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Evolution of sexual signals

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

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Summary

Sexual signals are key components in mate attraction and mate choice and their evolution is likely to play an important role in speciation. Sexual attraction through sex pheromones is best studied in moths, where females emit species-specific pheromone blends. This species-specificity stems from the presence/absence and ratios of the different components. For example, the two closely related noctuid moth species *Heliothis subflexa* and *Heliothis virescens* produce similar sex pheromone blends, with one major difference: acetate esters (hereafter also referred to as "acetates"). Acetates are absent in *H. virescens* and present in *H. subflexa*, where they play a dual function: attracting conspecific males, while repelling the sympatrically occurring species, *H. virescens*.

Past research has shown that *H. subflexa* females have higher acetate levels when *H. virescens* are present, and lower in their absence. This finding suggests that high acetate levels may be selected in sympatry to avoid cross-attraction, but may be costly and selected against when *H. virescens* are absent and the chance of cross-attraction is thus reduced.

In this thesis I investigated both within and between species variation in acetate levels, by studying the genetic architecture responsible for this variation and looking at the potential costs associated with high acetate levels. Previous genetic analyses have identified two major QTLs underlying variation in acetate levels (on Chromosome 20 and 28). In **Chapter 2** and **Chapter 3**, I identified candidate genes that are involved in acetate variation in both QTLs.

In **Chapter 2**, I searched for genes responsible for acetate variation between *H. subflexa* and *H. virescens*. To do so, I used a line in which the previously identified QTL on Chromosome 20 from *H. virescens* was introgressed into the genomic background of *H. subflexa*. Females from this introgressed line have acetates in their

sex pheromone blend, but in much lower levels than wild-type *H. subflexa*. By conducting comparative transcriptomic analyses with the introgressed line, wild-type *H. subflexa*, and *H. virescens,* we identified four candidate genes involved in acetate biosynthesis: two lipases and two esterases. We demonstrated that when these candidate genes were knocked-out with CRISPR/Cas9 in the introgressed line, the acetate levels increased. Hence, the lower level of acetates found in the introgressed line could be explained by the overexpression of *H. virescens* genes that are involved in acetate degradation. These results suggest that acetate degradation plays an important role in the tuning of pheromone blend composition.

In **Chapter 3**, we discovered three esterases in tandem within the other previously identified QTL (on chromosome 28). One of these esterases, CXE21, carried a naturally occurring transposable element. Since esterases degrade acetate in alcohol, we thus expected higher level of acetates when this transposable element was present. However, we found the opposite result: lower levels of acetates were observed when the transposable element was present. Moreover, when we used another knock-out method (CRISPR-Cas 9 technique), we obtained the same result. This finding suggests that CXE21, instead of degrading acetates, may actually synthetize acetates. Hence, the role of esterases in general may change depending on the context: an esterase should degrade acetates (like LipX or LipZ in our case), but when water is absent an esterase can do the opposite reaction and synthesize acetates (like CXE21 in our case).

In addition, we selected for high and low levels of acetate in *H. subflexa* over 10 generations. In **Chapter 4** I investigated how this affected the pheromone blend. We demonstrated that acetate levels responded well to artificial selection, while the quantity of pheromone produced and the other important pheromone compounds for male attraction (Z9-11:Ald, Z11-16:Ald and Z11-16:OH) did not change in response to acetate selection. Moreover, we found that the genetic variation of the acetates, which were under

selection, became dissociated from the other pheromone components. In other word, the acetates could respond to selection independently from the other pheromone components due to changes in the genetic covariance structure. This mechanism allows acetates to be modulated without indirect effect on the other pheromone components, which may have promoted intra-specific variation in acetate levels.

Finally, in **Chapter 5** I investigated whether high acetate levels come with a cost. As costs may stay hidden under ideal laboratory rearing conditions, I subjected the two selection lines to food stress. I demonstrated that in a reduced diet treatment, females with high acetate ratios had longer developmental times and a lower fertility than females that had less acetates.

Previous behavioral experiments demonstrated that *H. subflexa* females with less than 5% acetates in their pheromone blend attract *H. virescens* males, thus, high acetate levels should be selected for when both species co-occur to avoid cross-attraction. However, as we found condition-dependent costs associated with high acetate levels, selection for less acetate may be favored when *H. virescens* are absent. Hence, intra-specific variation in the *H. subflexa* female sex pheromone blend may result from a balance between costly acetates and the benefit of deterring heterospecifics. I propose that *H. subflexa* acetate levels may be modulated by up- and down-regulation of some genes highlighted in **Chapter 2** and **3** (LipX and CXE21) and I hypothesize that the absence of acetates from the pheromone blend of *H. virescens* females and other Noctuidea, may be explained by an increased acetates degradation allowed by the duplication of esterase and lipase genes.

In conclusion, to comprehend the evolution of sexual communication, it is essential to discover the genetic architecture responsible of both within and between species variation in sexual signal together with the selective pressures that shaped sexual signal variation.

Samenvatting

Seksuele signalen zijn sleutelcomponenten in het aantrekken en kiezen van partners en hun evolutie speelt waarschijnlijk een belangrijke rol in soortvorming. Seksuele aantrekking door seksferomonen wordt het meest bestudeerd bij nachtvlinders, waar vrouwtjes soortspecifieke feromoonmengsels uitzenden. Deze soortspecificiteit komt voort uit de aan- of afwezigheid en de verhoudingen van de verschillende componenten. Zo produceren de twee nauw verwante nachtvlindersoorten *Heliothis subflexa* en *Heliothis virescens* vergelijkbare feromoonmengsels, met één belangrijk verschil: acetaatesters (hierna ook "acetaten" genoemd). Acetaten zijn afwezig in *H. virescens* en aanwezig in *H. subflexa*, waar ze een dubbele functie hebben: het aantrekken van soortgenoten en het afstoten van de sympatrisch voorkomende soort, *H. virescens*.

Eerder onderzoek heeft aangetoond dat vrouwtjes van *H. subflexa* een hoger acetaatgehalte hebben als *H. virescens* aanwezig is, en een lager acetaatgehalte als ze afwezig zijn. Deze bevinding suggereert dat hoge acetaatgehaltes geselecteerd kunnen worden in sympatrie om kruisaantrekking te voorkomen, maar duur kunnen zijn en tegen geselecteerd kunnen worden wanneer *H. virescens* afwezig is en de kans op kruisaantrekking dus kleiner wordt.

In dit proefschrift heb ik zowel binnen als tussen soorten variatie in acetaatspiegels onderzocht, door de genetische architectuur die verantwoordelijk is voor deze variatie te bestuderen en te kijken naar de mogelijke kosten die geassocieerd worden met hoge acetaatspiegels. Eerdere genetische analyses hebben twee belangrijke QTL's geïdentificeerd die ten grondslag liggen aan variatie in acetaatniveaus (op chromosoom 20 en 28). In hoofdstuk 2 en hoofdstuk 3 heb ik kandidaatgenen geïdentificeerd die betrokken zijn bij acetaatvariatie in beide QTLs.

In hoofdstuk 2 heb ik gezocht naar genen die verantwoordelijk zijn voor de acetaatvariatie tussen *H. subflexa* en *H. virescens*. Hiervoor gebruikte ik een lijn waarin de eerder geïdentificeerde QTL op chromosoom 20 van *H. virescens* werd geïntrogresseerd in de genomische achtergrond van *H. subflexa*. Vrouwtjes van deze geïntrogresseerde lijn hebben acetaten in hun seksferomoonmengsel, maar in veel lagere gehaltes dan wild-*type H. subflexa*. Door vergelijkende transcriptoomanalyses uit te voeren met de introgressed lijn, wild-type *H. subflexa* en *H. virescens*, identificeerden we vier kandidaatgenen die betrokken zijn bij de biosynthese van acetaat: twee lipasen en twee esterasen. We toonden aan dat wanneer deze kandidaatgenen werden uitgeschakeld met CRISPR-Cas9 in de introgressielijn, de acetaatniveaus toenamen. Het lagere acetaatgehalte in de geïntrogresseerde lijn kan dus verklaard worden door de overexpressie van *H. virescens* genen die betrokken zijn bij acetaatafbraak. Deze resultaten suggereren dat acetaatafbraak een belangrijke rol speelt bij het afstemmen van de samenstelling van feromoonmengsels.

In hoofdstuk 3 ontdekten we drie esterases in tandem binnen de andere eerder geïdentificeerde QTL (op chromosoom 28). Eén van deze esterases, CXE21, droeg een natuurlijk voorkomend transponeerbaar element. Aangezien esterases acetaat afbreken in alcohol, verwachtten we dus een hoger acetaatgehalte wanneer dit transponeerbare element aanwezig was. We vonden echter het tegenovergestelde resultaat: er werden lagere acetaatgehaltes waargenomen wanneer het transponeerbare element aanwezig was. Bovendien verkregen we hetzelfde resultaat toen we een andere knock-out methode gebruikten (CRISPR-Cas9 techniek). Deze bevinding suggereert dat CXE21, in plaats van acetaten af te breken, acetaten kan synthetiseren. De rol van esterases in het algemeen kan dus veranderen afhankelijk van de context: een esterase moet acetaten afbreken (zoals LipX of LipZ in ons geval), maar als er geen

water is kan een esterase de tegenovergestelde reactie uitvoeren en acetaten synthetiseren (zoals CXE21 in ons geval).

Daarnaast selecteerden we voor hoge en lage acetaatgehaltes in *H. subflexa* over 10 generaties. In hoofdstuk 4 onderzocht ik hoe dit het feromoonmengsel beïnvloedde. We toonden aan dat het acetaatgehalte goed reageerde op kunstmatige selectie, terwijl de hoeveelheid geproduceerd feromoon en de andere belangrijke feromoonverbindingen voor mannelijke aantrekking (Z9-11:Ald, Z11-16:Ald en Z11-16:OH) niet veranderden in reactie op acetaatselectie. Bovendien vonden we dat de genetische variatie van de acetaten, waarop geselecteerd werd, los kwam te staan van de andere feromooncomponenten. Met andere woorden, de acetaten konden onafhankelijk van de andere feromooncomponenten reageren op selectie door veranderingen in de genetische covariantiestructuur. Door dit mechanisme kunnen acetaten gemoduleerd worden zonder indirect effect op de andere feromooncomponenten, wat intra-specifieke variatie in acetaatniveaus kan hebben bevorderd.

Tot slot heb ik in hoofdstuk 5 onderzocht of hoge acetaatgehaltes kosten met zich meebrengen. Omdat de kosten verborgen kunnen blijven onder ideale laboratoriumomstandigheden, onderwierp ik de twee selectielijnen aan voedselstress. Ik toonde aan dat vrouwtjes met een hoog acetaatgehalte in een behandeling met verlaagd dieet een langere ontwikkelingstijd en een lagere vruchtbaarheid hadden dan vrouwtjes met minder acetaten.

Eerdere gedragsexperimenten toonden aan dat *H. subflexa* vrouwtjes met minder dan 5% acetaten in hun feromoonmengsel *H. virescens* mannetjes aantrekken, dus zou er geselecteerd moeten worden op hoge acetaatgehaltes wanneer beide soorten samen voorkomen om kruisaantrekking te voorkomen. Omdat we echter toestandsafhankelijke kosten geassocieerd met hoge acetaatgehaltes vonden, kan selectie voor minder acetaat bevorderd worden wanneer *H. virescens* afwezig is. Intraspecifieke variatie in het vrouwelijke feromoonmengsel van *H. subflexa* kan dus het resultaat zijn van een balans tussen kostbare acetaten en het

voordeel van het afschrikken van heterospecifieke individuen. Ik stel voor dat de acetaatniveaus van *H. subflexa* gemoduleerd kunnen worden door up- en down-regulatie van enkele genen die in hoofdstuk 2 en 3 naar voren zijn gekomen (LipX en CXE21) en ik stel als hypothese dat de afwezigheid van acetaten in het feromoonmengsel van H. virescens vrouwtjes en andere Noctuidea verklaard kan worden door een verhoogde acetaatafbraak die mogelijk wordt gemaakt door de duplicatie van esterase- en lipasegenen.

Concluderend, om de evolutie van seksuele communicatie te begrijpen is het essentieel om de genetische architectuur te ontdekken die verantwoordelijk is voor zowel de variatie in seksuele signalen binnen als tussen soorten, samen met de selectieve druk die de variatie in seksuele signalen heeft gevormd.

Author contributions

Chapter 2: *Lipases and carboxylesterases are involved in interspecific pheromone differences between two moth species*

Arthur de Fouchier, Elise Fruitet, Rik Lievers, Peter Kuperus, Jennifer Emerson, Fred Gould, David G. Heckel, Astrid T. Groot

AF, EF, FG, ATG and DGH conceptualized the study; AF, EF, RL, PK and JE performed the experiments; AF, EF and DGH analyzed the data; AF, EF, DGH and ATG wrote and edited the paper; All authors approved the submitted manuscript.

Chapter 3: *An esterase affects pheromone components important for reproductive isolation between two closely related moth species*

Elise Fruitet, Astrid T. Groot, David G. Heckel

All authors contributed to the design and conceptualization of the study; EF and DGH performed the experiments; EF analyzed the data; EF wrote the first draft and TB, ATG and DGH contributed substantially to the revisions; All authors approved the submitted manuscript.

Chapter 4: *Experimental evolution of a pheromone signal*

Thomas Blankers, Elise Fruitet, Emily Burdfield-Steel, Astrid T. Groot

EF, TB, EBS and ATG contributed to the design and conceptualization of the study; EF, TB and EBS performed the experiments; TB analyzed the data; TB wrote the first draft and EF, EBS and ATG contributed substantially to the revisions; All authors approved the submitted manuscript.

Chapter 5: *High acetate levels in H. subflexa sex pheromone blend are associated with reduced fitness in a stressful environment*

Elise Fruitet, Rick De Jong, Thomas Blankers, Astrid T. Groot, Emily Burdfield-Steel

EF, TB, EBS and ATG contributed to the design and conceptualization of the study; EF and RJ performed the experiments; EF analyzed the data; EF wrote the first draft and TB, EBS and ATG contributed substantially to the revisions.

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Arthur de Fouchier

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Biography

Elise Clara Ella Fruitet was born on the 6th of March 1995 in France, in the southern city of Montpellier where she grew up. As a kid she wanted to become a forest ranger. In 2012, she started a Bachelor's degree in Biology of the Organisms at the *Université de Montpellier*. At the end of her Bachelor she did an internship on sexual avoidance through olfactory recognition in two sub-species of mice *Mus musculus* and *Mus domesticus* at the *Institut des Sciences de l'Evolution de Montpellier*, supervised by Guila Ganem. Between 2015-2017, she continued her studies at the *Université de Montpellier* with a Master's degree in Evolution and Ecology. In the course of these years, she did a former internship on the fitness effects of sexual selection and selfing under experimental evolution regimes of a hermaphrodite (*Physa acuta*) under the supervision of Patrice David at the *Centre d'Ecologie, Fonctionelle et Evolutive in Montpellier* and a latter one on Whole Genome Duplication event in anther smut fungi (*Microbotryum spp.*) parasite of *Dianthus* at the *Laboratoire d'Ecology, Systematique et d'Evolution in Orsay (France)* supervised by Tatiana Giraud and Ricardo Rodriguez de la Vega. After several years studying in her homeland, she decided to look for a PhD abroad, in order to live new experiences. In December 2017, she got an International Max-Planck Research School fellowship; the 1st of March 2018 she started her PhD on sexual communication in Moths under the supervision of Astrid Groot and David Heckel. Her research was carried out mostly at the *Institut for Biodiversity and Ecosystem Dynamics, University of Amsterdam (The Netherlands)* but also at the *Max-Planck Institut for Chemical Ecology, in Jena (Germany)*. While writing her thesis, she became a mother and thus went through the last steps of her PhD with the joyful – yet demanding - company of her beloved newborn, Timothy. The upcoming horizons of her life are still uncharted.

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