Evolution of sexual signals
Within and between species variation in a dual function sex-pheromone component in two noctuid moths
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Publication date
2023

Citation for published version (APA):

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1

General Introduction
I. Divergence in sexual signal can lead to reproductive isolation and speciation

One of the core questions of evolutionary biology is to explain the origin of biodiversity. This enigma is based on a fundamental question: how do new species evolve? The answer to that puzzling question is usually a combination of natural selection, sexual selection and genetic drift which lead to speciation (Kirkpatrick and Ravigné 2017; Servedio and Boughman 2017). Speciation is a slow process that traditionally is thought to happen when isolating barriers reduce the gene flow among populations (Coyne and Orr, 2004) and can be defined as “the splitting of one species in two or more” (Panhuis et al., 2001).

A frequent mechanism underlying reproductive isolation and subsequent speciation is divergence in sexual signals (Coyne and Orr, 2004; Mayr and Pasteur, 1963; Ritchie, 2007; Smadja and Butlin, 2009). Sexual signals are used to transmit information between sexes within a species and can be used for mate choice. Assortative mating may occur when a specific trait phenotype is preferred which can lead to premating isolation and drive speciation (Servedio and Boughman, 2017). Divergence in sexual signals can also prevent hybrid matings and thus be involved in reinforcement of prezygotic barriers (Coyne and Orr, 2004; Ritchie, 2007). To comprehend the evolution of reproductive isolation and subsequent speciation, investigating variation in sexual signals and its evolvability is essential.

A great diversity of sexual signals can be found in nature, targeting all the senses (for example visual, acoustic, tactile or chemical) and sometimes multiple senses at the same time (aka multi-modal signals). The signal can take a multitude of forms
including bright colors, horns, complex songs, elaborated dance or sex-pheromones (Anderson, 1994). Studying the evolution of sexual signals is complex because it is driven by selection acting in different ways as described below.

II. The evolution of sexual signals

Sexual signals often consist of multiple components (Candolin, 2003; Rowe, 1999) which can share genetic or developmental basis which can constrain the response to selection of sexual signal. Moreover, sexual signal/components can be involved in different functions, like mate attraction and/or repulsion of rivals or heterospecifics (Wiens and Tuschhoff, 2020). For example, sex pheromones are usually made of multiple chemical components which will attract conspecifics while the presence of certain compounds may repeal co-occurring heterospecifics (Ferveur, 2005; Jacquin-Joly and Groot, 2018). Similarly, variation in pitch and rhythm creates unique love songs to which conspecific mates should respond while deterring heterospecifics (Blankers et al., 2015) or rivals (Wilkins et al., 2015). In addition, the signal/component may convey information that reflect the quality of the sender. Sexual signals can be used both in species recognition and mate choice.

Sexual signals as species recognition signals (interspecific)

Sexual signals are often used to locate conspecific mates while avoiding heterospecifics (i.e species recognition; Ryan and Rand 1993; Anderson 1994). Indeed, sexual signals are usually species-specific and differ significantly between closely related species to prevent cross-attraction (Ritchie, 2007; Wiens and Tuschhoff, 2020; Wilkins et al., 2013). Signals used for species recognition are believed to be under strong stabilizing selection as deviation from
the population mean may lead to the attraction of no, or wrong, mates (Johansson and Jones, 2007; Löfstedt, 1993).

If the primary function of the signal is to assess mate compatibility and location, the signal must be reliable and easy to discriminate from other signals in the environment. In other words, individuals producing signals that are not or poorly recognized by receivers are selected against. Since long period of stabilizing selection should erode the variance of sexual signals, the evolvability of sexual signals is hypothesized to decrease over time. However, closely related species often have divergent signals and preferences, and accumulating evidence shows that intra-specific variation in sexual signals is common (De Pasqual et al., 2021; Groot et al., 2014; Ptacek, 2000; Rundle et al., 2005).

Changes in the environment may lead to switch in the preference and directional selection may act on signals until the population mean signal matches the new preference. For example, when sexual signals from different species overlap, communication interference can occur, leading to sexual harassment or hybridization which may result in reduced fitness (Verrell 1994; Gröning and Hochkirch 2008). To avoid communication interference, sexual signals may diverge between closely related species that live in sympatry, which is known as reproductive character displacement (Brown et al., 1956; Crampton et al., 2011; Higge et al., 2000; Kirschel et al., 2009; Lemmon, 2009).

Reproductive character displacement is thought to be a signature of selection favoring discrimination among signals. If species experience different degrees of communication interference across their range, for example in the case of partial sympatry between sister species, reproductive character displacement can lead to increased intra-specific variation (Hoskin et al., 2005).
Sexual signals as mate preference signals (intraspecific)

In addition to character displacement, intra-specific variation in sexual signals may result from covariance with other traits, such as fitness and immune status, which allows receivers to prefer not only compatible, but also high-quality mates (Andersson and Simmons, 2006; Coyne and Orr, 2004; Ritchie, 2007; Schaefer and Ruxton, 2015; Wiens and Tuschhoff, 2020). Sexual signals involved in mate preference are expected to be under directional selection when higher or lower values of a signal indicate higher or lower fitness (Johanson and Jones, 2007).

The expression of (exaggerated) sexual signals may be costly, demanding the investment of a lot of resources (Hill, 1996; Ryan, 1988). In addition, sexual signals can also be exploited by predators/parasites to more easily locate their prey/host, leading to a decreased fitness in the signaller (Marshall et al., 2015; Sheldon et al., 1996; White et al., 2022). Because of these different costs, individuals may vary their investment in sexual signals according to their condition (Jennions et al., 2001).

As condition of individuals may vary with the conditions and resources in their environment, the cost/benefit balance of sexual signal expression can change depending on the habitat. Thus, variation in habitat may generate intra-specific variation. The ability to modulate investment in sexual signals depending on the environment could be beneficial and plasticity may be selected (Cornwallis and Uller, 2010). However, the evolution of adaptive phenotypic plasticity may be limited by the genetic architecture (Lande, 2009).

In conclusion, understanding the evolution of sexual signal is particularly complicated because a single multi-component signal may be susceptible to a variety of selection pressures that might fluctuate depending on the environment. By investigating the genetic
architecture responsible for sexual signal variation, we may predict how trait variation can respond to selection.

III. Genetic basis of sexual signal

The genetic architecture can be defined as the translation from genotype to phenotype and its variational properties (Hansen, 2006). Genetic variation can originate from random mutations or chromosomal recombinations. Gene duplications may also happen, after which different evolutionary paths may emerge to specialized or novel functions (Zhang, 2003).

To investigate the genetic basis responsible for signal variation between populations or species, genetic mapping (such as Quantitative trait loci (QTL) analyses) is commonly used to identify the location and the number of loci associated with the phenotypic variation. Sexual signals may be controlled by many loci with small phenotypic effects (polygenic) or a few loci with major phenotypic effects. These different genetic architectures have consequences for the tempo and mode of speciation (Arbuthnott, 2009; Templeton, 1981). Moreover, sexual signal variation can result not only from protein coding differences but also from differential gene expression. Sexual signal expression may be modulated depending on the environment and individual condition. Comparative transcriptomic analyses between species, sexes or body parts, can highlight differently expressed candidate genes that affect sexual signals.

The development and improvement of next-generation sequencing provides access to an increasing number of genomic and transcriptomic sequences, which can help in the identification of candidate genes. However, how genotypes map to phenotypes is still largely unknown and gene(s) underlying variation in sexual signals remain quite elusive. Functional analysis of candidate genes by gene silencing or knockdown using RNAi or CRISPR-Cas9 technique should improve our comprehension of the genetic architecture involved in sexual signal variation.
IV. Sexual communication through sex pheromone in moths

Moths are an excellent example to study the evolution of sexual signals, as well as their involvement in reproductive isolation and speciation. First, Lepidoptera are among the most diverse group of animals, with about 180,000 described species, among which 155,000 are moths (Allison and Cardé, 2016; Mallet, 2014). Second, sexual communication in moths has been intensively studied, because many moth species are important agricultural pests.

To improve pest management programs, insect chemical ecology research has focused on the identification of female sex-pheromone, because pheromone lures with the appropriate sex pheromone blend are used to monitor their presence and abundance and to disrupt the mating of pest species. By now, the composition of female sex pheromone blends of over 2000 moth species has been identified (see Pherobase.com). Typically, when female moths are ready to mate, they extrude their pheromone gland to emit a long-range sex-pheromone that attracts males from a distance (See figure 1, Ando et al., 2004). The pheromone blend is detected by the antennae of males who will follow the plume upwind with a characteristic zig-zag flight until it reaches the female (Allison and Cardé, 2016; Baker and Vickers, 1997).

Figure 1: Photos of Heliothis subflexa and Heliothis virescens

A) Calling H. virescens female. Photo By Jan van Arkel.
B) Adult H. subflexa. Photos by Laila Kee
V. Composition of moth sex pheromones

Moth sex pheromones are mostly composed of three types (Type I, II and III). The most common pheromone compounds are carbon chains (C_{10}-C_{18}) with a terminal alcohol, aldehyde, or acetate ester functional group, also named Type I pheromones (reviewed by Jurenka, 2017, 2004; Tillman et al., 1999). These compounds are synthesized de novo in the female pheromone gland (Ando et al., 2004). Type II pheromones consist of polyunsaturated hydrocarbons, in addition to zero, one, or two epoxide functions (Ando et al., 2004; Löfstedt et al., 2016). They are derived from linoleic or linolenic acid precursors obtained from the diet (Stanley-Samuelson et al., 1988) and represent 15% of the known pheromones. In addition, a few moth species produce methyl-branched compounds, which are referred to as Type III pheromones (Löfstedt et al., 2016).

Although the first sex pheromone identified in the silkworm moth consisted of one compound (i.e.: bombykol, Butenandt, 1959), moth sex pheromones usually consist of a multi-component blend. The major component is produced in large amounts and generally initiate the male attraction and minor components are produced in smaller quantities but increase the attraction. Even if the pheromone blend of different species may share the same compounds, variation in their ratios and the presence/absence of other specific compounds make each blend species-specific (Ando et al., 2004; Tillman et al., 1999).

VI. Enzymes underlying sex pheromone biosynthesis

Detailed biochemical studies have unraveled different key enzymes involved in the synthesis of Type I pheromone compounds as subsequently described in a chronological order and schematized in
Figure 2 (reviewed by Tillman et al. 1999; Jurenka 2004; Groot et al. 2016). First, desaturases introduce one or more double bonds to the carbon chains, which are then converted to alcohols by fatty acyl reductases (FARs). The alcohol can be directly emitted in the pheromone blend, oxidized to aldehydes by alcohol oxidases or acetylated to acetate esters by acetyltransferases. The created aldehyde and acetate esters can be emitted or be reverted back into an alcohol by aldehyde reductase or acetate esterase.

As the enzymes involved in the sex pheromone biosynthetic pathway are members of large protein families and may play multiple roles in different tissues, the identification of candidate genes involved in sex-pheromone variation has been challenging. For example, even though acetate esters (hereafter referred to acetates) are present in the pheromone blend of various species (Byers, 2006), the genes involved in their synthesis are still elusive (Ding and Löfstedt, 2015; Groot et al., 2013).

**Figure 2: Enzymes involved in sex-pheromone production.**
Arrows indicate the direction of the reaction, the enzyme involved in the reaction is indicated above the arrow in grey. FARs: fatty acyl reductases. X-XX: Carbon chain of unspecified length and possible double-bond. Ald: aldehyde functional group, OH: alcohol functional group, and OAc: acetate ester functional group.
VII. Dual function of acetates in two Noctuid moth

Understanding acetate variation is of particular interest, because acetates can have a dual function: attracting conspecific males while deterring heterospecific males of closely related species (Figure 3). This is for example the case in two closely related noctuid species of the genus Heliothis (newly renamed Chloridea, (Pogue, 2013), but for the sake of continuity I will stick to Heliothis in this thesis).

Figure 3: Dual function of acetate esters in H. subflexa. Hs: H. subflexa, Hv: H. virescens

Both Heliothis subflexa and H. virescens share the same major component (Z11-16:Ald) which is critical for male attraction. Three acetate-esters, (Z)-7-hexadecenyl acetate (Z7-16:OAc), (Z)-9-hexadecenyl acetate (Z9-16:OAc), and (Z)-11-hexadecenyl acetate (Z11-16:OAc), referred to as acetates hereafter, are produced by H. subflexa females, which increase the attraction of conspecific males but repel H. virescens males (Baker and Vickers, 1997; Groot et al., 2007, 2006; Vickers, 2002). The presence of acetates decreases thus
the chance of cross-attraction and hybridization between the two species (Groot et al., 2009b, 2006).

In addition to this interspecific variation, intraspecific variation in the acetate levels of *H. subflexa* has been found, which correlates with the presence of *H. virescens* (Groot et al., 2009b), as summarized here. During three consecutive years, variation in the female sex pheromone blend and male responses of both *H. subflexa* and *H. virescens* were quantified over different geographic regions of the US (Groot et al., 2009b).

In North Carolina where both species co-occur, *H. subflexa* produced high levels of acetates, while in Mexico where *H. virescens* were almost absent, low amounts of acetates were found. This finding suggests that high acetate levels may be costly, because low acetate levels are found when the risk of cross-attraction is reduced. This intraspecific variation is partly due to genetic differences, as even after the two populations of *H. subflexa* were reared under the same lab conditions for multiple generations, differences in acetate levels remained. Hence, *H. subflexa* seems a perfect model to compare the genetic architecture responsible of both the intra- and inter-specific variation in acetate level.

Discovering the genetic basis of acetate variation could enlighten us on how dual function sex pheromone components has evolved and the role it played in the speciation process. Previously, two main QTLs involved in acetate variation were discovered on chromosome 20 and 28 by backcrossing hybrids of *H. subflexa* and *H. virescens* (Groot et al., 2009a, 2004a; Sheck et al., 2006). One of these QTL has been shown to also be involved in the intraspecific variation of acetates (Groot et al., 2013), which suggests that within and between species variation in acetate levels can be partly explained by a shared genetic architecture.

The genes responsible for the variation in acetate levels have not been characterized yet. However, we do know that despite the differences in the female sex pheromone blend, both species’
genomes have genes involved in acetate production, because acetates have been discovered in the hairpencils of male *H. virescens* (Teal and Tumlinson, 1989) and the legs of both sexes (Zweerus et al., 2023). The proportion of acetates in the sex-pheromone blend of *H. subflexa* may result from a balance between acetate production by acetyl-transferase(s) and acetate degradation by esterase(s) (see Fig 2).

VIII. Outline of the thesis

The main 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 investigated the genetic architecture of inter- and intra-specific variation in acetate levels, as well as the evolutionary potential of intra-specific variation both in the context of artificial, unidimensional selection and under food stress condition to mimic selection pressures experienced in the wild.

Three main questions are addressed in this thesis: can we identify specific genes responsible of acetate level variation in the previously identified QTLs? Can we artificially select for higher or lower acetate ratios in *H. subflexa* and how does this affect the rest of the pheromone blend? Are high acetate levels in *H. subflexa* associated with condition-dependent costs?

First, I investigated which specific genes are responsible for acetate level variation both between *H. virescens* and *H. subflexa* and among *H. subflexa* populations. In Chapter 2, I focused on the genes involved in inter-specific variation in acetate levels. To do so, we used a line in which a previously identified QTL, known to influences acetate levels, from *H. virescens* was introgressed into the genomic
background of *H. subflexa*. By conducting a combination of transcriptomic and gene deactivation analyses, we aimed to identify genes responsible of acetate variation. In **Chapter 3**, I tested the involvement of one esterase in variation in acetates in *H. subflexa*, which is situated on the other previously identified QTL known to affects acetate levels. To test the effect of this enzyme, I worked with two gene deactivation techniques: a naturally occurring transposable element and CRISPR-cas9.

Together with colleagues, I conducted experimental selection on *H. subflexa* for either high or low acetate levels. In **Chapter 4**, I deciphered how a multi-component signal evolves when selection acts on one specific group of compounds. Specifically, we aimed to determined how acetate ratios and the genetic co-variance among pheromone components evolved in response to selection favoring higher or lower rates of acetates. In **Chapter 5**, I aimed to uncover other potential selection pressures acting on acetate levels by assessing trade-offs between acetate levels and other life history traits in *H. subflexa* using individuals from the created selection line. To determine whether condition-dependent costs are associated with high acetate levels, I manipulated the diet of the animals. Finally, in **Chapter 6**, I discuss the main results of all chapters in relation to the main aims of the thesis and I place them in a broader scientific context.
Chapter 1

References


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