SEXUAL DIMORPHISM IN DROSOPHILA CUTICULAR HYDROCARBONS AND THEIR ROLES AS MATING SIGNALS

By

Haosu Cong

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ABSTRACT

Sexual dimorphism exemplifies the remarkable diversity and aesthetic beauty in nature, with mating signals representing a key aspect of this phenomenon. These signals have evolved to encompass multimodal sensory modalities, and understanding their evolution necessitates exploring the complex interactions among various selective pressures.

This dissertation examines the intricate relationship between sexual dimorphism and mating signals, utilizing cuticular hydrocarbons (CHCs) in *Drosophila* species as a model system. We investigated three pivotal questions: 1) Is there a correlation between the evolution of sexual dimorphism and the evolution of mating signals, and can the degree of sexual dimorphism predict the functional roles of these signals? 2) What genetic mechanisms underlie the evolution of exaggerated female traits? 3) What phenotypic trade-offs are associated with the evolution of mating signals?

Employing the Bray-Curtis dissimilarity index, we initially assessed the degrees of sexual dimorphism in CHC profiles across *Drosophila* species and tested the impact of CHC perception on male courtship interest. Our findings did not support a correlation between the degree of sexual dimorphism and the use of CHCs for mate recognition. Next, we focused on a species exhibiting pronounced sexual dimorphism, identifying a candidate gene with female-biased expression in adult oenocytes, likely responsible for the production of exaggerated female traits. This expression pattern is attributed to *cis*-regulatory changes, characterized by two specific modules: one related to oenocyte expression and another to sex-biased expression. Lastly, the costs associated with producing methyl-branched cuticular hydrocarbons (mbCHCs) in transgenic *D.*

mojavensis lines was tested. Our findings do not reveal direct developmental trade-offs associated with the production of mbCHCs, but suggest a positive correlation between mbCHC production and reproductive fitness. While mbCHCs are correlated to individual fitness, they have not evolved to function as reliable signals influencing mate preferences.

This dissertation contributes to addressing unresolved questions regarding the evolution of sexual dimorphism and mating signals, offering novel insights into the genetic mechanisms and potential evolutionary costs associated with these traits.

This dissertation is dedicated to every one of you reading it.
Thank you for engaging with my work and me.

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Each day, I enjoy getting an iced coffee in the early afternoon, surrounded by blooming flowers, changing leaves, and butterflies, wasps, and lady beetles landing on my shoulder. My taste in music has shifted from C-POP girl group EDM to R&B. I love smiling at everyone I encounter on the street and wishing them good luck, whispering those thoughts in my heart.

I have experienced the joy of spring rain, the annoyance of summer cicadas, the charm of fall breezes, and the dance of snowflakes. I cherish river, having been inspired since my trip to Cincinnati along the Ohio River, and I often walk along the Grand River Avenue and Red Cedar River. My favorite karaoke song now is “River” in Mandarin, as rivers have always brought me peace.

So, future self, no matter what happens, I trust in you—we trust in you. Please continue to trust yourself and be proud of who you are. Life is a journey, and time is a river, both filled with surprises and excitement, flowing into our hearts.

I am not waiting for spring or the sea,
Just yearning for the same flower that blossoms in me.
But it's hard to depict,
So I weave tales of spring,
And listen to whispers of the sea.

When leaves not long for raindrops’ grace,
The wind brings them down to kiss the earth's embrace.
But when my heartbeat fails to match your palm’s rhyme,
Know that you’re still needed by my arms, in time.
I bet you dove too deep into the ocean of my gaze,
Where waves of longing crash into silent, endless maze.

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Evolution of sexual dimorphism under complicated interactions of selective forces

Describing nature necessitates acknowledgment of its inherent beauty and diversity. Among the estimated 1.5 million species within Earth's biodiversity, over half are classified as insects (Stork *et al.* 2015). Insects display significant intraspecific diversity, characterized by a wide range of variations in shape, size, life history, and ecological niches. Sexual dimorphism, which refers to the differences between sexes, is a prevalent phenomenon in nature and can manifest in morphological, physiological, and behavioral traits. Our understanding of these ubiquitous forms of intraspecific diversity has prompted numerous studies over the decades, focusing on the traits and mechanisms that underlie evolutionary processes. In this dissertation, I aim to further investigate the evolution of sexual dimorphism, offering novel insights and understandings.

Sexual dimorphism can be categorized into three types: primary, secondary, and ecological sexual dimorphism (Williams and Carroll 2009) (**Figure 1.1A**). Traits associated with primary sexual dimorphism are directly related to reproduction, such as reproductive organs and insect genitalia. In contrast, secondary sexual dimorphism refers to traits that are not directly involved in reproduction. Ecological sexual dimorphism arises when divergent ecological functions and niches are established between the sexes.

The evolution of sexual dimorphism is complex, which resulted from two main reasons. First, intricate interactions among selective forces (e.g., between sexual

selection and natural selection) may influence the trajectory of the evolution of sexual dimorphism in multiple directions. Second, different stages of the evolution of sexual dimorphism can be driven by divergent selective forces, from its initial occurrence to its elaboration and maintenance at an optimal degree of expression.

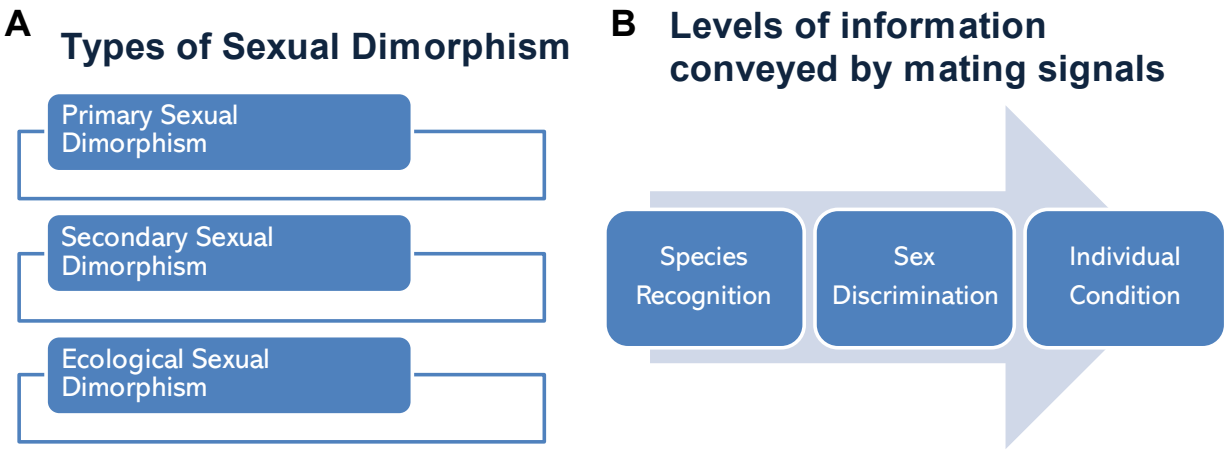


Figure 1.1 Introduction of (A) types of sexual dimorphism and (B) levels of information conveyed by mating signals. Three types of sexual dimorphism are classified as the direct association with reproduction and predicted primary selective pressure. Three levels of information were suggested to be conveyed according to the primary role of the mating signals.

Although secondary sexual dimorphism is not directly related to reproduction, these traits have been suggested to play a role in final mate choice. Specifically, they may serve one of two functions: 1) facilitating intrasexual competition for potential mates, or 2) acting as mating signals for the opposite sex, conveying information for mate recognition and choice. For example, in the male horned beetle *Allomyrina dichotoma* (Coleoptera: Scarabaeidae), horn length is proposed as a reliable indicator of fighting ability, which influences access to potential female mates and subsequent reproductive success (Karino *et al.* 2005). In other organisms in the animal kingdom, traits classified as secondary sexual dimorphism serve as mating signals that affect

mate choice. The darkness of a lion's mane is positively correlated with the lioness' preferences for mates (West and Packer 2002). Similarly, the number of eyespots on peacocks' tails has been shown to positively correlate with mating success among peahens (Dakin and Montgomerie 2011).

These sexually dimorphic traits, particularly those that are exaggerated in males, have been extensively discussed and investigated for their potential roles as mating signals across the animal kingdom. The reasons for the extensive study of male exaggerated traits can be attributed to two main factors: 1) the historically anthropomorphic view that females are the choosy sex, and 2) the relative rarity of exaggerated traits in females. Traditionally, females have been regarded as the "choosy" sex in mate choice. However, recent studies have suggested that the roles of males and females can be dynamic and context-dependent, with males exhibiting choosiness under certain conditions (Edward and Chapman 2011).

In contrast, while male exaggerated traits have received significant attention, understanding of female exaggerated traits remains limited. This lack of understanding can be largely attributed to the rarity of such traits in nature.

1.2 Evolution of diversified roles of mating signals under combinational selective forces

Mating signals can be broadly categorized based on physical characteristics, the media that convey the signals, the types of receptors and organs involved, and the modes of information processing. These categories include visual signals (such as body size and pigmentation), chemical signals (odors and tastes), acoustic signals, and mechanical signals (Halfwerk *et al.* 2019; Mitoyen *et al.* 2019).

In the class Insecta, the evolution of mating signals is taxonomically divergent, with different lineages employing distinct types of signals. For instance, fireflies (Coleoptera: Lampyridae) have evolved bioluminescent flash signals in response to sexual selection and mate choice (Lewis and Cratsley 2008). Similarly, male crickets (Orthoptera: Gryllidae) produce species-specific calling songs to attract females, demonstrating the use of auditory signals in mating behavior (Doherty and Hoy 1985).

Chemical signals, particularly insect pheromones, have been extensively studied since the first pheromone, bombykol, was isolated from the genitalia of female silkworm moths (*Bombyx mori*). Bombykol is known to elicit mating behavior in male moths (Karlson and Butenandt 1959). The evolution and diversity of mating signals have garnered significant interest from researchers over the decades. This diversity can primarily be understood through two aspects: 1) the use of combination of multiple signals, and 2) the levels of information conveyed by these signals.

While studies often focus on single sensory modalities, multimodal mating components are prevalent in the animal kingdom. Multimodal mating signals involve the integration of multiple sensory modalities that influence a receiver's mating decisions. These integrated signal components provide receivers with enhanced benefits for assessing the fitness of potential mates. Multimodal mating signals fundamentally demonstrate that a single display can be perceived through different sensory modalities. For example, frog calls, which serve as acoustic signals, are frequently accompanied by visual signals such as vocal sac movements and/or water surface vibrations (Halfwerk *et al.* 2019). Another well-studied instance of multimodal mating components is found in *Drosophila melanogaster*, where a variety of sensory modalities—including visual,

chemical, acoustic, and mechanical signals—play roles in mate recognition and choice (Fan *et al.* 2013) (**Table 1.1**). Research has investigated the diversity of sensory modalities employed by the two sexes across various species, revealing evolutionary patterns that reflect taxonomic divergence (Wen and Li 2011).

Behavior	Orient→	Tap→	Sing→	Lick→	Attempt→ Copulation	Copulation
Senses Used	Vision Olfaction	Gustation	Hearing	Gustation	Mechano-sensation	Mechano-sensation
Sensory Structures Used	Eyes Antennae	Foreleg Tarsi	Antennae	Labellum	Genitalia	Genitalia Legs

Table 1.1 Series of *Drosophila* courtship behavior and sensory modalities involved.

Mating signals can convey three categories of information between the producer and receiver: species recognition, sex discrimination, and mate quality (Johansson and Jones 2007) (**Figure 1.1B**). Species-specific signals play a crucial role in species recognition, often observed through reproductive character displacement during reinforcement processes. The Jewelwing damselflies (*Calopteryx aequabilis* and *C. maculata*) serve as a classic example for investigating reinforcement, where wing coloration has been identified as a character displacement that enables sympatric male Jewelwings to discriminate between species (Mullen and Andrés 2007).

Sex-specific traits or signals also facilitate sex discrimination. In addition to bombykol, which is exclusively produced by females, various blends of chemical signals exhibiting sexual dimorphism in insects contribute to this process. For instance, males

of Hawaiian swordtail crickets (genus *Laupala*) exhibit different behavioral responses to varying profiles of cuticular hydrocarbons deposited on conspecific females' antennae (Stamps and Shaw 2019).

Furthermore, mating signals can indicate mate conditions or qualities, encompassing factors such as fitness, fecundity, and mating status. In the funnel-web spider (*Agelenopsis aperta*), males demonstrate a strong preference for pheromones produced by unmated females over those from mated females (Singer and Riechert 1995). Thus, mating signals can evolve to serve single or multiple roles, influenced by a combination of selective pressures.

The complex interactions of various selective pressures complicate the correlation between the evolution of sexual dimorphism and mating signals. Nevertheless, understanding this relationship is essential for gaining novel insights into the evolution of sexually dimorphic mating signals in nature.

Even when a mating signal appears to serve one primary role, the exact information reflected by the signal can vary and become complex. For instance, mating signals may directly reflect the quality of the signaler through mate assessment, a concept supported by the theory of honest signaling, which has been empirically tested in several systems (Steiger and Stökl 2014). Evolving such honest signals can be costly; in addition to the basic costs associated with signal transmission, there are strategic costs incurred in generating these signals (Harper and Smith 2003). An argument has been made that signals must be costly to be considered honest, positing that the evolution of honest signals should balance the potential costs of cheating at equilibrium (Számadó 2011). Empirical studies on honest signaling have primarily

focused on measuring costs or trade-offs associated with the production of signaling traits. For example, research on the body size of female *Lobesia botrana* (Lepidoptera: Tortricidae) indicated that males prefer larger females with bigger glands capable of producing sex pheromones, which are associated with significantly longer signaling times (Harari *et al.* 2011). Further investigations using novel methodologies such as genetic tools are expected to enhance our understanding of the evolution of mating signals.

Among all mating signals, the evolution of chemical communication has been extensively studied, particularly in insects. Chemical communication is posited to be the oldest and most ubiquitous form of communication in nature. It is unique primarily due to its modes of transmission and sensory processing. Chemical signals can function in the absence of the signaler and can persist over long distances and durations. The sensory modalities associated with chemical signals are generally discrete, reflecting the availability of specific receptors (Steiger *et al.* 2011; Baeckens 2019). The number of genes encoding chemoreceptors varies widely among arthropods; for example, the fig wasp (*Ceratosolen solmsi*) possesses only five gustatory receptors, whereas the German cockroach (*Blattella germanica*) has 431 annotated gustatory receptors (Robertson 2019). Given the ubiquitous nature of chemical communication, studying its evolution not only enhances our understanding of this signaling sensory modality but also contributes to insights into the divergent evolution of multimodal signaling systems.

The evolution of chemical communication is primarily hypothesized to be influenced by sensory exploitation, wherein a biased sensory perception is predicted to exist within the chemical receiver. Through selective forces, this bias becomes

enhanced and stabilized, leading to the evolution of discrete chemical signal receptors (Steiger *et al.* 2011).

Pheromones, which are chemical signals released by organisms for intraspecific communication, have been documented across approximately 3,000 compounds in insect species (Symonds and Elgar 2008). Depending on their effective range and the associated sensory organs and receptors, insect pheromones can be categorized into volatile and contact pheromones. Volatile pheromones typically function over longer distances, while contact pheromones are utilized in close-range interactions (Duffy *et al.* 2018). Insect species may evolve to use either type, with both types potentially present within a single species, especially to elicit mating behavior. For example, in *Bagrada hilaris* (Heteroptera: Pentatomidae), studies have demonstrated the presence of both long-range volatile pheromones and potential contact pheromones associated with short-range courtship behavior (Guarino *et al.* 2008).

However, contact pheromones, or short-distance pheromones, do not possess the traditionally recognized characteristics associated with long-distance signaling. Conversely, the evolution of contact pheromones may resemble that of other short-distance signals, such as visual signals. Therefore, the evolutionary pathways of contact pheromones may diverge from those of volatile signals, particularly regarding the related selective forces. Understanding this divergence is crucial for a comprehensive understanding of the evolution of chemical signals. Moreover, the “intermediate” phase of contact pheromones may provide novel insights into the evolution of multimodal mating signal systems.

1.3 Using *Drosophila* as a model to investigate questions in evolution of sexual dimorphism and mating signals

In summary, there are several existing gaps in our understanding of the evolution of sexual dimorphism and mating signals. We have outlined the complex interactions of multiple selective forces that influence both processes. The **first** question that arises is whether there is a correlation between these evolutionary processes. Additionally, we briefly discussed the dynamics of the "choosy" role between the sexes, which exemplifies this innovative understanding in evolutionary biology. While male exaggerated traits have been extensively studied, female exaggerated traits have received comparatively less attention. This raises a **second** question: what novel insights can we gain by investigating the evolution of female exaggerated traits?

Furthermore, the evolution of honest signaling and its associated costs has predominantly been studied under laboratory conditions, often through enforced artificial selection. This leads to a **third** question: what novel tools can be employed to provide new insights into these evolutionary processes? The evolution of signaling, particularly chemical communication, is crucial for understanding multimodal signaling components. Contact pheromones, as enhanced short-distance sensory cues, represent an intriguing system for investigation. This dissertation aims to address these questions and provide novel insights into these important but missing questions.

To tackle the aforementioned questions, *Drosophila* species present distinct advantages. First, the genus *Drosophila*, belonging to the family Drosophilidae, comprises approximately 1,450 species, including the well-studied model organism *D. melanogaster* (Markow and O'grady 2005b). The diverse biology and ecological roles of

these species serve as essential resources for investigating evolutionary processes. Second, the tools developed for *Drosophila* research are well-established and versatile, making it easy to conduct various bioassays and genetic studies. This adaptability enhances the potential for innovative research in evolutionary biology.

1.4 Courtship behavior of *Drosophila*

Courtship behavior in *Drosophila* males generally follows a stereotypical sequence; however, significant diversity in courtship behavior exists across species (Wen and Li 2011). Typical *Drosophila* courtship behavior involves utilization of an integration of visual, acoustic, and chemical cues (Wicker-Thomas 2007). Various behavioral components are observed across *Drosophila* species, including orientation, tapping, wing displays (such as flicking, waving, semaphoring, scissoring, and vibrating), circling, chasing, licking, and mounting (Spieth 1974).

Different sensory modalities are thought to be involved in each step of the courtship ritual. For instance, the courtship begins when a male encounters a female by orienting toward her. The male utilizes his compound eyes to detect dynamic signals (such as movements and locomotor actions) and static signals (such as body pigmentation and color) from the female, which sequentially stimulate his subsequent behaviors (Cook 1973; Cook 1979). The male then taps the female's dorsal abdomen with his front legs, where contact pheromones are perceived. Gustatory signals are perceived through gustatory receptors on his foreleg tarsi during this action. To pursue copulation, the male will chase the female, display his wings, and vibrate (Bennet-Clark *et al.* 1976; Von Schilcher 1976). This may also be accompanied by the male vibrating his abdomen to produce substrate-borne sounds transmitted to the female (Fabre *et al.*

2012). Additionally, females can produce sounds prior to courtship, which guide the male in navigating toward her (Ejima and Griffith 2008). Thus, acoustic signals, perceived through auditory organs, are universally employed by both sexes. The male then extends his proboscis to lick her genitalia and attempts to copulate by bending his abdomen, utilizing both gustatory and tactile signals (**Table 1.1**).

While a range of stimuli is proposed for *D. melanogaster*, the utilization of sensory modalities shows taxonomic divergence in other non-*melanogaster* species across the *Drosophila* genus. Species closely related to *D. melanogaster* within the *melanogaster* subgroup exhibit highly similar courtship rituals and sensory components (Cobb *et al.* 1989). Conversely, species in the *montium* subgroup typically display wing vibrations after mounting and do not engage in the licking stage (Spieth 1952; Hoikkala and Crossley 2000). Furthermore, in the *mulleri* subgroup, independent evolution of courtship rituals has been observed; for instance, *D. leonis* and *D. nigrospiracula* do not incorporate tapping in their courtship behavior (Alonso-Pimentel *et al.* 1995). Notably, no records indicate that *D. pegasa* exhibits any of the aforementioned courtship behaviors (Wasserman *et al.* 1971). The diversification of behavioral responses during courtship across *Drosophila* species provides abundant research opportunities but also presents challenges for relevant comparative studies.

1.5 *Drosophila* cuticular hydrocarbons (CHCs) as mating signals

Among the various mating signals, hydrocarbons deposited on the insect cuticle can function as contact pheromones, perceived during tapping through the gustatory organs, foreleg tarsi, and proboscis. Cuticular hydrocarbons (CHCs) play a critical role in chemical communication, particularly in mating behavior (Stocker 1994; Boll and Noll

2002; Bray and Amrein 2003). A well-studied example is the comparison between two closely related species, *D. melanogaster* and *D. simulans*. *Drosophila melanogaster* exhibits qualitative differences in CHC profiles between the sexes, with females producing sex-specific CHCs such as 7,11-heptacosadiene (7,11-HD), while *D. simulans* shows no qualitative differences in CHC compositions between sexes (Jallon and David 1987).

The roles of CHCs as mating signals have been extensively investigated in this species pair. The female-specific 7,11-HD in *D. melanogaster* serves as an aphrodisiac to conspecific males, eliciting dose-dependent responses in male courtship rituals that convey information about female quality (Antony *et al.* 1985). Conversely, this compound acts as an anti-aphrodisiac, discouraging courtship from *D. simulans* males, thereby playing a role in species recognition (Seeholzer *et al.* 2018; Ahmed *et al.* 2019).

Diversity in CHC profiles can be observed across *Drosophila* species at the population, sex, and individual levels. CHC profiles consist of a blend of components with varying chemical structures, including differences in carbon chain length, branching patterns, and the number and position of double bonds. Generally, *Drosophila* CHCs can be categorized into n-alkanes, monoenes, dienes, alkatrienes, and methyl-branched alkanes (**Figure 1.2**). For instance, a population-level comparison reveals differences between two strains of *D. melanogaster*, Canton-S and Tai-Y, where 7-tricosene is the primary CHC component in Canton-S males but is rarely produced by Tai-Y males (Scott 1994).

Sexually dimorphic CHC profiles can be species-specific, exhibiting qualitative and/or quantitative differences. Individual-level variations in CHC profiles can be

attributed to phenotypic plasticity, influenced by factors such as age, diet, mating status, health, and other physiological conditions (Cortot *et al.* 2022). In the context of contact pheromones, intraindividual variations in CHC profiles may also serve as honest signals reflecting sexual attractiveness, contributing to mate assessment and choice. For example, short-chain CHCs induced by a high-yeast diet can result in less attractive females (Fedina *et al.* 2012). Variations in CHC profiles arise from both genotypic and phenotypic factors, and deciphering the evolution of divergent CHC blends will enhance our understanding of the evolution of chemical signaling.

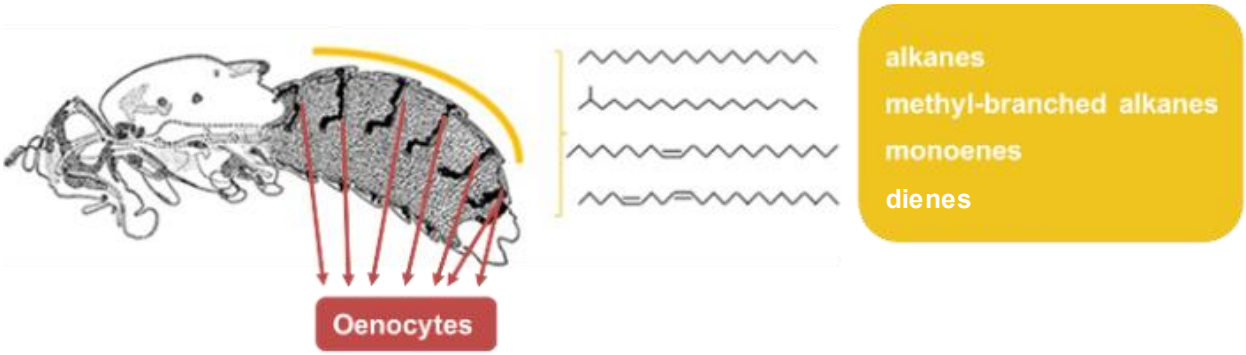


Figure 1.2 Side view of oenocytes (Left) and **Types of cuticular hydrocarbons in *D. melanogaster* (Right)** adapted from (Chung and Carroll 2015).

However, there is still a lack of understanding regarding whether the sexual dimorphism observed in CHC profiles is driven by or correlated with the role of CHCs in mate recognition and mate choice. Further comparative studies exploring the relationship between sexual dimorphism and signaling roles of CHCs are needed.

1.6 CHC biosynthesis in *Drosophila*

In *Drosophila* species, cuticular hydrocarbons (CHCs) are synthesized within oenocytes located beneath the dorsal abdomen cuticle and subsequently transported to the insect cuticle (Ferveur *et al.* 1997; Schal *et al.* 1998; Fan *et al.* 2013) (**Figure 1.2, Figure**

1.3A). The biochemical reactions and pathways involved in CHC biosynthesis have been elucidated through studies utilizing radiolabeled precursors (Dillwith *et al.* 1981; Dillwith *et al.* 1982; Blomquist *et al.* 1987). A series of enzymes involved in the fatty acid synthesis pathway have been implicated in CHC production. Fatty acyl-CoA molecules may undergo desaturation processes, which are facilitated by specific enzymatic proteins known as desaturases. Elongases facilitate chain-length elongation, resulting in the production of long-chain fatty acyl-CoA. Additionally, reductases convert fatty acyl-CoA to aldehydes, while a single cytochrome P450 enzyme catalyzes the decarboxylation process to yield final products as hydrocarbons (Blomquist and Ginzel 2021).

The genes related to CHC synthesis are rapidly evolving across *Drosophila* species, making the prediction of genes responsible for specific CHC blends challenging (Finet *et al.* 2019). In *D. melanogaster*, however, the genes involved in CHC synthesis have been well characterized. For instance, two key enzymes are essential for the production of the female-specific CHC, 7,11-heptacosadiene (7,11-HD). The desaturase gene *DesatF* is responsible for introducing a second double bond between the 7th and 8th carbons (Chertemps *et al.* 2006). Additionally, the elongase gene *EloF* has been suggested to specifically target the production of 27-carbon and 29-carbon dienes (Chertemps *et al.* 2007).

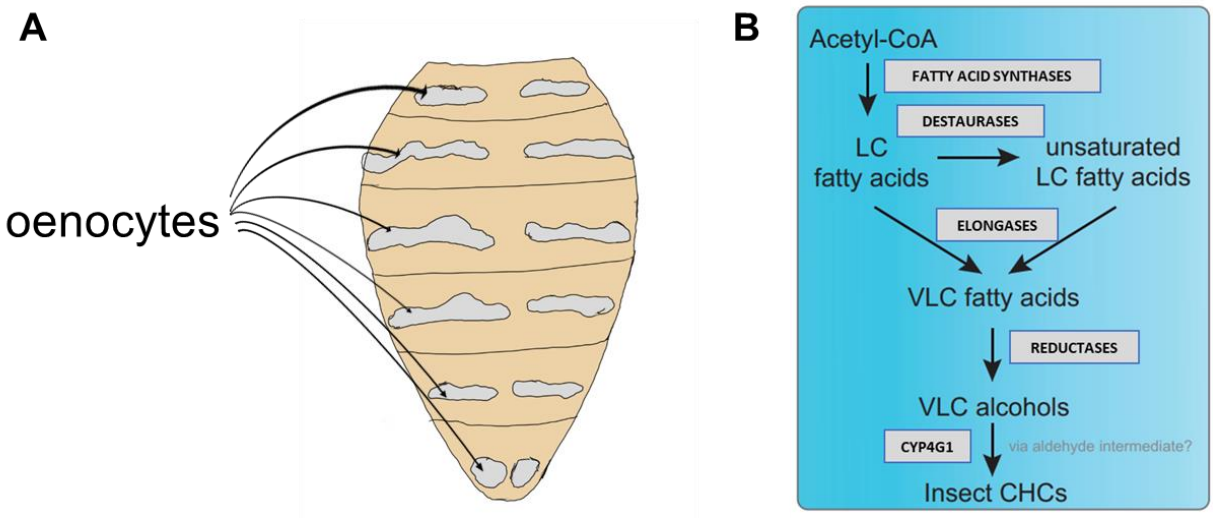


Figure 1.3 (A) Vertical view of dissected *Drosophila* oenocytes and (B) Biosynthesis pathways of *Drosophila* CHCs adapted from (Finet *et al.* 2019).

Genetic variations involved in CHC synthesis are precisely regulated, contributing to the variations and diversity of CHC profiles at multiple levels. In *D. melanogaster*, interpopulational variations in CHCs can be observed in the positioning of double bonds. For instance, in the Canton-S strain, the primary CHC components are 7-tricosene (7-T) in males and 7,11-heptacosadiene (7,11-HD) in females, establishing the 7-positioned double bond as a characteristic feature of this strain's CHC profile. In contrast, the Tai-Y strain exhibits a female-specific polymorphism where the major compound 7,11-heptacosadiene (7,11-HD) is replaced by 5,9-heptacosadiene (5,9-HD). Here, females produce low levels of 7,11-HD but high levels of its positional isomer, 5,9-HD (Scott 1994). Previous genetic studies have suggested that the female-specific expression of the desaturase gene *desat2* may account for this polymorphism. *Desat2* encodes a desaturation reaction specific to the 9-position, leading to the production of 9-positioned unsaturated hydrocarbons in females (Coyne *et al.* 1999; Dallerac *et al.* 2000).

Sex-specific expression patterns are also observed in other genes related to CHC synthesis. Both *eloF* and *desatF* are named for their female-biased expression patterns in *D. melanogaster*, and both contribute to the sexual dimorphism of CHC profiles. This has raised questions regarding the evolutionary mechanisms underlying such sex-specific expression patterns. A comprehensive study investigated *cis*-regulatory changes in the orthologs of *desatF* across *Drosophila* species. The findings indicated rapid evolution and frequent alterations in the *cis*-regulatory elements of these alleles, including gene inactivation, losses of gene expression, and transitions in sex-specific expression patterns. Furthermore, the study suggested that the sex-specific expression of these genes may arise from the gain of a binding site for DOUBLESEX, a transcription factor involved in sex determination in *Drosophila* species (Shirangi *et al.* 2009).

This established understanding of genetic variations and the evolution of sex-specific expression of CHC synthesis genes provides both technical feasibility and theoretical knowledge for future studies. However, whether other genetic mechanisms underlying the evolution of these sex-specific gene expression patterns remains unknown. Given the current insights into female-biased expression patterns, novel understandings of female exaggerated traits could be further explored.

1.7 Other roles of *Drosophila* CHCs

Cuticular hydrocarbon (CHC) synthesis can incur significant costs due to the involvement of additional enzymes and the gain of highly specific and precise gene expression patterns. It has been suggested that methyl-branched alkanes, a common group within CHC blends, require greater metabolic investment for production compared

to linear-chain alkanes (Nelson 1993). Beyond their role in mating signals, CHCs are also essential constituents of the *Drosophila* cuticular wax layer, functioning primarily for waterproofing (Chung and Carroll 2015). More specifically, the dual roles of methyl-branched alkanes in influencing both desiccation resistance and mate choice have been documented in *Drosophila* (Chung and Carroll 2015). The evolution of CHCs is theoretically predicted to be driven by interactions between natural selection and sexual selection (Blows 2002). Consequently, an equilibrium is hypothesized to exist between the evolution of these dual roles of CHC blends, with elaboration on one role—such as mating signals—potentially eliciting antagonistic or synergistic responses in the evolution of the other role.

In addition to the equilibrium established between desiccation resistance and mating signals, the evolution of CHC profiles may also involve trade-offs with other primary traits. As previously mentioned, the sensory components involved in mate choice can honestly signal the sender's fecundity, mating status, fitness, and related conditions (Fedina *et al.* 2012). Selection pressures related to desiccation resistance have been shown to result in decreased fecundity (Kwan *et al.* 2008), prolonged development time, reduced adult viability (Chippindale *et al.* 1998), and increased longevity (Rose *et al.* 1992). Generating mating signals can also be costly, potentially offsetting energy invested in development and fecundity. Given that CHCs serve both roles, understanding the trade-offs associated with the production of costly CHC components is critical for our comprehension of adaptation and sexual selection.

However, two key questions remain unanswered: 1) How do CHCs contribute to the evolution of the equilibrium between desiccation resistance and mating signals? and

2) What are the trade-offs associated with costly CHC synthesis? Investigating the evolution of honest signaling is particularly significant, especially regarding the potential costs of signaling. While previous studies have utilized the selection of *Drosophila* strains under laboratory conditions to predict trends, the application of advanced genetic tools will offer novel insights into these questions.

1.8 Summary and research objectives

In summary, in this dissertation, by using CHCs in *Drosophila* as a model, I aim to investigate the evolution of sexual dimorphism of CHCs and their related roles as mating signals, with the following aims: **1)** to determine the correlation between mating signals and sexual dimorphism, **2)** to determine the genetic mechanism underlying female exaggerated traits, **3)** to assess the potential trade-offs in costly CHC production.

Through these aims, this dissertation seeks to provide novel insights into the evolution of sexual dimorphism and mating signals in *Drosophila*.

CHAPTER 2: SEXUAL DIMORPHISM IN CHCS AND THEIR ROLES IN *DROSOPHILA* COURTSHIP

I would like to acknowledge the following colleagues, since this chapter could not have been accomplished without the contributions made by them.

Dr. Mei Luo

- Assisted in performing non-choice mating assay

Dr. Rajanikanth Chowdanayaka

- Assisted in checking the results of non-choice mating assay

Nadia Sbisa

- Assisted in checking the results of non-choice mating assay

Dr. Zinan Wang

- Assisted in analyzing CHC profiles of the perfumed flies

2.1 Introduction

Species in *Drosophila* utilize multimodal sensory modalities for mate assessment, to enhance reproductive success (Mitoyen *et al.* 2019). During the process of mate assessment, two components are likely to occur, mate recognition and mate choice. Mate recognition is the process that consists of identifying and assessing a potential mate (Ryan and Rand 1993), which further can be divided into two separate components: species recognition and sex recognition within species (Blows and Allan 1998b). Mate choice is the stage where focal individuals can get access to multiple potential mates and make final mate decisions. Recently, dynamic sex role where both sexes can be choosy to conduct mate choice has been investigated and discussed (Edward and Chapman 2011). Both mate recognition and mate choice evolved as a

consequence to maximize reproductive success through investments into reproduction with suitable and better mates (Bateman 1948).

Mating signals can serve the roles for conveying complex levels of information to facilitate mate recognition and mate choice. A series of sensory modalities can be used by individual *Drosophila* to conduct both processes and make mating decisions (**Table 1.1**). Signals used for mate recognition and mate choice have been studied in *Drosophila* species, but few studies have investigated the separate roles of mating signals involved in mate recognition and mate choice. The following question was raised: whether the mating signal traits involved in both processes are distinct or the same.

In the 1990s, divergent signal functions and corresponding evolutionary processes were proposed, driven by intra- and interspecific individual preferences (Andersson 1994). Recently, the concept of mating signals being processed and evaluated serially has been proposed. In this model, “static” signal components are likely to be processed first and often evolve during the process of speciation. “Dynamic” components with high interindividual variations are processed secondarily. The proposed model was tested on the cricket calling song, and the author suggested that further tests, with potentially generalized results across taxa, are necessary (Gray 2022).

Among all the sensory modalities used in *Drosophila*, determining the specific roles of mating signals has been of great importance in understanding the dynamic evolution of multimodal components. The functions of specific mating signals have been tested in specific species, but not in a comparative study across *Drosophila.* For

example, *Drosophila* courtship song includes two distinct components, with one component primarily involved in species recognition, and another component evolved rapidly under mate choice and sexual selection. The independent loss of both components has been suggested in species from the *repleta* group (Ewing and Miyan 1986). In contrast, a morphological visual signal, head-width, is the only sensory modality used by *D. heteroneura* for mate choice and sexual selection. There is no evidence to support such a trait is also contributing to mate recognition as premating reproductive isolation barrier between species (Boake *et al.* 1997).

In addition to acoustic and visual signals, CHCs as gustatory cues perceived during courtship rituals, are suggested to be involved in both processes of mate recognition and mate choice within and between two *Drosophila* species (Blows and Allan 1998a). However, comparative studies across *Drosophila* genera are required to understand the role of CHCs in courtship are needed.

Furthermore, unlike other sensory modalities, CHC profiles show variations between sexes at different levels, which makes the chemical signal unique model in the *Drosophila* multimodal system. Firstly, within CHC profiles, one or more CHC components can be sex specific. Secondly, with the presence of unsaturated double bonds or branches at different locations on the carbon chains, some isomers of the same CHC components can be produced only by males or females. Lastly, females and males may produce the same CHC components, but some components may show differences in quantitative amounts between sexes. The levels or types of differences in CHC sexual dimorphism are different among species, and there are no taxonomic similarities that have been suggested. This left the understanding the different displays

of sexual dimorphism in *Drosophila* CHCs unresolved.

Moreover, with different displays of CHC profiles across *Drosophila* species, whether such variations are correlated with the role of CHCs in courtship is unknown. Degree of sexual dimorphism has been commonly used in comparative studies focusing on morphological traits (Ralls 1977; Arak 1988; Ralls and Mesnick 2009; Zorba *et al.* 2011). Understanding about degrees of sexual dimorphism in physiological traits, coupled with understanding the evolution of such traits under selection or their sex-dependent functions, is still lacking.

In this study, we selected nine species to investigate the roles of CHCs in courtship in each species (*D. melanogaster*, *D. simulans*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. willistoni*, *D. mojavensis*, and *D. repleta*). The nine species were selected across the *Drosophila* genus, spanning both the *Sophophora* and *Drosophila* subgenus and representing different lineages. Quantifying sexual dimorphism by degrees of sexual dimorphism will provide novel tools to identify potential correlation pattern across phylogeny correspondingly. Additionally, investigating whether we can use degrees of sexual dimorphism to predict use of CHCs in male courtship is also to contribute our knowledge in the evolution of mating signals.

2.2 Results

2.2.1 Variations in degrees of CHC sexual dimorphism across *Drosophila* species

We first determined the levels of CHC sexual dimorphism across the nine *Drosophila* species. The CHC profiles of both males and females of these species have been described in our previous work (Wang *et al.* 2022). We adapted the previously

described data with further specifying different isomers, and used the Bray-Curtis dissimilarity statistic to determine the dissimilarity of CHC profiles between the male and female CHCs of each species (**Figure 2.1**). Across these nine species, the Bray-Curtis dissimilarity index ranged from 0.08 in *D. pseudoobscura*, where the CHC profiles of both sexes in this species appeared to be the least sexually dimorphic to 0.95 in *D. erecta* where the degree of CHC dimorphism was maximum (**Figure 2.1**). In *D. erecta*, the female CHC profile comprises of 26- to 33-carbon long CHCs, including two long chained dienes, C31:2 and C33:2, while the male profile comprises of CHCs with 21- to 28-carbon long CHCs without any dienes. Among these nine species, five species (*D. simulans, D. yakuba, D. ananassae, D. pseudoobscura* and *D. mojavensis*) do not possess CHC components that are unique to one sex. The other four species (*D. melanogaster*, *D. erecta*, *D. willistoni*, and *D. repleta*) possess sex specific CHC components or isomers either in one or both sexes (**Table S2.1**).

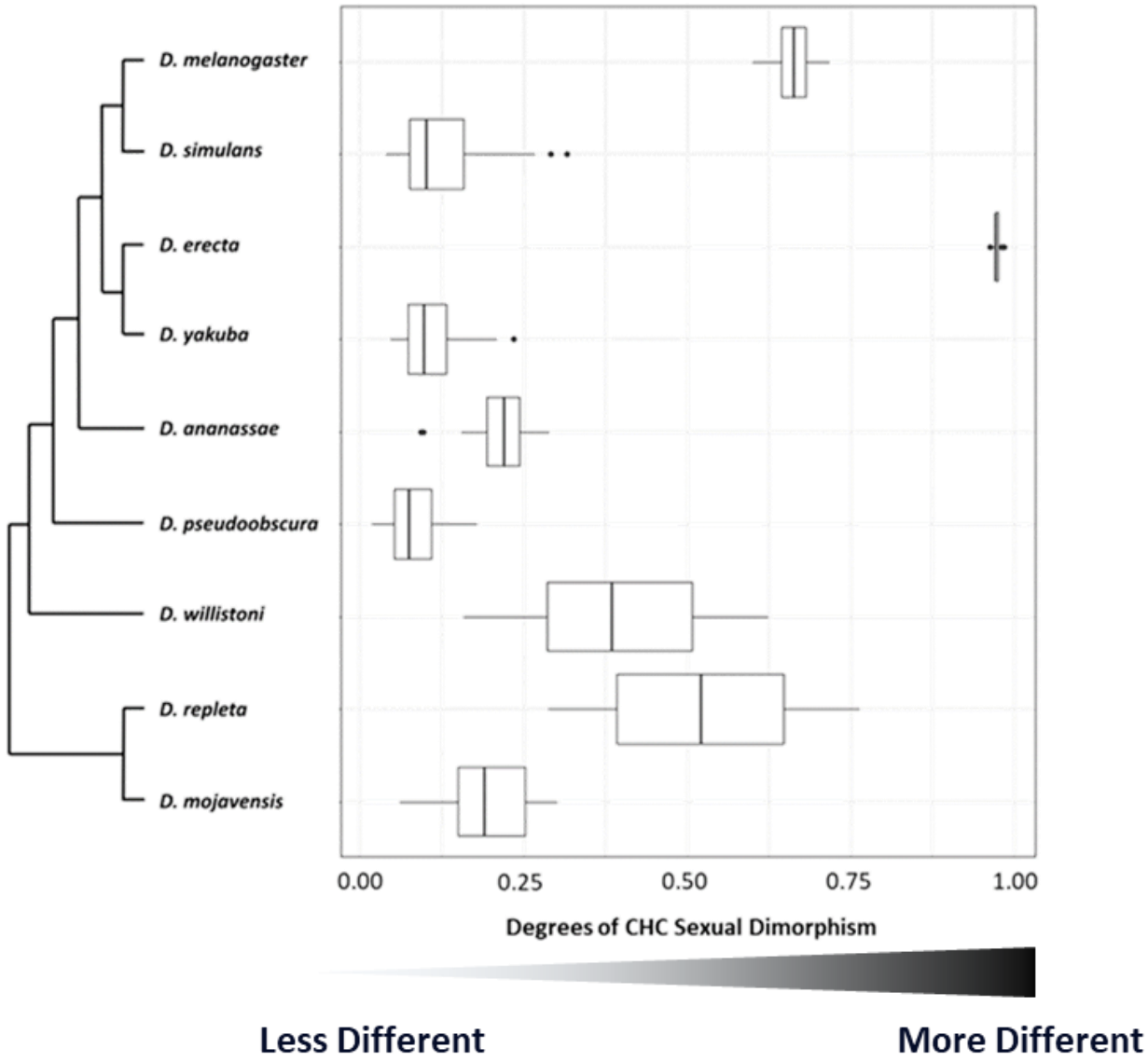


Figure 2.1 Divergent degrees of CHC sexual dimorphism across *Drosophila* species. Sexual dimorphism in CHC profiles across nine *Drosophila* species were calculated using the Bray-Curtis dissimilarity index (0 = no dissimilarity, 1 = highly dissimilar). Mean values range from 0.08 (*D. pseudoobscura*) to 0.97 (*D. erecta*), 0.66 (*D. melanogaster*), 0.12 (*D. simulans*), 0.11 (*D. yakuba*), 0.21 (*D. ananassae*), 0.39 (*D. willistoni*), 0.20 (*D. mojavensis*), 0.53 (*D. repleta*).

2.2.2 Effects of foreleg tarsi removal on male courtship interests

Drosophila males perceive female CHCs using specific gustatory receptors on the foreleg tarsi by tapping on the female abdomen (Fan *et al.* 2013; Ahmed *et al.*

2019). After tapping, wing display and copulation trials are followed as a continuous courtship ritual in serial order (**Table 1.1**). To determine whether CHC inputs are crucial for continued courtship interest, we removed the foreleg tarsi from the males of these nine species and assayed for changes in male courtship behavior towards conspecific females. We used the presence or absence of wing display as the indicator of male ‘s continuous courtship interest, as all these species utilize wing display (vibrational courtship song) as part of their sequential courtship ritual after perceiving chemosensory cues by tapping (Markow and O'grady 2005a).

Our results showed that *D. melanogaster* males did not show any significant change in continuous courtship interests (χ^2=0.27, p = 0.60). In contrast, *D. simulans* males showed a significant decrease in male continuous courtship interests after tarsi removal (χ^2=10.16, p < 0.01). Males of the remaining six species (*D. yakuba, D. erecta, D. ananassae, D. pseudoobscura, D. willistoni,* and *D. mojavensis*) also exhibited significantly reduced male courtship interests following tarsi removal, with the exception of *D. repleta* (**Figure 2.2**).

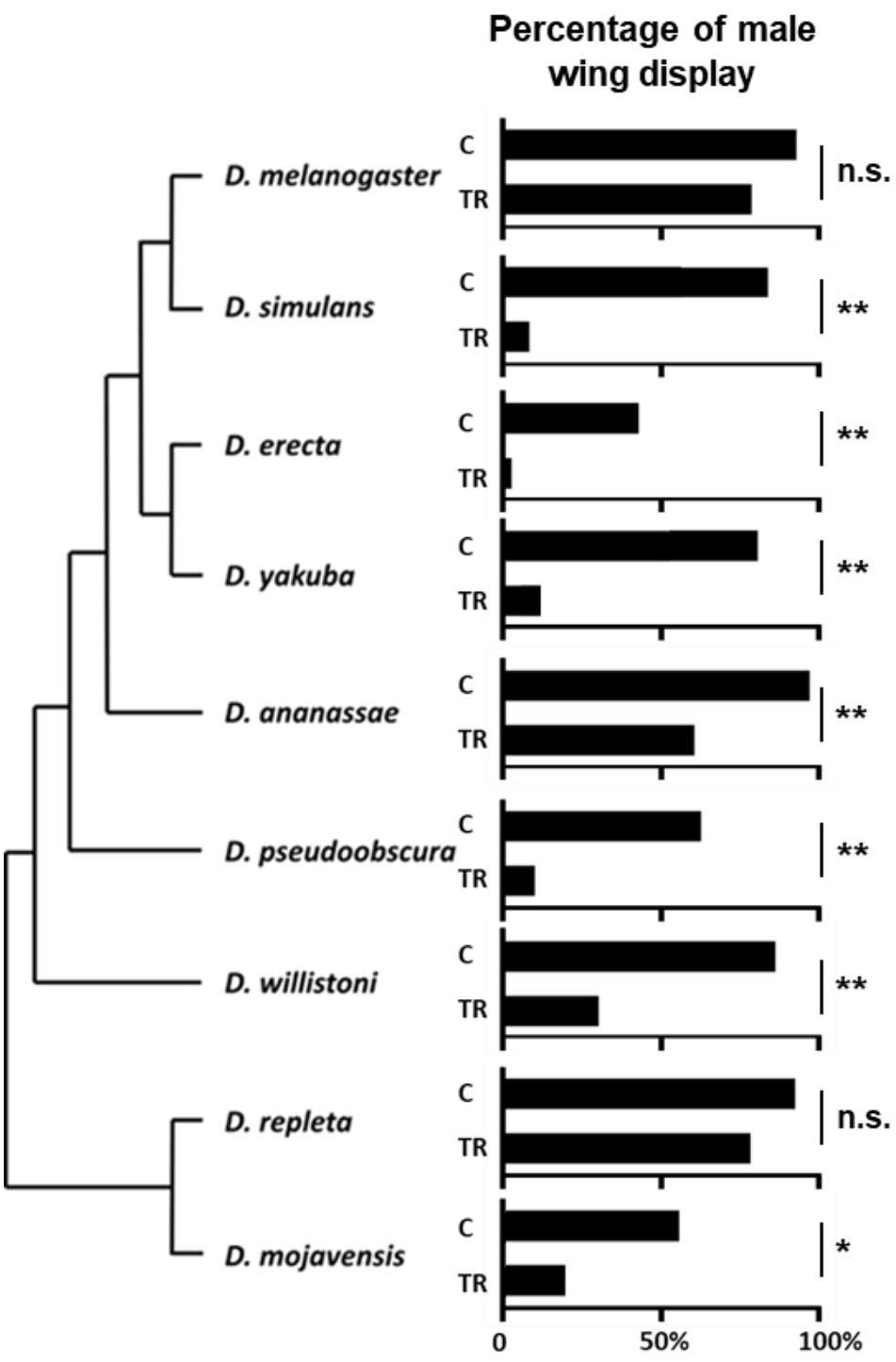


Figure 2.2 Tarsi removal affected male courtship interests in most *Drosophila* species. Wing display % in no-choice mating assays. Loss of tarsi leads to significant reduction in courtship interests across *Drosophila* species, except *D. melanogaster* and *D. repleta.* C = Control, TR = Tarsi Removed. The *Chi*-Square test was used to determine any significant differences in wing displays. n.s = not significant *$p < 0.05$; **$p < 0.01$.

To exclude the possibility that the decrease in courtship interests exhibited by some of these species was due to other potential reasons caused by tarsi removal (e.g., injury) rather than the inability of males perceiving female CHCs, we complemented the results further with a CHC perfuming assay. Female CHCs from the two species with the highest and lowest degrees of CHC sexual dimorphism, *D. erecta* and *D. pseudoobscura* were extracted and coated on genetically modified *D. melanogaster* female flies without CHCs (CHC- *D. melanogaster*). Experiments showed that *D. erecta* and *D. pseudoobscura* male flies display courtship interests towards these CHC- *D. melanogaster* female flies that have been coated with conspecific female CHCs at a significantly higher rate compared to uncoated CHC- *D. melanogaster* females (**Figure 2.3**). This suggests that CHC inputs are important for continuous male courtship interests in these two species and would even lead to male attempting copulation towards heterospecific females if the CHC blend is correct.

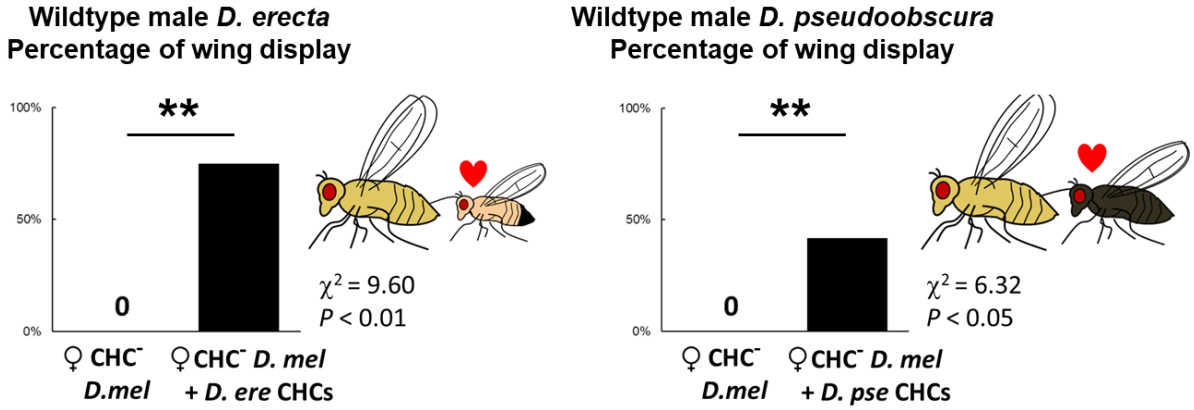


Figure 2.3 Detection of CHC is important in maintaining *Drosophila* courtship interests. Female CHCs from *D. erecta* and *D. pseudoobscura* coated on female CHC- *D. melanogaster* are able to elicit courtship interests from conspecific males. *Chi*-Square test was used to determine any significant differences in wing displays. *$p < 0.05$; **$p < 0.01$.

2.2.3 Foreleg tarsi removal resulted in longer courtship latency in *D. melanogaster* and *D. repleta*

As for the two *Drosophila* species *D. melanogaster* and *D. repleta* that did not show significant changes in courtship interests after tarsi-removal, we further tested other possible roles of CHCs in their courtship. We hypothesized that although CHCs are not essential for continuous courtship interests, CHCs are important in reducing male courtship latency. This hypothesis was based on that CHCs can possibly convey information of mate quality, and thus contribute to more efficient mate decision process done by males. For both species, we measured and compared courtship latency between tarsi removed males and wild type males. Mean courtship latency of *D. melanogaster* wild type males is 3.87 min, compared with 10.45 min for tarsi removed males ($t = 3.76$, df = 15.35, $p < 0.01$; **Figure 2.4A**). Mean courtship latency of *D. repleta* wild type males is 4.62 min, compared with 18.63 min for tarsi removed males ($t = 2.84$, df = 21.35, $p < 0.01$; **Figure 2.4B**). These results support our hypothesis, suggesting that while CHCs are not directly responsible for maintaining continuous courtship interests in these two species, the perception of CHC is contributing to a faster reproductive static with shorter mating latency and thus may be involved in communicating mate quality in these two species.

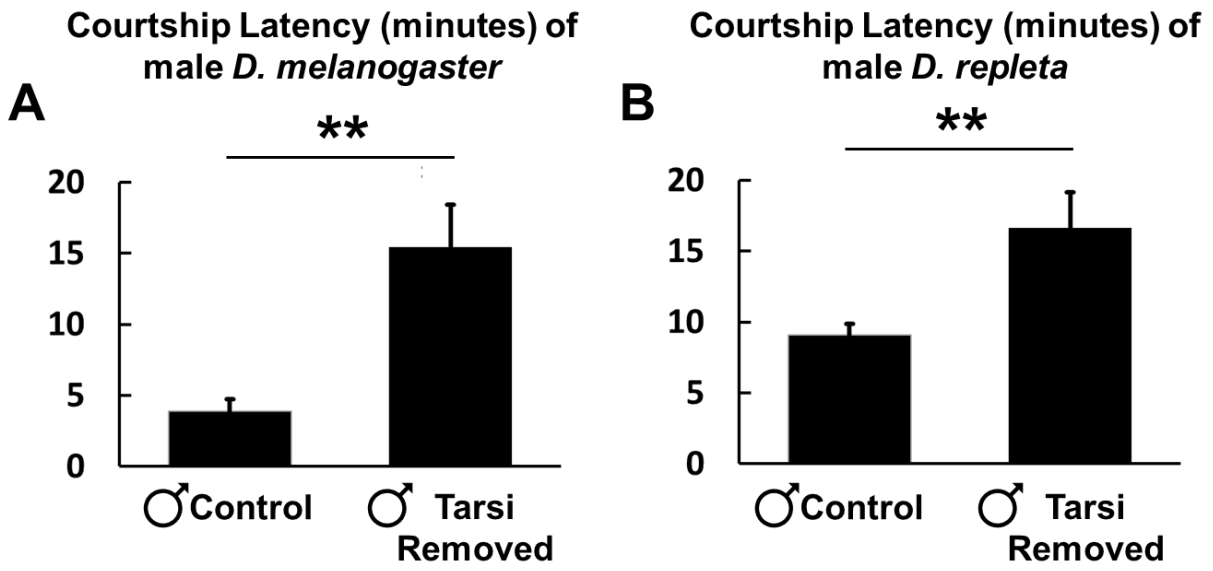


Figure 2.4 Loss of detection of CHCs resulted in increased courtship latency in both *D. melanogaster* (A) and *D. repleta* (B). Courtship latency is the duration between the introduction of test male to the unmated female and the first courtship wing display by the males. The student's t-test was used to determine any significant differences in courtship latency. ***p* < 0.01.

2.2.4 No correlation between the degree of CHC sexual dimorphism and use of female CHCs as signals for courtship

To determine if the degree of sexual dimorphism in CHC profiles can be used to indicate the importance in male courtship, we performed a simple linear regression analysis to determine if the degree of CHC sexual dimorphism could explain changes in courtship behavior after tarsi removal among the selected species. The results of the regression indicated degrees of CHC sexual dimorphism can only explain 9.53% of the variation in utilization of CHCs for male courtship interests [F (1,7) = 0.7222, p = 0.4235]. Our analysis showed that there is not enough evidence to support a correlation between levels of CHC sexual dimorphism and the change in courtship interests after tarsi removal (**Figure 2.5**). This suggests that the degree of sexual dimorphism in CHC profiles may not be informative for predicting whether female CHCs are important for

maintaining courtship interests across the *Drosophila* species studied.

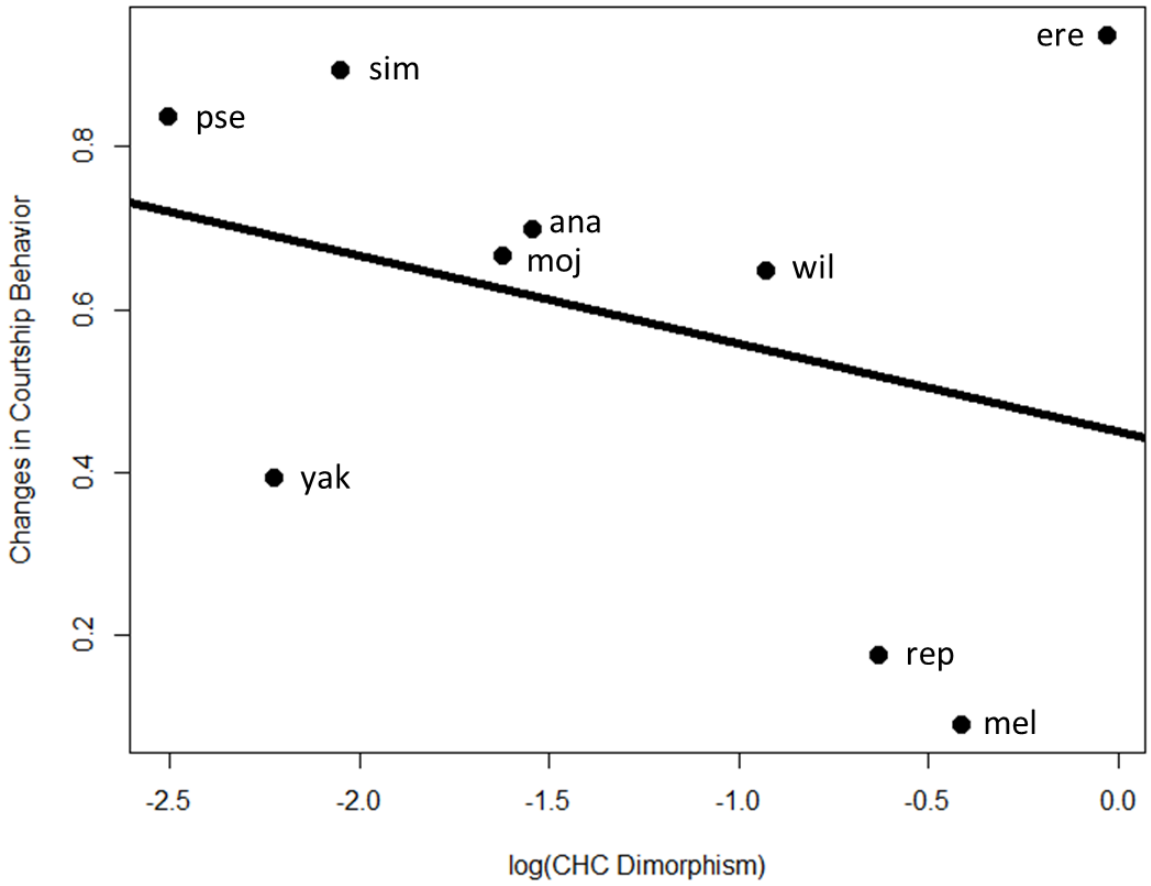


Figure 2.5 Degree of sexual dimorphism in CHC profiles is not enough to be used to inform if female CHCs are important for male courtship. Regression of levels of CHCs sexual differences and courtship interests changes after tarsal removal in males. Changes in Courtship Interests ~ 0.4502 + -0.1081*log (Degrees of CHC dimorphism). *mel = D. melanogaster*, *sim = D. simulans*, *yak = D. yakuba*, *ere = D. erecta*, *ana = D. ananassae*, *pse = D. pseudoobscura*, *wil = D. willistoni*, *moj = D. mojavensis*, *rep = D. repleta.*

2.3 Discussion

Mate recognition and mate choice have been suggested to be discrete processes for the perception and evaluation by signal perceivers, where mating signals are evolved distinctively (Blows and Allan 1998b). Recent studies have argued this with testing the model of “serial processing and order-of-operations”, where mating signals are perceived at first, followed by a series of recognizing (mate recognition) and choosing

(sexual selection) mates. The importance of determining the evolution of mating signal with differentiating potential roles becomes more crucial for understanding evolutionary divergence (Gray 2022).

In this study, we selected nine *Drosophila* species across phylogeny and investigated the role of CHCs in courtship. Firstly, we found qualitative and quantitative differences in sexual dimorphism across the *Drosophila* genus. The CHC dissimilarities range from 0.08 in *D. pseudoobscura* to 0.95 in *D. erecta.* The diversity in CHC degrees of sexual dimorphism indicates the complicated evolutionary processes occurred on this trait. In addition, further individual study in the evolution of sexual dimorphism in each species is of importance in further understanding independent evolution across different lineages, especially the evolution of the most CHC sexual dimorphic species, *D. erecta.*

Our results suggest that in most of the selected species, perception of CHC is necessary for males to maintain courtship interests, moving onto wing display as a serial matter. This suggests female CHCs in these species play a crucial role in signal recognition responsible for primary courtship interest. Two test species, *D. melanogaster* and *D. repleta* do not require CHC input for occurrence of wing display and following steps in the courtship rituals. However, the loss of perception of CHCs does result in a decrease in courtship latency in the corresponding species, suggesting the importance of CHC inputs in efficient courtship display and further enhanced reproductive fitness of the population. It is likely that CHCs in these species convey information about the quality of potential mates. An example is the female-specific 7,11-HD in *D. melanogaster*, which is an aphrodisiac that can increase male courtship occurrence in this species (Antony *et al.* 1985), but CHC is not necessary for courtship

initiation in this species (Fan *et al.* 2013; Shahandeh *et al.* 2018; Ahmed *et al.* 2019). We suggest that, other signals such as visual, acoustic, and olfactory signals (Colyott *et al.* 2016) may be more important for the mate decision in *D. melanogaster* and *D. repleta*. Or different reproductive tactics independently evolved in these species, where multimodal signal components are important in efficient signal recognition, but none of the individual signals are necessary for initiating courtship.

The important role of CHCs in courtship can be also supported by the perfuming assay, where the coated conspecific CHC blend on to *D. melanogaster females* can still initiate courtship behavior of *D. erecta* and *D. pseudoobscura* males. This suggests that the perfumed CHC blends can weaken the established premating reproductive isolation barrier, and that other mating sensory modalities are not necessary basis for males making mate decision.

In conclusion, our experiments and analyses do not support the hypothesis that the degree of CHC sexual dimorphism is predictive of the roles of CHCs as mating signals for courtship. This further provides empirical evidence to suggest that the rapid evolution of sexual dimorphism may be caused by or constrained by other important pleiotropic functions of the traits, not only resulting from the role of mating signal recognition.

2.4 Materials and Methods

Drosophila species

D. simulans (14021-0251.195), *D. yakuba* (14021-0261-01), *D. erecta (*14021-0224.01), *D. ananassae (*14024-0371.13), *D. pseudoobscura* (14011-0121.94), *D. willistoni* (14030-0811.24), *D. mojavensis* (15081-1352.10), and *D. repleta* (15084-1611.13) were

obtained from the National *Drosophila* Species Stock Center. *D. melanogaster* was a gift from Dr. Sean Carroll's lab (University of Maryland). All species were reared on standard cornmeal medium (Flystuff 66-121 Nutri-Fly Bloomington Formulation) at 25°C, except for *D. pseudoobscura* which was reared at 18°C, which has been suggested to be the optimal development condition for such species.

Foreleg tarsi removal and no-choice courtship assay

To test whether males of different *Drosophila* species utilize CHCs as signals for mate recognition, tarsal segment of the forelegs from adult unmated males were removed using micro-scissors under light anesthesia 24 hours before these males were used for courtship assays. No-choice mating assays were performed at 25°C (except for *D. pseudoobscura* at 18°C) in the morning between a single unmated sexually mature female and a single unmated sexually mature male either with tarsi removed or intact tarsi. The age of sexual maturity was predetermined from pilot tests (**Table S2.2**) and is based on a previous study (Pitnick *et al.* 1995). To begin each assay, a single female and single male were separately aspirated into each well of a Plexiglas mating chamber (Ejima and Griffith 2011). See Table S2.3 for sample sizes for no-choice mating assays. The mating pairs were recorded for one hour using a cell phone camera and courtship occurrences were manually determined. Wing display was tested for the males, to determine their continuous courtship interests, as part of their sequential courtship ritual after perceiving chemosensory cues by tapping (Markow and O'grady 2005a). Courtship latency was determined as the duration between the introduction of test male to the unmated female and the first courtship wing display by the males. Each recorded video was checked, and the presence of wing display of each test male was determined, by

three different observers individually.

CHC- flies and CHC coating

To generate CHC- flies, we crossed the 5′*mFAS*-GAL4 driver line (which expresses GAL4 in adult oenocytes) with the UAS-*Cyp4g1* RNAi line as described in a previous study (Wang *et al.* 2022). For the coating experiments, CHCs from fifty sexually matured unmated females (either *D. erecta* or *D. pseudoobscura*) were extracted using 600-800 µl hexane and pipetted into a 2ml glass vial. The hexane was evaporated under a nitrogen gas flow, leaving the compound as a residue coating the bottom of the vial. Groups of ten CHC- *D. melanogaster* unmated female flies were transferred to the coated vials and subjected to two high vortex pulses lasting 20s, with one 20s pause between two pulses. Six flies from each group were used for behavioral tests and the remaining four flies were subjected to GC-MS analysis protocols as described previously (Wang *et al.* 2022; Wang *et al.* 2023) to determine effective transfer of the CHC extracts onto the flies.

Statistical Analyses

The CHC measurements for the different *Drosophila* species and sex were adapted from a previous study, with further specifying different isomers in this study (Wang *et al.* 2022). In this study, we use the Bray–Curtis dissimilarity statistics (Taft *et al.* 2015) to analyze the degree of similarity/dissimilarity between male and female CHC profiles of these species. Ranging from 0 to 1, the Bray–Curtis dissimilarity statistic measures the dissimilarity between two datasets (0 = highly similar, 1 = highly dissimilar), and we use this statistic as a measurement of the level of CHC sexual dimorphism in a given species. Bray-Curtis dissimilarity was calculated using the `vegan` package in R

(Oksanen *et al.* 2013). In no-choice mating assay, $\chi 2$ tests were used to determine significance in the courtship occurrence between intact males and tarsi removed males, and were conducted using the `chisq.test()` function in R (Chung *et al.* 2014). Student's *t*-tests were used to determine significant changes in courtship latency between intact males and tarsi removed males, and were performed using the `t.test()` function in R. A simple linear regression analysis was used to test the correlation between changes in courtship interest in males after tarsi being removed and the degree of sexual dimorphism in the CHC profiles of the same species, using `lm()` function in R. All analyses were conducted in RStudio (Rstudioteam 2022).

CHAPTER 3. GENETIC MECHANISMS UNDERLYING CHC EXAGGERATED DIMORPHISM IN FEMALE *DROSOPHILA ERECTA*

I would like to acknowledge the following colleagues, since this chapter could not have been accomplished without the contributions made by them.

Dr. Jian Pu

- Assisted in producing several GFP reporter constructs and corresponding transgenic fly lines

Samantha Kalchik

- Assisted in producing some GFP reporter constructs and corresponding transgenic fly lines

Nadia Sbisa

- Assisted in producing some GFP reporter constructs and corresponding transgenic fly lines

3.1 Introduction

Sexual dimorphism exhibits considerable diversity in nature, rendering it a compelling area of study for evolutionary biologists. A prominent example of sexually dimorphic traits is the elaboration of male morphological features, such as the peacock's tail (*Pavo cristatus*) and the lion's mane (*Panthera leo*) (Williams and Carroll 2009). Two primary reasons account for the extensive research focused on male sexually dimorphic traits. First, historical perspectives have often positioned females as the choosier sex, by investing more in offspring care. As a result, male exaggerated ornaments have evolved under the pressures of female mate choice and male-male competition (Warren *et al.* 2013). Second, studies of male exaggerated ornaments dominate the literature partly

because female exaggerated traits are predicted to be rare, with their evolution influenced by natural selection for resource access, rather than solely through sexual selection (Tobias *et al.* 2012). The rarity of female exaggerated traits in nature has resulted in a limited understanding of their evolutionary mechanisms.

Three primary viewpoints have emerged to explain the evolution of female exaggerated traits. The first posits that female ornamental traits arise from shared genomic heritage with males, leading to correlated inheritance. This concept, initially proposed by Darwin (Darwin 1871), is supported by empirical evidence demonstrating a correlation between the degree of trait exaggeration and genomic sharing in females with conspecific males (Amundsen 2000). The second viewpoint, particularly relevant in sex-role-reversed and polyandrous species, suggests that a reversed sexual selection force—male mate choice—drives the elaboration of female traits (Clutton-Brock 2007). Empirical evidence indicates that females can enhance their fitness by attracting mates (Rosvall 2011). The third perspective emphasizes the role of female competition for non-sexual resources, suggesting that other selective pressures contribute to trait elaboration, that is being supported by empirical tests (Stankowich and Caro 2009). Despite these proposed theories, the evolution of female exaggerated traits remains underexplored, particularly regarding the mechanisms involved.

In addition to the limited understanding of female exaggerated traits, knowledge about the evolution of physiological sexually dimorphic traits is also scarce, as highlighted in the second chapter. Notably, we observed divergent degrees of sexual dimorphism in cuticular hydrocarbon (CHC) profiles across selected *Drosophila* species. The Bray-Curtis dissimilarity index, with a maximum score of 1.00 indicating extreme

sexual dimorphism, showed that *D. erecta* scored 0.95.

The characterization of CHC profiles in the *melanogaster* subgroup was first conducted by Jallon and David, who noted that female *D. erecta* specifically produces very long CHC components (31–33 carbons), absent in males, leading to significant differences between the sexes (Jallon and David 1987). Our results from a Principal Component Analysis (PCA) confirmed this. The results indicated that 97.7% of the variation could be explained by the first two principal components. CHC profiles from all male species within the *melanogaster* subgroup formed a distinct cluster, while CHC samples from *D. erecta* females were distinctively separated from this cluster (**Figure 3.1A**). This separation suggests that the highest degree of sexual dimorphism in *D. erecta* is attributable to female CHC blends.

Furthermore, the major components of a chemical blend are hypothesized to serve sociochemical functions, eliciting behavioral responses among individuals (Symonds and Elgar 2008). A comparative analysis of CHC profiles between sexes also indicated that the pronounced sexual dimorphism observed in *D. erecta* females is due to the presence of these very long CHCs, likely representing an independent evolutionary gain in this lineage (**Figure 3.1B**). In this study, we propose to use the very long CHCs (31–33 carbons) as a model to investigate the represented female exaggerated trait in *D. erecta*.

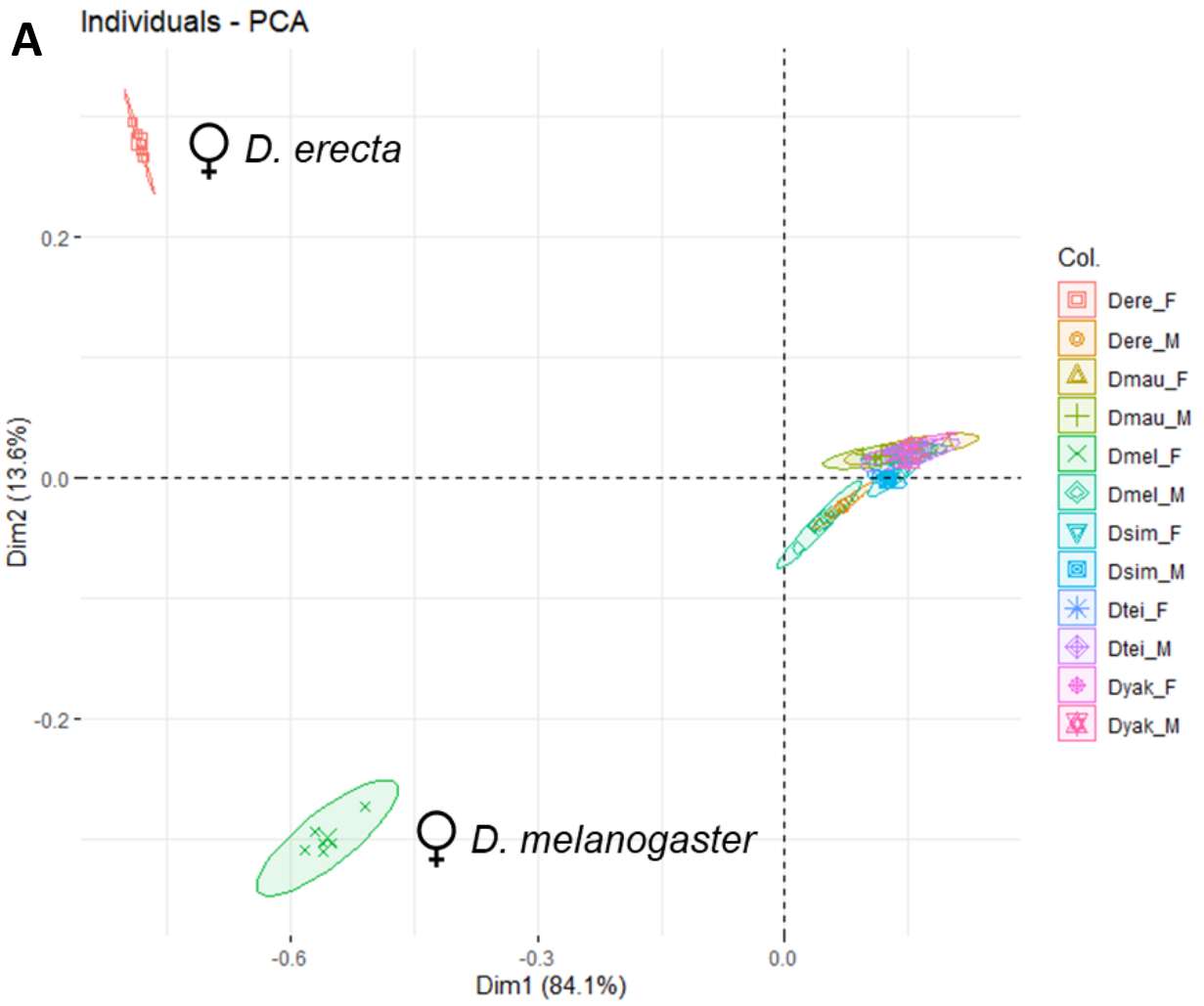
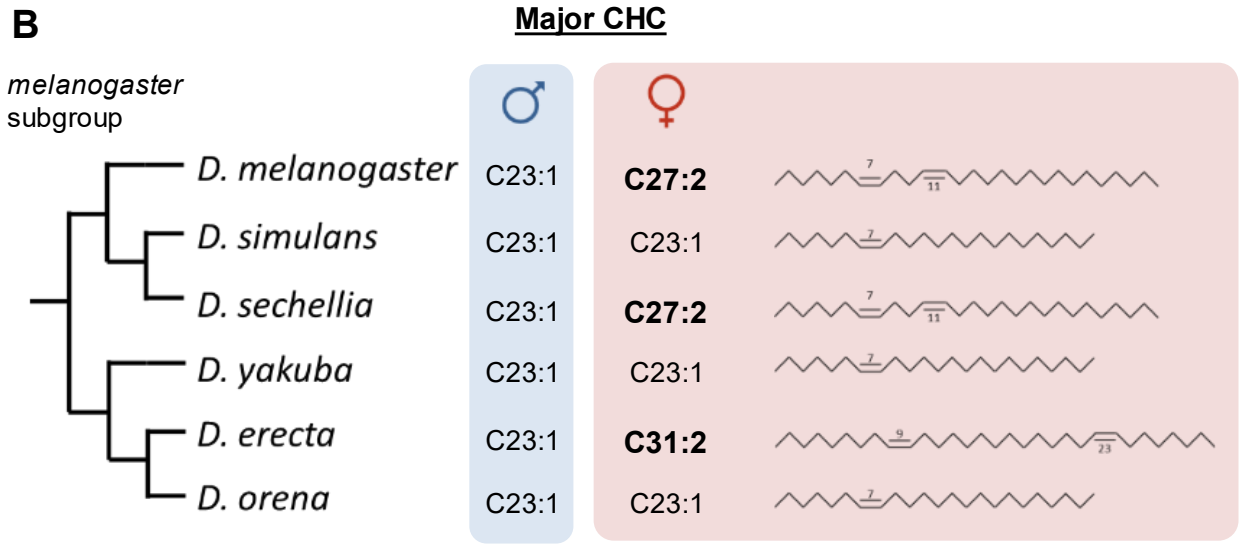


Figure 3.1 The very long CHCs represent female exaggerated traits in *D. erecta*. (**A**) Principal component analysis (PCA) of CHC profiles of *Drosophila* species in *melanogaster* subgroup. Percentages of individual chemicals (amount of each compound divided by total amount of all CHCs) in each species were used for the PCA analyses. 97.7% of the variations can be explained by PC1 and PC2. *Dere = D. erecta*, *Dmau = D. mauritiana, Dmel = D. melanogaster, Dsim = D. simulans, Dtei = D. teissieri, Dyak = D. yakuba, F = Female, M = Male.* (**B**) Comparison of major CHC compound between sexes across *Drosophila* species in *melanogaster* subgroup. Chemical structures shown on the right reflect the major CHC compound in females only. Different isomers with different double bond positions can be found across different populations. Diagrams here only suggest the differences in chain lengths across species.

Figure 3.1 (*cont'd*)



Understanding the evolution of female exaggerated traits is crucial, particularly the genetic mechanisms underlying their production. While the genetic mechanisms underlying male-specific traits are relatively well-established in various model organisms, including *Drosophila*, female traits remain less understood. For example, in *D. biarmipes*, the male-specific wing spot plays a role in courtship rituals, suggesting its contribution to female mate choice (Singh and Chatterjee 1987). The *yellow* protein, involved in melanin biosynthesis, is expressed at low levels during wing development in *D. melanogaster* and *D. pseudoobscura*, but at high levels in the wing spots of male *D. biarmipes* (Wittkopp *et al.* 2002). The evolution of this trait involved changes in the *cis*-regulatory elements of the *yellow* gene, with gains in binding sites for transcription factors being a key mechanism (Gompel *et al.* 2005).

Similarly, genetic mechanism underlying sexual dimorphism CHC profiles has been studied in *D. melanogaster*, characterized by the female-specific production of 7,11-HD (Jallon and David 1987). The fatty acid desaturase *DesatF* is responsible for adding a second unsaturated double bond in the production of this CHC component,

with its expression localized to the oenocytes of female flies (Chertemps *et al.* 2006). Rapid evolution in the cis-regulatory regions of *desatF* has been suggested to explain this specific expression pattern (Shirangi *et al.* 2009), including the gain of binding sites for *DOUBLESEX* (*dsx*), a crucial transcription factor for sexual differentiation in *Drosophila* (Hopkins and Kopp 2021).

Doublesex operates across a range of somatic cells in *Drosophila*, contributing to the production of sexually dimorphic traits through its two isoforms with sex-specific sequences. Furthermore, the process of gaining sex biased expression usually involves gaining *dsx* expression in tissues and modifying the repertoire of *dsx* targets through changes in binding sites within enhancers (Hopkins and Kopp 2021). While *dsx* is essential for the production of many sexually dimorphic traits, recent studies highlight the role of hormonal inputs in sexual differentiation, such as higher levels of 20-hydroxyecdysone in female butterflies correlating with enlarged wing spots (Bhardwaj *et al.* 2018).

In summary, the evolution of sexual dimorphism can often be attributed to *cis*-regulatory changes, particularly through the gain or loss of *dsx* binding sites. In *D. melanogaster*, rapid changes in enhancer sequences of the CHC synthesis gene *desatF* have been implicated in the production of female-specific CHC components. However, whether this mechanism applies universally, especially to the very long CHC components in female *D. erecta*, remains unknown. Investigating the genetic mechanisms underlying these traits will enhance our understanding of female exaggerated traits.

A novel model of the evolution of highly specific gene expression has been

previously proposed (Pu *et al.* 2021), which involved dissecting regulatory sequences and using GFP reporter systems to create transgenic *Drosophila* lines. By comparing homologous regulatory sequences across lineages, we identified stepwise evolutionary changes, including gains or losses of specific modules. This methodology can similarly be applied in our proposed study to provide evidence for the genetic mechanisms underlying the production of the very long CHCs in *D. erecta*.

In *Drosophila*, CHC synthesis shares fatty acid synthesis pathways that include several processes and involves families of enzymes (**Figure 1.3B**). Fatty acid synthetases produce medium-chain carbon backbones, while desaturases introduce unsaturated double bonds. Elongases extend these medium-length chains to longer chains, and reductases, alongside a cytochrome P450 enzyme, finalize CHC production (Chung and Carroll 2015). We hypothesize that at least one elongase gene is responsible for the production of the female-specific very long CHCs in *D. erecta*, responsible for the production female exaggerated traits in this species.

3.2 Results

3.2.1 Nineteen elongase genes were found in *D. erecta* genome, with an independent loss of *EloF*

To test our hypothesis regarding the candidate fatty acid elongase gene responsible for producing the very long-chain cuticular hydrocarbons (CHCs) in female *D. erecta*, we first referred to the elongase gene involved in the synthesis of the female-specific CHC, 7,11-HD, in *Drosophila melanogaster*. The gene *EloF* has been identified as being crucial for the production of 27- and 29-carbon dienes (Chertemps *et al.* 2007).

Using the coding sequence of *EloF* obtained from the annotated *D. melanogaster*

genome, we conducted a comparative analysis with the *D. erecta* genome. Our phylogenetic analyses and synteny comparisons revealed that the ortholog of *EloF* is absent from the *D. erecta* genome at the same genomic locus where it is located in *D. melanogaster* (**Figure 3.2A**).

To further explore the possibility that the ortholog of *EloF* might exist in *D. erecta* but not at the same locus, we performed an exhaustive search for all elongase genes within the *D. erecta* genome. This was achieved using an iterative BLAST search pipeline developed in previous work in our lab (Luo *et al.* 2020). We applied the same methodology to search for elongase genes across five other species within the *melanogaster* subgroup. Twenty elongase genes were identified in *D. melanogaster* genome, whilst only nineteen were found in *D. erecta* and *D. orena* genome. Together, our findings indicate that the ortholog of *EloF* is the only elongase gene absent in the genomes of both *D. erecta* and *D. orena*, suggesting a potential independent loss of this gene in these species (**Figure 3.2B**). This suggests that EloF is not the candidate gene responsible for producing the very long CHCs in *D. erecta*. This loss may provide insights into the evolutionary divergence of CHC profiles and highlight alternative pathways for producing very long-chain CHCs in female *D. erecta*. Further functional studies are needed to identify other elongase candidates that could play a role in synthesizing these unique CHCs in this species.

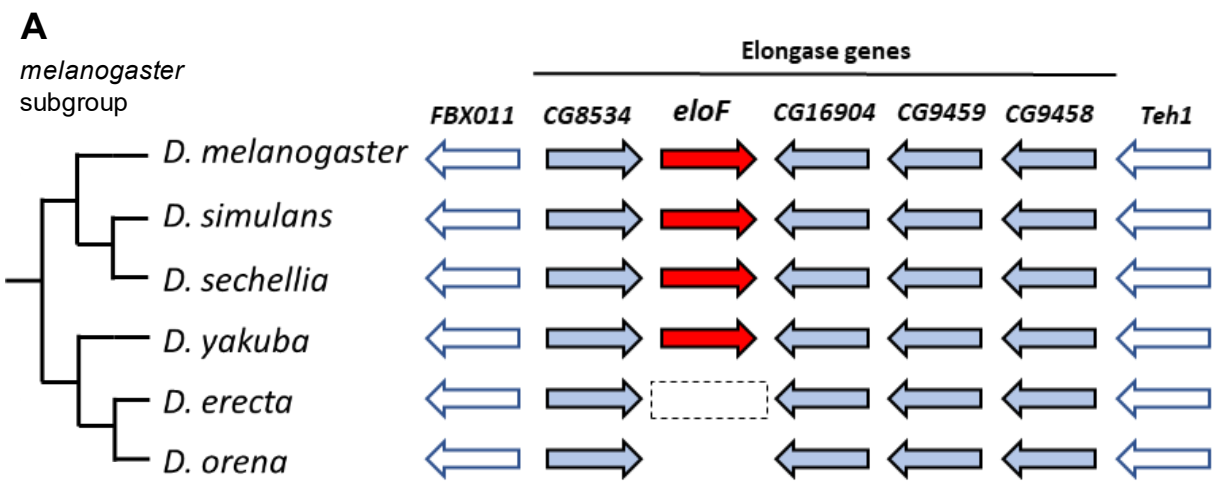
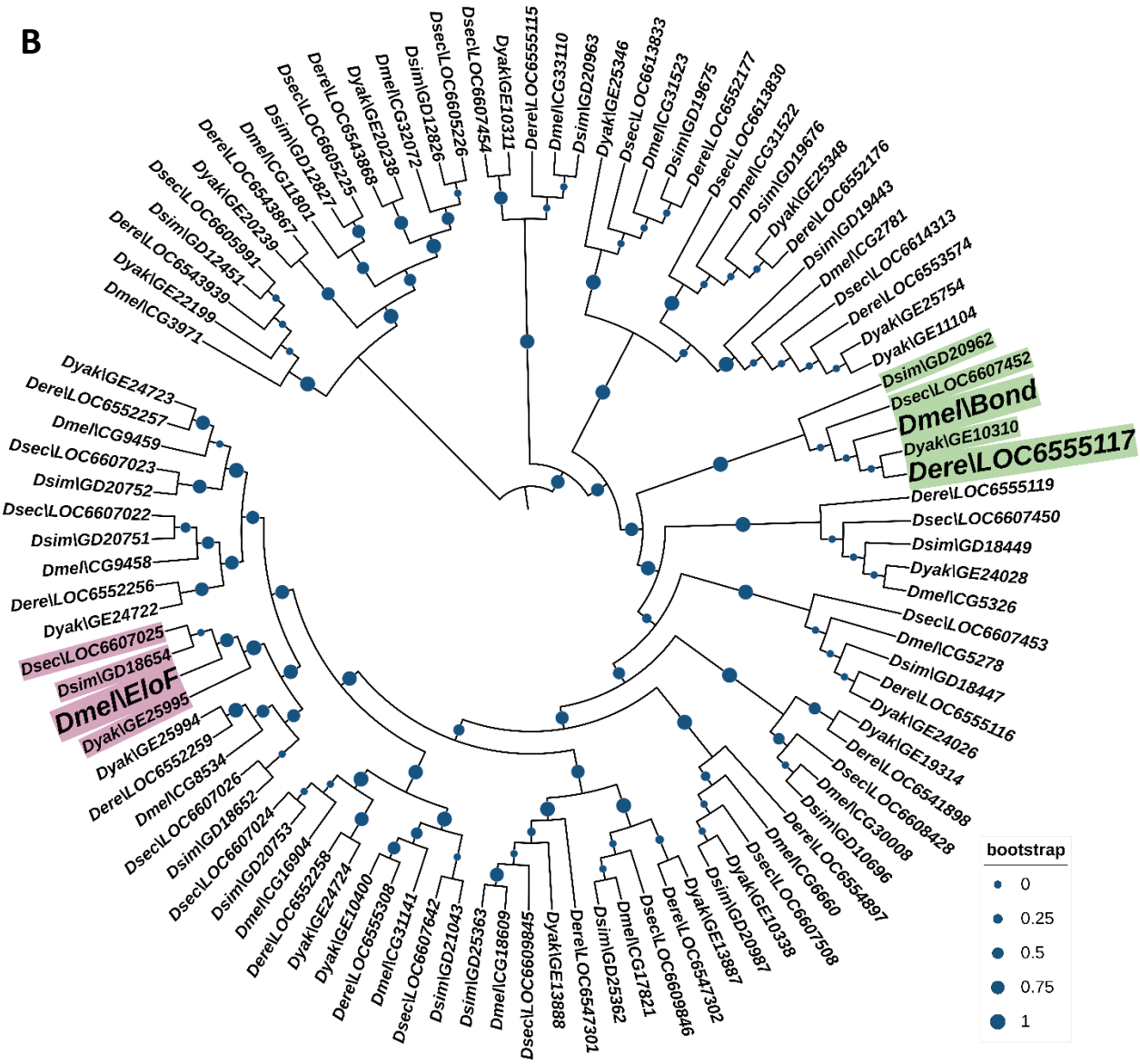


Figure 3.2 *EloF* is not present in the genome of *D. erecta*. (**A**) Synteny genome comparison of *EloF* and other genes in the corresponding cluster. The dotted line box indicates the ortholog of *EloF* is not found in the genomes of *D. erecta* and *D. orena*, suggesting an independent loss of *EloF*. (**B**) Phylogeny of all elongases found in the genomes from species across *melanogaster* subgroup. Amino acid sequences of all elongases were used for the analysis. RAxML maximum-likelihood analyses were conducted under the LG + Γ model. Different numbers of elongase genes were found across the species, 20 in *D. melanogaster (Dmel)*, 20 in *D. simulans (Dsim)*, 20 in *D. sechellia (Dsec)*, 21 in *D. yakuba (Dyak)*, and 19 in *D. erecta (Dere)*. Orthologs lie in the cluster of *Dmel*/*EloF* are labeled in pink. Orthologs lie in the cluster of *Dmel*/*Bond* are labeled in green. An ortholog of *Dmel*/*EloF* is not found in *D. erecta* and sibling species *D. orena*.

Figure 3.2 (*cont'd*)



3.2.2 Only one elongase gene shows female biased expression in *D. erecta* oenocytes

Our results indicate that the *D. erecta* genome contains 19 fatty acid elongase genes, compared to 20 in *D. melanogaster*. To identify the specific elongase gene responsible for producing the very long cuticular hydrocarbons (CHCs) in female *D. erecta*, we hypothesized that at least one elongase gene would be female-biasedly expressed in

the oenocytes of this species.

To test this hypothesis, we screened the expression patterns of all 19 elongase genes in the *D. erecta* oenocyte *in situ* hybridization by using DIG-labeled RNA probes. The biosynthesis of CHCs is known to occur specifically in the oenocytes of *Drosophila* (Billeter *et al.* 2009). Among the 19 elongase genes examined, we found that only one gene, *LOC6555117*, exhibited female-biased expression in the oenocytes of *D. erecta* (**Figure 3.3**).

Several other elongase genes were detected with oenocytes expression, but they displayed either male-biased expression (*LOC6552258* and *LOC6552177*) or expression in both sexes (*LOC6541898* and *LOC6554897*) (**Figure 3.3**). Consequently, we selected *LOC6555117* as the candidate gene for further investigation into its role in producing the unique very long CHCs in female *D. erecta*. This selection sets the stage for subsequent functional studies aimed at determining the involvement of this gene in CHC biosynthesis.

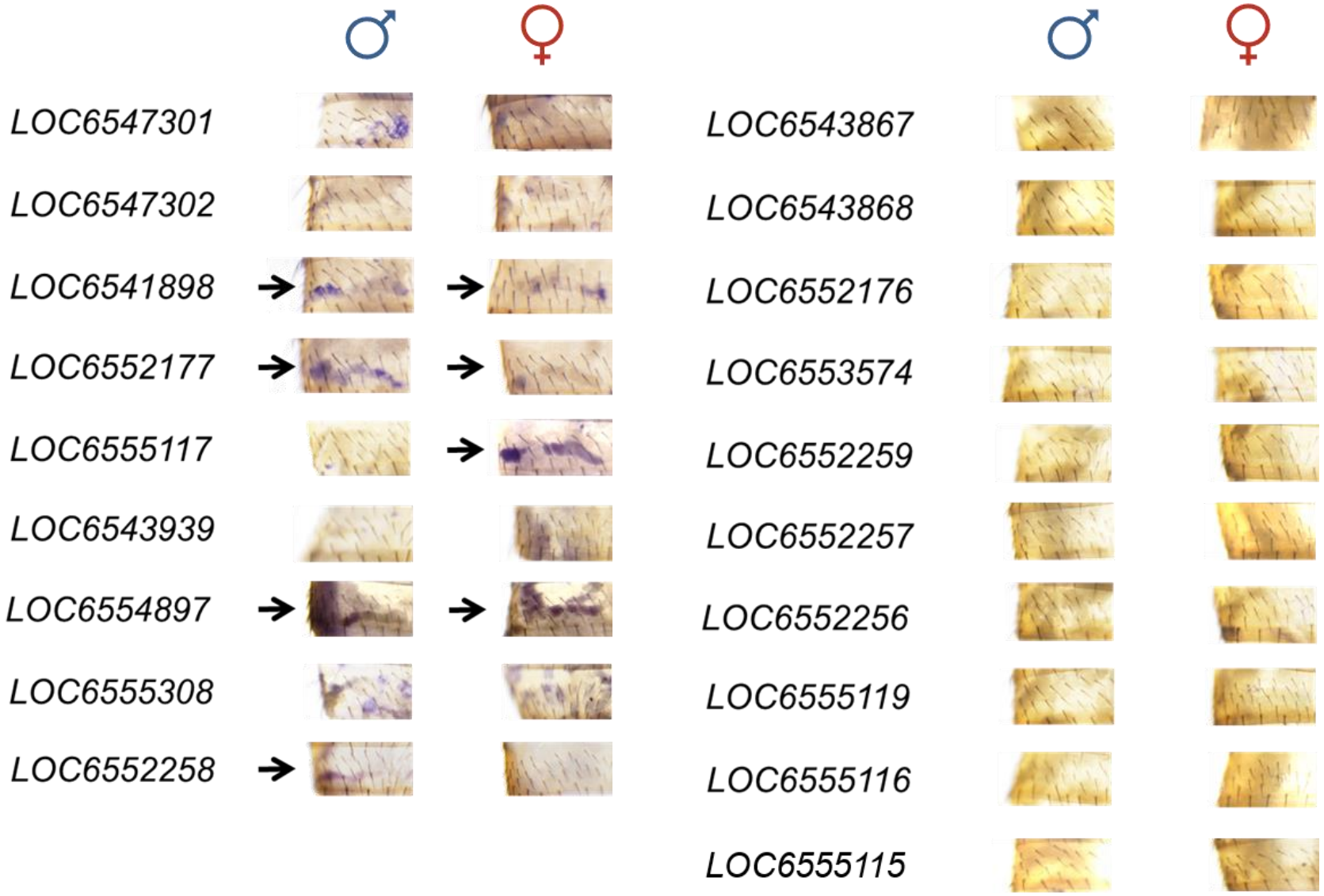


Figure 3.3 Expression of elongase genes in 4-day old *D. erecta* oenocytes. Expression of 19 elongase genes are individually tested by using *in situ* hybridization, with gene specific probes. Arrows show gene expression in adult oenocytes, and only one gene shows female biased gene expression pattern.

3.2.3 *Cis*-regulatory changes underlie the female biased expression

With the candidate gene *LOC6555117* showing female-biased expression in oenocytes, we aimed to uncover the mechanisms underlying this expression pattern. In *D. melanogaster*, the gene *DesatF* has been implicated in cuticular hydrocarbon (CHC) biosynthesis, with its sex-specific expression attributed to changes in *cis*-regulatory sequences (Shirangi *et al.* 2009). We hypothesized that the female-biased expression of *LOC6555117* in *D. erecta* is similarly due to changes in its *cis*-regulatory regions.

To test this hypothesis, we constructed GFP reporter vectors using non-coding DNA sequences surrounding the *LOC6555117* gene. Our results indicated that the enhancer responsible for driving oenocyte expression in females is located within the 5' non-coding region (designated as *ereA*) of *LOC6555117* (**Figure 3.4A**). The GFP expression driven by *ereA* exhibited a strong female-biased pattern.

In contrast, the homologous region from the sibling species *D. orena* yielded only weak GFP expression in both sexes (**Figure 3.4B**). This observation aligns with the *in situ* hybridization results for the gene expression in these two species (**Figure 3.4C**), further supporting our conclusion that *cis*-regulatory differences account for the female-biased expression of *LOC6555117* in *D. erecta*. This finding underscores the importance of regulatory sequence changes in shaping sexually dimorphic gene expression.

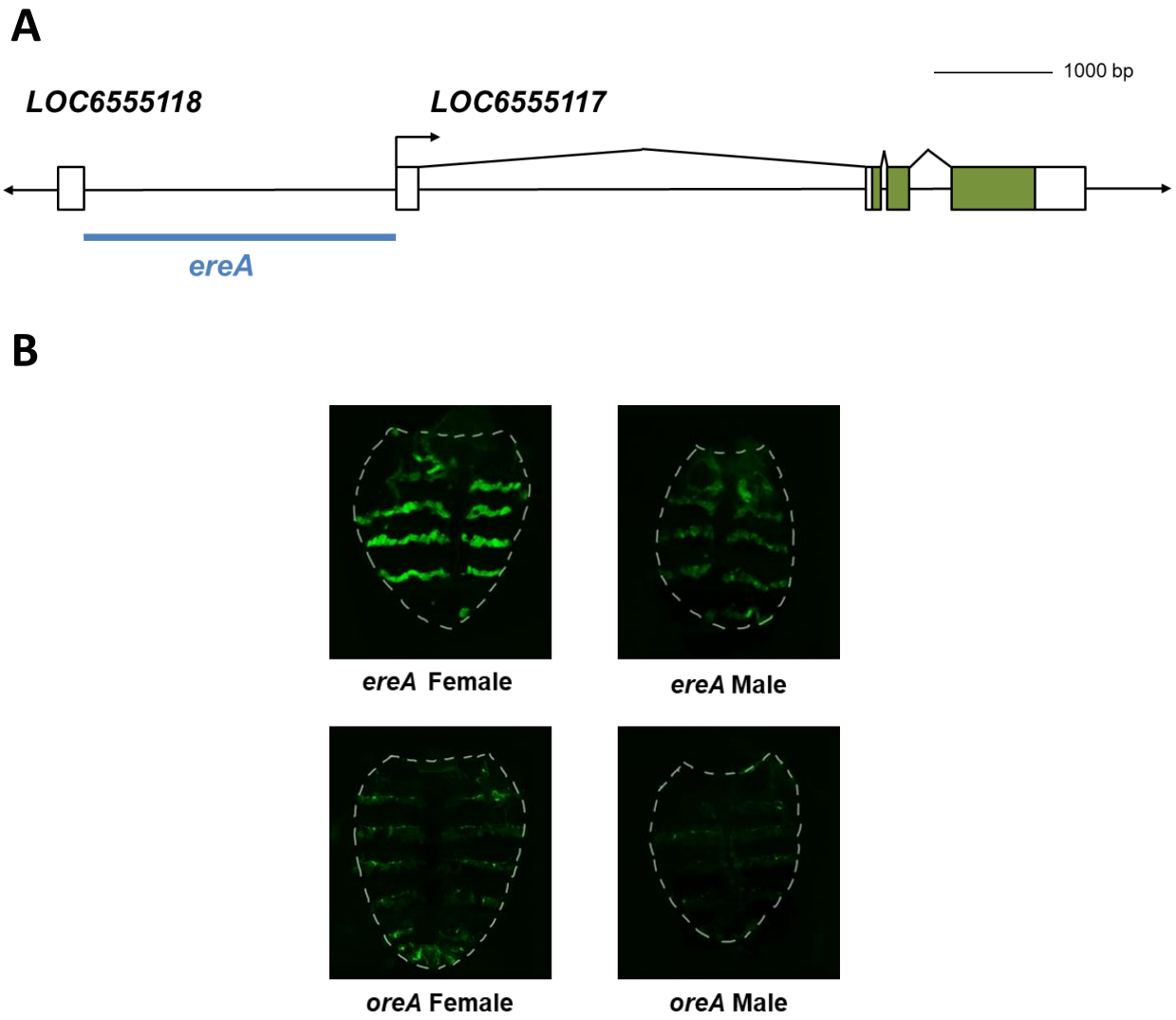
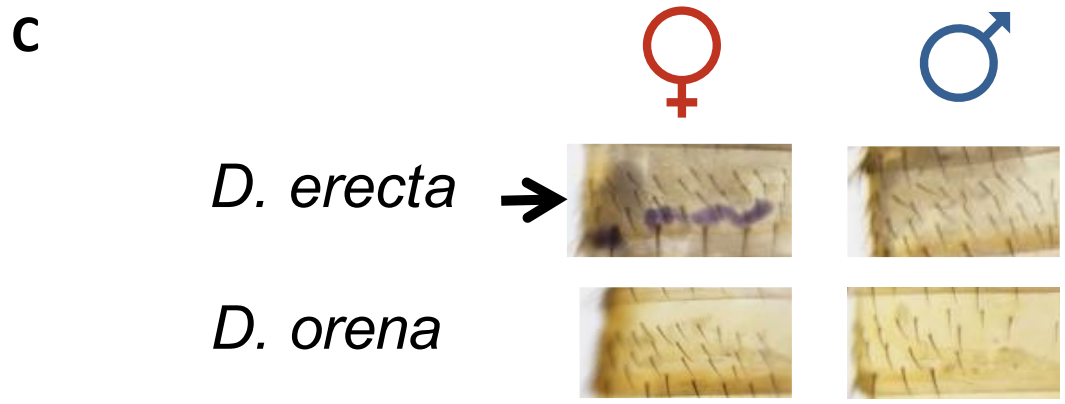


Figure 3.4 *Cis*-regulatory modifications are responsible for the female-biased expression. (**A**) Schematic view of the test region (*ereA*) on *D. erecta* genome. *ereA* is located on the 5' end of the *LOC6555117* gene. (**B**) Gene expression pattern of the GFP reporter protein driven by *ereA* and *oreA* (homologous fragment of *ereA* in sibling species, *D. orena*). *ereA* drives highly female biased expression pattern in oenocytes, whilst *oreA* drives very weak expression pattern. (**C**) Gene expression of *LOC6555117* in the oenocytes of 4-day old adult *D. erecta* and *D. orena*. *In situ* hybridization with specific RNA probes were used to test gene expression. Arrows suggest gene expressions. These results suggest the same trend of gene expression as GFP reporter constructs.

Figure 3.4 (*cont'd*)



3.2.4 An oenocyte expression module and a sex related module are identified in the regulatory region

Having established that the female-specific expression of *LOC6555117* is attributed to *cis*-regulatory changes, we proceeded to identify the specific regulatory modules within the regulatory region. We systematically dissected the 5' non-coding region of *LOC6555117* into three overlapping segments: *A1*, *A2*, and *A3*, constructing corresponding GFP reporter constructs. Among these, *A2* exhibited sexually non-biased GFP expression in oenocytes, indicating the presence of an oenocyte-specific driving module (**Figure 3.5**).

Further dissection of *A2* led us to a minimum 397 bp sequence, *A2.2*, which successfully recapitulated the oenocyte-specific expression pattern (**Figure 3.5**). This was confirmed by building additional constructs *A2.2a*, *A2.2b*, and *A2.2c*, none of which drove strong GFP expression recapitulating *A2.2* pattern in adult oenocytes (**Figure S3.1**).

Next, to explore the reason behind the female-biased expression in the *ereA* GFP reporter construct, we hypothesized the existence of a sex-related module. To test

this, we generated two more constructs: *A1+A2* and *A2+A3*. The *A2+A3* construct displayed strong female-biased GFP expression in oenocytes, whereas *A1+A2* showed non-sex-biased expression. This suggested that a sex-related module is present in the *A3* region (**Figure 3.5**).

We further dissected A3 into three sub-regions: *A3.1*, *A3.2*, and *A3.3*. Both additionally narrowed constructs, *A2+A3.1* and *A2+A3.1+A3.2*, could drive non-sex-biased GFP expression, suggesting that the sex-related module is not contained within *A3.1* or *A3.2* alone. Consequently, we concluded that the sex-related module resides in A3.3, identified as a minimum 400 bp sequence. This work highlights the intricate regulatory architecture underlying female-biased expression of *LOC6555117* and contributes to our understanding of the genetic mechanisms underlying sexually dimorphic gene expressions.

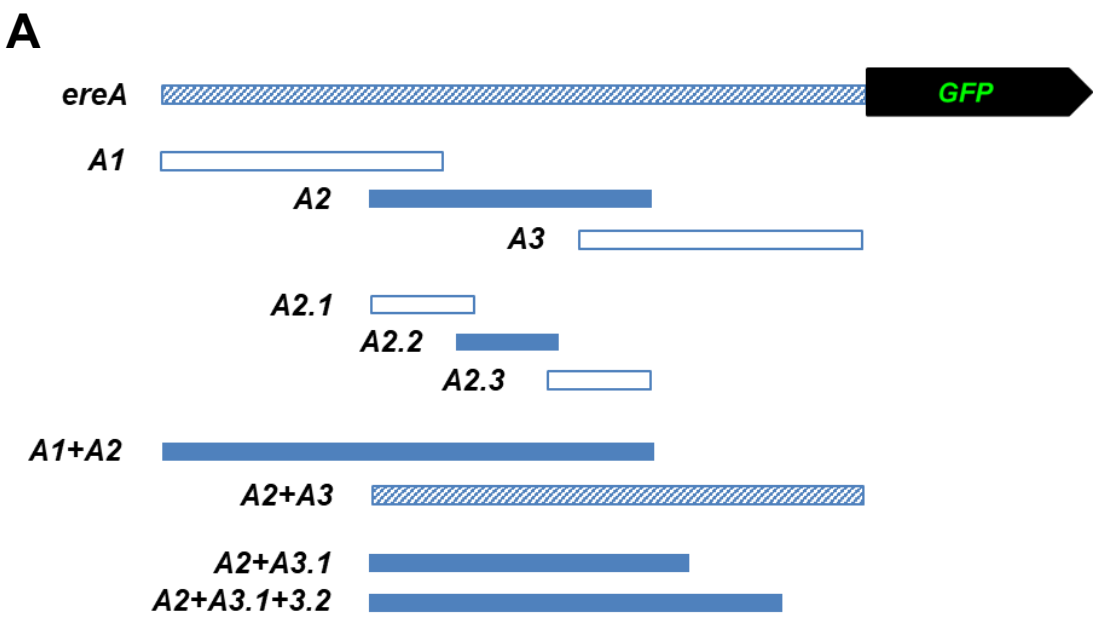
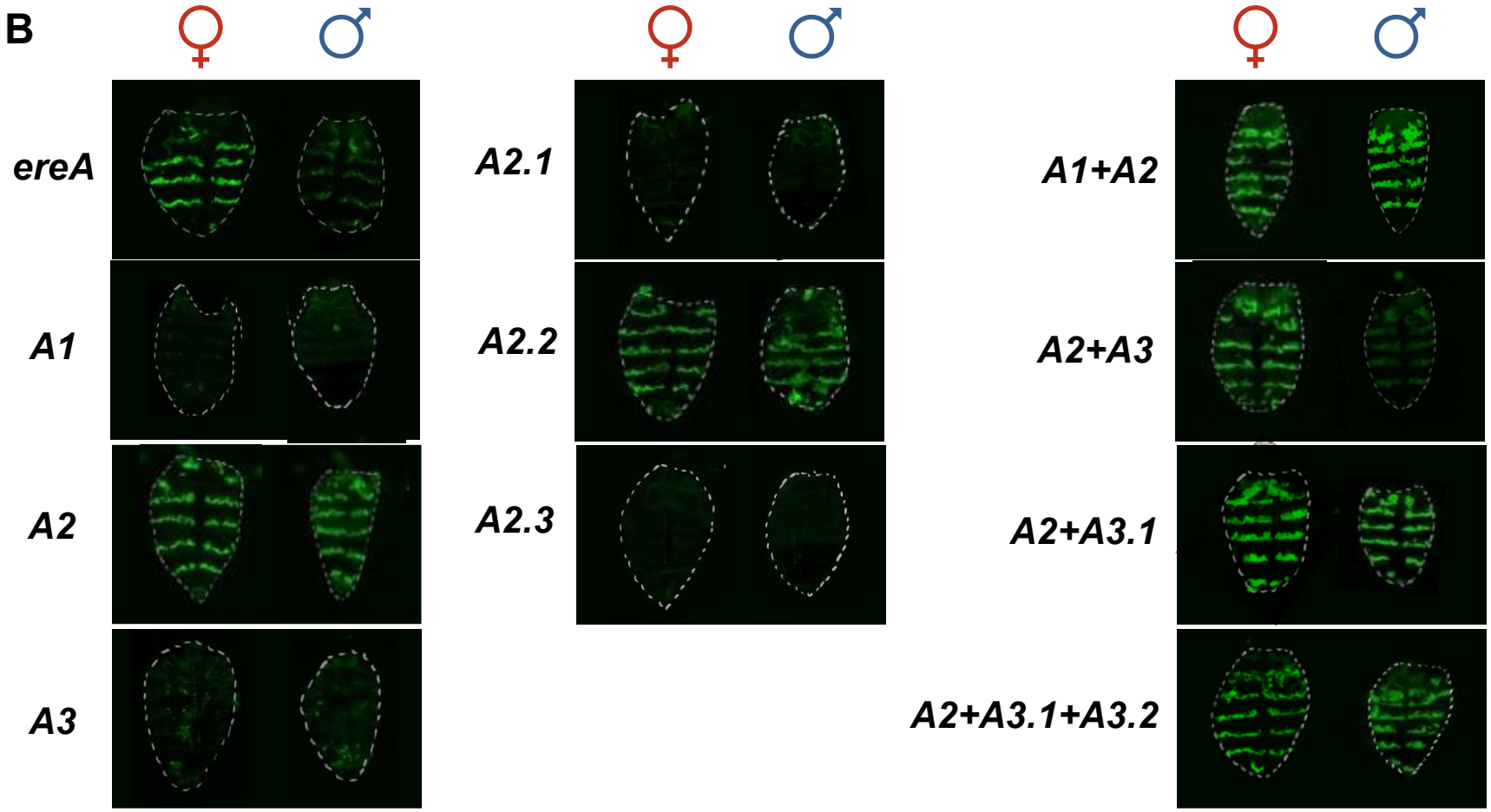


Figure 3.5 Identification of an oenocyte expression module and a sex-related module in the regulatory region. (A) Schematic dissection of all GFP reporter constructs. Striped box = female biased expression, solidly filled box = non-sex biased expression, no filled box = no expression. **(B)** GFP reporter protein expression in oenocytes corresponding to the different overlapping constructs. The A2.2 fragment is the minimum region that can recapitulate a sexually monomorphic oenocyte expression. The A2+A3 fragment can recapitulate the female biased oenocyte expression pattern, and a sex related module is suggested in A3.3. The results together suggest that an oenocyte expression module and a sex related module are responsible for the female biased gene expression.

Figure 3.5 (*cont'd*)



In summary, our dissection of the 5' end *cis*-regulatory region of *LOC6555117* identified a minimum 397 bp sequence, *A2.2*, which serves as a module driving oenocyte-specific, non-sex-biased gene expression. Additionally, we discovered a minimum 400 bp sequence, *A3.3*, functioning as a sex-related module responsible for generating female-biased expression, operating in conjunction with *A2.2*. Notably, these two modules are separated by a 915 bp sequence within the *D. erecta* genome. This delineation of regulatory elements enhances our understanding of the genetic architecture underlying sexually dimorphic gene expressions.

3.2.5 A weaker oenocyte expression module leads to the weak expression *in D. orena*

The final question we addressed was what underlies the weaker expression of the ortholog gene of *LOC6555117* in the sibling species *D. orena* (**Figure 3.6**). After confirming that *A2.2* is the minimum sequence capable of driving oenocyte expression, we obtained the homologous sequence from *D. orena*, designated as *oreA2.2*. The GFP reporter construct for *oreA2.2* demonstrated weak GFP expression, indicating that this sequence serves as a less effective oenocyte expression module in the *D. orena* genome. This finding helps explain the observed weaker gene expression in this sibling species (**Figure 3.4**).

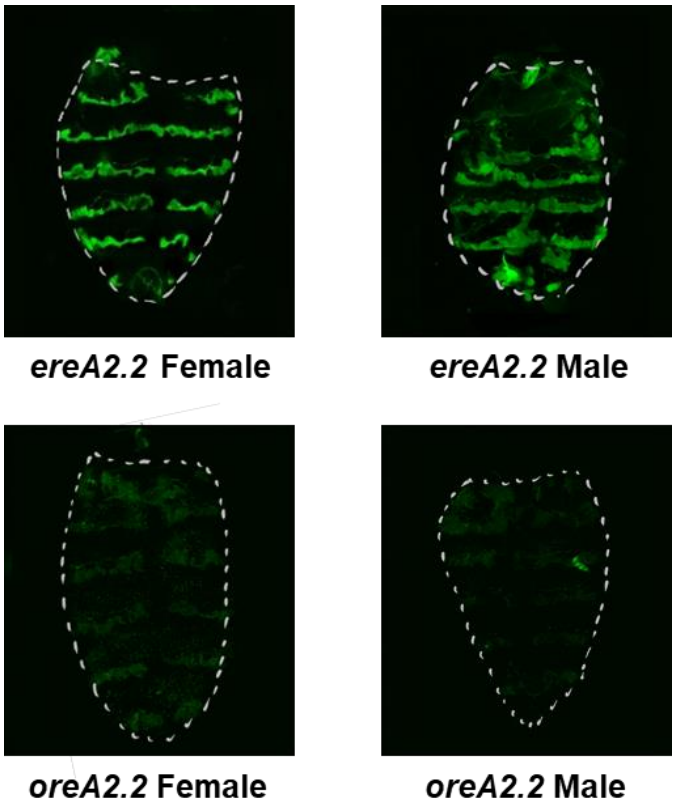


Figure 3.6 A weak oenocyte driving module is found in *oreA2.2.* The homologous fragment of *A2.2* in *D. orena* is named after *oreA2.2* and cloned into GFP reporter construct, which can drive weak GFP expressions in oenocytes, suggesting changes in the fragment underline the weak gene expression in oenocytes of *D. orena*.

3.3 Discussion

Sexual dimorphism in cuticular hydrocarbon (CHC) profiles across *Drosophila* species reflects complex selective pressures that shape the evolution of the traits. Among the species examined, *D. erecta* exhibits the most pronounced sexual dimorphism in its CHC profiles (**Figure 2.1**). Comparative analyses within the *melanogaster* subgroup have confirmed that *D. erecta* independently evolved the capacity to produce very long, female-specific CHCs, which are representative of exaggerated female traits. However, the mechanisms underlying the evolution of female exaggerated traits remain poorly understood in general due to their rarity in nature.

In this study, we investigated the genetic mechanisms underlying the evolution of

female exaggerated CHC profiles in *D. erecta*. By screening the expression patterns of all elongase genes in the oenocytes of *D. erecta*, we identified an elongase gene, *LOC6555117*, likely involved in the biosynthesis of these very long CHCs. Notably, *LOC6555117* is the only elongase gene exhibiting female-biased expression in the oenocytes of *D. erecta* (**Figure 3.3**). Our analysis revealed that this female-biased expression is attributable to *cis*-regulatory changes located in the 5' non-coding region of the gene. Through systematic dissection of the regulatory region and the buildups of corresponding GFP reporter constructs, we identified that the female-biased expression results from the functional interaction between an oenocyte-driving module and a sex-related module (**Figure 3.5**). In contrast, the ortholog of *LOC6555117* in the sibling species *D. orena* shows weak expression in oenocytes of both sexes, where changes in the homologous *cis*-regulatory module were suggested to contribute to the weak expression (**Figure 3.6**).

Although we successfully identified the sex-related module in A3.3, we could not conclusively determine whether the transcription factor *dsx* responsible for this sex-related function. Previous studies have shown that *dsx* is responsible for sex-specific gene expression of other CHC biosynthesis-related genes in *D. melanogaster* (Hopkins and Kopp 2021). The consensus target sequence of *dsx* binding domain in *D. melanogaster* has been predicted by several studies (Burtis *et al.* 1991; Erdman *et al.* 1996; Narendra *et al.* 2002). We utilized the Erdman-Burtis Consensus sequence, "RNNACWAWGTNNY," to query the 5' regulatory region of *LOC6555117*. Although we did not find any specific alignment of the consensus sequence in *A3.3*, alignments were present in other regions of the regulatory sequence (**Figure S3.2**). Despite this, we

cannot rule out the possibility that *dsx* contributes to the female-biased expression pattern of this gene. Furthermore, additional investigation suggests that hormonal mediation may play a significant role in this expression pattern. Our preliminary results show that *LOC6555117* is expressed strongly in both sexes of 1-day-old *D. erecta* without sex biases, indicating that sex-biased expression may be age-related and likely under hormonal mediation (**Figure S3.3**).

Our findings indicate the presence of two distinct types of modules driving oenocyte expression in these closely related species: one that facilitates strong expression in *D. erecta* and another that produces very weak expression in *D. orena* (**Figure 3.6**). However, the specific evolutionary gains and/or losses of these modules remain unclear. We hypothesize that the strong oenocyte expression module in *D. erecta* represents an independent evolutionary gain, but requires further investigation. Additionally, while our data indicate that *cis*-regulatory changes are responsible for the female-biased expression of *LOC6555117*, coding changes in the candidate gene may also contribute to the production of very long CHCs in female *D. erecta*. Preliminary data generated from RNA interference (RNAi) targeting the gene *Bond* (the ortholog of *LOC6555117* in *D. melanogaster*) in a transgenic *D. melanogaster* line suggest that knocking down *Bond* expression reduces two major female-specific CHC compounds, underscoring its importance in maintaining sexual dimorphism in CHC profiles (**Figure S3.4**). Future work examining the effects of manipulating *LOC6555117* expressions on the CHC profiles of *D. erecta* will provide valuable insights into answering the question.

Indeed, *LOC6555117*, with its female-biased oenocyte expression in *D. erecta*, is our candidate gene responsible for the production of very long CHCs. However, direct

evidence of its functional role must be obtained through gene knockdown, knockout, or overexpression. In the absence of such evidence, other CHC biosynthesis-related genes may also play a role, particularly those previously shown to influence CHC length, such as fatty-acyl CoA reductase (Rusuwa *et al.* 2022). We have also observed sex-biased expression in several reductase genes in *D. erecta* oenocytes as preliminary results (**Figure S3.5**).

Interestingly, *Bond*, the ortholog of *LOC6555117* in *D. melanogaster*, has also been implicated in the biosynthesis of another *Drosophila* sex pheromone, CH503 (Ng *et al.* 2015). Furthermore, additional regulatory modules for *Bond* have been identified in other non-coding regions, reflecting rapid evolutionary changes in enhancers across species (Pu *et al.* 2021). Thus, we propose that *Bond* may function as a "toolkit" gene, incorporating rapid evolutionary modifications in *cis*-regulatory regions, likely contributing to the correlation between gene function and specification processes, particularly in the diversification of pheromone evolution.

To enhance our understanding of the evolution of this female exaggerated trait, the ecological role of very long CHCs in *D. erecta* must be further explored. Initially, we proposed three parallel hypotheses: 1) *D. erecta* and its sibling species *D. orena* are thought to have evolved sympatrically (Linz *et al.* 2013), establishing a premating reproductive isolation barrier (Lee and Watanabe 1987); therefore, very long CHCs may contribute to mate recognition as a premating reproductive barrier. 2) As honest signals, the production of these very long CHCs could be condition-dependent, with quantitative variations influencing female sexual attractiveness and male mate choice. This hypothesis is based in previous studies that have suggested that the dose effect of

major CHC components as aphrodisiacs elicits varying male courtship behaviors (Billeter *et al.* 2009). 3) These very long CHCs may also be involved in other essential physiological processes, such as desiccation resistance, with evidence indicating that CHC chain length is positively correlated with desiccation resistance (Ferveur *et al.* 2018). Thus, the elaboration of these very long CHCs in females may also result from natural selection alone, as suggested in other study systems (Okada *et al.* 2021).

In summary, this study provides novel insights into the genetic mechanisms underlying the evolution of female exaggerated traits, specifically the very long CHCs in female *D. erecta*.

3.4 Materials and Methods

Drosophila species and strains

The *D. erecta* line were obtained from the National *Drosophila* Species Sock Center (NDSSC). The *D. orena* line was gifted by Dr. Mark Rebeiz (University of Pittsburgh) The *D. melanogaster attP40*, *UAS-Bond RNAi* strain was obtained from the Bloomington *Drosophila* Stock Center. The *D. melanogaster G3-GAL4* strain was obtained from our previous work (Wang *et al.* 2023). All species and strains were reared on standard cornmeal medium (Flystuff 66-121 Nutri-Fly Bloomington Formulation).

In situ hybridization in adult oenocytes

Adult oenocytes from 4-day old adults were dissected in Phosphate-Buffered Saline (PBS). RNA probes were made from 4 cDNA mixtures of 4 to 5 day-old adults using the primers listed in **Table S3.1**, as described previously (Pu *et al.* 2021). The procedure of performing in situ hybridization of 4-day old adult oenocytes was adapted from a previous study (Finet *et al.* 2019).

Data Collection

Elongase genes were identified in five complete *Drosophila* genomes by using *D. melanogaster* protein sequences to preform tblastn. The five genomes were retrieved from the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/). Repeated tblastn were used following a previously described searching pipeline to make sure all elongase genes from each species were identified (Luo *et al.* 2020). Genomic information of the five species was also used to perform synteny analyses to determine the presence or absence of ortholog of *EloF* in the cluster in the genome of *D. erecta*.

Phylogenetic analysis

Amino acid and DNA sequences were aligned with MUSCLE with manual adjustments (Luo *et al.* 2020). Maximum-likelihood searches were performed using Phylogeny.fr (http://phylogeny.lirmm.fr/phylo_cgi/contacts.cgi) using a gamma distribution for final likelihood evaluation. One-thousand bootstrap replicates were conducted for support estimation, (Dereeper *et al.* 2008).

Generation of GFP reporter constructs and transgenic flies

All GFP reporter constructs were produced by firstly using PCR amplifying the test fragments from the genomes of *D. erecta* or *D. orena*. The fragments were then cloned into the GFP reporter vector *pS3aG* via the *AscI* and *SbfI* sites (all primers listed in **Table S3.1**). All constructed were micro-injected into the *D. melanogaster attP40* strain, which were using the PhiC31 integrase system to get integrated into the genome.

Imaging

All *in situ* hybridization and GFP images were captured using the Nikon SMZ18

dissecting stereo microscope system, as described previously (Pu *et al.* 2021). For GFP imaging, samples were prepared by dissecting four-day old adults in PBS and transferred on slides with glycerol mountant [80%(vol/volinwater) glycerol, 0.1 M Tris (pH 8.0)].

CHC profiles analyses

Offspring of crossing the *D. melanogaster G3-GAL4* and *UAS-Bond RNAi* strains was generated to test the changes in CHC profiles in *D. melanogaster* after knocking down *Bond* expression in adult oenocytes. CHCs were extracted typically from five four- to five-day-old offspring adult flies soaked in 100 µl hexane containing hexacosane (C26; 25 ng/ul) as internal standard for ten minutes. Extracts were directly analyzed by the GC/MS (7890A, Agilent Technologies Inc., Santa Clara, CA) coupled with a HP-1ms column 30 m by 0.25 mm (i.d.) with a 0.25 µm film thickness (J&W Scientific, Folsom, CA). Mass spectra were acquired in Electron Ionization (EI) mode (70 eV) with Total Ion Mode (TIM) using the GC/MS (5975C, Agilent Technologies Inc., Santa Clara, CA). The peak areas were recorded by MassHunter software (Agilent Technologies Inc., Santa Clara, CA). Helium was the carrier gas at 0.7 ml/min and the GC thermal program was set as follows: 60 °C for 4 min, 15 °C/min to 200 °C, 5 °C/min to 280 °C, then held for 10 min. Straight-chain compounds were identified by comparing retention times and mass spectra with authentic standard mixture (C6-C40) (Supelco 49452-U, Sigma-Aldrich, St. Louis, MO). Methyl-branched alkanes, alkenes, dienes and cis-Vaccenyl acetate were then identified by a combination of their specific fragment ions on the side of functional groups (methyl branch or double bonds) and retention times relative to linear-chain hydrocarbon standards. Each individual CHC peak was quantified using its

comparison with peak area of internal standards.

Statistical Analyses

PCA analyses were performed using the `prcomp()` function in R. The student's *t*-tests were used to determine significant changes in CHC profiles between test lines, and were performed using the `t.test()` function in R. All analyses were conducted in RStudio (Rstudioteam 2022).

CHAPTER 4. PHENOTYPIC TRADEOFFS OF PRODUCING COSTLY CHCS IN *DROSOPHILA MOJAVENSIS*

I would like to acknowledge the following colleagues, since this chapter could not have been accomplished without the contributions made by them.

Ishu Kudapa

- Assisted in performing two-choice mating assay and development assay

Nathaniel Fellows

- Assisted in testing reproductive fitness and longevity

Bella Balabuszko-Reay

- Assisted in performing development assay

Dr. Rajanikanth Chowdanayaka

- Assisted in performing two-choice mating assay

Jordy Hernandez

- Assisted in testing reproductive maturity and latency

Zhuo Chen

- Assisted in performing two-choice mating assay

Dr. Zinan Wang

- Assisted in performing two-choice mating assay and development assay

4.1 Introduction

Interactions among individuals in natural environments can lead to conflicts over limited resources. In particular, conflicts between males and females often relate to mating (Darwin 1871). A common reproductive strategy involves individuals mating with higher-quality partners to maximize reproductive fitness. Consequently, selection may favor

signals that indicate the presence of high-quality mates. Substantial research has focused on understanding the mechanisms underlying the evolution of mating signals, particularly regarding the maintenance of signal reliability, commonly referred to as "honest signaling" (Husak *et al.* 2015).

Multiple perspectives and intense debates have emerged to explain the reliability of these signals. Signal reliability is thought to evolve when the cost-to-benefit ratio for the signal sender is low, ultimately leading to an equilibrium. The theory positing that the cost to the sender is crucial for maintaining signal reliability is known as the "handicap" principle, which is widely accepted (Zahavi 1975). Grafen first tested the "handicap" theory using a mathematical model, suggesting that 1) larger or more intense signals incur higher production costs and 2) higher-quality senders can produce larger signals at lower marginal costs (Grafen 1990). Empirical studies have largely supported these predictions (Murai *et al.* 2009). For instance, in barn swallows (*Hirundo rustica*), it was observed that short-tailed males experienced greater survival challenges compared to long-tailed males when artificial tail extensions were introduced (Moller *et al.* 1995).

However, it has become increasingly evident that existing studies predominantly focus on testing the "handicap" theory, necessitating further insights into the precise costs associated with honest signaling (Kotiaho 2001; Getty 2006; Husak *et al.* 2015). Understanding the costs incurred by signal senders is essential for elucidating the evolution of signal reliability under the "handicap" theory, thereby addressing the cost-to-benefit ratio to produce honest signals. Nonetheless, the limited research tools available have hindered the exploration of novel perspectives.

Husak et al. highlighted this interdisciplinary gap, noting that behavioral

ecologists often predict potential costs for senders through mathematical models but conduct fewer functional tests. Conversely, functional morphologists have focused on examining the biomechanical mechanisms underlying signal traits without a solid theoretical framework. Husak et al. emphasized the significance and advantages of integrating more functional approaches in the investigation of animal signaling costs (Husak *et al.* 2015).

This study aims to address the important but underexplored issue of the costs associated with producing reliable signals. The costs related to signal production are largely attributed to increased energy allocation, where significant alterations in energy investment can lead to multiple phenotypic consequences (Royle *et al.* 2006).

Several major phenotypic trade-offs associated with energy allocation for signaling have been proposed and tested. For example, there is a fitness cost, where the selection pressure to secure high-quality mates is exerted through the production of reliable signals. In *Lobesia botrana* (Lepidoptera: Tortricidae), the major components of female chemical profiles are suggested to serve as honest signals of quality; larger females producing more sex pheromones is preferred by males, but producing the chemical signals is suggested to suffer cost in survival for both large and small females (Harari *et al.* 2011). Additionally, trade-offs in growth can occur due to signaling, resulting from constraints related to underlying biomechanical and physiological correlations in various systems (Blake 2004). A study on field crickets (*Teleogryllus commodus*) examined trade-offs between calling effort, with life history traits and longevity, suggesting that the heavy investment in producing mating calls by males resulted in early adulthood death in this species (Hunt *et al.* 2004).

The evolution of cuticular hydrocarbons (CHCs) has been influenced by multiple selective pressures, with experiments involving artificial selection indicating interactions between natural and sexual selection across various systems (Blows 2002; Berson *et al.* 2019). Our findings in the first chapter did not support the notion that CHC profiles can be predicted by their roles as mating signals (**Figure 2.5**), highlighting the complexity involved in the evolution of CHC production. The multifaceted nature of CHCs arises from their various functions. In addition to serving as mating signals, CHCs in the insect cuticular wax layer also play roles in preventing water loss (Chung and Carroll 2015; Leeson *et al.* 2020; Mitchell *et al.* 2023) and conveying insecticide resistance (Balabanidou *et al.* 2016; Chen *et al.* 2020; Pu *et al.* 2020). Given their multiple functions, natural and sexual selection may interact synergistically or antagonistically in the evolution of insect CHCs, potentially resulting in trade-offs (Blows 2002; Berson *et al.* 2019). We propose that insect CHCs serve as an excellent model for exploring evolution driven by complex selective pressures. This study will specifically use CHCs in *Drosophila* to investigate the potential constraints and costs associated with CHC production for signaling purposes.

In our previous work, methyl-branched CHCs (mbCHCs) were identified as key mediators of desiccation resistance across *Drosophila* specie (Chung *et al.* 2014; Wang *et al.* 2022). *D. mojavensis* produces long-chained mbCHCs as its major compounds, which are believed to confer high desiccation resistance (Wang *et al.* 2023). A transgenic line of *D. mojavensis* (*Dmoj/mElo* elongase gene knockout) developed in this study produces approximately 50% less major mbCHCs compared to the wild type, specifically producing no 2-methyl-hexacosane (2MeC32) and lower amounts of 2-

methyl-triacontane (2MeC30) (**Figure S4.1**). CHC production has been demonstrated to be an important sensory modality that elicits male courtship behavior and contributes to mate recognition (**Figure 2.2**). Therefore, we first tested whether the major constituents, mbCHCs, act as honest signals indicating mate quality, potentially reflecting abilities in stress response. Moreover, metabolic studies have suggested that mbCHC production is costlier compared to other linear-chained CHCs (Nelson 1993). However, the phenotypic consequences of this costly production have yet to be determined. Consequently, we investigated the costs associated with producing long-chained mbCHCs in terms of life history traits, longevity, and reproductive fitness. By employing advanced genetic manipulation techniques, this study aims to provide novel insights into the costs of signaling.

4.2 Results

4.2.1 Quantitative changes in mbCHCs do not affect mate preferences

Having confirmed that cuticular hydrocarbon (CHC) input is essential for maintaining male courtship interest in *D. mojavensis* (**Figure 2.2**), we further investigated whether the methyl-branched CHCs (mbCHCs) produced by female *D. mojavensis* have evolved as honest signals indicating individual mate quality. To address this question, we conducted two-choice mating assays, exposing wild-type focal individuals (ISO1) to potential mates from two lines: one producing lower levels of mbCHCs (M3.5, *Dmoj/mElo* knockout) and the other being wild-type (ISO1). This design allowed us to assess potential differences in mate preferences between the sexes.

Our results indicated that focal individuals from the ISO1 line did not exhibit significant differences in mate preferences toward the two lines (**Table 4.1**). Considering

that the primary difference between the two chosen lines is the quantity of mbCHCs produced, we suggest that variations in mbCHC levels are not correlated with sexual attractiveness in *D. mojavensis*. This further implies that mbCHCs, as major constituents of CHC profiles, do not serve as reliable indicators of mate quality for either sex of *D. mojavensis*.

Focal Individual	*n*	ISO1 chosen	M3.5 chosen	df	χ2	*P*
ISO1 ♂	43	19	24	1	0.744	0.39
ISO1 ♀	56	27	29	1	0.036	0.85

Table 4.1 Quantitative changes in mbCHCs do not mediate mate preferences in *D. mojavensis*. Two-choice mating assays were used to test the mate preferences. ISO1 = wildtype *D. mojavensis*, M3.5 = transgenic line (*Dmoj/mElo* knockout) of *D. mojavensis* with lower amount of mbCHCs production. *Chi*-square tests were used to determine significant differences in mate preferences. No significant differences were found in either sex, suggesting quantitative changes of mbCHCs did not affect mating preferences.

4.2.2 No tradeoffs between development and mbCHC production

Methyl-branched cuticular hydrocarbons (mbCHCs) have been shown to elicit sustained courtship interest in males as mating signals (**Figure 2.2**), but do not reflect mate quality (**Table 4.1**). Additionally, mbCHCs mediate desiccation resistance under extreme stress conditions, but not under intermediate stress (Wang *et al.* 2023). In light of this, we aimed to investigate the potential costs associated with the production of excessive mbCHCs.

We first hypothesized that development might be negatively impacted as a cost of producing mbCHCs. This could occur for two reasons: 1) energy may be redirected from growth to signaling, and 2) growth could be inhibited due to "pleiotropic" effects stemming from metabolic changes required for signaling. To test this hypothesis, we examined developmental traits, including egg-to-adult viability and egg-to-adult

development duration. We anticipated that, the M3.5 (*Dmoj/mElo* knockout) would show higher egg-to-adult viability and shorter egg-to-adult development duration compared to the wild-type (ISO1), due to reducing the unnecessary compensatory growth for increased energy intake.

However, our results revealed no significant differences in either developmental trait: egg-to-adult viability ($t = 0.78$, $p = 0.44$; **Figure 4.1A**), female egg-to-adult development duration ($t = 1.60$, $p = 0.11$; **Figure 4.1B**), and male egg-to-adult development duration ($t = 0.46$, $p = 0.65$; **Figure 4.1C**). These findings suggest that there are no developmental costs associated with the changing production of mbCHCs in *D. mojavensis*.

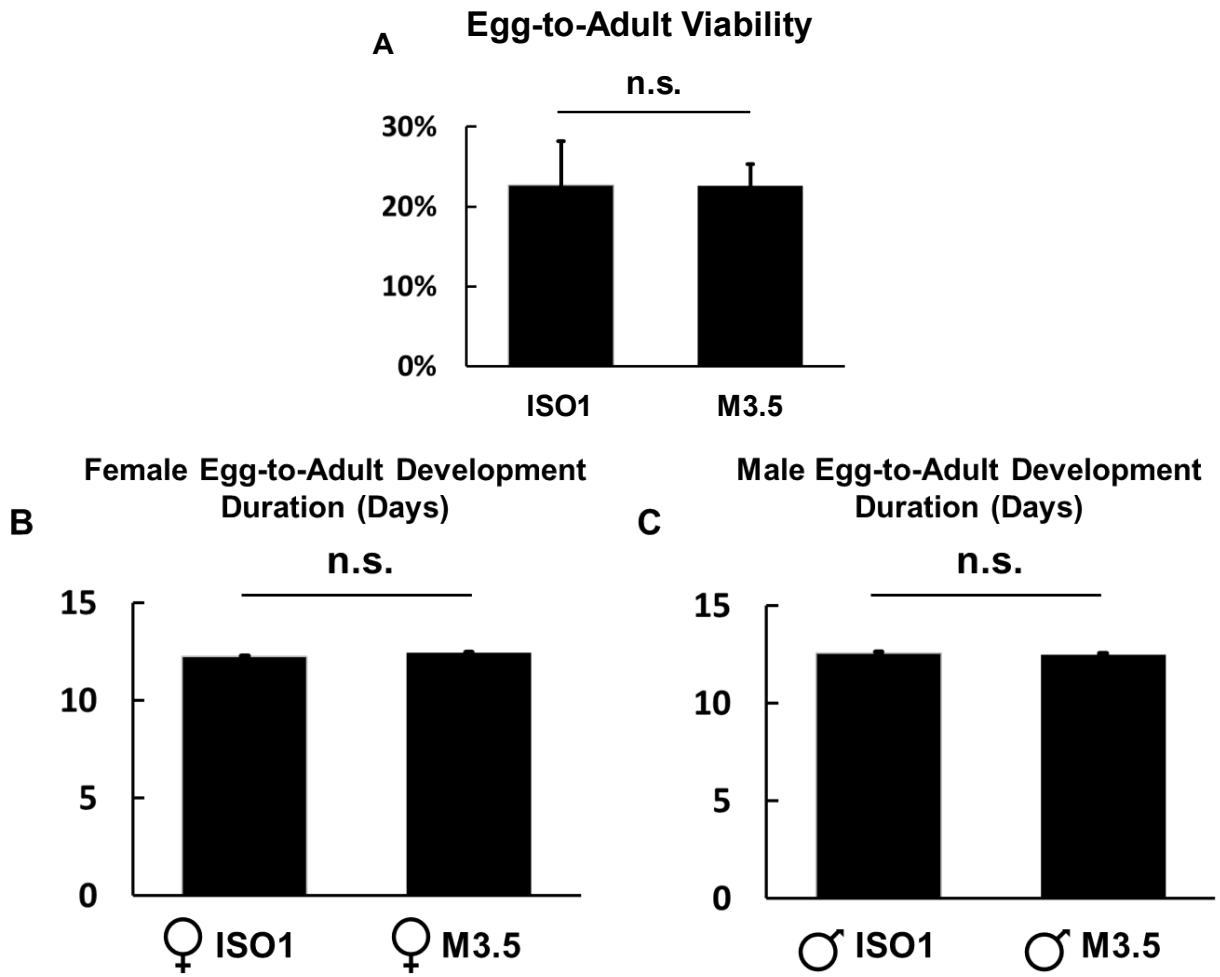


Figure 4.1 Development related life history traits were not affected by changes in mbCHCs production. ISO1 = wildtype *D. mojavensis*, M3.5 = transgenic line (*Dmoj/mElo* knockout) of *D. mojavensis* with lower amount of mbCHCs production. Student's *t*-tests were used to determine any significant differences in these phenotypic traits between the two test lines. No significant difference was found in egg-to-adult viability (**A**), female egg-to-adult development (**B**), and male egg-to-adult development (**C**). n.s = not significant.

4.2.3 Reproductive performance was negatively impacted in the transgenic line with decreased production of mbCHCs

We then investigated the potential costs associated with producing methyl-branched cuticular hydrocarbons (mbCHCs) on reproductive traits, specifically examining reproductive latency and reproductive fitness. We hypothesized that due to limited resource storage in adults and subsequent energy competition, trade-offs would exist

between reproduction and CHC synthesis. Consequently, we anticipated shorter reproductive latency and higher reproductive fitness in the M3.5 (*Dmoj/mElo* knockout) compared to wild-type (ISO1). However, our findings revealed a significant increase in reproductive latency for the M3.5 line, with an average of 6.08 days required to produce the first offspring, compared to 5.39 days for the ISO1 line ($t = 2.92$, $p < 0.01$; **Figure 4.2A**). Furthermore, the M3.5 line exhibited a significant reduction in offspring numbers, with five virgin pairs averagely producing 175.33 viable adults, compared to 234.11 viable adults produced by the ISO1 line ($t = 3.01$, $p < 0.01$; **Figure 4.2B**). These results do not support the existence of a trade-off between energy investment in reproduction and mbCHC synthesis. In fact, the negative changes observed in reproductive traits in the *Dmoj/mElo* knockout line suggest a potential role for *Dmoj/mElo* in reproduction-related physiological processes.

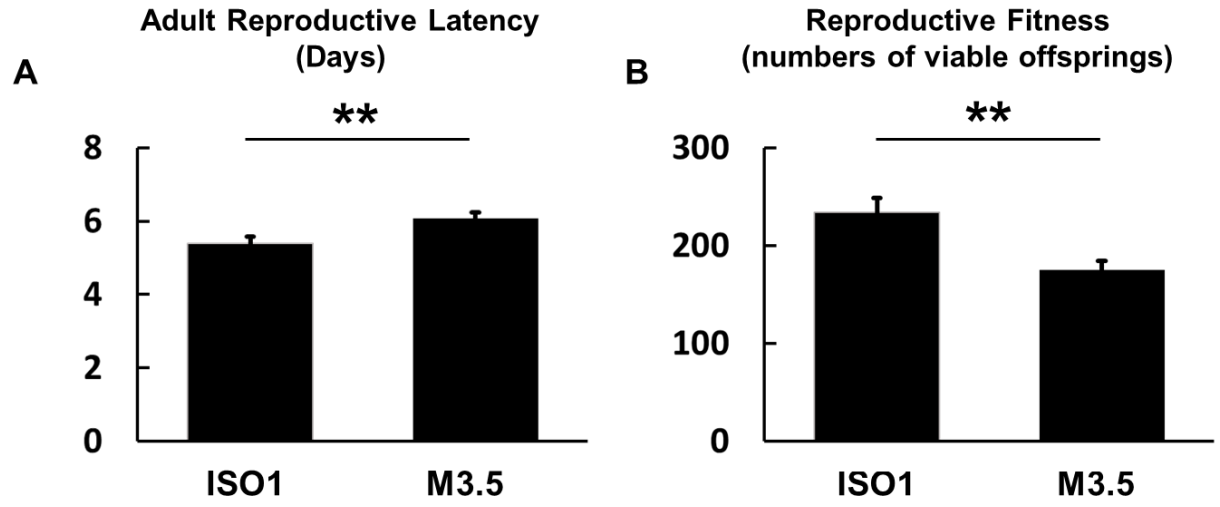


Figure 4.2 Negative impact on reproductive performance in transgenic lines with reduced mbCHC production. ISO1 = wildtype *D. mojavensis*, M3.5 = transgenic line (*Dmoj/mElo* knockout) of *D. mojavensis* with lower amount of mbCHCs production. Student's *t*-tests were used to determine any significant differences in these phenotypic traits between the two test lines. M3.5 show significantly increased reproductive latency after emergence before first offspring produced (**A**), and significantly reduced reproductive fitness by five pairs of virgin flies (**B**). ***p* < 0.01.

4.2.4 Decreased longevity in transgenic lines with reduced mbCHC production

Additionally, methyl-branched cuticular hydrocarbons (mbCHCs) have been shown to play a crucial role in desiccation resistance in *D. mojavensis* (Wang *et al.* 2023), and empirical evidence suggests that desiccation resistance is positively correlated with increased longevity in *Drosophila* (Rose *et al.* 1992). Consequently, we hypothesized that the M3.5 line would exhibit weaker survivorship (shorter longevity) compared to the ISO1 line, which could also contribute to higher reproductive fitness tested above. To test this hypothesis, we assessed the longevity of both lines and compared their 50% mortality rates (LT50). Our results indicated no significant difference in LT50 between the two lines for either sex (males: $t = 0.70$, $p = 0.51$, **Figure 4.3A**; females: $t = 1.88$, $p = 0.10$, **Figure 4.3B**). Additionally, we compared the difference in longevity between the two lines. Males of both lines did not show significant difference ($p = 0.15$; **Figure 4.3C**). Additionally, females of ISO1 line demonstrated significantly longer survival times than their M3.5 counterparts ($p < 0.01$; **Figure 4.3D**). Therefore, we suggest that the higher reproductive fitness observed in the ISO1 line can be partially attributed to females' significantly extended lifespan, while acknowledging that *Dmoj/mElo* may still play a functional role in reproductive processes, contributing to the increased reproductive latency.

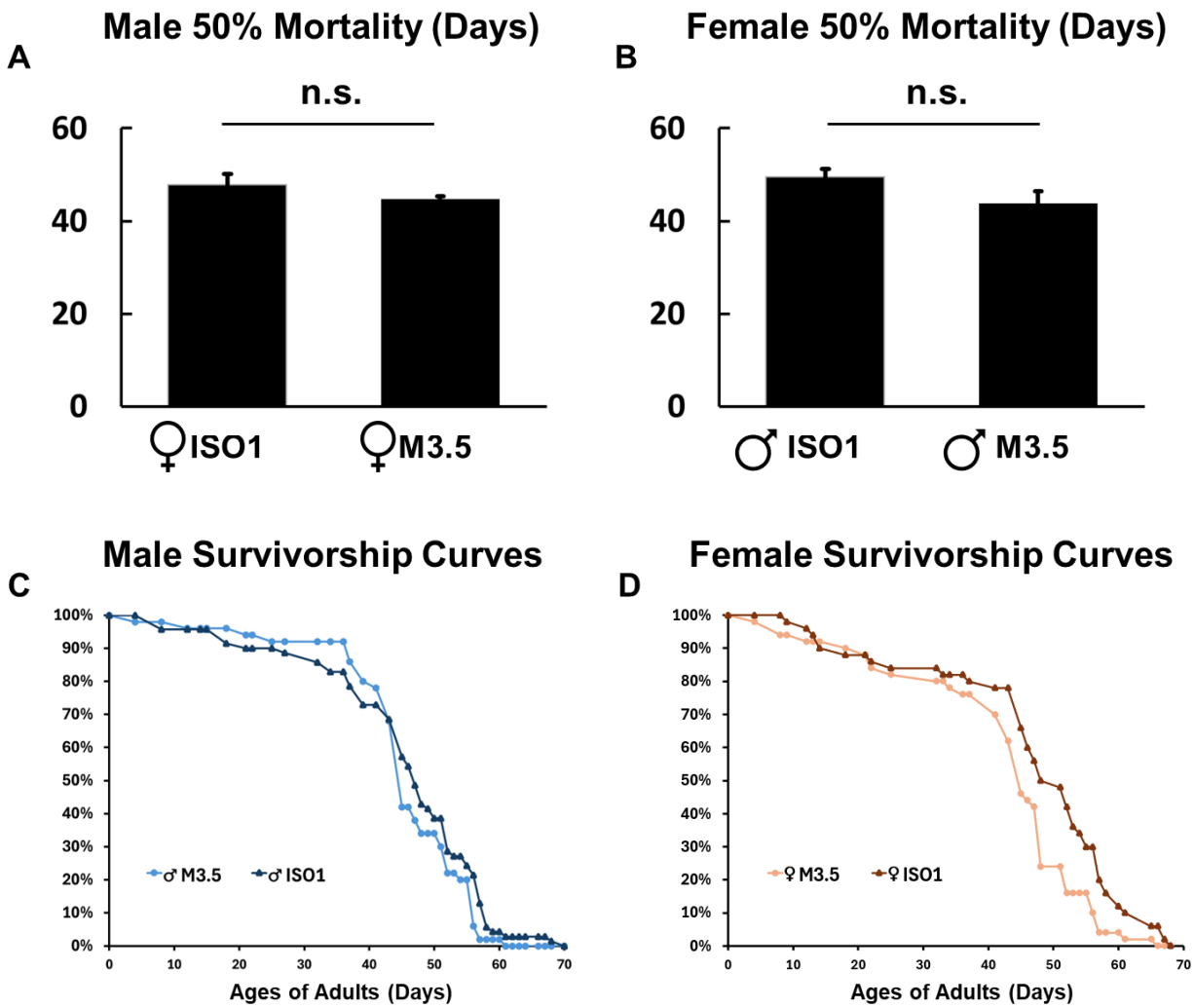


Figure 4.3 Lifespan of wildtype and transgenic lines of *D. mojavensis*. ISO1 = wildtype *D. mojavensis*, M3.5 = transgenic line (*Dmoj/mElo* knockout) of *D. mojavensis* with reduced mbCHCs production. Student's *t*-tests were used to determine any significant differences in these phenotypic traits between the two test lines. No significant difference between 50% mortality (LT50) in males (**A**) and females (**B**) between the two test lines. n.s = not significant. Kaplan-Meier approach was used to test the significance in survivorship. The percentage of surviving adults were shown from the enclosed day. No significant difference was found between males ($p = 0.15$) (**C**). ISO1 females show significant better surviving ability than M3.5 females ($p < 0.01$) (**D**).

4.3 Discussion

The production of reliable signaling is a topic of considerable discussion among

evolutionary biologists, posited to be influenced by complex interactions between natural selection and sexual selection. Among the various theories, the "handicap" theory is the most widely accepted, explaining the evolution of reliable signaling through the costs versus benefits incurred by signalers (Zahavi 1975). Therefore, understanding the absolute costs associated with the production of mating signals is essential for comprehending the underlying evolutionary processes. However, empirical evidence regarding the costs of signal production remains limited, primarily due to a lack of interdisciplinary approaches and appropriate research tools (Husak *et al.* 2015).

In this study, we utilized two lines of *D. mojavensis* from previous research: ISO1 (wildtype) and the genetically modified M3.5 line, which produces lower amounts of methyl-branched cuticular hydrocarbons (mbCHCs) due to *Dmoj/mElo* knockout. MbCHCs have been demonstrated to elicit sustained courtship interest from conspecific males, functioning as signals for mate recognition. We first aimed to determine whether mbCHCs could serve as indicators of individual mate quality in a dose-dependent manner. Results from two-choice mating assays did not support this hypothesis. We argue that these findings are robust, as further testing under heat stress conditions (37°C) is unnecessary, given that elevated temperatures are known to negatively impact courtship behavior in *D. mojavensis* (Patton and Krebs 2001; Shaible 2020).

Nonetheless, our results do not rule out the possibility that other CHC components may mediate sexual attractiveness and convey mate quality. Markow and Toolson suggested that the quantities of two dienes—pentatriacontadiene (C35:2) and heptatriacontadiene (C37:2)—may vary with rearing temperature and exhibit condition-specific plasticity, affecting sexual attractiveness in this species (Markow and Toolson).

However, in our study, the knockout of *Dmoj/mElo* did not alter the levels of these dienes (Wang *et al.* 2023). This indicates that the evolution of CHC profiles is shaped by complex selective pressures that may act on specific types of CHCs. In this context, mbCHCs primarily mediate desiccation resistance under natural selection, while the longer CHCs—dienes—may convey sexual attractiveness. Future studies across different species would enhance our understanding of the selective forces shaping CHC evolution.

Given the model prediction that producing mbCHCs incurs significant costs (Nelson 1993), we further examined the potential costs of mbCHC production in terms of phenotypic consequences. Our findings indicated no developmental costs associated with mbCHC production; however, the genetically modified M3.5 line demonstrated reduced reproductive success and female longevity. It is noteworthy that reduced longevity was observed only in females, indicating the potential of sexual antagonism in the evolution of mbCHCs production shaped by natural selection in this species. A similar hypothesis has been posited that natural or sexual selection may drive different evolutionary trajectories in CHC profiles between sexes in another mbCHC producer, *D. birchii* (Blows 2002). Empirical evidence is essential for testing this hypothesis.

Long-chain mbCHCs (2Me-C28) have been implicated in desiccation resistance in *D. melanogaster* (Wang *et al.* 2023). We therefore posed the question of whether natural aging processes (survivorship) and stress resistance (desiccation resistance) could be attributed to these specific long-chain mbCHCs. We investigated the survivorship of different lines of *D. melanogaster* that produce varying amounts of long-chain mbCHCs by employing the UAS-GAL4 system to either upregulate (overexpress)

or downregulate (RNAi knockdown) the gene *Dmel/CG18609* in adult oenocytes. Our results showed significantly improved survivorship in both modified gene expression directions, suggesting no direct relationship between long-chain mbCHC production and aging (survivorship); instead, the primary role appears to be in stress resistance (*Dmel/CG18609* overexpression females: $p = 0.012$; males: $p < 0.01$; *Dmel/CG18609* RNAi females: $p < 0.01$; males: $p < 0.01$; **Figure S4.2**). A sexually dimorphic trend was observed in both mbCHC-related studies, indicating a potential antagonistic pleiotropy of natural selection in both sexes.

Collectively, these results suggest a positive correlation between mbCHCs and mate quality, with higher amounts of mbCHCs being related to higher reproductive fitness and better survivorship; however, they do not appear to have evolved into honest signals for mate choice and mate preferences. Further investigation into the mechanisms governing the production of the dienes C35:2 and C37:2 would yield valuable insights into the broader context of CHC evolution. Additionally, biomechanical and physiological trade-offs are frequently observed in various systems (Vanhooydonck *et al.* 2001; Vandame *et al.* 2002; Blake 2004). The differential expression of *Dmoj/mElo* in our study likely contributes to these phenotypic changes, suggesting potential genetic architectures associated with CHC biosynthesis pathways that underlie the complex evolutionary processes involved in signal production.

In conclusion, the evolution of CHC production, which serves multiple functions, may involve interactions between natural selection and sexual selection that can be synergistic or antagonistic. Honest mating signals could emerge when evolutionary trajectories align, whereas deception may arise under conflicting pressures.

Determining the costs of signal production is crucial for understanding the "handicap" theory, and we propose that CHCs in *Drosophila* serve as excellent models for further investigations into the independent evolution of chemical signaling.

4.4 Materials and Methods

Drosophila species and strains

Two test lines of *D. mojavensis*, wildtype (ISO1) and a line that produces no 2MeC32 and lower amounts of 2MeC30 (M3.5, *Dmoj/mElo* knockout), were obtained from a previous study in the lab (Wang *et al.* 2023). To generate lines of *D. melanogaster* with *Dmel/mElo* overexpression or RNAi for the additional longevity tests, we crossed the 5′*mFAS*-GAL4 driver line (which expresses GAL4 in adult oenocytes) with the UAS-*mElo* overexpression and UAS- *mElo* RNAi lines as described in a previous study (Wang *et al.* 2022). All flies were reared and tested at 25℃ with 12h: 12h light: dark cycle, on standard cornmeal medium (Flystuff 66-121 Nutri-Fly Bloomington Formulation).

Two choice mating assays

The protocol was adapted from a previous study (Chung *et al.* 2014). Ten-day-old virgin flies were used in the tests, consistent with the previous methods reported in Chapter 2 for this species. Two lines of test flies were painted with blue or orange acrylic paints (DecoArt®) under CO_2 anesthesia 24hr before the tests. The color of paints used for the two lines were constantly shifted across all tests to eliminate the potential effects caused by coloration. In each setup, two chosen individuals from each line were introduced first, and then the focal individual from opposite sex was introduced as the start point of the test. Mate preferences of females were determined by the first male

they mated with. Mate preferences of males were determined by the first female they showed courtship rituals (**Table 1.1**) towards.

Development Assays

The protocol was adapted from a previous study (Etges 1990). Two batches of actively reproducing adults from each test line were reared in fly cages. Eggs laid were collected and randomly grouped into 40 on cover slides. The eggs were then transferred to fresh food vials for development assays. Egg-to-adult viability was determined by the total viable adults emerged from each vial divided by 40 [*n* (ISO1) = 17, *n* (M3.5) = 19]. Egg-to-adult duration was determined by the number of days, from the day when eggs were collected, to the day newly emerged adults were collected, where two sexes were recorded separated [*n* (ISO1-M) = 73, *n* (M3.5-M) = 91, *n* (ISO1-F) = 78, *n* (M3.5-F) = 85].

Reproduction Assays

Virgin flies collected on the day of emergence, from the previous development assays were used for the reproduction related traits tests. Each replicate consists of five females and five males from each line introduced into single fresh food vial. Reproduction latency was determined by the number of days, from the day adults emerged, to the day the first egg or larvae was observed [*n* (ISO1) = 23, *n* (M3.5) = 24]. The observations were made and checked by two individuals separately. After the first offspring was observed, the flies were regularly transferred into fresh food vials, with 2-or-3-day intervals. All food vials with the offsprings produced by same parental individuals form a cohort. The reproductive fitness was determined by the number of all viable adults collected from a cohort [*n* (ISO1) = 9, *n* (M3.5) = 9].

Longevity Assays

The protocol was adapted from a previous study (Linford *et al.* 2013). Virgin flies collected from the previous development assays were used for testing longevity. Each replicate experiment consists of either ten males or females from each test line transferred into single fresh food vial. Flies were regularly transferred into fresh food vials, at every 2-or-3-day intervals. 50% mortality (LT50) was determined by duration between the day of the start of the experiment and the day half (5) test individual died [*n* (ISO1-F) = 5, *n* (M3.5-F) = 5, *n* (ISO1-M) = 7, *n* (M3.5-F) = 5]. Longevity was determined by the number of alive days after emergence for each individual [*n* (ISO1-F) = 50, *n* (M3.5-F) = 50, *n* (ISO1-M) = 70, *n* (M3.5-F) = 50; *n* (*Dmel/CG18609* overexpression female) = 78, *n* (*Dmel/CG18609* RNAi female) = 76, *n* (*Dmel/CG18609* overexpression male) = 77, *n* (*Dmel/CG18609* RNAi male) = 75, *n* (overexpression control female) = 75, *n* (overexpression control male) = 72, *n* (RNAi control female) = 66, *n* (RNAi control male) = 75].

Statistical Analyses

The student's *t*-tests tests were used to determine significant changes in phenotypic traits between test lines, and were performed using the `t.test()` function in R. Kaplan-Meier approach was used to test the significance in survivorship through the `survival` and `survminer` packages in R, adapted from a previous study (Linford *et al.* ; Therneau 2015; Kassambara *et al.* 2016). All analyses were conducted, and figures were produced in RStudio (Rstudioteam 2022).

CHAPTER 5. GENERAL DISCUSSION AND FUTURE RESEARCH DIRECTIONS

5.1 Summary of the research projects

Sexual dimorphism highlights remarkable beauty and diversity in nature, with mating signals serving as common representations of this phenomenon. Multimodal mating signals have been suggested in various systems across the animal kingdom, and both sexual dimorphism and mating signals can be classified into various types, each suggesting different levels of information conveyed either inter- or intraspecifically (**Figure 1.1**). The evolution of sexual dimorphism and mating signals is influenced by complex interactions among multiple selective pressures, prompting significant interest and discussion among evolutionary biologists. This focus stems from our fascination with nature and aesthetics, as well as the crucial roles these traits play in central topics related to reproductive isolation and speciation.

Despite this interest, understanding these intricate evolutionary processes presents challenges, and empirical evidence needs to be further explored across diverse study systems. Importantly, there are still unresolved questions that must be addressed. In this dissertation, we investigated three important questions in evolutionary biology: **1**) Is there a correlation between the evolution of sexual dimorphism and the evolution of mating signals? Can the degree of sexual dimorphism be used to predict the functional roles of these traits as mating signals? **2**) To understand the evolution of sexual dimorphism, what genetic mechanisms underlie exaggerated female traits? **3**) To understand the evolution of mating signals, what phenotypic trade-offs exist as costs associated with the evolution of these signals? (**Figure 5.1**).

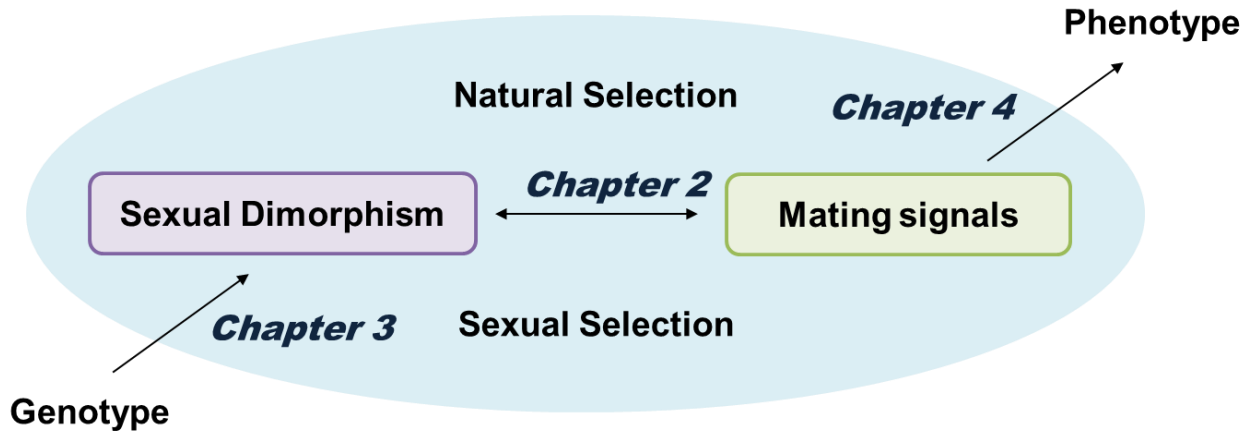


Figure 5.1 Overview of the research projects and dissertation.

We utilized cuticular hydrocarbons (CHCs) in *Drosophila* species as a model to investigate these questions. Previous studies on CHCs have indicated both 1) the presence of varying levels of sexually dimorphic CHC profiles across species and 2) the role of CHCs in *Drosophila* as contact pheromones for chemical communication (Ferveur 2005).

Initially, we assessed the degrees of sexual dimorphism using the Bray-Curtis dissimilarity index and tested the effects of CHC perception on maintaining male courtship interest across species. Our findings did not provide sufficient evidence to support a correlation between the degree of sexual dimorphism and the use of CHCs for male mate recognition (**Chapter 2**). Next, we focused on the *Drosophila* species exhibiting the highest degree of sexual dimorphism in CHCs, *D. erecta*, investigating the genetic mechanisms underlying the evolution of the exaggerated female traits. We identified a candidate gene *LOC6555117* with female-biased expression in adult oenocytes that is likely responsible to produce this trait. The observed female-biased expression pattern is attributed to *cis*-regulatory changes, with two specific modules identified: an oenocyte expression module and a sex-biased expression module

(**Chapter 3**). Finally, we explored the potential costs of producing methyl-branched cuticular hydrocarbons (mbCHCs) in transgenic lines of *D. mojavensis*, which generate mbCHCs as the major compounds in their CHC profiles. Our results do not show direct developmental tradeoffs of producing mbCHCs, but indicate that mbCHC production is positively correlated with reproduction and longevity. Even though mbCHCs are positively correlated with individuals' fitness, it did not evolve into serving as honest signals to mediate mate preferences. (**Chapter 4**).

Based on these results, I proposed several specific directions for future research in each chapter. The findings presented in this dissertation are expected to address the proposed questions and provide novel insights into the evolution of both sexual dimorphism and mating signals.

5.2 Evolution of mating signals: interactions among multimodal sensory modalities and plasticity with environmental change

Multimodal sensory modalities are proposed to function as a series of mating signals, potentially conveying different levels of information (**Table 1.1**). Mating signals have traditionally been categorized into visual, acoustic, chemical, and mechanical signals, based on the sensory modalities used for information perception. The evolution of each type of mating signal is influenced or constrained by the underlying genetic architecture, which has been extensively studied. However, future research should focus on the specific mechanisms by which each signal operates in natural contexts, potentially in a condition-dependent manner.

Firstly, within the framework of generalized sensory modalities, specific mating signals can be transmitted and perceived differently. For instance, within the realm of

chemical signals, both volatile pheromones (Karlson and Butenandt 1959) and contact pheromones (Guarino *et al.* 2008) have been identified. Due to the chemical characteristics of these compounds, their functions in nature differ significantly; volatile chemicals typically facilitate long-range interactions, while contact pheromones are necessary for close-range interactions (Duffy *et al.* 2018). It is plausible that long-distance volatile pheromones evolved for mate recognition, conveying information about species and sex, whereas short-distance contact pheromones indicate mate quality, contributing to mate choice and preferences. For example, in the multicolored Asian ladybeetle, *Harmonia axyridis* (Coleoptera; Coccinellidae), volatile pheromones are utilized for long-distance selection, while non-volatile contact pheromones serve short-distance selection (Brown *et al.* 2006; Durieux *et al.* 2012). Distinct short- and long-distance mating signals have also been observed in other sensory modalities, such as the two calling song classes in dark-eyed juncos (*Junco hyemalis*) (Titus 1998). Investigating the parallel evolution of these short- and long-distance signals, which convey similar information, may provide novel insights, particularly regarding the evolution of contact pheromones and visual signals related to fecundity. It would also be intriguing to determine whether the parallel evolutions among sensory modalities are biomechanically and physiologically correlated in a synergistic or antagonistic manner, potentially compensating for the loss of one sense or providing contrasting information that reflects different aspects of mate quality.

Secondly, it is crucial to understand how environmental changes shape the future evolutionary trajectories of mating signals. Effective signaling relies on the appropriate environmental context for information transmission. Changes in environmental

conditions can positively or negatively affect individual signal modalities, making it essential to test the plasticity of these signaling traits. Additionally, the interactions between individual mating signals and other signal traits, which may exhibit varying levels of resilience under environmental changes, require further investigation. In particular, climate change and urbanization have received increasing attention as significant environmental changes. The work of Heinen-Kay highlights how urbanization affects sexual communication and mating signals in the Anthropocene (Heinen-Kay *et al.* 2021), suggesting that similar studies across a broader range of taxa could enhance our understanding of this issue, especially in arthropods.

Finally, I emphasize the importance of understanding the plasticity of reproductive strategies in response to perception of divergent mating signals. Sociosexual environments can influence mating strategies, providing evidence of plasticity (Cong and Wang 2021). For instance, in *Drosophila*, both mating latency and duration can be altered by the population's sex ratio (Dore *et al.* 2021). If sociosexual factors are reflected in signaling, then investments in mating are likely to evolve plasticity as a response to the perception of these signals. Addressing these issues will further our understanding of population dynamics and related ecological processes.

5.3 Evolution of sexual dimorphism: further dissecting traits with specific characteristics

In addition to the previously proposed avenues for future research in the evolution of sexual dimorphism, such as investigating the evolution of female exaggerated traits in light of the dynamic nature of sexual roles, the complex drives of sexual dimorphism remain poorly understood. In Chapter 4, we proposed the possibility that different types

of cuticular hydrocarbon (CHC) compounds produced by *D. mojavensis* may serve distinct functions, each influenced by different selective forces. MbCHCs primarily mediate desiccation resistance under natural selection, while the longer CHCs—dienes—may convey sexual attractiveness in this species.

To further elucidate these potential drives, it would be beneficial to dissect sexual dimorphism into secondary sexual characters into multiple, more specific traits. For example, in the case of the lion's mane—a secondary sexual character—female mate choice is influenced by two primary axes of variability: mane length and coloration. Research has suggested that mane length primarily affects male-male competition, while coloration primarily responds to environmental changes (West and Packer 2002).

This framework can be applied to other sexually dimorphic traits, where multiple characteristics can be identified. For instance, body coloration may be broken down into both coloration intensity and coloration area, while mating calls can be categorized by call frequency and amplitude. I propose that this methodological approach could enhance our understanding of the evolution of traits with a wide range of phenotypic variations, such as the chemical blends produced by pheromone glands.

5.4 Evolution of insect CHCs: other variations in CHC profiles and potential interdisciplinary collaborations

Beyond the scope of mating signals and sexual dimorphism, several other CHC-related inquiries remain unexplored. First, as major components of insect cuticles, CHCs have been implicated in various other roles, including mediating insecticide resistance (Pu and Chung 2024), reflecting solar radiation to mitigate heat (Hadley 1994), and providing defense against pathogen penetration and infection (Gołębiowski *et al.* 2008).

Further investigation into these functions is required to systematically elucidate the evolutionary processes related to CHC production.

Additionally, the evolution of CHC biosynthesis enzyme families remains unknown. While the rapid gain and loss of reductase genes has been documented (Finet *et al.* 2019), the evolution of elongase genes— the focus of this dissertation— remains largely unknown. Preliminary synteny analyses have yielded initial insights but require further investigation. My current hypothesis posits that elongase genes also undergo rapid evolution across species, particularly orthologs involved in producing mating signals pertinent to speciation (**Figure S5.1**). What's more, investigating the roles of reductases and lipid transporters in CHC biosynthesis and deposition will be insightful, especially concerning how these gene families influence CHC profiles and their associated functions.

More specifically, several variations in CHC profiles are less studied, including: 1) changes in CHC profiles with aging, 2) mechanical transfers during interindividual physical contact, particularly during mating, and 3) intraindividual variations of CHCs across different body parts due to chemical diffusion. Throughout an individual's lifespan, CHC profiles continuously change as adults age, a phenomenon demonstrated in *D. melanogaster* (Jallon and Wicker-Thomas 2003). Investigating how these changing profiles correlate with physiological aging and how the variations in chemical composition reflect aging status as honest signals presents a promising avenue for research. In Chapter 3, we demonstrated that elongase *LOC6555117* exhibits female-biased expression through *in situ* hybridization in 4-day-old adults (**Figure 3.4C**). We also examined gene expression in 1-day-old adults and found no sex-biased expression

(**Figure S3.3**). Future research should focus on elucidating the genetic mechanisms underlying this specific temporal expression pattern.

Furthermore, mechanical transfer during mating has been suggested to occur (Weddle *et al.* 2013), and it is important to investigate how sex-specific compounds may have evolved to facilitate this mechanical transfer and serve as signals of mating status. While variations in CHC profiles across species and populations have been extensively studied, intraindividual variations in CHCs among body parts have only recently been examined (Sprenger *et al.* 2021). Both the mechanisms underlying these differences (potentially involving CHC diffusion) and the ecological implications of such intraindividual variations require further investigation. For instance, an insecticide-resistant strain of *Anopheles gambiae* was shown to produce a thicker layer of CHCs with differing compositions compared to susceptible strains, particularly on their legs (Balabanidou *et al.* 2016; Balabanidou *et al.* 2019). Understanding intraspecific CHC variations could provide valuable insights for future applied research.

Despite the known roles of CHCs as chemical signals, how diverse CHC compounds are perceived across *Drosophila* species remains to be elucidated. The perception of CHCs is generally discrete, with gustatory receptors implicated in their recognition. Substantial research has focused on identifying the gustatory receptors responsible for CHC perception, with Gr32a identified as the sole receptor required for recognizing *D. melanogaster* CHCs to date (Fan *et al.* 2013). Exploring how this single receptor processes divergent CHC compounds, how gustatory receptors coevolve with varying CHC production across species, and how other chemical signalers perceive CHCs are all valuable areas for future investigation, with valuable inputs from

neurobiology.

In addition, with the potential collaboration with biochemists and biophysicists, the dynamics of multicomponent mixture of CHC blends could be addressed. CHCs in ants were suggested to be solid-liquid mixtures presented on the cuticle (Menzel *et al.* 2019). The biphasic CHC layer guarantees both the roles of chemical communication and desiccation resistance. It is notable to hypothesize that solid phase incorporated with the role of desiccation resistance as exhibiting less plasticity, whereas the compounds in liquid phase can actively respond to ecological changes and serve as mating signals. Further exploration and characterization in the multi-leveled biphasic CHC blends become necessary in understanding the roles of specific CHC compounds from the perspective of chemical producers (Blomquist and Ginzel 2021).

In summary, studying insect CHCs offers significant insights into fatty-acid synthesis pathways, aging, fecundity, and stress responses, particularly through the model organism *Drosophila*. These findings can also address applied questions, such as pesticide resistance, arthropod adaptation to environmental changes, and the development of novel chemical control tools in agricultural systems. Interdisciplinary approaches are encouraged to broaden the scope of future research related to insect CHCs.

Final Thoughts

I have always been finding deep inspiration and enlightenment in the field of evolution. In nature, conflicts are constant, particularly when competition arises between the sexes. To resolve these sexual conflicts, the evolution of complex genetic architectures that give rise to sexually dimorphic traits often occur. These traits may evolve further to

facilitate communication between the sexes, addressing the underlying conflicts. While the production of sexually dimorphic traits and communication signals demands additional metabolic costs, it ultimately leads to the resolution of conflicts and the generation of greater diversity in nature—an outcome that initially stimulated human curiosity and investigation. In essence, resolving conflicts, such as sexual conflict, requires both effort (genetic architecture) and investment (metabolic cost), and it results in balanced beauty and diversity (sexual dimorphism). Throughout this process, honest and open communication (signaling) remains a crucial element in resolving conflicts and promoting the prosperity of life (fitness). This is the lesson I have learned from nature and evolution. C'est la vie (This is life).

While exploring the evolution of chemical communication, I was struck by the scarcity of studies on this subject compared to visual and acoustic signals. I cannot definitively say this imbalance stems from biases of human sensory, as we do not rely on chemical communication as often. However, if the ultimate goal of scientific advancement is to challenge assumptions and not take things for granted, I would encourage a broader perspective and more innovative thinking beyond the conventional frameworks.

Evolution engraves deep within,
The shape of my life was born to pin.
Not by the way how I faced the years,
And how I smile through the future fears.

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APPENDIX

	D. erecta ♂	D. erecta ♀
C21	31.2 ± 21.1	undetected
C22	17.6 ± 2.5	undetected
C22:1	15.4 ± 1.9	undetected
C23:1 (a)	234.9 ± 63.3	undetected
C23:1 (b)	805.3 ± 54.7	undetected
C23:1 (c)	33.8 ± 8.4	undetected
C24:1 (a)	5.7 ± 1.5*	undetected
C24:1 (b)	3.4 ± 1.4*	undetected
C24:1 (c)	2.1 ± 1.3*	undetected
2Me-C24	31.5 ± 4.6	undetected
C25:1 (a)	61.8 ± 7.2	undetected
C25:1 (b)	72.5 ± 3.8	undetected
2Me-C26	60.9 ± 10.3	17.5 ± 1.7
C27	6.8 ± 3.9*	undetected
2Me-C28	8.4 ± 1.5	105.7 ± 15.3
C29:1 (a)	undetected	11.1 ± 1.6
C29:1 (c)	undetected	10.4 ± 2.9*
2Me-C30	undetected	22 ± 7.3
C31:1 (d)	undetected	83.4 ± 10.4
C31:2 (d)	undetected	194.2 ± 18
C33:2 (g)	undetected	107.8 ± 24.2
C33:2 (b)	undetected	51.6 ± 12.2

	D. melanogaster ♂	D. melanogaster ♀
C21	14 ± 1.6	undetected
C22	15.4 ± 2.2	9.5 ± 1.2
C22:1	8.8 ± 0.9	undetected
C23:1 (a)	217.5 ± 16.2	132.2 ± 12.7
C23:1 (b)	631.2 ± 34.9	56.7 ± 6.7
C23:1 (c)	54.2 ± 3.6	undetected
C24:1 (a)	undetected	19.4 ± 0.9
C24:1 (b)	6.9 ± 2.4*	undetected
C24:1 (c)	2.5 ± 1.6*	undetected
2Me-C24	26.3 ± 2	10.1 ± 1.7
C25:1 (a)	45.5 ± 3.8	157.4 ± 7.4
C25:1 (b)	136.4 ± 16.1	83.2 ± 7.3
C25:2	undetected	11.9 ± 1
2Me-C26	44.7 ± 2	86.5 ± 10.8
C27:1	undetected	43.5 ± 11.4
C27:2	undetected	302.2 ± 9.8
2Me-C28	17.7 ± 1.7	40 ± 3.2
C29:2	undetected	185.7 ± 11.1

Table S2.1 Quantified CHC profiles across selected *Drosophila* species. Quantitation of the CHCs (ng/µL) of nine *Drosophila* species from 5 or 6 sets of five adult flies was adapted from (Wang *et al.* 2022) with specifying different isomers. (*) indicates that this CHC is not detected in all samples. Mean ± SEM is shown.

Table S2.1 (*cont'd*)

	D. repleta ♂	D. repleta ♀
2Me-C28	218.6 ± 22.6	126.2 ± 11.9
C29:2 (e)	109.3 ± 36.9	undetected
C29:2 (f)	143.4 ± 40.7	undetected
2Me-C30	119.9 ± 24.7	195.2 ± 29.4
C31:2 (e)	13.9 ± 4.3*	undetected
C31:2 (f)	100.6 ± 17.3	undetected
2Me-C32	2.2 ± 1.1*	undetected
C33:1 (a)	2.4 ± 1.2*	undetected
C33:2 (b)	21.9 ± 5.1	42.9 ± 5.8
C33:2 (c)	33.7 ± 10.5*	108.6 ± 20.3
C35:2 (a)	5.6 ± 2.2*	13.2 ± 3.1
C35:2 (b)	20.4 ± 7.2*	54.2 ± 13.2
C35:2 (c)	95.2 ± 26.1	308.3 ± 32.1
C37:2 (b)	9.6 ± 6.1*	41.4 ± 25.1

	D. yakuba ♂	D. yakuba ♀
C21	33.7 ± 3.2	41.2 ± 2.2
C22	32.2 ± 2.1	36.2 ± 3.3
C22:1	23.7 ± 1.3	24.6 ± 1.3
C23:1 (a)	278.5 ± 14	297.3 ± 21.2
C23:1 (b)	1254.8 ± 71.7	1270.6 ± 63.1
C23:1 (c)	undetected	7.1 ± 7.1*
C24:1 (a)	16.8 ± 2.9	15.6 ± 2.1
C24:1 (b)	3.3 ± 2.1*	5.6 ± 2.7*
C25:1 (a)	17.6 ± 1.3	14.5 ± 1.9
C25:1 (b)	42.3 ± 3	24.6 ± 4.2
2Me-C26	89.1 ± 14.7	42 ± 4.9
C27	4.4 ± 3*	17.6 ± 1.9
C27:1 (b)	10.2 ± 4.4*	21.6 ± 2.7
2Me-C28	7.1 ± 2.3*	40.1 ± 5.3

	D. mojavensis ♂	D. mojavensis ♀
2Me-C28	15.3 ± 0.6	14.7 ± 1.2
2Me-C30	97.6 ± 6.1	95.3 ± 7.7
2Me-C32	7.5 ± 0.4	7.6 ± 0.7
C33:1 (a)	5.5 ± 0.3	5.4 ± 0.7
C35:1 (a)	3.8 ± 0.4	3.6 ± 0.6
C35:1 (b)	7.2 ± 0.5	6.4 ± 1
C35:2 (a)	6.9 ± 1	5.6 ± 0.8
C35:2 (b)	92.8 ± 8.8	60.3 ± 10.1
C35:2 (c)	86.8 ± 18.5*	60.2 ± 10.4
C37:2 (a)	13.9 ± 0.7	14.8 ± 1.8
C37:2 (b)	28.2 ± 1.2	27.6 ± 6.1

	D. pseudoobscura ♂	D. pseudoobscura ♀
C25:1 (d)	44.8 ± 3.8	57.6 ± 4.9
C25:2 (d)	64.3 ± 5.9	49.4 ± 2.7
C26:1	45.8 ± 1.4	45.7 ± 2.6
C26:2 (b)	37.1 ± 4.5	30.9 ± 2.8
2Me-C26	50.6 ± 4.5	37 ± 3.4
C27:1 (b)	29.5 ± 4.1	24.3 ± 3
C27:2 (e)	1941.9 ± 76.5	1788.6 ± 87.9
2Me-C28	234 ± 16.7	219.9 ± 15.6
C29:2(h)	44.4 ± 4	39.6 ± 5.7
2Me-C30	26.5 ± 2.1	30.3 ± 4.4

Table S2.1 (*cont'd*)

	D. simulans ♂	D. simulans ♀
C22	26.5 ± 3.5	31.5 ± 2.8
C22:1	21.5 ± 1.7	26 ± 3
C23:1 (a)	294.6 ± 25.8	295.5 ± 23.7
C23:1 (b)	1344.2 ± 81.7	1608.9 ± 57.1
C23:1 (c)	94.7 ± 6.4	131.8 ± 6
C24:1 (a)	2.7 ± 1.7*	4.6 ± 2.1*
C24:1 (b)	30.1 ± 5.5	31.4 ± 3.5
C24:1 (c)	19.2 ± 2.8	21.9 ± 2.2
2Me-C24	4.6 ± 2.9*	3.6 ± 2.3*
C25:1 (a)	28 ± 3.5	41.1 ± 5.5
C25:1 (b)	93.6 ± 11.5	93.9 ± 13.8
C25:1 (c)	25.7 ± 2.8	17.1 ± 1.9
2Me-C26	111.2 ± 12	73.3 ± 10.5
C27	1.4 ± 1.4*	18.2 ± 3
2Me-C28	12.9 ± 1.2	31.1 ± 2

	D. ananassea ♂	D. ananassea ♀
2Me-C28	43.6 ± 5	51 ± 11.5
C29:1 (d)	7.4 ± 1.7	4.7 ± 2.3*
2Me-C30	100.1 ± 11.9	130.5 ± 7.9
C31:1 (a)	15.1 ± 0.9	23 ± 1.4
C31:1 (b)	16.1 ± 2.1	17.2 ± 0.4
C31:2 (e)	19.6 ± 1.7	16.6 ± 2.1
C31:2 (f)	79.7 ± 8.1	64.3 ± 12.2
C31:2 (g)	57 ± 4.9	48.2 ± 6.4
C31:2 (h)	37.8 ± 3	32.7 ± 5.7
C33:2 (b)	5.6 ± 0.8	8.2 ± 1.2
C33:2 (c)	15.4 ± 2.7	17.2 ± 2.5
C33:2 (d)	22.7 ± 2.7	25.8 ± 5.6
C33:2 (e)	14.3 ± 2.6	undetected
C35:2 (a)	5.5 ± 2.2	12.7 ± 2.7
C35:2 (b)	5.3 ± 2	8.6 ± 1
C35:2 (c)	5.9 ± 2.5	9 ± 1.6
C35:2 (d)	8.3 ± 5.1	14.6 ± 2.7

D. simulans	4
D. yakuba	4
D. erecta	2
D. ananassae	4
D. pseudoobscura	4
D. willistoni	2
D. mojavensis	10
D. repleta	8
D. melanogaster	4

Table S2.2 Age of tested adults (days) in the no-choice mating assays. The ages were initially referred to a previous study in the age of sexual maturity (Pitnick *et al.* 1995), and determined by results of pilot tests.

	n (Conspecific Intact ♂)	n (Conspecific Tarsi-removed ♂)
D. repleta ♀	40/36 for Courtship Latency	37/30 for Courtship Latency
D. mojavensis ♀	42	42
D. melanogaster ♀	46/36 for Courtship Latency	46/30 for Courtship Latency
D. erecta ♀	35	36
D. simulans ♀	31	34
D. yakuba ♀	39	38
D. ananassea ♀	60	62
D. willistoni ♀	37	46
D. psedoobscura ♀	40	40

	n (Intact *D. erecta* ♂)
CHC- ♀ *D. melanogaster* coated with *D. erecta* CHCs	8
CHC- ♀ *D. melanogaster*	8

	n (Intact *D. psedoobscura* ♂)
CHC- ♀ *D. melanogaster* coated with *D. psedoobscura* CHCs	17
CHC- ♀ *D. melanogaster*	15

Table S2.3 Sample sizes of no-choice mating assays. Number of tested males are shown. All tested individuals are sexually matured males with limited exposure to conspecific females.

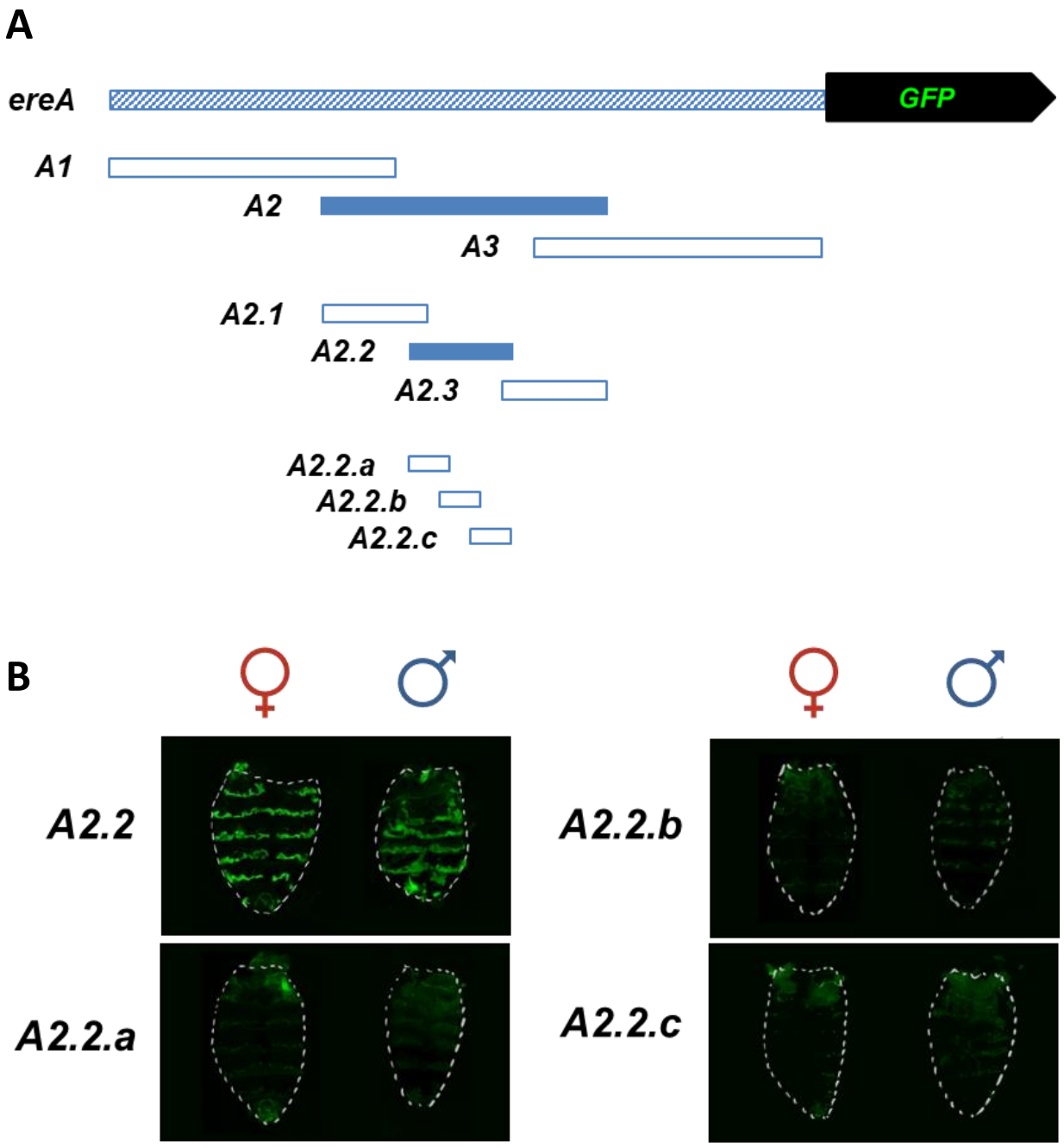


Figure S3.1 Identification of an oenocyte expression module. (A) Schematic dissection of all GFP reporter constructs. Striped box = female biased expression, solidly filled box = non-sex biased expression, no filled box = no expression. **(B)** GFP reporter protein expression in oenocytes corresponding to the different overlapping constructs. The A2.2 fragment is the minimum region that can recapitulate a sexually monomorphic oenocyte expression. Further dissecting A2.2 into A2.2.a, A2.2.b and A2.2.c do not show any strong GFP expression in the corresponding GFP reporting constructs.

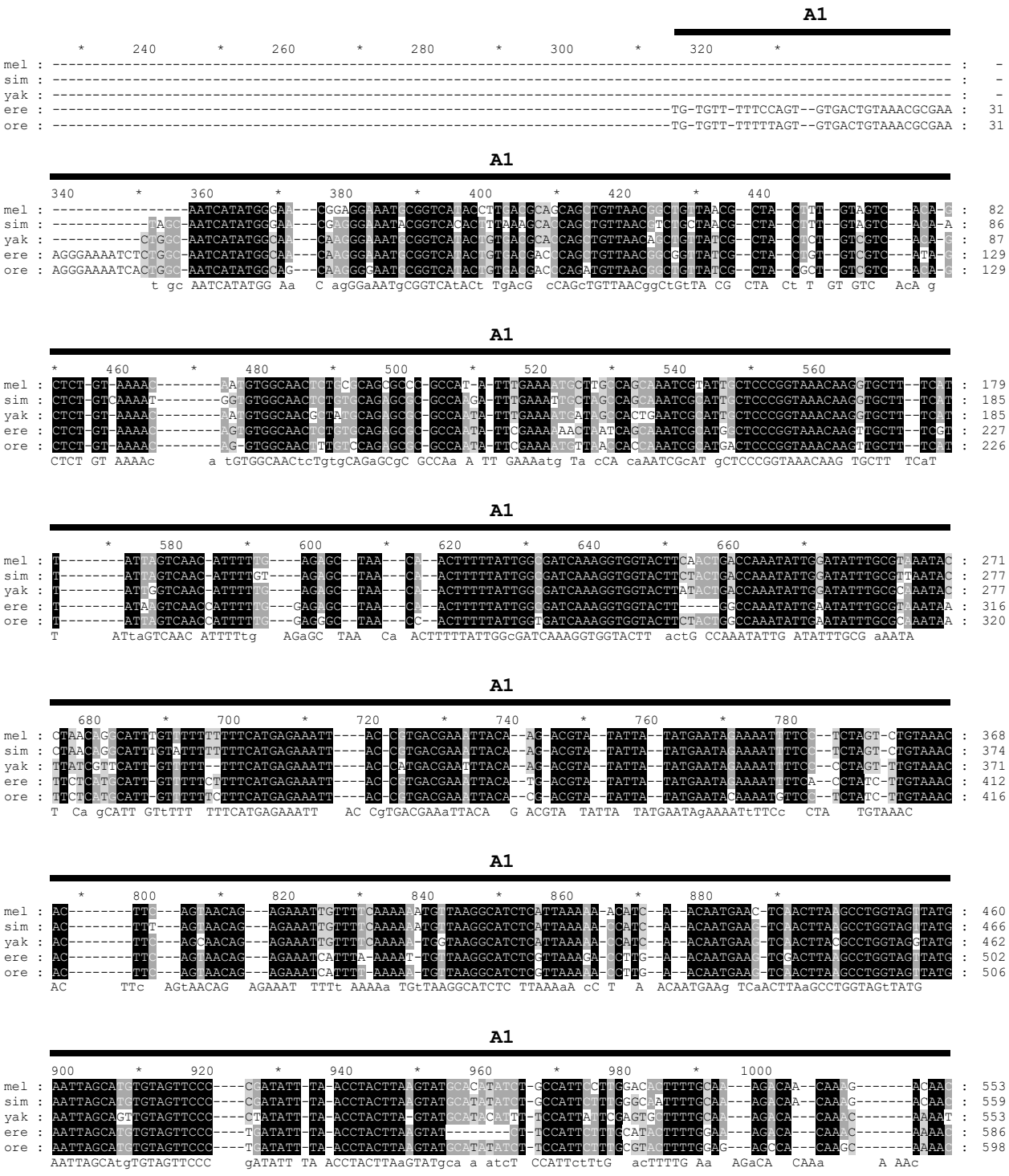


Figure S3.2 Full sequence alignment of *ereA* with homologous fragments from other species. The designed overlapping test fragments were labeled above each line of the sequence alignments. Black The predicted Erdman-Burtis Consensus of *dsx* binding site is highlighted in yellow, which lies in an overlapping region of *A2* and *A3*.

Figure S3.2 (*cont'd*)

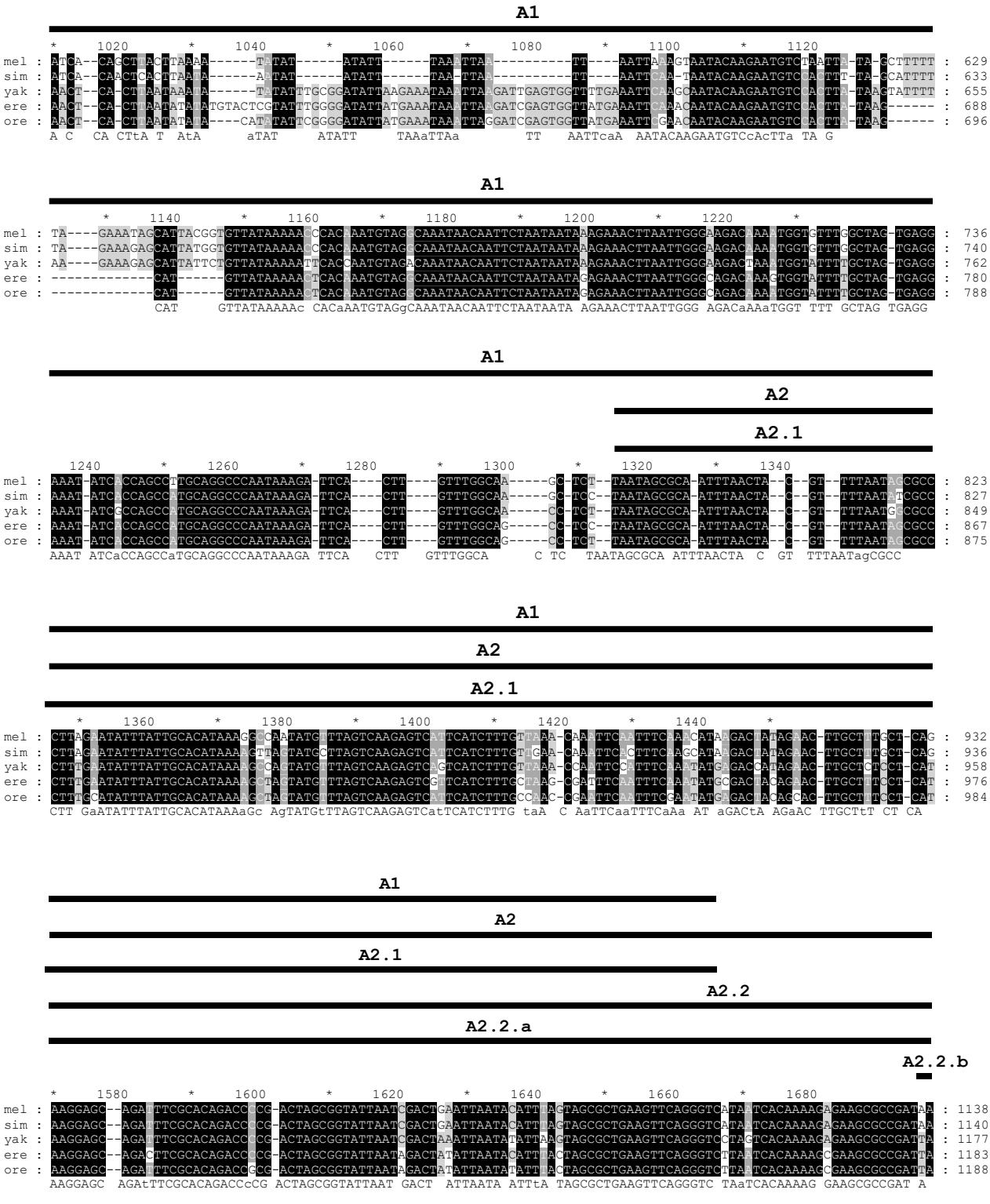


Figure S3.2 (*cont'd*)

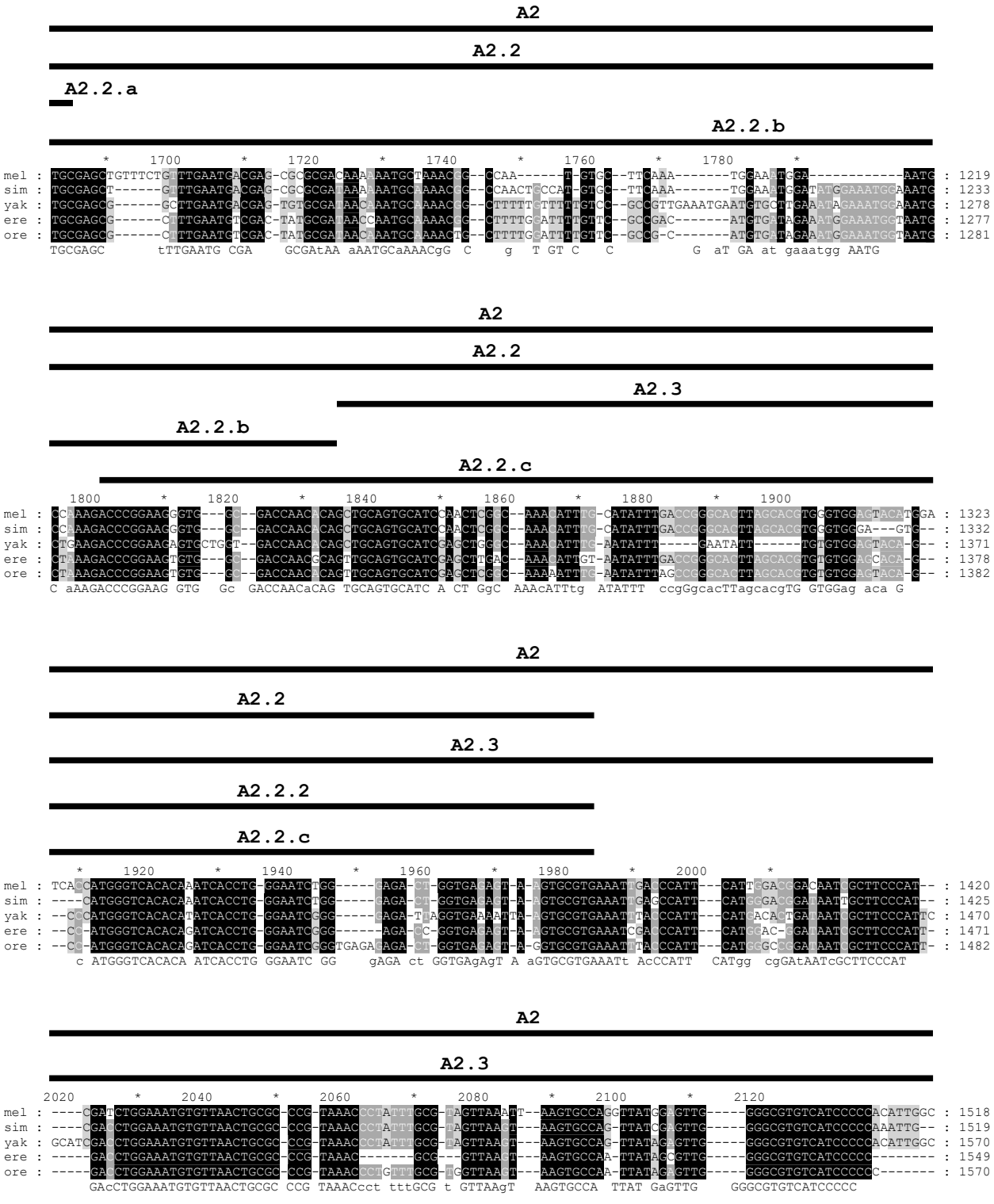


Figure S3.2 (*cont'd*)

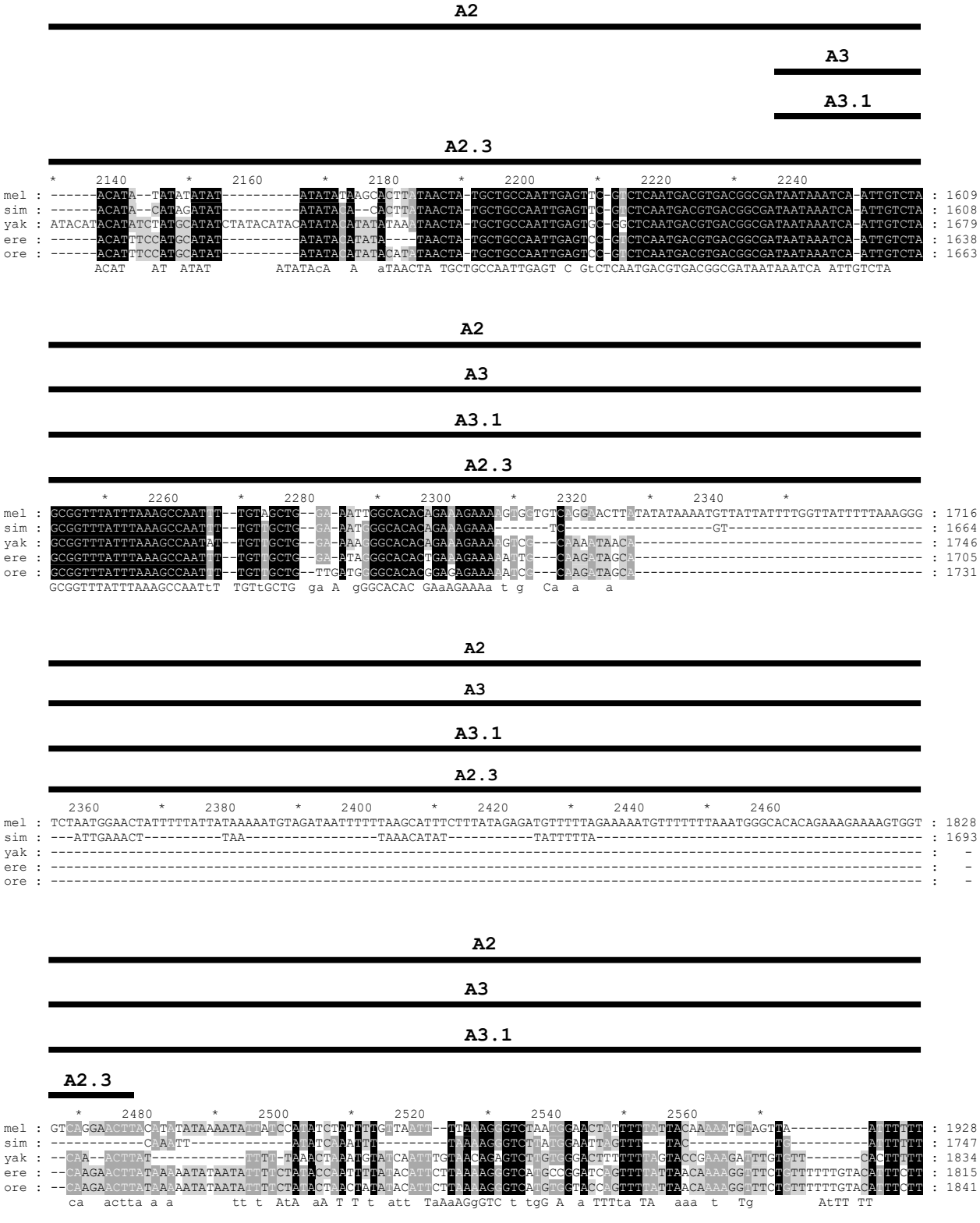


Figure S3.2 (*cont'd*)

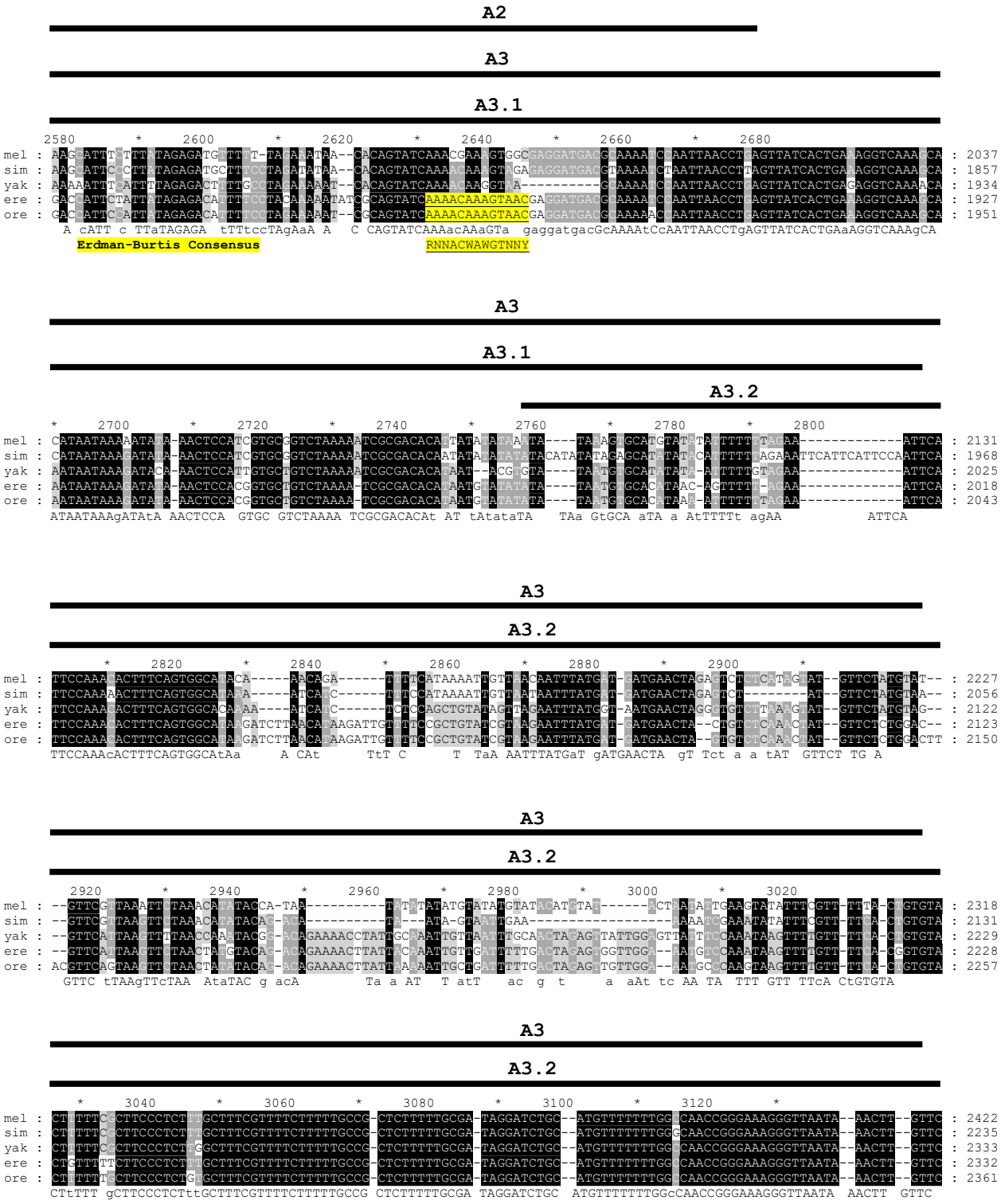
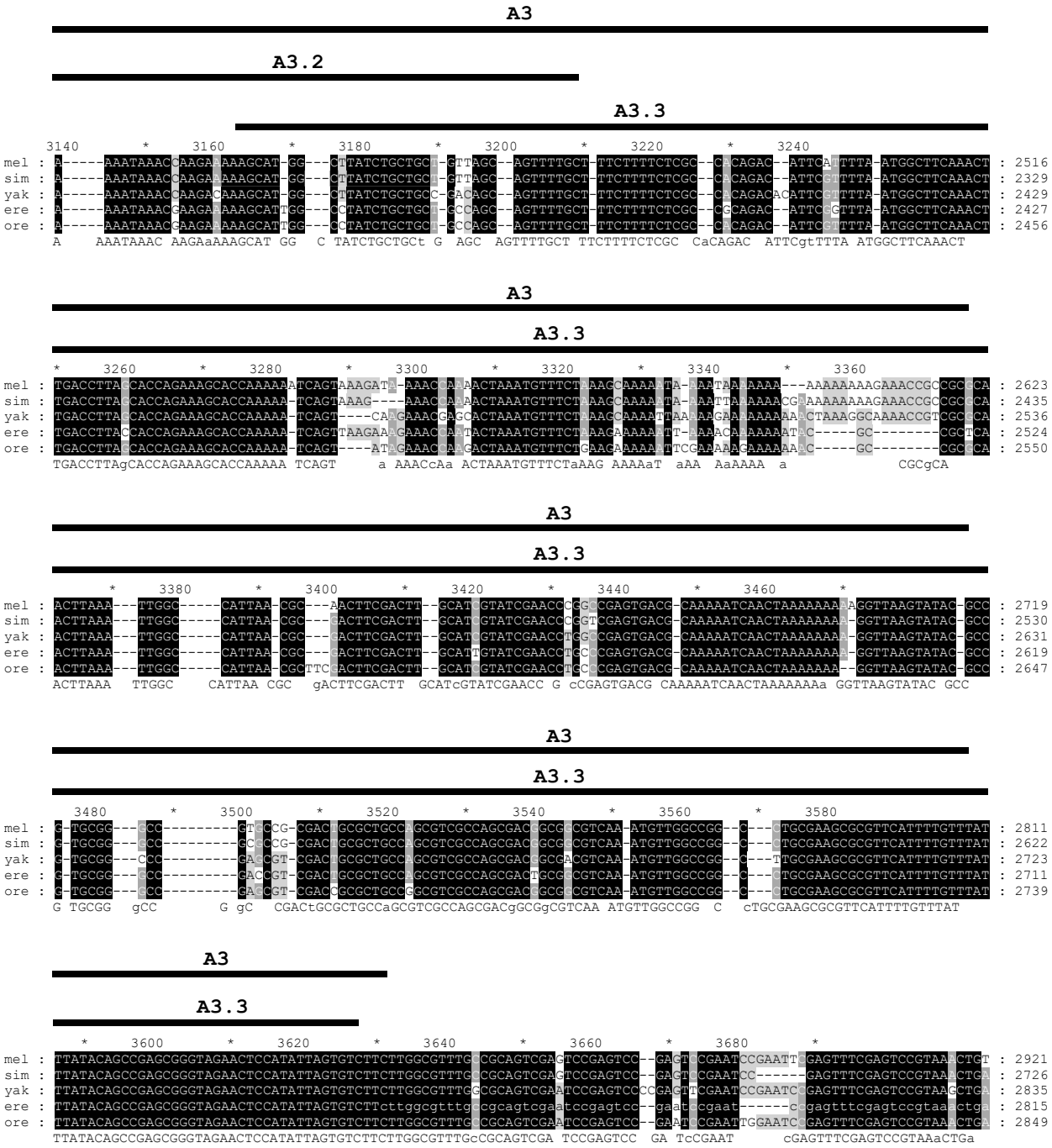


Figure S3.2 (*cont'd*)



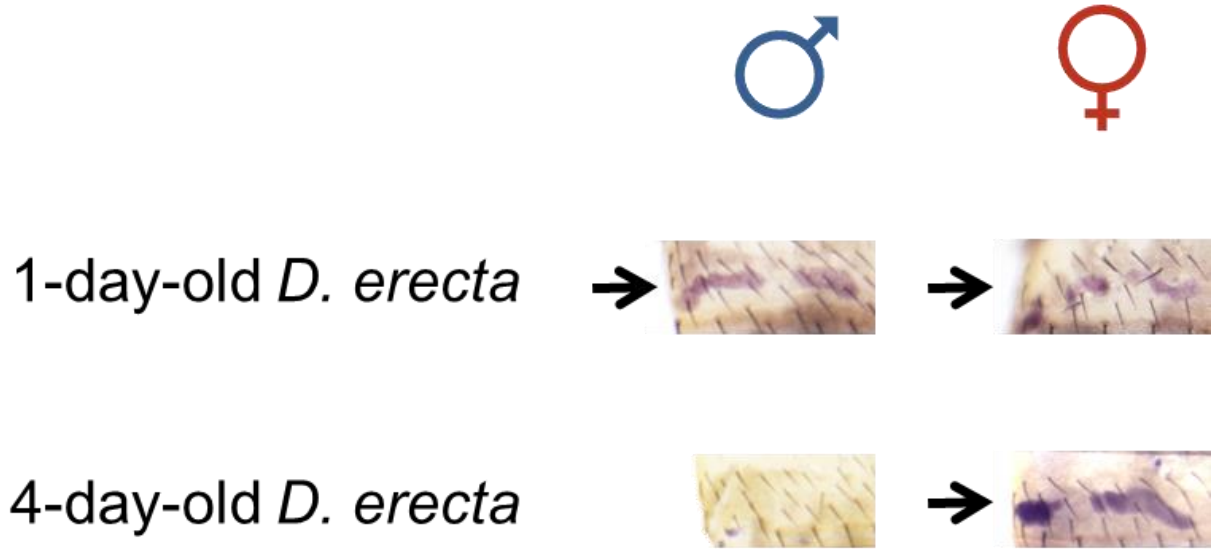


Figure S3.3 Expression of elongase gene *LOC6555117* in one-day- and four-day-old *D. erecta* oenocytes. Expressions are tested by using *in situ* hybridization, with gene specific probes. Arrows show gene expression. Female biased expression is found in 4-day-old *D. erecta* oenocytes, and non-sex biased expression is found in 1-day-old *D. erecta* oenocytes.

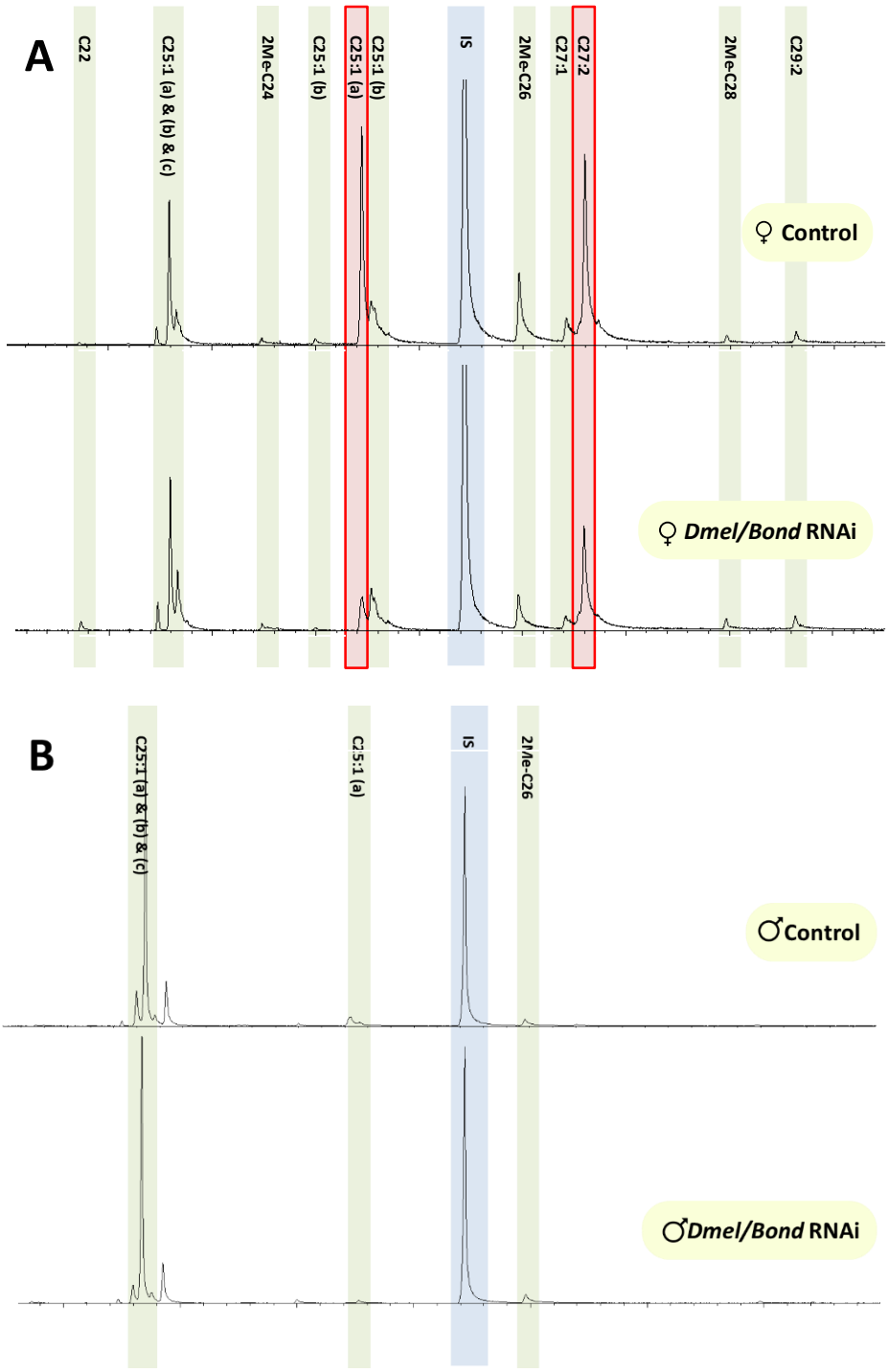


Figure S3.4 Chromatograms of CHCs profiles for *D. melanogaster* with *Dmel/Bond* RNAi. (A) Two compounds, C25:1 (b) and C27:2, were less produced with RNAi knocking down the expression of *Dmel/Bond* in female. (B) No CHC profile changes were detected in male after RNAi knockdown. Majorly detected CHC compounds were labeled. Non-labeled detections are not CHCs. "IS" stands for "internal standard" for quantity analyses. Colors of labels indicate status after RNAi knocking down, red with outline = Changed, green = Unchanged, blue = Internal Standard.

A

Name	Sequence
Dere_LOC6541898_F	ATGGAGGTGTCAGCAAGTCCAAAT
Dere_LOC6541898_R	TAGTATCGCCTTACGTAGTCCG
Dere_LOC6547302_F	ATGAATTTCACACTATTTGAAATT
Dere_LOC6547302_R	TGAAAAATAAGTATGTTAGCCAAC
Dere_LOC6543867_F	TAAATTTGGGGGGGAAAATATC
Dere_LOC6543867_R	TCCATTTAGGACCGTAGCGC
Dere_LOC6543868_F	CAGAGATCAGGTATAAAGGCGC
Dere_LOC6543868_R	GATAAATAAAGGGTCAGCATGACG
Dere_LOC6543939_F	ACATGGGTGTTCTACTACTGCGGC
Dere_LOC6543939_R	CAAAAAGATGAGAGGCTGCTTGCG
Dere_LOC6552176_F	ATGGCCTTAATTATGAAATACATCG
Dere_LOC6552176_R	GCATGAACCATTCTCCGGGATGTG
Dere_LOC6552177_F	CGACTTTTACAAAGCGAAATATCTC
Dere_LOC6552177_R	TTATTTGACTTTTCGCTGATGCAG
Dere_LOC6553574_F	AGAACGACTGCAACTACCCGAT
Dere_LOC6553574_R	TCACTTGTTGCCGGCGTTCACG
Dere_LOC6552259_F	TTCTTCAACTCCAAAATGGCTG
Dere_LOC6552259_R	GTGAGTACGCAGCGCATGTCG
Dere_LOC6552258_F	GGTGTCATCTACGTCATAAG
Dere_LOC6552258_R	AATATTTGTTGATTACAAATCAAT

Table S3.1 Primers used for molecular cloning (A) *in situ* hybridization RNA probes and (B) fragments for GFP reporter constructs. Sequences colored with red indicate designated cutting sites for restriction enzymes for cloning uses.

Table S3.1 (*cont'd*)

Name	Sequence
Dere_LOC6552257_F	TGTTCCACCGACGCCGATAACAC
Dere_LOC6552257_R	TTGGACATGCAGCTGAAGTTATAG
Dere_LOC6552256_F	ATGCTAATCGAAGCTTATAAACC
Dere_LOC6552256_R	AGTGGACAGCGAAAAAGCACATG
Dere_LOC6555119_F	AACTTCCCCAAGTCCATTGCCGCT
Dere_LOC6555119_R	ATACCTAGGGGTAGGATATGG
Dere_LOC6555117_F	CCAAGTCTGTGGACGGCGGCAGT
Dere_LOC6555117_R	AGGTAAAAGTAAAAGGTAATGGT
Dere_LOC6555116_F	AAGTCTCGTGGGTAGCGGATG
Dere_LOC6555116_R	CCAGGACAGCACGAAGAAGAGGT
Dere_LOC6555115_F	CGCCCAGTTCGTGCTGTGCATCT
Dere_LOC6555115_R	GACTGCTTTCGCTATTCAATTCAG
Dere_LOC6554897_F	ATGCGGCACAACATGGTGGC
Dere_LOC6554897_R	CCGTGTGATCCACTGGCTGGC
Dere_LOC6555308_F	AAATTGAATAGTAGAAAATAAATATC
Dere_LOC6555308_R	GACGGTCATGCAGCGAAAGTT
Dere_LOC6547301_F	AACACTGACTCAGCTCTGCC
Dere_LOC6547301_R	TCCGAAGTCGAATTTGAGAAAGT

Table S3.1 (*cont'd*)

B

Name	Sequence	Cloning Purposes
Dere-BondA-F	CCGGGCGAATTCGCCGGCGCGCCTGTGTTTTTCCAGTGTGACTGT	ereA, oreA, A1+A2
Dere-BondA-R	CGGTTGCGATCGCTTCCTGCAGGGACACTAATATGGAGTTCTACC	ereA, oreA, A2+A3
Dere-BondA1-R	CCTGCAGGGACCCTGAACTTCAGCGCT	A1, A2.1
Dere-BondA2-F	GGCGCGCCTAATAGCGCAATTTAACTAC	A2+A3, A2.1, A2+A3.1, A2+A3.1+A3.2
Dere-BondA2-R	CCTGCAGGTCAGTGATAACTCAGGTTAATT	A1+A2
Dere-BondA3-F	GGCGCGCCTAATAAATCAATTGTCTAGCGGT	A3
Dere-BondA2.2-F	GGCGCGCCCTCTCGGCGTTCTGATCAGC	A2.2, A2.2a, oreA2.2
Dere-BondA2.2-R	CCTGCAGGCACGCACTTACTCTCACCG	A2.2, A2.2c, oreA2.2
Dere-BondA2.3-F	GGCGCGCCGTTGCAGTGCATCGAGCTTG	A2.3
Dere-BondA2.3-R	CCTGCAGGAAGTTCTTGTGCTATCTTGC	A2.3
Dere-BondA2.2a-R	CCTGCAGGGCATAATCGGCGCTTCGCTTTTG	A2.2a
Dere-BondA2.2b-F	GGCGCGCCAATCACAAAAGCGAAGCGCCG	A2.2b
Dere-BondA2.2b-R	CCTGCAGGTGCGTTGGTCGCCACACTTC	A2.2b
Dere-BondA2.2c-F	GGCGCGCCACCCGGAAGTGTGGCGACCAAC	A2.2c
Dere-BondA3.1-R	CCTGCAGGAATGAATTTCTAAAAACTGTTATGTGC	A2+A3.1
Dere-BondA3.2-R	CCTGCAGGGCAAAACTGCTGGCAGCAGCAG	A2+A3.1+A3.2

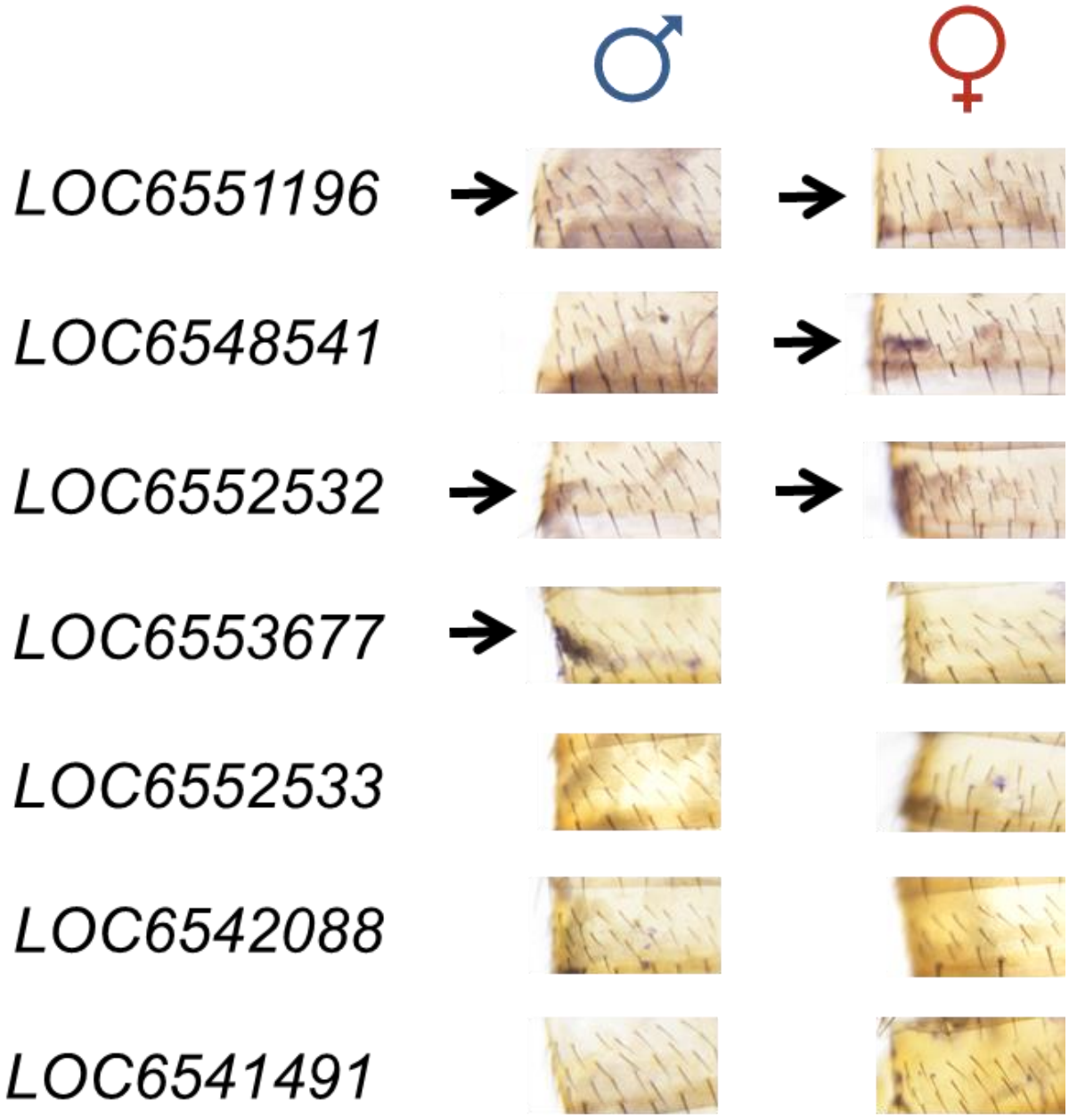


Figure S3.5 Expression of reductase genes in four-day-old *D. erecta* oenocytes. Expressions are tested by using *in situ* hybridization, with gene specific probes. Arrows show gene expression. Sex biased expression is found in *LOC6548541* and *LOC6553677*.

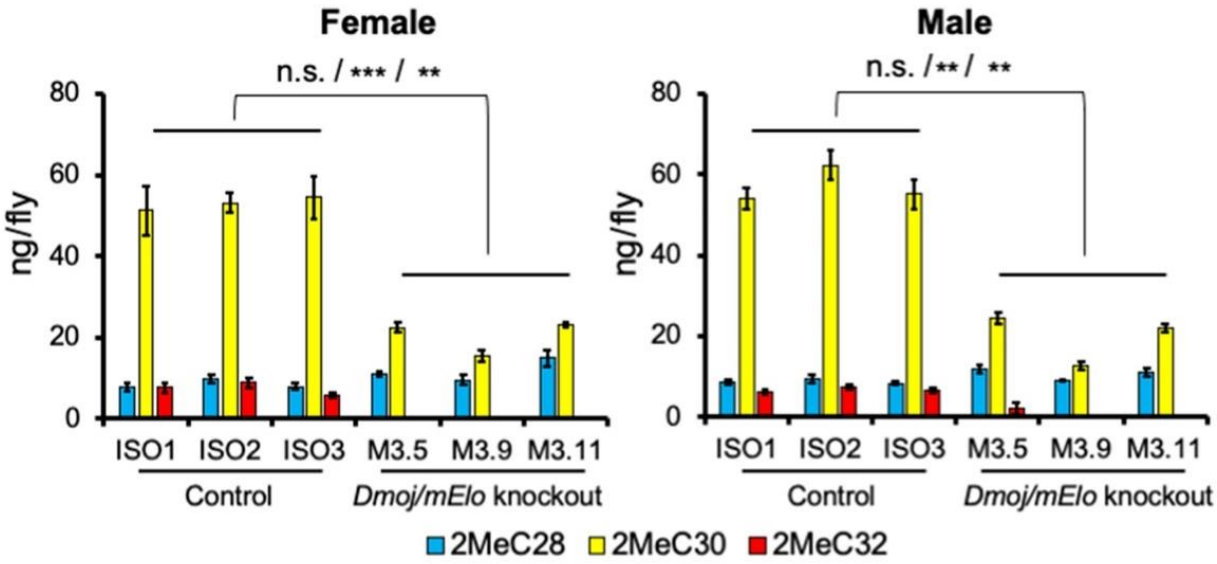


Figure S4.1 Quantitative changes of major mbCHCs in transgenic line of *D. mojavensis* (*Dmoj/mElo* knockout) adapted from (Wang *et al.* 2023). n.s. = not significant; ** $p < 0.01$; *** $p < 0.001$.

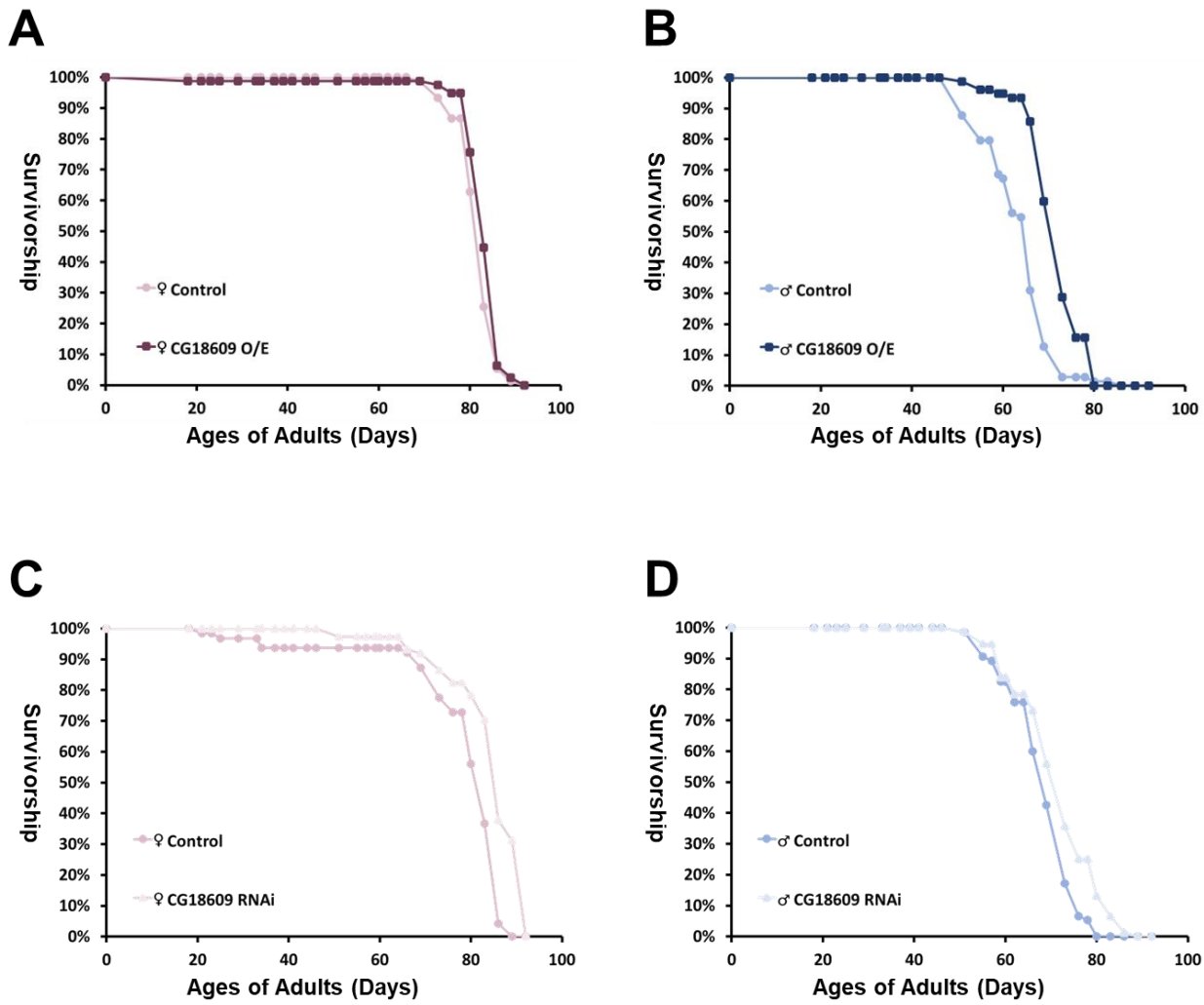


Figure S4.2 Survivorship of *D. melanogaster* with different levels of mbCHCs productions. (A) No significant difference in survivorship in females with overexpressing *CG18609* ($p = 0.012$). **(B)** Males from the line of *CG18609* overexpression survived significantly better ($p < 0.001$). Both females **(C)** and males (D) from the line of *CG18609* RNAi survived significantly better (both sexes, $p < 0.0001$). The percentage of surviving adults was shown from the enclosed day. Lines of transgenic *D. melanogaster* were adapted from (Wang *et al.* 2023). Control = *D. melanogaster* with normal level of mbCHCs production, *CG18609* O/E = Overexpressing *CG18609* in *D. melanogaster* oenocytes, leading to increasing amounts of longer chained mbCHCs. *CG18609* RNAi = Knocking down *CG18609* expression in *D. melanogaster* oenocytes, leading to reduced amounts of longer chained mbCHCs. Kaplan-Meier approach was used to determine the survivorship ability and any significant difference.

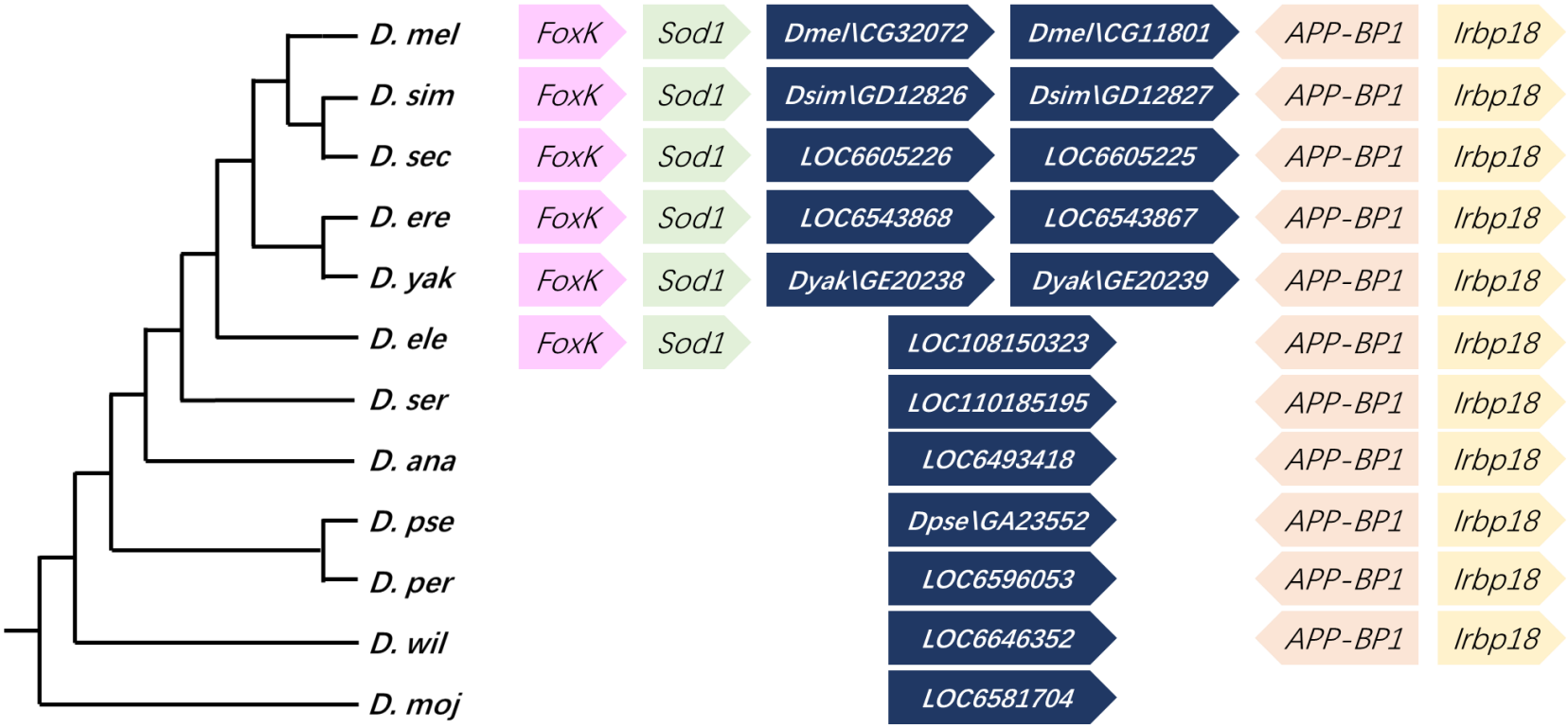


Figure S5.1 Synteny genome comparison of selected elongase genes across *Drosophila* species. Genes labeled with dark background and light font are elongase genes. Genes labeled with light background and dark font are marker genes. *D. mel = D. melanogaster*, *D. sim = D. simulans*, *D. yak = D. yakuba*, *D. ere = D. erecta*, *D. ana = D. ananassae*, *D. pse = D. pseudoobscura*, *D. wil = D. willistoni*, *D. moj = D. mojavensis*, *D. ele = D. elegans, D. ser = D. serrata, D. per = D. persimilis.* Blank areas indicate the absence of elongase orthologs in the genomes of the corresponding species, and the marker genes utilized aligned to the genomes but not close enough to be informative.

Figure S5.1 (*cont'd*)

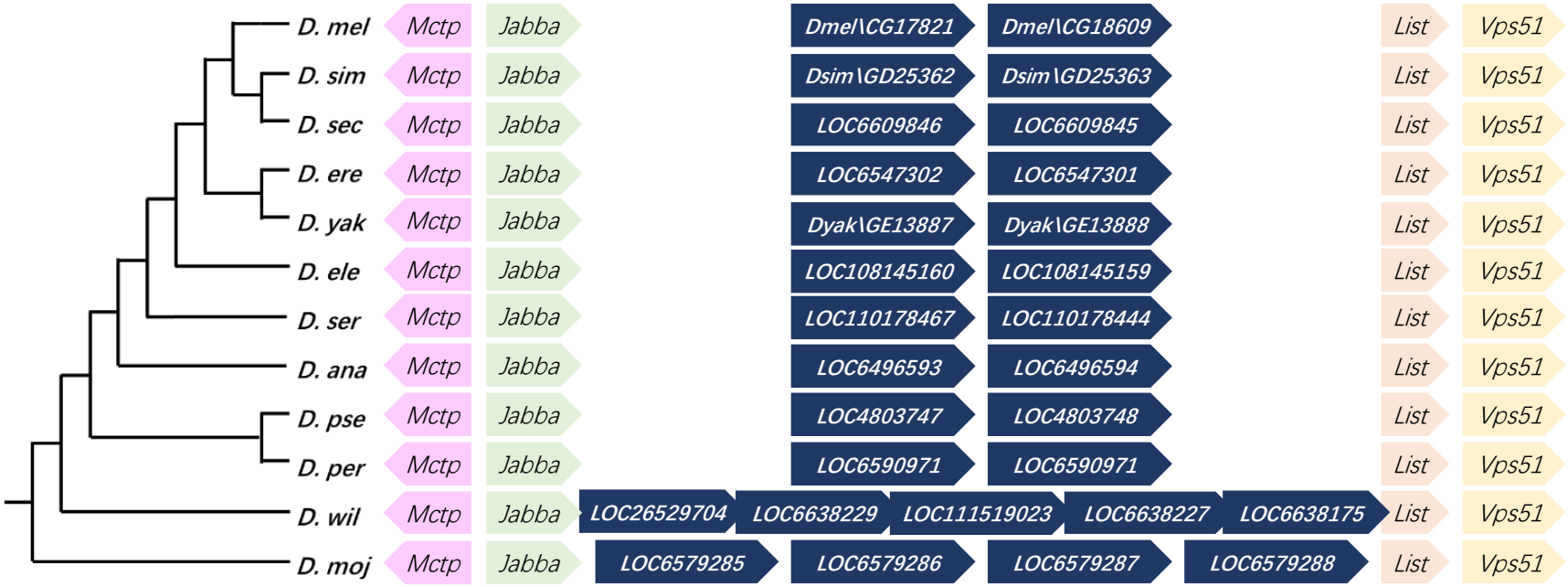


Figure S5.1 (*cont'd*)

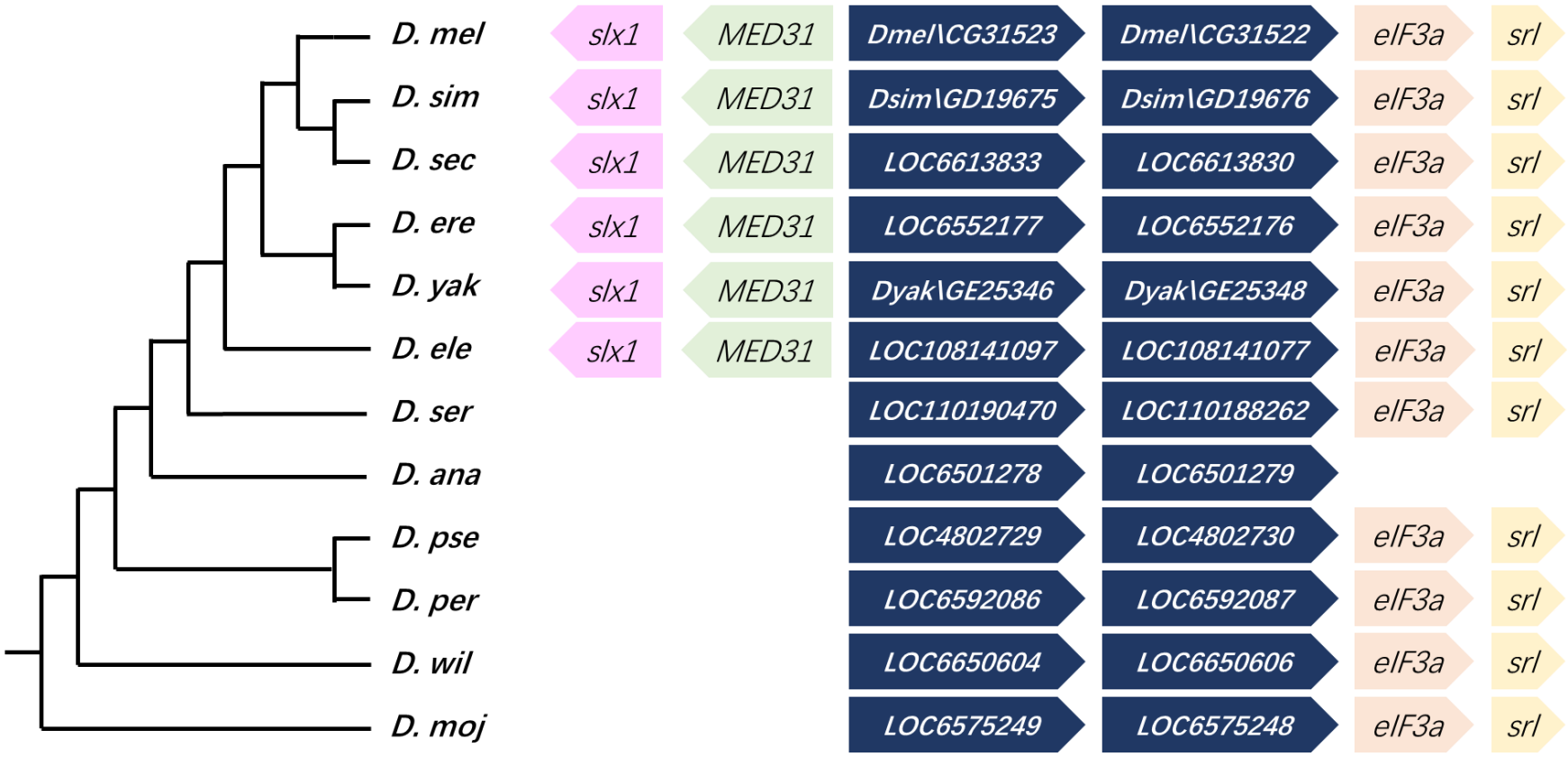


Figure S5.1 (*cont'd*)

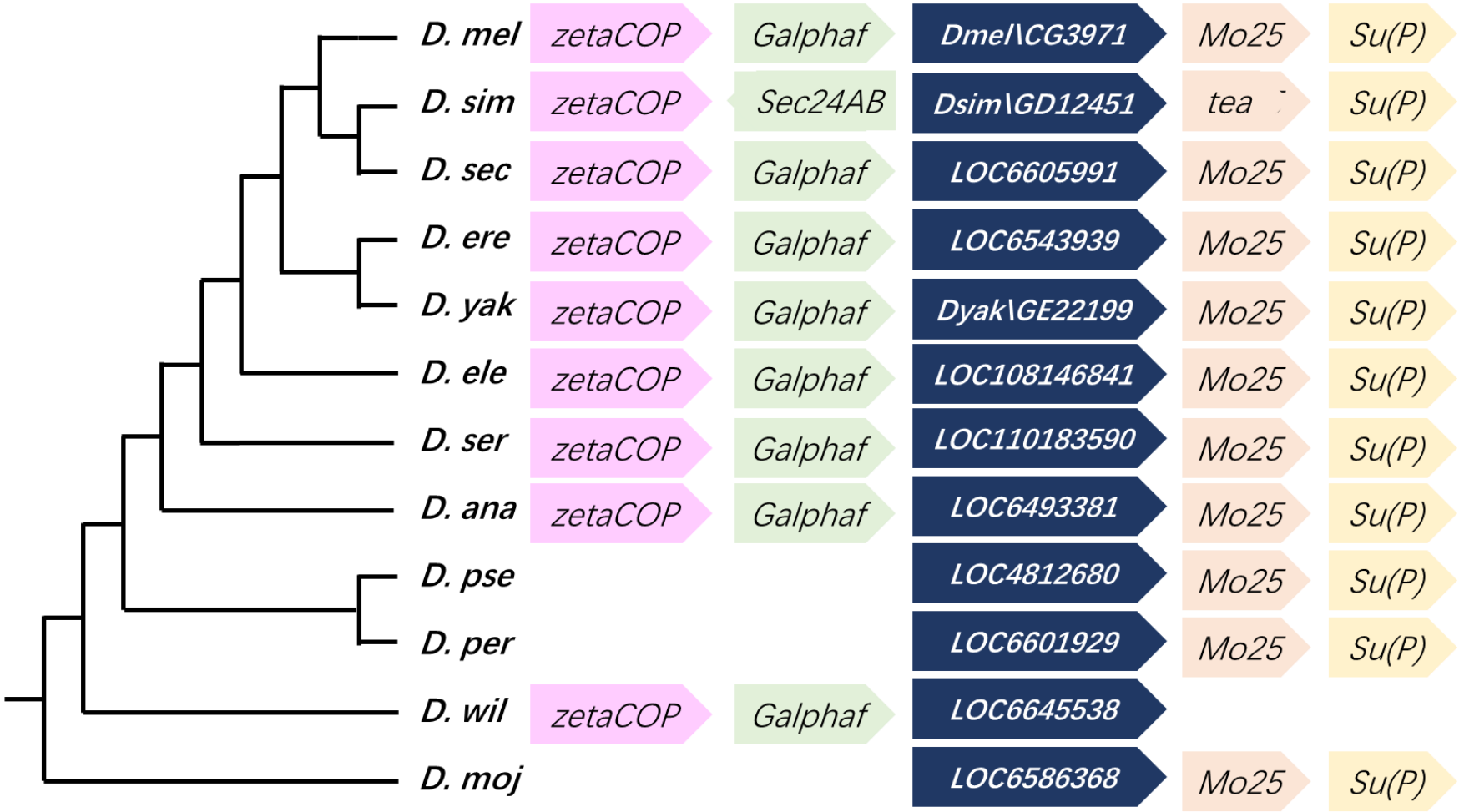
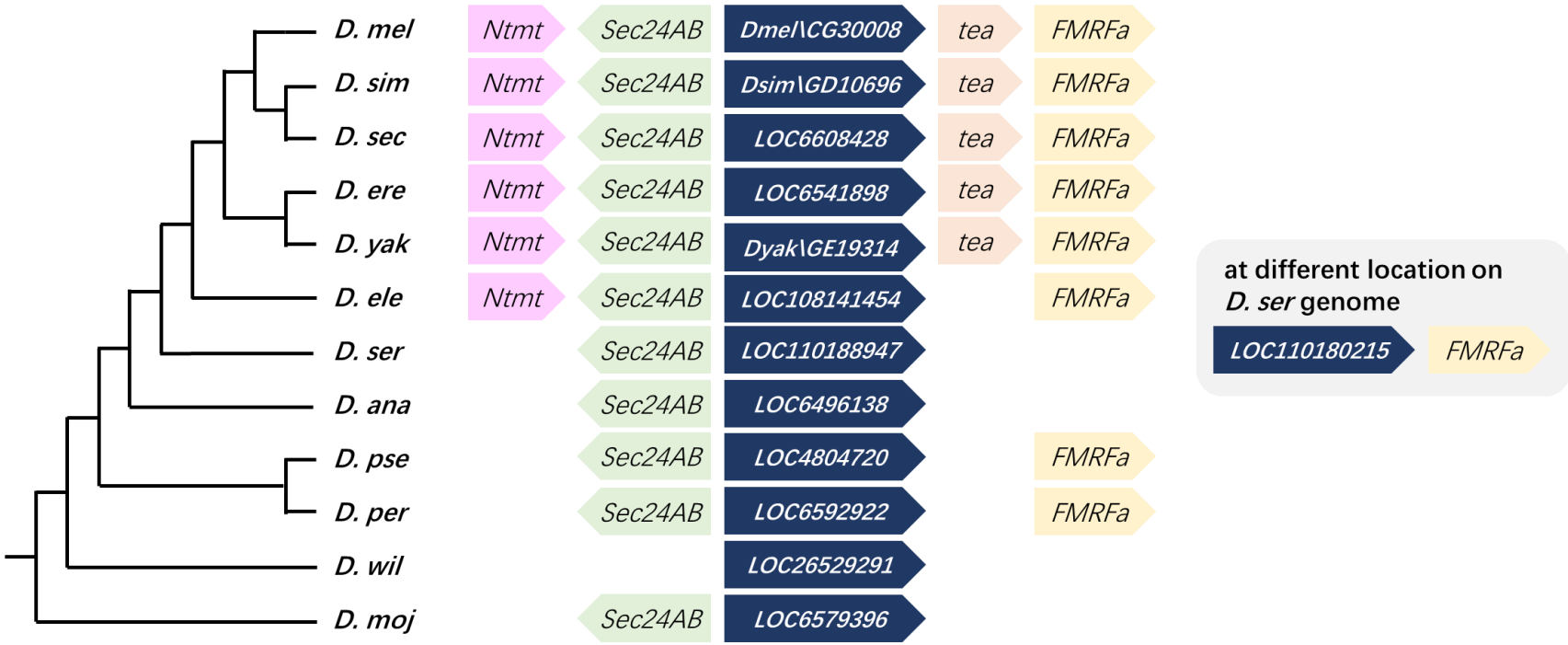


Figure S5.1 (*cont'd*)



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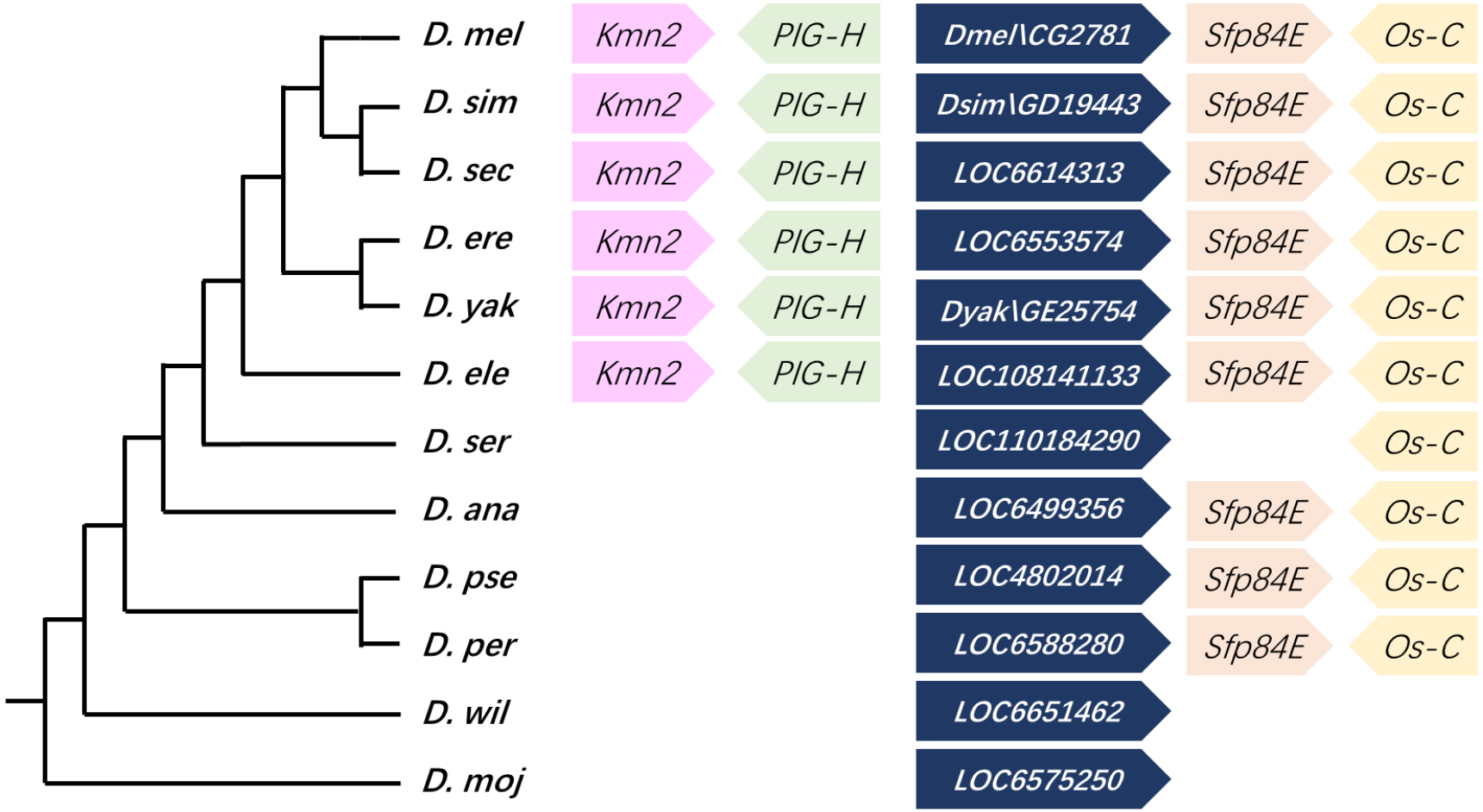

Figure S5.1 (*cont'd*)

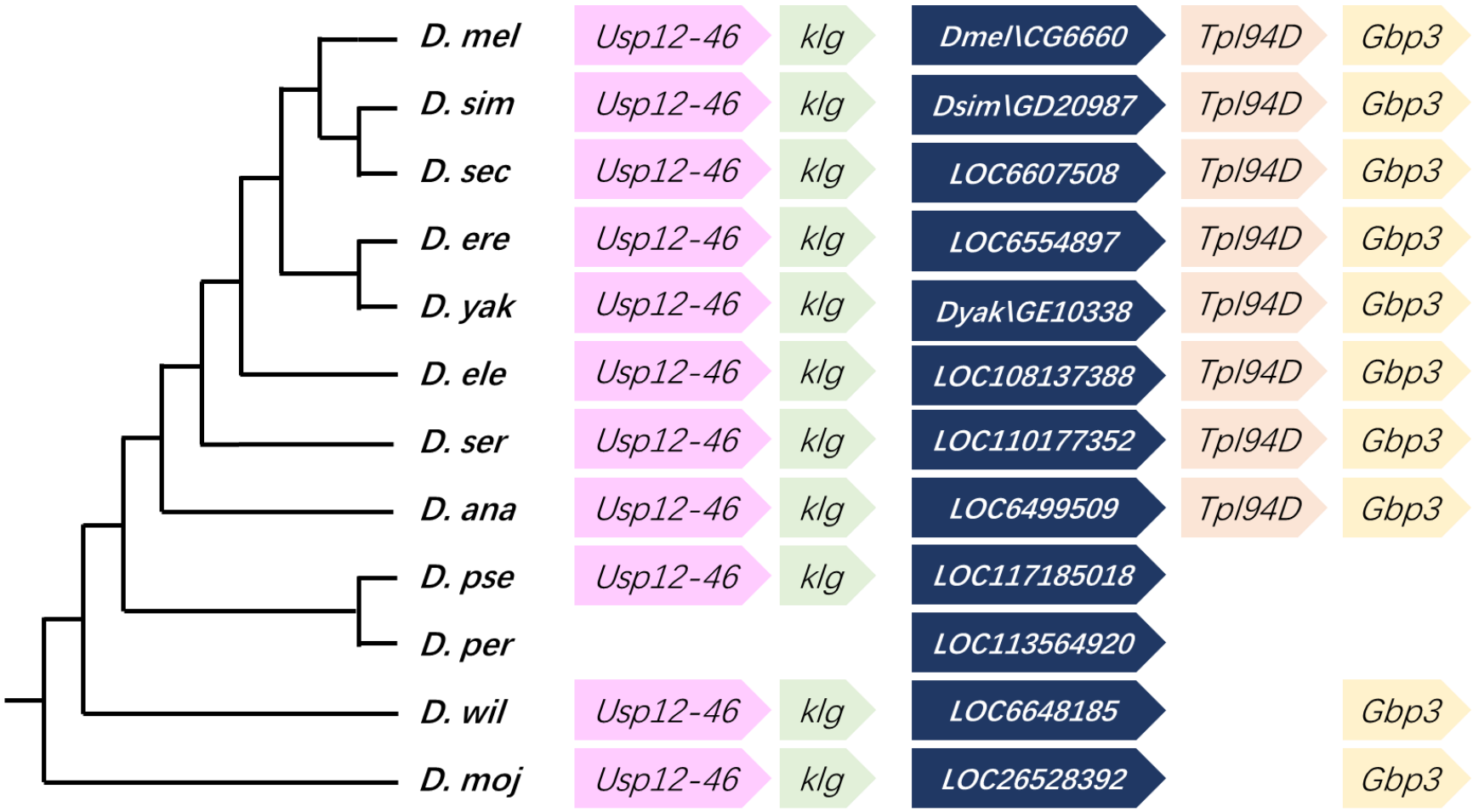

Figure S5.1 (*cont'd*)

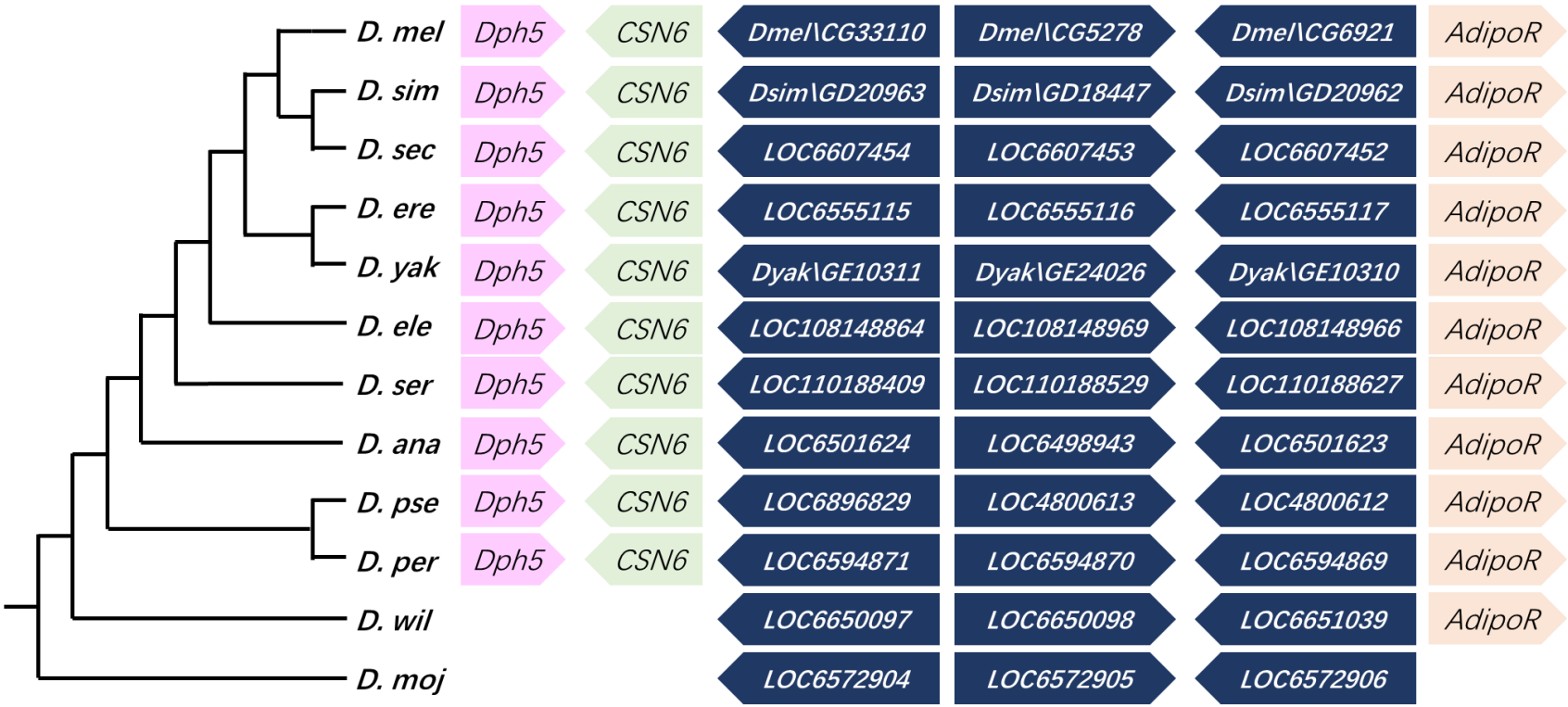

Figure S5.1 (*cont'd*)

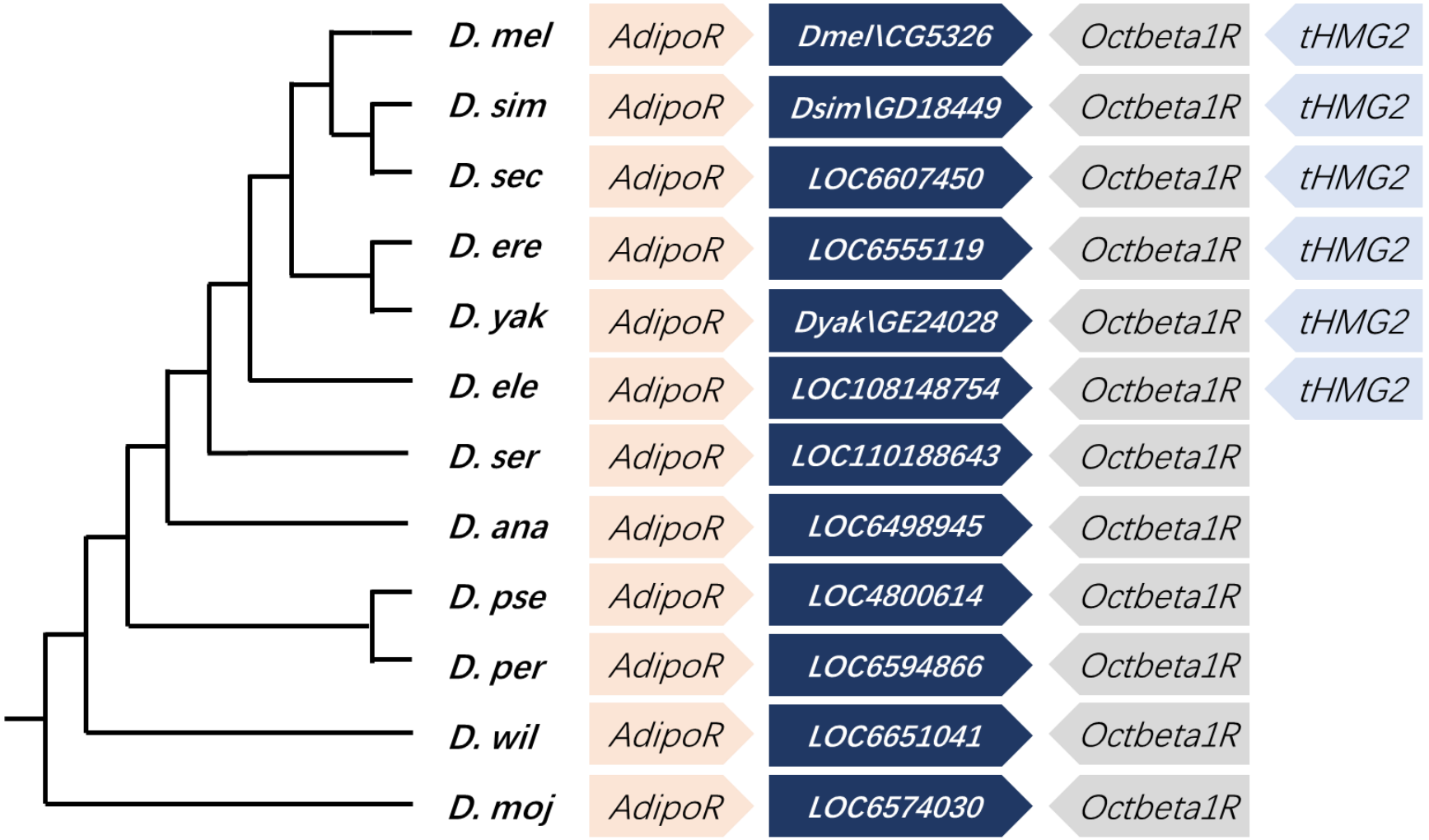

Figure S5.1 (*cont'd*)

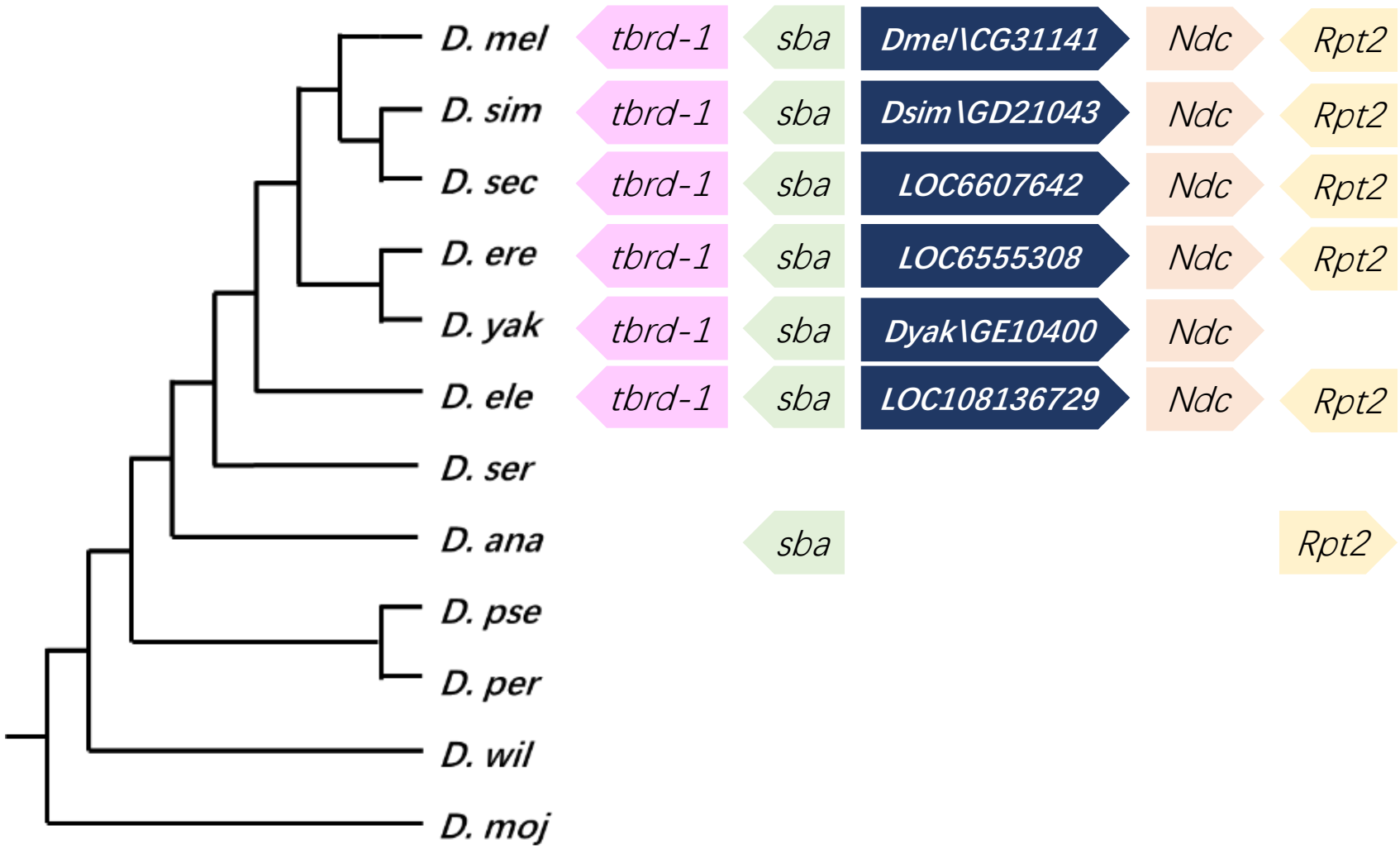

Figure S5.1 (*cont'd*)