Genetic Contributions of *desatF* and *eloF* to Courtship Mating Behavior and Cuticular Hydrocarbon Production in *Drosophila simulans* and *D. sechellia*

BY

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Abstract

Sexual isolation occurs between *Drosophila simulans* and *D. sechellia* due to differences in cuticular hydrocarbon (CHC) productions. A wide variety of hydrocarbons are produced, but *D. simulans* males and females predominantly produce 7-tricosene (7-T), a 23 carbon monoene, while *D. sechellia* males produce 7-T and *D. sechellia* females produce 7,11-heptacosadiene (7,11-HD), a 27 carbon diene (Coyne et al. 1994). An asymmetric mating pattern occurs due to hydrocarbon differences: *D. simulans* males only court *D. simulans* females and *D. sechellia* males court both *D. simulans* and *D. sechellia* females (Cobb and Jallon 1990). Previous quantitative trait locus (QTL) studies (Gleason et al. 2005; Gleason et al. 2009) identified *desatF* and *eloF* as candidate genes contributing to production of *D. sechellia* pheromone 7,11-HD. In this thesis, the effect of *D. sechellia* alleles in a *D. simulans* background is measured for *desatF* and *eloF* by (1) monitoring mating behavior response through copulation success and latency and (2) identifying the differences in CHC biosynthesis through elongation and desaturation changes. Behavioral analyses indicated that there was no significant effect on courtship for the genes independently. Analysis of CHC production differences indicates a more pronounced effect of *desatF* and *eloF* on pheromone biosynthesis. In elongation from 23 to 25 carbons, females carrying the *eloF* gene produced increased amounts of 7-pentacosene, indicating *eloF* effects hydrocarbon elongation. Absence of further elongation to 27 carbons suggests involvement of other elongases for synthesis to 7,11-HD. Females carrying the *desatF* gene produced increased amounts of 7,11-pentacosadiene, indicating *desatF* is responsible for increasing dienes. Only when females carried *D. sechellia* alleles at both loci did production of 7,11-HD occur. However, the amount of 7,11-HD was significantly lower than amounts produced by *D. sechellia* females. Evidence from this study indicates *desatF* and *eloF* are genes

present in the hydrocarbon biosynthesis pathway and are probably necessary for female *D. sechellia* pheromone production. However, *D. sechellia* alleles of *desatF* and *eloF* are not sufficient for production of 7,11-HD indicating involvement of other biosynthesis genes to fully produce the *D. sechellia* female pheromone.

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Introduction

Reproductive isolation between populations, which may lead to speciation (Dobzhansky 1951; Mayr 1963), can occur through premating or postmating mechanisms. Although postmating mechanisms have been studied, few genes affecting premating isolation have been identified (Noor 2003; Orr et al. 2004; Noor and Feder 2007). Mechanisms of premating isolation can play an important role in the behavior of males and females during courtship especially in the areas of mate choice and species recognition.

The causes of sexual isolation can be identified though investigation of where breakdown in courtship occurs and how species identify individuals. Courtship includes multiple sensory modalities and many specific steps that must be completed for copulation to occur. Each stage in the courtship ritual provides significant cues that detail how an individual should proceed. These cues can be visual, acoustic or chemical. During courtship, individuals send and receive many different signals that allow them to make choices about mates that will be the most successful based on species recognition and compatibility (Antony 1982; Greenspan and Ferveur 2000; Ejima and Griffith 2008). Signals received can identify individuals of a similar species and give clues to the receptively of that individual based on sexual maturity and mating status (Hebets and Papaj 2005). These signals allow a male or female to make a choice about whether or not they should pursue courtship with this other individual.

Courtship uses many complex signals involving multiple modalities for interpretation and identification of species (Bennet-Clark et al. 1974; Cobb and Jallon 1990; Ejima and Griffith 2008; Gleason et al. Submitted). One modality especially important to courtship is the detection of chemosensory signals (Antony 1982; Cobb and Jallon 1990; Coyne et al. 1994; Coyne and Charlesworth 1997; Grillet et al. 2006). Pheromones are a type of chemosensory signal and

changes to pheromone signals or the gustatory receptors that receive them can result in discrimination between species. Changes to pheromones that identify species can occur due to minor modifications of genes. For example, in *Drosophila melanogaster* and *D. simulans*, pheromone production in *D. melanogaster* females is caused by expression of two genes that are inactive in *D. simulans* females (Chertemps et al. 2006; Chertemps et al. 2007; Legendre et al. 2008; Shirangi et al. 2009). Identifying the genetic changes involved in reproductive isolation can aid in understanding how speciation occurs and how specific isolation mechanisms, such as pheromones, prevent successful courtship and copulation among species of *Drosophila*.

Courtship in *Drosophila*

In *Drosophila*, courtship behaviors have been extensively studied (Spieth 1974; Stocker and Gendre 1989; Tomaru et al. 1998; Tauber and Eberl 2002) and in *Drosophila melanogaster* there are five basic steps that the males and females must go through for copulation to occur (Spieth 1952, 1968, 1974). To start the courtship ritual, a male will approach a female and orient towards her. Once this visual cue is received, the male will walk up to the female and tap her abdomen with his forelegs. This process allows the male to pick up on tactile and gustatory signals from the female. The gustatory receptors located on the forelegs pick up on chemical cues, such as cuticular hydrocarbons, which are present on the female and allow the male to "taste" the female (Cobb and Jallon 1990; Howard et al. 2003; Ferveur 2005). This step of the courtship ritual allows the male to determine specific cues about the individual with whom he is interacting: the individual's sex, species and mating status. Once a female of the correct species and mating status is located, the male will produce an acoustic signal by vibrating his wings to produce a species-specific courtship song directed towards the female (Kyriacou and Hall 1982;

Ritchie and Phillips 1998; Ritchie et al. 1999). If the male and female accept each other as mates then copulation will occur. However, if either individual rejects the other, the steps of this courtship ritual are repeated until successful copulation occurs or courtship breaks off entirely. *Drosophila melanogaster, D. simulans*, and *D. sechellia* are closely related members of the *melanogaster* subgroup in the subgenus *Sophophora*. *D. simulans* and *D. sechellia* use the same courtship ritual outlined above for *D. melanogaster*, but the two former species adhere to an asymmetric mating pattern. *D. simulans* are sexually monomorphic and males will only mate with *D. simulans* females, but *D. sechellia* are sexually dimorphic and males will mate with both *D. simulans* and *D. sechellia* females (Cobb and Jallon 1990).

Detection of Signals in Courtship

Many species of *Drosophila* use different methods for identifying individuals of compatible species from non-compatible ones. In the different subgroups of *Drosophila*, the types of signals important to courtship vary (Cobb and Jallon 1990; Coyne et al. 1994; Gleason and Ritchie 2004; Ferveur 2005; Gleason et al. Submitted). Species within a group can require the detection of single or multiple signals in order to identify other individuals of their species. Signals can range from differences in courtship song, where the length of the interpulse interval varies (Tomaru et al. 1995; Ritchie et al. 1998), to the production of pheromones (Coyne et al. 1994; Howard et al. 2003; Ferveur 2005), where chemical cues can vary between sexes or species. Each sex many use similar senses to identify potential mates or they may use different signaling pathways. For example, *D. nebulosa* males rely on sight while females primarily use smell to correctly identify a potential mate (Gleason et al. Submitted). For *D. simulans* and *D. sechellia*, a breakdown in courtship process occurs at the tapping stage of the courtship ritual

(Cobb and Jallon 1990) due to differences in pheromone production. The male picks up on gustatory signals from the female caused by the presence of specific hydrocarbons located on the cuticle layer of the female's abdomen.

Cuticular Hydrocarbons in *Drosophila simulans* and *Drosophila sechellia*

Cuticular hydrocarbons (CHC) are long carbon chains produced in the oenocytes that function as contact pheromones. In *D. simulans* and *D. sechellia* many different CHC are produced that give important chemical messages to neighboring flies and potential mates. Even though there are an abundance of CHC present on each fly, two main CHC produced in *D. simulans* and *D. sechellia* play a role in mating isolation between the two species: 7-tricosene (7-T) and 7,11-heptacosadiene (7,11-HD). 7-T is a monoene with 23 carbons in length and one double bond at the 7th carbon position. 7,11-HD is a diene with 27 carbons in length and two double bonds at the 7th and 11th carbon positions. Each of the species produces a different profile of CHC and the main pheromones produced by each sex vary (Cobb and Jallon 1990). *D. simulans* males and females produce the same pheromone while *D. sechellia* males and females produce significantly different profiles. Males of *D. sechellia* and males and females of *D. simulans* predominantly produce the shorter, more saturated CHC 7-T. *D. sechellia* females predominantly produce the longer, less saturated CHC 7,11-HD (Cobb and Jallon 1990).

CHC Production and Effect on Mating Pattern of *D. simulans* and *D. sechellia*

Differences in CHC expression patterns lead to the asymmetric mating pattern found between *D. simulans* and *D. sechellia* because 7,11-HD adversely affects mating. In pheromone transfer experiments (Coyne et al. 1994; Coyne 1996; Coyne and Charlesworth 1997), when a

single *D. simulans* female, which produces 7-T, is crowded with *D. sechellia* females, which produce 7,11-HD, the cuticular hydrocarbons on the *D. sechellia* females rub off onto the *D. simulans* female making the latter resemble the former through smell. When courtship tests with *D. simulans* males follow this transfer protocol, the adverse effect of the *D. sechellia* pheromone is revealed (Coyne et al. 1994). Even though *D. simulans* males will normally court and mate with *D. simulans* females, the addition of the 7,11-HD pheromone through external transfer decreases the attraction of the males towards the females. The same courtship aversion behavior occurs when topical application of 7,11-HD is applied to *D. melanogaster* females that lack oenocyte cells, the site of pheromone production (Billeter et al. 2009). The knockdown of oenocyte cells (*oe-*) in *D. melanogaster* females eliminates production of 7,11-HD, thereby increasing the likelihood of courtship by *D. simulans* males. However, if a topical application of 7,11-HD is applied to *oe-* females; courtship with *D. simulans* males is again adversely affected. Thus, 7,11-HD is an anti-aphrodisiac to *D. simulans* males, which do not court females that produce this CHC.

Candidate Genes for Cuticular Hydrocarbon Expression

Previously a quantitative trait locus (QTL) study was performed to identify genomic regions affecting the amount of CHC production that differs between *D. simulans* and *D. sechellia* (Gleason et al. 2005; Gleason et al. 2009). Crossing *D. simulans* females with *D. sechellia* males produced hybrid F1 individuals. The F1 female offspring were then backcrossed to a *D. simulans* male to produce the F2 individuals used in the mapping population for the QTL study. The CHC from the F2 offspring of this backcross were extracted using hexane and then each individual was DNA genotyped for 46 markers that spanned the three chromosomes. After

the CHC extractions were analyzed using gas chromatography, a genetic linkage map was assembled (Figure 1).

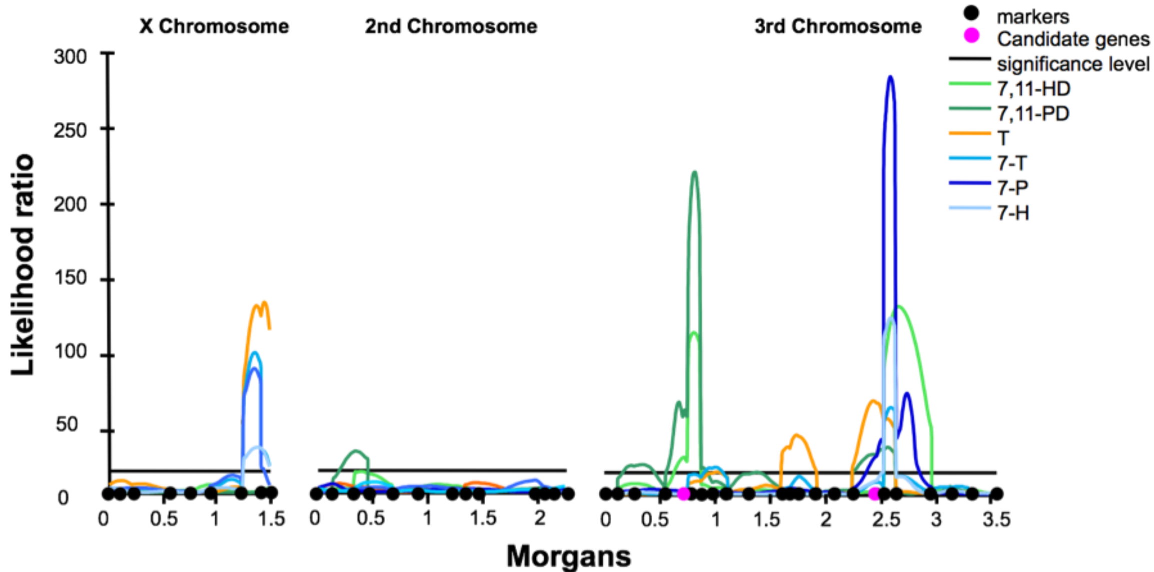

Figure 1. **QTL Map of CHCs that differ between *D. simulans* and *D. sechellia*** (from Gleason et al. 2009). Three major QTL are present: one on the X chromosome and two on the third chromosome.

For the six most abundant CHC present in the backcrossed flies, three major QTLs are present across the chromosomes. One is located on the X chromosome and two are present on the third chromosome: one on the right arm and the other on the left. Genes involved in CHC biosynthesis pathway of *D. melanogaster*, specifically the desaturation and elongation process, are located under the two QTLs on the third chromosome. Of the potential biosynthesis genes, *desatF* and *eloF* were identified as candidates effecting desaturation and elongation of CHC, respectively. Without any obvious candidate genes on the X chromosome, we decided to focus only on the QTLs identified in the third chromosome.

The relationship between ratios of CHC and the marker genes along the third chromosome was used to identify possible QTL effecting the elongation and desaturation changes between the species (Gleason et al. 2009). Mapping the ratio of 7, 11-pentacosadiene

(7, 11-PD) to 7-pentacosene (7-P), which shows the effect of desaturation by comparing the amount of 25-dienes to 25-monoenes, produced two QTLs. The area under one of these peaks colocalizes with the desaturation gene *desatF*. Mapping the ratio of 7-P to 7-tricosene (7-T), which shows the effect of elongation by comparing the amount of 25 carbon to 23 carbon compounds, produced one major QTL. The area under this peak corresponds to a cluster of elongation genes including *eloF*.

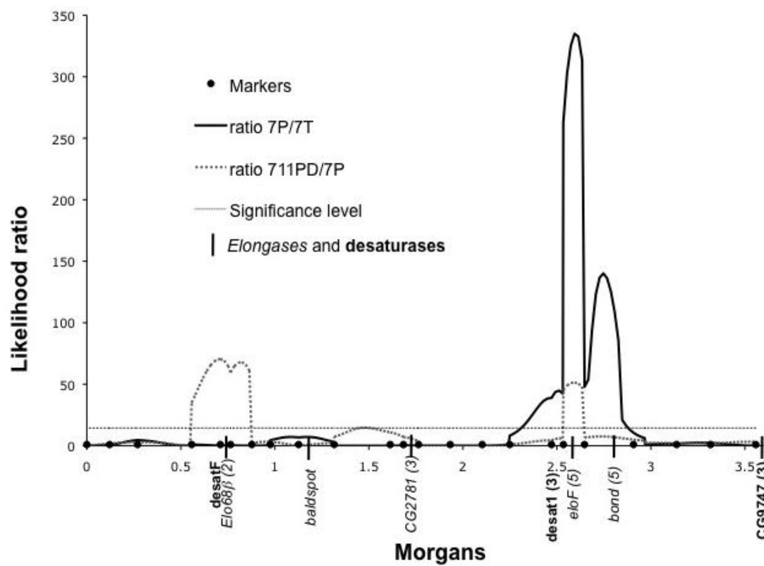

Figure 2. **QTL map of CHC ratios showing effects of elongation and desaturation**. Ratios corresponding to elongation are shown in bold and ratios showing desaturation are shown as a dashed line (Gleason et al. 2009). Markers that are named represent the CHC biosynthesis genes involved in elongation and desaturation located on the third chromosome.

Epistatic Effect of *D. sechellia* Alleles on Cuticular Hydrocarbon Production

The two candidate genes, *desatF* and *eloF*, are involved in producing 7,11-HD in *D. melanogaster* females; this is the same predominant CHC found in female *D. sechellia*. In the QTL study that aimed to identify regions contributing to pheromone production (Gleason et al. 2009), *desatF* was one of the original markers used to make the genetic linkage map while *eloF* was not included. To study the relationship between the two QTL that identified the candidate

genes *desatF* and *eloF*, markers *Sod* and *Metallothionein A* (*MtnA*) were used because they were located closest to the right and left QTL, respectively, on the third chromosome. Each of the loci individually has a slight effect on 7,11-HD production, but there is an epistatic effect between the two QTL, as shown in Figure 3 (Gleason et al. 2009). With both QTL present together as *D. sechellia* alleles, the female *D. sechellia* pheromone is produced. By identifying the genotypes of the markers *Sod* and *MtnA* in the backcross individuals from the QTL study (Gleason et al. 2009), the effect of these loci can be determined. If both markers, *Sod* and *MtnA*, carry the *D. simulans* genotype or if the individual is heterozygous for one marker only, only a small amount of 7,11-HD is produced (Figure 3). The heterozygous expression of one marker will only slightly raise the amount of 7,11-HD produced compared to *D. simulans*. However, when both markers have a heterozygous allelic state the amount of 7,11-HD increases substantially.

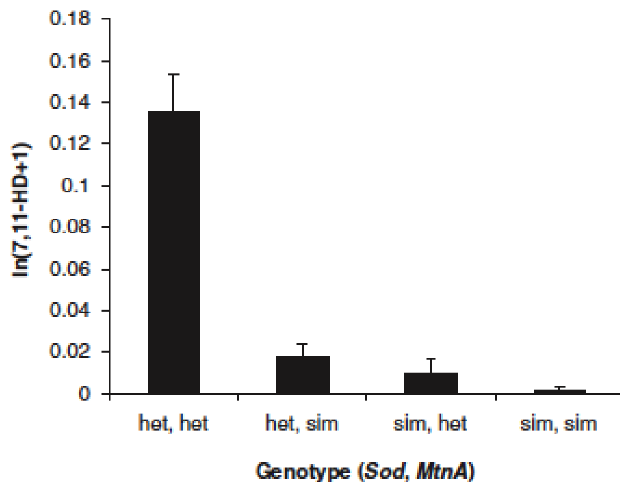

Figure 3. **Epistatic interaction between *D. sechellia* and *D. simulans* in the production of 7, 11-heptaocosadiene**. The amount of 7,11-HD is given for each allelic state at the loci for *Sod* and *Metallothionein A* (*MtnA*) in the individuals from the QTL study (from Gleason *et al.*, 2009).

Biosynthesis of Major Cuticular Hydrocarbons

The genes, *desatF* and *eloF*, were identified as possible candidate genes affecting the differential expression of CHC due to their function in *D. melanogaster*, a closely related species of *D. simulans* and *D. sechellia*. As seen in Figure 4, *desatF* and *eloF* are essential components of the pathway producing different forms of CHCs in the oenocytes.

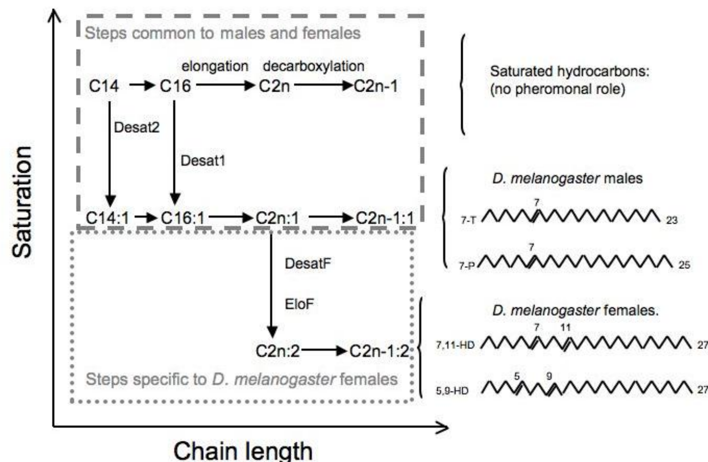

Figure 4. **Biosynthesis pathway of CHC in *D. melanogaster*** (Legendre et al. 2008). The syntheses of the CHC originate from a common precursor and are differentially expressed between the sexes.

For the synthesis of the major pheromones present in *D. melanogaster* males (7-T) and *D. melanogaster* females (7,11-HD), the CHC originate from a common precursor fatty acid, myristic acid (C14), a 14 carbon unsaturated fatty acid. At this stage the fatty acid goes through a series of desaturation and elongation reactions to increase the number of double bonds and increase the length of the hydrocarbon, respectively. The interaction of *desat2* with C14 will cause a desaturation event and produce the fatty acid myristoleic acid. After multiple elongation events, the fatty acid will reach the required length of carbons. At this point, synthesis divides between the steps necessary to make the male or female pheromone. The male CHC is elongated to a 24 carbon pheromone with one double bond. Then through a decarboxylation step, 7-T is

produced. For females, the CHC must go through an additional desaturation and elongation event driven by the interaction of the fatty acid with the feminine expressed genes *desatF* and *eloF*, respectively. With these two events the fatty acid becomes 28 carbons long with two double bonds. After a final decarboxylation step, one carbon is lost and the female pheromone, 7,11-HD is produced (Legendre et al. 2008).

Goals of this thesis

A QTL study was performed to identify genomic regions affecting production levels for CHC that differ between *D. simulans* and *D. sechellia* (Gleason et al. 2005; Gleason et al. 2009). Evidence from the study points towards *desatF* and *eloF* as potential candidate genes for the production of 7,11-HD in *D. sechellia* females. To test the hypothesis that these candidate genes are responsible for 7,11-HD production, this study measures the effect of replacing *desatF* and *eloF* in *D. simulans* with the *D. sechellia* alleles. The effect of *D. sechellia* alleles for *desatF* and *eloF* is measured using two methods: (1) monitoring the mating behavior response through copulation success and courtship latency and (2) identifying the role of *desatF* and *eloF* in CHC biosynthesis by identifying elongation and desaturation changes that the *D. sechellia* alleles make to the hydrocarbon profile.

Effect on mating behavior

Levels of courtship between *D. simulans* males and females with introgression of *D. sechellia* alleles in a *D. simulans* background could have a slight reduction from courtship levels observed between *D. simulans* males and *D. simulans* females because of changes in CHC production. Desaturation and elongation events occurring due to *D. sechellia* alleles of *desatF*

and *eloF*, respectively, can alter the type of CHC produced. Evidence from the QTL study (Gleason et al. 2009) implies that these candidate genes are active in CHC production in *D. sechellia* because *desatF* and *eloF* map to the genomic regions that correlate with 7,11-HD production and these genes are directly linked to 7,11-HD synthesis in the closely related species, *D. melanogaster* (Legendre et al. 2008). Thus, the presence of *D. sechellia* alleles of these genes should increase the number of longer, two double bond compounds in these species. Any female carrying *D. sechellia* alleles for *desatF* and *eloF* will have altered CHC production that causes *D. simulans* males to not court them. The decrease in copulation will occur due to production of 7,11-HD, the anti-aphrodisiac pheromone for *D. simulans*. However, copulation will only decrease when the female carries homozygous *D. sechellia* alleles for both *desatF* and *eloF* because of the epistatic interaction identified between the loci containing these genes (Gleason et al. 2009).

Inclusion of single *D. sechellia* alleles into a *D. simulans* background will not produce enough change to the hydrocarbon profile to completely disrupt the number of copulation events between *D. simulans* males and altered females because these females lack one of the genes necessary to produce the *D. sechellia* pheromone. However, courtship latency could increase due to desaturation or elongation changes affecting accurate identification of the chemosensory signal by *D. simulans* males. A change in copulation success and latency could occur between *D. simulans* males and females carrying heterozygous alleles for both *desatF* and *eloF*. The strength of this response depends on the dosage dependence of the *D. sechellia* alleles for production of 7,11-HD. If a hybrid allelic state is enough to produce 7,11-HD then copulation will decrease while copulation latency increases, but if a homozygous *D. sechellia* state for *desatF* and *eloF* is required to produce 7,11-HD then copulation and latency will remain

relatively the same as levels of *D. simulans* males with *D. simulans* females. If these genes are not sufficient to produce 7,11-HD then copulation of *D. simulans* males with the altered females will be similar to levels observed with *D. simulans* females.

Effect on CHC production

The effects on these candidate genes on copulation are mainly due to the differences they provoke in CHC production. The types of hydrocarbons produced by a female are controlled by the allelic states of the CHC genes. As described above, genes *desatF* and *eloF* are likely candidates for hydrocarbon synthesis due to their similar roles in *D. melanogaster* for the production of 7,11-HD. If only one of the candidate genes is expressed as a *D. sechellia* allele while the other remains in a *D. simulans* allelic state, the hydrocarbon profile will change slightly, but remain more like *D. simulans* than *D. sechellia*. Each of the candidate genes has a different effect on the type of hydrocarbons synthesized when individually expressed as a *D. sechellia* allele. Presence of the *D. sechellia* allele of the desaturase gene, *desatF*, decreases saturation levels of hydrocarbons thereby increasing the number of diene hydrocarbons while decreasing the monoenes. The *D. sechellia* allele for the elongase gene, *eloF*, increases the chain length of the hydrocarbon from the *D. simulans* length of 23 carbons to the 27 carbon length more prominent in *D. sechellia* females.

A homozygous *D. sechellia* allele for *desatF* expressed with a homozygous *D. simulans* allele for *eloF* will increase the number of double bonds present in the hydrocarbon, but will not increase CHC length to the 27 carbons present in *D. sechellia* females. If the genotypes were reversed with *D. simulans* allele for *desatF* and *D. sechellia* allele for *eloF*, the chain length increases while the saturation levels remain the same. *D. sechellia* allelic states for the two

genes independent of one another can produce measureable differences in hydrocarbon production, as predicted above, but, these changes would not be strong enough to reduce the mating response of *D. simulans* males with altered females. Because of epistatic effects between the QTL containing these candidate genes, only when *desatF* and *eloF* are expressed together as homozygous *D. sechellia* alleles will the production of the *D. sechellia* female pheromone, 7,11-HD, occur.

The predicted behavioral response of *D. simulans* males towards altered females coupled with the change in hydrocarbon production indicates the possible importance of *desatF* and *eloF* in *D. sechellia* CHC production. If the predicted candidate genes are responsible for production of *D. sechellia* female pheromone, 7,11-HD, a major pheromone profile change will only occur when an altered *D. simulans* female carries homozygous *D. sechellia* alleles of *desatF* and *eloF*. Females producing the 7,11-HD pheromone will show a decrease in copulation by *D. simulans* males. If these genes are not sufficient to produce 7,11-HD or if there are other genes involved in CHC biosynthesis, homozygous *D. sechellia* expression of *desatF* and *eloF* will not produce enough 7,11-HD to observe a change in copulation by *D. simulans* males.

Materials and Methods

Fly Lines

Flies were maintained in a 25 x 95 mm polystyrene vial with 9 mL of standard cornmeal-molasses media at 25° C on a 12:12 light-dark cycle. Three different species stocks were used in these experiments. For wildtype *D. simulans,* we used strain 14021-0251.169 (hereafter sim169) from the UC San Diego Species Stock Center. Also used was a line of *D. simulans* (hereafter simF2) with one recessive marker per chromosome arm including *forked* (f^2), *net* (*n*t), *plum* (*pm*), *scarlet* (*st*), and *ebony* (*e*). For wildtype *D. sechellia* (hereafter sec)*,* we used the David 4A line. Both of these latter two lines were used in previous investigations of the genetics of species differences (Gleason and Ritchie 2004; Gleason et al. 2005; Gleason et al. 2009).

In addition to the species stocks, we used a set of recombinant inbred lines (RILs) that had been developed between *D. simulans* and *D. sechellia.* These lines have a *D. simulans* background with random insertions of *D. sechellia* genes throughout the genome (J. M. Gleason, personal communication). In order to produce the RILs, a simF2 female was first crossed with a sec male. The F1 offspring are thus hybrids of *D. sechellia* and *D. simulans*. Because the males of this cross are sterile, only the F1 females were used to continue the line. The F1 hybrid female was backcrossed to a simF2 male. This produced F2 offspring that are genetically different due to random recombination events that occurred during mating. The F2 offspring were brother-sister crossed for 20 generations with selection against the recessive phenotypes of the simF2 line. Selection against the recessive phenotypes was performed to maintain as much of the *D. sechellia* genome as possible. Because the F1 hybrid females were backcrossed to

D. simulans, the resulting lines have a small amount of *D. sechellia* in a predominantly *D. simulans* background. Each line contains individuals that are genetically identical to each other, but every line is genetically different from the other lines.

All of the RILs were genotyped for the presence of the two candidate genes: *desatF* and *eloF*. We found four lines had *D. sechellia* alleles of *desatF*: G101A, M103J, M128J, M132A. Two lines had the *D. sechellia* alleles of *eloF*: M10J, S119. Due to the manner in which the RILs are produced by introgression of *D. sechellia* alleles into a *D. simulans* genome there are other genes within the background that have the *D. sechellia* genotype. These lines are not purely *D. simulans* with replacement only at the *desatF* or *eloF* genes by *D. sechellia* alleles. We decided to focus on the *desatF* line M128J and the *eloF* lines M10J and S119 because these lines were healthier and produced more viable offspring than the other lines at the beginning of the project.

Producing Individuals Containing *D. sechellia* alleles of both *desatF* and *eloF*

We attempted to develop a line homozygous for the *D. sechellia* alleles at both *desatF* and *eloF*. We first crossed five females from a *desatF* line with five males from an *eloF* line, along with the reciprocal cross. The crosses reproduced for seven days at which time the parental flies were cleared. Roughly ten days after the cross was set up the hybrid offspring began to eclose. The hybrid crosses between parental *desatF* and *eloF* lines created offspring that were heterozygous for both candidate genes. The hybrid offspring were sibling mated for seven days. Because *Drosophila* males do not recombine we decided to make one locus at a time homozygous. F2 individuals that were homozygous for the *D. sechellia* allele of *eloF* (or *desatF*) and heterozygous at *desatF* (or

eloF) were crossed with each other. The F3 offspring were wing genotyped (Gleason et al. 2004) as described below. Individuals that were homozygous *D. sechellia* at both *eloF* and *desatF* were used in subsequent cuticular hydrocarbon experiments.

Origin and Genotype of All Individuals Used in Courtship and CHC Experiments

Individuals with homozygous *D. sechellia* alleles at the loci for *desatF* and *eloF* originated from the six original lines: sim169, simF2, M10J, S119, M128J and sec. For a description of the genotype, origins and abbreviation of each of the individuals used in all courtship and CHC experiments, see Table 1 below. The *D. simulans* individuals came from vials of sim169 and simF2 that were maintained by standardizing the number of parents to control for the size and number of offspring. The *D. sechellia* individuals, who came from vials of sec, were maintained in the same manner as the *D. simulans* individuals. The individuals with a *D. sechellia* allele for *eloF* and a *D. simulans* allele for *desatF* came from crosses of the RILs M10J or S119. The individuals with a *D. sechellia* allele for *desatF* and a *D. simulans* allele for *eloF* came from crosses of the RIL M128J. The genotypes for the RILs M10J, S119 and M128J are individually specified at the loci for *eloF* and *desatF*, but due to the manner in which the RILs were created, through random recombination events, there are other portions of the genome that carry *D. sechellia* alleles; however, the majority of the genome is *D. simulans*. Each of the hybrid individuals comes from a cross between a *desatF* line and an *eloF* line (Table 1). The species hybrid differs from the previous four hybrids because the former is a cross between a sim169 female and a sec male that produces individuals heterozygous for all

loci across the genome. The latter hybrids are only specified heterozygous between the *D. simulans* and *D. sechellia* alleles for the *desatF* and *eloF* loci.

To produce individuals homozygous *D. sechellia* at both *desatF* and *eloF*, an intermediate cross was needed with individuals that were homozygous *D. sechellia* at *desatF* and heterozygous at *eloF* (D2E1) or heterozygous at *desatF* and homozygous *D. sechellia* at *eloF* (D1E2). The D2E1 and D1E2 individuals were made through crosses between females from hybrid 1 with males from hybrid 1. Other crosses were made between respective hybrid males and females as seen in Table 1. The final cross to create the individuals homozygous *D. sechellia* at both the *desatF* and *eloF* loci (D2E2) was made between D1E2 males and females. The D1E2 individuals used in the D2E2 cross originated from a cross between hybrid 1 individuals only because individuals in this cross were the healthiest and produced offspring while other crosses between hybrids were not as viable.

Table 1. Genotype, origin and abbreviation for all individuals used in copulation and CHC tests.

Name	Type of Individual	Genotype *desatF*	Genotype *eloF*	Predominant Genetic Background	Line(s) used to produce group
sim169	*D. simulans*	*sim*	*sim*	*sim*	sim169
simF2					simF2
eloF 1	eloF	*sim*	*sec*	*sim*	M10J
eloF 2					S119
desatF	desatF	*sec*	*sim*	*sim*	M128J
hybrid 1	hybrid	het	het	*sim*	M10J x M128J
hybrid 2					M128J x M10J
hybrid 3					M128J x S119
hybrid 4					S119 x M128J
species hybrid	species hybrid	het	het	het	sim169 x sec
D2E1	*desatF* locus *D. sechellia* homozygous *eloF* locus heterozygous	*sec*	het	*sim*	hybrid 1 x hybrid 1
					hybrid 3 x hybrid 3
					hybrid 4 x hybrid 4
D1E2	*eloF* locus *D. sechellia* homozygous *desatF* locus heterozygous	het	*sec*	*sim*	hybrid 1 x hybrid 1
					hybrid 2 x hybrid 2
					hybrid 4 x hybrid 4
D2E2	both loci homozygous	*sec*	*sec*	*sim*	D1E2 x D1E2 D1E2 individuals originated from a hybrid 1 cross
sec	*D. sechellia*	*sec*	*sec*	*sec*	sec

sim = *D. simulans*, *sec* = *D. sechellia*, het = heterozygous
In D2E1, D1E2 and D2E2 individuals: D = *desatF*, E = *eloF*, 1 = heterozygous allele between *D. simulans* and *D. sechellia*, 2 = homozygous *D. sechellia* allele

Wing Genotyping of All Individuals for *desatF* and *eloF*

Genotyping of individuals was done by extraction of DNA from wings (Gleason et al. 2004). Wing genotyping was used before phenotypic analysis or crosses to avoid using many individuals of genotypes that were not of interest. To collect a wing for DNA extraction, flies were anesthetized under CO_2 within six hours of eclosion. Using

forceps, one wing per individual was removed as close to the body as possible. Flies were housed individually in a 16.5 x 95 mm polystyrene vial with about 2 mL of standard cornmeal-molasses media for five to ten days for maturation. The wing was placed in a 0.6 mL Eppendorf tube to which was added 10 µl Squishing Buffer (10mM Tris, pH 8.2; 1mM EDTA; 25mM NaCl) and proteinase K (final concentration 200 µg/mL). The wing was broken up with a pipette tip and incubated for one hour at 37°C followed by 95°C for two minutes. 5 µL of the wing DNA prep was used in a 25 µL PCR reaction. The genotype of the individual was identified following agarose gel electrophoresis of the PCR products using primers for the *desatF* and *eloF* alleles. The *desatF* forward primer was 5'- AAC TCA TTC GAT CGC CAT TC-3' and the reverse primer was 5'- CGC ATC AGA TTC GTA AAG CA-3' producing a size difference of 108 bases. The *eloF* PCR used the forward primer 5'- ATT GCC ATG CTG GCG ATT TG-3' and the reverse primer 5'- GAC AGG ATC CTC CGA AAT GA-3' producing a size difference of 41 bases. Flies having the correct genotype were subsequently mated through the process described above to produce offspring with the *D. sechellia* homozygous alleles at *desatF* and *eloF*.

Copulation Tests

The presence of *D. sechellia* alleles for *desatF* and *eloF* in a mostly *D. simulans* background in females was used to measure the effect of the candidate genes on copulation by *D. simulans* males. The effect that these loci have on courtship interactions between *D. simulans* and *D. sechellia* were measured through the copulation success rate and the copulation latency of sim169 males with each female from the different genotype

groups. Copulation was measured with a *D. simulans* male because replacement of the *desatF* and *eloF* loci with *D. sechellia* alleles should produce 7,11-HD, an anti-aphrodisiac for *D. simulans*. If *desatF* and *eloF* are the genes responsible for 7,11-HD production there will be an absence of copulation between *D. simulans* males and altered females. The *D. simulans* line sim169 was used instead of simF2 for copulation tests because the latter carries recessive markers that make it hard to maintain as a healthy stock and the males do not court consistently. The effect of each of the *D. sechellia* alleles for *desatF* and *eloF* was measured separately (desatF, eloF 1 and eloF 2) and with hybrid *desatF* and *eloF* (hybrid 1 through 4 and species hybrid). The copulation success and latency of each of these two groups was compared to the copulation success and latency of the sim169 males with wildtype *D. sechellia* (sec) and *D. simulans* (sim169) females. Individuals used in these courtship experiments are listed in Table 1 and their origin is described above. The copulation success and latency times of the individuals that were *D. sechellia* homozygous for one locus and heterozygous for the other locus (D2E1 and D1E2 females) and *D. sechellia* homozygous for both loci (D2E2 females) were unable to be completed because of the low number of individuals obtained that fit these genotypes.

Before starting the copulation tests, the lines were standardized in order to control the number and size of offspring and for overcrowding. For each of the groups used in the copulation tests (sim169, simF2, eloF 1, eloF 2, desatF, hybrid 1, hybrid 3, hybrid 4, species hybrid and sec) five virgin females and five virgin males from each respective group were placed in a 25 x 95 mm polystyrene vial with about 9 mL of standard cornmeal-molasses media for seven days. Once offspring emerged, virgin females from

each group (the test females) and virgin males from sim169 were collected within 6 hours of eclosion. The sexes and groups were housed separately with up to 10 individuals in a 16.5 x 95 mm polystyrene vial with about 2 mL of standard cornmeal-molasses media. The virgin males and virgin females were allowed to mature for seven to ten days before use in the copulation test.

The copulation test measured successful copulation and copulation latency time of a sim169 male with each of the different test females. All copulation tests were performed between one and four hours post lights on. Tests were performed under controlled conditions by using an incubator that maintained the temperature at 25° C and the humidity at 50%.

To start the copulation test, one test female and one sim169 male were aspirated into a 16.5 x 95 mm polystyrene vial containing about 2 mL of standard cornmeal-molasses food. Once the male and female were together, the vial was placed in the incubator set at 25° C and 50% humidity. Because copulation in *D. sechellia* and *D. simulans* lasts roughly twenty minutes, the vials were checked every ten minutes for successful copulation. Copulation latency was scored as the time from the start of the test, when male and female were place together in the vial, to the ten minute interval that showed copulation. All courtship tests lasted for a total of 60 minutes.

Analysis of the Copulation Test Data

We measured the copulation success rate and copulation latency time of sim169 males with each test female. Copulation percentages were found by taking the number of successful copulations over the total number of copulation tests attempted. To find

significant differences between groups of females, copulation percentage data was analyzed using a Fisher's exact test with a confidence interval of 95%.

The copulation latency data only included sim169 males that had successful copulation with the tested female. Copulation latency times were determined from the start time of the test to the ten minute interval where copulation occurred. The copulation latency times were analyzed using an ANOVA with a *post-hoc* Tukey test with a 5% error rate. Because we made comparisons among nine genotype groups, a Bonferroni correction of 0.00139 was used to measure for a significant effect of genotype on copulation latency time.

Cuticular Hydrocarbon (CHC) Profiles

To test the effect of the presence of the *D. sechellia* genes in a *D. simulans* background on CHC profiles, we compared the groups in Table 1 above to known CHC expression profiles of *D. sechellia* and *D. simulans* females. We wanted to measure the effect of each of the *D. sechellia* loci *desatF* (desatF) and *eloF* (eloF 1 and eloF 2) separately, *desatF* and *eloF* together as hybrid alleles (hybrid 1 through 4 and species hybrid), each locus fixed to *D. sechellia* homozygous separately (D2E1 and D1E2) and *desatF* and *eloF* together as homozygous *D. sechellia* alleles (D2E2).

Fly groups used in the CHC Profile experiment were standardized using the same method as the Copulation Test experiments. The genotype groups produced by crosses between the hybrid males and females to make the homozygous and heterozygous loci (D2E1 and D1E2) and the crosses between the D2E1 or D1E2 males and females were not standardized in order to increase the likelihood of producing females of the desired

genotype by increasing the number of offspring in each cross. Cuticular hydrocarbons were extracted from all the genotype groups in Table 1 as described below. CHC were extracted from the individual *desatF* (desatF) and *eloF* (eloF 1 and eloF 2) loci lines as well as from the individuals used in the process to make the cross containing both *D. sechellia* alleles of *desatF* and *eloF* (D2E2). These include the individuals that genotyped as homozygous *D. sechellia* for *eloF* and heterozygous for *desatF* (D1E2), as well as the reciprocal individuals, homozygous *D. sechellia* for *desatF* and heterozygous for *eloF* (D2E1).

To extract CHC from the flies, virgin females were collected from each genotype within six hours after eclosion. Each female was housed individually in a 16.5 x 95 mm polystyrene vial containing about 2 mL of standard cornmeal-molasses food. When the females were five to ten days old, the CHC were extracted. The females were immobilized by placing on ice for five minutes before being immersed in 70 mL of heptane containing 770 ng of hexacosane as an internal standard. The heptane extracts from each group were analyzed with a Perichrom gas chromatograph with a flame ionization detector. The gas chromatograph used a BPI capillary column. The oven temperature was programmed to change from 280°C to 300°C at 3°C/minute (Antony 1982; Wicker and Jallon 1995; Dallerac et al. 2000). The retention times of the CHC for each individual were compared to known *D. sechellia* and *D. simulans* profiles in order to identify the different CHC peaks present. Finding the area under the curve and normalizing to the amount of internal standard, hexacosane, present determined the total hydrocarbon amount for each of these peaks. Because the absolute amounts of

hydrocarbons vary for each individual the total amount of each CHC was determined as a percentage relative to the sum of 23 – 29 carbons.

CHC Profile Analysis of Data

The hydrocarbon analysis produced percentage amounts for 28 different types of CHC present on the cuticle of the fly. Only eight of the 28 CHC were used in subsequent analyses because these were the main hydrocarbons showing variation between individuals with *D. simulans* alleles to individuals with *D. sechellia* alleles. Percentage values for each of the eight hydrocarbons were transformed using an arc sine transformation to make the data points more normally distributed. Even though *D. simulans* and *D. sechellia* produce statistically different hydrocarbon profiles, the intraspecific variation of individuals within a particular species is also significantly different (Coyne et al. 1994; Coyne 1996). Thus, in the analyses of hydrocarbon profiles in this study, ratios are used rather than absolute amounts of hydrocarbons produced.

Ratios were made of the arcsine transformed percentages to measure elongation differences (7-P to 7-T and 7-H to 7-P), desaturation differences (7, 11-PD to 7-P) and to compare the ratio of *D. sechellia* to *D. simulans* pheromone (7,11-HD to 7-T). One was added to the ratio values before a log was taken for each value. All subsequent analyses were done with these transformed values. Differences in CHC expression ratios were compared among all the genotype groups from Table 1 using an ANOVA with a *post-hoc* Tukey test with a 5% error rate. Because we made comparisons among 14 groups, a Bonferroni correction of 0.0005495 was used to find the significant differences between the genotype groups.

Results and Discussion

Effect of *desatF* and *eloF* on courtship success

To measure the effect that *D. sechellia* alleles of *desatF* and *eloF* have on the courtship success of sim169 males with each of the genotype groups of females, the percentage of males that copulated with each type of female was determined. To identify if the genotype of the female had an effect on whether a sim169 male would copulate with her, a Fisher's Exact Test was used to analyze the results. This analysis measured the number of sim169 males that copulated with sim169 females and compared this value to the number of sim169 males that copulated with each of the test females. Not all of the genotype groups from Table 1 are represented in Figure 5 because of the low number of females possessing the required genotypes.

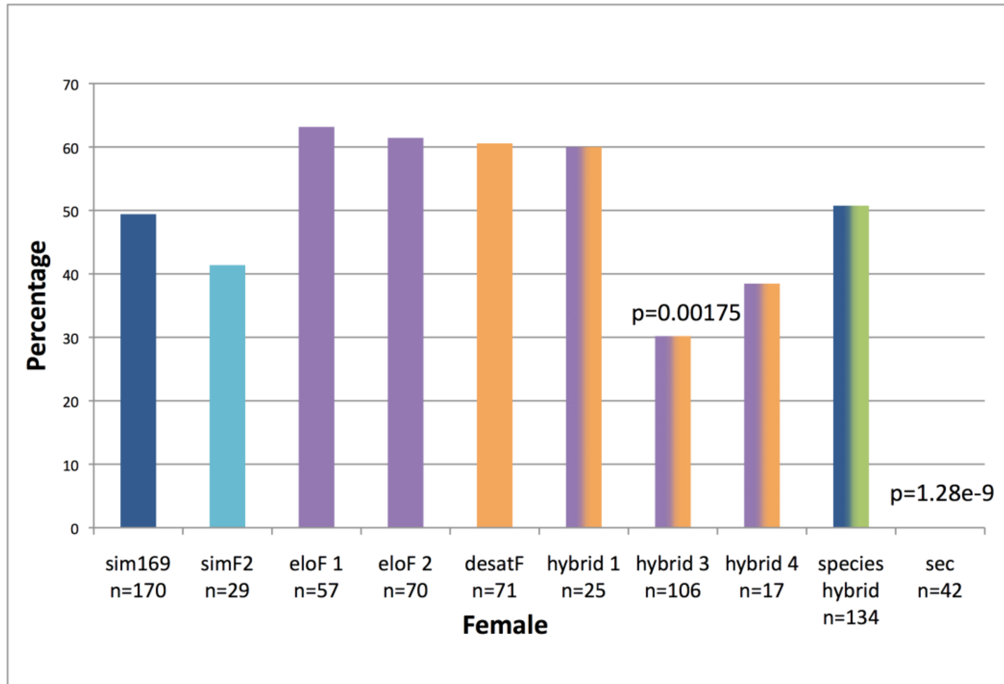

Figure 5: **Copulation percentage of sim169 males with altered females**. The percentage of pairs from each genotype group that copulated is compared to the pairing of sim169 males with sim169 females. One of the genotype groups, hybrid 3, showed a significant decrease in copulation (Two-Tailed Fisher's Exact Test, p=0.00175). None of the sim169 males copulated with sec females. The number of pairs tested is given below each genotype group.

Two sets of *D. simulans* females are present in these copulation tests, sim169 and simF2. The simF2 females are genetically identical to the *D. simulans* parental line used to make the RILs and because of this they carry a set of five recessive markers, which makes them hard to maintain as a healthy stock. For the copulation percentage tests, sim169 males were used instead of simF2 males because the simF2 line is difficult to maintain and males do not court consistently. However, using the sim169 males to measure copulation percentages does not seem to have a biased effect on the results because sim169 males showed similar copulation rates, 49% and 41% with the *D. simulans* females, sim169 and simF2, respectively (Figure 5).

Females carrying either the *eloF* or *desatF D. sechellia* allele were mated as often as the sim169 females (Figure 5). For the hybrid females, the hybrids 1, 3 and 4 have heterozygous alleles for *desatF* and *eloF*, in an otherwise *D. simulans* background, while the species hybrid females are heterozygous for alleles from both species throughout the genome. The hybrid females had a variable copulation percentage rate that ranged from 30-60% (Figure 5). The sim169 males only showed a significant decrease in copulation with hybrid 3 females. The other hybrid females were similar to the sim169 female copulation rate. As was expected from previous study (Coyne et al. 1994), sim169 males did not copulate with *D. sechellia* (sec) females.

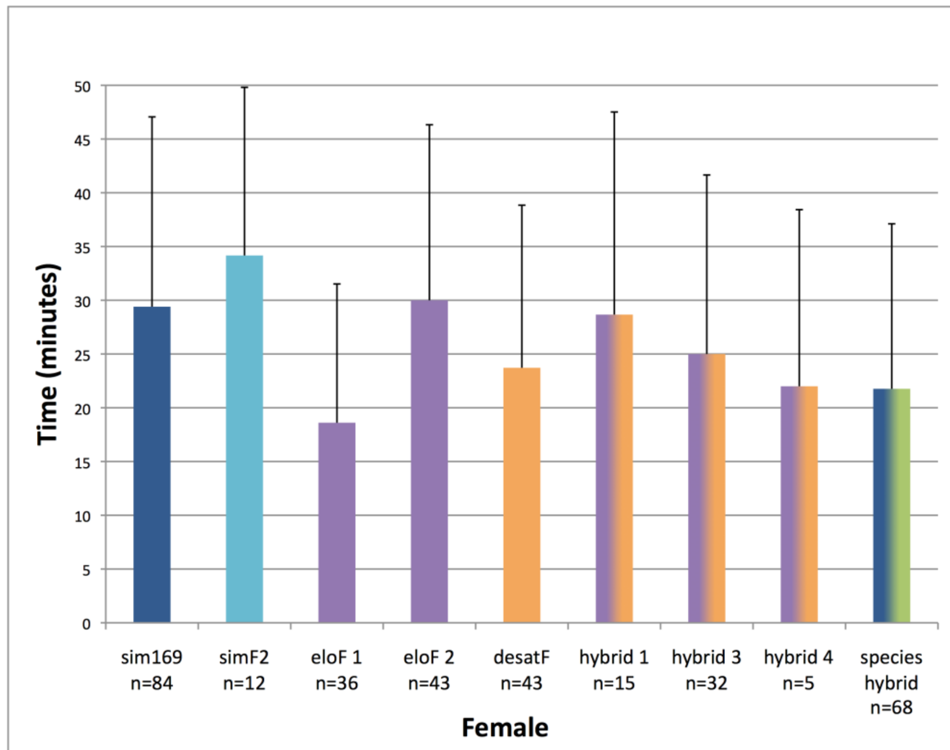

Figure 6: **Copulation latency of sim169 males with altered females**. The copulation latency time of pairs of sim169 males with females from each genotype group are compared by an ANOVA and Tukey test followed by a Bonferroni correction of 0.00139. The sim169 males showed similar copulation latency times with all genotype groups of females. Error bars represent one standard deviation of the data. The number of pairs tested is given below each genotype group.

The copulation latency time for the males that showed successful copulation to sim169 females was measured to determine if any of the females produced pheromones that would make them more or less attractive to the sim169 males measured by a decrease or increase, respectively, in time to copulation (Figure 6). Comparisons of copulation latency times for sim169 males to each of the female genotype groups showed the pairs of males and females had similar copulation latency times to one another with no significant differences.

Overall there was very little variation in the copulation percentages and latency times for each of the genotype groups tested. The copulation percentages of *D. simulans*

males with each group of females are relatively similar, with the exception of one of the hybrid groups (hybrid 3). This varies from the predicted outcome and results found in previous literature where it has been demonstrated that *D. simulans* males show reduced copulation with hybrid females (Cobb and Jallon 1990; Coyne et al. 1994). Not even the species hybrid, which has been shown to produce a measureable amount of the *D. sechellia* pheromone 7,11-HD, (Coyne et al. 1994; Coyne 1996; Gleason et al. 2005; Gleason et al. 2009) showed a significant decrease in copulation when compared to copulation percentages with *D. simulans* females (sim169 and simF2).

Results observed in the copulation tests of this study differ from previous literature where *D. simulans* males copulated less frequently with F1 hybrid females than with *D. simulans* males (Coyne et al. 1994). In our assay, the copulation rates for the two types of females were the same. Differences in the number of copulation events could be due to differences in the mating assay used in each experiment. The assay presented in this thesis paired one male and one female for 60 minutes while Coyne et al. (1994) paired four males and two females for 30 minutes. Due to the difference in number of individuals used in the two assays it would be expected that Coyne et al. (1994) would have higher copulation with hybrid females compared to our single male and female assay because of the number of individuals involved in the mating assay. However, our mating assay with a single male and female pairing had a greater amount of copulation. Observation of results that are inverse to what we predicted indicates that differences in the number of flies used in the mating assay does not explain why our species hybrid females do not have reduced copulation by *D. simulans* males.

The length of the copulation test could also have an effect on the copulation rate. Because our assay time was longer, if the majority of copulations with the hybrid females occurred in the second 30 minutes, this would explain the difference. While there is a large variation in copulation latency times for the single male and female assay used in this study, the majority of copulation was observed in the first 30 minutes of testing. Therefore, the longer time for the copulation test used in this study did not increase the likelihood that copulation would occur.

Another explanation for the differences observed in this study and Coyne et al. (1994) is that different strains of *D. simulans* produce variable levels of the hydrocarbons that are used in synthesis of the anti-aphrodosiac pheromone, 7,11-HD. Different strains of *D. simulans* were used in this study and Coyne et al. (1994) to produce the F1 hybrids used in the copulation tests. CHC production differs among strains of the same species (see Effect of *desatF* and *eloF* on Elongation of CHC Chain Length). The *D. simulans* strains, simF2 and sim169, produce statistically significantly different amounts of the 27 carbon compound (7-H) with simF2 producing an increased amount. The low levels of 7-H found in sim169 can affect the total amount of 7,11-HD received by *D. simulans* males during courtship because low numbers of compounds at the required length can reduce production levels of 27-dienes. Species hybrids used in the copulation tests for this study were produced from crosses between sim169 females and *D. sechellia* males. Because sim169 has a lower amount of 7-H present when hybrids are produced from these females the amount of 7,11-HD produced may be lower than the amount produced by a hybrid cross using another strain of *D. simulans* with a higher level of 7-H production.

To test for the effect of intraspecific variation in hydrocarbon production, copulation tests would need to be measured with many different strains of *D. simulans*. Hybrid F1 females would need to be produced from a wide variety of *D. simulans* strains and copulation rates would be measured with *D. simulans* males to observe if other strains have intermediate copulation rates (as observed in Coyne et al. (1994)) or levels similar to *D. simulans* females (as observed in Figure 5).

Lack of the other genotypes, especially those homozygous at one locus and heterozygous at the other locus or homozygous at both loci also makes it difficult to draw definitive results about the absolute effect of *desatF* and *eloF* in *D. sechellia*. Data is needed from these missing genotypes in order to see a clear effect of the candidate genes on copulation percentages and latency times. Further copulation tests between *D. simulans* males and females that are homozygous *D. sechellia* at both loci are needed to fully understand the effect *desatF* and *eloF* play in altering and reducing mating behavior.

Effect of *desatF* and *eloF* on Elongation of CHC Chain Length

Female *D. simulans* and *D. sechellia* have distinct CHC expression profiles in which each produces a signature amount of the species-specific pheromones. *D. simulans* mainly produces 7-T, the shorter, more saturated hydrocarbon, while *D. sechellia* produces 7,11-HD, the longer, less saturated compound. All of the hydrocarbons present on the cuticles of the females come from a common precursor fatty acid. The addition of the different *D. sechellia* alleles into the mainly *D. simulans* background should cause changes to *D. simulans* CHC profiles. These changes will

affect the type and amount of CHCs present. If *desatF* and *eloF* are the only genes responsible for the biosynthesis of 7,11-HD, the addition of the *D. sechellia* alleles into a *D. simulans* background will shift the CHCs towards a more *D. sechellia*-like expression profile.

Comparisons measuring the effect of the candidate genes on CHC production have additional genotype groups not present in the courtship tests. Analyses include females heterozygous at one locus and homozygous *D. sechellia* at the other locus and females that are homozygous *D. sechellia* at both loci.

The effect of the candidate genes, *desatF* and *eloF*, on the elongation process was measured by comparing the ratio of 7-pentacosene (7-P) to 7-tricosene (7-T), which compares production levels of hydrocarbons 25 carbons in length to those that are 23 carbons in length without increasing the number of double bonds. A higher ratio value indicates an increase in 7-P production. Two previous studies (Gleason et al. 2005; Gleason et al. 2009) used this ratio comparison to identify the genomic regions contributing to desaturation and elongation of CHCs.

Analysis of the different genotypes created two distinct groups of females producing increased amounts of 7-P (Figure 7) as indicated by the increase in the ratio of 7-P to 7-T. The genotype groups with two copies of the *eloF D. sechellia* allele (eloF 1 and eloF 2, Figure 7) and the hybrid group of females (with one copy of the *eloF D. sechellia* allele) produce similar ratios of 7-P to 7-T and these levels of 7-P production are statistically significantly higher than the ratio of 7-P to 7-T present in *D. simulans* females. Because the *eloF* and hybrid groups do not produce significantly different ratios

of 7-P to 7-T, this suggests that the elongation to 25 carbons is not dependent upon the dosage of *D. sechellia* alleles.

Increased 7-P production, shown by the higher ratio amount of 7-P to 7-T, is exclusive to individuals carrying the *D. sechellia* allele of the elongase gene, *eloF* because the desatF individuals have ratio levels similar to *D. simulans* while the eloF and hybrid individuals have intermediate ratio levels between *D. simulans* and *D. sechellia*. The desatF group carrying a *D. simulans* allele for *eloF* and *D. sechellia* allele for *desatF* shows ratio levels of 7-P to 7-T similar to both *D. simulans* females (sim169 and simF2) indicating that the *desatF* gene does not affect chain length of the hydrocarbon. Females possessing a heterozygous *desatF* and homozygous *D. sechellia eloF* genotype (D1E2), and the inverse (D2E1), produced CHC production results similar to the hybrid and eloF genotype groups.

However, the ratio levels of 7-P to 7-T caused by carrying the *D. sechellia eloF* allele are still statistically significantly lower than the ratio amount normally produced by *D. sechellia* females (Figure 7). The intermediate ratio levels of 7-P to 7-T produced by these former groups indicates that candidate gene, *eloF*, while directing synthesis of some hydrocarbons into the elongated form, does not fully produce the carbon chain lengths observed in *D. sechellia* females, which produce a much higher ratio of 25 to 23 carbon chains.

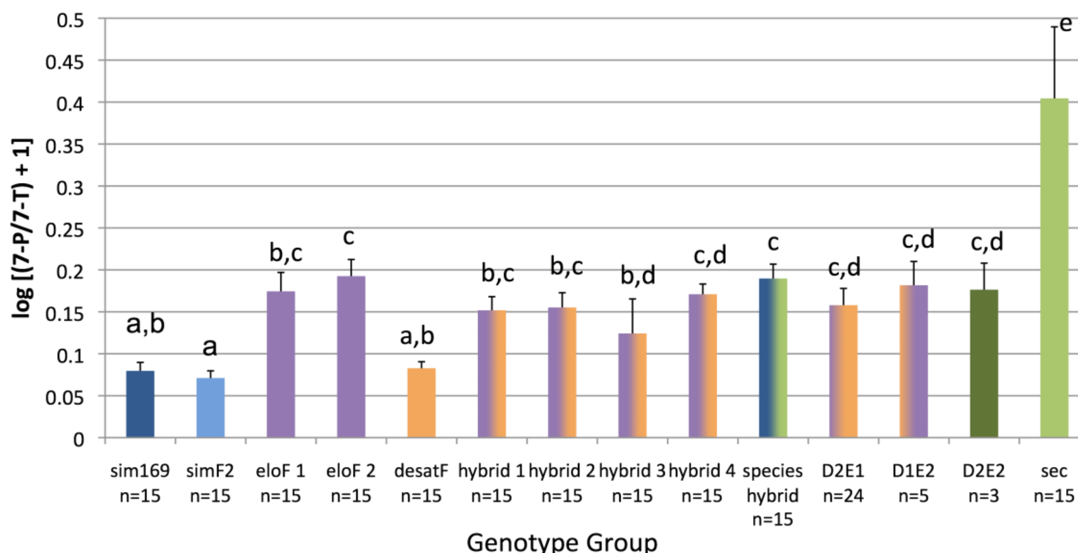

Figure 7: **Effect of *desatF* and *eloF* on hydrocarbon elongation from a 23 to 25 chain length.** The ratio of 7-P to 7-T is compared to measure the effect of the candidate genes, *desatF* and *eloF* on the elongation process from a 23 to 25 carbon chain length. Females with *D. sechellia* alleles of *eloF* and hybrid females display a significant increase in elongation. Different letters indicate statistically significant differences between pairs of female genotype groups by Wilcoxon's signed-ranks tests with Bonferroni correction for multiple tests. Error bars represent one standard deviation of the data. The number of females analyzed is given below each genotype group.

Another elongation comparison was made to measure the total effect a *D. sechellia* allele for candidate gene, *eloF*, has on elongation of the fatty acid precursor to the final hydrocarbon length of 27 carbons (Figure 8). Measuring the ratio of 7-heptacosene (7-H) to 7-P illustrates a shift from 25 to 27 carbons without a change in the number of double bonds. A higher ratio value indicates an increase in production of 7-H. The *D. simulans* line simF2 produces a higher ratio of 7-H to 7-P than the *D. simulans* line sim169. All of the other genotypes in between D. *simulans* and D. *sechellia* showed similar levels of 7-H to 7-P (Figure 8). As in the previous comparison, even though a greater ratio of 27 to 25 carbon hydrocarbons is produced the levels are still statistically significantly lower than the ratio produced by *D. sechellia* females. The reason for the

intermediate levels is likely the same as for elongation to 25 carbons that *eloF* is not the only gene involved in biosynthesis and thus does not complete elongation to 27 carbons.

Due to the lack of significant differences in production of the 7-H, as shown by the similar ratios of 7-H to 7-P across the genotype groups, the *eloF D. sechellia* allele only has an effect on elongation up to a specific chain length of 25 carbons. The cessation of effect on elongation difference indicates that other elongases must be included in the process in order for the pheromone to be extended to its final length of 27 carbons. In the QTL study that identified the genomic regions contributing to elongation and desaturation, the locus identified on the third chromosome dealing with elongation covered an area of the genome with a wide number of elongase genes (Gleason et al. 2005; Gleason et al. 2009) it is possible that while *eloF* is important to one step of the elongation process another elongase located in this region completes CHC synthesis to the 27 carbon length of 7,11-HD.

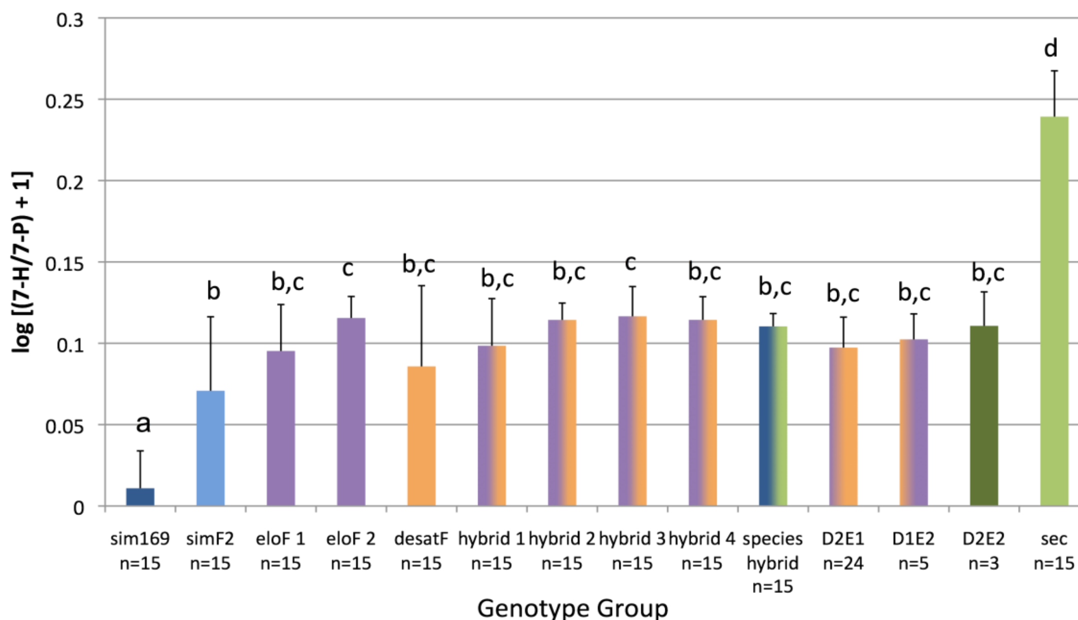

Figure 8: **Effect of *desatF* and *eloF* on hydrocarbon elongation from a 25 to 27 chain length.** The ratio of 7-H to 7-P is compared to measure the effect of the candidate genes, *desatF* and *eloF* on the elongation process from a 25 to 27 carbon chain length. No effect of *D. sechellia* alleles on elongation is apparent at this stage. Different letters indicate statistically significant differences between pairs of female genotype groups by Wilcoxon's signed-ranks tests with Bonferroni correction for multiple tests. Error bars represent one standard deviation of the data. The number of females analyzed is given below each genotype group.

Effect of *desatF* and *eloF* on Desaturation Levels of CHC

To test for the effects of the candidate genes on desaturation levels, a comparison was made of the diene and monoene pheromones at various carbon lengths. A higher value indicates that more dienes were produced than monoenes. No 23-dienes were produced so ratio comparisons were only made of 25-dienes to 25-monoenes and 27-dienes to 27-monoenes. The latter comparison did not produce any distinct groups to illustrate the effect of the candidate genes due to a large amount of variance in the data (data not shown). The large amount of variance could be due to the lack of sufficient hydrocarbons of the full length (27 carbons) because the majority of the genotypes did not express significant amounts of 7-H compared to levels of 7-P (Figure 8) and therefore

the additional desaturation on the 27 carbon chains may not have as large of an effect on these pheromones.

The comparison of the 25 dienes to the 25 monoenes illustrates the effect of the candidate genes on the desaturation process (Figure 9). The females with the *D. sechellia* allele for *desatF* produced a significantly greater ratio of 7, 11-PD to 7-P than the *D. simulans* (sim169 and simF2) and *eloF* (eloF 1 and eloF 2) females. The species hybrid females and the homozygous *D. sechellia desatF* and heterozygous *eloF* females (D2E1) produced a significantly higher ratio of 7, 11-PD to 7-P than the *D. simulans* females, but did not show as much increase in the amount of desaturation as the *desatF* females, desatF. The latter group produced a significantly greater ratio of 7, 11-PD to 7-P compared to the two former groups, species hybrid and D2E1.

The difference in the desaturation levels for the females carrying homozygous *D. sechellia desatF* or heterozygous *desatF* suggests dosage dependence for the amount of *desatF D. sechellia* alleles present and the amount of dienes expressed (Figure 9). When two *desatF D. sechellia* alleles are present, as in desatF and D2E1 females, there is a significantly higher ratio of 25 dienes to monoenes. If copy number of the alleles is important to amount of pheromone produced, the hybrid groups should produce 7, 11-PD amounts that are intermediate between the *D. simulans* and *D. sechellia* levels. But some hybrid groups (hybrid 1, hybrid 2, hybrid 4 and species hybrid) do not produce ratio levels of 7, 11-PD to 7-P that are statistically significantly different from *D. simulans* levels. Some of the hybrid females (hybrid 3) do produce a small ratio of 7, 11-PD to 7-P, statistically significantly higher than *D. simulans* levels. But variation in the data is so

great that firm conclusions about effects of the *D. sechellia* allele for *desatF* on desaturation levels in hybrids cannot be drawn.

When comparing the ratio of 7,11-PD to 7-P in desatF females to the ratio found in *D. sechellia* females, the former actually produces a statistically significantly greater amount of dienes than the amount produced in *D. sechellia* females (Figure 9). The greater ratio of 7,11-PD to 7-P produced by the desatF females is likely due to the way hydrocarbons are synthesized in a series of successive steps. *D. sechellia* females would continue synthesis of the hydrocarbons towards the production of the longer hydrocarbon 7,11-HD, rather than stop synthesis at the shorter 7, 11-PD. The large ratio of 7,11-PD to 7-P in the desatF females suggests that the *desatF* locus is an important step in hydrocarbon synthesis for addition of a second double bond because replacement of a *D. simulans* desatF locus region with a *D. sechellia* allele greatly increases the likelihood for diene production.

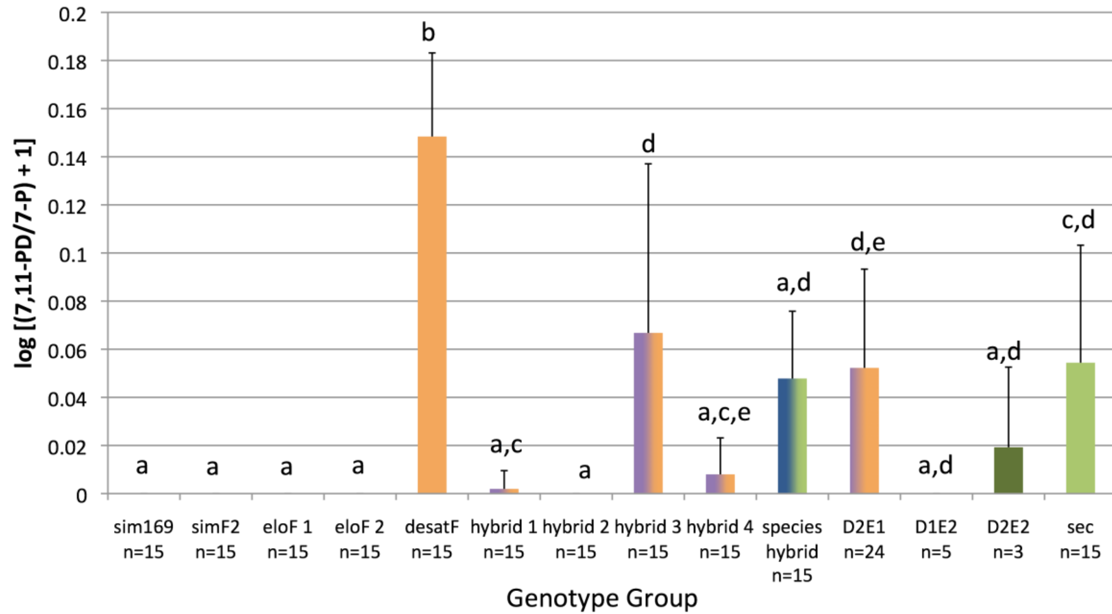

Figure 9: **Effect of *desatF* and *eloF* on hydrocarbon desaturation of a 25 carbon chain length.** The ratio of 7, 11-PD to 7-P is compared to measure the effect of the candidate genes, *desatF* and *eloF* on the desaturation process. Females with *D. sechellia* alleles of *desatF* and hybrid females display an increase in desaturation levels, but the effect varies with the genotype group. The desatF group of females shows the greatest increase in desaturation of a 25 carbon chain length. Different letters indicate statistically significant differences between pairs of female genotype groups by Wilcoxon's signed-ranks tests with Bonferroni correction for multiple tests. Error bars represent one standard deviation of the data. The number of females analyzed is given below each genotype group.

Epistatic Interaction between *D. sechellia* alleles for *desatF* and *eloF* on CHC

Comparisons of the ratio of 7, 11-heptacosadiene (7,11-HD) to 7-T were made to interpret the interaction between *D. sechellia* alleles of *desatF* and *eloF* and determine the effect that these loci have on producing the *D. sechellia* hydrocarbon, 7,11-HD (Figure 10). A higher ratio value indicates an increase in the production of 7,11-HD. Females from the groups with only the desatF or eloF genotype are not able to produce significant amounts of 7,11-HD. Instead these groups are statistically similar to the ratio levels of 7,11-HD to 7-T produced by *D. simulans* females. For example, in the desatF females, the *eloF* locus carries a *D. simulans* allele while the *desatF* locus carries a *D.*

sechellia allele, based on predicted effects elongation will remain *D. simulans*-like at 23 carbons while the amount of dienes will increase. But even when these changes occur, as seen in the elongation effect of the eloF genotype (eloF 1 and eloF 2, Figure 7) and the desaturation effect of the desatF genotype (desatF, Figure 9), the changes in pheromone production do not drive CHC synthesis towards 7,11-HD. A single locus with *D. sechellia* allele replacement does not produce a significant change in the pheromones necessary for identification as a *D. sechellia* female.

Only when the two loci are carried together with at least heterozygous alleles does any measure of 7,11-HD occur. The hybrid groups of females show a significant increase in the ratio of 7,11-HD to 7-T when compared to the *D. simulans* (sim169 and simF2), *desatF* (desatF) and *eloF* (eloF 1 and eloF 2) females. Requirement for the genotype of both loci to be *D. sechellia* suggests an epistatic interaction between the genomic regions where *desatF* and *eloF* occur confirming the results observed in Figure 3. The two loci interact together to produce the *D. sechellia* CHC, but neither locus can produce this pheromone on its own. As in the comparisons for elongation and desaturation, the ratios of 7,11-HD to 7-T are significantly less than levels produced by *D. sechellia* females. The hybrid female groups produce levels of 7,11-HD slightly intermediate between *D. simulans* and *D. sechellia*, but some hybrids are more similar to *D. simulans* (hybrid 2 and hybrid 3). Only the species hybrid group displays a 7,11-HD production level that is statistically different from the levels produced by *D. simulans* and appears intermediate between the two species. Species hybrid females, because they carry heterozygous alleles across the entire genome, are more likely to have the *D. sechellia* allele for the genes needed in CHC production. This suggests candidate genes, *desatF* and *eloF*, are

not the only genes contributing to differences in CHC production levels between *D. simulans* and *D. sechellia*. Because QTL studies can only identify a region that contributes to a phenotype, it is possible that one of the other elongase genes around *eloF* or an unidentified desaturase gene near *desatF*, are the actual genes controlling biosynthesis of 7,11-HD.

In particular, the females from the D2E2 group, which have homozygous *D. sechellia* alleles for both of the candidate genes, do not produce the same amount of 7,11-HD as *D. sechellia* females. Variation of the individual females from this genotype group makes D2E2 females similar to both *D. simulans* and hybrid females. The large amount of variation occurs with the D2E2 females because when the hydrocarbons were analyzed only three females were produced with the correct genotype. Two of the females produced pheromone profiles similar to *D. simulans* while the other produced a more *D. sechellia*-like expression profile with a level of 7,11-HD similar to *D. sechellia* (personal observation, data not shown). Because of the manner in which these D2E2 females were produced through multiple combinations of various RILs, it is possible that a random recombination event may have occurred in the female that was able to produce a pheromone profile similar to *D. sechellia*. Sequencing of this individual and the other two D2E2 females would be needed to compare the changes that occurred in the desaturation and elongation genomic regions of the D2E2 *D. sechellia*-like female, which allowed for 7,11-HD production.

The comparison of the elongation and desaturation differences reveals the importance of the epistatic interaction between the loci containing the candidate genes *desatF* and *eloF*. Even if these genes are not the only biosynthesis genes involved in

pheromone production, interactions between these genomic regions still illustrate the effect the *desatF* and *eloF* locus have on the biosynthesis step involved in producing *D. sechellia* CHCs. *D. sechellia* allele expression of one locus affects response at the other locus to a degree where only dual expression of the *D. sechellia* allele at both loci will cause production of the *D. sechellia* pheromone, 7,11-HD.

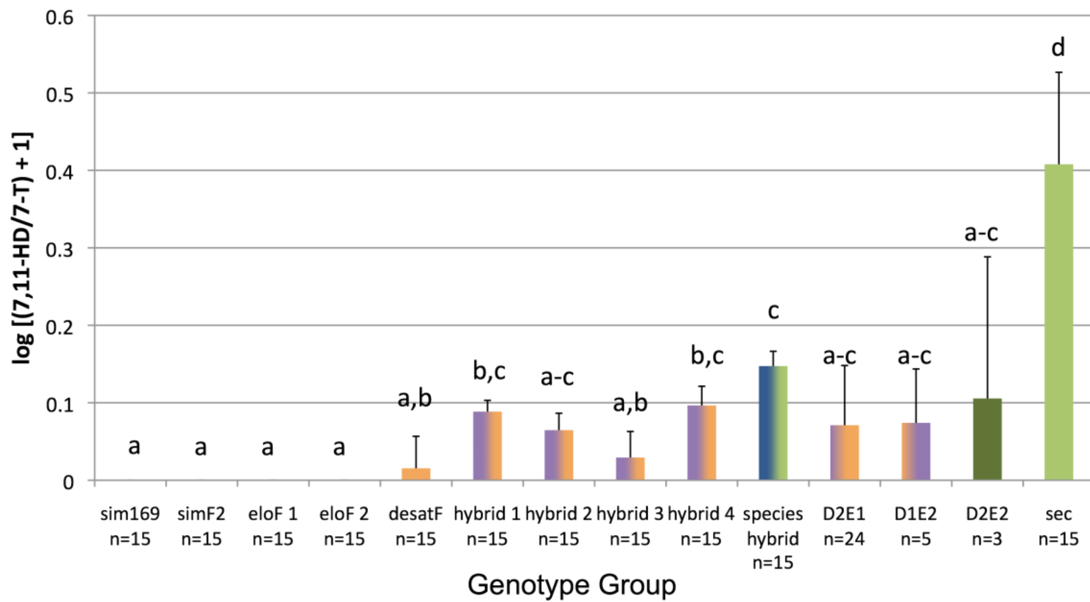

Figure 10: **Effect of *desatF* and *eloF* on expression of D. sechellia female hydrocarbon 7,11-HD.** The ratio of 7,11-HD to 7-T is compared to measure the effect of the candidate genes, *desatF* and *eloF* on the production of *D. sechellia* pheromone 7,11-HD. The comparisons show an epistatic interaction between loci where only females carrying *D. sechellia* alleles for both candidate genes produce 7,11-HD. Different letters indicate statistically significant differences between pairs of female genotype groups by Wilcoxon's signed-ranks tests with Bonferroni correction for multiple tests. Error bars represent one standard deviation of the data. The number of females analyzed is given below each genotype group.

Conclusions

D. simulans and *D. sechellia* have an asymmetric mating pattern in which *D. sechellia* males will copulate with *D. sechellia* and *D. simulans* females, but *D. simulans* males will only copulate with *D. simulans* females. The differences in the mating behavior of these two species occur due to differences in CHC production. *D. sechellia* females produce 7,11-HD, a chemosensory pheromone that is an anti-aphrodisiac to *D. simulans* males. A QTL study identified genomic regions on the third chromosome contributing to differences in CHC production between *D. simulans* and *D. sechellia* (Gleason et al. 2005; Gleason et al. 2009). These genomic regions contained *desatF* and *eloF*, genes present in the closely related species *D. melanogaster*, which are responsible for CHC biosynthesis of the female pheromone 7,11-HD (Legendre et al. 2008). The effect of these genes on copulation success and CHC production was measured by replacement of the *desatF* and *eloF* loci in *D. simulans* with *D. sechellia* alleles of the genes.

The copulation tests, which measured the copulation percentage and latency of *D. simulans* males with altered females, only included the effect of each locus individually, with females carrying a *D. sechellia* allele at *desatF* or *eloF* and with females carrying hybrid alleles at both loci. Overall, there was very little effect of these genes on the copulation success rate with *D. simulans* males or the average latency times to copulation (Figure 5 and 6). Not even the hybrid females, which have been observed in previous literature to induce an adverse mating response from *D. simulans* males (Cobb and Jallon 1990; Coyne et al. 1994), produced a significant reduction in copulation. This would suggest that *D. sechellia* alleles of *desatF* and *eloF*, even though they are responsible for

production of 7,11-HD in *D. melanogaster*, might not be the only genes responsible for the same pheromone in *D. sechellia*. However, the full effect of these candidate genes is difficult to determine because the test does not include females carrying homozygous *D. sechellia* alleles at both loci. Measuring the mating response of *D. simulans* males with females carry this genotype would reveal more about the effect of these genes on courtship with *D. simulans*. If these genes act in a fashion similar to *D. melanogaster* and produce 7,11-HD then D. simulans males would display reduced courtship with females carrying homozygous *D. sechellia* alleles at both loci.

These differences in mating behaviors between *D. simulans* and *D. sechellia* develop from differences in the prominent pheromones produced by each species. *D. simulans* produce 7-T, a 23 carbon monoene, while *D. sechellia* produce 7,11-HD, a 27 carbon diene. Comparisons of the hydrocarbons produced by each genotype group used to make the individuals homozygous *D. sechellia* at both loci reveals the effect of each gene individually and the epistatic effect between the gene regions. Elongation differences were determined by measuring the effect of the genes on production of longer hydrocarbons by comparing the ratios of 7-P to 7-T and 7-H to 7-P. In elongation to 25 carbons, females carrying the *eloF* gene produced increased amounts of 7-P, indicating that *eloF* has an effect on hydrocarbon elongation (Figure 7). However, the absence of an effect on elongation to 27 carbons suggests the involvement of other elongases to synthesize hydrocarbons to the full length of 7,11-HD (Figure 8). The genomic region where *eloF* is located includes a cluster of five elongases. Because *eloF* does not complete synthesis of the hydrocarbon, one of these other elongase genes could be responsible for elongation to 27 carbons. Desaturation differences were determined by

measuring the effect of the genes on the production of dienes by comparing the ratio of 7, 11-PD to 7-P. Females carrying the *desatF* gene produced increased amounts of 7, 11-PD, indicating that *desatF* is responsible for increasing the number of dienes (Figure 9). Dosage dependence was also observed in which females that carried two copies of the *D. sechellia* allele for *desatF* had increased production of 7, 11-PD compared to females that only had one copy. An epistatic interaction was observed between the loci carrying *desatF* and *eloF* by comparison of the ratio of the *D. sechellia* 7,11-HD to the *D simulans* 7-T (Figure 10). Only when females carried *D. sechellia* alleles at both loci did production of 7,11-HD occur. However, the amount of 7,11-HD was significantly lower than amounts produced by *D. sechellia* females. Especially when measuring the 7,11-HD amounts produced by D2E2 females, which carry *D. sechellia* alleles for *desatF* and *eloF*, and *D. sechellia* females. The large production difference suggests that *desatF* and *eloF* are not the only genes involved in CHC synthesis. The effect of the other elongases located near *eloF* must be investigated in order to determine the relationship they have with *desatF* and whether interactions between these other elongases and *desatF* are able to produce *D. sechellia* 7,11-HD.

The difference in CHC production between *D. simulans* and *D. sechellia* leads to sexual isolation between the two species because the latter species produces 7,11-HD, a pheromone that is an anti-aphrodisiac to the former species. Identification of the gene changes that occurred in *D. simulans* to inactivate the pathway responsible for production of 7,11-HD is important to identify how these species diverged from one another. While this study demonstrates the effect that *desatF* and *eloF* have on changing CHC production, the evidence points toward involvement of other synthesis genes that are

required for females to produce the amount of 7,11-HD needed to reduce courtship by *D. simulans* males. Further investigation of the genomic regions identified on the third chromosome is needed to explore the effect of all the biosynthesis genes, especially the group of elongases near *eloF*, which may be involved in altering CHC production.

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