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Molecular and functional characterization of an aldehyde oxidase from the greater wax moth (*Galleria mellonella*) as odorant degrading enzyme

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Dedico esta tesis a mi Madre.

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Summary

Odorant degrading enzymes (ODEs) are proposed to degrade/inactivate volatile organic compounds (VOCs) in millisecond scale time. Thus, ODEs play an important role in the insect olfactory system as a reset mechanism. Through transcriptomic approaches, several putative ODEs have been reported, but a few functionally characterized. It is likely that the inhibition of these enzymes could incapacitate the olfactory system and, consequently, disrupt chemical communication promoting and complementing the integrated pest management. One main example is the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae), in which males produce the sex pheromone (nonanal and undecanal, as major components) for attracting females. Nevertheless, the aldehyde-based sex pheromone appears to be unsuitable for ethological control, due to chemical instability, short-range volatility and common presence in beehives. Therefore, the aim of this work was to characterize at molecular and functional level an ODE, which degrade the sex pheromone components from *G. mellonella* as an alternative target for further control strategies. In order to accomplish this objective, putative sequences for ODEs were obtained from the comparison of two transcriptomes. Likewise, to unravel the profile of volatiles that *G. mellonella* must be faced besides the sex pheromone blend, VOCs were trapped from honeycombs and the identification was made by Gas Chromatography coupled with Mass Spectrometry (GC-MS). The identified volatiles were used to evaluate the enzyme activity of antennal extracts. To perform the sex- and tissue-biased expression as well as the relative expression of identified ODEs, semiquantitative RT-PCR and quantitative RT-PCR were used, respectively. According to the results from the transcriptome comparison, two ODEs-encoding genes were obtained and classified as *GmelAOX2* and *GmelAOX3*. On the other hand, *GmelAOX2* showed a sex-biased expression and both *GmelAOX2* and *GmelAOX3* performed a higher

relative expression in males rather than females. Moreover, 74 compounds were identified through GC-MS, such as terpenes, aldehydes, alcohols and esters. The functional assay revealed that antennal extracts had the strongest enzymatic activity to undecanal (4-fold) compared to benzaldehyde (control). Finally, our data suggest that these enzymes have a crucial role for metabolizing sex pheromone compounds as well as plant-derived aldehydes, which are related to honeycombs and the life cycle of *G. mellonella*.

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CHAPTER I

General Introduction

1.1. Introduction

It is estimated that in the last 50 years, pollinators have increased the yield of several pollinator-dependent crops by 300% (Aizen and Harder, 2009). Honeybees (e.g. *Apis mellifera*), are essential to produce the natural sweet substance known as honey, which represents a product with a high nutritional value, due to its content of proteins, sugars and vitamins. Moreover, it has secondary metabolites (e.g. polyphenols) with antioxidant and pharmacological properties (García, 2018), that makes this product of high impact on the market. For instance, the world production of honey reached 607,989 tons exported, where China, Argentina and Ukraine were the main producers in 2016 (García, 2018). On the other hand, Chile had 9,000 tons exported and about 36 million dollars in profits from 2012 to 2015, only in natural honey (ODEPA, 2015). In 2016, Chile had a production of 7,137 tons, ranking 21st worldwide. However, this production decreased to 5,212 tons, down to 29th place by 2017 (ODEPA, 2018). In fact, Apiculture has shown a decline in the number of *A. mellifera* intended for honey production, and each year global exports of natural honey diminished by 7% its value from 2017 to 2018 (Neumann and Carreck, 2010; Workman, 2019).

Undoubtedly, the improper use of pesticides has caused an imbalance in the population of these pollinators. For instance, neonicotinoid-type insecticides are among the most used, nonetheless they may not only affect pest insects but also non-target organisms, such as solitary bees, bumble bees and honey bees (Blacquière *et al.*, 2012; Straub *et al.*, 2019). In addition, the spread of pests, such as the small hive beetle, *Aethina tumida* (Evans *et al.*, 2003), parasitic mites, *Varroa destructor* and *V. jacobsoni* (Ramsey *et al.*, 2019; Bokaie *et al.*, 2014) and the great wax moth, *Galleria mellonella* (Kwadha *et al.*, 2017), have apparently enhanced the decline of bee populations (Klein *et al.*, 2007). The latter, *G. mellonella* L. (Lepidoptera: Pyralidae, Galleriinae) highlights for being a

worldwide distributed pest feeding on hive wax, pollen, and honey (Nielsen and Brister, 1979). Generally, the larval population of *G. mellonella* has been controlled with the application of pesticides, such as pyrethroids (deltamethrin) (Kurt and Kayis, 2015) and phosphorous insecticides (Ali *et al.*, 1973). However, the application of pesticides along with diseases in bee colonies is believed to have contributed the attack of *G. mellonella* larvae by making colonies more vulnerable (Romel *et al.*, 1992).

Specific and environment-friendly methods have been developed as strategies for controlling pest insects, such as the use of semiochemicals through ethological approaches that target insect behavior. These molecules can modulate intra- and interspecific communication between two organisms of the same or different species, respectively (i.e., pheromones and allelochemicals, respectively). For example, mating disruption technique is used to control populations of the grapevine moth, *Lobesia botrana*, based on the main component of the sexual pheromone, (*E*,*Z*)-7,9-dodecadienyl acetate (Arn *et al.*, 1988). In the case of *G. mellonella*, its sex pheromone (synthesized by males) consists mainly of a mixture of two aldehydes, nonanal and undecanal (Leyrer and Monroe, 1973; Romel *et al.*, 1992), and a third minor component, 5,11-dimethylpentacosane (Svensson *et al.*, 2014). However, no methods of sexual confusion have been reported with success for the monitoring and/or control of *G. mellonella*. Indeed, Flint and Merkle (1983) showed no significant differences in female attraction by using traps with the sex pheromone components (nonanal and undecanal). Although, male pheromone gland extract showed a major female attraction compared to the synthetic compounds, this has not been proven in field trials.

With the advent of new bioinformatics tools, many genes have been allowed to be identified. In relation to the genes associated with insect communication (this is described in the next sections), the most studied are the coding genes for odorant-biding proteins

(OBPs) and odorant receptors (ORs), which are involved in the transport and reception of semiochemicals compounds, respectively. A third group are odorant-degrading enzymes (ODEs) that have been less studied. All these proteins are targets to design new bioactive molecules to be implemented in pest management. The aim of this research is to characterize an odorant-degrading enzyme (ODE) that degrades the major sex pheromone components of *G. mellonella* at molecular and functional level.

1.2. *Galleria mellonella*: Biology, Semiochemistry and Economic Importance

1.2.1. Biology of *Galleria mellonella*

G. mellonella has a biological cycle from 30 to 60 days, which is temperature-dependent (between 26 to 38°C). Its adult stage can last from 7 to 15 days, and in the case of females, they lay 400 up to 1800 eggs, 4 to 10 days after having emerged (Zamorano, 2009). It has been observed that these moths can have up to 4 generations in one year (Parra *et al.*, 2006). Adults have sexual dimorphism, where males are smaller and lighter in color compared to females (Kwadha *et al.*, 2017). Another remarkable characteristic are the palps, where the female projects them forward, while the male presents them curved and downward (Smith, 1965). When the populations of *G. mellonella* reach their sexual maturity, they fly near of combs to mate, being the females the ones that later return to these bee hives to leave their eggs. Noteworthy, this occurs during 18:00 and 00:00 hours as it corresponds to its maximum activity, and where honeybees present their lowest activity. When the larvae form the silk threads inside the hive, the bees begin to have difficulty moving and become trapped. It is known as “galleriasis”, which causes the bees to leave the honeycomb or starve to death (Whitcomb, 1965).

1.2.2. The semiochemistry of *G. mellonella*

Unlike most Lepidoptera, this moth has a different form of mating, interestingly, the male produces an acoustic courtship behavior that attracts virgin females and then the male releases the sex pheromone from wing lateral glands to initiate mating. Kwadha *et al.* (2017) have proposed that the adaptation of this moth in terms of its sexual communication is due to the females need to invest their energies into reproductive processes (e.g. egg production and locate oviposition sites), instead to produce sex pheromone. Hence, the use of acoustics signal by males to attract females as the first approach could be useful to increase the survival chances of the wax moth's progeny. On the other hand, several studies have shown the components of the sex pheromone blends, for instance, Roller *et al.* (1968) identified undecanal from the male lateral glands. Then, the sex pheromone was identified by Leyrer and Monroe (1973) as a mixture of two main aldehydes components, nonanal and undecanal (7:3). Moreover, the blend has minor components, such as hexanal, heptanal, octanal, decanal, undecanol and 6,10,14-trimethyl-2-pentadecanol (Lebedeva *et al.*, 2002), though no behavioral activity has been proved for a synthetic mixture so far. On the contrary, Svensson *et al.* (2014), identified the 5,11-dimethylpentacosane as a novel minor sex pheromone component of *G. mellonella*, which showed behavioral activity along with nonanal and undecanal. Overall, semiochemicals offer an alternative for this pest, owing to their sustainability, easy applicability and a relative low cost compared to conventional strategies for pest controlling.

1.2.3. Economic importance and control methods

G. mellonella is considered a worldwide honeybee pest and in Chile has been widely reported from Arica to South-central areas where the temperatures are warmer during the year, enhancing their development. The importance of the wax moth lies in the deterioration of hives and hive products, leading to the reduction of the bee population or total abandonment of the hive (Charrière and Imdorf, 1997). During its larval stage, *G. mellonella* feeds not only on wax stored by bees, but also on pollen and honey (Nielsen and Brister, 1979). It has been reported that a few days are enough for the larva to devastate a hive (Jafari *et al.*, 2010), until adult females find more unprotected hives at dusk to deposit hundreds of eggs, starting the process of larval development again.

The most effective natural enemies of *G. mellonella* are the bees themselves, however, the attack of *V. destructor* and other mites can weaken the hives, allowing this moth to enter (Whitcomb, 1965). The traditional method for controlling the population of *G. mellonella* has considered the use of pesticides, such as pyrethroids. Nevertheless, at present their use is less recommended due to their persistence and global effect towards the so called collony colapse disorder (Godfray *et al.*, 2014). Thus, alternative methods for managing this moth have been studied and developed. For example, studies by Jafari *et al.* (2010) have reported the use of the sterile male technique, where gamma rays are used for the sterilization of moth males with the subsequent inability of females to produce offspring. Although the technique seems to work for *G. mellonella* with optimal rates of release of sterile males, normal males and females in 4:1:1 ratio, respectively, it has been determined that this still requires prior application of insecticide in order to decrease the moth population. On the other hand, the insecticidal activity of azaridactine from alcoholic extracts of Neem-X®, a biopesticide derived from the *Azadirachta indica* tree, was evaluated (Contreras, 2011). The results of that study suggest that high

concentrations of azaridactine (30 to 60 ppm) added to the diet of the larvae could negatively affect the feeding of these, generating decrease in fertility. Likewise, the use of extracts based on *Bacillus thuringiensis* L. as insecticidal agents towards *G. mellonella* larvae, was evaluated by Zamorano (2009). However, their results appear discouraging as no extract concentration exceeded 12% mortality.

1.3. Olfactory systems in insects

In insects, the detection and processing of chemical cues through olfaction is crucial for successful mating, avoidance of harmful compounds, and location of either oviposition sites or food sources (Choo *et al.*, 2013; Li *et al.*, 2018). For instance, pollinators need to find floral resources using a sophisticated system capable of detecting and distinguishing volatile organic compounds (VOCs) in a short timescale (below 500 milliseconds (ms)) (Rusch *et al.*, 2016). Unlike other senses, such as touch, vision, or hearing, the use of VOCs by insects (e.g. aphids, beetles, flies and moths) to communicate messages over relatively long distances is an advantage (El-Shafie and Romeno, 2017). These volatile chemicals are called semiochemicals and mediate interactions between organisms of the same species (i.e., pheromone) and different species (i.e., allelochemicals). These chemicals are recognized through a systematic cascade of events that occur in chemosensory organs named sensilla, which can be found in maxillary palps, legs and mainly covering the antennae (Zhou, 2010; Pelosi *et al.*, 2017). Chemoreception is mainly related to four key protein families (Figure 1) such as odorant-binding proteins (OBPs), chemosensory proteins (CSPs), chemosensory receptors and odorant degrading enzymes (ODEs) (Pelosi *et al.*, 2006, Pelosi *et al.*, 2017; Mei *et al.*, 2018; Song *et al.*, 2018). Upon entry of odorants through cuticular pores, OBPs and CSPs transport these hydrophobic molecules across an aqueous lymph (sensillar lymph) to ORs. OBPs can be

divided in three groups (Zhou, 2010): pheromone binding proteins, PBPs; general odorant binding proteins, GOBPs; and antennal binding protein x, ABPx (Krieger, *et al.*, 1996; Wang *et al.*, 2020). The chemosensory receptors can be divided in odorant receptors, ORs; ionotropic receptors, IRs; gustatory receptors, GRs; and sensory neuron membrane proteins, SNMPs (Klein, 1987; Pelosi and Maida, 1995; Isono and Morita, 2010; Zhou, 2010; Leal, 2013). Thus, a chemical signal is transduced into an electrical stimulus when ORs are activated (Sato and Touhara, 2008) leaving these semiochemicals in the sensillar lymph (Figure 2). One concern that arises is the final destination of these chemicals after receptor activation: If these compounds are not degraded, then they could accumulate in the peripheral space interfering with the olfactory system in insects (Choo *et al.*, 2013). Indeed, there are enzymes known as ODEs, which operate on the recovery of sensitivity in the olfactory system to detect new odorants (Sakurai *et al.*, 2014; Zhang *et al.*, 2017; Li *et al.*, 2018).

Early studies reported the identification and isolation of an enzyme, called antennae-specific esterase (ApolPDE), from the sensillar lumen of the giant silkmoth, *Antheraea polyphemus* (Vogt and Riddiford, 1981). Interestingly, through a kinetic study of ApolPDE, authors showed that the pheromone of *A. polyphemus*, (*E,Z*)-6,11-hexadecadienyl acetate (*E*6,*Z*11-16:OAc), had an estimated half-life of 15 milliseconds (ms), suggesting ApolPDE as a pheromonal deactivator (Vogt *et al.*, 1985). Later, Ishida and Leal. (2008) supported this rapid degradation, where ≈30 ms were necessary to reset the olfactory system of the japanese beetle, *Popillia japonica* through the study of the sex pheromone degradation using a recombinant enzyme. ODEs are considered as the least specific compared with OBPs, CSPs and ORs, therefore, they represent more generally targetable proteins for behavioral inhibition (Vogt, 2005). However, more scientists have

addressed the identification of these enzymes, as the beginning of more in-depth studies in relation to insect control and management.

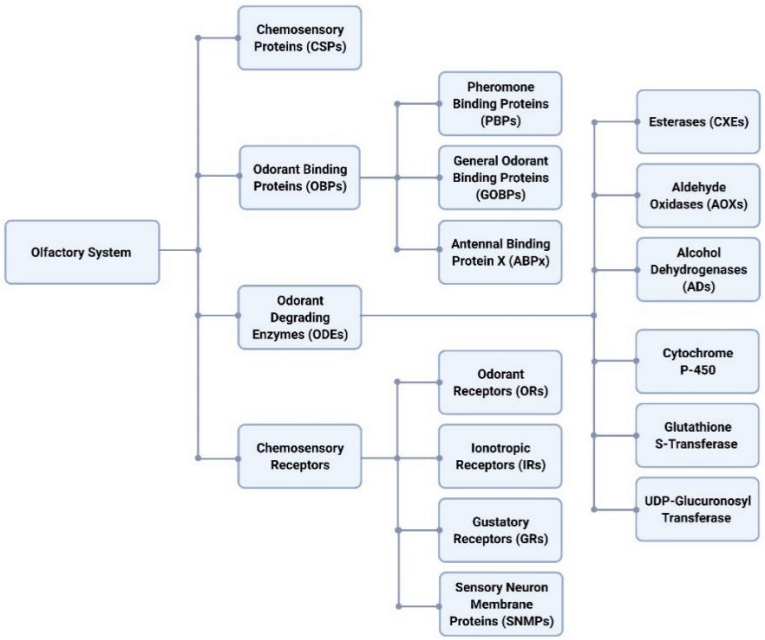

Figure 1. Schematic organization of proteins present in the olfactory system of Lepidoptera. It is possible to identify the four families, CSPs, OBPs, ODEs, and chemosensory receptors (Vogt, 2005; Pelosi *et al.*, 2006; Rytz *et al.*, 2013; Pelosi *et al.*, 2017).

1.4. Odorant degrading enzymes (ODEs)

Many pheromone compounds have been identified and synthesized especially in Lepidoptera and other orders, such as Diptera and Coleoptera (El-Sayed, 2006), since the discovery of the first compound of the sexual pheromone (*E*,*Z*)-10,12-hexadecadien-1-ol (bombykol) from the silk moth, *Bombyx mori* (Butenandt *et al.*, 1959). Lepidoptera use acetate, aldehyde and alcohol type compounds as sex pheromone for their intraspecific

communication (Ando *et al.*, 2004). Hence, it is common to find that most of mating disruption techniques used today are based on these type of chemicals (Arn *et al.*, 1992; Witzgall *et al.*, 2010). Nowadays, advances in transcriptomic methods have provided to scientists the identification of several ODEs (such as, carboxylesterases, CXEs; aldehyde oxidases, AOXs; alcohol dehydrogenases, ADs), some with tissue- and/or sex-biased expression through semi-quantitative PCR and quantitative PCR (qPCR) experiments. For example, Huang *et al.* (2016) found candidate genes for 12 carboxylesterases (CXEs), 2 aldehyde oxidases (AOXs) and 6 alcohol dehydrogenases (ADs) in codling moth, *Cydia pomonella*, which were predominantly expressed in antennae. Moreover, Zhang *et al.* (2017) described 18 putative CXEs genes and 4 AOXs genes in the rice leaffolder, *Cnaphalocrosis medinalis*, where 3 CXEs and 1 AOX were enriched in the antennae. This last gene (*CmedAOX2*) was significantly expressed in antennae of males than females, suggesting that the encoded protein could be involved in aldehyde-type sex pheromone degradation. ODEs could be participating in the olfaction process of *G. mellonella*, and especially AOXs, due to aldehyde-type compounds are the main components of their sex pheromone.

1.5. Problem statement

Considering that, 1) The worldwide economic importance of *G. mellonella* to beekeeping industry and the current methods for its monitoring and controlling remain without efficient results. 2) The role that olfactory system has in the insect communication is essential for reproductive success and host location, where three major proteins (such as, OBPs, ORs and ODEs) are crucial for transporting, transducing, and degrading of semiochemicals. Thus, ODEs the less studied so far at both molecular and functional level and no information is available for *G. mellonella* to date. 3) Advances in bioinformatics

techniques (e.g. transcriptomic) have made it possible to identify in several order of insects (such as, Diptera, Coleoptera and Lepidoptera) candidate encoding-genes for odorant degrading enzymes (ODEs). But there is no information about these enzymes in *G. mellonella*, however, the head transcriptome has been assembled in our laboratory, which will be helpful for identifying the candidates ODEs of this moth. The results obtained in this research will provide valuable information in basic science for researchers working in chemical ecology and insect physiology. Therefore, the identification and functional study of ODEs would provide the necessary information to corroborate or reject the use of these enzymes as targets through their inhibition and, subsequently, disruption of chemical communication for insect pest control. This could interfere with the female moth's ability to find mates and reproduce. Such novel approaches have the potential to diminish the overuse on routine agricultural chemicals, which cause concerns about adverse effects. We believe that *G. mellonella* represents an interesting model of study due to: 1) Its inverse sexual communication where males release the sex pheromone blend (nonanal and undecanal, as the main components); 2) Straightforward protocols for rearing and 3) Available information from our laboratory. According to the above, the following hypothesis arises:

1.6. Hypothesis

G. mellonella has female- and antennae-specific expression of at least one aldehyde-related odorant degrading enzyme that show enzymatic activity toward sex pheromone (i.e., nonanal and undecanal) rather than bee-hive volatiles.

1.7. General objective

To characterize an odorant-degrading enzyme (ODE) that degrades the major sex pheromone components of *G. mellonella* at molecular and functional level.

1.8. Specific objectives

I. To evaluate sex- and tissue-specific expression of AOXs of *G. mellonella* through semi-quantitative PCR technique.

II. To determine the relative expression levels of AOXs in antennae of both sexes through quantitative PCR (qPCR).

III. To evaluate enzymatic activity of sex-biased AOX towards sex pheromone and bee hive-derived volatiles.

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CHAPTER II

An Overview of Antennal Esterases in Lepidoptera

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An Overview of Antennal Esterases in Lepidoptera

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Abstract

Lepidoptera are used as a model for the study of insect olfactory proteins. Among them, odorant degrading enzymes (ODEs), that degrade odorant molecules to maintain the sensitivity of antennae, have received less attention. In particular, antennal esterases (AEs) (responsible for ester degradation) are crucial for intraspecific communication in Lepidoptera. Currently, transcriptomic and genomic studies have provided AEs in several species. However, efforts in gene annotation, classification, and functional assignment are still lacking. Therefore, we propose to combine evidence at evolutionary, structural and functional level to update ODEs as well as key information into an easier classification, particularly of AEs. Finally, the kinetic parameters for putative inhibition of ODEs are discussed in terms of its role in future integrated pest management strategies.

Keywords: Lepidoptera, olfactory system, antennal esterases, transcriptomic, semiochemicals, inhibition

2.1. Introduction

In insects, the detection and processing of chemical cues through olfaction is crucial for successful mating, avoidance of harmful compounds, and location of either oviposition sites or food sources (Choo *et al.*, 2013; Li *et al.*, 2018). For instance, pollinators need to find floral resources using a sophisticated system capable of detecting and distinguishing volatile organic compounds (VOCs) in a short timescale (below 500 milliseconds (ms)) (Rusch *et al.*, 2016). Unlike other senses, such as touch, vision, or hearing, the use of VOCs by insects (e.g. aphids, beetles, flies and moths) to communicate messages over relatively long distances is an advantage (El-Shafie and Romeno, 2017).

These volatile chemicals are called semiochemicals and mediate interactions between organisms of the same species (i.e., pheromone) and different species (i.e., allelochemicals). These chemicals are recognized through a systematic cascade of events that occur in chemosensory organs named sensilla, which can be found in maxillary palps, legs and mainly covering the antennae (Zhou, 2010; Pelosi *et al.*, 2017). Chemoreception is mainly related to four key protein families (Figure 1) such as odorant-binding proteins (OBPs), chemosensory proteins (CSPs), chemosensory receptors and odorant degrading enzymes (ODEs) (Pelosi *et al.*, 2006, Pelosi *et al.*, 2017; Mei *et al.*, 2018; Song *et al.*, 2018). Upon entry of odorants through cuticular pores, OBPs and CSPs transport these hydrophobic molecules across an aqueous lymph (sensillar lymph) to ORs. OBPs can be divided in three groups (Zhou, 2010): pheromone binding proteins, PBPs; general odorant binding proteins, GOBPs; and antennal binding protein x, ABPx (Krieger, *et al.*, 1996; Wang *et al.*, 2020). The chemosensory receptors can be divided in odorant receptors, ORs; ionotropic receptors, IRs; gustatory receptors, GRs; and sensory neuron membrane proteins, SNMPs (Klein, 1987; Pelosi and Maida, 1995; Isono and Morita, 2010; Zhou, 2010; Leal, 2013). Thus, a chemical signal is transduced into an electrical stimulus when

ORs are activated (Sato and Touhara, 2008) leaving these semiochemicals in the sensillar lymph (Figure 2A).

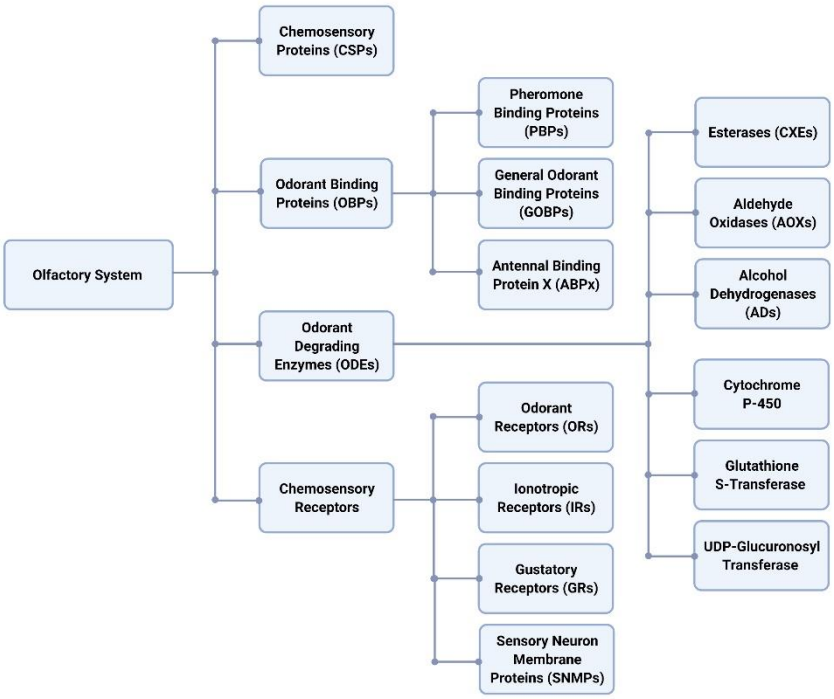

Figure. 1. Schematic organization of proteins present in the olfactory system of Lepidoptera. It is possible to identify the four families, CSPs, OBPs, ODEs, and chemosensory receptors (Vogt, 2005; Pelosi *et al.*, 2006; Rytz *et al.*, 2013; Pelosi *et al.*, 2017).

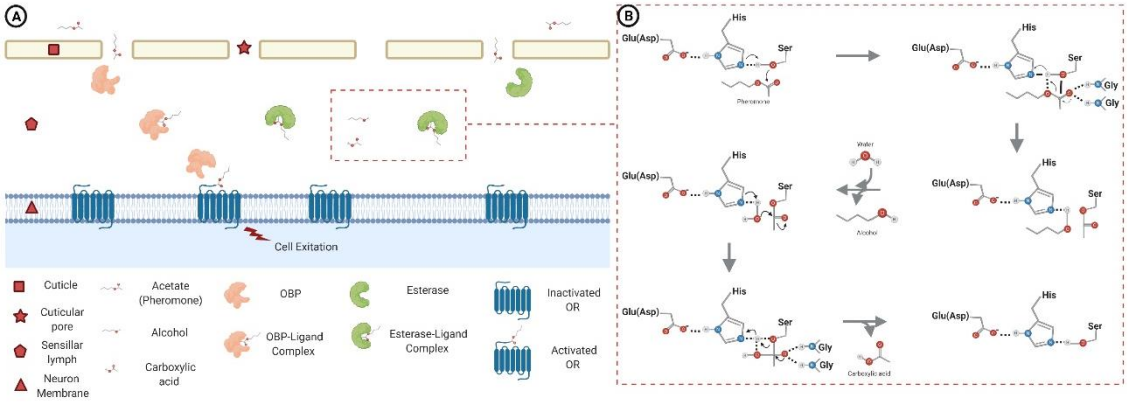

Figure. 2. A) Schematic representation of the olfactory mechanism in sensilla of Lepidoptera with emphasis on esterases. Compounds from the environment pass through cuticular pores toward the sensillar lymph. Here, OBPs bind and transport these molecules to ORs located in the dendritic membrane of olfactory neurons where they are activated. After cell excitation, the molecules are degraded by the action of ODEs (esterases). These enzymes can even act when the molecules enter to the sensillar lymph (Leal, 2013). B) Reaction mechanism of the esterases in Lepidoptera. The ester hydrolysis occurs in a two-step reaction plus water addition. There is first a nucleophilic attack produced by the serine hydroxyl on the carbonyl carbon of the pheromone. The reaction is then stabilized by the histidine and this amino acid is stabilized by the glutamic acid at the same time. A molecule of alcohol is then released, and the enzyme is acetylated. Second, the water molecule has affinity with the histidine residue and then acts as a nucleophile on the acetylated enzyme. Finally, a carboxylic acid is released, and the enzyme is free to start a new reaction. Importantly, there are two conserved glycines participating in the stabilization of the transition states in the oxyanion hole (Montella *et al.*, 2012).

One question that arises is the final destination of these chemicals after receptor activation: If these compounds are not degraded, then they could accumulate in the peripheral space interfering with the olfactory system in insects (Choo *et al.*, 2013). Indeed, there are enzymes known as ODEs, which operate on the recovery of sensitivity in the olfactory system to detect new odorants (Sakurai *et al.*, 2014; Zhang *et al.*, 2017; Li *et al.*, 2018).

Early studies reported the identification and isolation of an enzyme called antennae-specific esterase (ApolPDE) from the sensillar lumen of the Giant silk moth, *Antheraea polyphemus* (Vogt and Riddiford, 1981). Interestingly, through a kinetic study of ApolPDE, the authors showed that in the presence of this enzyme, the pheromone (*E*,*Z*)-6,11-hexadecadienyl acetate (*E*6,*Z*11-16:OAc) has an estimated half-life of 15 ms suggesting that ApolPDE is a pheromonal deactivator (Vogt *et al.*, 1985). Ishida and Leal. (2008) later supported this rapid degradation where ≈30 ms were necessary to reset the olfactory system of the Japanese beetle, *Popillia japonica*, through the study of the sex pheromone degradation using a recombinant enzyme. In this context, understanding the main actors in the inactivation of chemical signals is fundamental to the discovery of new molecules capable of disabling this mechanism (Leal, 2013). According to Vogt. (2005), ODEs can be a target for behavioral inhibition because they degrade many different types of volatile compounds. Scientists have identified these enzymes as the beginning of more in-depth studies related to control and integrated pest management (IPM).

Despite the increasing amount of reported ODEs, no evolutionary analyses have been performed on these proteins among Lepidoptera. Furthermore, the structural features of the enzymes that could explain their selectivity have not yet been studied. Considering the diversity of acetate esters reported as sex pheromone compounds (463 acetate esters have been identified in the Pherobase database, https://www.pherobase.com/), the main

focus of this review is the structure and evolutionary traits of antennal esterases in Lepidoptera, which are responsible for the degradation of acetate ester-type pheromone components.

Thus, this text will offer a wider spectrum of new enzymes identified through bioinformatics techniques (i.e., transcriptomic) to attach a function through further functional studies. We propose specific guidelines that might help to clarify whether an ODE can be classified into a pheromone degrading enzyme (PDE) or not. The last step includes directed studies for this type of enzymes due to their participation in the degradation of pheromones, which in turn have a role in the behavior of organisms of the same species. In this way, new alternative and less costly pest management strategies could be implemented.

2.2. Odorant Degrading Enzymes and Their Role in Sexual Communication

The olfactory system of insects has evolved to such an extent that it can process hundreds of compounds with different chemical structures from the environment to produce a change in behavior. Particularly, Lepidoptera emerged from a basal linage called non-Ditrysia to a new linage called Ditrysia since the Mesozoic era (over 100 million years); the species-specific pheromone components have similarly evolved. The sex pheromone that is generally emitted by females is crucial to attract a conspecific partner and achieve reproductive success. It is therefore not surprising that there are considerable structural differences in the blends of sex pheromones (Ando *et al.*, 2004). We advise reviewing Löfstedt *et al.* (2016) for a complete understanding of the different types of pheromones in Lepidoptera.

Broadly, four groups of sex pheromones are described: Type 0 pheromones are structurally analogous to plant volatile compounds with short-chain ketones and alcohols.

They are considered as primitive because they are also identified in non-Ditrysia species. The leaf miner moth, *Eriocrania semipurpurella*, is an example of non-Ditrysia moths and uses (2*S*, 6*Z*)-6-nonen-2-ol and (2*R*, 6*Z*)-6-nonen-2-ol as sex pheromone (Yuvaraj *et al.*, 2017). Type I pheromones are biosynthesized de novo from acetate with C_{10}-C_{18} alcohols, aldehydes, and esters. Type II pheromones are biosynthesized from decarboxylation and epoxidation from dietary linolenic or linoleic acids where C_{17}-C_{25} polyunsaturated hydrocarbons are part of their structure (Millar, 2000). Type III pheromones contain one or more methyl branches in their structure with C_{17}-C_{23} saturated and unsaturated hydrocarbons. Many sex pheromone compounds have been identified especially in Lepidoptera and other orders such as Diptera and Coleoptera (El-Sayed *et al.*, 2006) since the discovery of (*E*,*Z*)-10,12-hexadecadien-1-ol (bombykol)— the sex pheromone of the silk moth *Bombyx mori* (Butenandt *et al.*, 1959).

Most mating disruption techniques used today for controlling and monitoring moth pests are based on these chemicals (Arn *et al.*, 1992; Witzgall *et al.*, 2010). For instance, *Tuta absoluta* is a pest that attacks tomato crops, and components of its sexual pheromone ((*E*,*Z*,*Z*)-3,8,11-tetradecatrienyl acetate and (*E*,*Z*)-3,8-tetradecadienyl acetate) have been tested in greenhouses to control this insect (Cocco *et al.*, 2012). Some studies have used inhibitors of ODEs such as trifluoromethyl ketones (TFMKs) to affect the pheromone detection and alter the behavior of moths. Malo *et al.* (2013) studied the male antennal response of the fall armyworm *Spodoptera frugiperda* against the inhibitor (*Z*)-9-tetradecenyl trifluoromethyl ketone (Z9-14:TFMK) through electroantennography assay. This inhibitor is analogous to the main pheromone component (*Z*)-9-tetradecenyl acetate (Z9-14:Ac) of *S. frugiperda*, and it can significantly reduce the antennal response from 2.51 ± 0.37 mV to 1.10 ± 0.24 mV. On the other hand, Bau *et al.* (1999) disrupted

the orientation flight of *S. littoralis* and *Sesamia nonagrioides* males in wind tunnel assays using TFMKs.

This background suggests that ODEs have an important role in the degradation of these pheromones. Consequently, one question that arises is if ODEs have evolved in order to degrade a wide range of these chemical cues or are limited to degrade a particular sex pheromone group for olfactory purposes. In this sense, transcriptomic analyses have provided the profile of ODEs that several moth could use for olfaction purposes. Acetates are the main sex pheromone components in Lepidoptera, and dozens of esterases in antennae have been reported as summarized in Table 1. Putative functions of ODEs have been suggested such as plant volatile and/or sex pheromone degradation though a few studies have actually addressed the functional role of some of these. In that sense, most enzymes characterized today are related to their sexual role in the degradation of pheromone components.

Transcriptomic advances have provided scientists a constantly growing database of sequences to identify ODEs including some with tissue-biased expression through reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative (RT-qPCR) experiments. For example, 18 carboxylesterase (*CXE*) genes have been identified in the rice leaffolder, *Cnaphalocrocis medinalis*, through its antennal transcriptome (Zhang *et al.*, 2017). Furthermore, the Egyptian armworm *S. littoralis* has an encoding-gene to *SlCXE7* that was 3-fold more expressed in males than females through RT-qPCR analysis (Durand *et al.*, 2011). A greater expression of these enzymes in male antennae suggests that ODEs participate in the modulation of pheromone concentration since it is the female who produces and releases these semiochemicals to attract her conspecific mate in most species of moths.

2.3. Evolutionary Traits of ODEs Across Lepidoptera

Insects are the most abundant and specious group of organisms. Nearly 150,000 species have been described in the Lepidoptera order alone. Considering their ecological impact, they have served as model systems to understand their mechanisms to locate mates and hosts plants as main examples. Furthermore, moths are important subjects of study within an evolutionary context due to their phenotypic plasticity, which comprises the ability of an organism (specifically a genotype) to respond to an environmental alteration with a change in its morphology, physiology, behavior or life history (Moczek, 2010). These evolutionary processes are related to structural or regulatory mutations and change an amino acid in the coding region of a protein or affect the gene expression, respectively (Albre *et al.*, 2012).

Genetic drift and natural selection can also contribute to the divergence of new enzymatic functions (Jones, 2017). In support of this, the cytochrome P450 enzymes (commonly involved in detoxification function) have had many mutations capable of catalyzing many chemical compounds (Bloom *et al.*, 2007). In this sense, Lepidoptera are a clear representation of speciation where these changes are often related to their olfactory system for conspecific mate recognition. The extensive list of sex pheromone compounds identified to date (e.g., 463 acetate esters, 390 aldehydes, 331 primary alcohols, 299 secondary alcohols, and 28 tertiary alcohols reported in the Pherobase database) serves as a clue to the different types of enzymes involved in their biosynthesis. New desaturases have emerged by gene duplication and then diverged towards new functions (Roelofs *et al.*, 2002). For instance, this has led to the evolution of castes and social organization in ants due to the expansion of the desaturase gene (Helmkampf *et al.*, 2014). In this context, diverse enzymes are needed to degrade the large number of semiochemicals present in the environment that insects have to interact.

ODEs have evolved from one gene family where catalytic and non-catalytic enzymes emerge (Oakeshott *et al.*, 2005). Some studies have performed phylogenetic analysis for CXEs, and it is important to emphasize that the results showed a monophyletic clade where the most representative PDE (ApolPDE) was presented (He *et al.*, 2014; Zhang *et al.*, 2016). In Figure 3, we show a PDE clade (Figure S1 Supplementary Material, a complete phylogenetic tree) constructed with some esterases in order to understand how these enzymes have evolved to degrade certain types of compounds. SlCXE13 from, *S. littoralis* (Durand *et al.*, 2010a); SlitCXE13 from *S. litura* (Zhang *et al.*, 2016); SexiCXE13, *S. exigua* (He *et al.*, 2014); SinfCXE13 from *Sesamia inferens* (Zhang *et al.*, 2014) and ApolPDE1 from *A. polyphemus* (Ishida and Leal, 2005) belong to Ditrysia moths that use Type I sex pheromones. These enzymes are phylogenetically closer compared to EsemCXE6 from *E. semipurpurella*, a non-Ditrysia moth that use Type 0 sex pheromone. Moreover, PJAPPDE1 from *P. japonica*, and DmelEST6 from the fruit fly, *Drosophila melanogaster* were added due to their enzymes have been functionally well studied (Ishida and Leal, 2008; Younus *et al.*, 2017). Lepidoptera, Coleoptera and Diptera belong to the Holometabola group, but it is unknown the origin of physiological and morphological innovations in insects (Misof *et al.*, 2014). Here, putative PDEs in Lepidoptera seem to have evolved from the ancient moth *E. semipurpurella*, as expected considering its non-Ditrysian origin. Overall, these results shed light on the evolution of these enzymes from *D. melanogaster*. It has been reported that DmelEST6 acts as an ODE with activity towards food esters, such as propyl, hexyl, heptyl, nonyl, decyl, neryl and geranyl acetates (Younus *et al.*, 2017). Therefore, it is proposed that Lepidoptera PDEs might have evolved from a common ancestor (i.e., DmelEST6), changing from catalytic activity toward food esters to specific acetate-based sex pheromone components. In this context, this issue could be considered when

classifying an esterase as a PDE; however, a necessary functional assay to confirm such a role is needed.

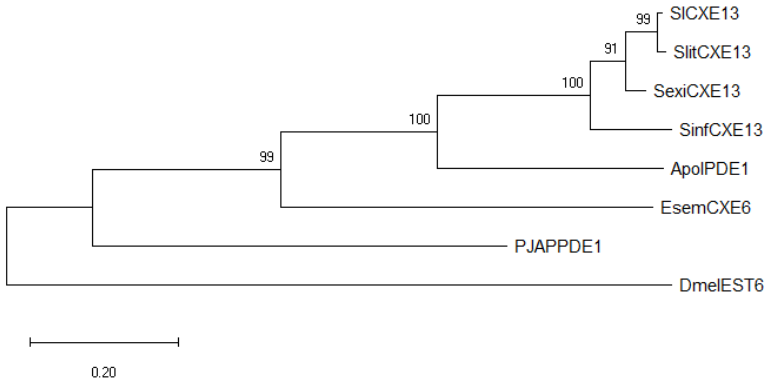

Fig. 3. Phylogenetic tree of esterases. SlCXE13 (S. littoralis), SlitCXE13 (S. litura), SexiCXE13 (S. exigua), SinfCXE13 (Sesamia inferens), ApolPDE1 (A. polyphemus), EsemCXE6 (E. semipurpurella), PJAPPDE1 (P. japonica) and DmelEST6 (D. melanogaster). Phylogenetic analyses for esterases were performed by using MAFFT sever for multiple sequence alignments and FastTree software for phylogenetic relationships based on maximum-likelihood method (Price *et al.*, 2010).

2.4. Antennal Esterases in Lepidoptera

Among insects, esterases have been classified in three major classes: I) as enzymes with neuro/developmental functions; II) intracellular enzymes with dietary detoxification functions, and III) secreted enzymes that use hormones or pheromones as substrates (Claudianos *et al.*, 2006). The latter is related to catalytically active enzymes that belong to *CXE* gene family and the α/β hydrolase superfamily (Punta *et al.*, 2012). Their function is to hydrolyze esters in a two-step reaction plus water addition (Figure 2B). First, an alcohol-type metabolite is produced from the hydrolysis of ester bonds to subsequently generate an enzyme in an acylated, carbamylated, or phosphorylated form

depending on its substrate (carboxylic, carbamic, or phosphoric ester, respectively). An acid-type metabolite is then formed and released due to water molecule addition so that the enzyme goes back to its active state (Montella *et al.*, 2012). Thus, these CXEs play important roles such as neurogenesis, development regulation, xenobiotic detoxification, and pheromones (Yu *et al.*, 2009). The first CXE related to olfaction in moths was the PDE from *A. polyphemus* (Vogt *et al.*, 1985); more recently, the CXEs identified in the tobacco cutworm *S. litura* (Zhang *et al.*, 2016), the light brown apple moth *Epiphyas postvittana* (Jordan *et al.*, 2008), and the black cutworm *Agrotis ipsilon* (Gu *et al.*, 2013) among others have been studied by RNA-seq approaches (Table 1).

To date, *CXE* genes have been localized in different tissues of several moth species according to RT-PCR and qRT-PCR experiments. On the other hand, native polyacrylamide gel electrophoresis (Native-PAGE) has been used to study the enzymatic activity of some esterases (He *et al.*, 2014). The expression in a specific tissue could shed lights on the function of a CXE. For example, Zhang *et al.* (2014) found five CXE genes in *S. inferens* where all of them (*SinfCXE10, SinfCXE13, SinfCXE14, SinfCXE18 and SinfCXE20*) were significantly expressed in different tissues such as pheromone glands, thoraxes, abdomens, and antennae. However, *SinfCXE10* was expressed specifically in the antennae; therefore, the authors propose that this gene could be involved in pheromone degradation particularly in (*Z*)-11-hexadecenyl acetate. Although CXEs are present in olfactory organs (e.g., antennae), it seems that their pheromone-degrading function is more related to sex-biased expression. Durand *et al.* (2011) confirmed that a CXE gene from *S. littoralis* (*SlCXE7*) was restricted to antennae rather than other tissues through *in situ* hybridization (ISH) technique. This was more significantly expressed in males than in females according to qRT-PCR. Depending on its sensillar location such as

pheromone-sensitive sensilla (i.e., trichodea, Str I), *SlCXE7* could play a role in pheromone signal termination as well as degrading odorant background noise.

On the contrary, He *et al.* (2014) reported one CXE gene from the beet armyworm, *S. exigua* (*SexiCXE4*), highly expressed in antennae and proboscises but had no sex-biased expression. *SexiCXE4* presented a higher preference (70-fold) to plant volatiles ((*Z*)-3-hexenyl acetate and hexyl acetate) than pheromone compounds (*Z*,*E*)-9,12-tetradecenyl acetate and (*Z*)-9-tetradecenyl acetate via *in vitro* functional assays. This suggests a role as a general odorant-degrading enzyme (GODE). Likewise, two CXEs genes (*EoblCXE7* and *EoblCXE13*) in the tea geometrid moth *Ectropis obliqua* were localized in pheromone-related sensilla (Str I) as well as sensilla basiconica (Sba) and gustatory sensilla styloconica (Sst) using fluorescence *in situ* hybridization (FISH) technique (Sun *et al.*, 2017). *EoblCXE13* showed a differential expression pattern where it was restricted to the base of Str I in males. The lack of male-biased localization in this study suggests that the CXE genes might be involved in the hydrolysis of host plant volatiles rather than pheromone components (He *et al.*, 2014). Despite the large amount of CXE expressed in the antennae of *E. obliqua*, no acetate ester-type sex pheromone degradation role could be attributed because this insect uses unsaturated hydrocarbons and enantiomers of epoxy hydrocarbons ((*Z*,*Z*,*Z*)-3,6,9-octadecatriene and 6,7-epoxy-(*Z*,*Z*)-3,9-octadecadiene) as sex pheromone (Type II sex pheromones) (Sun *et al.*, 2017). Likely, an epoxide hydrolase could participate in the degradation of the pheromone of *E. obliqua*, by catalyzing the hydrolysis of epoxide-like compounds to diols as many epoxy hydrolases do with epoxide-containing lipids (Morisseau, 2013), but further studies are needed.

2.5. Structural Features of Esterases

The ability of proteins to bind to chemical compounds strongly depends on their amino acid constitution and conformation such as domain arrangement, conformational dynamics, as well as the shape and amount of binding sites. Naturally, enzymes are not the exception with several enzymes as targets for the identification of substrates as well as competitive and non-competitive inhibitors or allosteric compounds in a drug-discovery approach.

A good example is the study of acetylcholinesterase (AChE) inhibitors such as organophosphorus compounds used as insecticides or toxic carbamates applied as pesticides (Čolović *et al.*, 2013). For insects and particularly moths, evolution has provided highly specific adaptations for sexual communication that has resulted in evolved structural features. For instance, conserved structural regions have been found for ORs with seven transmembrane domains, increased sequence identity towards the C-terminal region, and, more interestingly, sequence motifs, such as LLLLECS, QQLIQ, and ILKTS in pheromone receptors (PRs) (Zhang and Löfstedt, 2015; Köblös *et al.*, 2018).

On the other hand, three and two conserved disulfide bridges play an important role giving structural stability in OBPs and CSPs, respectively (Pelosi *et al.*, 2006). As a third player in perireceptor events, ODEs have not been structurally characterized so far from a wide viewpoint. In these cases, some enzymes appear to have preserved domains where acid (i.e., aspartic or glutamic acid) and base (i.e., histidine, arginine or lysine) side chains of residues are part of the active sites (Jimenez-Morales *et al.*, 2012).

After the identification of *A. polyphemus* CXE as a PDE (ApolPDE), structural investigations revealed that these enzymes share common features among moths. For instance, they have a conserved pentapeptide “G-X-S-X-G” (X represent any amino acid)

motif that is characteristic of esterases (Cygler *et al.*, 1993; Yin *et al.*, 2011) and common catalytic triad "S-E(D)-H" that can catalyze the hydrolysis of esters—an important group of pheromone compounds with 10 to 18 carbon atoms and one or two unsaturated carbons (Löfstedt and Kozlov, 1997; Oakeshott *et al.*, 1999). The absence of one of these residues causes these hydrolases to be transformed into catalytically inactive proteins (e.g. neurotactin or neuroligin); thus, they will be assigned in recognition or signal processing functions for neurodevelopment (Oakeshott *et al.*, 2005). Moreover, CXEs bear an oxyanion hole formed with the amine group (–NH) from the "G-G-A" motif that stabilizes high-energy intermediates and the transition state through hydrogen bonding in the active site (Zhang *et al.*, 2002; Zhang *et al.*, 2017). This feature is conserved in all esterase families in both vertebrates and invertebrates (Oakeshott *et al.*, 1999).

On the other hand, some studies have found N-glycosylation sites that could help to improve resistance against proteolysis, reduce non-specific protein interactions, and increase the protein solubility and stability (Fonseca-Maldonado *et al.*, 2013; Sun *et al.*, 2015). The idea that CXEs are secreted into the extracellular medium is based on the presence of a N-terminal signal peptide (Oakeshott *et al.*, 2005). This latter characteristic is relevant because they can be found in the sensillar lymph that can interact with the compounds that enter through the cuticular pores. Although CXEs do not share many similarities in the DNA sequences, they do have homology in their structure because the residues are conserved in the catalytic sites. Therefore, this family of proteins likely originated from a common ancestor (Nardini and Dijkstra, 1999).

As mentioned in the previous sections, bioinformatic techniques have appeared as an alternative in the search for new enzymes. Thus, several antennal transcriptomes have been published and are very useful because they have free access. Figure 4 shows the modeled structures of the ApolPDE1 from *A. polyphemus*, EsemCXE6 from *E.*

semipurpurella (public RNAseq raw data were downloaded from NCBI database, https://www.ncbi.nlm.nih.gov/, under the experiment SRX2627820), SinfCXE13 from *S. inferens*, and SlitCXE13 from *S. litura*. No crystallized CXEs structures have been published yet for Lepidoptera; however, three crystallized structures in Diptera with the access code 4FNM (Jackson *et al.*, 2013), 5CH3 (Correy *et al.*, 2016), and 5THM (Younus *et al.*, 2017) in the protein data bank were used as templates in molecular modelling. These structures confirm the conservation of these enzymes in relation to their binding site regardless of their non-Ditrysia or Ditrysia origin.

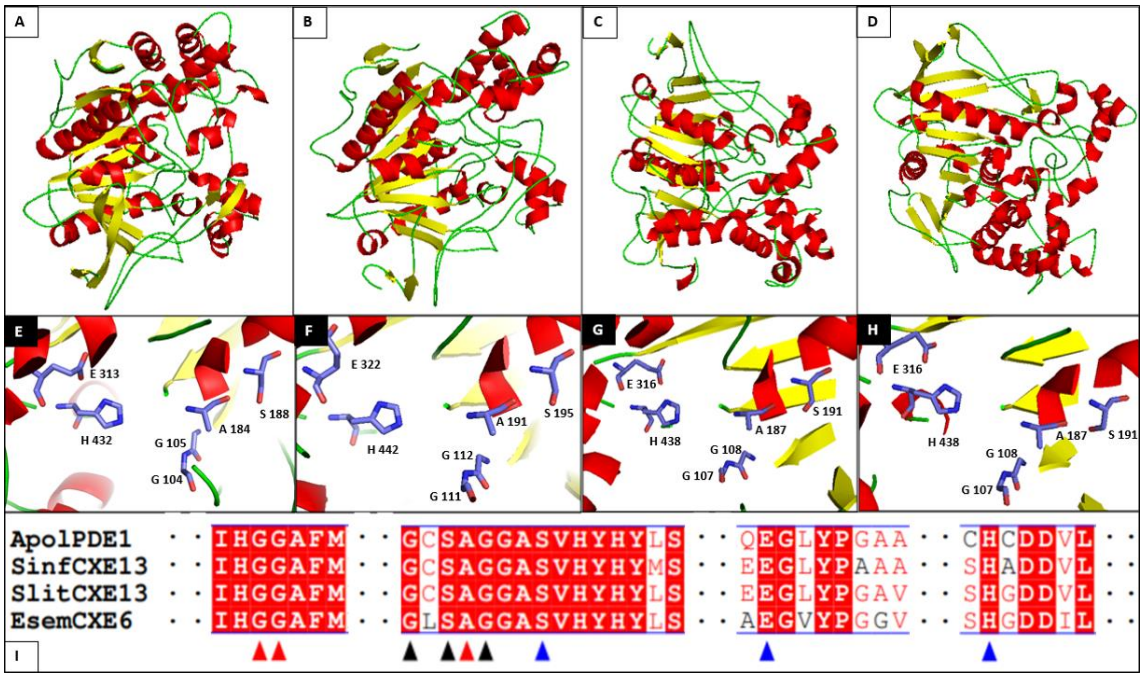

Fig. 4. Modeled structures of esterases in Lepidoptera and partial alignment. A) ApolPDE1 from A. polyphemus, B) EsemCXE6 from E. semipurpurella, C) SinfCXE13 from *S. inferens*, and D) SlitCXE13 from *S. litura*. E-H) Active site of ApolPDE1, EsemCXE6, SinfCXE13, and SlitCXE13, respectively. I) Partial alignment of amino acids sequences. Amino acids not shown are represented by two sequential dots. Oxyanion hole (G104-G105-A184) is indicated by red arrows. G181-X-S183-X-G185

motif is indicated by black arrows. The catalytic triad (S188-E(D)313-H432) is indicated by the blue arrows. The program Modeller 9.15 (Sali and Blundell, 1993; Webb and Sali, 2016) was used to build the three-dimensional structures and 4FNM (ApolPDE1), 5CH3 (SinfCXE13 and SlitCXE13), 5THM (EsemCXE6) as templates were used to obtain these modeled structures. Moreover, molecular dynamic (MD) simulations were performed using the NAMD 2.9 (Phillips *et al.*, 2005) so as to achieve a refinement of the modeled structure via the root mean square deviation (RMSD).

SinfCXE13 and SlitCXE13 have been reported as enzymes expressed in the antenna, but they are not tissue-specific. Interestingly, these two are within the phylogenetic clade of enzymes secreted that use pheromones as substrates and are close to ApolPDE1 (He *et al.*, 2014; Zhang *et al.*, 2016). So far, putative esterase sequences from the transcriptome of *E. semipurpurella* have not been published; therefore, as a complement to this work, 17 CXEs transcripts were identified according to a phylogenetic analysis (data not included): Only EsemCXE6 was in the same clade as ApolPDE1, SinfCXE13, and SlitCXE13. Here, is possible to visualize the α-helices (red helical ribbons), β-sheets (yellow arrows), and loops (green smooth ropes) in all modeled structures (Figure 4A-D), which is consistent with those values reported for esterases (Montella *et al.*, 2012). Interestingly, these characteristics are typical of α/β hydrolases and their folding gives a conformation of globular proteins. Furthermore, these sequences have a signal peptide indicating that these enzymes enter a secretory pathway, but they are not necessarily secreted to the extracellular environment (Nielsen, 2017). Despite the evolution of these moths, the antennal esterases conserved the residues in their active site that are responsible for the catalysis of chemical compounds (Figure 4E-H).

2.6. Antennal Esterases Inhibition in Integrated Pest Management

Antennal esterases are ubiquitous in the olfaction process and can control the levels of stimuli in the sensilla through the rapid catabolism of semiochemicals-mainly acetate-type pheromones. Therefore, inhibition of these enzymes emerges as a complement to IPM because current strategies are costly, e.g., the use of synthetic pheromones for mating disruption (Guerrero and Rosell, 2005). Several analogous compounds to sex pheromones have been tested in Lepidoptera as reported by Reddy and Guerrero (2010), where TFMKs appear to be good candidates for enzyme inhibition. These molecules enter through cuticular pores (Figure 2A) toward the sensillar lymph where OBPs can bind these inhibitors. Indeed, Campanacci *et al.* (1999) showed that the (*Z*)-11-hexadecenyl trifluoromethyl ketone was efficient in displacing the sex pheromone by binding to the recombinant PBP from *Mamestra brassicae* (MbraPBP1) through functional assays. Likewise, TFMKs can interact with ODEs inside the sensilla where it may form stable hydrates acting as transition-state analogues of pheromones. In brief, the inhibitory activity of TFMKs is due to a stable hemiacetal of tetrahedral geometry that is formed between the conserve serine residue of the antennal esterases with the highly electrophilic carbonyl (Durán *et al.*, 1993; Wiedemann *et al.*, 1998). Some studies have been performed with TFMKs and electrophysiological assays in *P. xylostella* (Prestwich and Streinz, 1988), *Thaumetopoea pityocampa* (Parrilla and Guerrero, 1994), *M. brassicae* (Renou *et al.*, 2002), *Cydia pomonella* (Giner *et al.*, 2009), and *S. frugiperda* (Malo *et al.*, 2013). Moreover, TFMKs have been used with antennal extracts of different moth species, e.g., *S. littoralis* (Durán *et al.*, 1993), *Ostrinia nubilalis* (Riba *et al.*, 2005), and *C. pomonella* (Giner *et al.*, 2009). All of these studies have shown that TFMKs had an effect on the catalytic activity of antennal esterases. On the other hand, behavior assays in tunnel wind with these inhibitors have shown a disruptive effect on the orientation

flight in males of *S. nonagrioides*, *S. littoralis*, and *C. pomonella* (Bau *et al.*, 1999; Giner *et al.*, 2009). In the field, it has been reported that TFMKs are effective pheromone antagonists for several insect pests such as *S. nonagrioides* (Riba *et al.*, 2001), *C. pomonella* (Giner *et al.*, 2009), *Zeuzera pyrina* (Muñoz *et al.*, 2011), *O. nubilalis* (Solé *et al.*, 2008), or *T. absoluta* (Dominguez *et al.*, 2016). The reduction in damage caused by *S. nonagrioides* and *O. nubilalis* in maize fields after application of *Z*11-16:TFMK was particularly remarkable, i.e., this is an analogue of the pheromone of the former species at a dose of 80 g/ha (Solé *et al.*, 2008).

Riba *et al.* (2001) further evaluated the biological toxicity of (*Z*)-11-hexadecenyl trifluoromethyl ketone (*Z*11-16:TFMK) and 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) in mice, and the results showed low toxicity (LD_{50} 1 g/kg after the 6^{th} day of the assay). Overall, an accurate identification and characterization of these enzymes could provide the basis for the development of putative inhibitory compounds that will be an alternative to IPM.

2.7. Concluding Remarks and Further Perspectives

The olfactory system is critical for reproductive success in many insect species. Some of these are of great economic importance in agriculture and forestry fields such as moths from the Tortricidae and Pyralidae family. Fortunately, the semiochemical compounds involved in host recognition, mating, or defense behaviors are being used to manage insect pests through environmentally friendly approaches, e.g., mating disruption and mass trapping. However, globalization has facilitated the dissemination of insect species throughout the world. How do insects adapt to these new environments? More specifically, how their olfactory system responds to these new conditions?

To answer these questions, it is important to understand the molecular basis and mechanisms involved in insect olfaction where proteins are the main players. Comprehensive studies have been performed for ORs, OBPs, and CSPs; ODEs have garnered less attention. These enzymes are novel targets for the use of species-specific chemicals in IPM. Therefore, we propose that an improved approach to classify a certain ODE into PDE would be crucial based on a highly specific sex pheromone communication. The evidence presented in this work suggests that a limited number of ODEs would actually be antennal- and pheromone-specific. Therefore, gene expression through qRT-PCR should consider comparing between sexes in antennae and then in the rest of tissues.

Phylogenetic analyses could help to filter ODEs close to already characterized PDEs (e.g., ApolPDE). Particularly, some studies (*S. inferens* and *S. littura*) found that a monophyletic clade (proposed as PDE clade) is present in moths. With this in mind, further ODEs that fall within this clade could be considered for characterization as putative PDEs. Finally, heterologous expression of the selected ODE(s) with purification and kinetic assays would be crucial for such task.

Alternatively, localization techniques such as FISH in specific sensilla could strongly support pheromone degradation function. Of note, transcriptomics has arisen as a useful tool to identify putative enzymes. Four modeled structures (ApolPDE1, SinfCXE13, SlitCXE13 and EsemCXE6) were shown to visualize their conformation and the residues of the active site, which resulted in conserved function. With the above issue addressed, the identification and characterization of these enzymes could provide the basis for the development of putative inhibitory compounds (e.g., TFMKs) that will be a complement to IMP strategies.

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CHAPTER III

Genome-wide identification of aldehyde oxidase genes in moths and butterflies suggests new insights into their function as odorant-degrading enzymes

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Genome-wide identification of aldehyde oxidase genes in moths and butterflies suggests new insights into their function as odorant-degrading enzymes

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Abstract

Aldehyde oxidases (AOXs) are common detoxifying enzymes in several organisms. In insects, AOXs act in xenobiotic metabolism and as odorant-degrading enzymes (ODEs). These last appear as crucial enzymes in the life cycle of insects, helping to reset their olfactory system, particularly in lepidopterans, which fulfill important ecological roles (e.g., pollination or destructive life cycles). A comprehensive understanding of their olfactory system has provided opportunities to study key chemosensory proteins. However, no significant advance has been made around lepidopteran AOXs research, and even less around butterflies, a recently evolved lineage. In this study we have identified novel AOX gene families in moths and butterflies in order to understand their role as ODEs. Eighteen genomes from both moths and butterflies were used for phylogenetics, molecular evolution and sequence analyses. We identified 164 AOXs, from which 91 are new. Their phylogeny showed two main clades that are potentially related to odorant-degrading function, where both moths and butterflies have AOXs. A first ODE-related clade seems to have a non-ditrysian origin, likely related to plant volatiles. A second ODE-related clade could be more pheromone-biased. Molecular evolution analysis suggests a slight purifying selection process, though a number of sites appeared under positive selection. ODE-related AOXs have changed a phenylalanine residue by proline in the active site. Finally, this study could serve as a reference for further evolutionary and functional studies around Lepidopteran AOXs.

Keywords: Lepidoptera, insect olfaction, aldehyde oxidase, genome, phylogenetic

3.1. Introduction

The study of gene evolution in insects has provided outstanding advances in the understanding of evolutionary processes, such as expansion or contraction of gene families (Li *et al.*, 2019). Particularly, lepidopterans represent an extraordinary target due to a clear diversification into moth and butterflies lineages (Kawahara *et al.*, 2019). Thus, the impact of gene evolution can be seen even within moths. For instance, regulation of desaturase genes in two sibling Helicoverpa species (i.e., *H. armigera* and *H. assulta*) results in reproductive isolation (Li *et al.*, 2015). Nowadays, the enormous amount of genomic and transcriptomic datasets for insects has provided an opportunity to elucidate novel genes and their evolutionary relationships (Oppenheim *et al.*, 2015), something that can support our understanding of ecological aspects of insects, such as behavior. For many insect species, behavior is mainly driven by olfaction.

Olfaction is primarily processed by insect antennae and their small hair-like structures called sensilla, in which a set of proteins work synergistically to maintain an extremely sensitive and dynamic system (Hansson and Stensmyr, 2011; Leal, 2013; He *et al.*, 2019). For instance, odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) function as transporters that carry odorants across the sensillar lymph (Zhou, 2010; Leal, 2013). These odorants reach an heteromeric complex of receptors, such as odorant receptors (ORs), an odorant receptor co-receptor (Orco) and a sensory neuron membrane protein (SNMP), as recently reported (Zhang *et al.*, 2020), to unleash depolarization in olfactory neuron membranes that triggers a behavioral response (Kaissling, 2013). Along with these olfactory proteins, odorant-degrading enzymes (ODEs), such as carboxylesterases (CXEs), glutathione-S-transferases (GSTs) and aldehyde oxidases (AOXs), are responsible for resetting the insect olfactory system through the degradation of odorant molecules (Chertemps and Meïbèche, 2021; Godoy *et*

al., 2021). Among ODEs, CXEs and GSTs have received particular attention due to their role in sex pheromone degradation in moths. For example, CXEs have been reported to degrade ester-type molecules (e.g., sex pheromones and plant volatiles) in moths *Plodia interpunctella*, *Spodoptera exigua*, *Grapholita molesta*, *Plutella xylostella*, and *Athetis lepigone* (He *et al.*, 2014a,b,c, 2015; Zhang *et al.*, 2017a; Liu *et al.*, 2019; Wei *et al.*, 2020; Wang *et al.*, 2021a). Likewise, GSTs have been well characterized in terms of function, being the delta class likely related to odorant degrading functions (Durand *et al.*, 2018). This is supported by the reported degrading function of *G. molesta* GST (GmolGSTd1) (Li *et al.*, 2018) and *Cydia pomonella* GST (CpomGSTd2) (Huang *et al.*, 2017). The AOXs, on the other hand, have received less attention so far. However, some species that use aldehydes as semiochemicals (i.e., chemicals that mediate communication between two organisms), have been studied, such as *Manduca sexta*, *Bombyx mori*, *Antheraea polyphemus*, *Amyelois transitella*, and *H. armigera*, among others (Riddiford, 1967; Kasang *et al.*, 1978; Coffelt *et al.*, 1979; Zhang *et al.*, 2012). Particularly, AOXs catalyze the oxidation of aldehydes to carboxylic acids (Garattini *et al.*, 2009; Garattini and Terao, 2012). In that sense, a few studies have functionally evaluated that process against aldehyde-based semiochemicals: an early study in *M. sexta* AOX reports that it catalyzes (E,Z)-10,12- hexadecadienal (bombykal) (Rybczynski *et al.*, 1989), and more recently, *A. transitella* AOX2 (AtraAOX2) was reported to hydrolyze plant volatiles (e.g., propanal, hexanal, and heptanal) as well as a sex pheromone component (*Z,Z*)-11,13-hexadecadienal (Choo *et al.*, 2013). Further evidence in terms of enzymatic activity of AOXs is still lacking. Nevertheless, important aspects of their function and structural features are underpinned by xanthine dehydrogenases (XDHs), an enzyme that catalyzes the oxidation of purines, pterin and aldehydes (Wang *et al.*, 2016). Among insects, lepidopterans have attracted special attention due to their

establishment as crop pests, some with worldwide distribution. It is known that moths rely heavily on the sense of smell (Weiss, 2001), developing long distance attraction based on volatile chemicals (e.g., sex pheromones) (Chemnitz *et al.*, 2015). In fact, hundreds of these volatiles have been identified since the first one reported for *B. mori*, the sex pheromone (*E,Z*)- 10,12-hexadecadien-1-ol (bombykol) (Butenandt *et al.*, 1959). On the contrary, butterflies rely heavily on visual cues and short-range chemical communication, understood as a multi- sensory integration (Costanzo and Monteiro, 2007), and hence have received less attention in terms of olfaction. Moreover, butterflies represent an interesting group for comparative studies considering their transition from moths approximately 98 Mya (million years ago) (Kawahara *et al.*, 2019). Thus, it is believed that comparing AOXs between moths and butterflies might deepen our understanding of their odorant-degrading function. Considering the difference in olfactory integration during the life cycle of moths and butterflies, we hypothesize that there is a specific clade of AOXs for both moths and butterflies that could be related to odorant-degrading function as well as both moth- and butterfly-specific gene expansions. Therefore, the objective of this study was to identify novel AOX genes from moths and butterflies using genomic and transcriptomic data and analyze them in terms of gene location, phylogeny, evolutionary processes, and structure.

3.2. Materials and Methods

3.2.1. Data Collection

Publicly available genomic data were retrieved from NCBI Genome database 1 and InsectBase 2 for major lepidopteran families, such as Bombycidae, Sphingidae, Noctuidae, Pyralidae, Crambidae, and Plutellidae for moths, whereas Nymphalidae, Pieridae and Papilionidae were used for butterflies (Table 1). Each fully represented

genome assembly with Reference Sequence (RefSeq) was downloaded from NCBI Assembly database at either contig, scaffold or chromosome level (Supplementary Table 1). Identification of Aldehyde Oxidase Family Bioinformatics pipeline BITACORA (Vizueta *et al.*, 2020) was used to identify already annotated AOX genes and potentially novel related genes from both moth and butterfly genomes. A database for AOX gene family was built using reported protein sequences for lepidopterans (Rybczynski *et al.*, 1989; Merlin *et al.*, 2005; Pelletier *et al.*, 2007; Choo *et al.*, 2013; Ou *et al.*, 2014; Zhang *et al.*, 2014, 2017b; Yang *et al.*, 2015; Huang *et al.*, 2016; He *et al.*, 2017; Xu and Liao, 2017; Wang *et al.*, 2021b).

Table 1. Summary of identified aldehyde oxidase genes in moths and butterflies.

Species	**AOX gene annotation[a,c]**				
Moths	**Total**	**Novel**	**Complete CDS**	**Average length (aa)**	**Functional annotation[b]**
B. mandarina	7	6	7	1272	1
B. mori	9	6	9	1266	3
M. sexta	19	18	16	1194	16
H. armigera	8	2	8	1335	8
S. frugiperda	20	20	19	1259	19
O. furnacalis	8	5	8	1298	8
P. xylostella	6	3	5	1271	5
A. transitella	6	5	6	1349	6
T. ni	14	14	11	1209	11

Butterflies	Total	Novel	Complete CDS	Average length (aa)	Functional annotation[b]
H. melpomene	2	2	2	2523	2
P. rapae	6	6	6	1301	6
B. anynana	11	11	10	1282	10
D. plexippus	10	10	7	1215	9
V. tameamea	6	6	5	1243	6
P. aegeria	9	9	8	1327	9
P. polytes	6	6	6	1231	6
P. xuthus	7	7	7	1265	7
P. machaon	7	7	6	1281	7

[a] Complete gene annotation available in Supplementary Table 1.

[b] Based on Interpro and BLAST searches.

[c] A complete annotation table can be found in Supplementary Table 1.

To identify family and structural domains for AOXs, InterPro server was used 3. The identified profile was used to search for HMM profile in PFAM database 4 (PF01315; ID Ald_Xan_dh_C). This process increased the likelihood of identifying sequences encoding members of the AOX gene family. Further processing included the trimming of isoforms (98% cutoff) using a provided script in BITACORA pipeline. Subsequently, BLAST searches were run with the identified proteins for manual annotation. Protein domain finder CDvist 5 (Adebali *et al.*, 2015) was used to identify conserved domains of AOXs, namely two (2Fe-2S), one flavin-containing region (FAD-binding domain) and one

molybdenum cofactor/substrate-binding domain. All proteins identified in this study are provided in Supplementary Table 1.

3.2.2. Sequence Analysis and Genome Structure

The genomic organization of identified AOX genes from both moth and butterfly species that use aldehyde-based semiochemicals was analyzed based on Vogt *et al.* (2015) and Xu and Liao (2017) including some modifications. Moths *Bombyx mandarina*, *P. xylostella*, and *A. transitella*, and butterflies *Heliconius melpomene* and *Bicyclus anynana*, were selected for this task. Annotated gene features (in GFF3 format) were retrieved from the gene identification protocol based on the BITACORA pipeline and analyzed manually. Thus, species name as well as source, start, end, strand and attributes were used for each AOX gene. Finally, gene localization was prepared in image editor Inkscape 0.48 software.

3.2.3. Data Preprocessing and Transcriptome Assembly

To both take advantage of transcriptomic data and include Tortricidae and Eriocraniidae families, we retrieved antennal RNA-seq data for moth *Lobesia botrana* (data from our laboratory) and non-ditrysian moth *Eriocrania semipurpurella* (SRR5328787). FASTQ files for both moths, one containing left-pair reads and other the right-pair reads, were used for assembly. Ribosomal RNA reads were removed by mapping the libraries using Bowtie2 v.2.3.3.1 (Langmead *et al.*, 2009) against a custom rRNA database created from insect ribosomal sequences downloaded from NCBI 6, and keeping non-mapped reads using SAMtools v.1.6 (Li *et al.*, 2009). Low-quality reads were removed based on their q-score composition using NGSQC Toolkit v.2.3 (Patel and Jain, 2012), and high quality reads were concatenated to build de novo transcriptomes

using Trinity v.2.6.5 (Grabherr *et al.*, 2011) with a P-value of 0.05 and fold-change value of 2.

3.2.4. Phylogenetic Analysis

A phylogeny for the identified AOX genes in moths and butterflies, including XDHs and AOXs from mosquitoes, beetles and bees as outgroups, was built. Full-length amino acid sequences that include conserved domains were aligned using MAFFT server 7 (Katoh *et al.*, 2019). GUIDANCE2 server 8 was used to check consistency of the multiple sequence alignment (Sela *et al.*, 2015). Briefly, the consistency of the alignment was measured with a score less than 0.5, in which sequences were deleted. It is worth noting that confidence scores near 1 and 0, suggest a highly and poorly consistent alignment, respectively. Finally, phylogenetic analysis was performed using maximum-likelihood method with FastTree software (Price *et al.*, 2010). To highlight clades, specific taxa and functional evidence, the phylogenetic tree was edited using FigTree software 9 and image editor Inkscape 0.48 software.

3.2.5. Molecular Evolution Analysis

In order to identify putative selective pressures on AOXs, a molecular evolution analysis was performed based on the methodologies reported by Engsontia *et al.* (2014) and Soffan *et al.* (2018). Two models were used through EasyCodeML software (Gao *et al.*, 2019) to elucidate selective pressures acting on the evolutionary process of 93 lepidopteran AOX genes, 9 XDHs and 11 AOXs from other insect orders. First, site model was applied to detect positive selection for a set of 113 sequences (Yang *et al.*, 2000). Additionally, a branch-site model was applied to test the presence of amino acids that evolved under positive selection in a specific clade represented by 8 AOX sequences

(most of them functionally studied). All the amino acid sequences were aligned by ClustalW 10, and converted to DNA alignment with PAL2NAL server 11. A maximum likelihood tree was prepared using the DNA alignment by FastTree software under default parameters. Briefly, the software estimated the ratio of normalized non-synonymous (dN) to synonymous (dS) (e.g., dN/dS or ω) substitution rate via the maximum likelihood method. The ω value indicates the mode of evolution, where $\omega > 1$ suggests evidence of positive selection with amino acid replacement, whereas $\omega < 1$ refers to purifying selection, and $\omega = 0$ indicates neutral selection. The specific models (M0, M3, M1a, M2a, M7, M8, and M8a) used under the "site model" method are described in detail in previous reports (Yang *et al.*, 2000; Yang and Nielsen, 2002; Swanson *et al.*, 2003). For the branch-site model, the 8 AOX sequences were labeled in the phylogenetic tree as foreground branch with the remaining clades as background branches. The change in ω was evaluated for a set of sites in each foreground branch through an alternative model, whereas neutral evolution was evaluated through a null model. Likelihood ratio tests (LRTs) were used to compare both models and significant results were determined using χ 2-tests. Finally, Bayes Empirical Bayes (BEB) analysis was used when LRT was significant to identify positive selected sites (PSSs) within each amino acid sequence (Yang *et al.*, 2005).

3.2.6. Sequence Analysis and Protein Structure Prediction

First, a multiple sequence alignment (MSA) was built with 7 AOX sequences also used in molecular evolution analyses, belonging to *A. transitella*, *B. mori*, *P. xylostella*, *H. armigera*, *Papilio xuthus*, *Papilio machaon*, and *B. anynana*, in which PSSs were identified and indicated (Supplementary Table 1). Sequences from *B. mandarina* AOX5 (BmanAOX5), *Drosophila melanogaster* AOX2 (DmelAOX2) and mammal AOXs, such as *Mus musculus* AOX2 (MmAOX2) and AOX3 (MmAOX3), and *Homo sapiens* AOX1

(HsAOX1), were also included. MSA was built in Multalin server 12 and ESPript 3.0 13 (Corpet, 1988). The amino acid sequence of AtraAOX2 and BanyAOX2 were submitted to BLASTp available on the NCBI website 14 for template selection. To optimize the structural information available for AOXs, a multiple template-based homology modeling approach was considered as it was reported to increase accuracy in predicted protein models (Sokkar *et al.*, 2011). First, multiple structure alignments were generated by SALIGN command, which is implemented in Modeler 10.1. Five hundred models of each AOX were obtained using Modeler 10.1 15. The best models were selected according to the lowest discrete optimized protein energy (DOPE) score provided by the software. The coordinates were analyzed via ProCheck 16 to check stereochemical quality. Lepidopteran AOXs were visualized through PyMOL software 17. The 3D structure of mammal AOXs were retrieved from Protein Data Bank 18 and DmelAOX2 was downloaded from AlphaFold database 19.

3.3. Results

3.3.1. Identification and Annotation of Aldehyde Oxidase Genes

Eighteen genome assemblies were retrieved from NCBI assembly database and InsectBase server. From those, 6 were assembled at chromosome level, whereas 11 at scaffold and 1 at contig level (Supplementary Table 1). The use of BITACORA pipeline resulted in a raw amount of 163 putative AOX genes for moths and 100 for butterflies. After homology searches through BLAST followed by conserved domain analyses, 99 AOX genes were left for moths and 65 for butterflies. The average amino acid length of AOXs is 1272 and 1407 for moths and butterflies, respectively. On the other hand, the moth species that showed a higher number of AOX were *Spodoptera frugiperda* with 20 sequences, *M. sexta* with 19 sequences and *Trichoplusia ni* with 14. In butterflies, *B.*

anynana and *Danaus plexippus* showed 11 and 10 AOX sequences, respectively. The specific number of AOX genes for each species is summarized in Table 1. Overall, 58 novel AOX genes were identified for moths whereas 33 AOX genes were identified for butterflies. BLAST hits for most of the novel AOX genes were either AOXs from other lepidopteran species or XDHs from the same species. In that sense, 6 new AOXs were identified for *B. mandarina* and *B. mori*, 16 for *M. sexta*, 17 for *S. frugiperda* and 10 for *D. plexippus*, as the greater numbers found. No novel AOX genes were found for butterflies *Papilio polytes* and *P. machaon.* Although most of the lepidopteran species studied here have a few annotated AOX genes, several of them are not fully annotated nor studied in terms of function. It is worth noting that the amount and length of AOX genes might be dependent on genome sequencing and annotation quality, therefore, previous estimates should be taken into account with caution.

3.3.2. Phylogenetic Relationships and Gene

Clusters Between Moths and Butterflies Phylogenetic analysis (Figure 1) suggests the presence of 5 main clades, 2 being related to either antennae specificity or odorant degradation (clades A and B in Figure 1). A clear diversification from insect XDHs and non-lepidopteran AOXs is observable. A clade with putative odorant-degrading function (labeled as A in Figure 1) appears to be a group of AOX genes evolved in ditrysian species, having the non-ditrysian moth *E. semipurpurella* EsemAOX1 at the base of the clade. Here, 9 moth AOX genes are reported to be enriched in antennae (Figure 1, species indicated with black circles next to their name), from which only M. sexta AOX1 (MsexAOX1) has been related to aldehyde-degrading function (Rybczynski *et al.*, 1989). A secondary odorant degrading-related clade (labeled as B in Figure 1) seems to have evolved by gene duplication. This clade includes 5 moth AOX genes enriched in antennae

(Figure 1, species indicated with black circles next to their name), and includes *A. transitella* (AtraAOX2), *P. xylostella* (PxylAOX3), and *B. mori* (BmorAOX5) which were functionally studied (Choo *et al.*, 2013; Zhang *et al.*, 2020; Wang *et al.*, 2021b). Furthermore, butterfly- and moth-specific AOX lineages were identified (highlighted in red and gray, respectively, in Figure 1), but no reported odorant-degrading function was found for these.

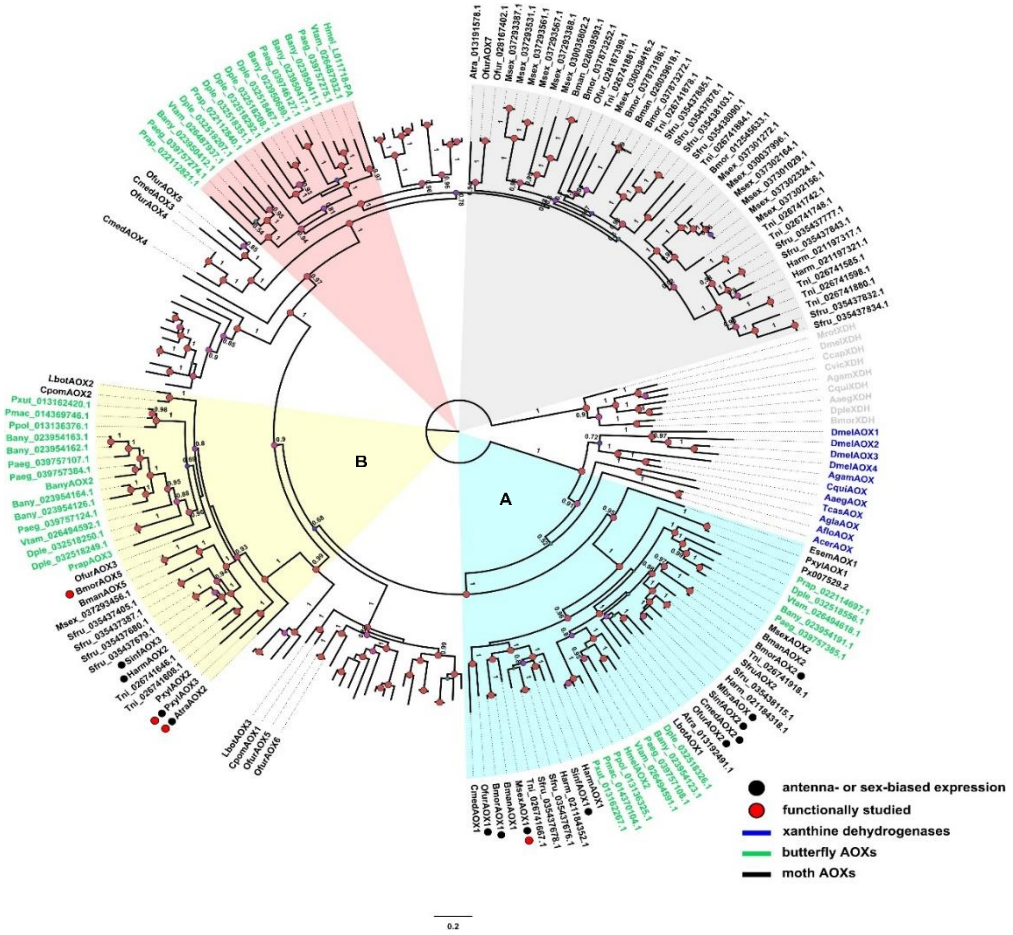

Figure 1. Phylogenetic tree of AOXs identified from moth and butterfly genomes as well as some AOXs and XDHs from other insect species. Clades A (light blue) and B (yellow) are highlighted as lineage with putative ODE function and lineage with ODE function, respectively. Species in green correspond to butterflies, species in black are moths, and

species in blue are AOX outgroups. The clade shaded in red color corresponds to butterfly specific AOXs with no ODE described function, and the clade shaded in gray correspond to moth specific AOXs with no ODE described function. Red circles next to the sequence name represent AOXs that have been functionally studied [MsexAOX1, (*E*,*Z*)-10,12-hexadecadienal; AtraAOX2, (*Z*,*Z*)-11,13-hexadecadienal; PxylAOX3, (*Z*)-11-hexadecenal; BmorAOX5, benzaldehyde, salicylaldehyde, vanillic aldehyde, propanal, and heptanal]. Black circles next to the sequence name indicate antennae- or sex-biased expression. Confidence scores are indicated as circles (> 70%) in nodes. All annotated genes and their amino acid sequences are in Supplementary Table 1.

Interestingly, butterflies that have aldehyde-related pheromones have AOXs present in at least one of the odorant-related clades (A or B). There are AOXs of some butterflies that are in these clades, but no aldehyde-related semiochemical has been reported yet, such as those present in *D. plexippus*, *Pararge aegeria*, *Pieris rapae*, *P. polytes*, *P. xuthus*, *P. machaon*, and *Vanessa tameamea*. On the contrary, *H. melpomene*, that uses aldehyde-based semiochemicals, showed only 1 AOX (HmelAOX2) in clade A. Likewise, *B. anynana* (with hexadecanal as a pheromone component), has 2 AOXs in clade A, while 5 in clade B. In terms of gene location, *B. mandarina*, *A. transitella* and *P. xylostella*, that use aldehydes as semiochemicals and have AOX genes related to ODE function, are far from other AOX genes, with the exception of PxylAOX3 (Figure 2). *H. melpomene* has 2 grouped AOX genes that suggest the same origin for both. Likewise, *B. anynana* has two big clusters of 4 and 7 AOX genes. Interestingly, from the bigger cluster of *B. anynana*, the 7 AOX genes were distributed in odorant-degrading clades A and B. Similarly, AOX1, AOX2 and AOX5 of *B. mandarina* that are grouped in a single cluster, are distributed in clades A and B. Besides HmelAOX2 present in clade A, gene

HMEL011718-PA clustered with HmelAOX2, which appeared in the previously mentioned butterfly-specific clade (red clade in Figure 1).

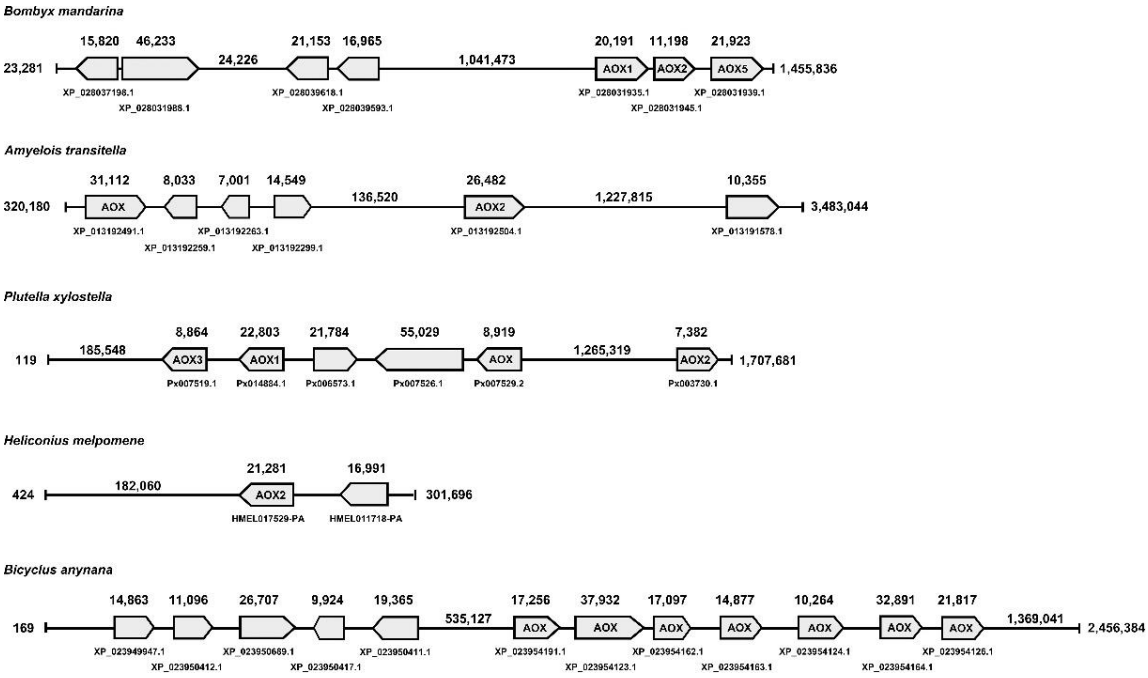

Figure 2. Gene location analysis for AOXs identified from lepidopterans that use aldehydes as semiochemicals, such as moths *B. mandarina*, *A. transitella*, *P. xylostella* and butterflies *H. melpomene* and *B. anynana.*

3.3.3. Selective Pressures on Aldehyde Oxidase Genes

Positive selection was first evaluated for a set of 113 sequences that included XDHs and AOXs of not only butterflies and moths, but also beetles, mosquitoes, and flies (Table 2). The four models implemented (e.g., M3 vs. M0, M1a vs. M2a, M7 vs. M8 and M8a vs. M8) showed significant differences according to LRT analysis. Interestingly, a purifying selection was suggested as site model (M0) resulted in $\omega = 0.89$. Additionally, a branch-site model was used to test selective pressures on specific sites (i.e., codons) among 8 closely related AOX sequences, including moths *A. transitella* AtraAOX2, *B.*

mori BmorAOX5, *P. xylostella* PxylAOX3, *H. armigera* HarmAOX2 and *Sesamia inferens* SinfAOX3, and butterflies *B. anynana* BanyAOX2, *P. xuthus* PxutAOX2 and *P. machaon* PmacAOX2 (Table 3). As expected, most of the enzymes were found to be under positive selection at many sites. For instance, AtraAOX2 resulted in 23.5% of their amino acids as PSSs, from which 105 sites showed either $P < 0.01$ or $P < 0.001$. Similarly, 22.2% of residues in BmorAOX5, 11.9% in HarmAOX, and 11.2% in SinfAOX3 were PSSs, with more than 20 sites identified with $P < 0.001$. In terms of butterflies, BanyAOX2 resulted in PSSs distributed in 40% of the entire sequence. However, less PSSs resulted for PxutAOX2 and PmacAOX2, representing only a 2–3% of the amino acid sequence length.

3.3.4. Link Between Function, Primary Sequence, and Protein Structure

To complement our previous methods that included annotation, phylogeny and molecular evolution analyses, a MSA was built followed by AOX structure prediction. The MSA was based on the same 8 lepidopteran AOX sequences detailed above, as well as *B. mandarina* BmanAOX5, *D. melanogaster* DmelAOX2, and mammal AOXs *M. musculus* MmAOX2 and MmAOX3, and *H. sapiens* HsAOX1, which have been well characterized in terms of structural features and active sites. As reference, the active site of MmAOX3 comprise Gln772, Glu1266, Lys889, Phe919 and Phe1014 (Terao *et al.*, 2020). From those, Gln772 and Glu1266 at positions equivalent to 739 and 1209 in Figure 3, were found to be conserved between all AOXs (red triangles in Figure 3). Interestingly, Phe919 (in vertebrates) at position 884 (in Lepidoptera) in Figure 3 was conserved among mammal AOXs, but replaced by Pro in all lepidopteran AOXs as well as DmelAOX2 (blue triangle in Figure 3). Lys889 (at position 855 in Figure 3) was also not conserved, with SinfAOX3, HarmAOX2, AtraAOX2, PmacAOX2, PxutAOX2 and PxylAOX3

having Gly, and BmorAOX5, BmanAOX5 and BanyAOX5 having Ser instead (purple triangle in Figure 3). In terms of structure, we could predict the 3D arrangements for AtraAOX2 and BanyAOX2, which were used to corroborate the identified conserved residues at the active site (Figure 4). The active sites equivalent to MmAOX3 Gln772 and Glu1266 were identified in both Lepidoptera species as well as in DmelAOX2 and vertebrate HsAOX1. Differences in conformation were observed, which are found in large structures that have not been relaxed through molecular dynamics. Nevertheless, our results are consistent with residue locations, supporting, for instance, the role that the insect specific Pro884 plays in the active site.

3.4. Discussion

In this study we identified a total of 164 AOX sequences from both moths and butterflies. In the context of an increasing amount of data from genomic studies, we have taken advantage of publicly available genome assemblies to identify and analyze AOX gene families in Lepidoptera. Particularly, AOXs are metal-containing enzymes that metabolize aldehydes into their corresponding carboxylic acids and other sub products (Krenitsky *et al.*, 1972). Their role in insect chemosensation has been studied since 1989 when *M. sexta* AOX (MsexAOX1) was reported to catalyze (*E,Z*)-10,12-hexadecadienal (bombykal), the sex pheromone of this species (Rybczynski *et al.*, 1989). However, reports about insect AOXs and their function toward aldehydes took more than 20 years to be published again, when *A. transitella* AOX2 (AtraAOX2) was comprehensively studied (Choo *et al.*, 2013).

Although AOX genes have been related to metabolism of xenobiotics in mammals as well as in Culex mosquitoes (Hemingway *et al.*, 2000; Coleman *et al.*, 2002; Terao *et al.*, 2020), recent efforts have been focused on insect AOXs that can act as ODEs in

olfactory organs, such as antennae and maxillary palps. Here, we report a profile of sequences related to AOX gene family that provides new data sets for several lepidopterans (Table 1 and Supplementary Table 1). For instance, our analyses revealed 5 full-length and 1 partial AOX sequences for *P. xylostella*, including the only identified AOX so far (PxylAOX3) (Wang *et al.*, 2021b). Similarly, 9 full-length sequences were identified for *B. mori*, including BmorAOX1, BmorAOX2, and BmorAOX5, the only AOXs reported so far (Pelletier *et al.*, 2007; Zhang *et al.*, 2020). Likewise, we report 2 new AOXs for *H. armigera*, in which 6 AOXs had previously been reported, including HarmAOX2, suggested to be a candidate pheromone-degrading enzyme (Xu and Liao, 2017). For butterflies, no AOX-related studies have been published to our knowledge. Hence, this study would be the first to report such enzymes in this group.

In terms of number of AOXs identified in both moths and butterflies, it is interesting to notice that generalist moth species, such as *S. frugiperda*, *M. sexta* and *T. ni* resulted in the highest number of AOXs. However, we could not establish a direct relationship between number of AOXs and the condition of generalist vs. specialist species, something that has been proposed for other chemosensory proteins, such as ORs (Venthur and Zhou, 2018). Thus, we can highlight that, overall, moths resulted in a similar number of AOXs compared with butterflies, excluding *S. frugiperda*, *M. sexta*, and *T. ni*. On the one hand, the amount of identified AOXs could have been determined by the unavailability of well-assembled genomes in both moths and butterflies. On the other hand, this can also be explained because moths are largely dependent on chemosensation (at short and long range) whereas butterflies use pheromones for short range communication and visual cues (Costanzo and Monteiro, 2007). In that sense, it can be suggested that butterflies have some AOXs related to odorant degradation and to a lesser extent for the metabolism of xenobiotics. This assumption is supported by our

phylogenetic analysis, where two clades related to odorant-degrading function showed the presence of both moth and butterfly AOXs.

Table 2. Positive selection analysis using site model on 113 Lepidopteran AOXs and XDHs sequences.

Model	np	LnL	Estimates of parameters	Models compared	LRT *p*-value
Site model					
M3	60	-10876.292891	p0: 0.46282; p1: 0.38177; p3: 0.15540; ω0: 0.24443; ω1: 1.46989; ω2: 4.81439	M3 vs M0	0.00E+0.00
M0	56	-11425.851842	ω: 0.89134		
M2a	59	-10882.888127	p0: 0.39310; p1: 0.41468; p2: 0.19222; ω1: 0.17566; ω2: 1.00000; ω3: 3.80060	M1a vs M2a	0.00E+0.00
M1a	57	-11050.821062	p0: 0.50743; p1: 0.49257; ω0: 0.15450; ω1: 1.00000		
M8	59	-10886.022772	p0: 0.78439; p: 0.53521; q: 0.46979 (p1: 0.21561); ω: 3.50001	M7 vs M8	0.00E+0.00
M7	57	-11067.720224	p: 0.50998; q: 0.38141		
M8a	58	-11036.136130	p0: 0.57595; p: 1.01933; q: 4.04743 (p1: 0.42405); ω: 1.00000	M8a vs M8	0.00E+0.00

Table 3. Positive selection analysis using branch-site model on 8 Lepidopteran AOX sequences.

Model	np	LnL	Estimates of parameters	LRT *p*-value[a]	PSSs[b]
Branch-site model					
#1 AtraAOX2	18	-19563.928883	p0: 0.34727; p1: 0.47396; p2a: 0.07560; p2b: 0.10317; ω0: 0.06748; ω1: 1.00000; ω2a: 0.06748; ω2b: 1.00000	0.00E+00	23V, 30P, 40T, 41M, 45L, 47I, 49K, 81L, 112C, 113R, 116D, 123K, 151E, 183N, 187R, 205G, 221R, 232S, 243V, 283N, 299E, 376L, 382V, 384N, 423V, 461L, 463F, 468E, 472F, 495L, 540A, 542Q, 546S, 547E, 554G, 562A, 564E, 596G, 605A, 615V, 624P, 643R, 657V, 659V, 661V, 662L, 706M, 735S, 740H, 762I, 764E, 771A, 788T, 791I, 794C, 805Q, 807R, 810C, 826V, 833T, 840I, 842N, 862E, 874S, 875V, 879V, 884K, 889V, 893H, 900T, 903K, 920H, 925I, 935S, 941K, 942I, 964F, 989I, 1005T, 1017G, 1048L, 1053Y, 1054I, 1076R, 1079N, 1089K, 1091E, 1092M, 1099K, 1103W, 1108L, 1123K, 1126S, 1165L, 1174G, 1188I, 1189F, 1192H, 1193S, 1250V, 1254N, 1257R, 1264H, 1267A, 1289Y

#2 PxylAOX3	18	-19576.412306	p0: 0.36546; p1: 0.47336; p2a: 0.07023; p2b: 0.09096; ω0: 0.07760; ω1: 1.00000; ω2a: 0.07760; ω2b: 1.00000	0.00E+0.00	25T, 35E, 75R, 76R, 84T, 105I, 110D, 112C, 140Q, 187R, 227S, 255I, 264A, 271D, 301Q, 308L, 312I, 313S, 317S, 319A, 336E, 339R, 343L, 354S, 380G, 391Q, 404D, 405M, 406R, 450N, 454N, 458H, 462A, 467T, 479Y, 491L, 499S, 535G, 536T, 540A, 543S, 554G, 632A, 643R, 676E, 715G, 722T, 769M, 785S, 836S, 844C, 894L, 896T, 909A, 914T, 961E, 974F, 1003M, 1020I, 1037E, 1055A, 1097T, 1107E, 1134A, 1135I, 1191K, 1219K, 1284A, 1286D
#3 BmorAOX5	18	-19563.931580	p0: 0.34710; p1: 0.47328; p2a: 0.07599; p2b: 0.10362; ω0: 0.06797; ω1: 1.00000; ω2a: 0.06797; ω2b: 1.00000	0.00E+0.00	23V, 30P, 40T, 41M, 45L, 47I, 49K, 81L, 112C, 116D, 123K, 124E, 151E, 183N, 187R, 205G, 221R, 232S, 243V, 283N, 299E, 376L, 382V, 384N, 423V, 461L, 463F, 468E, 472F, 495L, 540A, 547E, 554G, 562A, 564E, 596G, 605A, 615V, 624P, 643R, 657V, 659V, 661V, 662L, 706M, 735S, 740H, 762I, 764E, 771A, 788T, 791I, 794C, 805Q, 807R, 810C, 826V, 833T, 840I, 842N, 862E, 874S, 875V, 879V, 884K, 889V, 893H, 900T, 903K, 920H, 925I, 935S, 941K, 942I, 964F, 989I, 1005T, 1017G, 1048L, 1053Y, 1054I, 1099K, 1103W, 1108L, 1123K, 1126S, 1165L, 1174G, 1188I, 1189F, 1192H, 1193S, 1250V, 1254N, 1257R, 1264H, 1267A, 1289Y

#4 HarmAOX2	18	-19623.819643	p0: 0.39374; p1: 0.51575; p2a: 0.03918; p2b: 0.05133; ω0: 0.08015; ω1: 1.00000; ω2a: 0.08015; ω2b: 1.00000	0.00E+0.00	94V, 245K, 314L, 315E, 364E, 374L, 386R, 401L, 445F, 458H, 467T, 499S, 596G, 607V, 621L, 630Y, 670I, 678L, 834T, 869C, 966E, 981M, 1070V, 1104R, 1116Y, 1139Q, 1142V, 1147Y, 1190D
#5 SinfAOX3	18	-19633.400356	p0: 0.39394; p1: 0.52825; p2a: 0.03324; p2b: 0.04457; ω0: 0.07706; ω1: 1.00000; ω2a: 0.07706; ω2b: 1.00000	0.00E+0.00	81L, 102I, 211K, 306L, 320I, 387N, 437A, 442N, 493G, 501E, 530S, 606T, 685K, 722T, 765S, 874S, 879V, 900T, 1048L, 1143L, 1198T, 1246G, 1270I, 1290E, 1295S

#6 BanyAOX2	18	-19536.056864	p0: 0.34728; p1: 0.44832; p2a: 0.08922; p2b: 0.11518; ω0: 0.06915; ω1: 1.00000; ω2a: 0.06915 ; ω2b: 1.00000	0.00E+0.00	Supp. Info.
#7 PxutAOX2	18	-19667.749019	p0: 0.33543; p1: 0.44553; p2a: 0.09408; p2b: 0.12496; ω0: 0.06042; ω1: 1.00000; ω2a: 0.06042; ω2b: 1.00000	0.00E+0.00	423V, 728V, 835I, 1141D

#8 PmacAOX2	18	-19668.210787	p0: 0.42216; p1: 0.54813; p2a: 0.01293; p2b: 0.01679; ω0: 0.07895; ω1: 1.00000; ω2a: 0.07895; ω2b: 1.00000	0.00E+0.00	284Y, 486F, 725G, 730K, 934K

[a]Significant difference according to likelihood-ratio test (LRT). [b]Positive selected sites included with $P > 0.99$ according to Bayes Empirical Bayes (BEB) analysis. Other amino acids under positive selection with less than 0.95 of significance are included in Supplementary information.

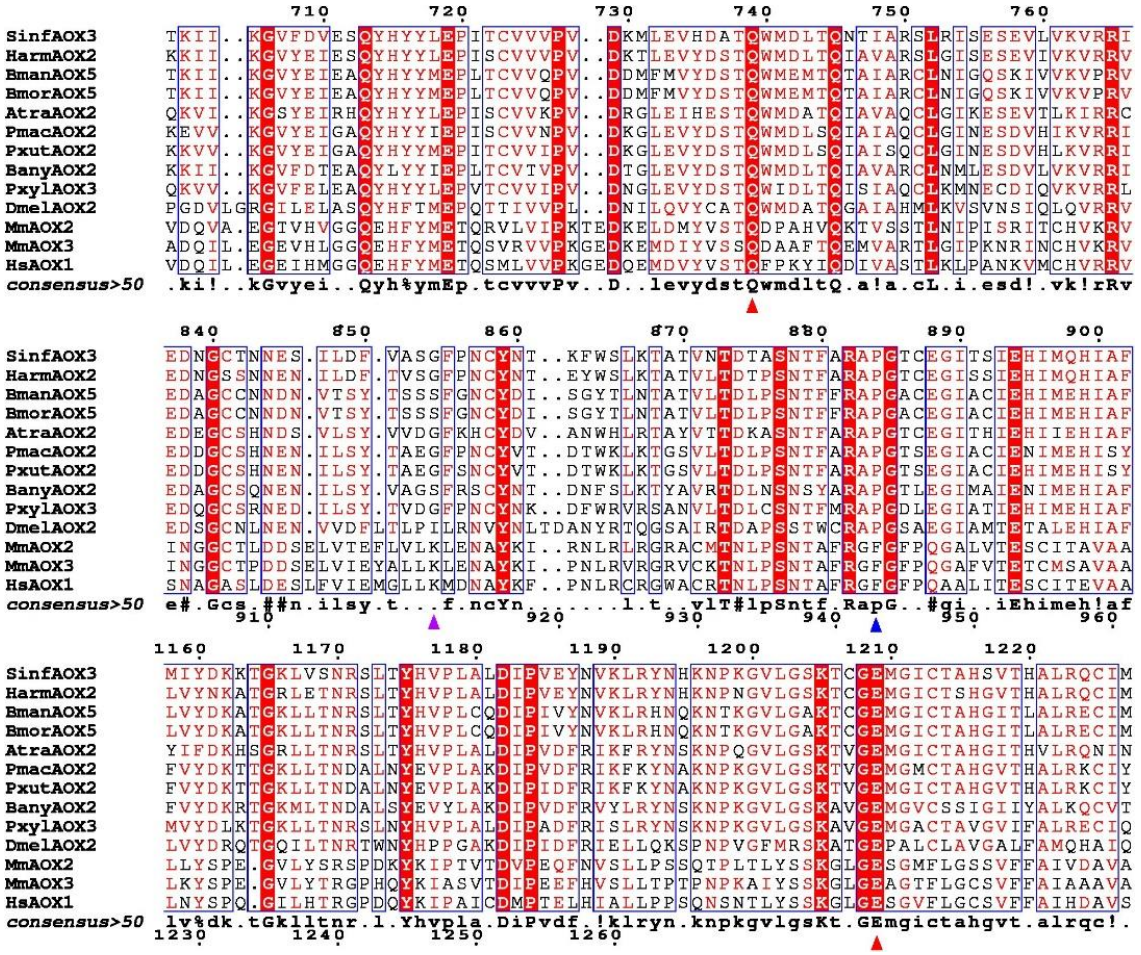

Figure 3. Fragment of a multiple sequence alignment between vertebrate and invertebrate AOXs. Identical residues are highlighted in white letters with red background. Similar residues are highlighted in red and framed in blue. Triangles indicate conserved sites according to Terao *et al.* (2020). Red triangles show conserved residues across all analyzed species. Blue triangle shows residue conserved only in insect species. Purple triangle shows an active site with different residues within Lepidoptera species. Full alignment can be found in Supplementary Figure 1.

To date, two studies have reported a phylogeny for insect AOXs with focus on moths *P. xylostella* and *H. armigera* (He *et al.*, 2017; Xu and Liao, 2017). Both analyses confirm XDHs as common ancestors followed by Dipteran AOXs, and support lepidopteran AOXs as more recently evolved enzymes. In that sense, our phylogenetic analysis is consistent with both. Furthermore, this analysis showed the two ODE-related clades already mentioned as well as a common ancestor in *E. semipurpurella* AOX1 (EsemAOX1), the only AOX identified from its antennal transcriptome. Interestingly, as an old lineage of moths (i.e., non-ditrysia), *E. semipurpurella* represents a model for evolutionary studies. Yuvaraj *et al.* (2017) showed that moth pheromone receptors could have evolved from plant volatile-related ORs, since two *E. semipurpurella* ORs (EsemOR3 and EsemOR5) that are phylogenetically close to plant volatile- responding ORs, respond to its sex pheromone (2*S*,6*Z*-6-nonen- 2-ol), which resemble plant volatiles. The lack of more AOXs in *E. semipurpurella* could indicate that gene duplication events in other moths, and likely butterflies, happened in response to the use of more specialized aldehyde-related volatiles, such as sex pheromones. Furthermore, those that are close to EsemAOX1, in ODE-related clade (clade A in Figure 1), could likely be more plant volatile-biased.

It can be argued that moths with functionally studied AOXs, namely BmorAOX5, PxylAOX3 and AtraAOX2, are not strictly related to sex pheromone degradation. In fact, AtraAOX2 has not showed specificity for *A. transitella* sex pheromone [(*Z*,*Z*)-11,13-hexadecadienal], being also able to catalyze aldehyde-related plant volatiles (Choo *et al.*, 2013). Similarly, PxylAOX3 was reported able to degrade sex pheromone (*Z*)-11-hexadecenal as well as plant-derived aldehydes, such as phenylacetaldehyde and nonanal (Wang *et al.*, 2021b). Butterflies, *H. melpomene* with (*Z*)-9-octadecenal, octadecanal, (*Z*)-11-icosenal, icosanal and (*Z*)-13-docosenal as sex pheromone components (Darragh

et al., 2017), and *B. anynana* with hexadecanal (Nieberding *et al.*, 2008), represent the only butterflies that use aldehydes as semiochemicals in our data sets. Interestingly, one AOX (i.e., HmelAOX2) was present in an ODE-related clade according to our phylogenetic analysis, while *B. anynana* had seven. It is worth noting that *H. melpomene* as pollinator (Andersson and Dobson, 2003) and *B. anynana* as a fruit-feeding butterfly (Lewis and Wedell, 2007), are both exposed to more aldehydes emitted by plants and fruits. For *B. anynana*, it is noticeable that the seven AOXs (from a total of 12) appear to have emerged independently from the rest. Something similar to what is found in the gene location of moth species, such as *B. mandarina*, *A. transitella* and *P. xylostella*, where their AOX genes potentially related to ODE function, are far from other AOX genes, with the exception of PxylAOX3.

From the two ODE-related clades in our phylogenetic analysis, clade B resulted highly supported by both functional studies and antennal-enriched expression (Figure 1). The fact that AOXs from butterflies were also present in this clade, further suggests that these could use aldehyde-based volatiles as semiochemicals. Although fewer studies have exploited the semiochemistry of butterflies compared with moths, increasing evidence suggests that several species of butterflies, including *H. melpomene* and *B. anynana*, use volatiles as semiochemicals. For example, an early study reported strong antennal responses of *H. melpomene* to several tropical plant-derived volatiles, such as linalool, linalool oxide I and II, oxoisophoroneoxide and phenylacetaldehyde (Andersson and Dobson, 2003). Recently, 55 compounds exclusive of androconia (specialized units where secretory glands are found) in sympatric Pieridae butterflies that would play a role in mating orientation, were reported (Nobre *et al.*, 2021). On the other hand, some moths and butterflies can share pheromone biosynthetic pathways. It has been reported that in *B. anynana* the synthesis of hexadecanal and (*Z*)-9-tetradecenol is mediated by conserved

fatty acyl Δ11-desaturases (Liénard *et al.*, 2014). In that sense, it is expected that other enzymes, such as AOXs, could be conserved between moths and butterflies. Therefore, it appears that AOXs in ODE-related clades could function for aldehyde-related semiochemicals whether derived from plants or conspecific species. Thus, more functional studies focused on both moths and butterflies would be necessary to support a monophyletic pheromone-degrading clade.

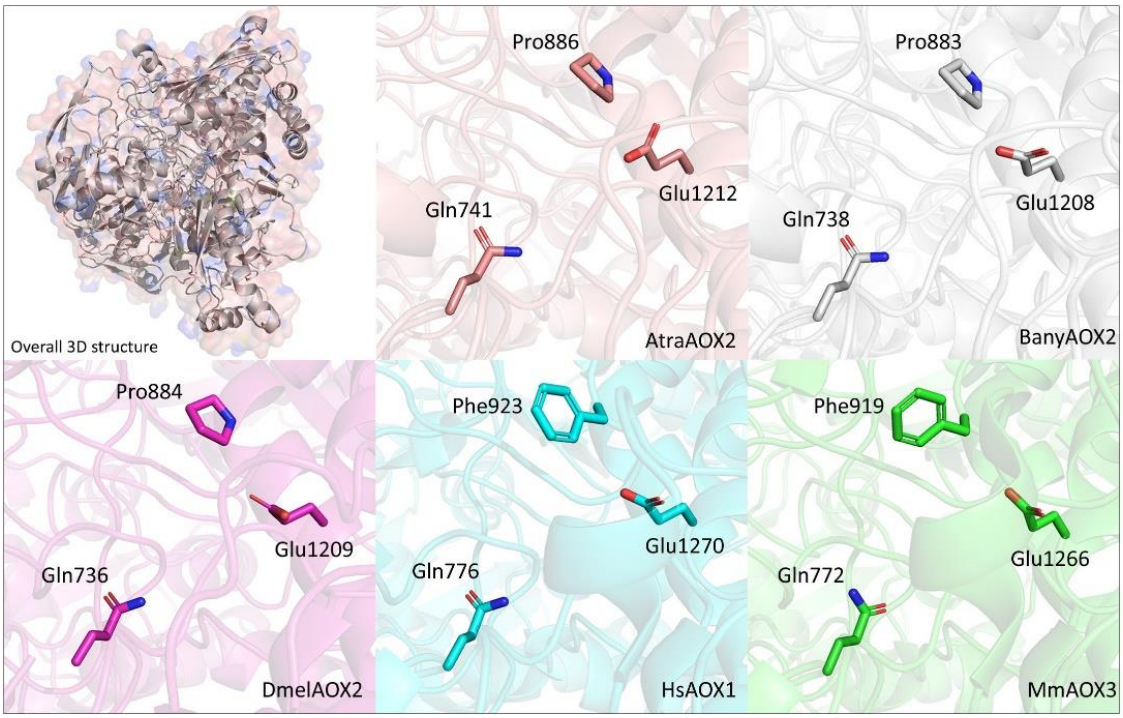

Figure 4. Visualization of the location of conserved residues in 3D representations of the active site of *A. transitella* AOX2 (AtraAOX2—orange), *B. anynana* AOX2 (BanyAOX2—gray), *D. melanogaster* AOX2 (DmelAOX2—pink), *H. sapiens* AOX1 (HsAOX1—cyan) and *M. musculus* AOX3 (MmAOX3—green). Residues and their positions are indicated in each AOX. Mammal AOXs were retrieved from Protein Data Bank (https://www.rcsb.org/) and DmelAOX2 was downloaded from AlphaFold database (https://alphafold.ebi.ac.uk/).

In general, the function of AOXs toward aldehydes might resemble the function of XDHs, which are their evolutionary ancestors (Kurosaki *et al.*, 2013; Wang *et al.*, 2016). High levels of similarity between vertebrate XDHs and AOXs have been reported (Terao *et al.*, 2020). For instance, sequence identity between mammal AOXs, namely HsAOX1 and MmAOX1, reaches 83%. On the other hand, sequence identity between lepidopteran and mammal AOXs is 30%. More specifically, among lepidopteran AOXs, sequence identity starts decreasing at 67%. This divergence within lepidopterans is evidenced in an important amount of PSSs among those phylogenetically close AOXs that were selected for our molecular evolution analyses. Nevertheless, and as expected, residues that are conserved were not PSSs, such as those from the active site.

Our MSA analysis revealed that highly conserved residues in vertebrate AOXs, namely Glu1266, Phe919, Lys889 and Gln772, may or may not be conserved in lepidopteran and *D. melanogaster* AOXs. Thus, Glu1266 (at position 1,209 in our MSA, Figure 3) that is reported to be crucial for catalytic activity resulted highly conserved (Coelho *et al.*, 2012), while Phe919 from vertebrates changes to Pro in insect AOXs (at position 884 in our MSA, Figures 3, 4). It is difficult to predict the effect of Pro instead of Phe in insect AOXs. The change from an aromatic side chain toward an aliphatic portion like the one present in Pro, might have some effects on selectivity and stability of aldehyde substrates. In our structural analyses, Pro884 was found in the active site of AtraAOX2, BanyAOX2 and DmelAOX2, keeping the region hydrophobic. In other studies, enzymes such as HCV NS5b polymerase, Pro197 along with Arg200, Cys366, Met414 and Tyr448, were reported to be crucial for ligand selectivity (Li *et al.*, 2010). On the contrary, Pro substitutions in human carbonic anhydrase II led to an increased rigidity of the enzyme and subsequent decreased catalytic activity (Boone *et al.*, 2015). In fact, it is well accepted that Pro restricts protein backbones with the lack of a hydrogen

bond donor, disrupting α-helices (Woolfson and Williams, 1990; Van Arnam *et al.*, 2011).

Overall, we believe this study represents the first to group a comprehensive set of AOX genes for several lepidopteran species. We have validated AOX sequences previously described and added 58 more in moths and 33 more in butterflies. We have also uncovered the potential importance of aldehydes as semiochemicals in butterflies, as reflected by the number of AOX present in this group. The information presented herein is a helpful reference for further evolutionary and functional studies in this highly biodiverse order.

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CHAPTER IV

Characterization of two aldehyde oxidases from the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) with potential role as odorant degrading enzymes

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Characterization of two aldehyde oxidases from the grater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) with potential role as odorant degrading enzymes

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Abstract

Odorant degrading enzymes (ODEs) are proposed to degrade/inactivate volatile organic compounds (VOCs) in millisecond scale time. Thus, ODEs play an important role in the insect olfactory system as a reset mechanism. The inhibition of these enzymes could incapacitate the olfactory system and, consequently, disrupt chemical communication promoting and complementing the integrated pest management. Here, we report two novel aldehyde oxidases, AOXs-encoding genes (*GmelAOX2* and *GmelAOX3*) thought transcriptomic analysis in the greater wax moth, *Galleria mellonella*. GmelAOX2 was clustered in clade with ODE function, according to phylogenetic analysis. Likewise, to unravel the profile of volatiles that *G. mellonella* might be face besides the sex pheromone blend, VOCs were trapped from honeycombs and the identification was made by Gas Chromatography-Mass Spectrometry. Semi-quantitative RT-PCR showed that GmelAXO2 have a sex-biased expression, and qRT-PCR indicated that both GmelAOX2 and GmelAOX3 have a higher relative expression in males antennae rather than females. A functional assay revealed that antennal extracts had the strongest enzymatic activity against undecanal (4-fold) compare to benzaldehyde (control). Our data suggest that these enzymes have a crucial role for metabolizing sex pheromone compounds as well as plant-derived aldehydes, which are related to honeycombs and the life cycle of *G. mellonella*.

Key words Aldehyde oxidases; apiculture; *Galleria mellonella*; relative expression; semiochemicals; transcriptomics

4.1. Introduction

In insects, mating or threats avoidance as well as host and food seeking are mainly result of the interaction between volatile organic compounds (VOCs) and a well-tuned olfactory system (Sato and Touhara, 2008; Zhou, 2010). Here, the transport, transduction and degradation of VOCs is carried out by olfactory proteins, such as odorant binding proteins (OBPs), odorant receptors (ORs) and odorant degrading enzymes (ODEs) (Kaissling, 2013; Leal, 2013., Pelosi *et al.*, 2017; Chertemps and Maïbèche, 2021). Briefly, chemical cues enter through cuticular pores placed in hair-like structures called sensilla on antennae, towards the sensillar lymph where OBPs bind and transport molecules to the ORs. Once receptors are activated, a transduction signal is started unleashing insect behavioral response. Finally, ODEs rapidly degrade the odorant stimuli in order to avoid accumulation of compounds in the peripheral space, leading to rapid signal termination (Leal, 2013). This allows to recover and maintain the sensitivity of the olfactory system by its resetting on a millisecond (ms) timescale, and thus receive new chemical signals (Ishida and Leal, 2005). Noteworthy, there are different families of ODEs involved in stimuli deactivation, such as carboxylesterases (CXEs), aldehyde oxidases (AOXs), glutathione-S-transferases (GSTs) and cytochrome P450 (CYPs). Particularly, CXEs and AOXs have been studied as ODEs (Chertemps *et al.*, 2015), in fact, the first ODE (a CXE) was classified as pheromone-degrading enzyme (PDE), being specifically present in male antennae of moth *Antherea polyphemus* (Vogt and Riddiford, 1981). This PDE was capable to rapidly degrade (in an estimated half-life of 15 ms) the sex pheromone (*E,Z*)-6,11-hexadecadienyl acetate according to *in vivo* and *in vitro* assays (Vogt *et al.*, 1985). Another PDE was identified in the scarab beetle *Popillia japonica*, being only expressed in male antennae. Authors reported that both the native and recombinant enzyme showed preference against the sex pheromone, (*R*)-japonilure,

rather than its enantiomer, (*S*)-japonilure (behavioral antagonist) (Ishida and Leal, 2008). On the other hand, the first AOX in moths was characterized in the tobacco hornworm *Manduca sexta*, and reported as a dimer from column chromatography with an estimated molecular weight of 295 kDa (Rybczynski *et al.*, 1989). This AOX was classified as a PDE (MsexAOX) because it had nearly 60% greater expression in male than female antennae. Additionally, kinetic parameters of MsexAOX showed preference for the pheromone compound, (*E,Z*)-10,12-hexadecadienal (bombykal) (K_m 5.4 µM) compared with other VOCs, such as propanal (K_m 6.8 µM) and benzaldehyde (K_m 225.1 µM) (Rybczynski *et al.*, 1989). Complementary, Merlin *et al.* (2005) proposed that MbraAOX-encoding gene from cabbage armyworm *Mamestra brassicae*, is active at sensillar lymph level and that its expression is restricted to olfactory sensilla (i.e. Sensilla trichoidea, Str I) through *in situ* hybridization (ISH). In functional terms, these enzymes can transform aldehyde-type semiochemicals into inactive forms, such as carboxylic acids (Guerrero and Rosell, 2005). Nowadays, several moth species use a blend of chemicals as sex pheromone, where aldehydes can act as either major or minor components. For example, (*Z,Z*)-7,11-hexadecadienal, (*E*)-11,13-tetradecadienal, (*E,E,Z,Z*)-4,6,11,13-hexadecatetraenal as major pheromone components for the moths citrus leafminer, *Phyllocnistis citrella*, the eastern blackheaded budworm, *Acleris variana* and the promethea moth, *Callosamia promethean*, respectively (Ando *et al.*, 1985; Gries *et al.*, 1994; Gago *et al.*, 2013). On the other hand, aldehyde-based pheromones can act as minor components, such as (*E*)-10-hexadecenal, (*E,E*)-8,10-dodecadienal and (*E,Z*)-6,11-hexadecadienal for the legume podborer, *Maruca vitrata*, the codling moth, *Cydia pomonella*, and *A. polyphemus*, respectively (Kochansky *et al.*, 1975; Ebbinghaus *et al.*, 1997; Downham *et al.*, 2003). Noteworthy, aldehydes can also act as behavioral

antagonists. For instance, females of *Bombyx mori* emit bombykal in its pheromone blend besides bombykol, as a behavioral antagonist (Kaissling *et al.*, 1978).

The fact that *A. polyphemus* and *B. mori* use aldehydes in their life cycle supported the presence of AOXs that could metabolyze these semiochemicals. Thus, Rybczynski *et al.* (1990) identified antenna-specific aldehyde oxidases (AOXs) in antennal extracts from *A. polyphemus* and *B. mori*, which were more abundant in males than females through polyacrylamide gel electrophoresis (PAGE). Likewise, Zhang *et al.* (2017) found four putative AOXs genes in the rice leaffolder, *Cnaphalocrosis medinalis* through transcriptomic analysis. Authors reported *CmedAOX2*-encoding transcript significantly more expressed in male than female antennae, indicating a putative degradation role for the sex pheromone blend, (*Z*)-11-octadecenal and (*Z*)-13-octadecenal. On the contrary, there are some exceptions, where AOXs can degrade other aldehyde-based pheromone compounds, with no involvement of sex-biased expression. For instance, recent studies in the cotton bollworm, *Helicoverpa armigera*, identified six full-length AOXs genes, from which HarmAOX2 was suggested as PDE for inactivating (*Z*)-11-hexadecenal and (*Z*)-9-hexadecenal through specific and significant expression in adult antennae of both sexes (Xu & Liao, 2017).

The greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), is an important pest of honeybee products (Kwadha *et al.*, 2017). Larvae of *G. mellonella* use honeycombs to make silken galleries disrupting the development and growth of bees, this event is called Galleriasis (Williams, 1997). Interestingly, its mating is highlighted by males producing an acoustic signal and a sex pheromone blend, mainly formed by aldehydes nonanal and undecanal (major components) (Leyrer and Monroe, 1973), that attract conspecific females. These aldehyde-type compounds have been reported from different sources, such as insect sex pheromone (Butenandt *et al.*, 1959; Choo *et al.*, 2013; Gago *et al.*,

2013; Zhang, *et al.*, 2014; Foster and Anderson, 2018), VOCs from plants (Hao *et al.*, 2018; González-Mas *et al.*, 2019, Giuffrè *et al.*, 2020), as well as in beehive products, such as honey, pollen, wax and propolis (Castro-Vázquez *et al.*, 2007; Lima-Neto *et al.*, 2017; Pattamayutanon *et al.*, 2017; Abd El-Wahed *et al.*, 2019). The usual methods to manage this pest are based on chemical insecticides, e.g. naphthalene, methyl bromide, paradichlorobenzene and carbon dioxide (CO_2), however, these compounds (except CO_2) represent health risk and lead to residues in honeybee products (Ritter and Akratanakul, 2006; Kwadha *et al.*, 2017). Furthermore, no environmental-friendly control methods have been reported for *G. mellonella*, so far. Whereas research in OBPs, ORs, and other olfactory proteins, such as ionotropic receptors (IRs), gustatory receptors (GRs) and chemosensory proteins (CSPs) have been made in *G. mellonella* from transcriptome (Zhao *et al.*, 2019; Lizana *et al.*, 2020), no ODEs have been studied in-depth. Therefore, the understanding of semiochemicals degradation mechanisms by AOXs in antennae of *G. mellonella* would provide the necessary information to corroborate or reject the use of these enzymes as targets through their inhibition and, subsequently, disruption of chemical communication for insect pest controlling. Here, we report the phylogenetic relation, relative expression and enzymatic activity of two novel AOXs-encoding genes, *GmelAOX2* and *GmelAOX3*, from the antennae of *G. mellonella*.

4.2. Materials and Methods

4.2.1. Insect rearing

Wild *G. mellonella* were obtained from honeycombs located at Quepe sector of La Araucanía region and their rearing was established according to the methodology reported by Zamorano (2009), using a diet based on a mixture of sugar in freshly boiled distilled water, to which glycerin and vitamins were added. The food source was based

on cereals Nestum® and wheat germ, both mixed in the proportions suggested by the same author. Foster boxes (plastic) were arranged with a rectangular top window, covered by a mesh bonded with silicone and stored in a growth chamber (ShelLab) at a temperature of 28 ± 1°C. Once eggs are obtained from moths, they were disposed in control tape towards the diet, facilitating its feeding and larval development. Larvae were individualized in plastic pots until adult stage.

4.2.2. Collection of honeycombs volatiles

Volatiles were trapped by using four sterilized borosilicate-glass chambers (Vernon *et al.*, 1977). Pieces of 5 x 5 cm of honeycomb were introduce and placed at the bottom of the chambers. In two upper outlets (per chamber) were located Porapak-Q (Divinylbenzene/Ethyl vinyl benzene) columns (100 mg). A positive/negative pressure air system was used according to Agelopoulos *et al.* (1999), and the air was dried and purified before pass through the glass chamber. The trapping of volatile compounds was carried out for a period of 24 hours. Then, compounds were desorbed from the Porapak-Q column with 1 mL of hexane and concentrated up to 50 µL under a nitrogen (N_2) flow (Parra *et al.*, 2009).

4.2.3. Honeycombs volatiles identification by GC/MS

The volatile compounds (1 µL) were analyzed using a gas chromatograph (Thermo Scientific Trace 1300) coupled to a mass spectrometer (GC-MS) (Thermo Scientific ISQ 7000) equipped with a HP-5 (5% cross-linked phenyl-methyl siloxane) capillary column (30 m, 0.25 mm, 0.25 µm). Helium (He) was used as the gas carrier, with a flow rate of 1 mL/min. Mass spectrum acquisition was performed in the mass range from 30 to 500 m/z. Ionization was performed by electron impact at 70 eV with an ion

source at 250 °C. The GC oven was programmed to remain from 40 °C for 2 min and increased at 4°C/min to 250 °C and held for 5 min. The temperature of GC injector, transfer line and detector were at 250 °C (Parra *et al.*, 2009). Tentative structural assignments were made by comparing their mass spectra with the MS library (NIST), as well as by comparison of their Kovats indices by using the *n*-alkanes (C_9-C_{21}) and (C_{21}-C_{40}) series with Kovats indices published in literature and the injection of standards (Sigma Aldrich, St. Louis, MO).

4.2.4. Identification of AOXs transcripts by comparing two transcriptome data of G. mellonella

The AOXs identification was assisted by using whole head (head plus antennae) transcriptome for *G. mellonella* (Lizana *et al.*, 2020) assembled in our laboratory, and the antennal transcriptome assembled by Zhao *et al.* (2019). Firstly, an in-house database of lepidopteran AOXs created with sequences reported in literature was used in order to make a local BLAST through makeblastdb script for nucleotide and protein with the data set obtained from the assembled transcriptomes. Subsequently, transcripts were identified by local searches using the Tools of NCBI BLASTx and BLASTn (Altschul *et al.*, 1997) between our database and the assembled data set of *G. mellonella*. BLAST hits with e-values <1.0E-5 were considered to be significant (Anderson & Brass, 1998), and genes were assigned to each contig based on the BLAST hits with the highest score value. The open reading frame (ORF) of each unigene was determined by using the ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder) and sequences with a number of amino acids (aa) >1000 were selected. These sequences were used as a database in order to compare them with the transcriptome assembled by Zhao *et al.* (2019) and the identification was carried out as mentioned above. In addition, InterPro platform was used to evaluate their

gene ontology (GO), and sequences were submitted to Expasy to calculate their molecular weight *in silico*.

4.2.5. Phylogenetic Analysis of G. mellonella AOXs

A phylogeny was built for the identified AOX transcripts in *G. mellonella*, including sequences identified in a previous study on AOXs Lepidoptera (Godoy *et al.*, 2022) and other insects were used. Full-length amino acid sequences that include conserved domains were aligned using MAFFT server7 (Katoh *et al.*, 2019). GUIDANCE2 server8 was used to check consistency of the multiple sequence alignment (Sela *et al.*, 2015). Briefly, the consistency of the alignment was measured with a score less than 0.5, in which sequences were deleted. It is worth noting that confidence scores near 1 and 0, suggest a highly and poorly consistent alignment, respectively. Finally, phylogenetic analysis was performed using maximum- likelihood method with FastTree software (Price *et al.*, 2010). To highlight clades, specific taxa and functional evidence, the phylogenetic tree was edited using FigTree software9 and image editor Inkscape 0.48 software.

4.2.6. Total RNA extraction, cDNA synthesis and primer design

Total RNA extraction was performed following the methodology proposed by Gu *et al.* (2015), using different tissue samples (antennae, n=100; legs, n=50; wings, n=50; body without legs, wings and heads, n=10) from males and females was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). Moreover, the RNA concentration was analyzed using a Quantus Fluorometer (Promega). The RNA integrity was checked by 1% agarose gel electrophoresis and samples were stored at -80°C until use. From the RNA samples, through the semi-quantitative RT-PCR technique and using a thermocycler

(GeneTechnologies), a stock of cDNA was generated at a concentration of 100 ng/µL for each tissue. AffinityScript qPCR cDNA Synthesis Kit (Stratagene, Cedar Creek, TX, USA) was used by following the manufacturer's instructions. Finally, primers used for every AOX transcript were designed using PrimerQuest® program (IDT, Coralville, Iowa, USA).

4.2.7. Analysis of tissue distribution by semi-quantitative RT-PCR

Amplification of GmelAOXs in several tissues was carried out following the reported by Gu *et al.* (2015) and Lizana *et al.* (2020) and the PCR mix is mentioned in Supplementary Information Table S1. Housekeeping gene, *β*-actin (Accession code *KP331524*), was used as endogenous gene and positive control for the analysis. PCR program for *β*-actin was performed under the following conditions: an initial denaturation step of 95 ºC for 2 minutes, followed by 35 cycles of: 1) denaturation step of 95 ºC for 30 seconds, 2) annealing step of 48 ºC for 30 seconds, 3) extension of 72 ºC for 1 minute, and a final extension for 10 min at 72 ºC. PCR products were be analyzed on 1% agarose gel and visualized after staining with SYBR™. PCR program for AOXs consisted of: an initial denaturation step of 95 ºC for 3 minutes, followed by 35 cycles of: 1) denaturation step of 95 ºC for 30 seconds, 2) annealing step of 50 ºC for 30 seconds, 3) extension of 72 ºC for 1 minute, and a final extension for 10 min at 72 ºC.

4.2.8. Analysis of GmelAOXs relative expression by qRT-PCR

All qRT-PCR reactions were performed using Brilliant II SYBR Green qPCR Master mix in a qPCR-compatible equipment. The following cycling conditions were used, being 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 30 s, 57 ºC for 30 s, and 72 ºC for 1 min. The presence of specific amplified PCR product was verified for each

reaction by melt curve analysis, 95 °C for 15 s, 55 °C for 1 min and 95 °C for 15 s. The specific primers used in this study were designed using PrimerQuest® program (IDT, Coralville, Iowa, USA) and their efficiencies (ranging from 90 to 110%) validated by standard curve with five 10× serial dilutions of antennal cDNA. Housekeeping gene, *β*-actin, was used as internal control. All the experiments were performed using three biological replicates, each with three technical replicates. The relative quantification was analyzed by the $2^{-\Delta\Delta Ct}$ based on Pffafl method (Pfaffl, 2001). Statistical differences were evaluated by t-test using the SPSS Statistics 22. The semi-quantitative PCR and qRT-PCR primers are listed in Supporting Information Table S2.

4.2.9. AOX activity from antennal extract

Enzymatic activity was performed using different aldehyde substrates according to Wang *et al.* (2021). Thiazolyl blue tetrazolium bromide (MTT) was used as the electron acceptor and phenazine methosulfate (PMS) was the electron donor. Then, the enzymatic activity was measured according to the purple insoluble MTT formazan formation. Protein concentration was measured by the Bradford method using bovine serum albumin as a quantitative standard. The reaction contained: fresh crude antennal extracts (75 µg), 3 mM aldehyde substrate dissolved in DMSO, 0.1 M potassium phosphate buffer (pH 8.0), 0.4 mM MTT, 0.1 mM PMS. Then, it was incubated at 30 °C for 1 h, and the reaction was quenched with 10% acetic acid. The reaction was determined at 570 nm using a spectrophotometer UV-5100B (Metash Instruments, Shanghai). All experiments were performed in triplicate.

4.3. Results

4.3.1. Volatile organic compounds in honeycombs

A total of 74 VOCs were identified from infested honeycombs. Table 1 lists the profile of volatiles according to retention time and their structure class. For instance, terpenes, such as α-pinene, camphene, β-pinene, α-terpinene, limonene, γ-terpinene, linalool, 3-terpineol, limonene-1,2-diol, solongifolene, (*E*)-thujopsene, α-caryophyllene, β-ionone, (+)-β-selinene, α-muurolene, β-bisabolene, α-cadinol, (*Z*,*Z*)-farnesol, murgantiol and sclarene, were identified. Likewise, aldehyde-type compounds were found, including octanal, nonanal, (*E*)-2-nonenal, undecanal, (*Z*)-2-dodecenal, α-sinensal, (*Z*)-10-hexadecenal and (*E*,*E*,*Z*,*Z*)-4,6,11,13-hexadecatetraenal. In addition, several esters were identified, such as hexyl acetate, ethyl phenylacetate, *n*-octyl acetate, linalyl butyrate, isobornyl butyrate, octyl tiglate, methyl cycloundecanecarboxylate, isobutyl decanoate, ethyl tetradecanoate, 3-hexenyl-(*Z*)-cinnamate, 10-undecenyl angelate, ethyl-(*Z*)-7-hexadecenoate, (*Z*)-4-hexadecenyl acetate, incensole acetate, methyl-(*Z*)-communate, 2-ethylhexyl-(*E*)-4-methoxycinnamate and integerrimine. Other types of compounds were identified, such as alcohols, ketones, ethers, alkanes, furans, carboxylic acids and phenols.

4.3.2. AOXs-related transcripts obtained by comparing two transcriptomes

According to the results from the comparison of the whole head transcriptome assembled in our laboratory and the antennal transcriptome reported by Zhao *et al.* (2019), it was possible to identify 2 AOX transcripts. From BLASTp analysis, sequences with unigenes DN3568 and DN34847 were matched with two aldehyde oxidases (Accession code *QPF77599.1* and *QPF77600.1*) of *G. mellonella*, which were retrieved from the NCBI database with an identity percentage of 99.13% and 99.18%, respectively. These

sequences were annotated as *GmelAOX2* and *GmelAOX3*, and can be found in Supplementary Information Table S3. Thus, *GmelAOXs* and *GmelAOX3* performed an ORF of 3816 nucleotides (nt) and 3675 nt, respectively. In addition, both sequences presented the three typical domain of AOXs, 1) two 2Fe-2S clusters, 2) FAD-binding and 3) molybdenum cofactor (Moco) binding, based on a search in InterPro platform. On the other hand, the results of the theoretical molecular weight in these enzymes according Expasy platform showed that GmelAOX2 and GmelAOX3 have subunits of 141 and 134 kDa, respectively. According to phylogenetic analysis (Figure 1), only GmelAOX2 have a linage related to the clade with ODE function, as well as being closely associated to AtraAOX2 from the navel orangeworm *Amyelois transitella*, with high bootstrap value (>70%).

Table 1. Beehive volatile components identified by GC-MS.

Compound	Compound classification	Source	Source Reference	Retention Time (RT)	K_{exp}	K_{ref}	Identification Reference
α-pinene	Terpene	Honey	Radovic *et al.* (2001)	10,63	935	922	Standard*
camphene	Terpene	Propolis	Pino *et al.* (2006)	11,10	951	953	Choi (2003)
β-pinene	Terpene	Propolis, Honeybees	Pino *et al.* (2006); Torto *et al.* (2005)	11,85	974	962	Standard*
α-terpinene	Terpene	Honey	Panseri *et al.* (2013)	13,22	1018	1018	Adams (1998)
limonene	Terpene	Propolis, Wax	Pino *et al.* (2006); Ferber and Nursten (1977)	13,42	1025	1024	Standard*; Adams (1998)
γ-terpinene	Terpene	Honey	Panseri *et al.* (2013)	14,54	1062	1062	Adams (1998)
linalool	Terpene	Honey	Pattamayutanon *et al.* (2017)	15,46	1090	1088	Standard*
3-terpineol	Terpene	Propolis	Pino *et al.* (2006)	16,61	1130	1130	Karioti *et al.* (2003)
limonene-1,2-diol	Terpene	Honey	Kaskoniene and Venskutonis (2010)	22,00	1321	1321	Hamm *et al.* (2005)

isolongifolene	Terpene	-	-	23,71	1387	1387	Adams (1998)
(*E*)-thujopsene	Terpene	-	-	24,71	1426	1426	Skaltsa *et al.* (2003)
α-caryophyllene	Terpene	Propolis	Pino *et al.* (2006)	25,38	1454	1454	Adams (1998)
β-ionone	Terpene	-	-	26,16	1485	1485	Standard*; Adams (1998)
(+)-β-selinene	Terpene	-	-	26,34	1492	1492	De Kraker *et al.* (2003)
α-muurolene	Terpene	Propolis	Pino *et al.* (2006)	26,53	1499	1499	Karioti *et al.* (2003)
β-bisabolene	Terpene	Propolis	Pino *et al.* (2006)	26,76	1509	1509	Adams (1998)
α-cadinol	Terpene	Propolis	Pino *et al.* (2006)	30,00	1652	1652	Tellez *et al.* (1999)
***(Z,Z)*-farnesol**	Terpene	Honeybees	Schmitt *et al.* (2007)	30,84	1689	1689	Apel *et al.* (2004)
murgantiol	Terpene	-	-	31,98	1743	1743	Zahn *et al.* (2008)
sclarene	Terpene	-	-	36,57	1967	1967	Adams (1998)
octanal	Aldehyde	Honey, Honeybees, Wax	Radovic *et al.* (2001); Torto *et al.* (2005); Ferber and Nursten (1977)	12,69	999	999	Zehentbauer and Reineccius. (2002)

nonanal	Aldehyde	Honeybees, *G. mellonella* pheromone, Wax	Schmitt *et al.* (2007), Kwadha *et al.* (2017); Ferber and Nursten (1977)	15,27	1085	1084	Standard*
(*E*)-2-nonenal	Aldehyde	Propolis	Mohtar *et al.* (2017)	17,26	1153	1155	Zehentbauer and Reineccius (2002)
undecanal	Aldehyde	Honeybees, *G. mellonella* pheromone	Schmitt *et al.* (2007), Kwadha *et al.* (2017)	21,00	1284	1284	Standard*
(*Z*)-2-dodecenal	Aldehyde	-	-	25,70	1467	1467	Marques *et al.* (2000)
α-sinensal	Aldehyde	Honey	Castro-Vázquez *et al.* (2007)	32,16	1752	1752	Adams (1998)
(*Z*)-10-Hexadecenal	Aldehyde	-	-	33,27	1804	1804	Marques *et al.* (2000)
(*E,E,Z,Z*)-4,6,11,13-hexadecatetraenal	Aldehyde	-	-	35,78	1926	1926	Gago *et al.* (2013)
hexyl acetate	Ester	Honeybees	Torto *et al.* (2005)	13,06	1012	1014	Jordan *et al.* (2003)
ethyl phenylacetate	Ester	Honey	Pattamayutanon *et al.* (2017)	19,87	1244	1244	Jordán *et al.* (2003)

n-octyl acetate	Ester	Honeybees venom	Abd El-Wahed *et al.* (2018)	20,00	1249	1248	Sotomayor *et al.* (2004)
linalyl butyrate	Ester	-	-	24,60	1422	1422	Adams (1998)
isobornyl butyrate	Ester	-	-	25,91	1475	1475	Tepe *et al.* (2004)
octyl tiglate	Ester	-	-	27,14	1526	1526	Adams and Dev (2010)
methyl cycloundecanecarboxylate	Ester	-	-	27,37	1536	1536	Ali *et al.* (2008)
isobutyl decanoate	Ester	Pollen	Lima-Neto *et al.* (2017)	27,58	1545	1545	Demyttenaere *et al.* (2003)
ethyl tetradecanoate	Ester	Honey	Pattamyutanon *et al.* (2017)	31,79	1734	1734	El-Sayed *et al.* (2005)
3-hexenyl-(*Z*)-cinnamate	Ester	-	-	35,21	1897	1897	Wang *et al.* (2006)
10-undecenyl angelate	Ester	-	-	32,57	1771	1771	Adams and Dev (2010)
ethyl-(*Z*)-7-hexadecenoate	Ester	-	-	36,79	1978	1978	McDaniel *et al.* (1992)
(*Z*)-4-hexadecenyl acetate	Ester	-	-	36,96	1987	1987	Marques *et al.* (2000)
incensole acetate	Ester	-	-	40,44	2189	2149	Hamm *et al.* (2005)
methyl-(*Z*)-communate	Ester	-	-	41,09	2228	2228	Adams (1998)

2-ethylhexyl-(*E*)-4-methoxycinnamate	Ester	-	-	42,94	2339	2339	De Simon *et al.* (2009)
integerrimine	Ester	-	-	43,97	2402	2402	Witte *et al.* (1993)
2-nonanol	Alcohol	Honeybees venom	Abd El-Wahed *et al.* (2018)	15,65	1096	1098	Adams (1998)
cinnamyl alcohol	Alcohol	Pollen	Lima-Neto *et al.* (2017)	21,87	1316	1312	Choi (2003)
undecanol	Alcohol	Honeybees	Degrandi-Hoffman *et al.* (2004)	24,22	1406	1402	Standard*
2,3-dimethoxybenzyl alcohol	Alcohol	-	-	25,25	1449	1449	Zhao *et al.* (2008)
guaia-3,10(14)-dien-11-ol	Alcohol	-	-	30,64	1680	1680	Morteza-Semnani *et al.* (2006)
2,6-di-tert-butyl-4-ethylphenol	Alcohol	-	-	32,33	1760	1760	Gómez *et al.* (1993)
(*Z*)-11-eicosen-1-ol	Alcohol	Honeybees, Honeybees venom	Schmitt *et al.* (2007); Abd El-Wahed *et al.* (2018)	41,48	2251	2250	McDaniel *et al.* (1992)
1-eicosanol	Alcohol	Honeybees	Suwannapong *et al.* (2011)	41,92	2277	2274	Standard*
2-acetylpyridine	Ketone	-	-	13,73	1035	1035	Leffingwell and Alford (2005)

artemisia ketone	Ketone	-	-	14,65	1065	1063	Tellez *et al.* (1999)
1,3-indandione	Ketone	-	-	22,96	1358	1358	Rostad *et al.* (1986)
1,6-dioxaspirodecane	Ether	-	-	14,17	1050	1050	Jaramillo *et al.* (2013)
2-methyl-coumaran	Ether	-	-	21,63	1306	1306	Harrison *et al.* 2009
decane	Alkane	Honeybees, Wax	Graham *et al.* (2009); Ferber and Nursten (1977)	12,75	1001	1001	Adams (1998)
(*E*)-decahydronaphthalene	Alkane	-	-	14,38	1057	1057	Adams (1998)
1,1-bi(cyclopentyl)	Alkane	-	-	15,15	1081	1081	Lai *et al.* (1995)
dodecane	Alkane	Pollen, Wax	Lima-Neto *et al.* (2017); Ferber and Nursten (1977)	18,69	1200	1199	Adams (1998)
tridecane	Alkane	Pollen, Wax	Neto *et al.* (2017); Ferber and Nursten (1977)	21,45	1299	1299	Adams (1998)
tetradecane	Alkane	Honey, Wax	Pattamayutanon *et al.* (2017); Ferber and Nursten (1977)	24,06	1399	1399	Adams (1998)
hexadecane	Alkane	Honey, Honeybees, Wax	Schmitt *et al.* (2007); Pattamayutanon *et al.*	28,85	1599	1600	Adams (1998)

			(2017); Ferber and Nursten (1977)				
heptadecane	Alkane	Honeybees, Wax	Schmitt *et al.* (2007); Ferber and Nursten (1977)	31,07	1699	1700	Adams (1998)
2-methyl-1-octadecene	Alkene	-	-	35,07	1890	1890	Zaikin and Borisov (2002)
5-methyldocosane	Alkane	-	-	41,49	2252	2252	Zaikin and Borisov (2002)
pentacosane	Alkane	Honeybees, Pollen	Schmitt *et al.* (2007); Lima-Neto *et al.* (2017)	45,43	2489	2485	Standard*
heptacosane	Alkane	Honeybees	Schmitt *et al.* (2007)	47,6	2598	2597	Standard*
5-methylfurfural	Furan	Honey, Pollen	Pattamayutanon *et al.* (2017); Panseri *et al.* (2013)	10,91	945	945	Jordán *et al.* (2002)
(*Z*)-linalool oxide	Furan	Honey, Pollen	Pattamayutanon *et al.* (2017); Panseri *et al.* (2013)	14,73	1068	1070	Choi (2003)
tetradecanoic acid	Carboxylic acid	Honey	Karabagias *et al.* (2019)	32,77	1781	1780	Pino *et al.* (2005)
***p*-chlorophenol**	Phenol	-	-	27,80	1555	1555	Kim and Kim, (2000)

RT= Retention time obtained from the mass spectrums; K_{exp}= Kovats determined by using the n-alkane series (C9-C18 and C21-C40); K_{ref}= Kovats based on the injection of Standards (*) and search in literature by the comparison with GC-MS spectrometry library. (-) = Non reference on source of the tentative compounds.

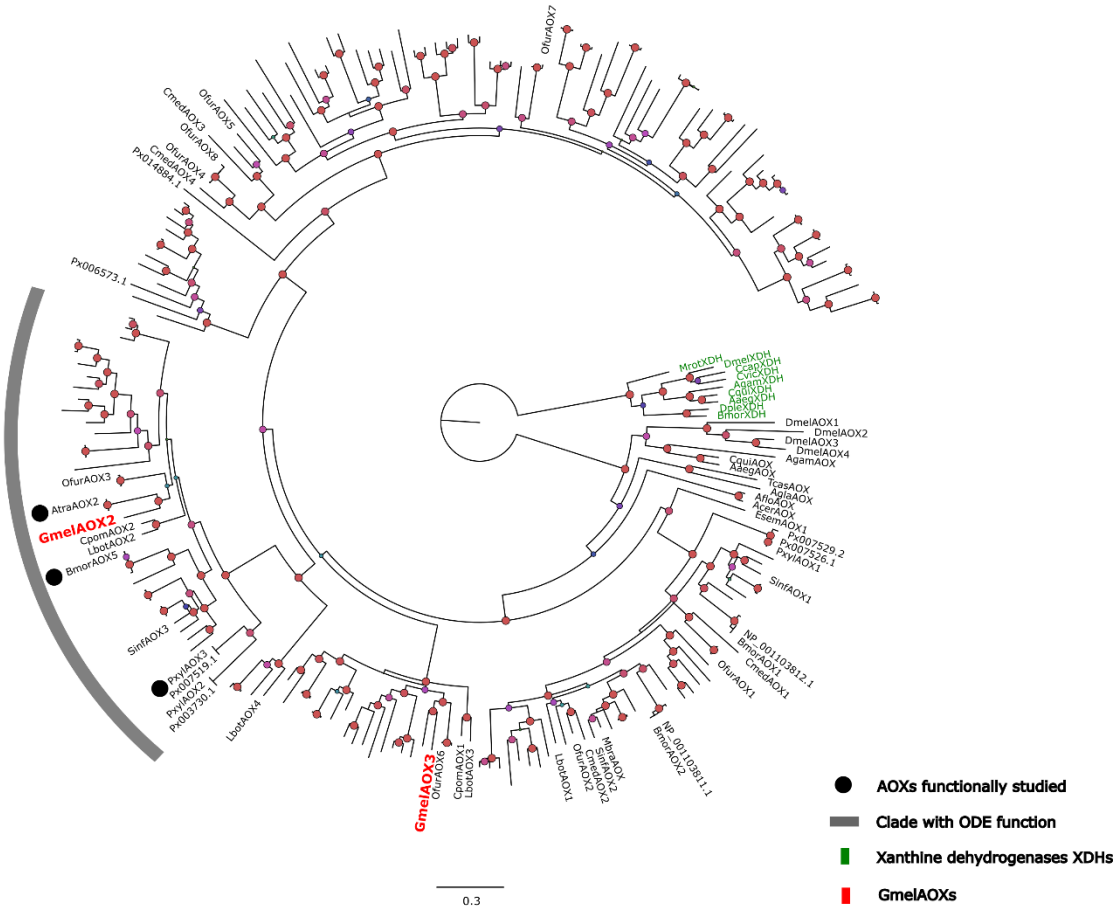

Figure 1. Phylogenetic tree of AOXs identified in *G. mellonella* transcriptomes as well as sequences from a previous report in lepidopteran (Godoy *et al.*, 2022). Black circles show functional AOX reported in *A. transitella* (AtraAOX2), *P. xylostella* (PxylAOX3) and *B. mori* (BmorAOX5). Grey line shows AOXs clustered in the clade with ODE function. In green are the xanthine dehydrogenases (XDHs) and in red are GmelAOXs.

4.3.3. Tissue distribution and relative expression levels of GmelAOX2 and GmelAOX3

To confirm the AOXs identification and clarify their tissue distribution in both sexes, semi-quantitative PCR was performed (Figure 2). Our results suggest that enzymes do not have a tissue-specific expression. Where *GmelAOX2* and *GmelAOX3* are

distributed in male body, with the latter also expressed in female body. Besides body, PCR products of both enzymes were presented in male rather than female antennae. According to qRT-PCR results, there were significant differences in relative expression of *GmelAOXs* in antennae from both sexes (Figure 3). The *GmelAOX2* showed a higher expression in males (6-fold) rather than females (1-fold). Similarly, *GmelAOX3* showed a higher expression in male antennae (2-fold) compared with female antennae (1-fold). The amplification of the housekeeping gene *β*-actin, where all samples (antennae, legs, wings and bodies) amplified is shown in Supplementary Information Figure S1.

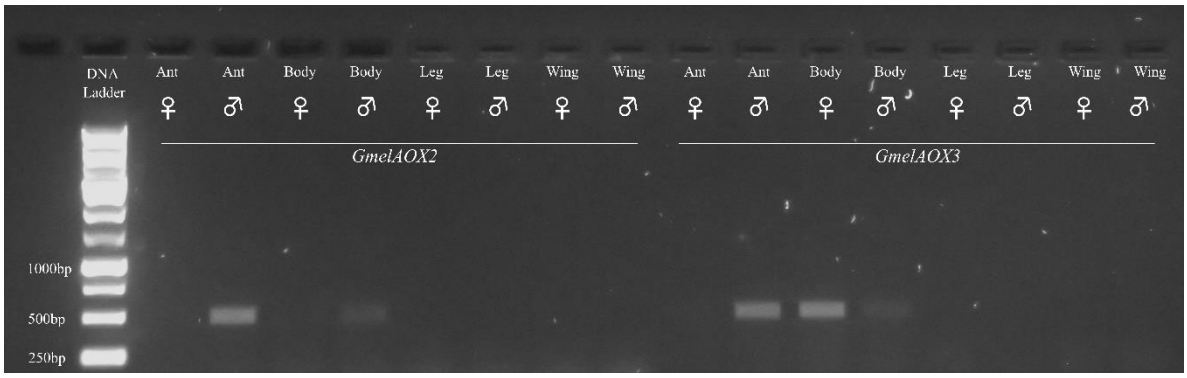

Figure 2. *Galleria mellonella* aldehyde oxidases (AOXs) transcript levels in different tissues tested by semiquantitative RT-PCR. All reactions were run under the same experimental conditions. Ant., antennae; Body (without head, wings and legs); Legs; Wings.

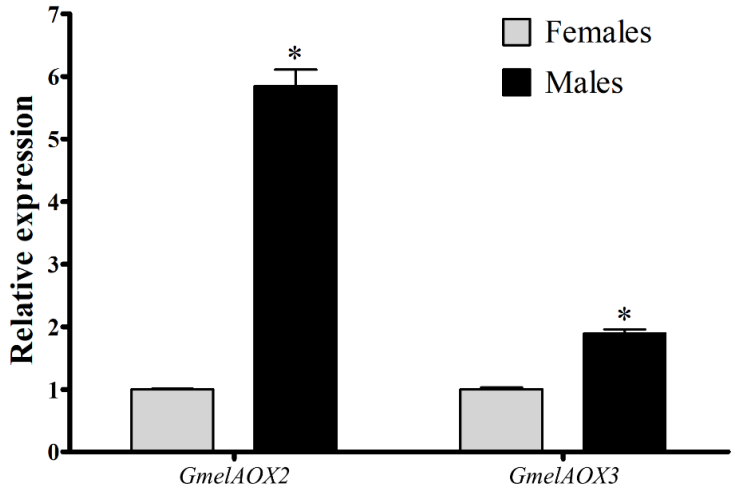

Figure 3. *Galleria mellonella* aldehyde oxidases (AOXs) transcript levels in antennae females and males tested by qRT-PCR. Grey and black bars indicate ♀ and ♂ respectively. Statistical differences are shown by asterisks (*, $p < 0.05$). Standard error is represented by error bars.

4.3.4. AOXs activity from antennal extract

In order to evaluate the enzymatic activity of GmelAOXs in antennal extracts, some aldehydes of the previously identified from honeycombs, as well as pheromone components, were selected. It is worth noting that some aldehydes were not commercially available, and other structurally similar compounds were included, such as *trans*-2-hexenal, hexenal and decanal. Also, undecane was include as the corresponding alkane of undecanal. The activity was presented as relative activity (%), where benzaldehyde was used as standard (100%) according to Choo *et al.* (2013). In Figure 4, shows the results obtained, where undecanal performed the highest activity, followed by decanal, undecane, nonanal, hexanal, *trans*-2-hexanal and octanal. Noteworthy, undecane showed the third highest activity even without presenting an aldehyde in its structure. GmelAOX2 could play a more active role in the degradation of aldehyde-type compounds compared

to GmelAOX3, due to its phylogenetic relationship with AtraAOX2 from the pyralid moth *A. transitella*.

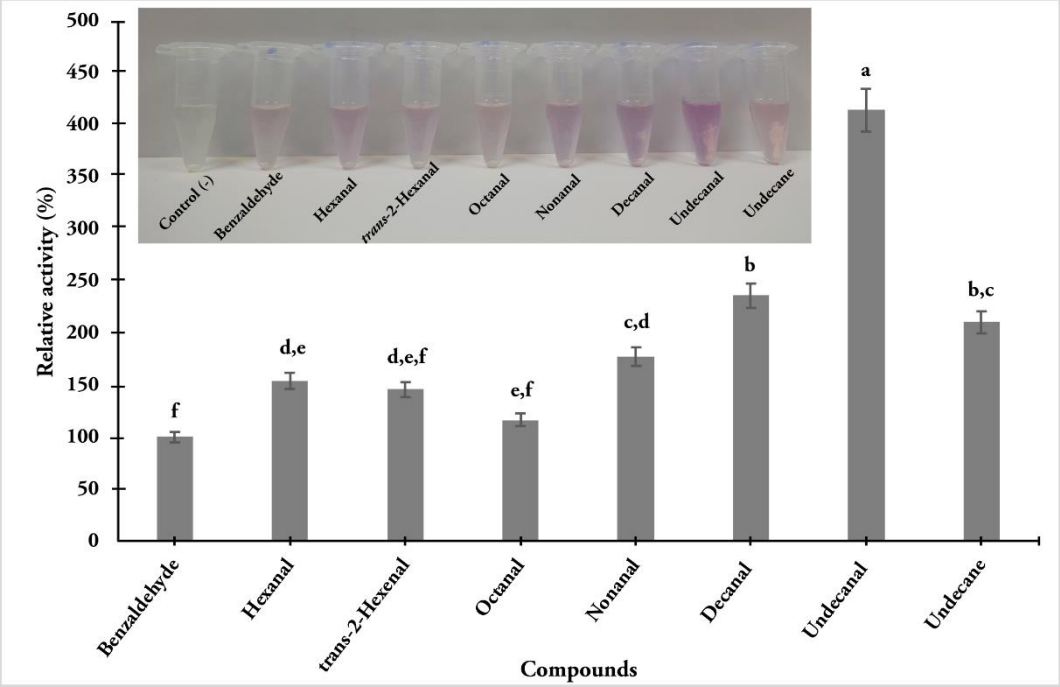

Figure 4. Relative activity of substrates oxidation by antennal extracts of G. mellonella. The oxidation of substrates (3 mM) by antennal extract (75 µg) was determined at 30 °C and quenched with 10% acetic acid. The reduction of MTT was measured spectrophotometrically at 570 nm. Activity is observed according to the purple insoluble MTT formazan formation. A blank was set as a negative control by adding buffer only. ANOVA followed by Tukey test for multiple comparisons for an average mean comparison. Different letters indicate significant differences ($p < 0.05$).

4.4. Discussion

Here, we identified several compounds in honeycombs which were infested with *G. mellonella*. Among these chemicals, nonanal and undecanal are reported as part of its

sex pheromone blend (Flint and Merkle, 1983; Kwadha *et al.*, 2017). Likewise, other aldehydes have been associated with bees and their products, for instance, octanal from honey and wax (Ferber and Nursten 1977; Radovic *et al.*, 2001; Torto *et al.*, 2005), (*E*)-2-nonenal with propolis (Mohtar *et al.*, 2017) and α-sinensal with honey (Castro-Vázquez *et al.*, 2007). On the other hand, (*Z*)-2-dodecenal is a volatile found in clementine oil (Chisholm *et al.*, 2003), (*Z*)-10-hexadecenal has been reported as pheromone in some lepidopteran of the Crambidae family (Boo, 1998; El-Sayed *et al.*, 2013), and (*E*,*E*,*Z*,*Z*)-4,6,11,13-hexadecatetraenal has been identified as the major sex pheromone of the promethea moth, *Callosamia promethea* (Gago *et al.*, 2013).

The *G. mellonella* is a ubiquitous pest to honeycombs and a nocturnal insect which lay their eggs inside honeycombs, especially when bees are less active (Nielsen and Brister, 1977). Moreover, when adults emerge from pupa stage they fly toward the trees to mate. So, this moth must rely on its olfactory system for detecting and decoding semiochemicals, and thus define its behavior. The clearance of compounds that remains in the sensillar lymph is a critical step in the olfaction process, which is carried out by ODEs, in order to lead the entrance of new stimuli in the antennae (Leal, 2013). Research around ODEs is limited, where the most studied have been reported in Lepidoptera, namely the antennal esterase SlCX7 in *Spodoptera littoralis*, that where capable to hydrolyze the pheromone and plant compounds (Durand *et al.*, 2011). In addition, He *et al.* (2015) identified an ODE gene from the polyphagous moth, *S. exigua SexiCXE10* which performed high activity for ester plant volatiles. In terms of AOXs, studies are even scarcer, where bioinformatics analyses have served to identify putative enzymes, for instance, four AOXs were reported in *C. medinalis*, three mainly found in adult abdomen and one enriched in antennae (Zhang *et al.*, 2017). Another authors identified three AOXs in *Sesamia inferens*, two were antennae-specific (*SinfAOX1* and *SinfAOX2*) and one

(*SinfAOX3*) expressed in antennae and abdomen (Zhang *et al.*, 2014). As well as, the phylogenetic relationships of these enzymes have allowed the clustering of AOXs with ODE function (Godoy *et al.*, 2022). Phylogenetic analysis showed that both *GmelAOX2* and *GmelAOX3* have evolved from xanthine dehydrogenases (XDHs) (Fig.1), similarly to other reported AOXs (Huang *et al.*, 2016; Zhang *et al.*, 2017), due to gene duplication events (Kurosaki *et al.*, 2013). Remarkably, *GmelAOX2* was grouped in the clade with ODE function, where other functionally studied AOXs (Choo *et al.*, 2013; Zhang *et al.*, 2020; Wang *et al.*, 2021) are found, shedding lights of its role as ODE.

As a result of semi-quantitative RT-PCR, tissue-specific expression was not performed because of *GmelAOX2* was expressed in body and antennae of males, and *GmelAOX3* was expressed in body of both sexes and male antennae only. However, we suggest that at least *GmelAOX2* have a sex-biased expression in male antennae according to RT-PCR, despite its slight expression in body. The expression of these enzymes in body might be associated to a detoxification process, due to the degradation of xenobiotic compounds, such as pesticides (Huang *et al.*, 2016). In fact, AOXs are capable to use several compounds as substrates, i.e. N-heterocyclics, N-oxides, azo dyes and aldehydes with different hydrocarbon chain size (Coleman *et al.*, 2002). In contrast to the expression of AOXs in the body, their expression in antennae would be involved in the degradation of sex pheromone components or aldehyde-type plant volatile, being potentially classified as pheromone degrading enzymes (PDEs) (Xu and Liao, 2017). Huang *et al.* (2016) showed an expression of two AOXs in *C. pomonella* (*CpomAXO1* and *CpomAOX2*) in several tissues (antennae, thoraxes, abdomens, legs and wings), but they propose a role in odorant degradation when these enzymes are expressed in antennae even if they are not specific to this tissue. Insect antennae are the main olfactory organs, therefore the expression of AOXs in these structures would be closely related in the degradation

process of semiochemicals (Xu and Liao, 2017). Thus, studies in *S. inferens* and *C. medinalis* have shown significant differences in the relative expression of these AOXs (*SinfAOX1*, *SinfAOX2* and *CmedAOX2*) according to qRT-PCR, where male antennae showed a higher expression (Zhang *et al.*, 2014; Zhang *et al.*, 2017). Here, *GmelAOX2* and *GmelAOX3* showed a higher expression in males (Figure 3). Interestingly, it is common for conspecific females to synthesize and release the sex pheromone to attract males (Ando *et al.*, 2004), however, in *G. mellonella* it is the male that produces the sex pheromone (Kwadha *et al.*, 2017). Therefore, a higher expression of AOXs in male antennae could help to rapidly degrade aldehyde-type compounds that are part of the sex pheromone blend of *G. mellonella* allowing the entrance of another chemical cues. Noteworthy, low mRNA expression does not always imply low protein levels or low enzyme activities (Newman *et al.*, 2006). Preliminarily, these results indicate that both aldehyde oxidases in *G. mellonella* could be classified as general odorant degrading enzymes (GODEs) instead of PDEs.

The AOXs function in insects towards semiochemicals degradation has been recently studied from pheromone glands extracts of *B. mori* (BmorAOX5), where they were active over several aldehydes (i.e., benzaldehyde, salicylaldehyde, vanillic aldehyde, heptanal and propanal) (Zhang *et al.*, 2020). Furthermore, Wang *et al.* (2021), evaluated the activity of a recombinant AOX (PxylAOX3) from the diamondblack moth *Plutella xylostella*, where it was capable of degrade its sex pheromone components and several aldehyde-type volatiles derived from plants. Although our efforts to express GmelAOXs in a bacterial system were unsuccessful (data not shown), antennal extracts were capable of metabolize aldehyde-type compounds, such as some of the identified from infested honeycombs. Data published by Leyrer and Monroe (1973) indicate that *G. mellonella* sex pheromone blend has two major components, nonanal and undecanal in a

ratio of 7:3. With this into account, the strongest activity on undecanal (4-fold) compare to benzaldehyde (control) in antennal extracts, is consistent. Despite nonanal is in major proportion than undecanal, 1.7-fold higher enzyme activity compared with control, was obtained. Also, hexanal, octanal and decanal, reported as minor sex pheromone components of *G. mellonella* (Kwadha *et al.*, 2017), performed more activity than control. Studies show that aldehyde-type VOCs have insecticide activity against some dipterans, such as hexanal and octanal as toxic compounds to *Drosophila sechellia* and *D. melanogaster*, respectively (Legal *et al.*, 1999). In addition, decanal has shown toxicity against the scarab beetle *Tribolium castaneum* (Tian *et al.*, 2022) as well as nonanal against *S. frugiperda* (Flores-Macías *et al.*, 2021). Undecanal was also reported as repellent against mosquito *Anopheles gambiae* (Omolo *et al.*, 2021). The degradation of these compounds by GmelAOXs shed light on the key role in the olfaction process of this insect. The fact that males present a higher expression of AOXs supports a metabolization of their own pheromone components. The antennal extracts also shown activity over *trans*-2-hexenal, a compound which also have been reported as insecticide on *T. castaneum* (Cui *et al.*, 2021). Although undecane was surprisingly active in antennal extracts, it is likely that other enzymes are present in the antennae, i.e. monooxygenases (Wojtasek and Leal, 1999; Scott and Wen, 2001) that are capable of transform the alkane to a corresponding alcohol (undecanol), and then it is dehydrogenated to the corresponding aldehyde (undecanal) by alcohol dehydrogenases (Zhang *et al.*, 2014; Huang *et al.*, 2016). Bearing this in mind, this study shows that AOXs presented in antennal extracts were able to metabolize several compounds. On the other hand, a few studies have shown that antennal esterases are specialized in the degradation of pheromones acting as PDEs, for instance, ApolPDE from *A. polyphemus* had the greater activity against its sex pheromone (Vogt and Riddiford, 1981). As well as, PjapPDE from

P. japonica performed a substrate preference for the sex pheromone rather than its antagonist (Ishida and Leal, 2005). Here, we demonstrated that the antenna extracts showed significantly higher activity against the undecanal compound (sex pheromone) compared to the other aldehydes tested in the enzymatic assay. Therefore, GmelAOX2 could be associated with a PDE role due to its phylogenetic relationship with ODEs that have a pheromone-biased degradation according our results.

In conclusion, we have demonstrated that two novel AOXs transcripts identify here are expressed *G. mellonella*, where *GmelAOX2* has a sex-biased expression. These enzymes have a crucial role for metabolizing sex pheromone compounds as well as plant-derived aldehydes, which are related to honeycombs and the life cycle of *G. mellonella*. Thus, to control this moth it is necessary search antagonists able to inhibit these enzymes, in order to incorporate new strategies on insect pest management.

4.5. Acknowledgments

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Chapter V

Concluding remarks and Further perspectives

5.1. Concluding remarks

Aldeyde oxidases (AOXs)-encoding genes of *G. mellonella* transcriptomes were identified and the activity of antennal extract against aldehyde-type compounds from its sex pheromone as well as those present in honeycombs were evaluated. Thus, two AOXs were identified (*GmelAOX2* and *GmelAOX3*) with no sex- and tissue-specific expression. Besides, *GmelAOX2* performed the highest relative expression levels in male antennae rather than female. According to volatiles released from honeycombs, different types of compounds were found, i.e., esters, alcohols and aldehydes. Therefore, the repertoire of enzymes might be acting in the matabolization of these compounds, in order to "reset" the antennae in insects.

G. mellonella has a reverse sexual communication because males synthesize and relase sex pheromone compounds to attrack females. Although both sexes are capable of express *GmelAOX2* and *GmelAOX3*, we demonstrated that these enzymes have a higher expression in males antennae. Likewise, aldehyde-type compounds have been reported to have insecticidal activity, males might increase the levels of AOXs in order to detoxify their olfactory system. We suggest that *GmelAOX2* have a sex-biased expression in males according to semiquantitative RT-PCR and qRT-PCR. Even though both AOXs appear to have evolved from xanthine dehydrogenases, *GmelAOX2* is associated to a linage with odorant degrading function, in fact it is closely related to the functional studied enzyme *AtraAOX2* from *A. transitella*.

Here, antennal extracts show a strong enzymatic activity against sex pheromone components, specially to undecanal compared to benzaldehyde used as control. This supports the role as ODEs, being able to degrade both sex pheromone components as well as potential toxic compounds. Finally, *G. mellonella* represent a model organism to study new strategies related to insect pest control. First, this moth is easily reared in captivity

with up to four generation annualy. Second, its semiochemistry is relatively known, where aldehyde are the main components of its sex pheromone. Third, sex pheromone antagonist compounds could be used as inhibitors of AOXs or others in the repertoire of proteins involved in the olfactory system, such as OBPs or ORs. Finally, our research and the reported literature suggest that bioinformatic, phylogeny, protein structure and enzymatic analysis can help in the search of new useful semiocemicals for pest controlling.

5.2. Further perspectives

The reproductive success in many insect species is based on the olfactory system. Fortunately, the semiochemicals compounds involved in host recognition, mating, or defense behaviors are being used to manage insect pests through environmentally friendly approaches, e.g., mating disruption and mass trapping. Nevertheless, globalization has facilitated the dissemination of insect species throughout the world. Therefore, it is crutial to understand how insects adapt to new environments and more specifically, how their olfactory system responds to these new conditions. To answer these questions, it is important to study the molecular basis and mechanisms involved in insect olfaction where proteins are the main players. Enzymes are novel targets for the use of species-specific chemicals in integrated pest management.

In apiculture, *G. mellonella* represent a ubiquitous insect pest and its control is mainly based in chemical methods. On the other hand, highlights as a model organism to study new strategies to control and manage insect pests. Through bioinformatics approaches, such as genomics and transcriptomics, several genes and transcripts related to olfaction have been identified. Besides, chemical ecology has allowed the understanding of the relationship between semiochemicals and *G. mellonella*. More

studies are certainly necessary to in detail unravelling the mechanisms involved in the olfaction processes. Likewise, sex pheromone components antagonists could be used as control agents in order to reduce environmental impacts caused by chemical pesticides. The recombinant production of these enzymes would be used to test new compounds, evaluating their affinity and binding capacity to certain volatile compounds associated with the behavior and life cycle of *G. mellonella*.

CHAPTER VI

Appendices

Table S1. Setup reaction for semiquantitative RT-PCR.

Semiquantitaive RT-PCR setup	25 µL reaction
Master Mix (Takara)	13 µL
Primer forward (10 µM)	0.5 µL
Primer reverse (10 µM)	0.5 µL
cDNA (100 ng/µL)	1 µL
H_2O (Molecular biology)	10 µL

Table S1. Setup reaction for qRT-PCR.

qRT-PCR setup	10 µL reaction
Brilliant II SYBR Green qPCR Master mix	5 µL
Primer forward (10 µM)	0.5 µL
Primer reverse (10 µM)	0.5 µL
cDNA (10 ng/µL)	1 µL
H_2O (Molecular biology)	3 µL

Table S2. Primer sequences for semiquantitative RT-PCR and qRT-PCR

PCR	Name	Sense	Sequence
Semiquantitative RT-PCR	AOX2	Forward	CCGCGCCGGATAAAGATAAA
	AOX2	Reverse	CCAGCCGCAATCGCTATAA
Semiquantitative RT-PCR	AOX3	Forward	CGTGGCGAACAAAGTGAAAG
	AOX3	Reverse	TCCACGCCCACTTCATAATC
qRT-PCR	AOX2	Forward	GCCGGTATAACTCTCACAGAA
	AOX2	Reverse	AGAAACAAGTCCGAGGGAAAG
qRT-PCR	AOX3	Forward	GGTCAAGGCGTGTATCCAATTA
	AOX3	Reverse	TGAGAGTTATACCGGCTCCTATC

Table S3. Transcriptome annotation from *G. mellonella*.

Species	Unigene code	Aminoacid sequence	Sequence length aa	Sequence lenght nt	ORF
Galleria mellonella	DN3568	MTCIEFTVNGKKCSVDVRTPRDMTLNAYLRYVLALPGTKAMCHEGGCGTC SVSVRARRETTGNTETFSVNSCLVLVFSCHGWDITTIEGVGNRHDGYSDI QNRFKAFNATQCGYCTPGWIMNLYSLQDKHLTTAELEKSFGSNTCRCTGY RPILDVIKSYAVDASPELCQRVKDIEDLKICDKNFRNCQRKCSTYSVGSD WSLLENSLPRTEKTIVLDFGKKKMYKVFDEEDIFDIFNKYGVESYQLVDG NTAKGIYETYEYPRVLIDISNVKSLKGYQFDQNLIIGANTSLEECRQIFN DVAHSNDDFSYLSEFAKHFESIAHIPVRNIGSLAGNLMLKHSMPSFPSDV FLLLSAVGAVVTVKNSNGQRTNLYMTHFLKYNMQGVLMLSISLPPLGASN IFKSYKIMPRNQNAVAIVNAAFLINMSSDNKTIKEATIVFGNISTEFIYA KHTEDYLRNKNCFDNKVLQGAIKELHDEICPEENDFYSKDIRKKLAIGLF YKFALSIAPSSIVDARYRSGGELLQRPISHGTQDFQTDSSLYPLNQPLPK LEALLQSSGEAQFVNDIPPFPLEVFGAFVLSTIHVGQIDSIDTTDVLKID GVLAVYTAKDIPGANSFIRPGVQLESENEEILVSSNVKYYGQPLAIVVAT SQELAANVANKVKVTYKNISFEAPVTTIDRAKKDSKRYVPSDESIDPKGR GSNVTKTIKGLYEVGTQFHYYMEPISCVVVPVEDGLEVYESTQWMDLSQI AIAQCLDIKESQILMKVRRCGGAFGGKISRNVQAATACALVAHKLTLPCR FILPMQTNMSIVGGRFPTQCDYEVGVDDDGKIQYLQATVVQDQGYSINDS VISYTAGGFPNCYNSDYMSVKFASVFTDLPCNTFMRAPGTSEGIICIENI MEHIAYGVQKDATDVRLTNMRKEDNDLPQLIDILKKEADYDERKKSIQDY NKSNRWIKKAIHISPMIFPVEYYGNYSAMVSIYRGDGTVTITTGGIEMGQ GLNTKAAQVCAHELNIPLRYISVLPSMSFVAANNVFSGSSITSESVCYSI IKACEILNKRLEPVKQKLTNPTWEQIAWKAGEDLVDLTAKYMMTDQEKDL SNYSAFGVAILETQLDVLTGRYELLRADILEDVGLSANPTIDVGQLEGGY IQGLGYFTTEKMVYDEHTGKKLSNRSLTYHVPLALDIPADFRVKFRYNSK NHKGVLGSKTVGEMGICTAYGVTHALRRCIMESRKESGYDPNEWINIDVP YTPESVLKALAVKLEEFVFKP	1271	3816	Complete

Galleria mellonella	DN34847	MCREGGCGACIVAVSQTHPDTKETHVFSVNSCLVHILSCHNWDITTIEGI GNKKNGYHAIQTRLAAFNGTQCGYCTPGWIMNMYSIDKRSNEKLSMRQIE DSFGSNMCRCTGYRSILDAFKSFASDLEPELKNKVQDLEDLHKLGCRNTC ERRCSALDEEWCIINKKSDTLLAVGGDGSRWYKAFTIKDVFKILSKEGVD SYRFVAGNTGQGVYPITTEPRVLIDISSIAAIKGTWTDGNLVIGAGMTLT EVMNEFQKWANENEDFAYLNEFYKHLSLVAHIPVRNIGTIAGNLSLKNKH NNFPSDLFLIFATVEAAITIVNDKLDKQCIDFQEFLKTDLTNKLITEVKL PPLPTTCLIKTYKIMARAQNVHAIVNAGFLFKLDTSNKIVSTNIVYGSMS PTFVNAVQTERALKDSQLYNEETLQKALSVLNTELSPDVDPPEASPQCRK TIALALFYKALLSLCPNVNPRYKSGGTELERELSKGTQTFDTDKSIWPLN KPVPKLEALSQCSGEARYSCDVNPGPRTVHVAFVLSDVSVAEIDRFDPSE ALKVSGVIAFLTAKDIPGKNTFTPTNVPWQEVDEEILASNKVLYYGQPVG LIVAISHNLAVSAAKLVKVYYKNNKAKPVLTIQDALTAPDKDKRIRKEVT TKATDRGQDIKQVIKGALSIPSQYHYTMETQSCTVIPTENGVEVRSATQW IDLIHVAVANMLSVQQNKVEVIVNRVGGAYGGKASRSSLIACAAALAATA CGRAASLVLPIDTNMAAIGKRQECLVEYELGVNNSGVIQYLNVSYYSDCG WSYNDTAGSAIASVLANLYDSSRWTITGYSVLTDKASNTWCRAPGTTEAI AIHEHLMERIAYATKLDPVDVRIANIAEKHSGIKDMIATLKYSSNYDTRK TEIATYNSENAWKKKGLKLSIMSFPIEYSWNFPVTVSVYHGDGTVAISQG GIEMGQGINTKIAQVCAYTLKIPLEKVSVIGSNSFVSPNAMCSNGSITSD CVAYATLRACKELLNRFEDIKENTNEPWEETVKKAFEKGINLQASYMTSP LDSLQCYDVYGVCAIEVELDVLTGTHVVKRVDLLEDAGISLSPDIDVGQI EGAFIMGLGLWTTEQLVYDQKTGRLLTDRTWTYHPPGAKDIPVDFRITLQ PNAPNPAGVLRSKATGEPALTLAVGVTFALHDAILDARKEFGYKDTEWLS VDVPYSVENILKAISPNYEYYKLY	1224	3675	Complete

Table S3. Transcriptome annotation from *G. mellonella*. *Continuation.*

Species	BLASTp best hit	Max Score	Total Score	Query Cover	E-value	Identity	Accession	2Fe-2S	FAD-binding	MoCo
Galleria mellonella	aldehyde oxidase 2 [Galleria mellonella]	2604	2604	100%	0.0	99.13%	QPF77599.1	yes	yes	yes
Galleria mellonella	aldehyde oxidase 3 [Galleria mellonella]	2471	2471	100%	0.0	99.18%	QPF77600.1	yes	yes	yes