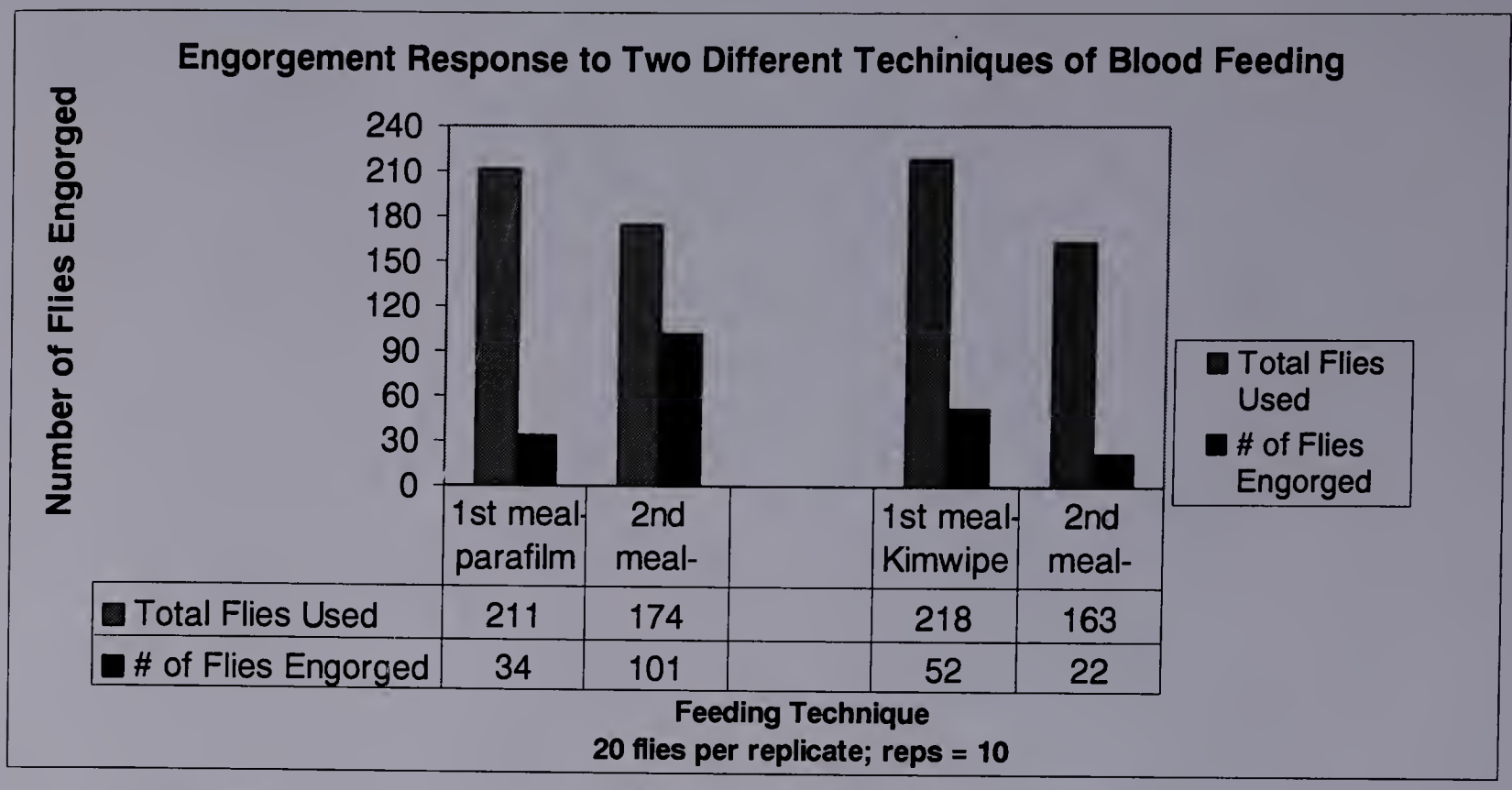

Effect of Feeding Technique and Blood Temperature on Engorgement by T. nigrovittatus
Percentage Engorged
100
90
80
70
60
50
40
30
20
10
0
Parafilm (Warm Blood)
Kimwipe (Warm Blood)
Kimwipe (Cold Blood)
65 flies per replicate; reps = 10

Figure 8. The engorgement response of female *T. nigrovittatus* during alternate and successive feeding assays. The data was analyzed using *t*-test to compare the mean percentage of flies that fed when exposed to parafilm first and blood-soaked Kimwipes second. There was a significant difference in mean percentage (± SEM) of flies that engorged using the parafilm assay first (15.8 ± 4.9) and the Kimwipe assay scond (59.5 ± 4.9) ($F_{1, 18} = 39.1$; $P < 0.0001$). There were 20 flies used per experimental group (i.e., parafilm and Kimwipes) and 10 replicates performed. A total of 218 flies used in experimentation in the first assay. Important to note is that the data is graphed as the number of flies that engorged out of the total flies exposed to blood using each feeding technique in order to amplify the differences between the techniques. The alternate design was also tested, where flies were exposed to blood-soaked Kimwipes first and then to parafilm membranes second. There was a significant difference in mean percentage (± SEM) of flies that engorged when exposed to blood-soaked Kimwipes first (74.7 ± 7.8) and parafilm second (32.0 ± 7.8) ($F_{1, 20} = 14.9$; $P < 0.0012$). There were 20 flies used per experimental group and 10 replicates performed. A total of 211 flies were used in experimentation during the assay. This study demonstrates that the lack of successful engorgement when flies are exposed to blood using an artificial membrane is not because the flies are not in the blood feeding mode. Rather, there are probably missing stimuli since the flies that failed to engorge using membranes fed more readily when exposed to blood-soaked Kimwipes.


Engorgement Response to Two Different Techiniques of Blood Feeding
Number of Flies Engorged
240
210
180
150
120
90
60
30
0
Total Flies Used
# of Flies Engorged
1st meal-parafilm
2nd meal-
1st meal-Kimwipe
2nd meal-
Total Flies Used 211 174 218 163
# of Flies Engorged 34 101 52 22
Feeding Technique
20 flies per replicate; reps = 10

CHAPTER V

THE EFFECT OF DROSULFAKININ ON PROTEIN AND CARBOHYDATE INGESTION BY THE BLOW FLY, *PHORMIA REGINA*.

Abstract

Sulfakinins, which are thought to be satiety factors in invertebrates, have previously been shown to inhibit feeding in the German cockroach and desert locust. This is the first study to examine the effect of sulfakinin as a feeding satiety factor in the black blow fly, *Phormia regina.* Additionally, this is also the first study to examine the effect of sulfakinin on two of its nutrient requirements (i.e., carbohydrates and proteins). I found that drosulfakinin I (DrmSKI) significantly inhibited carbohydrate feeding by 44% at the most effective dose (10 nmol) in female flies. Statistically, there was no significant effect on males; however, injections of 10 nmol DrmSKI reduced carbohydrate feeding by 34% compared to the sham. Drosulfakinin had no effect on protein feeding and no significant inhibition was detected in females or males. The results of this study lend further support to the idea that carbohydrates and proteins are regulated by separate control mechanisms, especially in Calliphoridae. Finally, feeding satiety for carbohydrates is probably not the result of sulfakinins alone.

Introduction

Despite the economical and medical importance of understanding feeding behavior in insects, there has been a lack of research focused on examining what role peptides and hormones have in feeding satiety. *Phormia regina* is arguably one of the most well understood insect models, especially in regards to feeding biology (see Dethier, 1976), and therefore is an excellent insect to use when studying feeding satiation. The blow fly requires two different essential nutrients, carbohydrates and proteins, and consumes them separately. Examining the satiation regulation of these two nutrients separately provides a better understanding of the effects of the invertebrate neuropeptide, sulfakinin. To date, what is known about satiety in *P. regina* is based on a neural mechanism of feedback from the stretch of the crop for sugar feeding (Gelperin, 1967) and protein feeding (Belzer, 1979). Nothing has been proposed for the chemical aspects of satiation in the blow fly.

Recently, sulfakinin has been identified as a feeding satiety factor in the German cockroach, *Blattella germanica* (Maestro et al., 2001), the desert locust, *Schistocerca gregaria* (Wei et al., 2000), and the salt marsh horse fly, *Tabanus nigrovittatus* (chapter III). Sulfakinins, at the most effective doses, reduced the meal size by 55% in the locust, 60% in the cockroach, and inhibited blood engorgement by 45.5% in the horse fly. The blow fly is a more attractive insect model to study this neuropeptide because it consumes the necessary nutrients separately. The goal of the present study is to examine the role of sulfakinins in both carbohydrate and protein satiation in *P. regina*.

Materials and Methods

Maintaining Flies. All flies used in the following experiments were reared and maintained according to Stoffolano (1974) under a 16:8 light/dark photoperiod with approximately 50% RH. All flies emerging within 24 h were considered one cohort, in order to standardize age and physiological state, and placed in a 23 cm^3 metal-screened cage. The flies were provided access to aqueous sucrose (0.126 M) for 48 h and then at the end of the second day post-emergence, the flies were deprived of sucrose 16-20 h prior to experimentation. Flies still had access to water during the starvation period.

Injection and Drug Preparation. Drosulfakinin I (DrmSKI) and drosulfakinin-II (DrmSKII) (R. J. Nachman, gift) was prepared in a stock solution of 80% acetonitrile and 20% water, made up to 0.01% trifluoroacetic acid and stored at -20^0 C. DrmSKI (FDD**Y[SO_3H]**GHMRFa) differs from DrmSKII (GGDDQFDD**Y[SO_3H]**GHMRFa) by the N-terminus extension of GGDDQ-. It is presumed that DrmSKII would have similar effects to DrmSKI as they only differ in the N-terminus.

Flies from one cohort were randomly assigned to the different treatment groups and a single run of all of treatments was considered one replicate. Flies were cold immobilized, separated by sex, and held on ice until injections were administered. Sham-injected and treatment flies were injected with a 1 µl of solution into the intersegmental membrane of the abdomen, as previously described in chapter III. Sham-injected flies were injected with *Phormia* saline (Chen and Friedman, 1975) and treatment flies were injected with sulfakinin (1, 4, 7, and 10 nmol) dissolved in *Phormia* saline. After an individual fly was injected it was placed back on ice until the entire experimental group (10 flies for each treatment) was injected with the solution. The entire injection process

for each group took less than 10 minutes. Each experimental group of flies was placed in a glass Petri dish and the feeding assay was started. There was zero mortality with all injections. All flies recovered from injections and resumed normal behavior in less than 10 minutes.

Feeding Assay. For the feeding assay, sucrose and whole beef liver were offered separately and examined in both males and females separately. For the sucrose assay, amaranth dye [shown by Thompson and Holling (1974) as non-stimulating] was added to 0.126 M sucrose in order to see the presence of the meal in dissections. Filter paper was cut in half and placed in the Petri dish so that two halves were side by side on the bottom of the dish. On one half of the filter paper, a piece of crumpled Kimwipe was added and the sucrose was pippetted onto the Kimwipe and filter paper until thoroughly soaked. The experimental group of flies was placed on the other half of filter paper. This design allowed flies to recover from their immobilization and have an area that was not soaked by the sucrose. For the beef liver assay, filter paper was again cut into half, but this time only one half was placed into the Petri dish. The approximately same-sized pieces of liver were placed on the non-filter paper side of the dish and then macerated with scissors. The experimental group of flies was added to the side of the Petri dish with the filter paper. The flies were allowed to feed *ad libitum* during both sucrose and liver assays for 40 minutes, which, based on preliminary experiments, had previously been determined as the optimal amount of time to observe an effect on food intake.

Dissection and Analysis. After the feeding assay was completed, flies were killed and later dissected. The crop of the fly was carefully removed, near the point where the crop duct ends and the crop sac begins, and then weighed. In order to determine the

weight of the meal ingested, the mean weight (mg) of an empty crop (previously determined to be 0.38 mg, see Appendix A) was subtracted from the weight of the full crop. The weight (mg) of the meal ingested can be converted to volume (µl) imbibed for both sucrose and liver (see Appendix B). It should be noted that only the crop was examined and statistically analyzed, though almost every fly had a meal in the midgut. Some flies are reported in the results section as having not fed (i.e., the percentage fed results), but this refers to no meal being found in the crop. The percentage of insects that fed was not reported by Maestro et al. (2001) or Wei et al. (2000), nor did the authors state whether insects that failed to feed were included in their analysis.

A total of 1,285 flies were used in experimentation for the DrmSKI assay, with 12 replicates for carbohydrates and 14 replicates for proteins performed. A total of 1,679 flies were used in experimentation for the DrmSKII assay, with 20 replicates for carbohydrates and 14 replicates for proteins performed. All statistical comparisons of the data were analyzed using ANOVA to compare the mean weight of the meal in the crop by treatment and the mean percentage of flies that fed by treatment. A Tukey-Kramer HSD test was used for further statistical analysis between the treatments (JMP, SAS Institute Inc., 2005). The percentage of difference between the sham-injected group and the treatment group was calculated by using the following formula:

$$\frac{\textit{\% engorged by treatment} - \textit{\% engorged by sham}}{\textit{\% engorged by sham}} \times 100 = \textit{\% Difference}$$

Results

Drosulfakinin I. DrmSKI had a statistically significant effect on sucrose ingestion by female *P. regina* ($F_{4, 156} = 4.89$; $P < 0.001$; Fig. 9). A Tukey-Kramer HSD test showed that only the 10 nmol dose was significantly different from the sham-injected group. Injections of 10 nmol DrmSKI, the most effective dose, reduced sucrose ingestion by 44% relative to the sham-injected group (Table 1). DrmSKI did not have a statistically significant effect on sucrose ingestion by male *P. regina* ($F_{4, 105} = 1.42$; $P = 0.23$; Fig. 9). However, at the most effective dose (10 nmol) sucrose feeding was reduced by 34% (Table 1). DrmSKI had no effect on protein ingestion by female or male *P. regina* (Female: $F_{4, 268} = 1.77$; $P = 0.14$; Male: $F_{4, 192} = 0.18$; $P = 0.95$; Fig. 10).

Not all flies had the presence of a sucrose or liver meal in the crop. Flies that did not have a meal in the crop were not included in the statistical analysis of the meal size, though the percentage of flies with a meal in the crop was recorded. For females exposed to sucrose, 51% of the controls had a meal. Thus, 49% of the controls failed to have a meal in the crop. For males exposed to sucrose, 55% of the controls had a meal. For females exposed to liver, 99% of the controls had the presence of a meal. When males were exposed to liver, 88% of the controls had the presence of a meal in the crop. The reason why some flies (i.e., 49% of the female control flies) failed to put a sucrose meal in the crop remains unclear. However, the percentage of flies that fed (i.e., had a meal in the crop) was not statistically different between the control and treatment groups for those exposed to sucrose (females: $F_{5, 36} = 0.29$; $P = 0.92$, males: $F_{5, 36} = 1.15$; $P = 0.35$; Fig.11) or exposed to liver (females: $F_{5, 36} = 2.3$; $P = 0.06$, males: $F_{5, 24} = 0.36$; $P = 0.87$; Fig.12).

Drosulfakinin II. Injections of DrmSKII did not have a significant effect on sucrose ingestion by female or male *P. regina* (Female: $F_{4, 177} = 0.58$; $P = 0.68$; Male: $F_{4, 150} = 0.65$; $P = 0.63$; Fig. 13). At the most effective dose (4 nmol), sucrose feeding was reduced by 16% relative to the sham-injected group in the females (Table 1). At the most effective dose (1 nmol) sucrose feeding was reduced by 24% (Table 1). Injections of DrmSKII had no effect on protein ingestion by females ($F_{4, 284} = 1.52$; $P = 0.20$; Fig. 14) or by males ($F_{4, 296} = 2.15$; $P = 0.07$; Fig. 14).

As stated above, not all flies had the presence of a meal in the crop. Flies that did not feed (i.e., no meal in the crop) were not included in the statistical analysis of the size of the meal. However, the percentage of flies with a meal in the crop was recorded and analyzed. For females exposed to sucrose, 51% of the controls had the presence of a meal. For males exposed to sucrose, 37% of the controls had fed. For females exposed to liver, 94% of the controls had fed. When males were exposed to liver, 93% of the controls had a meal in the crop. The percentage of flies that had a meal in the crop was not statistically different between the control and treatment groups for those exposed to sucrose (females: $F_{5, 54} = 0.74$; $P = 0.60$, males: $F_{5, 54} = 0.66$; $P = 0.65$; Fig. 15) or to liver (females: $F_{5, 36} = 0.55$; $P = 0.73$, males: $F_{5, 36} = 0.36$; $P = 0.87$; Fig. 16).

Discussion

Sulfakinin in insects has been shown to have myotropic effects on the gut, stimulate the release of digestive enzymes, and inhibit feeding (Schoofs and Nachman, *in press*). Thus, sulfakinins appear to be physiologically (and structurally) homologous to the gastrin-cholecystokinin system in vertebrates (Schoofs and Nachman, *in press*).

However, sulfakinins have not expressed the same biological action in all of the insects that have been examined thus far, such as the lack of myotropism in the gut of two different species of blow flies (Duve et al., 1994; Haselton et al., 2006). This study demonstrates that sulfakinins do not act as a satiety factor for all nutrients (i.e., carbohydrates vs. proteins).

The percentage of inhibition (34% for males, 44% for females) for flies injected with DrmSKI and exposed to sucrose (a carbohydrate source) is comparable to the previous research examining satiation in the desert locust (55%), in the German cockroach (60%), and inhibition of engorgement in the female salt marsh horse fly (45.5%). That DrmSKI had a greater effect on carbohydrate feeding in females and not protein feeding makes biological sense since females need a sizable prótein meal for egg development. Since both nutrient types are directed to the crop, reducing the size of the carbohydrate meal allows for more space in the crop for protein when it is encountered, especially as protein in more difficult to encounter in nature. This ultimately allows for more space in the abdominal cavity for egg production as well. There was very little, if no observed inhibition at any of the doses of DrmSKI or DrmSKII when flies were exposed to liver (a protein source). At various concentrations of each nutrient, for both sexes and both drugs, 81% of the cases (or 13 of the 16 possible combinations) showed a decrease in feeding for the sucrose assays, while only 25% (or 4 of the possible 16 combinations) showed a decrease in feeding for the protein assays (Table 1). This supports the assertion that sulfakinins, at least for females, affect sucrose feeding more than protein feeding. Factors affecting protein satiation may be controlled by another

mechanism(s), such as foregut and abdominal stretch receptors for protein inhibition in blow flies (Gelperin, 1967; Belzer, 1979).

DrmSKII inhibited sucrose ingestion in females at a lower dose (4 nmol instead of 10 nmol in DrmSKI) and the percentage of inhibition (16%) was considerably lower compared to DrmSKI (44%). The percentage of inhibition (24%) when males were exposed to sucrose and injected with DrmSKII was also lower compared to the effect of DrmSKI (34%). Like the females, the most effective dose (1 nmol) was at a lower concentration compared to the effect of DrmSKI (10 nmol).

Reporting the percentage of feeding is important as an indicator of successful feeding throughout all treatments. Neither Maestro et al. (2001) or Wei et al. (2000) reported the percentage of feeding or stated whether insects that failed to feed were included in their analysis of the inhibition of meal size. In the current study only 51% of the female controls (for both DrmSKI and DrmSKII) put a sucrose meal in the crop, meaning 49% failed to. It is unclear why some flies failed to feed for the sucrose assays, yet almost all of the flies (> 90%) exposed to liver stored a meal in the crop. The difference between the sucrose and liver assays may be due to the importance of protein for reproduction. The percentage of flies that had a sucrose or liver meal in the crop was not statistically different between the controls and treatments, lending further support to the idea that sulfakinins are not influencing whether the flies feed or not, but rather affect the size of the meal once the insect begins feeding.

Protein satiation and carbohydrate satiation are likely controlled separately in most of the Diptera (Stoffolano, 1979; Belzer, 1978; 1979; Simpson and Bernays, 1983), thus lending further support to differing effects of sulfakinin on the separate nutrients.

Furthermore, other insect feeding control mechanisms have been identified by several authors. The neural mechanisms (i.e., stretch receptors, chemosensory adaptation, etc.) outlined by Bernays and Simpson (1982) and biogenic amines (i.e., serotonin) (Dacks et al., 2003) have all been shown to affect short-term feeding satiety in insects. Specifically, Bowden and Dethier (1986) showed that abdominal stretch receptors functioned in terminating a meal and determining the meal size. In addition, work by Aguilar et al. (2004) compared the anti-alimentary activity effects of perisulfakinin (PSK) and leucomyosuppressin (LMS) in *B. germanica*. The authors showed that both PSK and LMS had myostimulatory effects on the gut. However, food accumulated in the foregut and decreased in the hindgut when injected with LMS, while there were no observable differences in food accumulation when injected with PSK. The authors postulate that the mechanisms for feeding inhibition by LMS and PSK are different and that LMS inhibits feeding because of the persistence of signals from the stretch receptors in the gut, whereas PSK probably inhibits feeding by acting on the CNS.

Sulfakinins have been shown to have myotropic actions on the gut of the cockroach and locust, yet it has not been demonstrated in the Diptera (Duve et al., 1994; Haselton, 1994). Research by Duve et al. (1994) and Haselton et al. (2006) showed that sulfakinins had no myostimulatory or myoinhibitory actions on the crop in Calliphoridae. The lack of a myotropic response by sulfakinins in flies suggests they may inhibit feeding by acting on the CNS, as proposed by Aguilar et al. (2004) in the cockroach. The difference between my findings and that of the other two laboratories might be due to the fact that I was examining satiety regulation in two very different types of feeders (i.e., cockroaches and locusts versus flies) with drastically different digestive systems. In other

words, cockroaches and locusts have a linearly arranged digestive system whereas the Diptera have a diverticulated system (i.e., a diverticulated crop for food storage). All food ingested by locusts and cockroaches must pass through the foregut (crop), midgut and hindgut with no diversion to different organs. When the blow fly ingests a meal, the food is diverted to both the diverticulted crop and midgut. When the midgut is full, the cardiac sphincter closes and all other food is stored in the crop (Simpson and Bernays, 1983). When the midgut empties, food is then re-shunted up to and into the midgut for digestion (Simpson and Bernays, 1983). With the exception of the effect of DrmSKI on sucrose feeding by females, we did not observe a strong dose-dependent inhibitory response with any of the other combinations of assays, so it may be that chemical satiation alone (i.e., sulfakinin) is not a strong enough satiety signal to produce significant feeding inhibition in this insect.

In conclusion, in *P. regina* sulfakinins (particularly DrmSKI) inhibit sucrose feeding, but not protein feeding. Therefore, the two nutrient sources probably are under different control mechanisms. Also, sulfakinins probably act additively in the blow fly (as CCK does in vertebrates) with other mechanisms, like stretch receptors and biogenic amines. One biogenic amine, serotonin, has been shown to inhibit sucrose feeding in the flesh fly (*Neobellieria bullata*: Dacks et al., 2003) and inhibit protein feeding in the blow fly (*P. regina*: Stoffolano, *unpublished data*). Sulfakinins are thought to be homologous to vertebrate cholecystokinin (CCK). In research using rats, Hayes and colleagues (2004) showed that cholecystokinin-induced satiety requires the inhibition of gastric emptying and showed that gastric distention induced CCK activation of serotonin (5-HT3) receptors (Hayes et al., 2006). In vertebrates, the biological actions of CCK are mediated

by vagal sensory nerve fibers (VSNF), and it is the release of serotonin by gastrointestinal stimulation that activates the VSNF (reviewed in Hayes et al., 2006). Further, Hayes and Corvasa (2005) demonstrated that CCK and serotonin act synergistically to inhibit feeding. Thus, it seems highly unlikely that sulfakinins act alone in invertebrates to produce satiety.

Table 1. The effect of sulfakinin on the percentage of feeding inhibition is shown. Negative percentages reflect a reduction in feeding, whereas positive percentages reflect an increase in feeding. Percentages are rounded to the nearest whole number. Flies that failed to feed (i.e., no meal found in the crop) were not included in this analysis. This is the same data that is presented in Figures 10, 11, 14, and 15, except this table is looking at the percentage of reduction in meal size, whereas the figures examine the amount of meal ingested.

Percentage of Feeding Inhibition

Sucrose	**DRMSKI-Female**	**DRMSKI-Male**	**DRMSKII-Female**	**DRMSKII-Male**
10 nmol	-44%	-34%	6%	-15%
7 nmol	-10%	-20%	-6%	-18%
4 nmol	-9%	6%	-16%	-12%
1 nmol	12%	-16%	-7%	-24%
Protein				
10 nmol	0%	1%	8%	18%
7 nmol	12%	3%	-11%	-4%
4 nmol	18%	4%	-7%	20%
1 nmol	8%	-3%	0%	9%

Figure 9. DrmSKI significantly inhibited sucrose feeding ($F_{4,\ 156} = 4.89$; $P < 0.001$) by females. There was a 44% feeding inhibition at the 10 nmol dose. DrmSKI did not have a statistically significant effect ($F_{4,\ 105} = 1.42$; $P = 0.23$) on sucrose feeding by males, however, at the most effective dose (10 nmol) feeding was inhibited by 34%. Out of all of the treatments, only the 10 nmol dose for females was significantly different from the sham-injected group. Small bars represent SEM.

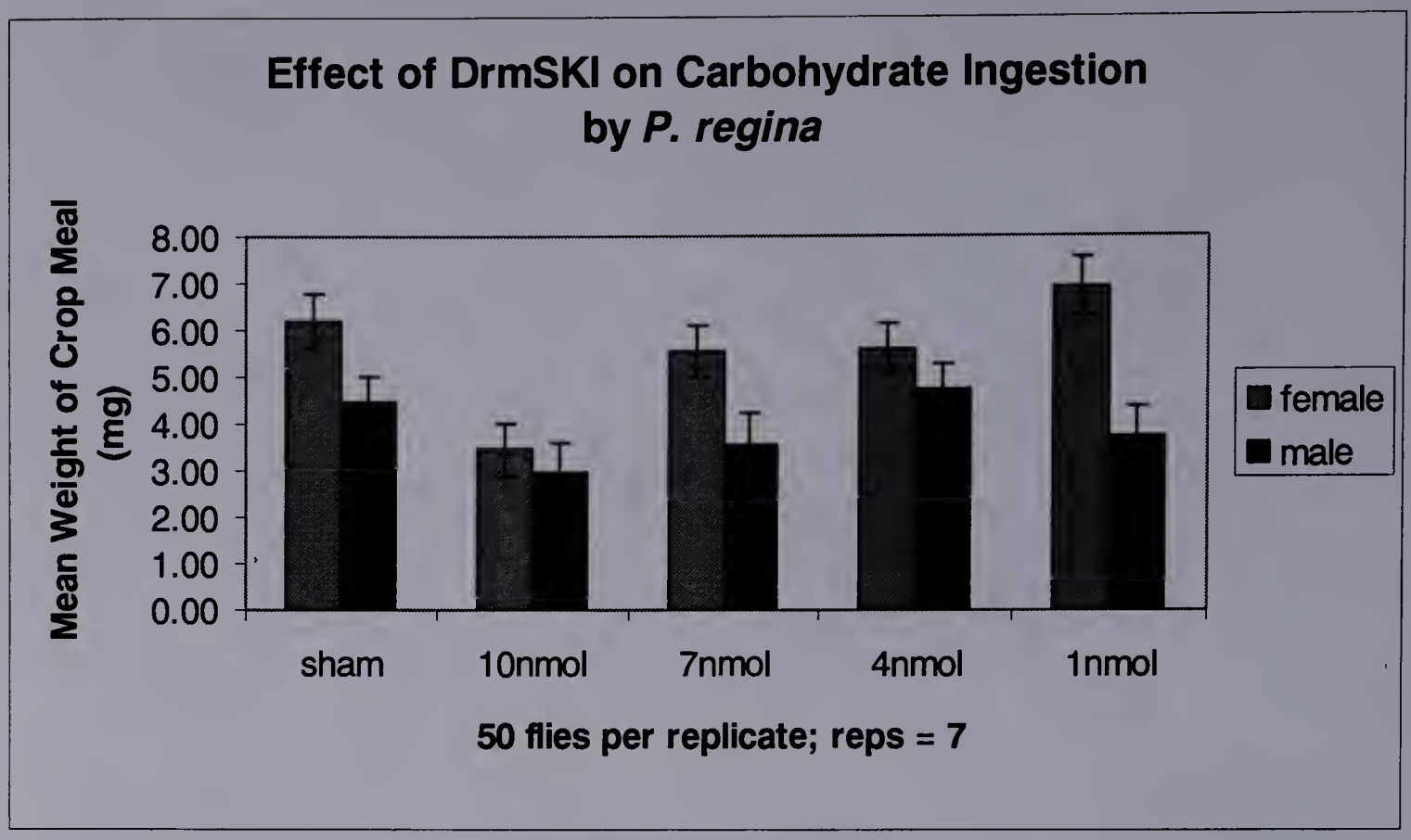

Effect of DrmSKI on Carbohydrate Ingestion by P. regina
Mean Weight of Crop Meal (mg)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
sham
10nmol
7nmol
4nmol
1nmol
female
male
50 flies per replicate; reps = 7

Figure 10. There was no significant effect of DrmSKI on ingestion for females ($F_{4, 268} = 1.77$; $P = 0.14$) or males exposed to liver ($F_{4, 192} = 0.18$; $P = 0.95$). Small bars represent SEM.

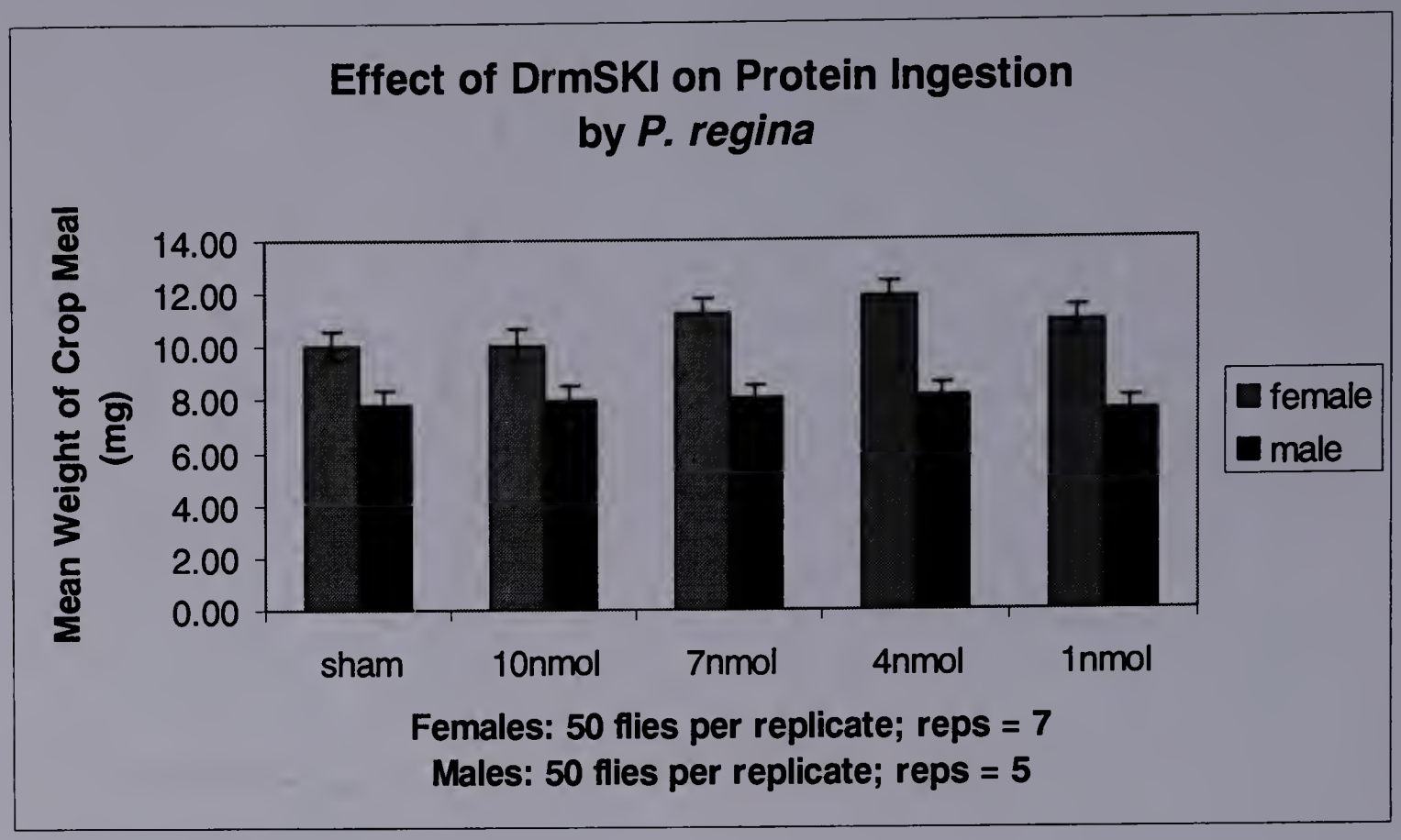

Effect of DrmSKI on Protein Ingestion
by P. regina
Mean Weight of Crop Meal (mg)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
sham
10nmol
7nmol
4nmol
1nmol
female
male
Females: 50 flies per replicate; reps = 7
Males: 50 flies per replicate; reps = 5

Figure 11. The effect of Drosulfakinin I on the percentage of flies fed on sucrose. There was no significant effect on the percentage of flies that imbibed a meal for either females ($F_{5, 36} = 0.29$; $P = 0.92$) or males ($F_{5, 35} = 1.15$; $P = 0.35$). Small bars represent SEM.

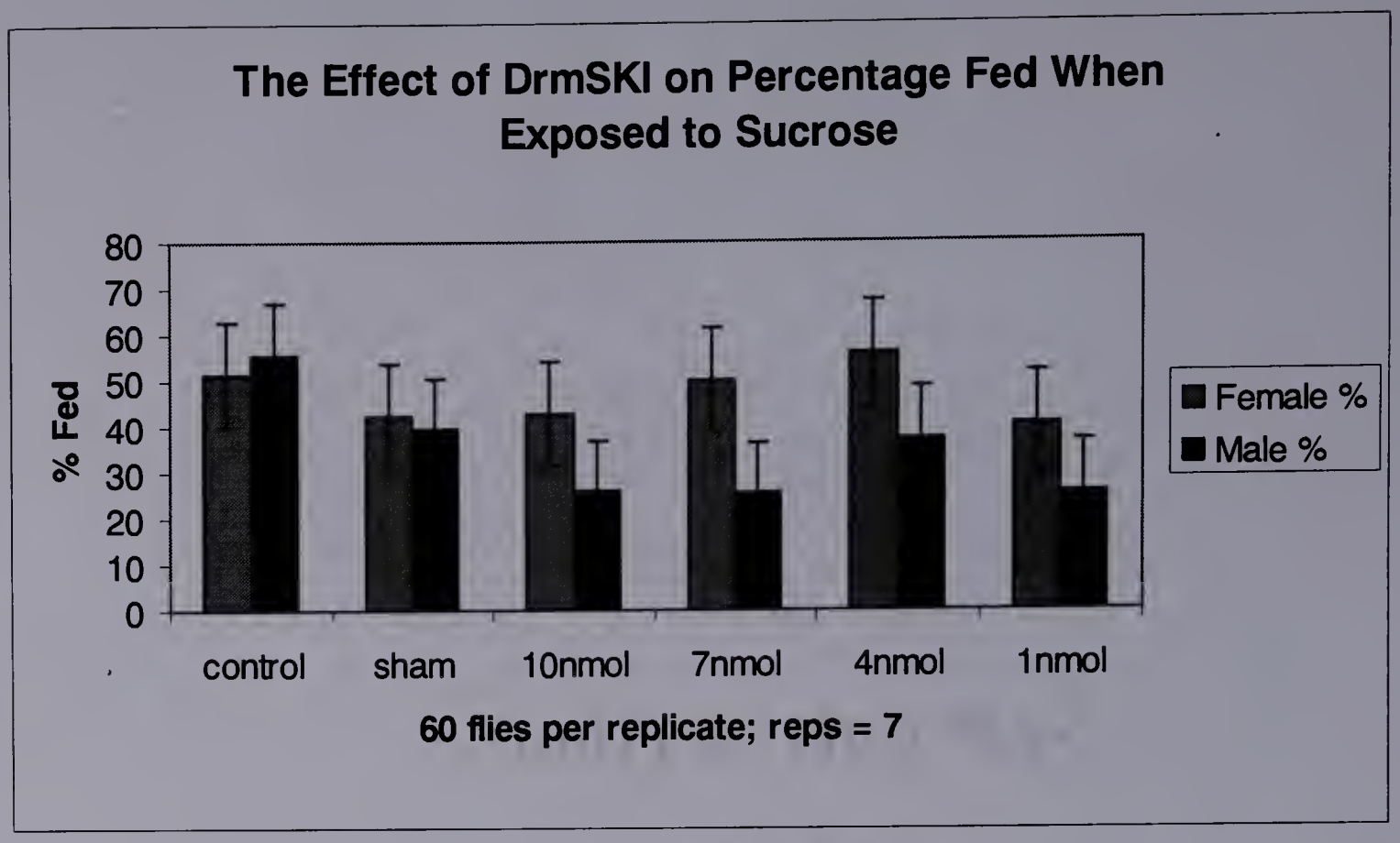

The Effect of DrmSKI on Percentage Fed When Exposed to Sucrose
% Fed
80
70
60
50
40
30
20
10
0
control
sham
10nmol
7nmol
4nmol
1nmol
Female %
Male %
60 flies per replicate; reps = 7

Figure 12. The effect of Drosulfakinin I on the percentage of flies fed on liver. There was no significant effect on the percentage of flies that imbibed a meal for either females ($F_{5, 36} = 2.3$; $P = 0.06$) or males ($F_{5, 24} = 0.36$; $P = 0.87$). Small bars represent SEM.

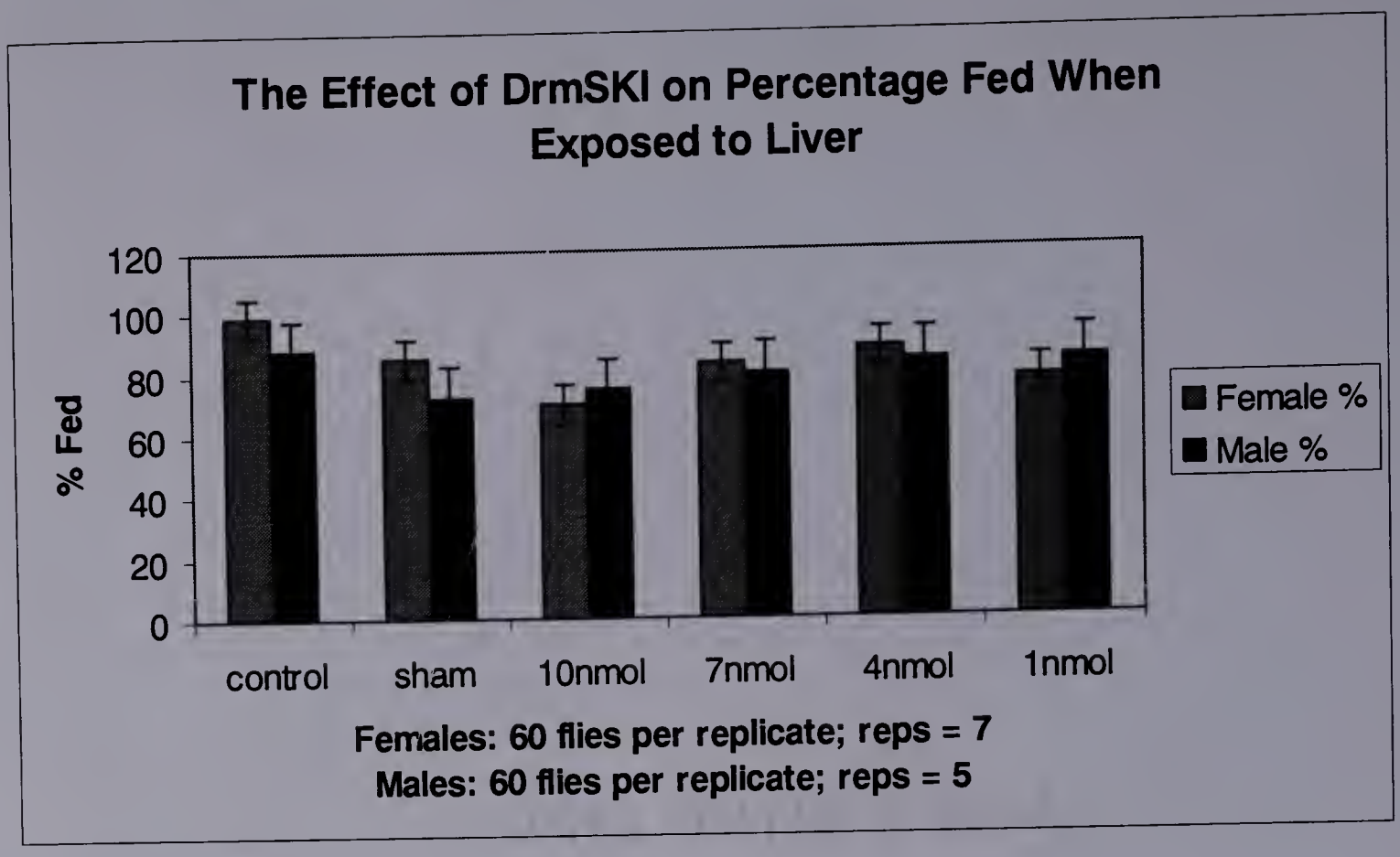

The Effect of DrmSKI on Percentage Fed When Exposed to Liver
% Fed
120
100
80
60
40
20
0
control
sham
10nmol
7nmol
4nmol
1nmol
Female %
Male %
Females: 60 flies per replicate; reps = 7
Males: 60 flies per replicate; reps = 5

Figure 13. DrmSKII had no significant statistical effect on sucrose feeding ($F_{4, 177} = 0.58$; $P = 0.68$) by females. At the most effective dose (4 nmol) feeding was inhibited by 16%, relative to the sham-injected group. There was also no statistically significant effect ($F_{4, 150} = 0.65$; $P = .63$) for males exposed to sucrose; feeding was inhibited by 24% at the 1 nmol dose compared to the sham-injected group. Small bars represent SEM.

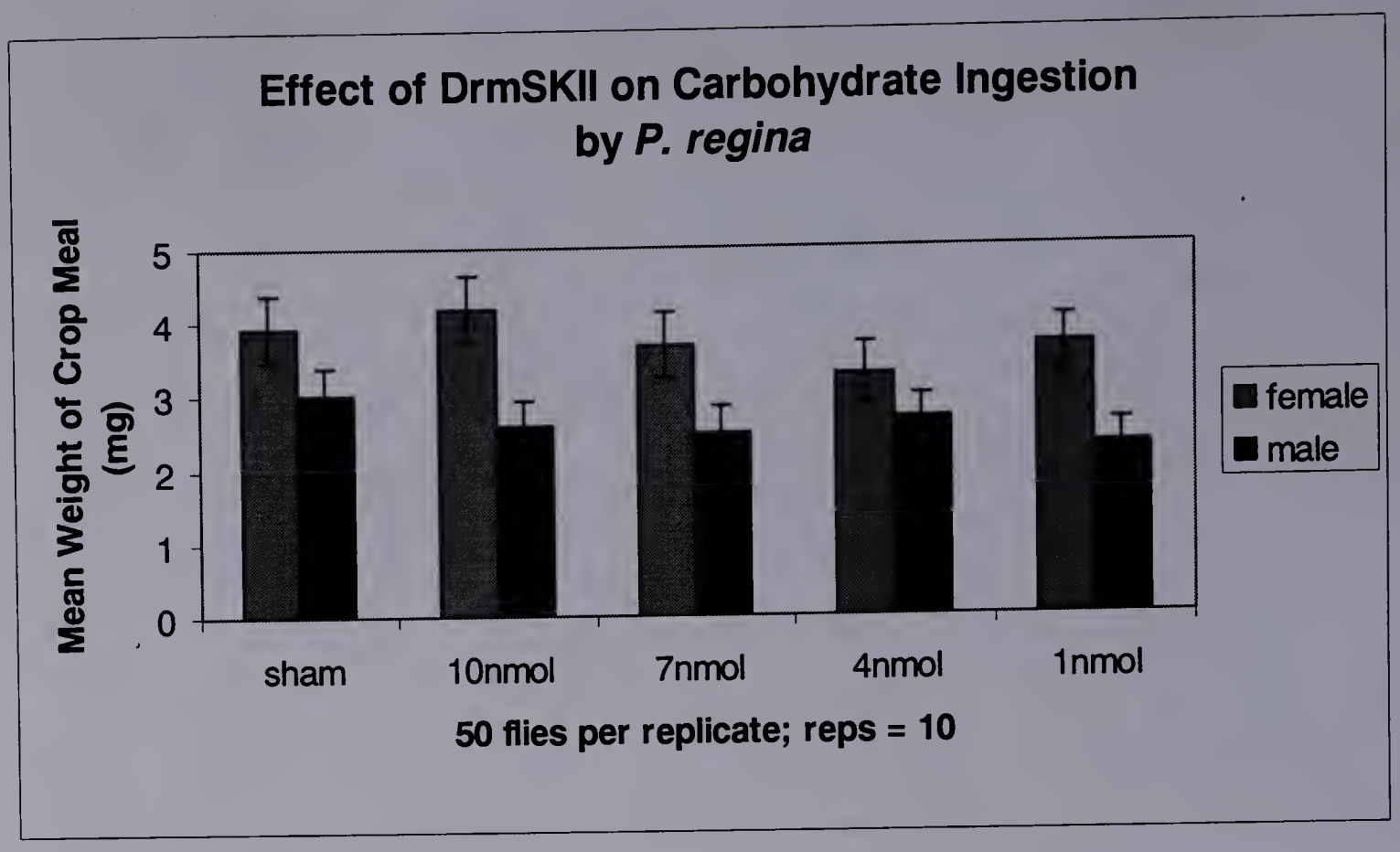

Effect of DrmSKII on Carbohydrate Ingestion by P. regina
Mean Weight of Crop Meal (mg)
0
1
2
3
4
5
sham
10nmol
7nmol
4nmol
1nmol
female
male
50 flies per replicate; reps = 10

Figure 14. DrmSKII had no significant effect on protein ingestion for females ($F_{4, 284} = 1.52$; $P = 0.20$) or males ($F_{4, 296} = 2.15$; $P = 0.07$). Small bars represent SEM.

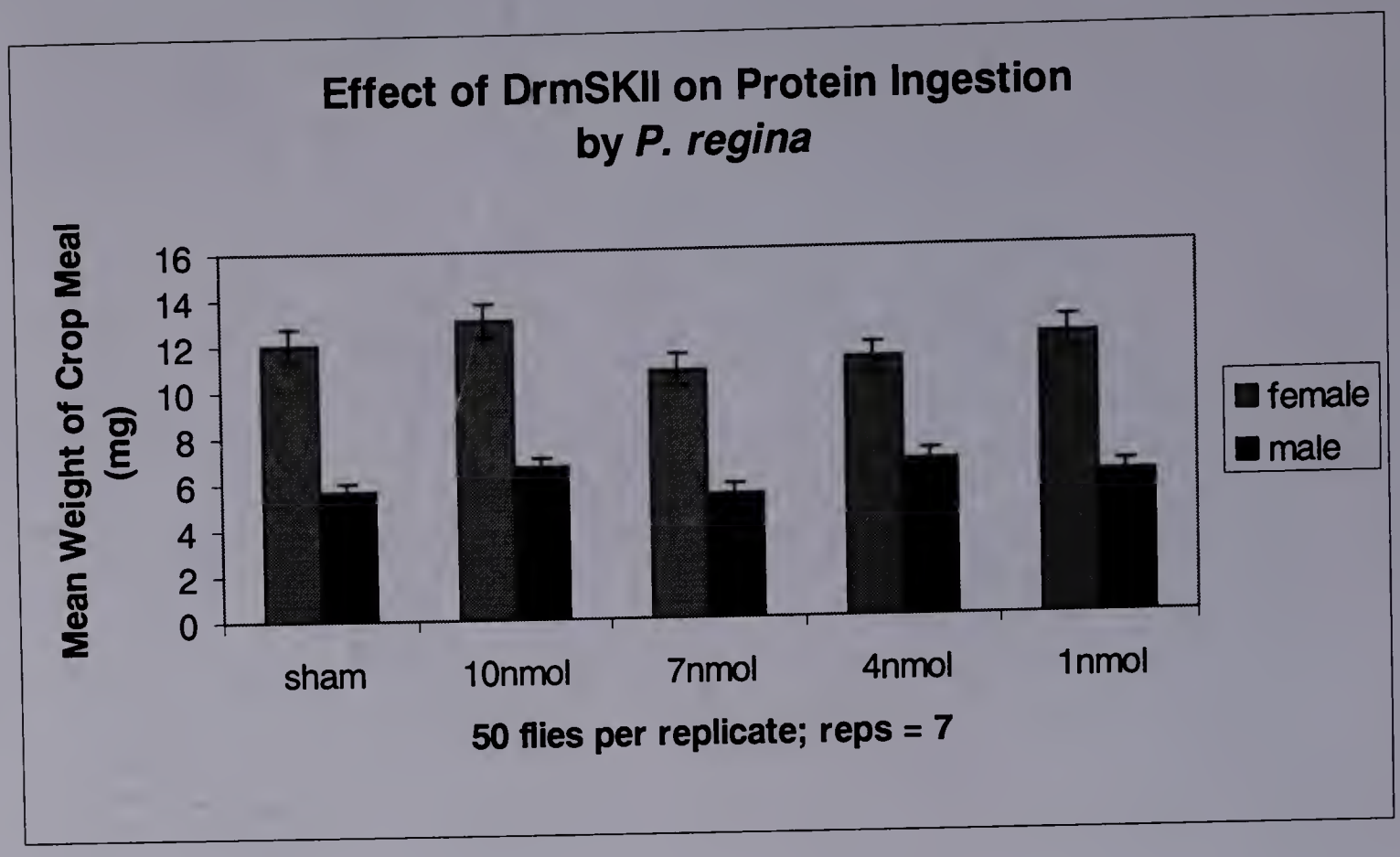

Effect of DrmSKII on Protein Ingestion by P. regina
Mean Weight of Crop Meal (mg)
16
14
12
10
8
6
4
2
0
sham
10nmol
7nmol
4nmol
1nmol
female
male
50 flies per replicate; reps = 7

Figure 15. The effect of Drosulfakinin II on the percentage of flies fed on sucrose. There was no significant effect on the percentage of flies that imbibed a meal for either females ($F_{5, 54} = 0.74$; $P = 0.60$) or males ($F_{5, 54} = 0.66$; $P = 0.65$). Small bars represent SEM.

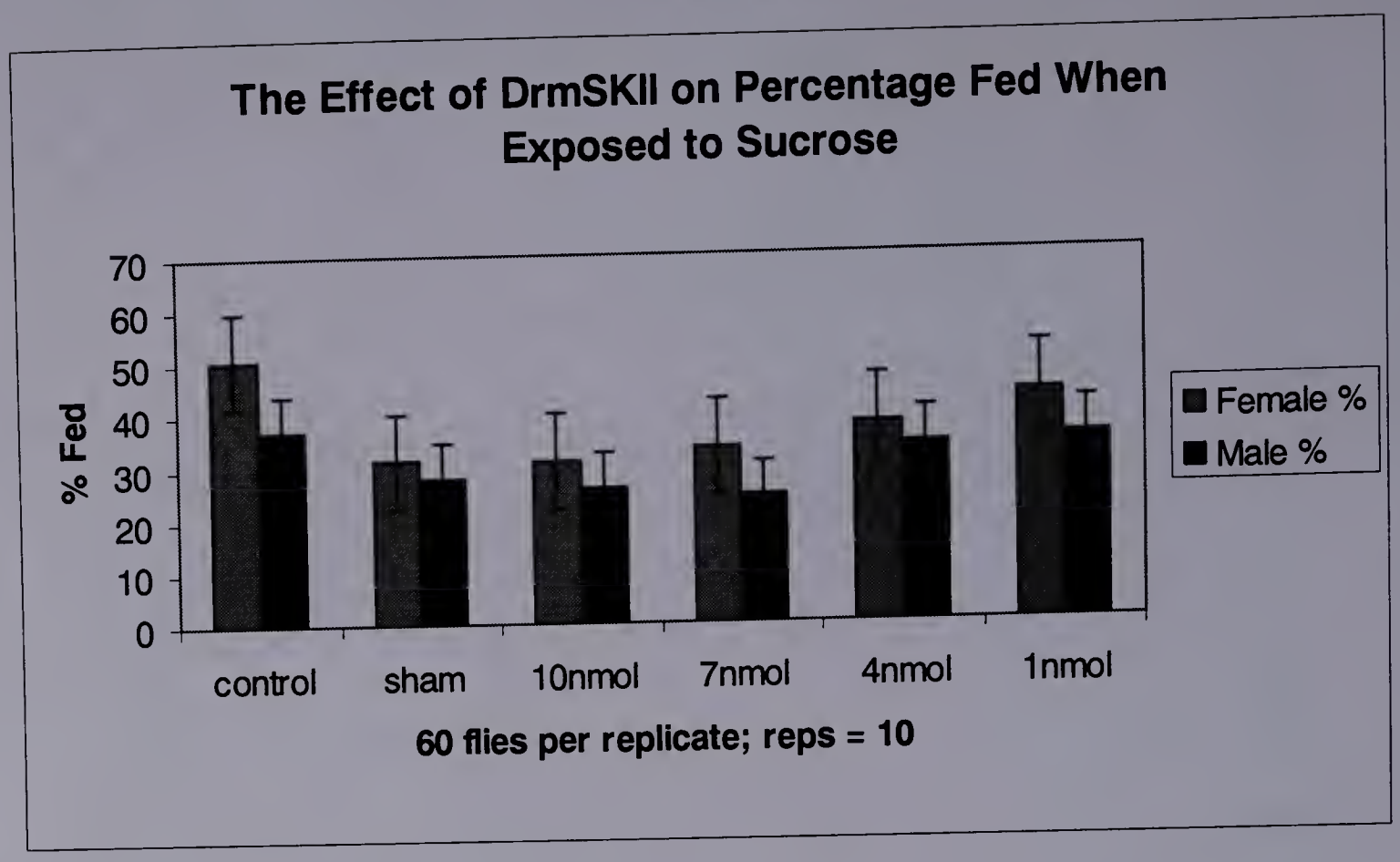
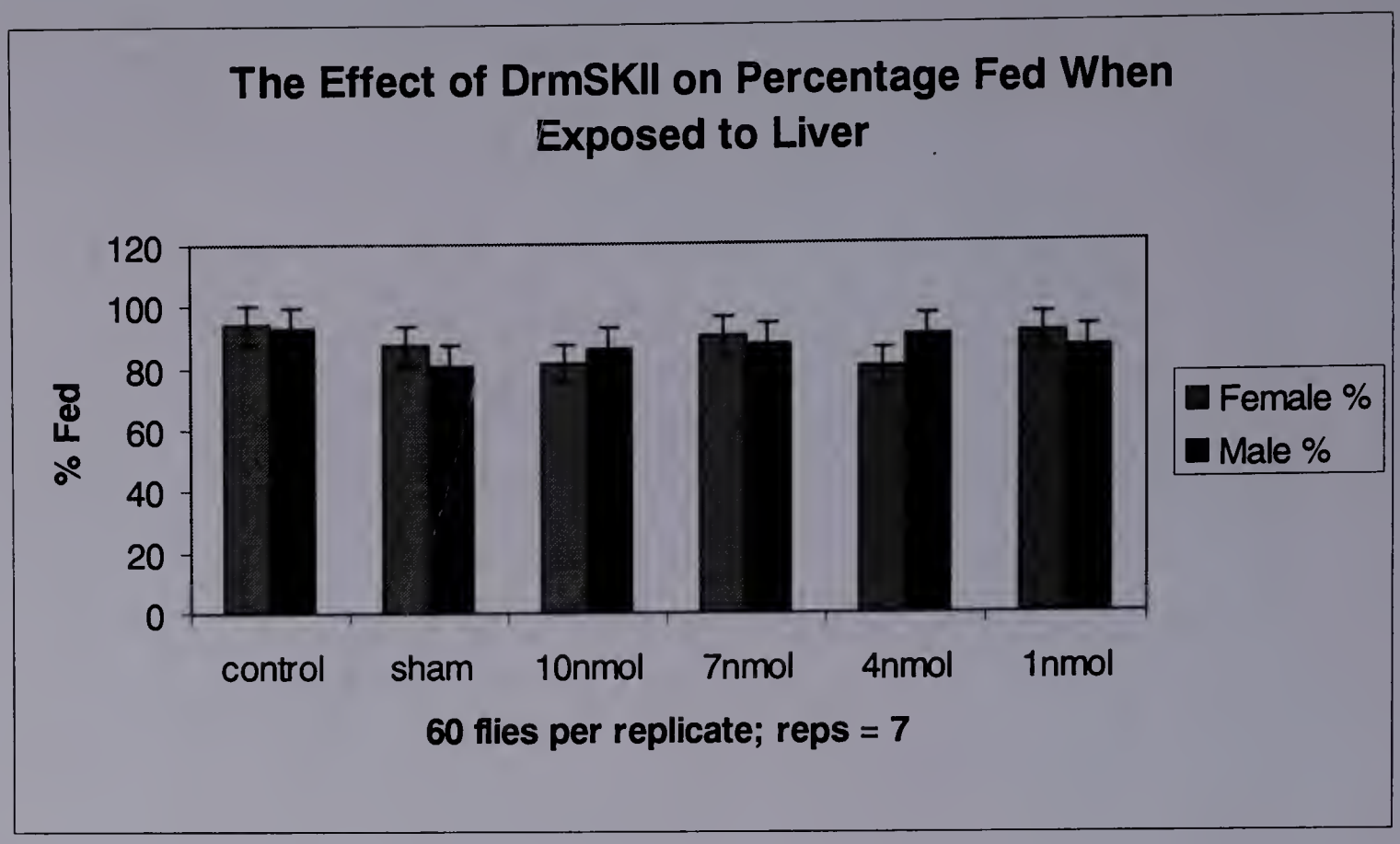

The Effect of DrmSKII on Percentage Fed When Exposed to Sucrose
% Fed
70
60
50
40
30
20
10
0
control
sham
10nmol
7nmol
4nmol
1nmol
Female %
Male %
60 flies per replicate; reps = 10

Figure 16. The effect of Drosulfakinin II on the percentage of flies fed on liver. There was no significant effect on the percentage of flies that imbibed a meal for either females ($F_{5, 36} = 0.55$; $P = 0.73$) or males ($F_{5, 36} = 0.36$; $P = 0.87$). Small bars represent SEM.


The Effect of DrmSKII on Percentage Fed When Exposed to Liver
% Fed
120
100
80
60
40
20
0
control
sham
10nmol
7nmol
4nmol
1nmol
Female %
Male %
60 flies per replicate; reps = 7

CHAPTER VI

GENERAL DISCUSSION

Despite the economic importance that insect feeding habits have on the quality of human life, there remain many aspects of feeding behavior undiscovered. The salt marsh horse fly and the black blow fly were used in experimentation in order to investigate several physiological and behavioral factors that affect feeding and satiety. Both an odor attractant (i.e., octenol) and the temperature of the blood meal were found to significantly stimulate blood feeding in the horse fly. The results of these experiments also demonstrate that the percentage of flies engorging fluctuates throughout season, with a peak in engorgement seemingly coinciding with peak horse fly abundance and activity on the marsh.

Little information is known about the role of sulfakinins as satiety factors in insects. There are currently only two papers (Maestro et al., 2001; Wei et al., 2000) published on the effect of sulfakinin on feeding inhibition. The horse fly and blow fly are excellent insect models to use because they require two different nutrients, proteins and carbohydrates. At the most effective dose, sulfakinin reduced the percentage of females engorging a blood meal by 45-58%. For the blow fly, sulfakinin (DrmSKI) significantly inhibited sucrose feeding by 44% in the females, and while not statistically significant, inhibited sucrose feeding by 34% in the males. Sulfakinin had no effect on protein feeding in the blow fly. The effect of sulfakinin on feeding in the Diptera is dependent on the type of nutrient source (i.e., carbohydrates and proteinaceous materials, which include blood) and even the feeding method used (i.e., parafilm membrane versus blood-soaked Kimwipes).

The horse fly and blow fly may be relatively closely related species, but have drastically different modes of feeding and probably drastically different mechanisms of control. Sulfakinin inhibited engorgement of any blood meal by the horse fly. Reflecting on how it might be working endogenously, sulfakinin may be released during the blood meal once the horse fly has a sufficient meal to produce a batch of eggs. After they are able to produce a batch of eggs host seeking is arrested and oogenesis takes about 7-10 days. They do not search or probe for another meal during egg production. So, biologically it makes sense that exogenous amounts of sulfakinin would inhibit the fly from taking an entire blood meal.

For the blow fly, exogenous injections of SK was affecting the size of the sucrose meal, yet had no effect on protein feeding. Exogenous sulfakinin probably has little to no effect on protein meal size in the blow fly because of the importance of protein for reproduction. For that reason, the blow fly probably has a lower threshold level for controlling protein feeding. In other words, they will readily accept a meal and probably need a much stronger cue or the additive actions from other mechanisms to inhibit protein feeding.

Like vertebrate satiation, feeding control in insects is surely not the result of one mechanism. Sulfakinins are probably acting with other peptides, hormones, biogenic amines, and neural mechanisms. It is important to continue to identify neuropeptides that are involved in the feeding regulation of insects and to continue the research necessary for understanding how these neuropeptides work and their mode of action. This is especially vital as the neuropeptides involved in feeding satiety have been suggested as possible alternative targets for insect control (Nachman et al., 2005). Understanding the

mechanisms that control feeding also requires knowing the factors that lead to successful feeding in insects, especially if satiety factors (i.e., neuropeptides) are going to be used in applied research with pesticides. The traditional methods for controlling insects have proven to be economically burdensome and many have negative environmental impacts on non-target organisms. For *T. nigrovittatus,* control efforts are even more important due to the fragility of the salt marshes they inhabit. Therefore, the use of naturally occurring neuropeptides to influence satiety, and more importantly engorging behavior, makes for an attractive proposal for future research, and tabanid control. It is imperative for future research to identify sulfakinin antagonists and receptor cells in order to make conclusions concerning the function of sulfakinin and its relationship to feeding satiety regulation, especially among the insects. The results of the experiments described in this thesis indicate that there are several behavioral factors that affect feeding in the salt marsh horse fly, *T. nigrovittatus*, and physiological factors, specifically sulfakinin affecting satiation of blood feeding in the horse fly and sucrose feeding in the blow fly.

APPENDIX A

MEAN WEIGHT OF AN EMPTY *P. REGINA* CROP

In order to determine the mean weight of an empty crop, adult flies from one cohort (3 days post-emergence) were fed 0.126 M sucrose and were then starved 16-20 hours before dissections. Thirteen randomly chosen flies were dissected and the crop was removed and placed on filter paper. The crop was then opened up and any liquid present was squeezed out with No. 5 forceps and blotted on the filter paper. The empty crop was weighed and recorded (see Table 2). The mean weight (± SEM) of an empty crop was determined to be 0.38 mg (± 0.05).

Table 2. Mean weight of an empty crop.

Fly	Weight (mg) of Empty Crop
1	0.40
2	0.30
3	0.10
4	0.30
5	0.10
6	0.80
7	0.40
8	0.50
9	0.60
10	0.30
11	0.40
12	0.30
13	0.40
Mean (± SEM)	0.38 (± 0.05)

APPENDIX B

CONVERSION DATA FOR MICROLITERS TO MILIGRAMS OF SUCROSE AND LIVER

In order to determine the volume of the meal imbibed by the fly, preliminary experiments were conducted in order to determine the weight (mg) of various microliters of 0.126 M sucrose (Table 3 and Fig. 18) and digested liver. These conversions are essential, especially when making comparisons with other investigators where they report only weight and not volume, or vise versa. Knowing the weight (mg) of the volume (µl) of nutrients and the weight of an empty crop (Appendix A) expedited the process of measuring the volume of meal imbibed by each individual fly during the feeding assays described in chapter IV. In order to determine how many µl of the meal was imbibed, I subtracted the weight of an empty crop from the weight of a crop with a meal in it. I was able to weigh the crops of the individual flies and convert that to the volume of meal imbibed instead of dissecting each crop open to remove and measure the contents. In order to get the weight of a liver meal, I fed several flies whole liver, *ad libitum*, for 30 min. After feeding, the flies were dissected and the liver meals that were consumed were extracted from the crops using a micropipette. All of the consumed liver meals from the flies' crops were combined together on parafilm in a Petri dish. Microliter increments were then weighed, repeated three times, and recorded (see Table 4 and Fig. 19).

Table 3. Conversion data for microliters to milligrams of 0.126M sucrose.

Microliter	Weight-a	Weight-b	Weight-c	Average (mg)
1	1.6	1.2	1.3	1.37
2	2.6	2.5	2.6	2.57
3	3.2	2.1	2.6	2.63
4	3.5	3.6	3.9	3.67
5	5.2	4.6	5.6	5.13
6	5.7	4.4	6.6	5.57
7	7.6	6.9	6.6	7.03
8	6.9	7.9	7.9	7.57
9	9.2	8.9	9	9.03
10	9.8	9.4	9.9	9.70
11	10.5	10.7	10.6	10.60
12	11.8	11.7	10.9	11.47
13	12.4	12.8	12.7	12.63
14	13.6	13.8	12.2	13.20
15	14.6	14.6	14.4	14.53
16	15.2	15.6	15.7	15.50
17	16.8	16.7	15.2	16.23
18	17.6	16.9	17.2	17.23
19	18.6	18.6	18.1	18.43
20	18.8	19.5	19.7	19.33

Figure 18. Conversion data (expressed as a regression line) for microliters to milligrams of 0.126M sucrose.

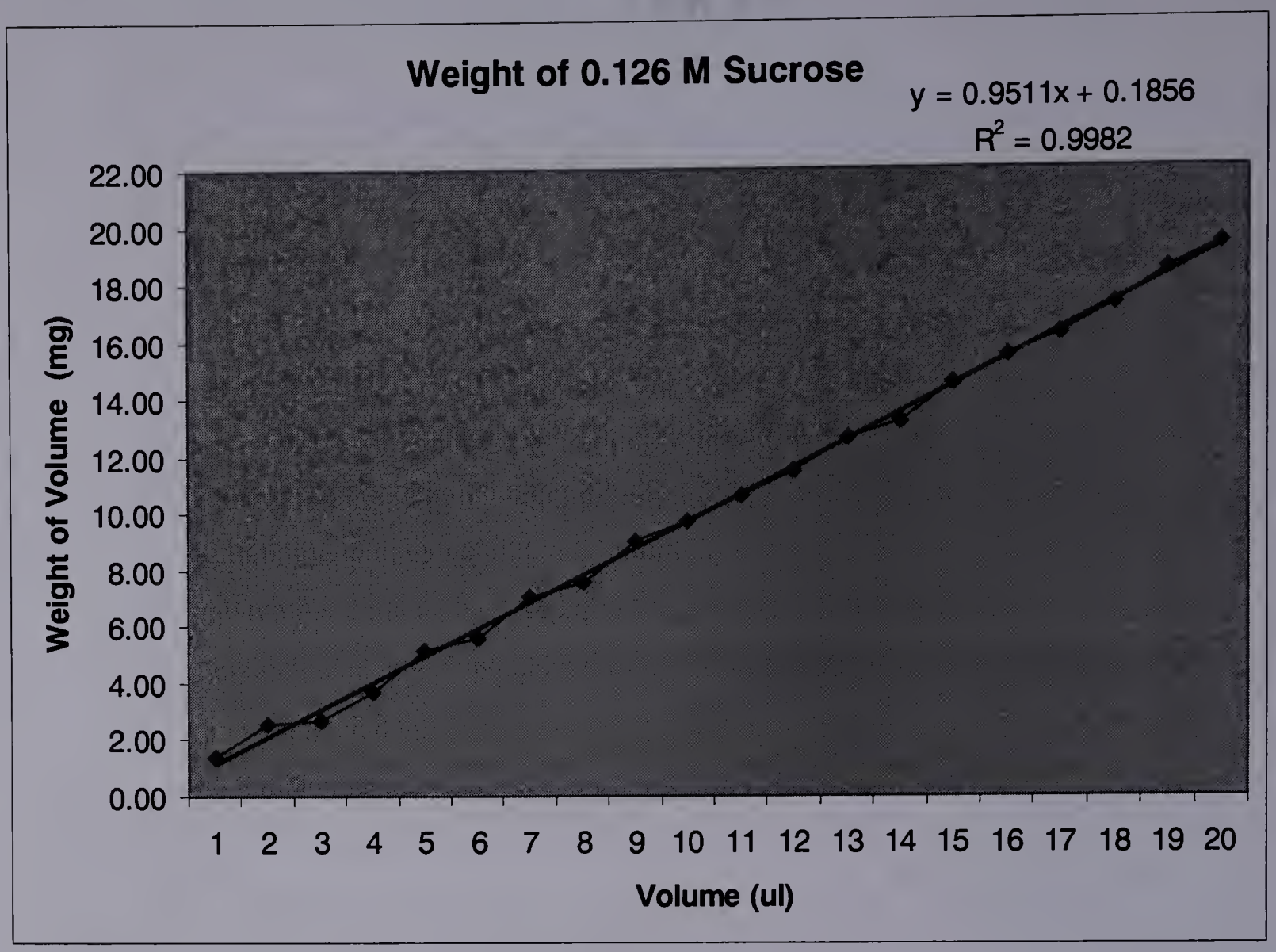
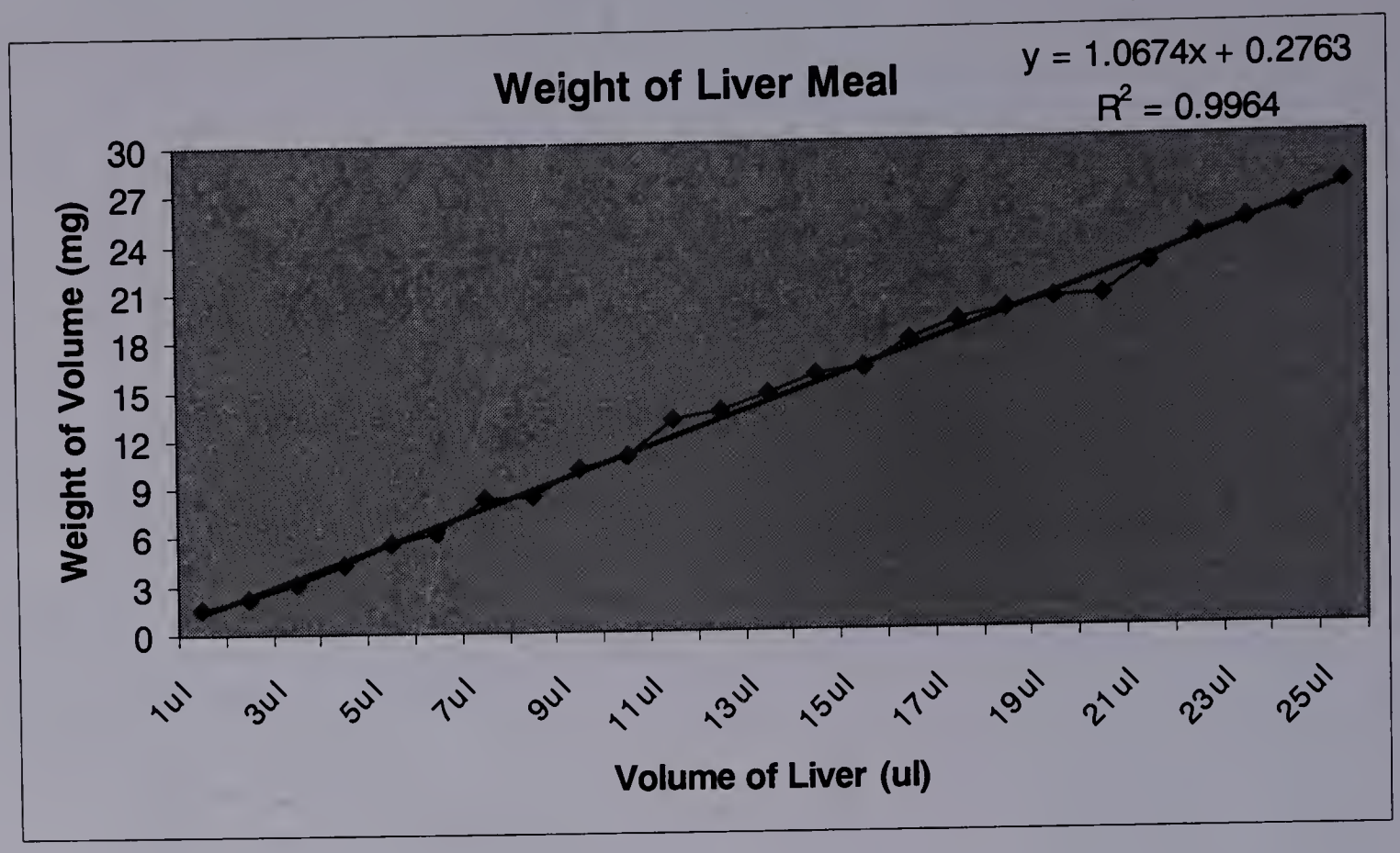

Weight of 0.126 M Sucrose
y = 0.9511x + 0.1856
R2 = 0.9982
Weight of Volume (mg)
22.00
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Volume (ul)

Table 4. Conversion data for microliters to milligrams of a liver meal. Whole beef liver was fed to the flies. The liver meal was extracted from the crops of the flies, combined together in a Petri dish, and then weighed in various microliter amounts.

Microliter	Weight-a	Weight-b	Weight-c	Average (mg)
1	1.5	1.4	1.7	1.53
2	2.3	1.9	2.2	2.13
3	3.1	3.1	2.9	3.03
4	4.7	4.5	3.6	4.27
5	5.3	5.7	5.6	5.53
6	6.9	6	5.7	6.20
7	8.6	8.4	7.7	8.23
8	8.9	7.9	8.2	8.33
9	10.2	10.4	9.6	10.07
10	10.7	10.9	10.7	10.77
11	13.2	13.7	12.2	13.03
12	14	13.5	13	13.50
13	14.6	14.4	14.8	14.60
14	15.8	15.5	15.8	15.70
15	16.2	15.9	16.3	16.13
16	18.2	17.4		17.80
17	18.9	19		18.95
18	19.6	19.5		19.55
19	20.5	19.9		20.20
20	20.9	19.8		20.35
21	22.2	22.3		22.25
22	23.9	24.1		24.00
23	25.6	24.2		24.90
24	25.4	25.9		25.65
25	27.5	26.7		27.10

Figure 19. Conversion data (expressed as a regression line) for microliters to milligrams of liver is shown.


Weight of Liver Meal
y = 1.0674x + 0.2763
R2 = 0.9964
Weight of Volume (mg)
30
27
24
21
18
15
12
9
6
3
0
1ul
3ul
5ul
7ul
9ul
11ul
13ul
15ul
17ul
19ul
21ul
23ul
25ul
Volume of Liver (ul)

BIBLIOGRAPHY

Adams, T. S. 1999. Hematophagy and hormone release. Annals of the Entomological Society of America 92: 1-13.

Aguilar, R., Maestro, J. L., Vilaplana, L., Chiva, C., Andreu, D., and Bellés, X. 2004. Identification of leucomyosuppressin in the German cockroach, *Blattela germanica*, as an inhibitor of food intake. Regulatory Peptides 119: 105-112.

Allen, S. A., and Stoffolano, J. G., Jr. 1986a. The effects of background contrast on visual attraction and orientation of *Tabanus nigrovittatus* (Diptera: Tabanidae). Environmental Entomology 15: 689-694.

____. 1986b. The effects of hue and intensity on visual attraction of adult *Tabanus nigrovittatus* (Diptera: Tabanidae). Journal of Medical Entomology 23: 83-91.

____. 1986c. The importance of pattern in visual attraction of *Tabanus nigrovittatus* Macquart (Diptera: Tabanidae). Canadian Journal of Zoology 64: 2273-2278.

Barnard, D. R., Bernier, U. R., Possey, K. H., and Xue, R. D. 2002. Repellency of IR3535, KBR3023, para-menthane-3,8-diol, and deet to black salt marsh mosquitoes (Diptera: Culicidae) in the Everglades National Park. Journal of Medical Entomology 39: 895-899.

Beglinger, C., and Degen, L. 2004. Fat in the intestine as a regulator of appetite-role of CCK. Physiology and Behavior 83: 617-621.

Belzer, W. R. 1978. Recurrent nerve inhibition of protein feeding in the blowfly *Phormia regina*. Physiological Entomology 3: 259-263.

____. 1979. Abdominal stretch in the regulation of protein ingestion by the black blowfly, *Phormia regina*. Physiological Entomology 4: 7-13.

Bernays, E. A., and Simpson, S. J. 1982. Control of food intake. Advances in Insect Physiology 16: 59-118.

Bosler, E., and Hansens, E. 1974. Natural feeding behavior of adult saltmarsh greenheads, and its relation to oogenesis. Annals of the Entomological Society of America 67: 321-324.

Bowden, E., and Dethier, V. G. 1986. Coordination of a dual inhibitory system regulating feeding behavior in the blowfly. Journal of Comparative Physiology A. 158: 713-722.

Brady, J. 1973. Changes in the probing responsiveness of starving tsetse flies (*Glossina morsitans* Westw.) (Diptera, Glossinidae). Bulletin of Entomological Research 63: 247-255.

Chen, A., and S. Friedman. 1975. An Isotonic saline for the adult blowfly, *Phormia regina*, and its application to perfusion experiments. Journal of Insect Physiology 21: 529-536

Chirov, P. A., and Alekseyev, A. N. 1970. K foziologii pitaniya slepnei (Diptera, Tabanidae). [On the physiology of feeding in the Tabanidae]. Byulleten' Moskovskogo Obshchestva Ispytatelei Prirody Otdel Biologicheskii 75: 60-67.

Dacks, A. M., Nickel, T., and Mitchell, B. K. 2003. An examination of serotonin and feeding in the flesh fly, *Neobellieria bullata* (Sarcophagidae: Diptera). Journal of Insect Physiology 16: 1-21.

Dethier, V. G. 1954. Notes on the biting response of tsetse flies. American Journal of Tropical Medical Hygiene 3: 160-171.

____. **1957**. Parasitological Reviews: The sensory physiology of blood-sucking arthropods. Experimental Parasitology 6: 68-122.

____. **1976**. The Hungry Fly. Harvard University Press. Cambridge, MA.

Dethier, V.G., Solomon, R.L., and Turner, L.H.. 1965. Sensory input and central excitation and inhibition in the blowfly. Journal of Comparative Physiology and Psychology 60: 303-313.

Duve, H., Rehfeld, J. F., East, P., and Thorpe, A. 1994. Localization of sulfakinin neuronal pathways in the blowfly, *Calliphora vomitoria*. Cell and Tissue Research 275: 177-186.

Freeman, J. V., and Hansens, E. J. 1972. Collecting larvae of the salt marsh greenhead *Tabanus nigrovittatus* and related species in New Jersey: comparison of methods. Environmental Entomology 1: 653-658.

Friend, W. G., and Smith, J. J. B. 1977. Factors affecting feeding by bloodsucking insects. Annual Review of Entomology 22: 309-331

Friend, W. G., and Stoffolano, J. G., Jr. 1983. Feeding responses of the horse fly, *Tabanus nigrovittatus*, to phagostimulants. Physiological Entomology 8: 377-383.

_____. **1984**. Feeding responses of the horse fly, *Tabanus nigrovittatus*, to physical factors, ATP analogues and blood fractions. Physiological Entomology 9: 395-402.

_____. **1991**. Feeding behavior of the horse fly, *Tabanus nigrovittatus* (Diptera: Tabanidae): effects of dissolved solids on ingestion and destination of sucrose or ATP diets. Physiological Entomology 16: 35-45.

Foil, L. D., and Hribar, L. J. 1995. Evaluation of tsetse attractants as baits for horse flies and deer flies (Diptera: Tabanidae) in Louisiana. Florida Entomology 78: 129-133.

Fonágy, A., Schoofs, L., Proost, P., Van Damme, J., and DeLoof, A. 1992. Isolation and primary structure of two sulfakinin-like peptides from the flesh fly, *Neobellieria bullata*. Comparative Biochemistry and Physiology. C, Comparative Pharmacology and Toxicology 103: 135-142.

Gatehouse, A. G. 1970. The probing response of *Stomoxys calcitrans* to certain physical and olfactory stimuli. Journal of Insect Physiology 16: 61-74.

Gelperin, A. 1967. Stretch receptors in the foregut of the blowfly. Science 157: 208-210.

____. 1971. Regulation of feeding. Annual Review of Entomology 16: 365-378.

Geary, N. 2004. Endocrine controls of eating: CCK, leptin, and ghrelin. Physiology and Behavior 81: 719-733.

Graham, N., and Stoffolano, J. G., Jr. 1983. Relationship between female size, type of egg mass deposited, and description of the oviposition behavior of the sibling species *Tabanus nigrovittatus* and *T. simulans* (Diptera: Tabanidae). Annals of the Entomological Society of America 76: 699-702.

Gwadz, R. 1969. Regulation of blood meal size in the mosquito. Journal of Insect Physiology 15: 2039-2044.

Haselton, A. T. 2005. Myotropic peptide hormones and serotonin in the regulation of feeding in the adult blow fly *Phormia regina*, and the adult horse fly *Tabanus nigrovittatus*. Dissertation. University of Massachusetts, Amherst.

Haselton, A. T., Yin, C.-M., and Stoffolano, J. G., Jr. 2006. The effects of *Calliphora vomitoria* tachykinin-I and the FMRFamide-related peptide perisulfakinin on female *Phormia regina* crop contractions, *in vitro*. Journal of Insect Physiology (*in press*).

Hayes, M. R., Chory, F. M., Gallagher, C. A., and Covasa, M. 2006. Serotonin trype-3 receptors mediate cholecystokinin-induced satiation through gastric distension. American Journal of Physiology-Regulatory Integrated Comparative Physiology (*in press*).

Hayes, M. R., and Covasa, M. 2005. CCK and 5-HT act synergistically to suppress food intake through simultaneous activation of CCK-1 and 5-HT3 receptors. Peptides 26: 2322-2330.

Hayes, M. R., Moore, R. L., Shah, S. M., and Covasa, M. 2004. 5-HT3 receptors participate in CCK-induced suppression of food intake by delaying gastric emptying. American Journal of Physiology- Regulatory Integrated Comparative Physiology 287: 817-823.

Hayes, R. O., Doane, O. W., Jr., Sakolsky, G., and Berrick, S. 1993. Evaluation of attractants in traps for greenhead fly (Diptera: Tabanidae) collections on a Cape Cod, Massachusetts, salt marsh. Journal of the American Mosquito Control Association 9: 436-440.

Hocking, B. 1971. Blood-sucking behavior of terrestrial arthropods. Annual Review of Entomology 16: 1-26.

Hopkins, B. A. 1964. The probing response of *Stomoxys calcitrans* (L.) (the stable fly) to vapours. Animal Behavior 12: 513-524.

Jaenson, T. G., Dos Santos, R. C., and Hall, D. R. 1991. Attraction of *Glossina longipalpis* (Diptera: Glossinidae) in Guinea-Bissau to odor-baited biconical traps. Journal of Medical Entomology 28:284-286.

Klun, J. A., Strickman, D., Rowton, E., Williams, J., Kramer, M., Roberts, D., and Debboun, M. 2004. Comparative resistance of *Anopheles albimanus* and *Aedes aegypti* to N,N-diethyl-3-methylbenzamide (Deet) and 2-methylpiperidinyl-3-cyclohexen-1-carboxamide (A13-37220) in laboratory human-volunteer repellent assays. Journal of Medical Entomology 41: 418-422.

Konan, Y. L, Sylla, M. S., Doannio, J. M., and Traore, S. 2003. Comparison of the effect of two excipients (karate nut butter and Vaseline) on the efficacy of *Cocos nucifera*, *Elaeis guineensis* and *Carapa procera* oil-based repellents formulations against mosquitoes biting in Ivory Coast. Parasite 10: 181-184.

Maestro, J., Aguilar, R., Pascual, N., Valero, M. L., Piulachs, M. D., Andreu, D., Navarro, I., and Bellés, X. 2001. Screening of antifeedant activity in brain extracts led to the identification of sulfakinin as a satiety promoter in the German cockroach. European Journal of Biochemistry 268: 5824-5830.

Magnarelli, L. A., and Stoffolano, J. G., Jr. 1980. Blood feeding, oogenesis, and oviposition by *Tabanus nigrovittatus* in the laboratory. Annals of the Entomological Society of America 73:14-17.

Mather, T. N., and Defoliart, G. R. 1984. Reduced feeding success on squirrels and chipmunks by older *Aedes triseriatus* females. Mosquito News 44: 471-475.

Meola, R. W., and Petralia, R. S. 1980. Juvenile hormone induction of biting behavior in *Culex* mosquitoes. Science 209: 1548-1550.

Nachman, R. J., Holman, G. M., Haddon, W. F., and Ling, F. 1986. Leucosulfakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. Science 234: 71-73.

Nachman, R.J., Isaac, R.E., Coast, G.M., and Holman, G.M. 1997. Aib-containing analogues of the insect kinin neuropeptide family demonstrate resistance to an insect angiotensin-converting enzyme and potent diuretic activity. Peptides 18: 53-57.

Nichols, R. 1992. Isolation and structural characterization of *Drosophila* TDVDHVFLRFamide and FMRFamide-containing neural peptides. Journal of Molecular Neuroscience 3: 213-218.

____. 2003. Signaling pathways and physiological functions of *Drosophila melanogaster* FMRFamide-related peptides. Annual Review of Entomology 48: 485-503.

O'Meara, G. F. 1987. Nutritional ecology of blood-feeding Diptera. In S. Slanksy, Jr. and J. G. Rodriguez (eds.), Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates. John Wiley and Sons Inc., USA. Pages 741-764.

Perich, M. J., Strickman, D., Wirtz, R. A., Stockwell, S. A., Glick, J. I., Burge, R., Hunt, G., and Lawyer, P. G. 1995. Field evaluation of four repellents against *Leptoconops americanus* (Diptera: Ceratopogonidae) biting midges. Journal of Medical Entomology 32: 306-309.

Rice, M. J. 1972. Recent Advances in Physiology of Tsetse flies and other Hematophagous Diptera. In C. Anderson and W. L. Kilama (eds.), Parasitoses of Man and Animals in Africa. Proceedings of the East African Medical Research Council Scientific Conference, Chapter 5, Paper 4.

Rudolfs, W. 1922. Chemotropism of mosquitoes. Bulletin of the New Jersey Agriculture Experimental Station 367: 1-23.

SAS Institute Inc. 2005. JMP Start Statistics, version 3rd ed. Brooks/Cole-Thomson Learning, Belmont, CA.

Schoofs, L., Holman, G. M., Hayes, T., and DeLoof, A. 1990. Isolation and identification of a sulfakinin-like peptide with sequence homology to vertebrate gastric and cholecystokinin, from the brain of *Locusta migratoria*. In A. R. McCaffey and I. D. Wilson (eds.), Chromatography and Isolation of Insect Hormones and Pheromones. New York, Plenum. Pages 231-241.

Schoofs, L., and Nachman, R. J. 2006. Sulfakinins. Handbook of Biologically Active Peptides. Elsevier, San Diego. (*in press*).

Simpson, S. J., and Bernays, E. A. 1983. The regulation of feeding: locusts and blowflies are not so different from mammals. Appetite 4: 313-346.

Stoffolano, J. G., Jr. 1974. Influence of diapause and diet on the development of gonads and accessory reproductive glands of the black blowfly, *Phormia regina* (Meigen). Canadian Journal of Zoology 52: 981-988.

____. 1979. Comparison of methods for feeding blood to *Tabanus nigrovittatus* in the laboratory. Annals of the Entomological Society America 72: 216-217.

____. 1983. Destination of the meal and the effect of a previous sugar or blood meal on subsequent feeding behavior in female *Tabanus nigrovittatus* (Diptera: Tabanidae). Annals of the Entomological Society America 76: 452-454.

Stoffolano, J. G., Jr., Angioy, A. M., Crnjar, R., Liscia, A., and Pietra, P. 1990. Electrophysiological recordings from the taste chemosensilla of *Tabanus nigrovittatus* (Diptera: Tabanidae) and determination of behavioral tarsal acceptance thresholds. Journal of Medical Entomology 27: 14-23.

Stoffolano, J. G., Jr., and Majer, J. 1997. Comparison between tsetse fly synthetic diet and bovine blood on reproduction and survivorship in *Tabanus nigrovittatus* (Diptera: Tabanidae). Journal of Medical Entomology 34: 738-740.

Strader, A. D., and Woods, S. C. 2005. Gastrointestinal hormones and food intake. Gastroenterology 128: 175-191.

Takken, W., and Kline, D. L. 1989. Carbon dioxide and 1-octen-3-ol as mosquito attractants. Journal of the American Mosquito Control Association 5: 311-316.

Thompson, B. H. 1976. Studies on the attraction of *Simulium damnosum* s. I. (Diptera: Simulidae) to its hosts. I. The relative importance of sight, exhaled breath, and smell. Tropenmedizin Und Parasitologie 4:455-473.

Thompson, A. J., and Holling, C. S. 1974. Experimental component analysis on blowfly feeding behaviour. Journal of Insect Physiology 20: 1553-1563.

Thompson, P. H., and Krauter, P. C. 1978. Rearing of Texas Tabanidae (Diptera). I. Collection, feeding, and maintenance of coastal marsh species. Proceedings of the Entomology Society of Washington 80: 616-625.

Tully, T., and Hirsch, J. 1983. Two nonassociative components of the proboscis extension reflex in the blow fly, *Phormia regina*, which may affect measures of conditioning and of the central excitatory state. Behavioral Neuroscience 97: 146-153.

Vale, G. A. 1977. The flight of tsetse flies to and from a stationary ox. Bulletin of Entomological Research 67: 297-303.

____. **1979.** Field responses of tsetse flies (Diptera: Glossinidae) to odours of men, lactic acid and carbon dioxide. Bulletin of the Entomology Research 69: 459-467.

____. **1980**. Field studies on the responses of the tsetse flies (Glossinidae) and other Diptera to carbon dioxide, acetone and other chemicals. Bulletin of Entomological Research 70: 563-570

Vale, G. A., and Hall, D. R. 1985. The role of 1-oten-3-ol, acetone, and carbon dioxide in the attraction of tsetse flies, *Glossina* spp. (Diptera: Glossinidae) to ox odour. Bulletin of Entomological Research 75: 209-217.

Veenstra, J. A. 1989. Isolation and structure of two gastric/cholecystokinin-like neuropeptides from the American cockroach homologous to the leucosulfakinins. Neuropeptides 14: 145-149.

Veenstra, J. A., Lau, G. W., Agricola, H. J., and Petzel, D. H. 1995. Immunohistological localization of regulatory peptides in the midgut of the female mosquito *Aedes aegypti*. Histochecmical Cell Biology 104: 337-347.

Warnes, M. L., and Finlayson L. H. 1985. Responses of the stable fly, *Stomoxys calcitrans* (Diptera: Muscidae) to carbon dioxide and other host odors II. Orientation. Bulletin of Entomological Research 75: 717-727.

Wei, Z., Baggerman, G., Nachman, R. J., Goldsworthy, G., Verheart, P., De Loof, A., and Schoofs, L. 2000. Sulfakinins reduce food intake in the desert locust, *Schistocerca gregaria*. Journal of Insect Physiology 46:1259-1265.