Evolution of sexual signals

Within and between species variation in a dual function sex-pheromone component in two noctuid moths

Fruitet, E.

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General Discussion

In this thesis, I focused on variation in acetate esters (hereafter referred to as acetates) in female *Heliothis subflexa*. These acetates serve a dual function by attracting conspecific *H. subflexa* males, while repelling heterospecific *H. virescens*. Both noctuid moths produce sex pheromone blends with the same components except for three acetates ((Z)-7-hexadecenyl acetate, (Z)-9-hexadecenyl acetate and (Z)-11-hexadecenyl acetate), which are absent from *H. virescens* female sex pheromone blend.

Geographic and temporal variation in the relative amount of acetates has been observed in natural populations of *H. subflexa*, and interestingly this variation correlates with the presence of the conspecific *H. virescens* (Groot et al., 2009b) – with higher amounts of acetates found in populations that co-occur with *H. virescens*. When both species co-occur high acetate levels may be selected to avoid cross-attraction. However, when *H. virescens* are absent lower acetate levels are observed which suggests that high acetate levels may be costly and are thus counter-selected when the risk of cross-attraction is low.

The aims of this thesis were *to i) identify genes underlying acetate variation both between H. subflexa and H. virescens and among H. subflexa populations* and *ii) characterize evolutionary potential of acetate variation in H. subflexa as well as the selective forces that drove this variation*. To do so, I used gene editing, artificial selection, and life history trade-off analysis to investigate the mechanism involved in both between and within species variation in acetate levels. This integrated approach has allowed me to shed light on both the causes and consequences of sexual signal variation while also contributing to our understanding of the speciation process.

To comprehend how both within and between species variation in acetate levels evolved, I first focused on the genetic basis of this variation. I investigated the genetic architecture that underlies *inter*specific variation in Chapter 2 and *intra*specific variation in Chapter 3 and Chapter 4.

Quantitative trait locus (QTL) analyses are commonly used to identify the location of one or multiple loci that affect a quantitative trait (such as weight, height or acetate levels in our case). Previously, by crossing of *H. subflexa* and *H. virescens* and mapping AFLP marker-based on the backcross families, two QTLs were identified as involved in acetate variation between both species (Groot et al., 2009a). Interestingly, one of these QTLs was also responsible for intra-specific variation in acetate levels in *H. subflexa* (Groot et al., 2013). We investigated specific genes within these QTLs and found two esterases and two lipases responsible for interspecific variation in the acetate levels (Chapter 2) and an esterase which increased the acetate levels in *H. subflexa* (Chapter 3).

Furthermore, since the acetates are part of a multicomponent blend, I wondered how selection on one component would affect the rest of the blend. To do so, we explored the evolutionary response of the *H. subflexa* sex pheromone blend to artificial selection on acetate levels (Chapter 4). In addition, in Chapter 5, I investigated if intraspecific variation in the acetate levels can be explained by condition-dependent costs associated with the composition of the blend.

Below I summarize our findings on the origin of both within and between species variation in acetate levels (**part 1 and 2**), speculate on evolutionary scenarios that could explain acetate levels variation (**part 3**) and suggest how future research could further investigate sex-pheromone evolution in general (**part 4**).

I. Genetic architecture that underlies acetate variation

Studies from the early 1980s revealed that the presence of acetates in the sex-pheromone was likely produced by oxidation of a precursor alcohol with the corresponding chain length (number of C atoms) and saturation (number of double bonds) (see Figure 1;

reviewed in (Jurenka and Roelofs, 1989; Morse and Meighen, 1987a). Genes controlling expression of acetyl transferases, which are the enzymes responsible for acetate synthesis, seemed the perfect candidates. Hence, it was assumed that differential expression or presence of specific acetyl transferase in the sex pheromone gland would explain variation in acetate levels in the sex pheromone blend. However, despite more than 25 years of research, no genes encoding the acetyl transferase that synthesize acetates in the sex pheromone blend have been identified in any moth species (Ding and Löfstedt, 2015; Groot et al., 2013). This is particularly surprising, because acetates are among the most common type I sex pheromone compounds in moths (Byers, 2006).

Nevertheless, the quantity of acetates released in the pheromone blend can be explained not only by creation of acetate by esterification of the alcohol, but also by degradation of acetate back to the alcohol through hydrolysis (see Figure 1). Indeed, enzymatic studies in the 1980s suggested that variation in the presence/absence or quantity of acetates between closely related species could be explained by increased/reduced acetate degradation by esterase(s) (Morse and Meighen, 1986, 1990). As pointed out by Foster et al. (2018), pheromone compounds are produced and broken-down again, the amount of pheromone released seems not to be limited by a costly synthesis of the compounds but rather by a (relatively) important degradation mechanism.

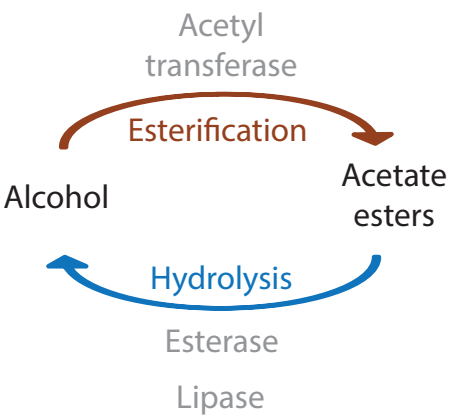

Figure 1: Acetate biosynthesis.
The enzymes involved in the different reactions are indicated in grey.

Previously, backcrosses of *H. virescens* and *H. subflexa* F1 hybrids into *H. subflexa* revealed two QTLs that together explained 53% of the variation in acetate levels between the species (Groot et al., 2009a; Sheck et al., 2006). The *H. virescens* allele at one of this QTL, thus coding for no/low acetate levels, was crossed into a *H. subflexa* genetic background which resulted in a so-called introgression line. Females from this introgression line produced acetates but in much lower amount in comparison to wild type female *H. subflexa*.

Is the absence of acetates in *H. virescens* explained by an increased acetate degradation?

In Chapter 2, we searched for candidate genes in the QTL that could explain interspecific variation in acetates between *H. subflexa* and *H. virescens*. We compared gene expression levels in the pheromone gland of the introgression line and in wild type *H. subflexa* and *H. virescens*. By doing so, we found two lipases and one esterase over-expressed in the introgression line and in *H. virescens*.

Since both lipases and esterases can degrade acetates, we investigated if the over-expression of those enzymes could explain the lower acetate levels found in the introgression line. Therefore, we used CRISPR/Cas9 gene editing to knock out the lipases and esterases. As hypothesized, deactivation of the targeted enzymes resulted in increased acetate levels. Hence, the lower amount of acetates found in the introgression line was due to increased acetate degradation.

These results found in Chapter 2 are in line with the findings from Teal and Tumlinson (1987) who applied acetates onto both *H. subflexa* and *H. virescens* pheromone glands, and found that acetates were degraded in both species, but much quicker in *H. virescens* compared to *H. subflexa* (Teal and Tumlinson, 1987). This

faster acetate degradation rate found in *H. virescens* suggests that there is increased hydrolysis activity in *H. virescens* pheromone glands compared to those of *H. subflexa*. However, even though we demonstrated that an increased hydrolysis significantly affected the acetate levels (Chapter 2), we still don't know if this mechanism alone is sufficient to explain the total absence of acetates in the *H. virescens* pheromone blend.

Although no acetates have ever been detected in the *H. virescens* pheromone gland, acetates were found on female legs and on the male hairpencils (Zweerus et al., 2023). Similar observations were made in *Helicoverpa armigera*: acetates were found on the female legs and the male hairpencils, despite the pheromone gland of females of this species not containing acetates (Zweerus et al., 2023). Since the absence of acetates in the pheromone gland seems tissue- and sex-specific, genomic comparison is uninformative and the identification of the genes responsible for acetates production may be more difficult than previously thought.

If species that do not have acetates in their pheromone blend are in fact producing those components in other tissues, these species must thus possess one or more genes encoding acetyltransferase activity. We can thus hypothesize that acetates are ubiquitously produced but may be degraded in the sex-pheromone gland. One explanation for the production of acetates followed by its degradation in the pheromone gland could be that the enzyme(s) that produce acetates cannot be under-expressed in the sex pheromone gland because they are involved in other physiological pathways.

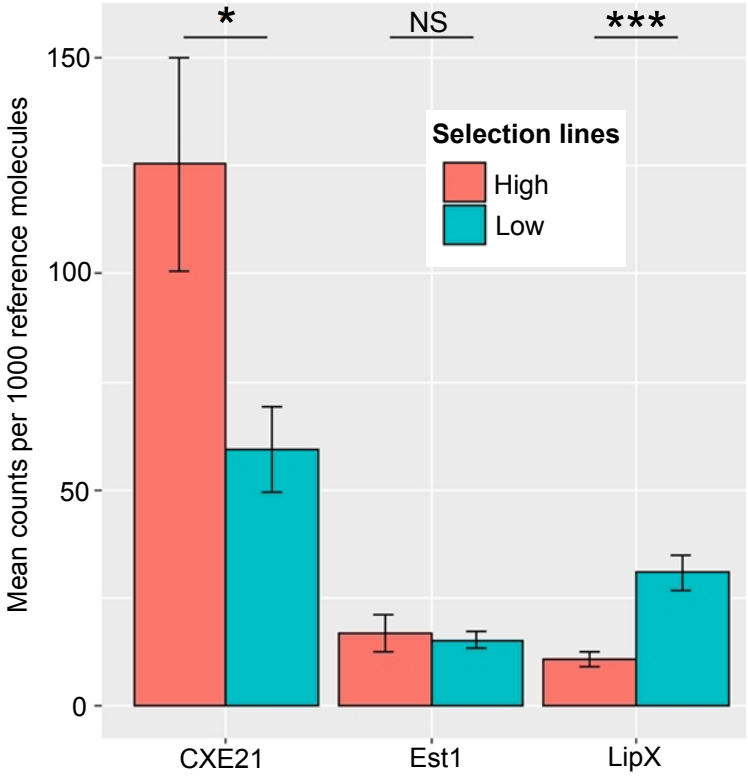

Figure 2: A Boxplot showing the mean target per 1000 reference molecules for each gene.

*Red: High producing acetate selection line, Blue: Low acetate producing selection line. * P < 0.05; *** P < 0.001. Figure copied from* (Vaskimo, 2022)

Does acetate degradation also play a role in intra-specific variation in acetate levels?

Four enzymes, two esterases and two lipases, were shown to affect the interspecific variation in the acetate levels (see Chapter 2). Thus, I hypothesized that these enzymes are also involved in acetate variation among *H. subflexa* populations. Since we showed in Chapter 2 that one of the two lipases, LipZ was not expressed in the *H. subflexa* pheromone gland, therefore, this enzyme should not be involved in intra-specific variation in acetate levels.

During my PhD project, we created two *H. subflexa* selection lines for high and low levels of acetates (as described in Chapter 4). In these lines we compared the expression levels of the previously identified genes between the two selection lines at generation nine through qPCR (Vaskimo, 2022). The result of this qPCR revealed that LipX was overexpressed in the Low acetate line compared to the High acetate line, while no significant difference in expression level was found for Est1 (Figure 2). Unfortunately, differences in expression level between the Low and High acetate selection lines were not performed for Est2, thus I cannot speculate about its involvement in intra-specific variation in acetate levels.

In conclusion, LipX appears to be involved in both between and within species variation, while Est1 and LipZ seem to only be responsible for inter-specific variation. Regulation of acetate levels through hydrolysis thus seems to be important for both intra- and inter-specific variation, but the enzymes responsible for this reaction are not always shared.

Esterases can degrade acetates, but may also produce acetates

The esterases that we identified Chapter 2 are located on QTL Chromosome 20 and involved in acetates degradation, which is the expected role of esterases. In Chapter 3 I describe another esterase that we discovered on QTL Chromosome 28 with an apparent opposite effect; when CXE21 is expressed, the acetate levels in the pheromone blend increase instead of decrease.

In line with this finding, a qPCR performed on *H. subflexa* selection lines demonstrated an overexpression of CXE21 in the High acetate line compared to the Low acetate line (Figure 2, Vaskimo, 2022). This additional result confirms that increased acetate levels correlate with an over-expression of CXE21, while lower acetate levels are associated with an under-expression of CXE21. One explanation for this opposite-to-expected result is that CXE21 esterase is synthesizing acetates instead of degrading them.

Although these results seem counter-intuitive at first, enzymes are usually able to conduct reversible enzymatic activities and these activities depend on the environmental conditions. Hence, enzymes with the same name can be responsible for opposite reactions. When water is present, esterases preferably degrade acetates into alcohol, but in hydrophobic environments esterases can synthesize acetates (Bornscheuer and Kazlauskas, 2005). Therefore, exclusion of water that would otherwise drive acetates degradation is essential to allow the production of acetates.

Since sex pheromone biosynthesis occurs in the sex pheromone glands, it is possible that the expression of specific esterases in different parts of the gland would allow for opposite reactions. For example, reactions may occur in the gland membrane (Teal and Tumlinson, 1987) which could provide a suitable non-aqueous environment and drive acetate synthesis, while acetate degradation would be favored inside the gland. Thus, variation in the

expression of esterases within the gland itself could permit a fine-tuning of the acetate levels in the pheromone blend.

Is CXE21 also involved in between species variation in acetate levels?

Since CXE21 is present on the QTL of chromosome 28, that was shown to be involved in acetate variation at both within and between species level (Groot et al., 2013), I wondered what role this enzyme could play in *H. virescens*. The coding sequence of CXE21 seems too similar between the two species to presume that this enzyme may bear a different function in *H. virescens* than it does in *H. subflexa*. However, differences in gene regulation rather than in the coding sequence may cause phenotypic differences, as has been found in many cases.

For example, in *Drosophila* cuticular hydrocarbons act as sex-pheromone and play an important role during courtship behavior. It has been demonstrated that the presence of diene hydrocarbons in *Drosophila melanogaster* and *D. sechellia* females was caused by the expression of desatF gene (Chertemps et al., 2006). In *D. simulans* females which produce only monoene and no diene hydrocarbon, desatF gene is present but not expressed (Chertemps et al., 2006).

In addition, variation in the gene expression allow differential expression of sexual signals between sexes (Williams and Carroll, 2009). To go back to our previous example, desatF gene is expressed in both females *D. melanogaster* and *D. sechellia*, but not in the males of those two species who produce only monoene hydrocarbons (Chertemps et al., 2006). Similarly, *H. virescens* males do produce acetates in their pheromone blend while acetates are absent from the female sex-pheromone blend. Hence, we can assume that although *H. virescens* females do not produce acetates, gene(s) coding for acetyl-transferase activity should be present but may be repressed in *H. virescens* females.

In Chapter 3 we showed that CXE21 is in a cluster with two other esterases, and this same cluster seems conserved in Noctuidae. Hence, I hypothesized that the CXE21 enzyme may be repressed in species that do not produce acetates in their pheromone blend. However, it seems that CXE21 is expressed in the pheromone gland and other tissues (such as the leg, thorax and male hairpencil) of at least two noctuid species that do not produce acetates: *H. armigera* and *H. virescens* (Zweerus et al., 2023). Further investigation would be needed to determine if the expression level of this esterase correlates with the presence/absence of acetates in the pheromone blend of more Noctuidae species.

Acetate levels can evolve independently from the other pheromone components

Sex pheromones are usually composed of multiple components that are likely to be genetically interdependent. Hence, to understand how sex pheromone signals evolve it is important to understand how selection on one component will affect the others. To answer this question, we artificially selected for high and low acetate levels in *H. subflexa* during 10 generations. Chapter 4 describes how the pheromone blend responded to this selection.

We found that acetates could respond to selection independently from the other pheromone components and that this was likely facilitated by changes in the genetic covariance structure. The genetic covariance structure is a statistical description of genetic correlations between traits, such as the different components of the pheromone blend. These genetic correlations are important, because if trait A is genetically correlated to trait B, trait B will experience indirect selection effects as a consequence of direct selection on trait A. Although often interpreted as stable, these correlations can vary over time and space (Eroukhmanoff, 2009).

In the selection lines, we observed that genetic variation in the acetates that were under selection became dissociated from genetic variation in the other components. Specifically, selection first seemed to break the genetic relationship between the acetates and the other pheromone components, resulting in changed acetate levels in subsequent generations without associated changes in the rest of the pheromone blend.

These findings correlate with the results obtained in Chapter 3, since the expression/repression of CXE21 affected the acetate levels, but not any of the other pheromone components. Thus, acetates can evolve without indirect selection responses in other sex pheromone components.

II. Costs associated with high acetate levels

Since organisms do not have access to unlimited resources, trade-offs between different traits can occur, which can result in intra-specific variation in sexual signals depending on how much resources are allocated to sexual signal expression (Selander, 1965). Investment in sexual signals may vary in function of the individual or environmental conditions (Iglesias-Carrasco et al., 2016; Jennions et al., 2001). As previously mentioned, the acetate levels of *H. subflexa* sex pheromone blend vary both geographically and seasonally; high acetate levels are found when *H. virescens* are present while when the chances of cross-attraction are reduced, lower acetate levels are observed (Groot et al., 2009b). Hence, I hypothesized that high acetate levels are associated with fitness costs.

Sex pheromones have been thought to not be costly for a long time (Greenfield, 1981; Johansson and Jones, 2007; Steiger and Stökl, 2014), although that idea is now being re-examined in multiple systems (Chemnitz et al., 2015; Foster, 2009; Rantala et al., 2003,

Harari et al., 2011, Jaffe et al. 2007). The production of sex pheromone may not be associated with direct energetic costs such as calling in frogs or insects (Ryan, 1988). However, investment in sexual signals may vary as a function of individual condition (Jennions et al., 2001). Hence, even components that have no clear reason to be costly may still be associated with the signaler condition. Condition-dependent costs may remain hidden under idealized lab conditions but should be revealed under stressful condition (Cotton et al., 2004; Reznick, 1985).

In Chapter 5 I highlighted condition-dependent costs associated with high acetate levels. To do so, I manipulated the resources available at the larval stage by providing only 25% of the nutrients compared to standard rearing. When resources were limited, I found an increased developmental time and reduced fertility per night in *H. subflexa* females with high acetate levels than females with low acetate levels. Moreover, females with high acetate levels had a higher risk of mating failure than females with low acetate levels. These findings indicate that high acetate levels are associated with different costs.

III. Evolution of the acetate levels

Combining the results from this thesis and the literature, below I will now speculate on the possible mechanisms that have driven the evolution of the observed inter- and intra-specific variation in acetate levels from the sex pheromone blend of *H. subflexa*, *H. virescens* and other Noctuidae.

Intra-specific variation in acetate levels maintained by a balance between avoiding cross-attraction and costly acetates

Sexual signals involved in species recognition are assumed to be under strong stabilizing selection (Johansson and Jones, 2007; Löfstedt, 1993), while signals used in mate choice are supposed to be under directional selection (Johansson and Jones, 2007). Importantly, the same signal can serve both functions and may thus be shaped by a complex of selection pressures.

In other words, the evolution of insect sex-pheromones may be influenced not solely by between-species interactions (such as communication interference), but also by within-species interactions (mate choice), as well as within-individual variation (diet, age, mating status). To understand how intra-specific variation in sexual signal is maintained we first need to disentangle which selection forces are occurring and in which direction are they acting on the sexual signal.

To avoid cross attraction, high acetate levels are selected in sympatry

It was previously shown that the presence of acetates in the pheromone blend of *H. subflexa* increases the attraction of conspecific males, while repelling males of a sympatrically occurring species, *H.* virescens (Groot et al., 2007, 2006; Vickers, 2002; Vickers and Baker, 1997). Moreover, the attracting effect of acetates seems to be increased in *H. subflexa* males that live in sympatry with *H. virescens*. For example, pheromone lures with a *H. subflexa* pheromone blend that contained the three acetates attracted significantly more *H. subflexa* males in North Carolina where both species co-occur than in Mexico where *H. virescens* is absent (Groot et al., 2007). In addition, *H. subflexa* females from North Carolina produce higher acetate levels and conspecific males are

assortatively attracted to local females (Groot et al., 2009b). Finally, *H. subflexa* females produce higher acetate levels when exposed to *H. virescens* pheromone during pupation and in the first three days after eclosion, in comparison to no odour or to their own (Groot et al., 2010). Hence, since *H. subflexa* acetate levels vary in relation to the presence of *H. virescens* and conspecific males seems to prefer local females, selection against cross-attraction may have driven an increase in acetate levels when *H. subflexa* and *H. virescens* live in sympatry.

...But high acetate levels are costly

The presence of *H. virescens* both geographically and seasonally affects the acetate levels of *H. subflexa* sex pheromone blend (Groot et al., 2009b). Since *H. subflexa* have blends with lower relative amounts of acetates when the chances of cross-attraction are reduced, I hypothesized that high acetate levels are associated with fitness costs. In Chapter 5 I showed that high acetate levels bear multiple condition-dependent costs. These are costs that stay hidden in the idealized rearing condition in the laboratory, where trade-offs in the allocation of resources are weak because nutrients are not limited. Since high acetate levels are costly, females with lower acetate levels may have a selective advantage when *H. virescens* are absent. Thus, intra-specific variation in acetate levels in *H. subflexa* may be maintained by balancing selection of high and low acetate levels, depending on the presence/absence of *H. virescens* (see Figure 3)

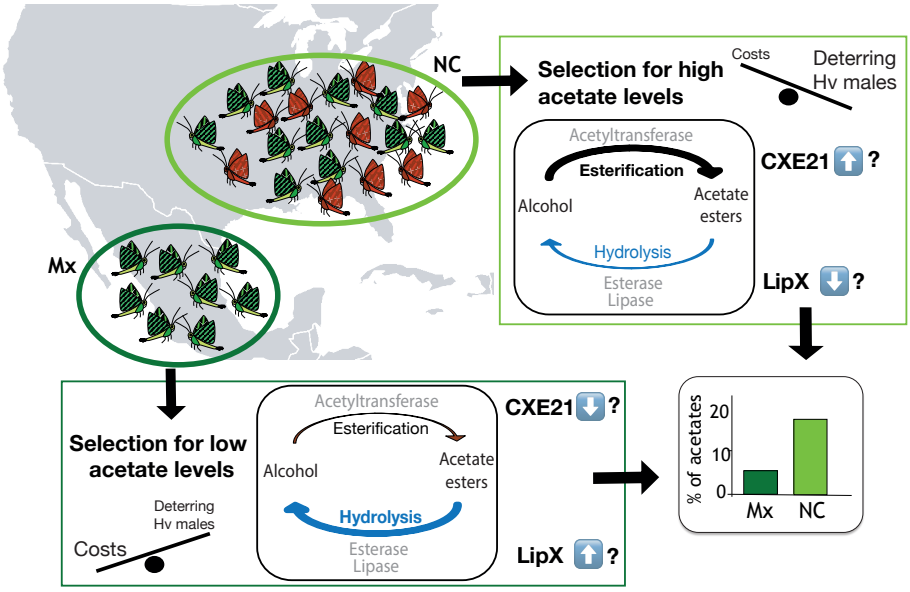

Figure 3: Hypothetical evolution of intra-specific variation in acetate levels in H. subflexa.

NC: North Carolina, Mx: Western Mexico. ↑: gene upregulated, ↓: gene downregulated. CXE21 is an esterase whose expression seems to increase the acetate levels and LipX is an lipase whose expression reduces the acetate levels.

How are acetate levels modulated in H. subflexa?

How could variation in acetate levels be accomplished? Possibly, the fine-tunning of acetate levels could be due to differential expression of enzymes involved in both synthesis and degradation of acetates. It would be interesting to investigate if the candidate genes I highlighted in this thesis are differentially expressed in *H. subflexa* populations of North Carolina and Mexico. As represented on Figure 3, I hypothesize that in the *H. subflexa* population of North Carolina which have higher acetate levels, CXE21 may be up-regulated while LipX may be down-regulated. In contrast, in Mexican populations which have lower acetate levels, CXE21 may be down-regulated and LipX up-regulated.

Variation in acetate levels between populations may be facilitated by the univariate response of acetates to selection, as highlighted in Chapter 4. Since acetate levels can be modulated without affecting the other components of the pheromone blend, acetates can easily respond to selection for either higher or lower rates without altering the rest of the blend. As any deviation from the existing pheromone blend could decrease the attraction of conspecific males, the possibility of modifying only the acetate levels without affecting the rest of the blend may have facilitated intra-specific variation in the acetate levels in this species.

Inter-specific variation explained by reinforcement of premating barriers

In general, to understand how sexual communication signals evolved, it is important to know how males respond to the pheromone blend. Changes in sexual signals may not alter the receiver's response right away, but reinforcement by selection against hybridization can favor receivers that discriminate conspecifics from heterospecifics. Therefore, establishing how variation in sexual signal affects the receiver response and in which context the variation is perceived by the receiver can give us valuable clues as to when premating barriers played a role in the speciation process.

H. virescens males are repelled by the presence of acetates unless the percentage of acetates is less than 5% of the total pheromone blend (Groot et al., 2006; Vickers and Baker, 1997). As a low percentage of acetates is not enough to deter *H. virescens* males, the presence/absence of acetates may have played a role as reinforcement of this prezygotic barrier in sympatry. To confirm this hypothesis, it would be interesting to investigate if the aversion for acetates observed in *H. virescens* males also occurs in areas where *H. subflexa* is absent.

Previously geographic variation in male responses to acetates have been found in another Noctuidae species in which acetates are absent from the female sex pheromone blend. *Helicoverpa armigera* females do not produce acetates but the presence of Z11-16:OAc in pheromone lures reduced male catch only in regions where a closely related species is present that does produce acetates : *Helicoverpa punctigera* in Australia and *Helicoverpa assulta* in China (Gao et al., 2020). This finding suggests that the absence of acetates in *H. armigera* is not always associated with a repulsion for acetates in the pheromone blend by males. Hence, avoidance of acetates by *H. armigera* males may have reinforced the pre-zygotic barriers.

The absence of acetates is a derived state

I hypothesize that the absence of acetates in female sex-pheromone blend is a derived state, based on the following arguments. First, according to a survey of Pherolist, a website that listed the pheromone blend of 1572 moth species and is now replaced by Pherobase, acetates are the most commonly used pheromone compound (Byers, 2006). Second, within the Noctuidae group most species sex-pheromone blends contain acetates, and only in a few *Helicoverpa* and *Heliothine* species acetates are absent (see Pherobase and Figure 5). Thus, the absence of acetates in the pheromone gland is an exception rather than the norm. Third, the absence of acetates in the female sex-pheromone gland of both *H. virescens* and *H. armigera* is tissue- and sex-specific, as acetates were found on legs of both males and females in these species (Zweerus et al., 2023). This finding suggests that even though females from those two species do not emit acetates in their sex pheromone blend, they carry gene(s) for acetyltransferase(s) allowing them to produce acetates. Hence, the absence of acetates from the *H. virescens* sex pheromone blend could be explained by the repression of acetyltransferase gene(s) in the sex pheromone gland and by acetate removal.

Evolution of acetate removal

To elucidate the role that acetate degradation may have play in Noctuidae sex pheromone biosynthesis, I conducted phylogenetic analyses on the genes that we identified in Chapter 2 to be involved in acetate level variation. Specifically, I built two phylogenetic trees, one for the lipases (Figure 4A) and a second for the esterases (Figure 4B), using genome and transcriptome sequences from Noctuidae.

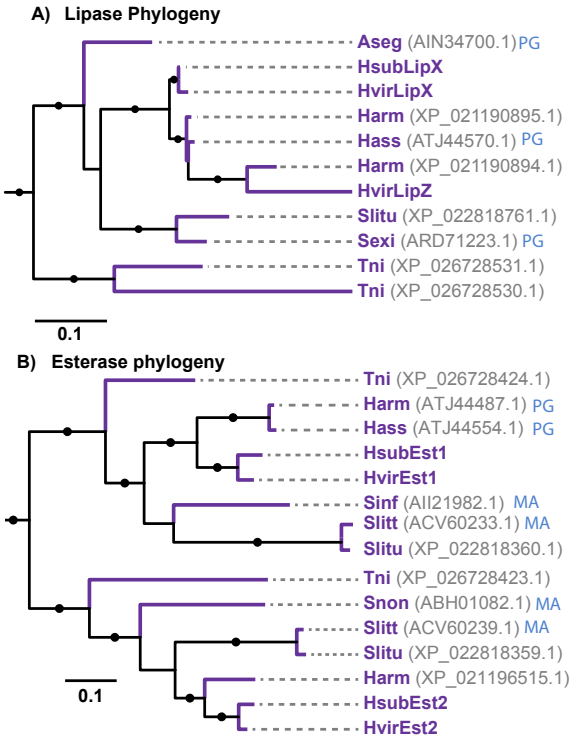

Figure 4: Maximum-likelihood phylogenies
A) Maximum-likelihood phylogeny of LipX and LipZ phylogenetic tree built from amino acid sequences of Noctuidae acidic lipases.
B) Maximum-likelihood phylogeny of Est1 and Est2 orthologues. Phylogenetic tree built from amino acid sequences of Noctuidae esterases. The scale bar represents 0.1 expected amino-acid substitutions per site Dots represent bootstrap support ≥ 85. In grey in brackets: sequence name in NCBI. In blue: the body part from which the transcriptomic sequence was collected, PG: pheromone gland and MA: male antennae.

Interestingly, the phylogenetic study of the lipases revealed that two Heliothinae moths without acetates, *H. virescens* and *H. armigera*, possess two lipases (LipX and LipZ), while in their close relatives with acetates, *H. subflexa* and *Helicoverpa assulta*, only LipX is expressed in the pheromone gland (Figure 4A, Li et al., 2017) Similarly, in two other Noctuidae that contain acetates in their pheromone blend, *Spodoptera exigua* and *Agrotis segetum*, only the

homolog of LipX is expressed. In *Spodoptera litura* whose sex pheromone blend consists only of acetates, LipX is present but not expressed in the pheromone gland (Zhang et al., 2015). Since LipX is expressed in species with acetates, it seems unlikely that LipX degrades all the acetates from the pheromone gland alone, but may participates in the degradation of acetates together with other enzymes. Differential expression of LipX could modulate the acetate levels and participate in fine-tuning the acetate levels.

Genomic data from *H. subflexa* confirmed that LipZ is present but not expressed, which suggests that a duplication event happened in a common ancestor of *H. virescens* and *H. armigera*. Gene duplication allows copies of a gene to each have their own evolutionary path or it may simply increase the total amount of gene product, in this case the acetate-hydrolizing lipases. To confirm that a duplication event happened, genomic data from *H. assulta*, *Helicoverpa zea* and *Helicoverpa gelotopoeon* would be needed. Unfortunately, only transcriptomic but no genomic data were available for *H. assulta*. Since the expression of LipZ correlates with the absence/presence of acetates in Noctuidae sex pheromone

blends, the duplication event may have allowed some Noctuidae species to degrade acetates instantly.

The phylogeny of the esterases suggest that a duplication event happened at the basal node of the Noctuidae clade (Figure 4B). Transcriptomic data from the pheromone gland of *H. assulta* and *H. armigera* revealed that only Est1 and not Est2 is expressed in their pheromone gland, although genomic data from *H. armigera* indicate that Est2 is present. Although *H. assulta* produces acetates while *H. armigera* does not, Est1 does not seem to be differentially expressed by both species (Li et al., 2017). In Chapter 2 we found higher expression of Est1 in the pheromone gland of *H. virescens* in comparison to *H. subflexa,* but this difference was not significant. Based on this finding, the expression pattern of Est1 and Est2 does not seem to correlate with the presence/absence of acetates in the sex pheromone blend of Noctuidea species. Hence, the specific role

of both esterases remains elusive at the Noctuidae level, but I suggest that these enzymes are involved in the fine tuning of acetate levels in the pheromone blend.

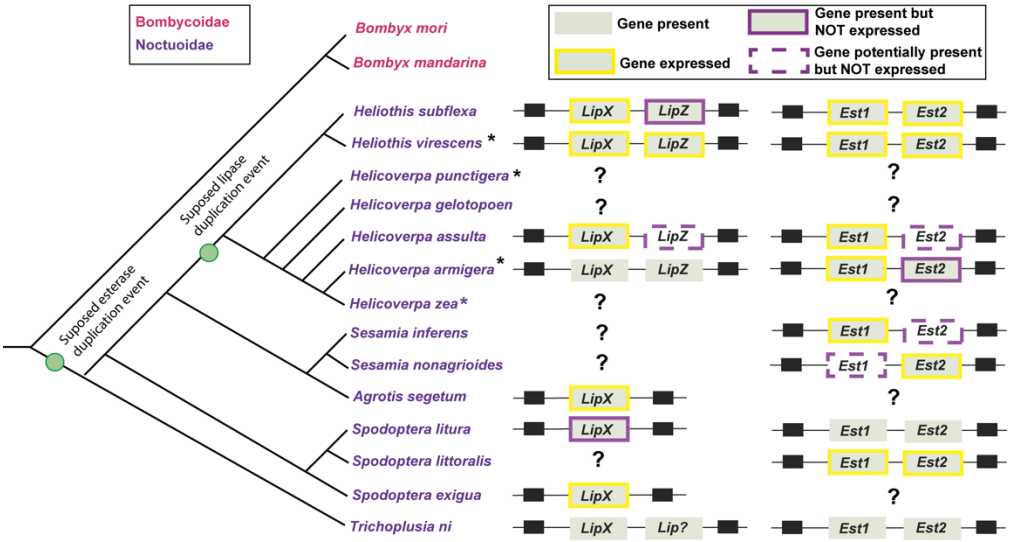

Figure 5: Hypothetical evolution of LipX, LipZ, Est1 and Est2 orthologues among Noctuidae.

Relatedness between the species studied is based on Kergoat et al., (2021) *phylogeny. In pink are superfamily Bombycoidae, in purple are Noctuoidea. * Indicates species that do not contain acetates in their female sex pheromone blend. Grey rectangles represent the presence of gene orthologues, when circled in yellow the gene is expressed while when circled in purple it is not expressed. White rectangles with a purple dotted line represent the expected presence of a gene not expressed which should be confirmed by genomic data. ?: no genomic or transcriptomic orthologue sequence were found in NCBI for that species.*

In conclusion, the pheromone blend composition may be fine-tuned by the expression/repression of enzymes involved in acetate degradation. I hypothesize that several duplication events of genes involved in the regulation of acetate degradation enzymes happened in the Noctuidae clade (see Figure 5). Based on the genomic and transcriptomic data available, I suggest that first an esterase duplication event happened at the basal node of the Noctuidae clade and second a lipase duplication event happened before the divergence of *Helicoverpa* clade from *Heliothis* clade. Since Noctuidae species that do not have acetates in their pheromone

blend seems present only after the lipase duplication event occurred, I hypothesize that gene duplication caused increased acetate degradation, which may have resulted in a complete removal of acetates from the pheromone blend in some species.

IV. Evolution of moth sex pheromone

To understand moth sex pheromone evolution, it is important to comprehend the genetic basis responsible for sex pheromone variation. Although moth sex pheromones have been intensively studied, their genetic basis remain poorly understood (reviewed in Groot et al., 2016). In fact, to our knowledge so far only one gene has been identified to be responsible of intra-specific sex-pheromone variation in a moth (Lassance et al., 2010). The hunt for genes involved in sex pheromone variation could be made more effective. First, more research should focus on enzymes involved in degradation, in addition to synthesis, of pheromone compounds. Second, genomic comparisons may not always be informative, because variation in sex pheromone composition may be explained mostly by differential expression/repression of enzymes rather than their presence/absence. Furthermore, once differential gene expression has been highlighted, it is important to functionally characterize the effect of the candidate gene(s) on the phenotype. This is particularly important, because enzymes may carry different functions depending on the environment they are expressed in and/or their interaction with other genes.

In addition to genetic variation, the environment can also play an important role in the variation in sex pheromone communication. For example, condition-dependent costs may promote variation in a sexual signal. However, trade-offs may stay hidden under ideal laboratory conditions, even if they are important in natural habitats and could explain variation in the signal composition seen in wild populations. Thus, stress factors should be used to reveal condition-dependent costs associated with sex-pheromone composition. In

addition, although type I pheromone are generally produced *de novo* in the female sex pheromone gland, the pheromone blend can be affected by the diet (Chapter 5, Foster, 2009). It could thus be interesting to investigate if being a specialist or a generalist influences the sex-pheromone blend composition. Larvae of specialist moths usually feed on plants that are toxic for generalist and can even use the plant defense mechanism as protection against predators or parasites (Nishida, 2002). Possibly, specialist species may produce costlier pheromone blend as the protection provided by their host plant may reduce that cost.

V. Conclusion

Throughout this thesis, I investigated how both intra- and inter-specific variation in acetate levels is maintained in two noctuid moth species. To do so, I ***i) identified genes underlying acetate variation both between H. subflexa and H. virescens and among H. subflexa populations*** and ***ii) characterized evolutionary potential of acetate variation in H. subflexa as well as some selective forces that drove this variation***. More precisely, I resolved three main questions: Can we identify specific genes responsible of acetate level variation in the previously identified QTLs? YES! Can we artificially select for higher or lower acetate ratios in H. subflexa? YES! Are high acetate levels in H. subflexa associated with condition-dependent costs? YES!

Although acetates are commonly found in numerous moth sex-pheromone blends and have been subject to many years of intensive research, very little was known about the genes involved in acetate levels variation. We discovered four enzymes involved in acetate degradation, two lipases and two esterases, which may be responsible for fine-tuning acetate levels in *H. subflexa* as well as acetate removal in *H. virescens*. Moreover, I found an esterase with an opposite-to-expected effect, whose expression seems to increase acetate levels and, thus, may also be involved in the fine-tuning of acetate levels in *H. subflexa*.

After selecting for high and low acetate levels, we discovered that acetate levels can be modulated without affecting the other pheromone compounds which may have facilitated *H. subflexa* intra-specific variation. Since the presence of acetates have a dual function, as they are attractant for *H. subflexa* males while deterring *H. virescens* males, when both species co-occur higher levels of acetate may have been selected to avoid cross-attraction. However, having high acetate levels is associated with condition-dependent costs and may thus be counter-selected when *H. virescens* is absent. Hence, intra-specific variation in acetate levels seems to be maintained by a balance between producing costly acetates and avoiding cross-attraction. More generally, environmental heterogeneity can generate fluctuating selection on sexual traits and create variation, it is thus important to characterize which selection pressures are occurring and in which context.

Finally, I hypothesized that the absence of acetates in *H. virescens* is a derived state, which can be explained by acetate degradation through an increased hydrolysis and that the deterring effect of acetates on *H. virescens* males may possibly have been selected for in sympatry to reinforce premating barriers.

As a concluding remark, discovering the genetic architecture responsible of both within and between species variation in sexual signal is essential to comprehend the evolution of sexual communication. However, progress in that field will be possible only by a combination of multiple approaches and by focusing less on prior assumptions. As learnt in this thesis, variation in sexual signals may have different to expected explanations.

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