When sexual signallers are choosers too

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SUMMARY

When sexual signallers are choosers too

Sexual selection is a potent evolutionary force and was proposed by Darwin because natural selection alone could not explain the evolution of exaggerated sexual signals, like colourful ornaments in birds. However, it is not only selection for extremes but also the selection for intermediate trait values that can affect signal evolution. When sexual signals are used for species recognition, the mean signal in a population is preferred to minimize cross-attraction of other species. Species-recognition signals are thus under stabilizing selection. Since stabilizing selection selects against variation that deviates from population mean, it is unclear how changes in sexual signals can still occur. Such changes are only possible when these sexual signals are also subject to other selection pressures for instance, when these signals are also used to select partners within a species.

Sexual signals for mate choice are commonly investigated with the assumption that one sex is the signaller while the other sex chooses. However, both sexes can be signallers and choosers. When sexual signallers are choosers too, mutual mate choice arises and increases the number and interactions of selection pressures. Mutual mate choice may thus alter the resulting selection pressures on sexual signals. Therefore, it is important to understand the role of both sexes in sexual communication.

In this thesis, I challenged the prevailing view that only one sex (i.e., the male) is choosing by providing evidence for female mate choice in a noctuid moth. While females of the tobacco budworm *Chloridea* (formerly *Heliothis*) *virescens* (Lepidoptera: Noctuidae) attract males via a long-range sex pheromone, males also emit a pheromone from elaborate structures, called hairpencils, at close range. Females use the male hairpencil pheromone for species recognition but whether these chemicals are used by the female to discriminately assess males of different quality was unclear. I thus investigated female mate choice in *C. virescens* and determined the male signal underlying female mate choice.

In chapter 2, I assessed whether female mate choice in *C. virescens* exists, and to which extent the male hairpencil pheromone affects female mating decisions. I provided evidence of female mate choice based on the identification of crucial female actions in close-range courtship and showed that females prefer to mate with larger males. Females that mated with larger males produced more offspring. Male size thus determines male quality and plays a key role in male attractiveness. While the male hairpencil pheromone has been suggested to underlie female mate choice, neither quantity nor the composition of the known male pheromone blend explained female mate choice in these experiments. This chapter thus shows that females are choosers too and that they choose males based on a signal that is related to male size. In addition, this chapter entails a detailed description of courtship behaviour, including a novel element that involves leg-to-leg contact, which I termed ‘grubbing’.

In chapter 3, I tested the extent to which female mating status affects female sexual behaviour and mate choice, in females that are not only choosers but also signallers. Theory predicts that virgin females should not be choosy because the risk of remaining unmated is high. However, this theory is based on non-signalling females. By signalling, females actively attract males and can adjust the arrival rate of males, so that these females may be choosy. I found that virgin *C. virescens* females signalled more than mated females, but virgin and mated signalling females were equally ready to (re)mate. In contrast to my expectation, I found that virgin signalling females, like non-signalling females, showed weaker mate preference than mated females. This can be explained by the fact that females can increase their fitness through multiple matings and by becoming more selective with every mating. These findings emphasize that female signalling should be considered as a crucial component of female mate choice.

While I found that the traditional male hairpencil pheromone does not underlie female mate choice (chapter 2), recently discovered new pheromone compounds in the male hairpencils might instead explain how females choose males. In chapter 4, I assessed if two newly described, nutrition-derived compounds in the *C. virescens* male hairpencil pheromone could be the signal underlying female mate choice. These compounds are the secondary plant metabolites methyl salicylate (MeSA) and δ-decalactone. Since it was known that at least MeSA is sequestered from the diet, I first tested how larval diet and adult feeding affects MeSA and δ-decalactone content in males. MeSA content was indeed related to larval diet, as males reared on full diet had a higher MeSA content than males reared on diet reduced in nutritional value. Delta-decalactone content was not related to the nutritional value of the larval diet, but varied significantly with adult feeding: males fed with sugar-water had higher amounts than males fed with plain water. Moreover, MeSA and δ-decalactone amounts correlated positively with male pupal mass. Therefore, I tested the hypothesis that female preference for larger males is reflected in a preference for higher amounts of MeSA and/or δ-decalactone. I found that male mating success was associated with higher δ-decalactone but lower MeSA content. Females may thus use the amounts of these compounds in the male hairpencil pheromone as a signal to gauge male size and to discriminate between males of different quality.

The discovery of tactile interactions between the male and the female during courtship (chapter 2) and the presence of pheromone compounds on moth legs suggested that moths may use information from leg-to-leg contact. In chapter 5, I analysed the chemical compounds on male and female legs of three closely-related moth species, and sought to determine the biological role of pheromone on heliothine moth legs. The identification and quantification of chemicals in leg extracts revealed that identical pheromone compounds were present on the legs in both sexes of all three species, with no striking interspecies or intersex differences. Surprisingly, I found acetate esters in leg extracts of species that lack these chemicals in their sex pheromone. I also found that known and putative pheromone biosynthesis genes are expressed in leg tissue, which suggests moth legs as an additional site of pheromone production. I then explored whether the pheromone compounds on legs act as oviposition-deterring signals, which does not seem to be the case. Finally, I tested these chemicals for antimicrobial properties, and found that two pheromone compounds prevent bacterial growth. Moth pheromone compounds on legs may thus not be for sexual chemical communication but function as antibiotics to protect the oviposited eggs or young larvae.

Taken together, I found that the communication system of *C. virescens* is based on mutual mate choice, because males choose females based on their sex pheromone and this thesis shows that females are choosers too. Thus, mutual mate choice in *C. virescens* exists, but this mutual choice is based on different types of signals: while females attract males via a sex pheromone, males signal their attractiveness not through the biosynthetically related male hairpencil pheromone, but through another signal that conveys information about male size. Possibly, this signal consists of δ-decalactone and methyl salicylate, two newly discovered compounds in the male hairpencil pheromone. Hence, male and female mate choice seem to exert independent selection pressures on different types of sexual signals. The different selection pressures and their interactions are likely to maintain variation in male and female sexual signals. Thus, when sexual signallers are choosers too, changes in species-specific sexual communication channels of moths may occur. Since I found that male size explains female mate choice and that diet and feeding have a significant effect on male attractiveness, it is likely that food sources are central to shifts in this sexual communication. In this thesis, I propose that sexual selection drives divergence in sexual signals if factors affecting male size (e.g., diet) are reinforced by natural selection. This research adds to our understanding of the sexual chemical communication of *C. virescens* and provides valuable insights into how moth sexual communication channels can evolve.

SAMENVATTING

Wanneer seksuele signaalgever ook kiezers zijn

Seksuele selectie is een belangrijke evolutionaire kracht en werd door Darwin voorgesteld omdat natuurlijke selectie het bestaan van overdreven seksuele signalen, zoals kleurrijke ornamenten bij vogels, niet kan verklaren. Het is echter niet alleen selectie voor extremen, maar ook de selectie voor intermediaire karakteristieken die de evolutie van signalen kan beïnvloeden. Wanneer seksuele signalen worden gebruikt voor soortherkenning, worden signaalwaardes die dicht bij het gemiddelde van een populatie liggen geprefereerd om aantrekking van andere soorten te minimaliseren. Soortenherkenningssignalen zorgen daardoor voor stabiliserende selectie. Aangezien stabiliserende selectie de individuen met signaalwaardes die afwijken van het populatie-gemiddelde weg selecteert, is het onduidelijk hoe veranderingen in seksuele signalen toch kunnen evolueren. Hiervoor is het bijvoorbeeld nodig dat de seksuele signalen ook onderhevig zijn aan andere selectiedrukken, zoals seksuele signalen die niet alleen gebruikt worden voor soortherkenning maar ook om partners binnen een soort te selecteren.

Seksuele signalen voor partnerkeuze worden gewoonlijk onderzocht onder de aanname dat het ene geslacht het signaal verstuurd (verzender) terwijl het andere geslacht kiest op basis van het signaal (ontvanger). In werkelijkheid kunnen vaak beide geslachten zowel seksuele signalen uitzenden als ook ontvangen. Wanneer de primaire verzenders ook partnerkeuzes maken, ontstaat wederzijdse partnerkeuze en neemt het aantal en het potentieel voor interacties van selectiedrukken toe. Wederzijdse partnerkeuze kan de resulterende selectiedruk op seksuele signalen dus doen veranderen. Daarom is het belangrijk om de rol van beide seksen in seksuele communicatie te begrijpen.

In dit proefschrift heb ik de heersende opvatting dat slechts één sekse (in het geval van nachtvlinders, het mannetje) kiest in twijfel getrokken door bewijs te leveren voor vrouwelijke partnerkeuze bij een nachtvlinder. Terwijl vrouwtjes van de tabaksworm *Chloridea* (voorheen *Heliothis*) *virescens* (Lepidoptera: Noctuidae) mannetjes aantrekken via een lange-afstandseksferomoon, scheiden mannetjes op korte afstand ook een feromoon uit via uitgebreide structuren, hairpencils genaamd. Vrouwtjes gebruiken het mannelijk feromoon voor soortherkenning, maar of deze chemische stoffen door het vrouwtje gebruikt worden om mannetjes van verschillende kwaliteit te onderscheiden was onbekend. Daarom onderzocht ik de vrouwelijke partnerkeuze bij *C. virescens* en bepaalde ik het mannelijk signaal dat aan de basis ligt van de vrouwelijke partnerkeuze.

In hoofdstuk 2 onderzocht ik of vrouwelijke partnerkeuze bij *C. virescens* überhaupt bestaat en in welke mate het mannelijke feromoon de vrouwelijke paringsbeslissingen beïnvloedt. Ik heb bewijs geleverd voor vrouwelijke partnerkeuze op basis van de identificatie van cruciale vrouwelijke acties tijdens de balts en aangetoond dat vrouwtjes bij voorkeur paren met grotere mannetjes. Vrouwtjes die met grotere mannetjes paarden, produceerden meer nakomelingen. De grootte van de mannetjes bepaalt dus de kwaliteit van de mannetjes en speelt een sleutelrol in de aantrekkelijkheid van de mannetjes. Hoewel gesuggereerd is dat het mannelijke feromoon de partnerkeuze van de vrouwtjes beïnvloedt, verklaarde noch de hoeveelheid noch de samenstelling van het bekende mannelijke feromoonmengsel de partnerkeuze van de vrouwtjes in deze experimenten. Dit hoofdstuk toont dus aan dat vrouwtjes ook kiezers zijn en dat zij mannetjes kiezen op basis van een signaal dat gerelateerd is aan de grootte van het mannetje. Bovendien bevat dit hoofdstuk een gedetailleerde beschrijving van het paringsritueel dat aan de paring voorafgaat, waaronder een nieuw element dat contact tussen de poten behelst en dat ik 'grubbing' heb genoemd.

In hoofdstuk 3 heb ik getest in hoeverre de paringsstatus van vrouwtjes van invloed is op seksueel gedrag en partnerkeuze, bij vrouwtjes die niet alleen kiezers zijn maar ook verzenders. Door te signaleren kunnen vrouwtjes de aanwas van mannetjes moduleren en zo de paringskans van het vrouwtje vergroten. De theorie voorspelt dat maagdelijke vrouwtjes niet kieskeurig zouden moeten zijn omdat het risico om ongepaard te blijven groot is. Echter, deze theorie betreft niet-signalerende vrouwtjes en gaat mogelijk niet op voor signalerende vrouwtjes. Door signalen af te geven kunnen maagdelijke vrouwtjes namelijk kieskeurig zijn, omdat zij hiermee actief mannetjes aantrekken. Ik vond dat maagdelijke *C. virescens* vrouwtjes meer signaleerden dan gepaarde vrouwtjes, maar dat maagdelijke en gepaarde signalerende vrouwtjes evenveel bereid waren om (opnieuw) te paren. Tegen mijn voorspellingen in, vond ik dat maagdelijk signalerende vrouwtjes, net als niet-signalerende vrouwtjes, een zwakkere paringsvoorkeur vertoonden dan gepaarde vrouwtjes. Dit kan verklaard worden door het feit dat gepaarde vrouwtjes hun fitness kunnen verhogen door vaker te paren en bij iedere paring selectiever te worden op mannelijke kwaliteit. Deze bevindingen benadrukken dat vrouwelijke signalen moeten worden beschouwd als een cruciale component van vrouwelijke partnerkeuze.

Hoewel ik ontdekte dat het traditionele mannelijke feromoon niet gebruikt wordt door vrouwtjes om mannetjes te kiezen (hoofdstuk 2), zouden recente, nieuw ontdekte feromooncomponenten in het mannetjesferomoon misschien wel gebruikt kunnen worden. In hoofdstuk 4 heb ik onderzocht of twee nieuw beschreven, van planten afkomstige componenten in het mannelijke feromoon van *C. virescens* het signaal zouden kunnen zijn dat vrouwtjes gebruiken voor hun partnerkeuze. Dit zijn de secundaire plantenmetabolieten methylsalicylaat (MeSA) en δ-decalacton. Omdat bekend is dat MeSA uit het dieet wordt opgenomen, heb ik eerst getest hoe het dieet van de rupsen en het voedsel van de volwassenen het MeSA- en δ-decalactongehalte van de mannetjes beïnvloedt. Het MeSA-gehalte hing inderdaad samen met het dieet van de rupsen: mannetjes die op een volledig dieet gekweekt werden, hadden een hoger MeSA-gehalte dan mannetjes die op een gereduceerd dieet gekweekt werden. Het δ-decalactongehalte varieerde niet met het dieet van de rupsen, maar wel significant met het voedsel van de volwassen dieren: mannetjes gevoerd met suikerwater hadden hogere hoeveelheden δ-decalacton dan mannetjes gevoed met gewoon water. Bovendien correleerden de hoeveelheden MeSA en δ-decalacton positief met de massa van de mannetjespoppen. Daarom testte ik de hypothese dat de voorkeur van de vrouwtjes voor grotere mannetjes zich weerspiegelt in een voorkeur voor hogere hoeveelheden MeSA en/of δ-decalacton. Ik ontdekt dat mannelijk paringssucces geassocieerd was met een hoger δ-decalactongehalte maar een lager MeSA-gehalte. Het is dus mogelijk dat vrouwtjes de hoeveelheden van deze stoffen in het mannelijke feromoon gebruiken als een signaal om de grootte van de mannetjes te meten en onderscheid te maken tussen mannetjes van verschillende kwaliteit.

De ontdekking van tactiele interacties tussen het mannetje en het vrouwtje tijdens de balts (hoofdstuk 2) en de aanwezigheid van feromoonverbindingen op de poten van de mannetjes en vrouwtjes nachtvlinders suggereerden dat nachtvlinders informatie van poot-tot-poot contact kunnen gebruiken. In hoofdstuk 5 analyseerde ik de chemische verbindingen op de poten van mannetjes en vrouwtjes van drie nauw verwante nachtvlindersoorten, en trachtte ik de biologische rol van feromoon op poten van heliothine nachtvlinders te bepalen. De identificatie en kwantificering van chemische verbindingen in extracten van de poten toonde aan dat identieke feromoonverbindingen aanwezig waren op de poten van beide geslachten van alle drie de soorten, zonder opvallende verschillen tussen de soorten of tussen de geslachten. Verrassend genoeg vond ik acetaat-esters in pootjes-extracten van soorten die deze componenten niet in hun seksferomoon hebben. Ik vond ook dat bekende en vermeende genen voor feromoonbiosynthese tot expressie komen in het pootweefsel, wat suggereert dat de poten van nachtvlinders een extra locatie voor feromoonproductie zijn. Vervolgens onderzocht ik of de feromooncomponenten op de poten fungeren als ovipositie-afschrikkende signalen, wat niet het geval lijkt te zijn. Tenslotte testte ik de feromooncomponenten op antimicrobiële eigenschappen en ontdekte dat twee componenten bacteriële groei verhinderen. Het is dus mogelijk dat de feromooncomponenten op de poten van nachtvlinders niet dienen voor seksuele chemische communicatie, maar functioneren als antibiotica ter bescherming van de pas gelegde eieren of jonge rupsen.

Alles bij elkaar genomen is het communicatiesysteem van *C. virescens* gebaseerd op wederzijdse partnerkeuze, want mannetjes kiezen vrouwtjes op basis van hun seksferomoon en dit proefschrift toont aan dat vrouwtjes ook kiezen tussen mannetjes. Er is dus sprake van wederzijdse partnerkeuze bij *C. virescens*, maar deze wederzijdse keuze is gebaseerd op verschillende soorten signalen: terwijl vrouwtjes mannetjes aantrekken via een seksferomoon, signaleren mannetjes hun aantrekkelijkheid niet via het biosynthetisch verwante mannelijke feromoon, maar via een ander signaal dat informatie over de grootte van het mannetje verschaft. Mogelijk bestaat dit signaal uit δ-decalacton en methylsalicylaat, twee recent ontdekte verbindingen in het mannelijke feromoon. Mannelijke en vrouwelijke partnerkeuze lijken dus een onafhankelijke selectiedruk uit te oefenen op verschillende soorten seksuele signalen. De verschillende selectiedrukken en hun interacties houden waarschijnlijk de variatie in mannelijke en vrouwelijke seksuele signalen in stand. Wanneer de verzenders ook de kiezers zijn en vice versa, kunnen veranderingen in soortspecifieke seksuele communicatiekanalen van nachtvlinders optreden. Aangezien ik ontdekt heb dat de grootte van het mannetje de partnerkeuze beïnvloedt en dat dieet een significant effect hebben op de aantrekkelijkheid van het mannetje, is het waarschijnlijk dat voedselbronnen centraal staan in verschuivingen in deze seksuele communicatie. In dit proefschrift stel ik voor dat seksuele selectie divergentie in seksuele signalen veroorzaakt als factoren die de grootte van de mannetjes beïnvloeden (bv. dieet) versterkt worden door natuurlijke selectie. Dit onderzoek draagt bij tot ons begrip van de seksuele chemische communicatie van *C. virescens* en verschaft waardevolle inzichten in hoe seksuele communicatiekanalen van nachtvlinders kunnen evolueren.

AUTHOR CONTRIBUTIONS

2 | Experimental evidence for female mate choice in a noctuid moth
Naomi L. Zweerus, Michiel van Wijk, Coby Schal, Astrid T. Groot
NLZ, MvW, CS and ATG conceived and designed the study, NLZ conducted the experiments, NLZ and MvW performed the statistical analyses. NLZ and ATG wrote the first draft, and all authors contributed substantially to the approved manuscript.

3 | Mating status affects female choice when females are signallers
Naomi L. Zweerus, Michiel van Wijk, Isabel M. Smallegange, Astrid T. Groot
NLZ and ATG designed the study, NLZ conducted the experiments, NLZ and MvW performed the statistical analyses. NLZ and IMS wrote the first draft, and all authors contributed to the writing of the approved manuscript.

4 | Nutrition-derived male sex-pheromone compounds affect female choice in a noctuid moth
Michiel van Wijk, Naomi L. Zweerus, Coby Schal, Astrid T. Groot
NLZ, MvW, CS and ATG designed the study, NLZ conducted the experiments, NLZ and MvW performed the statistical analyses. ATG wrote the first draft of the manuscript. All authors provided critical feedback and contributed substantially to the final manuscript.

5 | More to legs than meets the eye: presence and function of pheromone compounds on heliothine moth legs
Naomi L. Zweerus, Laura J. Caton, Lotte de Jeu, Astrid T. Groot
NLZ and ATG conceived and designed the study, LJC, LdJ and NLZ carried out the experiments and analysed the data. NLZ wrote the first draft and all authors contributed substantially to the revisions.

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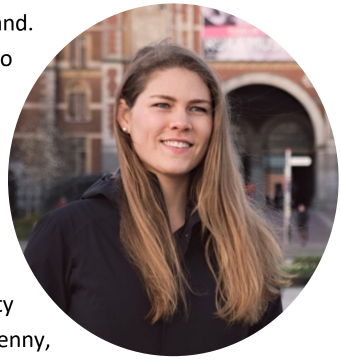
Cheers!

ABOUT THE AUTHOR



Naomi Linda Zweerus was born on May 28 1990 in Switzerland. When she was a baby of 8 weeks, her family moved to Singapore where she spent the first three years of her life. Back in Switzerland, she attended school and finished high school with the Swiss Matura and the Bilingual International Baccalaureate (IB) in Zurich in 2010. Naomi took a gap year during which she travelled, worked one winter season as a ski instructor, and did an internship in the Department of Molecular Life Sciences of Prof. Konrad Basler at the University of Zurich. The enthusiasm of her daily supervisor, Dr. Fabian Jenny, for empirical research was so inspiring that she decided to study biology herself. In 2014, Naomi obtained a BSc degree in Biology from the University of Zurich, and graduated with an MSc degree in Ecology from the same university in 2016. Due to her passion for lab work and the motivation to work in an international environment, she sought a PhD position abroad. Her dream came true as she got accepted in the same year as a PhD candidate at the University of Amsterdam in the group of Prof. Astrid Groot. Naomi joined the PhD council of the PE&RC graduate school (PPC) in 2016 and chaired the council for year in 2019. Based on this experience, she took on a part-time job as a PhD Programme coordinator for PE&RC in 2019 and supported the team until 2022.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)
- On the circumstances of female mate choice

Writing of project proposal (4.5 ECTS)
- Understanding sexual selection: the role of male premating signals in potential mutual mate choice

Post-graduate courses (3.6 ECTS)
- ICE Graduate course in insect chemical ecology; Penn State University, USA (2017)
- Meta-analysis; WGS, WUR, the Netherlands (2018)

Laboratory training and working visits (0.4 ECTS)
- Research visit to the laboratory Department of Entomology and Plant Pathology, North Carolina State University (2017)

Invited review of (unpublished) journal manuscript (1 ECTS)
- Proc B: sexual selection (2019)

Competence strengthening / skills courses (2.1 ECTS)
- Competence assessment; WUR
- Efficient writing strategies; WUR
- Career lunches; UvA
- Science communication workshop; UvA

Scientific integrity / ethics in science activity (0.6 ECTS)
- Scientific integrity; online; WUR (2020)

PE&RC Annual meetings, seminars and the PE&RC weekend (3 ECTS)
- Workshop: tipping points in pest management (2016)
- PE&RC Weekends first years, midterm, last year (2016, 2018, 2020)
- PE&RC Days (2016, 2017, 2018, 2019)

Discussion groups / local seminars or scientific meetings (8.4 ECTS)
- IBED soup lunch meetings (2016-2020)
- NEV Entomologendag (2017, 2020)
- NLSEB Annual meeting of the Netherlands Society for Evolutionary Biology (2018, 2019)
- NAEM Netherlands Annual Ecology Meeting (2017, 2020)
- P(h)D Events organized by IBED P(h)D council (2016-2020)

International symposia, workshops and conferences (5 ECTS)
- ESEB; the Netherlands (2017)
- EMPSEB; Portugal (2019)

Lecturing / supervision of practicals / tutorials (3 ECTS)

- Evolutionary biology (2017)
- Evolution and behaviour (2018, 2019, 2020)

BSc/MSc thesis supervision (10.5 ECTS)

- The effect of mating status on choosiness in female *C. virescens*
- Immuno-gene expression of infected moths
- Chemical compounds on moth legs
- Antimicrobial properties of pheromone compounds