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Investigation into the sex pheromone of the adult female odd beetle, *Thylodrias contractus* Motschulsky, 1839

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Abstract

Insects of many species depend on the chemical cues in sex pheromones to identify potential reproductive partners. Previous studies have shown that odd beetle, *Thylodrias contractus* utilize sex pheromones at the onset of reproductive physiology that leads to mating behavior. This is a study to make strides to identify the sex pheromone(s) for the odd beetle, *T. contractus*. In this study, efforts to isolate the sex pheromone of this pest insect began with observations of the mating behavior of this insect. This was followed by dissections of adult virgin females in an attempt to reveal which parts of the anatomy were producing the pheromone. In addition, several sampling methods were used to help identify this pheromone on a Gas Chromatograph. These methods included blowing filtered-air across live beetles onto an absorbent column, solvent washes of adult beetles and a sampling technique using solid phase microextraction (SPME) on live insects. Finally, an analysis of the headspace above live adult virgin females using a Gas Chromatograph/ Mass Spectrometer (GC-MS) was made. Comparison of the Gas Chromatograph results and the results of the GC-MS has narrowed the possibilities of what compounds may be included in the female attractant pheromone.

Key Words: Odd beetle, *Thylodrias contractus*, pheromone, monitoring

1. Introduction

1.1 *Thylodrias contractus* Motschulsky, 1839

The odd beetle, *Thylodrias contractus* is capable of causing the biodeterioration of museum objects (Alpert, 1988). *T. contractus* are one of the most common pests found in direct association with natural history collections (Jacobs, 1995). This beetle has been suggested to be both synanthropic and cosmopolitan (Mertins, 1981). *T. contractus* are known to eat all types of hide, skin and pinned insects that are stored in collections or on exhibit in museums (Alpert, 1988).

1.1.1 Sex Pheromone

Insects of many species depend on the chemical cues in sex pheromones to identify potential reproductive partners (Schal, Fan and Blomquist, 1992). The adult male *T. contractus* readily reacts to the sex pheromone of the adult virgin female *T. contractus*. After successful mating, the female no longer produces the sex pheromone (Mertins, 1982).

1.1.2 Monitoring

Historically, monitoring for *Thylodrias contractus* within a museum has delivered imprecise results that make it difficult to locate the source(s) of an infestation. The challenge of locating the source of an infestation is due in part to the fact that the most

common monitoring tool has been the use of un-baited sticky traps. These traps have no attraction to the insect, but instead rely on the chance that the insect will simply blunder into the trap (VanRyckeghem, 2011). Museum staff and pest management companies currently lack a readily available monitoring lure for the dermestid odd beetle, *Thylodrias contractus* (VanRyckeghem and Kelley, 2011). Pest monitoring programs with sex pheromones can reveal the location of infestations (Pierce, 1994). Identification of the sex pheromone(s) and the potential to synthesize the sex pheromone of *T. contractus* would be beneficial in monitoring this pest insect. Sex pheromone lures placed in sticky traps tend to give much more detailed information of where infestations are located and capture a much higher percentage of the adult male insect pests (VanRyckeghem and Kelley, 2011). Sex pheromones for museum pest species such as the tobacco beetle, (*Lasioderma serricorne),* clothes moths, (*Tineola bisselliella* and *Tinea pellionella),* and carpet beetles, (*Attagenus unicolor* and *Anthrenus verbasci)* have drastically improved the capture rate of these species in sticky traps in museum settings and have allowed assertive Integrated Pest Management (IPM) specialists to locate, remove and/or treat sources of infestation before they spread into greater areas of the museum collection (VanRyckeghem and Kelley, 2011).

1.2 Pheromone Discovery

The process of determining the chemical compounds that make up sex pheromones or other semiochemicals produced by biological organisms can be multifaceted. In this study, I began by first observing the mating behavior of the adult *T. contractus*. This was followed up by attempting to identify the part of the anatomy that contained the sex pheromone producing gland in the adult female. In theory, knowledge of the specific body part that produces the pheromone would allow a more focused analysis on a Gas Chromatograph (GC). When testing small-scale preparative separations of volatiles and semivolatiles, gas chromatography is the standard method of analysis (Millar and Haynes, 1998).

1.2.1 Methods of Pheromone Discovery

In other sampling, three different techniques to collect pheromone for analysis were used in this study. The first technique was done by performing solvent washes of adult beetles and beetle body parts. The second technique isolates the airborne pheromone from adult insects by trapping the organic volatiles onto a porous matrix. The third technique is Solid Phase Microextraction (SPME). SPME is commonly used to isolate volatile organic compounds from live, adult insects. SPME is a sensitive extraction technique used in various matrices for volatile components (Borg-Karlson and Mozuraitis, 1996, Frérot et al, 1997). I tested samples using all three of these techniques and then ran the collected material through a GC for analysis.

1.2.2 Gas Chromatograph – Mass Spectrometer Analysis

A final means of analysis in this study used two commonly coupled instruments, the gas chromatograph and the mass spectrometer (GC-MS). A GC-MS can help identify

semiochemicals compounds in pheromones (Millar and Haynes, 1998, Borg-Karlson and Mozuraitis, 1996). The MS-GC was used on an SPME sample from the aeration of live adult virgin female odd beetles, *T. contractus*. The mass spectrometer breaks the compounds from the aeration sample into fragments and separates out the ions. The ions are analyzed based on the abundance of each and recorded as a spectrum and on the basis of mass/charge ratio (Millar and Haynes, 1998).

2. Materials and Methods

2.1 Insect Colony

The insects used in this study were from a laboratory culture derived from an infestation within a natural history museum. The culture has been maintained since 2007 under ambient room temperature, light, and humidity conditions in wide mouth, glass, 1 pint canning jars (Ball Corporation, Broomfield, CO, USA). These jars were covered to contain the insects with open-topped metal screw lids fitted with Whatman® No. 1, 90 mm filter paper (Gorham, 1991). The filter paper served as a means to allow the passage of air in and out of the containers. The larval diet consisted of dried insect cadavers which were primarily the ground-up larvae and pupae of *Musca domestica*.

2.2 Separation of Pupae

Individual insects for observation were removed from the cultures in the pupal stage and isolated individually in ½ dram glass vials (Figure 1). They were observed daily until adult emergence. After eclosion, individual males and females were removed from the vials and were kept isolated from other adults to ensure virginity. Insects were held in glass petri dishes until investigations were performed. All males and females used in this study were observed less than seven days after eclosion. The female *T. contractus* has a peak attraction to males 48 hours after eclosion (Mertins, 1982). Absorptive paper exposed to virgin females, remains attractive to males for an entire 16 months following exposure (Mertins, 1982).

2.3 Dissections of Adult Virgin Females

Dissections of different anatomical parts of the larviform adult virgin females were performed and the reactions of sexually active adult virgin males to these body parts were recorded. The dissected parts were divided into four areas which included: head, thorax and legs, the first anterior (1 -7) tergites, and the single remaining (8^{TH}) posterior tergite also referred to here as the abdominal tip (Figure 2). Dissection of the virgin females was performed using a Nikon® stereoscopic microscope with magnification up to 40X and an external high intensity light source. The dissections were performed using micro-dissecting needles and a micro-dissecting spatula.

2.4 Observations Parameters

Observations were performed with the insects in 100 mm X 10 mm open plastic Petri dishes. To prevent escape, the interior outside edge of the Petri dish was painted with a liquid fluon and allowed to dry before the insects were placed inside. The bottom surface of the petri dishes was coated with a single sheet of filter paper (Whatman No. 1, 90 mm). This provided better traction for the insects to walk in the dish. Observations were performed under ambient room lighting with some additional desk lighting. Observations of courtship were videotaped and later studied to record detailed observations.

2.4.1 Observation Responses

Observations of the adult male responses to the different dissected parts of the female anatomy were recorded into one of the following categories: Strong Response, Definite Response, Slight Response and No Response. An observation of Strong Response meant that the majority of males in the arena had immediate and extended behavioral changes when exposed to the dissected body part. A Definite Response indicated that at least one male would interact with the dissected body part for 10 seconds or more before moving away. A Slight Response indicated that the male beetle would react to the dissected body part for 2 seconds or less before moving away. An observation of No Response meant that the adult males did not exhibit any behavioral changes at all to the dissected body part within the arena. All behavioral changes noted by the males in this study were attractive in nature.

2.5 Gas Chromatograph Analyses of Solvent Washes using Hexane

The solvent wash of the insects was performed using hexane in a standard 500 ml LDPE wash bottle with a bent nozzle. Hexane from the bottle was squeezed through the nozzle using hand-pressure. Approximately 1.5 ml of hexane was sprayed over a live virgin female being held in a ½ dram clear-glass vial with a Teflon®-lined screw cap. The beetle was held in the hexane solution for a period of 24 hours prior to CG analysis. This solvent wash technique has been performed in the pursuit of pheromone identification in other insect species (Howse et al. 2013).

2.6 Gas Chromatograph Analyses of Solvent Washes using Dichloromethane (DCM)

Solvent washes using dichloromethane (DCM) were also performed. A wash of the glassware used to hold live virgin female from 24 – 72 hours after eclosion was performed using a wash bottle containing DCM with a bent nozzle. The DCM (2 ml) was sprayed down all sides of the 1 dram vial. The wash was performed immediately after the female was removed from the vial. DCM was used since it is a one-carbon compound that provides a clean, single peak in the GC analysis.

2.7 Gas Chromatograph Analyses of Porapak Q Samples

Porapak Q® (Waters Corporation, Milford, MA, USA) is a standard testing material that has been used to identify and isolate insect pheromones in the past (Borg-Karlson and Mozuraitis, 1996, Millar and Haynes, 1998). The testing of the airspace above adult insects used a porous absorbent matrix column to collect the potential pheromone. The columns were filled with Porapak Q 50/80 mesh. Test samples originated from a tank of breathing-quality compressed air that was regulated and run through a 36 cm tall by 1.3 cm diameter condenser column (Chemglass Life Sciences, Vineland, NJ, USA) filled with glass wool moistened with distilled water. The air sample was then run through a second 36 cm tall X 2.5 cm diameter condenser column (Chemglass) containing activated charcoal held in the column by thin layers of glass wool on each end of the charcoal. After the regulated air was filtered through the 2 columns, it was then passed across a short glass chamber containing the adult virgin females resting on a 1 cm diameter sheet of filter paper. Volatiles from the head space located above containers of adult virgin females were collected and captured in the Porapak Q columns for future investigation (Figure 3 and Figure 4). These columns were then analyzed using a GC. Volatiles were extracted sequentially with 0.5 ml of hexane, then a solution of hexane:ether at a ratio of 95:5, and lastly 0.5 ml of dichloromethane. One microliter samples of the extracts were placed into the GC column.

2.8 Gas Chromatograph Analyses of SPME Samples

The SPME sampling was performed using a Solid Phase Microextraction holder (Supelco® manufactured by Sigma-Aldrich, St. Louis, MO, USA) along with a DVB/CAR/PDMS 50/30 μm fiber assembly (Supelco). The live insects were held in a 4 ml clear-glass screw-top vial using an open plastic cap with a PTFE Red Rubber liner. The insects were held for a period of 24 hours with the exposed SPME fiber prior to GC analysis. The SPME fiber was conditioned according to manufacturer's specifications at a temperature of 270° C for 0.5 hours prior to exposure to the headspace above the live insects.

2.9 Gas Chromatograph Equipment and Procedures

The gas chromatograph used to analyze the samples was a Shimadzu® model GC-17A FID.

2.9.1 Standard Method of Gas Chromatograph Analysis

The standard method used on the GC started with a column temperatures of 35° C held for 2 minutes. The column temperature was then ramped upward at 10° C per minute until a temperature of 260° C was reached. The temperature of 260° C was then held for 5.5 minutes. This entire analysis lasts for 30 minutes.

2.9.2 SPME Method of Gas Chromatograph Analysis

The GC method used on SPME fibers started with a column temperature of 35° C for 3 minutes. The column temperature was then ramped up at a rate of 3.6° C per minute until 200° C was reached. The column temperature was held at 200° C for 11.2 minutes. This entire SPME analysis lasts for 60 total minutes.

2.9.3 Known Chemical References on the Gas Chromatograph Analyses

As a means to map known chemical references on the GC, an n-Hydrocarbon mix (85:15), 1 ml, 1000 μg/ml in methylene chloride:carbon disulfide was used under the brand name: TraceCERT® (Supelco, Sigma-Aldrich).

2.10 Gas Chromatograph – Mass Spectrometer Methods

The GC-MS analysis of the aerated headspace above live virgin females was performed by the independent company, CHEMIR-EVANS Analytical Group (2672 Metro Blvd. Maryland Heights, Missouri). The GC resolves the sample components based on volatility, and the MS detects and identifies the components. In the MS analysis, the resolved sample components are ionized and separated in a mass analyzer. The fragmentation pattern of a sample component and its computer-library match enables sample identification.

3. Results

3.1 Mating Behavior

In my observations of the mating behavior of adult *T. contractus,* the males immediately engaged the females and begin the courtship process 100% of the time in the presence of virgin females. The process would follow a predictable sequence as seen in the ethogram of the adult male mating behavior in (Figure 5). In my observations of seven successful mating courtship rituals, males rubbed back and forth on the dorsal side of the females at an average of 80 times per male. A rub consisted of an anterior to posterior swaying of the male's body while maintaining contact between his sternum and the dorsal side of the female. In all circumstances where the rubbing occurred, females that had previously been walking actively about the Petri dish suddenly stopped and became still for the next 6 – 8 minutes of the mating process. Brush-like setae on the second abdominal sternum of the adult male *T. contractus* may produce a type of pheromone used in the mating process (Mertins, 1982). A diagram of the rubbing positions can be seen in Table 1. Rubs were in the 12:00 o'clock position 78% of the time, the 11:00 o'clock position 10.5% of the time, and in the 1:00 o'clock position 9 % of the time. Nearly 98% of the time, the positioning of the male was at or near the 12:00 o'clock position. The only diversion from this sequence occurred when multiple males would engage a single virgin female at the same time. In these cases, the males would bypass any rubbing behavior on the head and thorax and would immediately begin abdominal distal bending in an attempt to copulate. In some

circumstances, it appeared that 2 – 3 males were able to successfully copulate with a single female simultaneously. If a single virgin male and a single virgin female were introduced into the Petri dish with no other beetles, the male would always begin the rubbing behavior with the glandular setal hairs on his sternum over the head, thorax and antennae of the female. This rubbing behavior would last for approximately one minute before the male would slide down posteriorly on the female and attempt copulation. If the male was rubbing the upper head area of the female at a 12:00 o'clock position, the most likely next behavior would be continuing to rub in that same position. The next most frequent behavioral shift would be rubbing that same female at the 11:00 o'clock position or a 1:00 o'clock position or any position nearest to the current position. In all of my observations, the male was always in a 12:00 o'clock position prior to shifting down posteriorly on the female to attempt copulation. Successful copulations would last between 1 and 8 minutes before the male would break away.

3.2 Dissections of Adult Females

The results of the analyses of the dissected parts of the virgin females revealed that the parts of the adult female anatomy that elicited a Strong Response from the males were the two sections containing the thorax/legs and the first seven anterior abdominal tergites. The head of the female elicited a Slight Response. The eighth abdominal tergite, (also called the abdominal tip in this study) elicited No Response. When multiple males were exposed to the thorax/legs or upper abdominal sections of the adult virgin females, several males were observed attempting to mate with the body part and some even attempted to mate with the other males that were in contact with the body part. I was unable to locate one specific area that was the single sex pheromone production area on the virgin female, but the thorax and upper abdomen sections had the strongest attractive qualities to the adult males.

3.3 Gas Chromatograph Analyses of Solvent Washes using Hexane

A GC analysis of hexane solvent wash of the anterior (1-7) tergites of the female was performed using a standard method on the GC. The tergites yielded high GC peaks at the 6.74, 7.44, 15.23 and 15.90 minute marks of the 30 minute analyses. These peaks were also compared to those of the mated and virgin female air samples. GC peaks for virgin males that were not common to the peaks for virgin females occurred at the 18.3 and 20.40 minute marks using the standard GC method of analysis. The GC analyses varied greatly over several samples of the hexane solvent washes from between one and seven whole virgin females. There were several common peaks among samples. A common peak at the 15.23 minute mark correlated with other solvent wash analytical peaks for the tip of the abdomen and tergites.

3.4 Gas Chromatograph Analyses of Solvent Washes using Dichloromethane (DCM)

A GC analysis of dichloromethane solvent wash of glassware that housed adult virgin female *T. contractus* can be found in Figure 8 and a comparison of the GC analysis of that wash and an n-Hydrocarbon standard on the GC can be seen in Figure 9.

3.5 Gas Chromatograph Analyses of Porapak Q Samples

Porapak Q samples were taken from the aeration in the headspace above virgin females and the GC analyses of these samples were compared to the GC analyses from mated female and virgin male *T. contractus*. All of these analyses used the standard analytical method on the GC (Figure 7).

3.6 Gas Chromatograph Analyses of SPME Fiber Samples

A GC analysis of an SPME fiber exposed to of the headspace or aeration of the virgin adult females for a 24 hour period can be seen in Figures 7 and 11. This evaluation used the SPME method of GC analysis. An overlay of the GC analysis of n-Hydrocarbon standards and the GC analysis of an SPME of a virgin, female odd beetle, *T. contractus* can be found in Figure 12.

4. Discussion

4.1 Relationship of Adult Male Mating Behavior to Female Sex Pheromone

The observation of the mating behaviors of adult beetles suggest that the sex pheromone from the adult virgin female *T. contractus* has a significant effect on the behavior of the adult virgin males of this species. In my observations, virgin males would immediately engage the virgin females at their first chance and begin the courtship process 100% of the time. The process would follow a predictable sequence as seen in the ethogram (Figure 5). Nearly 98% of the time, the positioning of the male was at or near the 12:00 o'clock position. It is possible that male-produced pheromone emanating from setal hairs on the ventral abdomen suppresses the activity of the female in preparation for the process of mating. According to the ethogram, males approached virgin females from a perpendicular position or directly from the rear 84% of the time. Although there may be some visual cues involved, this does not contradict my findings that the strongest pheromone producing body parts on the female are the thorax and upper abdomen. These two areas are the most frequently approached areas by males to initiate the mating process. My observations of the mating process strongly suggests that the female *T. contractus* is very likely producing an active sex pheromone that attracts males to her in the mating process.

4.2 Physical Location of Sex Pheromone Production in Adult Females

The dissection of the virgin females did not offer conclusive evidence of where the sex pheromone is produced and/or emitted in adult virgin female *T. contractus*. The reaction of the males to the dissected parts of the anatomy did suggest that the males were most attracted to the thoracic and upper abdominal sections more than other sections. The head of the female did have a very slight attraction to some males but there was no attraction to the abdominal tip. The lack of response to the abdominal tip was unexpected. In Lepidoptera, this area commonly contains a structure where pheromones are produced and emitted. In studying the anatomy of the abdominal tip of female *T. contractus*,

anatomical structures that are most likely associated with oviposition, appeared to possibly have glandular setae that could potentially produce and emit sex pheromone. This was not the case. Further studies are necessary to determine the exact locations of sex pheromone production on the females. Since I was unable to isolate the exact location of pheromone production on the dissected female body parts, I was limited in the collection of precise information that I could gather from GC analyses that could lead to an identification of the sex pheromone.

4.3 Discussion of Solvent Washes Using Hexane

Looking only at the GC analyses of the different hexane solvent washes of whole, adult insects or dissected body parts, I do not see a definite trend that points to discovery of the potential sex pheromone of the female odd beetle, *T. contractus*. There are several reasons that GC analyses from hexane washes can be unpredictable. The large volume of solvent used in the wash must be removed in the analysis while attempting to minimize the loss of the volatile semiochemicals. The solvent peaks on the GC analysis can sometimes obscure the semiochemicals peaks (Millar and Haynes, 1998). For this reason, I am discarding the results from the hexane solvent washes in this study.

4.4 Discussion of Common Dermestid Beetle Pheromones

First-hand knowledge of GC analyses of the sex pheromones of a wide range of insects and information provided by an online database of insect pheromones and semiochemicals (El-Sayed, 2014) has shown that the vast majority of sex pheromone semiochemicals are of a molecular weight ranging from 6 to 18 carbon molecules. Published semiochemical compounds that make up the female sex pheromones of dermestid beetles reveal compounds that are of a molecular weight ranging from 10 to 16 carbon molecules (Mayer and McLaughlin, 1991). The female sex pheromone for *Anthrenus flavipes* has a molecular weight of 10 carbon molecules in the compound: (Z)-3-Decenoic acid. The 2-component female sex pheromone for the varied carpet beetle, *Anthrenus verbasci* both have a molecular weight of 12 carbon molecules where the first component is: (Z)-8-Dodecen-1-ol acetate and the second component is: (E)-8- Dodecen-1-ol acetate. The female sex pheromone for black carpet beetle, *Attagenus megatoma* has a molecular weight of 14 carbon molecules in the compound: (E,Z)-3,5-Tetradecadienoic acid. The 2-component female sex pheromone for *Trogoderma glabrum* both have a molecular weight of 16 carbon molecules where the first component is: [R(E)]-14-Methyl-8-hexadecenal and the second component is: [R(E)]-14-Methyl-8-hexadecen-1-ol. The 2-component female sex pheromone for the khapra beetle, *Trogoderma granarium* both have a molecular weight of 16 carbon molecules where the first component is: [R(Z)]-14-Methyl-8-hexadecenal and the second component is: [R(E)]-14-Methyl-8-hexadecenal (Mayer and McLaughlin, 1991).

4.5 Discussion of Solvent Washes Using Dichloromethane

GC analyses of the dichloromethane solvent washes taken from the glassware that housed virgin females and the GC Analyses of the dissected body parts of virgin females were performed. These analyses revealed consistent and similar results. Only two noticeable sample peaks were found in all of these GC analyses. The first peak came almost immediately following the known eluant peak of the dichloromethane solvent at 3.30 minutes. The early peak was located at a position that is close to compounds that have three to four carbon molecules. The second, later peak came at 24.99 minutes (Figure 8). Based on the comparison of the later peak to the n-Hydrocarbon standards as seen in Figure 9, this eluant of the solvent wash reveals a compound that is likely a 23 to 24 carbon bond molecule. Both of the sample peaks from the dichloromethane wash are outside of the general range of insect pheromones. This late peak is theorized to be more related to hydrocarbons from the cuticle rather than related to sex pheromone. Based on these concepts, the dichloromethane washes did not give me information to help with the identification of the sex pheromone of *T. contractus*. Just like the hexane solvent washes, the GC analyses of dichloromethane solvent washes can be unpredictable (Millar and Haynes, 1998).

4.6 Discussion of Samples Taken from Porapak Q System

The collection of volatiles in the headspace above insects via aeration of the insects has advantages over the analyses of solvent washes. Aeration gives a more representative sample of the organism. The aeration samples can be considered more “clean” since they are not complicated by the solvent in the solvent extracts and thus contain only the sample volatiles (Millar and Haynes, 1998). Although the Porapak Q samples are considered advantageous over the solvent extracts, they can pose a problem if the volatile compounds are polar or they have a low molecular weight (Millar and Haynes, 1998). A comparison of the peaks in the GC analysis that occurred in both the mated and virgin females was carried out. Since we know that mated females no longer attract males, analytical peaks that were found in both mated and virgin females were cancelled out as representations of potential sex pheromone (Figure 7). Similar GC peak patterns to those remaining peaks that were left after the cancellation process in the Porapak Q samples were seen in the GC analyses of the SPME fibers from adult virgin female *T. contractus*. Ultimately, I used the SPME analyses to help me find potential compounds that could be pheromones. I had more consistent analyses when using the SPME method. A single, consistent analytical peak or peaks was/were not seen throughout the Porapak Q samples. Some of this inconsistency may have been due to contamination of the GC from earlier pheromone samples run through it. Regardless of why I had no consistency in the Porapak Q samples, I felt it necessary to discard most of this data because I did not have reproducible results.

4.7 Discussion of Samples Taken from SPME Fibers

The aeration of adult beetles using GC analyses of SPME fibers exposed to adult beetles yielded consistent results that aided in determining which GC peaks had the greatest

potential to be the female sex pheromone of *T. contractus*. The SPME method aided in seeing more detail and separation between analytical peaks than the standard method. I was able to reproduce the same analytical peaks on five separate trials of virgin females exposed to SPME fibers over a 24 hour period. In different SPME trials, I compared the GC analyses of virgin adult females to mated adult females. With these comparisons, I was able to determine which peaks on GC analyses were exclusive to adult virgin females and also in the range of molecular weight (6 – 18 carbon molecules) similar to other known sex pheromones of stored-product insects. Since adult, male *T. contractus* are attracted to adult, virgin females but lose that attraction after mating, we can assume that the GC peaks on the analyses of virgin adult females that are not found in the other adult beetles can be associated with the sex pheromone of the virgin adult female. By means of direct comparisons of the GC analyses of virgin vs. mated females, it is possible to identify those analytical peaks that are exclusive to the virgin female *T. contractus*. These comparisons with SPME samples can be seen in Figure 6. Once I identified those peaks on the GC from the SPME samples, I was able to extrapolate the molecular weight of the compounds at those peaks by overlapping the GC analysis of the known, n-Hydrocarbon mix in the TraceCERT standards directly over the GC analyses of the SPME of the virgin females as seen in Figure 11. From that information, I was able to look at the GC-MS analysis to find which chemical compounds were the most likely to correspond with the GC peaks that represented the sex pheromone.

4.7.1 Potential Sex Pheromones Derived from SPME Fiber Samples

Five potential peaks on the GC analysis of the SPME fibers stood out as having the best potential to be the sex pheromone of *T. contractus*. These five peaks, as seen in Figure 6, were found only in the virgin female SPME analyses and these five peaks were reproduced over multiple trials. These peaks occurred at the times of 41.5, 43.4, 43.6, 45.7 and 46 minutes using the SPME method on the GC. Comparing these peaks to potential compounds found in the GC-MS analysis based on the fragmentation pattern of a sample component, and its computer-library match, reveals that several chemical compounds have high potential to be related to the sex pheromone of the virgin female *T. contractus*. The report provided by CHEMIR-EVANS also provided the percent probability that those compounds are the chemicals being eluted at that specific time. The peak that eluted at 41.5 minutes has a 50% probability to be trans-B-Ionone, CAS [79-77-6]. The peak that eluted at 43.4 minutes has a 62% probability to be S-Dihydroactinidiolide CAS [17092-92-1]. The peak that eluted at 43.6 minutes has a 73% probability to be n-Hexadecanoic acid, methyl ester CAS [112-39-0]. The peak that eluted at 45.7 minutes has a 70% probability to be n-Hexadecanoic acid CAS [57-10-3]. Finally, I see a direct match at 46.00 minutes (Figure 10) to a known compound where the SPME analysis of the virgin female eluted at the same time as 9,12 (z,z) Octadecadienoic acid CAS [60-33-3], which is an 18 carbon straight chain molecule.

4.8 Further Research into the Discovery of the Sex Pheromone of *T. contractus*

4.8.1 Research of Potential Synthetic Compounds

Dilutions of the above compounds, once acquired through purchase or synthesis and offered to adult, male *T. contractus*, may indicate whether or not these compounds are the sex pheromones of female *T. contractus*. Research into the detailed observations of the interactions of adult males to these individual compounds or combinations of these compounds should be carried out. Positive reactions to any of these compounds could indicate the identity of the sex pheromone of the female *T. contractus*.

4.8.2 Research Using Nuclear Magnetic Resonance and Fractionation of Solvent Washes

If none of the above compounds elicit positive male response, future analysis of the female pheromone using a nuclear magnetic resonance (NMR) would complement the MS analysis and would be beneficial. Fractionation of solvent washes is also another alternative method that could be utilized to isolate the sex pheromone of this beetle.

4.8.3 Research Using Electroantennograms

Electroantennogram (EAG) studies use the antennae of insects as a means to record the antennal response to volatile compounds. EAG analysis can be extremely useful in the identification of insect sex pheromones, (Roelofs and Comeau, 1971). In my pursuit of identifying the volatile compounds that make up the sex pheromone of the female *T. contractus*, I was unable to utilize an EAG due to the fact that the antennae of adult male *T. contractus* are less than 1 mm in length. Unfortunately, the EAG equipment which I had access to, was incapable of working with antennae of this size. Access to a lab with capabilities to run small-scale electroantennographic detection coupled with gas chromatography (EAD-GC), with subsequent pheromone identifications confirmed by re-analyzing the extract with a coupled gas chromatograph-mass spectrometer (GC-MS) using the same column and GC operating parameters as used in the GC-EAD analysis, would prove extremely valuable.

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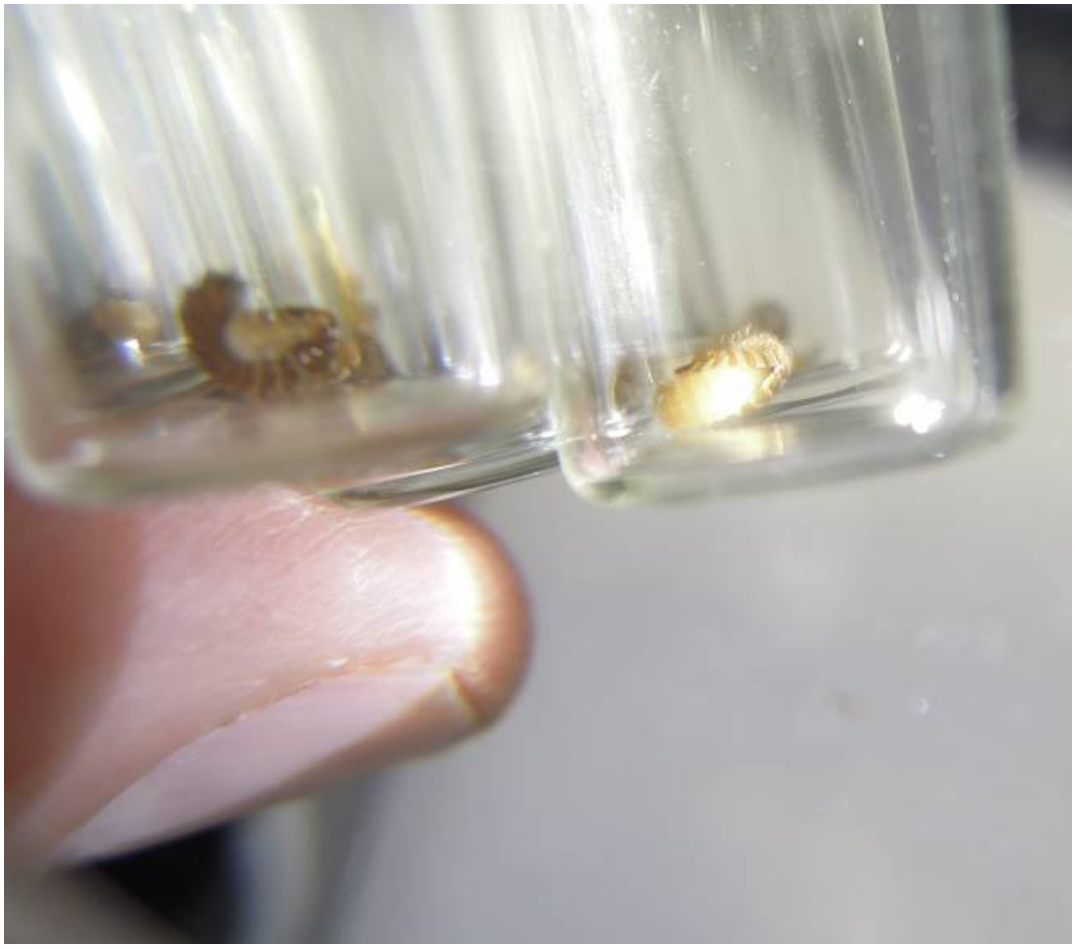


Figure 1: Pupae from cultures of *Thylodrias contractus* were separated out into glass vials prior to eclosion to ensure virginity.

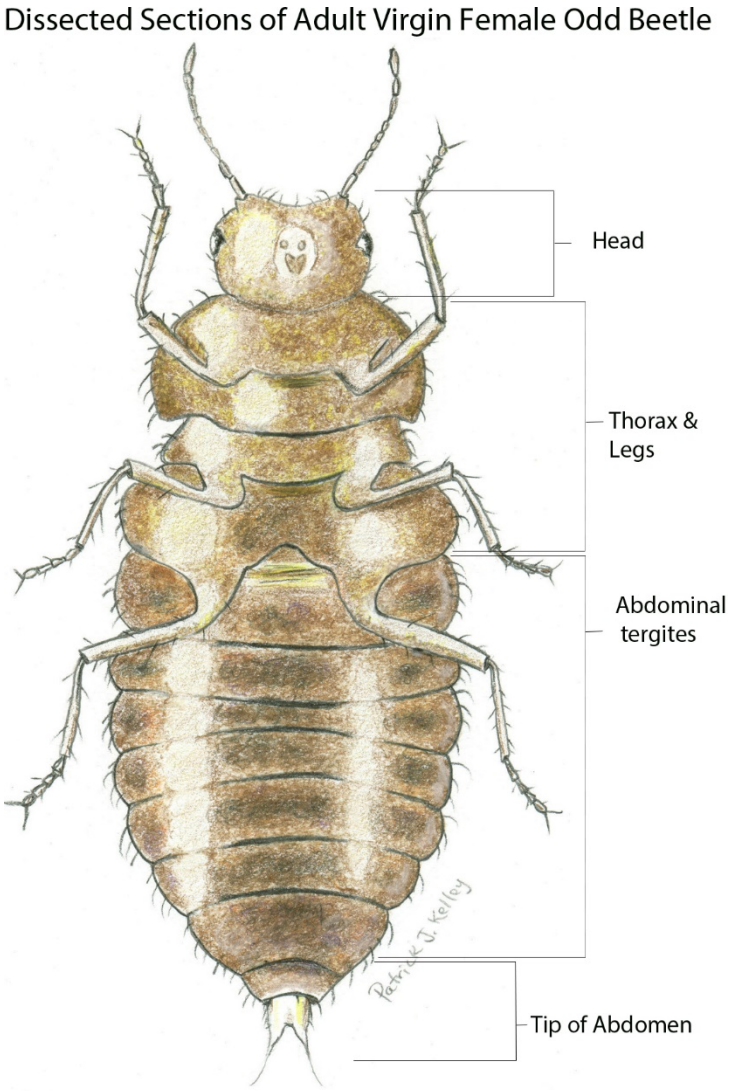


Figure 2: Drawing of an adult female odd beetle, *Thylodrias contractus* female showing the four areas that were dissected and presented to sexually active males to observe their response.

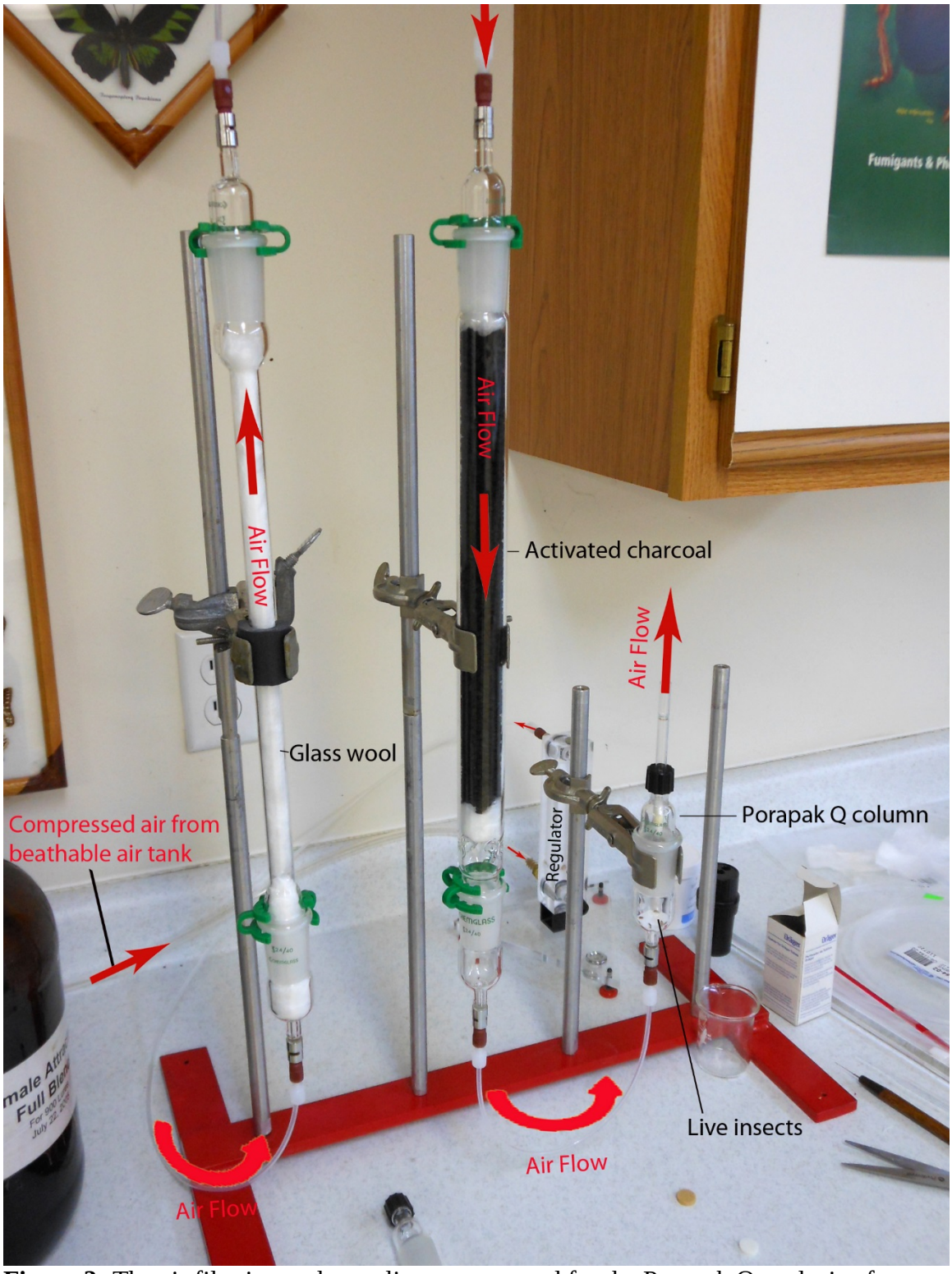


Figure 3: The air filtering and sampling system used for the Porapak Q analysis of *Thylodrias contractus*

Figure 4: Multiple adult virgin female *Thylodrias contractus* placed together to collect potential sex pheromone in the head space above with the Porapak Q column.

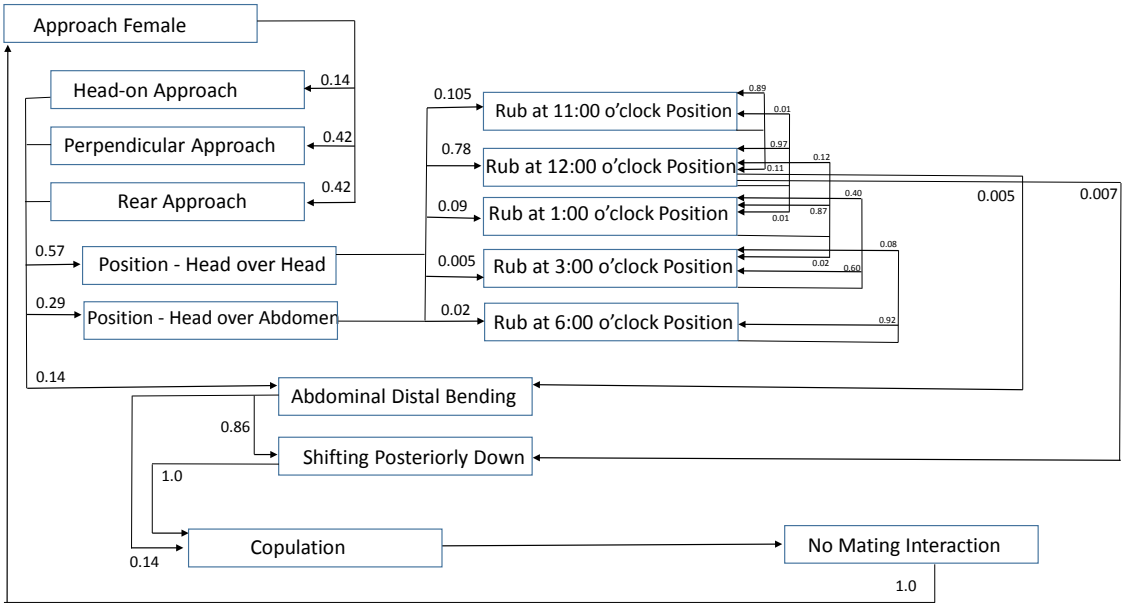
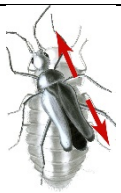
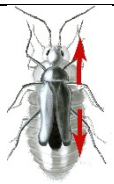
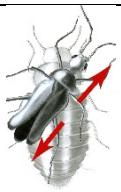


Figure 5. Ethogram representing the courtship behaviors of male *Thylodrias contractus*.

Name of Behavior	Description of Behavior	Image of Behavior
Head–on Approach	Male approaches female from a head-on position	
Perpendicular Approach	Male approaches female from a perpendicular position	
Rear Approach	Male approaches female from the posterior side of the female	
Position Head Over Head	Male adjusts or rotates his body so his head is positioned over the female's head	
Position Head Over Abdomen	Male adjusts or rotates his body so his head is positioned over the female's abdomen	
Rubbing in 11:00 o'clock Position	Male on top of female with head at a 11:00 o'clock position with respect to female's head and rubbing the female's body in that same direction	
Rubbing in 12:00 o'clock Position	Male on top of female with both heads in a line when viewing dorsally. Male begins rubbing the female's body in a parallel direction with her body	
Rubbing in 1:00 o'clock Position	Male on top of female with head at a 1:00 o'clock position with respect to female's head and rubbing the female's body in that same direction	
Rubbing in 3:00 o'clock Position	Male on top of female with head at a 3:00 o'clock position with respect to female's head and rubbing the female's body in that same direction	
Rubbing in 6:00 o'clock Position	Male on top of female with head at a 6:00 o'clock position with respect to female's head and rubbing the female's body in that same direction	

Abdominal Distal Bending	Male extends and arches abdomen	
Shifting Posteriorly Down	While keeping parallel to the female with heads aligned, the male backs into a position closer to the distal abdominal end of the female	
Copulation	Coupling begins	
No Mating Interaction	Male disregards female	No Image

Table 1. Mating behaviors exhibited in adult, male odd beetle, *Thylodrias contractus*.

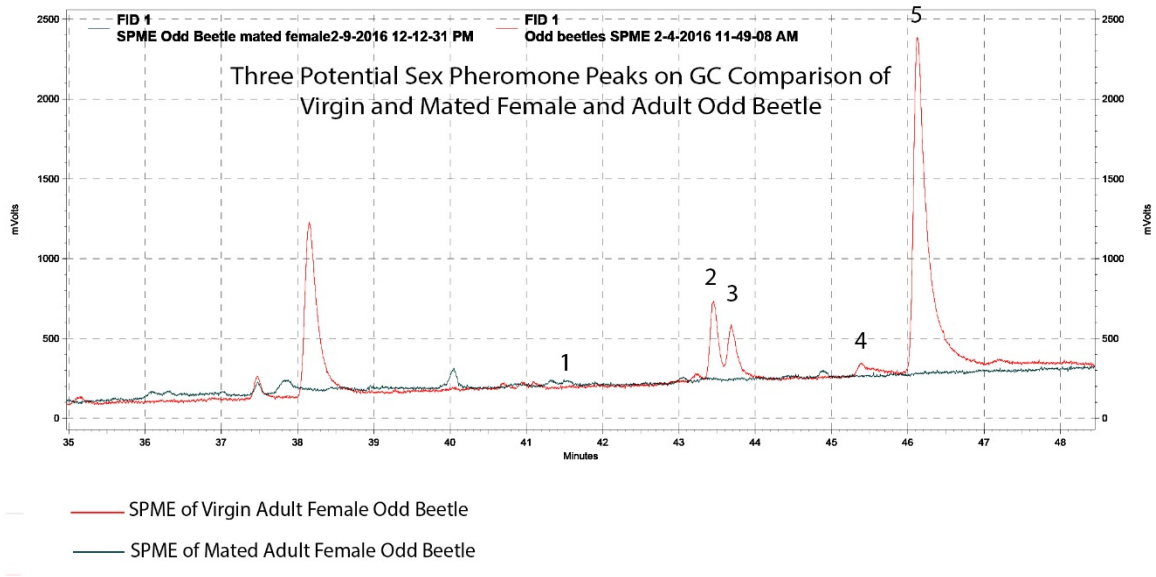


Figure 6. Comparison of GC analysis of the SPME samples of virgin female adult odd beetle, *T. contractus* and mated female adult odd beetle, *T. contractus*. This comparison suggests that the five chemical compounds peaks marked and shown along the red line could potentially be the female sex pheromone in the analysis of the virgin females that is no longer present in those same females after mating has occurred. The peak at 38 minutes was discarded since it was not duplicated in other trials with virgin females.

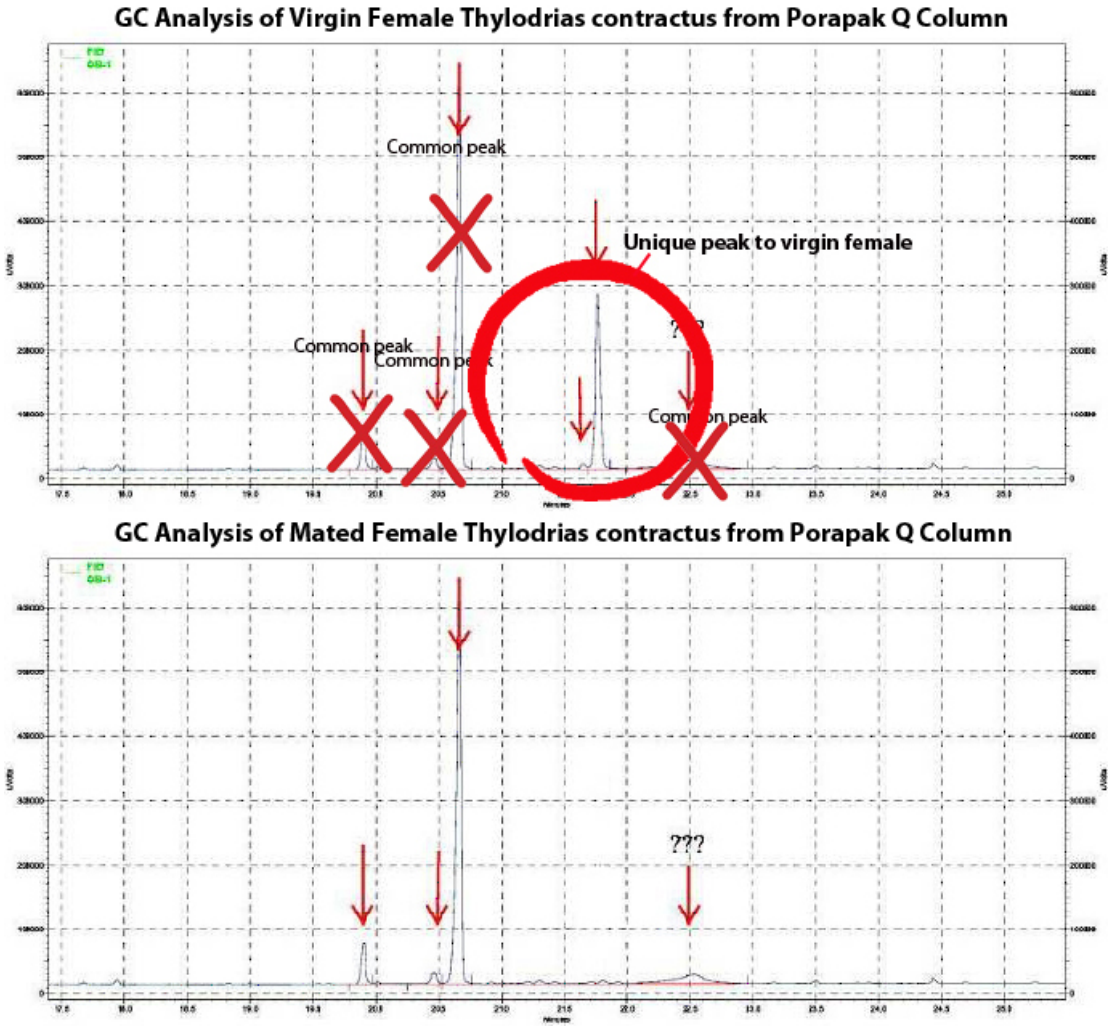
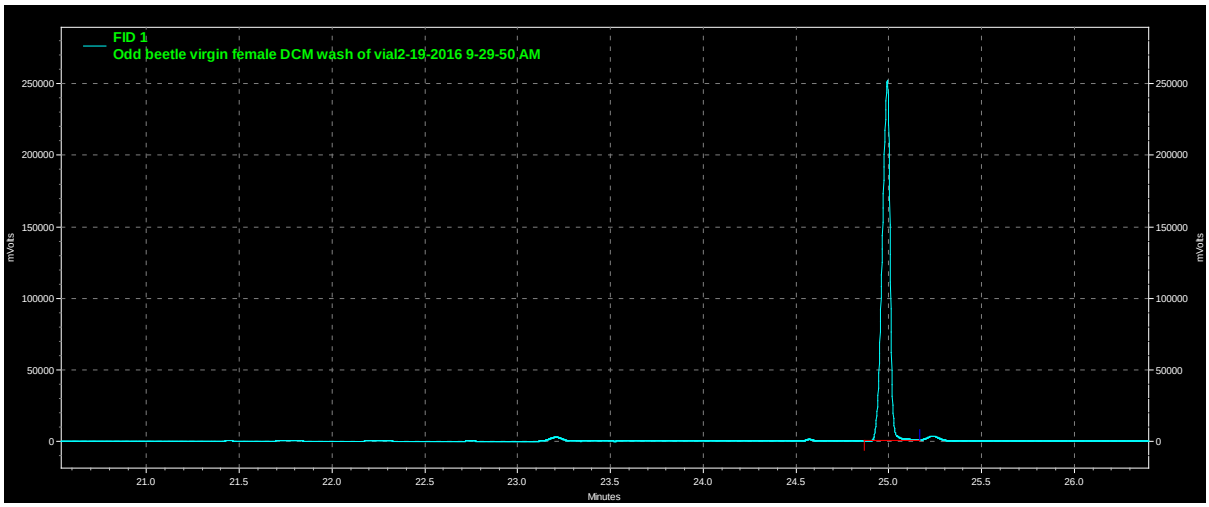
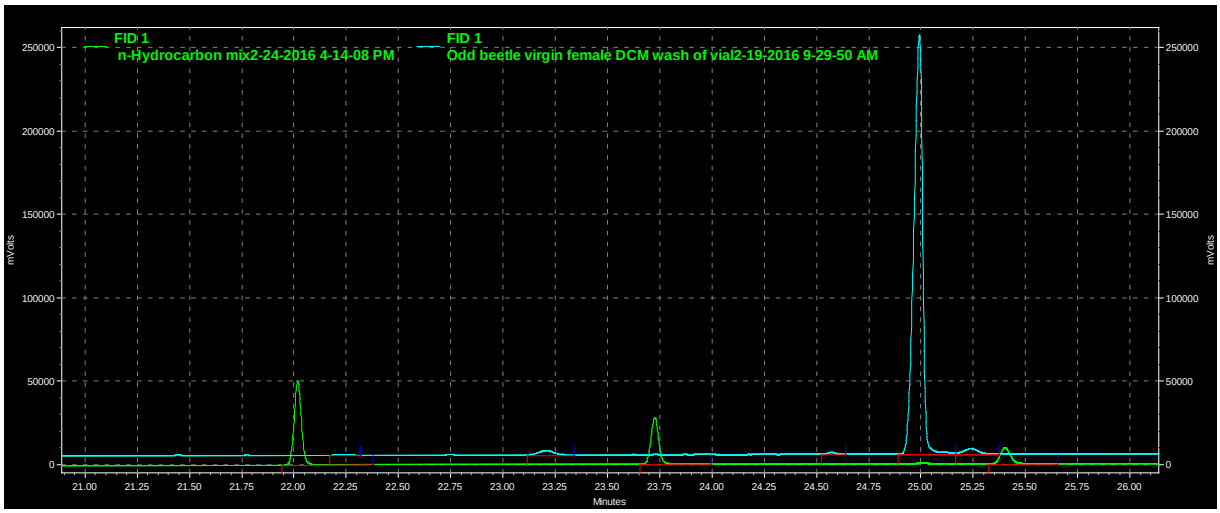


Figure 7: Method used to determine the potential sex pheromone of the adult female *Thylodrias contractus.* Determination was based on the comparison of the GC analysis of the absorbed odors produced by virgin females to that of mated females. When similar peaks occurred in both, it was assumed that these did not correspond to the sex pheromone and were disregarded as potential pheromone. The analysis on top shows a strong peak in the virgin female that was not present in the mated female analysis. The circled area represents that peak with potential for pheromone.



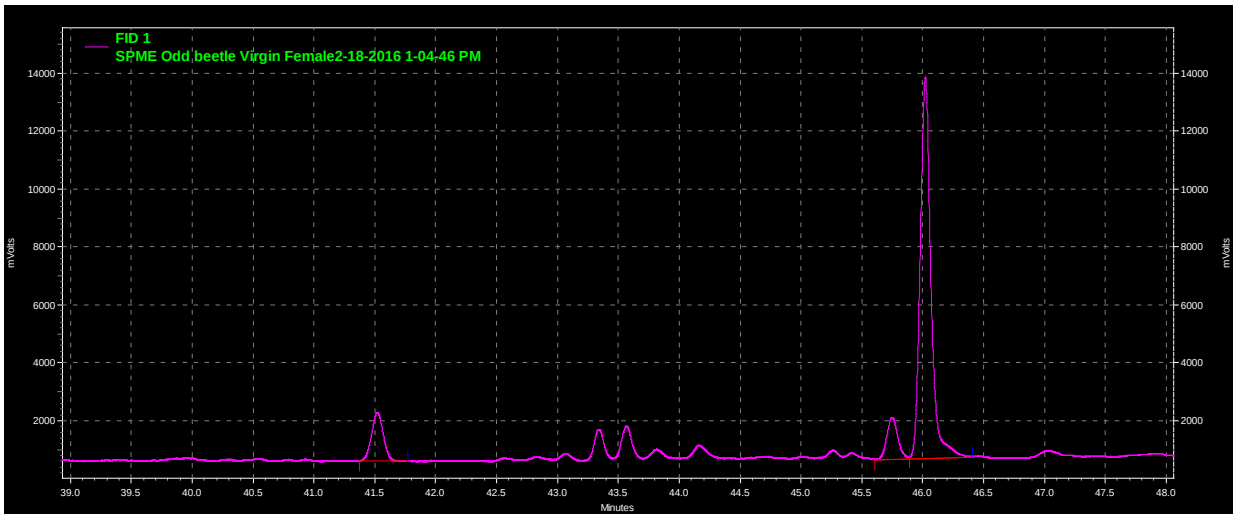
Name	Retention Time	Height	Height Percent	Area	Area Percent
	24.992	251713	100.00	739601	100.00
Totals		251713	100.00	739601	100.00

Figure 8: GC analysis of dichloromethane (DCM) wash of glassware that housed a virgin adult female odd beetle, *T. contractus* for a 48 hour period.



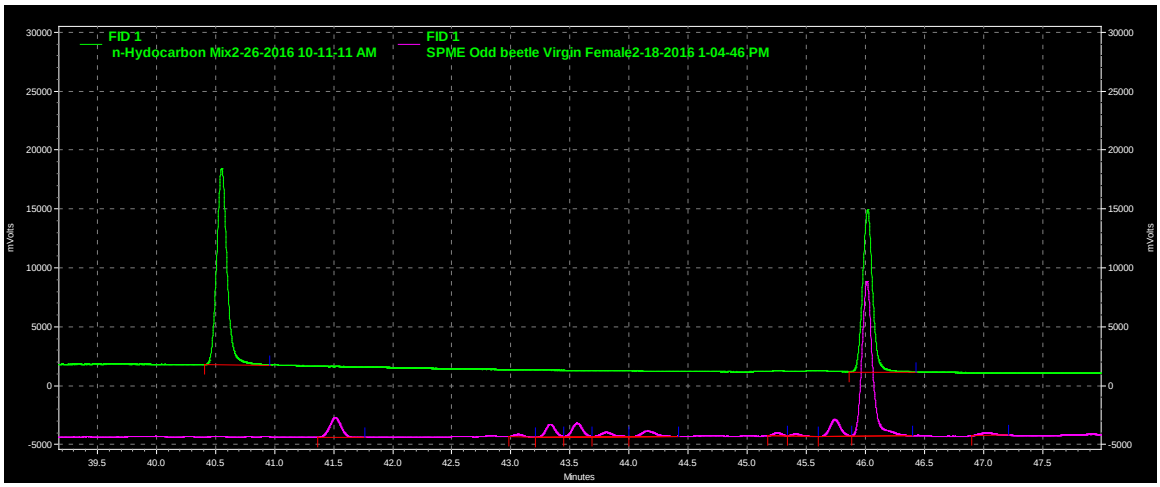
Name	Retention Time	Height	Height Percent	Area	Area Percent
n-Nonane	5.638	451618	26.48	907665	24.52
n-Decane	7.425	422577	24.77	894741	24.18
n-Dodecane	10.631	297842	17.46	633679	17.12
n-Tetradecane	13.426	168413	9.87	375652	10.15
Internal Standard	15.907	115347	6.76	282027	7.62
n-Hexadecane	18.139	92113	5.40	211280	5.71
n Octadecane	20.165	69873	4.10	169468	4.58
n-Eicosane	22.017	50362	2.95	122627	3.31
n-Docosane	23.727	27952	1.64	71988	1.95
n-Tetracosane	25.400	9674	0.57	31937	0.86
Totals		1705771	100.00	3701064	100.00

Figure 9: Overlay of n-Hydrocarbon standard on GC to GC analysis of dichloromethane (DCM) wash of glassware that housed an adult, virgin, female odd beetle, *Thylodrias contractus*.



Name	Retention Time	Height	Height Percent	Area	Area Percent
	2.412	968	4.52	8443	5.61
	36.659	722	3.37	15102	10.04
	37.966	1462	6.82	8480	5.63
	41.517	1671	7.80	11163	7.42
	45.744	1429	6.67	8998	5.98
n-Octadecane	46.022	13147	61.35	80725	53.64
	49.715	1218	5.68	8460	5.62
	54.946	814	3.80	9121	6.06
Totals		21431	100.00	150492	100.00

Figure 10: GC analysis of SPME of adult, virgin female odd beetle, *Thylodrias contractus.*



Name	Retention Time	Height	Height Percent	Area	Area Percent
n-Nonane	8.023	30813	20.07	132733	14.81
n-Decane	12.141	25688	16.73	132128	14.74
n-Dodecane	20.399	20938	13.64	137037	15.29
n-Tetradecane	27.867	19476	12.69	133454	14.89
Internal Standard	34.539	18850	12.28	119006	13.28
n-Hexadecane	40.551	16687	10.87	102126	11.39
n-Octadecane	46.022	13763	8.97	81402	9.08
n-Eicosane	51.467	7291	4.75	58409	6.52
Totals		153506	100.00	896295	100.00

Figure 11: Overlay of n-Hydrocarbon standards on a GC to GC analysis of SPME sample taken for 24 hours from an adult, virgin female odd beetle, *Thylodrias contractus.*