MECHANISMS UNDERLYING DESICCATION RESISTANCE IN *DROSOPHILA* SPECIES

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ABSTRACT

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Adaptation to various and extreme environments is key to long-term species persistence. Reducing water loss is important for organisms adapting to different terrestrial environments. In *Drosophila* fruit flies and other terrestrial insects, their small body size and large surface areas to volume ratios make them vulnerable to desiccation stress. Their ability to prevent water loss is crucial for their survival. Previous studies have suggested that cuticular water loss accounts for the majority of water loss in insects and hypothesized that differences in cuticular hydrocarbon (CHC) content accounted for differences in desiccation resistance between mesic and desert species. However, the specific association between different CHC components and desiccation has not been established, and the genetic mechanisms underlying the evolution of these CHC components that confer high desiccation resistance have not been elucidated. This dissertation investigated how the evolution of CHCs in insects affected desiccation resistance and elucidates the genetic mechanisms underlying their evolution. With a comprehensive association study of desiccation resistance and CHCs in 46 *Drosophila* species and 4 species in closely-related genera, the analyses showed that mbCHC chain lengths were important predictors of desiccation resistance and longer mbCHCs contributed to higher desiccation resistance. This dissertation further investigated the genetic and molecular mechanisms underlying longer chain mbCHCs and higher desiccation resistance in a desert *Drosophila* species, *Drosophila mojavensis*. A fatty acyl-CoA elongase

gene, *mElo (**m**ethyl-branched CHC **Elo**ngase)*, was identified in *Drosophila* species for the elongation of mbCHCs. Overexpression experiments in *D. melanogaster* demonstrated that coding changes in *mElo* from *D. mojavensis* lead to longer mbCHCs and higher desiccation resistance. Further experiments using CRISPR-Cas9 to knock out *mElo* from *D. mojavensis* showed that knockout of this gene decreased the production of the longest mbCHCs and significantly reduced desiccation resistance at their ecological-relevant temperature. Results from this dissertation elucidate the molecular and evolutionary mechanisms that enable species to reduce water loss and maintain water balance as our planet gets warmer and more arid in the next few decades.

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To many people who have helped me, instructed me, and cared for me.
To days and nights that I have spent in the laboratory doing experiments.

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PREFACE

In this dissertation, I tried to integrate my results and analyses in a complete story and to understand how a trait evolves to help insects survive from aridity, universal stress in terrestrial ecosystem. Some of the results, in particular those in Chapter 4 and appendices, are still preliminary, which, I hope, can facilitate future research.

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CHAPTER 1. INTRODUCTION

Insects, which form the bulk of biodiversity on Earth, are an integral part of our ecosystem and provide ecological and economic benefits to humans (Losey and Vaughan 2006). As our planet experiences rapid and consistent warming due to climate change, this has led to unpredictable drought events in many habitats and increasing desiccation stress on many insect species (Ault 2020, Berdugo et al. 2020). Due to the small body size and high surface-area-to-volume ratio (Kühsel et al. 2017), insects are vulnerable to water loss and desiccation, especially when also facing increasing temperature (Ramsay 1935, Bubliy et al. 2012). To withstand desiccation stress in habitats with various levels of humidity, insects have evolved diverse strategies to reduce and/or tolerate water loss. However, whether and how insects could evolve and adapt to increasing desiccation stress due to climate change are less clear.

To answer these questions, understanding current adaptations of insect species to diverse environments is crucial and could shed light on how the species would evolve (Hoffmann and Sgrò 2011, Schindler and Hilborn 2015). In this chapter, I will review the literature on strategies of desiccation adaptation in insect species and identify common physiological and genetic mechanisms underlying the evolution of desiccation resistance. This knowledge can be applied to inform management efforts in pest management science and conservation biology.

Desiccation resistance evolves rapidly in insects

The importance of desiccation resistance for terrestrial organisms including insects has been recognized a long time ago (Matthew 1915, Cloudsley-Thompson 1975). By exposing

insects in dry conditions and recording the time to mortality, empirical studies have determined the desiccation resistance for many different insect species. These studies showed a wide range of desiccation resistance across insect taxa, in particular, some of the species which are phylogenetically distant have evolved high desiccation resistance and have adapted to extremely arid environments. Examples include the desert locust *Schistocerca gregaria* Forsskål (Orthoptera: Acrididae) and the cactophilic fruit fly, *Drosophila mojavensis* (Patterson and Crow) (Diptera: Drosophilidae) (Cloudsley-Thompson 1975). This suggests the potential of insect species for evolving higher desiccation resistance when dealing with drier environments.

Understanding the evolutionary pattern of a trait across the phylogeny can also be informative (Adams and Collyer 2019). It can reveal how factors such as habitat types and climates could influence insects' adaptation to desiccation. For example, a recent study examined the desiccation resistance of 82 ant species (Hymenoptera: Formicidae) that were sampled from diverse microhabitats in a single community and reported their desiccation resistance varied from 0.7 to 98 h (Bujan et al. 2016). This approximately 100-fold variation in desiccation resistance of ant species suggested local adaptations to microhabitats could drive rapid evolution of their desiccation resistance. Another example is the evolution of desiccation resistance in *Drosophila* species. Similarly, a wide range of desiccation resistance from 4 to 135 h was reported in 95 *Drosophila* species that were collected worldwide (Kellermann et al. 2012). Both studies showed that between closely-related insect species, the levels of desiccation resistance can be very different, and high desiccation resistance has evolved independently in distant species.

Physiological and genetic mechanisms underlying desiccation resistance in insects

Although the physiology of insect desiccation resistance has been widely studied in different species, their genetic mechanisms are less investigated. The mechanisms underlying how insects physiologically adapt to dry environments such as deserts can not only provide insights into how the tiny organisms survive and thrive in terrestrial ecosystems but also predict how the species could evolve when facing increasing desiccation stress. Although most traits in organisms are controlled by multiple genes with complicated interactions, it is hypothesized that some key genes are playing major roles in the evolution of those traits (Boyle et al. 2017). In this section, I sought to understand how those key genes underlie physiological adaptation to desiccation.

The question regarding how insects deal with desiccation has been firstly investigated in one of the earliest insect physiological studies by Vincent Wigglesworth, one of the pioneer insect physiologists (Locke 1996). This study showed that the presence of a lipid layer on the body surface of insects, later named Cuticular Hydrocarbons (CHCs), was able to prevent water transpiration and resist desiccation (Wigglesworth 1945), which was later found to be a major mechanism for all insect taxa to prevent water loss and develop desiccation resistance (Blomquist and Bagnères 2010, Chung and Carroll 2015). Water loss is a major challenge when facing desiccation. Studies in various insect species, including the lubber grasshoppers (Orthoptera: Romaleidae) (Quinlan and Hadley 1993), the desert harvest ant (Hymenoptera: Formicidae) (Johnson and Gibbs 2004), the house fly (Diptera: Muscidae) (Montooth and Gibbs 2003), and different fruit fly species (Diptera: Drosophilidae) (Gibbs and Matzkin 2001, Gibbs et al. 2003a), have determined the major mechanisms of desiccation resistance as conserving

water from cuticular water loss, respiratory water loss, and excremental water loss, as well as tolerating water loss (dehydration tolerance) (**Figure 1.1**) (Benoit et al. 2007). In this chapter, I will illustrate these general mechanisms of desiccation resistance that are present in the insect taxa, along with the hygrosensation, which could top-down regulate these mechanisms (**Figure 1.1**). For each of the mechanisms, I will also discuss potential evolutionary trajectories for the insects adapting to drier environments.

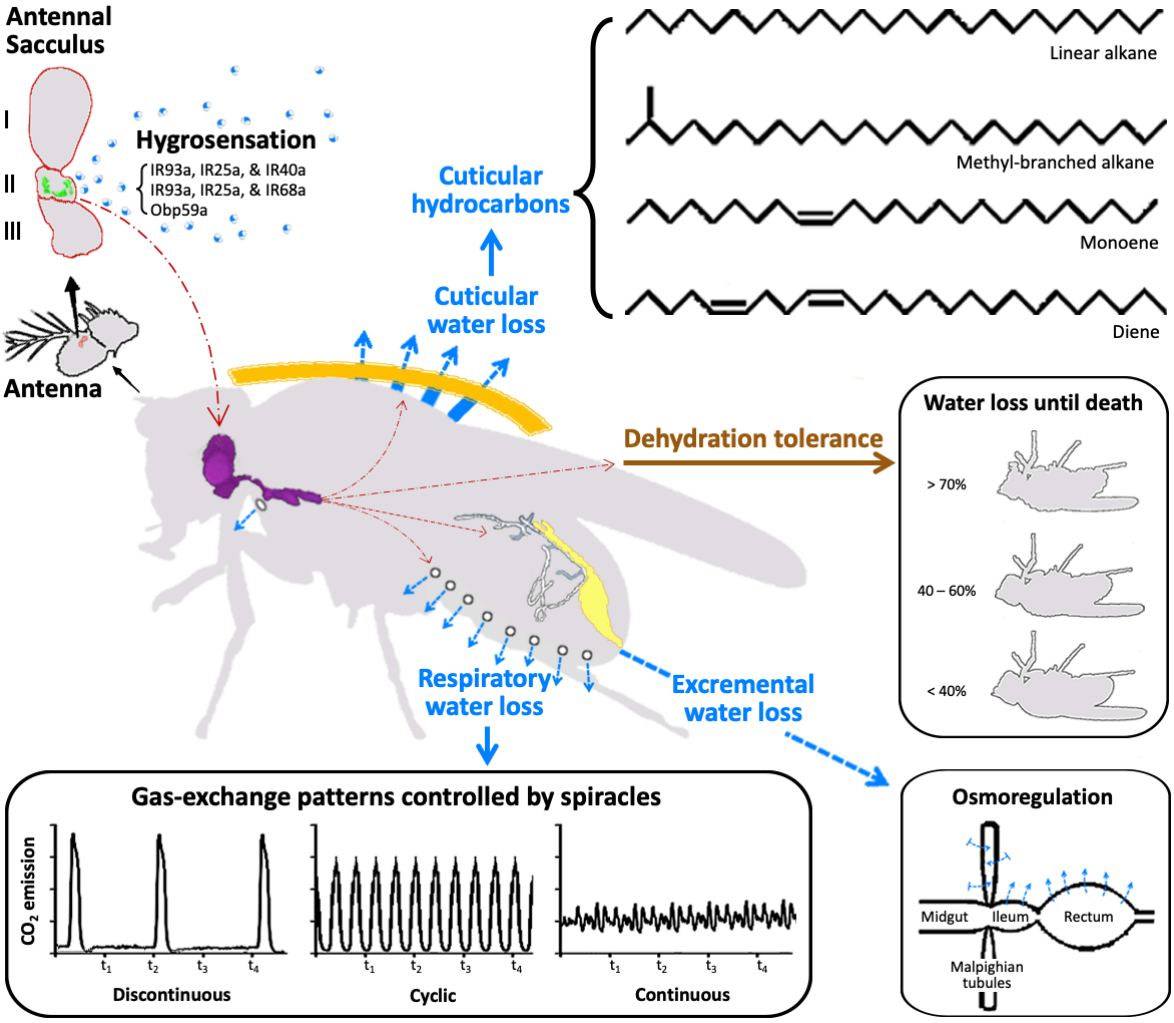


Figure 1.1. Insect physiology associated with desiccation adaptation. When facing desiccation stress, it is important for insects to prevent water loss. Cuticular hydrocarbons (CHCs) are a hydrophobic lipid layer on the body surface of the insect species, which contains different types of hydrocarbons. This lipid layer is able to prevent cuticular water loss, the major part

Figure 1.1. (cont'd)
generating desiccation stress. Through the control from spiracles, there are three major gas-exchange patterns observed in insect species, of which the discontinuous gas exchange pattern cyclically closes the spiracles and presumes to help reduce respiratory water loss. Insects use Malpighian tubules, ileum, and rectum to maintain water secretion and reabsorption so as to prevent excremental water loss. The dehydration tolerance, the capability of tolerating water loss, is another important mechanism for insects' adaptation to desiccation. Studies have been found across insect taxa, the species have a wide range of dehydration tolerance and certain species such as the Antarctic midge are able to survive in an anhydrous status with more than 70% body water reduced. In addition, hygrosensation is an essential modality for insects perceiving the humidity changes in the environment. One of the mechanisms is to use two combinations of different ionotropic receptors (including IR93a, IR25a, and IR40a; and IR93a, IR25a, and IR68a), coupled with the odor binding protein (Obp59a) in the same sensilla in the region of antennal sacculus to sense the dry air and wet air, respectively. The hygrosensation can further trigger different physiological and behavioral responses and help them adapt to desiccation.

Cuticular water loss and cuticular hydrocarbons

Cuticular water transpiration is the major route of water loss that generates desiccation stress to insects (Gibbs et al. 2003a). The use of CHCs has been found to be a general strategy in insects to resist cuticular water loss. The function of CHCs in desiccation adaptation has been demonstrated by knocking down a P450 oxidative decarbonylase gene, an essential gene in the last step of CHC synthesis converting all CHC precursors to the final hydrocarbons (**Figure 1.2**), in several insect species such as *D. melanogaster* and *L. migratoria* (Qiu et al. 2012b, Yu et al. 2016). The knockdown experiments resulted in the loss of most CHCs and drastically reduced desiccation resistance. CHCs are hydrophobic and contain a mixture of hydrocarbons with various chemical functional groups such as methyl groups and double bonds (**Figure 1.2**). The genetic mechanisms underlying the synthesis and transportation of CHCs have been widely studied, due to the pleiotropic roles in desiccation resistance, chemical communication, and insecticide resistance (Chung and Carroll 2015, Balabanidou et al. 2018). In the last few decades, studies in many different insect species showed complex pathways for CHC *de novo*

syntheses in the adult oenocytes (**Figure 1.2**) (Ginzel and Blomquist 2016). Briefly, the synthesis of CHCs co-opts the fatty acid synthesis pathway and starts from two types of fatty acid synthases (FAS), the microsomal and cytosolic FASs, incorporating acetyl-CoA, (methyl)malonyl-CoAs, and required amino acids (for example, Valine, Isoleucine, or Methionine) to form fatty acyl-CoA precursors. Different desaturases and elongases expressed in the oenocytes lead to the production of mixed fatty acyl-CoAs with different lengths and saturation levels. The further two steps are specific to CHC synthesis in the oenocytes and involve reductases and P450 decarbonylases for the final CHC products. These different enzymes can influence the efficiency of each other during the CHC synthesis. In addition to genes directly participating in the CHC synthesis, other genes in diverse physiological processes including steroid synthesis gene (Chiang et al. 2016), transportation genes (Broehan et al. 2013), pigmentation genes (Massey et al. 2019, Lamb et al. 2020, Noh et al. 2020), and cuticular thickening and translocation genes (Balabanidou et al. 2018) can also affect the abundance and composition of insect CHCs.

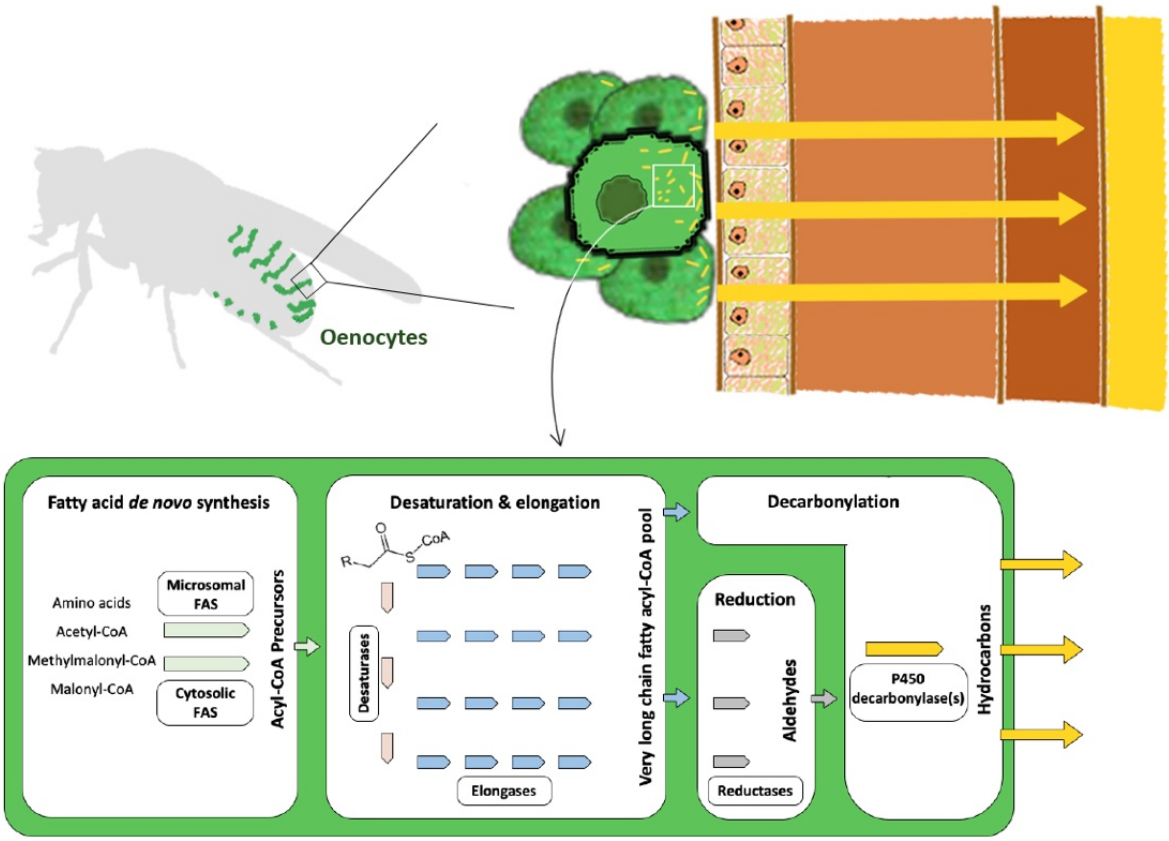


Figure 1.2. The abundance and diversity of cuticular hydrocarbons in insects are regulated by both the synthesis network and transportation. Cuticular hydrocarbons (CHCs) are synthesized in the oenocytes, a cluster of cells usually under insects' abdominal cuticles. The synthesis co-opts from the fatty acid *de novo* synthesis pathway and starts from two types of fatty acid synthases (FASs), the microsomal and cytosolic FASs, incorporating acetyl-CoAs, (methyl)malonyl-CoAs, and required amino acids (for example, Valine, Isoleucine, or Methionine) to form fatty acyl-CoA precursors. Diverse desaturases and elongases expressed in the oenocytes lead to the production of mixed fatty acyl-CoAs with different lengths and levels of saturation. The further two steps are specific to CHC synthesis in the oenocytes and involve reductases and P450 decarbonylase(s) for converting the fatty acyl-CoAs to the final CHC products. The CHC products are then transported through different layers of insect cuticle and accumulate on the outmost layer. The processes in CHC transportation and development of cuticles such as pigmentation and cuticle thickening can also influence the composition of the hydrocarbon mixture.

The composition of CHCs is complicated and evolving rapidly across species, which could lead to changes in physical properties such as the melting temperature of this layer that correlates with desiccation resistance (Gibbs and Pomonis 1995, Menzel et al. 2019). Though it

is still not fully understood how the physical properties of CHCs affect the ecology and biology of insects, an association has been found that CHCs with higher melting temperature either from longer CHCs or more saturated CHCs correlate with higher desiccation resistance (Jezovit et al. 2017, Menzel et al. 2017). This association has been partially demonstrated in a recent functional study in two sibling *Drosophila* species, *Drosophila birchii* and *Drosophila serrata*, with similar CHC composition except for the methyl branched CHC (mbCHCs) (Chung et al. 2014). By knocking out the mbCHC-specific FAS gene (*mFAS*), this study demonstrated that the presence of mbCHCs drastically increases insects' desiccation resistance and unsaturated CHCs are necessary to desiccation resistance.

In a recent study, Krupp et al. (2020) used the RNAi-mediated gene knockdown technique to eliminate the oenocytes in adult *D. melanogaster* leading to no CHCs phenotype (CHC^{-}) and then coated a mixture of synthetic CHCs on the flies which rescued their desiccation resistance to the original level. This study demonstrated that desiccation resistance is an adaptive trait dependent on the CHCs. In addition, Krupp et al. (2020) also separately coated saturated and unsaturated CHC mixture back to CHC^{-} flies, the results showed that only the coating of saturated CHCs lead to a significant increase in the desiccation resistance, supporting the hypothesis that saturated CHCs are important to the desiccation resistance. However, the coating of saturated CHCs only resulted in a small increase in the desiccation resistance and cannot rescue the original level of desiccation resistance for the flies, suggesting that saturated CHCs alone are not sufficient to prevent water loss efficiently. In another study that investigated the effects of unsaturated CHCs in the desiccation resistance of *D. melanogaster* using an evolution experiment and RNAi knockdown techniques, higher desiccation resistance

was also associated with the increased portion of unsaturated CHCs (Ferveur et al. 2018). Taken together, this evidence leads to a hypothesis that, instead of saturated CHCs alone, the interactions between the saturated and unsaturated hydrocarbons could be critical in preventing cuticular water loss.

Another important property is the CHC lengths which positively correlate with the melting temperature and efficiency in preventing water loss. This has been repeatedly reported in multiple groups of insects. However, the genetic and molecular mechanisms are less understood. In the CHC synthesis pathway, the elongases in the CHC synthesis are potential candidate genes that control the length variations of CHCs (Teerawanichpan et al. 2010). Several elongase genes in *Drosophila* flies have been shown to elongate different types of CHCs. For example, *EloF* (Chertemps et al. 2007, Combs et al. 2018), *CG30008*, and *CG18609* (Dembeck et al. 2015) are responsible for the elongation of dienes, monoenes, and mbCHCs in *D. melanogaster*, respectively. How do the CHC lengths contribute to the desiccation resistance? To answer this question, using RNAi knockdown to reduce the expression of these elongase genes in the oenocytes can be a start to exploring the effects of CHC lengths on the desiccation resistance. For most insect species, CHC lengths vary from 20 to 40 carbons and their length variation is positively associated with desiccation resistance. For example, the desert *Drosophila* species such as *Drosophila mojavensis (Toolson et al. 1990)* and *Drosophila buzzatii* (De Oliveira et al. 2011) have the longest CHCs in both mbCHCs (ranging from 28 to 32 carbons) and unsaturated CHCs (33 to 37 carbons), while most mesic *Drosophila* species have the CHC length varied from 21 to 30 carbons) (Jezovit et al. 2017). The number of elongase genes also varies across species. Within the *Drosophila* species, the number of elongase genes

in the genomes is estimated from 15 to 21, while some other species have fewer elongase genes. For example, *L. migratoria* only has 7 elongase genes (Zhao et al. 2020). This difference suggests that gene duplication and gene loss could occur to elongase genes, which may be a mechanism underlying evolution in CHC lengths across species. In addition, potential changes in the coding sequences and regulatory regions in these elongase genes could further affect CHC length and desiccation resistance.

In addition to modifying CHC composition, another way for insects to adapt to drier environments can be the increases in production of overall CHCs. Since the first report of *Cyp4g1* as an overall control in CHC synthesis in *D. melanogaster* (Qiu et al. 2012a), studies on the CYP4G homologs in many different insect taxa have shown this gene and its homologs are functionally conserved in the CHC synthesis and necessary to CHC production. The overexpression of CYP4G homologs in the oenocytes leads to increased CHC abundance, which has been detected in different species when undergoing desiccating hardening (Koto et al. 2019, Wang et al. 2019a, Chen et al. 2020), suggest this be a parallel evolution. The overexpression of CYP4G homologs can be one of the first adaptive mechanisms facing desiccation stress. Except for the CHC synthesis pathway, different physiological processes can alter CHC abundance and influence desiccation resistance. For example, cuticular melanization involves different pigmentation genes, which influence CHC abundance in a length-dependent manner. A previous study showed that loss-of-function mutants of *ebony* and *tan* in *D. melanogaster* induced different length-dependent changes: the *ebony* loss-of-function mutant increased amounts of CHCs longer than 25 carbons and the *tan* mutant only increased CHC production shorter than 25 carbons (Massey et al. 2019). The effect of *ebony* in CHC production

is found to be conserved in *D. americana* and *D. novamexicana* (Lamb et al. 2020). Though increases in CHC production are a common strategy in desiccation adaptation, the molecular and genetic mechanisms underlying this phenotype can be different.

As the major strategy in preventing water loss, the evolution of CHCs is also affected by many other factors, such as chemical communication, abiotic and biotic factors, and insects' physiology. In addition to preventing water loss, CHCs also play an important role in chemical communication between sexes, individuals, or species (Chung and Carroll 2015, Jezovit et al. 2017). For example, the abundance and composition of CHCs can evolve in opposite directions with a trade-off in the desiccation resistance, e.g., more unsaturated CHCs in the female *D. melanogaster* to avoid interspecific mating with its sympatric sibling *Drosophila simulans* comparing (Seeholzer et al. 2018), or less saturated CHCs in the male sagebrush crickets to sacrifice some level of desiccation resistance in favor of mating success (Steiger et al. 2013). These studies suggest that the evolution of CHCs is meanwhile influenced by chemical communication and the interactions with other species. In addition, CHCs have been found affected by abiotic factors such as temperature and related physiological processes, resulting in a high variation in the CHC abundance and composition even among populations of the same species (Phillips et al. 1990, Frentiu and Chenoweth 2010, Dembeck et al. 2015). Therefore, to understand how the CHCs would evolve to help insect species better survive from desiccation, the other factors should also be taken into consideration.

Respiratory water loss and discontinuous gas-exchange cycle

Insects have a unique respiratory system. They obtain oxygen from the air passively diffusing through the cyclically opened spiracles and networked tracheae (Chown and Nicolson

2004). A disadvantage of this respiration is water loss when the spiracles are opening, posing inevitable desiccation stress. Although it is difficult to directly measure respiratory water loss due to the confound of cuticular water loss, studies have been using CO_2 ventilation detected in a flow-through respirometry as a proxy to examine the opening of spiracles and estimate the respiratory water loss (Lighton 1994). Since gas exchange positively correlates with the metabolic rate, when facing desiccation stress, insects tend to be in a quiescent state so as to minimize metabolism and then the respiratory water loss (Chown 2011, Gusev et al. 2014). By comparing the CO_2 emission pattern across insect taxa, a discontinuous gas-exchange cycle (DGC) has been observed conditionally used in several groups of insect species in Blattodea, Hymenoptera, Lepidoptera, and Orthoptera combining with cyclic and continuous gas-exchange patterns (Marais et al. 2005, White et al. 2007, Woods and Smith 2010), suggesting that the use of DGC could be an evolutionary novelty with a fitness advantage. The DGC pattern maintains closed spiracles but rhythmically open for a very short period for a burst of CO2 emission (Figure 1) (Quinlan and Gibbs 2006, Huang et al. 2015). As presented in the comparison of three gas exchange patterns in Figure 2, DGC possesses a longer period of spiracular closure compared with the continuous and cyclic ones. When the spiracles are closed, respiratory water loss is presumed to cease.

While many studies have found the DGC is a heritable trait that confers higher desiccation resistance in some species such as *Erynnis propertius* (Lepidoptera: Hesperiidae) (Williams et al. 2010) and the speckled cockroach, *Nauphoeta cinereal* (Blattodea: Blaberidae) (Schimpf et al. 2009, Schimpf et al. 2013), it is still contentious about whether this is an adaptive trait for all insect species because DGC does not consistently conserve water among all

taxa. Some studies found either the abolishment of DGC by gradually ramped hypoxia in the carpenter ant, *Camponotus vicinus* (Hymenoptera: Formicidae) (Lighton and Turner 2008), or the presence of DGC in the queens of seed-harvester ant, *Pogonomyrmex barbatus* (Hymenoptera: Formicidae), did not alter respiratory water loss (Gibbs and Johnson 2004), suggesting the use of DGC for other purposes (Hetz and Bradley 2005). Moreover, an experimental evolution study on the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae), showed the properties of DGC did not account for the increased desiccation resistance for this species (Talal et al. 2016). Even though the lack of consensus has invoked multiple hypotheses on the evolutionary explanation of DGC (details see the review in Contreras et al. (2014) and Matthews and Terblanche (2015)), there are plenty of cases showing the contribution of DGC to reduce respiratory water loss and therefore, higher desiccation resistance, suggesting that the evolved modulation of DGC characteristics could confer a fitness advantage under desiccating stress and potentially help insects adapt to dryer environments.

The spiracular activity is mostly controlled by inner valves under the spiracular opening consisting of several muscles functioning as the opener and closer components (Wasserthal and Fröhlich 2017). Comparative studies for closely related species in insect orders with DGC patterns such as Lepidoptera (Schmitz and Wasserthal 1999) and Blattodea (Chaudhari 2016) showed that a conserved valve system has been found with similar morphology, though with certain variations in the size and shapes. More interestingly, the species without DGC, as in *D. melanogaster* larva (Wang et al. 2018), has a valve system with related muscular control. Using RNAi knockdown techniques, Wang et al. (2018) showed several genes, including *tyn*, *pch*, *lmd*, and *blow*, underlie the development of this valve system, as well as muscles connecting to the

valve. These genes may be a morphological adaptation underlying the spiracular control. However, since all the species have similar spiracular structures, suggesting the cyclic control and DGC pattern could be an adaptive trait that may help species conserve respiratory water loss. In addition, studies have shown the muscular control for the valval opening or closure is regulated by the O_2 and CO_2 pressure modulated by connected inhibitory and excitatory axons (Miller 1962, Matthews and White 2011), suggesting the evolution in the neural circuit is also important in the gas-exchange pattern. Could the perception of humidity also affect the specular control? The induced DGC in some xeric species such as the desert locusts suggests this could be a possible adaption to a dryer environment (Matthews and White 2013, Groenewald et al. 2014). More investigations in the mechanisms of humidity perception and neural control are required to better understand how the gas exchange patterns can evolve for water conservation and desiccation adaptation

Excremental water loss and osmoregulation

It is conserved in the animal kingdom to maintain osmo-homeostasis. By controlling water intake and excrement, organisms can maintain suitable water content in the body and withstand desiccation stress. In insects, the osmo-homeostasis is mainly controlled by Malpighian (renal) tubules and hindgut (ileum and rectum) and regulated by endocrine hormone (Chapman 2013). The Malpighian tubules are slender tubes usually found in the posterior part of the alimentary canals and are functionally analogous to the mammalian kidney which absorbs solutes, wastes, and water from the surrounding hemolymph. The absorbed substances were then delivered to the hindgut with important ions and water actively re-

absorbed by the ileum and rectum. This process is important for waste excrement and detoxification, as well as iono- and osmo-regulation (Beyenbach et al. 2010).

The water excretion in the Malpighian tubules and absorption in the hindgut is usually conducted by water transporting channels such as the aquaporins (Spring et al. 2009), and coordinated by multiple neuropeptides. Ubiquitous knockdown of aquaporins in the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae) (Drake et al. 2015) and *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Yao et al. 2018) have led to increased water conservation and desiccation resistance, suggesting aquaporins are the major channels for water transportation in insects. How is water flux through aquaporins regulated to maintain the osmo-homeostasis? Studies showed the diuretic neuropeptides, including kinin, capa, and DH31, are able to bind with receptors on the epithelium of Malpighian tubules in insects and stimulate the transcellular chloride transport and V-Type ATPase activities, along with transcellular water flux through the aquaporins (Cabrero et al. 2014, Cabrero et al. 2020). RNAi knockdown of these peptides is able to increase desiccation resistance in several different insect species (Terhzaz et al. 2015, Cannell et al. 2016, Alford et al. 2019). Therefore, one of the regulations in water flux locates at Malpighian tubules and reduced expression of diuretic neuropeptides can conserve water excretion. The second control could be the water reabsorption in the hindgut, while the mechanisms are less explored. The previously mentioned ATPase channels and water transporting channels are expressed on the apical surface of the ileum and rectum (Patrick et al. 2006), suggesting a potentially similar mechanism with coupled iono- and osmo-regulation. Early endocrine studies in the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae), have found neuropeptides such as chloride transport stimulating hormone (CTSH) and ion transport

peptide (ITP) help maintain the re-absorptive properties of the hindgut (Audsley et al. 1992, Phillips et al. 1996). Interestingly, later experiments on *D. melanogaster* found *ITP* gene had a higher expression when exposed to arid conditions, which did not only increase water reabsorption in the hindgut and repress excretion but also promotes thirst and drink more water in the laboratory (Gáliková et al. 2018). Taken together, these studies suggest that osmo-homeostasis, though could be regulated by different sets of neuropeptides in different insects, is important for insects withstanding different levels of desiccation stress.

Dehydration tolerance

In addition to preventing different types of water loss, an inherent trait that helps survive from dry environments is the percentage of water loss that a species can tolerate, which is defined as dehydration tolerance (or desiccation tolerance). In the scenarios that water loss occurs unavoidably, the dehydration tolerance can directly decide the species' survivability. The desert *Drosophila* species were found to have higher dehydration tolerance than mesic ones, which can tolerate around 5% more water loss (Gibbs and Matzkin 2001). This difference in the dehydration tolerance has also been observed in many groups of closely related insect species such as mosquitoes (Benoit et al. 2010), springtails (Holmstrup et al. 2001), and butterflies (Mazer and Appel 2001). The largest difference was found in several species of midges (Diptera: Chironomidae). The Antarctic midge, *Belgica antarctica* (Benoit et al. 2007), and the African sleeping midge, *Polypedilum vanderplanki* (Cornette et al. 2010), are able to tolerate 70% and 95% of water loss, respectively, and be alive in an anhydrous status, while other midge species such as *Polypedilum nubifer* (Gusev et al. 2014) can barely survive from

little water loss. These comparisons suggest desiccation tolerance evolves rapidly across species and can be an important strategy to adapt to dry environments.

Comparative investigations using these different groups of insect species suggest several types of biomolecules contribute to desiccation tolerance, including late embryogenesis abundant (LEA) protein, amino acids such as proline, sugars such as trehalose, polyols, antioxidants, and heat shock proteins (Sogame and Kikawada 2017, Thorat and Nath 2018). However, these biomolecules can also contribute to other abiotic tolerance including thermal tolerance (MacMillan and Sinclair 2011, Teets and Denlinger 2013, Wang et al. 2019b). This could be a general mechanism to protect cell and tissue structures against different stresses.

Current investigations to understand the genetic and molecular mechanisms underlying dehydration tolerance are still limited. Most studies use different -omics techniques to identify important genomic and transcriptomic elements that may contribute to this trait. These elements are usually specific to species. For example, a unique presence of compact clusters of anhydrobiosis-related genes was found in the genome of *P. vanderplanki*, while this was not found in any other insect species (Gusev et al. 2014). In these compact clusters, dehydration-related genes such as the antioxidant enzymes, protein-repair methyltransferases, and aquaporins are duplicated multiple times and compactly arranged together. The extra copies also share a low similarity in protein sequences. This suggests that multiple gene duplication events could underlie the high dehydration tolerance in *P. vanderplanki*. Considering the complicated evolutionary events, this type of evolution may not be a norm for other species to evolve when dealing with the increasing aridity.

Some mechanisms in dehydration tolerance may be general in most insect species, including the induced expression of heat shock proteins, up-regulation of genes in cellular recycling pathways, and down-regulation of genes for metabolic depression. These induced changes are not only found in anhydrous midge species (Teets et al. 2012, Gusev et al. 2014), but also other species such as the desert *Drosophila* species (Matzkin and Markow 2009) and mosquitoes (Wang et al. 2011). Though these induced changes can be found in most species, the extent of these differs. Comparisons between sibling midge species, *P. vanderplanki* and *P. nubifer*, showed a similar number of metabolic-related genes is present in both midge species but with distinct expression patterns when facing desiccation (Gusev et al. 2014). This suggests that the regulation of gene expression can be another mechanism for different levels of dehydration tolerance.

Hygrosensation and top-down effects

Hygrosensation is an essential sensory modality that allows animals to detect variations in humidity in the environment (Filingeri 2015). For organisms facing unpredictable dryer environments, it is extremely important to accurately sense the relative humidity and to trigger corresponding behavioral and physiological responses to withstand the stresses. In insect species, the hygrosensation has been found mainly accomplished by two types of neurons on the antenna to respond to an increase and a reduction in the humidity (Yokohari and Tateda 1976, Itoh et al. 1984), respectively. Some early studies in ticks, cockroaches, and *D. melanogaster* suggested the hygrosensation was associated with a thermosensitive mechanoreceptor, a TRP channel that hypothesized to use water vapor pressure under different temperatures to sense humidity (Yokohari and Tateda 1976, Liu et al. 2007, Tichy and

Kallina 2010). However, recent studies in *D. melanogaster* identified two neighboring hygrosensation neurons housed in no-pore sensilla at antennal sacculus, a three-chambered pocket on the posterior surface of the antenna (**Figure 1**), which also involves two different combinations of ionotropic receptors (IRs) (Enjin et al. 2016, Knecht et al. 2016, Knecht et al. 2017). The first combination consists of IR25a, IR93a, and IR40a to trigger a neural response for hydrated flies seeking dry air, while the second one includes IR25a, IR93a, and IR68a and activates moist-seeking in dehydrated flies. Further studies showed within the shafts of these sensilla, there is an abundantly expressed gene, *Obp59a*, encoding a small soluble protein and responsible for the humidity sensing (Sun et al. 2018). Interestingly, the knockout of *Obp59a* from *D. melanogaster*, which made the flies not perceive humidity normally, almost doubled the flies' desiccation resistance (Sun et al. 2018), suggesting the sensation of no humidity environment from the disrupted hygrosensation results in physiological responses and increased desiccation resistance.

The mechanisms underlying hygrosensation in most insect species are still less investigated, while distinct preferences of humidity were reported between closely related species. The preferred humidity level for the desert *D. mojavensis* is around 20%, but the rainforest species *D. teissieri* and wide-spread *D. melanogaster* prefer to stay in the environments with 70-85% relative humidity (Gibbs et al. 2003b, Enjin et al. 2016). This difference was also widely observed in many insect taxa such as parasitoid wasps (Tee and Lee 2015) and ants (Greenslade 1972). How do insects evolve different preferences to humidity? The characterized *IR* and *Obp* genes for hygrosensation in *D. melanogaster* can also be found in the genomes of most other *Drosophila* species, with 50 – 70% protein sequence similarities.

This suggests they could have a similar capability of sensing humidity. As evolutionary changes in neural circuits can influence insects' perception and behavioral consequences on the same olfactory or visual inputs (Hansson and Stensmyr 2011, Kelber 2016), I hypothesize that distinct preferences to humidity in insect species could result from changes in their perception, instead of hygrosensation receptors.

The endocrine regulation and neural control for the top-down effects provide another perspective to appreciate the mechanisms underlying desiccation adaptation. For remote signaling from the brain to different tissues, the use of peptide hormones has found an important regulatory pathway that could undergo rapid evolution. A recent study reported that the signaling pathway of the inotocin, an oxytocin/vasopressin-like peptide, is able to regulate the expression of the decarbonylase *Cyp4g1*, the last step in the CHC synthesis pathway, in the ant species *Camponotus fellah* (Koto et al. 2019). When the ant worker initiates foraging outside the nest, of which the humidity is lower than inside the nest, the overexpress of inotocin receptor in oenocytes was found leading to higher *Cyp4g1* expression and more overall CHCs, which confers higher desiccation resistance. This study showed that the inotocin is an important signaling pathway in regulating CHC synthesis and desiccation adaptation in ant species. However, through mining the genes in the inotocin signaling pathway in the genomes of around 200 species, Liutkeviciute et al. (2016) reported that the inotocin pathway was not found in the genomes from several orders, including Trichoptera, Lepidoptera, Siphonaptera, Mecoptera, and Diptera, suggesting this pathway be lost in their common ancestor around 280 million years ago. Interestingly, preliminary results in the laboratory showed the dipteran *D. melanogaster* with *Obp59a knockout* have unusually higher CHC amounts, suggesting that the

incapability of sensing humidity is able to trigger the over-production or accumulation of CHCs with an unknown mechanism and increase the desiccation resistance. This evidence suggests a different signaling pathway in regulating CHC syntheses in Diptera and probably also in the insects from other orders without inotocin pathway. Putative pathways could include the very conserved insulin/insulin-like growth factor signaling (IIS) (Kuo et al. 2012) and Pigment-Dispersing Factor (PDF) (Krupp et al. 2013) which were found regulating CHC syntheses and sex attractiveness in *D. melanogaster*. IIS-related genes were also found differentially regulated in *D. melanogaster* and *D. mojavensis* when experiencing desiccating stress (Matzkin and Markow 2009, Nuzhdin et al. 2009), however, more experiments are needed to determine the role of the IIS pathway in regulating CHC syntheses and desiccation adaptation.

The endocrine system can also regulate osmo-homeostasis in insects. Most of the current studies are still focusing on model species. In *D. melanogaster*, capa is a peptide that was found mediating osmo-homeostasis in Malpighian tubules and defecation in *D. melanogaster*. When *D. melanogaster* is facing desiccation stress, its *capa* gene is up-regulated and can inhibit water secretion through defecation (Terhzaz et al. 2015). Further comparisons of transcriptional activities of *capa* in two mosquito species and three other *Drosophila* species showed *capa* had different expression patterns. Its expression level was not changing in a desert *D. mojavensis* when facing desiccation stress. However, *D. mojavensis* have a more frequent and less amount defecation pattern compared with *D. melanogaster* under desiccation stress, suggesting *capa* signaling pathway does not apply to osmo-regulation in *D. mojavensis* and a different mechanism is involved. The hygrosensation and cascading top-down effects are diverse and regulated in a complicated manner. Although current evidence shows

rapid evolutionary changes in this top-down regulation, there are still a lot of unknowns regarding the genetic and molecular mechanisms.

Summary

In this chapter, I comprehensively reviewed mechanisms underlying insect adaptation to desiccation. When facing drier environments, the ability to prevent water loss is crucial for insects better surviving. To prevent water loss from the cuticle, spiracle, and defecation, insects have been using versatile strategies including the use of cuticular hydrocarbons, discontinuous gas-exchange cycles in spiracular control, and osmoregulation in the Malpighian tubules and hindgut. Except for preventing water loss, insects also developed mechanisms to tolerate water loss. Though some species have already evolved special mechanisms to survive from an anhydrous state by losing most of the body water, general mechanisms have been observed in diverse taxa, for example, the use of different biomolecules to maintain complete cellular and tissue structure. In addition, the genetic and molecular mechanisms in hygrosensation of insect species have been recently dissected which could shed light on how insects sense, perceive, and respond to aridity. Comparative studies about insects' desiccation resistance and diverse strategies in desiccation adaptation suggested a rapid evolution in the desiccation adaptations, which pose the importance for investigations of evolutionary mechanisms.

The novelties for adaptation to extreme environments usually require multiple evolutionary changes, for example, the dehydration tolerance in the Antarctic midge. This makes most species difficult to rapidly gain this special strategy to adapt to changing environments. Therefore, I hypothesize that only a few of the traits could repeatedly evolve in species for their adaptation and the use of CHCs to prevent cuticular water loss is one of them.

As genes underlying CHC syntheses could have complicated interactions, evolutionary changes in one or a few of them could drastically alter CHC profiles (Gleason et al. 2009, Holze et al. 2021). Recent studies showed complicated composition in CHC layers of different insects. Some properties such as the length, abundance, and presence of mbCHCs are important to desiccation resistance, but it is still less clear how the other properties and other types of CHCs contribute to desiccation resistance.

In addition, as CHCs play important roles in chemical communication in insects, the evolutionary trajectories of CHCs may be influenced by other factors than aridity. CHCs in some insect species may evolve to be less effective to prevent water loss for other advantages, for example, higher mating chances (Steiger et al. 2013). This pleiotropic role of CHCs may bias our understanding regarding how their properties contribute to desiccation resistance. Therefore, to better understand how CHCs could evolve for insects' adaptation to desiccation, I investigated the correlation between CHCs collected from different species and used functional experiments to test whether there are causal effects between CHC properties and desiccation resistance.

The knowledge can be applied to pest management science and conservation biology. The mechanisms in the desiccation adaptation could have a complicated interaction with pest control strategies such as chemical control and biological control. For example, CHCs as the outmost lipid layer on the body surface of insect pests are also the very first defending layer against the penetration of insecticides (Chen et al. 2020). If a thicker CHC layer evolves for species adapting to drier environments, this could prevent insecticide penetration and facilitate the development of insecticide resistance (Pu et al. 2020). Biological control is one of the most

efficient pest management strategies including the release of natural enemies or protecting existing natural enemies (Naranjo et al. 2015). However, when facing changing environments including increasing aridity, the evolutionary trajectories in natural enemies and their pest hosts may diverge and lead to unexpected consequences (Thomson et al. 2010). In addition, the evolutionary mechanisms underlying desiccation adaptation can also be used to track adaptive progress in endangered species and inform appropriate strategies for evolutionary rescue (Vander Wal et al. 2013).

Desiccation has been one of the most important forces driving the evolution of all terrestrial organisms, and currently increasing global average temperatures (Ault 2020) are stressing species in many areas. I propose to study the evolutionary mechanisms underlying current desiccation adaptations, which will help better manage pests and conserve species. Beyond desiccation adaptation, the evolutionary mechanisms that lead to novel traits and higher fitness can pave the roads for future studies that better understand how species can evolve and face new challenges.

Research objectives

Among all strategies that insects deploy to withstand desiccation stress, the use of CHCs to prevent water loss plays a major role (Gibbs and Matzkin 2001, Gibbs et al. 2003a). In this dissertation, I aimed to investigate the physiological and genetic mechanisms underlying CHC syntheses and insect desiccation resistance.

Objective 1. Investigate the association between CHCs and desiccation resistance in *Drosophila* species

Objective 2. Elucidate the genetic mechanisms underlying the longest mbCHC and highest desiccation resistance in a desert *Drosophila* species

Objective 3. Determine different factors affecting CHC synthesis in *Drosophila* species

CHAPTER 2. PROPERTIES OF CHCS UNDERLYING THE EVOLUTION OF DESICCATION RESISTANCE

In this chapter, I sought to understand associations between CHCs and desiccation resistance in insect species. This chapter could not have been accomplished without help from several colleagues:

Dr. Jian Pu

- Helped set up desiccation assays
- Helped score fly mortalities in some desiccation assays

Dr. Joseph Receveur

- Helped with data cleaning, Random Forest analysis, and visualization

Cole Richards

- Helped collect and sort flies in preparation of CHC collection and desiccation assays

Haosu Cong

- Helped score fly mortalities in some desiccation assays

Introduction

A central goal in biology is to understand how phenotypic evolution leads to organismal adaptation to different habitats. There are diverse mechanisms for dealing with abiotic challenges in many different taxa including insects, plants, and mammals (Wingfield 2013, Colinet et al. 2015, Basu et al. 2016). However, these different traits, especially physiological responses to the abiotic adaptation, are rarely compared interspecifically to understand the physiological basis and potential evolutionary trajectories. A comparative perspective among

species about phenotypic evolution could provide insights to predict the responses to future environmental changes (Hoffmann and Sgro 2011).

Desiccation is one of the most critical challenges for organisms surviving and thriving in terrestrial environments, especially as Earth's climate is increasingly warmer and drier (Ault 2020). As the most abundant organisms, insects have relatively small body sizes and therefore, a high surface-area-to-volume ratio, which renders them vulnerable to water loss (Kühsel et al. 2017). Early studies in insects' strategies for resisting desiccation indicated that water conservation, or the prevention of water loss, contributes the most to the desiccation resistance (Addo-Bediako et al. 2001, Gibbs and Matzkin 2001). In insects, water is lost via three major routes: cuticular water evaporation, respiratory loss, and defecation (Gibbs et al. 2003a, Chown et al. 2011). Variation in cuticular water loss is the major contributor to water loss and desiccation resistance across many insects (Gibbs and Rajpurohit 2010, Chown et al. 2011, Johnson et al. 2011). In some extreme cases, for example, in the queen of the harvest ant *Pogonomyrmex barbatus* (Hymenoptera: Formicidae), cuticular water transpiration accounts for 97% of increased water loss (Johnson and Gibbs 2004). Therefore, to resist desiccation, it is necessary to minimize cuticular water loss.

The use of cuticular hydrocarbons (CHCs) has been found as a generic mechanism for insects preventing cuticular water loss and desiccation resistance. They are synthesized in cells called oenocytes with different groups of genes (Blomquist and Bagnères 2010). The CHCs form a hydrophobic layer on an insect's body surface and they are able to constrain water transpiration. It has been hypothesized that the physical properties of CHCs such as the melting

temperature and quantity are positively correlated with their water-proofing capabilities and desiccation resistance (Gibbs and Pomonis 1995, Gibbs 2002a). Increased overall amounts of CHCs could form a thicker lipid layer and confer higher desiccation resistance, which has been demonstrated by overexpressing the synthesis genes in different insect species (Qiu et al. 2012a, Yu et al. 2016, Chen et al. 2020). In addition, CHCs with higher saturation and/or longer carbon chains have higher melting temperatures and can help prevent water loss (Gibbs 1998, Jezovit et al. 2017). Another study genetically removed oenocytes from *Drosophila melanogaster* adults to eliminate CHC synthesis (Oe^{-}), and then independently coated saturated and unsaturated CHCs on Oe^{-} flies to examine the contribution of CHCs to the desiccation resistance (Krupp et al. 2020). The results showed that the coating of saturated CHC mixture led to a significant but minor increase in their desiccation resistance. However, the chemical composition of CHCs suggested that saturated CHCs alone may not be sufficient to explain their association (Krupp et al. 2020). In addition, the coating of saturated CHCs alone was not able to return desiccation resistance to the original level (Krupp et al. 2020). Another study reduced unsaturated CHCs in *D. melanogaster* by knocking down the desaturase genes leading to decreased desiccation resistance (Ferveur et al. 2018). Taken together, both saturated and unsaturated CHCs play an important role in preventing water loss. Due to the complexity of CHC chemical composition, the contribution of CHC chemical properties to the desiccation resistance is largely unclear.

Drosophila species have been excellent models to investigate environmental adaptation and evolution, including the association between desiccation resistance and CHCs. Previous studies have examined the desiccation resistance for different *Drosophila* species and showed a

high interspecific variation (Matzkin et al. 2009, Kellermann et al. 2012), suggesting a rapid evolution in diverse mechanisms underlying desiccation resistance. Since CHCs are the generic mechanism for insects including *Drosophila* species for preventing water loss (Gibbs et al. 2003a), the evolution of the chemical composition and quantity of CHCs among *Drosophila* species could underlie their variation of desiccation resistance. Within the *Drosophila* genus, CHCs consist of four major types based on chemical functional groups, including linear alkane, methyl-branched alkane, monoene, and diene (Chung and Carroll 2015). The mixture of different types, lengths, and quantities of CHCs could lead to different levels of desiccation resistance. Although recent studies have demonstrated certain types of CHCs such as methyl branched CHCs (Chung et al. 2014) by manipulating the gene expression in the oenocytes with molecular biology techniques, it is less understood how the co-occurrence of different CHCs helps resist desiccation.

In this chapter, we used *Drosophila* species as a model to investigate how CHCs contribute to desiccation resistance. We surveyed the cuticular hydrocarbon profiles and desiccation resistance for 46 *Drosophila* species, using three *Scaptodrosophila* species and one *Chymomyza* species as the outgroups. We examined the correlation between several CHC properties and desiccation resistance in these species. We also used random forest regression analysis and principal component regression analysis to understand how different CHCs could contribute to desiccation resistance. Results from this chapter can help understand how the evolution of a trait can lead to species adapting to diverse environments.

Results

In this chapter, I measured the desiccation resistance, body weight, and CHC profiles for 46 *Drosophila* species, as well as of three *Scaptodrosophila* species and one *Chymomyza* species as the outgroup (**Figure 2.1; Appendix I**). Desiccation resistance of these species ranges from 3 to 60 h, suggesting variability in their ability to withstand desiccation stresses. I mapped the evolution of desiccation resistance through the phylogeny and showed that the common ancestor of these species had an intermediate level of desiccation resistance which further evolved to lower and higher levels of resistance (**Figure 2.2**). The evolutionary trajectory of desiccation resistance is consistent with findings by other researchers (Kellermann et al. 2012).

Cuticular hydrocarbons of these species also exhibit variation in both chemical composition and quantity (**Figure 2.1**; **Appendix I**). The CHC profiles of most species contain mbCHCs, monoenes, and dienes, while alkanes are phylogenetically restricted to one species clade (**Figure 2.1**). I performed a correlation analysis for these different types of CHCs and for each of the CHCs across these species. Results showed a strong and positive correlation in the quantity and length of mbCHCs and dienes, as well as an intermediate and positive correlation between mbCHCs and monoenes (**Figure 2.3**).

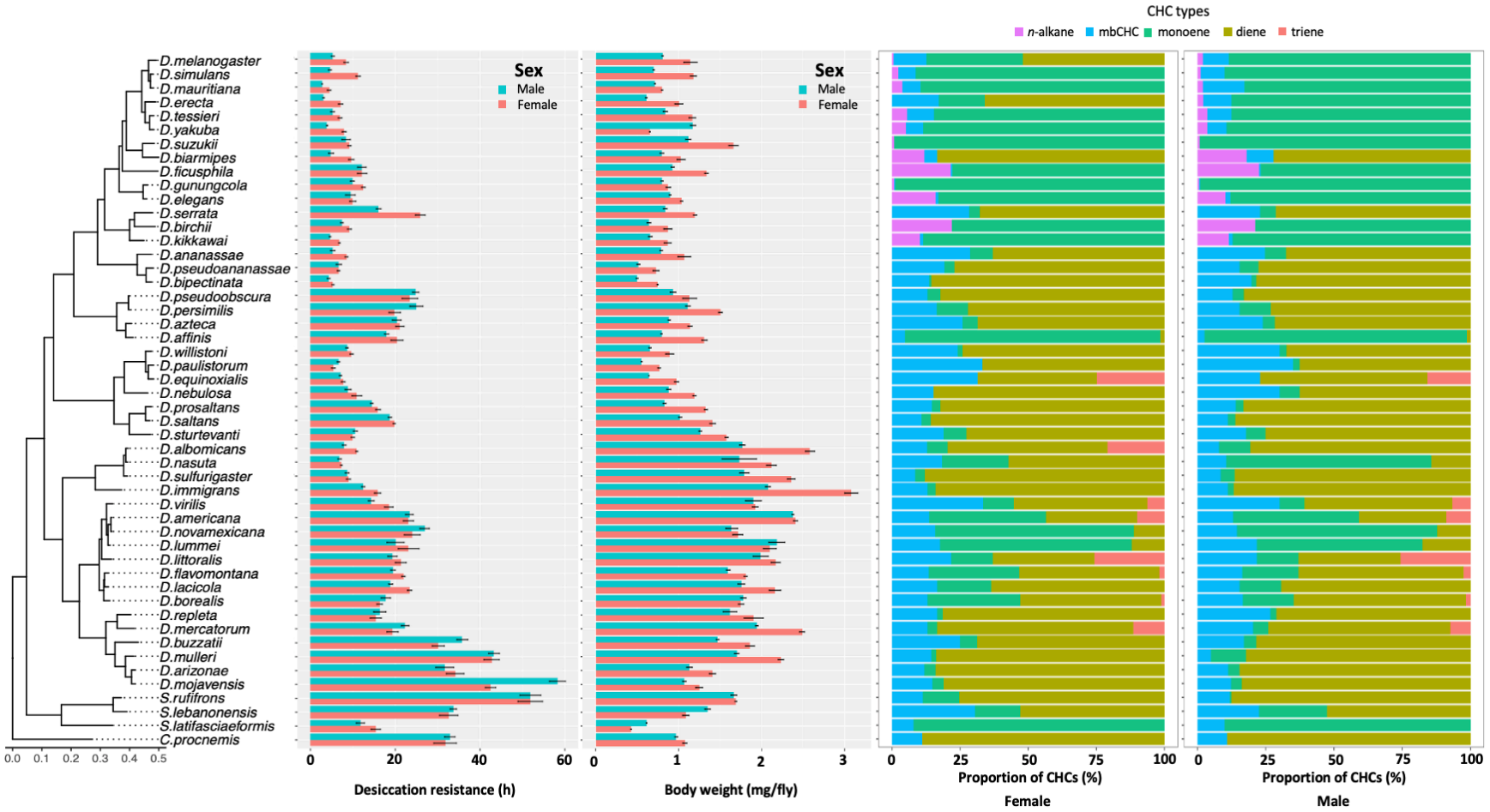


Figure 2.1. Desiccation resistance, body weight, and proportions of different types of CHCs in females and males of 46 *Drosophila* species, as well as three *Scaptodrosophila* species and one *Chymomyza* species.

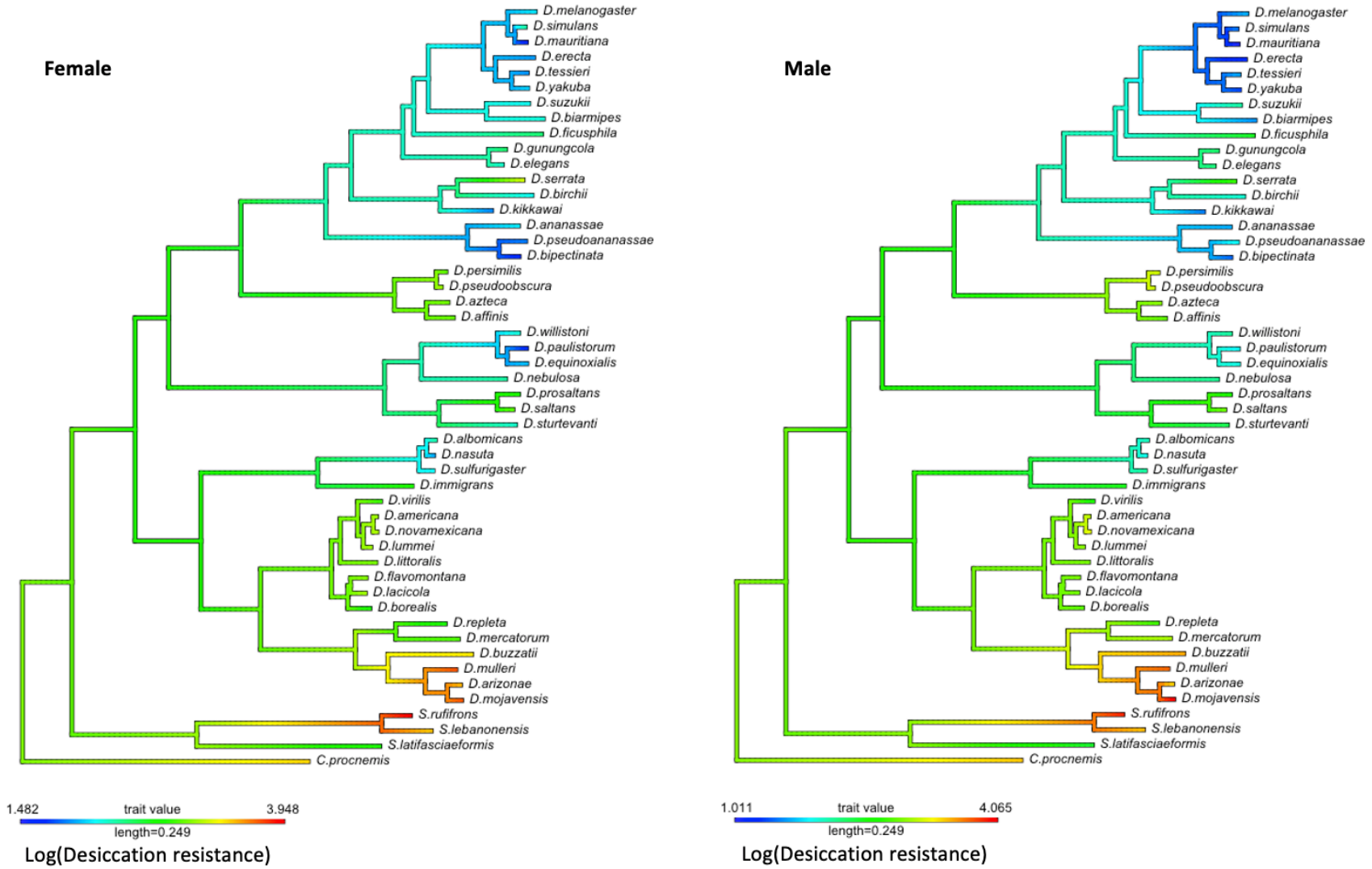


Figure 2.2. Evolutionary trajectories for desiccation resistance in females and males of 46 *Drosophila* species, as well as three *Scaptodrosophila* species and one *Chymomyza* species.

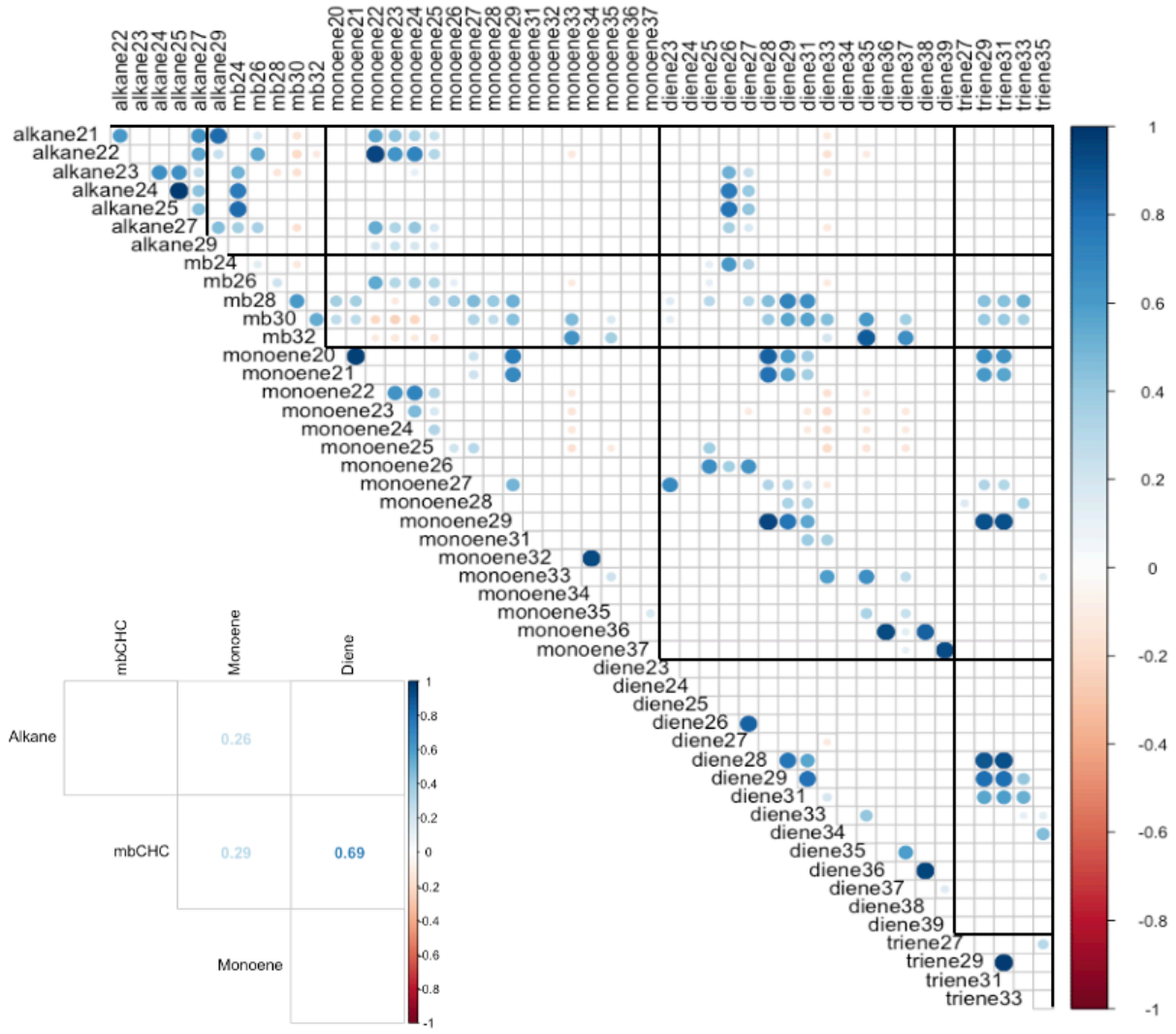


Figure 2.3. Correlation analyses for the quantities of each type of CHC (left) and each single CHC (right).

To determine if an association between different CHC properties and desiccation resistance exists, I used two different methods to analyze the data. In the first method, I aggregated these CHCs based on their chemical and structural properties and performed correlation analyses with desiccation resistance. For the second method, with the help of Dr. Joseph Receveur, I used machine learning methods including random forest and principal

component regression analysis to understand how the combination of important CHCs could predict desiccation resistance across these species.

Quantity of CHCs cannot predict desiccation resistance

Generalized linear regression between the total quantity of CHCs, bodyweight, and desiccation resistance showed that bodyweight is an important predictor for desiccation resistance(t = 9.6, P < 0.001), while the total quantity of CHCs does not correlate with desiccation resistance (P = 0.8) (**Figure 2.4**).

As different types of CHCs have varied melting temperatures, I also aggregated the quantity of CHCs based on their type and performed linear regression analyses with desiccation resistance. For all these models, bodyweight was included as a term. Results showed for all the four types of CHCs, only the total quantity of dienes has a positive correlation with desiccation resistance (t = 7.2, P < 0.001) (**Figure 2.5**). The total quantities of other three types of CHCs either have negative correlation or no correlation with desiccation resistance (Alkane: t = -5.9, P < 0.001; mbCHC: P = 0.05; monoene: t = -5.8, P < 0.001). These analyses showed the quantities of CHCs may not be a good predictor for desiccation resistance.

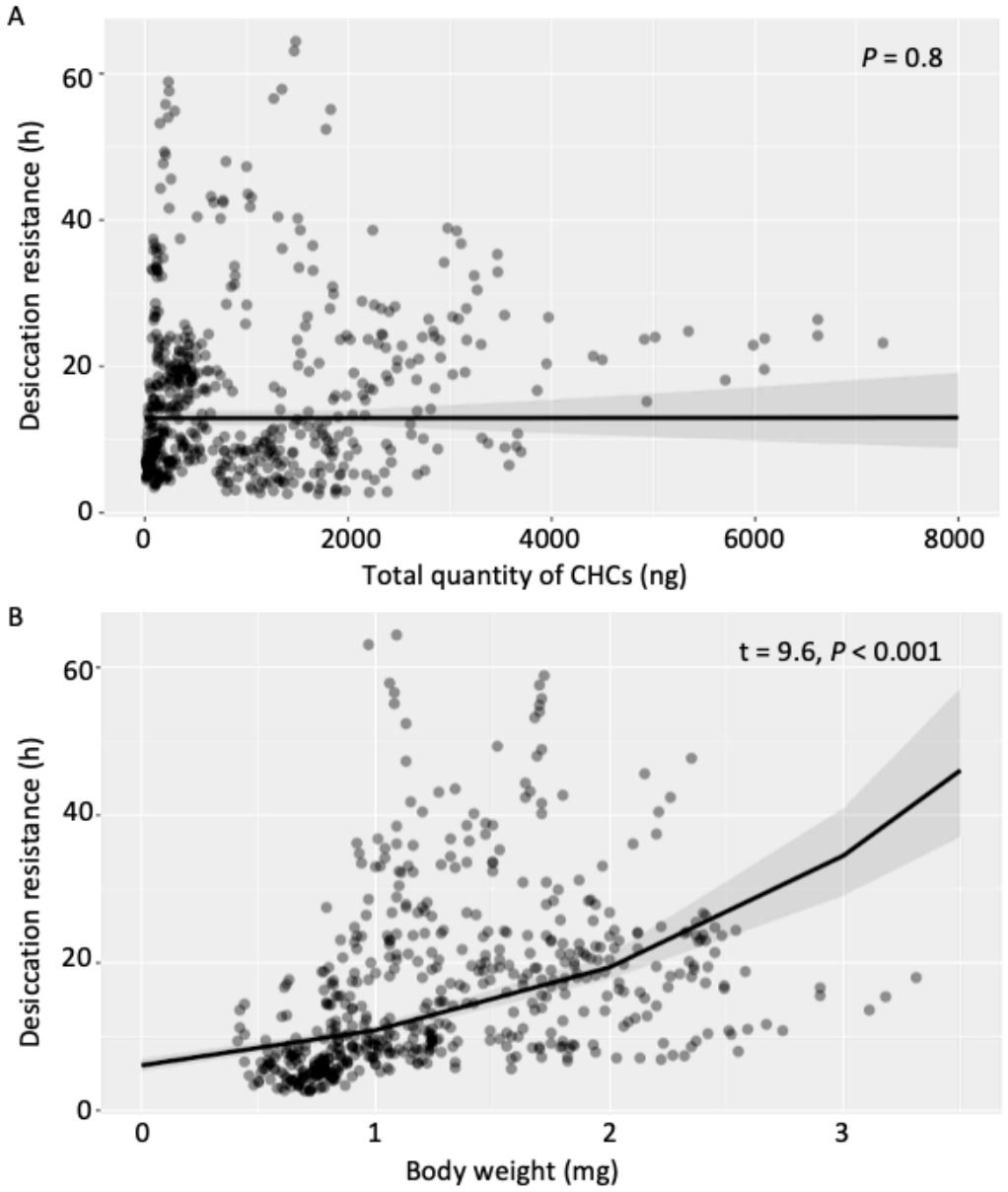


Figure 2.4. Bodyweight positively correlates with desiccation resistance, but the total quantity of CHCs does not have any correlation with desiccation resistance. Linear regression with the model log(Desiccation resistance) ~ TotalQuantity + Bodyweight was used to determine the correlation between these variables. Results showed that the body weight had a positive correlation with desiccation resistance (t = 11.9, P < 0.001), while the total quantity of CHCs (P = 0.8) and the interaction term (P = 0.8) do not correlate with desiccation resistance.

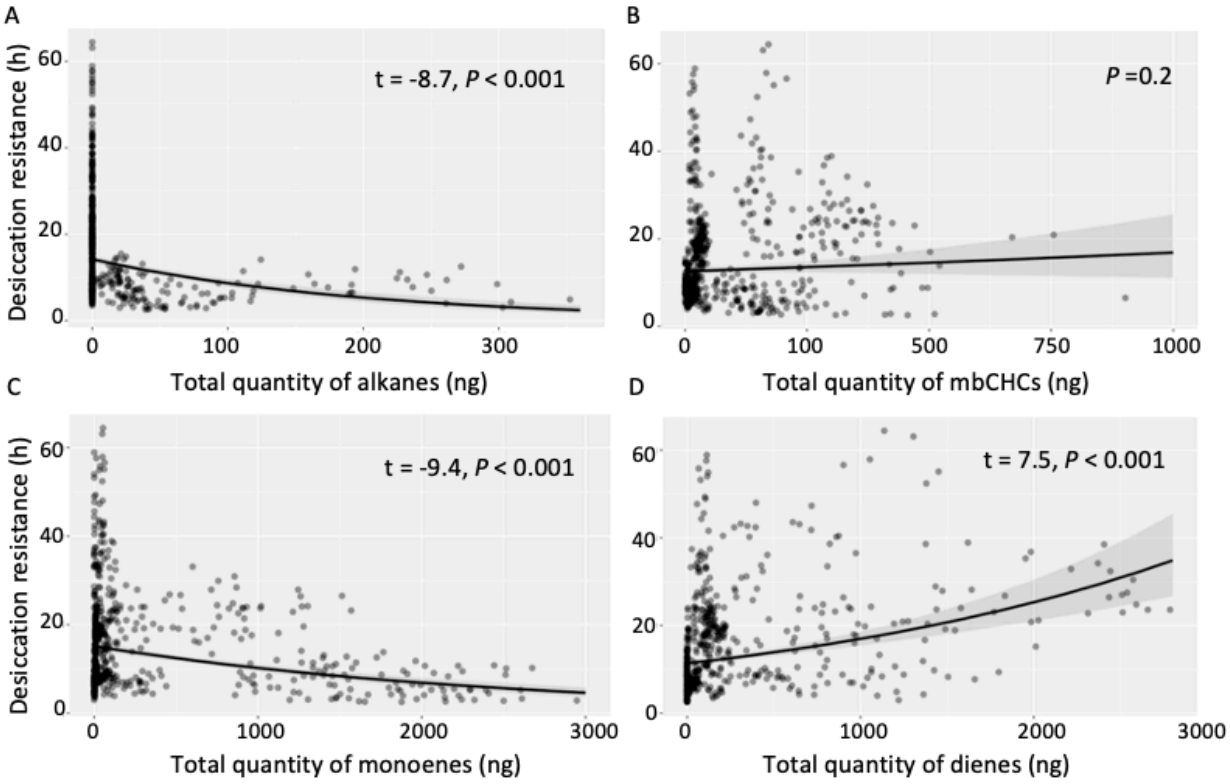


Figure 2.5. The total quantity of dienes positively correlates with desiccation resistance. Linear regression with the model log(Desiccation resistance) ~ Total_Quantity + Bodyweight was used to determine the correlation between these variables. Test statistics for the coefficient and *P*-value for the term of Total_Quantity are labeled in each plot of the model prediction.

Lengths of CHCs positively correlate with desiccation resistance

In addition to the total quantity of CHCs, a generalized linear model is applied to test how the lengths of carbon chains in CHCs correlate with desiccation resistance. As multiple CHCs with different lengths can be found in most *Drosophila* species and usually one of them has the highest quantity (**Appendix I**), I defined the CHC with the highest quantity as the major CHC for each type. The results showed that longer mbCHCs (t = 4.5, P < 0.001) and monoenes (t = 7.9, P < 0.001) correlated with higher desiccation resistance, while the lengths of dienes (t = -

2.6, *P* = 0.01) negatively correlated with desiccation resistance (**Figure 2.6**). This suggests mbCHCs and monoenes could play major roles in desiccation resistance.

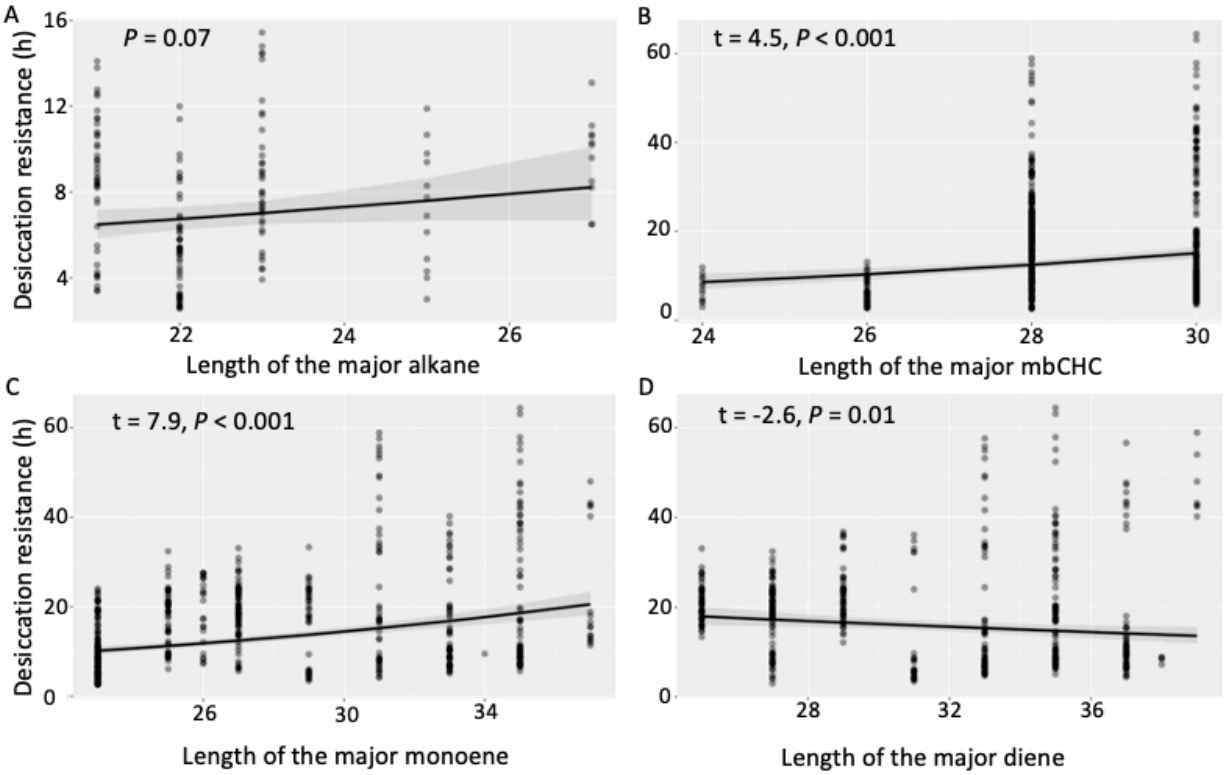


Figure 2.6. Species with longer mbCHCs and monoenes have higher desiccation resistance. Linear regression with the model log(Desiccation resistance) ~ Length was used to determine the correlation between lengths of the major CHCs and desiccation resistance. Test statistics for the coefficient and *P*-value is labeled in each plot of the model prediction.

The abundance of methyl branched CHCs are predictive for desiccation resistance

Although the CHC length is important for desiccation resistance, it is less clear how these different CHCs could predict desiccation resistance. The random forest analysis with the Out-Of-Bag (OOB) method was used to determine the important CHC chemicals to the prediction of desiccation resistance. To do this, I first test if CHC compositions can be used to classify species. The random forest classification was able to discriminate between species with

a 98.5% success rate. A few misclassified species were from the same clades in the phylogeny with similar CHC profiles, including species *D. teissieri, D. yakuba*, *D. simulans*, *D. erecta*, *D. melanogaster*, *S. rufifrons*, and *S. lebanonensis*. CHC contributors to the overall random forest model include 51 chemicals that are listed in **Figure 2.7**.

The random forest regression modeling was further used to determine how CHCs can predict desiccation resistance. The results showed that CHC composition was able to explain 87.5% of the variation in desiccation resistance with a Root Mean Square Error (RMSE) of 4.2 (**Figure 2.8A**). The abundance of mbCHCs (ranging from 2MeC26 to 2MeC32) had the highest importance to the desiccation resistance in the regression model, while a number of other CHCs (see importance plot below) did not substantially contribute to the accuracy of the model for desiccation resistance (**Figure 2.8B**).

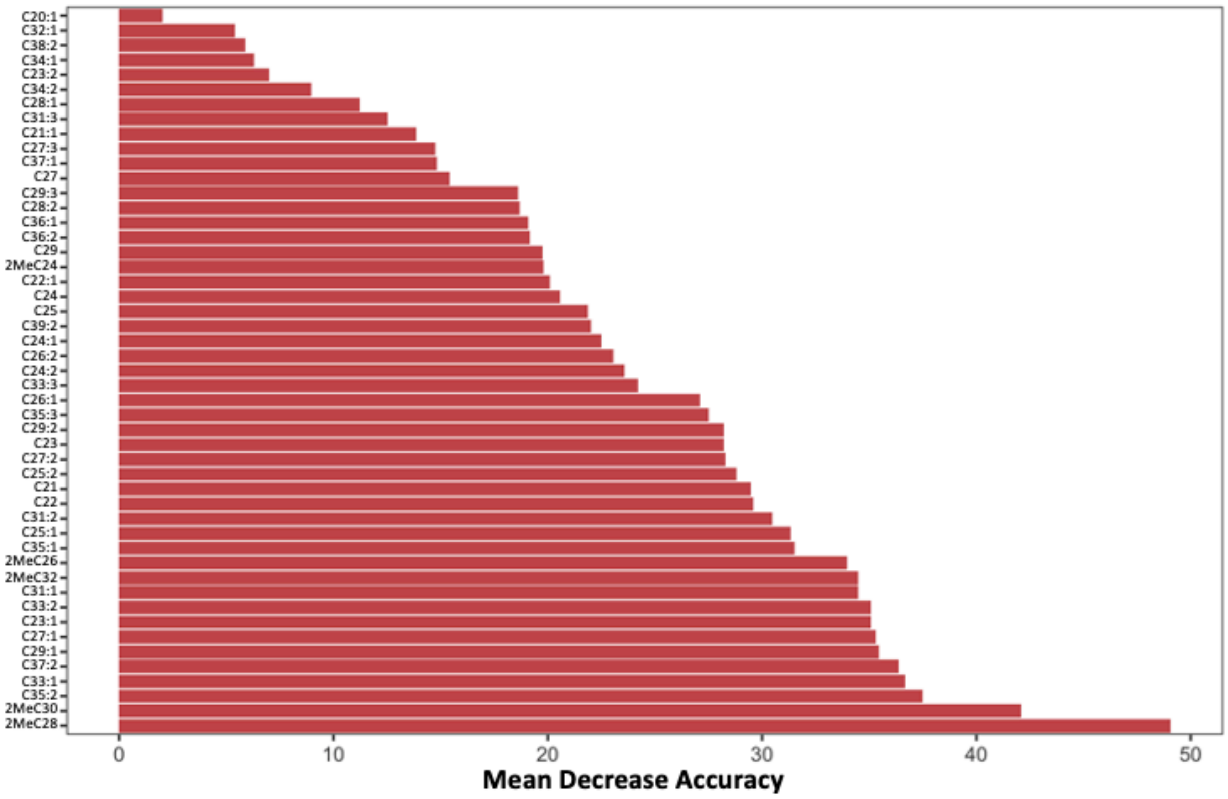


Figure 2.7. CHC profiles between species are distinct. Random Forest classification was able to discriminate between species with a 98.5% success rate. The top CHC contributors to the overall random forest model are listed.

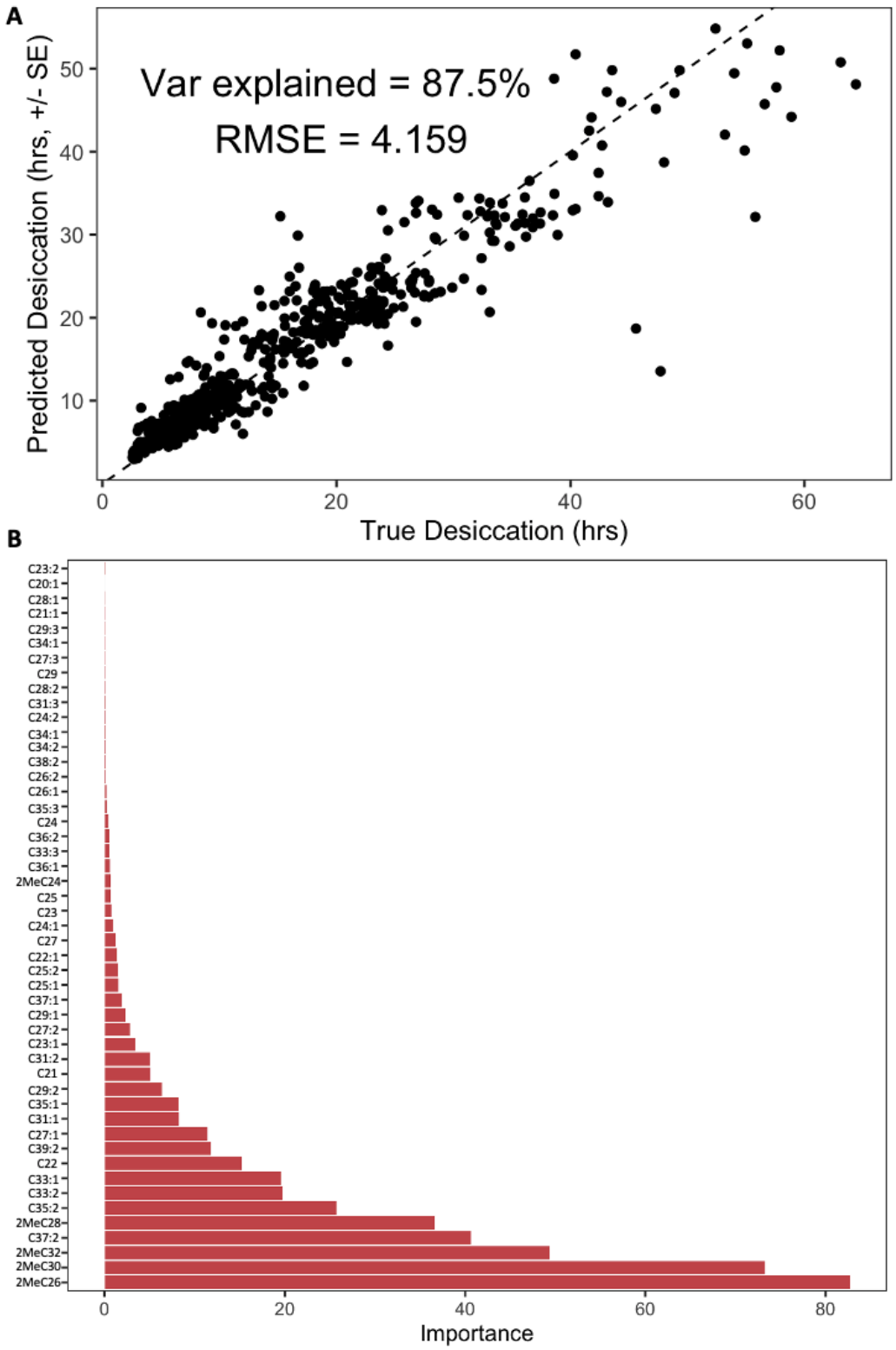


Figure 2.8. The abundance of mbCHCs is predictive of desiccation resistance. A. Random forest regression modeling of CHC abundance was able to explain 87.5% of the variation in desiccation

Figure 2.8 (cont'd)
resistance with a Root Mean Square Error (RMSE) of 4.159. B. The abundance of 2MeC26, 2MeC30, and 2MeC32 had the highest importance to the desiccation resistance regression model, while a number of CHCs did not substantially contribute to the accuracy of the model for desiccation resistance.

Longer mbCHCs have a higher contribution to desiccation resistance

The top 15 CHC variables from the random forest analysis and body weight were applied to the principal component regression analysis. Due to the collinearity between these CHCs (**Figure 2.3**), principal component analysis was first performed to convert all variables to orthogonal components (**Figure 2.9 A**). The top four components could explain the 23.9%, 20.5%, 11.5%, and 8.4% of the variance in all variables, and the correlations between these components and selected variables are shown in **Figure 2.9 B-C**. The partial least squared regression was further used to determine how these components correlate with desiccation resistance. Ten-fold cross-validation with the lowest Root Mean Square Error of Prediction (RMSEP) was used to determine the number of components used for the regression analysis. The analysis showed the use of five components in the model is optimal for the regression analysis which has the lowest RMSEP (**Figure 2.10A**). To determine how each single CHC contributes to desiccation resistance, the coefficients from the principal component regression were then converted to the model with the selected 15 CHCs and the bodyweight as variables (**Figure 2.10B)**. Since mbCHCs are important to desiccation resistance, the effects from mbCHCs of different lengths are further evaluated here. Shorter mbCHC, 2MeC26, has a negative coefficient, while the coefficients of longer mbCHCs, 2MeC28, 2MeC30, and 2MeC32, are positive (**Figure 2.10B**). In addition, the coefficients of 2MeC30 and 2MeC32 are larger than

2MeC28 (**Figure 2.10B**). This shows species with longer CHCs could have higher desiccation resistance.

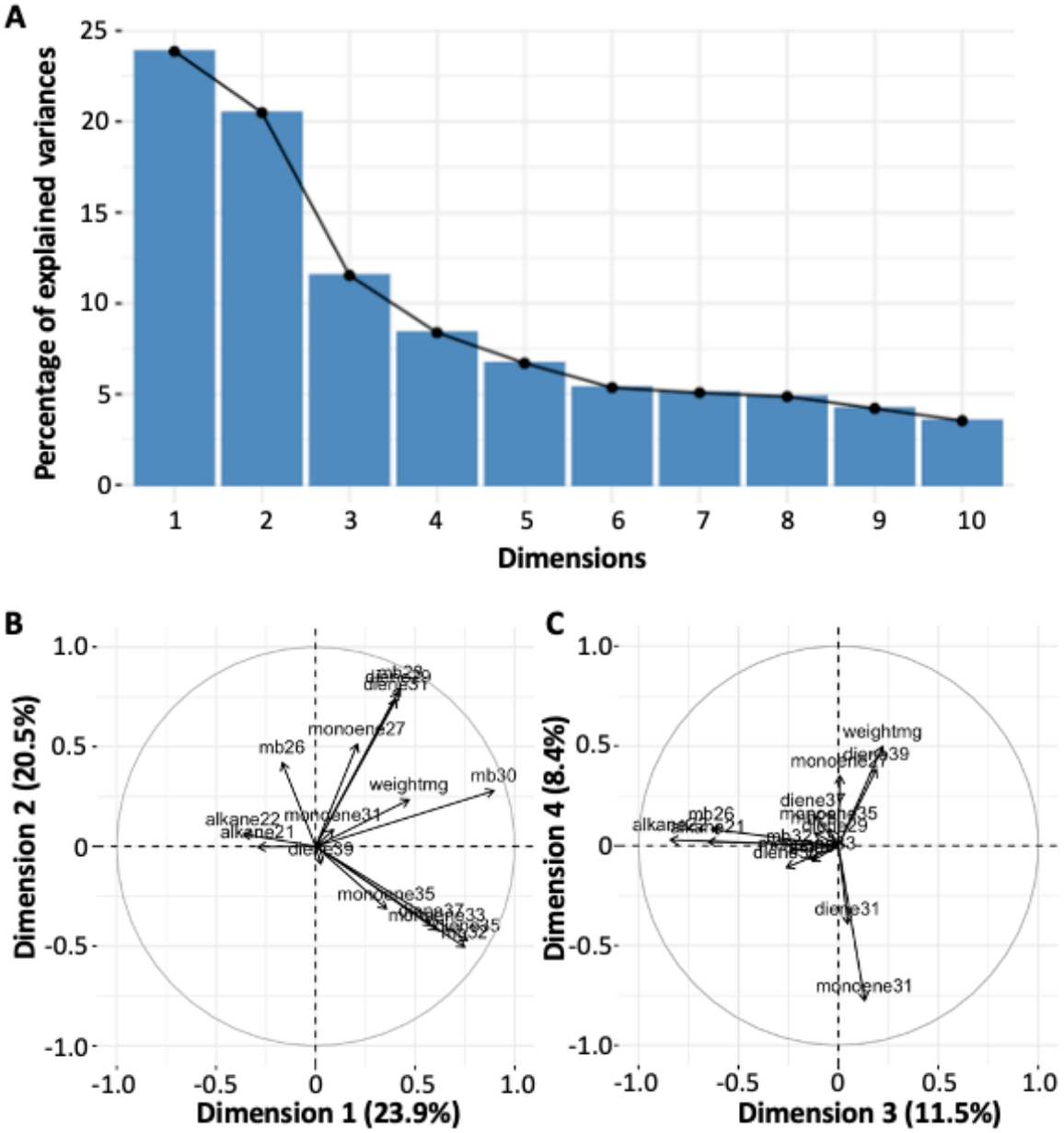


Figure 2.9. Scree plot from the principal component analysis (A) and correlations between variables and the top four components (B-C).

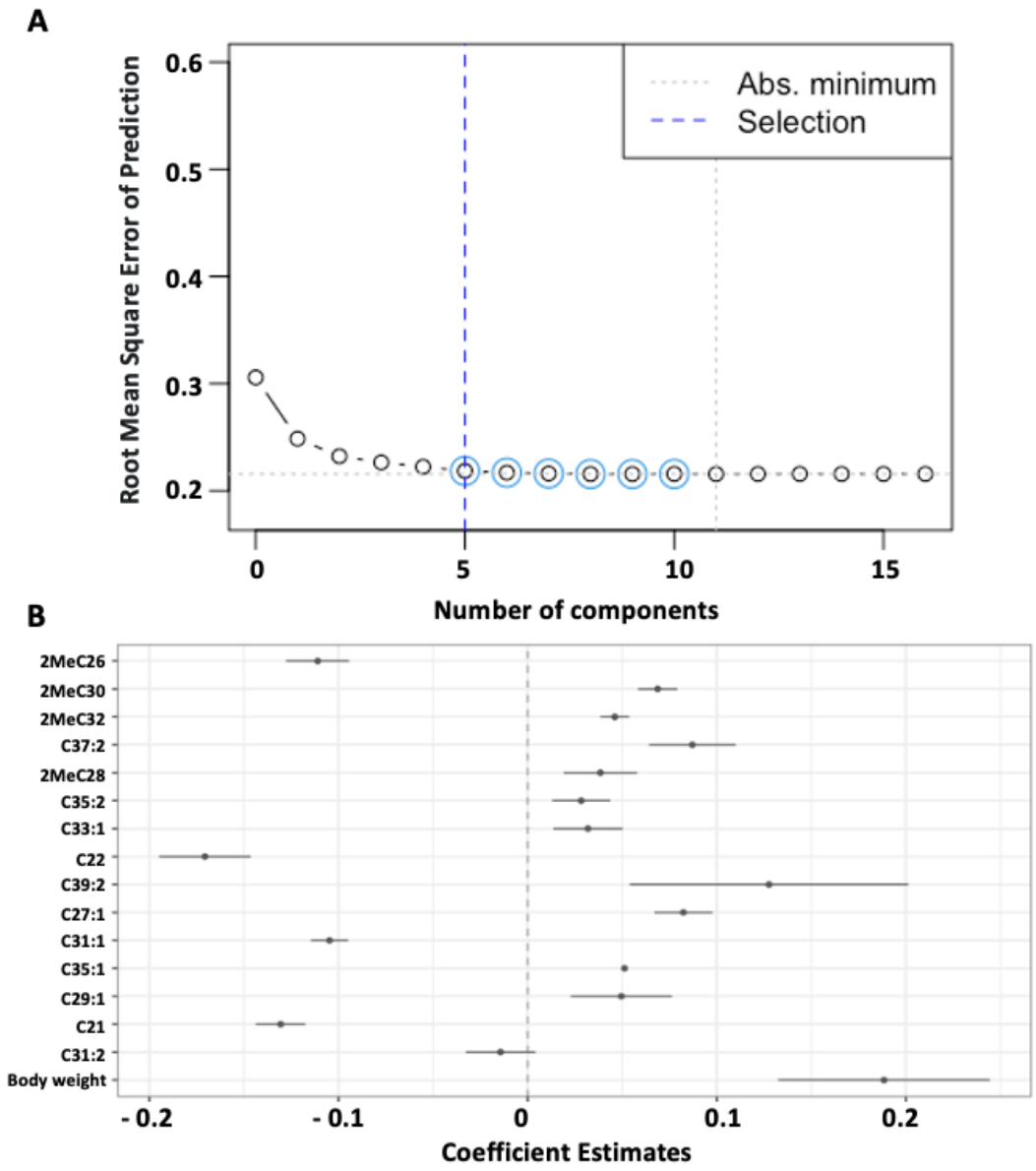


Figure 2.10. Longer mbCHCs predict higher desiccation resistance. Principal component regression analysis was used to determine how the important mbCHCs predict desiccation resistance. A. Ten-fold cross-validation with the use of Root Mean Square Error of Prediction (RMSEP) showed five components are optimal for the prediction. B. Converted coefficients for selected CHCs and body weight from the principal component regression model.

Sexual dimorphism in CHC profiles does not correlate with differences in desiccation resistance between sexes

The ancestral reconstruction suggested that the common ancestor of these 50 species could have intermediate levels of CHC sexual dimorphism (**Figure 2.11A**). This dimorphism between sexes could be further exaggerated in a few species including *D. erecta* and *S. latifasciaeformis*, while multiple species independently could lose this dimorphism such as *D. pseudoobscura*, *D. mojavensis*, *D. serrata*, and species in the virilis group (**Figure 2.11A**). However, there is no phylogenetic constraint in CHC sexual dimorphism (Pagel's λ = 0.18, P-value = 0.47), suggesting it could evolve rapidly between species. To determine if CHC differences between sexes would be the main factor leading to their differences in desiccation resistance, I used generalized linear regression to test the correlation between them. The results showed no correlation between sexual dimorphism in CHCs and desiccation resistance (P = 0.2). This suggests differences in CHCs between sexes do not contribute to their differences in desiccation resistance, and differences in desiccation resistance between sexes may be due to other physiological factors.

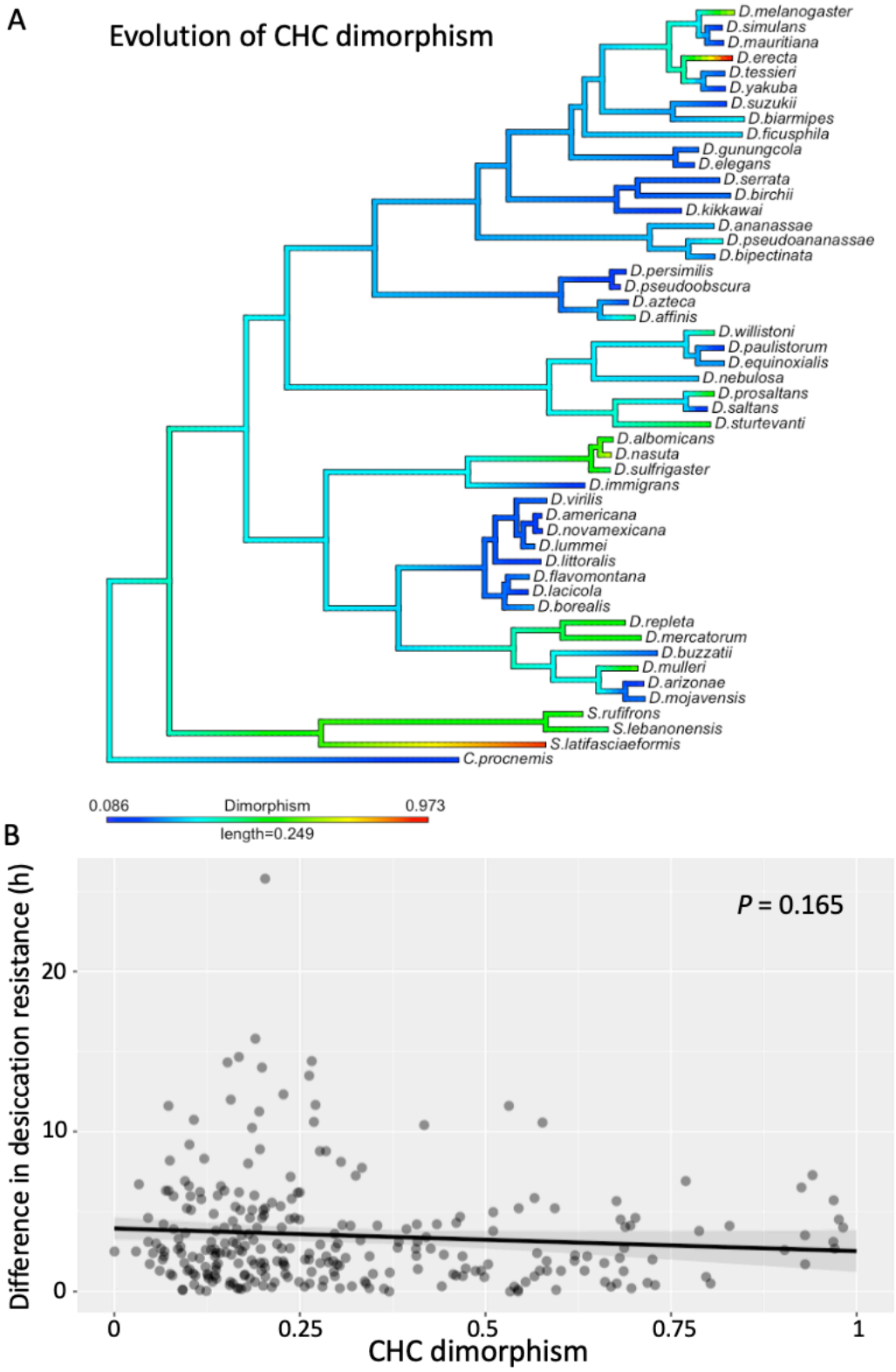


Figure 2.11. Sexual dimorphism in CHCs does not correlate with differences in desiccation resistance between sexes. A. Ancestral state reconstruction of levels of CHC dimorphism across

Figure 2.11 (cont'd)
Drosophila species. B. Regression analysis between levels of CHC dimorphism and difference in desiccation resistance between sexes.

Naked fly assays showed potentially synergistic contribution of mbCHCs and unsaturated CHCs on desiccation resistance

A preliminary trial was conducted to test how the coating of synthetic CHCs affects desiccation resistance. This trial showed single coating of either mbCHC or unsaturated CHC did not lead to increases in desiccation resistance, while coating of the combination of one mbCHC and one unsaturated CHC led to around 20% increase in desiccation resistance (One-Way ANOVA: $F_{(9,151)} = 10.6$, $P < 0.001$; *Post hoc* comparison using Dunnett's method; **Figure 2.12**). This suggests a potentially synergistic effect from mbCHCs and monoenes/dienes. Future experiments with naked fly assays using wider ranges of CHC quantities or CHCs with longer carbon chains can help understand how the quantity and length of CHCs affect desiccation resistance.

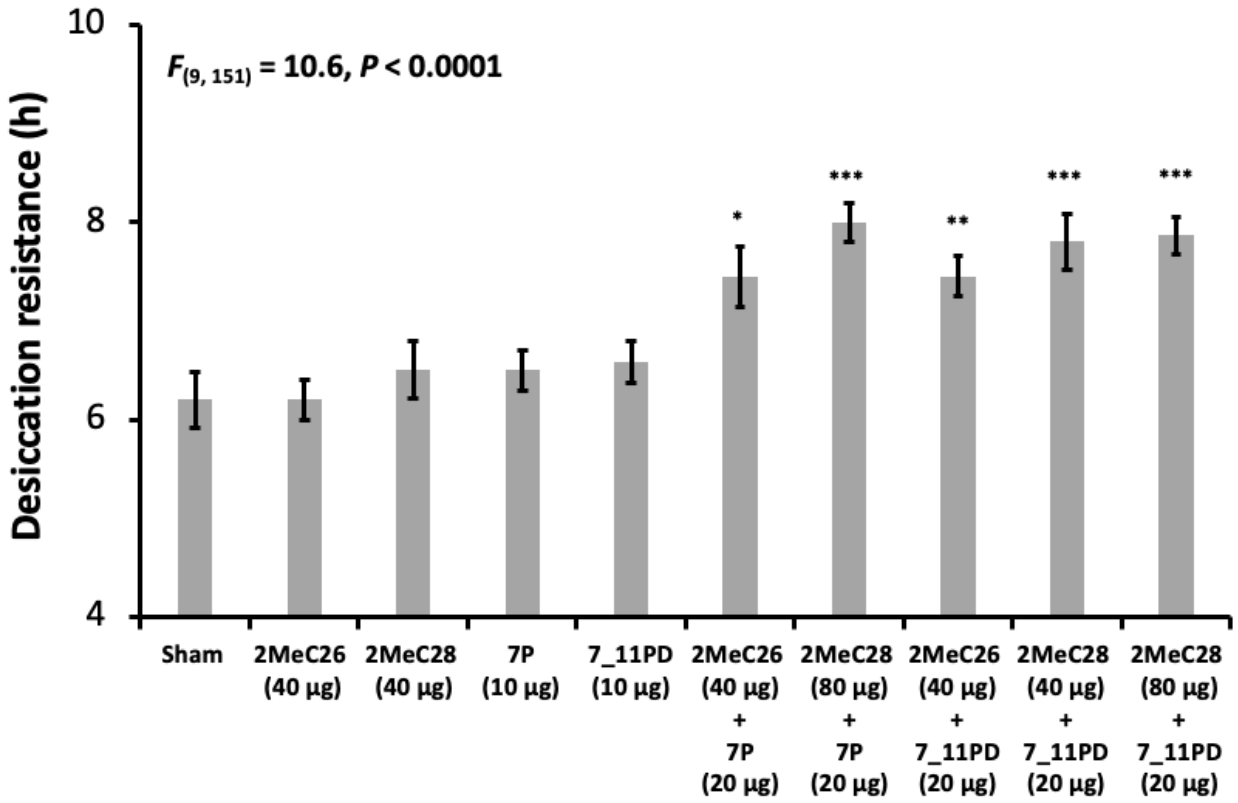


Figure 2.12. Naked fly assay on *D. melanogaster* showed potentially synergistic effects between mbCHCs and unsaturated CHCs on desiccation resistance. One-way ANOVA showed desiccation resistance between these coated flies was not statistically the same ($F_{(9,151)} = 10.6$, $P < 0.001$). *Post-hoc* comparisons were performed using Dunnett's method at *alpha* = 0.05. The treatments labeled the same letters are not significantly different.

Discussion

CHCs are a lipid layer composed of hydrocarbons with different structures and carbon-chain lengths. The dual roles of CHCs in preventing water loss and chemical communication could allow CHC properties to be selected differently. Which part of CHCs is more important to desiccation resistance? How do the changes in CHC composition influence desiccation resistance? In this chapter, I investigated desiccation resistance and CHC composition across 50 *Drosophila* and their sibling species and analyzed correlations between CHC profiles and desiccation resistance. My results showed that mbCHCs are present in most *Drosophila* species

and are important for predicting desiccation resistance. Specifically, longer chain mbCHCs contribute to higher desiccation resistance.

Across species, there was no significant correlation between the total quantity of CHCs and desiccation resistance. However, I found that the lengths of mbCHCs and monoenes are correlated with desiccation resistance. Further analyses showed the importance of longer mbCHCs in conferring higher desiccation resistance. Although mbCHCs do not have the highest melting temperatures (Chung and Carroll, 2015), these CHCs are saturated hydrocarbons and could still allow the CHC layer to maintain a solid phase at higher temperatures. This suggests that when in comparison with increasing abundance, changes in carbon-chain lengths of mbCHCs could be more important to their ability in withstanding the desiccation stress. The synthesis of mbCHCs involved several types of genes (Chung and Carroll, 2015). In *Drosophila* species, mbCHCs are synthesized by a single fatty acyl-CoA synthase distinct from another fatty acyl-CoA synthase which synthesizes the other non-branched CHCs (Chung et al. 2014), but the terminal enzymatic reaction for all CHCs is catalyzed by a single CYP4G decarbonylase gene (Qiu et al. 2012). In some other Dipteran species such as mosquitoes, two distinct CYP4G decarbonylase genes are shown to be involved in the terminal enzymatic reaction of mbCHCs and non-branched CHCs (Kefi et al. 2019). This evidence shows that the synthesis pathway or involved enzymes for mbCHC production are independent of the production of linear CHCs. The requirement of a different set of enzymes for the syntheses of mbCHCs suggests a high importance to this type of CHCs.

Why are alkanes, the type of hydrocarbons with the highest melting temperature and potentially the highest contribution to desiccation resistance, not prevalent in these species? A hypothesis that could explain this is the conflict between the syntheses of unsaturated linear hydrocarbons and alkanes. Their syntheses share the same enzymes in the initial steps in the pathway and the same precursors, so increases in one component would reduce the other one. Unsaturated hydrocarbons function as contact pheromones in regulating different behaviors such as mating and aggression. In females and males of *D. melanogaster,* monoenes and dienes are used for these two types of behaviors (Chertemps et al. 2007, Krupp et al. 2008). These behaviors could be as important as the ability to prevent water loss. Therefore, the use of linear unsaturated hydrocarbons could conflict with the use of linear alkane to prevent water loss. Alternatively, the use of mbCHCs may be able to avoid this conflict. It would be difficult to test this hypothesis. One method to test the correlation between this pattern could be from the survey of CHCs in insect species distributed from Hexapoda and determine the ancestral state of CHC compositions, as well as their evolutionary history.

Although mbCHCs have been found important in desiccation resistance, some correlation has been found between unsaturated CHCs and desiccation resistance. The lower melting temperatures in unsaturated CHCs allow them to be volatile and then be important in chemical communication (Chung and Carroll, 2015). The naked fly assay in this study showed the combination of mbCHCs and unsaturated hydrocarbons could have higher efficiency in preventing water loss, compared with mbCHCs alone. The naked fly assay is novel because it only alters gene expression in oenocytes without affecting the other physiological processes. This suggests the need for both saturated and unsaturated CHCs to potentially form an

effective layer for desiccation resistance. In this experiment, I only used mbCHCs with 26 and 28 carbons and this length difference may not be large enough to observe effects from different lengths. Future experiments that incorporate longer mbCHCs and unsaturated CHCs can functionally test different hypotheses regarding how different CHCs could contribute to insects' desiccation resistance, as well as their functions in chemical communication.

In this chapter, I showed that mbCHCs are the most important CHCs in desiccation resistance and longer chain mbCHCs are associated with higher desiccation resistance. It may need both mbCHCs and unsaturated CHCs for better preventing water loss and withstanding desiccation stress. In addition, the CHC data generated in this study can provide important resources for future research regarding how the evolution of CHCs underlies chemical communication in insect species.

Methods and Materials

Drosophila species

In this study, 46 *Drosophila* species, as well as three *Scaptodrosophila* species and one *Chymomyza* species as the outgroup were either ordered from the National Drosophila Species Stock Center (drosophilaspecies.com; NDSSC) or gifted from the Wittkopp Lab at the University of Michigan. Details are listed in **Table 2.1**. All species have been reared on standard cornmeal medium (DSSC Cornmeal).

Table 2.1. List of species used in this study

Genus	Species	Sources and strain code from NDSSC
Drosophila	*D. mojavensis*	15081-1352.10
Drosophila	*D. arizonae*	15081-1271.41
Drosophila	*D. aldrichi*	15081-1251.23
Drosophila	*D. mulleri*	15081-1371.01
Drosophila	*D. buzzatii*	15081-1291.63
Drosophila	*D. mercatorum*	15082-1521.38
Drosophila	*D. repleta*	15084-1611.13
Drosophila	*D. americana*	15010-0951.00
Drosophila	*D. novamexicana*	15010-1031.14
Drosophila	*D. lummei*	15010-1011.01
Drosophila	*D. virilis*	15010-1051.87
Drosophila	*D. littoralis*	15010-1001.11
Drosophila	*D. lacicola*	15010-0991.13
Drosophila	*D. borealis*	15010-0961.00
Drosophila	*D. montana*	15010-1021.23
Drosophila	*D. flavomontana*	15010-0981.00
Drosophila	*D. nasuta*	15112-1781.00
Drosophila	*D. albomicans*	15112-1751.00
Drosophila	*D. sulfurigaster*	15112-1811.04
Drosophila	*D. immigrans*	15111-1731.03
Drosophila	*D. equinoxialis*	14030-0741.00
Drosophila	*D. paulistorum*	14030-0771.11
Drosophila	*D. willistoni*	14030-0811.24
Drosophila	*D. nebulosa*	14030-0761.06
Drosophila	*D. prosaltans*	14045-0901.07
Drosophila	*D. saltans*	14045-0911.01
Drosophila	*D. sturtevanti*	14043-0871.16
Drosophila	*D. azteca*	14012-0171.03
Drosophila	*D. affinis*	14012-0141.02
Drosophila	*D. persimilis*	14011-0111.46
Drosophila	*D. pseudoobscura*	14011-0121.94
Drosophila	*D. bipectinata*	14024-0381.21
Drosophila	*D. ananassae*	14024- 0371.13
Drosophila	*D. serrata*	14028-0681.00
Drosophila	*D. kikkawai*	14028-0561.14
Drosophila	*D. birchii*	14028-0521.00

Table 2.1 (cont'd)

Drosophila	*D. elegans*	Gifted from the Wittkopp Lab
Drosophila	*D. gunungcola*	Gifted from the Wittkopp Lab
Drosophila	*D. biarmipes*	14023-0361.09
Drosophila	*D. suzukii*	Gifted from Rufus Lab
Drosophila	*D. erecta*	14021-0224.01
Drosophila	*D. tessieri*	14021-0257.01
Drosophila	*D. yakuba*	14021-0261-01
Drosophila	*D. mauritiana*	14021-0241.151
Drosophila	*D. simulans*	W501 (14021-0251.195)
Drosophila	*D. melanogaster*	Canton-S (Sean B. Carroll Lab)
Scaptodrosophila	*S. latifasciaeformis*	11030-0061.01
Scaptodrosophila	*S.lebanonensis*	11010-0011.00
Scaptodrosophila	*S.rufifrons*	11040-0071.00
Chymomyza	*C. procnemis*	20000-2631.01

Experimental Design

To investigate the contribution of cuticular hydrocarbons to desiccation resistance, a cohort-based design was used so that all variables were measured at the cohort level. For each species, five to six cohorts (replicates) were established and three measurements were conducted for each sex of the F1 progeny, including desiccation resistance, cuticular hydrocarbons, and body weight (**Figure 2.13**). Each cohort in each species was established by pooling five females and five males on standard cornmeal medium in environmental chambers at 25°C and 12L:12D photoperiod. To maximize the food and spatial availability and minimize the competition between the progenies, the flies of each cohort were transferred to fresh food every 5 days. The flies from F1 progeny were collected daily once they emerged from pupae, separated by sex, and maintained on the fresh cornmeal medium. All flies used for the measurements were four- to five-day-old.

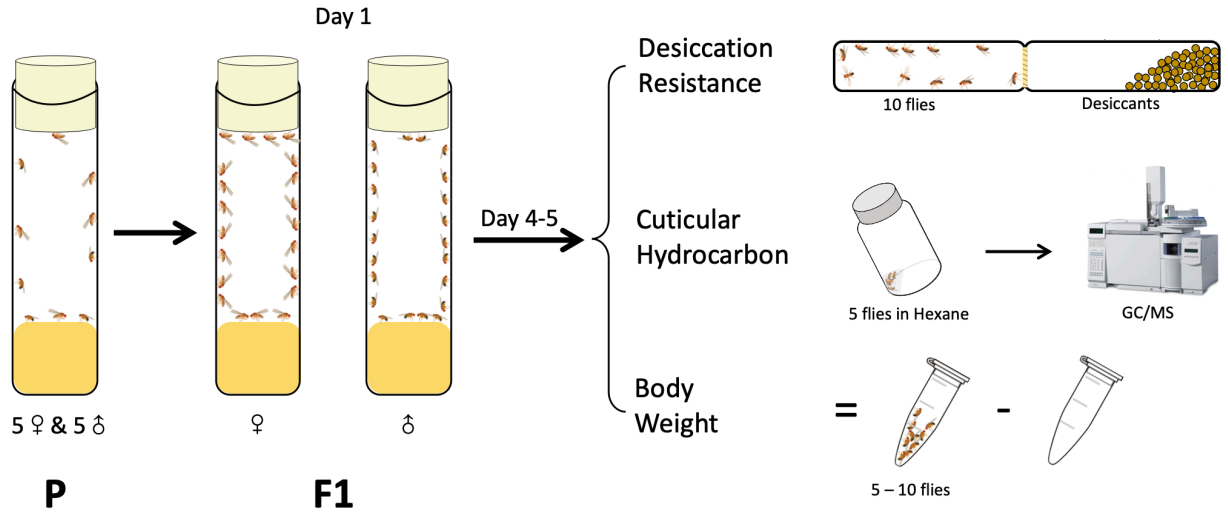


Figure 2.13. The diagram for the cohort-based experimental design. To establish each cohort, five pairs of females and males were reared on standard cornmeal medium. Three variables including the desiccation resistance, cuticular hydrocarbon, and body weight were measured for both sexes of the F1 progeny at four- to five-day-old.

Bioassays for desiccation resistance were performed consistent with previously published protocol (Figure 1) (Chung et al. 2014). Briefly, ten adults of the same sex from each cohort were placed in a vial sealed to another vial containing 10 g silica gel (#S7500-1KG; Sigma-Aldrich, St. Louis, MO). The experiments were conducted in the same environmental chambers at 25°C and 12L:12D photoperiod. Flies were scored hourly after 2 hours and dead flies were counted which was determined by leg movement. For each cohort, one vial was scored and the averaged time in hours until all flies died was recorded as the “desiccation resistance”.

Cuticular hydrocarbons (CHCs) were extracted and analyzed following previously published protocol (Figure 1) (Lamb et al. 2020). Briefly, five males and five females (four- to five-day-old) from each cohort were respectively soaked for 10 mins in 200 µl hexane containing hexacosane (C26; 25 ng/ul) as internal standard. Extracts were analyzed by GC/MS (7890A, Agilent Technologies Inc., Santa Clara, CA) equipped with a DB-17ht column (30 m by

0.25 mm (i.d.) with a 0.15 µm film thickness; Agilent Technologies Inc., Santa Clara, 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) with peak areas integrated 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: 100°C for 4 min, 3°C/min to 325°C.

To identify the CHC samples' chemical composition, we compared their retention time to the mass spectrum of an authentic standard mixture (C6-C40) (Supelco® 49452-U, Sigma-Aldrich, St. Louis, MO) (**Appendix I**). Methyl-branched alkanes, alkenes, and dienes 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. Except for these four mentioned types of hydrocarbons, trienes have been found only in *D. americana* and *D. novamexicana* in low amounts, so trienes were not used for further analyses. Individual CHC peak was quantified using its comparison with the peak area of the internal standard and presented as nanogram per fly (ng/fly).

Bodyweight was also incorporated in the data collection and analysis because it is positively correlated to body water content and desiccation resistance (Gibbs and Matzkin 2001). Bodyweight was determined as the difference between the Eppendorf tube containing five to ten alive flies and the same empty Eppendorf tube. Bodyweight was expressed in micrograms for each cohort.

Sexual dimorphism

CHC dimorphism has been observed in several species such as *D. melanogaster*, *D. erecta*, and *D. nasuta*, due to the presence and/or absence of certain types of CHCs, different lengths of CHCs of the same types, or different quantities of the same CHCs. To determine if CHC dimorphism contributes to differences in desiccation resistance between sexes, I tested the correlation between these two variables. I used the Bray-Curtis dissimilarity analysis to calculate the extent of dimorphism between sexes for all surveyed species. I then mapped the evolution of CHC sexual dimorphism across *Drosophila* species and tested its phylogenetic signals using Pagel's λ using *phylosig* from the r package 'phytools'. Results can lead to understanding whether a phylogenetic constraint in CHC sexual dimorphism should be taken into consideration during the correlation analysis. As CHC dimorphism was found not constraint by the phylogeny, I used generalized linear regression to test the correlation between dimorphism and desiccation resistance.

Naked fly assays

To generate flies without CHCs, 5'*mFas-Gal4* (Chung et al. 2014) and *UAS-Cyp4g1.RNAi* lines were crossed reciprocally and females of the F1 offspring were collected for CHC coating and further desiccation assays. This assay is able to test the contribution from a single CHC or combination of any CHCs to desiccation resistance. Synthetic mbCHCs, 2MeC26 and 2MeC28, were obtained from Dr. Jocelyn Millar (University of California - Riverside). Synthetic unsaturated CHCs, 7-C25:1 (referred to as 7P), and 7,11-C25:2 (referred to as 7_11PD), were from Sigma-Aldrich. To coat CHCs on flies, 2-mL glass vials containing CHCs dissolved in hexane were first evaporated under nitrogen. Ten flies of the same sex were then transferred into each

vial, following shaking for 20 seconds on, 20 seconds off, and 20 seconds on. Since preliminary trials showed the uses of 40 µg 2MeC26, 80 µg 2MeC28, 10 µg 7P, and 10 µg 7_11PD can lead to similar amounts of coating, in this experiment, I coated each of these CHCs on the flies, as well as combinations of two CHCs. As this experiment was limited by the number of trials and fly availability, treatments were set up with different quantities. This resulted in the consequence that desiccation resistance obtained between treatments was not comparable. Therefore, desiccation resistance of coated flies was only compared with the control, 'Sham'. To do this, one-way ANOVA with Dunnett's test was used to determine if these flies have different desiccation resistance from the control.

Statistics

All analyses were conducted in R (Version 4.0). Linear regression analyses were conducted using 'glm' function. Random forest discrimination analysis and random forest regression analysis were performed using the 'ranger' and 'randomForest' packages (Liaw and Wiener 2002, Wright and Ziegler 2015). Regression models were built using both Out Of Bag (OOB) error and test/training sets (70:30 split). To determine how useful each CHC variable in the prediction of desiccation resistance, the importance of top predictor CHCs was quantified using permutation importance (Altmann et al. 2010). To understand how CHCs are associated with desiccation resistance, I performed several analyses to investigate the contribution of different CHC variables such as the total quantity and lengths of the major peak in each type of CHCs. Principal component regression analysis was conducted using 'factoextra' and 'pls' packages (Wehrens and Mevik 2007).

CHAPTER 3. GENETIC MECHANISMS UNDERLYING DESICCATION RESISTANCE

In this chapter, I sought to elucidate genetic mechanisms underlying the elongation of mbCHCs and desiccation resistance in *Drosophila* species. Supporting results and data were listed in Appendix II. I would like to acknowledge the contribution from several colleagues which are listed below.

Dr. Jian Pu

- Helped with desiccation assays
- Helped with *Drosophila* microinjection

Eliana Giannetti

- Helped genotype *D. mojavensis GI20347*-knockout flies during the backcross

Haosu Cong

- Helped score fly mortalities in desiccation assays

Introduction

Adaptation to various and extreme environments is key to long-term species persistence. Among the diverse environments on Earth, one of the key features that shape the phenotypic evolution of species is the wide range of different abiotic factors such as temperature and humidity (Fei et al. 2017, Dillon and Lozier 2019, Rezende et al. 2020). Studies have uncovered various mechanisms underlying organismal adaption to extreme levels of abiotic factors. To survive from the extreme cold in the Antarctic regions, the Antarctic fish has evolved a special type of glycoproteins in their blood system to avoid being frozen (Devries

1971, Cheng and Chen 1999), while the Antarctic midge can enter an anhydrous status to survive from being frozen (Kawarasaki et al. 2014, Thorat and Nath 2018). Another example is that desert insects such as the cactophilic fruit flies, the desert harvester ants, and the desert tenebrionid beetles have evolved controllable spiracles coupling with reduced metabolic rates in their physiology to minimize water loss from respiration in extremely arid deserts (Ahearn 1970, Gibbs 2002b, Johnson and Gibbs 2004). Although some of these special adaptations can repeatedly evolve in distantly-related species across the phylogeny (Chen et al. 1997, Chown 2002), these adaptations usually require multiple changes in complicated physiological and behavioral processes, sometimes along with compensations from other physiological changes (Tobler and Plath 2011, González-Tokman et al. 2020). This makes the evolutionary specialties less applicable to many other species in ambient environments, especially when the environments are also changing rapidly.

As environments are getting warmer and more arid due to climate change, organisms must adapt to these changes to survive and thrive in these new conditions. Comparative studies have shown that across species, their capabilities of adapting to the abiotic factors in diverse environments are usually continuous and changing gradually (Kellermann et al. 2012, Bujan et al. 2016). This suggests the potential of common mechanisms that could account for the gradual changes in their capabilities of withstanding stresses from varied levels of abiotic factors. However, although studies reported the association between polygenic evolution and adaptations to changing environments (Barghi et al. 2019, Kellermann and van Heerwaarden 2019, Dowle et al. 2020), the physiological and genetic mechanisms that modulate organismal adaptations to varied levels of abiotic factors in diverse environments are less elucidated.

Previous studies suggest that ecological selection, leading to parallel or convergent evolution between different species, could result from evolutionary changes in the same gene (Zhen et al. 2012) or even from the same mutation in a single gene (French-Constant 1994), leading to the hypothesis that the molecular changes underlying adaptation are predictable (Losos 2011, Stern 2013). Therefore, investigating the genes and mechanisms underlying high desiccation in species living in arid habitats may allow us to determine how species may evolve in response to environmental change (Hoffmann and Sgrò 2011, Schindler and Hilborn 2015).

Insects, which form the bulk of biodiversity on Earth, are integral to our ecosystem. Their high surface-area-to-volume ratio accelerates water loss from the cuticle (Kühsel et al. 2017), making them vulnerable to desiccation, especially when temperatures increase and humidity levels decrease. However, many insect species have adapted to and occupied habitats with the humidity ranging from relatively low levels such as mountains and grasslands to extremely low levels such as deserts (Gibbs 2002b, Wang et al. 2021). To withstand desiccation stress, insects use a waxy layer on the cuticle, named cuticular hydrocarbons (CHCs), as one of the major strategies to prevent water loss (Wigglesworth 1933, Billeter et al. 2009, Qiu et al. 2012a). This cuticular layer is mainly made up of hydrocarbons synthesized in specialized cells underneath the cuticle called oenocytes (Lockey 1988, Billeter et al. 2009, Makki et al. 2014). In *Drosophila* and most other insects, these CHCs, with chain lengths ranging from approximately 21 to 50 carbons, consist of linear alkanes, alkenes (monoenes and dienes), and methyl-branched alkanes (mbCHCs) (Howard and Blomquist 2005). The varying ability of this CHC layer to prevent water loss depends on its chemical composition, which in turn determines its melting temperature (Gibbs and Pomonis 1995, Gibbs 2002a). The melting temperature is the

temperature at which the hydrocarbon melts, a physical property positively correlated with the water-proofing ability (Gibbs et al. 1997). Linear alkanes have the highest melting temperature, followed by methyl-branched alkanes (mbCHCs), monoenes, and dienes (Chung and Carroll 2015). This is due to branching and unsaturation lowering the melting temperature of the hydrocarbon (Gibbs and Pomonis 1995). Increasing CHC chain length increases melting temperature and can potentially lead to higher desiccation resistance. However, only a few studies showed the association between CHC chain-length and insects' desiccation resistance. For example, the desert drosophilid, *D. mojavensis*, produces a higher than average proportion of longer-chain CHCs (Jallon and David 1987, Etges and Jackson 2001), and is much more desiccation-resistant than other *Drosophila* species. Furthermore, in two independent laboratory selection experiments, *D. melanogaster* flies selected for desiccation resistance evolved longer-chain CHCs than control flies (Gibbs et al. 1997, Kwan and Rundle 2010). Taken together, this information shows that longer chain CHCs, especially mbCHCs, are more important for insects developing desiccation resistance. This is consistent with findings in Chapter I. However, the underlying genetic and evolutionary mechanisms are less clear.

In this chapter, I used a desert-dwelling insect species, *D. mojavensis*, as a model to investigate the genetic and evolutionary mechanisms underlying its highest desiccation resistance and longest mbCHCs. For *Drosophila* species possessing mbCHCs in their CHC layer, there is a length variation ranging from 24 carbons (2MeC24) to 32 carbons (2MeC32) (**Figure 3.1**). *Drosophila mojavensis* is a cactophilic species dwelling in desert habitats such as the Mojave Desert in the U.S. Among all tested *Drosophila* species, *D. mojavensis* is the species with the highest desiccation resistance (Matzkin et al. 2007, Kellermann et al. 2012). The mbCHCs in

D. mojavensis is also the longest with 2MeC30 being the major peak (**Figure 3.1**). Starting from investigations in the model species, *D. melanogaster*, I found a fatty acyl-CoA elongase gene, *CG18609*, which I named it *mElo*, that can elongate mbCHCs. I overexpressed the homologous elongase genes in the *D. mojavensis mElo* cluster and located the *mElo* ortholog, of which overexpression in *D. melanogaster* leads to longer-chained mbCHCs and higher desiccation resistance. To further characterize the function of this gene, I knocked out *Dmoj/mElo* from *D. mojavensis* using CRISPR-Cas9 and showed that this gene is responsible for the elongation of longer mbCHCs in this desert species. Desiccation assay on the knockout strains showed reduced desiccation resistance at its ecologically relevant temperature. Furthermore, I investigated the evolution of *mElo* cluster and *mElo* orthologs in other *Drosophila* species. These results suggest complicated evolutionary history in this gene cluster and coding changes in *mElo* may contribute to the variations in different levels of desiccation resistance in *Drosophila* species.

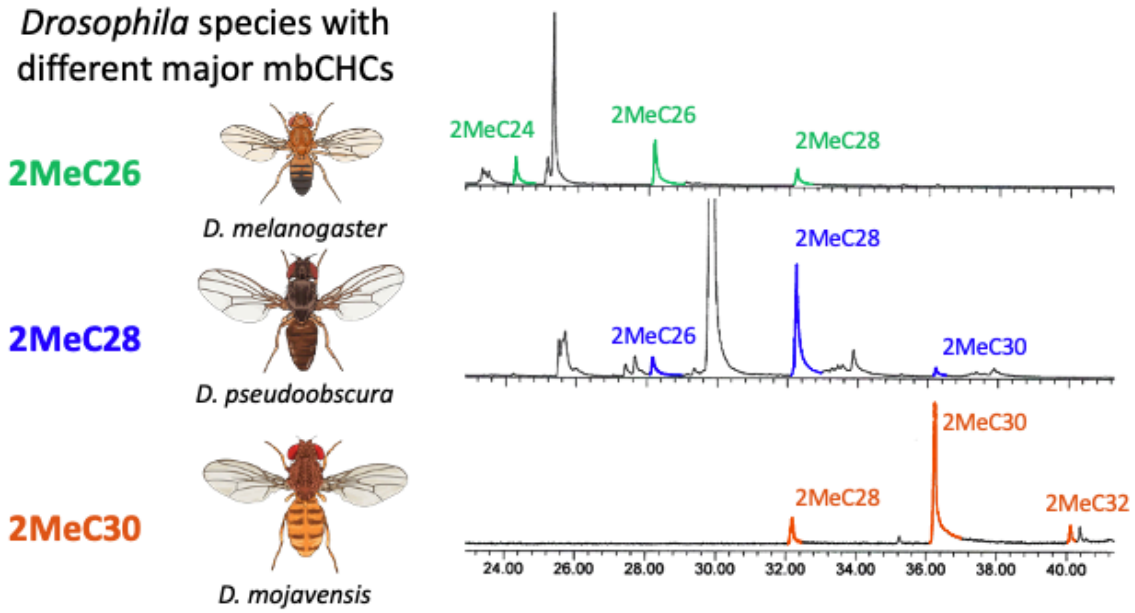


Figure 3.1. Chromatograms of mbCHCs for three *Drosophila* species, *D. melanogaster*, *D. pseudoobscura*, and *D. mojavensis*, of which their major mbCHCs have different lengths.

Results

The ancestor of Drosophila species could have medium lengths of mbCHCs

To understand how the trait of mbCHC lengths evolves in *Drosophila* species, I reconstructed the ancestral state of their major mbCHCs using the Maximum Likelihood method with the results from Chapter I. The results showed the ancestor of *Drosophila*, *Scaptodrosophila*, and *Chymomyza* genera had a 99% likelihood that the major mbCHC is 2MeC28 (**Figure 3.2**). In several clades such as ananassae, willistoni, and repleta group, their major mbCHCs could independently evolve from 2MeC28 to 2MeC30, while the lengths of the major mbCHCs in melanogaster group could be reduced to 26 carbons (**Figure 3.2**). I further aimed to investigate the molecular mechanisms underlying the evolution of mbCHC lengths in *Drosophila* species.

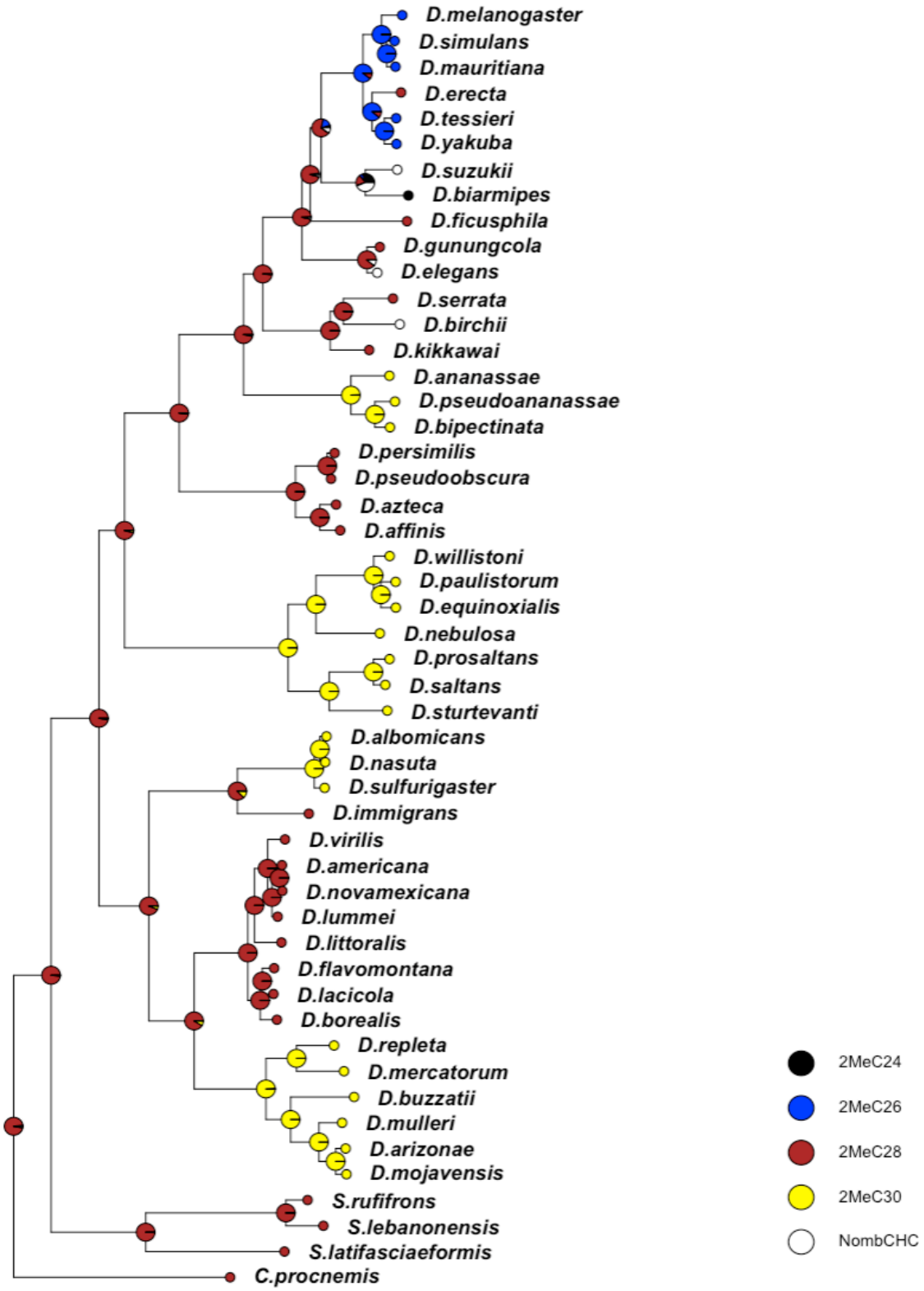


Figure 3.2. Ancestral state reconstruction for the lengths of major mbCHCs in the surveyed 50 species in Chapter 1.

mElo (CG18609) is an elongase for methyl-branched CHCs in D. melanogaster

Previous study showed a fatty acyl-CoA elongase gene, *CG18609*, is associated with the elongation of mbCHCs (Dembeck et al. 2015). To characterize the function of *CG18609*, *in situ* hybridization was first used to determine its expression patterns, following with an oenocyte-specific RNAi knockdown. The results showed *CG18609* is expressed in oenocytes of both male and female adults (**Fig. 3.3A**). Knockdown of *CG18609* in oenocytes almost eliminates the production of 2MeC28 in *D. melanogaster* (Female: $t_{(18)} = 17.1$, $P < 0.001$; Male: $t_{(17)} = 13.1$, $P < 0.001$) and reduces the production of 2MeC26 in males (Female: $P = 0.6$; Male: $t_{(17)} = 5.6$, $P < 0.001$), while the levels of 2MeC24 were increased by around eight times for both sexes (Female: $t_{(18)} = 10.0$, $P < 0.001$; Male: $t_{(17)} = 11.8$, $P < 0.001$) (**Fig. 3.3B-C**), suggesting that *CG18609* be involved in the elongation of 2MeC24 to 2MeC26 and 2MeC28.

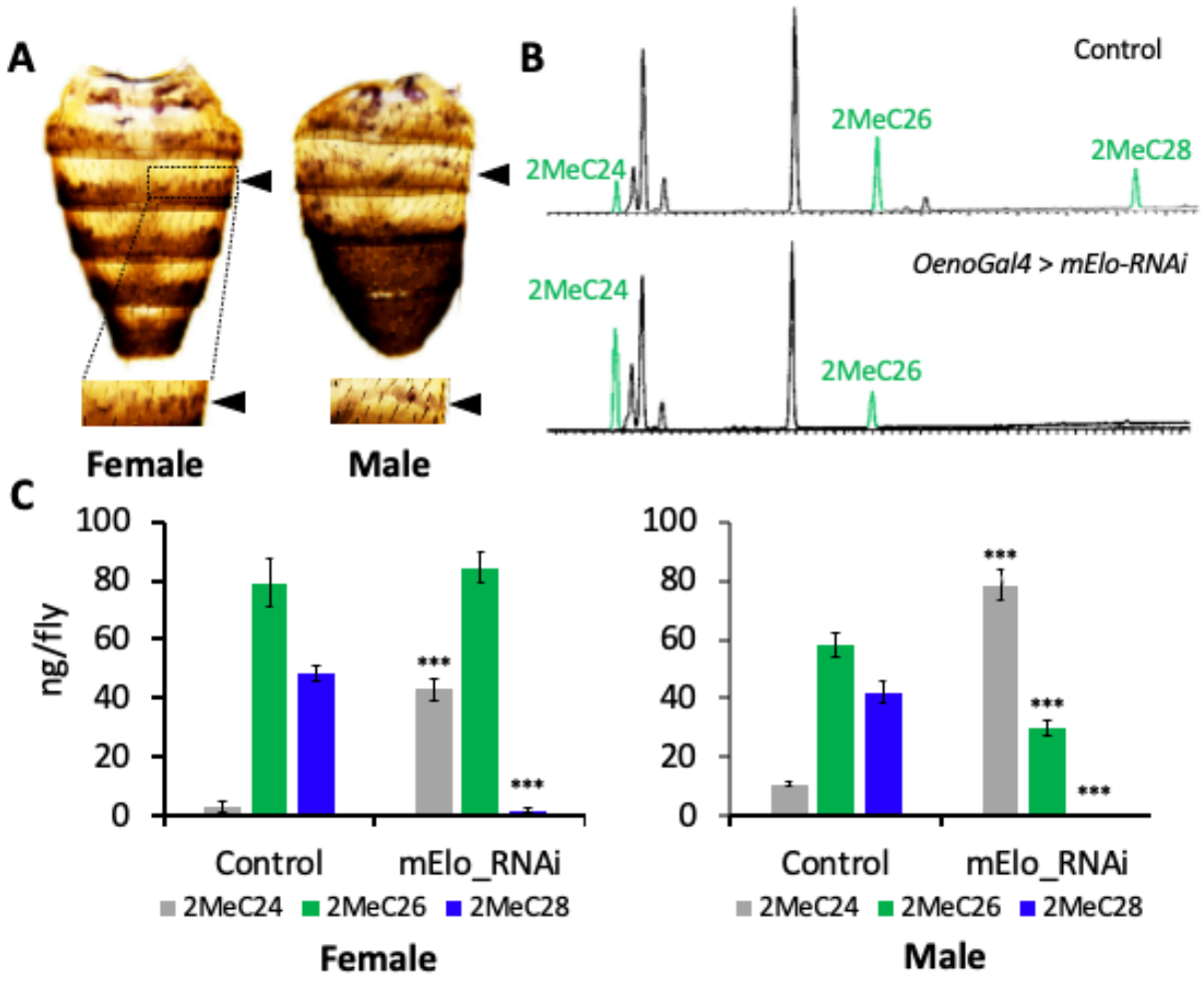


Figure 3.3. *CG18609* is involved in mbCHC elongation in *D. melanogaster*. A. *In situ* hybridization of *CG18609* in adult oenocytes of *D. melanogaster* showed this gene is expressed in adult oenocytes. B. Chromatogram of mbCHCs in male *D. melanogaster* with oenocyte-specific RNAi knockdown of *CG18609*. C. Amounts of mbCHCs in *D. melanogaster* with oenocyte-specific RNAi knockdown of *CG18609*. The oenocyte-specific RNAi knockdown was performed by crossing the RNAi line of *CG18609* with the oenocyte-specific GAL4 line (*Oeno*-Gal4). The student's *t*-test was used to compare the difference of each of mbCHCs between the knockdown treatment and control at *alpha*=0.05.

I further characterized the role of *CG18609* in *D. melanogaster* by knocking out this gene using the CRISPR-Cas9 technique and examining its effects on mbCHCs. Before the knockout experiment, I first performed a ubiquitous knockdown of *CG18609* using the ubiquitous Gal4 line (y[1] w[*]; P{w[+mC]=tubP-GAL4}LL7/TM3, Sb[1] Ser[1]; BSDC #5138) to determine whether the knockout can lead to viable phenotype. The ubiquitous Gal4 driver locates at the

third chromosome, which is over a balancer that generates a stubby phenotype. In the F1 generation, the count of the wild type (which has *CG18609* ubiquitously knocked down) and the stubby phenotype (which does not have any knockdown effects) can be compared to determine if the ubiquitous knockdown of *CG18609* could lead to a phenotype with lower fitness.

The lengths of mbCHCs in the ubiquitous knockdown of *CG18609* showed reduced 2MeC28 and increased 2MeC24, which is consistent with those in oenocyte-specific knockdown of *CG18609* (**Figure 3.4A; Table A2.2**). In addition, the numbers of wild-type and stubby flies in the offspring of the ubiquitous knockdown are the same (Chi-square test; P = 1.0) **(Figure 3.4B)**, suggesting the feasibility of knocking out *CG18609* from *D. melanogaster*. I then used CRISPR-Cas9 and homologous recombination to knock out *CG18609* from *D. melanogaster* with dsRED and *attP* sequences inserted (**Figure 3.4C**). In this experiment, the successful knockout can be screened by the presence of red fluorescence (as shown in the pupa in **Figure 3.4D**). I generated two independent strains with *CG18609* knocked out that had the same mbCHC phenotype as the *CG18609*-knockdown strains (**Figure3.4E; Table A2.3**). Results in these characterized the function of *CG18609* and demonstrated that this gene is the elongase gene in *D. melanogaster* for the elongation of mbCHCs. I named this gene *mElo* (which stands for ***m****bCHC* ***Elo****ngase*).

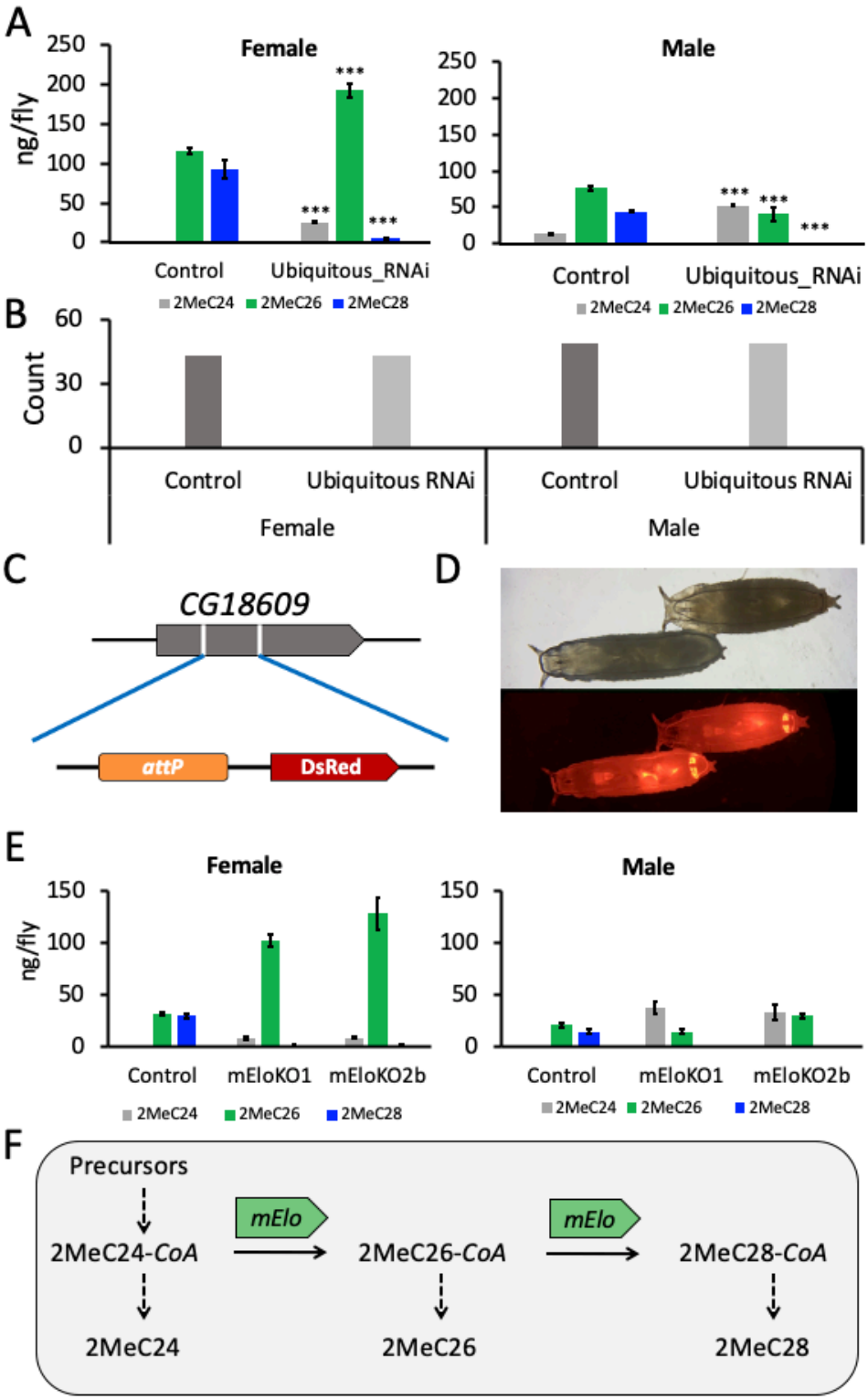


Figure 3.4. *CG18609* (which I named *mElo*) is an elongase for methyl-branched CHCs in *D. melanogaster*. A. Amounts of mbCHCs in *D. melanogaster* with ubiquitous knockdown of *mElo*.

Figure 3.4 (cont'd)
The ubiquitous knockdown was performed by crossing the RNAi line of *mElo* with the ubiquitous GAL4 line (*Tub-Gal4;3rd Chr/Stub # 5138*). The student's *t*-test was used to compare the difference for each of mbCHCs between the knockdown treatment and control at *alpha*=0.05. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. B. Numbers of the F1 offspring from ubiquitous knockdown of *mElo* with and without Tubby phenotype (resulted from the balancer chromosome from the ubiquitous GAL4 line). The *Chi*-square test was performed to determine the difference between the two phenotypes at *alpha* = 0.05 and no significant difference was detected. C. The scheme of CRISPR-Cas9 knockout with homologous recombination in *mElo* in *D. melanogaster*. D. Example of red fluorescence in the pupa of a CRISPR-Cas9 knockout strain. E. Amounts of mbCHCs in two strains of *D. melanogaster* with *mElo* knocked out using CRISPR-Cas9 system. F. The model regarding the function of *mElo* in mbCHC synthesis in *D. melanogaster*.

Gene expansion occurred in D. mojavensis mElo locus for CHC elongation

To search for the candidate elongase genes for mbCHC elongation in *D. mojavensis*, I identified *mElo* locus in the genome of *D. mojavensis* by anchoring conserved syntenic genes. In *D. melanogaster mElo* locus, I found another elongase gene, *CG17821*, while in *D. mojavensis mElo* locus, there are four elongase genes (*GI20343, GI20344, GI20345,* and *GI20347*) **(Fig. 3.5A**). *In situ* hybridization for these genes showed that *CG17821* was not expressed in adult oenocytes of *D. melanogaster* **(Fig. 3.5B**), while in both female and male *D. mojavensis,* three of the four elongase genes were expressed in adult oenocytes. I constructed a phylogenetic tree with the Maximum Likelihood method on the GTR model using the coding sequences to determine the phylogenetic relationship of the six elongase genes in *D. melanogaster* and *D. mojavensis mElo* loci. The results showed that *GI20343*, *GI20344*, and *GI20345* are likely to be the homologs of *CG17821* (**Fig. 3.5C**), suggesting a potential gene duplication event from *CG17821*. Only *GI20347* is homologous to *mElo* (**Fig. 3.5C**). I hypothesized that *GI20347* could be the ortholog of *mElo* in *D. mojavensis*.

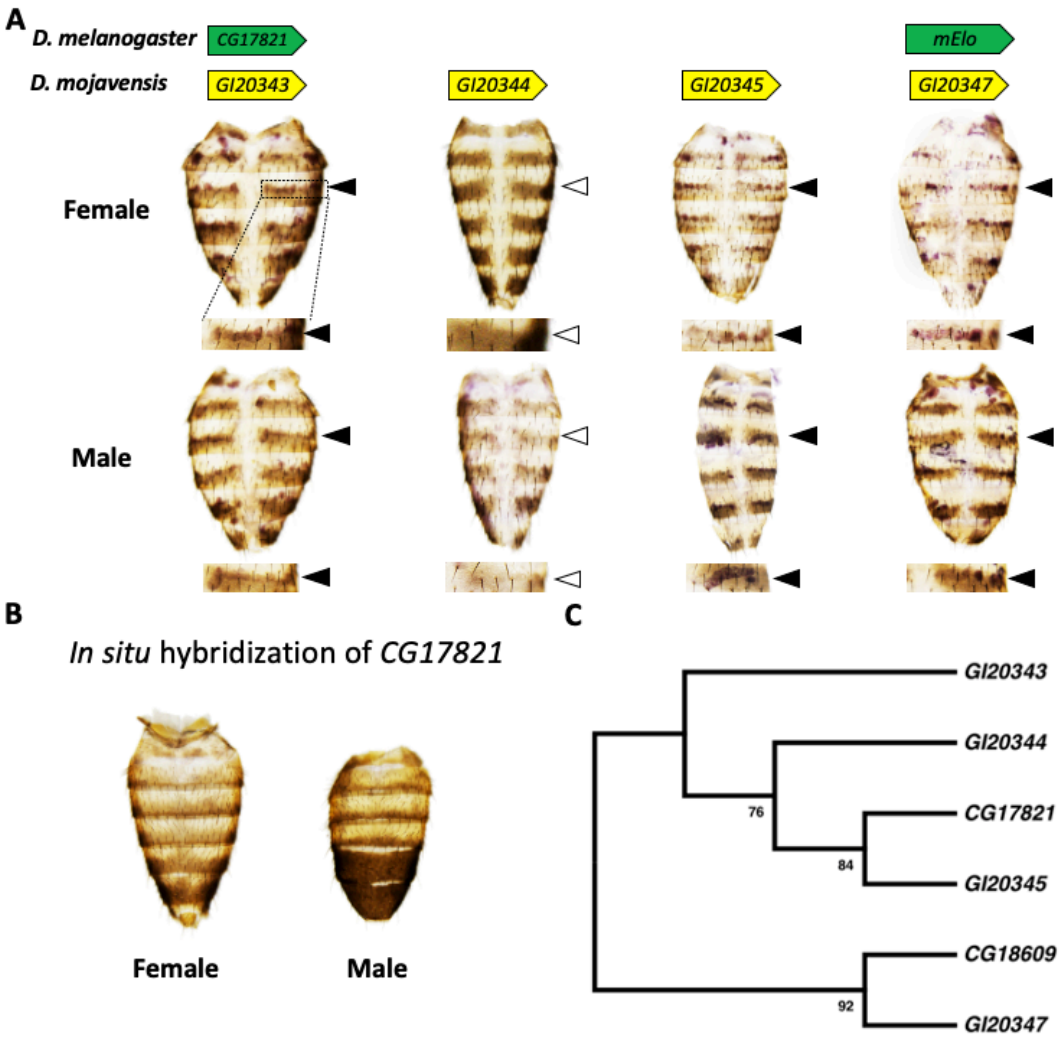


Figure 3.5. Gene expansion of elongase genes in *D. mojavensis mElo* locus. (A) *In situ* hybridization results of the four genes in *D. mojavensis mElo* locus in adult oenocytes showed only three of them have oenocyte expression; (B) *In situ* hybridization results of *CG17821* in adult oenocytes showed only this gene does not express in adult oenocyte; (C) Phylogenetic analysis of the elongase genes in *mElo* loci of *D. melanogaster* and *D. mojavensis* suggests *GI20347* is the potential ortholog of mElo and the gene duplication event could come from *CG17821*. The phylogenetic analysis used the Maximum Likelihood method with GTR model and 1000 bootstraps.

Overexpression of GI20347 in D. melanogaster leads to longer mbCHC and higher desiccation resistance.

As gene duplication is an important evolutionary process leading to novel function (Kondrashov 2012, Magadum et al. 2013), I hypothesized that at least one gene in this cluster

contributes to the production of 2MeC30 in *D. mojavensis*. To test this hypothesis, I made UAS lines of *D. melanogaster* in the laboratory, each of them carrying the genomic sequence of each identified elongase gene in *D. mojavensis mElo* locus, as well as *Dmel/mElo*. By crossing these UAS lines with an oenocyte-specific Gal4 driver, each of the elongase genes was overexpressed in the oenocytes of *D. melanogaster*. This experiment showed that the overexpression of *Dmel/mElo* in *D. melanogaster* can further elongate mbCHCs with significantly reduced 2MeC24 and increased 2MeC28 **(Figure 3.6 A-B; Table A2.4)**. The overexpression of two of the elongase genes in *D. mojavensis mElo* locus, *GI20345* and *GI20347*, also allowed transgenic *D. melanogaster* to produce longer mbCHCs. In particular, the overexpression of *GI20347* led to the production of a moderate amount of 2MeC30 **(Figure 3.6 A-B; Table A2.4).** This suggests that *GI20345* and *GI20347* could have different efficiencies of mbCHC elongation and may synergistically contribute to the longer chain mbCHC in *D. mojavensis*. In addition to the changes in mbCHCs, the overexpression experiments showed that *GI20345* can also elongate monoenes (**Table A2.4**).

To determine how the changes in mbCHC lengths affect the desiccation resistance, we performed desiccation assays on the *D. melanogaster* overexpression strains that have different mbCHC lengths. The results showed that transgenic flies with *GI20347* overexpression are significantly more desiccation resistant compared to the control (**Figure 3.6 C-D**), suggesting that a higher amount of 2MeC30 significantly increases desiccation resistance and *GI20347* is a candidate gene contributing to the high desiccation resistance in *D. mojavensis*.

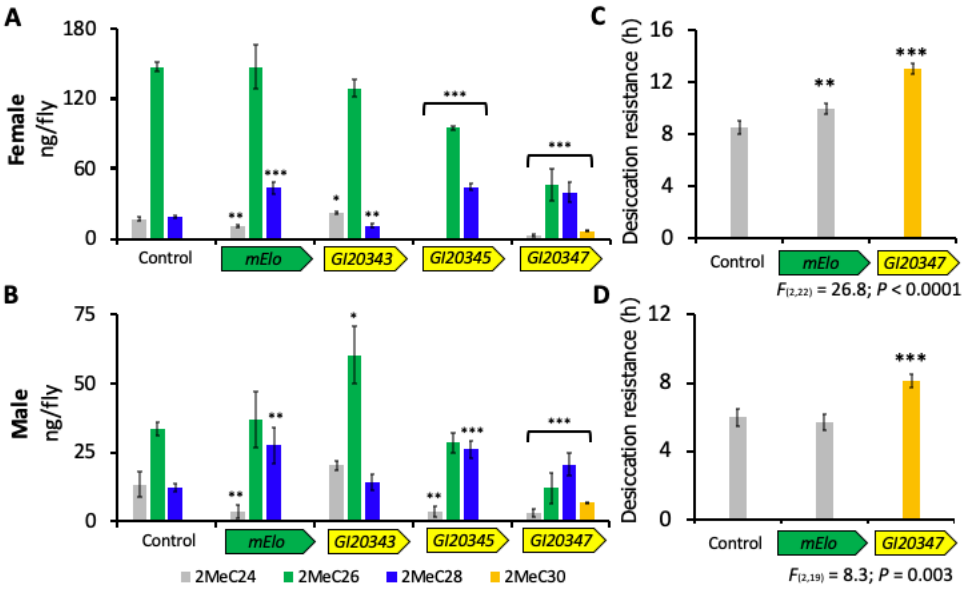


Figure 3.6. Overexpression of *GI20347* in *D. melanogaster* oenocytes leads to longer mbCHCs and increased desiccation resistance. A-B, Amounts of mbCHCs in female and male *D. melanogaster* with the elongase gene from *mElo* loci overexpressed in adult oenocytes. The amount of each mbCHCs in the overexpression strains was compared with control to determine the differences using the student's *t*-test at *alpha*=0.05. C-D, Desiccation resistance of female and male *D. melanogaster* strains with *mElo* and *GI20347* overexpression. One-way ANOVA was used to determine the differences of each CHC between the strains of *D. melanogaster*, followed by *post hoc* comparisons using Dunnett's method. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Two elongase genes in D. mojavensis mElo locus additively contribute to longer mbCHCs

The overexpression experiments in *D. melanogaster* showed that both *GI20345* and *GI20347* could elongate mbCHCs but to different lengths. Since elongase is a type of enzyme that could interfere with each other when reacting on the same substrate (Fritzler et al. 2007, Gregory et al. 2011), the presence of *mElo* in *D. melanogaster* with the overexpression of similar genes could affect the functions of those genes. To further determine the roles of *GI20345* and *GI20347* in mbCHC elongation, I performed the overexpression experiments in the background without *mElo*. To do this, I swapped the X-chromosome of mEloKO strain with the one from the balancer line (y[1] w[*] P{y[+t7.7]=nos-phiC31\int.NLS}X; sna[Sco]/CyO; BDSC

#34770) which carries a *P*-element insertion for *PhiC31* integrase expression in the early embryo, so that we can express the elongase genes in this strain without the effects of original *mElo*. We named the resulting line (*w[1118] /PhiC31integrase; CG18609[KO-dsRED-attP]*) the *mEloKO* strain. To perform the overexpression experiment in the same genetic background, we then made a Gal4 line in the *mEloKO* strain using the enhancer sequence of *GI20345* (*5'GI20345*) which can drive gene expression in the oenocytes (**Figure A2.1 -A2.2**), as well as three UAS lines in *mEloKO* background, each of which can be used to overexpress *mElo*, *GI20345*, and *GI20347*, respectively.

The strain *mEloKO* has the same phenotype as the knockdown of *mElo* with the reduction of 2MeC26 and 2MeC28 and the accumulation of 2MeC24 (**Figure 3.7A-B**). The overexpression of *mElo* rescued the CHC phenotype with 2MeC26 being the major peak (**Fig. 2.6A-B**). The overexpression of either *GI20345* or *GI20347* in the *mEloKO* background showed elongation of mbCHCs, but they led to different lengths of mbCHCs (**Figure 3.7A-B; Table A2.5**). Compared with the overexpression of *mElo*, the overexpression of *GI20345* leads to a similar pattern of mbCHCs that 2MeC26 is the major peak. But the lower 2MeC24 and higher 2MeC26 and 2MeC28 in *GI20345* overexpression compared with *mElo* suggested that *GI20345* could have higher efficiency in the elongation of mbCHCs to 2MeC26 and 2MeC28. More interestingly, the mbCHCs profiles in *GI20347* overexpression had higher 2MeC24 and 2MeC28 but lower 2MeC26 than *mElo* overexpression, as well as the production of 2MeC30, suggesting this gene had a preferred elongation from 2MeC26 to 2MeC28 and 2MeC30. The phylogenetic relationship of the six elongase genes in *D. melanogaster* and *D. mojavensis mElo* locus showed that only *GI20347* is clustered with *mElo*, suggesting it is the ortholog of *mElo* in *D. mojavensis*

and the evolutionary changes in the coding sequences of this gene underlies the synthesis of longer chain mbCHCs in *D. mojavensis*.

The observation that both *GI20345* and *Dmoj/mElo* have different preferences in mbCHC elongation allowed me to hypothesize that these two genes worked additively to elongate mbCHCs from 2MeC24 to 2MeC30. To test this hypothesis, I generated two transgenic strains that can overexpress both genes together in the adult oenocytes (**Figure A2.4**). The results showed that *GI20345* and *Dmoj/mElo* elongate mbCHCs with higher 2MeC26, 2MeC28, and 2MeC30 (**Figure 3.7C-D; Table A2.6**). Taken together, the results showed that *GI20345* and *GI20347* have different efficiencies in mbCHC elongation and additively contribute to the longer chain mbCHC in *D. mojavensis* (**Figure 3.7E**).

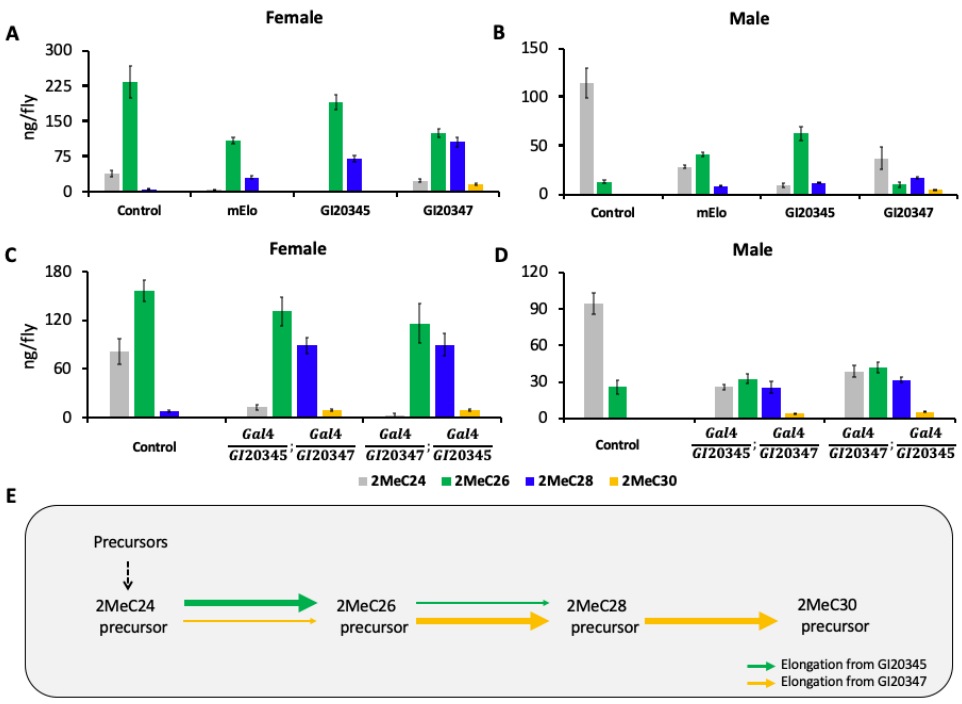


Figure 3.7. *GI20345* and *GI20347* in *D. mojavensis mElo* locus additively contribute to longer mbCHCs. A-B. Amounts of mbCHC of female and male *D. melanogaster mEloKO* strain with the

Figure 3.7 (cont'd)
overexpression of *mElo*, *GI20345*, and *GI20347* in the oenocytes. C-D. mbCHC profiles of female and male *D. melanogaster* strains with the overexpression of both *GI20345* and *GI20347* in the oenocytes. As mbCHCs in these overexpression strains have drastic changes with the produce of mbCHCs that are not observed in the control, there is no need to perform statistical analyses here. E. The model of *GI20345* and *GI20347* additively contribute to mbCHC elongation.

Knockout of GI20347 leads to shorter mbCHCs and reduced desiccation resistance at an ecologically relevant temperature

Although both *GI20345* and *GI20347* can elongate mbCHCs in the overexpression experiments in *D. melanogaster*, the phylogenetic relationship of these genes and higher elongating efficiency in *GI20347* suggest that *GI20347* could be the elongase at the last step of mbCHC elongation in *D. mojavensis*. To further characterize the function of *GI20347* in *D. mojavensis*, I aimed to knock out *GI20347* from *D. mojavensis* and determine its roles in mbCHC synthesis and desiccation resistance. I generated three *GI20347* mutant strains using the CRISPR/Cas9 technique with non-homologous end joining. These three mutant strains have mutations on the third exon of *GI20347*, including 5 bp insertion, 90 bp deletion, and 10 bp deletion, named M3.5, M3.9, and M3.11, respectively. To eliminate potential off-targets from the gene knockout, all the three strains were backcrossed with the parental wild-type flies for at least five generations and then crossed to be homozygous. All homozygous mutant strains are viable. To examine the effects of *GI20347* knockout on CHCs and desiccation resistance, I also established three iso-female lines, ISO1, ISO2, and ISO3, from the parental population as the control. In all the three mutant strains, 2MeC32 was eliminated and 2MeC30 was significantly reduced (**Figure 3.8A; Table A2.7**). This result demonstrates that *GI20347* is the elongase gene at the last step of mbCHC elongation in *D. mojavensis*.

To examine how the changes in mbCHC lengths affect the desiccation resistance of *D. mojavensis*, I subjected all the knockout strains and the control to desiccation assay at 27°C, a temperature consistent with previous assays. However, the results showed no significant difference in desiccation resistance between the knockout strains and the control at this temperature (**Figure 3.8B**). *D. mojavensis* is a desert-dwelling species of which the habitat has annual average temperatures ranging from 35 to 40°C (NOAA.com). Since the ability of CHCs in preventing water loss is associated with their melting temperature (Wigglesworth 1945, Gibbs 2007), I hypothesize that the rest of 2MeC28 and 2MeC30 in *GI20347* knockout *D. mojavensis* could still be efficient in preventing water loss at 27°C. Therefore, I asked whether the shortened mbCHCs in *D. mojavensis GI20347* knockout can reduce its desiccation resistance at its ecologically relevant temperature. I then repeated the desiccation assay at 37°C. At a higher temperature, the wild type *D. mojavensis* can survive for less period, which is around 18 hours, compared with 60 hours at 27°C (**Figure 3.8C**). More interestingly, the results showed that the desiccation resistance of the three knockout strains can only survive for around 8 hours (**Figure 3.8C**), suggesting 2MeC30 and 2MeC32 are important for *D. mojavensis* to survive in arid and hot environments, such as deserts.

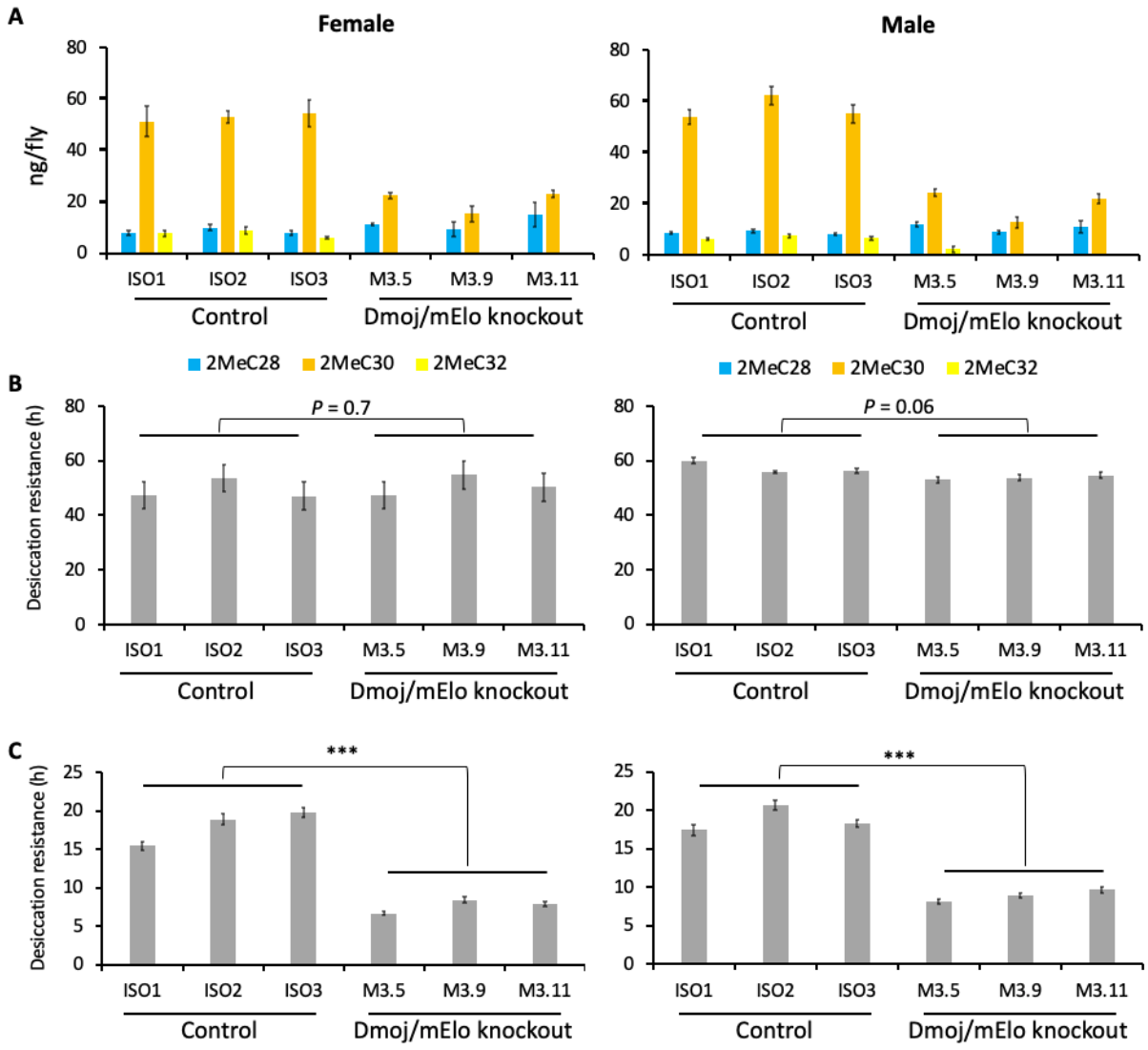


Figure 3.8. The knockout of *GI20347* reduces mbCHC lengths and desiccation resistance at the ecologically relevant temperature of *D. mojavensis*. A. Amounts of mbCHC of female and male *D. mojavensis with GI20347* knocked out. B. Desiccation resistance of *D. mojavensis with GI20347* knocked out at 27°C. C. Desiccation resistance of *D. mojavensis with GI20347* knocked out at 37°C, a temperature that relevant to the habitats of *D. mojavensis*. To determine whether desiccation resistance was affected by the knockout of *GI20347* gene, a linear mixed effects model was used to compare the two groups of flies with the iso-female and independent knockout strains being random effects. The significant difference between the wildtype and knockout flies was tested using paired contrast at *alpha* = 0.05. *** indicates $P < 0.001$.

A single mElo ortholog is present in Drosophila species

The overexpression of *Dmel/mElo* and *Dmoj/mElo* in *D. melanogaster* led to different lengths of mbCHCs either in *attP40* background (**Figure 3.9 A-B**) or *mEloKO* background (**Figure 3.9 A-B**), suggesting that the coding differences in *Dmoj/mElo* ortholog lead to the function that contributes to the production of 2MeC30. I asked whether this difference in *mElo* orthologs also applies to other *Drosophila* species and contributes to the length variation of mbCHCs. To answer this question, I investigated elongase genes in *mElo* loci across *Drosophila* species. Based on the conserved anchoring genes in *D. melanogaster mElo* locus (*Jappa*, *Hsst*, *CG33998*, and *List*), I located *mElo* loci in the genomes of 18 *Drosophila* species, as well as 5 species outside *Drosophila* genus as the outgroup. *mElo* loci can be identified in all these species, but three species in the outgroup do not have any homologs of *mElo* (**Figure 3.9**), suggesting *mElo* and its homologs in this locus could be originated from the common ancestor of *Scaptodrosophila*, *Drosophila*, and *Chymomyza* genus, and the ancestor of *Drosophila* genus could have two elongase genes in this locus. Different numbers of elongase genes between the clades were identified in *mElo* loci, suggesting multiple independent gene duplication events occurred in this loci, for example, in willistoni, virilis, and mojavensis groups (**Figure 3.9**).

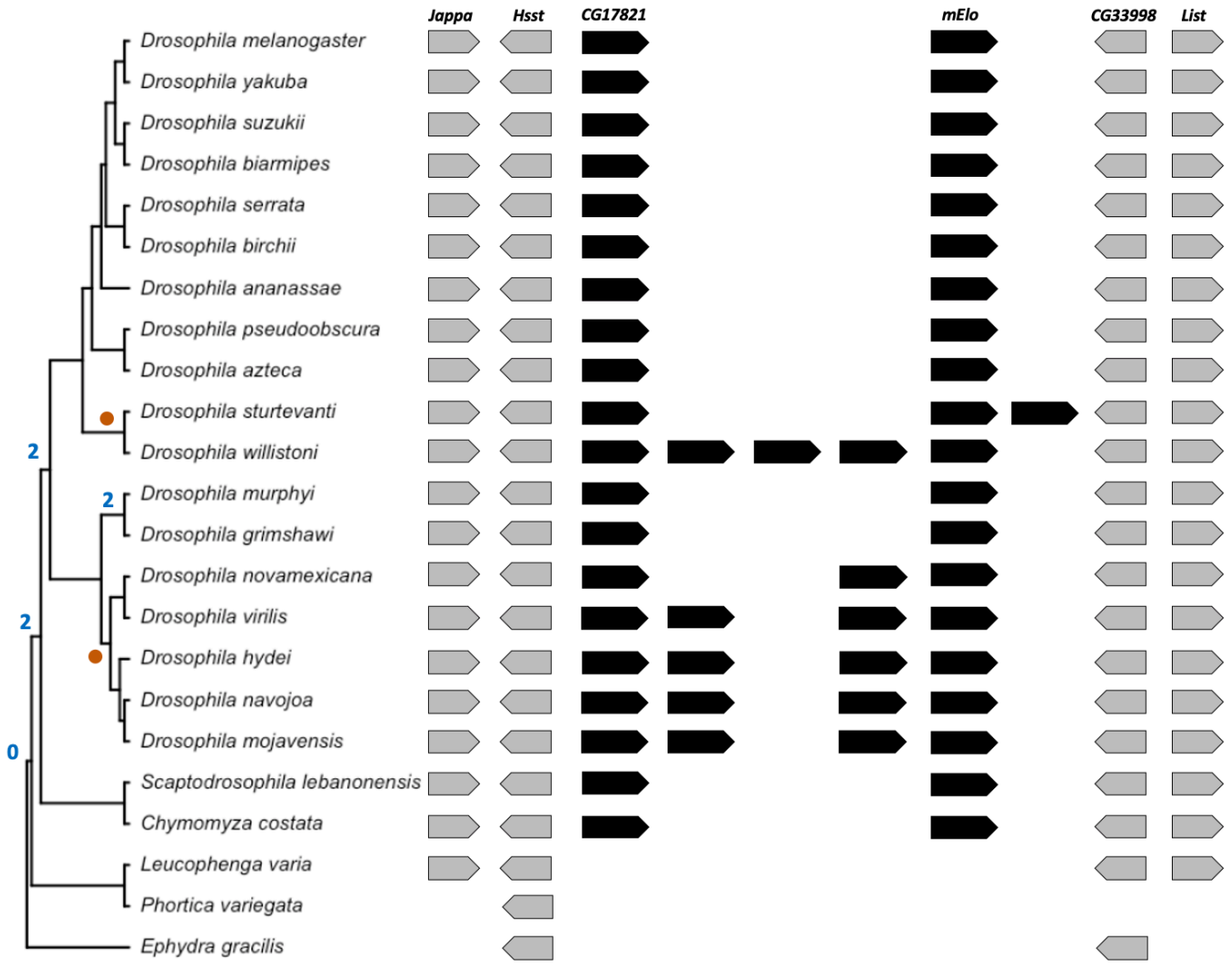


Figure 3.9. *mElo* loci of 18 species in *Drosophila* genus and 5 closely-related species. The locus was identified using the synteny information from *D. melanogaster*. Most sequences were obtained from NCBI GENOME database. If not available in NCBI, tBLASTn

Figure 3.9 (cont'd)
of amino acid sequences of *Jappa*, *Hsst*, *CG33998*, and *List* was used to locate *mElo* locus and tBLASTn of amino acid sequences of *mElo* and *CG17821* was further used to determine homologs of elongases in located loci. The labeled numbers on the phylogeny are inferred numbers of elongase genes in the ancestors and red dots indicate potential gene duplication events.

To determine the relationship of these elongase genes, I performed a phylogenetic analysis using the Maximum Likelihood method with LG substitution matrix on their protein coding sequences (**Figure 3.10**). The results showed for each species in the *Drosophila* genus, there could be only one *mElo* homolog, while the other elongase genes could be homologs of *CG17821*. Except for *D. sturtevanti*, *mElo* ortholog is the last elongase gene in this loci, suggesting the gene duplication events in species in *Drosophila* genus were independent. Interestingly, the two elongase genes in *mElo* loci in the outgroup species, *S. lebanonensis*, and *C. costata*, were found both homologous to *mElo* in the *Drosophila* genus (**Fig. 3.10**). This suggests the first elongase genes in the two outgroup species have different origins from those in *Drosophila* genus. This leads to a hypothesis that the ancestor of *mElo* locus could only have one ancestral homolog of *mElo*, but independent gene duplication events occurred in *Scaptodrosophila* spp, *Chymomyza* spp, and *Drosophila* spp, respectively. Although the evolutionary trajectory in *mElo* loci is complicated, it is ascertained that *mElo* is conserved in this loci. The coding changes in protein sequences of *mElo* could contribute to the evolution of mbCHC lengths and desiccation resistance in *Drosophila* species.

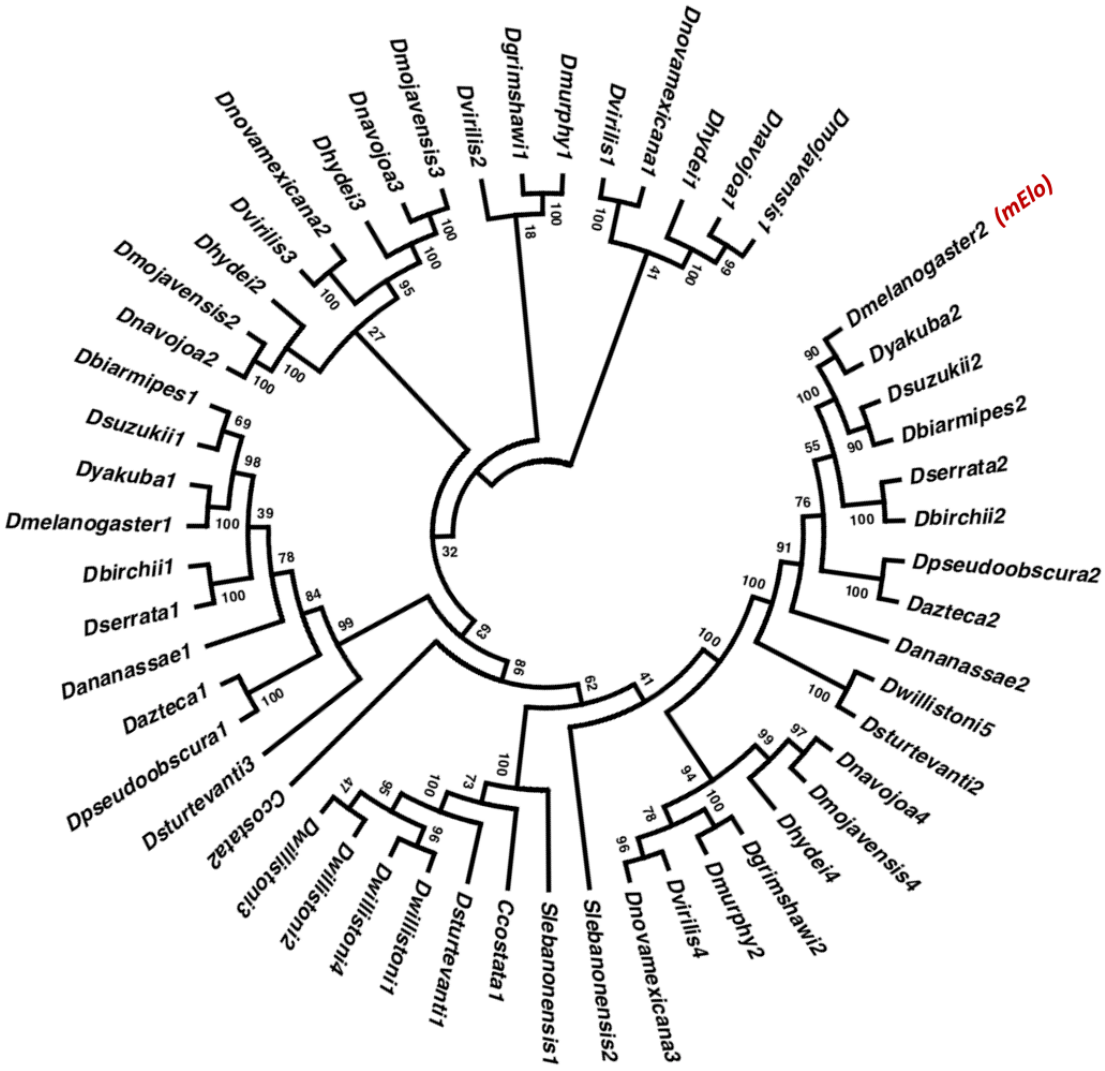


Figure 3.10. Phylogenetic relationship of elongase genes in *mElo* loci from 18 species in *Drosophila* genus and 5 closely-related species. The maximum Likelihood method with the LG substitution matrix was used for the phylogenetic tree construction.

Model: mElo loci from different Drosophila species contribute to the variation in desiccation resistance in Drosophila species

Results from this chapter demonstrated that longer chain mbCHCs can confer higher desiccation resistance and the longest mbCHCs in *D. mojavensis* underlie their adaptation to the hot and arid desert environments. The model for findings in this Chapter is displayed in **Figure 3.11**. The ancestral status had 2MeC28 as the major mbCHC and evolutionary changes

including gene duplication of elongase gene in *mElo* locus and coding changes in *mElo* gene underlie the evolution of longer mbCHCs and higher desiccation resistance. Results from this study can help understand how species adapt to hot and arid environments and also predict how species can evolve when facing warmer and drier environments due to climate change.

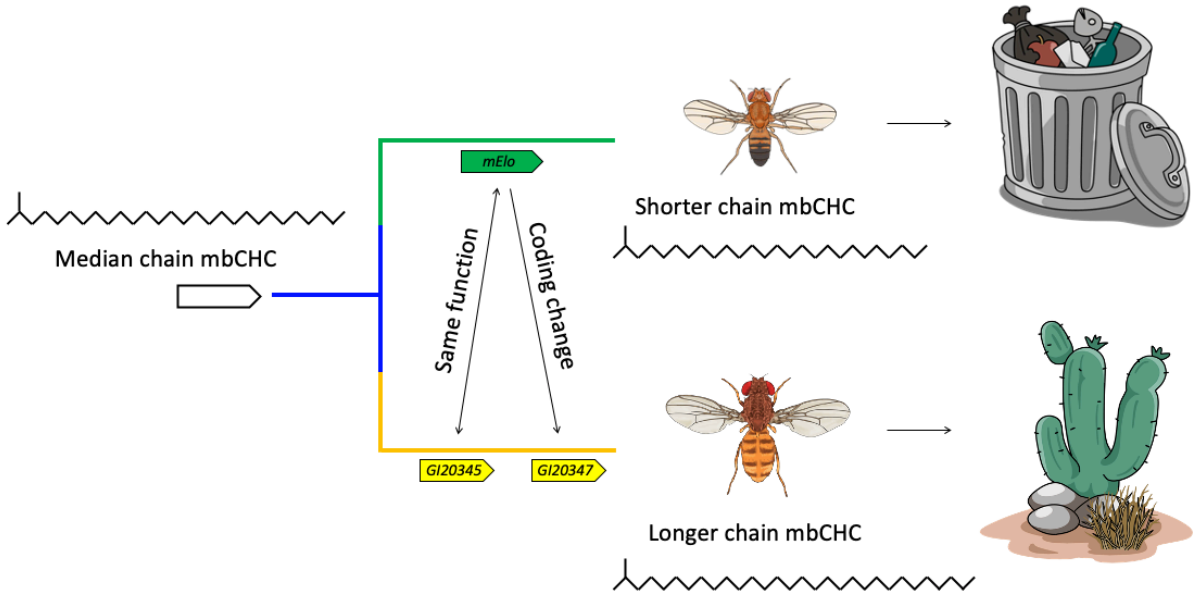


Figure 3.11. *Drosophila* species that dwell in different habitats have different lengths of mbCHCs. The ancestral state of the major mbCHC is 2MeC28, a mbCHC with median carbon-chain length. During the evolution, different *Drosophila* species has evolved different lengths of mbCHCs that are suitable for them adapting to their habitats. This study showed that *D. melanogaster*, a species dwelling in metropolitan areas, has shorter chain major mbCHCs with 26 carbons and lower desiccation resistance, while *D. mojavensis*, a desert-dwelling species, has the longer major mbCHCs with 30 carbons and higher desiccation resistance.

Discussion

Understanding how species evolve different levels of ability to adapt to various abiotic factors is key to predicting the evolutionary trajectory of species facing environmental changes, such as the increasing aridity resulting from climate change. In this chapter, I elucidated the genetic mechanisms underlying the positive association between mbCHC lengths and desiccation resistance across *Drosophila* species. I used *D. mojavensis*, the most desiccation resistant *Drosophila* species and also the species with the longest mbCHCs, as the model here to understand genes underlying elongation of mbCHC. Starting from the model species *D. melanogaster*, I found the fatty acyl-CoA elongase gene, *mElo*, for the elongation of medium chain length mbCHCs in *D. melanogaster*, while the locus of *mElo* in *D. mojavensis* underwent a gene duplication event and contains four elongase genes. Among these four genes, two of them, *GI20345* and *GI20347*, synergistically contribute to the elongation of longer mbCHCs: *GI20345* has the same function of *Dmel/mElo*, while *GI20347* has higher elongation efficiency. Results from desiccation assays on *D. melanogaster* with *GI20347* overexpression showed longer chain mbCHCs can increase desiccation resistance, while the assays on *D. mojavensis* with *GI20347* knockout at different temperatures demonstrated that the even longer chain mbCHCs in *D. mojavensis* contribute to their desiccation resistance at higher temperatures.

The length of mbCHCs is a modulator for desiccation resistance in Drosophila species

mbCHCs are important for insects in preventing water loss and evolving high desiccation resistance (Chung et al. 2014). The survey of CHC profiles across *Drosophila* species showed mbCHCs are a type of CHCs present in most Drosophila species but with length variation from 24 to 32. Although a correlation can be found from Chapter I that species that possess longer

mbCHCs have higher desiccation resistance, the complex physiology and ecology that may contribute to desiccation resistance across species could confound this correlation. However, results in this chapter functionally demonstrate that longer chain mbCHCs contribute to higher desiccation resistance. For example, the shift of major mbCHCs from 2MeC26 to 2MeC28 and the gain of 2MeC30 in *D. melanogaster* with *GI20347* overexpression led to higher desiccation resistance compared with the control, and the loss of 2MeC30 and 2MeC32 leads to reduced desiccation resistance in *D. mojavensis* with *GI20347* knocked out. One thing worth discussing here is that the mbCHC composition in *D. melanogaster* with *GI20347* overexpression is similar to that in species with intermediate levels of desiccation resistance, such as *D. pseudoobscura* and *D. americana*, suggesting the median chain mbCHCs in these species is a factor contributing to their higher desiccation resistance than those with shorter chain mbCHCs, for example, *D. melanogaster* and *D. yakuba*. These results demonstrate the causality underlying longer chain mbCHCs and higher desiccation resistance in *Drosophila* species. In another word, the carbon-chain lengths of mbCHCs are a modulator that contributes to the variation of desiccation resistance in insect species.

A 'pseudo' gene duplication event leads to longer chain mbCHCs in D. mojavensis

Gene duplication following neofunctionalization is an important evolutionary process for novel phenotypes (Magadum et al. 2013), which also applies for physiological adaptation to changing environments (Kondrashov 2012). During this evolutionary process, the duplication leads to two copies of a functional gene, one copy maintaining the original function, while the other copy could evolve a similar but new function. This process could contribute to the evolution of novel traits for species adapting to novel environments.

In this chapter, I showed that there are two elongase genes in *mElo* locus in *D. mojavensis* that could underlie the elongation of mbCHCs, while there is only one in *D. melanogaster* for mbCHC elongation. Phylogenetic analysis on these elongase genes shows that *GI20345* and *GI20347* were clustered with two different genes, *CG17821* and *mElo*, suggesting *GI20345* and *GI20347* could originate from different elongases. The evolutionary history of these elongase genes in *Drosophila* species is more complicated than I expected. I speculate that the promiscuous enzymatic activities of fatty acyl elongases could allow an elongase gene to evolve higher flexibility and may elongate different types of fatty acyl-CoAs (Agaba et al. 2004, Pereira et al. 2004). My results about the overexpression that *GI20345* can elongate both mbCHCs and monoenes is another experimental evidence in supporting this speculation.

When comparing *mElo* loci with the outgroup species of *Drosophila* genus, it suggests that elongase genes in *mElo* loci originated from the common ancestor of *Drosophila*, *Scaptodrosophila*, and *Chymomyza* genera, and the ancestral *mElo* locus of *Drosophila* genus could have two elongase genes. Comparing the current state of *mElo* loci across *Drosophila* species which had different numbers of elongase genes, multiple gene duplication and gene loss occurred during the evolution. For mbCHC elongation in *D. mojavensis*, even though this is not a real gene duplication event that two mbCHC-related elongases were originated from the same elongase gene, the results in this chapter still show that *GI20345* replaces the function of *mElo* and *GI20347* evolve to a new function that leads to longer chain mbCHCs. So here, to differentiate from the traditional naming system, I decide to call this process 'pseudo gene duplication' because all the three elongase genes, *GI20343*, *GI20344*, and *GI20345*, evolved from the first elongase gene in the ancestral *mElo* locus, while *GI20347* had coding changes

from the second elongase gene in the ancestral *mElo* locus with higher elongating efficiency in mbCHC elongation.

Adaptation to desiccation is conditional to other abiotic factors

One of the interesting findings in this dissertation is the temperature-dependent effects of long-chained mbCHCs in *D. mojavensis*. The *GI20347* knockout in *D. mojavensis* reduced 2MeC30 and 2MeC32 but did not change desiccation resistance at 27°C. However, its desiccation resistance was reduced to half at 37°C. One of the explanations for this difference should attribute to the presence of 2MeC28 and 2MeC30 in the *GI20347* knockout *D. mojavensis*, which can still protect from water evaporation. The ability of CHCs to prevent water loss has been found associated with their melting temperature (Wigglesworth 1945, Gibbs 2002a), a physical property that determines the phase (from liquid to solid) of the lipid CHC layer. Therefore, when temperature increases, CHCs with longer chains are less liquefied to better prevent water loss. In addition to this theory, deserts, the habitat of *D. mojavensis*, are usually dry and hot, with the average temperature ranging from 35 to 40°C. Therefore, to survive in desert environments, longer chain CHCs are necessary to withstand the desiccating stress at high temperatures.

Methods and Materials

Fly strains

The *y w; attP40* strain was used for *in situ* hybridization and transgenesis at the 2nd chromosome in *D. melanogaster*, and *y w; 86FB* strain was used for transgenesis at the 3rd chromosome. *D. mojavensis wrigleyi* (15081-1352.29) were ordered from the National Drosophila Species Stock Center (NDSSC; https://www.drosophilaspecies.com). The *oeno*GAL4

line (*PromE(800) line 2M*) was a gift from Joel Levine (Billeter et al. 2009). The balancer lines (w[1118]/Dp(1;Y)y[+]; CyO/nub[1] b[1] sna[Sco] lt[1] stw[3]; MKRS/TM6B, Tb[1] (BDSC #3703) and y[1] w[*] P{y[+t7.7]=nos-phiC31\int.NLS}X; sna[Sco]/CyO; BDSC #34770) were obtained from the Bloomington *Drosophila* Stock Center. All flies were maintained at room temperature on standard *Drosophila* food (Bloomington formulation, Genesee Scientific). All *D. melanogaster* GAL4/UAS-RNAi experiments were performed at 27^{o}C.

In situ hybridization and Imaging

In situ hybridization was performed on oenocytes of five-day-old adults using RNA probes as described in (Shirangi et al. 2009). Primers that were used for synthesizing probes were listed in **Table A2.8**. All *in situ* hybridization images were captured using the Nikon SMZ18 dissecting stereo microscope system.

Generation of constructs

Primers or oligos used for generating all these constructs were listed in **Table A2.8**.

Overexpression constructs

The overexpression constructs were cloned in *PhiC-31* site-specific transformation vector, *pWalium10-MOE* (Ni et al. 2009). The genomic DNA of *Dmel/CG17821*, *Dmel/CG18609* (*Dmel/mElo*), *Dmoj/GI20343*, *Dmoj/GI20344*, *Dmoj/GI20345*, *Dmoj/GI20347* (*Dmoj/mElo*), and *Dpse/GA15013 (Dpse/mElo)* were amplified by PCR from genomic DNA of corresponding species and then cloned into *pWalium10-MOE* vector using either NdeI/XbaI sites or *EcoRI/XbaI* sites.

Site mutagenesis constructs

Site mutagenesis of the 168th amino acid of both *Dmel/mElo* and *Dmoj/mElo* was introduced using primers with corresponding mutations using PCR and then cloned into *pWalium10-MOE* vector using EcoRI/XbaI sites. The resulting constructs were named *UAS-Dmel/mElo*^{L168F} and *UAS-Dmoj/mElo*^{F168L}.

Oenocyte-specific 5'GI20345-GAL4 construct

To locate the enhancer sequence that drives *Dmoj/GI20345* expression in oenocytes, GFP reporter constructs were first generated by PCR amplification of DNA sequences surrounding *GI20345* from *D. mojavensis* genome and then cloned into the GFP reporter vector *pS3aG* via the *AscI* and *SbfI* sites (Shirangi et al. 2009). The resulting constructs were then individually injected into the *Xout D. melanogaster* line and integrated into the genome using the *PhiC31* integrase system.

Since *5'GI20345* were found driving oenocyte expression in *D. melanogaster*, the construct *5'GI20345-pS3aG* was further used to generate oenocyte specific GAL4 construct. To do this, the GFP sequence was first cut out from *5'GI20345-pS3aG* construct using *SpeI* and *SbfI*. Then the GAL4 sequence which amplified from the pBPGUw (Addgene plasmid #17575) vector was subcloned into *5'GI20345-pS3aG* construct using *SpeI* and *SbfI*. Resulting construct was named *5'GI20345-pS3aGAL4.*

Generation of mElo knockout by CRISPR/Cas9 genome engineering in D. melanogaster

CRISPR/Cas9-mediated homology-directed repair (HDR) was used to generate a knockout of *Dmel/mElo*. The program, flyCRISPR Optimal Target Finder

(http://tools.flycrispr.molbio.wisc.edu/targetFinder/), was used to identify optimal CRISPR target sites (Gratz et al. 2014). Target-specific sequences for *Dmel/mElo* were synthesized as oligonucleotides, phosphorylated, annealed and ligated into the *BbsI* sires of *pU6-BbsI-chiRNA* (Addgene plasmid #45946) (Gratz et al. 2013) (5': mElO-gRNA1-BbsI-F and mElO-gRNA2-BbsI-F, 3': mElO-gRNA2-BbsI-F and mElO-gRNA2-BbsI-R; **Table A2.8**). To make the replacement donor, approximately 1kb homology arms flanking the cut sites were amplified by PCR using primers mElo_RightHomo_AscI_F and mElo_RightHomo_XhoI_R for the 5' homology arm and primers mElO_LeftHomo_EcoRI_F and mElO_LeftHomo_NotI_R for the 3' homology arm (**Table A2.8**). The replacement donors were cloned sequentially into the corresponding cut sites of the dsDNA donor vector *pHD-DsRed-attP* (Addgene plasmid #51019). The two gRNA constructs and the replacement donor construct were co-injected into the *D. melanogaster* strain (*w[1118]*; *PBac{y[+mDint2]=vas-Cas9}VK00027*; BDSC #51324) which carrying a vasa-Cas9 transgene on 3rd Chromosome. The dsRed fluorescence in the eyes was used to screen positive progeny, which were then crossed to *w[1118]* to remove the *vasa-Cas9* transgene before being back-crossed 5 generations and being made homozygous using the double balancer line *w[1118] /Dp(1;Y)y[+]; CyO/nub[1] b[1] sna[Sco] lt[1] stw[3]; MKRS/TM6B, Tb[1]* (BDSC #3703). The replacement of *Dmel/mElo* with dsRED/attP by HDR was confirmed by PCR using the primers DmelCG18609-EcoRI-F and DmelCG18609-XbaI-R (**Figure A2.5**). The resulting line is designated as *w[1118]; CG18609[KO-dsRED-attP]*.

Generation of w[1118] / PhiC31integrase; CG18609[KO-dsRED-attP].

To inject transgenes into *w[1118]; CG18609[KO-dsRED-attP]*, we integrated X chromosome from the balancer line (y[1] w[*] P{y[+t7.7]=nos-phiC31\int.NLS}X; sna[Sco]/CyO;

BDSC #34770) following the crossing diagram in **Figure A2.3.** The new line has the *nanos* enhancer driving *PhiC31* integrase in early stages during embryogenesis which can help future transgenesis. The resulting line is designated as *w[1118] /PhiC31integrase; CG18609[KO-dsRED-attP]* and named as *mELoKO* strain. All overexpression constructs made as described above can be injected individually into *w[1118] / PhiC31integrase; CG18609[KO-dsRED-attP]* strain. All primers used for this experiment are listed in **Table A2.8**.

Drosophila microinjections

Transgenesis in *D. melanogaster* was performed using standard protocols as described in http://gompel.org/methods. Embryos were collected from the egg-laying chamber every 30 mins and were aligned for microinjection. For *PhiC31* transgenesis, the concentrations of constructs that were used for injection were ranging from 150 to 400 ng/µL. For CRISPR/Cas9 genome editing, a mix of two sgRNA-encoding constructs (150 ng/µL) and a donor vector (400 ng/µL) was injected. All concentrations are given as final values in the injection mix.

Generation of mElo knockout by CRISPR/Cas9 genome engineering in D. mojavensis

To generate *Dmoj/mElo* mutant alleles in *D. mojavensis*, we used a non-homologous end joining mediated strategy by injecting the mixture of Cas9 protein (#CP01; PNA Bio) and sgRNAs into the embryos of this species. Following the protocol in Khallaf et al. (2020), we co-injected two sgRNAs targeting *Dmoj/white* (5'-ATCCGGGCGAACTGCTGGCC-3' and 5'-GATCAGGAGCTATTGATACG-3'). *Dmoj/mElo* specific sgRNAs were designed using the online tool CRISPR Design (http://crispr.mit.edu) and two sgRNAs were selected including 5'-CCGGCGGTCACTTCAATTGCCTC-3' and 5'-GCGCGTTTACAATCTTGGCCAGG-3'. All sgRNAs were

generated following the protocol in Kistler et al. (2015), with *in vitro* transcription using T7 Megascript Kit (Ambion) and purification using a MegaClear Kit (Ambion). The final injection mixture is composed of 300 ng/µL Cas9 protein and four sgRNAs, each 75 ng/µL.

To screen for the offspring of *D. mojavensis* carrying *Dmoj/mElo* mutant alleles, I used T7E1 assay (NEB #E3321) to determine potential mutations for each single fly following an established protocol (Zhu et al. 2019). The following sequencing on the mutant alleles showed three independent mutations in the offspring with 5 bp insertion, 90 bp deletion, and 10 bp deletion on *Dmoj/mElo* in the location of the second sgRNA, respectively. To diminish potential side effects, we backcrossed the females from the mutant strains with the parental males for at least five generations. During the backcrosses, we used a restriction enzyme AciI that was specific to the three mutations to screen for the flies carrying mutant alleles. All the three strains can result in viable homozygous offspring, which we named the three corresponding strains M3.5, M3.9, and M3.11.

Cuticular hydrocarbon extraction and analyses

CHC extraction, GC/MS analysis, CHC identification, and quantification were performed as described in Lamb et al. (2020) and Savage et al. (2021). The GC thermal program was set as follows: start from 100 °C, 5 °C/min to 200 °C, and 3 °C/min to 325 °C. For each sex in each reciprocal cross, three extractions were conducted as replicates and the results were pooled for further statistical analyses, so six replicates were performed for each cross. In cases that targeted CHCs were eliminated, statistical analyses were not conducted. To compare the quantities of CHCs between treatments, the student's t test or one-way ANOVA (following *post hoc* comparison using Dunnett's method) were used at *alpha* = 0.05.

Desiccation assay

Desiccation assays were performed as described in Chung et al. (2014). Silica gel (S7500-1KG) was ordered from Sigma-Aldrich. For each genotype, six replicates were conducted, each three from each reciprocal cross. In the overexpression experiments, desiccation resistance between treatments was compared using one-way ANOVA, following with *post hoc* comparison using Dunnett's method. To determine the difference in desiccation resistance between *D. mojavensis mElo*-knockout strain and the wild type strain, a linear mixed effects model was used to compare the two groups of flies with the iso-female and independent knockout strains being random effects. All the contrasts were conducted at *alpha* = 0.05.

CHAPTER 4 FACTORS AFFECTING SYNTHESES OF CUTICULAR HYDROCARBONS IN *DROSOPHILA* SPECIES

In this chapter, I sought to investigate several different factors that could affect the syntheses of cuticular hydrocarbons in *Drosophila* species. Results in this chapter were accomplished with help from several collaborators, of which contribution is listed below. There are also a few exploratory experiments that either produced negative results or did not provide a meaningful conclusion, they were gathered in Appendices III – V.

Eliana Giannetti

- Helped with *in situ* hybridizations of elongase and reductase genes in *D. mojavensis* oenocytes

Dr. Abby Lamb

- Generated *ebony* loss-of-function mutants in *D. novamexicana* and *D. americana*.

Dr. Jian Pu

- Helped with *D. melanogaster* dissection.

Introduction

The syntheses of CHCs in insects' oenocytes are complicated and can be affected by different genetic, physiological, and behavioral factors (Chung and Carroll 2015, Blomquist and Ginzel 2021). Considering the importance of CHCs in chemical communication and desiccation resistance, understanding how these different factors influence CHC syntheses can help predict their effects on biological functions. In this chapter, I aimed to investigate multiple factors that

underlie the evolution of CHC syntheses in different *Drosophila* species, including 1) elongases and reductases that contribute to CHC syntheses in *D. mojavensis*; 2) effects of pigmentation genes on CHC syntheses in *D. novamexicana* and *D. americana*; 3) effects of grooming behaviors on CHC syntheses in *D. melanogaster*. Results from this chapter can shed light on how CHC compositions between species and even individuals can be so dynamic and informative.

Results

Multiple elongases and reductases contribute to CHC syntheses in D. mojavensis

Gene duplication of elongase genes occurred in the *D. mojavensis* genome, which could contribute to the synthesis of longer chain mbCHCs (**Chapter 3**). One caveat in the results shows that the knockout of *GI20347* cannot fully eliminate the synthesis of 2MeC30, while the overexpression of GI20345 in *D. melanogaster* did not produce any 2MeC30. This conflict leads to the hypothesis that other genes contributing to the long chain mbCHCs such as 2MeC30.

Considering the promiscuity in the enzymatic activity of elongases, I cannot exclude the possibility that other elongases are involved in the elongation of mbCHCs in *D. mojavensis*. D. melanogaster mElo knockout females still have longer mbCHCs (**Chapter 3 Figure 3.4**), suggesting there could be a potential elongase gene that only expressed in females' oenocytes for mbCHC elongation. Previous screening by Dr. Henry Chung showed among all elongase genes that expressed in the oenocytes of *D. melanogaster* (unpublished data), only one of them, *eloF*, has female-specific expression, consistent with findings in other studies (Chertemps et al. 2006, Chertemps et al. 2007, Combs et al. 2018). The function of *eloF* has been investigated in vitro by Chertemps et al. (2006), which can elongate dienes in females.

However, the knockdown of *eloF* in oenocytes does not only reduce dienes, but also all alkanes including mbCHCs. This information suggests the female-specific elongase gene, *eloF*, could be the other elongase gene for mbCHC elongation in *D. melanogaster* females, which can help locate the candidate genes in *D. mojavensis*.

Another possibility could be the synergistic effects from the combination of elongases and reductases. Although the role of reductase in mbCHCs has been chemically determined, which can convert fatty acyl-CoA precursors to aldehydes before being decarboxylated to CHCs (Qiu et al. 2012a), how these genes contribute to diverse CHCs and how they evolve across species is largely unknown. Previous work by Dr. Henry Chung showed that an elongase and a reductase synergistically led to the production of a longer chain monoene in an African population of *D. melanogaster* (Unpublished data). Based on this information, I hypothesize that evolutionary changes in reductases can also contribute to the longer chain CHCs (especially mbCHCs) in *D. mojavensis*. In addition, Finet et al. (2019) screened the expression of reductases in *D. melanogaster* oenocytes and examined the birth-and-death of reductase genes in 12 *Drosophila* species. This study shows that there are both female- and male-specifically expressed reductases in *D. melanogaster* and a rapid evolutionary gain and loss of reductase genes in different *Drosophila* species, suggesting evolutionary changes in reductases could also contribute to the evolution of CHCs.

To investigate whether there are other elongases and reductases in *D. mojavensis* contributing to the syntheses of long chain mbCHCs, I used *in situ* hybridization to survey their expression in adult oenocytes. Combining with BLAST search and synteny-based search, I found 17 elongase genes in the *D. mojavensis* genome. Finet et al. (2019) showed that there are 13

reductase genes in the *D. mojavensis* genome. *In situ* hybridization shows seven elongases and two reductases are expressed in oenocytes in both sexes (**Figure 4.1 – Figure 4.2; Appendix VII-VIII**). Interestingly, the *eloF* cluster in *D. mojavensis* does not contain any elongase genes (**Figure 4.1**). Since *D. mojavensis* also has high amounts of long-chained dienes in its CHC profile, this suggests independent evolution in other elongase genes in *D. mojavensis* for the elongation of dienes. However, whether there are elongase genes beyond those in *D. mojavensis mElo* locus for mbCHC elongation is unknown. To answer this question, future research can focus on overexpressing the other elongase genes that were found expressed in adult oenocytes in *D. melanogaster* and examining changes in CHC profiles.

In situ hybridization of reductase genes showed only two of them are expressed in adult oenocytes, which could be the candidate genes for synthesizing longer chain CHCs in *D. mojavensis*. One of them, *GI15313*, is the ortholog of *CG4020* in *D. melanogaster*. Among all reductases in *D. melanogaster*, *CG4020* is the only one that has a female-specific oenocyte expression (**Figure. 4.2**) (Finet et al. 2019). As *D. melanogaster* female with *mElo* knocked out still has longer mbCHCs and dienes, I hypothesized that *CG4020* could be the reductase gene for synthesizing mbCHCs and dienes in this species. Furthermore, *GI15313* could be the candidate reductase gene for producing the very long chain mbCHC and potentially dienes. Future studies for further functionally testing this hypothesis can either overexpress *GI15313* in *D. melanogaster* or knock down its expression in adult oenocytes of *D. mojavensis*.

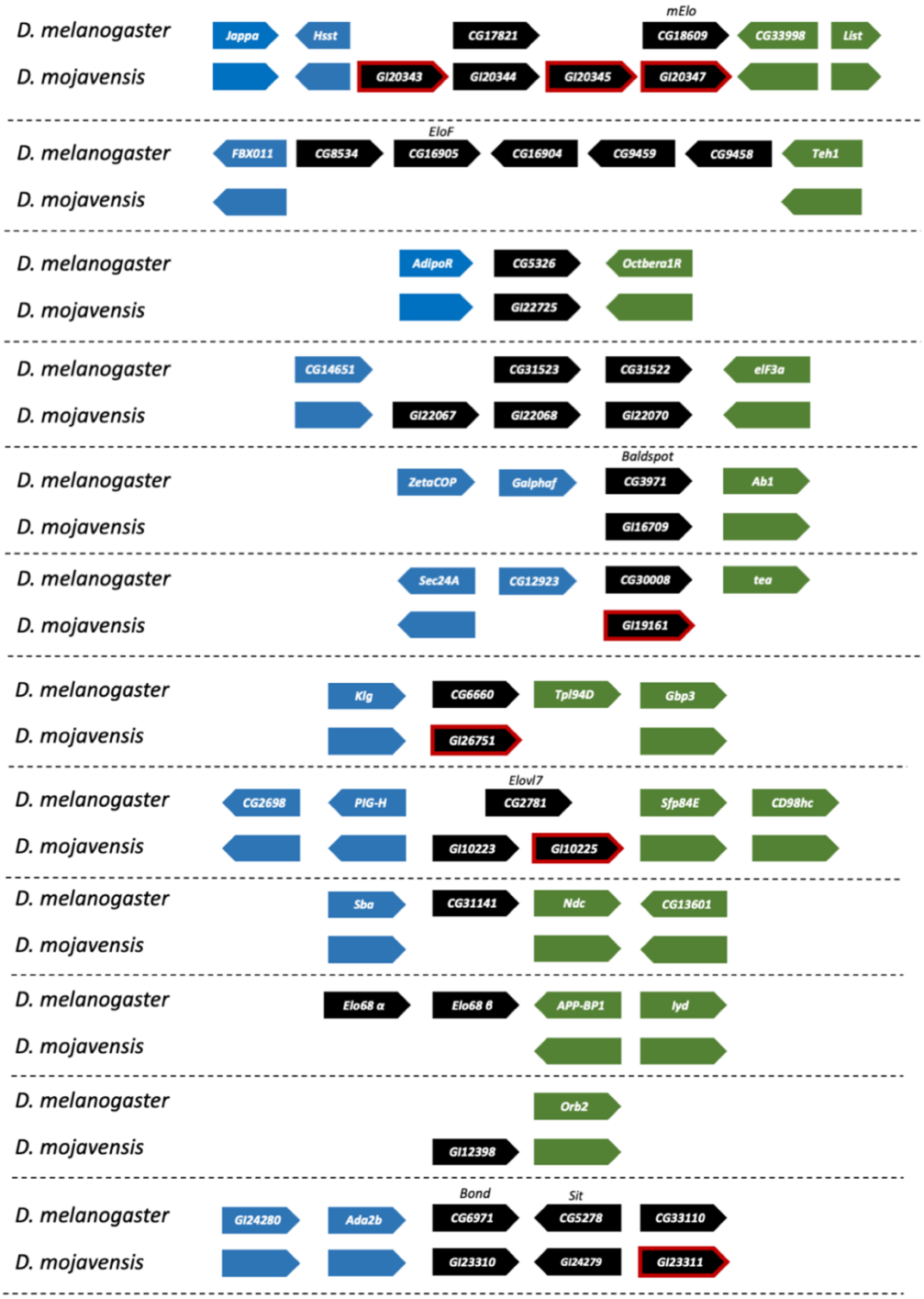


Figure 4.1. Summary of elongase genes in the *D. mojavensis* genome. Screened with *in situ* hybridization, genes that are expressed in adult oenocytes are marked with the red border. Staining pictures of adult oenocytes from *in situ* hybridization are listed in Appendix VI.

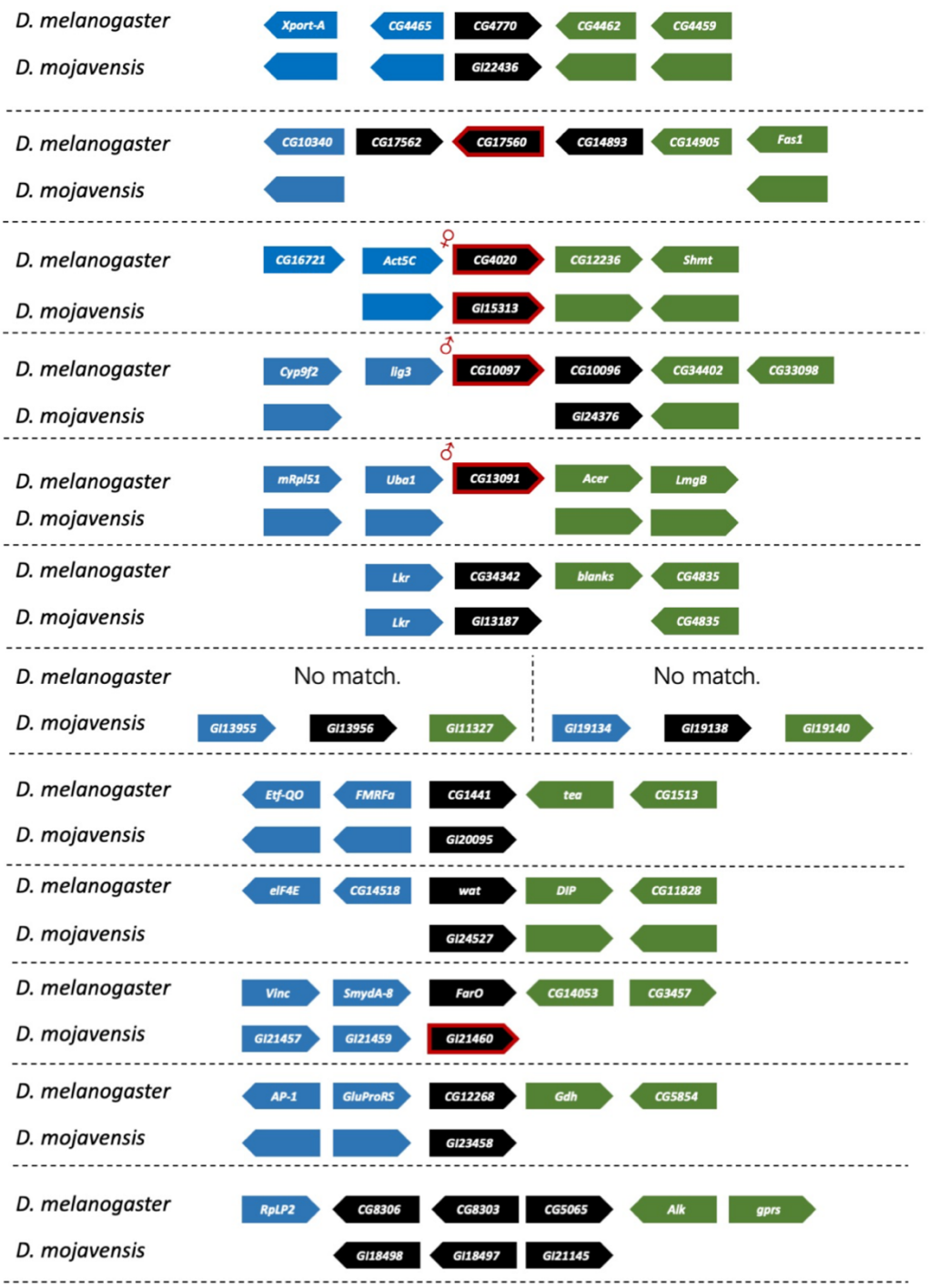


Figure 4.2. Summary of reductase genes in the *D. mojavensis* genome. Screened with *in situ* hybridization, genes that are expressed in adult oenocytes are marked with the red border. Staining pictures of adult oenocytes from *in situ* hybridization are listed in Appendix VII.

The pigmentation gene ebony contributes to CHC syntheses in D. mojavensis

Pigmentation biosynthesis genes *ebony* has recently been shown to affect the abundance of CHCs in *D. melanogaster* (Massey et al. 2019). *ebony* is a gene for synthesizing beta-alanyl-dopamine (Borycz et al. 2002). Defective *ebony* can also lead to a darker cuticle phenotype resulting from the accumulation of dark pigments on the cuticle (Wittkopp et al. 2003). Although it is unclear how this gene mechanistically affects CHC abundance, Massey et al. 2019 generated *ebony* loss-of-function mutant in *D. melanogaster* and showed the loss of *ebony* increases the abundance of CHCs. To understand whether this also applies to other *Drosophila* species, I collaborated with Abby Lamb from the University of Michigan and examined the CHC differences in *ebony* loss-of-function mutants in *D. novamexicana* and *D. americana*. In this section, Abby generated *ebony* loss-of-function mutants for both species using the CRISPR-Cas9 technique and made hemizygous strains with a single functional copy of *ebony* as the control. The comparison between *ebony* loss-of-function mutant and the control showed a general increase in CHC abundance for *D. novamexicana* but only a few monoenes and dienes increased in *D. americana* (**Figure 4.3A-B**). Furthermore, we asked whether the two *ebony* alleles from the two species could affect CHC abundance differently. To answer this question, we used a reciprocal hemizygosity test and generated reciprocal hemizygotes (F1 hybrids) of these two species that contain either allele of *ebony* from them. We then compared the CHC profiles for the two reciprocal hemizygotes. The results showed that both reciprocal hemizygotes with one *ebony* allele (F1Ne and F1Ae) have fewer CHCs compared with the control without *ebony*, but no significant differences between the two reciprocal hemizygotes (**Figure 4.3C**). This suggests that both *ebony* alleles from *D. novamexicana* and *D. americana*

could lead to reduced CHC abundance but allelic divergence in these two species does not have a detectable effect on CHC abundance. Details about the generation of *ebony* loss-of-function mutants in these two species and effects can be found in Lamb et al. (2020).

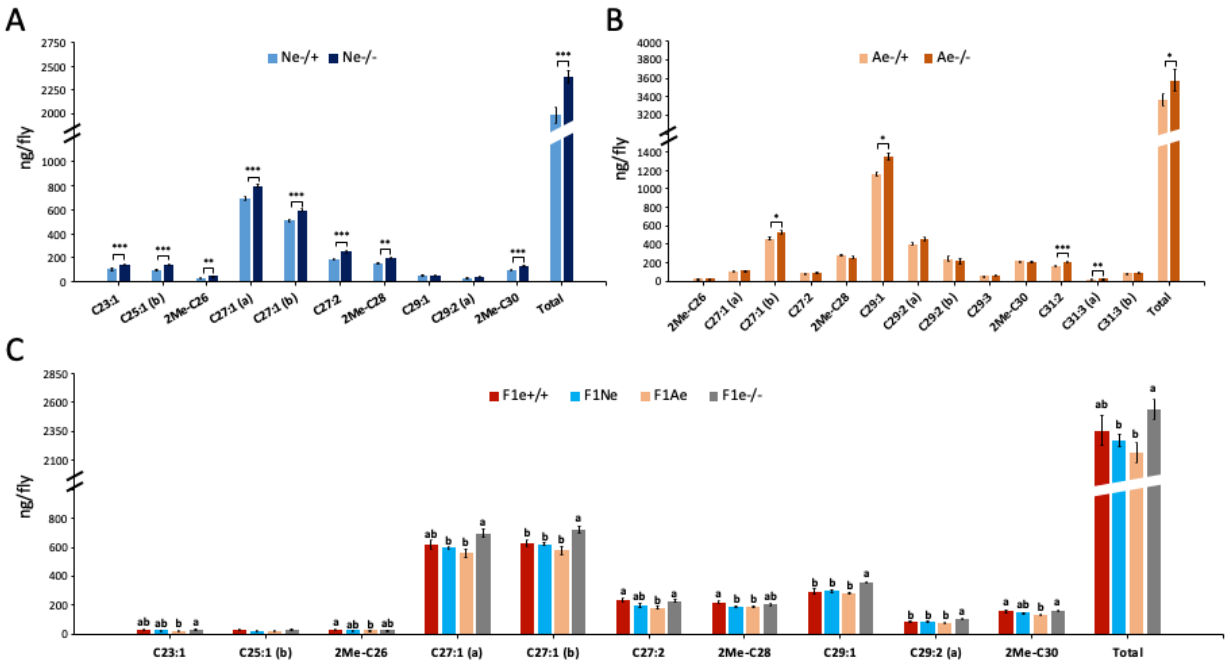


Figure 4.3. *ebony* loss-of-function mutant increases CHC abundance in both *D. novamexicana* and *D. americana*. A-B. The abundance of individual CHC compounds (ng/fly ± Standard Error (SE)) for *D. novamexicana* and *D. americana* mutants. Ne-/+ and Ne-/- refer to heterozygous and homozygous *D. novamexicana* mutants for an *ebony* null (e^-) allele. Ae-/+ and Ae-/- refer to heterozygous and homozygous *D. americana* mutants for an *ebony* null allele. Student's t-test was performed for each pair of individual CHC at *alpha* = 0.05. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. C. The abundance of individual CHC compounds (ng/fly ± SE) for reciprocal F1 hybrids that contain one *ebony* allele from *D. novamexicana* (F1Ne) and *D. americana* (F1Ae), both alleles (F1e+/+), and homozygous for *ebony* null allele. One-way ANOVA was performed for each set of individual CHC, followed by *post hoc* comparison using Tukey's method at *alpha* = 0.05. For each CHC compound, the F1 hybrids shared with the same letter are not significantly different.

Grooming behaviors may modulate CHC abundance as a response to acclimate to heterogeneous environments

The real environment is heterogeneous rather than being constant (Allouche et al. 2012). Studies on species' adaptation in the laboratory are usually conducted in either constant levels of such abiotic factors as temperature and humidity or a setup with artificial fluctuation (Nakahira and Arakawa 2006, Prasifka et al. 2015). In daily life in nature, all organisms would experience heterogeneous environments with varying levels of abiotic factors such as temperature and humidity. For ectotherms including insects of which physiology changes according to the environment, they could experience short-term stresses from varied levels of abiotic factors in heterogeneous environments, for example, different levels of desiccation stress. Insects use CHCs to prevent water loss and then resist desiccation stresses. In nature, if a fruit fly entered an area that is dryer, how does it cope with this sudden change? Is there a common mechanism for species quickly adjusting their physiology and acclimating to the heterogeneous environment?

A previous study that subjected *D. melanogaster* to rapid desiccation hardening showed that *D. melanogaster* could rapidly accumulate CHCs on their cuticle, suggesting a potential means of coping with rapid environmental changes (Stinziano et al. 2015). Another study shows when the humidity sensing ability was abolished in *D. melanogaster* by knocking out *Obp59a*, the small soluble protein in antennae for hygrosensation, the loss of humidity response can lead to accumulation of CHCs and increases in desiccation resistance (Sun et al. 2018). This suggests that when sensing low humidity, *D. melanogaster* is able to adjust the abundance of its CHCs and quickly acclimate to dryer environments. However, how *D. melanogaster* quickly accumulates CHCs as a response is unknown.

One caveat in the synthesis of CHCs is that insects synthesize CHCs in the tissue oenocytes that locate under the abdominal cuticle (Chung and Carroll, 2015). However, all produced CHCs need to coat the whole body surface to prevent water loss. A hypothesis about this process is that grooming behaviors could allow insects to physically spread CHCs from the abdomen to other parts of the body surface. Grooming behavior has multiple biological functions (Zhukovskaya et al. 2013, Hamiduzzaman et al. 2017). Although this behavior has been studied, it has not been connected with the changes in CHC abundance. Grooming in *D. melanogaster* includes several specific behaviors that mainly use its forelegs and hindlegs to clean reachable body parts (Szebenyi 1969, Qiao et al. 2018). To determine how grooming affects CHC abundance, I removed different body parts from 1-day-old *D. melanogaster*, including the pairs of forelegs, midlegs, hindlegs, and wings, to inhibit its grooming behaviors and compared their CHC abundance at the 4^{th} day. The results showed that for both males and females, removal of forelegs and hindlegs which abolished the grooming behaviors significantly increased the overall abundance of CHCs (**Figure 4.4**). This increasing trend is consistent for each single CHC (**Figure 4.4**). While the removal of midlegs and wings that does not affect grooming behaviors, CHC abundance was the same as the control (**Figure 4.4**). This suggests grooming can reduce the overall abundance of CHCs, which is important for desiccation resistance. Based on this data, I hypothesized that when insects sense reduced humidity in the air in heterogeneous environments, they could adjust the frequency in grooming to accumulate CHCs and quickly acclimate to the dryness. To test this hypothesis, I propose future experiments to quantify the frequency and time for *D. melanogaster* grooming at setups with different levels of humidity and temperature. In addition, I propose to compare the grooming

behaviors for *D. melanogaster Obp59a* knockout mutant with the control. This can directly test whether the increased CHC abundance in this mutant results from reduced grooming behaviors.

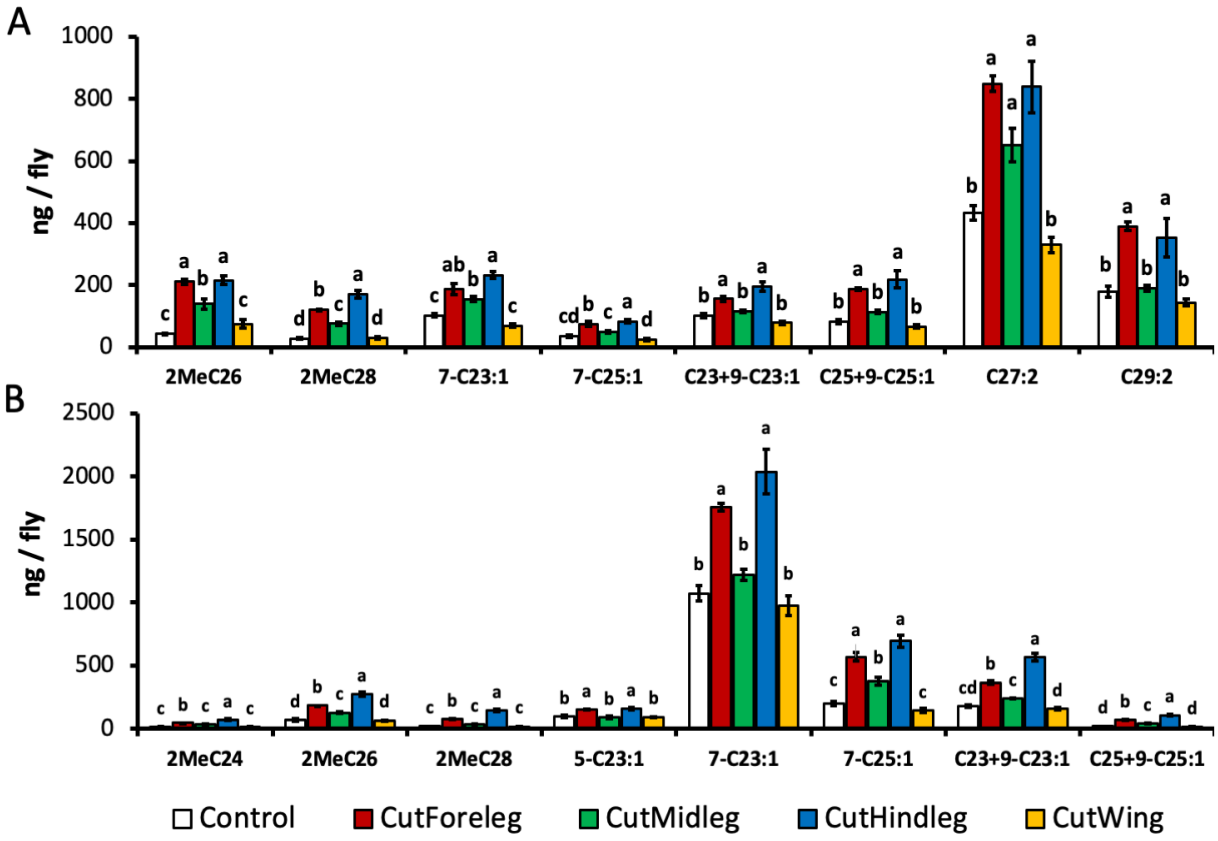


Figure 4.4. Cuticular hydrocarbons of female (A) and male (B) *D. melanogaster* with one body part removed. Differences in CHC abundance between the removal of different body parts were determined using one-way ANOVA, followed by *post hoc* analyses using Tukey's method at *alpha* = 0.05. For each CHC, the bars labeled with different letters are significantly different.

Discussion

In this chapter, I investigated several factors that could affect the synthesis of CHCs. I first screened the expression of all elongases and reductases expressed in adult oenocytes of *D. mojavensis*. Identified genes with oenocyte expression can be used for further study in

understanding how *D. mojavensis* evolve the longest chain CHCs. Secondly, I showed the expression of a pigmentation gene, *ebony*, could reduce CHC abundance in *D. novamexicana* and *D. americana*. At the last, I showed grooming is a behavior that could reduce CHC abundance in *D. melanogaster*. I hypothesized that grooming behaviors could be used to modulate the overall amount of CHCs on insect body surfaces so that insects can acclimate to heterogeneous environments.

Cuticular hydrocarbons are important chemicals for communication and preventing water loss in insects. The genes underlying CHC syntheses have been found undergoing complicated evolutionary changes. When comparing elongases in *D. melanogaster* and *D. mojavensis*, except for the gene duplication in *mElo* locus, I also identified a couple of gene duplication and gene loss events. The *eloF* locus in *D. melanogaster* has five elongase genes but none of them can be found in *D. mojavensis*. However, both *D. melanogaster* and *D. mojavensis* have a high proportion of dienes in their CHC profile, suggesting independent evolution of elongases for elongating dienes in both species. The function of reductase genes is less investigated in *Drosophila* species (Holze et al. 2021). The oenocyte-specific RNAi screening of reductases in *D. melanogaster* showed the reductases do not have specific regulation on CHCs (Dembeck et al. 2015), however, in a rice pest species, the brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), one of its reductases was found specific to synthesize linear alkanes (Li et al. 2020). This suggests there is still certain specificity in reductase genes and evolutionary changes in reductases in *D. mojavensis* could also contribute to the CHC evolution. To understand how the other elongases and reductases evolve to lead to long CHCs

in *D. mojavensis*, future studies can co-overexpress both candidate elongase and reductase genes in *D. melanogaster* to examine the synergistic effects on CHC synthesis.

In addition to evolutionary changes in the synthesis genes, I also identified two other factors, expression of pigmentation genes and grooming behaviors, which can reduce CHC abundance in *Drosophila* species. Pigmentation in insect species has been studied for decades and variation in the pigmentation has been observed inter- and intra-specifically (True 2003, Davis and Moyle 2019). In this chapter, I only showed that the presence of *ebony* could reduce CHC abundance. Similar effects of other pigmentation genes such as *tan* on CHC abundance have been reported (Massey et al. 2019), but whether how the interactions of these different pigmentation genes affect CHC abundance is not clear.

This is the first study to connect grooming behavior and CHC abundance regulation. Grooming is a very interesting behavior that has been associated with several other important biological functions, such as disease/pest defense (Zhukovskaya et al. 2013, Hamiduzzaman et al. 2017), olfaction, and courtship facilitation (Callahan 1975, Wada-Katsumata and Schal 2019). Regulation of CHC abundance through grooming can be easily controlled by the grooming frequencies. Different frequencies of grooming of insects in different environments have been reported (Westhus et al. 2014). Therefore, it is reasonable to hypothesize that insects could use grooming behavior to actively regulate their CHC abundance. This regulation could further benefit insects' acclimation to heterogeneous environments, as well as facilitate mating behaviors that are affected by CHCs.

Methods and Materials

In situ hybridization, CHC extraction, and identification are the same as described in Chapter 2 and Chapter 3. Primers used for generating the RNA probes are listed in Table 4.1.

Table 4.1.Primers used for generating the RNA probes

Primers	Sequences (5' – 3')
GI23311-ISHprobe-F	GCGGCAATGCGACATTCCCC
GI23311-ISHprobe-R	TTACAGCGCCTTCAGTTTGTGCG
GI24279-ISHprobe-F	ATCCCCGCACCAATGACTGG
GI24279-ISHprobe-R	TTTGCAGGTTGGTGATGTACTTC
GI22067-ISHprobe-F	ACCGCATATGGCTGCTGGTG
GI22067-ISHprobe-R	TCACTTGTTGCCCGCATTGAAC
GI22068-ISHprobe-F	ATGGTCAATATCTGCTGGTGGT
GI22068-ISHprobe-R	CATACAGGCACCCTTGTTGGTG
GI22070-ISHprobe-F	ATGGTGCACGCCTTCCAATTG
GI22070-ISHprobe-R	TTCCGGTGTTTCAGCTCCTGG
GI16709-ISHprobe-F	ATTGAGCAAGACCGTGTGTGC
GI16709-ISHprobe-R	CTACTGTGCCTTCGCCTTGC
GI19161-ISHprobe-F	CTGCACATAACGGAGTTTACGAT
GI19161-ISHprobe-R	AATTTCACCTCAGCTGCTTCAG
GI26751-ISHprobe-F	GTGTTCATAGTGCTGCGCAAG
GI26751-ISHprobe-R	CAACTGAGTTTCGCCTTGGC
GI12398-ISHprobe-F	CTGGCGTCTGCCTTTTGGTTG
GI12398-ISHprobe-R	AATGACAGCATGTAGGCCACTC
GI22725_ISHprobe_F	ATGGCGCGTGCTGTTTGGC
GI22725_ISHprobe_R	TATCTGGAGGATGGTGATGTAC
GI13187_ISHprobe_F	GTCAACGTACAAGGATCCATTTC
GI13187_ISHprobe_R	CGGTGGCCATGGCAATTAAATC
GI13956_ISHprobe_F	GGCAGATATACCGGTGTATAAC
GI13956_ISHprobe_R	GCTTTTGGTATGGTTTCCAATGG
GI15313_ISHprobe_F	TCATCTCCACGCTGGAGGAGC
GI15313_ISHprobe_R	CCTCACAAAGTGCAGCACTGG
GI18497_ISHprobe_F	CCAGAAGTACAATGAGAAGACC
GI18497_ISHprobe_R	CTATGGATGGCCTAACTATGGC
GI18498_ISHprobe_F	CTCGCCCATCACAGATTTCTATG
GI18498_ISHprobe_R	TTGGGCTCCAGTTGCTTGAGC
GI21145_ISHprobe_F	GCAAGCGTCCCAATACGTATAC
GI21145_ISHprobe_R	GCACGCTGGACATGGCATATCT

Table 4.1 (cont'd)

GI19138_ISHprobe_F	TTTCACCATCGGCCTATGAGCC
GI19138_ISHprobe_R	AAGCGCTTTTGGCCCAAAATG
GI20095_ISHprobe_F	CTTGCGCAATGCCGTCTTCATG
GI20095_ISHprobe_R	CACATCGACGGGCAAGAAGTC
GI21460_ISHprobe_F	CGCCAAAGGAGCTGCTCATATAC
GI21460_ISHprobe_R	CCTTTGATCTCCTCAATCATGCCC
GI22436_ISHprobe_F	CGTTTTCTGTACGTATCCACTGC
GI22436_ISHprobe_R	TCCGCCTTGTGCACCATCATG
GI23458_ISHprobe_F	ATTTTCAGCAAGCTGTTGGAGAAG
GI23458_ISHprobe_R	TTGGGAGTTATTTCCGCTAGGC
GI24376_ISHprobe_F	GTTTTTGCCTTGCTGTTCAAATCG
GI24376_ISHprobe_R	TAATGCTTGGCCGAACGATGC
GI24527_ISHprobe_F	GCCGCCCATATATAACTATGTGC
GI24527_ISHprobe_R	CGCTTAATGCGCTTGATGGATTC

CHAPTER 5. DISCUSSION

In this dissertation, I investigated how the evolution of insect CHCs can contribute to different levels of desiccation resistance in *Drosophila* species, as well as the underlying genetic and physiological mechanisms. In Chapter 2, I surveyed desiccation resistance and CHC profiles for 50 *Drosophila* and sibling species. Association analyses between them showed one class of CHCs, methyl-branched CHCs (mbCHCs), is important to predict desiccation resistance in these species. Furthermore, it revealed a correlation that species with longer mbCHCs could have higher desiccation resistance. In Chapter 3, to further investigate the physiological and genetic mechanisms underlying this correlation, I used a desert-dwelling species, *D. mojavensis*, as my model in this study and demonstrated that evolutionary changes in a locus containing fatty acyl-CoA elongase genes lead to the production of the longest mbCHCs in *D. mojavensis* and contribute to its highest levels of desiccation resistance. In Chapter 4, as CHCs have been demonstrated an important factor in determining insects' desiccation resistance, I further investigated a few factors that could regulate syntheses and abundance of CHCs in insects, including other fatty acyl-CoA elongase and reductase genes expressed in adult oenocytes of *D. mojavensis*, effects of pigmentation genes on CHC production, and effects of grooming behaviors in CHC abundance. This dissertation can help us understand how the evolution of a trait can evolve to modulate the ability of insects in withstanding desiccation stress and contribute to their adaptation to diverse and changing environments.

Temperature but not aridity could drive phenotypic evolution for desert adaptation

One of the surprising results in this study is the different effects of *mElo* knockout on desiccation resistance in *D. mojavensis* at two temperatures. The knockout strain had a striking

decrease in its resistance level at 37°C, an ecologically-relevant temperature to this desert species, while no effects were observed at an ambient temperature (27°C). Higher temperatures could increase water evaporation. More importantly, higher temperatures could lead to phase changes of insect CHCs and then a rapid increase in water loss. However, when the environmental temperature is far lower than the melting temperature of CHCs, the CHC layer with either original CHCs or shortened CHCs is able to efficiently prevent water loss. As shown in desiccation assays, *mElo* knockout strain of *D. mojavensis* can survive for the same amount of time as the wildtype *D. mojavensis* at 27°C. This leads to a hypothesis that the evolution of some traits for withstanding desiccation stress (such as the lengths of mbCHCs) in insects are driven by the temperature, instead of the aridity (or precipitation) in the environments. In this dissertation, another piece of evidence that may also be able to support this hypothesis is that the longest chain mbCHCs were also observed in species living in hot and humid rainforests, for example, *D. ananassae* and *D. willistoni*. Although their habitats are humid rainforests, it is also important for them to survive from the heat front in the middle of a day using long CHCs that have high melting temperatures. However, this is solely my speculation. To test this hypothesis, one could elucidate the evolutionary trajectories of water loss rates in different species and correlate this finding with their habitats. This may uncover how temperature drives the phenotypic evolution of traits in preventing water loss.

Although long chain mbCHCs are important to prevent water loss in a hot environment, a question remains interesting but unclear – how low temperatures drive the evolution of adaptation to desiccation. High temperatures can increase water evaporation, while low temperatures could also result in low humidity in the air. Different from high temperatures, low

temperatures could result in less stress in water evaporation and therefore, a CHC layer with lower melting temperature, but to survive from both stresses of cold and aridity, insects may need to evolve a different set of traits. The Antarctic and Arctic areas are the coldest and the most arid environments on Earth, but there are still arthropod species dwelling in these areas such as the Antarctic midge, *Belgica antarctica* Jacobs (Diptera: Chironomidae), and the Arctic springtail, *Megaphorura arctica* (Tullberg) (Onychiuridae: Collembola). A study compared CHCs in the Antarctic midge before and after desiccation exposure (Benoit et al. 2007). This study showed CHCs in this species have medium lengths and did not change after a rapid desiccation hardening (Benoit et al. 2007). This fits my hypothesis that long chain mbCHCs may only be a useful trait to withstand desiccation stress in hot environments.

Therefore, the traits for adapting to the same stress, such as desiccation, could evolve differently when facing other different stresses. This dissertation focuses on the evolution of CHCs, in particular the lengths of mbCHCs, which are found to be affected by both temperature and humidity. Here, I use a diagram to illustrate how these two abiotic factors could shape the evolution of mbCHC lengths (Figure 5.2). Basically, in warm or hot environments, mbCHCs would either maintain the same lengths as the ancestral state or evolve to be longer when the temperature is increasing and producing higher stress. However, in cold environments, the lengths of mbCHCs do not need to be longer and the need for CHCs in chemical communication could lead to more diverse evolutionary changes in their lengths. This illustration has only taken one trait and two abiotic factors into consideration and the evolutionary trajectories have already been complicated. The realistic scenario regarding the adaptation of species to a specific type of environment would be even more intricate.

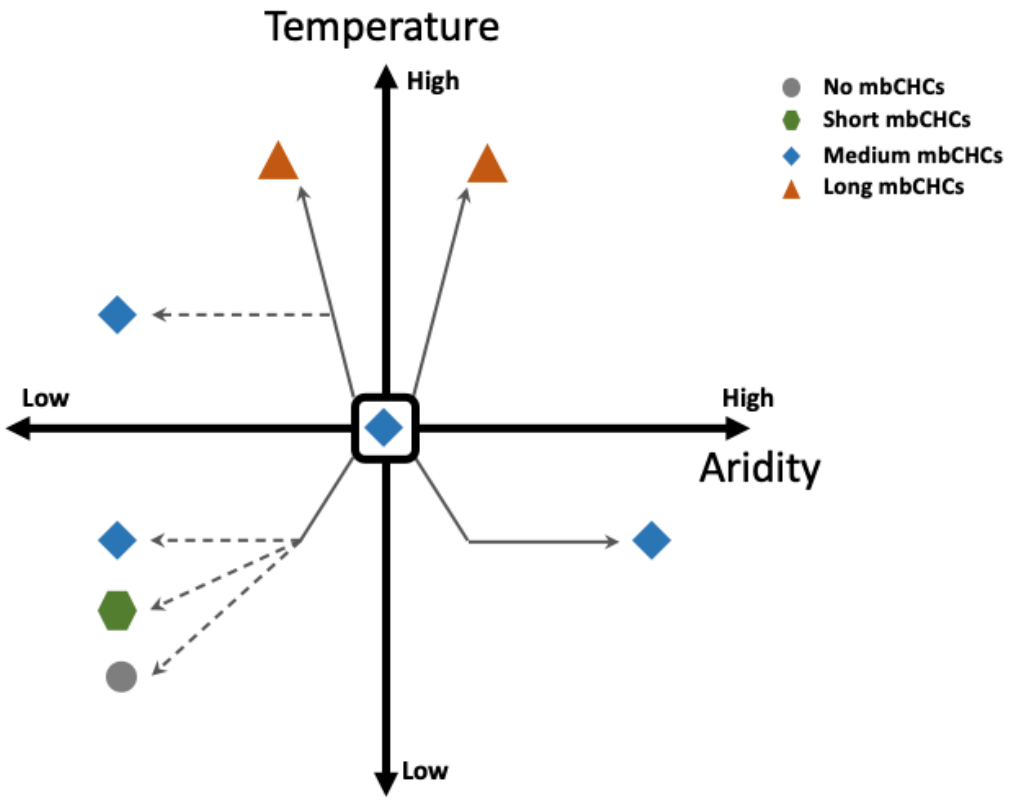


Figure 5.1. Illustration about how the temperature and aridity could influence the evolution of mbCHC lengths, a trait that is important for insect desiccation resistance. The ancestral state of mbCHC lengths would be medium. High temperature could drive the evolution of long chain mbCHCs, while at lower temperatures, the chain lengths of mbCHCs could be less important for withstanding desiccation stress.

Model about insects developing resistance against desiccation and other stresses

This dissertation elucidates an important mechanism underlying how insects withstand desiccation stress. However, as also introduced in Chapter 1 (**Figure 1.1**), desiccation resistance in insects involves multiple traits, including routes of preventing water loss and traits for tolerating water loss. To adapt to terrestrial environments with different levels of aridity, it is the combination of these traits that contribute to insects' desiccation resistance. To illustrate how insects generally develop desiccation resistance and adapt to different terrestrial

environments, I propose a model here borrowing the idea from the Cannikin Law (or, Wooden Bucket theory). This theory states the capacity of a wooden bucket is determined by its shortest stave, instead of the other longer staves. This theory is widely acknowledged in management science. In my opinion, it also applies to characterize insects' physiology in adapting to different environments. Taking desiccation resistance as an example, the resistance level is analogous to the capacity of a wooden bucket and each of their traits is a stave (**Figure 5.2A**). Under the umbrella of Cannikin Law, the level of desiccation resistance in an insect species is determined by the weakest trait of preventing and/or tolerating water loss. In the other words, only when all traits in a species have the maximum ability to prevent/tolerate water loss, for example, the desert species, *D. mojavensis*, can this species well adapt to dry environments. To better illustrate this model, I used several wooden buckets standing for several cases regarding insects with different levels of desiccation resistance (**Figure 5.2 B-D**).

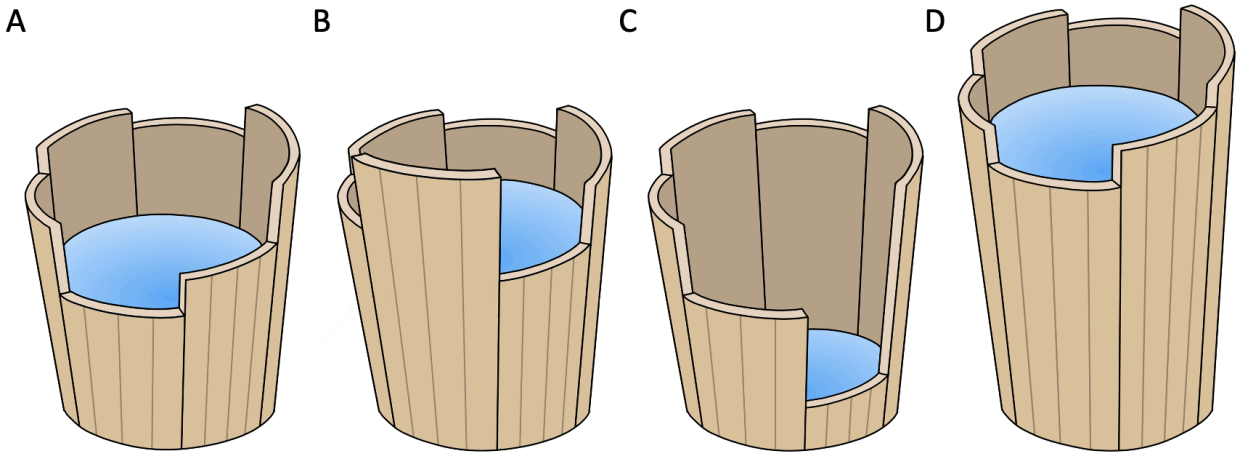


Figure 5.2. Application of Cannikin Law to explain trait evolution in insects' desiccation resistance. This application suggests that insects use multiple traits to combinatorially withstand desiccation stress and the level of desiccation resistance in a species is determined by the weakest trait of preventing and/or tolerating water loss. A. A bucket standing for a medium level of desiccation resistance, determined by the weakest trait (the shortest stake).

Figure 5.2 (cont'd)
B. Trait evolution that results in higher efficiency in preventing water loss (a longer stake) may increase its desiccation resistance, but the increment is constrained by the weakest trait. C. Loss of one trait can strikingly reduce desiccation resistance. D. Only all traits have high levels of efficiency in preventing or tolerating water loss, the species can develop a high desiccation resistance. The figure was drawn by Yuzhang Shan in the Department of Laboratory Medicine at Zhongshan Hospital of Fudan University, Shanghai, China.

One caveat observed in this dissertation is that the findings of the effects of long chain mbCHCs on desiccation resistance cannot apply to all species. For example, *D. ananassae*, *D. willistoni*, and *D. sulfrigaster* all have 2MeC30 as the major mbCHC (**Appendix I**), but they have low desiccation resistance (**Figure 2.1**). Similarly, some species with a relatively large body size were also found with low levels of desiccation resistance, such as *D. albomicans* and *D. americana*. This suggests all these traits including the use of CHCs are only necessary for insects to develop a high level of resistance and each of them alone would not be sufficient. The application of Cannikin Law in insect desiccation resistance can explain these observations which cannot be explained by the findings from this study. Even though *D. ananassae* and *D. willistoni* have the longest mbCHCs, the other traits in these species could make them lose water rapidly or less able to tolerate water loss. This could be the scenario in **Figure 5.2C** that the shortage in one trait would cause the species to be susceptible to desiccation stress.

Can we predict the phenotypic evolution of other species when facing climate change?

One of the central goals in biology is whether we can predict the phenotypic evolution of species when facing changing environments. Although longer chain mbCHC itself may not be able to allow for a mesic species directly adapting to the desert, functional experiments in this dissertation demonstrated that elongated mbCHCs in *D. melanogaster* indeed increased its desiccation resistance by around thirty percent. Could this trait evolution apply to other species

when facing warmer and drier environments due to climate change? To answer this question, two factors are required to be understood. First, what are the costs (or constraints) of this trait? Second, is the molecular mechanism underlying this trait evolution easy to apply to other species?

Except for preventing water loss, the evolution of CHCs is also influenced by chemical communication in insects. Chung et al. (2014) demonstrated the presence of mbCHCs has dual roles in affecting desiccation adaptation and mating success in two sibling *Drosophila* species, *D. serrata* and *D. birchii*. Although it is not clear how the length variation in mbCHCs would affect mating, some behavioral studies showed insect species have a preference for mbCHC with certain lengths but not the others (Adams and Tsutsui 2020). Combining with findings in this dissertation, an interesting conflict has been uncovered: for example, *D. serrata* females prefer to mate with males perfumed with a shorter mbCHC (2MeC26) (Chung et al. 2014), while the longer mbCHC (2MeC28) is the major peak in *D. serrata* and confers higher desiccation resistance. This suggests in *D. serrata*, mating success could be a constraint in its evolution to longer mbCHCs and higher desiccation resistance. Is this a universal constraint in insects? Does species always maintain the trait of shorter mbCHCs in favor of higher mating success? In addition to mating success, are there other physiological costs in, for example, longevity or reproduction for insect species investing in longer chain mbCHCs? The transgenic *Drosophila* strains generated in my dissertation are perfect models for answering these questions. I propose to use these strains to further investigate whether there are constraints in these species with different mbCHC lengths. Characterizing such constraints can also help us understand why and how some species can only survive from their niches.

Another important aspect is to understand how easily other species could evolve such traits for adapting to dryer environments. In this dissertation, I replaced *mElo* in *D. melanogaster* with the elongase genes from *D. mojavensis mElo* locus. Though the coding change was found in *Dmoj/mElo* for its higher efficiency in mbCHC elongation, the results showed the expression of *Dmoj/mElo* itself is not sufficient to produce longer chain mbCHCs. Two hypotheses were proposed based on results in Chapter 3 and Chapter 4 that gene duplication in elongase genes and coding change in a reductase gene could underlie the longest mbCHCs in *D. mojavensis*. This suggests that more than one evolutionary change is required for the production of the longest mbCHCs. In *D. mojavensis*, one extra elongase gene was gained *via* gene duplication event in *mElo* locus followed by gain of gene expression and potential coding changes. It seems difficult to have multiple evolutionary changes in a short period, however, I would argue that there are potentially alternative routes for achieving this. Taking the synthesis of longer mbCHCs as an example, two or more elongase genes may be required. For most insect species, the elongase genes that are used for CHC synthesis are only a small portion of their elongase genes in the genomes (such as findings in *D. melanogaster* and *D. mojavensis* in this dissertation). Therefore, instead of undergoing a gene duplication event, *cis*-regulatory evolution in other elongase genes could allow an independent gain of elongase expression in the oenocytes for mbCHC elongation. This hypothetical scenario is plausible because *cis*-regulatory changes have been found to evolve rapidly in species contributing to many morphological, physiological, and behavioral changes (Wray 2007, Hill et al. 2021) and adaptation to changes in the environments (Juneja et al. 2016, Dowle et al. 2020). Therefore, I hypothesize that insect species could evolve longer mbCHCs in face of warmer and drier

habitats due to climate change, which, however, may be resulted from different mechanisms. On the other hand, this also suggests that gene duplication following related genetic changes could also underlie the evolution of other traits for the adaptation of organisms to changing environments.

Final comments

In the past millions of years, organisms have evolved countless traits that allow them to live in diverse and extreme environments. They have now occupied each small niche on Earth, from volcanoes to the polar, from alpines to deep oceans, from rainforests to deserts. It is fascinating to learn from these organisms about their adaptational strategies, as well as underlying physiological and genetic mechanisms. Over the last few decades, from bacteria living in volcano vents, we learned their special enzymes are functional at extremely high temperatures; from fishes living in deep oceans, we learned the loss of body cavities prevents them from being crushed by intense pressure; from insects living in deserts, we learned they evolve longer cuticular hydrocarbons to prevent water loss at high temperatures… These intricate mechanisms in different species provide a precious treasure for us to explore. Insights gained from them will allow us to foresee how species would evolve in the future and inspire discoveries in many other disciplines.

APPENDICES

APPENDIX I: Chromatograms of the alkane mixture standard and cuticular hydrocarbons for 50 Drosophila and sibling species

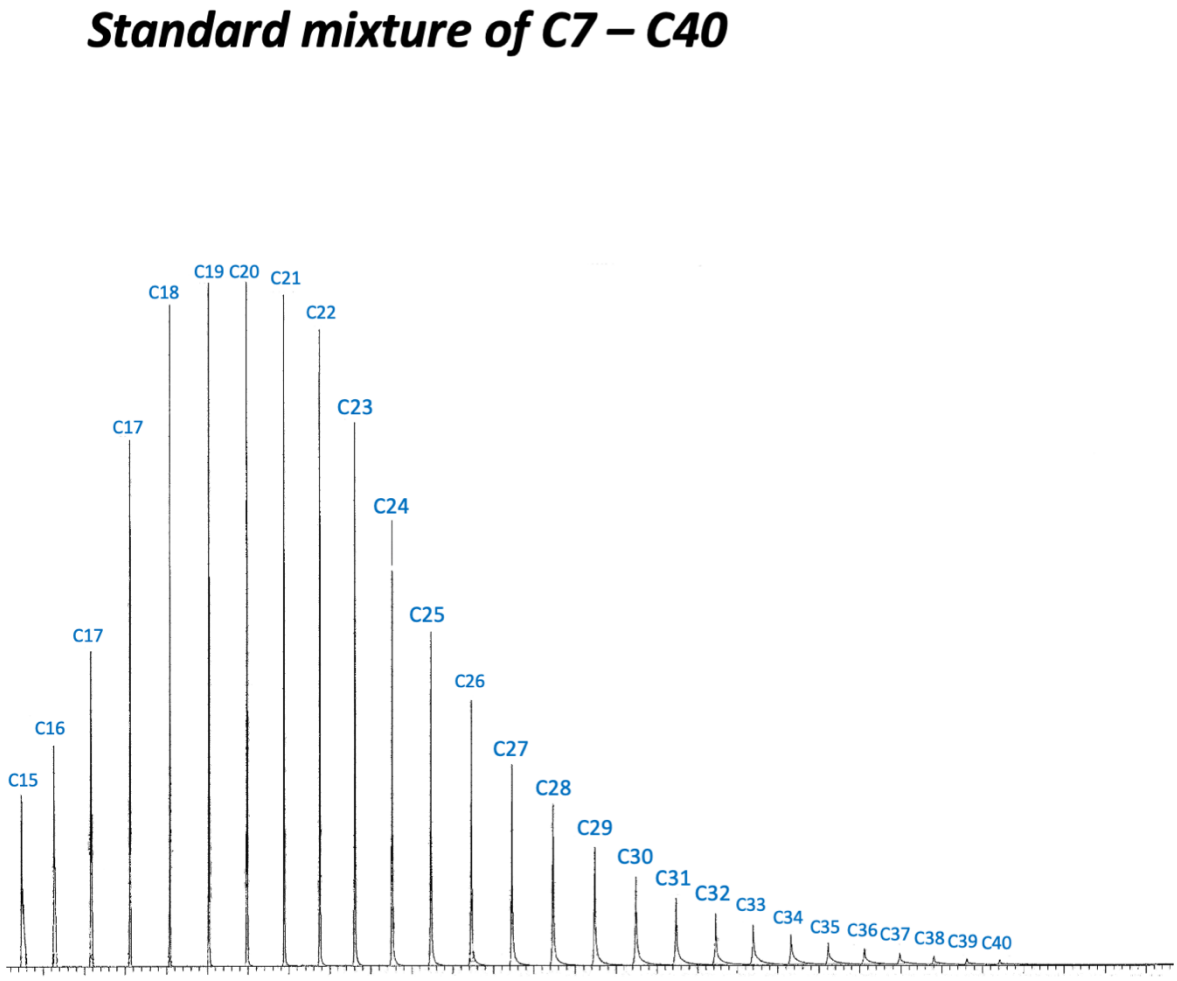
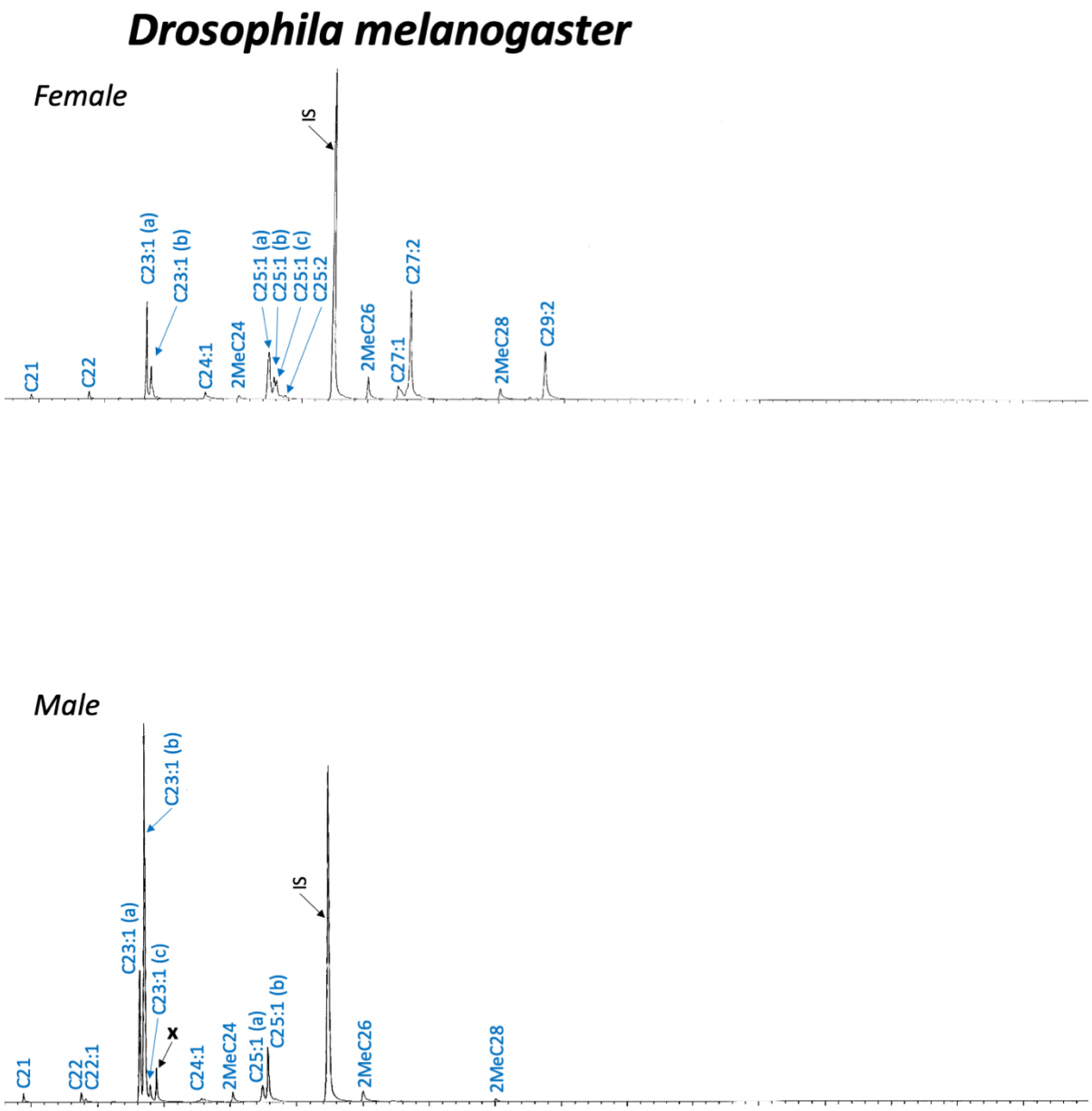
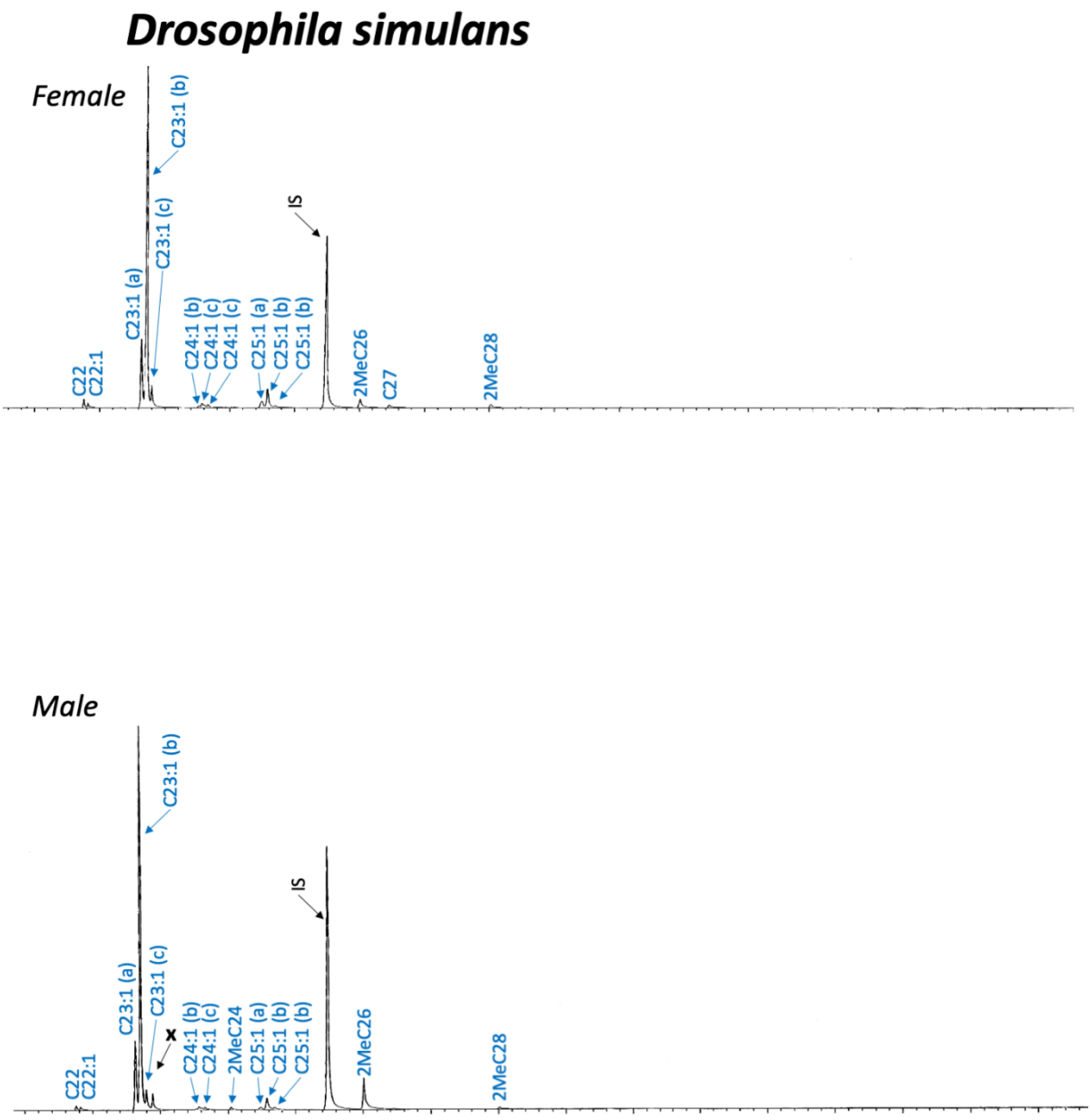


Figure A1.1. Chromatogram of the standard mixture of linear alkanes from C7 – C40.



Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.2. Chromatograms of CHCs for female and male *Drosophila melanogaster*.



Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.3. Chromatograms of CHCs for female and male *Drosophila simulans*.

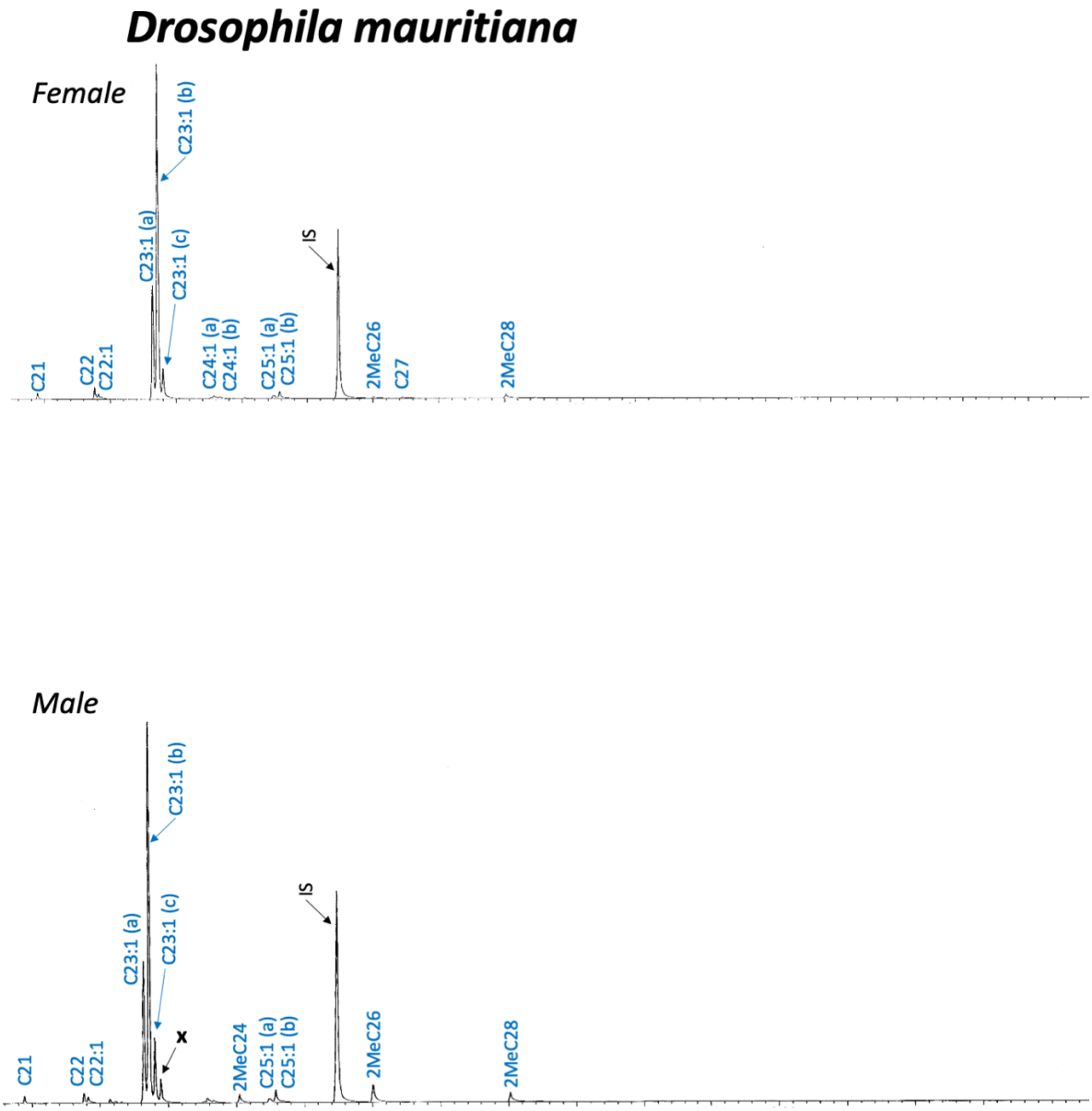
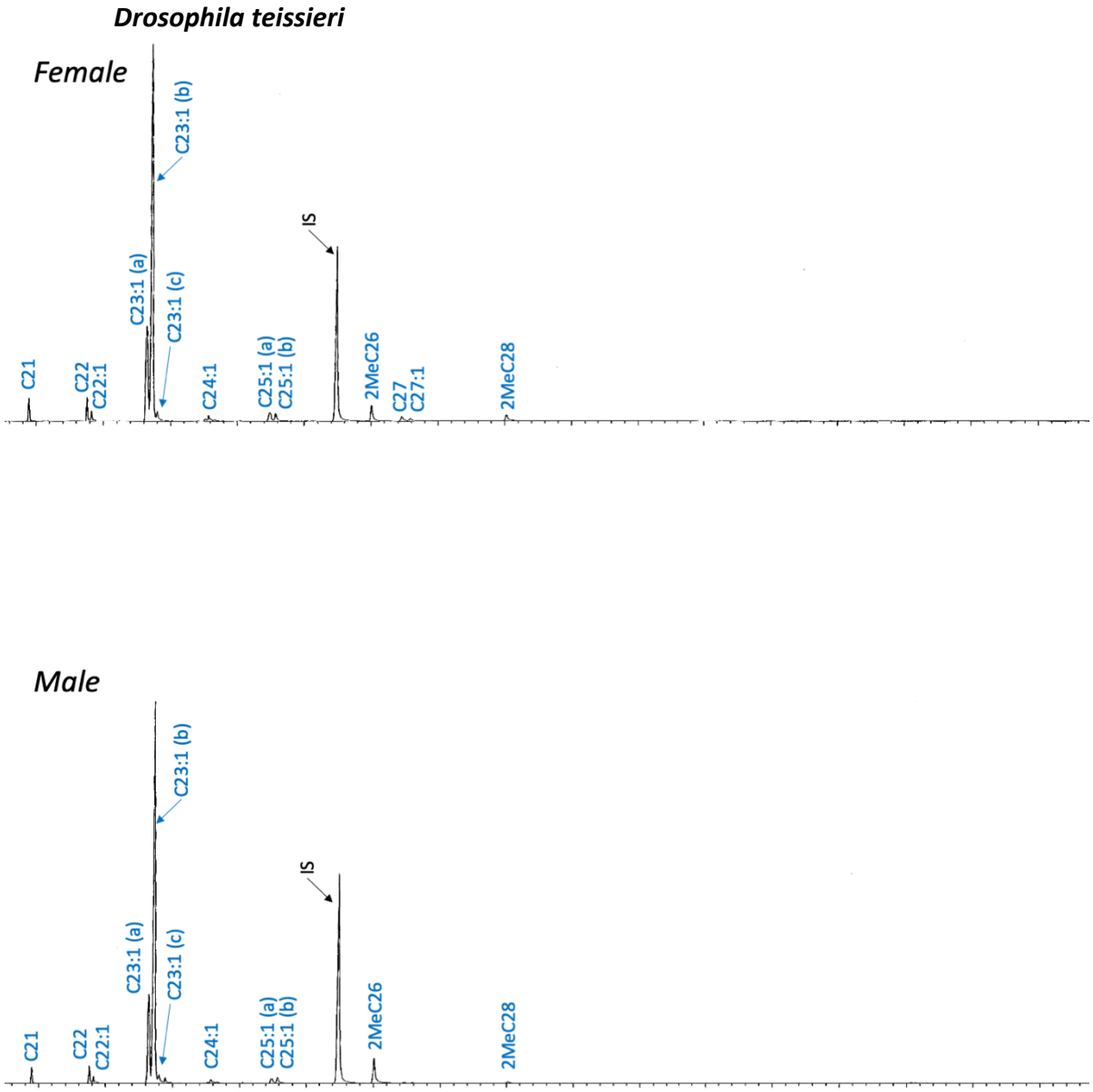
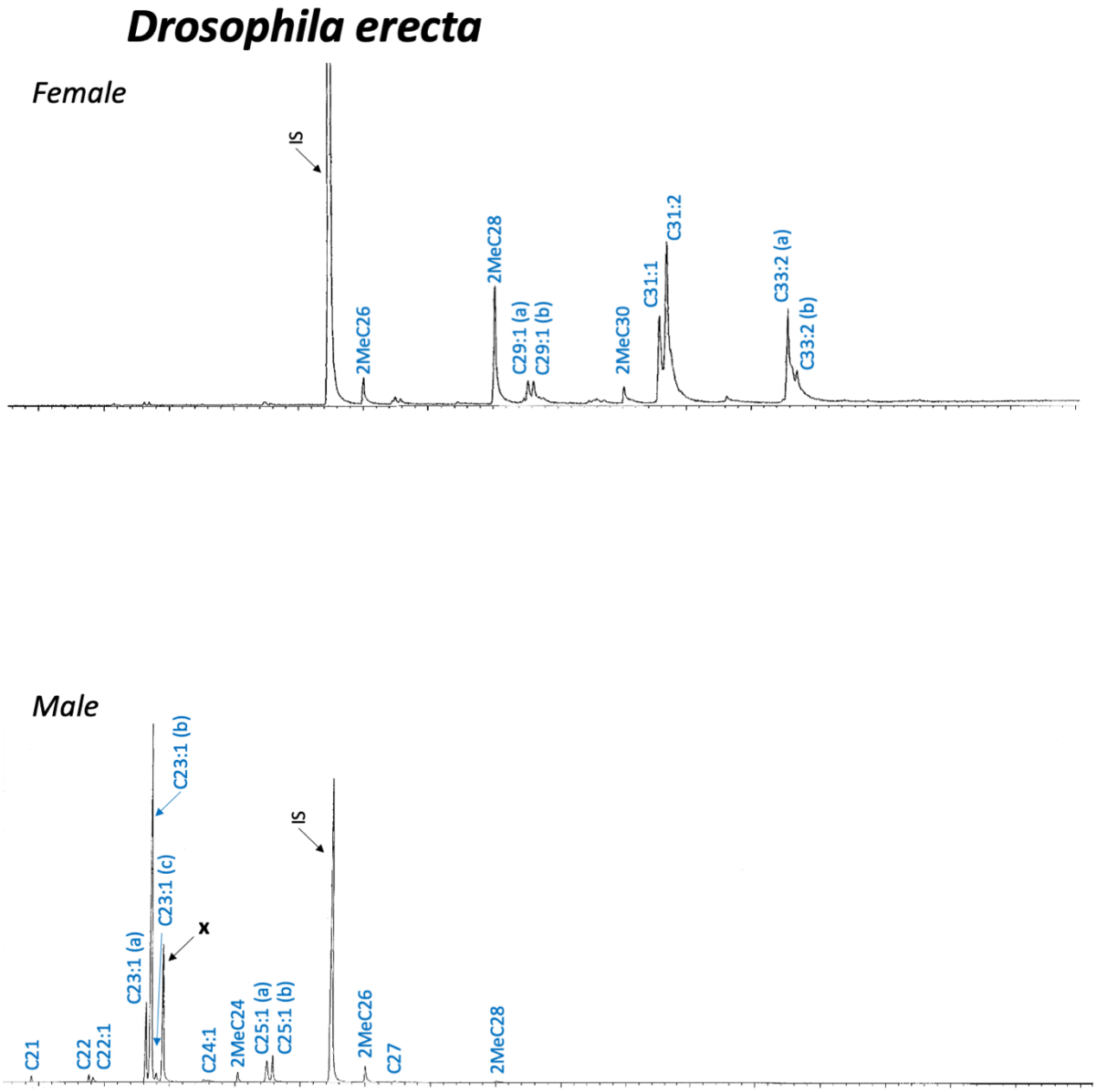


Figure A1.4. Chromatograms of CHCs for female and male *Drosophila mauritiana*.



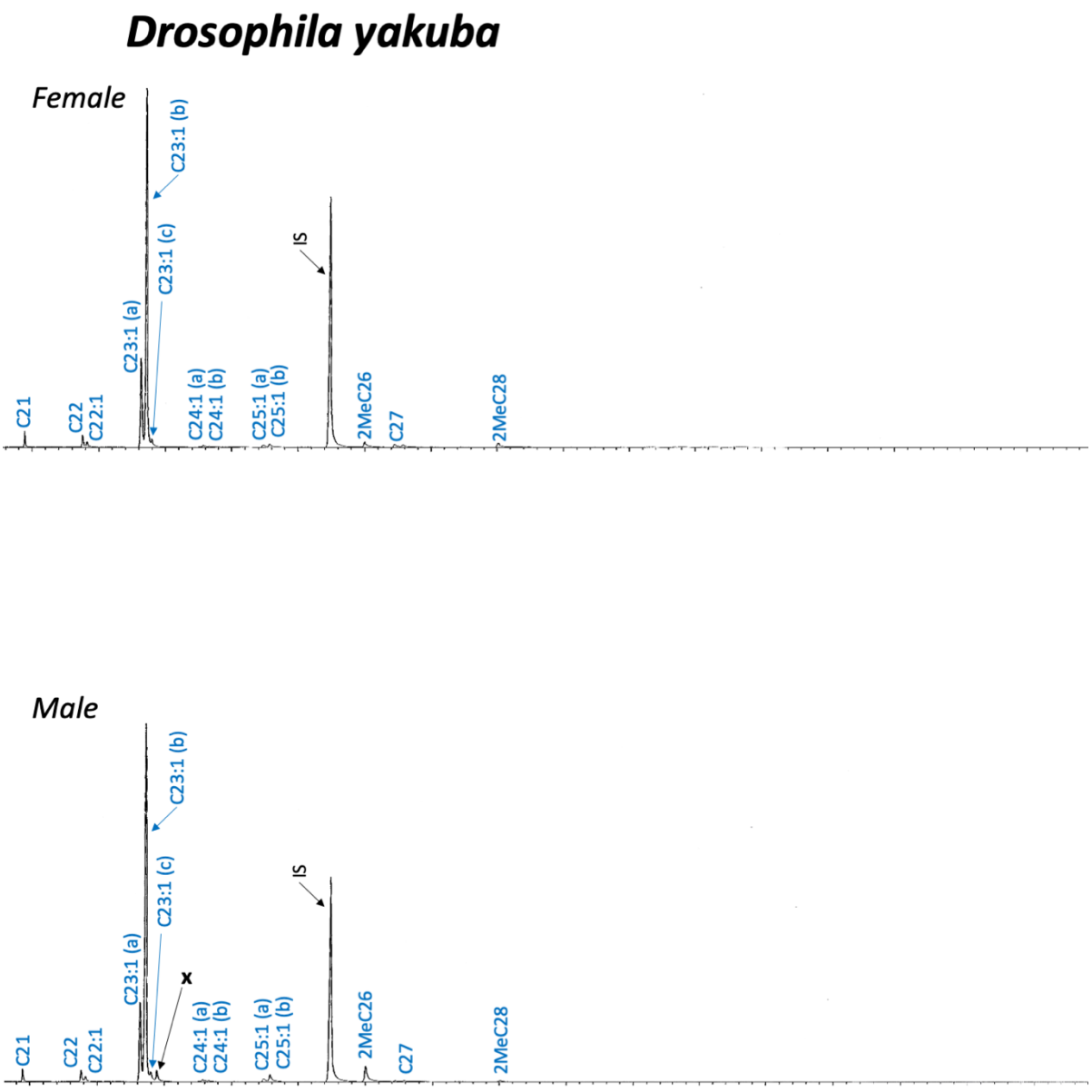
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.5. Chromatograms of CHCs for female and male *Drosophila teissieri*.



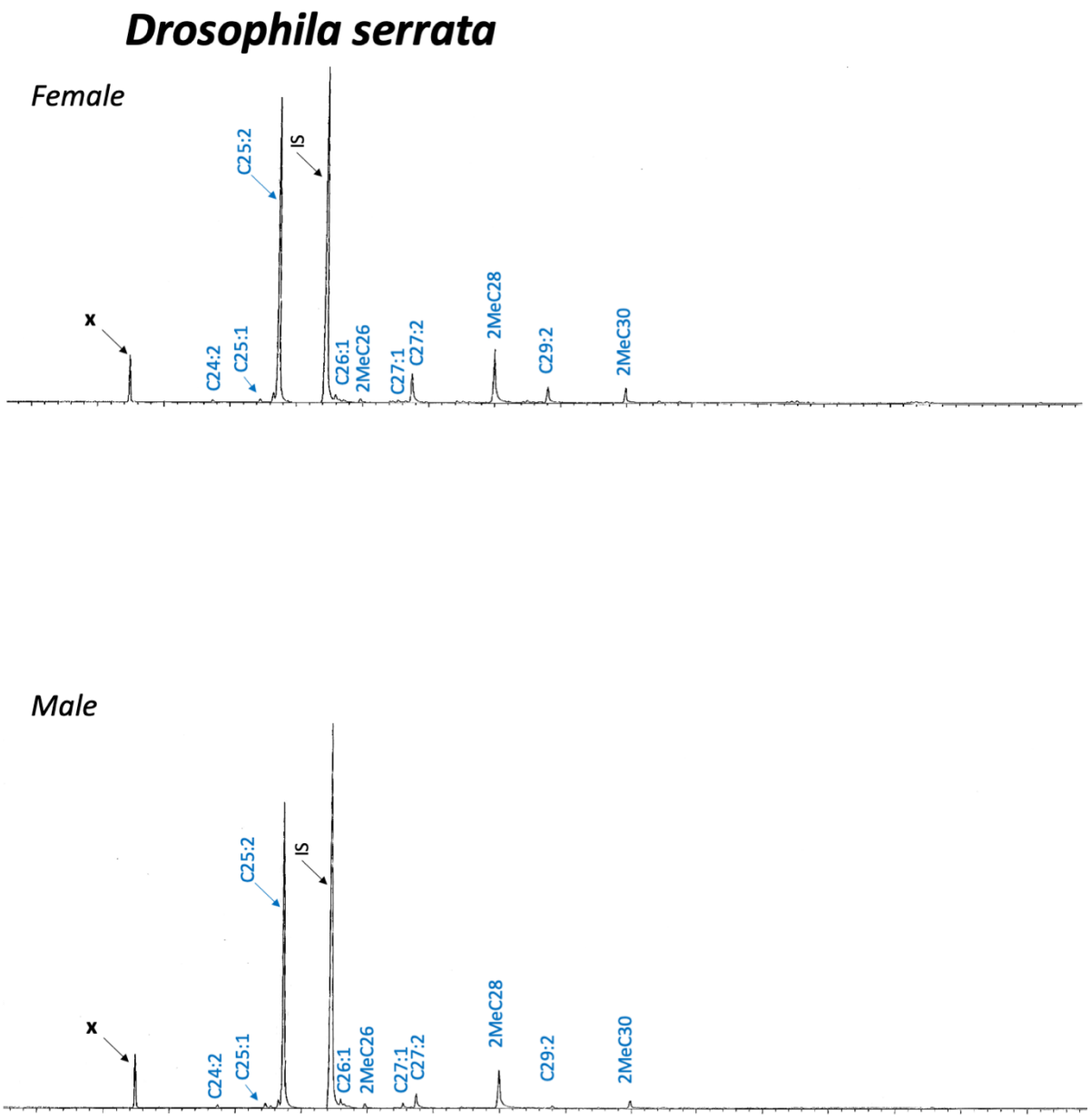
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.6. Chromatograms of CHCs for female and male *Drosophila erecta*.



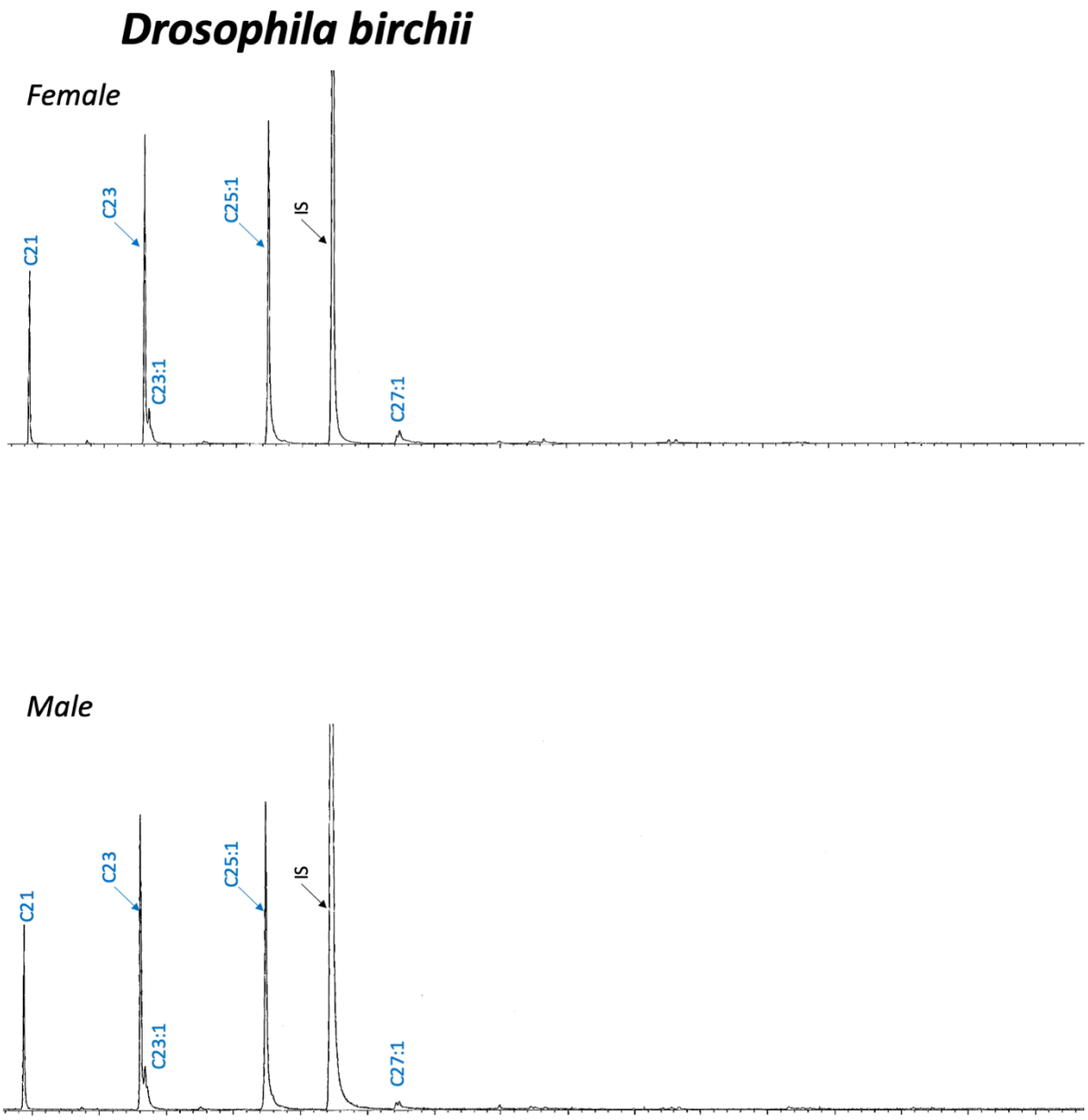
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.7. Chromatograms of CHCs for female and male *Drosophila yakuba*.



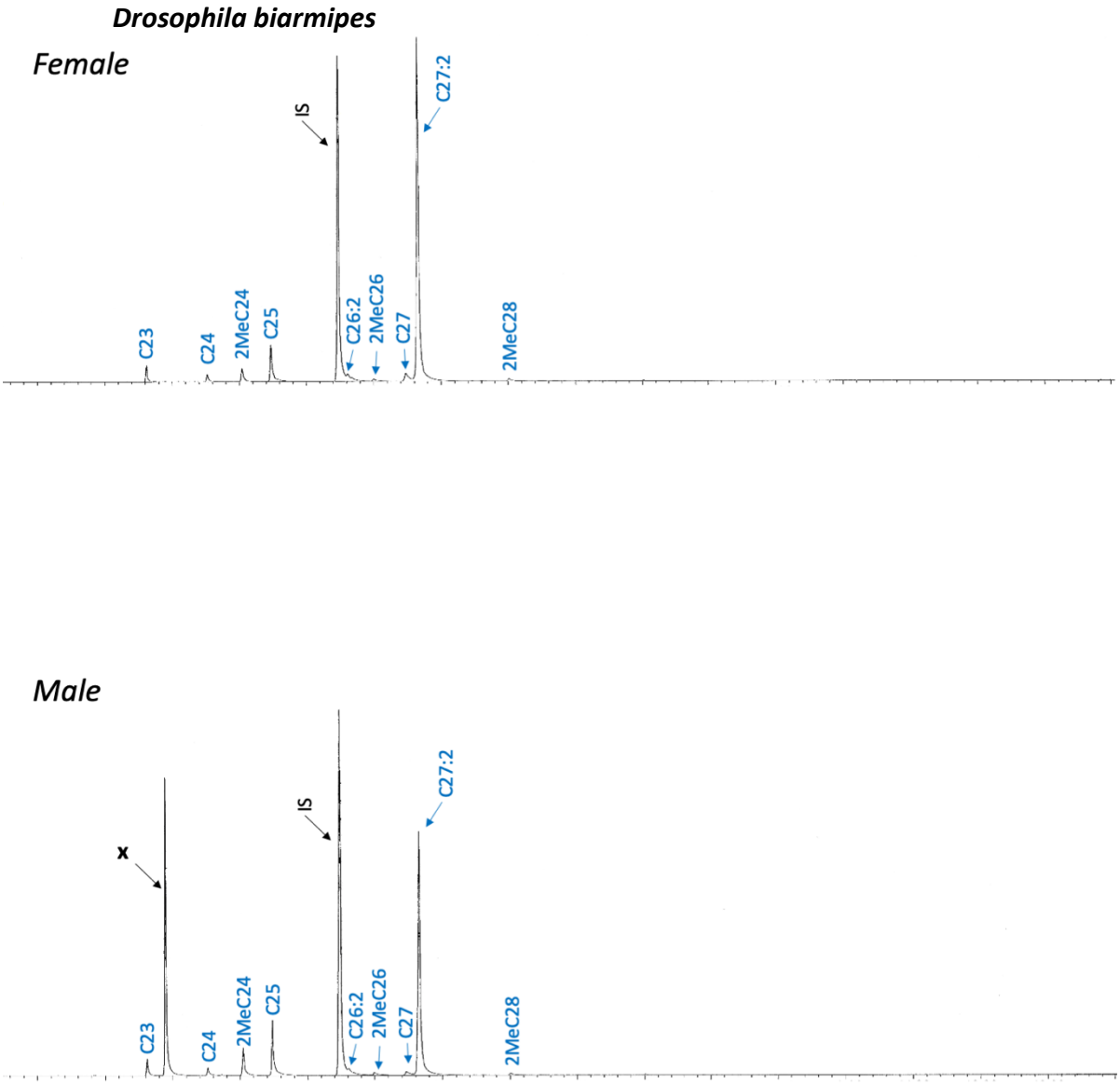
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.8. Chromatograms of CHCs for female and male *Drosophila serrata*.



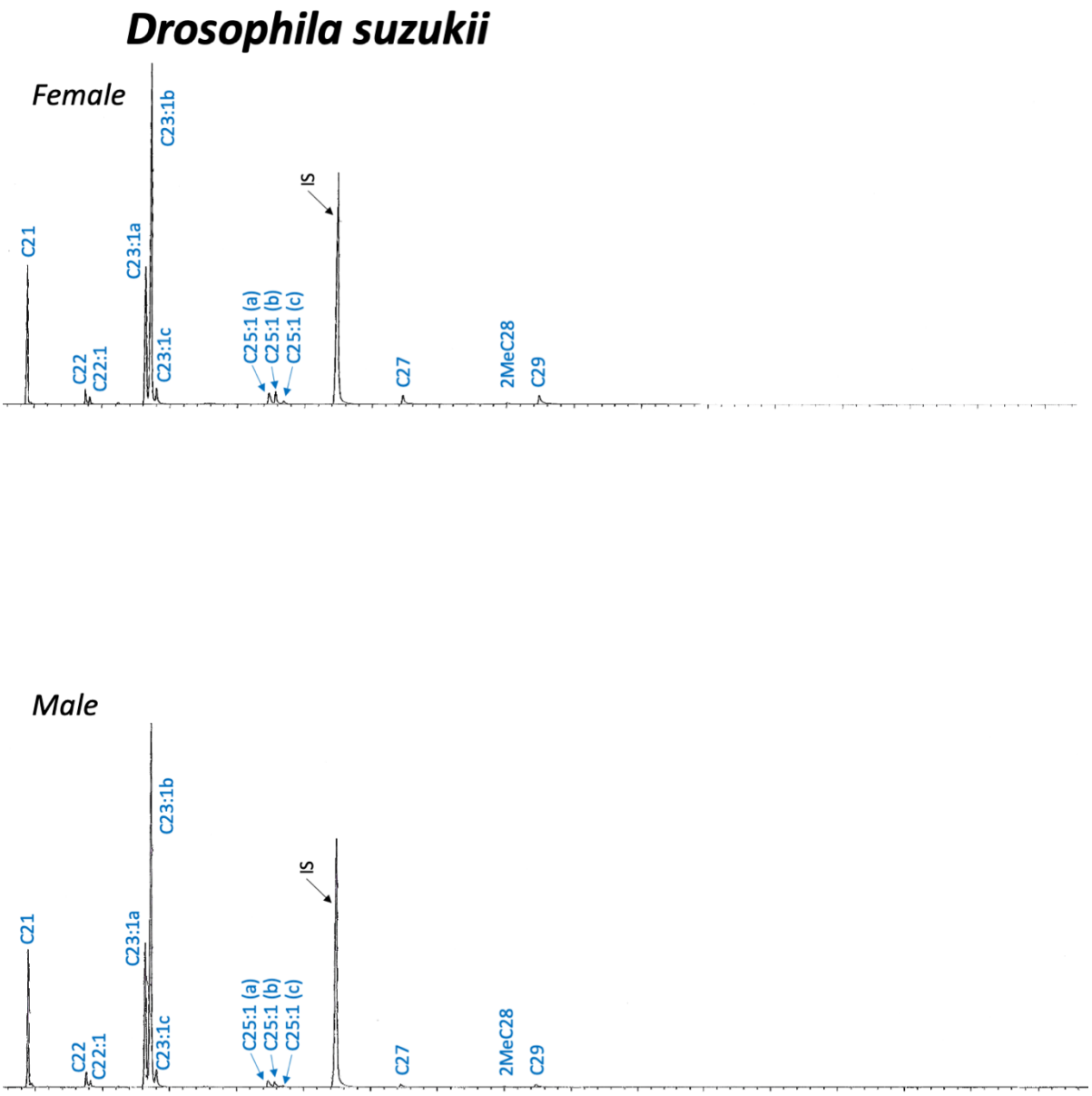
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.9. Chromatograms of CHCs for female and male *Drosophila birchii*.



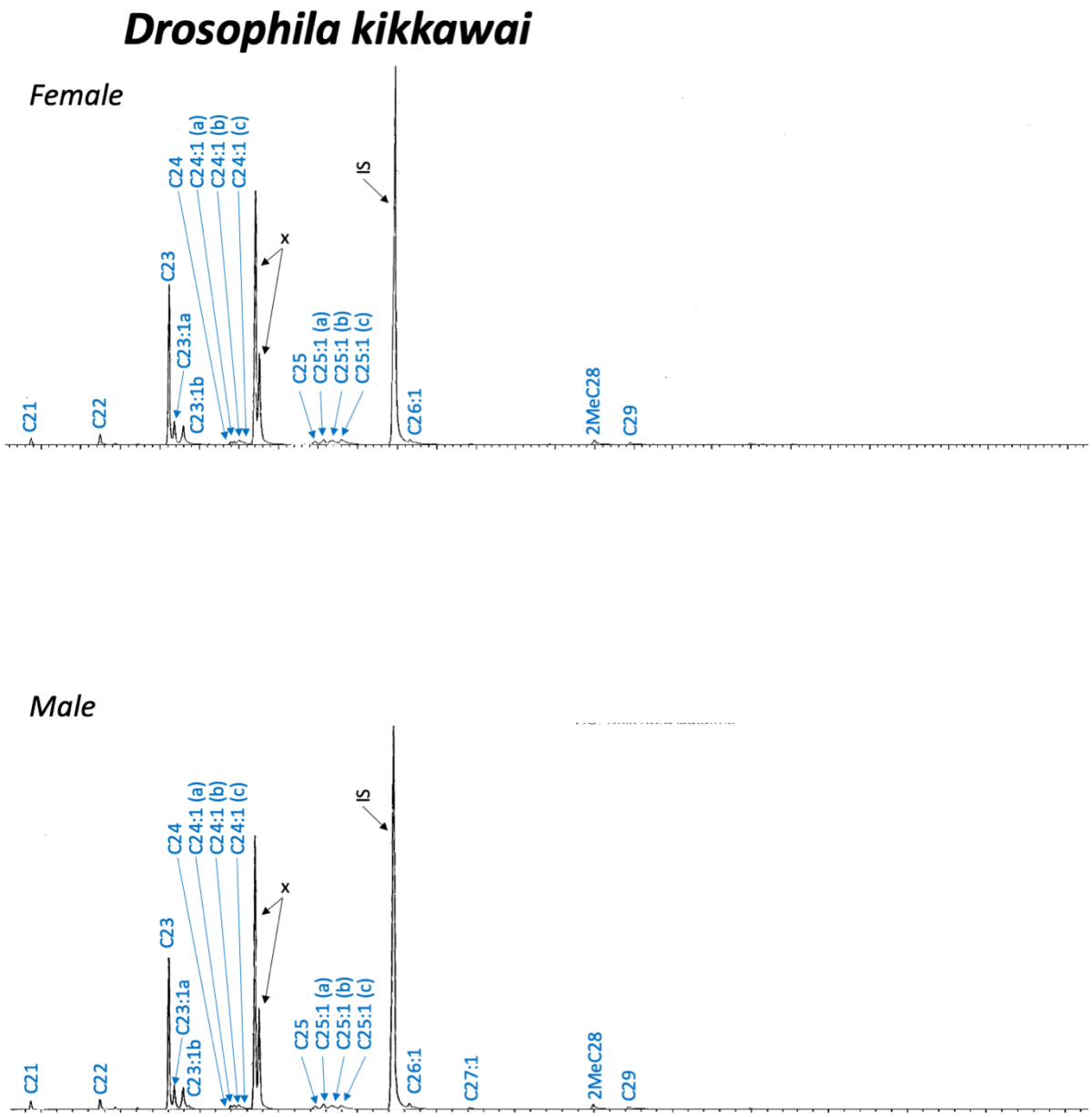
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.10. Chromatograms of CHCs for female and male *Drosophila biarmipes*.



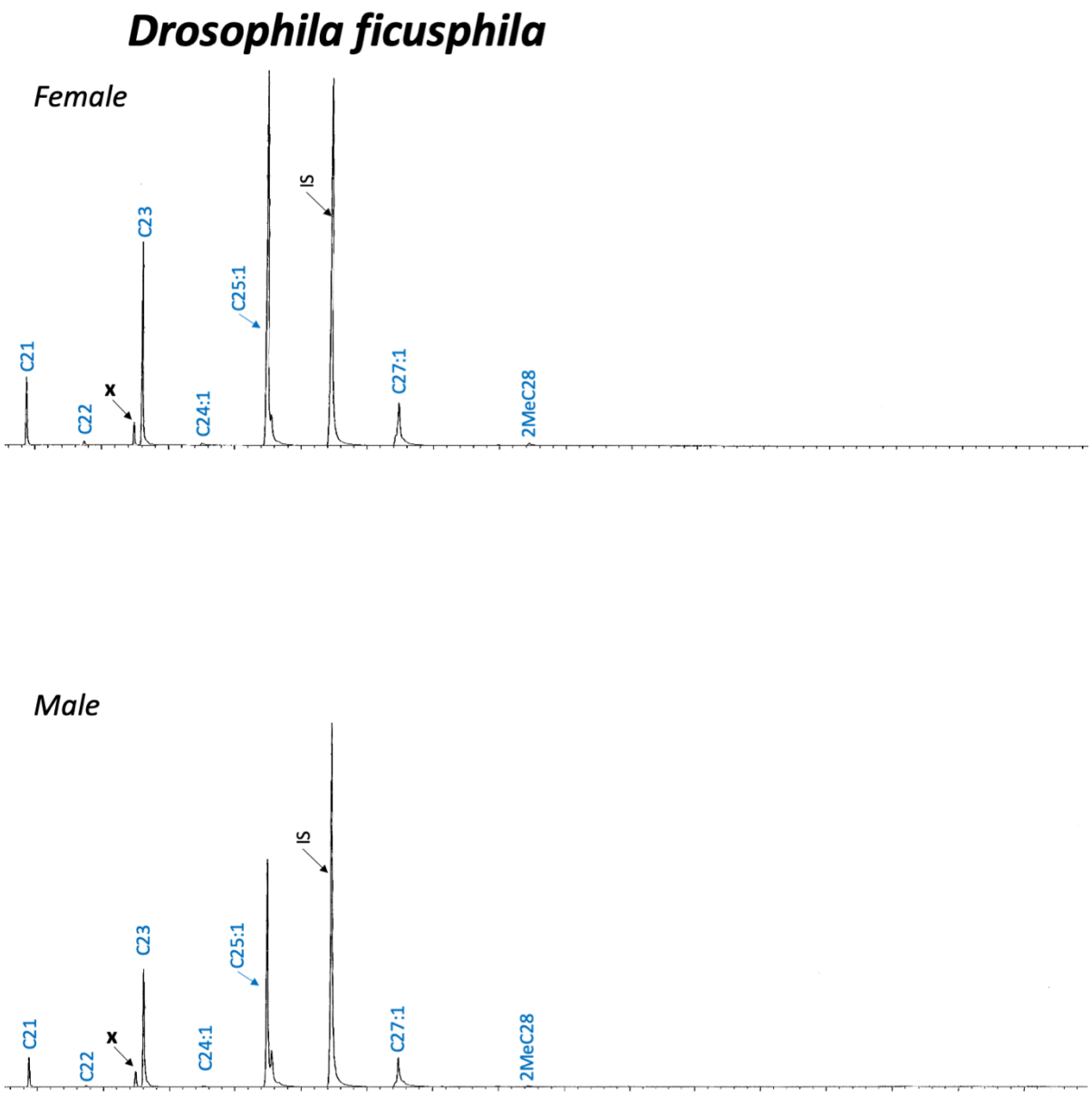
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.11. Chromatograms of CHCs for female and male *Drosophila suzukii*.



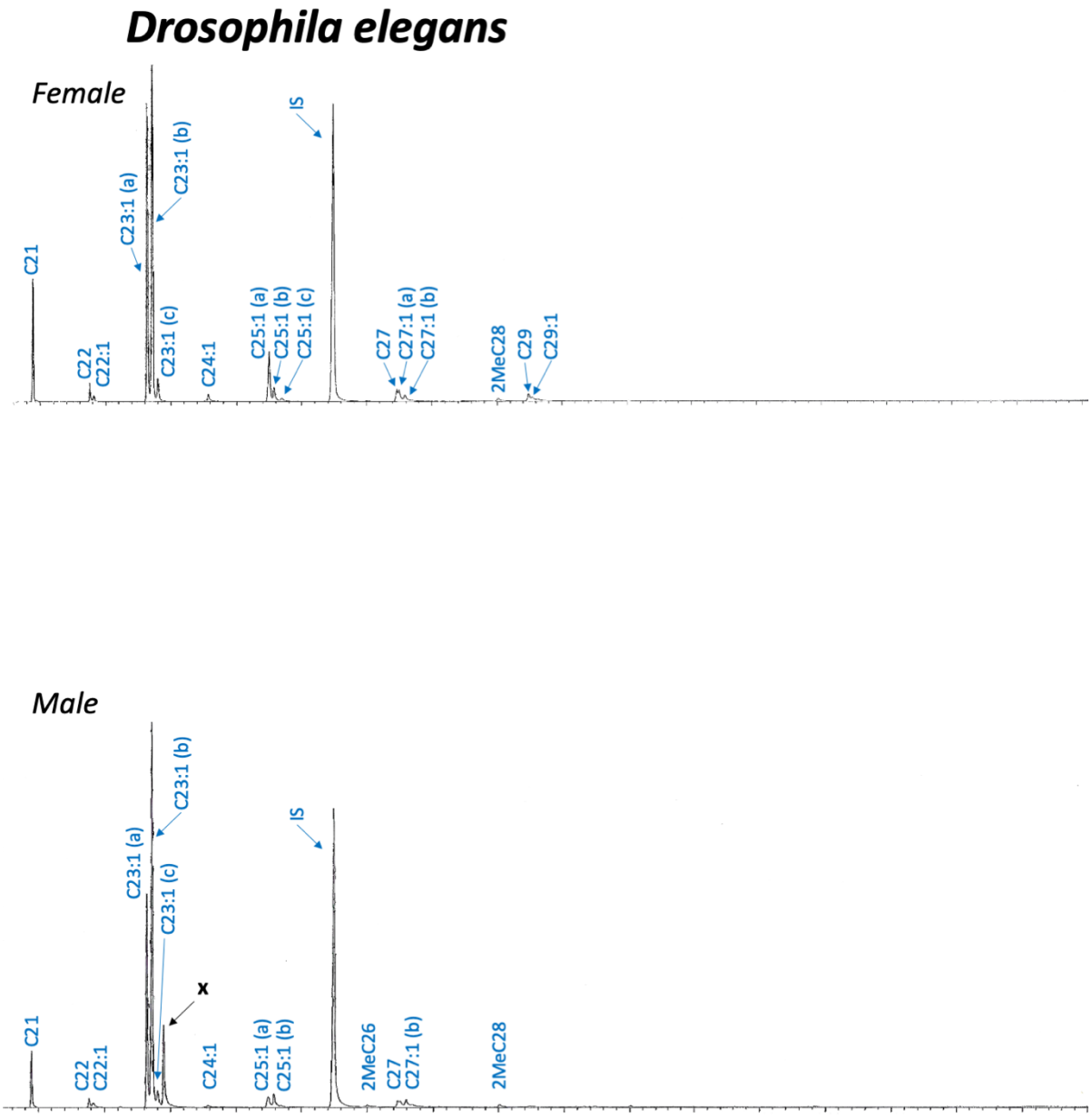
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.12. Chromatograms of CHCs for female and male *Drosophila kikkawai*.



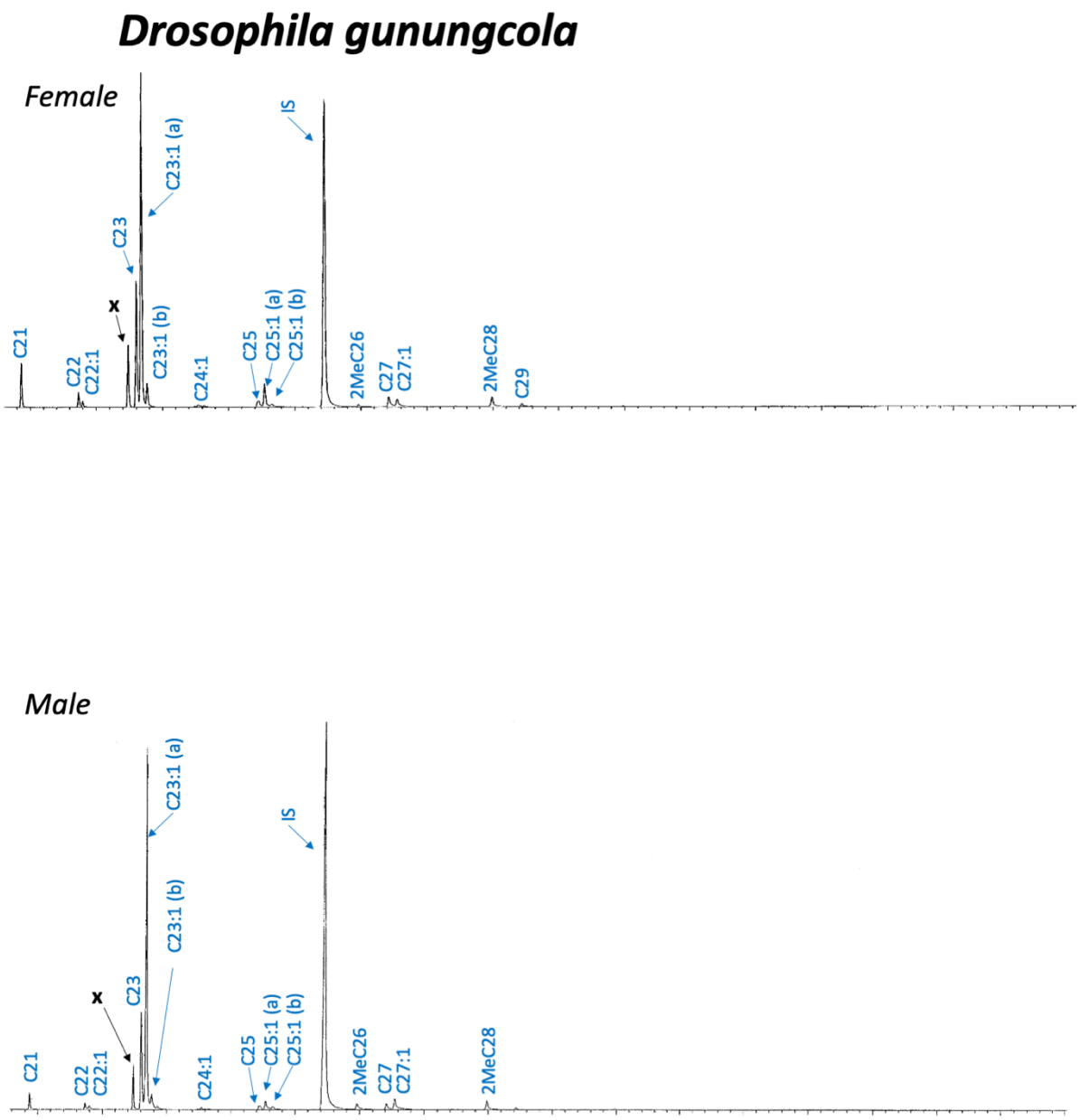
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.13. Chromatograms of CHCs for female and male *Drosophila ficusphila*.



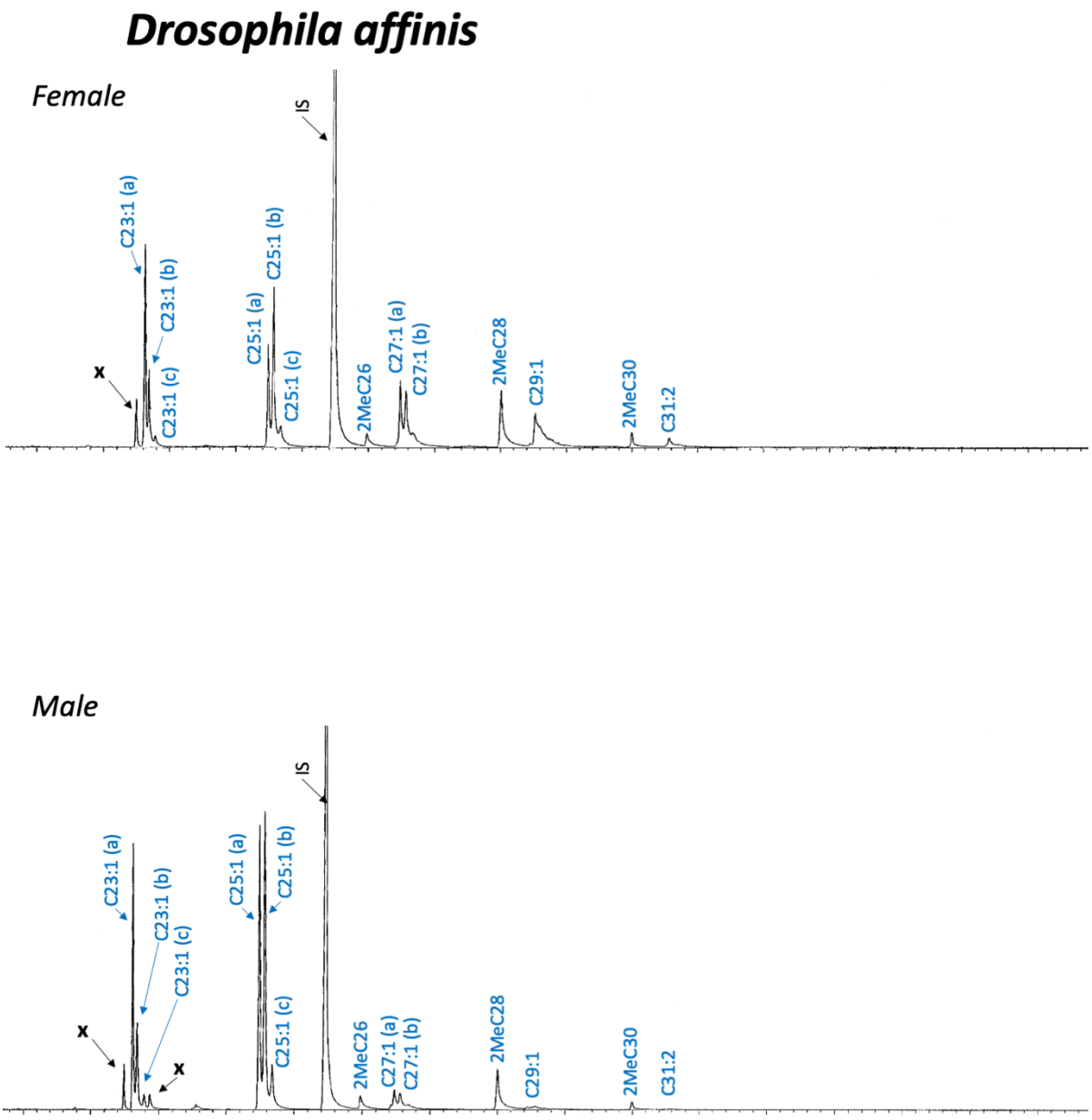
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.14. Chromatograms of CHCs for female and male *Drosophila elegans*.



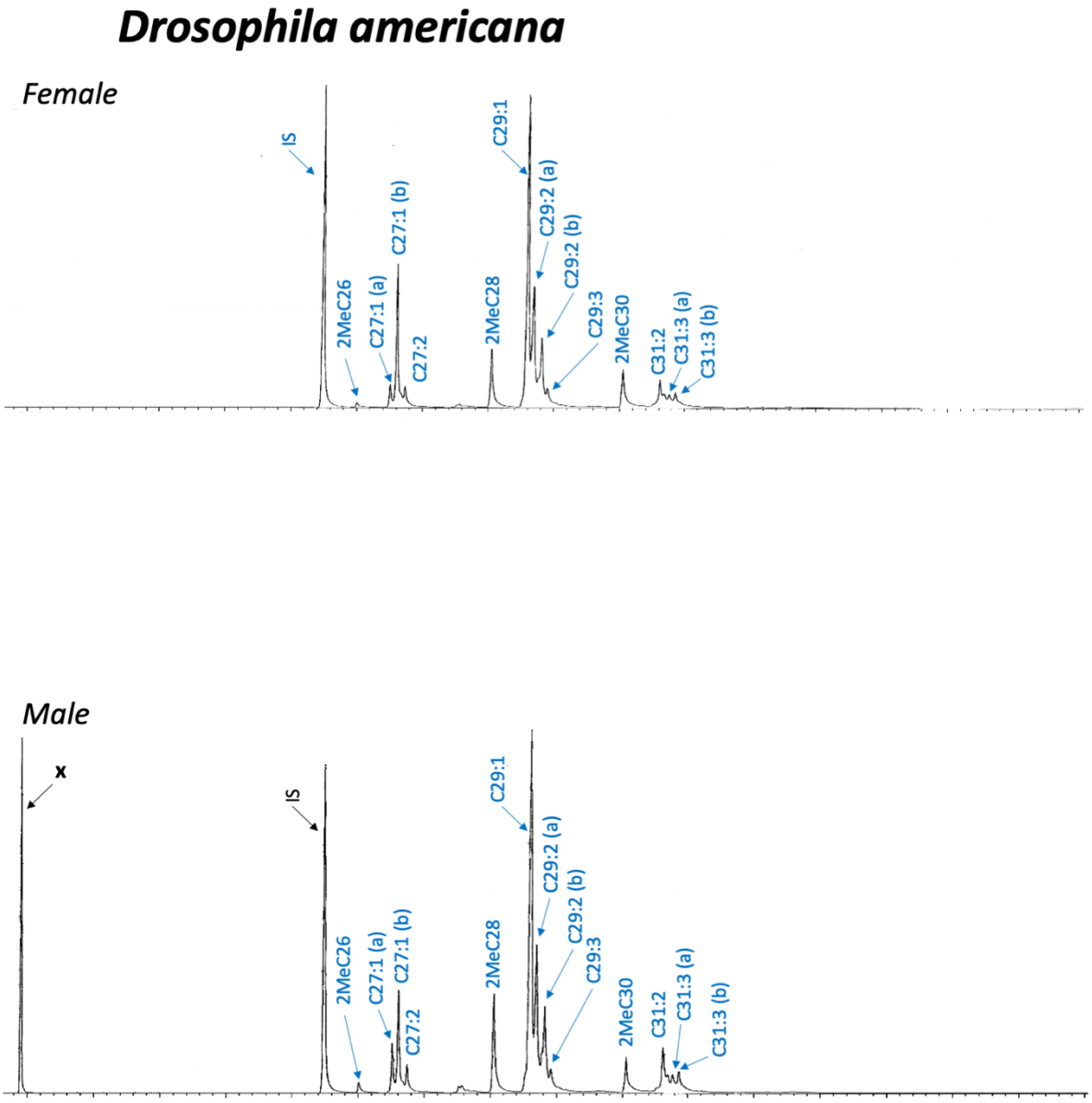
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.15. Chromatograms of CHCs for female and male *Drosophila gunungcola*.



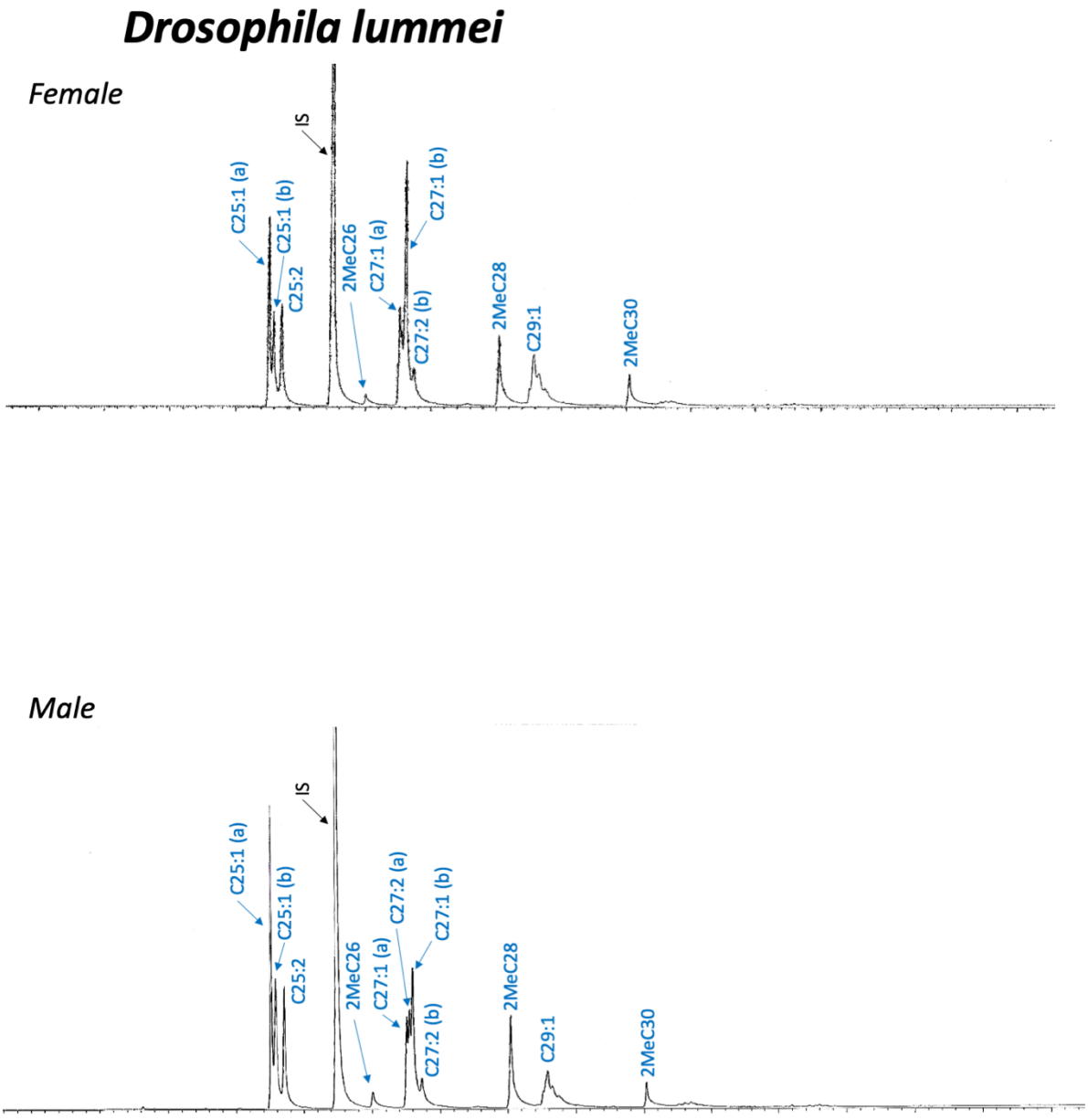
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.16. Chromatograms of CHCs for female and male *Drosophila affinis*.



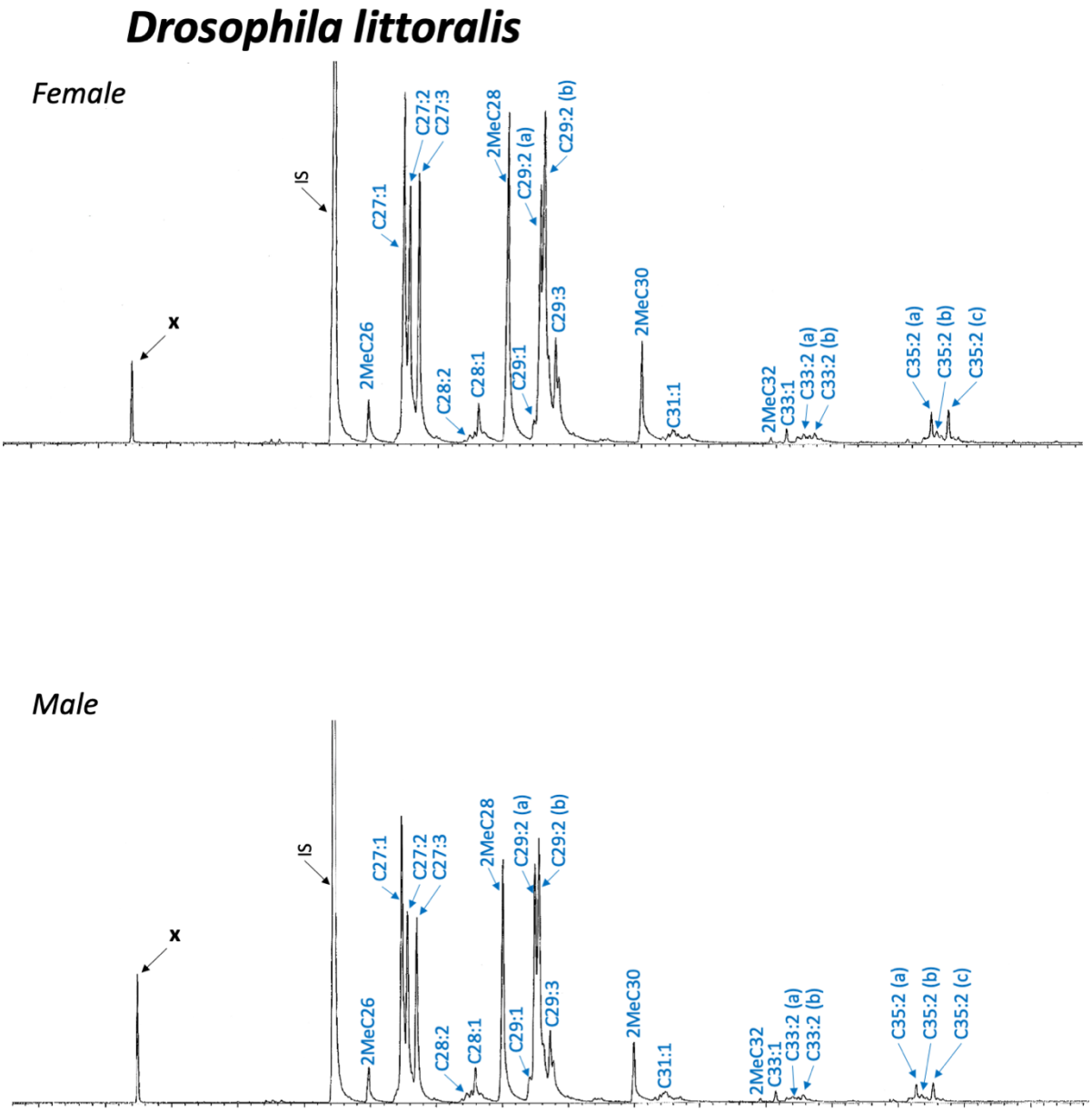
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.17. Chromatograms of CHCs for female and male *Drosophila americana*.



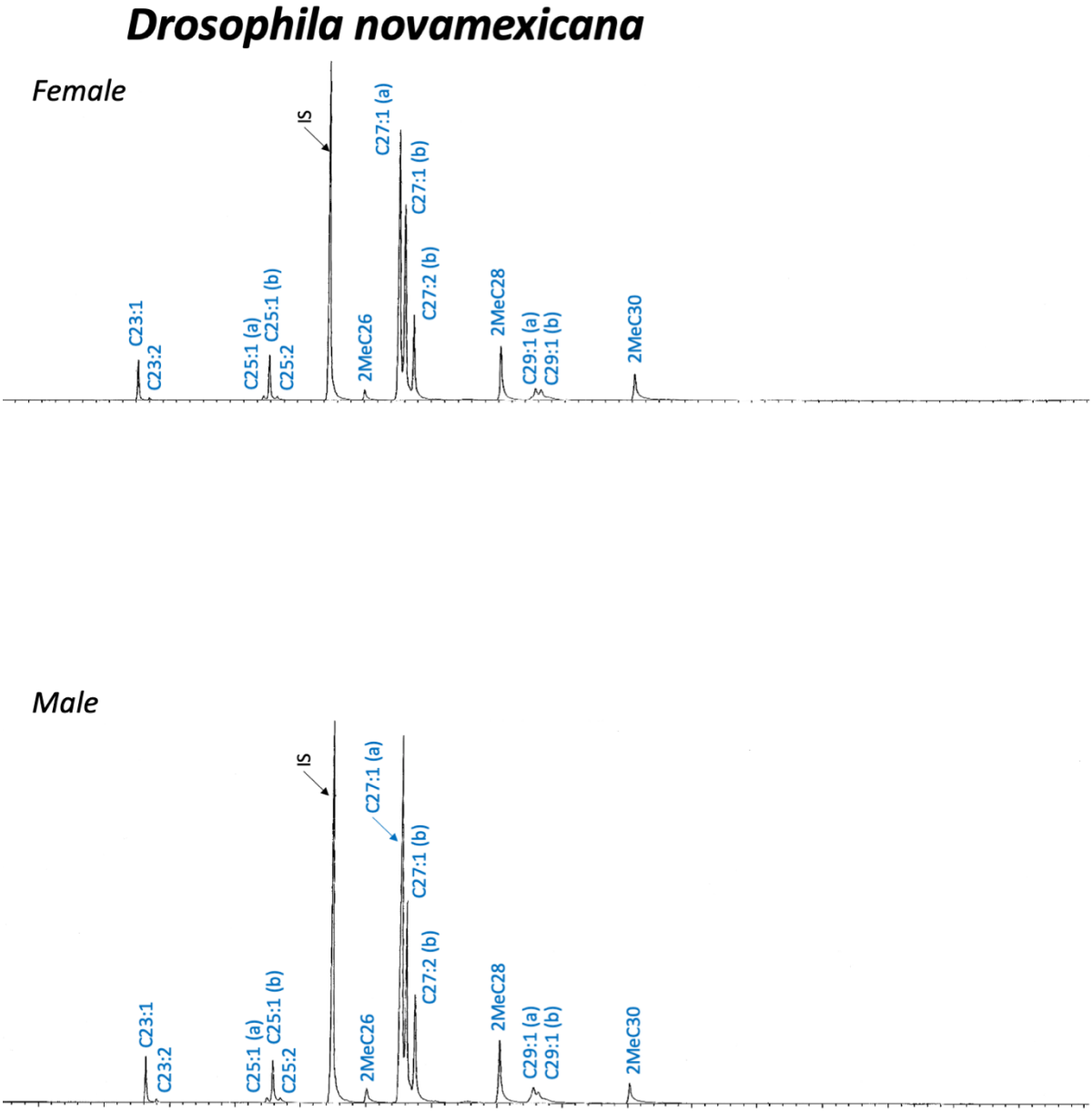
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.18. Chromatograms of CHCs for female and male *Drosophila lummei*.



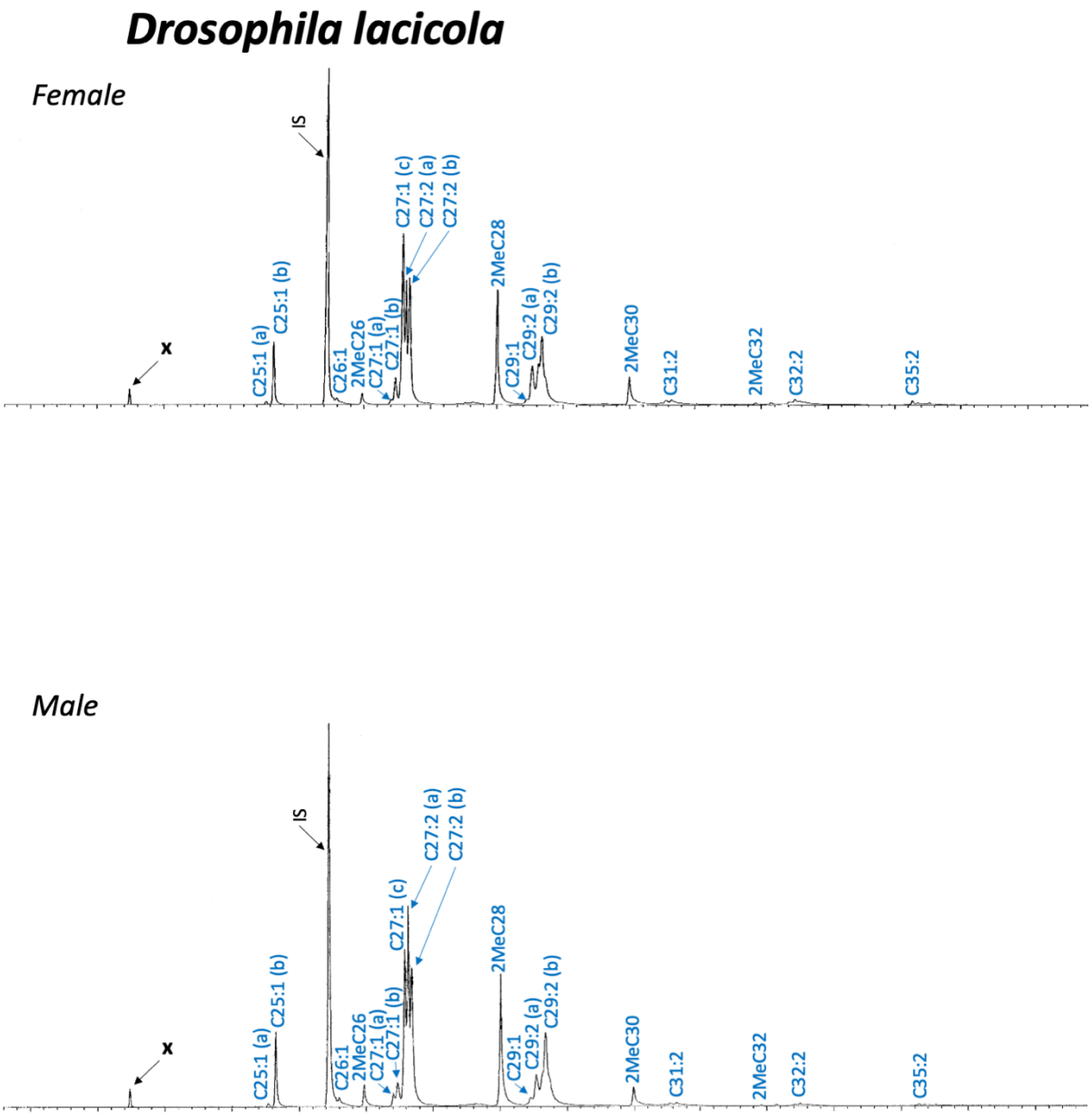
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.19. Chromatograms of CHCs for female and male *Drosophila littoralis*.



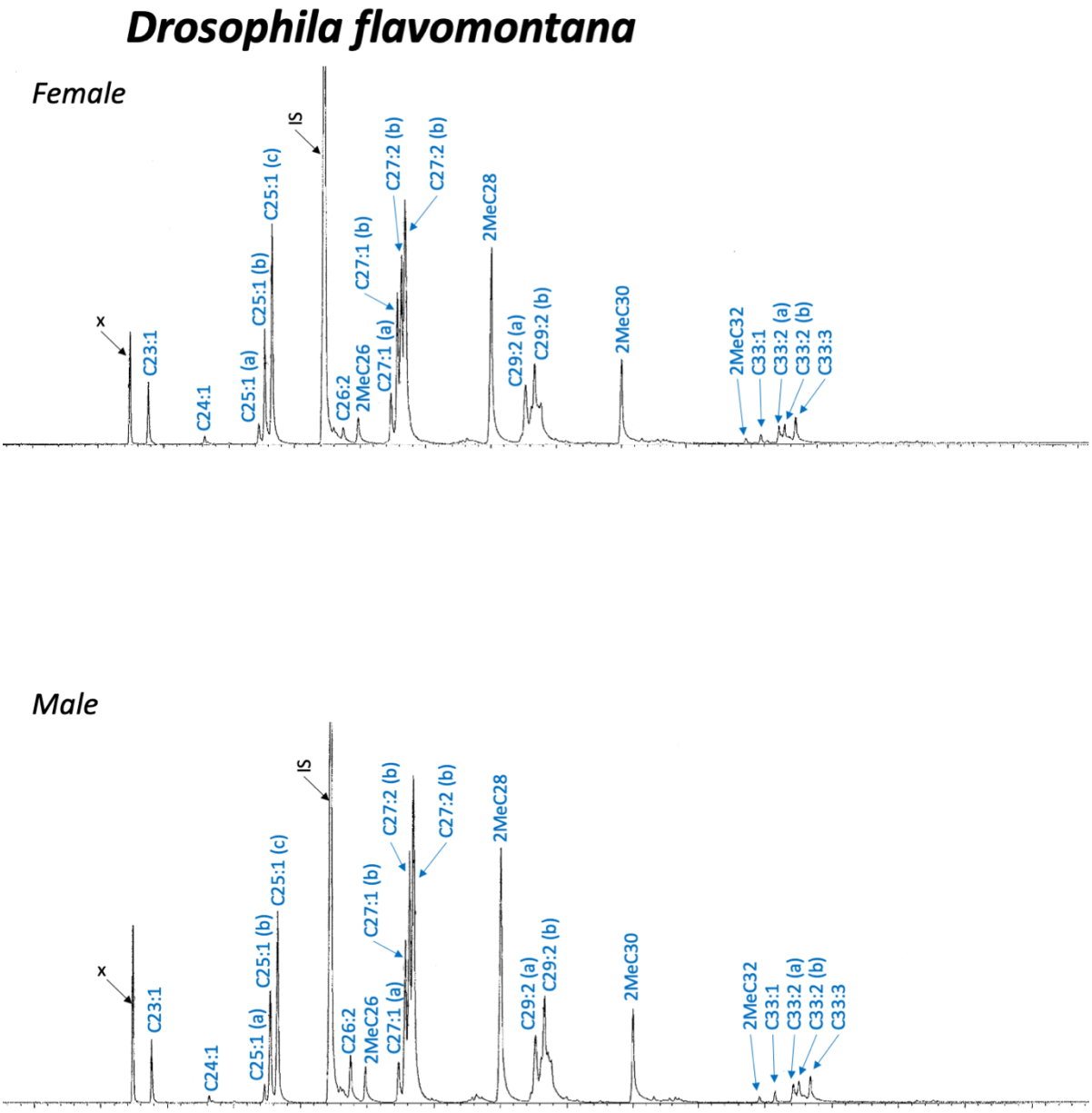
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.20. Chromatograms of CHCs for female and male *Drosophila novamexicana*.



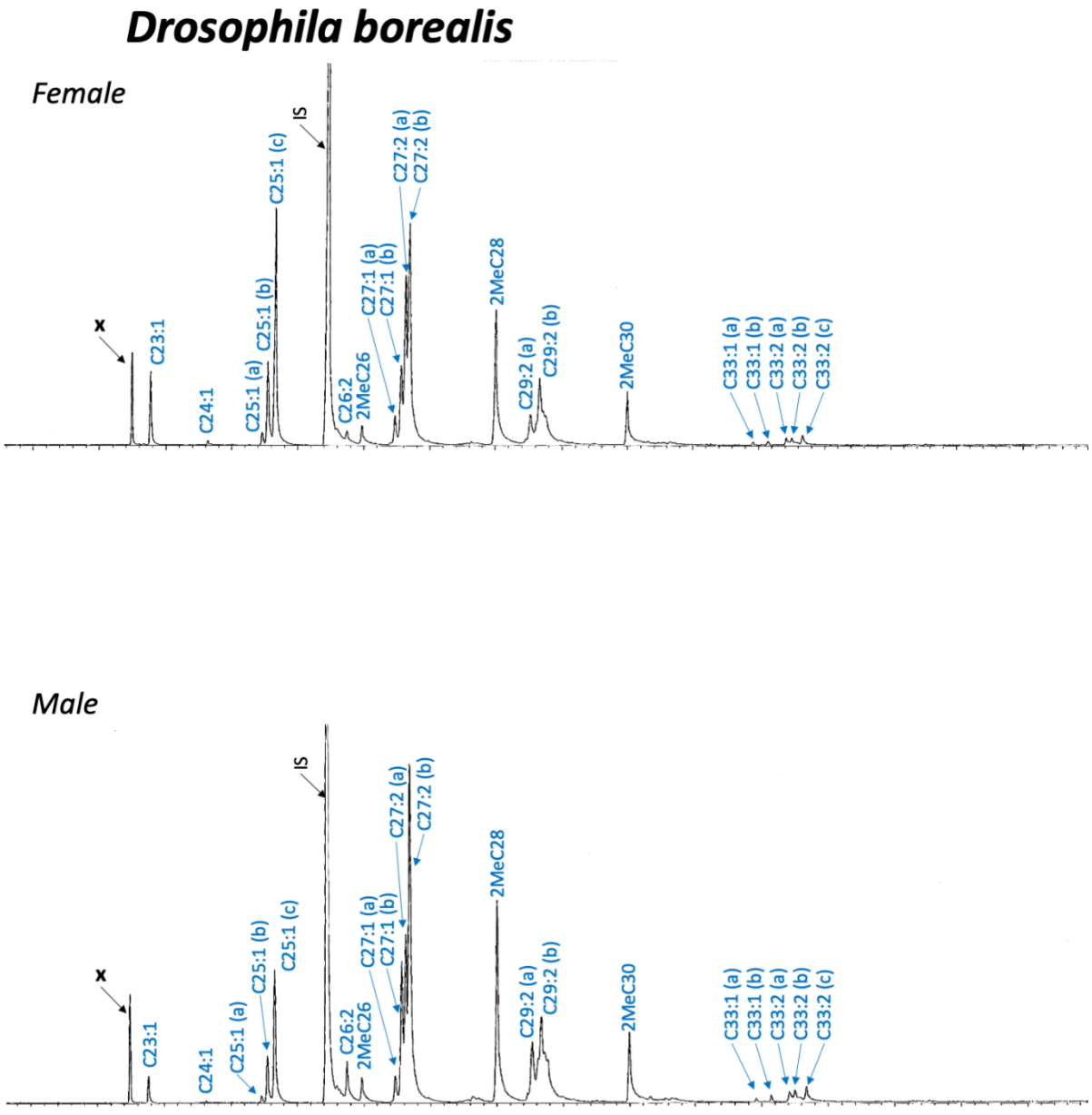
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.21. Chromatograms of CHCs for female and male *Drosophila lacicola*.



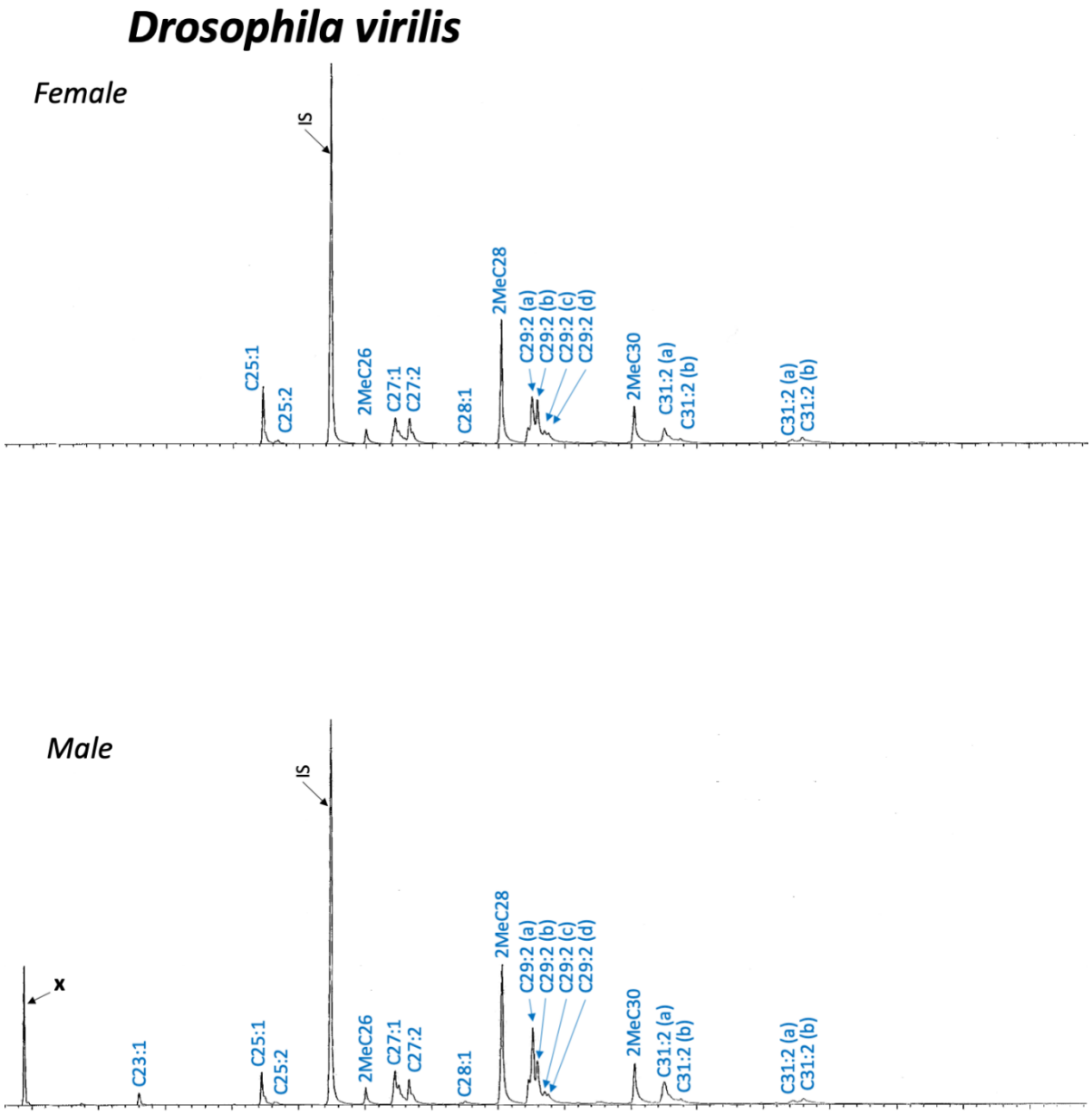
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.22. Chromatograms of CHCs for female and male *Drosophila flavomontana*.



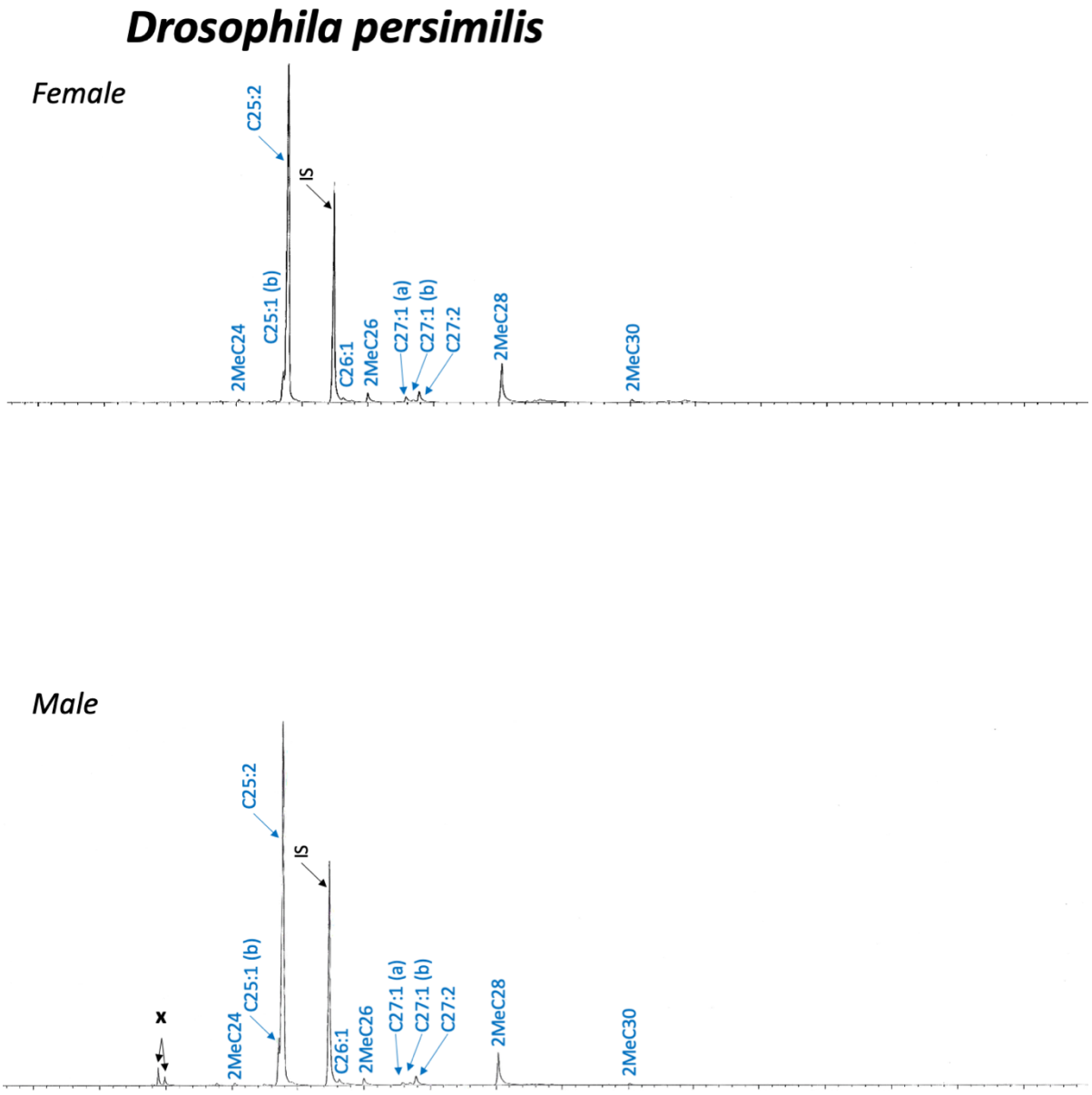
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.23. Chromatograms of CHCs for female and male *Drosophila borealis*.



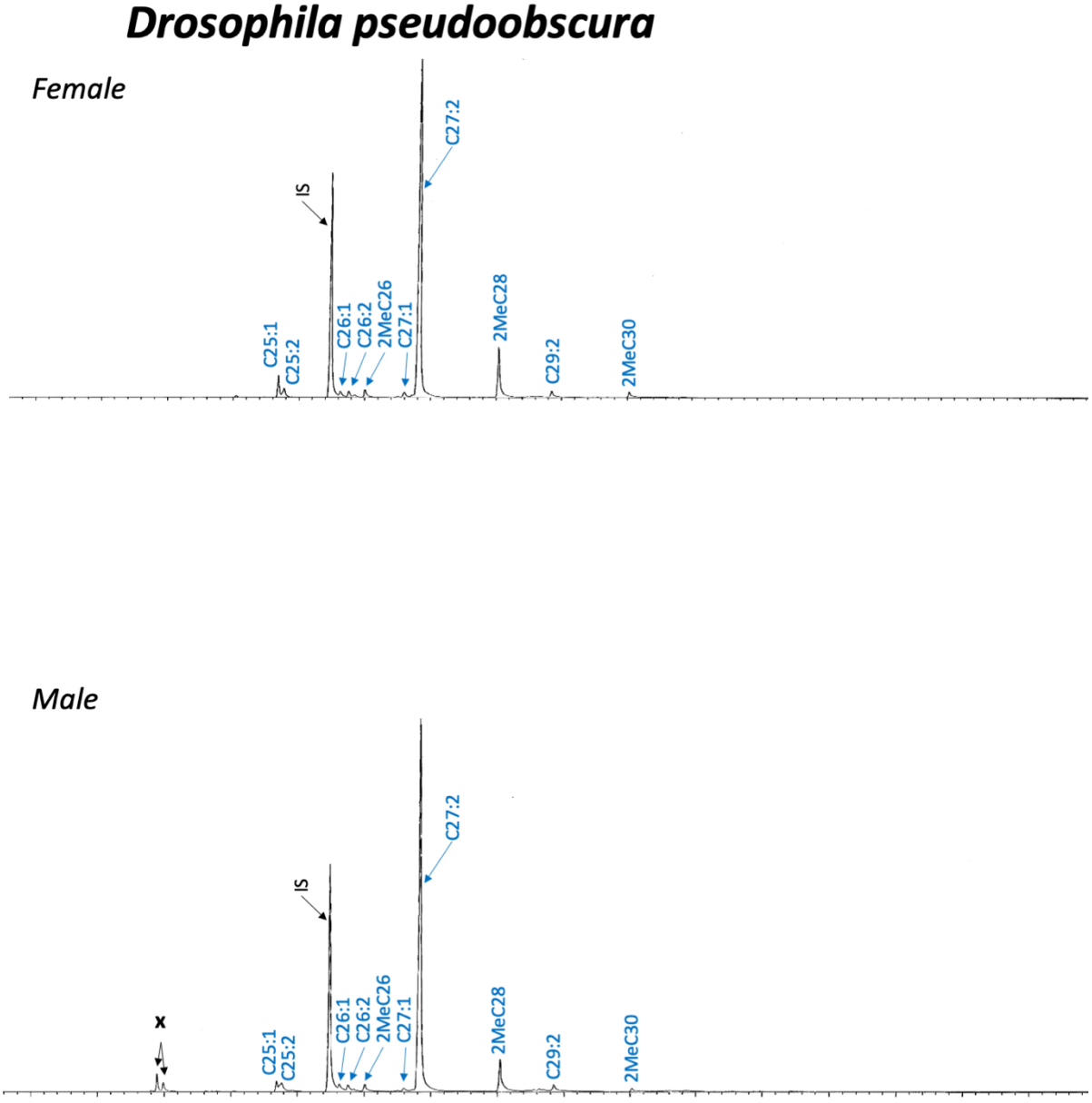
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.24. Chromatograms of CHCs for female and male *Drosophila virilis*.



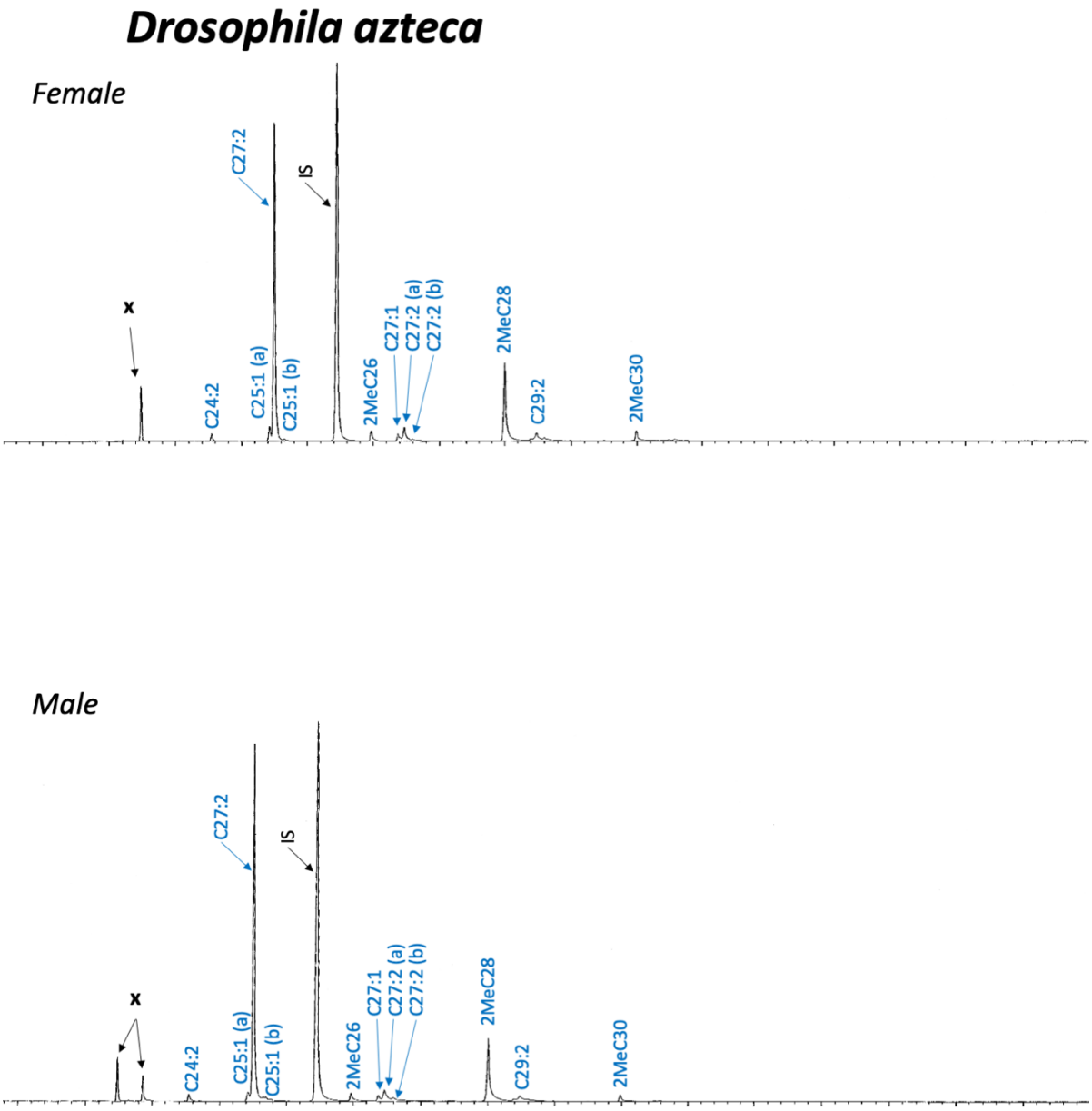
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.25. Chromatograms of CHCs for female and male *Drosophila persimilis*.



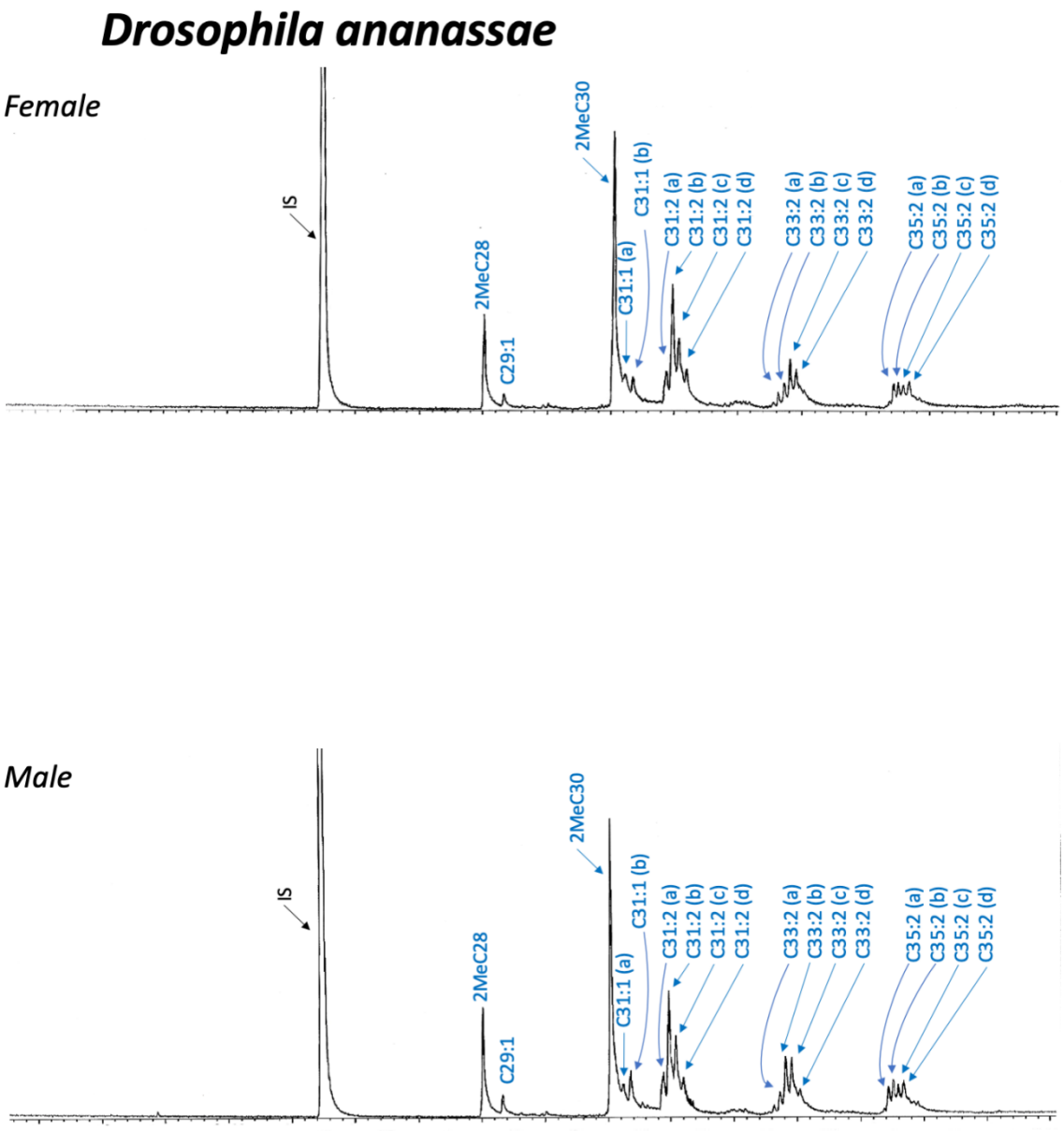
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.26. Chromatograms of CHCs for female and male *Drosophila pseudoobscura*.



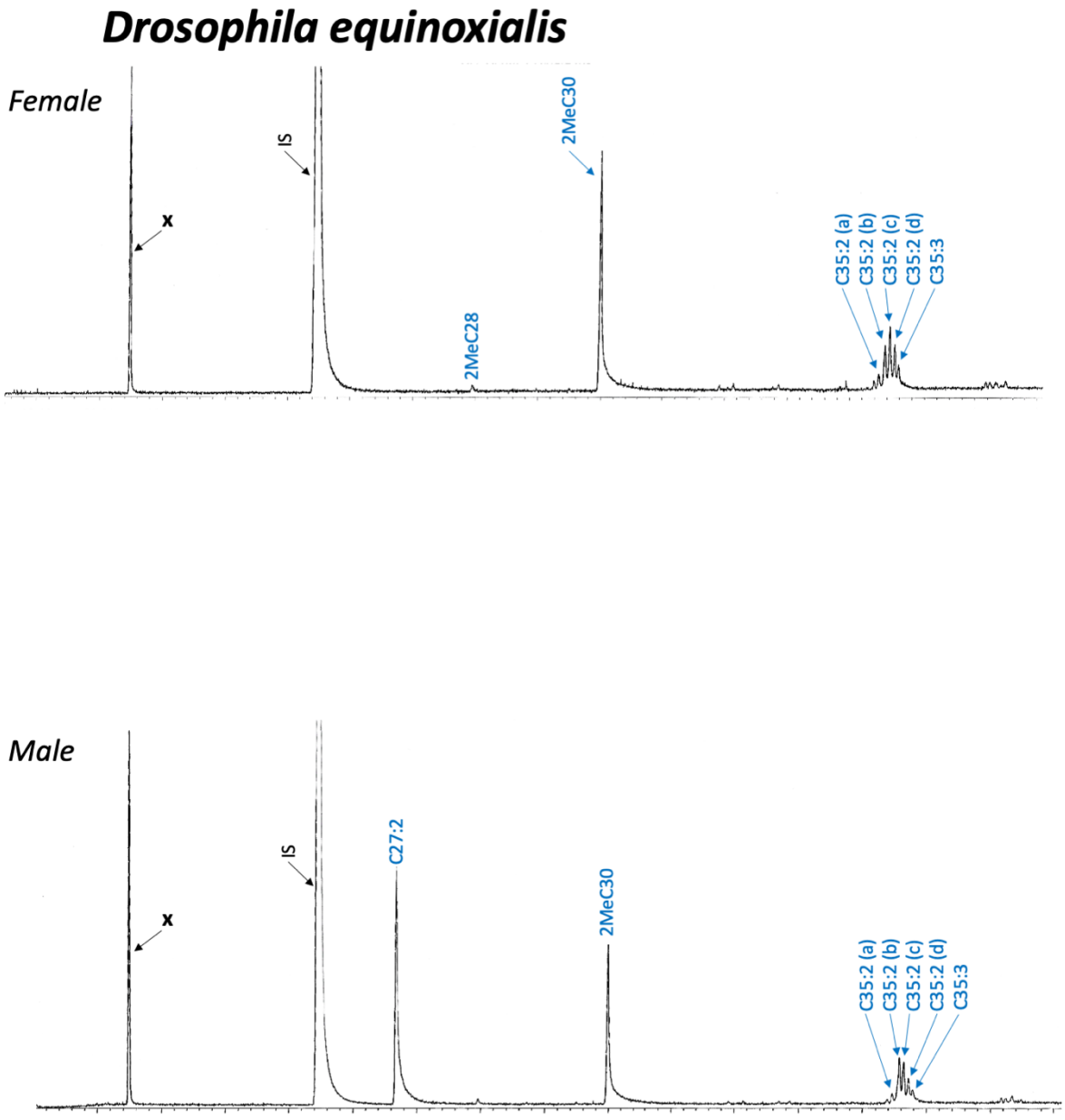
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.27. Chromatograms of CHCs for female and male *Drosophila azteca*.



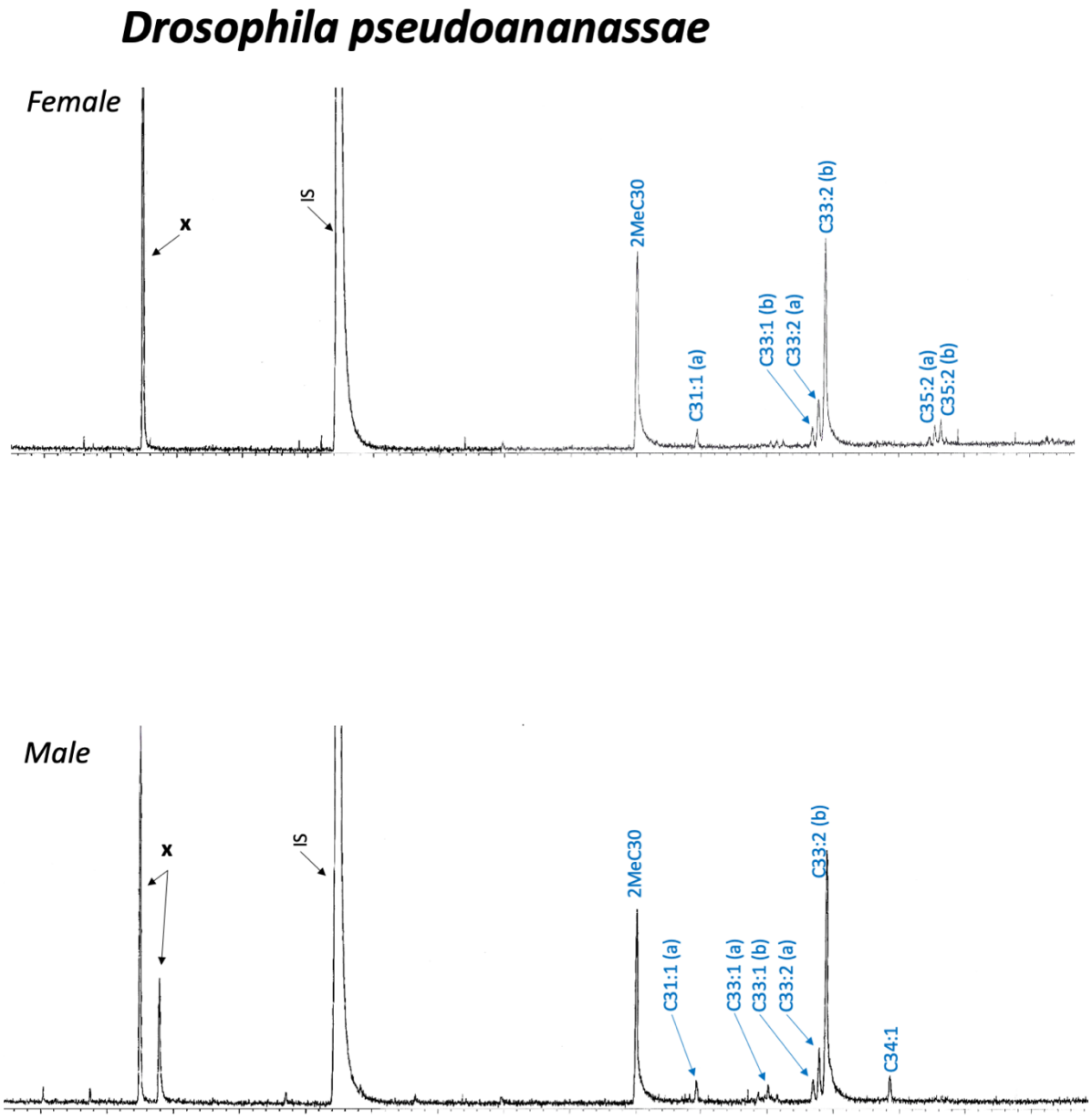
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.28. Chromatograms of CHCs for female and male *Drosophila ananassae*.



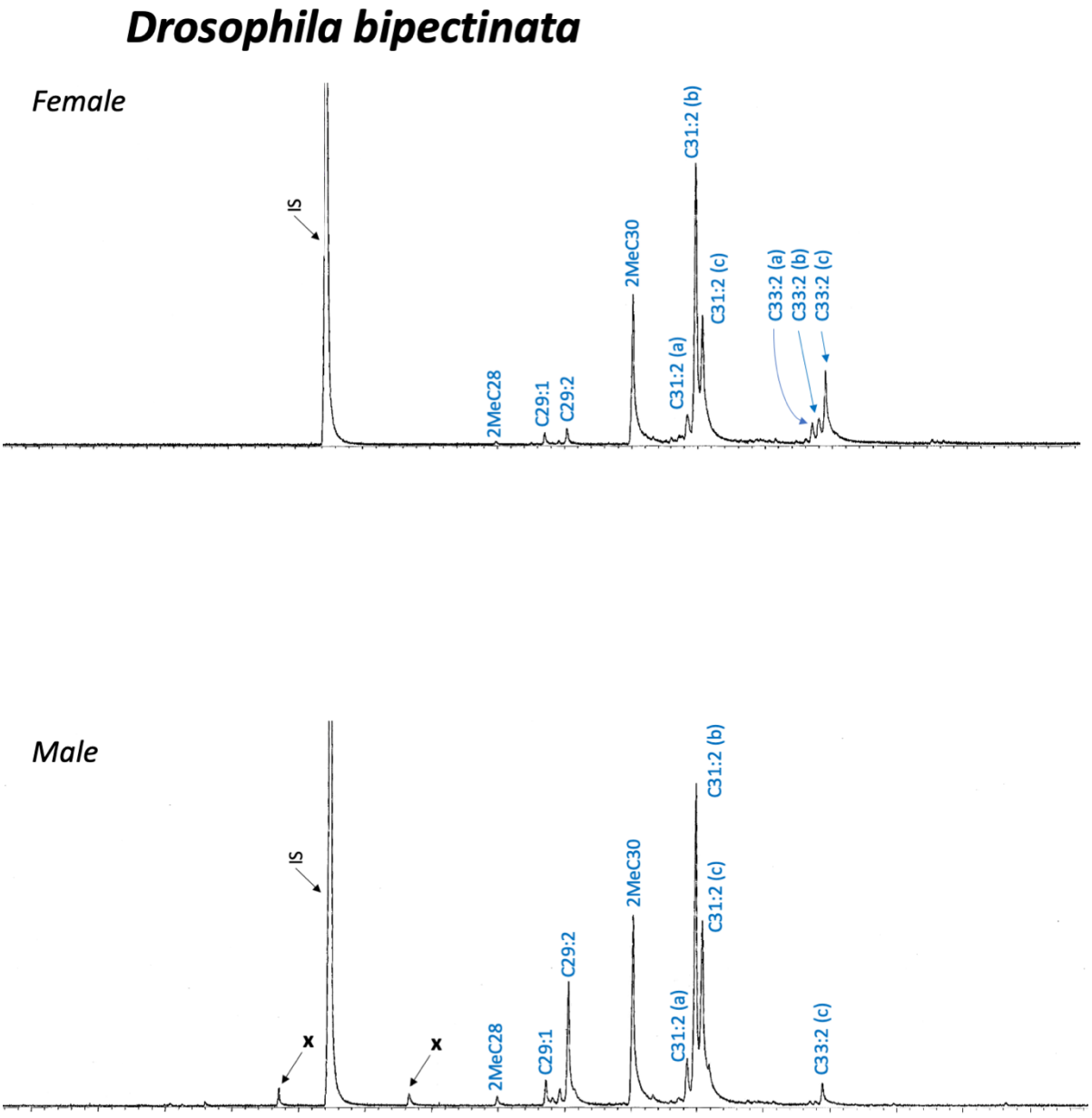
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.29. Chromatograms of CHCs for female and male *Drosophila equinoxialis*.



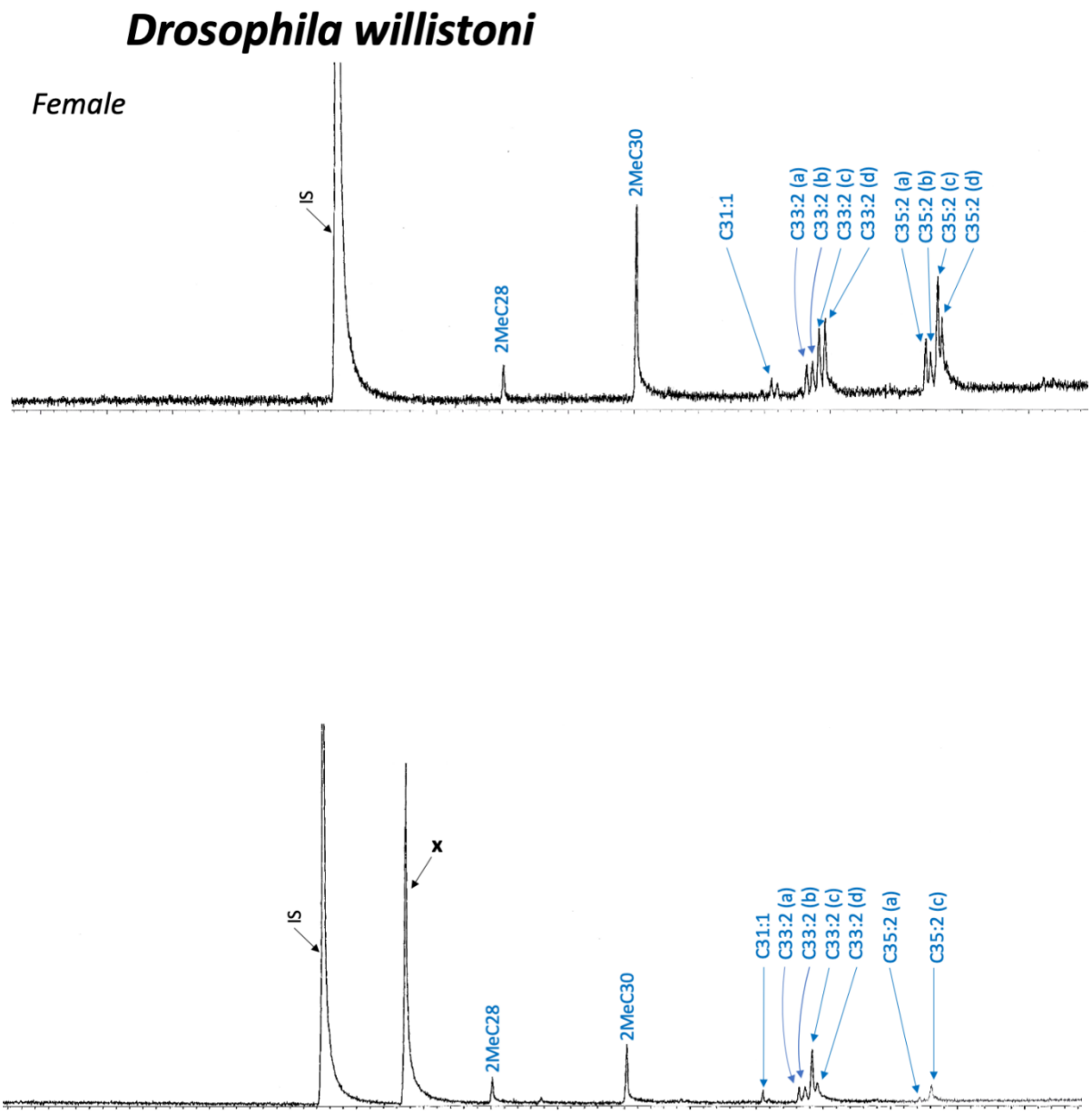
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.30. Chromatograms of CHCs for female and male *Drosophila pseudoananassae*.



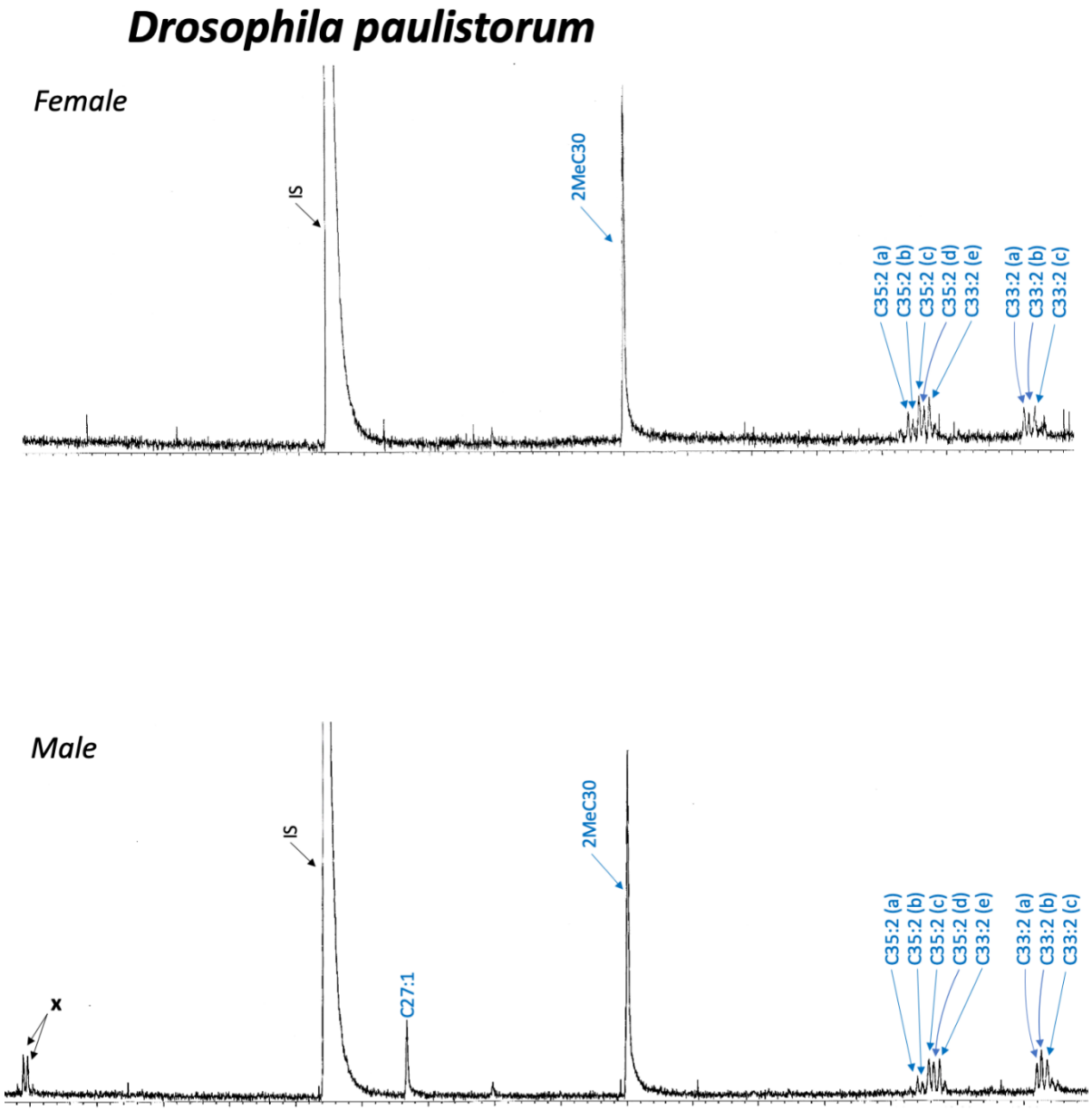
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.31. Chromatograms of CHCs for female and male *Drosophila bipectinata*.



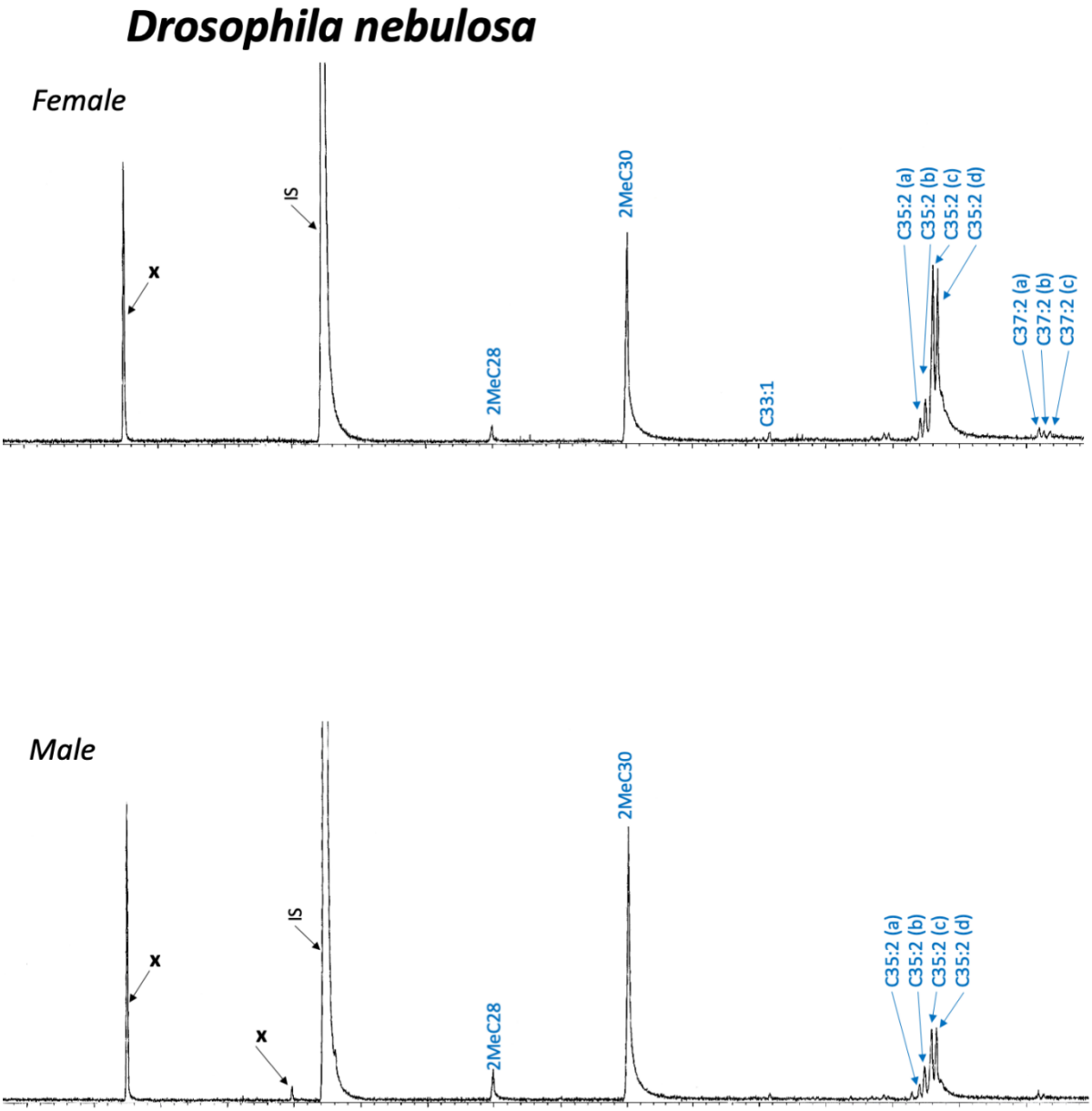
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.32. Chromatograms of CHCs for female and male *Drosophila willistoni*.



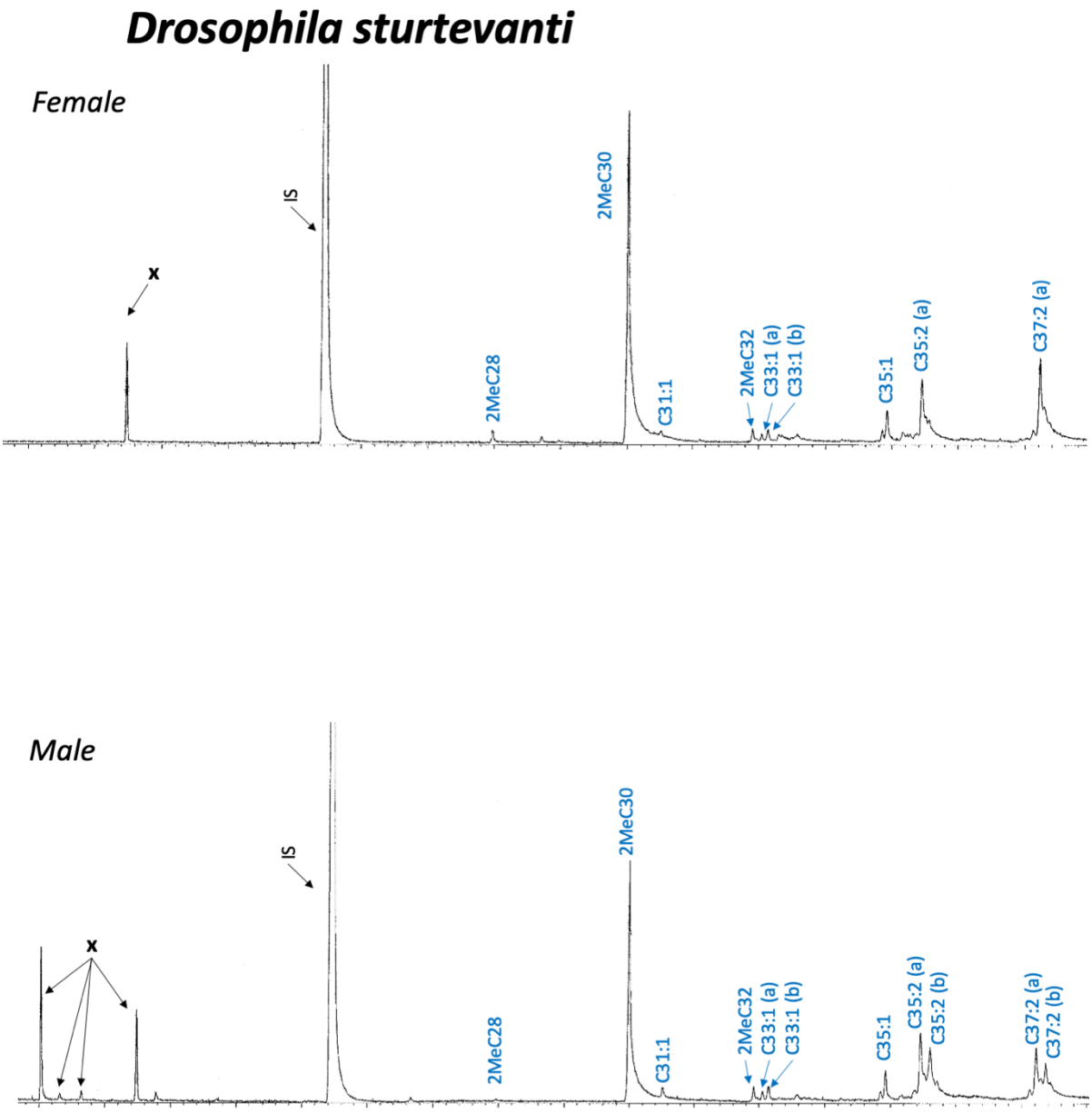
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.33. Chromatograms of CHCs for female and male *Drosophila paulistorum*.



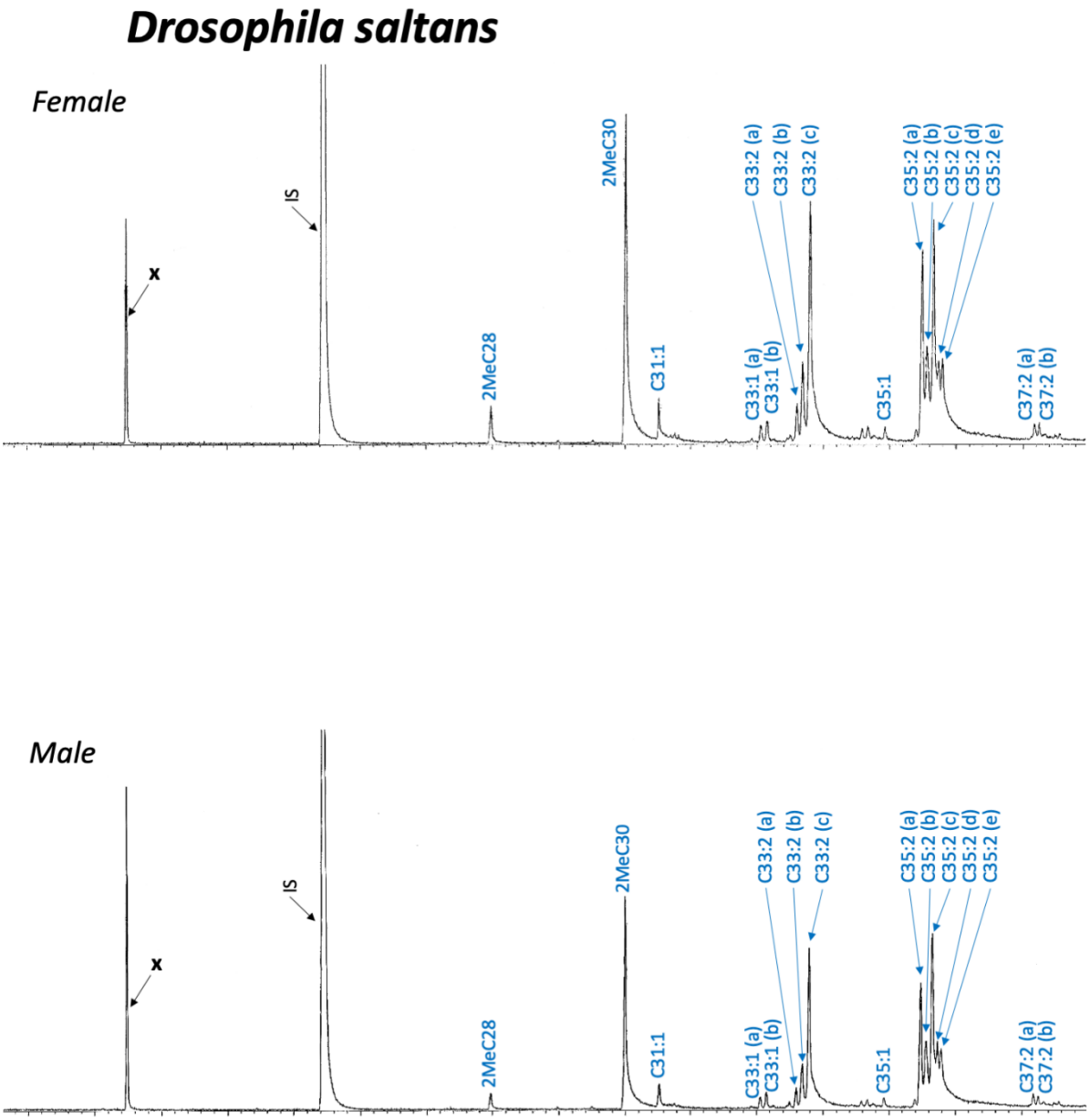
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.34. Chromatograms of CHCs for female and male *Drosophila nebulosa*.



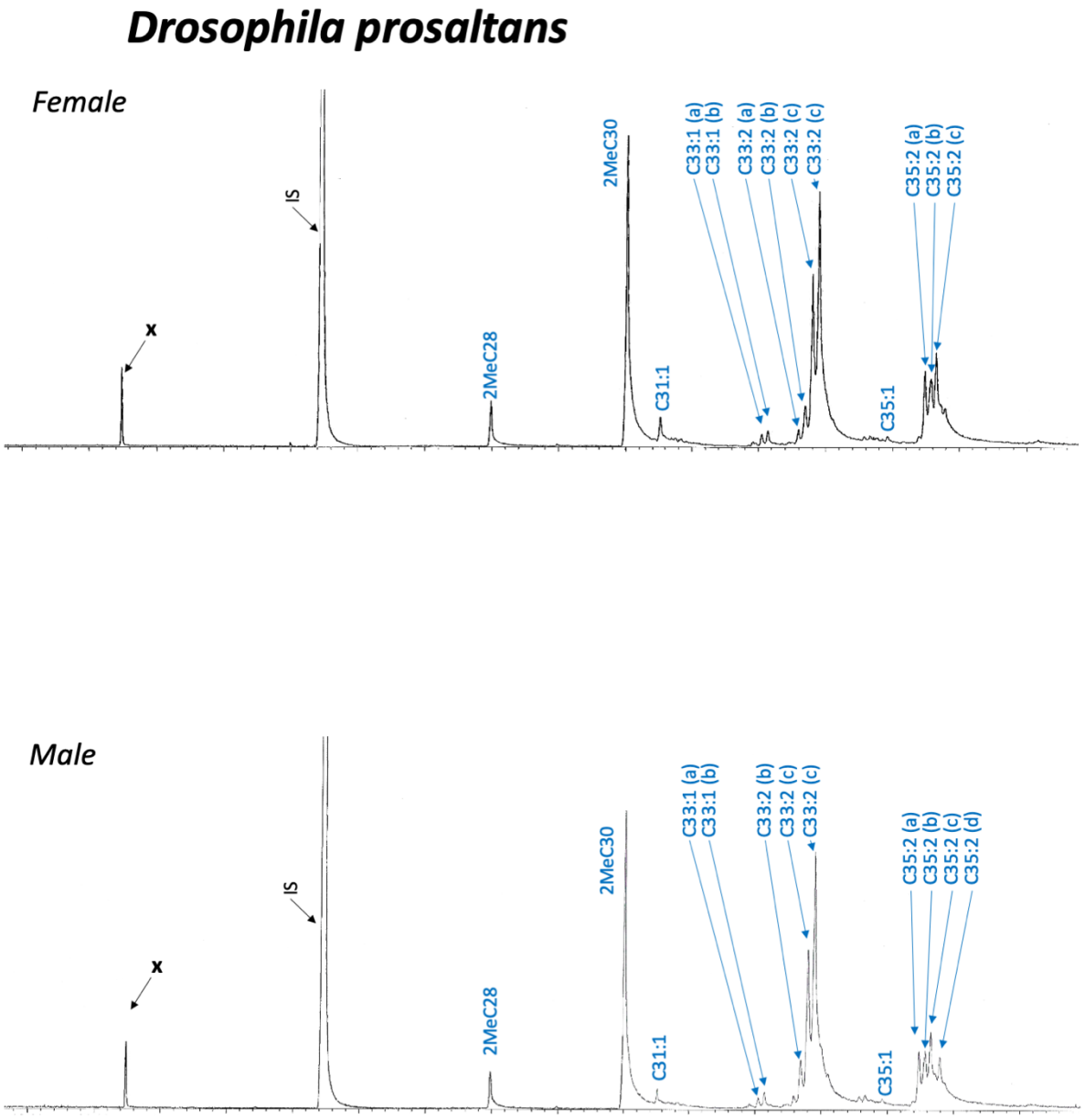
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.35. Chromatograms of CHCs for female and male *Drosophila sturtevanti*.



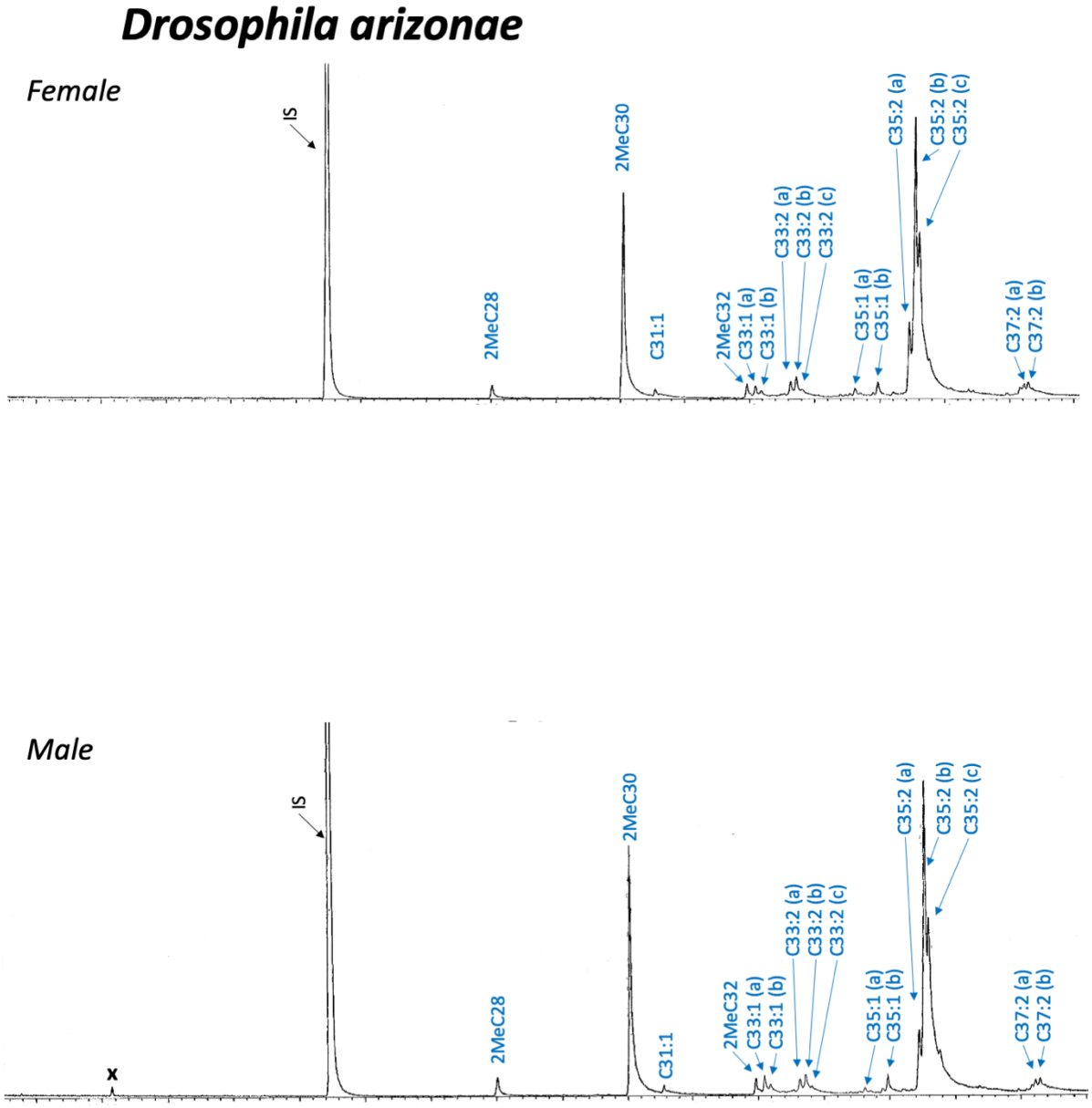
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.36. Chromatograms of CHCs for female and male *Drosophila saltans*.



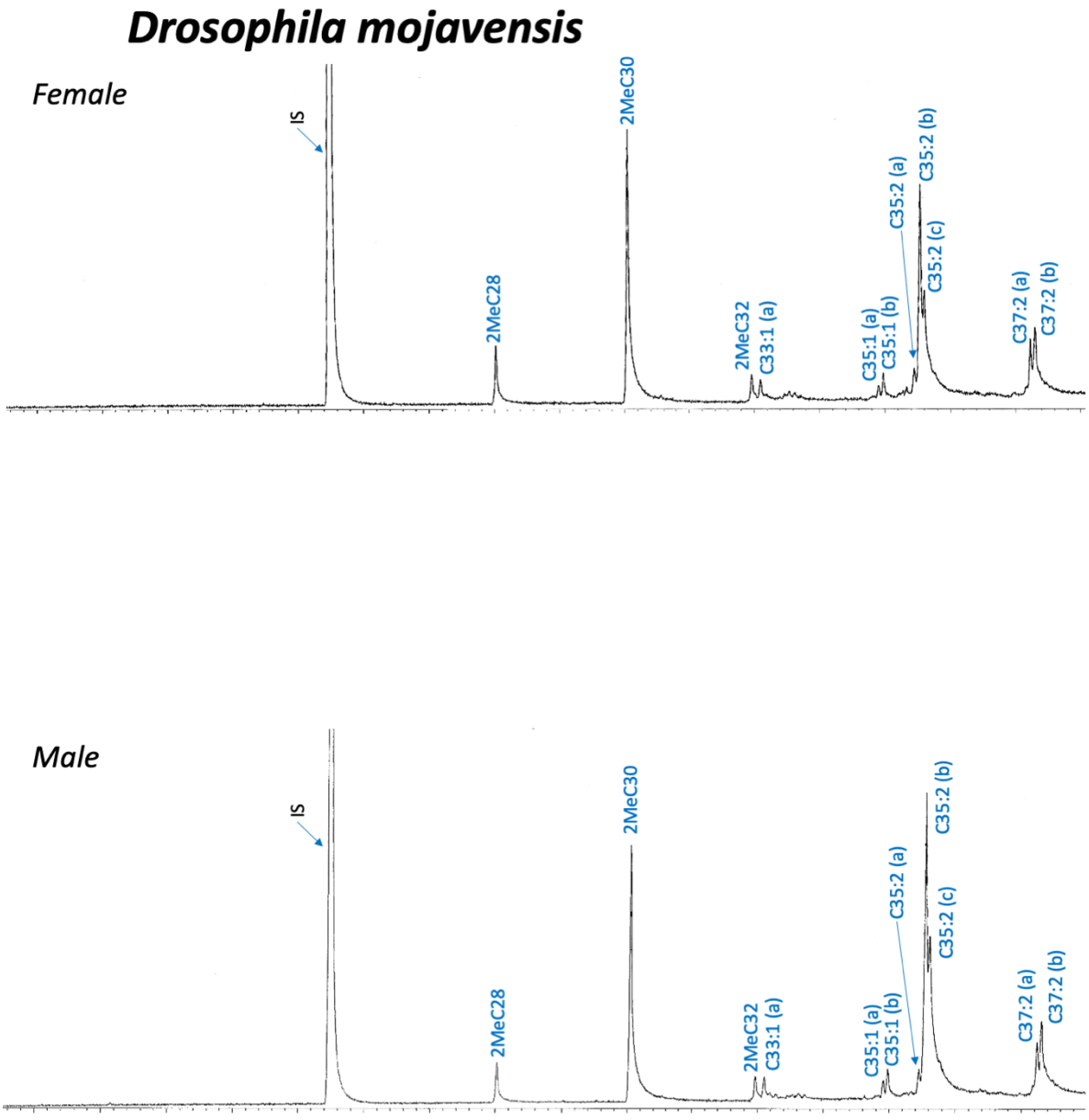
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.37. Chromatograms of CHCs for female and male *Drosophila prosaltans*.



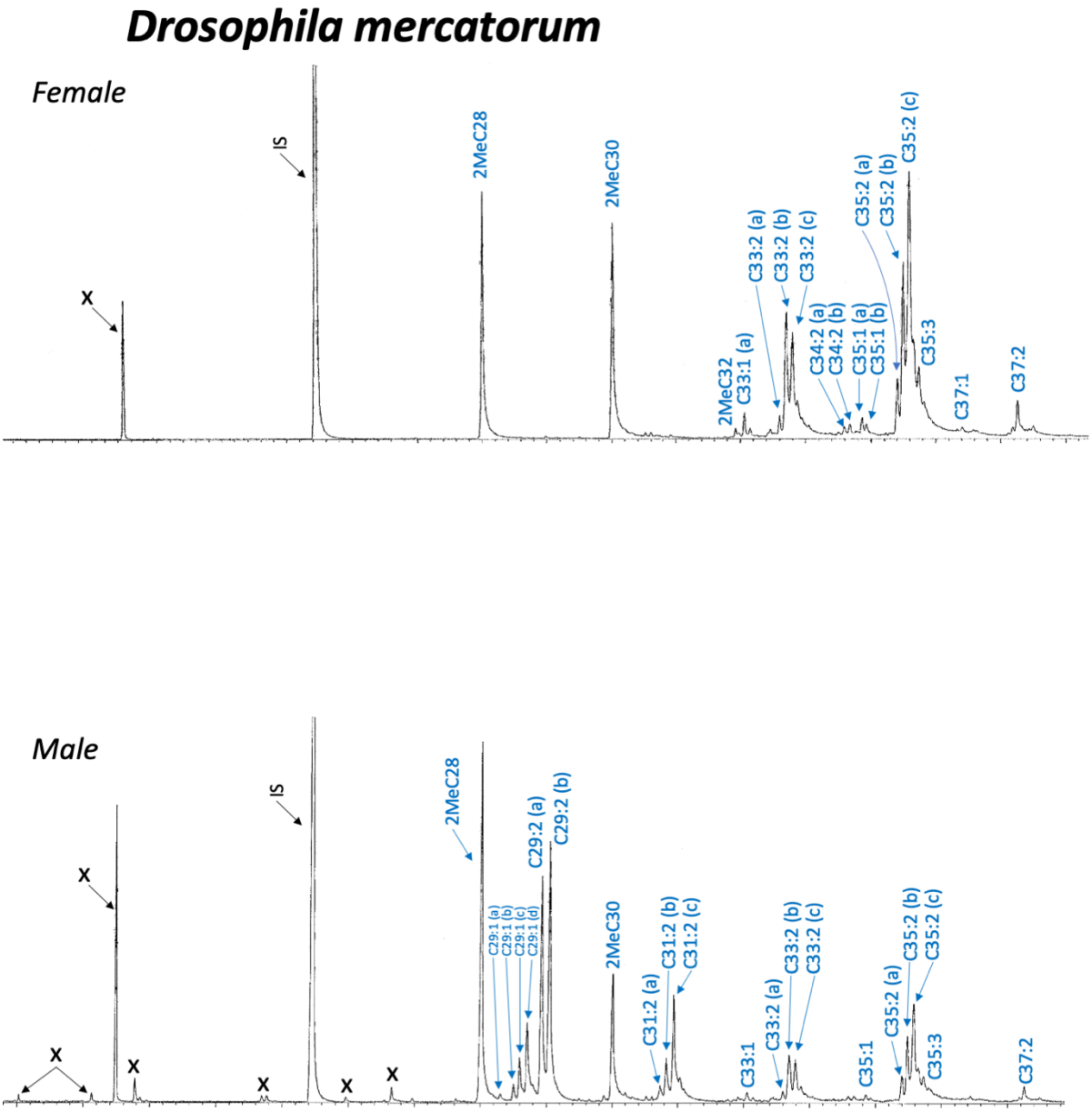
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.38. Chromatograms of CHCs for female and male *Drosophila arizonae*.



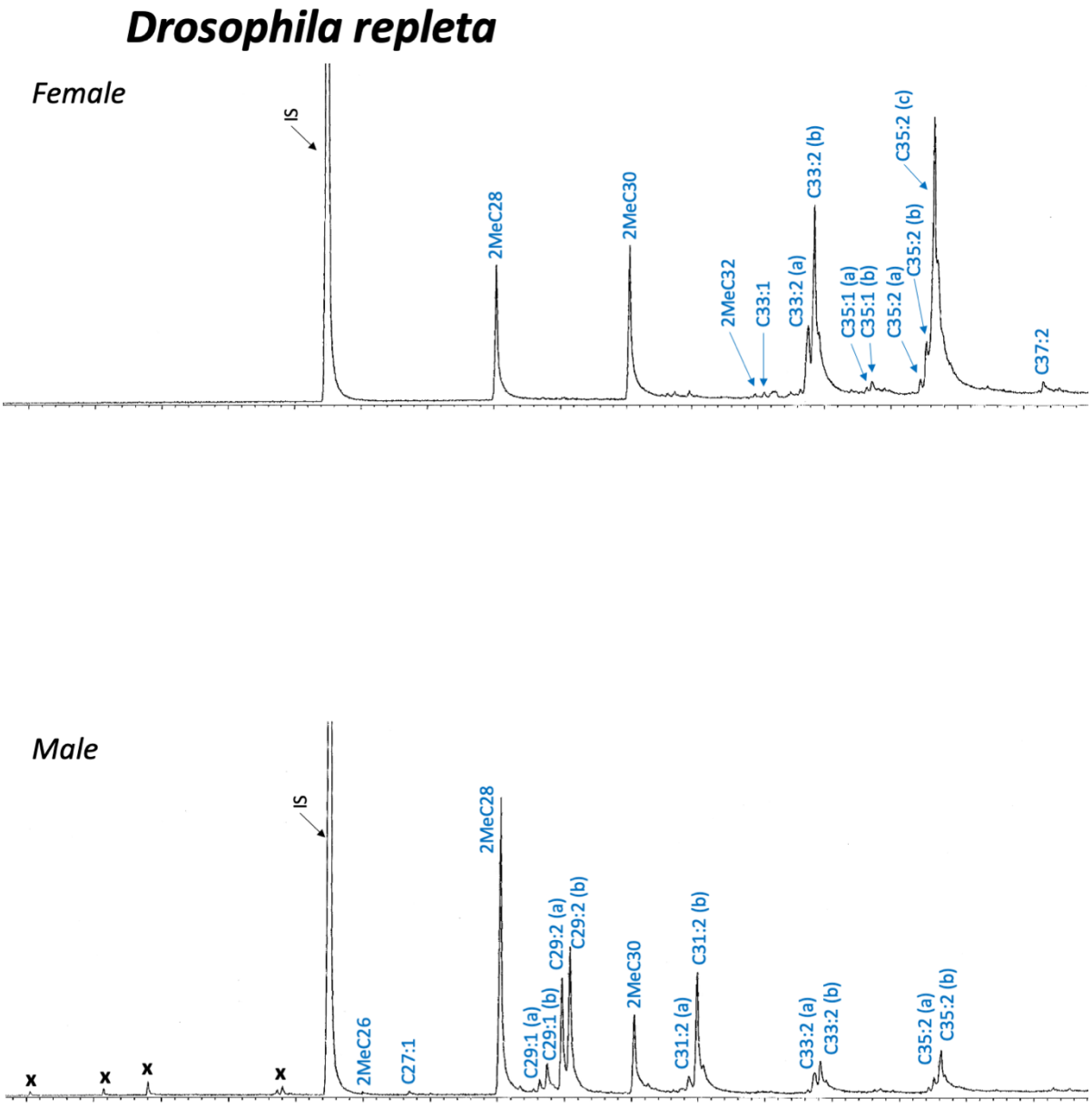
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.39. Chromatograms of CHCs for female and male *Drosophila mojavensis*.



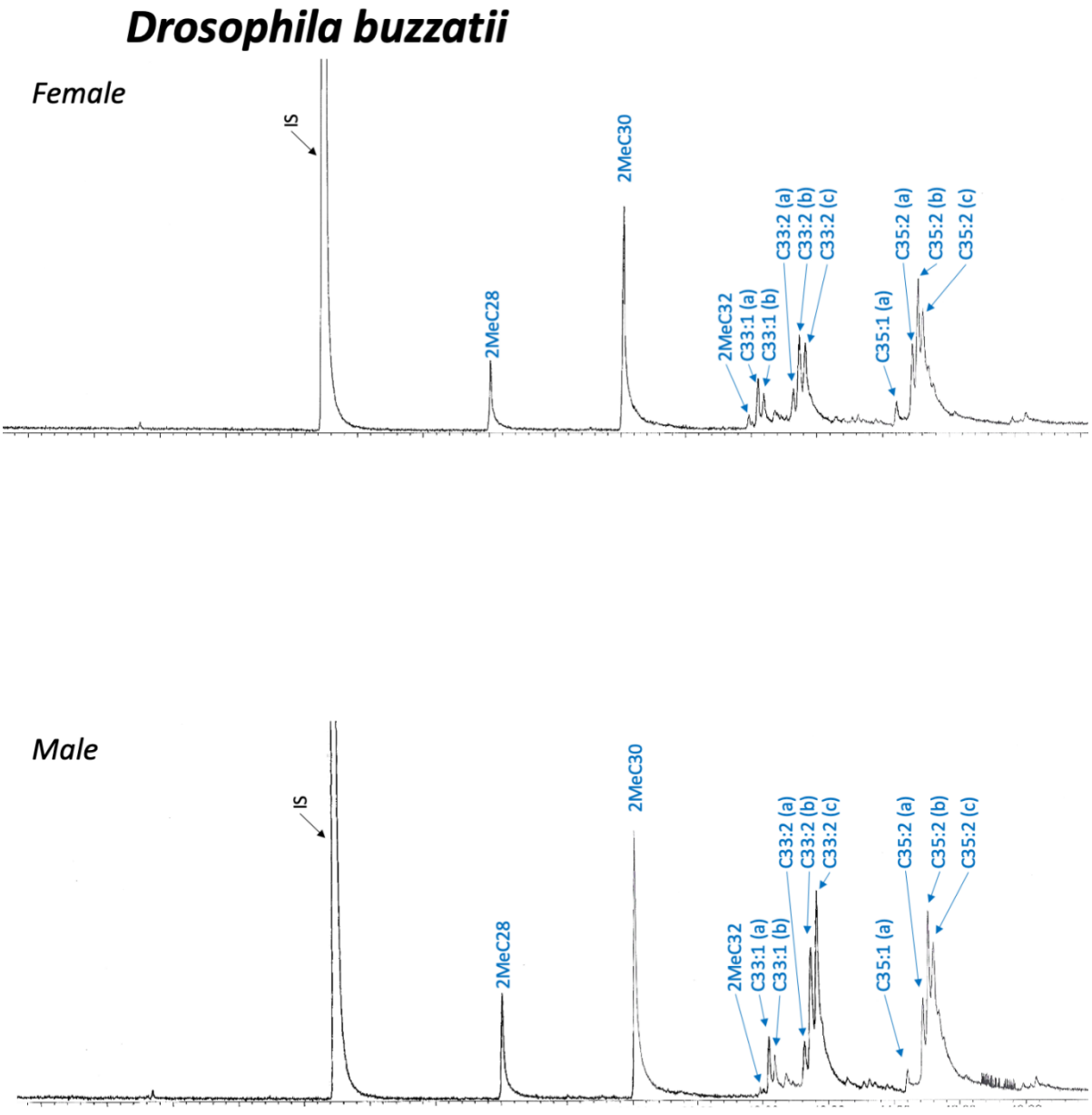
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.40. Chromatograms of CHCs for female and male *Drosophila mercatorum*.



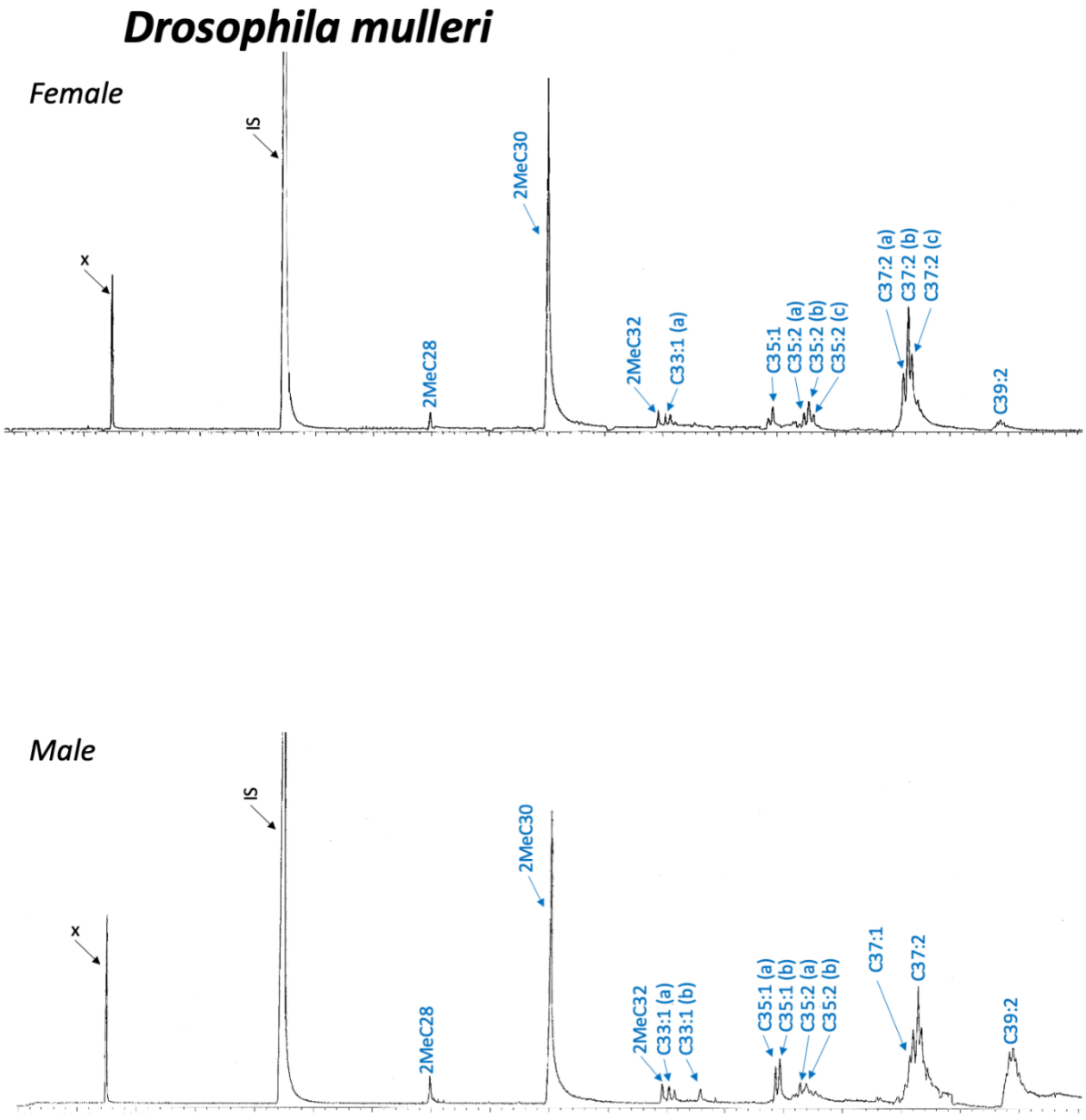
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.41. Chromatograms of CHCs for female and male *Drosophila repleta*.



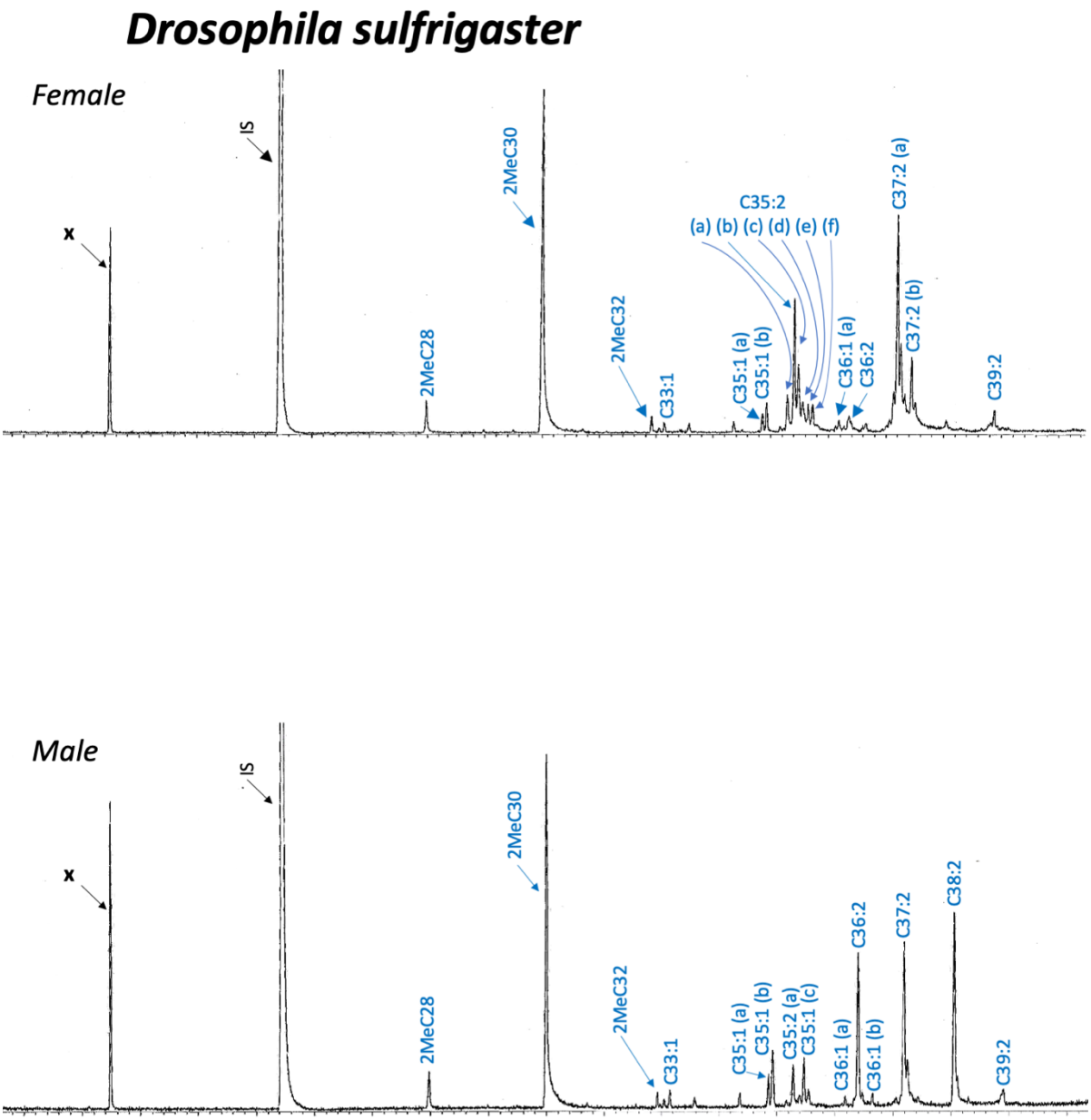
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.42. Chromatograms of CHCs for female and male *Drosophila buzzatii*.



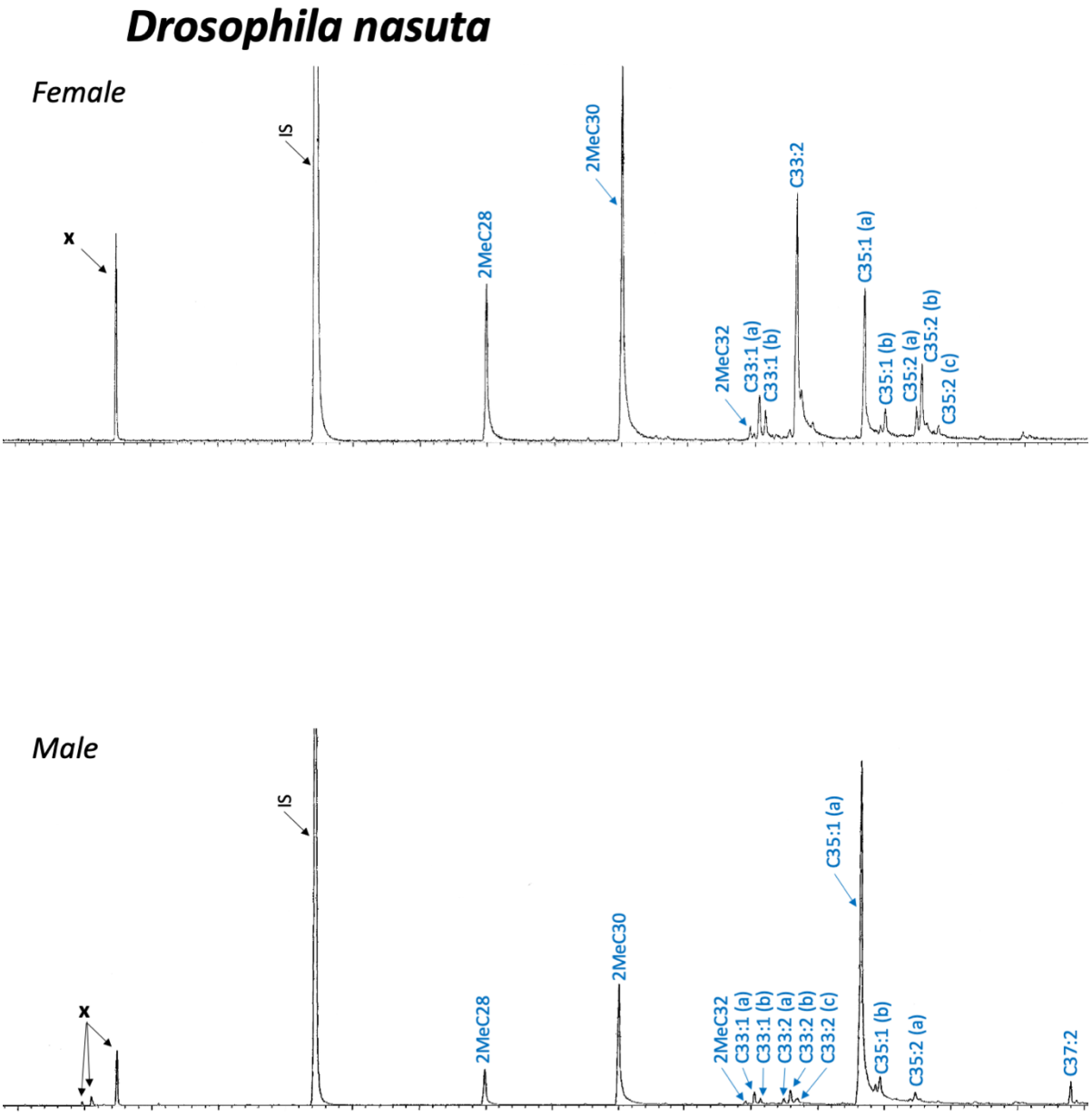
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.43. Chromatograms of CHCs for female and male *Drosophila mulleri*.



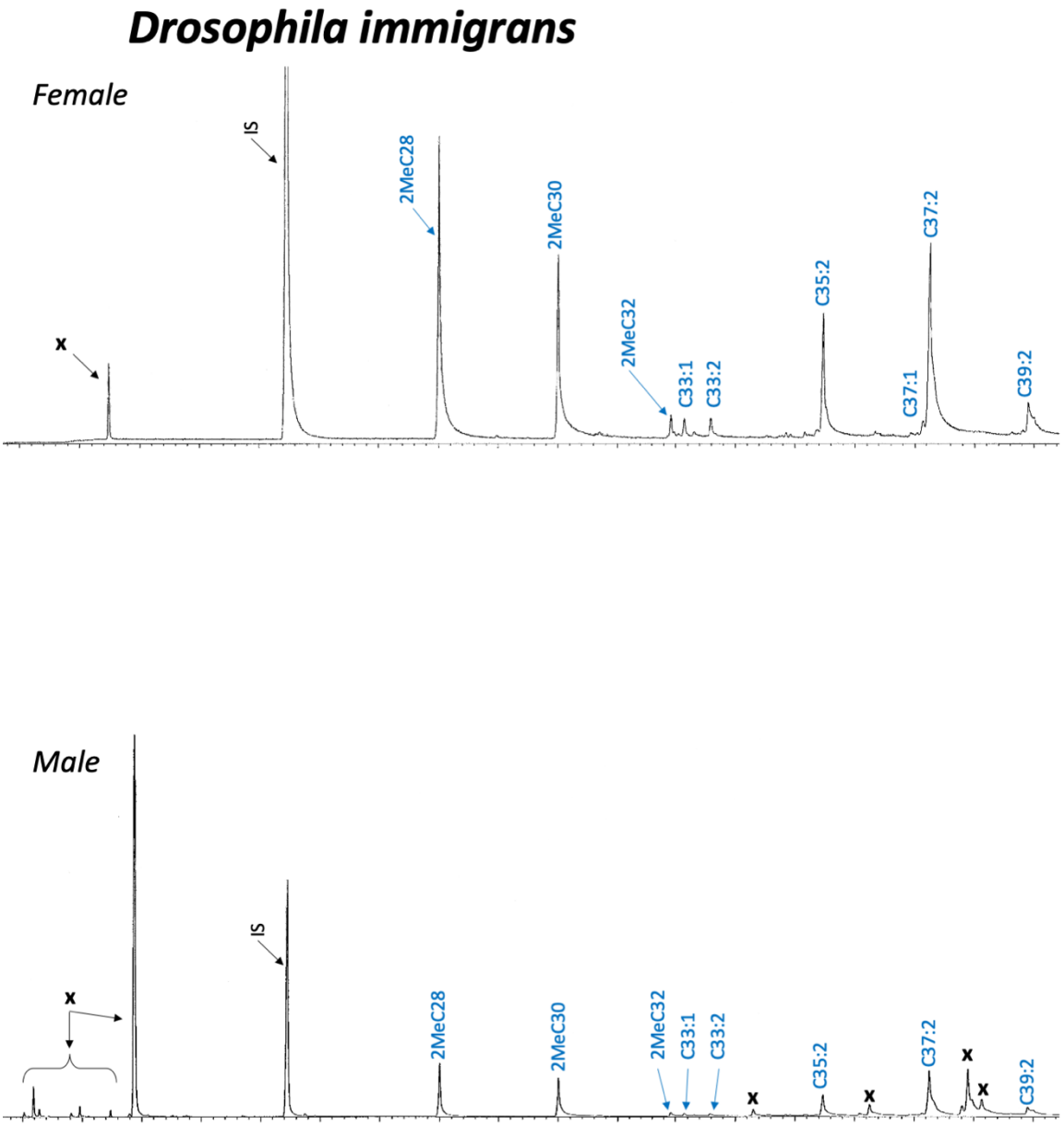
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.44. Chromatograms of CHCs for female and male *Drosophila sulfrigaster*.



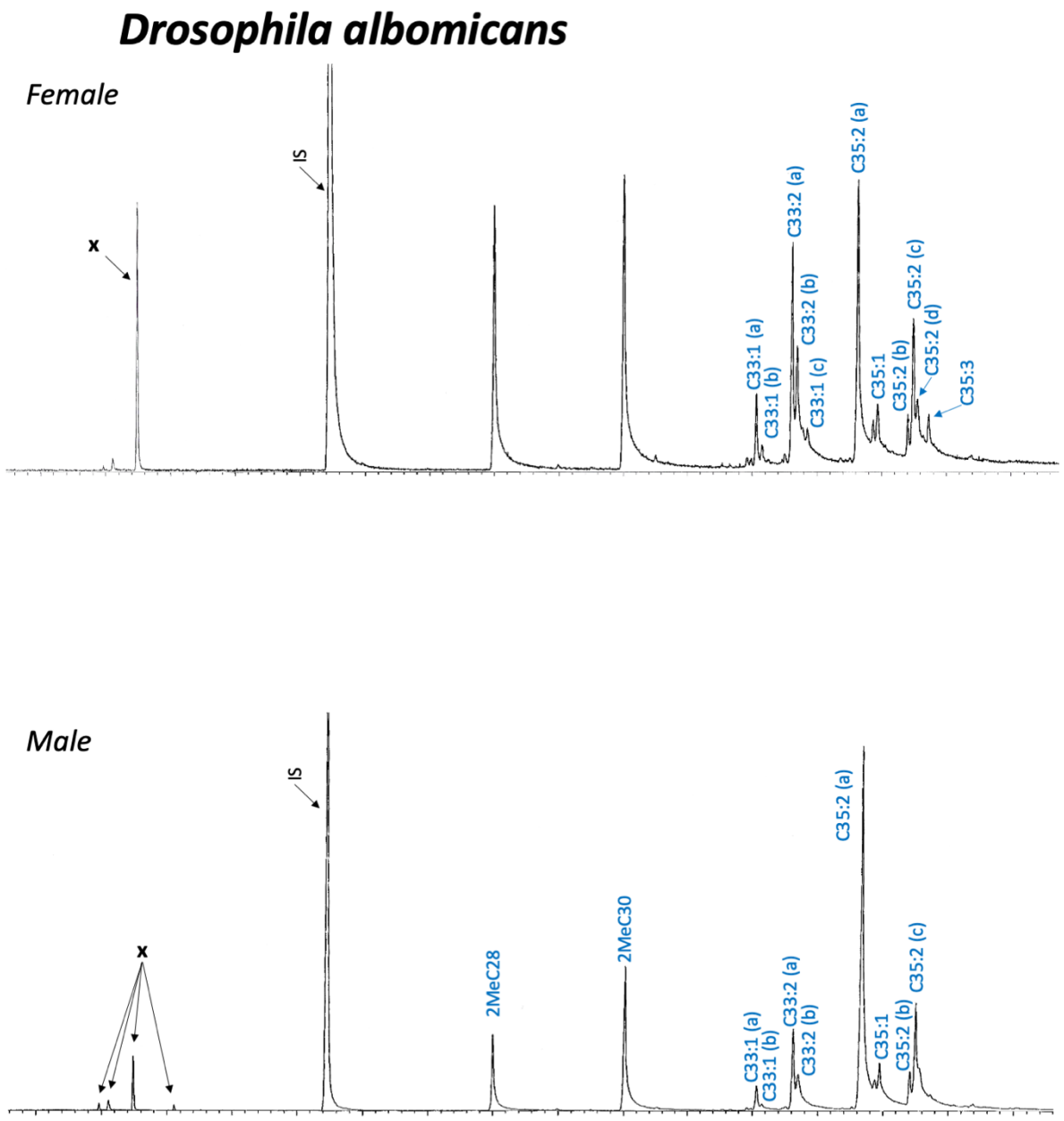
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.45. Chromatograms of CHCs for female and male *Drosophila nasuta*.



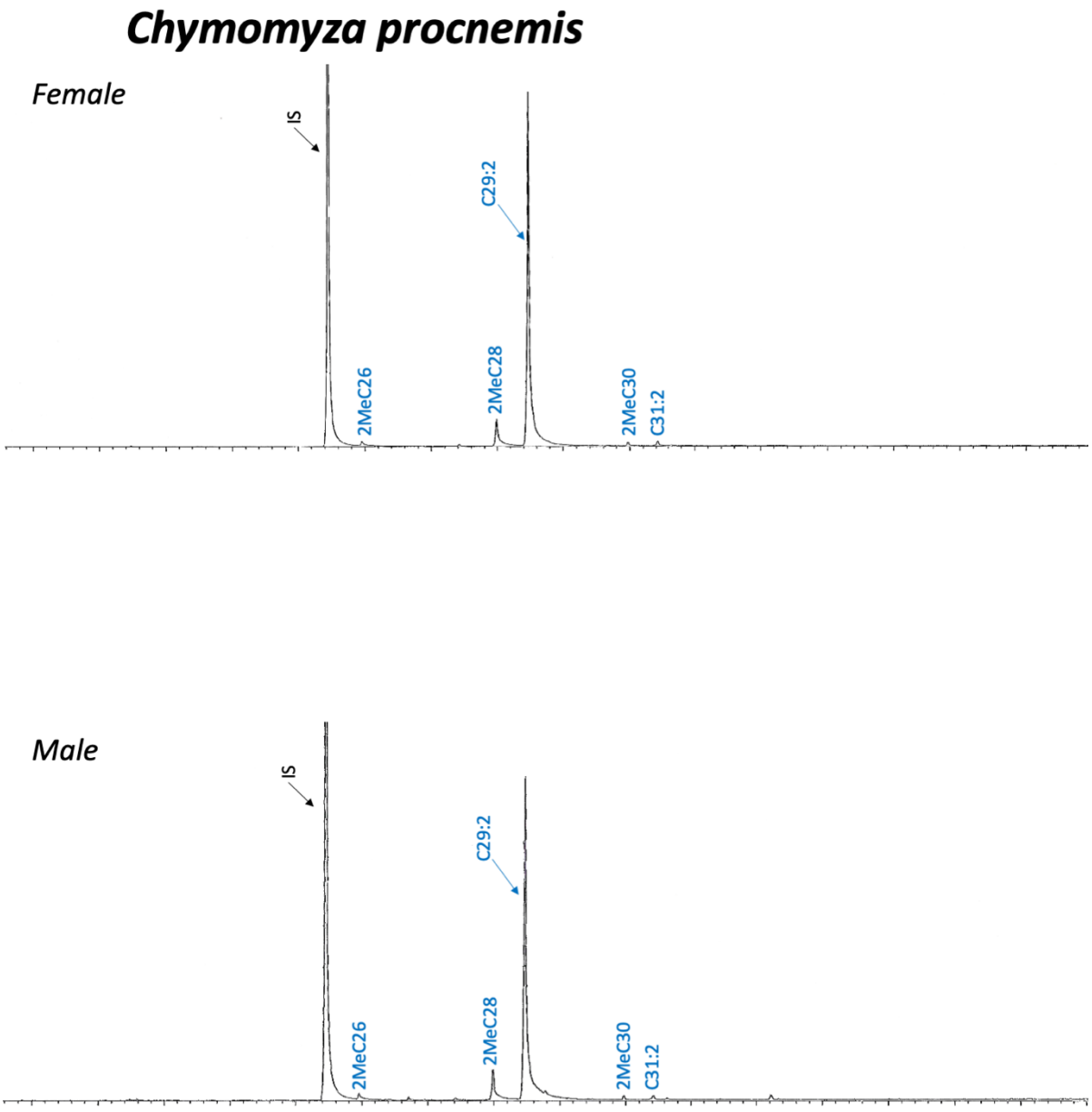
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.46. Chromatograms of CHCs for female and male *Drosophila immigrans*.



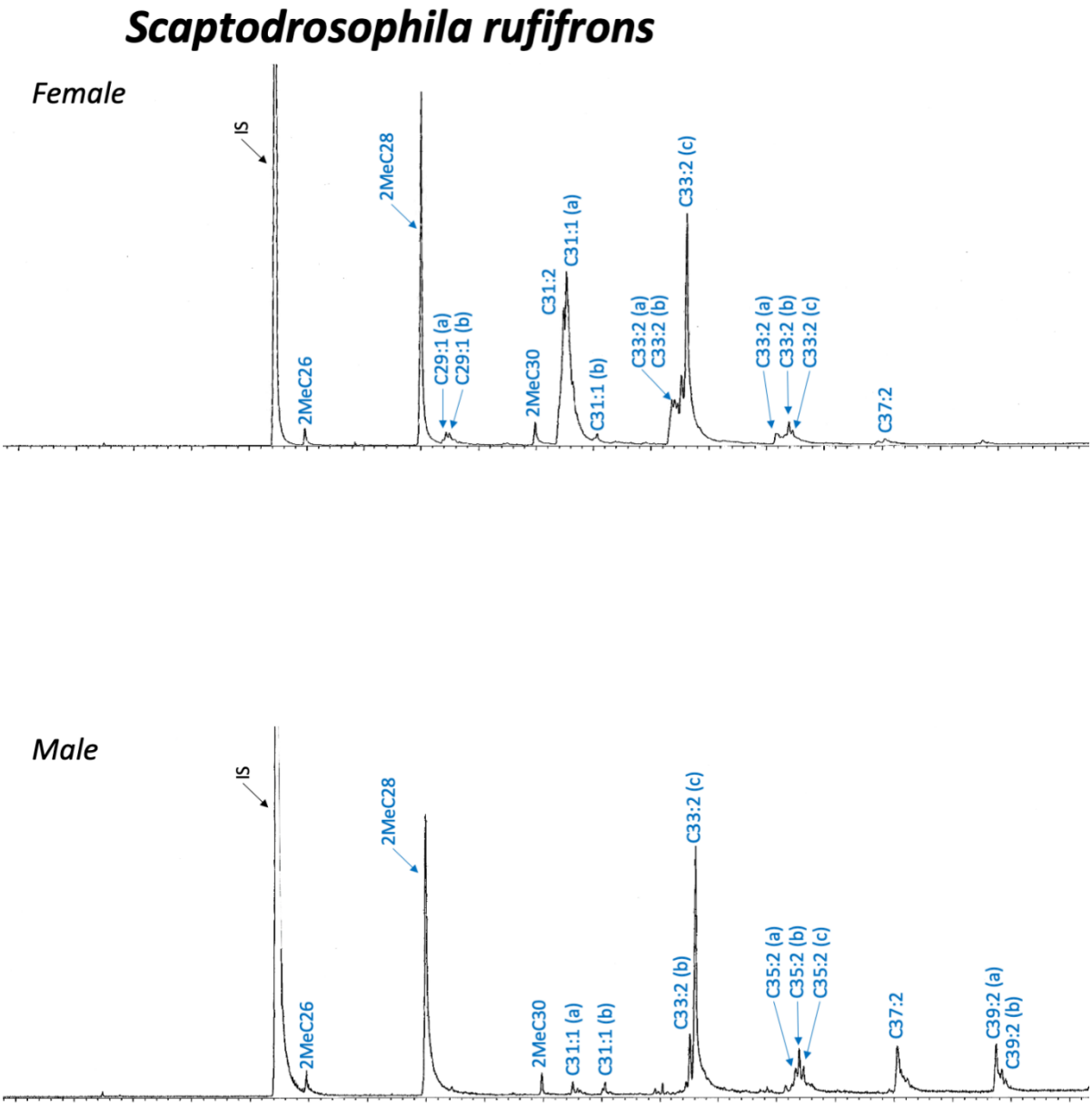
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.47. Chromatograms of CHCs for female and male *Drosophila albomicans*.



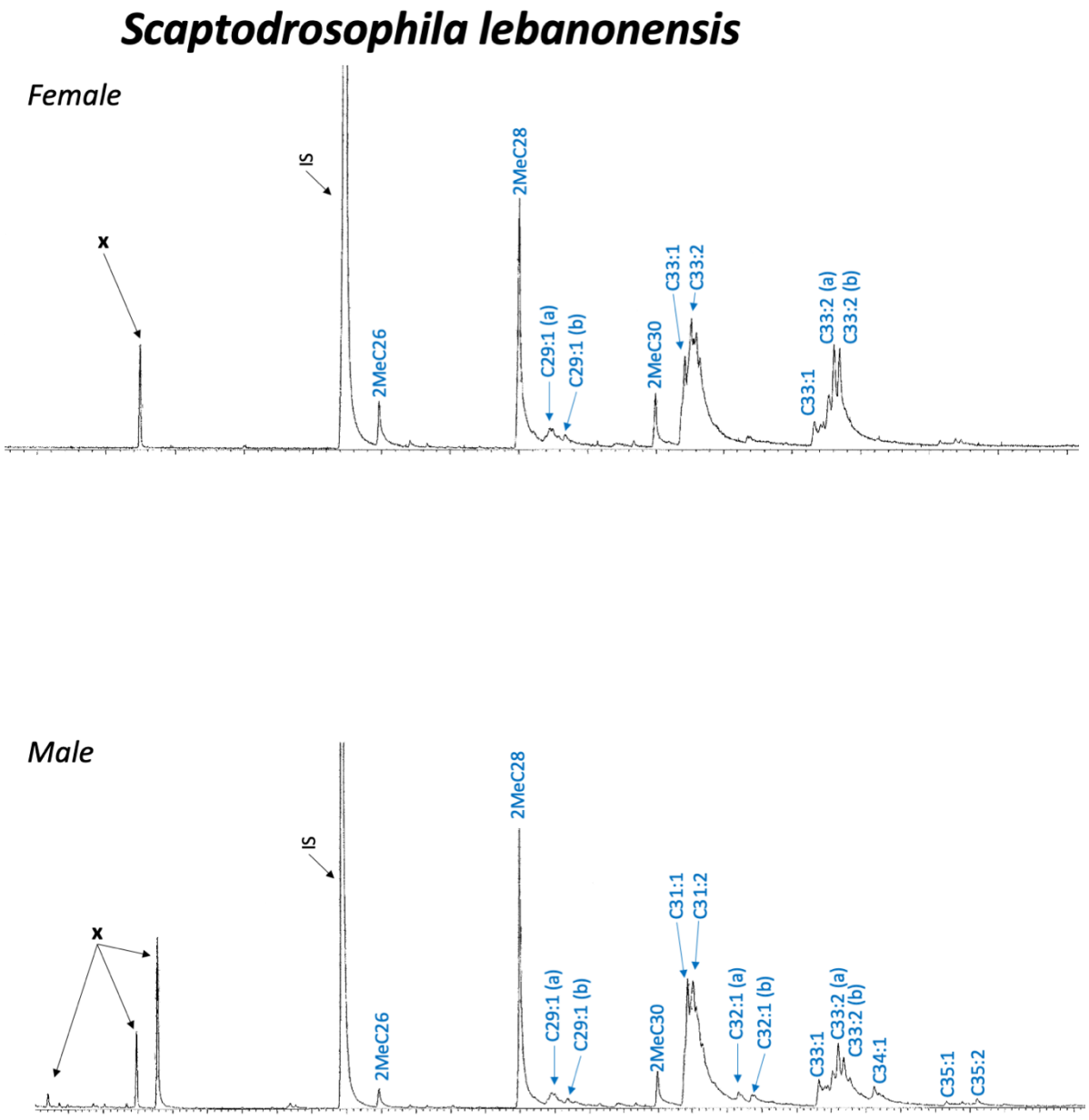
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.48. Chromatograms of CHCs for female and male *Chymomyza procnemis*.



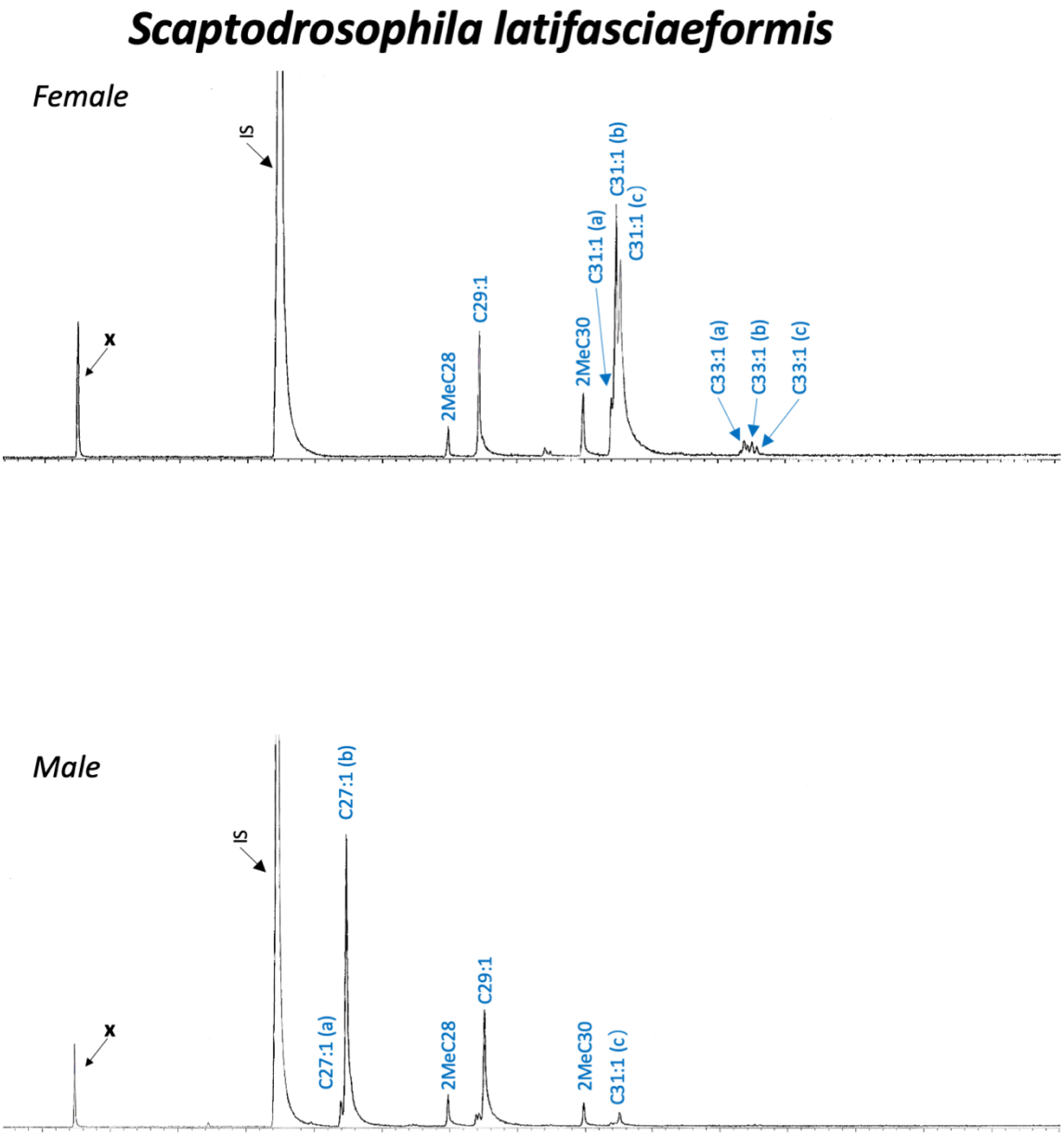
Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;

Figure A1.49. Chromatograms of CHCs for female and male *Scaptodrosophila rufifrons*.



Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.50. Chromatograms of CHCs for female and male *Scaptodrosophila lebanonensis*.



Note: IS refers to Internal Standard, which are C26 with 1000 ng / fly;
x refers to non-CHC chemicals

Figure A1.51. Chromatograms of CHCs for female and male *Scaptodrosophila latifasciaeformis*.

APPENDIX II: Supplemental materials for Chapter 3.

Table A2.1. CHC profiles for *OenoGAL4 > UAS-Dmel/mElo-RNAi.* The abundance is in ng / fly.

Female_CHCs	Control	mElo_RNAi
C23 + 9-C23:1	121.4 ± 5.5	149.6 ± 8.8
7-C23:1	60.2 ± 4.9	67.3 ± 5.2
2MeC24	10.0 ± 2.1	43.2 ± 3.7
C25 + 9-C25:1	144.0 ± 6.7	234.7 ± 11.3
7-C25:1	134.0 ± 7.6	140.8 ± 15.7
2MeC26	79.3 ± 8.4	84.5 ± 5.1
7,11-C27:2 + 7-C27:1	329.8 ± 22.3	310.6 ± 22.3
2MeC28	48.5 ± 2.5	6.5 ± 0.3
7,11-C29:2	74.9 ± 6.8	69.9 ± 3.9
Male_CHCs	**Control**	**mElo_RNAi**
C21	8.3 ± 0.9	12.6 ± 0.7
C22	16.3 ± 1.0	25.8 ± 2.2
C22:1	9.2 ± 0.6	13.4 ± 1.4
C23 + 9-C23:1	246.9 ± 23.7	404.2 ± 42.1
7-C23:1	649.3 ± 56.9	913.5 ± 72.6
5-C23:1	56.2 ± 5.5	84.7 ± 8.5
2MeC24	10.8 ± 0.9	78.4 ± 5.4
C25 + 9-C25:1	79.4 ± 6.3	101.3 ± 7.2
7-C25:1	195.5 ± 25.3	301.0 ± 27.8
2MeC26	58.0 ± 6.3	30.0 ± 2.7
C27	11.6 ± 1.2	0
2MeC28	42.1 ± 3.4	0

Table A2.2. CHC profiles for *UbiquitousGAL4* > *UAS-Dmel/mElo-RNAi*. The abundance is in ng / fly.

Female_CHCs	Control	mElo_RNAi
C23 + 9-C23:1	76.6 ± 7.5	48.0 ± 2.0
7-C23:1	29.3 ± 6.5	21.7 ± 1.8
2MeC24	0	25.2 ± 0.8
C25 + 9-C25:1	94.8 ± 7.5	126.0 ± 3.5
7-C25:1	94.2 ± 32.2	70.6 ± 3.6
2MeC26	116.3 ± 4.3	192.7 ± 8.8
C27	105.8 ± 36.7	47.7 ± 1.5
7,11-C27:2 + 7-C27:1	766.3 ± 152.6	720.3 ± 122.3
5,9-C27:2	40.4 ± 0.1	46.8 ± 2.5
2MeC28	92.9 ± 11.8	5.8 ± 0.3
7,11-C29:2	292.9 ± 1.2	176.0 ± 11.3
Male_CHCs	**Control**	**mElo_RNAi**
C23 + 9-C23:1	177.9 ± 2.1	207.7 ± 12.1
7-C23:1	700.7 ± 9.9	619 ± 49.3
5-C23:1	66.9 ± 1.0	57.9 ± 4.9
2MeC24	13.6 ± 0.9	52.5 ± 16.3
C25 + 9-C25:1	48.2 ± 1.8	66.5 ± 1.1
7-C25:1	314.0 ± 8.7	306.4 ± 7.5
2MeC26	76.3 ± 1.3	40.5 ± 9.1
C27	10.0 ± 0.2	0
2MeC28	44.3 ± 1.8	0

Table A2.3. CHC profiles for two strains of *D. melanogaster* with *mElo* knocked out (*mEloKO1* & *mEloKO2b*). The two strains and the control have *PhiC31_integrase* on the X chromosome. The abundance is in ng / fly. *mEloKO1* was used for further transgenesis.

Female_CHCs	**Control**	***mEloKO1***	***mEloKO2b***
C23 + 9-C23:1	78 ± 4.8	76.1 ± 2.9	57.4 ± 5.9
7-C23:1	38.6 ± 0.7	38.5 ± 1.8	36.1 ± 3.9
2MeC24	0	9.3 ± 1.4	9.3 ± 1
C25 + 9-C25:1	103.8 ± 7.5	150.1 ± 4.3	167.3 ± 8.9
7-C25:1	68.6 ± 3.7	86.2 ± 13.1	110.8 ± 2.5
2MeC26	32.3 ± 1.9	102.2 ± 6	127.7 ± 15.9
C27	100.6 ± 6.4	71.4 ± 6.6	102.1 ± 15.8
7,11-C27:2 + 7-C27:1	492.4 ± 8.1	430.8 ± 34.6	470.3 ± 25.9
5,9-C27:2	86 ± 2.7	0	1.9 ± 1.9
2MeC28	30 ± 2.4	1.7 ± 1	1.8 ± 1.2
C29	0	2.1 ± 1.3	1 ± 1
7-C29:1	3.8 ± 1.3	0.8 ± 0.8	3.1 ± 1.9
7,11-C29:2	307.5 ± 19	205.6 ± 16.7	289.9 ± 42.9
Male_CHCs	**Control**	***mEloKO1***	***mEloKO2b***
C21	0	23.7 ± 5.4	0
C22	14.7 ± 1.7	29.1 ± 5.1	14.7 ± 2.1
C22:1	3.3 ± 1.9	8 ± 4.7	1.5 ± 1.5
C23 + 9-C23:1	216.6 ± 10.2	265 ± 20	201.9 ± 6.5
7-C23:1	727.8 ± 32.1	1138.4 ± 108.5	655.7 ± 50.1
5-C23:1	67.5 ± 2.1	82.6 ± 7.4	51 ± 2.9
2MeC24	0	38 ± 5.7	33.1 ± 7.1
C25 + 9-C25:1	58.6 ± 7	23.4 ± 2.9	64.2 ± 13.5
7-C25:1	215.6 ± 10.8	77.9 ± 14.7	258.8 ± 33
2MeC26	20.9 ± 2.2	14.7 ± 2.3	30.1 ± 2.2
C27	12 ± 1.3	0	0
2MeC28	14.9 ± 2.1	0	0

Table A2.4. CHC profiles for *OenoGAL4* > *UAS-O/E* of *CG18609* (*mElo*), *GI20343*, *GI20345*, and *GI20347* in the *attP40* background. The abundance is in ng / fly.

Female_CHCs	Control	*CG18609*	*GI20343*	*GI20345*	*GI20347*
C21	0 ± 0	0 ± 0	6.6 ± 0.5	5.1 ± 1.6	0 ± 0
C22	7 ± 0.4	7.3 ± 0.4	0 ± 0	2.8 ± 1.8	7.5 ± 0.7
C23 + 9-C23:1	179.6 ± 9.7	164 ± 7	130.8 ± 9.7	144.3 ± 6.5	167.8 ± 15.6
7-C23:1	67.9 ± 3.9	72.9 ± 5.2	55.3 ± 9.4	58.7 ± 6.1	64.4 ± 4.1
2MeC24	17.3 ± 1.8	10.9 ± 1.5	22.9 ± 1.1	0 ± 0	3.2 ± 1.4
C25 + 9-C25:1	189 ± 17.2	149.3 ± 8.8	125 ± 10.6	215 ± 124.3	145.9 ± 18.9
7-C25:1	51.5 ± 18.9	23.8 ± 1.5	62 ± 19.7	4.3 ± 2.7	52.3 ± 15.6
7,11-C25:2	29.8 ± 2	30.7 ± 3.2	14.1 ± 2.5	51 ± 7.4	29.8 ± 2.4
2MeC26	147.6 ± 3.8	147.6 ± 18.9	129.4 ± 7.8	95.7 ± 1.7	46.4 ± 13.6
C27	13.5 ± 1.3	29.7 ± 4.6	11.4 ± 4.3	80.1 ± 6.5	48.4 ± 9.9
7,11-C27:2 + 7-C27:1	449.3 ± 21.3	496.2 ± 44.3	369.6 ± 60.2	483.3 ± 55.1	402.1 ± 26.8
2MeC28	19.1 ± 1.2	44 ± 5.2	11.6 ± 1.2	44.8 ± 2.5	39.8 ± 8.5
7,11-C29:2	94 ± 2.7	107.3 ± 7.9	63.7 ± 9.2	158.1 ± 16.3	126.5 ± 13.6
2MeC30	0 ± 0	0 ± 0	0 ± 0	0 ± 0	7 ± 0.7
Male_CHCs	**Control**	***CG18609***	***GI20343***	***GI20345***	***GI20347***
C21	11.2 ± 0.8	11.2 ± 1.5	10.3 ± 1.3	7.5 ± 0.7	5.4 ± 1.2
C22	22.9 ± 1.2	26.1 ± 2.7	20.9 ± 1.9	19 ± 1.4	21.5 ± 1.3
C22:1	9.1 ± 4.3	10 ± 0.7	0 ± 0	0 ± 0	5.6 ± 1.2
C23 + 9-C23:1	286.9 ± 14.9	254.1 ± 5.8	241.9 ± 25.2	156.2 ± 8.4	283.4 ± 12.1
7-C23:1	793.6 ± 30.6	799.8 ± 35.8	795.1 ± 81.3	322.4 ± 26.9	633.6 ± 14.1
5-C23:1	65.1 ± 2.2	63.3 ± 2.3	53.2 ± 10.8	39.7 ± 5.1	54.7 ± 1.3
2MeC24	13.4 ± 4.6	3.7 ± 2.3	20.5 ± 1.7	3.7 ± 1.8	3.1 ± 1.4
C25 + 9-C25:1	57.6 ± 10.8	43.1 ± 3.5	41.1 ± 5.4	59.3 ± 3.7	52.9 ± 2.9
7-C25:1	169.3 ± 24.2	120 ± 8.2	128.9 ± 22.1	209.5 ± 24.5	178.1 ± 9.8

Table A2.4 (cont'd)

2MeC26	33.7 ± 2.3	37 ± 10.3	60.3 ± 10.3	28.6 ± 3.8	12.2 ± 5.5
C27	12.8 ± 1.9	15.2 ± 1.4	4.8 ± 2.2	32.1 ± 6.2	14.6 ± 0.6
7-C27:1	0 ± 0	0 ± 0	0 ± 0	187.9 ± 45.6	0 ± 0
2MeC28	12.3 ± 1.5	27.6 ± 6.7	14.3 ± 3	26.2 ± 3.1	20.7 ± 4.2
2MeC30	0 ± 0	0 ± 0	0 ± 0	0 ± 0	6.9 ± 0.2

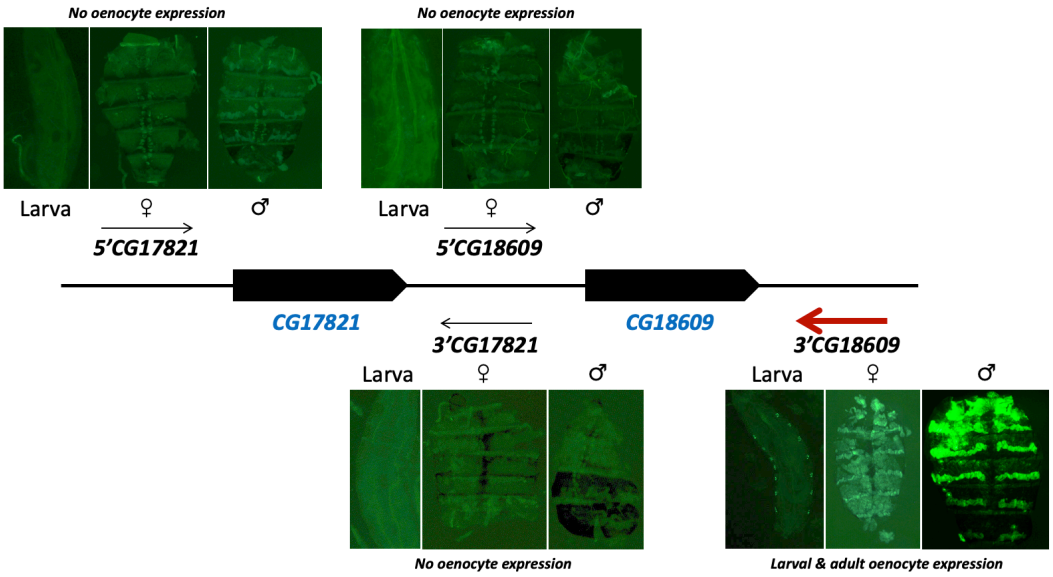


Figure A2.1. *3'CG18609* regulate oenocyte expression of *CG18609* in larval and adult oenocytes. GFP reporter assays of sequences surrounding elongase genes in the *D. melanogaster mElo* locus.

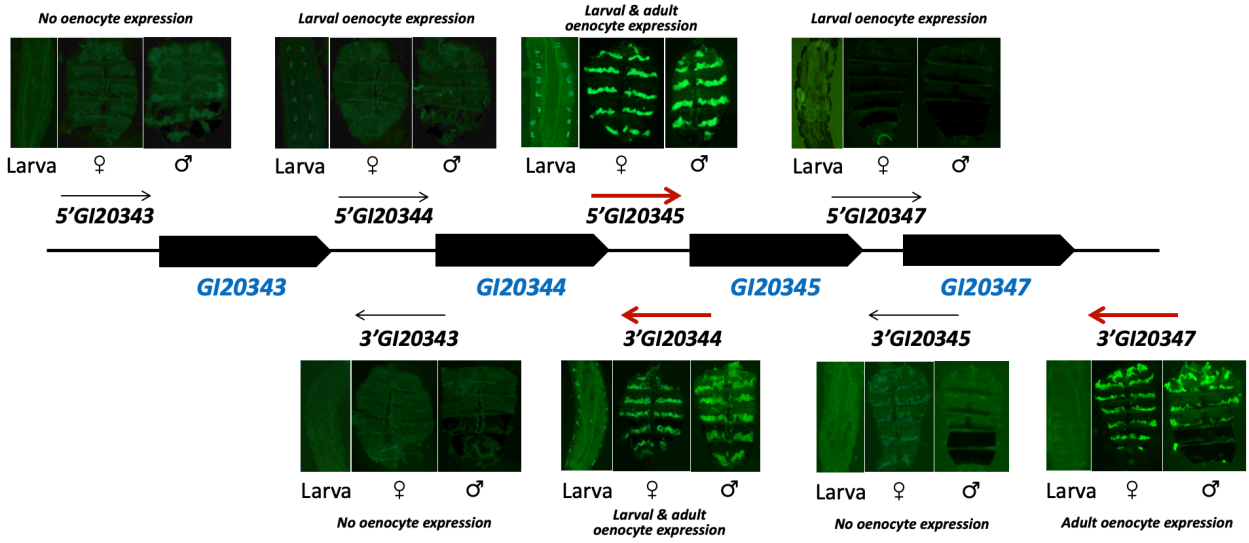


Figure A2.2. *3'GI20347* regulates oenocyte expression of *GI20347* in larval and adult oenocytes. GFP reporter assays of sequences surrounding elongase genes in the *D. mojavensis mElo* locus.

Table A2.5. CHC profiles for *5'GI20345-GAL4* > *UAS-O/E* of *mElo*, *GI20345*, and *GI20347* in the *mEloKO* background. The abundance is in ng / fly.

Female_CHCs	Control	*mElo*	*GI20345*	*GI20347*
C21	3.2 ± 1.4	6.6 ± 0.4	3.5 ± 0.8	0 ± 0
C22	3.3 ± 1.5	7.8 ± 0.7	5.2 ± 0.5	0.6 ± 0.6
C23 + 9-C23:1	274.1 ± 45.1	298.9 ± 21	98.4 ± 42.4	91.1 ± 9.4
7-C23:1	19 ± 12.1	7.3 ± 4	94.4 ± 43.5	2.4 ± 2.4
2MeC24	38.9 ± 6.4	5.8 ± 0.4	0 ± 0	24.3 ± 3.6
C25 + 9-C25:1	577 ± 89.8	343.4 ± 42.4	193.5 ± 19.5	209.9 ± 22.6
2MeC26	233.5 ± 33.4	109.6 ± 7.4	190.7 ± 16	126 ± 9.5
C27	198.1 ± 27.7	163.2 ± 22	299.7 ± 26	171.6 ± 20.9
7-C27:1	11.9 ± 7.7	0 ± 0	57 ± 12.5	23.3 ± 17.5
7,11-C27:2	259.1 ± 70.4	226.5 ± 15.7	190.8 ± 39.7	105.4 ± 18.4
5,9-C27:2	34.9 ± 11.6	0 ± 0	1.6 ± 0.7	21.3 ± 7
2MeC28	6.6 ± 1.5	31.1 ± 2.9	71.3 ± 6.6	106.3 ± 11
C29	2.3 ± 1.5	3.6 ± 2.4	39.1 ± 15.3	7 ± 2.2
7-C29:1	0 ± 0	2.6 ± 2.6	21.9 ± 9.8	6.5 ± 3.5
7,11-C29:2	126 ± 26.1	74.9 ± 14.7	198.9 ± 10.9	107.5 ± 14
2MeC30	0 ± 0	0 ± 0	0 ± 0	17 ± 2.8
Male_CHCs	**Control**	***mElo***	***GI20345***	***GI20347***
C21	43.4 ± 15	35.4 ± 4.8	17.8 ± 2.1	13.2 ± 1.6
C22	46.8 ± 13.4	34.3 ± 4.2	23.3 ± 1.8	20.2 ± 2.1
C22:1	12.8 ± 2.3	5.5 ± 2.6	1.4 ± 0.9	3.7 ± 0.9
C23 + 9-C23:1	508.1 ± 96.1	299.2 ± 27.5	157.2 ± 12.5	243.2 ± 22.4
7-C23:1	1287.9 ± 124.8	1139.8 ± 91.7	391.3 ± 48.3	876.7 ± 103.4
5-C23:1	62.6 ± 6	39.7 ± 12.6	45.9 ± 7.3	38.5 ± 8.1
2MeC24	115 ± 15.4	28.7 ± 2.1	9.8 ± 2	37.2 ± 11.3
C25 + 9-C25:1	53.5 ± 4.2	36.2 ± 3.1	88.6 ± 4.9	34.1 ± 4.4
7-C25:1	60.5 ± 8.8	37.5 ± 5.8	210.8 ± 26.1	31.9 ± 3.2
2MeC26	13.2 ± 1.4	41.5 ± 2	62.9 ± 7	10.4 ± 2.8
C27	0 ± 0	3.3 ± 1.5	13.4 ± 2.8	5.7 ± 2.2
7-C27:1	0 ± 0	0 ± 0	131.8 ± 6.2	0.6 ± 0.6
2MeC28	0 ± 0	9 ± 0.6	12 ± 0.7	17.3 ± 1.4
7-C29:1	0 ± 0	0 ± 0	4.7 ± 0.3	0.5 ± 0.5
2MeC30	0 ± 0	0 ± 0	0 ± 0	5.2 ± 0.5

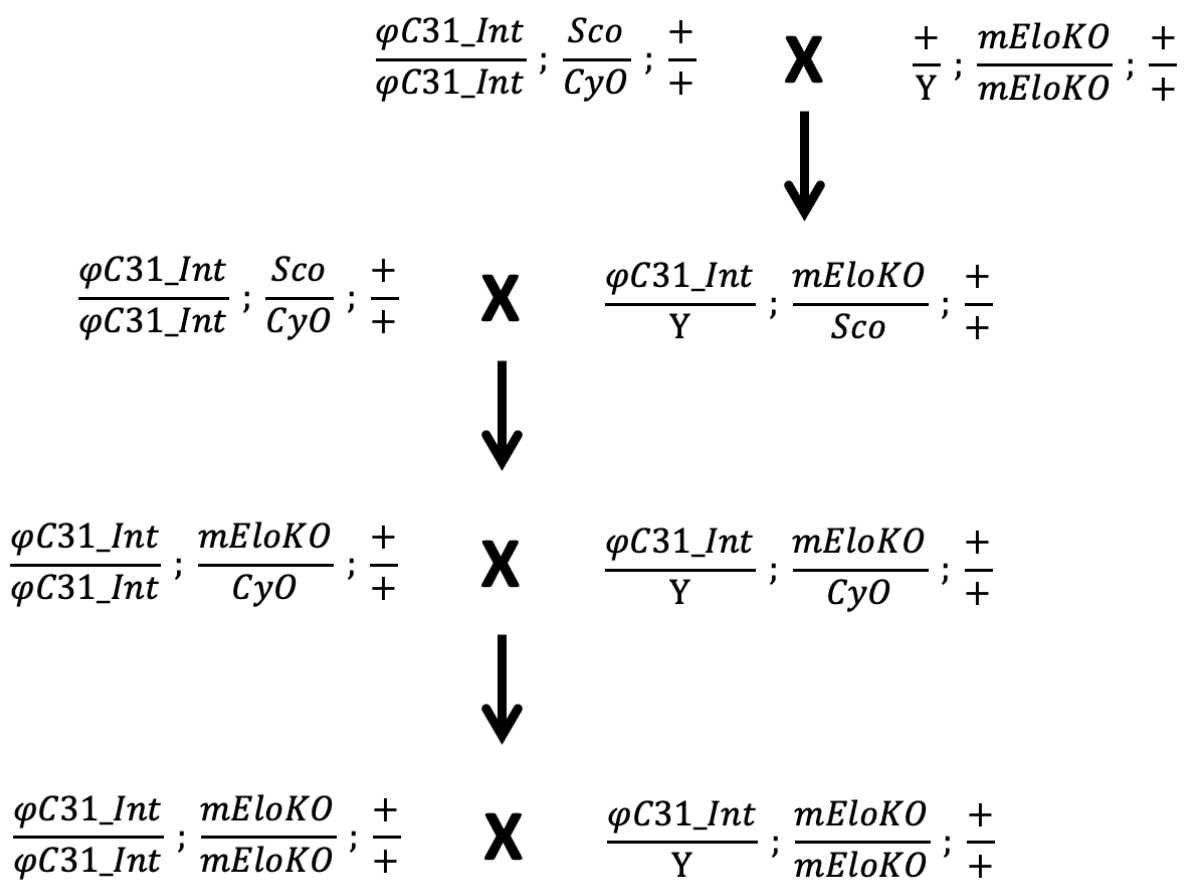


Figure A2.3. Crossing scheme for generating *D. melanogaster mEloKO* strains with *φC31* integrase on the X chromosome.

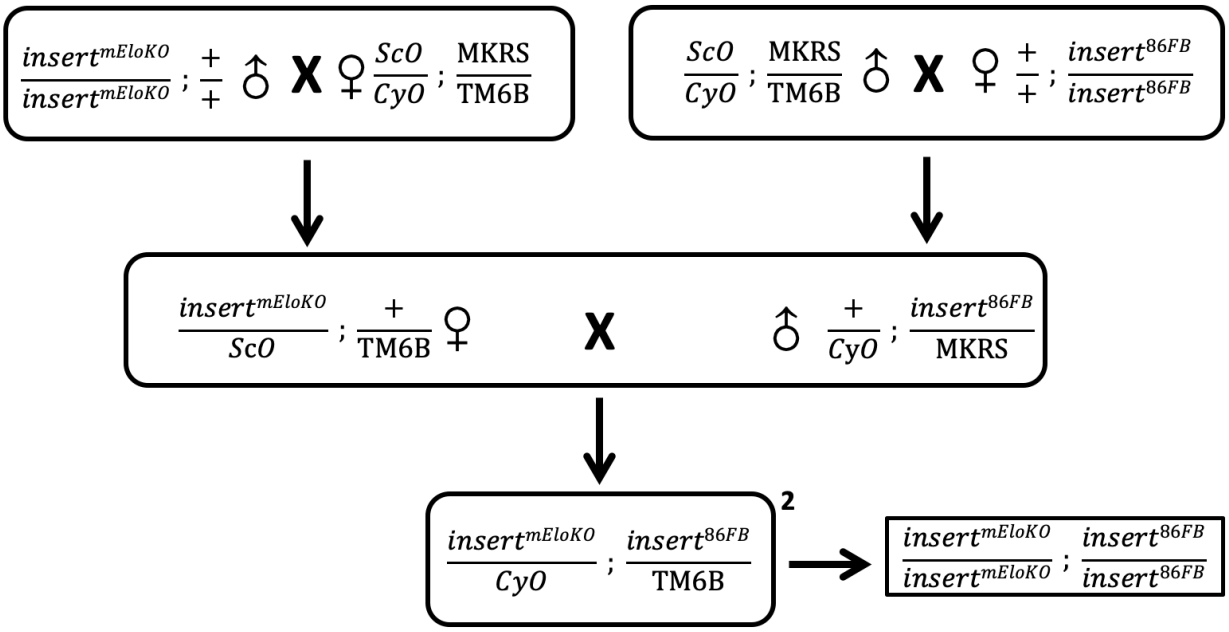


Figure A2.4. Crossing scheme for generating *D. melanogaster* UAS strains with inserts on both the 2nd and 3rd chromosomes.

Table A2.6. CHC profiles for *5'GI20345-GAL4 ; 5'GI20345-GAL4* > *UAS-O/E* with both *GI20345* and *GI20347* on the 2nd and 3rd chromosomes and with *GI20347* and *GI20345* on the 2nd and 3rd chromosomes. The abundance is in ng / fly.

Female_CHCs	Control	GI20345;GI20347	GI20347;GI20345
C21	1.8 ± 1.1	0 ± 0	0 ± 0
C22	2.6 ± 1.6	2.4 ± 1.6	0 ± 0
C23 + 9-C23:1	108 ± 11	94 ± 12.5	70.1 ± 14.3
7-C23:1	52.4 ± 6.2	38.5 ± 6.7	22.2 ± 1.9
2MeC24	81.3 ± 15.7	13.1 ± 3.3	2.9 ± 2.9
C25 + 9-C25:1	163.1 ± 14.3	106.1 ± 14	59.4 ± 10.6
2MeC26	93.1 ± 20.8	59.7 ± 20	1.2 ± 1.2
C27	156.5 ± 12.8	131 ± 18	116.3 ± 24.8
7-C27:1	28.2 ± 3	57.3 ± 11.2	61.2 ± 6.6
7,11-C27:2	0 ± 0	14.1 ± 14.1	37.1 ± 12.1
5,9-C27:2	380.1 ± 43.8	318.9 ± 38	228.5 ± 39.1
2MeC28	8.2 ± 1.9	88.9 ± 9.4	90 ± 13.7
C29	0 ± 0	5.9 ± 2.1	8.3 ± 1.2
7-C29:1	0 ± 0	5.8 ± 3.3	4 ± 1.9
7,11-C29:2	66 ± 3.5	152 ± 24.4	128.7 ± 11.8
2MeC30	0 ± 0	9.4 ± 1	9.4 ± 0.8
Male_CHCs	**Control**	**GI20345;GI20347**	**GI20347;GI20345**
C21	15.3 ± 1.3	6.5 ± 2.1	8.6 ± 0.6
C22	24.7 ± 1.5	16.4 ± 2.1	15.8 ± 1.5
C22:1	9.1 ± 0.4	4 ± 1.3	4.7 ± 2.7
C23 + 9-C23:1	254.7 ± 10.6	172.2 ± 17	179.7 ± 16.6
7-C23:1	778.2 ± 32.1	494.1 ± 20.2	484.4 ± 33.4
5-C23:1	42 ± 1.5	37.1 ± 2.8	42.3 ± 3.2
2MeC24	94.6 ± 8.7	25.7 ± 2.4	38.6 ± 4.9
C25 + 9-C25:1	34.5 ± 3.5	38.9 ± 2.8	79.7 ± 8.6
7-C25:1	41.5 ± 6.2	94.6 ± 11.5	182.1 ± 14.5
2MeC26	25.7 ± 5.4	32.4 ± 3.9	42 ± 4.2
C27	0 ± 0	9.3 ± 0.5	12.2 ± 2.1
7-C27:1	0 ± 0	19.3 ± 5.6	40.9 ± 4.8
2MeC28	0 ± 0	25.4 ± 4.7	31.5 ± 2.2
7-C29:1	0 ± 0	1.5 ± 1.5	0.6 ± 0.6
2MeC30	0 ± 0	3.9 ± 0.2	5.3 ± 0.3

Table A2.7. CHC profiles for *D. mojavensis* with mElo knocked out. ISO1, ISO2, and ISO3 are iso-female lines established from the parental population. M3.5, M3.9, and M3.11 are independent *mElo*-knockout lines with 5 bp insertion, 90 bp deletion, and 10 bp deletion on the third exon of *mElo*. The abundance is in ng / fly.

Female_CHCs	**ISO1**	**ISO2**	**ISO3**	**M3.5**	**M3.9**	**M3.11**
2MeC28	7.9 ± 0.9	10 ± 1	7.8 ± 0.8	11.1 ± 0.6	9.5 ± 2.9	14.9 ± 4.7
2MeC30	51.3 ± 6	53.1 ± 2.5	54.4 ± 5.2	22.5 ± 1.2	15.4 ± 3.1	23 ± 1.4
2MeC32	7.7 ± 1.3	8.9 ± 1.3	5.9 ± 0.6	0 ± 0	0 ± 0	0 ± 0
C33:1	3.1 ± 1.3	0 ± 0	1.3 ± 1.3	7.7 ± 0.9	2.2 ± 3.1	8.2 ± 3
C35:2 (a)	25.3 ± 2.1	27.2 ± 1.8	28.3 ± 1.5	30.1 ± 2.5	22.5 ± 4.4	29.9 ± 8.3
C35:2 (b)	5.9 ± 2.6	11.5 ± 3	16.2 ± 1.8	8.7 ± 0.5	1.2 ± 2.7	6.2 ± 5.9
C37:2 (a)	12.7 ± 2	16.6 ± 1.6	17.4 ± 1.6	14 ± 0.9	7.7 ± 1.7	17.1 ± 3.4
C37:2 (b)	15.1 ± 2.8	19.7 ± 2.5	27.8 ± 2.9	16.4 ± 1	9.4 ± 2.7	20.8 ± 5.6
Male_CHCs	**ISO1**	**ISO2**	**ISO3**	**M3.5**	**M3.9**	**M3.11**
2MeC28	8.6 ± 0.5	9.4 ± 0.9	8.2 ± 0.5	12 ± 1	8.9 ± 0.7	11 ± 2.5
2MeC30	53.9 ± 2.7	62.3 ± 3.7	55.2 ± 3.6	24.3 ± 1.4	12.7 ± 2	21.9 ± 2
2MeC32	6.3 ± 0.6	7.5 ± 0.7	6.5 ± 0.7	2.2 ± 1.4	0 ± 0	0 ± 0
C33:1	3.8 ± 1.1	1.2 ± 1.2	0 ± 0	6.2 ± 0.2	2.6 ± 3.8	7 ± 4.3
C35:2 (a)	46.7 ± 2.8	54.1 ± 4.2	46.8 ± 2.2	45.3 ± 7.5	23.9 ± 3.8	42.4 ± 10.6
C35:2 (b)	5.3 ± 3.4	0 ± 0	12 ± 7.3	12.8 ± 5.5	4.2 ± 6.5	6.2 ± 8.8
C37:2 (a)	9.5 ± 1	8.3 ± 1.3	9.1 ± 1.4	9.7 ± 2	5.5 ± 1	7 ± 1
C37:2 (b)	15.8 ± 1.9	14.8 ± 1.9	12 ± 1.3	18.9 ± 4.2	7.5 ± 1.9	8.3 ± 2.3

Dmel/mElo genomic span

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mEloKO sequences

5′ – TTTACCGGCGATTGTGATAGTTTTGGGTTACCTGCTGCTGATTTTCAAAGTGGGACCCGATTTCATGAGATCTCGTAAGCCCTACAATATGCGAAAAGCCATGTTGATCTACAACTTCTGTCAAGTTCTAATGAATTCTGGCATTTTTTTAATGG
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CTACTTTTACTTTATCAACAAGGTGATCGATCTGATAGACACGATATTCTTTGTGCTGAGAAAGAGCAACAAGCAAATCACCGTACTCCATGTATACCATCATGTATTCATGGTTTTGGGTGTGCCATTGACATACTATTTCTACGGACCCGGAGGACAATAC
AACCTGATGGGATATCTCAACTCATTTGTACATGTGGTGATGTACGCCTACTATTTCGCATCTGCTTGGTATCCAAACGTGAAGAGCACATTTTGGTGGAAAGAGTACATCACCAAGCTGCAGTTCCTACAGTTCATGATCCTCTTCGCCCAATCTGTACTGA
CATTGTGGCTAAATCCCGGTTGCCGTTTTCCCAAGGTCCTGCAGTACGTTCAGCTGGGAGGTTCCGTTTCCATGATGACCATGTTTGGAAACTTTTACTATCAAACCTATGTCAAAGCCAAAAGTAAAGAGCAGTGATTTATAAATACAATGCAGTTCTCTT
TACGCAATTGAGCCGGTCTCAATCGAGTGTACTGAGACTGAGCGTTTTATTCTGGGTCTCTCATTCCGAAATGTAGGACAGACATCGCAGTTCAAATAGCTGAATAATACATTATGTGATTTGATTTGGCTTCATTTCGTTAAAACAAACTGGATTGTTTCAA
GACTGCTGAAACAGTCGCCAGCGGTTCCTCAAACAACAATCAACCATTTAATAATGCTCCGATACTTGCGCA TACCTCAAGCGGGTAAGTTCGGCAATGTGCTGATTACTACAAGTATATCACTTCTTTGAAATACTCAACAGATCCTAACCCAATTCCGCT
GGCTGGATCACCATGGCCTATCACACTGATTTTGATAGCATATCTGTTGTTTGTCCTTAAATTGGGCAAGATCTTTATGAGA|*ggacatatgcaCACCTGCgatcgtagtgccccaactggggtaacctttgagttctctcagttgggggcgtagataacttcgtataatgtatgctat*
acgaagttatcgtacgGGATCTAATTCAATTAGAGACTAATTCAATTAGAGCTAATTCAATTAGGATCCAAGCTTATCGATTTCGAACCCTCGACCG CCGGAGTATAAATAGAGGCGCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACAC
GTCGCTAAGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACAATCGGCTCGAAGCCGGTCGCCACCatgGCCTCCTCCGAGGACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCGTGAACGGCCACGAGTTC
GAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACATCC
CCGACTACAAGAAGCTGTCCTTCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTcCAGGACGGCTCCTTCATCTACAAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCGACG
GCCCCGTAATGCAGAAGAAGACTATGGGCTGGGAGGCgTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAAGGGCGAGATCCACAAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTACATGGCCAAGAA
GCCCGTGCAGCTGCCCGGCTACTACTACGTGGACTCCAAGCTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAGCAGTACGAGCGCGCCGAGGGCCGCCACCACCTGTTCCTGTAGgggccGCGACTCTAGATCATAATC AGCCATACCACAT
TTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTTAACTTGTTTATTGCAGCTTATAATGGTTACaaataaaGCAATAGCATCACAAATTTCACaaataaaGCATTTTTTTCACTGCA
TTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAaccggtataacttcgtataatgtatgctatacgaagttataGAAGAGCactagtaaagatctccatgcataa|CGTTGGACTGCTGAACTCCTTGGTGCACACGGTCATGTATTTCTACTACTTTCTGTCTTCAG
AATATCCCGGAGTGAGGGCCAATATCTGGTGGAAGAAGTATATAACATTGACACAGCTCTGCCAGTTCTTCATGCTGCTCAGTTATGCCATCTATGTGCGATTCTTTTCACCGAATTGTGGCGTTCCACGCGGTCTTCTCTATCTAAATATGGTGCAAGGCGT
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AACCTTAATCGTATACAGAAAGTAAAATAATTTTCCATTCCACGTTAAGTCGCGTGTTTCAACAAATTTCCATGAAAATGATTGCCGATGGGCGGTCGAAATGTATCGAAAACCTAATAACATAAAGCTCAAGCGACCGGCGACTGGAAAAATTGCCAACA
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ATAAAACATTTCTAAAAGCTCCTATTGAAAGTAAAGGTTTTTTTTTTTGTGGAAATAAGAGAACACCAATAATGTATGCTAAAGAGAAATTTCGACTCAAAAAAGGTCGGAATTTCAATCAAGAGCTCTGCAA –3′

Figure A2.5. Sequences for the knockout of *mElo* and homology-directed repair in *D. melanogaster*

Table A2.8. Primers or Oligos used for this study

Name	Primers (5' to 3')
mElo-gRNA1-BbsI-F	CTTCGCAAGATCTTTATGAGAAAC
mELO-gRNA1-BbsI-R	AAACGTTTCTCATAAAGATCTTGC
mElo-gRNA2-BbsI-F	CTTCGGAGGCCATGTCAATGCCGT
mElo-gRNA2-BbsI-R	AAACACGGCATTGACATGGCCTCC
mEloLeftHomo_EcoRI_F	gcGAATTCCCATGCTGTCCTCGGATCAT
mEloLeftHomo_NotI_R	gcGCGGCCGCTCTCATAAAGATCTTGCCCAATTT
mEloRightHomo_AscI_F	gcGGCGCGCCCGTTGGACTGCTGAACTCC
mEloRightHomo_XhoI_R	ggCTCGAGTTAGTACACACGGTTCTTTCCT
5'DmelCG17821-AscI-F2	gcGGCGCGCCACACTAGGCGTATACGTAACGTGG
5'DmelCG17821-SbfI-R2	ggCCTGCAGGGGTGTCTCCGTCGAATGC
3'CG17821-AscI-F	gcGGCGCGCCGTTTCAGCAGTCTTGAAACAATCC
3'CG17821-SbfI-R	ggCCTGCAGGTTTATAAATACAATGCAGTTCTC
5'DmojGI20343-G6-F	gcGGCGCGCCctgtggtgtgctgtggtgg
5'DmojGI20343-G6-R	ggCCTGCAGGatccgagtggtaaacgcgact
5’DmojGI20344-AscI-F2	gcGGCGCGCCACCTTTAGAAATAATGTCGCAAAAT
5’DmojGI20344-SbfI-R2	ggCCTGCAGGATGAATTACACTAGAAATATTTGTTACCTG
5’DmojGI20345-AscI-F2	gcGGCGCGCCAATATTTCTTAAAATAAAAAAT
5’DmojGI20345-SbfI-R2	ggCCTGCAGGTTTGCACTGCACTGTTTTCC
5’DmojGI20347-AscI-F	gcGGCGCGCCTAGAAATGTAGCTTATTTTTG
5’DmojGI20347-SbfI-R	ggCCTGCAGGGGTTAAAAGCCACCTTTCGTC
3’DmojGI20347-AscI-F2	gcGGCGCGCCTCATGCGCTTTTGGCTGG
3’DmojGI20347-SbfI-R2	ggCCTGCAGGGAGCTCCAAACAAATGTAAGCAT
3’DmojGI20343-SbfI-R	ggCCTGCAGGACCTTTAGAAATAATGTCGCAAAAT
3’DmojGI20343-AscI-F	gcGGCGCGCCATGAATTACACTAGAAATATTTGTTACCTG
3’DmojGI20344-SbfI-R	ggCCTGCAGGAATATTTCTTAAAATAAAAAAT
3’DmojGI20344-AscI-F	gcGGCGCGCCTTTGCACTGCACTGTTTTCC
3’DmojGI20345-SbfI-R	ggCCTGCAGGTAGAAATGTAGCTTATTTTTG
3’DmojGI20345-AscI-F	gcGGCGCGCCGGTTAAAAGCCACCTTTCGTC
DmelCG18609-EcoRI-F	ggGAATTCATGCTCCGATACTTGCGCATAC
DmelCG18609-XbaI-R	ggTCTAGACTACGATTGCTTTGCGTTGATTTTCG
DmelCG17821-NdeI-F	ggCATATGATGAACTTCACACTATTGGATTTATTATT
DmelCG17821-XbaI-R	ggTCTAGATCACTGCTCTTTACTTTTGGCTTT
DmojGI20343-NdeI-F	ggCATATGATGCACGCATCGAATTCAAGTC
DmojGI20343CDS_XbaIF	ggTCTAGAATGCACGCATCGAATTCAAGTC
DmojGI20345-NdeI-F2	ggCATATGATGGGCGTCGATATAATCGAAC
DmojGI20345-XbaI-R2	ggTCTAGACTATTGAGTTTTCTTCGATTTTGGC
DmojGI20347-EcoRI-F	ggGAATTCATGCTCAATATTTTCAATATTC

Table A2.8 (cont'd)

DmojGI20347-XbaI-R	ggTCTAGATTACATTTGTTTGGAGCTCTTC
GI20344 ISH Probe F	GGGGTGTACCGAGTTATCATTAAT
GI20344 ISH Probe R	TTACTGCGTTTTCAATGTCTTGGG
GI20347 ISH Probe F	GGCATTTGTGATCTGC
GI20347 ISH Probe R	CTTGATATAGAATTTGCCAAAC
Dmoj\w_sgRNA3_F	GAAATTAATACGACTCACTATAggccagcagttcgcccggatGTTTTAGAGCTAGAAATAGC
Dmoj\w_sgRNA4_F	GAAATTAATACGACTCACTATAGgatcaggagctattgatacgGTTTTAGAGCTAGAAATAGC
Dmoj\mElo-sgRNA5-F	GAAATTAATACGACTCACTATAGgaggcaattgaagtgaccgcGTTTTAGAGCTAGAAATAGC
Dmoj\mElo-sgRNA3-F	GAAATTAATACGACTCACTATAGGcgcgtttacaatcttggccGTTTTAGAGCTAGAAATAGC

APPENDIX III: Cuticular hydrocarbons of spotted wing *Drosophila* that collected in Michigan in 2014, 2015, and 2018 do not differ from each other

In 2018, a field observation was made about a field-collected pest insect, spotted wing Drosophila (SWD; *Drosophila suzukii*) by investigators in Dr. Rufus's lab in the Department of Entomology at Michigan State University. This observation shows that SWD strains collected from the field in 2014, 2015, and 2018 had different levels of desiccation resistance, even though these strains have been reared in the laboratory conditions for different periods ranging from a few months to four years. At the moment, a hypothesis was proposed that within a few years, the higher desiccation resistance that evolved in the population in Michigan could be contributed by the evolution of CHCs. To test this hypothesis, I obtained those three SWD strains from Dr. Rufus and examined the differences in their cuticular hydrocarbons. The results showed that there are no significant differences between the strains collected in 2014, 2015, and 2018 (**Figure A3.1**).

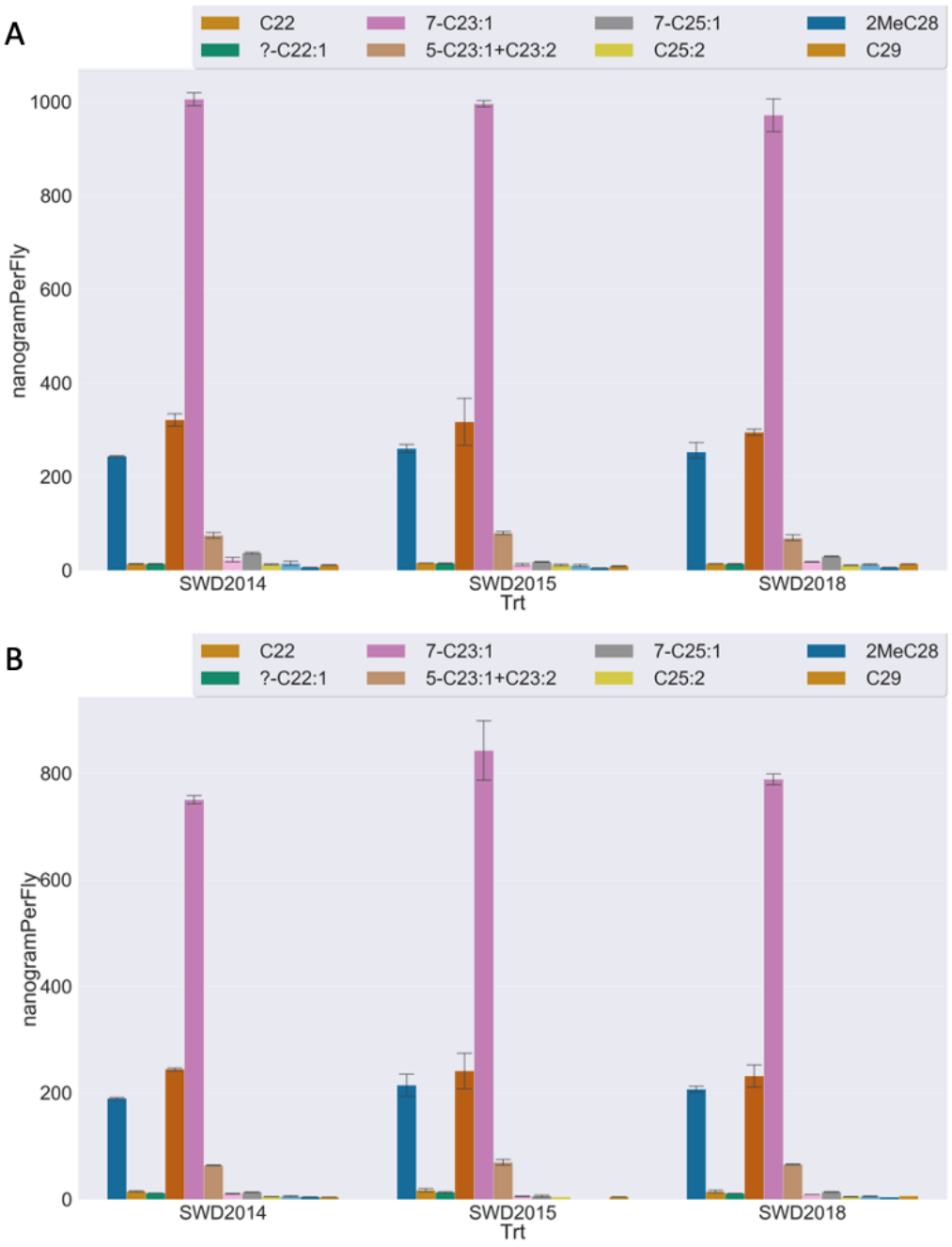

Figure A3.1. Cuticular hydrocarbon profiles of spotted wing *Drosophila* (SWD) that collected in 2014, 2015, and 2018. Although significant differences in desiccation resistance were observed in the three field-collected strains, no significant differences were observed in the cuticular hydrocarbons. CHC extraction, identification, and quantification were performed as mentioned in Chapter 1. For each of identified CHCs, one-way ANOVA was performed to determine the difference between the strains at *alpha* = 0.05.No significant differences were found for all CHCs.

APPENDIX IV: Characterization of cuticular hydrocarbons in the black soldier fly, *Hermetia illucens* L. (Diptera: Stratiomyidae)

The black soldier fly is famous for its capability in decomposing organic material and high potential for application in waste management (Tomberlin et al. 2002). Many studies on the mass rearing of this species showed the requirement of high relative humidity in this species (Holmes et al. 2012, Chia et al. 2018). This renders me interested in the CHC composition of this species.

To characterize the CHC composition of the black soldier fly, the CHCs of the black soldier fly were extracted at 2-day-old and 4-day-old for both females and males. The flies were obtained as pupae from Tomberlin lab in the Department of Entomology at Texas Agricultural and Mechanical University. After the flies emerged, I put them at 25°C. Since the black soldier fly has a much larger body size than fruit flies, I used 1 mL hexane with 25 ng/µL C26 as an internal standard to extract the CHCs from each single fly. The other operations were the same as described in Chapter I. Identification for methyl branched alkanes was based on Carlson et al. (1998).

Interestingly, I found all CHCs in the black soldier fly are linear alkanes and methyl branched alkanes (**Figure A4.1A**). CHCs of the black soldier fly are sexually monomorphic (**Figure A4.1A-B**). As the flies age, their CHCs also accumulate (**Figure A4.1B**). This fits the conclusion from Chapter 1 that a species needs both saturated and unsaturated CHCs to form a lipid layer with higher efficiency in preventing water loss. This could partially explain why the black soldier fly is sensitive to desiccation.

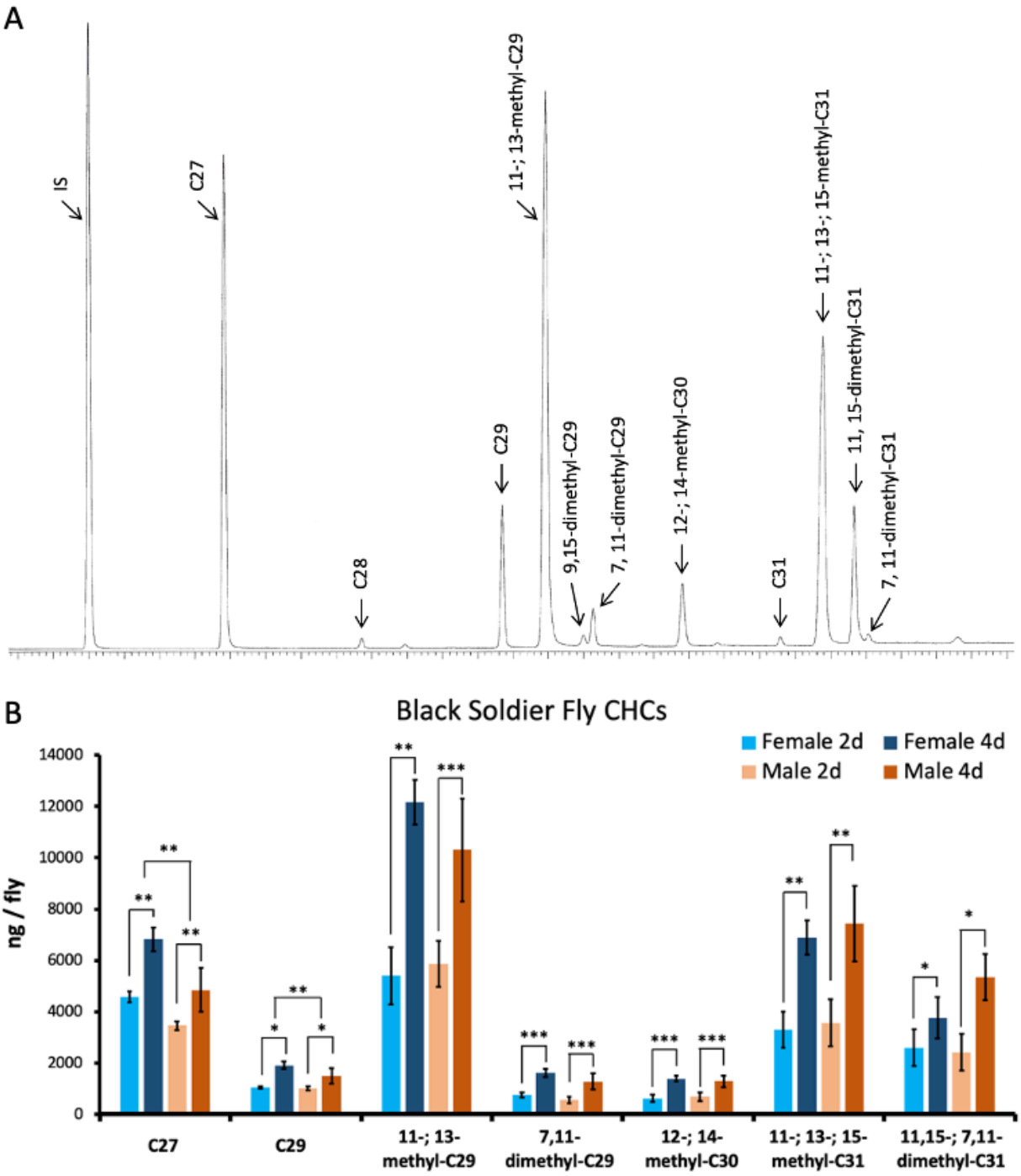

Figure A4.1. Cuticular hydrocarbon (CHCs) profiles of the black soldier fly. A. The chromatogram of CHCs in a female black soldier fly. B. Quantities of CHCs for both male and female black soldier flies at 2-d- and 4-d-old. CHC extraction, identification, and quantification were performed as mentioned in Chapter 1. For each identified CHC, one-way ANOVA followed by *post hoc* analysis was performed to determine the difference between the sexes and ages at *alpha* = 0.05.

APPENDIX V: *Drosophila mojavensis Cyp4g15* does not contribute to CHC synthesis

Mosquitoes, the notorious human-biting pests, are also one of the groups with the longest CHCs in Diptera. One of the recent studies showed in *Anopheles gambiae*, there are two functionally different decarbonylase genes in CYP4G family contributing to the CHC syntheses (Kefi et al. 2019). This study also showed one of CYP4G genes can specifically decarbonylase and produce very long chain dimethyl branched CHCs with 45 – 47 carbons. Although dimethyl branched CHCs are not found in *Drosophila* species, this published data allowed me to hypothesize that another CYP4G gene gained expression in oenocytes for synthesizing long chain CHCs in those *Drosophila* species with very long chain CHCs, including *D. mojavensis*. I searched the genome of *D. mojavensis*. Except for the conserved *Cyp4g1* (*GI11123*), there is another CYP4G gene, *Cyp4g15* (*GI14748*), in the genome of *D. mojavensis*. To test this hypothesis, I used *in situ* hybridization to test whether *Cyp4g15* is expressed in adult oenocytes for CHC syntheses. In this experiment, I used *Cyp4g1* as the control for *in situ* hybridization. Results showed that *Cyp4g15* is not expressed in the oenocytes, noting *Cyp4g1* has a very strong expression in oenocytes (**Figure A5**).

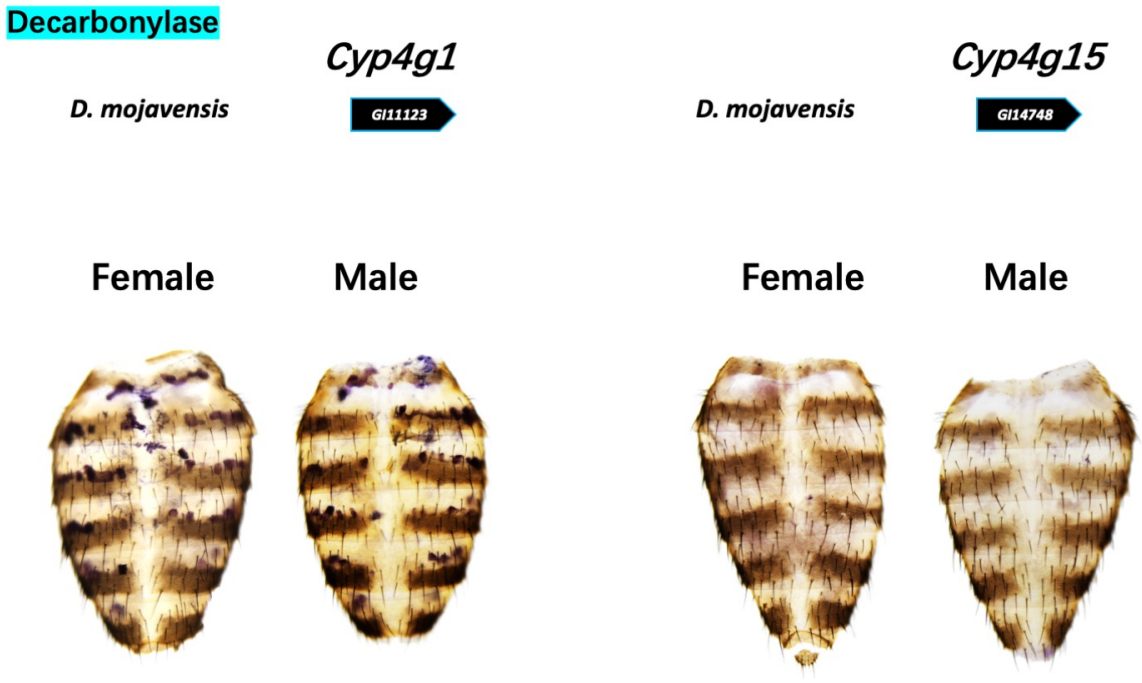

Figure A5.1. *In situ* hybridization of *D. mojavensis Cyp4g1* and *Cyp4g15* in adult oenocytes. Primers for generating the RNA probes are 5'-CTGAACAAGTACGGCGAGACG-3' and 5'-CTGGGCATACTCGAAACTCTTG-3' for *Dmoj/Cyp4g1*; and 5'-CTGTTTTCAAAACAGTCATACGC-3' and 5'-CCAATAGAATCTCCACAGTTGCC-3' for *Dmoj/Cyp4g15*.

APPENDIX VI: *In situ* hybridization of elongase genes in adult oenocytes of *Drosophila mojavensis*

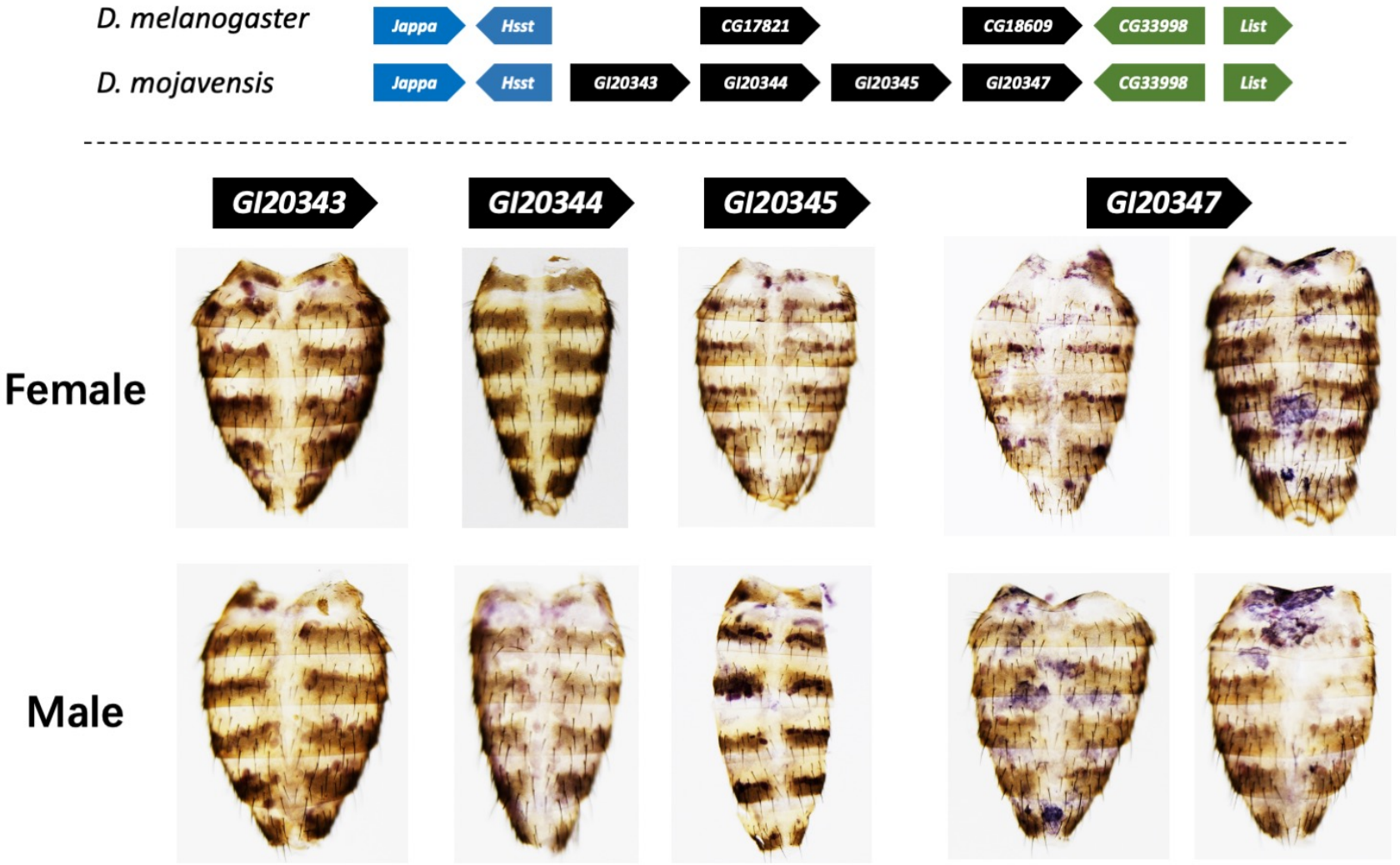

Figure A6.1. *In situ* hybridization of *GI20343*, *GI20344*, *GI20345*, and *GI20347* in adult oenocytes of female and male *D. mojavensis*.

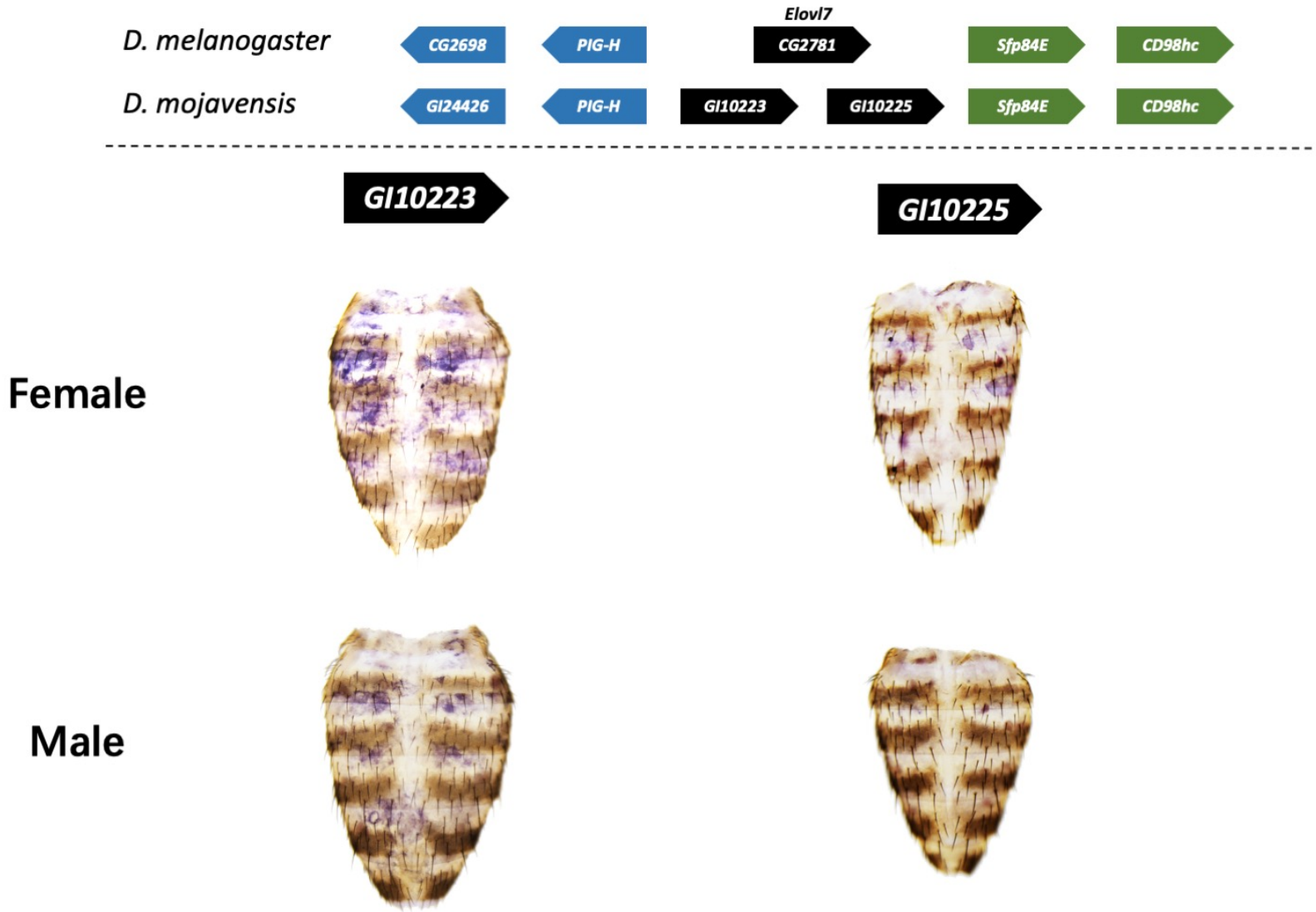

Figure A6.2. *In situ* hybridization of *GI10223* and *GI10225* in adult oenocytes of female and male *D. mojavensis*.

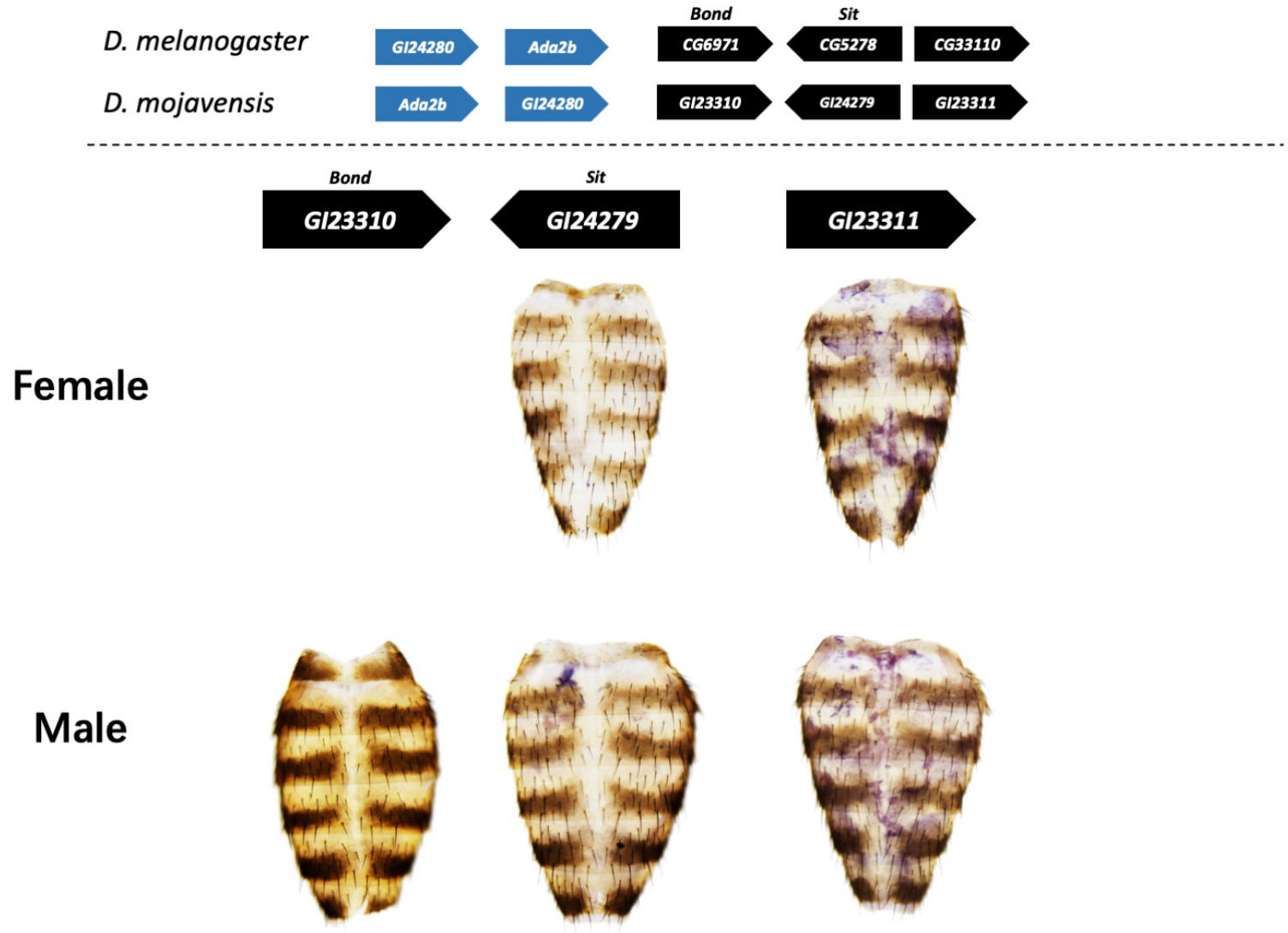

Figure A6.3. ***In situ*** **hybridization of** ***GI23310*****,** ***GI24279*****, and** ***GI23311*** **in adult oenocytes of female and male** ***D. mojavensis*****.**

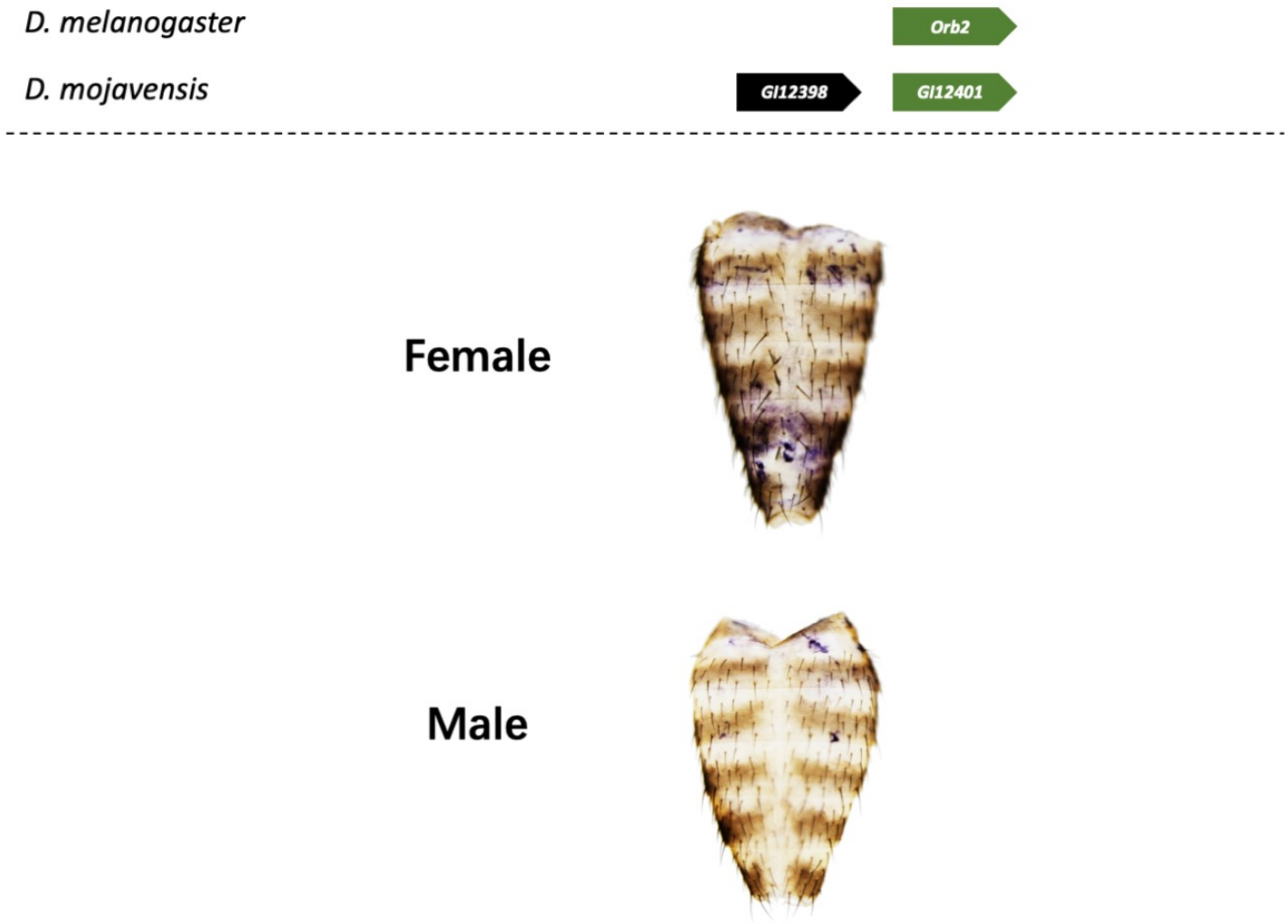

Figure A6.4. *In situ* hybridization of *GI12398* in adult oenocytes of female and male *D. mojavensis*.

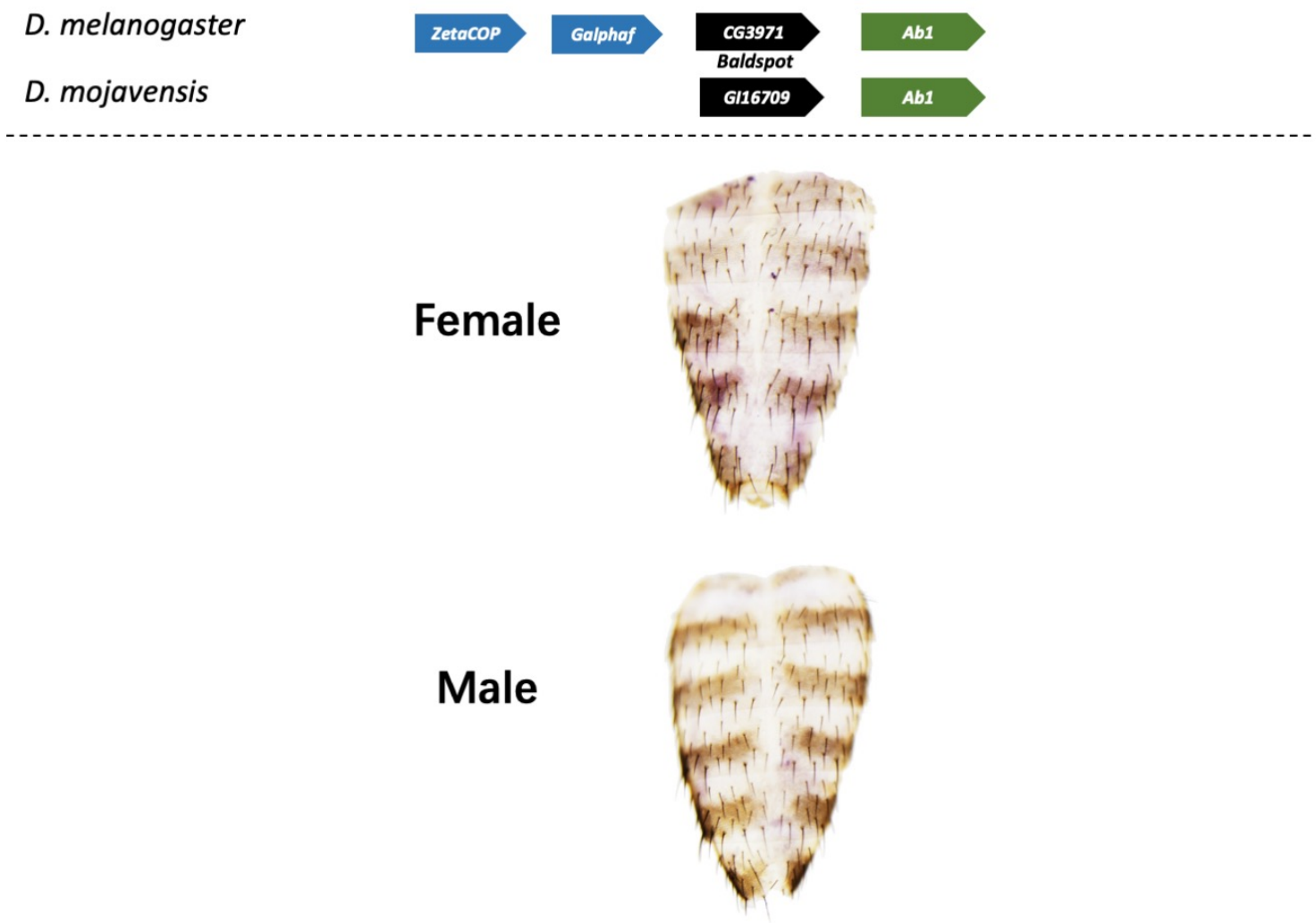

Figure A6.5. *In situ* hybridization of *GI16709* in adult oenocytes of female and male *D. mojavensis*.

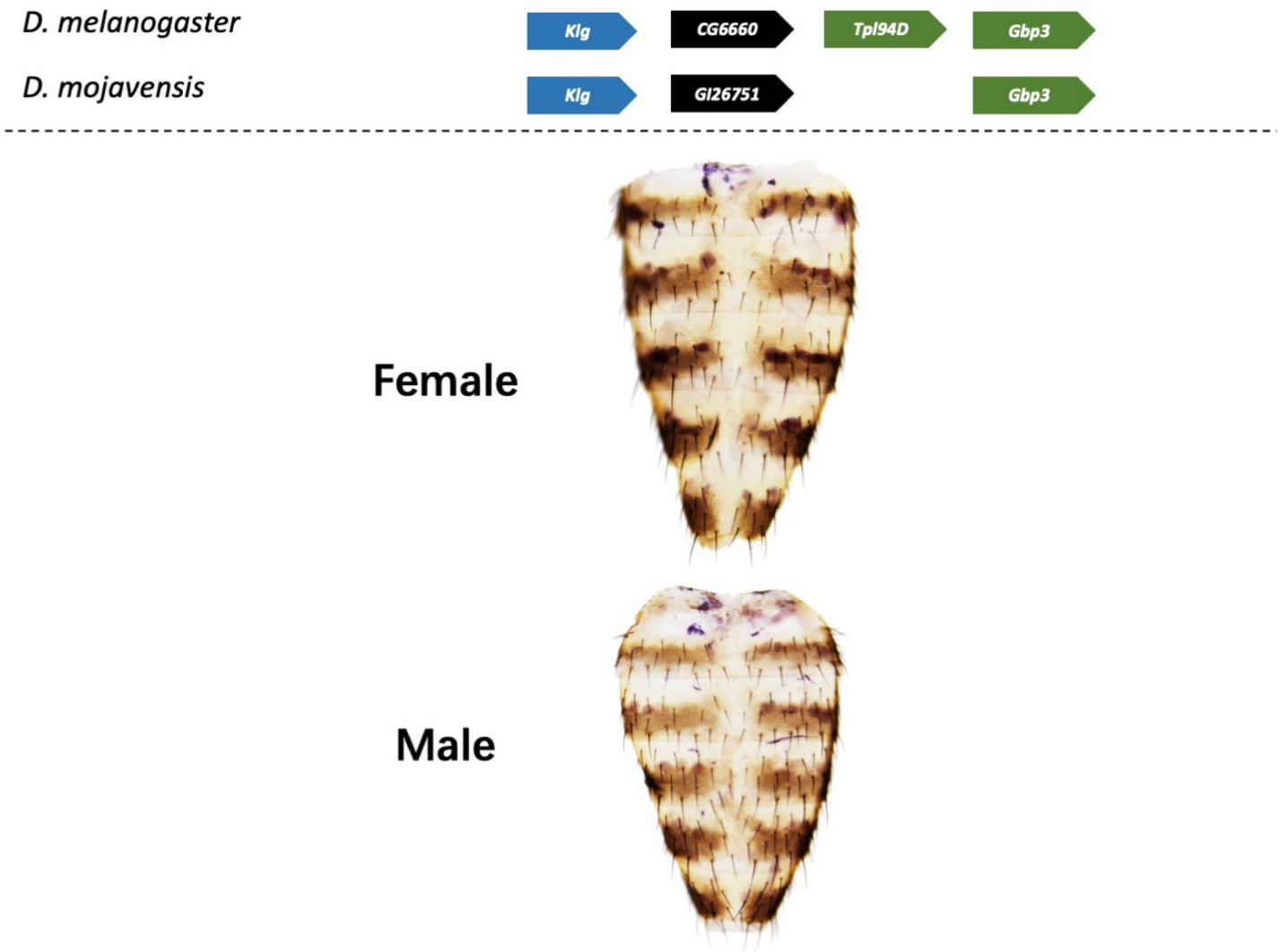

Figure A6.6. ***In situ*** **hybridization of** ***GI26751*** **in adult oenocytes of female and male** ***D. mojavensis*****.**

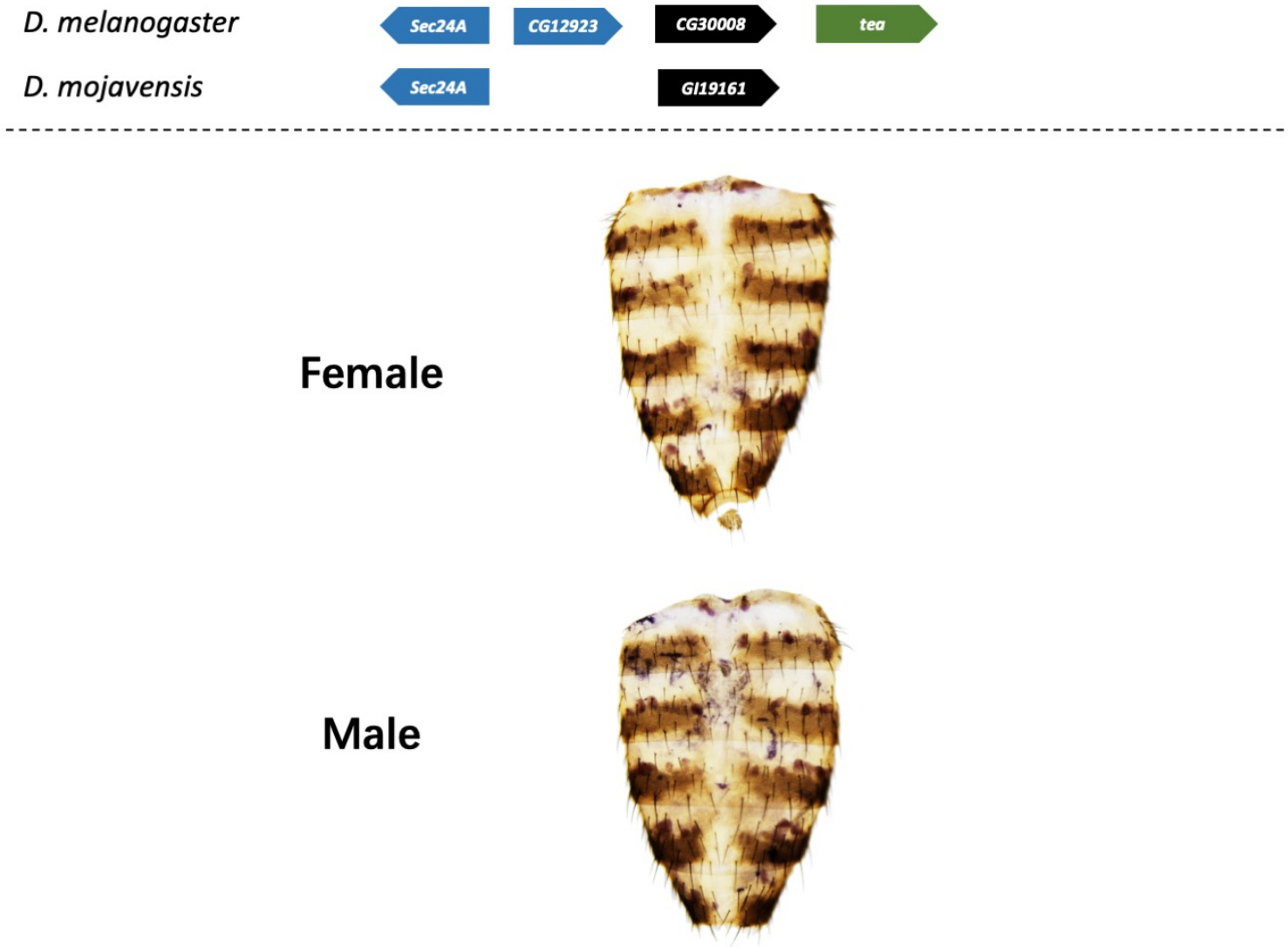

Figure A6.7. *In situ* hybridization of *GI19161* in adult oenocytes of female and male *D. mojavensis*.

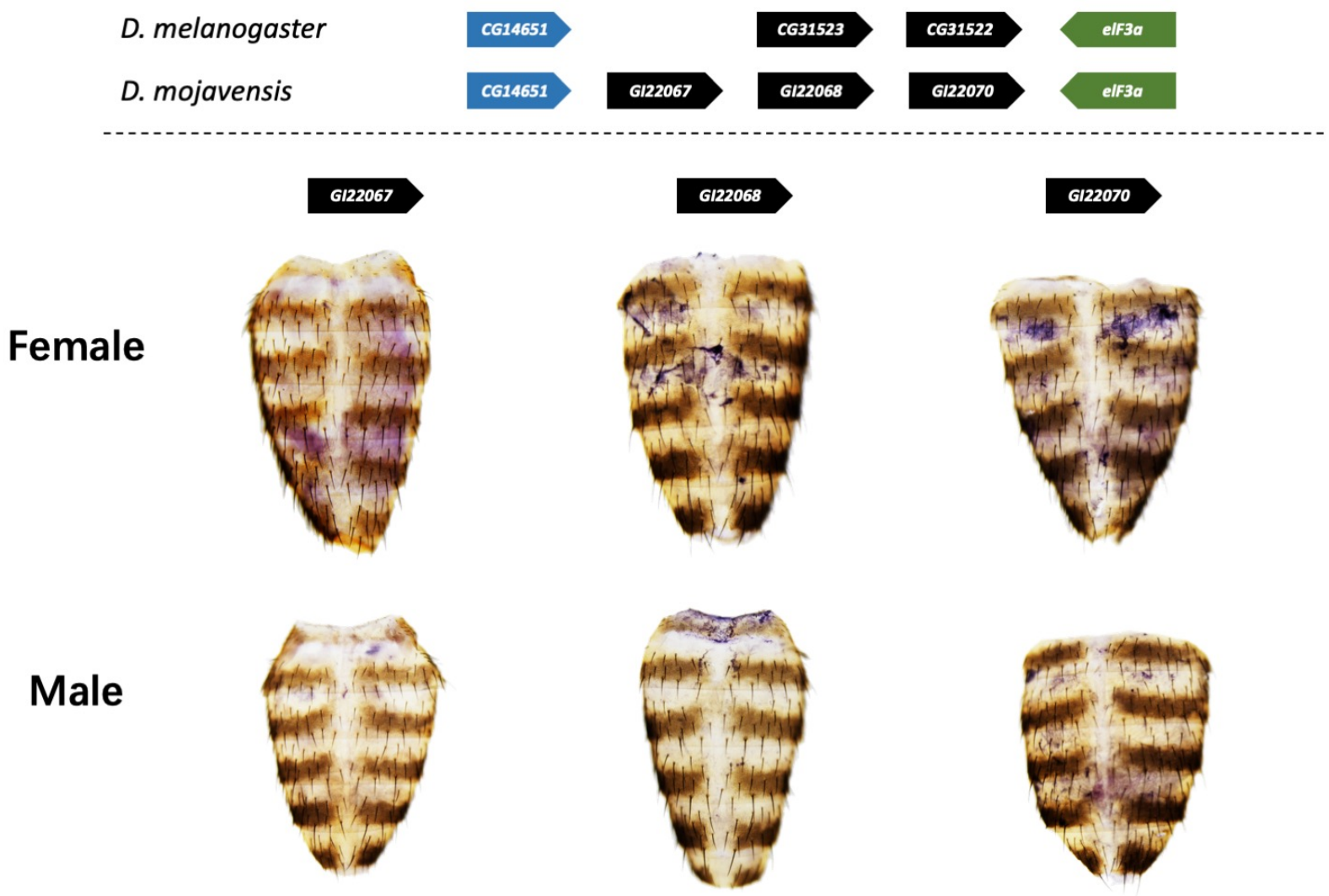

Figure A6.8. ***In situ*** **hybridization of** ***GI22067, GI22068*****, and** ***GI22070*** **in adult oenocytes of female and male** ***D. mojavensis*****.**

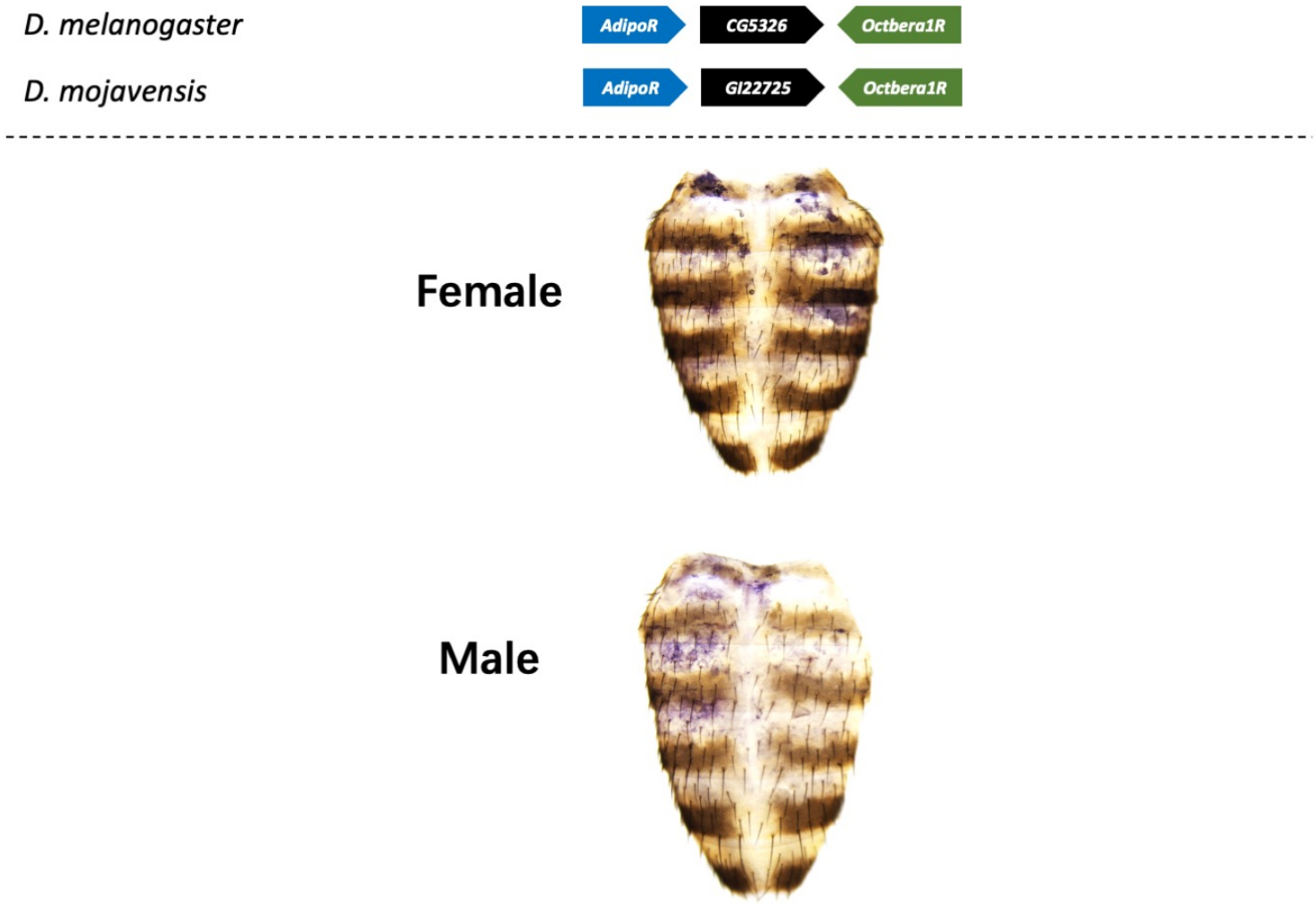

Figure A6.9. *In situ* hybridization of *GI22725* in adult oenocytes of female and male *D. mojavensis*.

APPENDIX VII: *In situ* hybridization of reductase genes in adult oenocytes of *Drosophila mojavensis*

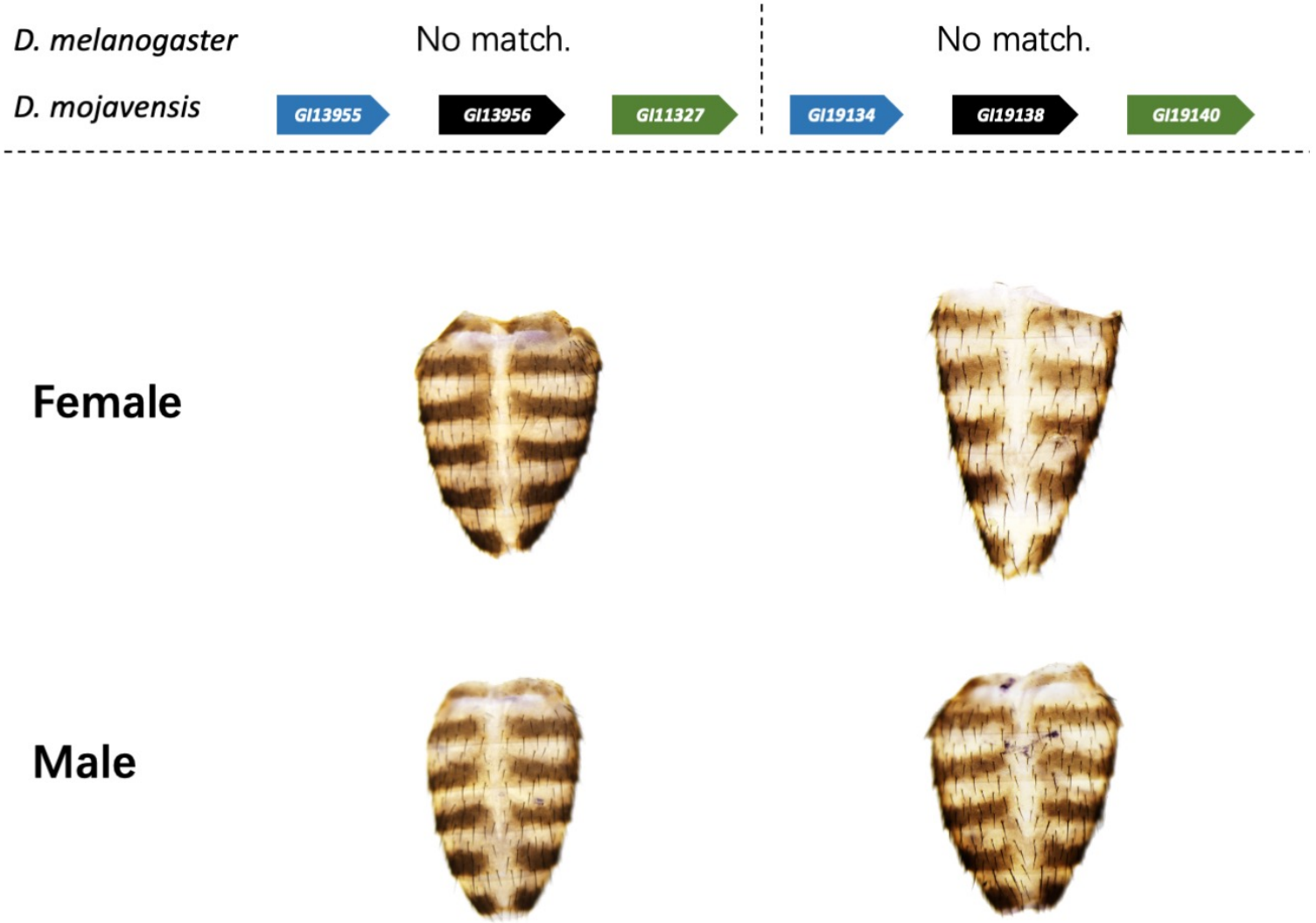

Figure A7.1. *In situ* hybridization of *GI13956* and *GI19138* in adult oenocytes of female and male *D. mojavensis*.

Figure A7.2. *In situ* hybridization of *GI20095* in adult oenocytes of female and male *D. mojavensis*.

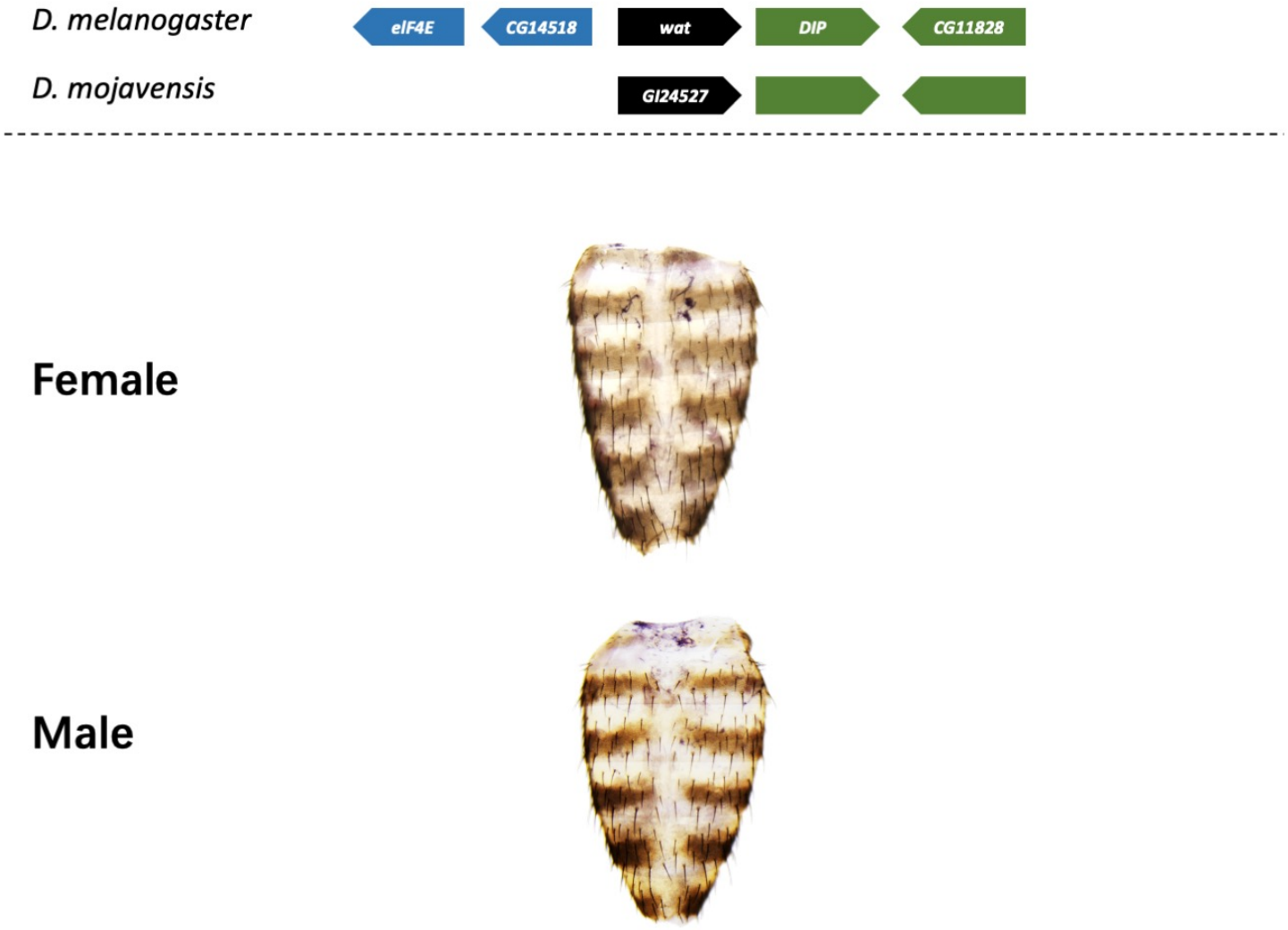

Figure A7.3. ***In situ*** **hybridization of** ***GI24527*** **in adult oenocytes of female and male** ***D. mojavensis*****.**

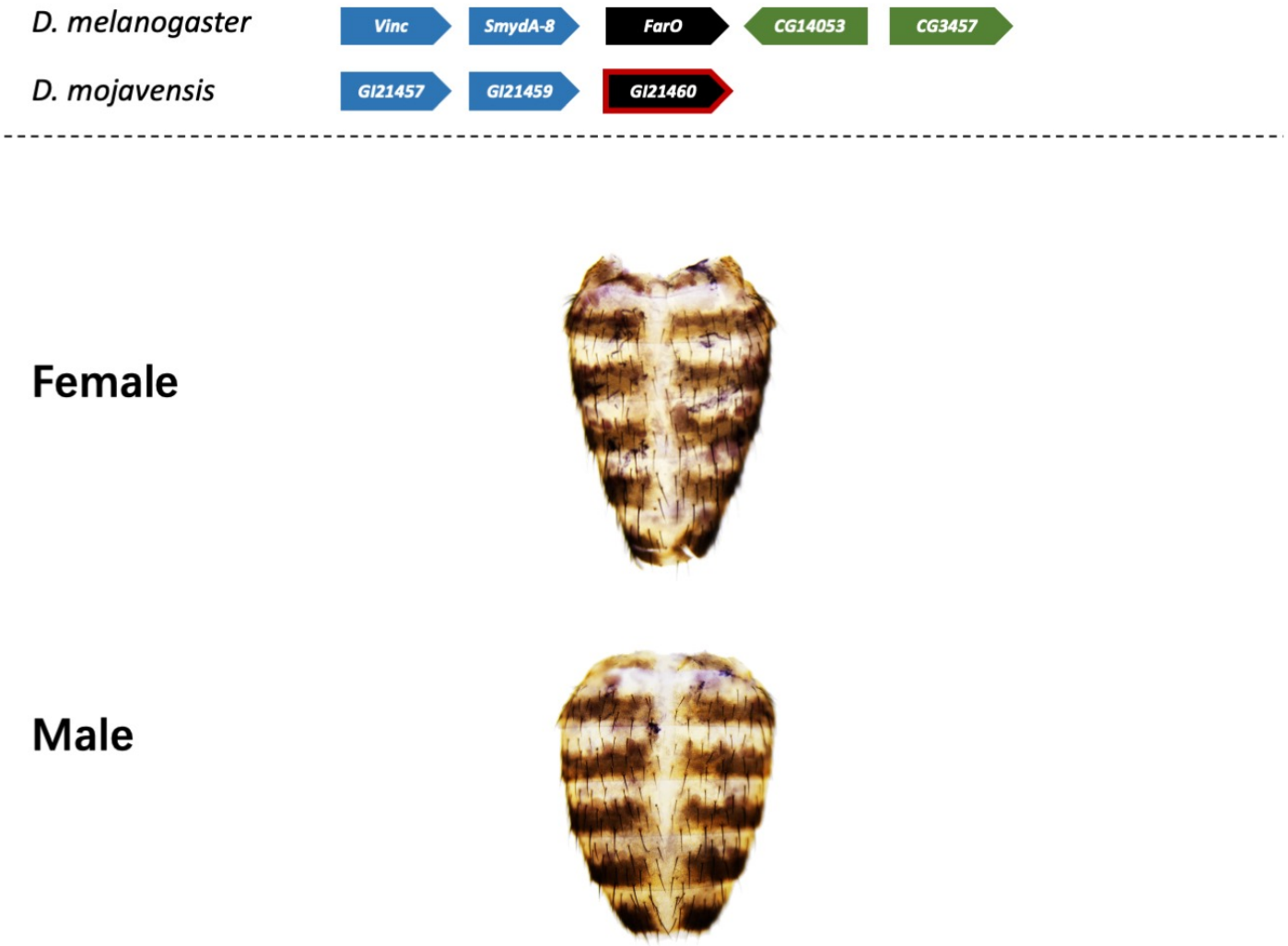

Figure A7.4. *In situ* hybridization of *GI21460* in adult oenocytes of female and male *D. mojavensis*.

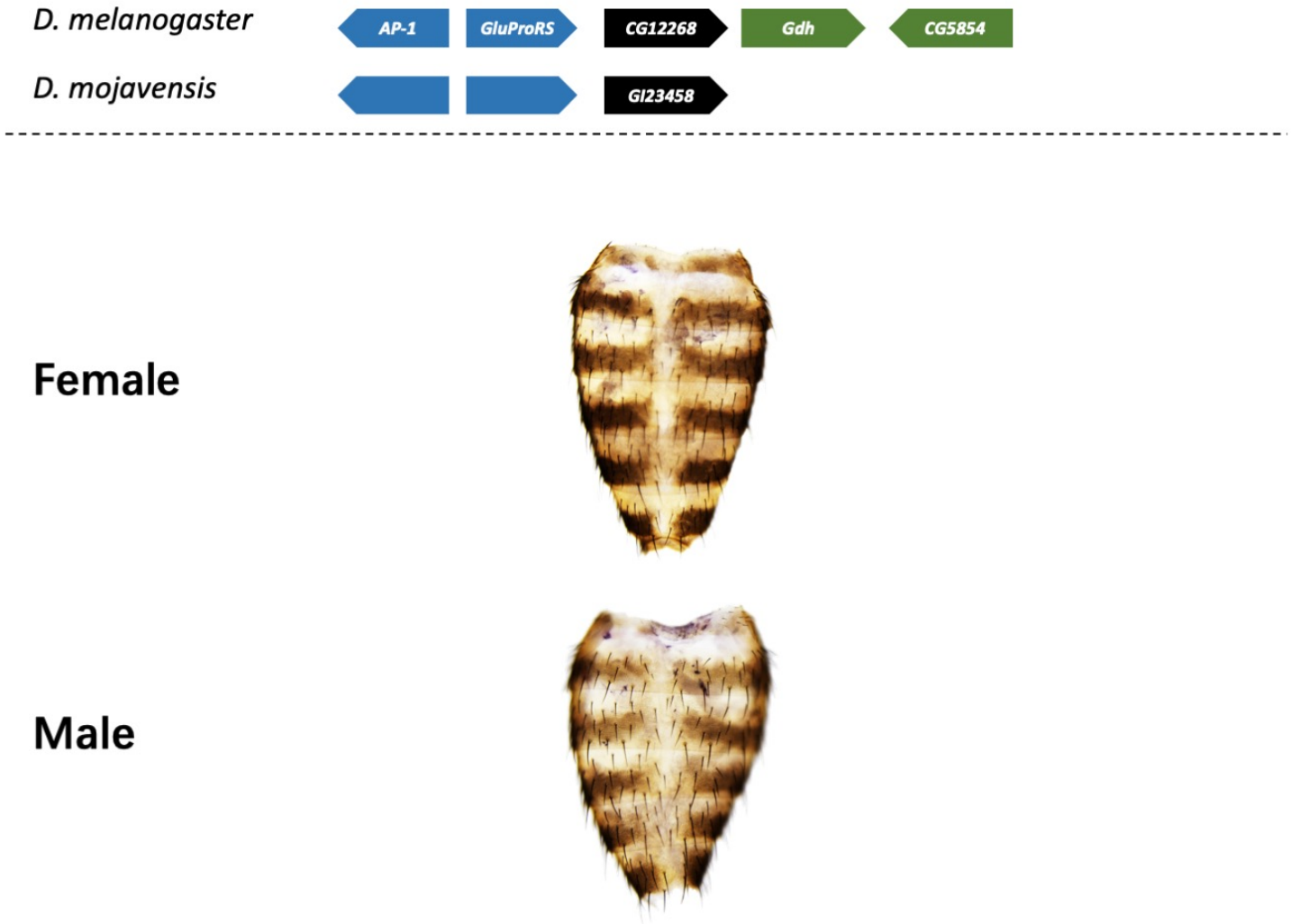

Figure A7.5. *In situ* hybridization of *GI23458* in adult oenocytes of female and male *D. mojavensis*.

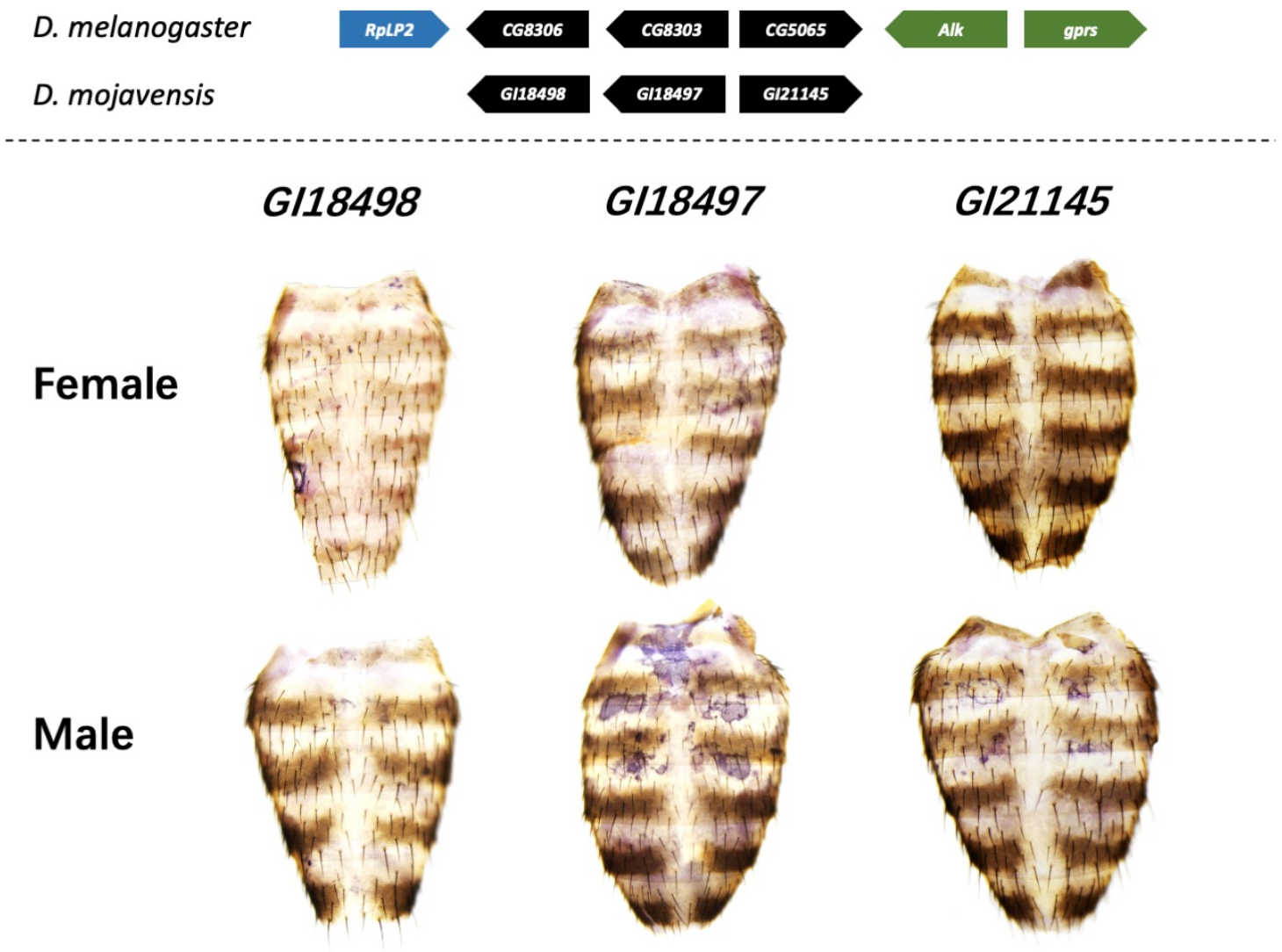

Figure A7.6. ***In situ*** **hybridization of** ***GI18498*****,** ***GI18497*****, and** ***GI21145*** **in adult oenocytes of female and male** ***D. mojavensis*****.**

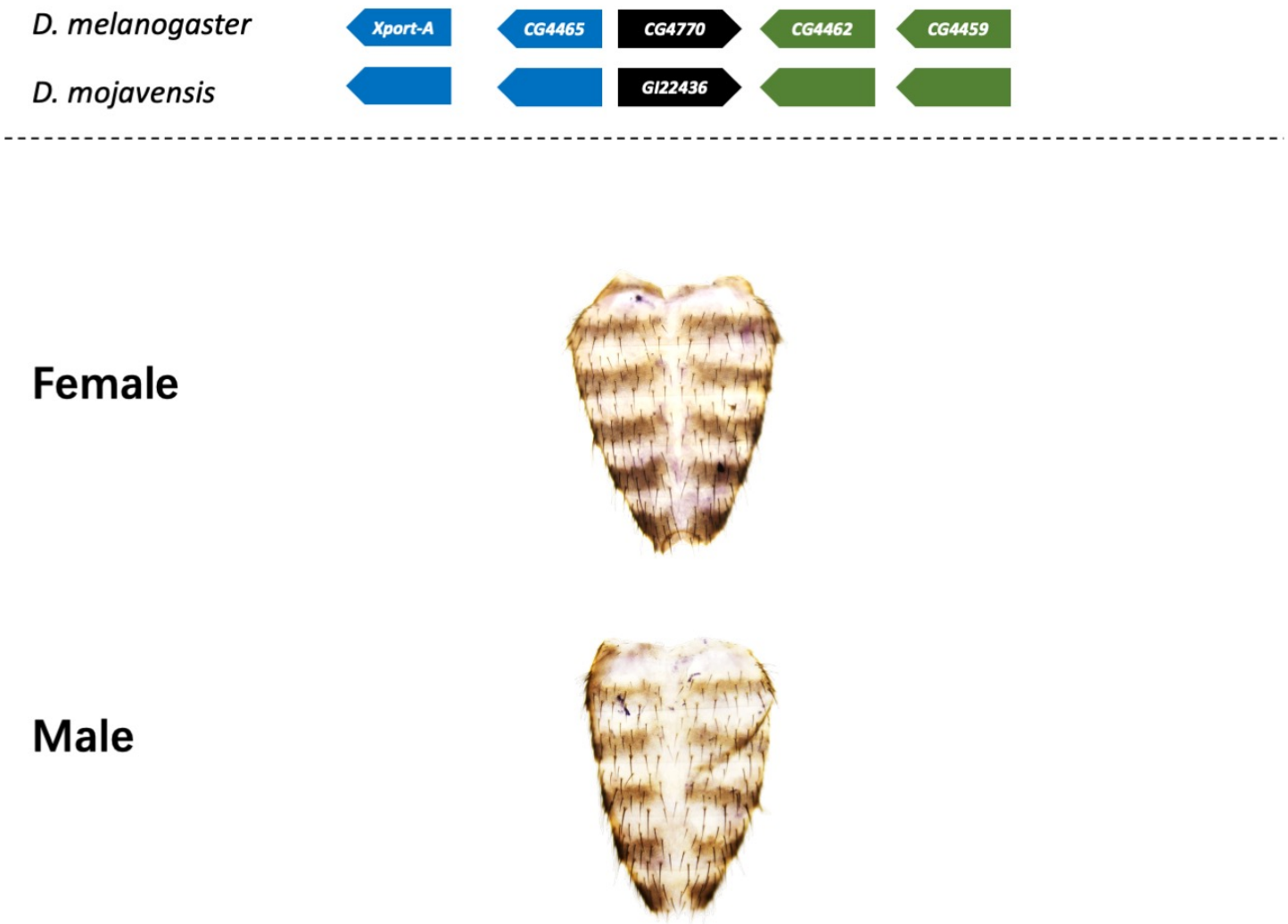

Figure A7.7. *In situ* hybridization of *GI22436* in adult oenocytes of female and male *D. mojavensis*.

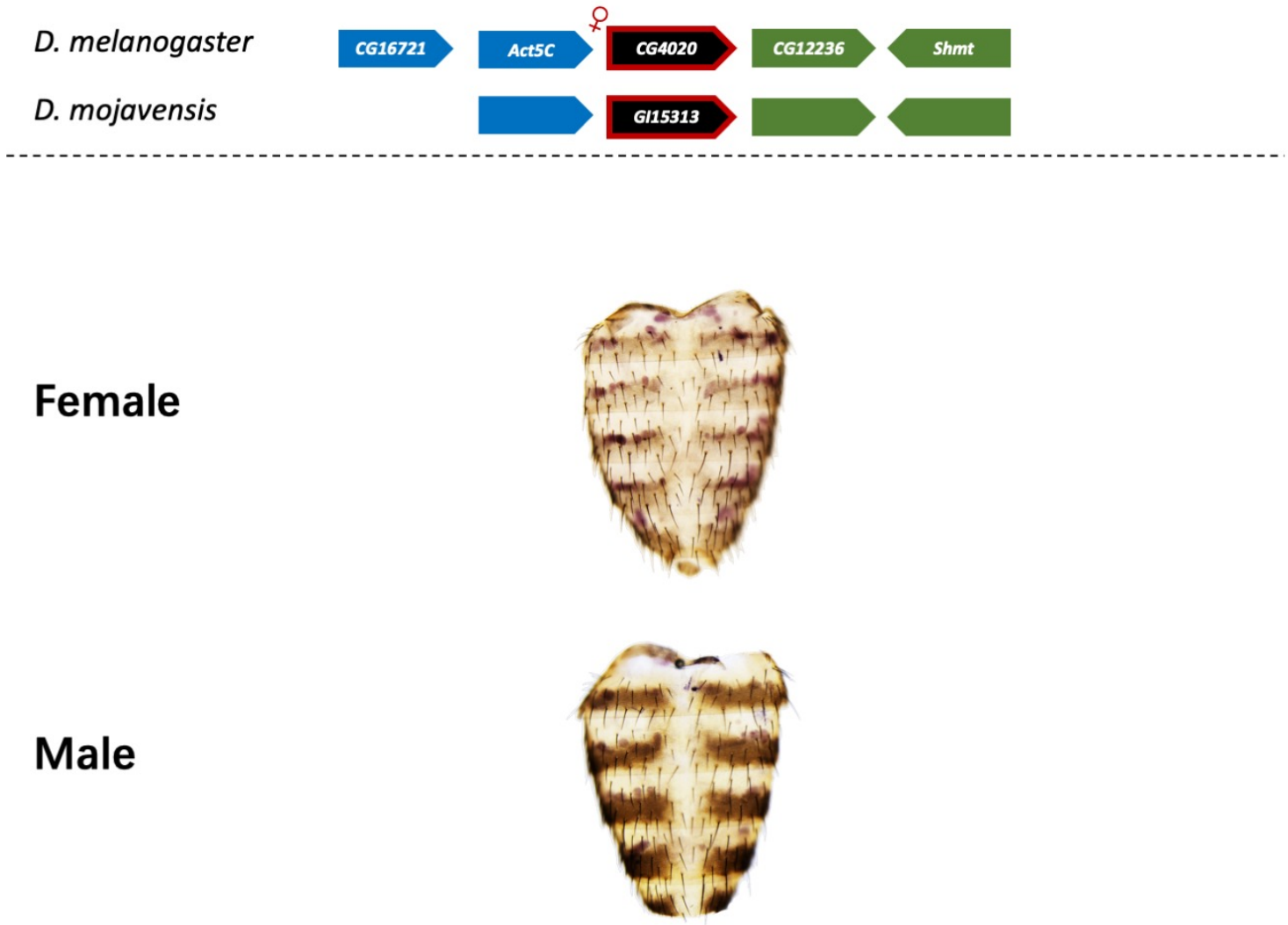

Figure A7.8. *In situ* hybridization of *GI15313* in adult oenocytes of female and male *D. mojavensis*.

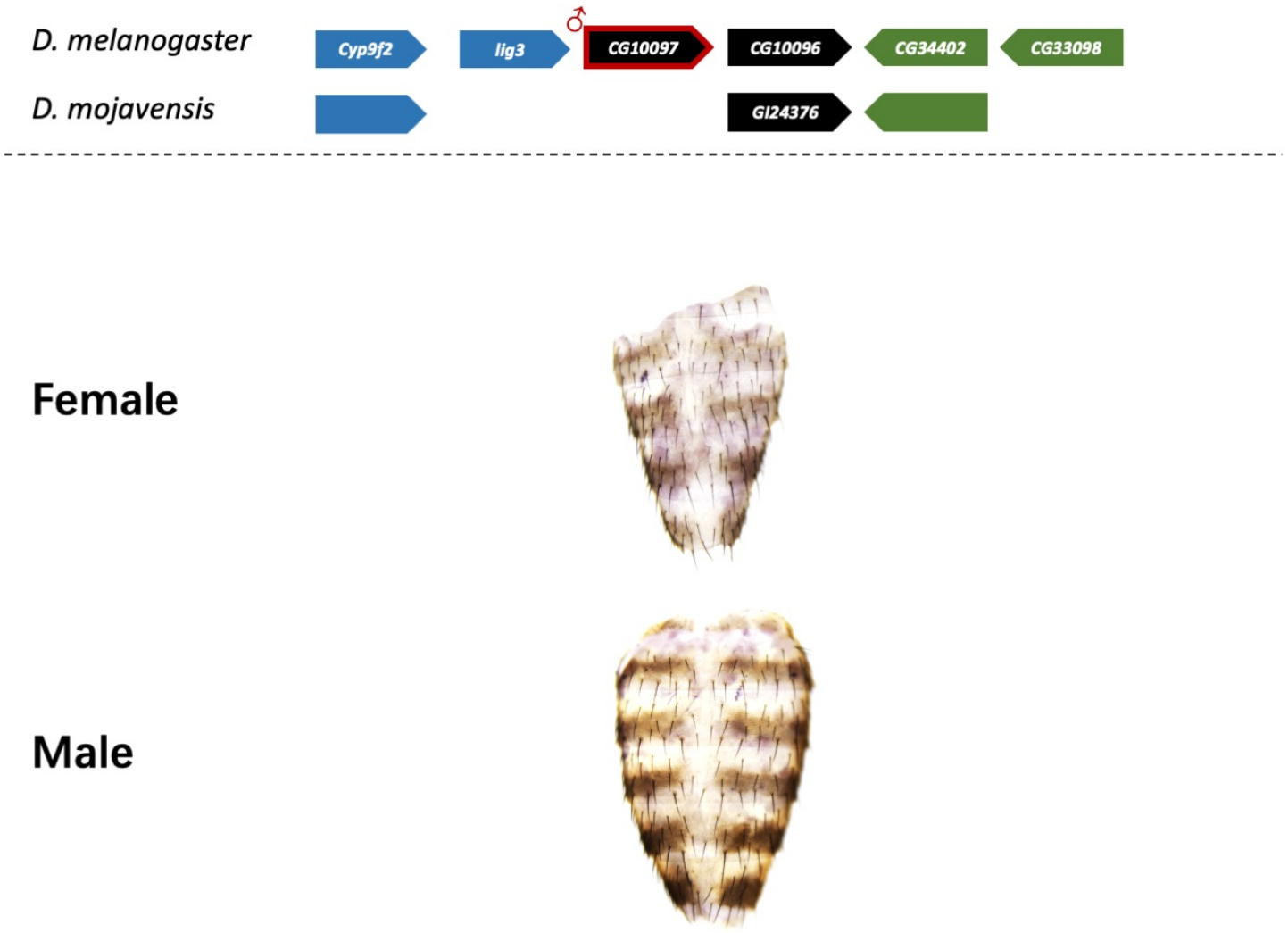

Figure A7.9. *In situ* hybridization of *GI24376* in adult oenocytes of female and male *D. mojavensis*.

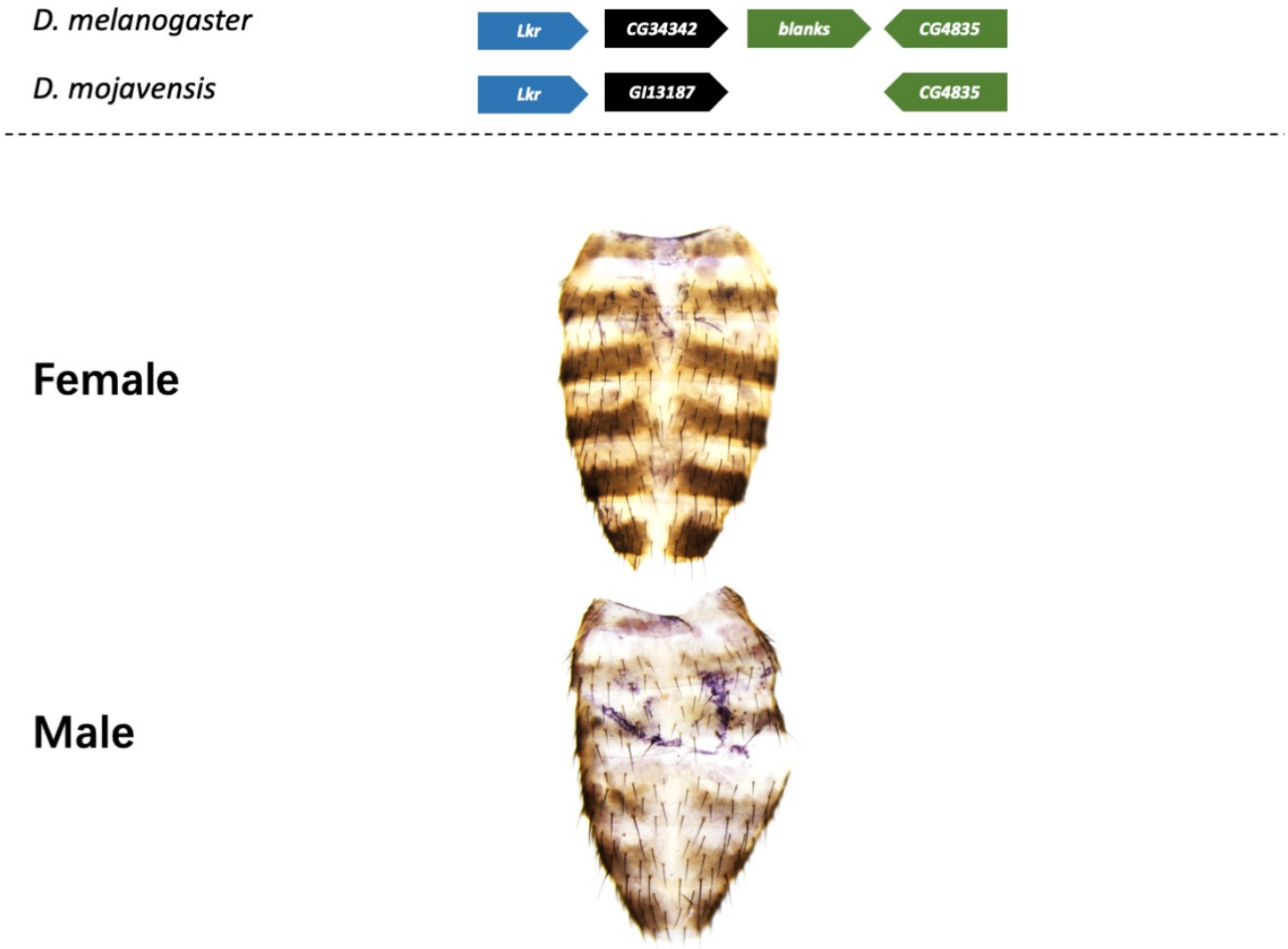

Figure A7.10. ***In situ*** **hybridization of** ***GI13187*** **in adult oenocytes of female and male** ***D. mojavensis*****.**

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