Release of aggregation pheromone by *Tribolium castaneum* (Coleoptera:Tenebrionidae): Differences among geographic populations, biosynthetic mechanisms inferred by RNAi gene silencing, and neuroendocrine effects on production

by

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Abstract

Intraspecific variation in pheromone release and response occurs commonly in many insect species. These variations could include differences in the isomeric composition of a given pheromone blend and the overall quantity produced. Feeding males of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae) produce an aggregation pheromone, 4,8-dimethyldecanal (DMD), that is attractive to both sexes. In this dissertation, I initially report the amount of aggregation pheromone released over 6-days by individual males from 14 populations of *T. castaneum* collected across North America, from Canada to Costa Rica. The populations studied also varied by their years in laboratory culture, from just one year for an Alabama population to over 50 yrs for a Kansas population. Pheromone released by single feeding males was obtained by headspace aeration and trapping the volatiles on the adsorbent Porapak-Q every 48 hrs for 6 days. Collected volatiles were eluted from the Porapak using hexane and analyzed by quantitative GC-MS. *T. castaneum* males produced varying amounts of DMD, from 1,547.5 ng for the Costa Rica population, to 99.3 ng for the Argyle, Manitoba population over a six-day period. Correlation analyses were done to examine any effects on pheromone release as a function of collection locality, years in colonization and morphometry of studied males for the pheromone data. Male body dimensions were measured from six populations and the amount of DMD produced was not significantly correlated with body length, width or area coverage for the beetles studied. The amount of DMD produced was not correlated with geographical location, the amount of time the populations have been cultured in the laboratory, or the size of the body regions measured. These findings are discussed in relation to previous studies that have quantified DMD from *T. castaneum*. Possible explanations for these differences are also explored.

The biosynthetic pathway of DMD was investigated by performing RNA sequencing on ventral abdominal cuticles of *T. castaneum*. Several different analyses of the data along with literature clues provided 26 candidate genes. RNA interference (RNAi) was used to evaluate the effect of suppressing the targeted transcript on DMD production using the quantitative procedure described previously. Out of the 26 candidates 5 had a significant effect on the amount of DMD produced. Suppression of fatty acid synthase decreased DMD production to 2 % of that from non-injected controls. RNAi targeting a lipase-3-like protein and a putative fatty acyl-CoA reductase each reduced DMD production to that of 36 % and 55 % the amount produced by non-injected controls, respectively. Beetles injected with dsRNA targeting a previously identified and characterized desaturase, TcasZ9desA, and an unknown hypothetical protein each produced only 39 % the amount as non-injected controls. This information is used to discuss a possible biosynthetic route of DMD.

Several neuroendocrine factors are known to affect pheromone production in several insect orders. Among these are juvenile hormone, its synthetic mimics, ecdysone and its derivatives. Adipokinetic hormone can mobilize an insect's lipids storage indicating starvation, which in T. castaneum prohibits DMD production. Biogenic amines can elicit a range of cellular responses and can orchestrate or modify physiological responses. Using the same system to collect and quantify DMD we tested these neuroendocrine factors ability to inhibit a feeding male from producing DMD, or to rescue a starved males inability to produce DMD. All of the compound tested, except dopamine, had no effect on DMD production in either fed or starved *T. castaneum* males. Dopamine showed an increase in the amount of DMD produced from feeding males but not statistically significantly more than control beetles. The results obtained here are

discussed in the light of DMD production being under the influence of hormone or endocrine factors.

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Table of Contents

List of Figures

List of Tables

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Chapter 1 - Introduction

Pheromones

A pheromone is a chemical signal used by members of the same species for communication. There are several types of pheromones which are classified according to the impacts on the physiology or behavior of either the receiver or sender. Some of these are sex pheromones, alarm pheromones and aggregation pheromones. Sex pheromones attract the opposite sex for mating. Among the Lepidoptera species studied, the predominant pheromones investigated are sex pheromones. These have carbon chains ranging from 10 to 18, characterized by unsaturated, straight-chain fatty acid derivatives. These typically feature several double bonds. Moth sex pheromones often exhibit a polar functional group, although some species lack this functional group. Pheromone precursors in this group are commonly stored as triacylglycerols (TAG) in a specific pheromone gland. (Matsumoto, 2010). The hydrolysis of these TAGs in the pheromone gland, coupled with subsequent enzymatic reactions, transforms the free fatty acids into the active sex pheromones (Foster & Anderson, 2019). Alarm pheromones warn other members of possible danger and can elicit defensive behavior. The honeybee, *Apis mellifera*, is a well-studied insect that utilizes alarm pheromones. These pheromones are produced in the mandibular gland and the stinger of worker bees (Pankiw, 2004) and the Koschevnikov gland (Breed et al., 2004). Aggregation pheromones are usually released by on sex and attract both sexes from the same species. The red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), produces an aggregation pheromone 4,8-dimethyldecanal (DMD) that is attractive to both sexes. This aggregation pheromone was found to be 4,8-dimethyldecanal (DMD) (Suzuki, 1980). DMD is a mixture of 4 optical isomers, ((4*R*,8*R*)-DMD, (4*R*,8*S*)-DMD, (4*S*,8*R*)-DMD and (4*S*,8*S*)-DMD) arises from the asymmetric

carbons at C-4 and C-8 (Suzuki, 1980; Suzuki et al., 1984). Synthetic DMD, when tested for activity, demonstrated diminished attractiveness compared to its naturally occurring counterpart. This was attributed to differences in enantiomeric composition and ratio (Suzuki, 1980; Suzuki et al., 1987). Bioassay data analyzed by Suzuki and Mori (1983) led to the conclusion that the naturally produced pheromone was predominantly comprised of the (4*R*,8*R*)-DMD isomer, given its activity closely mirroring that of the naturally occurring pheromone. Over 30 years later the natural stereoisomeric composition of DMD was found to be a 4:4:1:1 mixture of (4*R*,8*R*)-, (4*R*,8*S*)-, (4*S*,8*R*)- and (4*S*,8*S*)-DMD (Akasaka et al., 2011; Lu et al., 2011). In order for the male beetle to synthesize DMD the food must be nutritional (Ming & Lewis, 2010).

Tribolium castaneum

The red flour beetle *Tribolium castaneum* is a worldwide pest of stored products. It will infest many different types of grains. *T. castaneum* is particularly damaging to commodities stored and processed in rice and flour mills (Arthur et al., 2015; Buckman et al., 2013; Campbell et al., 2010a, 2010b, 2015; Hawkin et al., 2013). These infestations extend beyond individual mills, rapidly spreading to other food processing plants and storage facilities (Semeao et al., 2013). *T. castaneum* is also adept at infesting cocoa beans (Jung et al., 2020). Monitoring levels of *T. castaneum* inside of a facility typically involves using a trap of some type containing a lure of synthetic DMD. (Campbell et al., 2002; Campbell & Arbogast, 2004). The DMD that is added to the lures deviates from the racemic mixture found in the naturally occurring pheromone, contributing to a decrease in trap effectiveness. This phenomenon is believed to be influenced by both intrinsic and extrinsic factors (Fedina & Lewis, 2007). *T. castaneum* long life cycle and can complete generation in less than 30 days at ideal conditions. Also, the beetle has a

high reproductive rate, long life and is easy to rear in a laboratory (Semeao et al., 2013). These qualities along with the observed damage *T. castaneum* does to the food supply makes it an important insect to study.

Pheromone biosynthesis in insects

The use of fatty acid derived pheromones is a common occurrence in several insect orders. Flies (Diptera) are known to use fatty acids derived pheromones. These typically have longer carbon chains than those used by Lepidopterans, but the same group of enzymes is responsible for their synthesis (Blomquist et al., 2018). Blattidae, specifically cockroaches, use pheromones similar in structure to those of Diptera (Jurenka, 2004). Hymenoptera, specifically workers and queens of *Apis mellifera* use multiple pheromones that are modified fatty acids (Slessor et al., 1990, 2005; Wu et al., 2017) . Along with Lepidoptera pheromones, Coleoptera pheromones have also been studied for years. The beetles which have the most data available regarding pheromones biosynthesis are the bark beetles. These beetles use pheromones that are synthesized with isoprenoids (Symonds & Gitau-Clarke, 2016).

The biosynthesis of beetle pheromones involves two main pathways: the mevalonate and the fatty acid pathways. While cholesterol biosynthesis in vertebrates is extensively studied within the mevalonate pathway, insects lack certain enzymes for the final steps of this process (Goldstein & Brown, 1990). In insects, the mevalonate pathway is well-known for the biosynthesis of juvenile hormone III (JH III), a compound crucial in insect development. Also originating from the mevalonate pathway are multiple pheromones from *Dendroctonus* and *Ips* species of bark beetles. A large majority of information available concerning beetle pheromones comes from studies with these bark beetles and their mevalonate pathway biosynthesized

pheromones. While the structure of DMD suggests a possible isoprenoid origin, Kim et al., (2005) used labeling experiments and chemical inhibitors to conclude that it was synthesized by the fatty acid pathway.

Over the past decade next generation sequencing (NGS) technologies have been used to study the pheromone biosynthesis from several insect orders. Due to their pest status and economic importance, most of the insects examined thus far have been Lepidopterans. A major advantage of using moths as a model is that females have a pheromone gland in which the pheromones are synthesized *de novo*. A transcriptome approach has been employed to study the genes relating to the biosynthesis of Lepidopteran pheromones from this gland in the: tobacco budworm, *Heliothis virescens* (Vogel et al., 2010) turnip moth, *Agrotis segetum* (Ding & Löfstedt, 2015), almond moth, *Ephestia cautella* (Antony et al., 2015), diamondback moth, *Plutella xylostella* (Cha, Jung, et al., 2017; He et al., 2017), legume pod borer, *Maruca vitrata* (Cha, Kim, et al., 2017), the pink bollworm moth, *Pectinophora gossypiella* (Dou et al., 2019), Chinese oak silkworm, *Antheraea pernyi* (Wang et al., 2020), a zygaenid moth, *Achelura yunnanensis* (Nuo et al., 2021), a walnut tree pest, *Atrijuglans hetaohei* (Zhu et al., 2022), the fall armyworm, *Spodoptera frugiperda* (Qu et al., 2022), corn earworm, *Helicoverpa zea* (Dou et al., 2020) and a tea pest, *Scopula subpunctaria* (Qian et al., 2020). Similar approaches of studying the pheromone producing glands or cells have also been carried out in Hymenoptera. These include the honeybee, *Apis mellifera* (Liu et al., 2014; Wu et al., 2017) and the red-tailed bumble bee, *Bombus lapidarius* (Tupec et al., 2019). Pheromones of Hemipterans have also been examined with a similar approach in the plant bug, *Adelphocoris suturalis* (Luo et al., 2014) and the vetch aphid *Megoura viciae* (Song et al., 2023). The list of species here is by no means a complete record of the transcriptome approach being utilized to study genes involved in the

biosynthesis of pheromones. One feature in common with these studies is that the known pheromone gland was used for sequencing. The location of the pheromone gland(s) or pheromone producing cells is not known in *T. castaneum*. However, the anatomical location has been narrowed down to the ventral abdominal cuticle (Lu et al., 2011).

Objectives

i. Determine the appropriate strain/population of *T. castaneum* to use for RNAi work. This involves measuring males from14 different geographical populations across North America and Costa Rica. By using a consistent reliable method of quantification, this study also intended to resolve the discrepancy in the reported amounts of DMD found in the literature over the past 40 years.

ii. Use next generation sequencing (Illumina) to compare ventral abdominal cuticles from fed males vs starved males vs fed females vs starved females. Uncover differentially expressed genes from the data analysis and select candidate genes that might be involved in pheromone biosynthesis. Selected candidate genes are then targeted for suppression using RNAi. Those RNAi beetles will than have the amount of aggregation pheromone quantified using GCMS.

iii. Examine the effects that several neuroendocrine compounds have on the production of DMD. These compounds are either injected into adult male beetles or topically applied. Any effects from these compounds will aid in determining if DMD biosynthesis is under any type of endocrine control or strictly regulated by involuntary metabolic processes.

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Chapter 2 - Geographic Variation in Aggregation Pheromone Released by Male *Tribolium castaneum* from Different North American Populations

Introduction

Males of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae), produce an aggregation pheromone that is attractive to both sexes (Arnaud et al., 2002; Suzuki, 1980, 1981). Only feeding males will produce 4,8-dimethyldecanal (DMD) (Ming & Lewis, 2010). Commercial traps use synthetic DMD to monitor *T. castaneum* populations in areas containing stored products (Campbell et al., 2002; Campbell & Arbogast, 2004; Toews et al., 2005). Various studies found that *T. castaneum* populations that infest commodities with different nutritional qualities will produce different amounts of DMD. As shown by Ming and Lewis (2010), the amount of DMD produced decreases when the nutritional quality of diet decreases. Population density, photoperiod, and the age of the beetle are other factors that influence the amount of DMD produced (Hussain, Aftab, 1993). Boake and Wade (1984) examined four different populations of *T. castaneum* for differential responses to each other's pheromones using a pitfall assay with virgin females. Those authors found that females from three of the populations preferred DMD from another population more than DMD produced by males from their population. Although they did not quantify the amount of DMD that was collected from males in their experiment, they suggested that differences among strains that had been reared in the lab for more than seven years might indicate a genetic basis for those differences.

Studies that quantified DMD produced by male *T. castaneum* vary substantially in the reported amount of pheromone released over a given time. Arnaud et al, (2002) used a solid phase micro-extraction (SPME) fiber to collect DMD from single 30-day old virgin male that were sealed in a glass vial for four days. The volatiles collected were quantified using a gas chromatography mass spectrometry system (GCMS). The average amount of DMD they reported was 0.71 ng/day. Kim et al, (2005) quantified the amount of DMD from 50 to 500 mixed sex adults and collected DMD with a column containing Super Q adsorbent, either every 5 days or 10 days. An internal standard was added to the collections and analyzed using GCMS. The amount of DMD reported in their controls ranged from only 4.36 to 22.9 ng/day. Duchl et al, (2011) utilized a system similar to that reported by Kim et al. (2005). Super Q was used to collect volatiles from mixed cultures of different density and analyzed with GCMS. They did not report an exact number for the amount of DMD produced. However, Kim et al. (2005) did report that the maximum amount of DMD produced after 2 days in experiments containing 500 adults was approximately 180 ng/hr for the large group of beetles. Considering 180 ng/hr and half of the adults males, and that the groups tested contained 50% males, one can make the assumption that the amount of DMD produced was (180 ng * 24 hrs) / 250 males = 17.28 ng/day. Ming and Lewis (2010) used SPME to collect volatiles produced from a single male after 4 days in an enclosed chamber. They quantified the amount of DMD using GCMS but generated an external standard curve from known amounts of DMD rather than adding an internal standard of a non-DMD compound. They reported an average amount of DMD produced of 54.8 ng/day. Bloch Qazi et al, (1998) similarly collected DMD from single adult, virgin male with Super Q over 48 hrs and quantified by generating a standard curve from known amounts of DMD. They used GC-FID for their quantification instead of GCMS. The amount of

DMD they report finding was 81.5 ng/day. Ming and Lewis (2010) claimed to use the same strain of beetles that were used in the Bloch Qazi et al study (1998). Hussain et al, (1993) collected volatiles from single adult beetles using Super Q every 48 hrs. An internal standard was added to the samples and quantified using GCMS. They reported an average amount of DMD produced from a single male as 635 ng/day. Lu et al, (2011) used a system almost identical to that of Hussain et al. (1993). They report the highest amount of DMD found in the literature reviewed here, an average 878 ng/day per beetle. The point behind this summary of past studies involving collecting and quantifying DMD is to highlight the discrepancies in amount of DMD collected. This happens even when researchers are using very similar methods.

Variation among studies in the amounts of DMD produced by male *T. castaneum* generate similar hypotheses for causation regarding the differences in the amount of DMD collected from *T. castaneum.* Boake and Wade (1984) were perhaps the first to mention that there might be differences in the amount of volatiles produced by different strains of *T. castaneum*. Two of the populations they examined were collected only 200 km apart in the same year. One of those strains had females that preferred volatiles produced from its own males compared to the other strain. The other strains females greatly preferred volatiles produced not from males of their own strain but from males of the first mentioned population. Bloch Qazi et al, (1998) compare their results to that reported by Hussain et al, (1993) and recognize the fact of different methods used for quantifying DMD, but also suggest that there might be differences in the strains of beetles used. Ming and Lewis (2010) discuss their results compared to that of Bloch Qazi et al, (1998), Hussain et al, (1993), and Arnuad et al, (2002). They also realize the differences in methods used, but their results were similar to Bloch Qazi et al, (1998) using the same line of beetles several years later. Ming and Lewis compared their study to that of Arnuad

et al, (2002) and they both used similar techniques in collecting and quantifying DMD but got drastically different results. In general, the literature on quantitative variation in release of DMD by male *T. castaneum* is highly variable and does not control for geographic or genetic differences among conspecific populations of these beetles.

The study here was to determine if there was any difference in the amount of DMD produced from populations of *T. castaneum* separated spatially and temporally. All other variables remain consistent for at least one year. This will exclude most types of environmental interference. By analyzing 14 geographically distinct populations from North and Central America, using the same pheromone collection and quantification methods for each population as well as the same handling and rearing conditions the question of variance in *T. castaneum* pheromone production might finally be answered. Therefore, the objective of this study was to determine if variation in pheromone produced by males of *T. castaneum* varies by the population of beetles used, and thus would suggest a genetic basis for variation in pheromone systems.

Materials and Methods

Insects

Tribolium castaneum from thirteen laboratory cultures originating from different geographic locations, and one highly inbred line, the GA-2 strain (Tribolium Genome Sequencing Consortium, 2008), derived from the GA-1 strain were used in this study (Table 2.1). All thirteen cultures had originated in the field and were reared in the laboratory for different periods of time. Table 2.1 summarizes the collection location and the number of years the cultures have been in the laboratory prior to this work. Several populations such as: Costa Rica, GA-1, and USDA did not have exact locations specified in the literature, so the coordinates are

approximated near a suspected area. Beetles were reared on Golden Buffalo organic whole wheat flour (Heartland Mills, KS, USA) supplemented with 5 % brewer's yeast at 29 ºC and 70 % RH. Cultures were maintained by the addition of ~ 80 adults to ~500 g of fresh rearing media in a glass pint canning jar with a filter paper lid and kept in an incubator with a 16:8 (light-dark) photoperiod. All populations used in this study were reared in our laboratory in the described way for no less than one year before the pheromone was collected. Pupae were removed from the cultures, sexed according to morphology of ventral terminal abdominal tergites and placed individually into small glass vials with rearing media and a small amount of cracked wheat, which provided footing. All beetles were 10-12 days post adult eclosion (PAE) when initially placed into the aeration chambers for pheromone collection.

Table 2.1. *Tribolium castaneum* population information.

Population names, original collection locations, two letter name codes, years beetles had been reared in the laboratory and approximate collection locations for all the populations used in this study. There are no specific collection locations available for the CR-1, GA-1, GA-2 and USDA populations. The location listed was in the middle of the suspected regional collection point. The * denotes that GA-2 was derived from GA-1 but is regarded as a separate lab strain here.

Population Name	Collection Location	Code	Yrs in Culture	Coords. (°N, °W)	References
Alabama	Red Level, AL	AL	1	31.41, 86.61	Cato et al. 2017
Arkansas	Jonesboro, AR	AR	1	35.82, 90.74	Cato et al. 2017
California	Williams, CA	CA	1	39.15, 122.15	Cato et al. 2017
CR-1	Costa Rica‡	CR	27	9.94, 84.13	Beeman et al. 1996
GA-1	Georgia, USA‡	G1	36	32.07, 81.19	Halisack and Beeman 1983
GA-2	Georgia, USA‡*	G2	18	32.07, 81.19	Richards et al. 2008
Hudson	Hudson, KS	HD	8	38.10, 98.66	Cato et al. 2017
KS-1	Kansas City, KS	KS	8	39.09, 94.69	Cato et al. 2017
NDG-2	Argyle, MB	NG	28	50.18, 97.46	Thomson et al. 1995; Beeman et al. 1996
Shellenberger	Manhattan, KS	SH	6	39.19, 96.58	Campbell J personal communications
USDA	Eastern Kansas‡	US	50+	38.59, 95.70	Akbar et al. 2004; Arthur 2009
Winnipeg	Winnipeg, MB	WN	2	49.86, 97.17	Cato et al. 2017
Z-2	Chickasaw, OK	Z2	28	35.04, 98.00	USDA collection, Manhattan, KS
Z-4	Cedar Rapids, IA	Z4	28	41.97, 91.72	USDA collection, Manhattan, KS

Collection of pheromones

Individual males that were 10-12 days old were placed in a 7.5 x 2.75 cm custom made cylindrical glass aeration chamber with ~0.7 g of rearing media and ~0.4 g of cracked hard red or soft white winter wheat (Fig. 2.1). The aeration chambers were set up on the bench. The temperature and relative humidity (RH) varied minimally over the duration of the experiments, and the average values were: ~28 ºC and 30 % RH. Two 60 W incandescent light bulbs were on over the entire duration of the pheromone collection. Constant lighting was used in order to maximize pheromone release (Hussain 1993). Air was drawn in from the bottom of the aeration chamber by first passing through a pre-filter composed of ~0.5 g of Porapak Q 50/80 mesh (Supleco: Sigma Aldrich). The filtered air then entered the chamber containing the single adult male and was drawn to the top of the chamber at a rate of ~150 mL/min. The pre-filtered air and any pheromones/volatiles given off by the beetle and food were then drawn into a collection filter composed of ~0.5 g of Porapak Q 50/80 mesh. Figure 2.1 shows a partial setup of the aeration chambers. Each beetle was in its chamber for a total of 8 days. The temperature 28.0 ºC (± 1.0 ºC) and relative humidity 28 % RH (± 5 %) of the room with the aeration chambers was monitored and recorded by a digital data logging device (Onset Data Loggers) and remained constant throughout all aerations. The first two days the beetles were allowed to adjust to the environment of the chamber while the vacuum pump and the lights were on, and no volatiles were collected during this period, after which a collection tube was placed a the top exhaust opening of the chamber. The collection filters were then removed every two days afterwards for a total of 6 days. This method allowed for collecting pheromones over a 6-day period for a total of 3 data points (referred to as the 1st, 2nd, and 3rd 48 hr time points) for each beetle. At the end of a 48 hr collection period, the top collection filter was removed and replaced with a clean filter.

The volatiles were eluted from the column by dripping HPLC grade-hexanes (Thermo Scientific, Waltham, MA, USA) into the top of the collection filter and allowing them to drip into a 1.8 mL glass screw-cap vial (Thermo Scientific, Waltham, MA, USA) in which the first 1.5 mL of solvent was collected. The collected volatiles were spiked with 570 ng of N-dodecane (Thermo Scientific, Waltham, MA, USA) as an internal quantitative standard, by adding 4 µL of a 142.5 ng/µL solution of dodecane and hexanes. The collections were stored in a 1.8 mL glass vials with a Teflon coated cap. Before analysis the samples were concentrated by placing under a gentle flow of ultra-high purity (UHP) nitrogen. Final volume for analysis was ~80 µL.

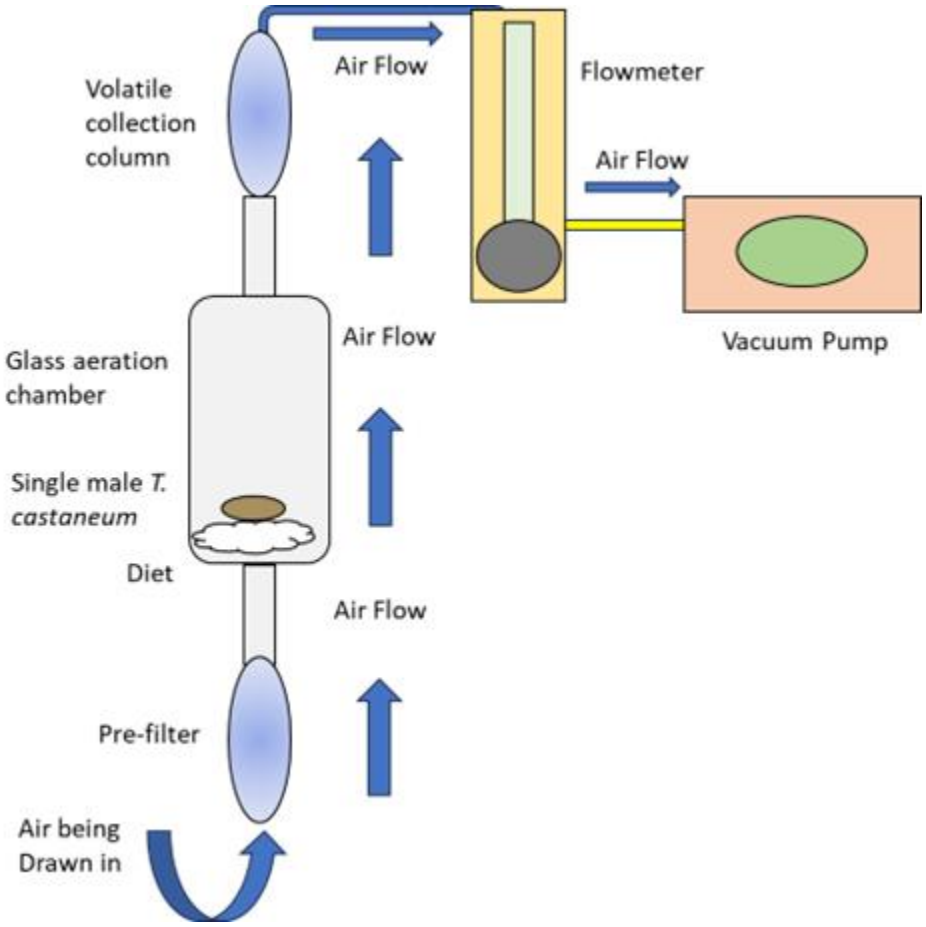


Figure 2.1. Aeration chamber setup that was used in these experiments.

Air is drawn in through the Porapak (pre-filter) column on the bottom of the chamber. The purified air is drawn into the aeration chamber which contains the diet and single beetle. Any volatiles produced by the beetle are collected on an additional Porapak column (volatile collection column) at the top of the chamber. A flow meter controls the rate of air that is drawn through the aeration setup for each chamber. The air is drawn through the system by a vacuum pump.

Pheromone quantification

All aeration samples were quantitatively analyzed by gas chromatography-mass spectrometry (GCMS) with an electron impact ionization (70 eV) using a Shimadzu GC-MS QP5050A (Shimadzu Scientific Instruments, Columbia, MO) with a J&W Scientific DB-1MS capillary column (Agilent Technologies, Santa Clara, CA, USA) (30 m x 0.25 mm x 0.25 um) in splitless mode, with UHP helium as the carrier gas. The injector and the heated transfer line to the MS were both set to 250 ºC. Oven temperature was programmed to start at 40 ºC for 0.5 min, then increased 10 ºC /min to 149 ºC, slowed to 1 ºC/min to 154 ºC, increased again to 10 ºC/min to 170 ºC, and then finished by increasing to 20 ºC/min to 240 ºC and held for 2 mins. The MS was set to full scan mode, recording mass fragments from 40 to 350 amu. The quantity of DMD for each sample was determined by comparing the area under the peak of the internal standard dodecane (representing 570 ng) to the area under the DMD peak (DMD area under the peak/dodecane area under the peak * 570 ng = ng of DMD). One µL of the concentrated pheromone sample was injected into the machine. Figure 2.2 shows an example of the total ion chromatogram (TIC) and mass spectrum (MS) for each peak generated from a typical sample run. Data was visualized using Microsoft Excel.

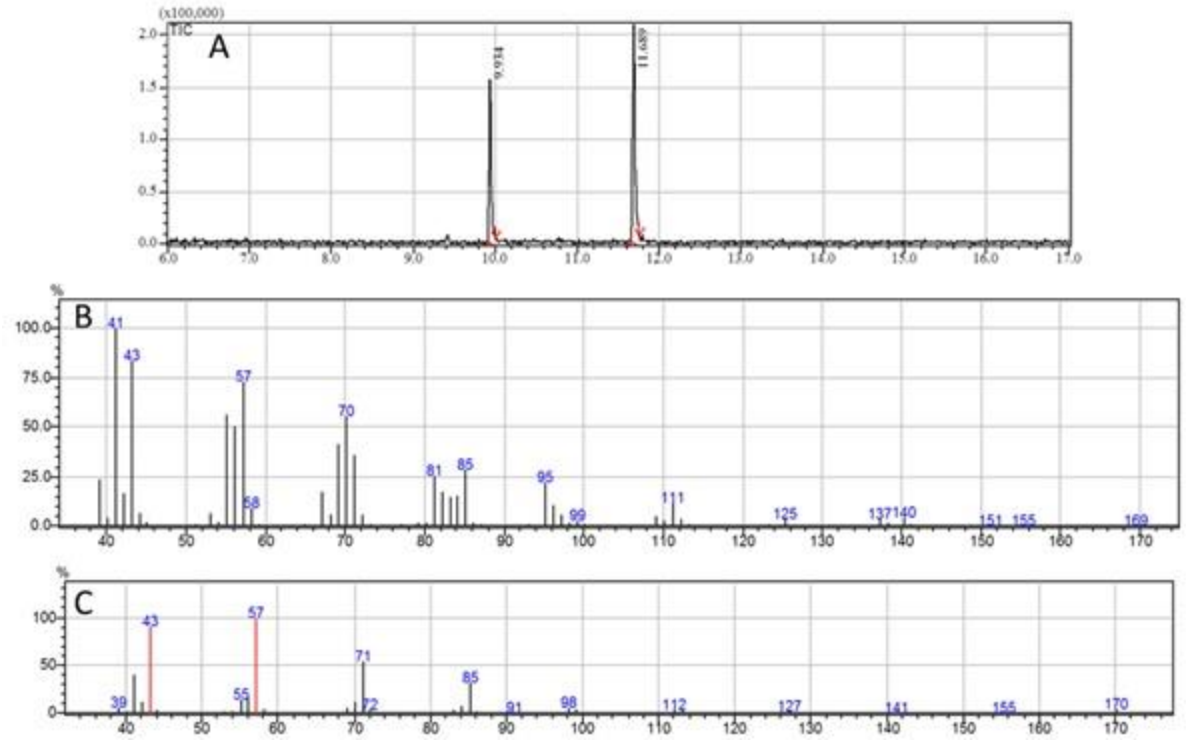


Figure 2.2. Representative total ion chromatogram (TIC) and mass spectra (MS) for the two peaks.

A. TIC with labeled peaks from the injection of 1 μL of the collected and concentrated beetle volatiles. The peaks labeled are the internal standard dodecane (peak at 9.9 mins) and DMD (peak at 11.6 mins). B. The MS generated from the dodecane peak at 9.9 mins and C. the MS generated from the DMD peak at 11.6 mins.

Beetle body measurements

Four male beetles from each of the Arkansas, Costa Rica, GA-2, USDA, and Z-2 populations were sexed as pupae and the mature adults were individually placed in glass vials with diet and cracked wheat. Males from the Goliath mutant strain of *T. castaneum* were also included in this set of experiments. Once the beetles were ~7 days PAE they were placed in 8 ºC for ~30 mins to slow their movement long enough to be photographed. The chilled beetles were individually photographed from the dorsal side using a ScopeTek (Lakeville, MN, USA) MDC560 digital camera connected to an Olympus (Shinjuku, Tokyo, Japan) SZX10 microscope. A stage micrometer was included in every picture. The photographs were analyzed in ImageJ,

version 1.51k (National Institute of Health (https://rsb.info.nih.gov/ij/). The width and length of both the pronotum and elytra were recorded in pixels, then compared to the number of pixels corresponding to 0.5 mm on the stage micrometer (Fig. 2.3).

After each beetle was photographed, they were returned to their vial and allowed to mature for several more days. Once they were 10-12 days PAE they were placed in the aeration chambers and DMD was collected and quantified as described previously.

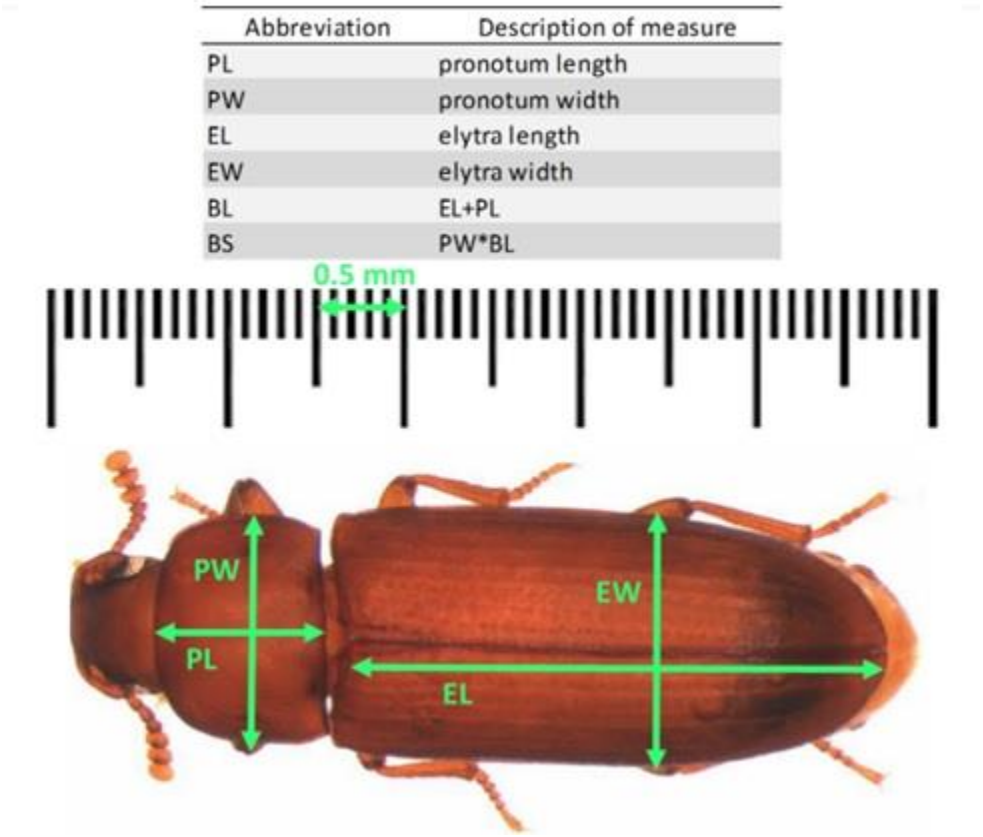


Figure 2.3. Example of how beetles' body parts were measured.

Pictures of each beetle included the stage micrometer as shown above. The number of pixels corresponding to each measurement was counted. Using the stage micrometer, the number of pixels corresponding to 0.5 mm based on the micrometer was used as the conversion factor in ImageJ.

Statistical analysis

A value of 1 ng was added to the raw data set in cases for which no measurable DMD was detected from a single beetle in a 48 hr aeration period by GC-MS following aeration. All raw data were then log transformed prior to further analyses and compared the differences of the mean log DMD produced between locations using the GLM for ANOVA, then followed by the LSD procedure for means comparisons using SAS 9.3. Means and standard errors for the raw data are reported. Correlation and regression analyses as well as data visualization were performed in Microsoft Excel.

Results

Quantification of 4,8-dimethyldecanal from 14 different geographical populations of *T. castaneum*

To identify the existence of any difference in the amount of DMD produced among different populations of *T. castaneum* we measured the amount of DMD produced by 12 male beetles individually, from 14 different populations. All the populations had been reared in our laboratory for at least 1 year prior to the DMD measurements. Cultures of beetles were maintained on the same diet and under the same environmental conditions during that time in our laboratory. The 14 populations measured in this study consisted of 5 groups that were significantly different from one another (Fig. 2.4). Over a six-day period, the CR-1 population produced the most DMD (1,547.5±188.9 ng) and the NDG-2 produced the lowest amount (99.3±30.8 ng), and this was not significantly different from the DMD amounts produced by beetles the Arkansas, Winnipeg, and GA-2 populations.

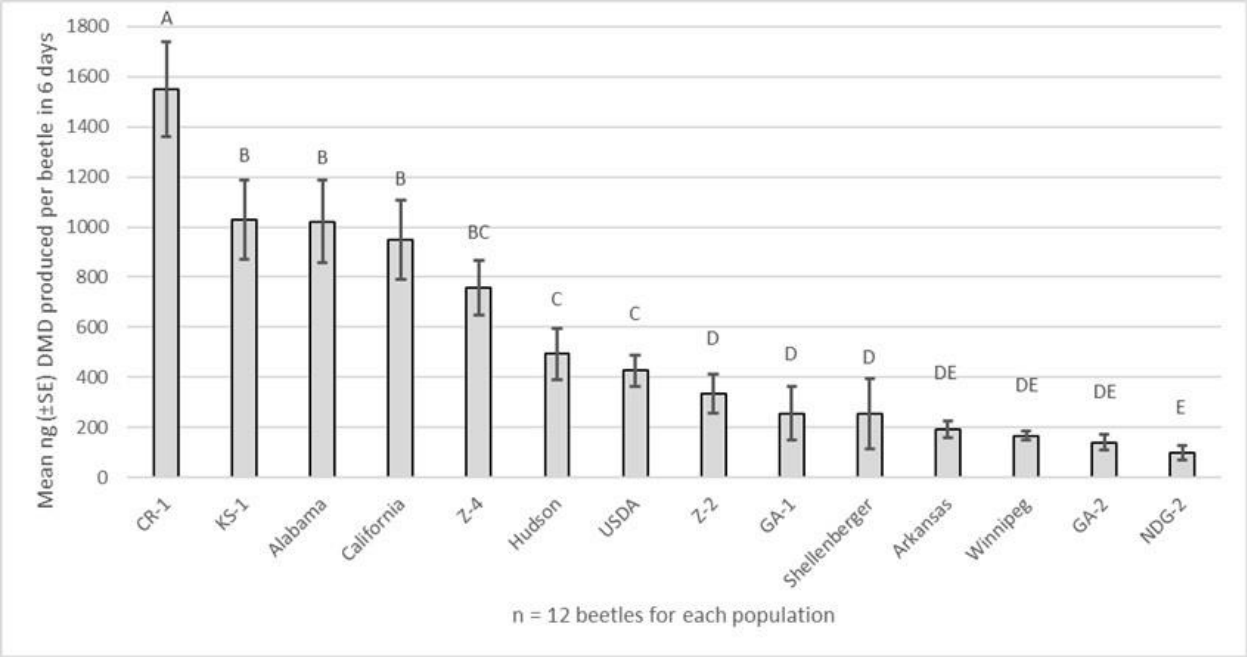


Figure 2.4. Mean amount of DMD produced in 6 days by a single male beetle from each population (n = 12 for each population).

The significance of means was calculated in SAS 9.3 with PROC GLM and t-statistics are least significant difference (LSD) tests for pairwise comparison. Statistical analysis was performed on log transformed data.

Influence of a population's original geographical location, time reared in culture and size of body regions on DMD production

A relationship existed between the mean amount of DMD produced by males of the 14 populations and the latitudinal coordinates (or estimated coordinates) of the populations' original collection location (Fig.2.5). However, exclusion of the CR-1 population from the analysis eliminated the significant correlation and significance that had existed (Fig.2.5). The mean amount of DMD produced by a single male beetle from each population was not correlated (r = 0.05, p = 0.86) to the longitudinal coordinates (or estimated coordinates) of their respective original collection location. Excluding the CR-1 population from the analysis increased the correlation coefficient but did not make the data significant (r = 0.32, p = 0.29). Interestingly,

the amount of time a population had been reared in the laboratory was not correlated to the mean amount of DMD produced by males from the populations tested (r = 0.09, p = 0.76). When data for the CR-1 population was removed from the correlation analysis of lab-rearing years, there was an increase in the correlation coefficient, but the relationship was still not significant (r = 0.28, p = 0.36).

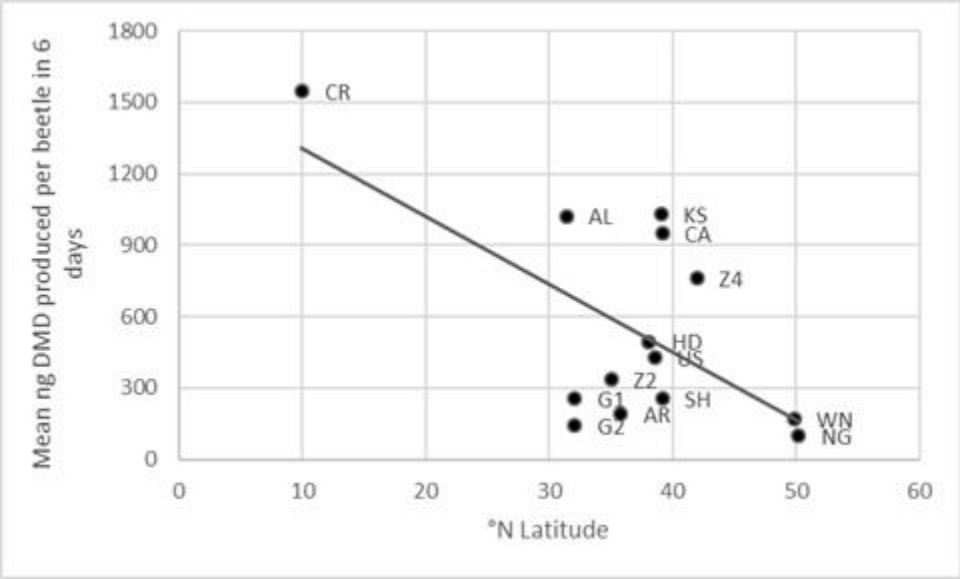
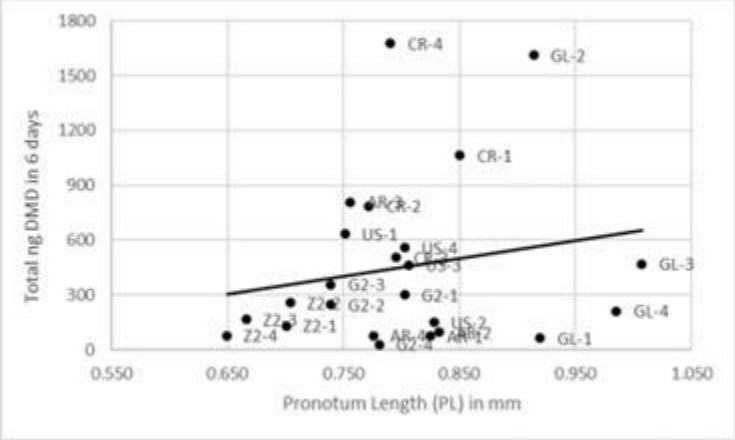


Figure 2.5. Plot of mean ng DMD produced to °N latitude of each populations' collection location.

Plot visualizes the correlation between DMD produced vs. original (or estimated) collection location (p = 0.02; r = -0.62). Removing the Costa Rica (CR) population from the regression (graph not shown) eliminates the significance (p = 0.47; r = -0.22). Analysis performed in Microsoft Excel.

The amount of DMD produced by male beetles (n = 4) was not significantly correlated to any of the morphometric variables measured on the adult males that were analyzed. (Fig.2.6). The mutant *Goliath* strain of *T. castaneum*, while much larger in size compared to the other populations, had a mean DMD production value of 589.6 ng (±351.4 ng) over a six-day period. This is about half as much as the mean produced by males from the CR-1 population

(1007.6±250.7 ng). Figure 2.7 compares the initial set of aerations from the populations of the beetles that had their body measurements taken to those that were actually measured. The amount of time between these 2 experiments was ~ 14 months. This example is evidence of how different populations continue to make a similar amount of DMD over any given time.



measurment	*r*	*p*-value
PW	0.180	0.400
PL	0.193	0.367
EW	0.146	0.495
EL	0.174	0.417
BL	0.183	0.393
BS	0.179	0.404

Figure 2.6. Plot of mean DMD produced by a single beetle to its pronotum length.

Plot of total ng DMD produced in 6 days by each beetle to the mm length of the pronotum. Plots of total DMD vs the body measurements listed looked similar due to the y-axis being identical. Small table insert shows the correlation coefficient and *p*-value of the regression analysis.

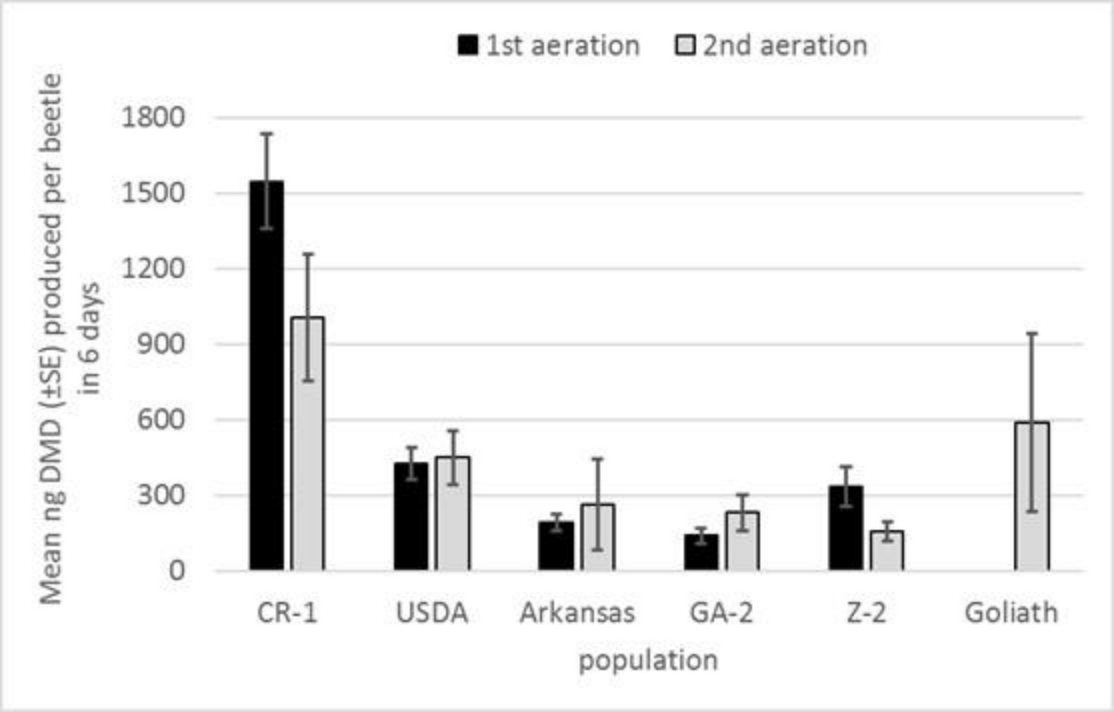


Figure 2.7. Amount of DMD collected from individuals of selected populations during the first and second aeration experiments.

1st aeration (black, n = 12) is the mean amount of DMD (±SE) determined from the original aeration experiments (same data that is in Figure 2.4). The second aeration (grey, n = 4) is from the experiments where the beetles were measured before they were placed into the chambers. Goliath was not measured the first time and thus lacks a black bar.

Discussion

These results demonstrate that similar-aged males of *T. castaneum* from different populations produce varying amounts of DMD by population from different geographic sources. All beetles placed in the aeration chambers were 10-12 days PAE. All DMD measurements were performed every 48 hrs over a six-day period in a consistent manner. The differences in the amount of DMD produced by males from different populations described here do not come as unexpected, as similar interpopulation difference have been found in other species for various semiochemicals. Different geographic populations of *Tribolium confusum* produce significantly different amounts of benzoquinones (Yezerski et al., 2000, 2004). Furthermore, throughout the

years multiple studies have quantified DMD in *T. castaneum*. The two main techniques used to collect the DMD reported by other groups are solid phase microextraction (SPME) (Arnaud et al., 2002; Ming & Lewis, 2010; Stevenson et al., 2017) and by absorption on Porapak columns (Duehl et al., 2011; Hussain, Aftab, 1993; Kim et al., 2005; Lu et al., 2011; Qazi et al., 1998) which was employed in this study. All these groups used GCMS to quantify the DMD collected except Bloch Qazi et al., (1998) who used GC with a flame ionization detector (FID). Although not specifically stated in every case, the amount of DMD produced per beetle in a 24 hr period can be deduced as values ranging from <10 ng (Kim et al., 2005) to >1 µg (Lu et al., 2011; Ming & Lewis, 2010; Qazi et al., 1998). Stevenson et al., (2017) Bloch Qazi et al., (1998) and Ming and Lewis (2010) used the same population of *T. castaneum* 12 years apart and obtained similar results for DMD produced by a single male in a 24 hr period, despite using different DMD collection techniques. However, Ming and Lewis (2010) used similar methods to that of Arnaud et al., (2002) with different populations of *T. castaneum*, and obtained very different results.

There was no correlation between the amount of DMD produced to the morphometric values determined for adult *T. castaneum* in this study. Similarly, neither the number of years a *T. castaneum* lab culture had been reared, nor the geographical coordinates of their original collection location were correlated with the amount of DMD produced. Chemical communication in Diptera is known to be modified over time in mass-rearing lab conditions (Sivinski et al., 1989). Although a correlation existed between DMD production and the latitude of the original collection locations, exclusion of the CR-1 population removed that significance (Fig. 2.5). Several possibilities exist in which a physical factor could have an influence on DMD production. The location of DMD production and site of its release remains unknown in *T.*

castaneum. The ventral abdominal cuticle is the area were the most DMD has been detected among different body parts of male *T. castaneum* (Lu et al., 2011).

A large amount of evidence proves that different geographical populations of bark beetles produced different amounts and mixtures of their aggregation pheromones. These species include: *Ips pini* (Cognate et al., 1999; Domingue & Teale, 2007; Hager & Teale, 1996; Miller et al., 1989, 1997), *Dendroctonus brevicomis* (Pureswaran, Hofstetter, Sullivan, Grady, et al., 2016; Pureswaran, Hofstetter, Sullivan, & Potter, 2016), and *Dendroctonus rufipennis* (Borden et al., 1996; Gries et al., 1988; Isitt et al., 2020). As Isitt et al., (2020) points out that these differences are likely genetic, in that study the possible reasons causing these genetic differences for bark beetles do not directly apply to *T. castaneum* populations reared in a laboratory for many years. Due to the exact same rearing conditions for the beetles in this study and the amount of DMD produced from each population, an underlying genetic cause(s) would provide the best explanation for these differences.

Most of the knowledge of pheromone biosynthesis in Coleoptera comes from the work done with several different species of Scolytidae Bark beetles biosynthesize their pheromones via the mevalonate pathway (Tittiger & Blomquist, 2017) and T. castaneum uses the fatty acid synthesis pathway (Kim et al., 2005), therefore comparing pheromone biosynthesis of Scolytidae to Tenebrionidae cannot be done directly.

One aspect this study was not able to address was the chirality of the DMD being produced. Lu et al., (2011) is the only report of the naturally occurring stereoisomeric composition of DMD. Using a wind tunnel assay, they found that the most attractive blend of synthetic stereoisomers was the same as the naturally produced ratio of ([(4*R*,8*R*)- /(4*R*,8*S*)-/

(4*S*,8*R*)-/(4*S*,8*S*)-DMD] = 4:4:1:1). However, only one population was tested in that attraction assay, and a different population was used to collect DMD for the stereoisomeric separation. Whether or not different mixtures of DMD stereoisomers are more attractive to one population compared to another have not been explored. Geographically separated populations of *T. castaneum* are more attracted to volatiles that had been absorbed onto paper discs from other populations then to the volatiles from members of their own population (Boake & Wade, 1984). The actual volatiles absorbed onto those discs were not quantified. There exists a possibility that those results could also be interpreted as individuals being less repelled by volatiles from other populations; or that one population released a more attractive mixture than the other. A set of behavioral and attraction experiments would complement this current study very well.

The work reported here clearly shows difference in the amount of DMD produced and released by male beetles from lineages originating from broadly different geographic locations. This work shows that environmental factors like rearing diet, temperature, photoperiod, and others in which all these populations were maintained in Manhattan, Kansas. These did not result in similar levels of pheromone production among these strains. Outside of some microbial or similar differences among beetles causing difference in pheromone production, the existence of genetic differences among these population would allow for some control over the amount of DMD produced and/or released by males. Preliminary studies were run in which F1 hybrids of males from two populations with very different pheromone release levels were measured. Results did not have any consistent pattern that could be based on a single-genetic basis for amounts of DMD produced. Multiple genes, perhaps responsible for variation in pheromone biosynthetic pathways in adult male *T. castaneum,* are likely responsible for the amount of DMD

released by males from different population. Future work on genetics and biosynthesis of DMD will be needed to provide more information on pheromone biology in *T. castaneum*.

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Chapter 3 - Using RNAi to Investigate the Biosynthetic Pathway of 4,8-dimethyldecanal

Introduction

Aggregation pheromones are important compounds in insect communication. In *Tribolium castaneum* only feeding males produce an aggregation pheromone that is attractive to both sexes which is called 4,8-dimethyldecanal (DMD). The location of the site of synthesis of DMD in the male beetle is not known. Studies previously determined that by washing only the ventral abdominal cuticle with solvents yielded the highest concentration of DMD (Lu et al., 2011).

Coleoptera have two main routes of known pheromone biosynthesis. The biosynthetic pathway of pheromones used by bark beetles (Scolytidae) is through the mevalonate pathway (Byers & Birgersson, 1990; Ivarsson et al., 1993; Lanne et al., 1989; Vanderwel, 1994). Tenebrionidae, which includes *T. castaneum*, use the fatty acid pathway to synthesize DMD (J. Kim et al., 2005). This is the same pathway used by many Lepidoptera. The main enzyme responsible for creating the backbone of these pheromones in Coleoptera and Lepidoptera is fatty acid synthase (FAS). Most of the early work on fatty acid derived pheromones came from studies involving the pheromone glands from different species of moths. The typical product of FAS is a carbon chain of 16C, although 14C and 18C (and longer) can occur (Bjostad & Roelofs, 1984; R. A. Jurenka et al., 1991, 1994; Tang et al., 1989). One way to generate a shorter carbon chain is to subject the FAS product to rounds of limited β-oxidation. This limited β-oxidation reduces the chain length by 2C each round (Bjostad & Roelofs, 1981, 1983; Ryan et al., 1982). The terminal carboxyl group of the fatty acid derived pheromone can be enzymatically modified

in several different ways. The functional group of DMD is an aldehyde. Production of an aldehyde requires the reduction of the fatty acyl precursor to an alcohol. This was thought to be a two-step reaction which requires a fatty acid reductase and an aldehyde reductase (Fang et al., 1995; Teal & Tumlinson, 1988). This process would produce an alcohol having an aldehyde intermediate, which would allow aldehydes to be produced directly from a reduction of a fatty acid. However, genes for this process have not been identified at the molecular level (Blomquist et al., 2021; Fang et al., 1995; R. Jurenka, 2004; Teal & Tumlinson, 1988) Another way to generate an aldehyde is by the oxidation of an alcohol. Evidence is available that this is done by a cytochrome P450 enzyme (Ahmad et al., 1987; Pennanec'h et al., 1997; Pho et al., 1996). The 2 methyl groups of DMD are inserted during the synthesis of the carbon chain. Methylmalonyl-CoA from propionate is substituted for malonyl-CoA, from acetate, in the growing chain (Kim et al. 2005).

In this study we compare transcriptome data between ventral abdominal cuticles from fed males, starved males, fed females and starved females. Differential expression analysis showed that there were multiple genes up regulated in the fed male tissues compared to the other treatments. Gene enrichment identified several genes in lipid metabolic processes. A later analysis of the transcriptome data and manual searching identified more possible candidates that could be involved in the biosynthesis of DMD. Candidate genes were then targeted for suppression with RNA interference (RNAi). The amount of DMD produced from these RNAi beetles was quantified using GCMS.

Materials and Methods

Sample preparation for Illumina sequencing

The GA-1 population (Haliscak & Beeman, 1983) was used for Illumina sequencing. For preparations of starved and fed beetles, the starved beetles were removed from their vial of diet and placed individually in an empty glass vial 24 hrs before dissection. The ventral abdominal epidermal layer including the cuticle was removed from each beetle by grabbing the anterior portion of the thorax and the posterior end of the abdomen and pulling in opposite directions. The freed abdomen was placed in PBS and all reproductive tissue, defensive glands and the digestive tract was removed, leaving the ventral abdominal cuticle and any attached material remaining. Epidermal layers from 25 individuals for each treatment were pooled in TRIzol (Ambian Applied Biosystems, USA). Each pool of 25 individual epidermal layers yielded the samples for 4 treatments starved males, starved females, fed males and fed females.

Total RNA was extracted from each sample of 25 pooled ventral abdominal cuticles using Trizol (Ambian Applied Biosystems, USA) according to the manufacturers protocol. RNA quality was tested using an Agilent Bioanalyzer prior to submission for sequencing. Illumina-based (GXII platform) rRNA-depleted transcriptome sequencing of a single RNA sample was carried out by Cofactor Genomics (St. Louis, MO, USA). Obtained sequences were single direction, 50 nt in length, and filtered for the sequences with a q-score higher than 30 and longer than 30 nt by using the FastX toolkit implemented in DNA Galaxy (Goecks et al., 2010). Assembly of the sequences to the reference genome Tcas3.0 was made in DNA Nexus (https://classic.dnanexus.com/home). The RPKM (read per kilobase per million mapped reads) was used for identifying the most highly expressed gens and the genes with undetectable transcript levels. These lists of genes were used for a GO-term enrichment test against the

official gene set (Tcas3.0 OGS) (H. S. Kim et al., 2010) in Blast2GO (Conesa et al., 2005) at the False Discovery Rate (FDR) of 0.05.

Illumina data was also analyzed with the Tcas5.2 genome assembly using Galaxy (https://usegalaxy.org/). The two files for each of the starved female, fed male and fed female treatments were concatenated, leaving one data file for each treatment. The FASTQ Groomer (tool version 1.0.4) was used on each file providing the fastqsanger output format. Using the FASTQ Trimmer (Galaxy tool version 1.0.0) the 5' and 3' reads were offset by: 0 bp and 15 bp for starved male, 0 bp and 5 bp for starved female and fed male, and 0 bp and 10 bp for fed female. After trimming, each file was separately aligned to the Tcas 5.2 reference genome (GCF_000002335.3_Tcas5.2_genomic.fna) with the Tcas 5.2 gene annotation model (GCF_000002335.3_Tcas5.2_genomic.gff) using TopHat (Galaxy tool version 0.9; tool version TopHat v2.0.14) with default parameters. The TopHat accepted hits file produced for each treatment was then analyzed by Cufflinks (Galaxy tool version 2.2.1.0; tool version Cufflinks v2.2.1) using the Tcas 5.2 reference annotation (GCF_000002335.3_Tcas5.2_genomic.gff) and all other parameters left on default. Cuffcompare (Galaxy tool version 2.2.1.0; tool version Cuffcompare v2.2.1 (4237)) was then used to compare the fed male Cufflinks assembled transcripts file to each of the other three treatments assembled transcripts file generated by Cufflinks separately, and each comparison used the Tcas 5.2 genome reference and reference annotation file (GCF_000002335.3_Tcas5.2_genomic.fna and GCF_000002335.3_Tcas5.2_genomic.gff) respectively, with all other parameters left on default. Using Cuffdiff (Galaxy tool version 2.2.1.3; tool version Cuffdiff v2.2.1 (4237)) the gene differential expression testing file was generated by taking the combined transcripts file generated by Cuffcompare for fed male vs. one of the other 3 treatments and the TopHat

accepted hits file for the fed male, and whichever treatment was used in the Cuffcompare step and running the program. The library normalization method was set to geometric, dispersion estimation method set to blind, and all other parameters left on default. The gene differential expression testing file produced from the fed male vs fed female and fed male vs starved male were downloaded and copied into the excel sheet which was used to analyze the DE genes in the analysis employing the Tcas 3.0 genome assembly.

Double stranded RNA (dsRNA) synthesis

Four virgin male KS-1 beetles (Romero et al., 2010) 14-16 days post adult eclosion were homogenized together in 250 µL of TRIzol reagent (Ambian Applied Biosystems, USA), and total RNA isolation and cDNA synthesis was performed as stated in the 'RNA extraction and cDNA synthesis' paragraph. Most of the PCRs to generate the dsRNA templates consisted of 12.5 µL of DreamTaq Master Mix (Thermo Scientific, Waltham, MA, USA), 0.75 µL of each forward and reverse T7-tagged primer, 10.5 µL of Nuclease-Free water (Thermo Scientific, Waltham, MA, USA), and 0.5 µL of a 5 times diluted cDNA template, for a total volume of 25 µL. The thermocycler conditions were: 95 ºC for 2 mins, followed by 35 cycles of 95 ºC for 45 secs, 60 ºC for 1 min, and 72 ºC for 45 secs; ending with 5 mins at 72 ºC. PCR products were run on a 1 % agarose gel to confirm the correct size and success of the reaction. Products were then purified with the DNA clean & Concentrator-25 kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol with the exception of eluting the PCR product with 15 uL of DEPC. Approximately 4 µL of the purified PCR product was run again on a 1 % agarose gel. The dsRNA was synthesized using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, Waltham, MA, USA). Each reaction consisted of 4 µL buffer, 8 µL rNTPs,

2 µL DEPC, 2 µL enzyme mix, and 4 µL purified PCR product, total volume was 20 µL. The reactions were mixed, briefly centrifuged, and placed at 37 ºC overnight. After the overnight incubation the PCR template was removed by adding 22.5 µL DEPC, 5.0 µL buffer and 2.5 µL RNase-free DNaseI (Thermo Scientific, Waltham, MA, USA) to each tube and incubated at 37.0 ºC for 45 mins. The dsRNA was then cleaned and concentrated using the phenol/chloroform method suggested by the manufacturer. The resulting pellet of dsRNA was suspended in 20 µL DEPC and measured with a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA).

Although most dsRNA templates were synthesized using the parameters above, several utilized different PCR conditions. A few used a Ta temperature of 63.0 ºC with an additional 15 secs added to the denaturation and extension steps. A couple of dsRNA templates were synthesized first by using a gradient plate cycle of 57.0-65.0 ºC. Products that were easily visualized on the 1 % agarose gel were pooled, diluted 10 times and used as a template in another PCR with the same conditions listed in the previous paragraph. Due to the recovery rate of the purified PCR products, the template volume in the dsRNA synthesis reactions could vary from 3 µL to 6 µL, with the difference being adjusted by removing/adding the appropriate volume of DEPC. All primers used in this study are listed in Table 3.1.

Table 3.1. Primers used in this study.

The T7* indicates the T7 promoter tag of TAATACGACTCACTATAGGG on the 5' end of the sequence. Primers names are derived from the GLEAN ID.

	Primers for dsRNA template		Primers for qPCR
Name:	Sequence 5'→3'	Name:	Sequence 5'→3'
699F	T7*CATTCCTACGCACTGGTTTC	699F	AATGCCAGCCGAAGAAGTG
699R	T7*TCCGACTCCCTTTCCTTCAA	699R	GCTGTATCCTCCGTTGGCG
3125F	T7*TTGGCTGAAACTGGAATC	3125F	AAAACATGGGAGCAACGC
3125R	T7*CAATAGATGAAGTGATGCG	3125R	GGAAGCATCGTATCTGGC
3175F	T7*CCTAAACAAAGGGCGTGAA	3175F	ACCACATACCGGAATCTGAAA
3175R	T7*CTTCGTCGGTCACCAAAT	3175R	GGTAGTTGTCTGACAGTTGTGCC
3176F	T7*GGTATTCGCTTGAAGTCTAC	3176F	CAAGTGAGTAATAATGGCTCGG
3176R	T7*CGCCATCGCACAAGTCCACA	3176R	GTTGCCACTAAAGCGGTCA
5622F	T7*CGTCGTCGCAAAACTAA	5622F	GTTGTTACGGCTTAGGGGTG
5622R	T7*GCAGTAAACTCTCGGTGTC	5622R	GTACCTTAGCGAGAACCAGATC
5624F	T7*GGAGCAGCGATTTGACTAT	5624F	AAATGCCGTGGTTAGGGA
5624R	T7*CAACCGTCGGCTCTATGT	5624R	TCCACTCAAACCCACTCCT
7042F	T7*ATTGTGGATTACGGCACTTTA	7042F	TTTGTAGCCCTAAGGACGAGG
7042R	T7*GTAAAAATCGGCGTGTCCTG	7042R	GCATAAGGTGGGTTCGGATT
7186F	T7*AGACCCACATAAAGATGCTGC	7186F	CTGAACTAACAAGCGAAGCA
7186R	T7*TTTGTTCCGTTTGCTCGTAT	7186R	AATGTAGGACCTGGCGGAC
7263F	T7*GCGATGATGTACGCTTGTGA	7263F	TTTGTAGCCCTAAGGACGAGG
7263R	T7*ATAAATACCGAACCTGGAAG	7263R	GCATAAGGTGGGTTCGGATT
8312F	T7*TCAAACAGTATTATGCGGCT	8312F	GGATAATACGGTCCTGGCG
8312R	T7*GTCTTTCAGAATACACCTCC	8312R	GTAAATCCCCGATGTTCATG
8471F	T7*ACATCTCGCCCTATCACCA	8471F	CATTGGGTGGTGGAACAGAGG
8471R	T7*AACGAAGAGTTTGTACGGCA	8471R	GGATTTAGCCATTCGTTCGTAA
10155F	T7*TGCGGCAGAGGATACAA	10155F	AACTTCGGCATGAGGTTTG
10155R	T7*AACGTCGAAGTACGAATG	10155R	TTCCAGCTTTGTTGTTGTGG
11134F	T7*AAGAACAACCCGACTG	11134F	AAGTGGCGATGTGCTGG
11134R	T7*CTGCCATGACTCAACTAT	11134R	TGACTAGACAGATTCAACCCAA
11173F	T7*TTTGCTGCTACTTTGG	11173F	TTTGTTCGTCATCATGGCTC
11173R	T7*TGACCATTTGCCTTAT	11173R	GGTCATAGCTGTTTCCCTCG
11231F	T7*CTACTTCGGCTTCCCCTTCT	11231F	CCCTGTTTCTTTTGACCC
11231R	T7*AAGTCTCAGTAACGCACGCT	11231R	TGGACTCAAGCAACAGCG
11471F	T7*CCTCAGCCAAGTTAGCAAC	11471F	ATGCCGACCCTTACAATGC
11471R	T7*GGTATAAGTCGGACAGGTCG	11471R	ACTTCCCCATAAATGAGCAG
11522F	T7*GCAGTTGACGCCTCTGTAG	11522F	ACTGGGTTAGTCGGAATGG
11522R	T7*CAAAACATTATGGACCAGATC	11522R	GCAAAATGTGTCTGAAGGAGC
13049F	T7*AACAAGGGCTCATTTATC	13049F	CTAATTTTGGAGGTGGGCG
13049R	T7*TCTAATATCCCACTCAGGT	13049R	TCGTAAGTCGGGCTGTGGT
15379F	T7*ATCTCTTCGTAAAGCCGT	15379F	CGCCAAATGCTCAACCC
15379R	T7*AAAGCGTAAGTGTTGGG	15379R	AATCGGTGAAATAGTCGTGGT
16415F	T7*CCACCGAGTCCATCACAAG	16415F	CAACCGTCGTGCCAATGTA
16415R	T7*GGAAAGTATGATGGTAGTTATGC	16415R	CAAATCATAAGCCCATCCG
31403F	T7*CATTACAAGGGCGACAC	31403F	GTCCTAATCTTCCACCCACTG
31403R	T7*CCAGTTGACAGAAGGCA	31403R	TGTCAATGTCGGGATTGTAGT
32112F	T7*AGAAATATACCCGATGT	32302F	CCATTGGGGTTGAGACGG
32112R	T7*GTCCGTGTTAGTGATGT	32302R	GGCAACTTCACTGGCGTG
33310F	T7*GTTCTGGCAGTTTTATC	33310F	CGTTGGGAACTACTTCTTGC
33310R	T7*AACCCCAACATCTCTTA	33310R	TGGAACCCACCCATTGTC
33365F	T7*ACTAATGGGATAAACGGG	33365F	ACAGATGGGTGGGCAAAG
33365R	T7*TATCTTCAGGGTTCAGCA	33365R	AGGCCAGCAACAATGAGG
		TcRSP3F	ACCGTCGTATTCGTGAATTGAC
		TcRSP3R	ACCTCGATACACCATAGCAAGC

Pupae injection of dsRNA

The KS-1 population (Romero et al, 2010) was used in all RNAi experiments. Pupae were collected in a glass petri dish. Once the eyes began to darken the pupae were placed dorsal side up on a piece of double sticky tape on a glass slide. The dsRNA was mixed with 5x dsRNA injection buffer in a 4:1 ratio (final buffer concentration of 0.1 mM $NaPO_4^{3-}$, 5 mM KCL, pH 7). Before injection, the slide of pupae was placed in a glass petri dish for 5 mins that was on top of ice. Pupae were injected in between the 3rd and 4th abdominal segments with 90 nL of the dsRNA/buffer solution, buffer only or non-injected. The total amount of dsRNA injected into each pupa ranged from ~ 100-200 ng. The slide of injected pupae and non-injected controls were placed in a glass petri dish with a small amount of flour, and the remaining surface of the double sticky tape was coated with flour so that no adhesive remained exposed. Once a pupa eclosed it was transferred to a glass vial with a small amount of rearing media and cracked wheat. All beetles were kept individually until they were 10-12 days PAE and used in the aeration experiments.

Analysis of RNAi efficiency by quantitative PCR (qPCR)

To examine the efficiency of the RNAi in transcript suppression, the beetles were removed from the aeration chambers after the 3rd, final, 48 hr DMD collection time point. The beetles were individually homogenized in 250 µL of TRIzol reagent (Ambian Applied Biosystems, USA) and total RNA was extracted according to the manufacturer's protocol. The total RNA was measured with a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Approximately 1 µg of total RNA was treated with RNase-free DNase to remove any DNA contamination. Approximately 500 ng of the DNase treated total RNA samples were converted

to cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) utilizing the T_{18} primer, according to the manufacturer's protocol. The RNAi targeted transcripts were quantified using a CFX connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each well consisted of 5.0 µL of Bullseye EvaGreen qPCR MasterMix (MidSci, Fenton, MO, USA), 0.4 µL of each primer, 3.2 µL of nuclease-free water (Thermo Scientific, Waltham, MA, USA), and 1 µL of 5 times diluted cDNA as a template; total qPCR volume was 10 µL/well. Ribosomal protein 3 (Rsp3) was used as an internal standard for normalization. For each of the RNAi aeration experiments, the qPCR plate consisted of all 6 of the individuals from the first RNAi target, and 5 individuals from each of the buffer and non-injected controls. The second plate consisted of all 6 individuals from the second RNAi target and also 5 individuals from each of the buffer and non-injected controls. The two plates shared 4 individuals from each of the buffer and non-injected controls, with the 5th sample being one that was unique to that plate. Each sample was run in triplicate or duplicate. The conditions of the qPCR were 50 ºC for 2 mins followed by 90 ºC for 2 mins; then 40 cycles of 95 ºC for 15secs, 59 ºC for 30secs and 72 ºC for 30secs. Afterwards the melt curve was analyzed with 60 ºC to 95 ºC at 0.5 ºC/5 sec incremental steps.

Confirmation of Illumina results by quantitative PCR (qPCR) for the candidate genes

Pupae from the KS-1 population were sexed and placed individually in glass vials with a small amount of rearing media and cracked wheat. When the beetles were 14-16 days PAE, 6 male and 6 female beetles were removed from the vials containing diet and placed individually in empty glass vials. After the 24 hr starvation period, each beetle was homogenized in 250 µL

TRIzol reagent. These served as the 24 hr starved treatments. For the fed treatments, 6 male beetles and 6 female beetles that remained on the rearing media were removed and individually homogenized in 250 µL TRIzol reagent. RNA extraction and cDNA synthesis was performed as described previously. The candidate gene transcripts were quantified using a CFX connect Real-Time PCR Detection System (Bio-Rad). Each well consisted of 5.0 µL of Bullseye EvaGreen qPCR MasterMix (MidSci, Fenton, MO, USA), 0.4 µL of each primer, 3.2 µL of nuclease-free water (Thermo Scientific), and 1 µL of 5 times diluted cDNA as a template; total qPCR volume was 10 µL/well. Ribosomal protein 3 (Rsp3) was used as an internal standard for normalization. Each plate consisted of 4 biological replicates of each treatment and each well performed in duplicate.

Pheromone collections

The collections and analysis of DMD from the RNAi beetles was identical to the method detailed in chapter 2.

Results

Illumina sequencing

Sequencing the ventral abdominal cuticle transcriptome from fed male, fed female, starved male and starved female resulted in over 5.5 mil, 3.0 mil, 9.8 mil and 11.9 mil 50 bp reads, respectively. Of these reads more than half of each sample were mapped to the *T. castaneum* 3.0 genome assembly. Focusing on genes that were found to be differentially expressed in fed males vs. starved males yielded 1159 genes that were two-fold down and 311 genes that were two-fold up. Those 311 genes were annotated with Blast2GO and had assigned potential functions. A total of 8 genes from the 311 two-fold up genes were classified as lipid metabolic processes from the enriched GO terms.

Using the Tcas5.2 genome assembly the Illumina sequencing data generated 181 genes up in fed male vs. starved male; 534 genes up in fed male vs. fed female. Homology searches for the 534 genes that were estimated to be up in males vs females were performed by manually BLASTing each to the NCBI non-redundant (nr) protein sequence database. Matches with an e-value of 10-3 with predicted poly-peptide sequences of a minimum length of 20 amino acids were sorted and classified into possible functional categories. Of these 534 genes, 14 were selected based upon predicted functions corresponding to possible lipid metabolic processes. Table 3.2 lists all the candidates tested in this study along with abbreviations used in future graphs and descriptions.

Table 3.2. All candidate genes tested in this study.

The GLEAN ID corresponds to the name of the primers in table 3.1. The 'abbreviations in this paper' column are not all official designations for each of the gene products listed here. The ones that do have official designations are: TcasZ9desA, TcasZdesB and Tcjhamat. The far right column is a designation for the experiment set. 1. Signifies that candidates were pulled out of the GO enrichment analysis using the Tcas3.0 reference genome. 2. Signifies that those candidates were from the analysis using the Tcas5.2 assembly and differential expression analysis using Galaxy. 3. Signifies that these candidates were selected based upon literature review as being possible genes associated with either fatty acid metabolism or endocrine regulation.

Beetlebase ID	Gene Description/Preferred Names:	Gene Symbol	Gene Length (bp)	CDS (bp)	Abbreviation in this paper	RNAi effect	exp. Set
TC000699	delta(7)-sterol 5(6)-desaturase erg31	LOC103315179	1369	55..1257	Δ7des-erg31	no	1
TC003125	luciferin 4-monooxygenase	LOC658413	1649	1..1635	LUC	no	2
TC003175	hypothetical protein	LOC103312227	403	23..382	hypo-3175	yes	2
TC003176	uncharacterized	LOC662840	363	43..294	unch-3176	no	2
TC005622	synaptotagmin 15	LOC661389	1833	143..1558	SYT15	no	2
TC005624	aldehyde dehydrogenase family 3 member B1	LOC661279	2390	842..2326	ALDH3B1	no	2
TC007042	phospholipase A1 member A	LOC662671	1977	326..1435	PLA1A-like	no	1
TC007186	uncharacterized	LOC661718	2539	35..2530	unch-7186	no	1
TC007263	lipase 3	LOC100141961	1423	110..1360	LP-3	yes	1
TC008312	dynactin subunit 5	LOC663200	687	78..626	DCTN5	no	2
TC008471	juvenile hormone acid O-methyltransferase	LOC662961	834	1..834	Tcjhamat	no	3
TC010155	protein Wnt-7b	LOC661936	1527	107..1177	Wnt-7b	no	2
TC011134	acyl-coenzyme A thioesterase 13	LOC103314417	686	169..588	ACOT13a	no	3
TC011173	acyl-coenzyme A thioesterase 13	LOC657079	460	26..445	ACOT13b	no	3
TC011231	cytochrome P450 CYP314A1	LOC661451	1713	125..1591	Cyp314a1	no	1
TC011471	Z9 acyl-CoA desaturase A	LOC658103	1062	1..1062	TcasZ9desA	yes	1
TC011522	fatty acid synthase	LOC103314884	7637	131..7282	TcFAS	yes	1
TC013049	regucalcin	LOC656103	1152	183..1112	RGN	no	2
TC015379	putative fatty acyl-CoA reductase CG5065	LOC656856	1764	231..1718	FAR-CG5065	yes	2
TC016415	Z9 acyl-CoA desaturase B	LOC657939	1053	1..1053	TcasZ9desB	no	1
TC031403	lipase member M-like	LOC103314455	2280	832..2070	LP M-like	no	2
TC032112	probable insulin-like peptide 7	LOC664355	782	267..701	Insl7	no	2
TC032302	probable medium-chain specific acyl-CoA dehydrogenase	LOC662897	1487	223..1470	MCAD	no	2
TC032595	15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like	LOC107397659	1056	100..1038	HPGD-15	no	2
TC033310	lysocardiolipin acyltransferase 1-like	LOC659586	1287	107..7798	LYCAT-1-like	no	2
TC033365	farnesol dehydrogenase-like	LOC658287	995	133..906	FDL-like	no	2/3
TC008261	ribosomal protein S3	LOC659167	861	93..818	RpS3	ND	NA

Confirmation of differentially expressed transcripts using qPCR

Candidate genes found to be differentially expressed in the NGS data (both from the Tcas3.0 and Tcas5.2, as well as literature derived candidates) were examined by qPCR (Figure 3.1 and Figure 3.2). Figure 3.1 contains the relative expression levels of all gene candidates that had a value less than 5.0 (±SE). Figure 3.2 contains the remaining candidates which had a relative transcript level value greater than 5.0 (±SE). The candidates Δ7des-erg31 and PLA1A-like in Figure 3.1 as well as all candidates in Figure 3.2 show the fed female (fed F) to have the highest transcript level out of the 4 templates examined. This is likely due to using whole body homogenates as templates for the qPCR instead of the ventral abdominal cuticle which was used to derive the differentially expressed gene candidates in the Illumina sequencing.

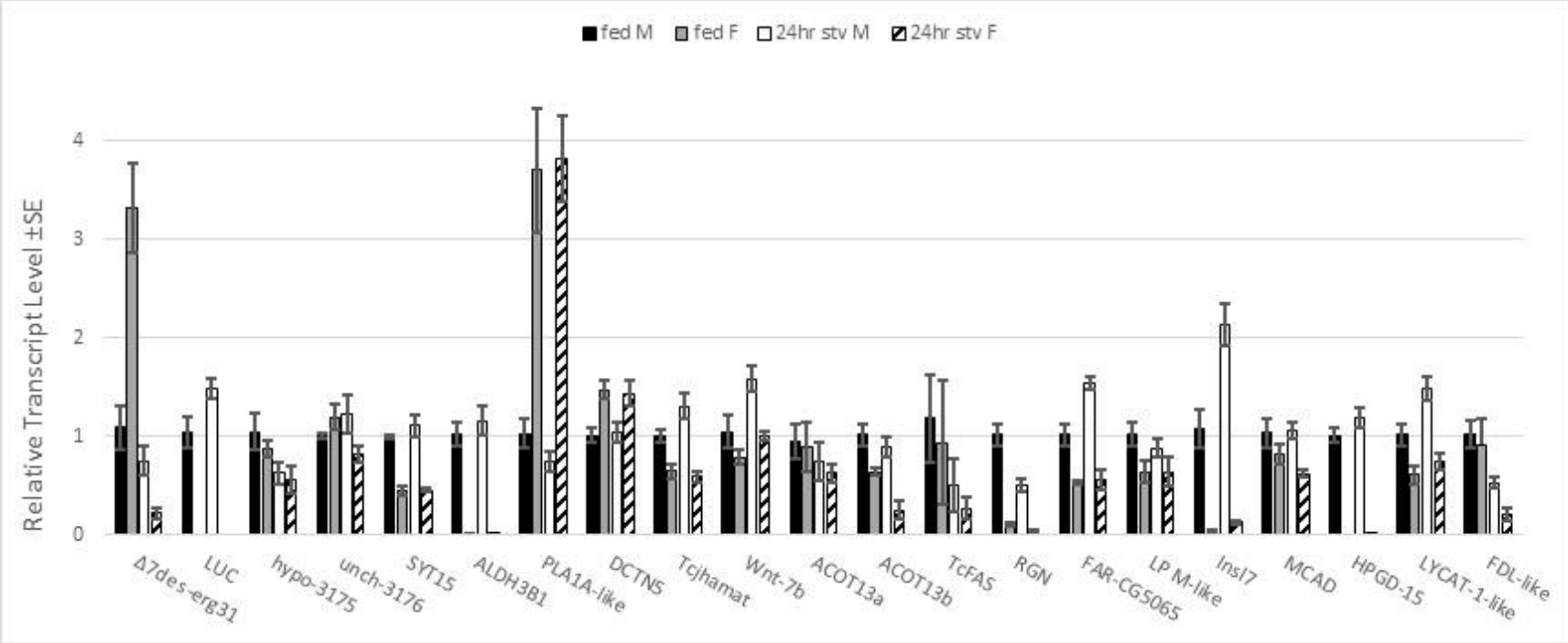

Figure 3.1. Candidate genes qPCR quantification.

qPCR of candidate genes relative mRNA expression levels normalized to that of *rsp3*. Each sample is from a total of 4 individual beetles, replicates (n = 4) except ACOT13a fed M, fed F and stv M where n = 3, and ACOT13b stv F where n = 3.

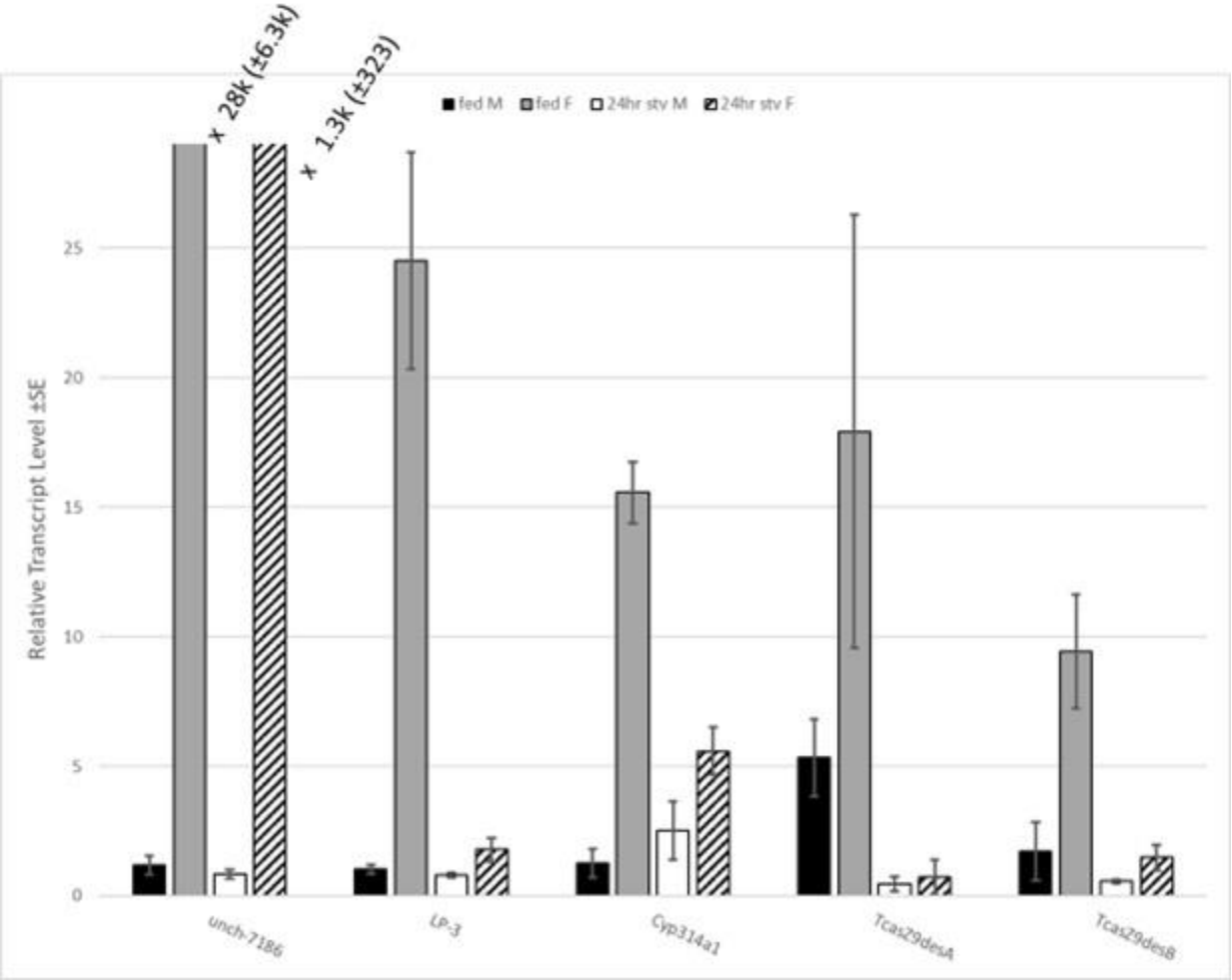

Figure 3.2. qPCR of the remaining candidate genes relative mRNA expression levels normalized to that of *rsp3*.

Each sample is from a total of 4 individual beetles, replicates n = 4. These 5 groups have much higher y-values compared to the other groups in Figure 3.1 and have been separated for overall aesthetics of both figures.

DMD production from the RNAi beetles

A total of 26 candidate genes were suppressed by injecting dsRNA into the late pupal stage (~72 hrs before adult eclosion). Once adult beetles were 10-12 days PAE they were placed in the aeration chamber and pheromone was collected for 6 days. Every 48 hrs the collection column was removed, and the pheromones were eluted and quantified by GCMS. Only 5

candidates' hypo-3175 (Figure 3.3), TcasZ9desA (Figure 3.4), TcFAS (Figure 3.5) and LP-3 and FAR-CG5065 (Figure 3.6)) showed a statistically significant reduction in DMD released compared to both non-injected and buffer injected controls. Suppressing the transcript for TcFAS showed the biggest decrease in DMD production with 6 beetles over the 6-day aeration period producing an average of 20.1 ng of DMD each, while the non-inject and buffer injected controls produced 996.5 ng and 1137.8 ng respectively (Figure 3.5). Suppressing the transcript for TcasZ9desA resulted in a ~50% reduction of DMD (814.8 ng) when compared to both the non-inject (2088.1 ng) and buffer injected (2493.3 ng) beetles (Figure 3.4). Targeting the transcript for hypo-3175 also caused a ~50% reduction in DMD (247.2 ng) when compared to both the non-inject and buffer injected individuals (624.6 ng and 829.9 ng respectively) (Figure 3.3). DMD was collected from beetles injected with dsRNA targeting LP-3 (435.5 ng) and another group of beetles were injected with dsRNA targeting FAR-CG5065 (659.3 ng). These two groups of beetles were assayed at the same time with a set of non-inject (1205.3 ng) and buffer injected (1342.7 ng) individuals (Figure 3.6). Both RNAi beetle groups were significantly different from the two control groups.

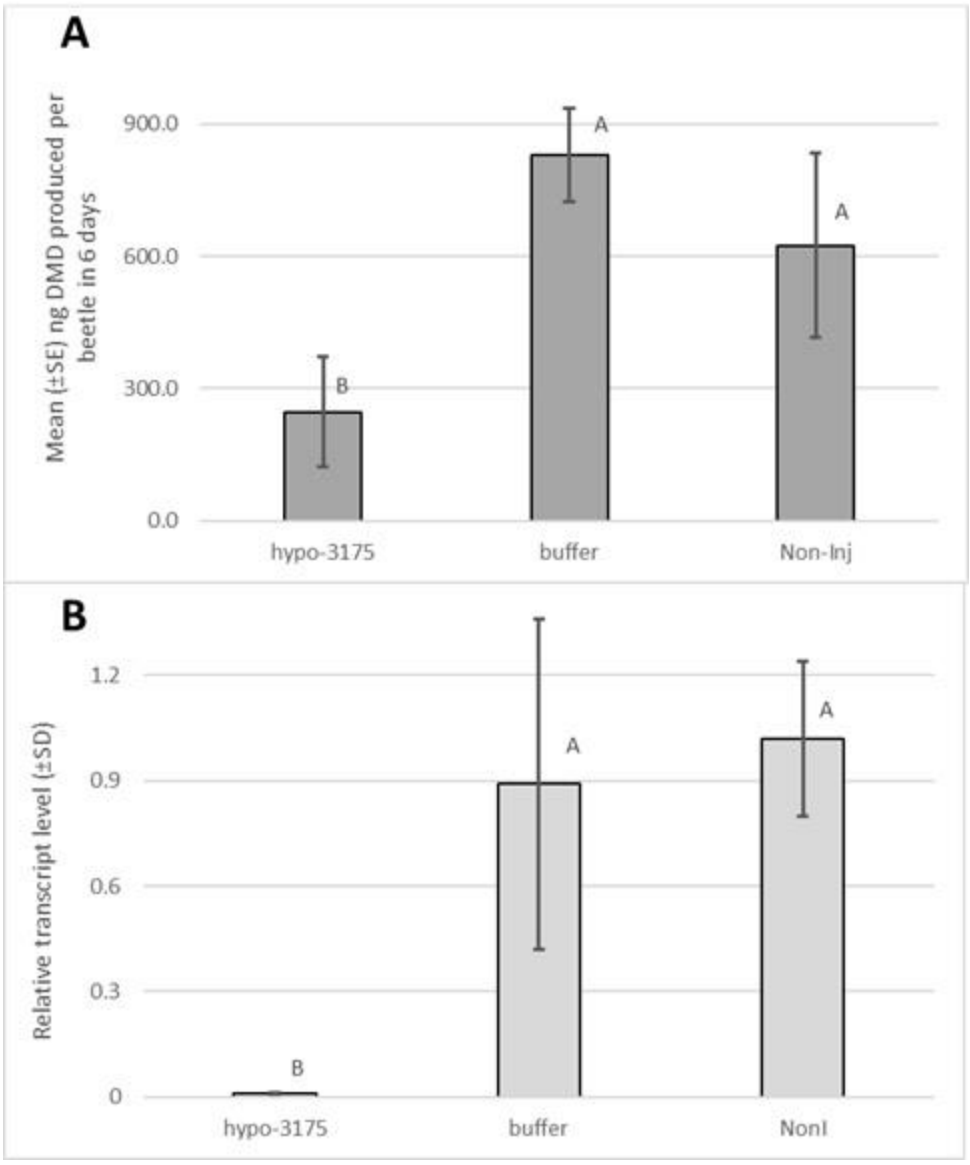

Figure 3.3. DMD produced from beetles injected with dsRNA targeting hyp-3175 and qPCR confirmation of suppression.

A. Mean (±SE) amount of DMD produced per beetle over a 6-day period for dsRNA injected targeting hyp-3175; dsRNA buffer injected only and non-injected individuals (n = 6 for each). B. Validation of RNAi efficiency of transcript suppression using the ΔΔCt method, based on the gene of interest and reference gene rps3, relative to the non-injected control expression levels. Error bars show ±SD (n = 6). The statistical significance was determined using the GLM model in SAS 9.3, different letters indicate a significant difference.

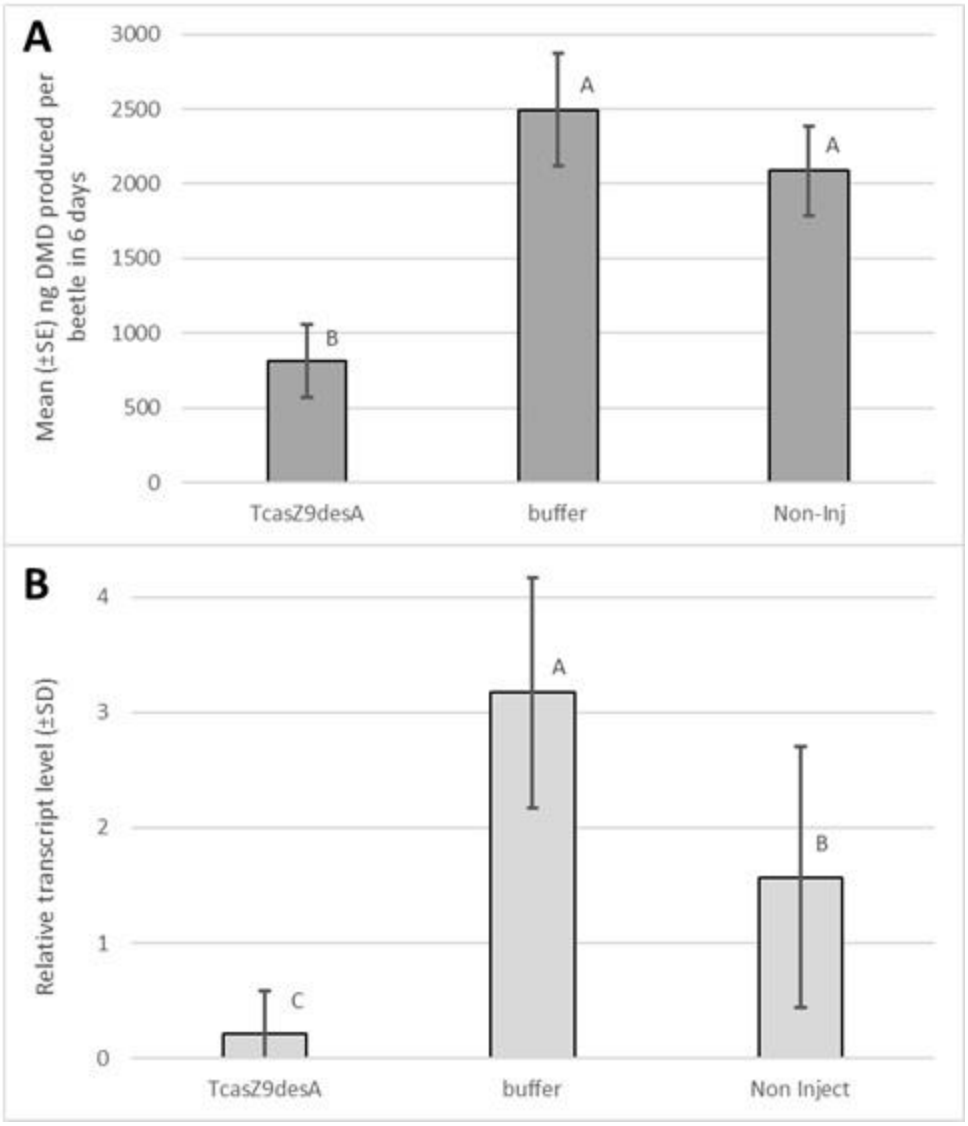

Figure 3.4. DMD produced from beetles injected with dsRNA targeting TcasZ9desA and qPCR confirmation of suppression.

A. Mean (±SE) amount of DMD produced per beetle over a 6-day period for dsRNA injected targeting TcasZ9desA, dsRNA buffer injected only, and non-injected individuals (n=6 for each). B. Validation of RNAi efficiency of transcript suppression for TcasZ9desA by quantitative RT-PCR. Relative expression was calculated using the ΔΔCt method, based on the gene of interest and reference gene *rps3*, relative to the non-injected control expression levels. Error bars show ±SD (n = 6). The statistical significance was determined using the GLM model in SAS 9.3, different letters indicate a significant difference.

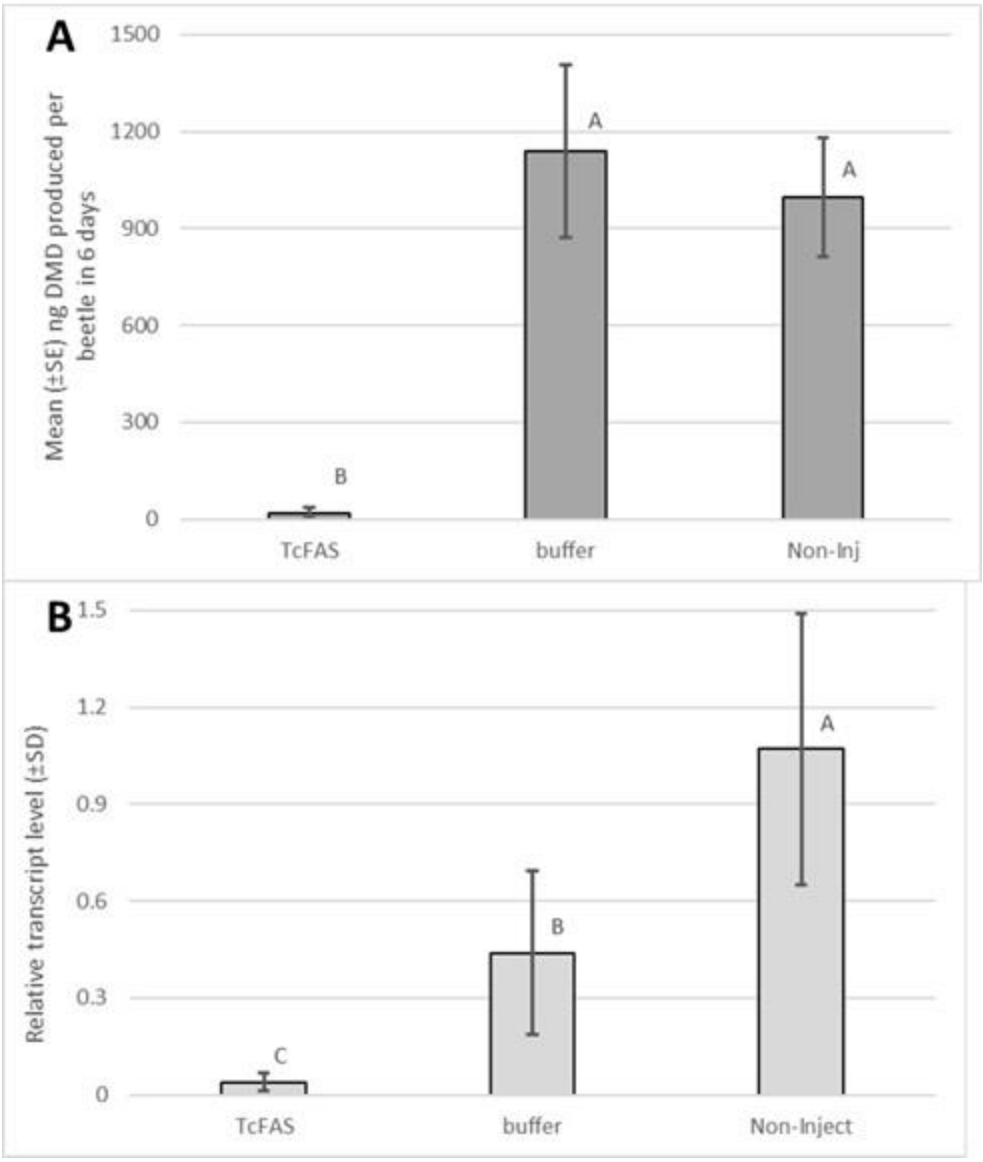

Figure 3.5. DMD produced from beetles injected with dsRNA targeting TcFAS and qPCR confirmation of suppression.

A. Mean (±SE) amount of DMD produced per beetle over a 6-day period for dsRNA injected targeting TcFAS, dsRNA buffer injected only, and non-injected individuals (n=6 for each). B. Validation of RNAi efficiency of transcript suppression for TcFAS by quantitative RT-PCR. Relative expression was calculated using the ΔΔCt method, based on the gene of interest and reference gene *rps3*, relative to the non-injected control expression levels. Error bars show ±SD (n = 6). The statistical significance was determined using the GLM model in SAS 9.3, different letters indicate a significant difference.

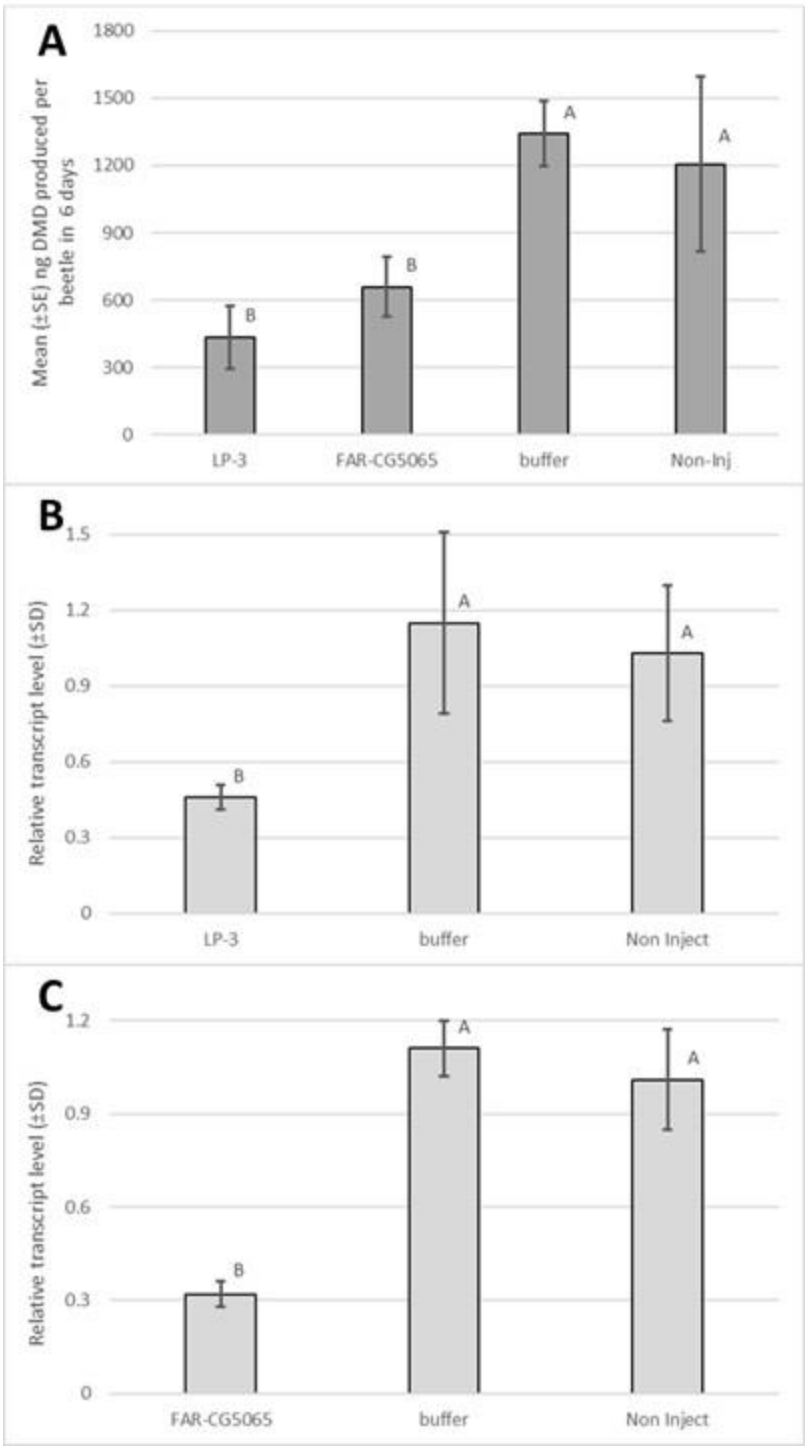

Figure 3.6. DMD produced from beetles injected with dsRNA targeting LP-3 or FAR-CG5065 and qPCR of suppression.

A. Mean (±SE) amount of DMD produced per beetle over a 6-day period for dsRNA injected targeting LP-3 or FAR-CG5065, dsRNA buffer injected only, and non-injected individuals (n=6 for each). B. Validation of RNAi efficiency of transcript suppression for LP-3 by quantitative RT-PCR. Relative expression was calculated using the ΔΔCt method, based on the gene of

interest and reference gene *rps3*, relative to the non-injected control expression levels. Error bars show ±SD (n = 6). The statistical significance was determined using the GLM model in SAS 9.3, different letters indicate a significant difference. (C) Validation of RNAi efficiency of transcript suppression for FAR-CG5065 by quantitative RT-PCR. Relative expression was calculated using the ΔΔCt method, based on the gene of interest and reference gene *rps3*, relative to the non-injected control expression levels. Error bars show ±SD (n = 6). The statistical significance was determined using the GLM model in SAS 9.3, different letters indicate a significant difference.

The other candidate genes did not show a significant difference between both the non-inject and buffer injected controls (Table 3.3). One instance occurred were the amount of DMD produced by the LYCAT-1-like RNAi beetles was significantly lower than that from the non-injected controls, but not the buffer injected controls. The opposite also occurred where the amount of DMD produced by the hypo-3176 RNAi beetles was significantly more than the non-injected controls but not different than the buffer injected controls. The effectiveness of the dsRNA was greater than 85% in suppressing the target gene transcripts for all candidates except for unch-3176, ACOT13A, LP-M-like, SYT15 and DCTN5 each of which were only 64 %, 58 %, 45 %, 62 %, and 71 % effective, respectively (Table 3.3).

Table 3.3. Candidates which RNAi had no effect on DMD production.

RNAi of candidates that showed no statistical significance on DMD production compared to both the non-injected and buffer injected controls. The IDs listed together inside of the outlined cells had DMD collected at the same time. The relative transcript levels were calculated using the ΔΔCt method, based on the gene of interest and the reference gene rps3, relative to the non-injected control relative transcript levels in each of the outlined cells.

dsRNA target ID	Mean ng DMD produced per beetle in 6 days (±SE)	Relative transcript level (±SD)
PLA1A-like	2079.3 (399.7)	0.15 (0.04)
unch-7186	1770.1 (202.2)	0.36 (0.07)
Non-Inject	1960.2 (150.2)	1.04 (0.33)
Buffer	1818.0 (237.4)	0.92 (0.35)
ACOT13B	827.6 (161.0)	0.02 (0.01)
ACOT13A	966.7 (192.0)	0.42 (0.15)
Non-Inject	792.4 (214.2)	1.01 (0.14)
Buffer	1043.7 (124.0)	0.90 (0.15)
Wnt-7b	940.8 (236.3)	0.14 (0.04)
LP M-like	1355.4 (294.6)	0.55 (0.24)
Non-Inject	1389.2 (303.9)	1.01 (0.16)
Buffer	1032.1 (170.0)	1.09 (0.09)
LYCAT-1-like	852.9 (136.6)	0.14 (0.04)
Insl7	1093.2 (159.3)	0.13 (0.05)
Non-Inject	445.7 (211.6)	1.02 (0.23)
Buffer	1212.3 (151.7)	1.10 (0.27)
LUC	1247.5 (85.4)	0.02 (0.02)
ALDH3B1	1633.6 (278.2)	0.06 (0.01)
Non-Inject	1533.6 (170.0)	1.01 (0.13)
Buffer	1408.0 (252.8)	1.13 (0.20)
MCAD	1286.9 (191.9)	0.15 (0.10)
RGN	1466.6 (219.2)	0.06 (0.02)
Non-Inject	1546.1 (442.7)	1.05 (0.34)
Buffer	1114.7 (175.2)	0.90 (0.25)
FDL-like	1183.1 (339.3)	0.01 (0.00)
SYT15	1107.9 (204.7)	0.38 (0.14)
Non-Inject	821.5 (131.7)	1.10 (0.54)
Buffer	949.9 (173.0)	0.83 (0.27)
Δ7des-erg31	1466.4 (270.3)	0.09 (0.06)
Tcjhamat	1179.7 (207.1)	0.01 (0.00)
Non-Inject	2466.3 (448.0)	1.20 (0.9)
Buffer	1685.7 (267.4)	0.80 (0.4)
HPGD-15	746.1 (181.2)	0.01 (0.00)
Non-Inject	697.6 (165.2)	1.07 (0.38)
Buffer	919.5 (145.5)	1.30 (0.33)

dsRNA target ID	Mean ng DMD produced per beetle in 6 days (±SE)	Relative transcript expression level (±SE)
Cyp314a1	1868.8 (252.5)	0.10 (0.02)
Non-Inject	2088.1 (300.8)	0.78 (0.18)
Buffer	2493.3 (375.9)	0.64 (0.08)
TcasZ9desB	1142.0 (267.5)	0.08 (0.10)
Non-Inject	996.5 (185.4)	1.10 (0.53)
Buffer	1137.8 (139.0)	0.34 (0.31)
unch-3176	1314.9 (349.9)	0.02 (0.01)
Non-Inject	624.6 (209.9)	1.01 (0.18)
Buffer	829.9 (106.6)	1.00 (0.36)
DCTN5	1991.1 (431.6)	0.29 (0.28)
Non-Inject	2297.9 (415.8)	0.91 (0.12)
Buffer	2134.5 (415.9)	0.91 (0.40)

Discussion

To determine possible genes involved in the biosynthesis of the aggregation pheromone 4,8-dimethyldecanl (DMD) of *Tribolium castaneum* we utilized an NGS approach to identify transcripts that were up regulated in feeding males compared to feeding females and 24 hr starved males and females. The initial results found 8 possible candidates associated with lipid metabolism. Another attempt at analyzing the NGS data using the more recent Tcas5.2 genome assembly and a different NGS pipeline located an additional 14 possible candidates. These 14 candidates were combined with 4 more from literature searches and the original 8 to give a total of 26 genes. These were targeted for transcript suppression using RNAi and subsequently the individuals had the amount of DMD produced quantified using the aeration method described previously. Out of the 26 candidates 5 exhibited a statistically significant decrease in the amount of DMD produced from the buffer injected controls and the non-injected controls. It should be noted that for every candidate gene, 20 pupae were injected with dsRNA and 20 pupae injected with buffer only. In total 1,040 pupae were injected. Only two pupae died and failed to make it to the adult stage. Those two pupae were buffer injected controls. Initial RNAi experiments were done in the late larva stage. The dsRNA targeting FAS was lethal. The injected larvae either died during the prepupae stage or died shortly after pupation. The suppression of fatty acid synthase (TcFAS) transcript had the most dramatic effect on DMD production. This result is somewhat expected. Kim et al. (2005) greatly reduced the amount of DMD produced from *T. castaneum* using a chemical inhibitor (2-octynoic acid) of the fatty acid pathway. In the same set of experiments, they also used mevastatin, an inhibitor of the mevalonate pathway, which did not have an effect on DMD production. The null effect of mevastatin demonstrated that DMD is not

made in a similar manner as aggregation pheromones from bark beetles (Barkawi et al., 2003; Sandstrom et al., 2006; Seybold et al., 1995).

The *de novo* biosynthesis of fatty acids is done by FAS. *T. castaneum* only has one copy of FAS in its genome. Suppressing the transcript for that gene not only eliminated production of DMD but also provided evidence that its synthesis cannot come from modifications of stored lipids or fatty acids. The biosynthesis of DMD and its precursors are tightly regulated to the nutritional status of the feeding male beetle (Ming & Lewis, 2010).

The structure of DMD resembles that of a methyl branched medium chain fatty acid, apart from DMD having an aldehyde group instead of an -OH. Also, the internal methyl groups in DMD help differentiate it from a straight-chain saturated fatty acid. These internal methyl groups have been studied in several insect species, mainly through the examination of hydrocarbon biosynthesis. In the cockroach *Periplaneta americana* ^{13}C labeled methylmalonate was incorporated into3-methylpentacosane, a component of its contact pheromone (Dwyer et al., 1981). The housefly *Musca domestica* also had ^{13}C labeled methylmalonate and ^{13}C labeled propionate incorporated into its methyl branched hydrocarbons (Dillwith et al., 1982). The position at which the ^{13}C labeled precursors were detected in the structure of the final compound(s) was evidence that the internal methyl groups were added during the chain elongation step (Schal et al., 1994). Utilizing this information about how the methyl groups are added to DMD and the data presented thus far, a picture of DMD synthesis can take shape. Combining the work of Kim et al. (2005) to the information gathered here, Figure 3.7 shows a possible way in which DMD might be synthesized in *T. castaneum*. The precursors for DMD, malonyl-CoA (from acetyl-CoA) and methylmalonyl-CoA (from propionyl-CoA) are provided to TcFAS. TcFAS uses these building blocks, starting with one malonyl-CoA followed by the

addition of one methylmalonyl-CoA. Another acetyl-CoA and another methylmalonyl-CoA are added. The growing chain is completed by the addition of four more units of malonyl-CoA. The final product released in this example is 10,14-dimethylpalmitoyl-CoA. The 16 C precursor then goes through 3 cycles of β-oxidation. The product from this set of reactions is the 10 C precursor 4,8-dimethyldeconyl-CoA. Indicated in Figure 3.7 the 16 C or 10 C DMD precursor could be loaded onto a glycerol backbone and stored as a triacylglycerol. This possibility provides one possibility to how a lipase (LP3) was able to significantly reduce DMD production from injection of dsRNA targeting that transcript. Additionally, this example in Figure 3.7 assumes that palmitic acid is the backbone of the DMD precursor. However, it is possible that stearic acid or even myristic acid could serve as the backbone of the final dimethylated product released from TcFAS. After the DMD precursor is released from TcFAS the putative fatty acyl-reductase would be able to reduce it to a fatty alcohol. In moths, this type of reductase that is involved in pheromone biosynthesis has a unique specificity and selectivity for fatty acyl substrates (Antony et al., 2016). If this TcFAR-CG5065 enzyme also has an ability to be more specific for one chain length than another that would expand its ability to act on the pre-DMD precursors at different positions in Figure 3.7. Sequence alignments and molecular modeling would provide valuable information regarding it's possible substrate specificities. Silencing the transcript for LP3 also had a significant impact on DMD production. Interestingly, the transcript level in the fed female was much higher than the other 3 treatments, while the fed male had the lowest relative transcript level out of the 4 treatments (Fig. 3.4B). This would suggest that LP-3 would not be unique to DMD biosynthesis.

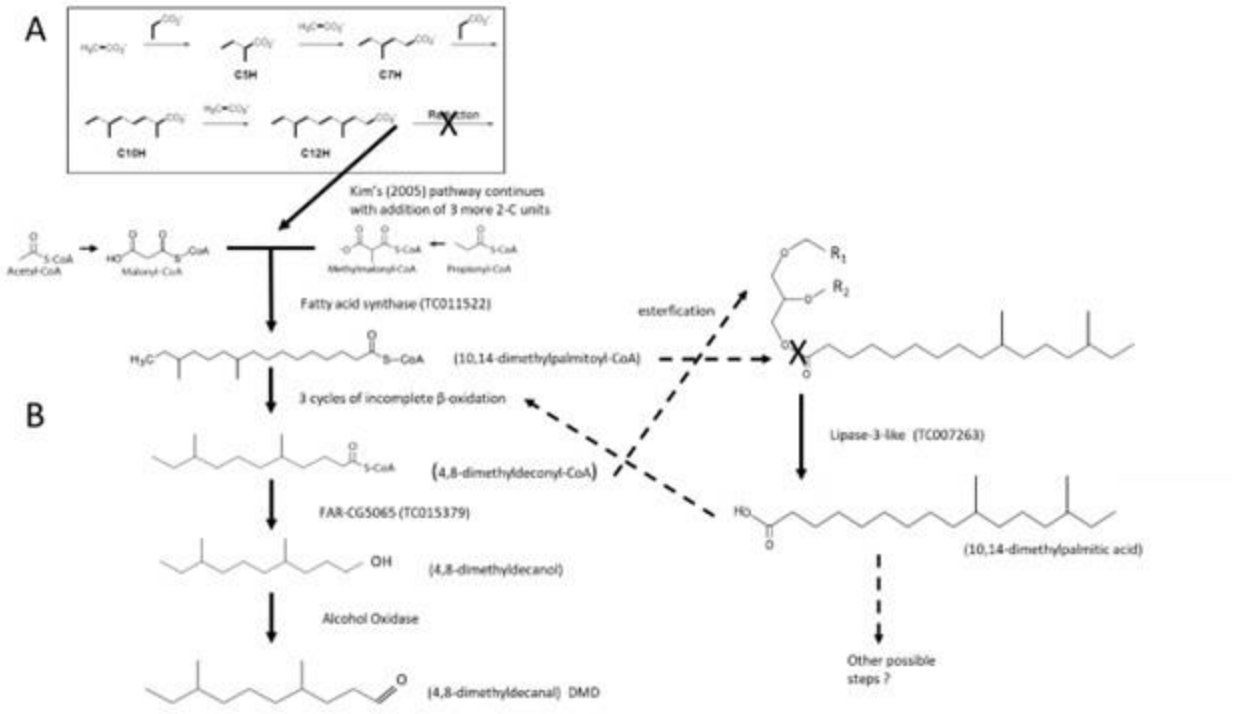

Figure 3.7. Hypothesized route(s) of DMD biosynthesis.

Hypothesized route(s) of DMD biosynthesis and possible locations of three of the candidate genes. A. The process of DMD biosynthesis begins as described by Kim (2005). Acetate units are condensed to form malonyl-CoA. Four malonyl-CoA's are added and then a unit of methylmalonyl-CoA is added to form the methyl group that will ultimately end up in the 4 position. B. Pathways hypothesized from the RNAi results in this study. The growing carbon chain process is performed by Fatty Acid Synthase (TC011522). The cycle continues in the subsequent steps by addition of: malonyl-CoA – methylmalonyl-CoA – malonyl-CoA. In this example the pre-DMD unit is a 16-carbon chain with methyl groups at positions 10 and 14 (10,14-dimethlypalmitoyl-CoA). This pre-DMD could be stored in a triacylglycerol (dashed arrow) or subjected to 3 rounds of β-oxidation. The product from the incomplete β-oxidation step would be a 10-carbon chain with methyl groups in the 4 and 8 position (4,8-dimethyldeconyl-CoA). This product could also be stored in a triacylglycerol (dashed arrow) or continue in the pathway. This 4,8-dimethyldeconyl-CoA is then reduced by another candidate gene FAR-CG5065 to form the fatty alcohol 4,8-dimethyldecanol. The final step shows 4,8-dimethyldecanol being oxidized by an alcohol oxidase to form DMD. If any of the pre-DMD compounds in this example are stored in triacylglycerol (TAG) then the suppression of Lipase-3 (TC007263) would inhibit their release and subsequently lower the amount of DMD detected in the aeration studies.

The remaining two candidates, which when targeted with RNAi significantly decreased DMD production, are difficult to find a place in the overall scheme of DMD production.

TcasZ9desA (TC011471) is a desaturase which has already been published on by (Horne et al.,

2010). This enzyme was found to introduce a cis-double bond in the delta-9 position of both stearic acid and palmitic acid, with a preference for stearic acid over palmitic acid. The reduction in DMD production from the suppression of that transcript might result from a more indirect effect. This could be to membrane destabilization, disruption of a feedback loop, or something else entirely. The remaining candidate causing a decrease in DMD production is a hypothetical protein according to NCBI. When the old beetlebase website was still on its original server, the tiling array data showed that only males had detectable transcript levels. Young males had a noticeably higher level than older males. However, in the data here the transcript for fed males was only slightly higher than the other 3 treatments (Fig 3.1A) Upon the release of the Tcas5.2 assembly it has been combined with TC003174 to form TC032302. TC032302 was tested as a candidate but had no effect on DMD production. The dsRNA template (and qPCR primers) was designed on the 5'-region of 32302 which coincided with the original TC003174 gene. The dsRNA primers and qPCR primers for hypo-3175 were on the far 3' end of TC03202 which would be on TC003175. TC032302 was tested as a candidate but had no effect on DMD production. BLASTing 3175 yields no good matches at all, however 3174 has several matches with one of the best being XP_004926890.1, which is short chain specific acyl-CoA dehydrogenase mitochondrial isoform X2 from *Bombyx mori*; (e^{value} -78).

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Chapter 4 - Neuroendocrine Factors Influencing Pheromone Production in *Tribolium castaneum*

Introduction

Neuropeptides are known to influence male produced aggregation pheromone production in several families of coleoptera. Juvenile hormone (JH) or one of its synthetic analogs, are some of the most studied endocrine factors affecting pheromone production. Males of the Colorado potato beetle, *Leptinotarsa decemlineata*, (Coleoptera: Chyromelida) produce an aggregation pheromone (*S*)-3,7dimethyl-2-oxo-oct-6-ene-1,3-diol [(*S*)-CPBI]. Antennal receptors of both sexes respond selectively to the (*S*)-enantiomer. Topical application of JH III increased production eightfold, while topical application of JH III and antennectomy increased production almost 200-fold (Dickens et al., 2002). Topical application of JH III or an analog to male bark beetles of *Ips paraconfusus*, (Hughes & Renwick, 1977) and *Ips cembrae* (Renwick & Dickens, 1979) (Scolytidae) increased production of three pheromones, ipsenol, ipsdienol and 2-phenylethanol in *I. paraconfusus* and 3-methyl-3-buten-10-ol in *I. cembrae*. Similar experiments involving topical applications of JH or an analog to male *Anthonomus grandis* (Curculionidae) also elicited an increase in production of two of its aggregation pheromone components (*Z*)-2-(3,3-dimethylcyclohexylidene)ethanol, (*cis*)-1-methyl-2-(1-methylethenyl) cyclobutaneethanol and 2-(1-methylethernyl)-5-methyl-4-hexen-1-ol (Dickens et al., 1988). All of the aggregation pheromones produced by these male beetles are made by the consumption of host plant compounds or by the mevalonate pathway which utilizes terpenes as building blocks. However, male *Tribolium castaneum* (Tenebrionidae) make an aggregation pheromone, 4,8-

dimethyldecanal (DMD) from the fatty acid pathway. Topical application of JH to male *T. castaneum* increased DMD production (Kim et al., 2005).

Biogenic amines are necessary components of an insect's nervous system. These compounds can act in multiple ways across most orders of insects. Three of these biogenic amines, dopamine, octopamine and tyramine, are known to illicit different types of responses in insects. Octopamine and tyramine are related amines that can have overlapping functions but are neuro-actively independent (Lange, 2009) and can also have opposing effects (Saraswati et al., 2004). Dopamine is associated with oogenesis and oviposition (Bloch et al., 2000; Boulay et al., 2001; Dombroski et al., 2003; Sasaki et al., 2007), JH metabolism (Gruntenko et al., 2005; Thompson et al., 1990), and olfaction (Macmillan & Mercer, 1987; Mercer & Erber, 1983). Dopamine and octopamine are also known to fluctuate from different types of stress in *T. castaneum* larvae (Hirashima et al., 1993). Octopamine is also able to modulate the activity of several hormones relating to metamorphosis in *Tribolium freemani*. These include but are not limited to juvenile hormone esterase and ecdysteroids (Hirashima et al., 1998).

Multiple insect hormones are known to interact with processes affecting the fat body. Adipokinetic hormone (AKH) is a member of a group of peptides that are established influencers of lipid metabolism. The AKH neuropeptides, from *Locosta migratoria* and *Schistocerca gregaria*, was one of the first insect neuropeptides to be sequenced (Stone et al., 1976). It is produced in the corpora cardiaca which is situated near the insect brain. During intense physical activity such as flying, AKH will mobilize the fat body for a source of energy (Gade et al., 1997). AKHs also can play roles in insect locomotion, immune responses, and reproductive processes (Kodrík, 2008; Plavšin et al., 2015; Van Der Horst, 2003).

Another insect hormone is 20-hydroxyecdysone (20HE) and its prohormone ecdysone (E). To trigger ecdysis in *T. castaneum*, 20HE is the main initiator that works in concert with several other hormones and peptides (Arakane et al., 2008). In other insect species such as *Bombyx mori*, (Hirashima et al., 1999), *Drosophila melanogaster* (Chiang et al., 2016) and *T. freemani* (Hirashima et al., 1998), 20HE and E are associated with levels of the biogenic amines previously listed above, mainly octopamine as well as JH, under stressful conditions.

The relationships of the neuroendocrine factors mentioned here make them ideal targets to test regarding pheromone production in *T. castaneum*. JH has already been tested with this regard (Kim et al., 2005). In this chapter the effects that these compounds might have on DMD production is examined by either topically applying the test compound or by injecting it into DMD producing adult male *T. castaneum*. Starving males that are not producing DMD are also treated with these compounds in an attempt to rescue the non-DMD producing phenotype.

Materials and Methods

Beetles used in this study

The KS-1 population (Romero et al., 2010) was used in all experiments involving hormones or neuropeptides. This is the same population that was used to examine the effects of DMD production by targeting candidate genes with RNAi from chapter 3. The KS-1 population has been in culture for at least a decade. In chapter 2 it was determined to be a moderate to high producer of DMD. Also, every beetle made detectable amounts of DMD whenever it was inside of the aeration chamber.

Adult injections of hormones and neuropeptides

Octopamine, tyramine, and precocene I was purchased from Sigma Aldrich (St. Louis, MO, USA). JH III, AKH1a, 20HE, E, methoprene and dopamine were kind gifts from Dr. Yoonseong Park's lab at Kansas State University (Manhattan, KS, USA). For all topical applications the volume was 0.5 µL. The compounds which were topically applied are: JH III, methoprene and precocene I, controls were applied with acetone only or had no treatment. All of these were dissolved in acetone. The concentration of these three compounds were each 1 mM for JH III, precocene I and high methoprene (hi meth), while low methoprene (lo meth) was 0.5 mM.

T. castaneum pupae were sexed and males placed individually in a glass vial with rearing media and a small amount of cracked wheat. Males that were 10-12 days post adult eclosion (PAE) were placed into the aeration chambers, which contained diet and cracked wheat, at the start of all experiments. The males were allowed to adjust to the environment of the chambers for the first, initial 48 hrs in all cases (this timepoint is referred to as the 0^{th} 48 hrs). The vacuum pump remained on pulling filtered air through the chamber, but no pheromone was being collected. For the experiments involving feeding males, diet was available inside the chambers the entire time. For the starvation experiments, beetles were placed on diet for the 1^{st} 48 hr collection point, diet was removed for the 2^{nd} 48 hr time point and diet was returned to only the controls for the 3^{rd} 48 hr time point; while treated beetles remained without diet.

Injections or topical applications occurred after the 2^{nd} 48 hr time point in all cases, except the testing of JHIII, which utilized a time frame of 24 hrs instead of 48 hrs. Topical applications were performed by removing the beetle from the chamber and placing it in a petri dish that was on ice. The beetle was cooled on the dish for ~180 secs. The beetle was then

transferred to a Sylgard dissecting dish and positioned so the ventral side was up. The volume of the compound being topically applied was 0.5 µL which was administered to the ventral abdominal cuticle. The application of acetone was monitored while it evaporated which took 30-40 secs. After all of the acetone evaporated the beetle was then placed back into the aeration chamber with or without diet and connected back to the aeration system. Injections of adults followed the same protocol as the topical applications, up to the point where the beetle was placed in the Sylgard (World Precision Instrument, Inc., Sarasota, FL, USA) dish. Injections were performed by first grasping and spreading the elytra with fine forceps while the beetle was laying ventral side up. Once the elytra were spread out the beetle was rolled about 120° towards the microscope objectives, so that the dorsal side was almost fully up. Using a very fine pulled glass needle, and a Nanoliter 2000 (World Precision Instrument, Inc., Sarasota, FL, USA) 50 nL of the test compound was injected on the left side, midway between the digestive tract and the spiracles on the 3rd abdominal segment. Test compounds injected (concentrations injected) were: 20HE, E, octopamine, tyramine, dopamine and AKH1a. All compounds being injected were dissolved in dsRNA injection buffer (final buffer concentration of 0.1 mM $NaPO_4^{3-}$, 5 mM KCL, pH 7). Non-injected control beetles in all experiments were manipulated the same as treated beetles. Once the control beetles started becoming active while still on the Sylgard dish, they were transferred back into their aeration chamber (with or without diet) which was then connected back to the aeration system.

Pheromone collections

The collection and quantification of DMD was carried out in a identical manner to that described in chapter 2. The only difference in this chapter compared to chapter 2 is that some of

the time points used to collect DMD is 24 hrs instead of 48 hrs. These 24 hrs timepoints are clearly indicated on the x-axis of any graph to which this difference applies.

dsRNA injections

RNAi was performed as previously stated in chapter 3. The data from candidate Tribolium castaneum juvenile hormone acid *O*-methyltransferase (TcJHAMT) (TC008471) (Minakuchi et al., 2008) is included in this chapter

Pheromone analysis

All pheromone analysis was performed in an identical manner to that which was described in chapter 2.

Results

Topical application of methoprene on DMD production in fed and starved beetles

To test whether the JH analog methoprene had an effect on DMD production, pheromone collections were taken before and after topical application (Fig. 4.1A). The amount of DMD produced by feeding males was not significantly different between the 4 test groups at both timepoints before the application. The 3rd 24 hr timepoint (1st time point after application, labeled on the graph as a tilted checkered lightning bolt) there was a significant reduction in DMD production for both doses of methoprene tested compared to the non-treated controls. However, the acetone only treatment was not significantly different from the non-treated controls or either dose of methoprene. There was an increase of DMD at the 4th 24 hr timepoint from all treatment groups. The group of beetles receiving the low dose of methoprene (0.5 µL of 0.5

mM) remained significantly less than the non-treated and acetone controls at the 4th 24hr timepoint.

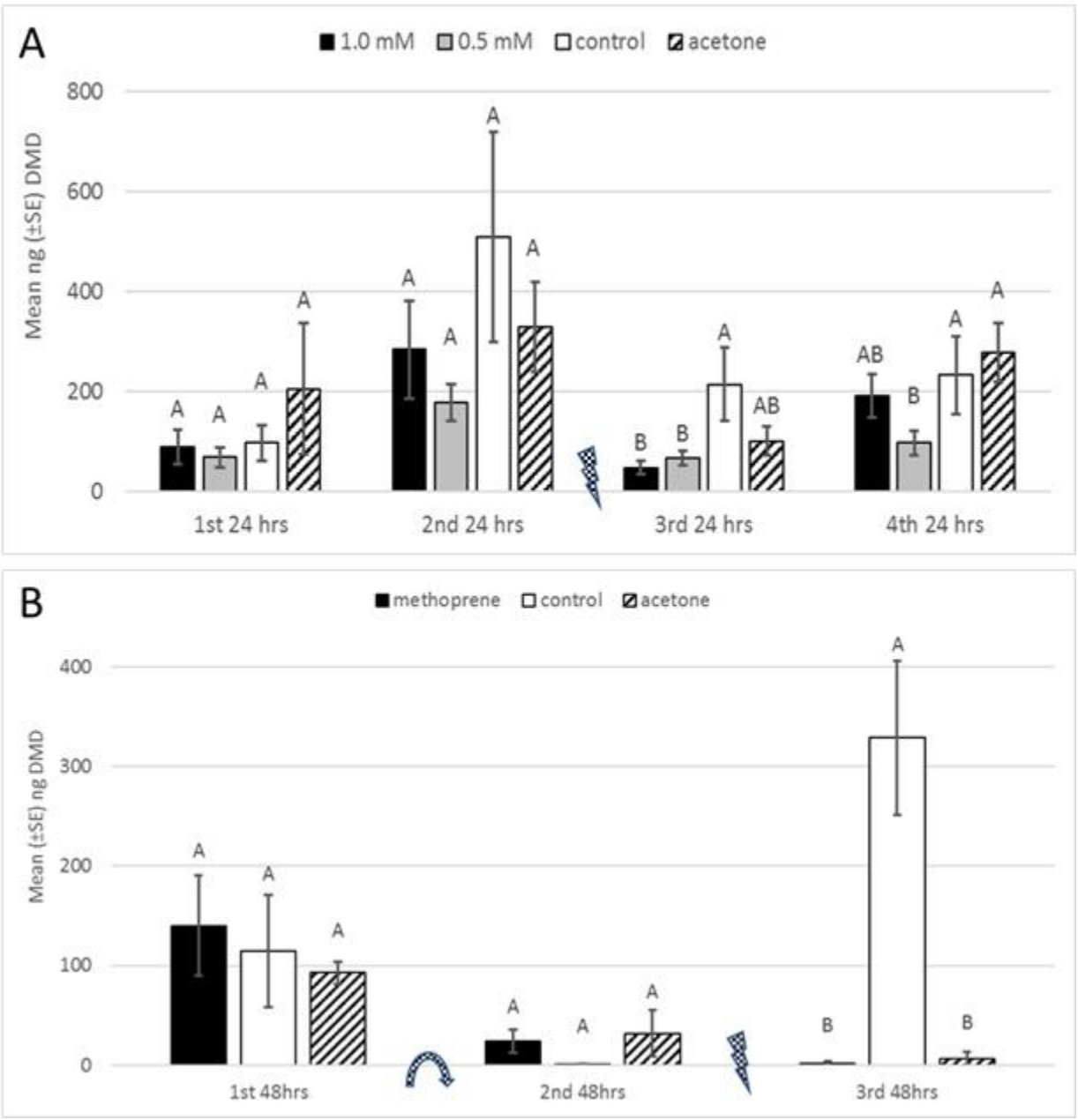

Figure 4.1. Effect of methoprene on DMD production in fed and starved beetles.

Mean ng of DMD (±SE) produced by *T. castaneum* males treated with methoprene. The small, checkered lightning bolt represents when methoprene or acetone only was topically applied, and for starved beetles when diet was returned to the chamber. The small, curved downward arrow represents when diet was removed from all beetles. A. Males were on diet throughout the

duration of the aeration period. B. Males were starved before being treated with methoprene or acetone only. Food was returned to the starved control beetles after the 2nd 48 hrs. Each bar represents the mean amount of DMD from 6 individuals with ± standard error. One-way ANOVA with completely randomized design ($P < 0.05$) was used for statistical analysis. Bars with the same letter are not significantly different from the others during the same time period.

Before the starvation period began, DMD was collected for a 48 hr period when the beetles were still on diet (Fig. 4.1B). Each group produced a similar amount of DMD during the 1st 48 hr period. A large decrease in DMD occurred in each group during the 2nd 48 hr period. This reduction was from the removal of food in the aeration chamber, labeled on graph with a curved, downward pointing checkered arrow. Before the 3rd 48 hr period began the beetles received either 0.5 µL of methoprene (0.5 mM), 0.5 µL of acetone only, or similar physical manipulation with no treatment (labeled on the graph as a tilted checkered lightning bolt). Afterwards there was the reintroduction of food to only the control beetles. This food that was given to the only the controls initiated a dramatic increase of DMD. Acetone and methoprene treated beetles had a continued decline in DMD production. Methoprene nor acetone was able to rescue the starved phenotype with an increase DMD.

Topical application of JHIII on 4,8-dimethyldecanal production in fed and starved beetles

The topical application of JHIII followed in an identical manner to that of methoprene. To test whether JHIII itself had an effect on DMD production, pheromone collections were taken before and after topical application of JHIII (Fig. 4.2A). During each time period when DMD was measured, there was no significant difference in DMD production amongst the three treatment groups.

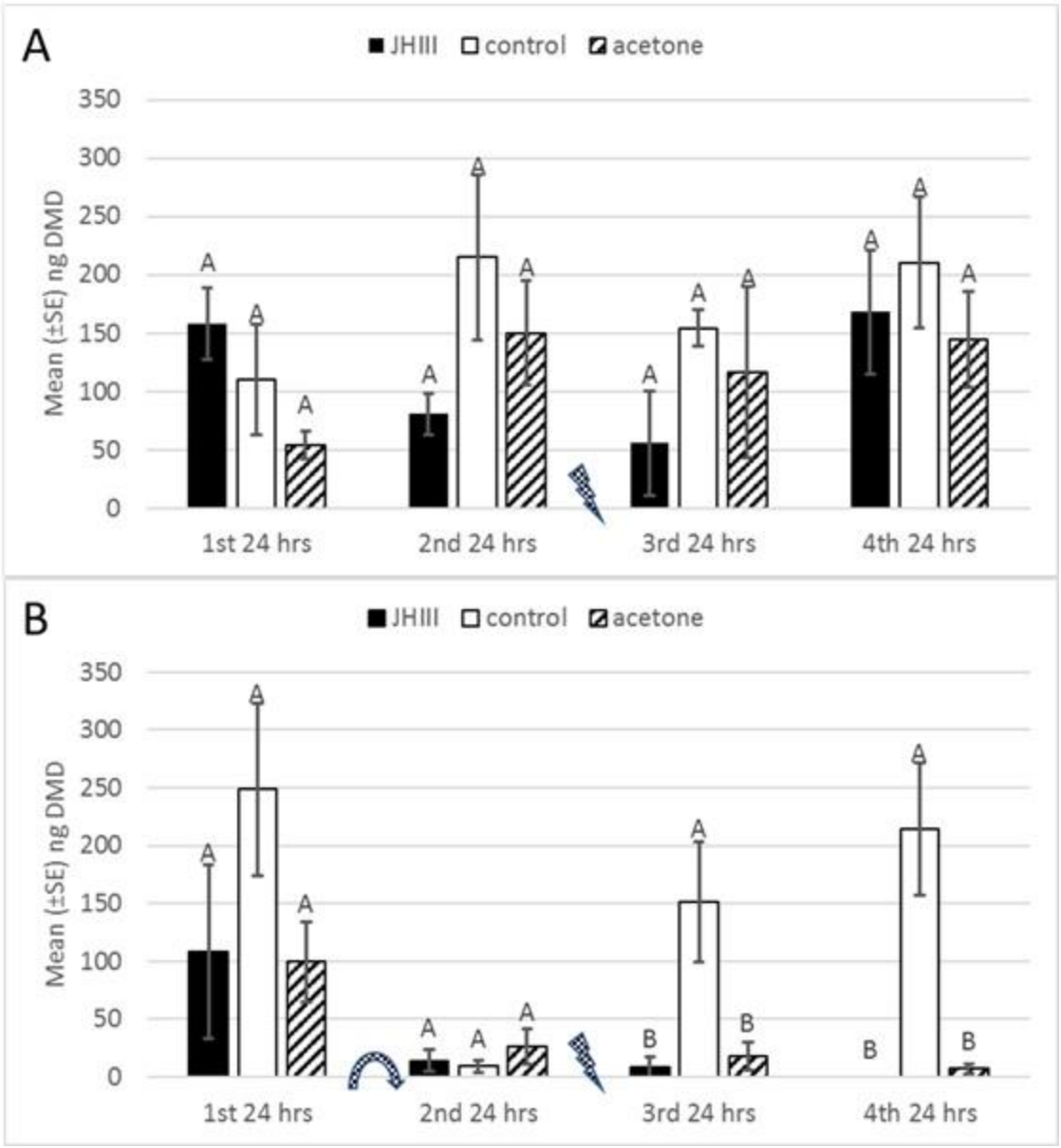

Figure 4.2. Effects of JHIII on DMD production in fed and starved beetles.

Mean ng of DMD (±SE) produced by *T. castaneum* males treated with JHIII. The small, checkered lightning bolt represents when JHIII or acetone only was topically applied, and for starved control beetles when diet was returned to the chamber. The small, curved downward arrow represents when diet was removed from all beetles. A. Males were on diet throughout the duration of the aeration period. B. Males were starved before being treated with JHIII or acetone only. Food was returned to only the starved control beetles after the 2nd 24 hrs. Each bar represents the mean amount of DMD from 4 individuals with ± standard error. One-way ANOVA with completely randomized design ($P < 0.05$) was used for statistical analysis. Bars with the same letter are not significantly different from the others during the same time period.

Before the starvation period began, DMD was collected for a 24 hr period when the beetles were still on diet (Fig. 4.2B). Non-treated controls altogether produced more DMD than the other two treatments, but this difference was not significant. After removal of the diet (2nd 24 hrs), labeled on graph with a curved, downward pointing checkered arrow, all groups had a decrease in DMD production. Topical application of JHIII or acetone only (labeled on the graph as a tilted checkered lightning bolt) had no effect on DMD production. The control beetles, which had food returned, increased the amount of DMD during this 3rd 24 hr period. The 4th 24 hrs were similar to the 3rd 24 hrs for each treatment group. Similar to methoprene, JHIII was also not able to initiate DMD production.

4,8-dimethyldecanal production from fed and starved beetles injected with octopamine or tyramine

Injection of octopamine or tyramine into feeding beetles caused a significant reduction in the amount of DMD produced, however so did the injection of buffer only (Fig.4.3A). During the 4th 48 hr period (2nd 48hrs after injection) all groups of beetles produced similar amounts of DMD. This suggests that the handling and injection trauma causes the beetles to reduce DMD production, or the effect is negative and indistinguishable from that of injection.

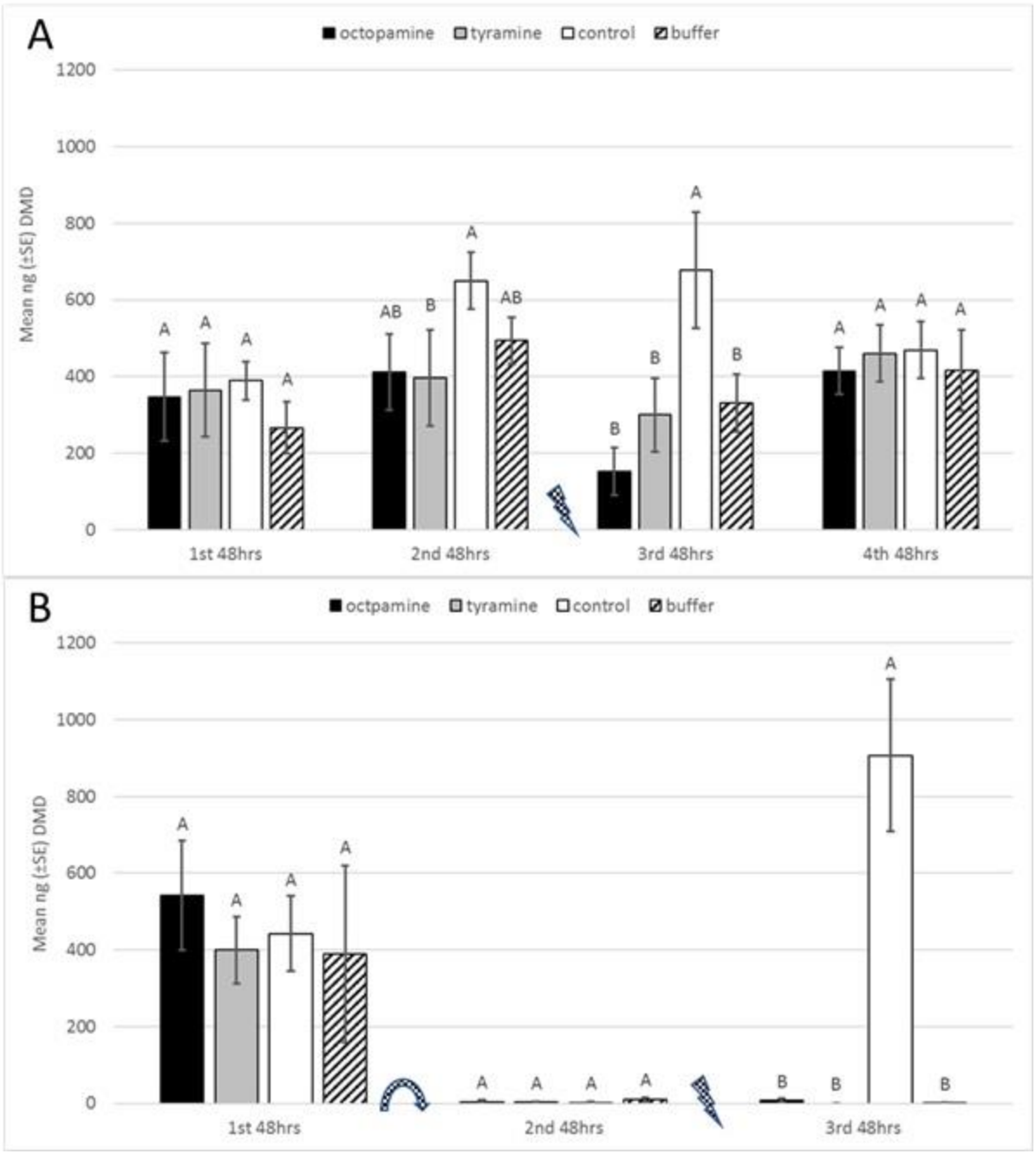

Figure 4.3. Effects of octopamine or tyramine on DMD production in fed and starved beetles.

Mean ng of DMD (±SE) produced by *T. castaneum* males injected with either octopamine, tyramine or buffer only. The small, checkered lightning bolt represents when the injection occurred, and for starved control beetles when diet was returned to the chamber. The small, curved downward arrow represents when diet was removed from all beetles. A. Males were on diet throughout the duration of the aeration period. B. Males were starved before being injected with octopamine, tyramine or buffer only. Food was returned to only the starved control beetles after the 2nd 48 hrs. Each bar represents the mean amount of DMD (±SE) from 6 individuals.

One-way ANOVA with completely randomized design ($P < 0.05$) was used for statistical analysis. Bars with the same letter are not significantly different from the others during the same time period.

Before the starvation period began, DMD was collected for a 48 hr period when the beetles were still on diet (Fig. 4.3B). Upon removal of the diet, all groups of beetles greatly reduced the amount of DMD compared to the previous 48 hrs. Return of diet to only the control beetles caused an increase of more than twice the amount of DMD compared to the 1st 48 hrs. The amount of DMD produced by the injection of either buffer, octopamine or tyramine groups was negligible. These results indicate that neither octopamine nor tyramine influence DMD production in *T. castaneum*.

4,8-dimethyldecanal production from fed and starved beetles injected with AKH1a or dopamine

Injection of AKH1a or buffer only into feeding beetles caused a significant reduction in the amount of DMD produced in the preceding 48 hrs compared to the non-injected controls. Injection of dopamine did not cause a significant reduction of DMD compared to that of the non-injected controls during the same time period (Fig. 4.4A). All treatment groups returned to similar output levels of DMD during the 4th 48 hr period.

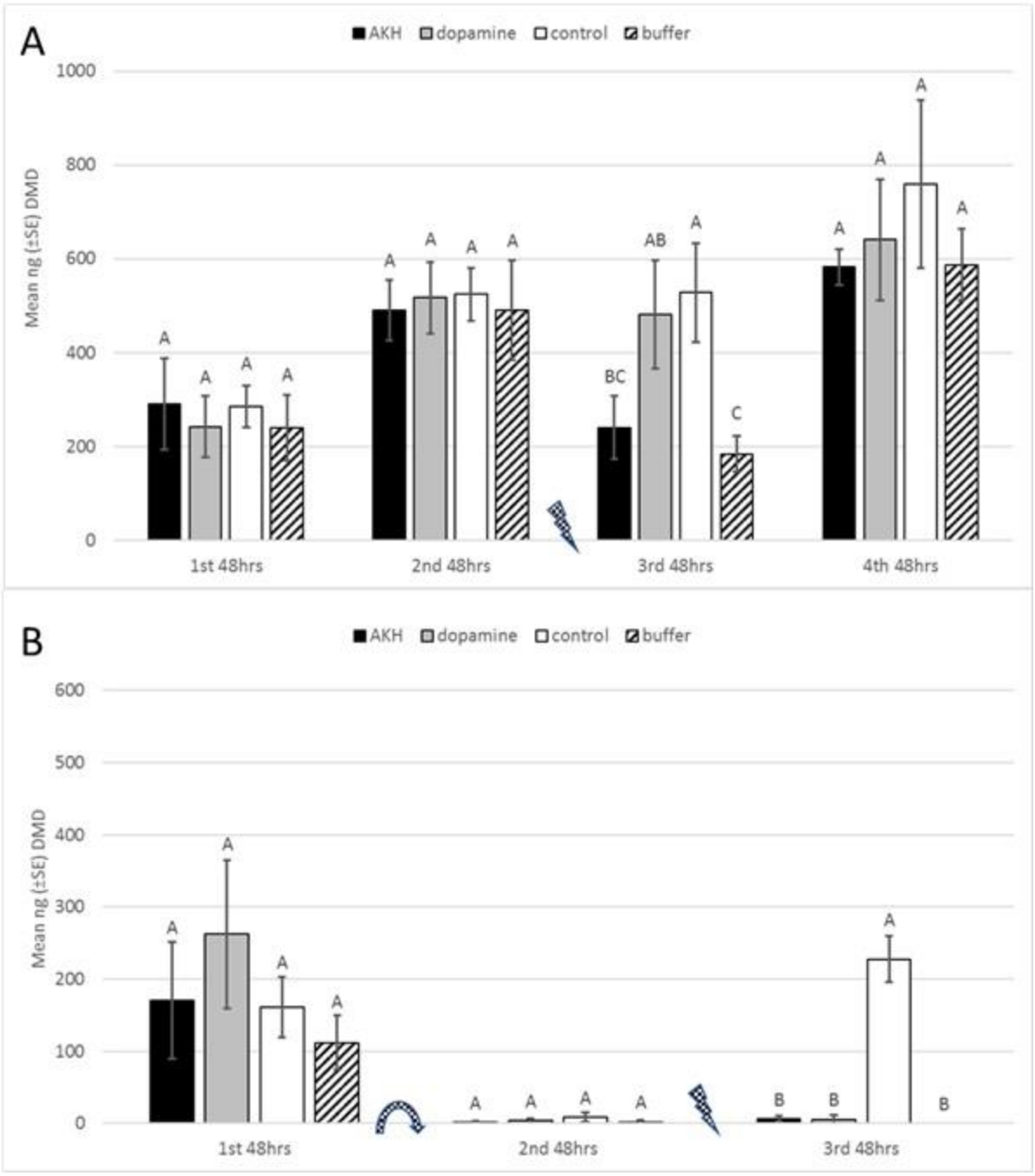

Figure 4.4. Effects of AKH1a or dopamine on DMD production in fed and starved beetles.

Mean ng of DMD (±SE) produced by *T. castaneum* males injected with either AKH1a, dopamine or buffer only. Small, checkered lightning bolt represents when the injection occurred, and for starved beetles when diet was returned to the chamber. The small, curved downward arrow represents when diet was removed from all beetles. A. Males were on diet throughout the duration of the aeration period. B. Males were starved before being injected with AKH1a, dopamine or buffer only. Food was returned to only the starved control beetles after the 2nd 48 hrs. Each bar represents the mean amount of DMD (±SE) from 6 individuals. One-way

ANOVA with completely randomized design ($P < 0.05$) was used for statistical analysis. Bars with the same letter are not significantly different from the others during the same time period.

Before the starvation period began, DMD was collected for a 48 hr period when the beetles were still on diet (Fig. 4.4B). Upon removal of the diet, all groups of beetles significantly reduced the amount of DMD compared to the previous 48 hrs. Return of diet to only the non-injected controls caused an increase of DMD similar to what was observed during the 1st 48 hrs. The injection of AKH1a or dopamine had no effect on DMD production in starved beetles.

Topical application of precocene I and JHIII on 4,8-dimethyldecanal production in fed beetles

The amount of DMD produced by feeding beetles that were either treated with a topical application of precocene I, JHIII or acetone only was not significantly different from that of the non-treated controls during any of the time points (Fig. 4.5). Although no significant difference in the amount of DMD produced was detected, precocene I treated beetles produced noticeably less DMD than the other treatment groups during the 4th 48 hr period.

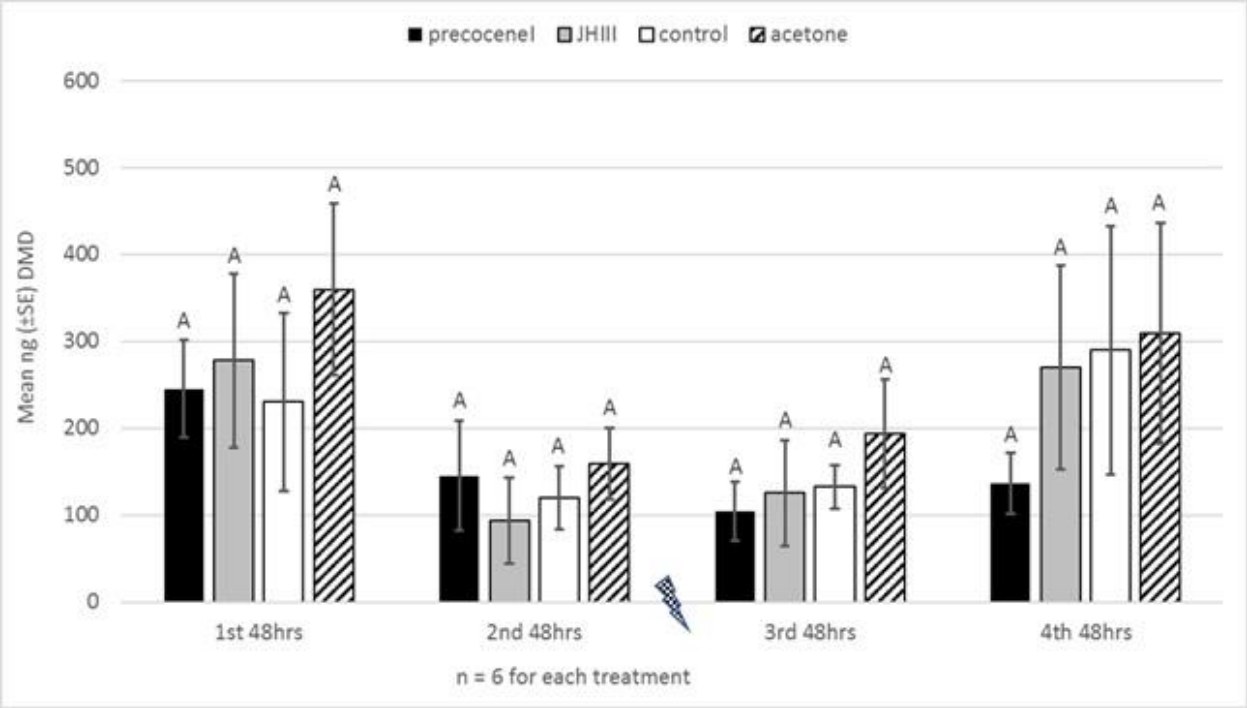

Figure 4.5. Effects of precocene I or JHIII on DMD production in fed beetles.

Mean ng of DMD (±SE) produced by *T. castaneum* males treated by topical application of precocene I, JHIII or acetone only. Small, checkered lightning bolt represents when the treatment occurred. Males were on diet throughout the duration of the aeration period. Each bar represents the mean amount of DMD (±SE) from 6 individuals. One-way ANOVA with completely randomized design ($P < 0.05$) was used for statistical analysis. Bars with the same letter are not significantly different from the others during the same time period.

4,8-dimethyldecanal production from starved beetles injected with 20-hydroxyecdysone or αEcdysone

Before the starvation period began, DMD was collected for a 48 hr period when the beetles were still on diet (Fig. 4.6). Upon removal of the diet, all groups of beetles greatly reduced the amount of DMD compared to the previous 48 hrs, with no significant difference in the level of reduction amongst the groups. Return of diet to the controls caused a mean increase of more than twice the amount of DMD than the 1st 48 hrs. Injection of 20HE, αE or buffer were not able to elicit DMD production in starved beetles.

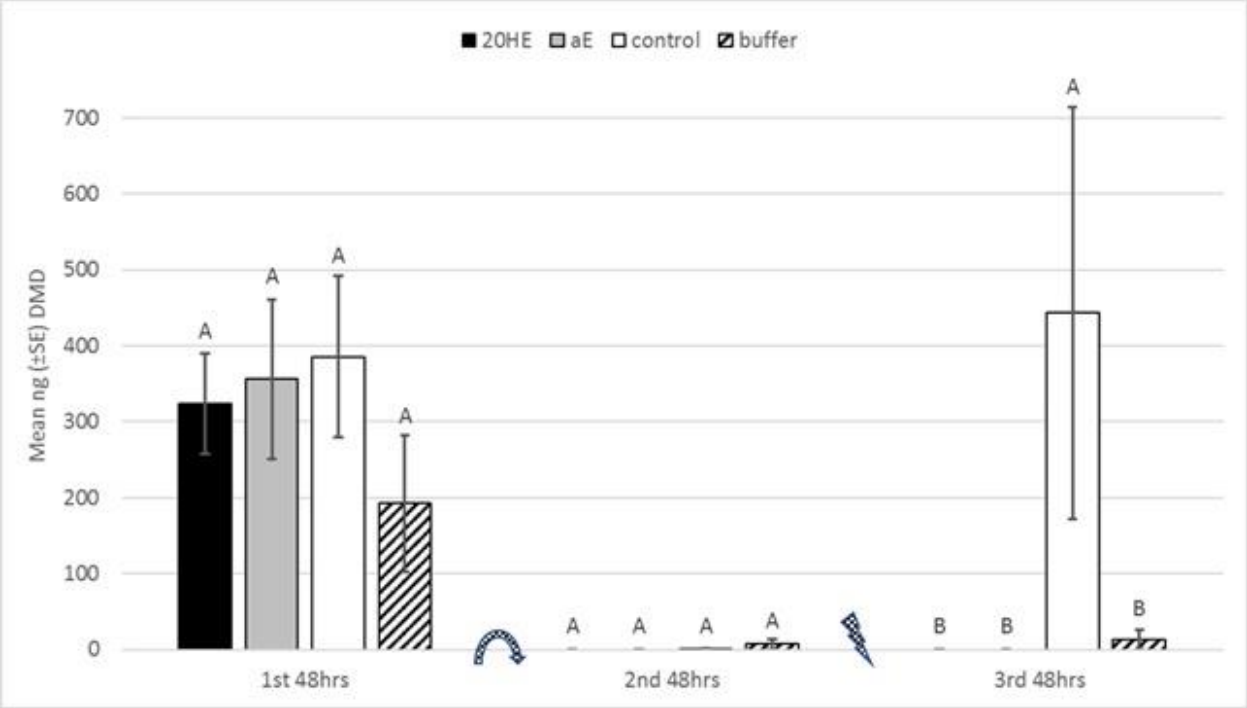

Figure 4.6. Effects of 20-hydroxyecdysone or ecdysone on DMD production in starved beetles.

Mean ng of DMD (±SE) produced by *T. castaneum* males injected with either 20HE, αE or buffer only. The small, checkered lightning bolt represents when the injection occurred, and when only the control beetles had diet returned to the chamber. The small, curved downward arrow represents when diet was removed from all beetles. Each bar represents the mean amount of DMD (±SE) from 6 individuals. One-way ANOVA with completely randomized design ($P < 0.05$) was used for statistical analysis. Bars with the same letter are not significantly different from the others during the same time period.

Effects of TcJHAMT (TC008471) RNAi on DMD production in feeding beetles

The function of JHIII on DMD production in feeding male beetles was further examined by injecting dsRNA of TcJHAMT or dsRNA buffer only into pupae which were >48 hrs from adult eclosion. This later time point was chosen so that pupal development would finish before the RNAi effects fully developed. After eclosion, the beetles were individually transferred to glass vials with diet and cracked wheat. Once the beetles were 10-12 days PAE they were placed into the aeration chambers for the initial adjustment period before DMD collection began. The beetles remained in the aeration chambers and DMD was quantified in the exact same manner as

chapter 3 (Fig. 4.7A). After the 3rd 48 hr aeration period (6th day) transcript levels from each beetle in the aeration setup were individually quantified to confirm the efficiency of RNAi with qPCR (see chapter 3 for methods) (Fig. 4.7B). The amount of DMD produced by TcJHAMT dsRNA injected beetles was less than the non-injected and buffer-injected beetles. However, DMD from RNAi beetles were only significantly different compared to the non-injected (Fig. 4.7A). The efficiency of the RNAi was very high. Beetles from each of the 3 treatments had significantly different levels of TcJHAMT transcript present amongst them (Fig. 4.7B). However, an almost complete suppression of TcJHMAT was not enough to eliminate DMD production in feeding males. These data suggest that the absence of endogenous levels of JHIII have a possible indirect effect on the production of DMD in feeding male *T. castaneum*.

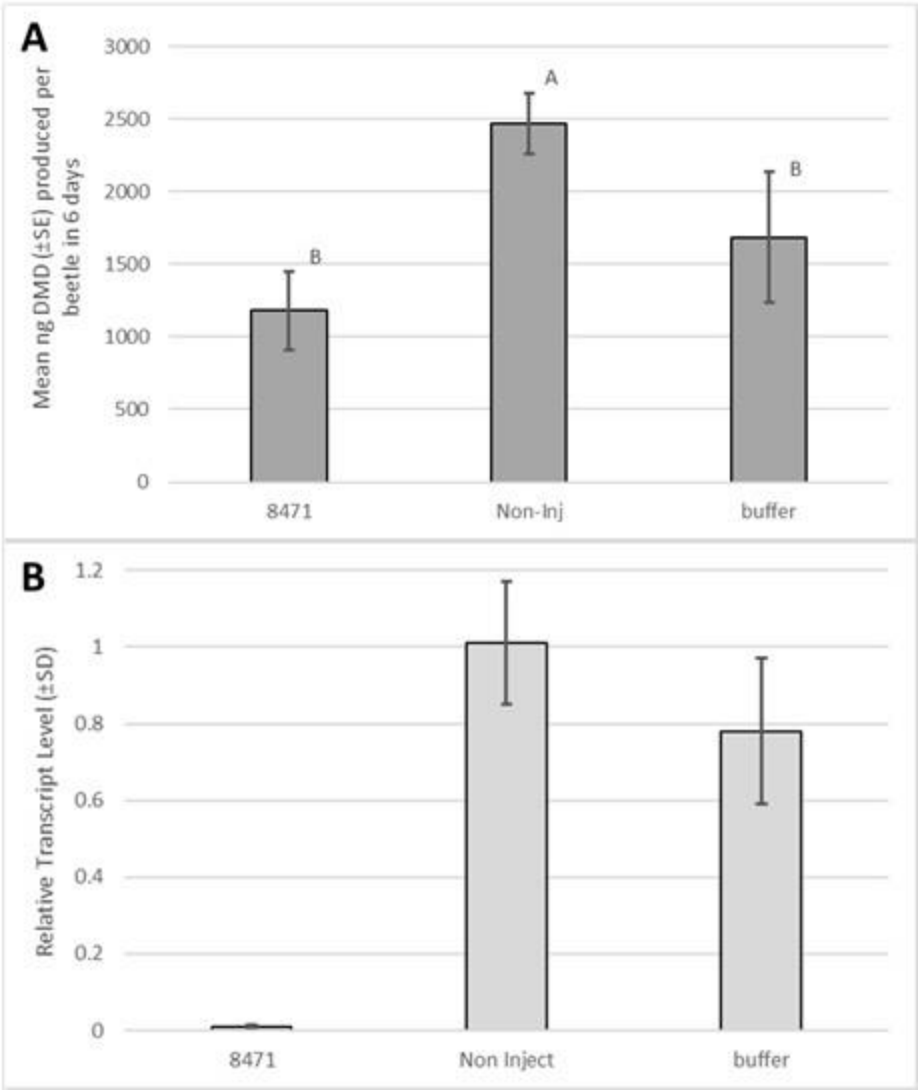

Figure 4.7. DMD produced from beetles injected with dsRNA targeting Tcjhamat and qPCR of suppression.

The mean amount of DMD (±SE) produced by beetles in 48 hrs that had been injected with dsRNA for TcJHAMT, dsRNA buffer, or non-injected. After the 6th day in aeration chambers beetles were removed and the transcript levels of 8471 were examined by real-time quantitative PCR (B). N = 6 for all groups in A (n = 6) for TcJHAMT, and n = 5 for non-inject and buffer injected. B. Transcript abundance of 8471 is relative to that of *T. castaneum rsp3*. One-way ANOVA with completely randomized design ($P < 0.05$) was used for statistical analysis. Bars with the same letter are not significantly different from the others.

Discussion

In this study, the effects of several hormones and endocrine factors were tested to determine if they had any effect on the release and/or production of the aggregation pheromone

4,8-dimethyldecanal (DMD) in male *T. castaneum*. Topical application of JHIII, Methoprene or the JH antagonist precocene I, had no effect on DMD production in individual male beetles that were either feeding or starved. These results contradict those from (Kim et al., 2005) and those from (Pierce et al., 1986). Kim et al. (2005) found that increasing amounts of JHIII resulted in decreasing amounts of DMD produced. Their DMD collection system was setup differently than the one described here. Additionally, in their setup, there were groups of multiple beetles present in each aeration chamber apparatus which could have contributed in some unknown way when increasing levels of exogenous JHIII were applied. Pierce et al., (1986) measured an increase in DMD production from beetles that were feeding on Methoprene treated oats for 20 days. Their DMD collection system was also different than the one used in this study. Additionally, the long term effect of methoprene consumption could promote effects not initiated with only one term high dose application. In *Tenebrio molitor* treatment of males with JHIII produced pheromones that females found more attractive than those not treated with JHIII (Rantala et al., 2003). Whether this increase was due to an increase of *T. molitor* pheromones, or a more attractive component, which might include different cuticular hydrocarbons, is not known. The results of the JHIII and methoprene assays also do not agree with those obtained from experiments with Scolytidae. Unfed *Ips pini* males produced more ipsdienol when treated with JHIII compared to untreated males (Lu, Fang, 1999). The dsRNA experiment targeting TcJHMAT (TC008741) did show a decrease in DMD production in injected beetles, this reduction was not significantly different to the beetles that were injected with buffer only. Also, topical treatment with the JH antagonist precocene I had no effect on DMD production. The global effect that juvenile hormone has on insects is likely to cause multiple physiological effects. Whether these effects are directly related to DMD production in *T. castaneum* seems unlikely.

The injection of either octopamine or tyramine was unable to increase the amount of DMD produced in feeding beetles or promote starved male beetles to produce any DMD. Multiple stressors, including starvation, increase the amount of octopamine and tyramine in larval *T. castaneum* (Hirashima et al., 1993). Some of these same stressors, namely starvation, caused extreme variation in the amount of non-DMD related semiochemicals released from adult *T. castaneum* (Sahu et al., 2023). The semiochemicals mainly identified were the benzoquinones which are produced in defensive glands in adult beetles as well as cuticular surface hydrocarbons. Additionally, Sahu et al. (2023) did not find any evidence of diet media affecting the overall amount of semiochemicals released.

Injection of dopamine into feeding T. castaneum males seemed to mask the effects of the physical trauma of the injection. The amount of DMD produced was the similar to that of the non-injected controls and significantly more than the buffer injected beetles. However the injection of dopamine into starving beetles was not able to force them into producing DMD.

The biogenic amines tested here, octopamine, tyramine and dopamine are likely constituents of a highly complex cocktail of pharmacological active compounds. None of which on their own seemed to be able to influence DMD production in *T. castaneum.* These compounds are likely modulated due to a response from a stimulus, whether positive or negative. The literature seems to suggest this as well. Most studies involve Lepidoptera. Due to the action of the pheromone biosynthesis activating neuropeptide (PBAN) in this insect order they are being neglected in this instance. There are many studies (excluding Lepidoptera) examining the properties of the three compounds tested here. In Hymenoptera, octopamine, tyramine and dopamine are associated with different behaviors and physiological processes (Beggs et al., 2007; Bloch et al., 2000; Dombroski et al., 2003; Friedman et al., 2018; Sasaki & Watanabe,

2022; Schulz & Robinson, 2001). In Diptera these biogenic amines also influence behavior in opposite ways (Saraswati et al., 2004) and be combined with pheromones to promote behavioral movements (Wicker-Thomas & Hamann, 2008). These results suggest that whatever (if any) signaling pathway is involved in the biosynthesis of DMD it is not likely strictly controlled by any of the biogenic amines tested here.

The function of AKH in insects is to mobilize the energy stored in the fat body. Injection of AKH1a into a feeding beetle (which would normally not need to use energy from fat) did significantly reduce the amount of DMD compared to the non-injected controls but was not significantly different than the buffer only injected beetles. Whether the trauma of the injection masked any true effect of AKH1a is not known.

The two ecdysones tested here, 20HE and αE, were only tested in starved beetles. This was due to them being the last compounds to be tested. Also 20HE is reported to lower food consumption in *T. castaneum* (Tatun et al., 2018, 2021) which might indirectly lower decrease DMD production causing a false positive. One neuroendocrine peptide that was not tested but theorized to decrease DMD production is sulfakinin (SK). Injection of synthetic SK into larvae of *T. castaneum* significantly reduced their food intake. Using dsRNA targeting the transcripts of SK and the SK receptor showed a dramatic increase in food consumption (Yu et al., 2013). Although these consumption assays were done in larvae, the SK peptide should have similar food intake regulatory properties in adult beetles. The SK peptide signaling system also cross-talks with the insulin system in *T. castaneum* (Lin et al., 2016). RNAi targeting the insulin-like receptor caused an increase in SK expression. It remains unknown if any neuropeptide signaling system is directly responsible for production and release of DMD, or if the hypothesized decrease in pheromone production is a due to the cessation of feeding. In chapter 3 one of the

candidate genes tested was an insulin-like peptide-7. There was no effect of DMD production with the suppression of this transcript.

The system used in this study works well but has its limitations. If one of the compounds tested here had an increase in DMD production on a feeding male followed by a recovery period of low DMD production, this setup would not be able to detect it. For example, the injection of AKH1a induced a large increase in DMD production (compared to non-injected controls) in the feeding beetles for the first 3-6 hrs which rapidly used all the available precursors, and then slowed down over the next 12 hrs, that increase would blend with the overall amount of DMD produced in the total 48 hrs. The natural variation of DMD production in each beetle over the course of the day also presents challenges in interpretating results. For example, the DMD measured from the topical application of JHIII to feeding males was lower than the non-treated controls and acetone only controls. However, the 1st and 2nd 24 hrs before the application of JHIII those beetles already showed a decreasing trend in DMD production. Interestingly adult T. castaneum that are wounded (injected with PBS) display increased amounts of methyl branched cuticular hydrocarbons (mbCHCs). This difference is also significantly different between sexes (Lo et al. 2023). In the injection experiments in this study it is possible that the wound generated from the injection of test compounds decreased the availability of DMD precursors. The reason for this increase of long chain mbCHCs is currently not known.

In this series of experiments, removing the diet from the beetles was the only action that consistently caused a reduction in DMD production. None of the compounds tested were able to induce DMD production in starved beetles other than the reintroduction of food. There is a large number of neuropeptides, endocrine factors and secondary messengers that were not tested. The production of DMD might not be strictly under the control of any endocrine-like compounds.

The biosynthetic pathway of DMD could be completely regulated by the availability of pheromone precursors directly obtained through the diet. As the name suggests, the function of aggregation pheromones is to signal other individuals to aggregate in a particular area. That could be interpreted as a signal for mating or a signal of the food having a quality suitable for sustaining the next generation of individuals. There is great potential for continuing research to uncover the mechanisms underlying the control of biosynthesis and release of DMD in male *T. castaneum*.

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