The Effect of Mating on the Pheromone System of the Yellow Mealworm Beetle, *Tenebrio molitor*.

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ABSTRACT

The mating behavior of the Yellow Mealworm beetle (*Tenebrio molitor*) is mediated by several pheromones, including the female produced sex pheromone, (R)-(+)-4-methylnonanol. 4-Methylnonanol serves both as a volatile male attractant and as a synergist for a non-volatile copulation-release pheromone. The objective of this study was to investigate the effect of mating on the activity of female extracts. Glass rod bioassays were conducted to compare the activity of extracts prepared from female beetles at various times after mating. Mating caused an immediate but temporary (less than one hour) inhibition of attraction. This was likely due to the deposition of an anti-aphrodisiac by the male that wore off relatively rapidly. Extracts prepared 0.5-1 hour after mating were often attractive but within 2 hours after mating a second inhibition of attraction was observed. This second effect was likely due to a drop in 4-methylnonanol titre. Additional bioassay results indicate that a longer mating interval, with multiple copulations, amplifies this drop in pheromone titre. Recent findings concerning sperm competition among *T. molitor* suggest that these mating effects could be of great adaptive significance. Preliminary *in vivo* and *in vitro* assays were conducted to determine if the decline in pheromone titre was due to a decrease in pheromone biosynthesis, an increase in pheromone internalization, or both. In the long term, a greater understanding of the regulation of pheromone production could be useful for the manipulation of pest populations.

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1. INTRODUCTION

Mate attraction is a process with almost as many mechanisms as there are numbers of species. It includes methods as diverse as the colorful plumage of the peacock to the pattern of light flashes in fireflies. The most common method in insects and many animals involves invisible chemicals called "pheromones" (Roelofs, 1995). Pheromones are substances that are secreted by one individual and perceived by another individual (of the same species) where they cause some specific effect, for example, a behavioural response (Karlson and Luscher, 1959). They resemble hormones in that they are produced and secreted by special glands (or specialized cells) but instead of being secreted into the blood they are released from the body. In effect, they represent a kind of intercellular communication at the organismal level with the signal and receptor in different individuals.

Insect pheromones can be single compounds or made up of highly specific blends of optical or geometric isomers (for review see Mori, 1996). Insect pheromones can be divided into at least six different behaviourally functional classes including: sex, aggregation, dispersal, alarm, recruitment, and maturation (Tillman *et al*., 1999).

This study involves the sex pheromones of the storage pest *Tenebrio molitor*, the Yellow Mealworm beetle, and specifically looks at their involvement in the events immediately following mating. This is an area of current interest as most studies to date have focused on the regulation and effects of pheromones before mating. *T. molitor* is a model organism for study for several reasons: it is easy to maintain cultures of the beetle in the lab; the beetles are large enough to manipulate surgically; and a large amount of information has been gathered in numerous fields of study using *T. molitor*. Another factor

that makes this beetle an interesting study subject is its multi-faceted pheromone communication system.

The mating behaviour of *T. molitor* is thought to be mediated by at least four types of sex pheromones including a female-produced male attractant (Valentine, 1931; Tschinkel *et al*., 1967; August, 1971), a male produced female attractant (Happ, 1969; August, 1971; Menon, 1976), at least one copulation release (CR) pheromone (Tanaka *et al*., 1986), and a male produced anti-aphrodisiac (Happ, 1969). The female produced attractant was identified by Tanaka *et al*. (1986, 1989) as (R)-(+)-4-methyl-1-nonanol.

It has been determined by bioassays that newly emerged females produce very little sex attractant but production increases as they mature until a peak around 4 or 5 days post-eclosion (Valentine, 1931; Happ and Wheeler, 1969). A parallel situation exists for the male with the male produced attractant reaching effective levels around the same time (5-8 days post-eclosion) (Menon, 1976). The two pheromones seem to work in concert to bring the two sexes together. The situation is further complicated by the actions of one or more CR pheromones. August (1971) reported that when the male's mouthparts were covered with wax, stimulation could still occur and the male attempted copulation but the genitalia were not extended. This suggests the presence of a non-volatile CR pheromone. Either 4-methylnonanol or the CR pheromone alone can elicit a copulation response at high concentrations but it is thought that under normal conditions the former acts as a synergist for the latter to release copulation (Tanaka *et al*., 1986; Hastings and Vanderwel, unpublished). The anti-aphrodisiac was suggested by Happ (1969) as a mechanism for one male to inhibit the response of other males to female scent. To summarize, the two attractants allow the male and female to find each other, 4-methylnonanol and the CR

pheromone together release the copulation response, and an anti-aphrodisiac prevents other males from finding the male's mating partner.

Studies of several insects have shown that pheromone production and release is regulated by many factors including both neural and endocrine mediated signals (for review see Schal *et al*., 1997). Regulatory cues include age of sexual maturity, migration, photoperiod, and host plant chemicals to name a few (Raina, 1988, 1997; McNeil *et al*., 1997). The effect of mating on pheromone production has been examined in several cockroach and moth species. A termination and/or decline of pheromone release in response to mating has been reported for the German cockroach, *Blatella germanica* and brown-banded cockroach, *Supella longipalpa* (Schal *et al*., 1997); the corn earworm moth, *Heliothis zea* (Raina, 1988); the gypsy moth, *Lymantria dispar* (Giebultowicz *et al*., 1991); the silkworm moth, *Bombyx mori* (Ando *et al*., 1996); and, the spruce budworm moth, *Choristoneura fumiferana* and obliquebanded leafroller moth, *Choristoneura rosaceana* (Delisle *et al*., 2000). In all of these cases, the mating-induced drop in pheromone levels could be due to decreased pheromone production or increased pheromone degradation (Kingan et al., 1995) and is thought to be triggered by either the mechanical stimulus of mating (neural) or some pheromonostatic factor (humoral) transferred by the male (Raina, 1988, 1997; Kingan *et al*., 1995). In some moths, longer mating durations led to a complete inhibition of pheromone production (Ando *et al*., 1996). As it becomes clear that insects do regulate their pheromones and behaviour in response to mating the focus has shifted to investigating the adaptive advantages of these mechanisms.

The adaptive significance of the regulation of post-copulatory behaviour is best considered from male and female perspectives separately. For the male, any mechanism

that inhibits further mating with his mate is likely an adaptation for improved sperm competition. Sperm competition was first defined as the competition within a single female between the sperm of two or more males for fertilization of the ova (Parker, 1970). Sperm competition mechanisms can be as important in sexual selection as the more familiar behavioural mechanisms usually characterized by male-male competition and female choice. Sperm competition has been suggested as an explanation for the evolution of internal fertilization as well as numerous other adaptations including the size differentiation between male and female gametes (Birkhead, 1996). In insects, multiple mating by the female (polyandry) has been observed in many species (Ridley, 1990) and evidence for sperm competition is available for at least five insect orders: Coleoptera, Diptera, Orthoptera, Hymenoptera, and Lepidoptera (for review, see Parker, 1970).

Studies of *T. molitor* have identified several sperm competition mechanisms at work. Gage and Baker (1991) found that males in the presence of a rival male increased ejaculate size, initiated mating more quickly and were also more likely to practice mate-guarding after copulation. These represent attempts to ensure first male sperm precedence (P_1). Gage (1992) suggested another mechanism in which males remove the sperm from a previous mating using penile spines in a 'scouring' mechanism. This should help to ensure second male sperm precedence (P_2), especially when the mating interval is short. Siva-Jothy et al. (1996) found that P_2 was high whether the mating interval was short (<10min) or long (24h). From this they concluded that sperm removal was not the primary mechanism by which *T. molitor* males ensure a high P_2. Drnevich *et al*. (2000) propose another mechanism that can actually ensure complete second male sperm precedence (P_2 = 1) if the mating interval is shorter than 5 minutes. They suggest that for these very short

mating intervals, the release of sperm from the first male's spermatophore is completely suppressed by the second mating. Other mechanisms have yet to be discovered to explain the $P_2 > 0.5$ that occurs for longer mating intervals. The high level of second male sperm precedence in *T. molitor* suggests that any mechanism that inhibits further mating by the female would be selectively favored.

The adaptive significance of regulated mating is less obvious for the female. Initially, sperm competition mechanisms were considered primarily from the male's point of view with the female taking a more passive role. More recently, the understanding of sperm competition has expanded to include the many adaptations associated with multiple matings by the female and the idea that females may have some physiological control over the paternity of their offspring after insemination (Birkhead, 1995). Some of the mechanisms practiced by the male during sperm competition may actually be disadvantageous to the female. Keller and Reeve (1995) suggest that females may mate with more than one male to ensure their eggs are fertilized by the highest quality sperm. The sperm from the male with the highest sperm quantity, fertilizing ability, or ability to out-compete another male's sperm would be most desirable if these traits can be passed on to offspring. Thus, it may be disadvantageous for the female's receptivity to be inhibited after one mating (as the male would prefer). However, once adequate (quantity and quality) sperm stores have been obtained, decreased pheromone levels could be a method of conserving energy and resources for egg production and laying.

The effect of mating on the production of pheromones in *T. molitor* has been examined previously (Happ, 1969; Happ and Wheeler, 1969) but only in a preliminary manner. Happ and Wheeler (1969) reported that mating accelerates pheromone production

in isolated females but inhibits it in crowded females. They also report that pheromone production in mated females continues to rise for several days after mating before falling with the onset of egg laying. Previous bioassays conducted in our lab (Vakili and Vanderwel, unpublished) indicate that there is a transient (about 12 hours) decrease in the CR activity of extracts prepared from mated females. No inhibition of CR activity was observed from extracts prepared 0.5 hour after mating. From this it was concluded that the observed decrease in activity was not due to an anti-aphrodisiac as reported by Happ (1969). Gas chromatographic analyses indicated that 4-methylnonanol levels in mature virgin females dropped from 40 ng per female to below our level of detection after mating. Since 4-methylnonanol acts as a synergist with the CR pheromone to release copulation it is possible that the drop in CR activity observed may actually be due to a decrease in 4-methylnonanol titre.

In this study, bioassays were conducted to investigate the effect that mating has on the pheromone bouquet of the female *T. molitor*. The basic procedure for a bioassay is to coat a glass rod with the surface extract of a female and observe the response of a virgin male to that rod. Since copulation release is not the primary action of 4-methylnonanol, the bioassays conducted in this study were modified to measure attraction and retention to the rods as well as ability to elicit a CR response. Furthermore, extracts were prepared from females immediately after mating to investigate the possibility of a short-lived anti-aphrodisiac. The effect of longer mating durations was also examined by bioassay. It was expected that the presence of an anti-aphrodisiac or a decrease in 4-methylnonanol titre would be observed as an inhibition of attraction as well as CR activity. Longer mating durations may amplify this inhibition.

Bioassay data can help to identify the effects of mating but in order to examine the mechanisms of these effects, more precise techniques are required. If, for example, 4-methylnonanol titre is being regulated, biochemical assays could be performed to determine the mechanism of this regulation. Mature Yellow Mealworms produce 4-methylnonanol through a modification of normal fatty acid biosynthesis. The biosynthetic route for this production was elucidated by Islam *et al*. (1999) and is shown in figure 1. The regulation of 4-methylnononal is thus an obvious starting point for biochemical study, because the structure of the female-produced male-attractant (4-methylnonanol) and its precursor (4-methylnonanoic acid) are known and radioactively labeled versions available.

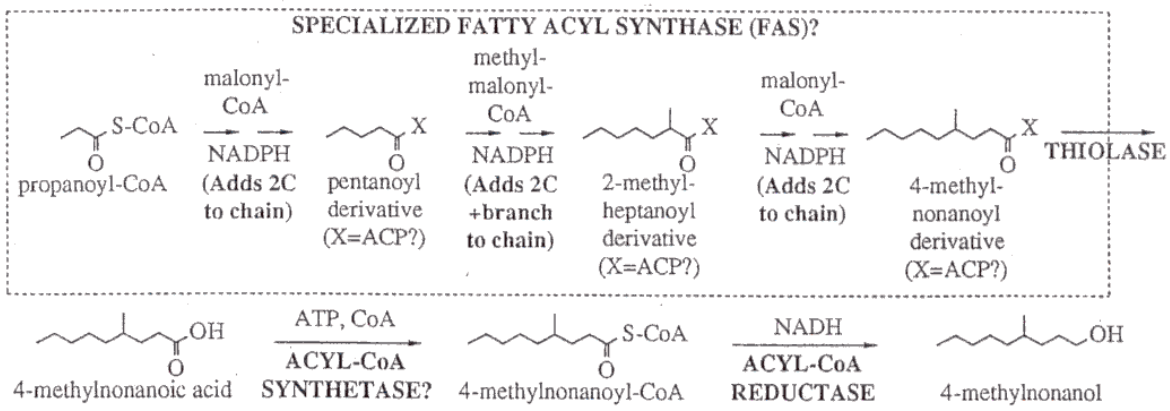

Figure 1: Putative biosynthetic route to 4-methylnonanol in female Yellow Mealworms. "ACP" = acyl carrier protein; "?" = aspect of pathway that must be confirmed. (Islam et al., 1999)

The first biochemical assay compared virgin and mated female beetles for their ability to internalize tritium labeled 4-methylnonanol. If females reduce their effective pheromone levels by internalizing the pheromone we would expect more label inside and less to be extracted from the surface. Next, the regulation of 4-methylnonanol biosynthesis

was examined. The biosynthesis of 4-methylnonanol could be regulated at many of the enzyme catalyzed steps shown in figure 1. The regulation of the last two steps was examined by *in vitro* and *in vivo* assays. First, the synthesis of ^{3}[H]4-methylnonanoyl CoA was attempted in the hope that crude homogenates of mated and virgin female beetles could be compared for their ability to reduce the ^{3}[H]4-methylnonanoyl CoA to ^{3}[H]4-methylnonanol (the *in vitro* assay). The ability of crude homogenates to synthesize ^{3}[H]4-methylnonanol from ^{3}[H]4-methylnonanoic acid was also assayed *in vitro*. *In vivo* assays were conducted to compare the ability of living virgin and mated females to make this same conversion after direct injection of ^{3}[H]4-methylnonanoic acid. We hypothesize that if either of the last two enzymatic steps in the biosynthesis of 4-methylnonanol is down-regulated by mating, this should result in decreased levels of labeled pheromone produced in the mated beetles.

A greater understanding of the effect of mating in Yellow Mealworms could contribute to a greater understanding of sperm competition mechanisms and the mating behaviours of other insects. In the long term, an understanding of the regulation of pheromone biosynthesis in *T. molitor* could be important for the implementation of new pest management strategies. Compounds that disrupt the mating systems of moths have already shown great promise in this area (for review see Minks and Carde, 1997; Wyatt, 1997).

2. METHODS

2.1 Rearing of Beetles

Tenebrio molitor larvae were obtained from Northwest Scientific Supply Ltd., Victoria, B.C.. The larvae were kept in large plastic containers with screened lids and fed with 4:2:1 wheat bran:rolled oats:wheat germ. Moisture was provided by moistening paper towels on the surface of the food (3-4 times weekly), or keeping sliced potatoes in the containers. Conditions were maintained at 28-30°C, 30-50% humidity, with a photoperiod of 12 hours light and 12 hours dark. The colonies were checked regularly, pupae removed, and their sex determined according to the method described by Bhattacharya and Waldbauer (1970). Males and females were maintained in separate containers in order to ensure virginity. The pupae were maintained in the same conditions as the larvae, checked regularly (every 2-3 days) and separated upon eclosion (emergence as adult). Emerged beetles were maintained under the same conditions as the larvae and pupae until they reached the appropriate age. In this way, virgin beetles of determined sex and age were obtained. Only mature beetles (5-10 days post-eclosion) with no obvious physical deformities were used in experiments.

2.2 Bioassays

Unless otherwise stated, all bioassays were conducted in an environment-controlled chamber at 25°C (±2°C), 50% humidity (±10%) and under a red heat lamp since the beetles are strongly photonegative to other wavelengths (Tschinkel, *et al*., 1967). All beetles were

allowed to acclimatize for at least 30 minutes before any bioassays were initiated. Experiments were conducted during the dark period typically near the end of it.

2.2.1 Single Mating Experiments

Mating: Male beetles were marked with a small spot of all-purpose liquid paper on their backs and given 30 seconds to dry. Each marked male was then placed under a glass petri dish with an unmarked female and allowed to mate. Mating was defined as the curving of the male abdomen under the female with a clear extension of the male genitalia (Tschinkel *et al*., 1967). Once a single clear mating event had occurred and the male and female had naturally separated the female was removed. Mated female beetles were extracted at 0, 0.5, 1 or 2 hours after removal from the petri dishes. The "0-hour" beetles were extracted immediately upon completion of the mating event whereas the "0.5-", "1-" and "2-hour" beetles were first kept in 20 mL vials with about 5 mL food for the appropriate amount of time. Virgin females were also extracted to provide a positive control. Pentane was used as a negative control.

Extraction: Each beetle was extracted in a 10 mL sample vial with 0.5 mL pentane. The vials were vortexed for 15 seconds, allowed to sit for 15 minutes, and then vortexed again for 15 seconds. The beetles were then removed and the extracts stored at –20°C in their sample vials with screw caps tightened.

Assay for extract activity: The bioassays were generally performed with matings and extractions conducted in the morning, and assays conducted in the afternoon of the same

day. Male beetles (5-10 days post-eclosion) were moved from incubation chambers to the environment-controlled chamber (same conditions as in mating procedure). Six male beetles were each placed under a glass petri dish and allowed to acclimatize for 15 minutes. The six extracts (pentane, virgin, 0 hour, 0.5 hour, 1 hour, and 2 hour) were randomized by one experimenter by covering their identities with stickers randomly labeled #1-6 so that the second experimenter did not know the identity of the extract in each assay. The first experimenter then coated glass rods with 30μL of each extract using a syringe, waited for 30 seconds to allow the pentane to evaporate, and placed them centrally under the petri dishes. The second experimenter recorded the start time and observed the male beetle's responses to the extract. Time and duration of all touches and copulation attempts were recorded for a 10 minute period for each extract. Three trials of these bioassays were conducted, twice with three repetitions and once with four repetitions of the procedure above.

2.2.2 Multiple Mating Experiments

These experiments were conducted using the same procedure for mating, extraction and assay as above except the number of times the female mated was varied instead of the amount of time after a single mating. In the first trial, 5 repetitions were used to compare extracts from female beetles after single matings (conducted as in 2 hour treatment above) and after a one hour mating duration (3-4 matings). The usual pentane and virgin controls were included. In the second trial, another 5 repetitions of these four treatments were performed but a 21 hour mating duration treatment was added as well. The once-mated beetles were extracted 2 hours after mating. The multiple-mated beetles were extracted 1

hour after the mating period was terminated or approximately 2 hours after the first mating. The 21 hour mated beetles were extracted 1 hour after the mating period was terminated or approximately 22 hours after the first mating. In all three cases extractions were performed at least one hour after the last mating and at least 2 hours after the first mating.

2.2.3 Analysis of Bioassay Results

For both the single and multiple mating experiments the data were analyzed as follows: Means of total touch time, total copulation time, first touch time and first copulation time were determined for all controls and treatments. The data were analyzed for treatment effects, block effects (where the block represents the day of each bioassay trial) and treatment*day interactions. This involved a type of analysis of variance (ANOVA) called a randomized complete block design that examines differences between treatments with block (day) as a factor. The analysis was performed using the SAS statistical package. If the F-test was significant for the treatment effect and no treatment*day interaction was present a Duncan's multiple range test was applied to determine which treatments were significantly different. If there were significant treatment*day interactions, an Orthogonal contrast test was conducted to determine the nature of these interactions for each block(day) individually.

2.3 Biochemical Assays

2.3.1 Purchased Chemicals

General: All solvents were purchased from BDH (Toronto, Ontario, Canada) or EM Science (Gibbstown, New Jersey, USA) and were of spectrophotometric grade or better.

For column chromatography, florisil (100-200 mesh, gas chromatography grade) was obtained from Spectrum Quality Products (Gardena, California, USA). The liquid scintillation cocktails Scintisafe Plus 50% and Scintisafe Econo 2 were obtained from Fisher Scientific (Fairlawn, New Jersey, USA). All other reagents were obtained from BDH (Toronto, Ontario, Canada) or Sigma (Oakville, Ontario, Canada) and were of ACS grade or better.

Synthesis of Tritium Labeled 4-Methylnonanoyl CoA: ^{3}[H]4-Methylnonanoic acid was custom-synthesized by Chemsyn Laboratories (Lenexa, Kansas, USA). Tris HCL and Tergitol 15-S-9 were purchased from Sigma (Oakville, Ontario, Canada). 5'Adenosine triphosphate (ATP, dipotassium salt) and Coenzyme A (CoA, trilithium salt dihydrate) were purchased from Calbiochem (San Diego, California, USA). Magnesium chloride hexahydrate was supplied by BDH. Coenzyme A synthetase (from Pseudomonas sp) was purchased from ICN Biomedicals Inc. (Aurora, Ohio, USA). Silica gel TLC plates (60 mesh, 250μm thickness) were purchased from EM Separations Technology (Gibbstown, New Jersey, USA).

***In vitro* Assay:** Nicotinamide adenine dinucleotide (NADH, disodium salt) nicotinamide adenine dinucleotide phosphate (NADPH, sodium salt), glycerol and protease inhibitor cocktail (for mammalian cell extracts) were purchased from Sigma (Oakville, Ontario, Canada). Zwittergent was purchased from Calbiochem (San Diego, California, USA).

2.3.2 Synthesis of Tritium Labeled 4-Methylnonanoyl CoA

The 4-methylnonanoyl CoA derivative was prepared using the enzymatic synthesis method described in Bacala (2000). ^{3}[H]4-Methylnonanoic acid was incubated with 3% (v/v) Tergitol 15-s-9, 60 mM $MgCl_2$, 40 mM ATP, 20 mM CoAS-H and 1 unit of Acyl Coenzyme A synthetase in Tris buffer (pH 8.0, 0.5 M) at 30°C. Thin layer chromatography (TLC) was used to estimate the percentage of successful conversion. TLC plates were spotted with a capillary tube and developed with 100:1 (diethyl ether:acetic acid). Once developed, the TLC plate was cut into strips and each strip counted in 5 mL Scintisafe Plus 50% on a Beckman LS6500 scintillation counter. Unreacted fatty acid migrates with the solvent front and acyl-CoA remains at the origin. Once sufficient incubation had occurred, the reaction was terminated with 25 μL concentrated HCL. The mixture was concentrated to 450 μL under a gentle N_2 stream and 50 μL acetonitrile added. The incubation mixture was then analyzed by high performance liquid chromatography (HPLC) with a solvent system of 95:5 citrate:acetonitrile, running at 2.0 mL/minute, with a gradient to 5:95 citrate:acetonitrile initiated at 17 minutes (2 %/minute). The column used was a Zorbax SB-C18 9.4 mm x 25 cm reverse-phase preparatory column.

2.3.3 Pheromone Internalization Assay

Assays were performed on mated and virgin females. Beetles were mated as per the procedure outlined for bioassays (2.2.1). Each female beetle (mated or virgin) was coated with 5 μL of ^{3}[H]4-methylnonanol in hexane (≈200,000 dpm) over the entire under side of the beetle. Coated beetles were kept in a scintillation vials for 15 minutes, then extracted. Extractions were performed exactly as described for the bioassays (2.2.1) to remove all

surface pheromones. Once the surface pheromones were extracted, the beetle was transferred to another vial containing scinti 50% plus fluid. The beetle was homogenized so that any ^{3}[H]4-methynonanol or degradation products that had been internalized could be measured. Internal and surface extractions were measured for radioactive levels on the Beckman LS6500 scintillation counter and mean levels compared by the two-sample Student's t-test (two-tailed).

2.3.4 *In vitro* Assay

Incubation: The initial plan was to incubate crude homogenates with ^{3}[H]4-methylnonanoyl CoA. However, the attempts to synthesize the CoA derivative were unsuccessful (as described in results) so the earlier precursor ^{3}[H]4-methylnonanoic acid was provided instead. Assays were performed on mated female beetles, virgin female beetles, and no protein controls. Mating was conducted exactly as described for the bioassay procedure (2.2.1) and the beetles were not assayed until at least 2 hours after mating. Each assay tube contained 2 mM ATP, 60 μM CoASH, 1 mM NADH, 1 mM NADPH, 20 μL protease inhibitor, and 0.05 mM zwittergent in 1mL homogenization buffer (50 mM phosphate, 1 mM DTT, 50 mM KCl, 10% glycerol (v/v), pH 7.4). The assay was initiated by the addition of 50 μL ^{3}[H]4-methylnonanoic acid (≈1,000,000 dpm). A beetle (virgin or mated) was added to each assay tube (except no protein controls) and crushed into a crude homogenate. The homogenate was then incubated for 30 minutes at 30°C on a shaking tray. The reaction was killed with 1 mL of 20% TCA and 1 mL of hexane added to prevent evaporation of 4-methylnonanoic acid or 4-methylnonanol. The crude homogenate was stored in the freezer (-20°C).

Extraction: Crude homogenates were transferred to centrifuge tubes and the beetle remains rinsed with two 1 mL volumes of hexane. The homogenate (plus hexane washes) was then extracted with three 1 mL volumes of hexane. Combined organic layers were washed with 1mL 10% aqueous Na_2SO_4 and dried on anhydrous Na_2SO_4.

Column Separation: Extracts of crude homogenates were run on 5g of 100-200 mesh florisil in a 50 mL glass burette (1cm diameter). Elution was performed by 50 mL 70:30 hexane:diethyl ether followed by 50 mL 100:1 ether:glacial acetic acid, and 10 mL fractions collected. The quantity of 4-methylnonanoic acid and 4-methylnonanol for each elution series was determined by adding 10 mL scintisafe Econo 2 to each fraction, vortexing, and counting in the LS6500 scintillation counter. The mean amount of 4-methylnonanoic acid converted to 4-methylnonanol was compared for mated, virgin, and no protein control by two-sample Student's t-test.

2.3.5 *In vivo* Assay

Assays were performed on mated and virgin females. Matings were performed as in the bioassay procedure (2.2.1). The beetles were not assayed until at least 2 hours after mating. Each beetle (mated or virgin) was injected with 0.5 μL (≈100,000 dpm) of ^{3}[H]4-methylnonanoic acid between the thorax and abdomen using a 2.5 μL syringe. Injected beetles were allowed to incubate at 30°C for 30 minutes before extraction.

Centrifuge tube extraction procedure: The following procedure is for two beetles per tube. Volumes were adjusted proportionally for larger numbers. Beetles were crushed in a 20 mL scintillation vial in 2.0 mL 3:2 pentane:isopropanol with a blunt glass rod. The

organic layer was transferred to a centrifuge tube and the beetle remains extracted three more times with 1.0 mL 3:2 pentane:isopropanol. The combined organic layers were washed with 5 mL 0.1M HCL in 10% aqueous Na_2SO_4, vortexed at high speed for 15-30 seconds, and centrifuged at ¾ speed (2500 rpm) for 10 minutes in an IEC HN-SII bench top centrifuge. The aqueous layer was then back-extracted three times with 2.0 mL pentane using the same vortexing and centrifuging technique. All organic layers were combined and then washed three times with 1.0 mL 10% aqueous Na_2SO_4. These washings formed emulsions upon vortexing and frequently required longer centrifuge periods or the addition of small amounts of ddH_2O. The final organic layer was dried on anhydrous Na_2SO_4.

Product identification: The extracts from the *in vivo* assays were run on a column exactly as described for the *in vitro* assay (2.3.4). A portion of each fraction (0.5mL) was added to 5mL scintisafe 50% plus fluid and counted to determine which fractions contained radioactive marker. These fractions were concentrated to 0.5 mL under a gentle N_2 stream, 0.5 mL HPLC eluent was added and any radioactive products were transferred into the HPLC eluent by evaporating all remaining liquid column eluent. The fractions that corresponded to 4-methylnonanol standards were analyzed by HPLC to determine if conversion of 4-methylnonanoic acid to 4-methylnonanol was the same for mated and virgin beetles. Two different solvent systems and columns were attempted: 1) Zorbax SB-C18 9.4 mm x 25 cm reverse-phase preparatory column. Mobile phase: 85:15 acetonitrile:1% aqueous acetic acid; 2) Zorbax SB-C18 4.6 mm x 15 cm reverse-phase analytical column. Mobile phase: 50:50 acetonitrile:1% aqueous acetic acid.

3. RESULTS

3.1 Bioassays

3.1.1 Single Mating Experiments

I. Mean total touch time

Analysis of the data revealed that there was a significant treatment*day effect for the mean total touch time due to different levels of response on different days ($p=0.0026$). This resulted in greatly different means for the same treatments on different days. Thus, differences between treatments were masked by the variability within treatments from day to day. When the pooled means were compared using the ANOVA and Duncan's multiple range tests, only the pentane treatment was significantly different from the other treatments (Figure 2). Figure 3 shows the means for each treatment separated by day. When each data set was examined independently the same pattern was observed for each (this pattern explains the significant treatment*day interaction). The data from individual days were analyzed using the Orthoganal contrast test to compare each treatment on each day to the virgin positive control. Virgin extracts had higher activity levels (larger mean touch times) for all three days with a significant difference ($p < 0.05$) on days 1 and 2. Activity dropped immediately upon mating, returned to virgin like levels or higher by 0.5 or 1 hour and then began to drop again within 2 hours.

II. Mean time to first touch.

Again, a significant treatment*day interaction was observed ($p = 0.0044$). Thus, the data were examined for each trial separately instead of being pooled (figure 4). The same

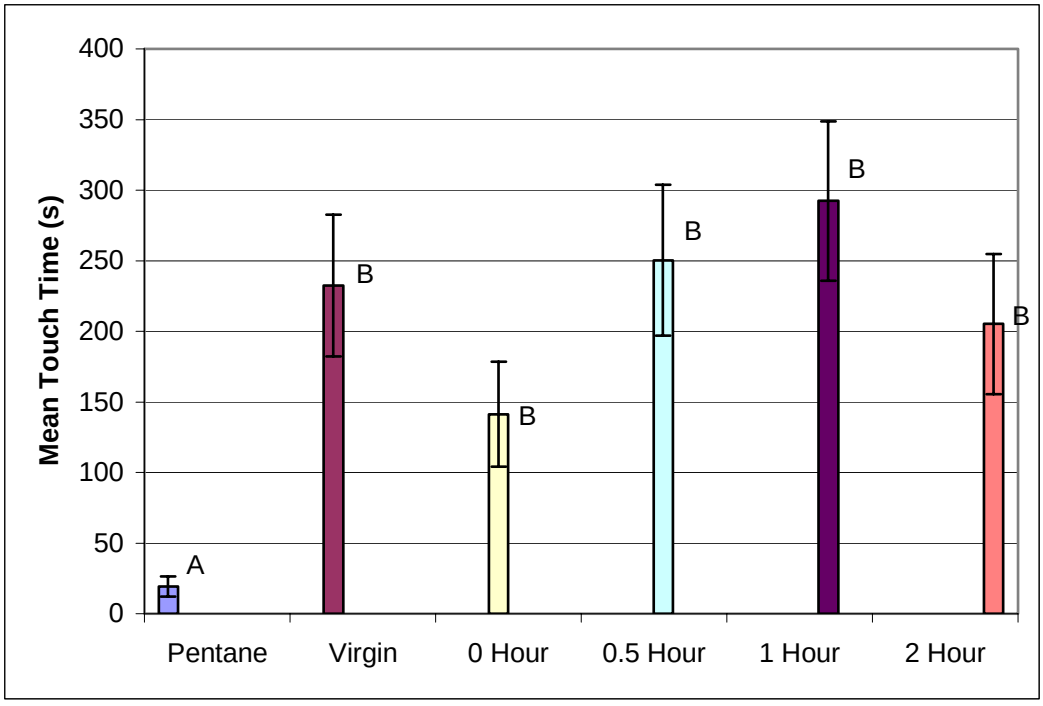

Figure 2: Effect of a single mating on the activity of female extracts: Females were extracted various times after mating (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The mean total time the males spent in contact with the rod (over a 600 second period) is presented, and compared to solvent and virgin extract controls. Treatments with the same letter are not significantly different. N = 10; Error bars indicate ± 1 standard error.

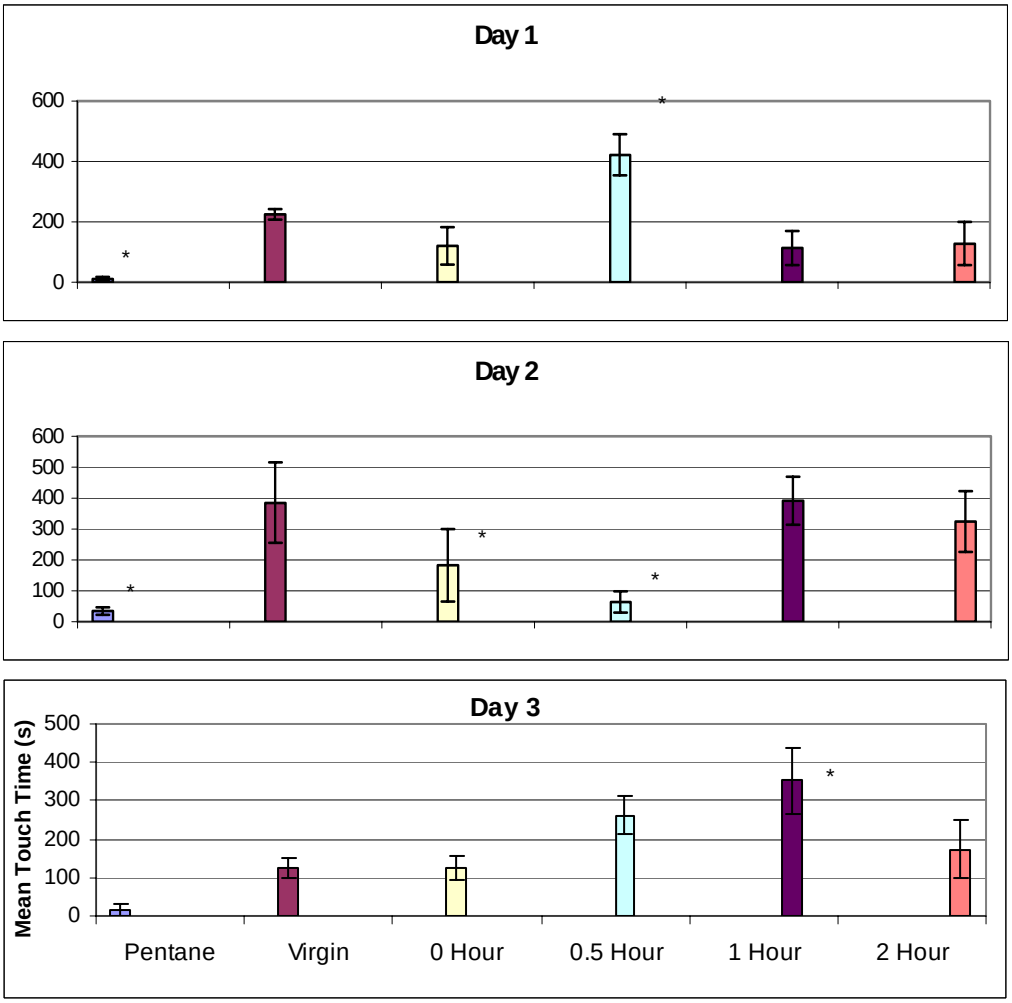

Figure 3: Effect of a single mating on the activity of female extracts – Data presented for each bioassay trial separately: Females were extracted various times after mating (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The mean total time the males spent in contact with the rod (over a 600 second period) is presented, and compared to solvent and virgin extract controls. Significant differences from the virgin control are indicated by an asterisk. N = 10; Error bars indicate ± 1 standard error.

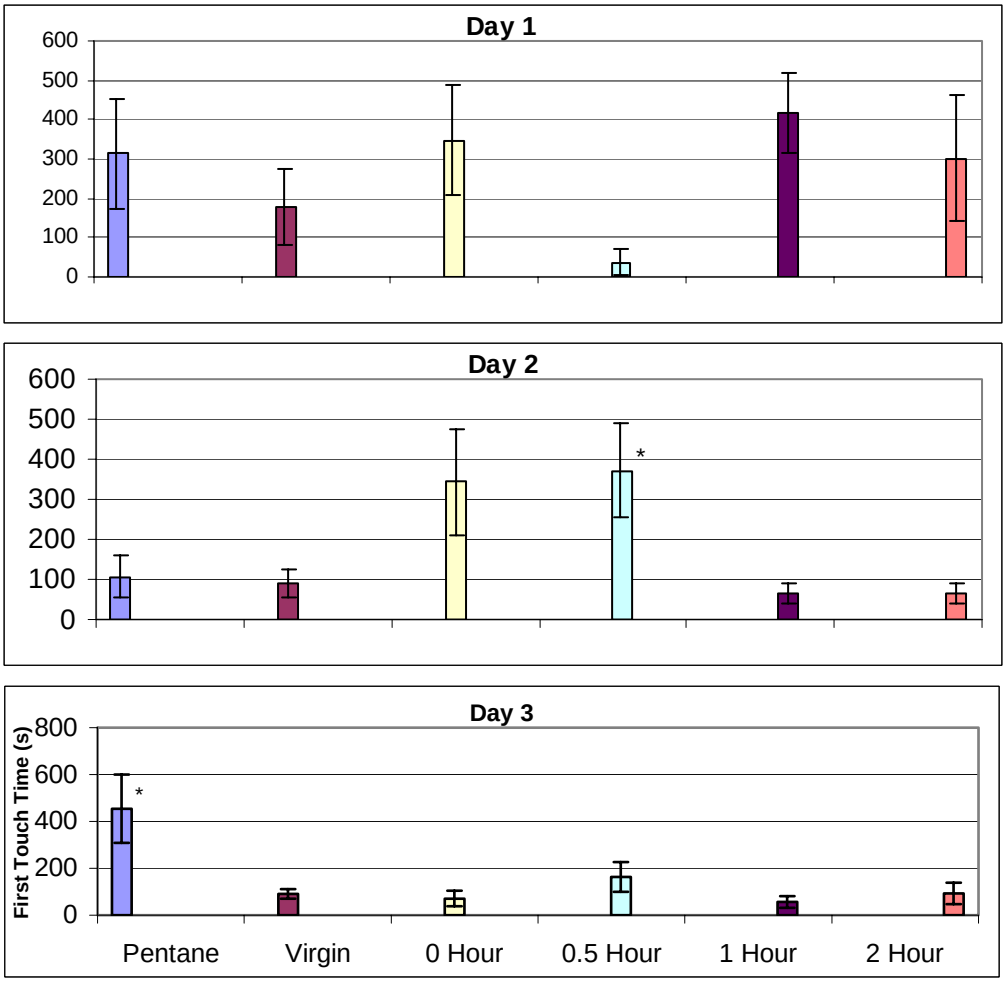

Figure 4: Effect of a single mating on the activity of female extracts – Data presented for each bioassay trial separately: Females were extracted various times after mating (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The mean total time before the males first touched the rod is presented, and compared to solvent and virgin extract controls. Males that never touched the rod were given a time of 600 seconds (the length of the observation period). Significant differences from the virgin control are indicated by an asterisk. N = 10; Error bars indicate ± 1 standard error.

general pattern was observed as for the total touch data. Virgin controls were more active than pentane as indicated by a lower mean time to first touch on all three days (significantly lower on day 3, $p \leq 0.05$). Activity declined for the 0 hour and/or 0.5 hour treatments resulting in higher times before the first touch (significantly higher on day 2, $p \leq 0.05$). Intermediate treatments between 0.5 and 2 hours after mating showed the mean time to first touch dropping again to virgin-like levels. Only on day 1 was the secondary inhibition of attraction between 1 and 2 hours clearly observed.

Instead of comparing means, a series of decay curves can be constructed. The percent negative response was plotted against one minute time intervals (for the 10 minute observation period) for each treatment. At time 0 all lines start at 100% because none of the beetles have responded yet. The faster the male beetles responded to a particular extract the steeper the curve. The greater the number of positive responses to a particular extract the closer the line gets to 0% negative response. As figure 5 shows, the pentane control had the highest percent negative response (30%) and one of the flattest slopes overall whereas the virgin control had the lowest percent negative response (0%) with one of the steepest slopes over all. For the other treatments, we see that the 0 hour and 0.5 hour decay curves are closer in shape and slope to the pentane whereas the 1 and 2 hour curves are closer to the virgin but perhaps not quite as attractive. However, these results were qualitative and no statistically significant difference could be shown for the curves.

III. Mean Total Copulation Time

Analysis of mean copulation times for all treatments showed no significant treatment effect ($p = 0.1351$) and also no significant treatment*day interaction ($p =$

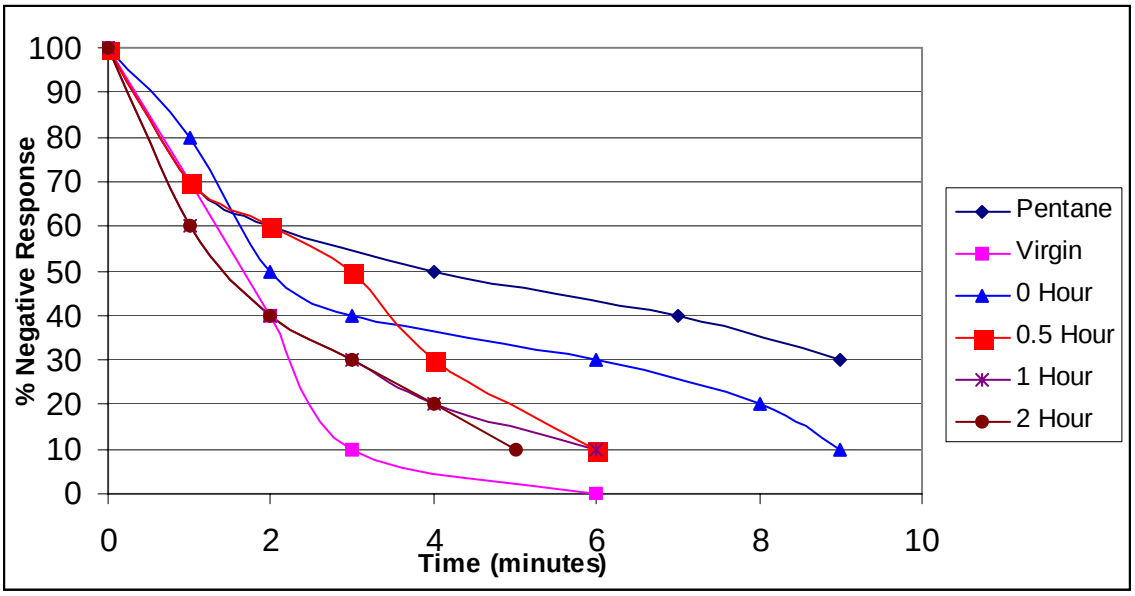

Figure 5: Effect of single mating on the activity of female extracts: Females were extracted various times after mating (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The curves show the percentage of beetles that had not yet responded at each one minute time interval. Extracts that elicit the strongest response will have steeper curves and reach a lower % negative response.

0.1995). These data indicate that mating does not have any significant effect on the activity of female extracts in terms of eliciting a copulation response. However, it should be noted that the pentane never elicited any response while all of the other treatments at least occasionally did. Furthermore, it is interesting to note that the 0 hour treatment did have a lower mean copulation time than the virgin extract and all other extracts were higher than the virgin (figure 6).

IV. Time to First Copulation

As for the mean touch time and time of first touch data, a significant treatment*day interaction was observed for the mean time to the first copulation ($p = 0.0211$). However, in this case it is difficult to observe the pattern creating this significant interaction. The Orthoganal contrast test showed that the virgin control was significantly ($p \leq 0.05$) more attractive (lower time to first copulation) on only one day (figure 7). The 0.5 hour treatment was sometimes significantly more active, sometimes significantly less. Similarly the 1 hour treatment was significantly more attractive than the virgin on one day, but as attractive or less on the other days.

3.1.2 Multiple Mating Experiments

I. Mean total touch time.

The results for the multiple mating experiments were analyzed in the same way as above. The effect of mating duration on the mean time spent touching the rod showed a significant treatment effect ($p = 0.0077$) and no treatment*day interaction, thus the pooled means could be used. Figure 8 shows the mean touch times for all five treatments. The

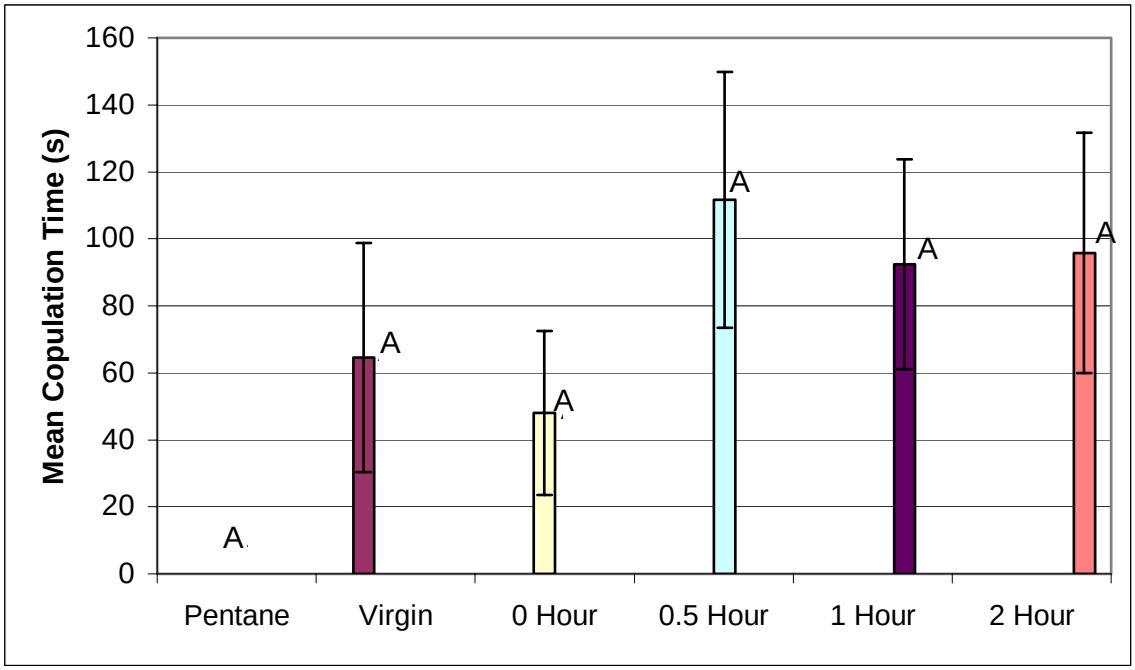

Figure 6: Effect of a single mating on the activity of female extracts: Females were extracted various times after mating (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The mean total time the males spent copulating with the rod (over a 600 second period) is presented, and compared to solvent and virgin extract controls. N = 10; Error bars indicate ± 1 standard error.

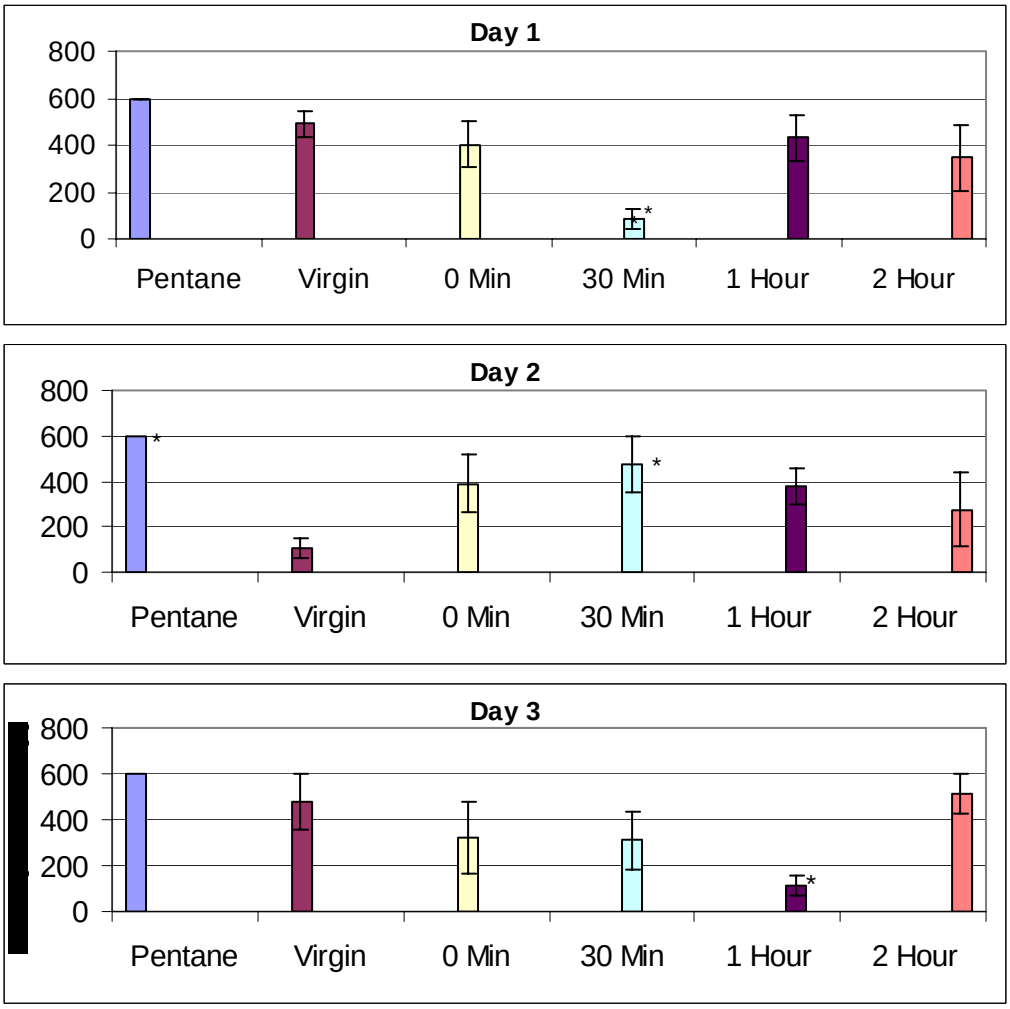

Figure 7: Effect of a single mating on the activity of female extracts – Data presented for each bioassay trial separately: Females were extracted various times after mating (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The mean total time before the males first copulated with the rod is presented, and compared to solvent and virgin extract controls. Males that never touched the rod were given a time of 600 seconds (the length of the observation period). Significant differences from the virgin control are indicated by an asterisk. N = 10; Error bars indicate ± 1 standard error.

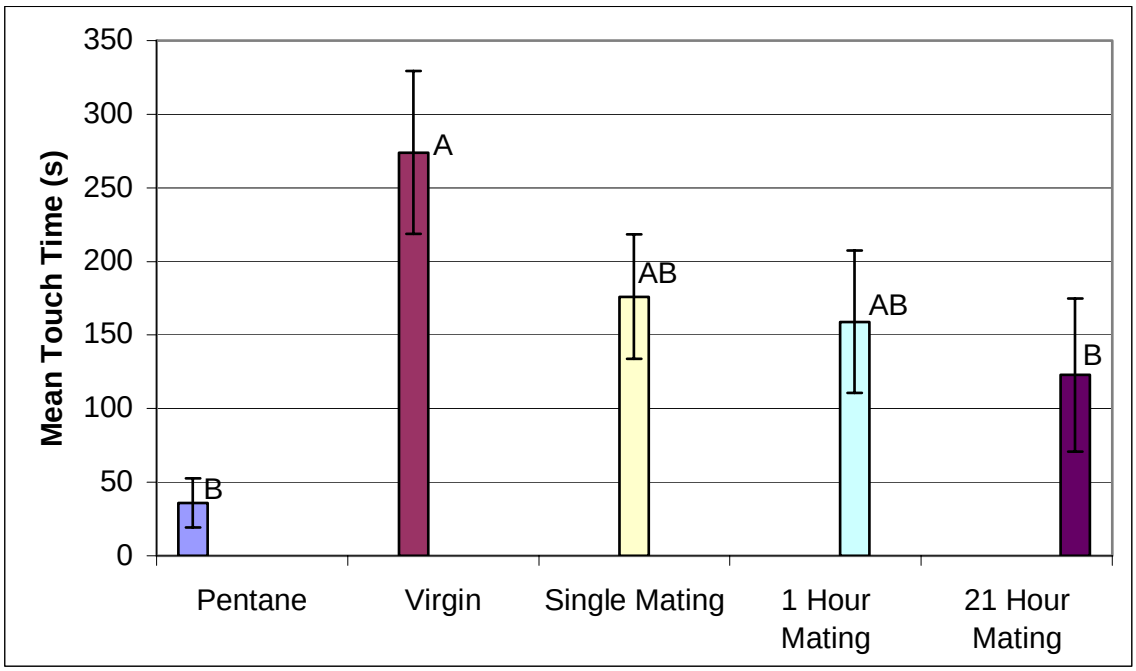

Figure 8: Effect of increased mating duration on the activity of female extracts: Females were extracted after various mating durations (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The mean total time the males spent in contact with the rod (over a 600 second period) is presented, and compared to solvent and virgin extract controls. N = 10 (except 21 Hour Mating, N = 5); Error bars indicate ± 1 standard error.

results show a clear trend towards decreased attractiveness (time spent touching the rod) as the mating duration increases, and after 21 hours of mating the extract was significantly less attractive than the virgin extract ($p \leq 0.05$).

II. Time to first touch

No significant treatment effect ($p = 0.4476$) or treatment*day interaction ($p \geq 0.05$) was determined for the mean time to first touch data. However, as Figure 9 shows, the trend was exactly the same as for the mean total touch time (figure 8). The virgin extract had the highest activity (observed here as lowest mean time to first touch). The longer the mating duration, the lower the activity levels observed. By the 21 hour mating duration, the mean time to first touch was essentially as long as that for the pentane control.

Decay curves for the time of first touch data were constructed for the multiple mating experiments and are shown in figure 10. The virgin control showed the lowest percent negative response and the steepest curve as expected. The single mating had a curve shifted towards that of the pentane control and the 1 hour and 21 hour matings had curves very similar to that of the pentane. Once again, these results were qualitative and no statistically significant difference could be shown for the curves although the general trend indicates that the activity of female extracts decreases with increased mating duration.

III. Mean total copulation time

The effect of mating on mean time spent copulating with the rod was found to have a significant treatment effect ($p = 0.0031$) and no significant treatment*day interaction ($p \geq 0.05$). Figure 11 shows the same trend of decreased activity as mating duration increases,

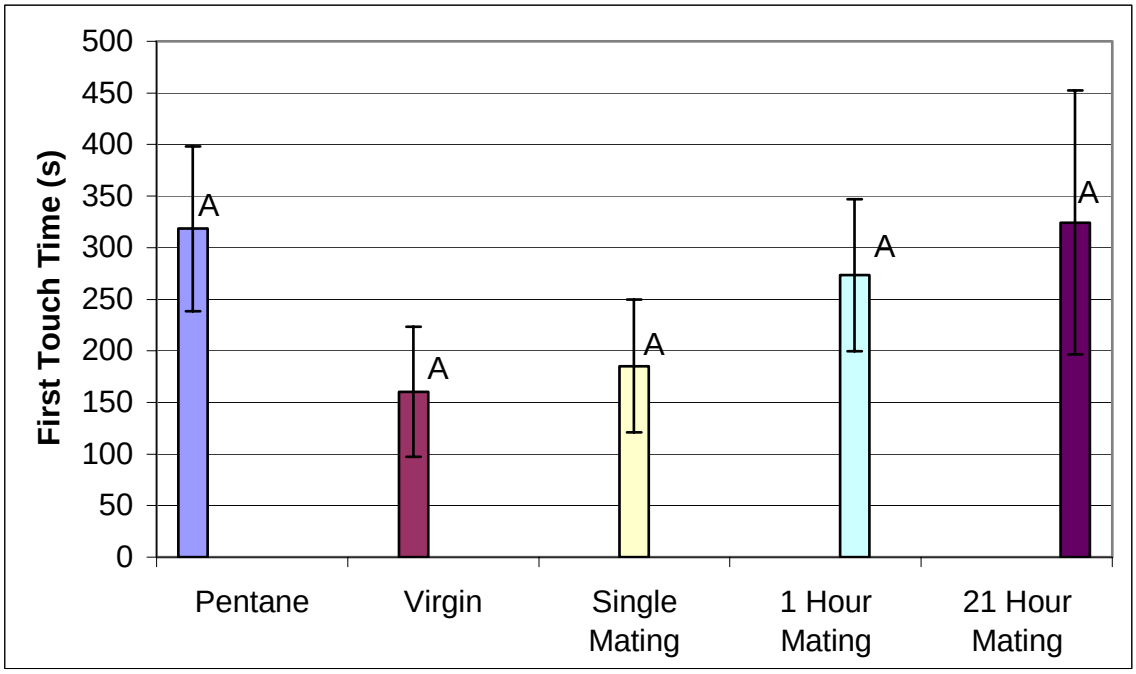

Figure 9: Effect of increased mating duration on the activity of female extracts: Females were extracted after various mating durations (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The mean total time before the males first touched the rod is presented, and compared to solvent and virgin extract controls. Males that never touched the rod were given a time of 600 seconds (the length of the observation period). Treatments with the same letter are not significantly different. N = 10 (except 21 Hour Mating, N = 5); Error bars indicate ± 1 standard error.

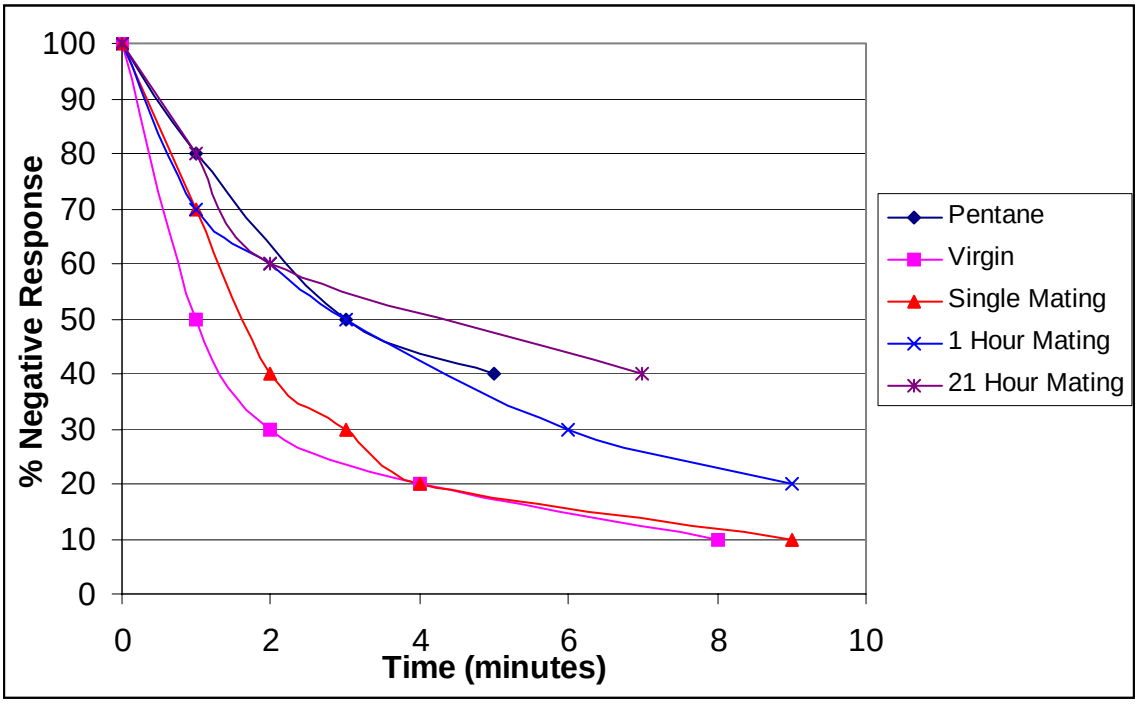

Figure 10: Effect of increased mating duration on the activity of female extracts: Females were extracted after various mating durations (as indicated) and the activity of the extracts to elicit responses from the male beetles was determined. The curves show the percentage of beetles that had not yet responded at each one minute time interval. Extracts that elicit the strongest response will have steeper curves and reach a lower % negative response.

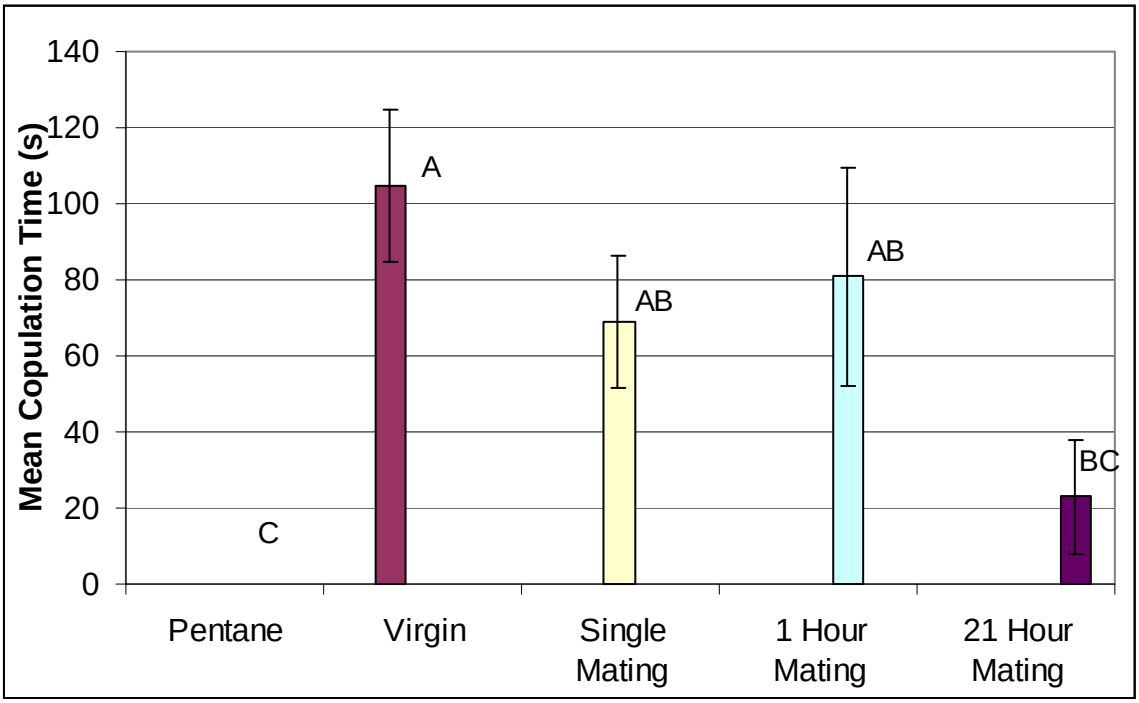

Figure 11: Effect of increased mating duration on the activity of female extracts: Females were extracted after various mating durations (as indicated) and the activity of the extracts to elicit responses from the male beetles was determined. The mean total time the males spent copulating with the rod (over a 600 second period) is presented, and compared to solvent and virgin extract controls. Treatments with the same letter are not significantly different. N = 10 (except 21 Hour Mating, N = 5); Error bars indicate ± 1 standard error.

in this case observed as decreased mean copulation time. The longer the mating period the shorter the average copulation response elicited. After 21 hours of mating the extract was significantly less attractive than the virgin extract and not significantly different from the pentane control ($p \leq 0.05$).

IV. Time to first copulation

As in the single mating experiments, the mean time to first touch data showed a significant treatment*day interaction. And, again as in the previous experiments it is difficult to observe the pattern that is creating this significant interaction. Figure 12 shows the results separated by day. In both trials, there was an increase in the mean time to the first copulation upon mating but on day 1 this occurred only after a single mating and on day 2 only after a 1 hour mating duration. Due to time constraints, the Orthoganal contrast test has not yet been performed on this data and therefore no significant differences between the treatments and the virgin control can be reported.

3.2 Biochemical Assays

3.2.1 Synthesis of Tritium Labeled 4-Methylnonanoyl CoA

Attempts to synthesize tritium labeled 4-methylnonanoyl CoA were unsuccessful. When the progress of the reaction was checked periodically by TLC there was no clear indication of product formation. Only the solvent front showed significantly high radioactivity indicating the presence of unreacted ^{3}[H]4-methylnonanoic acid only. The incubation mixture was analyzed by HPLC to confirm the suspected lack of product and to recover the purified acid. Figure 13 shows the HPLC elution profile for the incubation

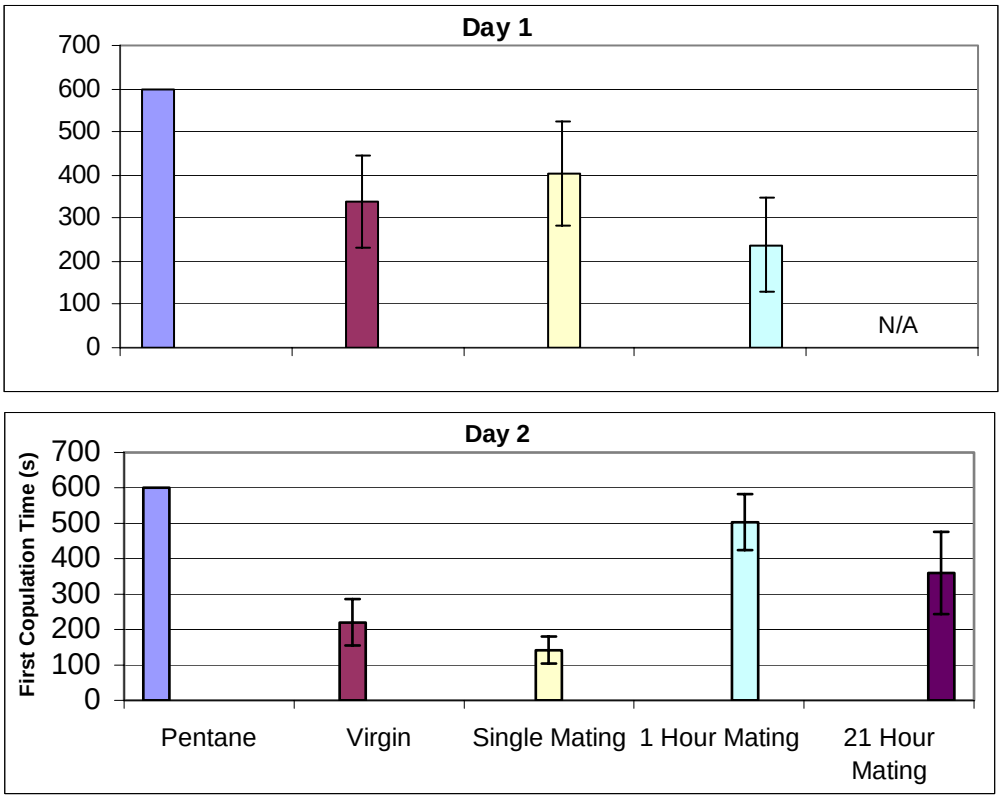

Figure 12: Effect of increased mating duration on the activity of female extracts – Data presented for each bioassay trial separately: Females were extracted after various mating durations (as indicated) and the ability of the extracts to elicit responses from the male beetles was determined. The mean total time before the males first copulated with the rod is presented, and compared to solvent and virgin extract controls. Males that never touched the rod were given a time of 600 seconds (the length of the observation period). N = 10 (except 21 Hour Mating, N = 5); Error bars indicate ± 1 standard error.

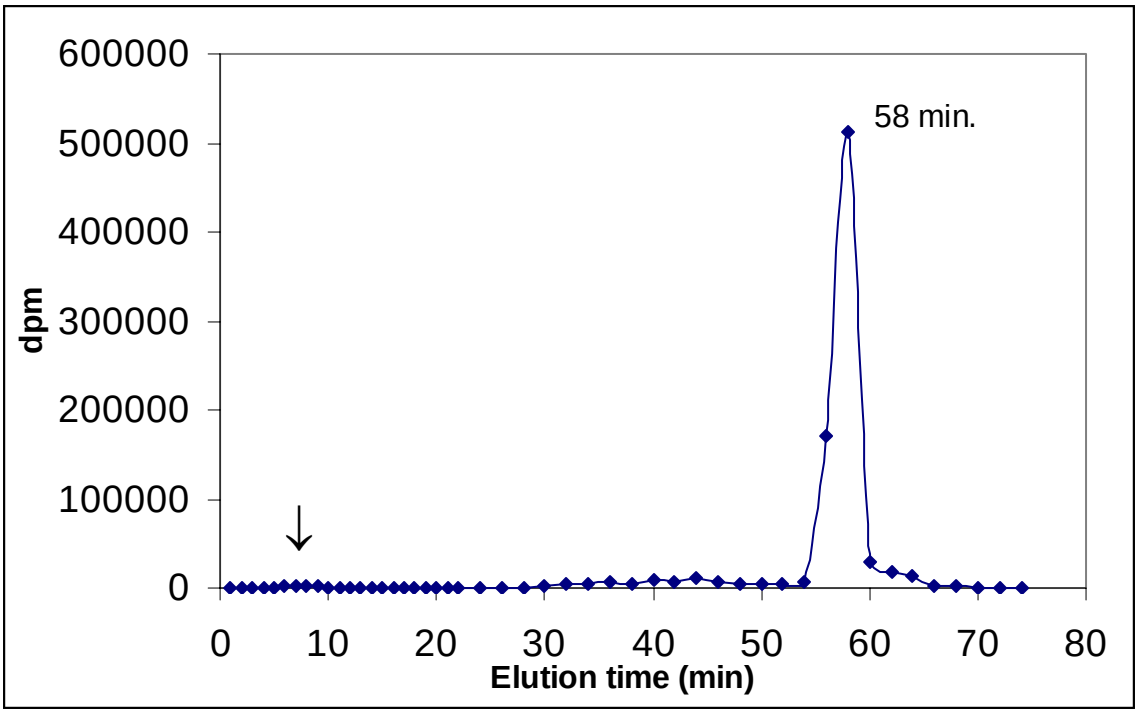

Figure 13: Elution profile for the preparation of ^{3}[H]4-methylnonanoyl CoA from ^{3}[H]4-methylnonanoic acid. Mobile phase: 95:5 citrate:acetonitrile, running at 2.0mL/minute, with a gradient to 5:95 citrate:acetonitrile initiated at 17 minutes (2%/minute).

mixture. The arrow at 7.75 minutes indicates the expected elution time of 4-methylnonanoyl CoA. Clearly the percent yield is virtually zero. A second attempt at this procedure yielded the same results.

3.2.2 Pheromone Internalization Assay

The assay results indicate that internalization is likely not a method which mated females use to decrease their pheromone titre. As figure 14 shows, the mated females actually internalized significantly less tritium labeled pheromone than the virgins did ($p = 0.001$). More of the pheromone remained on the surface or was volatilized by the mated females but no significant difference was detected ($p = 0.129$).

3.2.3 *In vitro* Assay

The assay results indicate that mating does not have an effect on the mean amount of 4-methylnonanoic acid converted to 4-methylnonanol by crude beetle homogenates. Figure 15 shows typical liquid column elution profiles for the no protein control (NPC), virgin, and mated treatments. The first peak represents 4-methylnonanol and the second peak represents 4-methylnonanoic acid. The profiles clearly show that the homogenates converted pheromone precursor to pheromone better than the NPC, but there was no difference between virgin and mated homogenates. Figure 16 shows the mean conversions for the three treatments. Again, conversion was significantly better for homogenates than the NPC ($p = 0.060$ for virgin treatment, $p < 0.001$ for mated treatment) but no difference was observed between virgin and mated beetle homogenates ($p = 0.930$).

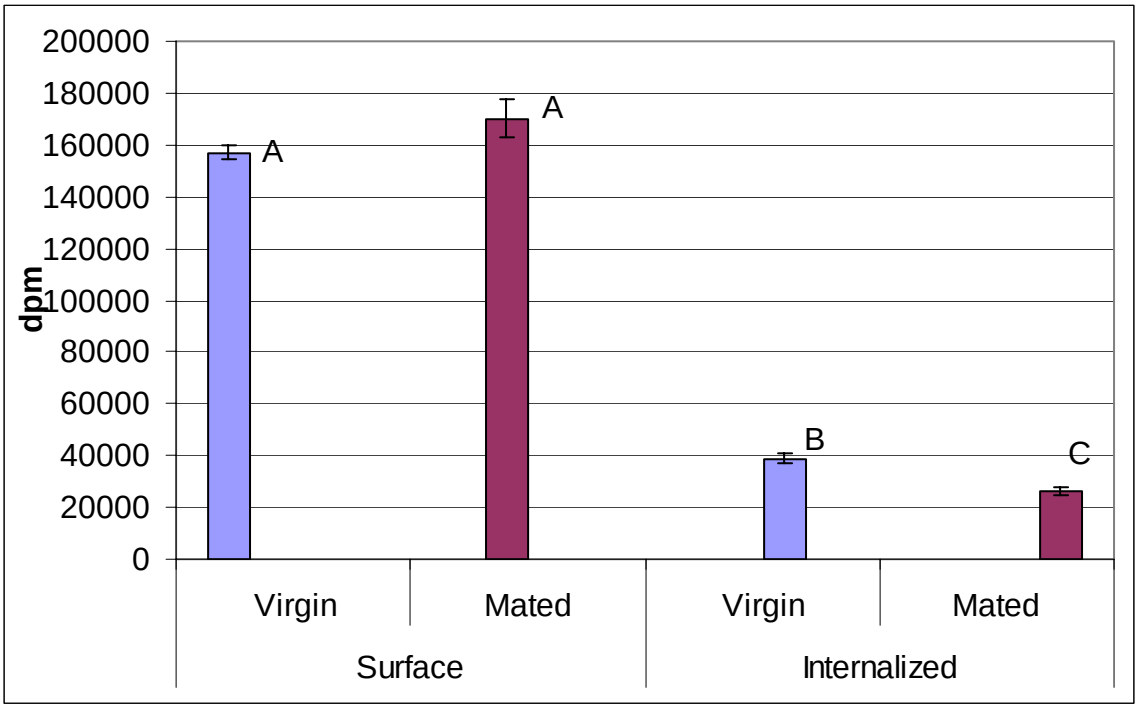

Figure 14: Effect of mating on the internalization of ^{3}[H]4-methylnonanol applied to the entire under-surface of virgin and mated females. Approximately 200,000 dpm was applied. Each pair of means (either for surface or internalized) were compared for the virgin and mated treatments by a two-sample Student's t-test (two-tailed). If the means significantly differed they were given different letters. N = 5; Error bars indicate ± 1 standard error.

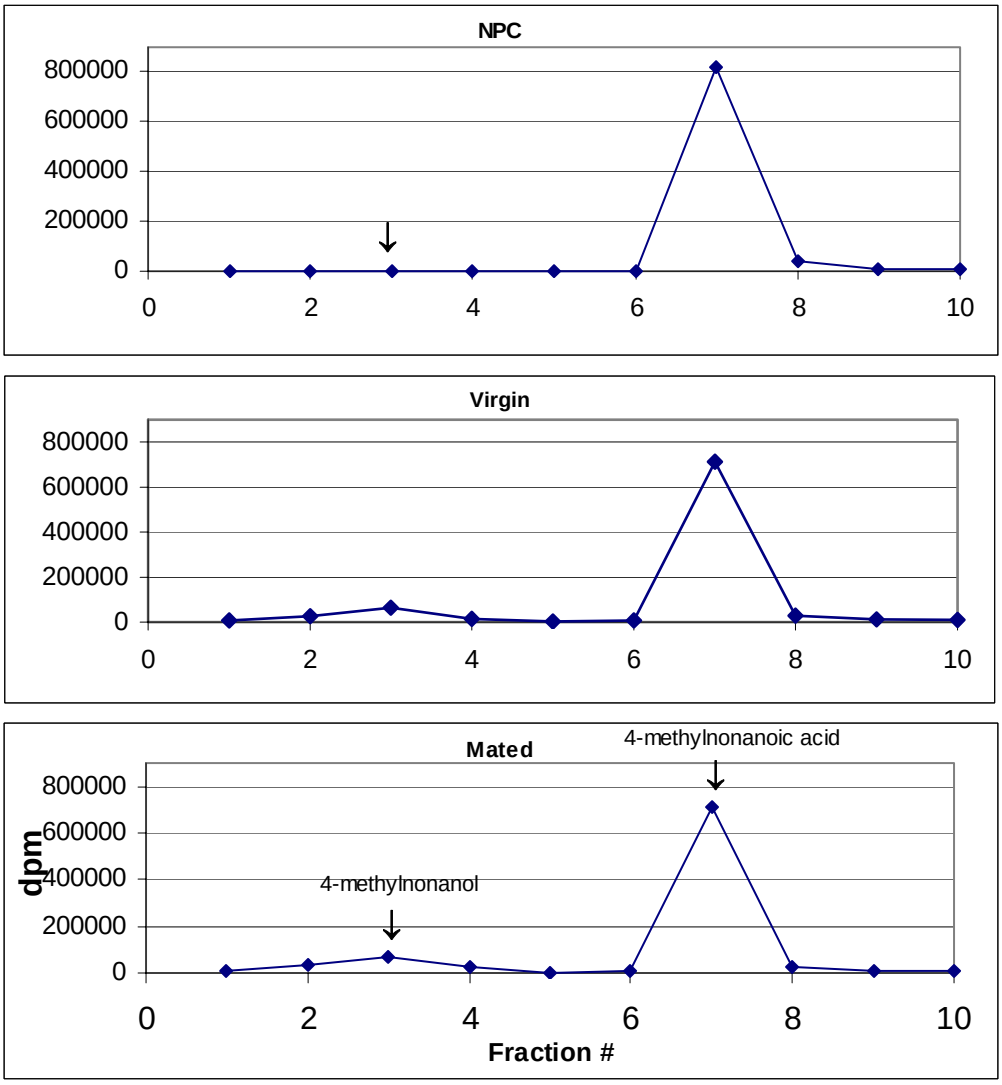

Figure 15: Effect of mating on the ability of crude beetle homogenates to convert 4-methylnonanoic acid to 4-methylnonanol in the presence of ATP, CoA, NADH, NADPH, protease inhibitor, zwittergent, and homogenization buffer (50mM phosphate, 1mM DTT, 50mM KCl, 10% glycerol (v/v), pH 7.4) at 30°C for 30 minutes. Identity of peaks is indicated in the mated panel; Arrow in no protein control (NPC) indicates absence of alcohol peak.

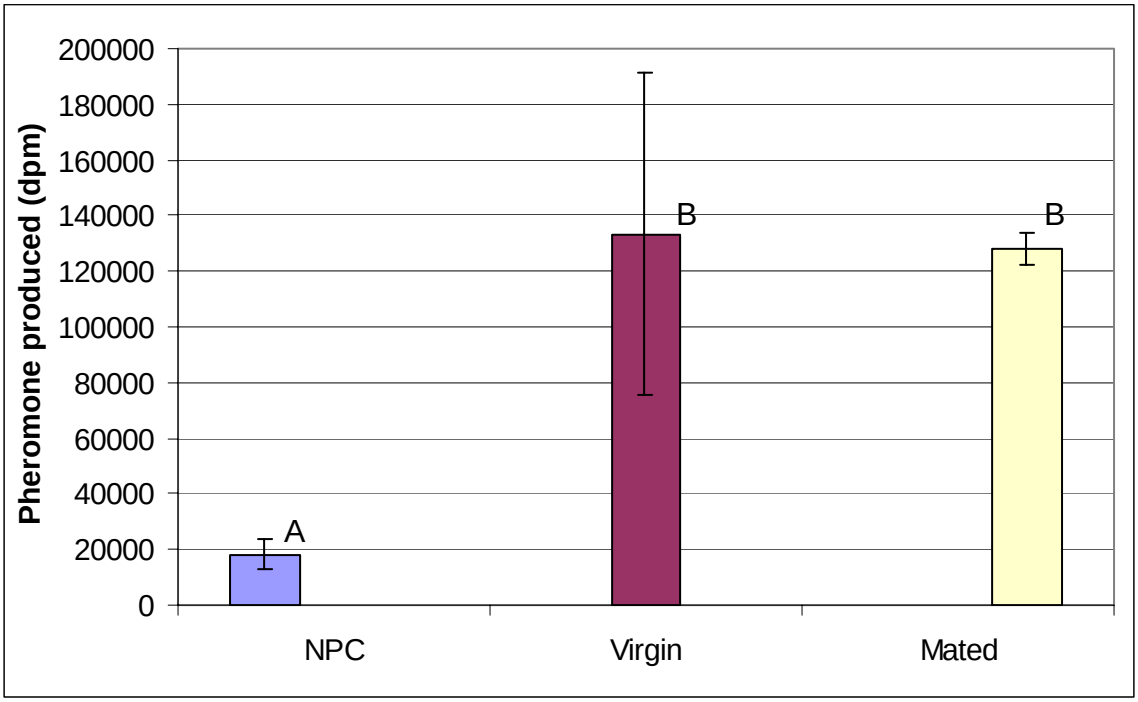

Figure 16: Effect of mating on the ability of crude beetle homogenates to convert 4-methylnonanoic acid to 4-methylnonanol in the presence of ATP, CoA, NADH, NADPH, protease inhibitor, zwittergent, and homogenization buffer (50mM phosphate, 1mM DTT, 50mM KCl, 10% glycerol (v/v), pH 7.4) at 30°C for 30 minutes. Mean amount of ^{3}[H]4-methylnonanol (pheromone) produced from ^{3}[H]4-methylnonanoic acid is presented. Treatments with the same letter did not significantly differ. N = 3; Error bars indicate ± 1 standard error.

3.2.4 *In vivo* Assay

No complete results for the HPLC characterization of extracts from virgin and mated females injected with ^{3}[H]4-methylnonanonoic acid were obtained. Using the preparatory column, the majority of the radioactive marker injected (or thought to be injected) was never recovered for the standards or the samples. Figure 17 shows representative elution profiles obtained for the standard and a sample (in this case a virgin extract). For the sample, small peaks were observed at many different elution times, even during the void volume. This prompted the switch to a different column, which in turn required a new elution solvent system. Using the analytical column, better standards could be obtained and all radioactive marker could be accounted for. However, as figure 18 shows, when a sample was run the same problems occurred. The small peaks observed represent only 1-2% of the radioactive marker that was thought to be injected. Due to these difficulties, the extracts from mated beetles were not run (because of the time invested in preparing them) and thus no meaningful comparison can be made between virgins and mated beetles. For all samples, an “oily” substance was observed when concentrated. It could not be determined definitively whether or not this portion contained any of the labeled product or not so it was injected as an emulsion. Furthermore, after injection, more radioactive counts (dpm) could be found in the HPLC syringe and on the sides of the sample vial than found in the eluent.

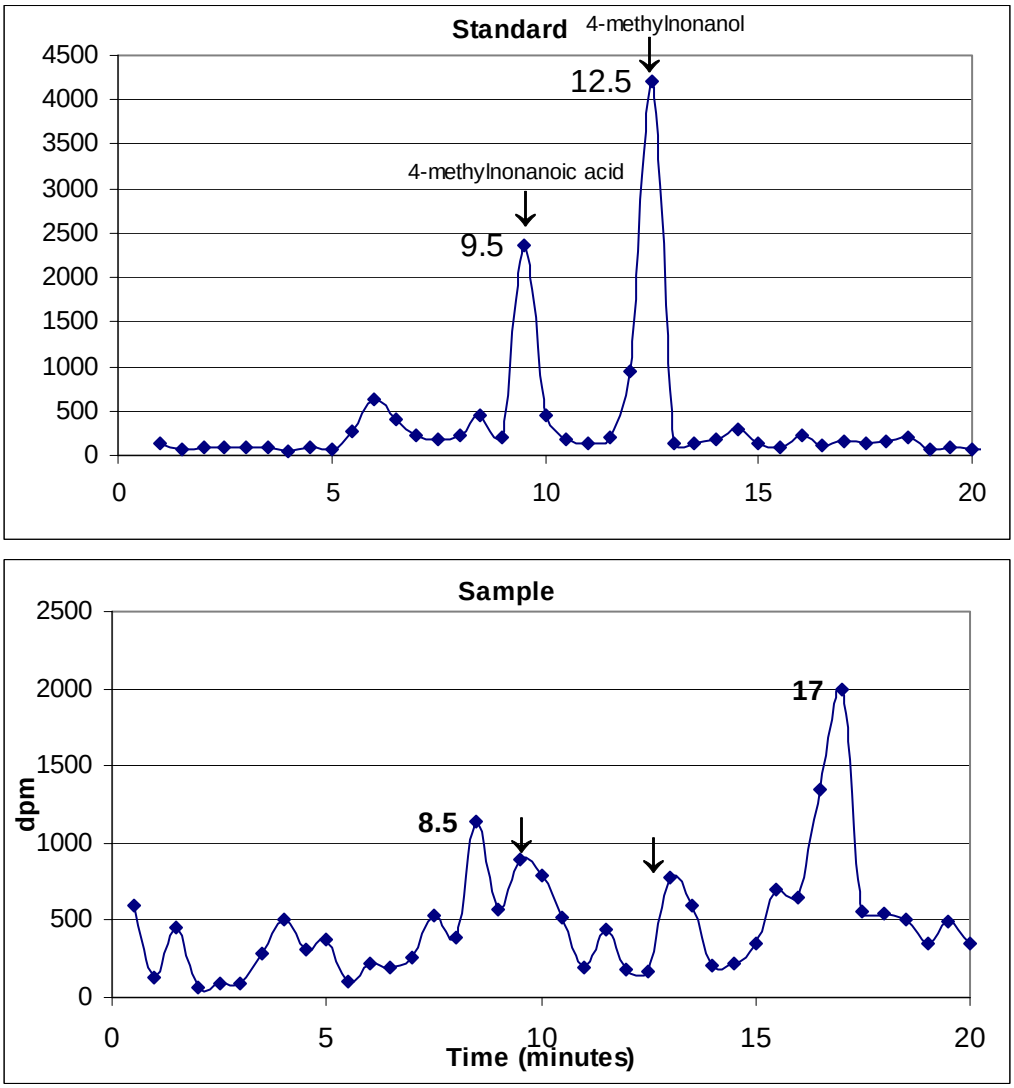

Figure 17: Representative HPLC elution profiles for *in vivo* assay results. A standard for both ^{3}[H]4-methylnonanoic acid and ^{3}[H]4-methylnonanol is shown in the panel above. The panel below depicts an extract from a virgin beetle that was injected with ^{3}[H]4-methylnonanoic acid. Arrows indicate the expected elution times of the tritium labeled acid and alcohol as indicated by the standard. HPLC analysis performed on a Zorbax SB-C18 9.4mm x 25cm reverse-phase preparatory column. Mobile phase: 85:15 acetonitrile:1% aqueous acetic acid.

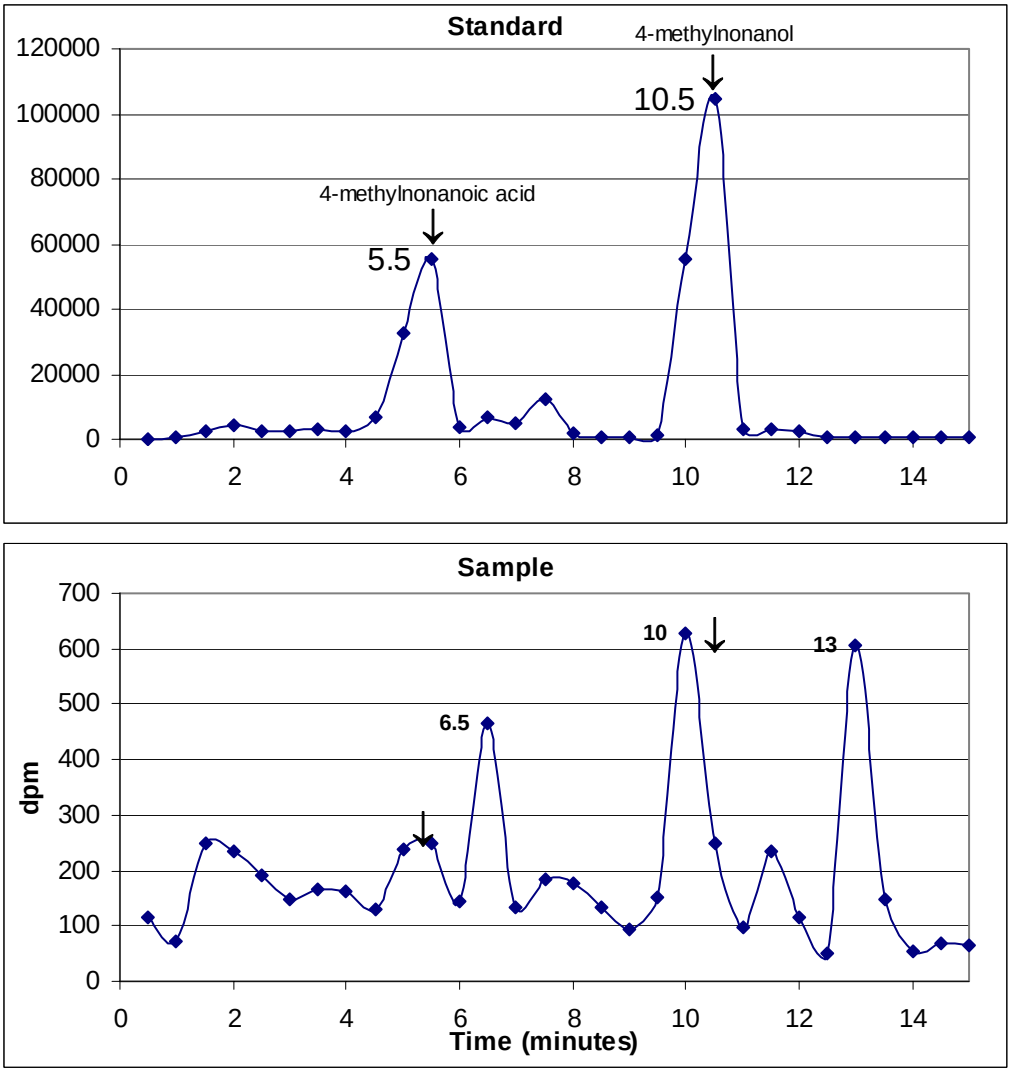

Figure 18: Representative HPLC elution profiles for *in vivo* assay results. A standard for both ^{3}[H]4-methylnonanoic acid and ^{3}[H]4-methylnonanol is shown in the panel above. The panel below depicts an extract from a virgin beetle that was injected with ^{3}[H]4-methylnonanoic acid. Arrows indicate the expected elution times of the tritium labeled acid and alcohol as indicated by the standard. HPLC analysis performed on a Zorbax SB-C18 4.6mm x 15cm reverse-phase analytical column. Mobile phase: 50:50 acetonitrile:1% aqueous acetic acid.

4. DISCUSSION

4.1 Bioassays

4.1.1 Single Mating Experiments

I. Mean total touch time

The bioassay results suggest that the attractiveness of the female *T. molitor* to males is regulated by two very different effects. Extracts of newly mated females (0 hour) were less able to maintain the interest of the males, compared to the virgin extract. This immediate effect likely indicates the involvement of an anti-aphrodisiac that is deposited by the male onto the female and inhibits the response of other males to the female. The effect was reversed by 0.5 to 1 hour after mating, indicating that the anti-aphrodisiac wears off quickly. Extracts taken 2 hours after mating were also less able to maintain the interest of the males, compared to the virgin extract. This second effect likely involves a decrease in pheromone titre beginning between 1 and 2 hours after mating.

The significant treatment*day interactions observed are due to varying levels of response on different days and could be the result of several factors. The mating response of *T. molitor* is greatly affected by relatively small changes in temperature and humidity. Male beetles will become almost completely inactive if the temperature falls too low or humidity rises too high (Hastings and Vanderwel, unpublished results; personal observation). Even in the environment control chamber, temperature fluctuated slightly and the humidity occasionally varied from 40-60%. The response of the male beetles may also vary with the time of day. The beetles are kept on a strict 12 hours light:12 hours dark photoperiod. Experiments were always conducted during the dark period but not always at

exactly the same time. Also, the females used to produce the extracts could have different basal pheromone levels. Happ and Wheeler (1969) reported that crowded females produced more sex attractant, and the females used in our experiments did not always come from colonies with the same degree of crowding. A larger number of repetitions of each treatment, all conducted on one day could avoid these day*treatment effects but may also introduce new sources of error due to fatigue from the longer observation period.

II. Mean time to first touch

The female-produced male-attractant, 4-methylnonanol, serves to attract males from a distance to the female. Thus, the "time to first touch" can be interpreted to reflect the 4-methylnonanol titre in the female extracts. If 4-methylnonanol titre drops or if its effects are masked by an anti-aphrodisiac, we would expect an increase in the amount of time it takes the male to find the rod. This increase can be seen qualitatively in the first touch decay curves, especially for the 0-hour treatment, suggesting the presence of an anti-aphrodisiac (figure 5). The comparison of means further supports the presence of an anti-aphrodisiac that wears off within 1 hour. The trend of decreased pheromone titre after 2 hours is observable but not statistically significant. Unfortunately, accurate determination of the mean time to first touch is complicated by the fact that some beetles never touch the rod while others accidentally touch it during its insertion under the petri dish. In order to further examine the effect of mating on the average time to first touch, a new experimental design is required. The bioassay could be improved by using a larger chamber, with the male beetles placed at a defined distance from the rod.

III. Mean total copulation time

Mating did not suppress the ability of the female extracts to elicit a CR response from the males. Even if there was an anti-aphrodisiac present and/or 4-methylnonanol titre declined, copulation could occur. The titre of the female-produced non-volatile CR pheromone may not be affected by mating, or it may take longer for the existing titre to dissipate due to its non-volatile nature. Also, the virgin males have their own CR pheromones (Tanaka *et al*., 1986) which obviously would still be present and unaffected by previous matings experienced by the female. Because of the small experimental area, the male beetles did eventually find the rod in virtually every case. Presumably, as long as a small amount of 4-methylnonanol was always present to act as a synergist with the CR pheromone, a CR response could occur in all treatments. However, it is surprising that all treatments except for the 0 hour actually elicited higher responses than the virgin. If it took longer to find the rod, we would have expected the average copulation time to decrease, because less of the 10 minute observation period would have been available. It was also observed that the 0.5 hour and 1 hour treatments were consistently more active for the total touch and first touch responses. In other words, after the anti-aphrodisiac effect wore off and before the drop in pheromone titre effect began, the extracts often appeared more attractive than the virgin control. Gage and Baker (1991) proposed a possible explanation for this in the observation that males accompanied by a rival male before or during mating were faster to initiate mating, inseminate more sperm per ejaculate, and were more likely to show post-copulatory guarding. They concluded that *T. molitor* adjust the number of sperm they inseminate according to perception of risk of sperm competition. It is possible that males in our bioassays could “perceive” the presence of another male by some scent

that was transferred to the rod from the extraction of a female that had mated with another male. This perception that another male is/was present may have prompted them to copulate more vigorously than those exposed to the virgin female extract alone. This corresponds to one of the major predictions of sperm competition that males will exercise control over their ejaculate size to balance the cost of sperm against the risk of losing sperm precedence to another male (Gage and Baker, 1991).

IV. Mean time to first copulation

The mean time to first copulation data is difficult to interpret, possibly due to both the limitations described for the time to first touch data and the "perception of sperm competition" effect described in the previous section. On one day (day 2) the virgin appeared to be the most active with significant increases in the time to first copulation occurring after mating. But, on the other days (day 1 and 3) the reverse was true with 0.5 hour and 1 hour treatments most active. It is possible that more consistent results could be obtained by examining the effect of mating on touch and copulation responses separately. A new experimental design was already suggested for the touch response. For the CR response perhaps a technique like that used by Tanaka *et al*. (1986) could be used. They coated a longer, bent rod and presented it directly to the beetle and scored it for CR activity. In this experimental design, the males did not have to "find" the rod, effectively separating the attraction effect from the CR effect.

4.1.2 Multiple Mating Experiments

I & II. Mean total touch time & mean time to first touch

The results of the multiple mating experiments are also consistent with the hypothesis that mating has an effect on the attractiveness of female extracts. The ability of female extracts both to attract and to retain the interest of the male was clearly decreased when the female was mated. Both types of response showed the same trend towards decreased activity of the extracts as mating duration increased. Since all of these extracts were taken at least 1 hour after the last mating event, it is assumed that a decrease in pheromone titre and not an anti-aphrodisiac is responsible for the observed decrease in extract activity.

The finding that longer mating durations may amplify the decrease in pheromone titre has, to my knowledge, not been reported for *T. molitor* but similar effects have been observed in several Lepidopteran species. Ando *et al*. (1996) report that for *Bombyx mori* a mating interval of one to six hours leads to a transient decrease in pheromone titre and a mating interval greater than six hours leads to a permanent inhibition of pheromone production. Giebultowicz *et al.* (1991) report similar findings for the gypsy moth, *Lymantria dispar*, which shows a transient suppression of pheromone production in response to the mechanical stimuli of mating and a permanent suppression once sperm transfer is complete and oviposition begins. It is likely that pheromone levels in *T. molitor* females are never completely inhibited as they have relatively long life spans and can go through several mating and egg laying periods (Menon, 1970). However, it makes sense that whatever mechanism is responsible for regulating pheromone titre in response to mating would respond more strongly to a longer mating duration. For example, if the

mechanism involves some factor transferred from the male to the female, more mating would result in more transfer and, therefore, a greater effect.

Another possibility that should not be ignored is that the 21 hour multiple mating treatment produced the least attractive extract because there was a longer time since the first mating. It is possible that the down-regulation of pheromone titre is greater after 21 hours than 2 hours. Further bioassays should be conducted to compare extracts from single and multiple matings after 21 hours and from mated females at various time periods longer than 2 hours. In either case, the results indicate that both an anti-aphrodisiac and a decrease in pheromone titre are involved in regulating the mating behaviour of *T. molitor*.

III & IV. Mean total copulation time & mean time to first copulation

Longer mating durations significantly decreased the ability of the female extracts to elicit a CR response. However, only the 21 hour mating treatment was significantly different from the virgin control and the single and 1 hour mating durations were not (figure 11). This is not necessarily inconsistent with the earlier observations that mating did not affect the ability of the extracts prepared after mating to elicit a CR response. Perhaps, after 21 hours, the levels of both the volatile 4-methylnonanol and the non-volatile CR pheromone have significantly decreased. It is also possible that after the 21 hour mating duration, the amplifying effect of the multiple matings had sufficiently decreased 4-methylnonanol levels to negatively affect CR activity. Thus, the decrease in CR activity upon longer mating durations could be just a function of the decreased 4-methylnonanol levels already proposed. Alternatively, it might represent another effect of mating not previously considered. The need to conduct bioassays that examine attraction and CR

activity separately may be even more important for the study of the effect of increased mating duration in *T. molitor*.

The experiments for mean time to first attempted copulation have all of the same flaws as described for the single mating experiments. The statistical analysis is not complete but is not likely to offer any surprises. The significant treatment*day interaction is consistent with the observation that mating seems to have some effect on CR response**,** but that effect is a complicated one.

4.1.3 Bioassays Summary

Mating apparently causes the deposition of an anti-aphrodisiac on the females, consistent with a previous report (Happ, 1969). This anti-aphrodisiac had only a transient effect, wearing off within one hour after mating. This finding has great adaptive significance especially in light of the recent findings concerning the sperm competition mechanisms of *T. molitor*. Drnevich *et al*. (2000) report that the interval between mating has a great effect on the sperm precedence of the second male to mate (P_2). Males that mated within 5 minutes after the first mating had higher sperm precedence and often achieved complete second male sperm precedence ($P_2 = 1$). If the interval was longer than 5 minutes the second male still had a high degree of sperm precedence but rarely achieved complete precedence. Drnevich *et al*. (2000) suggest that sperm release from the first male's spermatophore might be inhibited by the second male during the first 30 seconds of mating. Chemical inhibition or physical damage to the spermatophore were suggested as possible mechanisms. Clearly, an anti-aphrodisiac would have an adaptive advantage if it could just stall the second male long enough to prevent mating within the first 5 minutes.

Mating also apparently causes a second, longer term effect: from 1-2 hours after mating the ability of female extracts to attract males decreases, indicating that the titre of the female-produced sex attractant drops. This differs from the findings of Happ and Wheeler (1969) who suggested that pheromone levels remain high for several days after mating before dropping with the onset of egg laying. The reason for this inconsistency is probably a difference in bioassay technique. They used live beetles instead of extract-coated rods, and assayed the scent of many females at once at an unknown amount of time after mating. Thus, if there was a short term, transient decline in 4-methylnonanol titre they may not have detected it. Experiments in our lab (Vakili and Vanderwel, unpublished results) also dispute the large time interval before egg laying. In contrast, it has been found that viable eggs are produced and deposited within only a few hours of mating.

The regulation of pheromone titre could be of adaptive significance to either the male, the female, or both. The significance for the male once again involves sperm competition. Even though the most crucial time seems to be before 5 minutes, the second male sperm precedence remains fairly high (P_2>0.5) with mating intervals even as long as 24 hours (*Drnevich et al.*, 2000). Therefore, if the male transfers some factor to the female to inhibit pheromone production it could give a selective advantage even if the effects are not realized until several hours later. Such a mechanism is not without precedent. Raina (1988) reported that the male *Heliothis zea* transferred a hemolymph born factor to the female during mating that effectively turned off pheromone production within 2 hours of mating.

The significance of pheromone regulation for the female is quite different. It may be in the female's best interest to mate more than once in a short period of time. *T. molitor*

are known to be polyandrous (Gage, 1992). Some of the possible benefits of multiple matings include: (1) ensuring adequate sperm stores; (2) ensuring mating with high quality males; (3) "bet hedging" against possibility of sterility of first male; and (4) increasing genetic variability of offspring (for review see Keller and Reeve, 1995).

Multiple mating also has its disadvantages. Apart from the time and energy that courtship and copulation require, mating also increases the risk of predation, parasite transmission, and in some insects, the negative impacts of seminal fluid products (for review see Keller and Reeve, 1995). Once the female has adequate sperm, it may provide a selective advantage to devote all of her energy and resources to egg laying instead of pheromone production and mating. Menon and Nair (1972, 1976) report that ovary maturation and pheromone production are related and both may be regulated by juvenile hormone (JH). They found that treatment with excess JH triggers yolk deposition (a stage of egg maturation) which in some way inhibits pheromone production. Results in our laboratory suggest that 4-methylnonanol biosynthesis is stimulated by JH (Islam, Bacala, and Vanderwel, unpublished). Gerber (1967) suggests that mating stimulates oocyte production by suppressing its inhibitory mechanism. Therefore, mating could indirectly inhibit pheromone production by stimulating egg production which somehow keeps excess JH "occupied". The reverse of this is thought to occur when *T. molitor* is irradiated in such a way that ovary maturation is inhibited but pheromone production is unaffected (Menon and Nair, 1972). The excess JH seems to be used for pheromone production instead of egg production. It is difficult to envisage the mechanism for these suggestions because JH is released into the blood stream as any other hormone, and has no control over which receptors it binds to. Perhaps the receptors themselves are regulated in response to mating.

In any case, it seems reasonable that some mechanism exists to inhibit pheromone production and/or increase pheromone degradation so that the female's energy and resources can be devoted to egg production, maturation and oviposition. Based on studies in other beetles it seems likely that JH would be involved somehow (for review see Vanderwel, 1994).

The results of this study indicate that mating has a complicated effect on the behaviour and physiology of *T. molitor*. Additional bioassays should be conducted to extend the findings of this study. The activity of extracts taken from females more than 2 hours after mating must be investigated to determine the peak time and duration of suppressed attraction . Furthermore, new bioassay experimental designs are required to investigate the touch and CR responses separately and more effectively. Now that bioassay data has identified some of the possible mechanisms at work, more precise techniques are required to further our understanding of these mechanisms. Bioassays are limited by the highly variable nature of the assay tool: that is, the living male beetle. Thus, variability is introduced by both the female beetle in the form of different pheromone production levels, and by the male in the form of different response levels. Both of these sources of variability are affected by a wide range of physiological and environmental factors. For these reasons, we began preliminary attempts at biochemical assays that can actually quantify the effects that mating has on 4-methylnonanol titre. *In vitro* and *in vivo* assays using radiolabeled 4-methylnonanol and its precursors may be able to identify the mechanisms for the regulation of 4-methylnonanol biosynthesis in response to mating.

4.2 Biochemical Assays

4.2.1 Synthesis of Tritium Labeled 4-Methylnonanoyl CoA

All attempts at the enzymatic synthesis of ^{3}[H]4-methylnonanoyl CoA were unsuccessful. Our inability to synthesize the CoA derivative is difficult to understand and no concrete explanations for this failure have been determined. Bacala (2000) reported low but reasonable yields (10-20%) with this enzymatic synthesis technique. The fact that virtually zero yields were observed indicates that perhaps the enzyme was no longer active or was accidentally denatured during the procedure. Galliard and Stumpf (1968) reported 50-70% yields of radioactive long-chain s-acyl CoA using an enzymatic preparation made from avocado tissue. Bishop and Hajra (1980) reported 75% yields of ^{14}C-Labeled fatty acyl CoA using a chemical synthesis involving conversion of the fatty acid to an acyl chloride by oxalyl chloride followed by condensation with an excess of CoA to form the acyl CoA derivative. Perhaps an adaptation of one of these procedures would yield better results.

4.2.2 Pheromone Internalization Assay

To my knowledge, pheromone internalization has not been reported as a mechanism for decreased attractiveness in response to mating. Nevertheless, it seemed a simple place to begin and raised some interesting questions. The findings of the pheromone internalization assays indicate that increased internalization is likely not a method by which mated females decrease their release of pheromone. It is interesting to note that the mated females actually internalized significantly less of the radioactively labeled pheromone

applied. Furthermore, it was found that the majority of the labeled pheromone that was not internalized did not remain on the beetle's surface. Approximately 75% of the label was actually volatilized and recovered from the surface of the vial, not from the beetle. This introduces the possibility of an as yet unhypothesized mechanism of pheromone regulation in which mated beetles 'release' pheromone at different rates, possibly involving some binding protein. This could have adaptive significance in countering the effects of an anti-aphrodisiac so that the females interest in multiple mating can be met (discussed above). This assay will be repeated but with amounts of pheromone internalized, retained on surface, and volatilized all examined separately. Furthermore, these components will all be analyzed (by HPLC) to determine if the 4-methylnonanol is being degraded into any other products.

4.2.3 *In vitro* Assay

In vitro reduction of ^{3}[H]4-methylnonanoic acid was observed indicating that this assay does have the potential to detect the regulation of the last steps of 4-methylnonanol biosynthesis in response to mating. The results of the *in vitro* assay indicate that decreased conversion of 4-methylnonanoic acid to 4-methylnonanol is not the regulated step responsible for the observed decrease in activity of mated female extracts. However, this result is not conclusive until the identity of the conversion products are determined. Both mated and virgin females have similar levels of radioactive marker in the fraction in which we expect 4-methylnonanol, but this liquid chromatography procedure is only a crude separation. If the mated females were producing a longer chain fatty acid instead of the alcohol, its increased non-polar chain could cause it to elute earlier, making it appear to be

alcohol. The *in vitro* assay will be repeated and attempts will be made to identify the conversion products by HPLC or other means.

4.2.4 *In vivo* Assay

The *in vivo* assays have great potential because they represent a method of monitoring the biosynthesis of 4-methylnonanol directly and quantifiably (in contrast to the bioassays) and reflect the actual conditions of the living beetle (in contrast to the *in vitro* assays). The inconclusive results obtained for the in vivo assays were entirely due to difficulties with the HPLC analysis. These difficulties may also hold back the analysis of the internalization and *in vitro* assay products and must be resolved. It has been observed by myself and others (Bacala, personal communication) that frequently the standards run well but extracts from beetles do not. This is likely due to the coelution of other beetle products from the extract in the liquid chromatography separation. These unknown products, both tritium labeled and otherwise, make loading and running the HPLC column problematic and the results difficult to interpret. Other separation methods including derivatization techniques may be needed to accurately analyze the samples from the *in vivo* and other assays.

4.2.5 Biochemical Assays Summary

Despite the difficulties encountered thus far, biochemical studies still represent the greatest potential for the development of an understanding of the regulation of 4-methylnonanol biosynthesis in response to mating in *T. molitor*. The results discussed above represent a very preliminary investigation of the regulation of 4-methylnonanol.

None of the regulation mechanisms proposed can yet be confirmed or discounted. Decreased pheromone biosynthesis or increased pheromone degradation still seem the most likely, based on studies in other insects (Kingan *et al*., 1995). Internalization does not seem to be a method for mated females to decrease pheromone titre but increased volatilization may be. The development of procedures for the synthesis of 4-methylnonanoyl CoA and for better HPLC analyses will greatly expedite the biochemical studies.

4.3 Significance of Work

Some might suggest that the study of the pheromone system of a single insect species is academic and unlikely to have great significance. In response to this attitude I would remind skeptics that it was the study of a single insect hormone, ecdysone, which led to the first suggestion that hormones can have a direct effect on the expression of genes (Law et al., 1992). Today, this is known to be one of the most important roles that hormones play in the development of human beings. Science is replete with such examples in which seemingly academic studies of a single organism uncover phenomena with implications across a broad spectrum of fields and species. The findings of this study could lead to a greater understanding of insect mating behaviour, sperm competition mechanisms and the regulation of pheromone biosynthesis, all of which might have implications for human systems. Furthermore, a greater understanding of the regulation of pheromone production could be useful for the manipulation of pest populations. Insect control by the use of synthetic chemicals designed to disrupt mating systems is already successfully practiced for many moth species (for review see Minks and Carde, 1997; Wyatt, 1997).

These systems offer the promise of more environmentally conscious pest management options for many agricultural crops.

5. CONCLUSIONS

Mating causes a decrease in the attractiveness of female extracts as measured by a glass rod bioassay: (1) Extracts of newly mated females (0 hour) were less able to attract and maintain the interest of the males, compared to the virgin extract. (2) Extracts taken 2 hours after mating were also less able to attract and maintain the interest of the males, compared to the virgin extract. (3) A single mating did not suppress the ability of the female extracts to elicit a CR response from the males. (4) Longer mating durations further decreased the ability of female extracts to attract and maintain the interest of the males. (5) After a 21 hour mating duration the CR response elicited by the extracts also decreased.

From these conclusions, the following interpretations were made: (1) Mating reduced the attractiveness of the female *T. molitor* through two effects. (i) An immediate effect involved the deposition of an anti-aphrodisiac that wore off within one hour. (ii) A second effect involved the decrease in 4-methylnonanol titre within one to two hours after mating. (2) Multiple mating amplifies the decrease in 4-methylnonanol titre. (3) The CR pheromone titre did not decrease after mating until much later (21 hours) or unless the female was mated more than once.

Conclusions were not stated for the biochemical studies due to the preliminary nature of these experiments. The assays must be improved and repeated before conclusions can be stated with confidence.

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