Sex pheromone biosynthesis in moth pests

From gene discovery to biotechnological production

Dam, Marie

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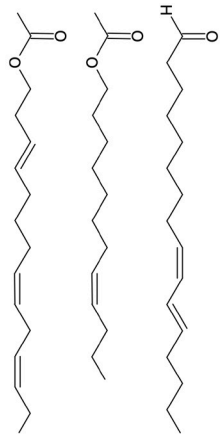

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Abstract:

Moths (Lepidoptera) are some of the most economically important pests in human agriculture. Conventional insecticides used for management promote insecticide resistance in addition to cause human- and environmental harm. This emphasises an inescapable need for sustainable alternatives and integrated practices. Moths rely on species-specific female-emitted sex pheromones for communication and reproduction, a chemical ecology paradigm that can be used for pest control, either by trapping males with pheromone baits or masking female pheromone trails with their own pheromones. Such an approach does not carry any risk for humans, other species or the environment, and has proven successful for many crops. Pheromones can be made biotechnologically with a potential for green and efficient production, using moth pheromone biosynthesis enzymes expressed in for example yeast.

The ultimate aim of this work has been to advance development of biotechnological production of pheromones for pest management. To this end, the yeast Yarrowia lipolytica was extensively engineered for efficient production of two pheromones, (*Z*)-11-hexadecenol and (*Z*)-9-tetradecenol, which can be applied in pest management of several moth species.

Another focus, serving to develop biotechnological pheromone production, has been to investigate sex pheromone biosynthesis in three economically important moth pests: the sugarcane borer *Diatraea saccharalis*, the oriental fruit moth *Grapholita molesta* and the tomato leafminer *Tuta absoluta*. The focus was on characterisation of genes for desaturases, which produce double bonds in the pheromones of these species. Methods used have been 1) the identification of pheromone precursors by gas chromatography/mass spectrometry analyses of fatty acids in the moth female pheromone gland, and to trace the fate of isotope-labelled pheromone precursors applied to the pheromone gland, 2) transcriptome and expression analysis of candidate genes ecoding enzymes in pheromone biosynthesis, and 3) functional characterisation of those genes in heterologous expression assays or by gene-deletion in the insect. For *D. saccharalis*, genes for both desaturases and a reductase involved in producing the main and minor pheromone components were characterised, and heterologous expression of genes and production of pheromone components demonstrated in yeast. For *G. molesta*, a unique desaturase has been identified, the first Δ8 desaturase in lepidopteran pheromone biosynthesis. The role of the desaturase was confirmed by CRISPR/Cas9 knock-out of the gene, creating a pheromone-deficient moth. For *T. absoluta*, the conventional biosynthesis pathway elucidation methodology has proved to be insufficient, revealing that either pheromone precursors or biosynthesis are different to what is common for most Lepidoptera. Interestingly, transcriptome analysis showed that this species does not have a desaturase in the lepidopteran-specific Δ11/10 lineage, otherwise frequently found in moth genomes. This collection of results provides insights into lepidopteran pheromone biosynthesis and evolution, especially of unusual pheromone structures in Tortricidae and Gelechiidae. The results also pave the way for more sustainable control of a pest on one of the world's biggest crops (*D. saccharalis* on sugarcane) and for studying pheromone biosynthesis in tortricid moths, major fruit pests around the world.

Key words: Lepidoptera, moths, sex pheromones, biosynthesis, desaturases, gene characterisation, transcriptomics, heterologous expression, biotechnology, integrated pest management

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*The world is full of magic things, patiently waiting for our **senses** to grow sharper. – W. B. Yeats*

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Abstract

Moths (Lepidoptera) are some of the most economically important pests in human agriculture. Conventional insecticides used for management promote insecticide resistance in addition to cause human- and environmental harm. This emphasises an inescapable need for sustainable alternatives and integrated practices. Moths rely on species-specific female-emitted sex pheromones for communication and reproduction, a chemical ecology paradigm that can be used for pest control, either by trapping males with pheromone baits or masking female pheromone trails with their own pheromones. Such an approach does not carry any risk for humans, other species or the environment, and has proven successful for many crops. Pheromones can be made biotechnologically with a potential for green and efficient production, using moth pheromone biosynthesis enzymes expressed in for example yeast.

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Popular science summary

Insects are the largest group of animals on our planet. More than one million insects have now been described, and it is expected that several million have yet to be discovered by humans. The study of insects is called entomology, and the origins of this word and the word insect are the same, meaning “cut up”, referring to the bodies of insects being divided in three parts: head, thorax (chest) and abdomen (rest of body following the chest). Insects are also characteristic by having wings and three pairs of legs. Their life cycle starts with an egg that develops into a larva, which in turn transforms into an adult via a pupal stage or via successive shedding of their exoskeleton skin.

The largest groups of insects are Coleoptera (beetles), Lepidoptera (moths and butterflies), Diptera (flies) and Hymenoptera (wasps, ants, and bees). Insects have successfully colonized almost all Earth´s terrestrial habitats and play many roles in natural ecosystems. This includes plant pollination, food for other animals, acting as decomposers and soil improvers, and cycling nutrients. In an anthropogenic (human) view, they provide ecosystem services including pollination of crops, honey, silk, food, and animal feed. They are also pests on our crops (e.g. aphids and locusts eating plants) and carry life-threatening diseases (e.g. mosquitoes spreading malaria). Human life and agriculture and our use of nature is affecting many insects in a negative way, causing decline in numbers and biodiversity.

Moth larvae eat many of our crop plants and are often controlled with insecticides. Unfortunately, this is not sustainable as insecticides can harm humans and the environment and causes resistance in the insects. New methods are needed to control moth pests. One way is developing solutions based on natural systems. Moth communication and mate finding relies on sex pheromones, and this can be used in pest control. Release of excess pheromones in a crop can prevent males from finding females, and no larvae will develop to eat the crops. Using pheromones instead of insecticides can be effective but is more expensive. Therefore, we need to develop cheaper production methods, such as biotechnological production with the use of insect enzymes as catalysts, in yeast that can be cultured at a large scale. We also need research to develop the technology for such production, i.e. discovery of insect enzymes to produce specific pheromones, and optimization of the foreign pheromone biosynthesis pathway once integrated into the yeast cell.

In the first part of this thesis work I show that yeast produced pheromones can be effective in the field. We produced a pheromone in the yeast *Yarrowia lipolytica* and used it successfully in field traps in Greece, for a pest on cotton, the cotton bollworm *Helicoverpa armigera*. *Yarrowia lipolytica* is a naturally abundant yeast, you can find it many places from in soil to meats and cheeses. It is very good at accumulating oils and lipids and is therefore a good host for production of lipid-derived moth pheromones. Extensive metabolic engineering of the yeast was necessary to produce a good amount of pheromone, i.e. we had to integrate several copies of the pheromone biosynthesis pathway and eliminate some of the host´s own use of lipids in its native processes, by deletion or down-regulation of genes.

In the second part of this thesis work, I study how pheromones are made in three moth species, with the aim of discovering enzymes that can be used for biotechnological production of pheromones. I do this by identifying lipids in the female pheromone gland, where the lipid-derived pheromones are made. The lipids present here can indicate which molecules pheromone biosynthesis starts from (pheromone "precursors"). When we know the pheromone precursors, we can draw a step-by-step biosynthesis pathway from seeing how they are incorporated into the pheromone. The precursors are applied externally to the pheromone gland, while the female is in the process of making pheromones. This can reveal the start, the end and the intermediate steps in pheromone biosynthesis, and from these intermediate molecules we can see which enzymatic functions are needed for the process. This lets us know which enzymes and genes we are looking for when we sequence all the genes in use in the pheromone gland. The genes can then be integrated into a yeast cell in the lab, and we can check if the genetically modified yeast is able to produce moth pheromones or intermediate molecules.

The sugarcane borer *Diatraea saccharalis* is a pest in the enormous sugarcane production taking place in Brazil. It is currently controlled using insecticides, which are not effective as larvae are protected inside the plant, and by release of huge numbers of parasitoid wasps that lay their eggs in the larvae and thereby kill them. Pheromones are not yet used as part of pest control. In my thesis, I uncover the sex pheromone biosynthesis pathway for this species, and characterize the key enzymes involved. I also show that we can produce the pheromone precursors in yeast.

The oriental fruit moth *Grapholita molesta* is a pest on peach production in China, but also on other fruit crops. Pheromones are already used in production areas to control the pest. In my thesis, I uncover the sex pheromone biosynthesis pathway for this species, particularly by deleting one enzyme from the genome and creating a moth that can no longer produce pheromone. This shows the key role of this enzyme in the pathway.

The tomato leafminer *Tuta absoluta* is a pest on tomato production, and has recently become an invasive pest in Europe, Africa and Asia, from its origin in South America. Pheromone traps are used in greenhouses to control this pest. In my thesis,

I describe many experiments we have done to try to reveal the pheromone biosynthesis pathway in this species. Its pheromone is more complex and comprise unusual molecules compared to the previous two moths, and it proved a challenge for us to solve this mystery with our conventional methodology. Therefore, work remains to be done. We have not been able to confirm which molecules are precursors for the pheromones, which enzymes are involved, or even if the pheromone is biosynthesised by any of the conventional pathways currently described for moth pheromone biosynthesis.

Populärvetenskaplig sammanfattning

Insekter är den största djurgruppen på vår planet. Mer än en miljon insektsarter har beskrivits, och man räknar med att flera miljoner ännu inte har upptäckts av människan. Studiet av insekter kallas entomologi, och ursprunget till detta ord och ordet insekt är detsamma, vilket betyder "uppskuren", med hänvisning till att insekternas kroppar är uppdelade i tre delar: huvud, mellankropp och bakkropp. Insekter kännetecknas också av att de har vingar och tre par ben. Deras livscykel börjar med ett ägg som utvecklas till en larv, som i sin tur förvandlas till en vuxen via ett puppstadium eller genom successivt fjällande av exoskelettets hud.

De största grupperna av insekter är Coleoptera (skalbaggar), Lepidoptera (fjärilar), Diptera (tvåvingar) och Hymenoptera (steklar). Insekter har framgångsrikt koloniserat nästan alla jordens terrestra livsmiljöer och har många funktioner i naturliga ekosystem. De pollinerar växter, är föda för andra djur, fungerar som nedbrytare och jordförbättrare samt bidrar till näringsämnenas kretslopp. Ur ett antropogent (mänskligt) perspektiv tillhandahåller de ekosystemtjänster som pollinering av grödor, honung, silke, livsmedel och djurfoder. De är också skadedjur på våra grödor (t.ex. bladlöss och gräshoppor som äter växter) och bär på livshotande sjukdomar (t.ex. myggor som sprider malaria). Mänsklig aktivitet, jordbruk och vår användning av naturen påverkar många insekter på ett negativt sätt och orsakar en minskning av antalet insekter och den biologiska mångfalden.

Fjärilslarver äter många av våra grödor och bekämpas ofta med pesticider. Tyvärr är detta inte hållbart eftersom dessa kan skada människor och miljö och orsaka resistens hos insekterna. Nya metoder behövs för att kontrollera dessa skadegörare. Ett sätt är att utveckla lösningar som bygger på naturliga system. Nattflyns kommunikation och partnersök är beroende av könsferomoner, och dessa kan användas för skadedjursbekämpning. Om man släpper ut ett överskott av feromoner i en gröda kan man hindra hanarna från att hitta honor, och inga larver kommer att utvecklas för att äta upp grödan. Att använda feromoner i stället för pesticider kan vara effektivt men är dyrare. Därför behöver vi utveckla billigare produktionsmetoder, till exempel bioteknologisk produktion med användning av insektsenzymer som katalysatorer, i jäst som kan odlas i stor skala. Vi behöver också forskning för att utveckla tekniken för sådan produktion, dvs. upptäckt av insektsenzymer för att producera specifika feromoner och optimering av biosyntesvägen för främmande feromoner när den väl har integrerats i jästcellen.

I den första delen av denna avhandling visar jag att jästproducerade feromoner kan vara effektiva i fält. Vi producerade ett feromon i jästen *Yarrowia lipolytica* och använde det framgångsrikt i fältfällor i Grekland, för en skadegörare på bomull, bomullsknölfly *Helicoverpa armigera*. *Yarrowia lipolytica* är en naturligt förekommande jästsvamp som kan hittas på många ställen, från jord till kött och ost. Den är mycket bra på att ackumulera oljor och lipider, och är därför en bra värd för produktion av lipidderiverade feromoner från nattflyn. Omfattande metabolisk modifiering av jästen var nödvändig för att producera en bra mängd feromon, dvs. vi var tvungna att integrera flera kopior av feromonets biosyntesväg och eliminera en del av värdens egen användning av lipider i sina naturliga processer, genom radering eller nedreglering av gener.

I den andra delen av denna avhandling studerar jag hur feromoner tillverkas hos tre nattfjärilar, med målet att upptäcka enzymer som kan användas för bioteknologisk produktion av feromoner. Detta gör jag genom att identifiera lipider i honans feromonkörtel, där de lipidderiverade feromonerna tillverkas. De lipider som finns här kan indikera vilka molekyler som feromonbiosyntesen startar från (feromonets s.k. prekursorer). När vi känner till feromonprekursorerna kan vi rita upp en biosyntesväg steg för steg genom att se hur de införlivas i feromonet. Förstadierna appliceras externt på feromonkörteln, medan honan är i färd med att tillverka feromoner. Detta kan avslöja början, slutet och de mellanliggande stegen i feromonbiosyntesen, och från dessa mellanliggande molekyler kan vi se vilka enzymatiska funktioner som behövs för processen. Detta gör att vi vet vilka enzymer och gener vi letar efter när vi sekvenserar alla de gener som används i feromonkörteln. Generna kan sedan integreras i en jästcell i labbet, och vi kan kontrollera om den genetiskt modifierade jästen kan producera fjärilsferomoner eller mellanliggande molekyler.

En art gräsmott, *Diatraea saccharalis* (sugarcane borer på engelska), är en skadeinsekt i den enorma sockerrörsproduktion som sker i Brasilien. Den bekämpas för närvarande med pesticider, som inte är särskilt effektiva eftersom larverna skyddas inne i växten, och genom utsättning av stora mängder parasitsteklar som lägger sina ägg i larverna och därmed dödar dem. Feromoner används ännu inte som en del av skadedjursbekämpningen. I min avhandling påvisar jag biosyntesvägen för könsferomoner för denna art, och karakteriserar de viktigaste enzymerna som är inblandade. Jag visar också att vi kan producera feromonets prekursorer i jäst.

Aprikosvecklare *Grapholita molesta* är en skadeinsekt framför allt inom persikoproduktionen i Kina, men även på andra frukter. Feromoner används redan i produktionsområden för att kontrollera skadeinsekten. I min avhandling beskriver jag biosyntesvägen för könsferomoner för denna art, genom att radera ett enzym från genomet och skapa en vecklare som inte längre kan producera feromon. Resultaten visar att detta enzym har en nyckelroll i processen.

Tomatmal *Tuta absoluta* är en skadegörare i tomatodlingar och har nyligen spridits från Sydamerika till Europa, Afrika och Asien, och kategoriseras som invasiv. Den kommer ursprungligen från Sydamerika. Feromonfällor används i växthus för att bekämpa denna skadegörare. I min avhandling beskriver jag många experiment som vi utfört för att försöka avslöja feromonets biosyntesväg hos denna art. Dess feromon är mer komplext och består av ovanliga molekyler jämfört med de två föregående insektsarterna, och det visade sig vara en utmaning för oss att lösa detta mysterium med vår konventionella metodik. Därför återstår mycket arbete. Vi har inte kunnat bekräfta vilka molekyler som är prekursorer för feromonerna, vilka enzymer som är inblandade, eller ens om feromonerna syntetiseras genom någon av de konventionella syntesvägar som tidigare beskrivits för biosyntes av feromoner hos nattfjärilar.

List of papers

Paper I

Holkenbrink C, Ding B-J, Wang H-L, **Dam MI**, Petkevicius K, Kildegaard KR, Wenning L, Sinkwitz C, Lorántfy B, Koutsoumpeli E, França L, Pires M, Bernardi C, Urrutia W, Mafra-Neto A, Ferreira BS, Raptopoulos D, Konstantopoulou M, Löfstedt C, Borodina I (2020) Production of moth sex pheromones for pest control by yeast fermentation. Metabolic Engineering 62:312-321. doi:10.1016/j.ymben.2020.10.001

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Paper IV

Dam MI, Wang H-L, Montes ZO, Konstantopoulou M, Löfstedt C Elucidation of the biosynthesis of the unusual sex pheromone of the tomato leaf miner *Tuta absoluta* requires new approaches. Manuscript

Author contribution to papers

Paper I

IB and CL conceived the study. CH, BJD, HLW, MID, KP, KRK, LW, CS and BL performed the experiments on pheromone production in yeasts. LF, MP, and BF carried out fermentation in controlled bioreactors and extracted the pheromones. CB, WU, and AM-N purified and oxidized the sample for activity tests. EK, DR, and MK performed laboratory and field tests of pheromones. IB drafted the manuscript and all the authors have contributed to writing.

Paper II

CL, MID, and HLW conceived and designed the study. CL, GPS, and PHGZ obtained funding. MID, BJD, HLW, GPS, JML and DJM conducted the experiments and analysed data. MID drafted the manuscript with inputs from all authors. All authors read and approved the final version of the manuscript.

Paper III

CL, MID, HLW and DGH conceived and designed the study or parts thereof. CL obtained funding. MID, BJD, KB, HLW and DP conducted the experiments and analysed data. MID drafted the manuscript with input from all authors. All authors read, revised, and approved the final version of the manuscript.

Paper IV

CL, MID and HLW conceived the study. CL and MK obtained funding. MID, HLW, ZMO and MK conducted the experiments and analysed data. MID drafted the manuscript with inputs from all authors. All authors read and approved the final version of the manuscript.

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Abbreviations

ACC	Acyl-CoA carboxylase
AFT1	Acetyl transferase from *Saccharomyces cerevisiae*
Bt	*Bacillus thuringiensis*
DAG	Diacyl glyceride
DGAT	Diacylglycerol acyltransferases
DMDS	Dimethyl-disulfide
ELO1	Elongase I in *Saccharomyces cerevisiae*
FAME	Fatty acid methyl ester
FAO1	Fatty alcohol oxidase in *Yarrowia lipolytica*
FAS	Fatty acid synthase
GC/EAD	Gas chromatography coupled to electroantennographic detection
GC/MS	Gas chromatography coupled to mass spectrometry
GPAT	Glycerol-3-phosphate acyltransferase
HFD	Fatty alcohol dehydrogenases in *Yarrowia lipolytica*
IPM	Integrated pest management
LPAT	lysophosphatidyl acyltransferase
OLE1	Δ9 fatty acyl desaturase in *Saccharomyces cerevisiae*
PEX10	The peroxisome biogenesis/synthesis gene in *Yarrowia lipolytica*
POX	acyl-CoA oxidases
Sf9	A specific *Spodoptera frugiperda* cell line
TAG	Triacyl glyceride

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Honglei. You are amazing. You make this world better. You understand what is important, “life is what is important” you said. You can do this and that, productivity and efficiency, but in the end and every day, life is what is important. You are good at listening, and thinking slowly and deeply about things, distilling (pun!) what is real and important. The family you help raise will be a force in this world. And thanks for all the wonderful tea.

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Tini. Wow. Your attitude and ever positive behaviour brought back some light for me, and that´s putting it lightly, too. Go, tiny ones, go! You make me think about how cool everything around us is. And you emphasize one of the main take-homes for me during this experience: it´s galaxies better not to work alone. I would always want you on my team! And you are an organising genius.

Dan and Jean-Marc and Zaide, thanks for being my bioinformatics gurus. You have met every expectation, teaching me exactly what I came here for. These are skills for the future.

Erling. Actually, most of all thank you for being an advocate for plants and birds of this world. And all the rest of your indispensable management.

Thank you, pheromone group present and past members, who all inspire and help me. Thank you, Glenn and Douglas, for contributing, and contributing to, the *Diatraea* project. Thanks to all you who have aided with scientific support in Ekologihuset and Biologihuset.

Other supervisors and mentors along the way: Wolfgang, David, Klas, Astrid, Jadranka and Marjorie. Thanks for general support, development of ideas and writing advice. You help me see what a nice environment working in science can be. New perspectives drive learning and understanding. And thanks to all the people I have asked about how you got your relationship to work in early days when you both were committed to the international scientific pursuit – the answer is: it is hard. And I have

learned that some people need less time with their partner and family than others. I like learning about others. You have to prioritise the life that is right for you.

Bec, Jana, Zaide, Emma, Twinkle. I probably told you that I feel like a social imposter. You make me think that is completely backwards. I will accept this. "*Now I know the things I know, and do the things I do, and if you do not like me so, to hell, my love, with you.*" Dorothy Parker says it well. Thank you for being excellent and sexy. Like a soft fox with its head in the wind. Thank you for discussions on philosophy and psychology. Let´s keep trying to understand others, and ourselves. Thank you, Qinyang, for your humour. You described my work as "Moth killing mission" after BLAM22, where I in my flash talk presented what I (really) do. I guess I can also be funny. In the spooky pheromone corridor! Moth-killing makes me think this is the darkest timeline… But that can be said for many things in this world. How alive it makes me feel when I can share things I am excited about, with you all.

Courses: I enjoyed all of it, I learned so much. Great teachers. Evolution! Statistics and R! Bioinformatics! All my dreams come true when I learn new things. And I got to go to amazing places in the world and meet new friends. Venkatesh at icipe in Nairobi – I was wonder-eying insects and birds with Megha and Devasena while you were happy to follow along (with philosophy). Amsterdam reprive in gloomy October. Parrots in the park! Thank you, Astrid and David, I am so grateful for this adventure into CRISPR.

Teaching: Humberto, Teresa, Aivars! I have enjoyed working with you immensely, and this teaching thing is part of my future.

Åsa Burman and her "Finish on time" course and book, which has led me down a rabbit hole of learning about happiness, productivity, mental health, and essentialism. Thanks to all those creators and authors out there crafting inspiring content about these topics. Journey before destination. Look at the side of wonder.

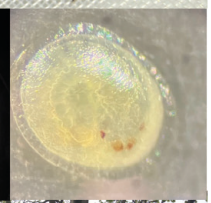
Thank you, Per Lundberg, for telling me in the "Introduction to Biology" course that I am not here to publish papers. I am not here to work for my supervisor. I am here for my own development. Thanks also for introducing the idea that possibly science writing does not have to be "dry". This motivates me to create new paths that do not follow the given framework or necessarily play by all the rules. I mean, we are here to create new paradigms, otherwise, where is the fun?

If any of you ever need help with anything, please ask me. I promise you, that will contribute greatly to my happiness. I believe I am quite good at (and enjoy) things such as researching and making fun presentations about interesting science topics or books, doing writing and figures (and colours!), cooking and baking, being a naturalist and appreciating the wonders of nature and the world around me. Also being direct (rude?) and getting to the point of things. I strive to understand both idealism and pragmatism. And I almost feel like a biologist now.



















Introduction

This is a review of the genetics behind sex pheromone biosynthesis in moths, how moths use sex pheromones for communication, and how sex pheromones can be used for pest control. It is also about how pheromones can be produced biotechnologically leveraging moth biosynthesis genetics, the necessity of developing such a production and alternative pest control methods, and the impact of insect pests.

Agricultural insect and moth pests and integrated pest management

Invasive species are at increasing rates becoming major threats to natural ecosystems, agriculture, and forestry. Large crop producers and developing countries are especially vulnerable to this pressure, and countries with large trade volumes or diverse crops are the greatest sources of invasive species affecting agriculture (Paini et al. 2016). Invasive insects are the animal group with the largest economic impact on human society. Yield loss and pest management costs are estimated to US$70 billion per year globally (Bradshaw et al. 2016). This is considered an underestimation, with studies lacking or sporadic, and especially the cost of reduction in ecosystem services is rarely studied. Some of the more costly insects are the formosan subterranean termite *Coptotermes formosanus* (Blattodea: Rhinotermitidae), the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae), the brown spruce longhorn beetle *Tetropium fuscum* (Coleoptera: Cerambycidae) and the spongy moth *Lymantria dispar* (Lepidoptera: Lymantriidae) (Bradshaw et al. 2016). *Coptotermes formosanus* comes from China and causes an estimated cost of US$1 billion per year worth of damage to wooden structures and live trees especially in the USA (Lax and Osbrink 2003). *Lymantria dispar*, introduced to USA from Europe in the 1800s, costs around US$3.2 billion per year in damage to trees and shrubs and the control to slow the spread (Tobin and Blackburn 2007). *Tetropium fuscum* causes damage to spruce trees, especially in Canada, costing US$4.5 billion per year (Bradshaw et al. 2016). *Plutella xylostella* is one of the most widely distributed moth pests, causing damage to cruciferous crops (Brassicaceae, the cabbage family) with an annual cost of control and yield loss estimated at US$4-5 billion (Zalucki et al. 2012). Insects are also economically

important pests on three of our largest food crops: maize, wheat and rice. The fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is a key pest on maize (also affecting rice, sorghum, soybeans and others), aphids (Hemiptera) on wheat, and several stem borers (Lepidoptera) on rice loss (Harrison et al. 2019; Savary et al. 2019).

The global eradication database GERDA has registered many government-lead programs targeting moth pests (caterpillars is the life stage causing damage), including against *L. dispar*, the codling moth *Cydia pomonella* (Tortricidae), the light-brown apple moth *Epyphias postvittana* (Tortricidae), the tomato leafminer *Tuta absoluta* (Gelechiidae), the cotton bollworm *Helicoverpa armigera* and the European grapevine moth *Lobesia botrana* (Tortricidae) (Suckling et al. 2017).

Spread and establishment of invasive insects is a complex process, but successes are largely due to the aid of human dispersal and global trade, and global warming (Garnas et al. 2016; Campos et al. 2017). Invasive insects are often good at adapting to new environments with fast population growth and multiple generations per year (Garnas et al. 2016). Preventing invasions may be impossible, because of global trade and lack of control standards. The only option might be development of management strategies, where early response and effective biosecurity can be cheaper than the cost of damages (Bradshaw et al. 2016; Garnas et al. 2016). Establishment of international protocols for biosecurity is one of the purposes of the International Plant Protection Convention (IPPC), the Food and Agriculture Organization of the United Nations (FAO) and others. Such protocols involve surveillance, quarantine, and phytosanitary measures such as irradiation-, heat-, methyl bromide-, high CO_2- or low O_2 treatments, at the level of harvesting, storage and transport of produce (Garnas et al. 2016).

Lepidopteran and other insect pests have traditionally been managed using chemical insecticides and Bt crops (insecticidal *Bacillus thuringiensis* proteins used in biocides and genetically modified crops), but this practice is considered unsustainable due to emerging resistance and/or negative effects on human health and environment, and many insecticides are being phased out (Vreysen et al. 2016; Suckling et al. 2017). The future paradigm of crop protection is by many governments and agricultural organisations seen as an integrated pest management (IPM) approach, using a multitude of methods and limiting the use of chemical insecticides. Strategies involve optimised cultural practices, crop diversity and rotations, using naturally resistant cultivars, priming of intrinsic plant defences, biological control with predators, parasitoids or entomopathogens (including Bt proteins), the release of sterile insects (including gene drives/super-Mendelian inheritance) and the use of semiochemicals (chemicals mediating inter- and intraspecific interactions, including pheromones) (Stenberg 2017).

Biological control with predators and parasitoids is one of the most studied approaches in lepidopteran IPM, targeting egg-, larval- or pupal stages of the pest insect. An example is how some control of *P. xylostella* is achieved in areas in Asia,

USA, Australia and New Zealand with a few species of hymenopteran parasitoids (Sarfraz et al. 2005; Furlong et al. 2013).

The use of semiochemicals in IPM includes both pheromones (mediating intraspecies communication) and allelochemicals (mediating interspecies interactions). The latter can be exemplified by control of *S. frugiperda* in maize plots in East Africa, where intercropped non-maize plants are used to repel the adult moth and attract it to a different host plant (Midega et al. 2018). Border plants can also be used to attract and increase the biodiversity of predators, like planting flower strips in apple orchards to control aphids (Jacobsen et al. 2022).

The use of pheromones targets the adult insect and affects intraspecies communication. This can be female sex pheromones that attract males, or aggregation pheromones that attract both sexes. Traps that retain the lured insects can be used for monitoring population densities or detection of newly invasive species, or for attract-and-kill (traps that also contain insecticides). This is widely used in agriculture, forestry and for stored products, not only for Lepidoptera but also Coleoptera and Diptera (Witzgall et al. 2010). Pheromones are species-specific and are not harmful to animals or beneficial insects, and they are used in such low quantities that no residuals are left on food crops upon human consumption (Witzgall et al. 2010). Pheromones can be used for species where the pupal or larval life stages are protected inside the plant, something that is difficult to achieve with conventional insecticides. The female sex pheromones of moths can be used for control by mating disruption. Release of synthetic pheromones in the field can mask the pheromone trail of a calling (emitting) female, thereby a male from finding her, and disrupting mating (**Figure 1**). Mating disruption was first demonstrated in the 1960s (Baker et al. 2016), and e.g. in Europe it is successfully used to reduce population densities of *C. pomonella* (Witzgall et al. 2008) and *L. botrana* (Ioriatti et al. 2008) in orchards and vineyards, respectively (Ioriatti et al. 2008; Witzgall et al. 2008). Witzgall et al. (2010) has reviewed the global use of sex pheromones in lures and for mating disruption, describing > 40 lepidopteran-, coleopteran- and dipteran species for which these pheromones are commercially available, and an area of 770 000 ha being under mating disruption at that time. An area of 100 000 ha grapevine was under *L. botrana* mating disruption management (EU and Chile).An area of 210 000 ha of apple and pear orchards was under *C. pomonella* mating disruption worldwide, which could be successfully controlled with 100 g of pheromone per ha (Witzgall et al. 2008).

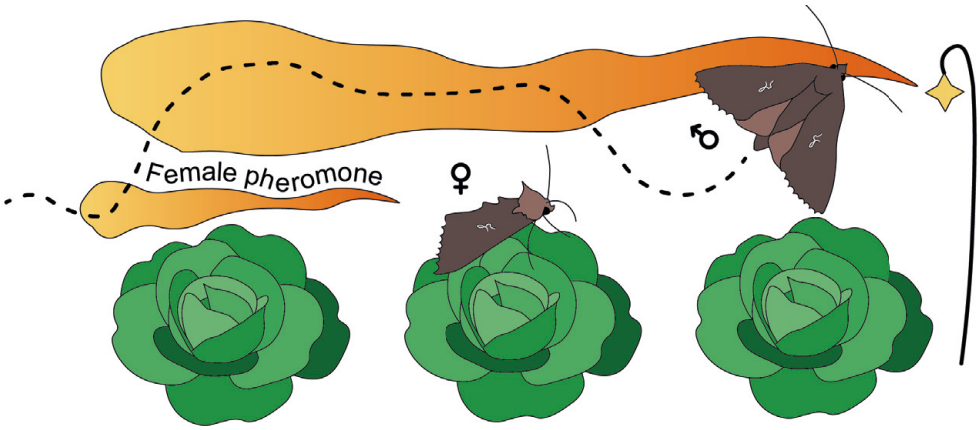


Figure 1 Mating disruption takes advantage of the pheromone communication and reproduction paradigm in moths. A male will follow the female sex pheromone trail upwind to locate her, but release of synthetic pheromone (yellow star dispensers) in the field can mask this trail.

The efficiency of mating disruption depends on the quality of the synthetic pheromone blend, pest population density, dispenser density and release rate and weather conditions. Pheromones dispensers are either manually applied, or dispersed by for example the Specialised Pheromone And Lure Application Technology SPLAT™ (Suckling 2015). The current largest mating disruption application is for *L. dispar* in USA, where pheromone-coated flakes are dispersed by plane (Tobin and Blackburn 2007). Challenges with the use of pheromones in IPM is especially that the technology development and production need a lot of research, can be expensive, and bringing a semiochemical product to market is difficult and takes time because of government regulations (Suckling 2015). Another downside to using pheromones compared to conventional insecticides is that often several pests are controlled at the same time with insecticides, something that is not achieved with highly selective pheromones that only target one species (Welter et al. 2005).

Insect communication and moth sex pheromones

The reason we can use pheromones to control insects is because many insect behaviours rely on these chemical signals (semiochemicals). Insects communicate with each other, interpret their heterogenous environments and modify their behaviours based on a sensory complexity responding to cues relying on vision, olfaction (smell), gustation (taste), humidity, temperature and/or mechanical stimuli (like hearing and airflow) (Gullan and Cranston 2014). Sex pheromones are chemical signals that affect mate-finding and reproduction. Pheromones can also

function as kairomones, i.e. signals being a disadvantage to the producer by attracting predators, or as allomones, i.e. signals being advantageous to the producer by repelling predators (Wyatt 2014). Pheromones are a good example of chemical ecology, the study of chemical cues that mediate interactions between organisms and their environment.

Moths rely on olfaction and volatile chemical signals for finding host plants and a mate. Sensilla on their antennae are porous and odours can enter and reach receptors in sensory neurons with the help of odorant-binding proteins and diffusion through the sensillum lymph. The receptors can be highly specific and sensitive to even very low amounts of ligand (Gullan and Cranston 2014). Moth sex pheromones are chemicals often based on the structure of naturally occurring and ubiquitous compounds, such as host plant compounds or common metabolites like fatty acids. A good review on moth pheromones is Allison and Cardé (2016), a collection of articles about their evolution, effect on behaviour, and application. Sex pheromones are in both quality (components/molecules) and quantity (component ratio) very species-specific blends of often similar compounds produced by female moths and released at a diel periodicity (depending on the light (photophase) and dark (scotophase) periods of the day) to attract males at a long distance by affecting their upwind flight orientation. Moth sex pheromones are classified based on their structure and biosynthesis, reviewed in Löfstedt et al. (2016). Approximately 75 % of compounds identified belong to the Type I class (**Figure 2**), which are straight-chain saturated or unsaturated fatty acids of length C_{10}-C_{18}, with a terminal alcohol, acetate or aldehyde group (like (*Z*)-hexadecenal (Z11-16:Ald) of *Helicoverpa armigera* (Noctuidae)). Some 15 % belong to the Type II class, unbranched and multiply-unsaturated hydrocarbons (mostly methylene-interrupted) of length C_{17}-C_{25} (like (*Z,Z,Z*)-1,3,6,9-nonadecatetraene (1,Z3,Z6,Z9-19:H) of the winter moth *Operophtera brumata* (Geometridae)), some with epoxy- or other functional groups. Other, minor, classes of sex pheromones are structures such as branched hydrocarbons with or without functional groups (Type III), short-chain saturated or unsaturated secondary alcohols or ketones (Type 0), structures with very unusual functional groups or terpenoid structures (unclassified). Approximately 2000 sex pheromones or attractants have been described for Lepidoptera, from 23 superfamilies, to which the vast majority of the 160.000 described species belong (**Figure 3**) (Löfstedt et al. 2016).

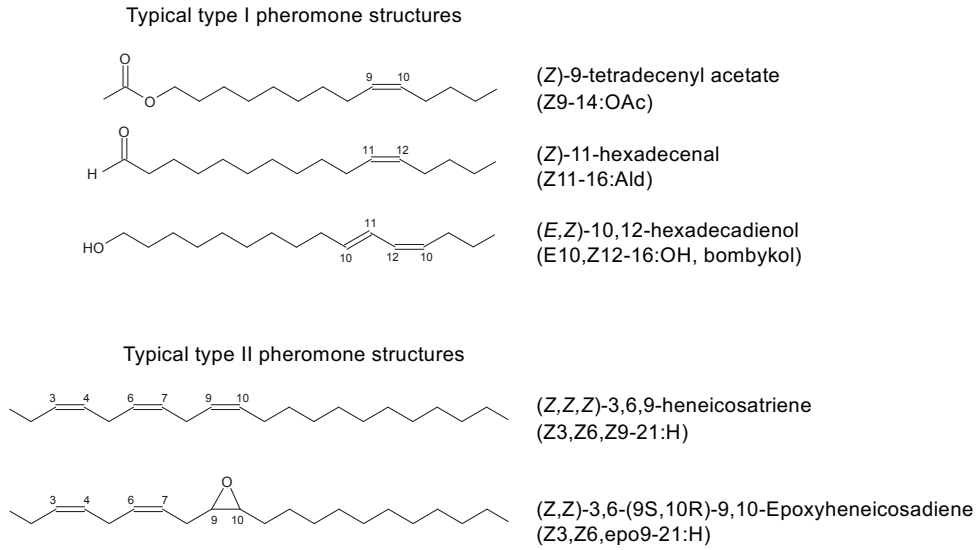


Figure 2 Structures of some typical Type I and Type II moth sex pheromone components. Z9-14:OAc is used by e.g. the fall armyworm *Spodoptera frugiperda* (Noctuidae), Z11-16:Ald by e.g. the cotton bollworm *Helicoverpa armigera* (Noctuidae) and bombykol by the silkworm moth *Bombyx mori* (Bombycidae). Z3,Z6,Z9-21:H is used by e.g. the Bella moth *Uthetheisa ornatrix* (Erebidae), Z3,Z6,epo9-21:H by the saltmarsh caterpillar moth *Estigmene acrea* (Erebidae).

Patterns in pheromone diversity can be used to corroborate and elucidate evolutionary relationships within Lepidoptera, where similar pheromone molecule structures can be seen within families. For example, within the superfamily Tortricoidea (> 10.000 species), where many species have been studied because they are important pests, the subfamily Tortricinae exhibit a general trend of Type I C_{14} pheromones with Δ^9 or Δ^{11} double bonds (like (*E*)-11-tetradecenyl acetate (E11-14:OAc) in *Epiphyas postvittana*) and the subfamily Olethreutinae has Type I C_{12} pheromones with Δ^8 or Δ^{10} double bonds (like (*E,E*)-8,10-dodecadienol (E8,E10-12:OH) in *Cydia pomonella*). Type I pheromones are seen throughout most of the lepidopteran order, a prominent exception is in the Papilionoid clade (the butterflies), where long-distance female-produced sex pheromones are not used at all – although similar compounds and biosynthesis can be found in male-produced courtship pheromones. Fewer Type II pheromones have been studied, but they have been found both in a basal lineage of the order and in more recent expansions, within the Pyraloid clade and Macroheterocera, evidence that they have evolved independently several times.

Sex pheromones also play a role in speciation, where for example a mutation in the biosynthetic pheromone pathway can lead to a difference in the pheromone blend quality or blend ratio, eventually resulting in reproductive isolation (Smadja and Butlin 2009; Lassance and Löfstedt 2013).

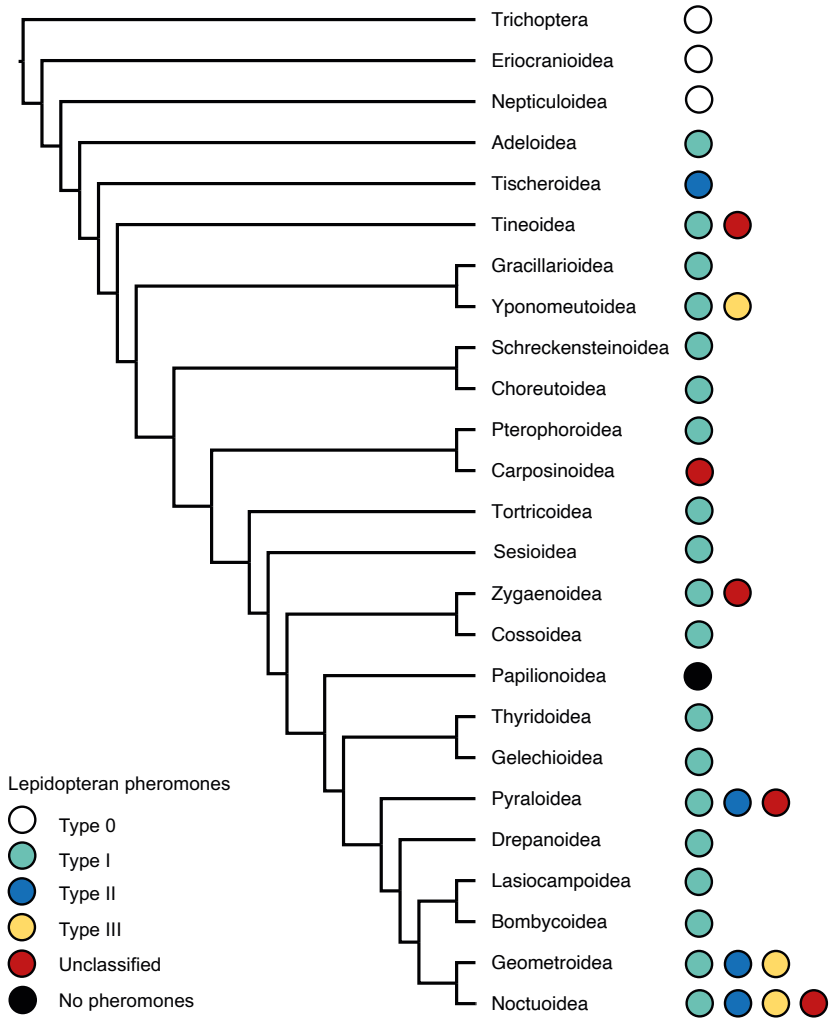


Figure 3 Phylogenetic hypothesis (cladogram) of Lepidoptera showing identified sex pheromone/attractant types in the different superfamilies, including the sister order Trichoptera (caddiesflies). Figure adapted from Löfstedt et al. (2016).

Sex pheromone biosynthesis and enzymes involved

Type I moth sex pheromones are synthesised *de novo* from acetate in a specialised pheromone gland in the female moth, located at the terminal segments of the abdomen (Percy-Cunningham and Macdonald 1987). Double bonds are mostly made by lepidopteran-specific Δ^9 or Δ^{11} fatty acyl desaturase enzymes (**Figure 4**) (Groot et al. 2016). These are evolved from the ubiquitous metabolic Δ^9 stearoyl-

CoA desaturase involved in regular fatty acid synthesis in most organisms (Wolf and Roelofs 1986; Liénard et al. 2008). The desaturases involved in Type I pheromone biosynthesis are preferentially expressed in the pheromone gland. Cis (*Z*) double bonds are most common, but trans (*E*) double bonds also occur. Desaturation is often proceeded or followed by controlled β-oxidation and chain shortening in the peroxisomes, removing two carbons at a time, or by chain elongation (Roelofs and Bjostad 1984). The final volatile pheromone or a precursor is formed by reduction of the terminal carboxyl group to an alcohol by enzymes specific to the lepidopteran expansion of the fatty acyl reductase family (Moto et al. 2003; Liénard et al. 2010a). The alcohol can be further modified into an aldehyde or acetate, by enzymes in the moth that has yet to be characterised.

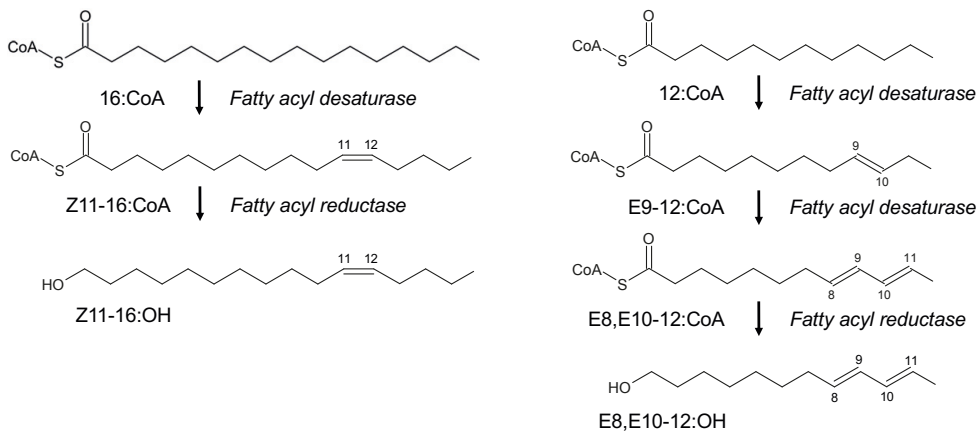


Figure 4 Moth pheromone biosynthesis pathways of Type I pheromone structures, taking place in the pheromone gland. Left: desaturation of hexadecanoic acid (16:CoA) to (*Z*)-11-hexedecenoic acid (Z11-16:CoA) and reduction to (*Z*)-11-hexedecenol (Z11-16:OH), e.g. in the cotton bollworm *Helicoverpa armigera* (Noctuidae) (Wang et al. 2005). Right: desaturation of dodecanoic acid (12:CoA) to (*E*)-9-dodecenoic acid (E9-12:CoA) and (*E,E*)-8,10-dodecadienoic acid (E8,E10-12:CoA) and reduction to (*E,E*)-8,10-dodecadienol (E8,E10-12:OH, codlemone) in the codling moth *Cydia pomonella* (Tortricidae) (Löfstedt and Bengtsson 1988).

The structure of the studied Type II pheromones suggest that these are synthesised by modification of essential larval diet-derived linoleic or linolenic acid that the insects probably cannot produce themselves (Löfstedt et al. 2016). This happens in oenocyte cells located in the abdominal cuticle (Ginzel et al. 2021). The synthesis can involve shortening or elongation of the carbon chain, additional desaturations, and final derivatisation to volatiles in the pheromone gland, like epoxidation by monooxygenases.

Synthesis of Type I pheromones in the pheromone gland is for many species controlled by a pheromone biosynthesis activating neuropeptide (PBAN), as is the transport of Type II pheromones into the pheromone gland (Matsumoto et al. 2010; Groot et al. 2016). Pheromone precursors have been shown in some species to be

stored as part of di- or triacylglycerides in lipid bodies (Matsumoto et al. 2002; Matsumoto et al. 2007; Foster et al. 2017).

Many desaturases involved in moth pheromone biosynthesis have been functionally characterised, especially those with specificity towards making Δ^9 and Δ^{11} double bonds, but also some that have Δ^5, Δ^6, Δ^{10}, Δ^{12}, Δ^{13}, Δ^{14} or terminal regiospecificity (Löfstedt et al. 2016). The desaturases can be specific or broad in their substrate selectivity, accepting either one or several fatty acid chain lengths. They can be specific with regards to the double bond position they produce, and also the ratio of *E/Z* isomers. Some desaturases involved in biosynthesis of two conjugated double bonds exhibit bifunctional properties, where one double bond is made followed by 1,4-dehydrogenation to make two double bonds. This is seen in *Cydia pomonella* (Tortricidae), where the desaturase *Cpo_CPRQ* uses 12:CoA as substrate to make (*E*)-9-dodecenoic acid (E9-12:CoA), and then turns this into (*E,E*)-8,10-dodecadienoic acid (E8,E10-12:CoA) (**Figure 4**) (Löfstedt and Bengtsson 1988; Lassance et al. 2021).

Knipple et al. (2002) analysed several lepidopteran desaturase sequences, and based on similarity divided them into lineages based on function, designated by a consensus signature motif (or several). NPVE are Δ^9 desaturases with preference for C_{18} substrates, KPSE are Δ^9 desaturases with preference for C_{16} substrates and XXXQ includes Δ^{11} desaturases and some with other regiospecificities (**Figure 5**). Another lineage are the front-end desaturases, which can introduce double bonds between an existing double bond and the carboxyl end of the fatty acid (Sperling et al. 2003). The specificity of desaturases likely depends on the shape and flexibility of the substrate channel. Aspects of this includes the opening where CoA in the acyl-CoA substrate interfaces with the enzyme, the residues at the distal end of the channel, the kink that allows introduction of a double bond and the placement and type of residues directly or indirectly in contact with the catalytic centre (Bai et al. 2015). Shorter substrates are expected to have more positional flexibility in the substrate channel and have been show to enable the production of trans double bonds at a higher rate in desaturases usually making only cis double bonds in longer fatty acyl chains (Sperling et al. 2003).

Studying the specificity of desaturases is useful both for elucidation of sequence-function relationships and for engineering enzymes for biotechnological applications. Ding et al. (2016a) have shown by gene mutation that stereospecificity (determining pheromone *E/Z* ratio) in the *Choristoneura rosaceana* (Tortricidae) and *C. parallela* desaturases is determined by a critical amino acid residue close to the catalytic centre. A similar site-directed mutagenesis study showed that the functionalities of the Δ^{11} and Δ^{14} desaturases in *Manduca sexta* (Sphingidae) could to some degree be switched by reciprocal exchange of one amino acid in the kink of the substrate channel (Buček et al. 2015). Exchanging amino acids in the distal end of the substrate channel can affect the substrate specificity (fatty acid chain length), as shown for a mouse desaturase where specificity could be changed to preferentially acting on C_{18} over C_{16} (Bai et al. 2015).

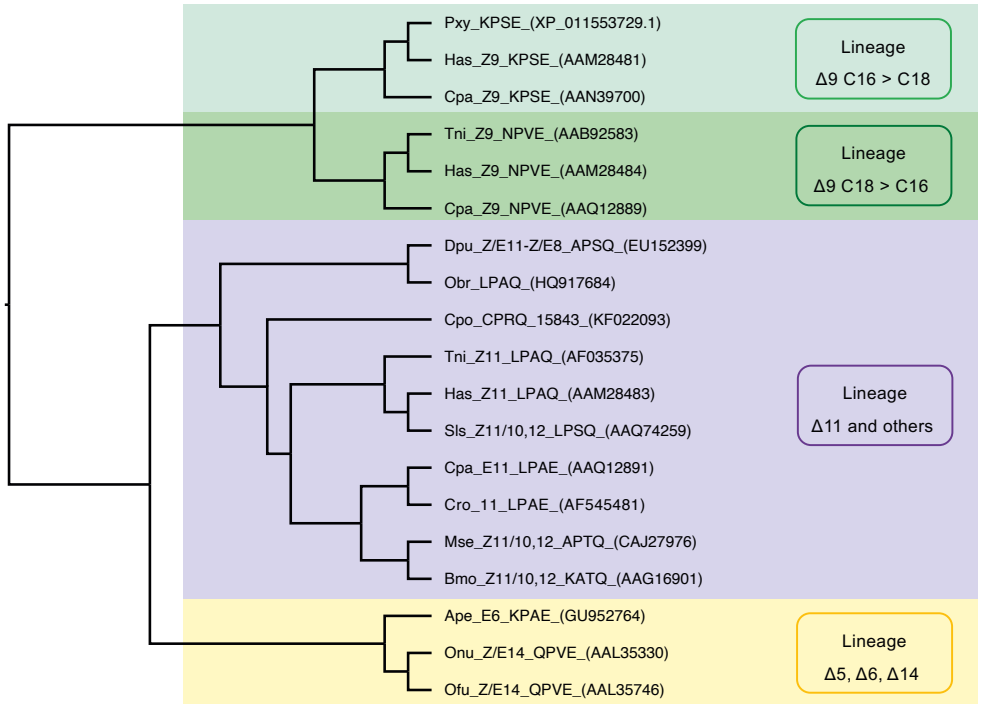


Figure 5 Simple example of phylogenetic hypothesis (cladogram) for the lepidopteran expansion of the fatty acyl desaturase gene family, based on protein sequences. Groups show functional lineages (double bond position), tip label is abbreviation of species name followed by desaturase function and/or motif and gene accession number. Ape, Chinese tussay silkworm *Antheraea pernyi* (Saturniidae); Bmo, silkworm moth *Bombyx mori* (Bombycidae); Cpa, spotted fireworm *Choristoneura parallela* (Tortricidae); Cpo, codling moth *Cydia pomonella* (Tortricidae); Cro, oblique banded leafroller *Choristoneura rosaceana* (Tortricidae); Dpu, Masson pine moth *Dendrolimus punctatus* (Lasiocampidae); Has, Oriental tobacco budworm *Helicoverpa assulta* (Noctuidae); Mse, tobacco hornworm moth *Manduca sexta* (Sphingidae); Obr, winter moth *Operophtera brumata* (Geometridae), Ofu, Asian corn borer *Osrinia furnacalis* (Crambidae); Onu, European corn borer *Ostrinia nubilalis* (Crambidae); Pxy, diamondback moth *Plutella xylostella* (Plutelliidae); Sls, Egyptian cotton leafworm *Spodoptera littoralis* (Noctuidae); Tni, cabbage looper *Trichoplusia ni* (Noctuidae).

The structure of lepidopteran desaturases is homologous to that of integral membrane desaturases that act on acyl-CoA substrates. Four trans-membrane helices are situated in the endoplasmic reticulum (ER) membrane, and conserved histidine residues surround the catalytic di-iron centre near the substrate channel, in the cytosol (Knipple et al. 2002). The desaturation reaction is oxygen- and NADH dependent and also involves NADH-cytochrome b5 reductase and cytochrome b5 for electron transport (Shanklin and Cahoon 1998).

The fatty acyl reductases in sex pheromone biosynthesis reduce fatty acyl-CoA to alcohol via aldehyde using NADPH electrons (Moto et al. 2003). These enzymes are located in the ER membrane in animals (Burdett et al. 1991). Like the desaturases, reductases can be both specific and broad in their substrate specificity,

but they are often less specific because they need to reduce all the structurally different precursors of the pheromone blend. The reductase can be responsible for the specific pheromone blend ratio, as seen for the *E*- and *Z*-strains of the European corn borer *Ostrinia nubilalis* (Crambidae), where allelic variation in the reductase gene causes the enzyme to selectively reduce the *E*- and *Z*- pheromone precursor, repectively, in the two strains (Lassance et al. 2010). Where the reductase has a broad specificity, the pheromone blend variation is determined by availability of precursors produced by the desaturases and controlled chain-shortening, as seen for several closely related *Yponomeuta* spp. (Yponomeutidae) (Liénard et al. 2010a).

Biotechnological production of pheromones

Fatty acyl desaturase enzymes of various specificities can be used to produce specific insect pheromones in a biological way, for use in pest control. The moth desaturases can be expressed in non-insect species that are used in the biotechnology industry to produce chemicals at large scales (heterologous systems, organisms expressing foreign DNA/genes). This can for example be genetically modified yeast cells fermented in bioreactors, or genetically modified plants grown in a field. This type of production is beneficial because it might be advantageous to chemical synthesis by needing less expensive substrates, solvents and catalysts, being more specific and producing less toxic waste (Wang et al. 2022). The use of pheromones in pest control is limited to a large degree by the high price of chemically synthesised pheromones compared to conventional insecticides, but biotechnological production can potentially reduce such costs.

All commercial pheromone products are currently produced by chemical synthesis, being available for > 40 species of Lepidoptera, beetles and flies (Witzgall et al. 2010). The agricultural pheromone market amounted to US$3.66 billion in 2022, and is expected to reach US$12.71 billion in 2030 (Fortune Business Insights 2021; Fortune Business Insights 2023). Commercially available semiochemicals are marketed by, among others, Russel IPM (Deeside, U.K.), Shin-Etsu Chemical Company (Tokyo, Japan), Isagro S.p.A. (Italy), ISCA Global (California, USA), Suterra LLC (Oregon, USA) and Provivi, Inc. (Colorado, USA).

100 g of active ingredient (pheromone codlemone) per ha is enough for successful mating disruption of the codling moth *Cydia pomonella* (Tortricidae) in apple orchards, 162 000 ha of which were under such treatment worldwide in 2006 (Witzgall et al. 2008). 25 tonnes of codlemone was produced in 2006, and the price was such that in a 4-year mating disruption program in Michigan, USA, farmers saved an estimated US$55-65 per ha, compared to orchards with conventional insecticide treatment (Witzgall et al. 2008; McGhee et al. 2011). This economy is the case for only a few widely used pheromones, for most the price is still higher than using conventional control methods.

Biotechnological fatty acid- and pheromone production, as an alternative to chemical synthesis, can be done from cheap renewable feedstocks or waste streams. This reduces the use of petrochemicals and takes advantage of the specificity inherent to enzymatic reactions (Li and Borodina 2015; Ortiz et al. 2020; Petkevicius et al. 2020; Löfstedt and Xia 2021). Fatty acids and derivatives are an integral part of all living organisms, natural participants in cell metabolism, cell signalling, membranes, energy storage and plant secondary metabolites. For decades metabolically engineered microbial cells have been used to produce pharmaceuticals and bulk- and fine chemicals, and fatty-acid derived compounds such as fuels, plastics, lubricants and food additives (Yu et al. 2014; Cheon et al. 2016). Fatty-acid derived compounds can be produced at high titres in oleaginous (naturally oil-producing) yeasts. 8 g/L straight-chain fatty alcohols were produced in lab-scale bioreactors in *Rhodotorula toruloides* (Fillet et al. 2015), and a yield of 25 % of dry cell weight of omega-3 fatty acids achieved in industrial-scale fermentation of *Yarrowia lipolytica* (Xie et al. 2015). Metabolic engineering of such non-model yeasts (the model yeast used in molecular biology research is baker´s yeast *Saccharomyces cerevisiae*) is made possible by recent advances in synthetic biology tools, and strategies for lipid overproduction targeting host genes such as *FAS* (fatty acid synthase), *ACC* (acyl-CoA carboxylase), *POX* (acyl-CoA oxidases), *PEX10* (the peroxisome synthesis gene), *DGA* (acyl-CoA dependent diacylglycerol acyltransferases) and *GPAT* (glycerol-3-phosphate acyltransferase) (**Figure 6**) (Tai and Stephanopoulos 2013; Darvishi et al. 2018; Zeng et al. 2018).

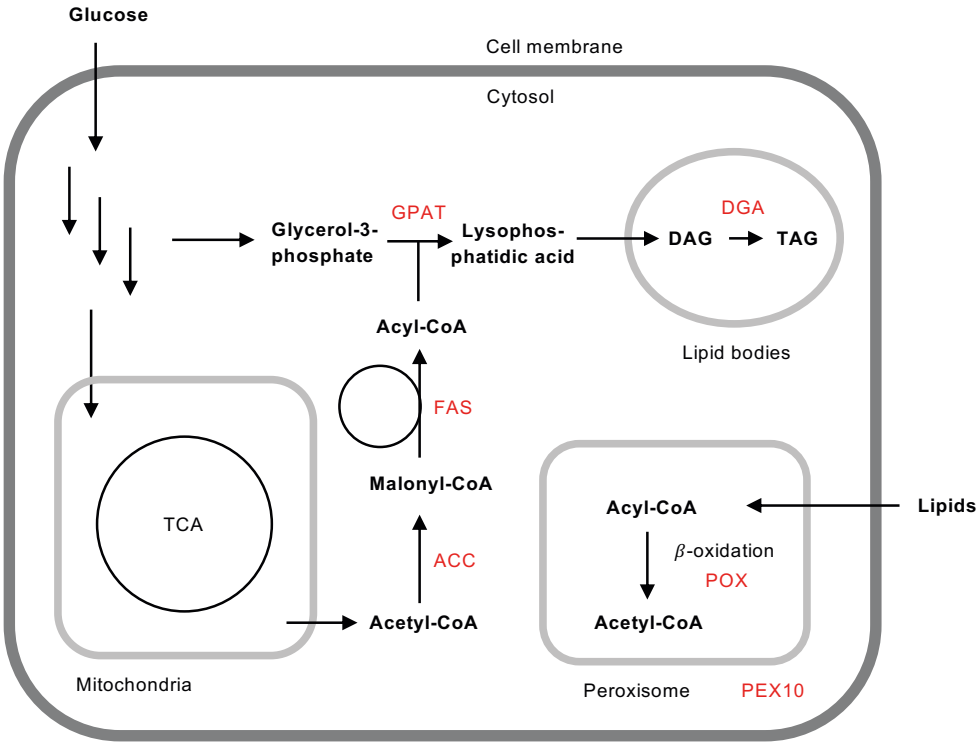


Figure 6 Schematic of *Yarrowia lipolytica* metabolism and genes (in red) often targeted in metabolic engineering for lipid overproduction and products that are derived from the acyl-CoA pool. This schematic is very simplified, with most intermediate molecules not shown, and arrows may represent several enzymatic steps. *FAS* (fatty acid synthase); *ACC* (acyl-CoA carboxylase); *POX* (acyl-CoA oxidases); *PEX10* (the peroxisome synthesis gene); *DGA* (acyl-CoA dependent diacylglycerol acyltransferases); *GPAT* (glycerol-3-phosphate acyltransferase); TCA, triacarboxylic acid cycle.

The production of pheromone precursors, and pheromone fatty alcohols and acetates have been demonstrated in yeast (Hagström et al. 2013; Ding et al. 2016b; Petkevicius et al. 2021; Petkevicius et al. 2022). If the final pheromone of a specific species is an aldehyde, only the precursor (fatty alcohol) is made in yeast, because the aldehyde is toxic to yeast. If the final pheromone is an acetate, also often only the precursor (fatty alcohol) is made, because the acetyltransferase (ATF) to do the next step is not yet known from any moth genome, although production has been demonstrated with *ATF1* from Saccharomyces *cerevisiae* (Ding et al. 2016b).

Plants can in a similar way be used for production of fatty-acid derived compounds and have for a long time been used for extraction of their natural lipid products for fuel (biodiesel) and oils. In engineered plants examples of commercial scale production of fine chemicals is oleic acid in safflower (Wood et al. 2018) and omega-3 fatty acids in rape (Petrie et al. 2010). Oil crops for large-scale production of non-native fatty acid-derived compounds should ideally be fast-growing

perennials with a high seed oil content, and species that do well in specific climates (Ortiz et al. 2020). Target genes commonly manipulated for higher flux towards fatty acid-derived products are thioesterases, WRINKLED1, DGAT (diacylglycerol acyltransferase), LPAT (lysophosphatidyl acyltransferase), GPAT and the use of seed-specific promoters (Haslam et al. 2016; Ortiz et al. 2020). Both stable and transient expression of insect pheromone biosynthesis genes have been demonstrated in plants, in tobacco *Nicotiana benthamiana* leaves or in seeds of the oilseed crop *Camelina sativa* (Nešněrová et al. 2004; Ding et al. 2014; Ortiz et al. 2020; Xia et al. 2021; Demski et al. 2022; Wang et al. 2022; Xia et al. 2022).

There are technical challenges to overcome before biotechnological production of pheromones is established to the same degree as production of insecticides. Both for yeast and plants, platform strains for high titre production of pheromones of various fatty acid chain lengths need to be developed, and high activity and specificity of enzymes ensured, possibly by enzyme engineering. For yeast, optimisation and upscaling of the fermentation process is also a challenge. Then, down-stream of biosynthesis, there are still high costs associated with purification and processing of the active ingredients. In yeast this could be circumvented by developing strains that can produce pheromone blends in natural ratios, combined with using the dried biomass directly in the field. Development of platform strains that can secrete the produced pheromones would also simplify the purification process. Pheromone-emitting plants used as intercrops could avoid the complex step of purification from seeds. This has been demonstrated with wheat engineered to emit pest aphid alarm pheromones (Bruce et al. 2015).

Other platforms that have been engineered to produce unsaturated fatty acids are bacteria and microalgae. Qin et al. (2023) have reviewed poly-unsaturated fatty acid (PUFA) production in plants, fungi, microalgae, and bacteria. Among all platforms, bacteria have the advantage of the fastest growth rate and largest toolbox for metabolic engineering, but it has a low ability to accumulate lipids. Microalgae show high accumulation of lipids and ease of engineering but have a slower growth rate and higher cultivation costs than yeasts, because of space needed for phototrophic cultivation.

Objectives of this PhD study

The objective of this PhD study has been to study sex pheromone biosynthesis in three moth species that are considered economically important pest insects with negative impact in agriculture. Research on moth sex pheromones is of applied importance because for many species, they can be used in sustainable pest management. This is an alternative to using conventional insecticides that promote insecticide resistance and are harmful to humans, beneficial insects, and biodiversity. Moth sex pheromones can be produced in biotechnological systems using insect enzymes, and the ambition of this work has also been to demonstrate the route from biosynthesis- and gene discovery in the insects to biotechnological production and field application. Elucidation of pheromone biosynthetic pathways is the main objective of the thesis, and this is a critical first step to enable a biotechnological production process.

To demonstrate the viability of biotechnological pheromone production, previously discovered insect desaturases and pheromone biosynthesis genes were expressed in the yeast *Yarrowia lipolytica*. The goal was to optimise production for increased titre by extensive metabolic engineering of the host strain´s fatty acid metabolism, purify pheromone products and demonstrate their effectiveness in field trials (**paper I**).

Then the focus of this thesis has been elucidation of the sex pheromone biosynthesis pathways in the sugarcane borer *Diatraea saccharalis* (Lepidoptera: Crambidae) (**paper II**), the oriental fruit moth *Grapholita molesta* (Lepidoptera: Tortricidae) (**paper III**) and the tomato leafminer *Tuta absoluta* (Lepidoptera: Gelechiidae) (**paper IV**). From this follows an attempt to reconstitute biosynthesis in a heterologous system that can be used in biotechnological production of the pheromones needed for pest control of each species.

Methods and experiments to achieve these objectives have included pheromone gland analysis of pheromones and fatty acids, *in vivo* isotope labelling studies, RNA-seq and transcriptome analysis including differential expression, and functional characterisation of pheromone biosynthesis genes by heterologous expression in yeast, insect, and plant systems.

Methods to study pheromone biosynthesis

The first step to elucidate the biosynthesis pathway of a certain sex pheromone is the identification of the molecules present in the species-specific sex pheromone blend. This information is confirmed by the development of a synthetic pheromone lure that elicits an optimal male response for it to be used in traps or for mating disruption. Then follows the identification of the genetic basis of the biosynthesis pathway in the insect and the characterization of the enzymes involved, which can make it possible to produce the active ingredients for the lure using biology and heterologous expression instead of synthetic organic chemistry.

Chemical ecology research involving moth pheromones started to have an increasing impact and popularity in the 1960s, after the first lepidopteran pheromone was identified from the silkworm moth *Bombyx mori* (Bombycidae) by Butenandt et al. (1959) at the Max Planck Institute in Munich. The fascinating story of the identification of bombykol in this domesticated moth is described in Hecker and Butenandt (1984). Unsuccessful primary attempts started in the late 1930s by collection of volatiles in the air above trapped, living females. The terminal abdominal segments (including the pheromone gland) were then dissected to isolate the pheromone (of > 500.000 individuals!), as it was believed this was the location of the mating signal because males elicited no response to females that had these parts removed. The compound structures were identified by various methods of chromatography, spectroscopy, and chemical micro-reactions, and by chemical synthesis of putative structures, testing which gave identical analysis results to the natural compounds.

The realisation that pheromones could be used to control pests, and the growing concern for the environment, lead to increased involvement of chemistry at entomological institutions, and a rush to identify new pheromones and develop practical applications (Baker et al. 2016). Around 2000 lepidopteran sex attractants or pheromones that elicit male responses had been identified by 2016 (Löfstedt et al. 2016), the methodology often employing GC/EAD (gas chromatography coupled to electroantennographic detection), GC/MS (gas chromatography coupled to mass spectrometry) and chemical synthesis of putatively active compounds.

The first biosynthetic pathways of sex pheromones were unraveled in the 1980s, by observing the incorporation pattern of topically applied isotope labelled precursors into the pheromones in living females (*in vivo* labelling experiments).

This type of experiments revealed the involvement of specific Δ^9 and Δ^{11} desaturase enzymes and controlled chain- shortening and elongation mechanisms (Roelofs and Bjostad 1984). With the advent of molecular techniques, it became possible to further characterise the desaturase enzymes. This could be done by reverse transcription of cDNA from pheromone gland-extracted mRNA and subsequent functional characterization of the coding sequences by expression in a desaturase-deficient yeast strain of baker's/brewer's yeast, *Saccharomyces cerevisiae* (Stukey et al. 1990). This was first demonstrated for the cabbage looper *Trichoplusia ni* (Noctuidae), confirming the Δ^{11} desaturase function involved in biosynthesis of the various pheromone components containing Δ^9, Δ^7 and Δ^5 double bonds (Knipple et al. 1998). Using the desaturase-deficient yeast allowed them to observe only the actions of the insect-derived desaturase without interference from the native yeast Δ^9 desaturase. The Δ^{11} function was postulated previously based on *in vivo* labelling experiments and various *in vitro* assays of pheromone gland extracts (Bjostad and Roelofs 1983; Wolf and Roelofs 1986).

The experimental workflow for the elucidation of sex pheromone biosynthetic pathways employed in this thesis work is described in Löfstedt and Xia (2021), and is outlined below (**Figure 7**). Laboratory-reared or acquired insects are kept in climate chambers at a suitable humidity (60-70 %), temperature (23-25 °C) and diel light cycle (light/dark). The light cycle is adjusted so that working hours is when the particular moth species produces and emits the most pheromone. This depends on if the species is nocturnal (night-active), diurnal (day-active) or crepuscular (active during twilight).

The first step in biosynthesis elucidation is looking for fatty acid pheromone precursors of the already known pheromone components in the pheromone gland (Type I structures are made here) or possibly in the abdominal cuticle (Type II structures are processed in the oenocyte cells). The presence of certain fatty acids, with specific double bonds, can reveal a preliminary pathway hypothesis. Pheromone glands of adult females are dissected at a time of peak calling/maximum pheromone emission, extracted in organic solvent and compounds present identified by GC/MS. The pheromone compounds can be detected in a heptane extract, while total fatty acids (including pheromone precursors) first need to be derivatised into **f**atty **a**cid **m**ethyl **e**sters (FAMEs).

In the second step in biosynthesis elucidation, deuterium-labelled forms of the potential precursors identified are topically applied to the pheromone gland of living females. After an incubation period, pheromone gland extracts are analysed by GC/MS to see which were incorporated into the pheromones, thereby confirming them as precursors. Gas chromatography works to separate the different compounds by size, because of their differential interaction with the column matrix. Larger compounds (longer fatty acids) have a longer retention time on the column. Other compound properties, like the position and number of double bonds, functional groups like an alcohol or acetate, and if any atoms are isotopes, like deuterium instead of hydrogen, also affects interaction with the column matrix and thereby

retention time. In the mass spectrometer the molecules are fragmented by ion bombardment, and the mass-to-charge ratio (*m/z*) of the now ionized fragments is determined in the oscillating electric fields of the mass analyser (e.g. a quadrupole). Compounds can be identified based on the fragment profile at a certain retention time. On a GC column of high polarity, the deuterated compounds will elute slightly earlier than their non-deuterated counterparts, giving a peak to the left in the chromatogram. The fragment ions containing deuterium will show a higher mass in the mass spectrum (**Figure 8**).

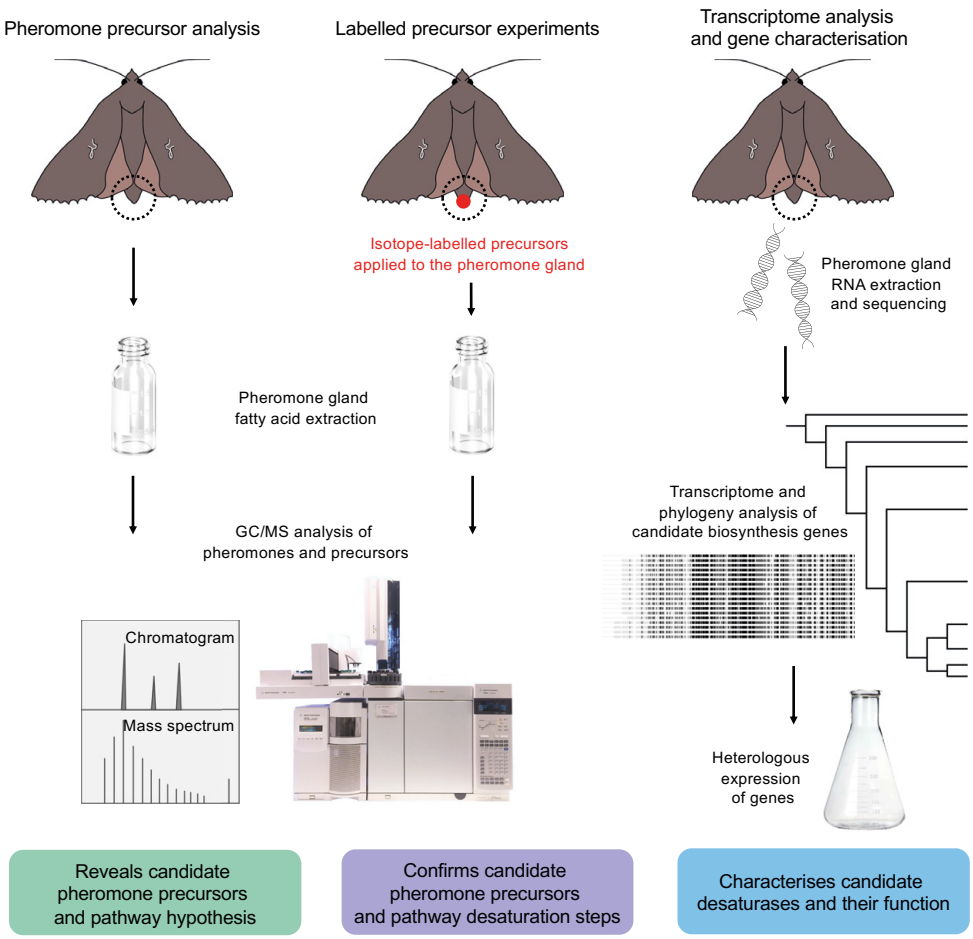


Figure 7 The experimental workflow for sex pheromone biosynthetic pathway elucidation in the Pheromone Group at Lund University.

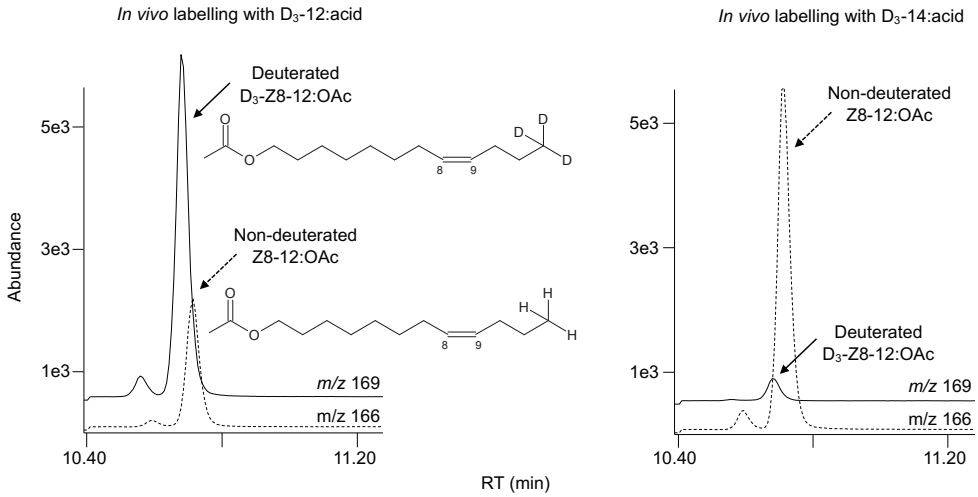


Figure 8 Example of result from isotope *in vivo* labelling experiment with *Grapholita molesta* (Tortricidae). After application of deuterated lauric acid (D_3-12:acid) to the pheromone gland, fatty acid extract is analysed by GC/MS (left panel). The chromatogram shows the pheromone pool divided between the non-deuterated peak of the pheromone (*Z*)-dodecenyl acetate (Z8-12:OAc, dashed line, characteristic ion *m/z* 166) and the slightly-to-the-left peak which is now labelled (D_3-Z8-12:OAc, solid line, ion *m/z* 169: +3 compared to the non-labelled because of the heavier deuterium atoms). X-axis shows retention time in minutes, y-axis abundance of ion fragments. Right panel shows similar experiment with deuterated myristic acid (D_3-14:acid), revealing much less label-incorporation. Figure modified from paper III in this thesis.

The third step is transcriptome analysis (RNA-Seq) of female pheromone gland tissue and differential expression analysis of genes related to pheromone biosynthesis in comparison to that of control tissue (male or other female tissues). Pheromone biosynthesis candidate genes are those fatty acyl desaturases that show high expression levels in the pheromone gland compared to the control tissue(s). Phylogenetic analysis of these particular enzyme sequences, together with those of other lepidopteran desaturases, can also indicate which are the most likely candidates to participate in pheromone biosynthesis, as many known pheromone biosynthesis desaturases cluster in the lepidopteran-specific $\Delta^{11/10}$ functional lineage (Liénard et al. 2008). Candidate genes are then cloned, expressed in a heterologous system and enzymes functionally characterised by which products are made (analysed by GC/MS). A yeast expression system, as described above, is fast and cheap, and pheromone precursors that are not native to the host but needed as substrates for the enzyme being assayed, can be supplemented to the growth medium. Sometimes expression of the insect enzymes within yeast cells does not produce the expected products. This was seen for the desaturase involved in pheromone biosynthesis in *Cydia pomonella* (Tortricidae), where only the monounsaturated precursor (*E*)-9-dodecenoic acid (E9-12:acid) was produced, and not the subsequent doubly unsaturated precursor (*E,E*)-8,10-dodecadienoic acid

(E8,E10-12:acid) (Lassance et al. 2021). Characterisation of this desaturase was instead achieved in a *Spodoptera frugiperda* (Noctuidae) cell line (Sf9), where the gene was introduced by infection with transgenic baculovirus. This system obviously shares a closer relationship with the species from which the desaturase gene originates, making it more likely to function properly. This expression can, however, be more time-consuming than yeast expression because of the required baculovirus production. Another heterologous system frequently used for gene characterization is the plant *Nicotiana* spp. transient expression system. Here the genes are introduced into the plant leaf tissue using *Agrobacterium tumefaciens*. Comparing all the three heterologous expression systems, yeast is the simplest, fastest and cheapest to work with, but there is a tendency for more false negatives than in the other two systems (Löfstedt and Xia 2021). The plant system is also fast if you already have the plants. The baculovirus expression vector system (BEVS) and infection of for example
Sf9 cells provides the most "native" insect environment.

Challenges with the pathway elucidation methodology appear at all levels. It can be that some precursors are only present transiently or in very low amounts in the pheromone gland and can be difficult to detect. It can also be that the isotope-labelled precursors are very difficult to synthesise or are simply not accepted as substrates via topical application. In transcriptome analysis, *de novo* assembly is still a challenge, even with a well-supported platform such as Trinity (Grabherr et al. 2011; Haas et al. 2013). Large gene families such as lepidopteran desaturases and reductases cannot always be well resolved, because of similar isoforms from recent gene duplication. Some of the challenges with expressing insect genes in heterologous hosts were mentioned above, and more research is needed to discover why some desaturases do not work as expected in the yeast expression system but do work in an insect cellular environment provided in the Sf9 cells or even in plants. We do not know enough about the desaturase or reductase mechanisms and native cellular environment within the pheromone gland to troubleshoot the functional assays effectively. Problems encountered in heterologous systems could involve faulty protein assembly, post-translational modification and membrane association, lack of supporting- or carrier proteins, a non-optimal substrate availability or transport and other missing environmental factors necessary for correct function of the insect enzymes.

An additional method in the toolbox for pheromone biosynthesis elucidation, is knock-out of candidate genes in the insect by the CRISPR/Cas technique. E.g., the main candidate desaturase knocked out in *Spodoptera exigua* (Noctuidae) resulted in a moth that could no longer produce pheromone, thereby confirming the key role of that desaturase in pheromone biosynthesis (Ahmed et al. 2021). The gene knock-out is made by injecting moth eggs with a Cas endonuclease protein, and guide RNA that will direct the endonuclease to the target desaturase, allowing it to make very specific DNA double strand cuts near PAM nucleotides (protospacer adjacent motif) in the target. The DNA repair by a non-homologous end-joining mechanism will

often result in a non-functional gene, and the adult will develop with a mutated germline if the injection was done early enough (Brady et al. 2021). The CRISPR/Cas method is more labour-intensive than any of the other biosynthesis elucidation methods described, taking several generations (**Figure 9**). It can also be a technical challenge to develop an optimal egg-injection protocol (Zhang and Reed 2017).

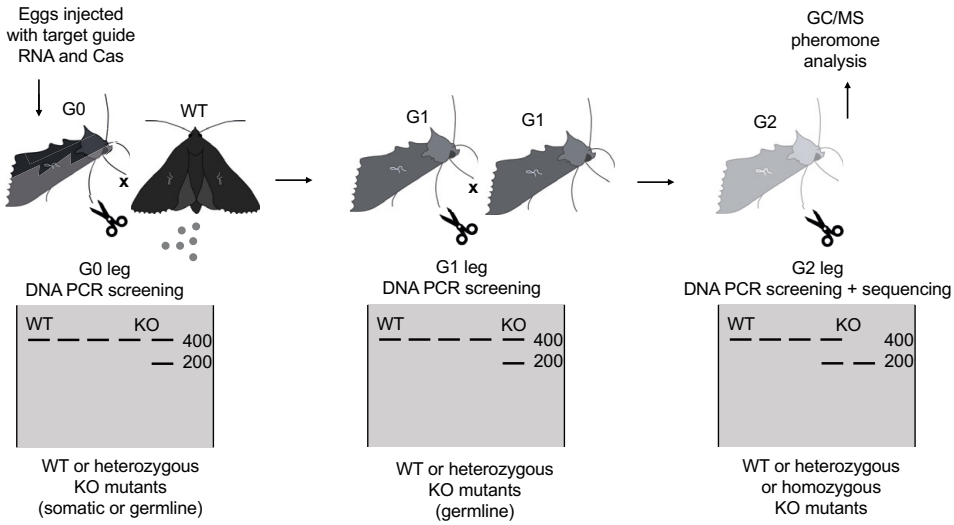


Figure 9 CRISPR/Cas workflow for knock-out of a moth desaturase gene. Adults surviving from injected eggs are designated generation G0, and PCR screening of target DNA reveals if knock-out was successful (in a heterozygote the lower band on the gel represents the mutant allele (KO), higher band the wildtype (WT) allele). These adults could have the mutation in somatic or germline cells. To reveal germline mutants, G0 is crossed to WT and G1 offspring are screened. Heterozygous germline mutants are crossed and G2 adults screened for a homozygous knock-out females, the pheromone gland of which are analysed by GC/MS to check if the deleted gene has affected the pheromone phenotype.

Study organisms – three little moths

Figure 10 From left to right: the oriental fruit moth *Grapholita molesta* (photo Buchner (2004)), the sugarcane borer *Diatraea saccharalis* (photo Marie Inger Dam) and the tomato leafminer *Tuta absoluta* (photo Buchner (2014)).

The sugarcane borer *Diatraea saccharalis* (Crambidae)

The sugarcane borer *Diatraea saccharalis* is a pale brown moth in the family Crambidae, originating from South America (**Figure 10**). The adult wingspan is 18-39 mm. Summer generation time can be as little as 25 days and overwintering takes place in the larval stage (Capinera 2001). The major female sex pheromone component of *D. saccharalis* has been identified as (*Z,E*)-9,11-hexadecadienal (Z9,E11-16:Ald) (Svatoš et al. 2001) and minor components as hexadecanal (16:Ald), (*Z*)-9-hexadecenal (Z9-16:Ald) and (*Z*)-11-hexadecenal (Z11-16:Ald) (Batista-Pereira et al. 2002; Kalinova et al. 2005; da Silva et al. 2021). Peak mating time in the laboratory is five hours into scotophase (Batista-Pereira et al. 2002).

Diatraea saccharalis is a widely distributed invasive species throughout southern USA, Central America, and tropical and subtropical zones of South America, and it is a key pest on sugarcane and maize (Francischini et al. 2019). The larva causes damage by eating leaves and boring into stalks, which in sugarcane production can diminish yields. The annual economic losses due to pest insects is estimated at more than US$4.5 billion in Brazil alone, the largest sugarcane producer in the world (Oliveira et al. 2014; Parra 2014; FAOSTAT 2021b). Insecticidal control is not efficient because the larvae are protected inside the plant and are present throughout the year (Batista-Pereira et al. 2002). Some reduction in *D. saccharalis* damage has been achieved with biological control, by mass release of egg- and larval parasitoids (Parra et al. 2010; Parra 2014). For example, in the state of São Paulo the economic

impact of *D. saccharalis* in areas under treatment was estimated at US$100 million/year in the 1980s compared to US$20 million/year in 2010 (Parra et al. 2010). The use of sex pheromones for monitoring or mating disruption has not yet been successfully employed for *D. saccharalis*, as only field tests with the major sex pheromone component has been done, and the results showed low attractiveness to males compared to conspecific females (Kalinova et al. 2005). In the laboratory, more complex blends improve attraction of males in wind tunnel assays (Kalinova et al. 2005; da Silva et al. 2021).

Biosynthesis of pheromone components similar to those of *D. saccharalis have* been described for several families. These indicate that biosynthesis of a structure such as Z9,E11-16:Ald could involve a Δ^{11} desaturase alone or in combination with a Δ^{9} desaturase, or a bifunctional desaturase. Both double bonds are introduced in sequence by a single desaturase belonging to the lineage of specific lepidopteran Δ^{11} desaturase, with a chain-shortening step in between, for pheromones of the currant shoot borer *Lampronia capitella* (Prodoxidae), (*Z,Z*)-9,11-tetradecadienol (Z9,Z11-14:OH) (Liénard et al. 2008), the light brown apple moth *Epiphyas postvittana* (Tortricidae), (*E,E*)-9,11-tetradecadienyl acetate (E9,E11-14:OAc) (Liu et al. 2002) and the tobacco cutworm *Spodoptera litura* (Noctuidae) (*Z,E*)-9,11-tetradecadienyl acetate (Z9,E11-14:OAc) (Xia et al. 2019). Two double bonds can also be introduced by a bifunctional desaturase via one double bond in the intermediate position, as seen for the codling moth *Cydia pomonella* (Tortricidae) ((*E,E*)-8-10-dodecadienol, E8,E10-12:OH) (Löfstedt and Bengtsson 1988) and the silkworm moth *Bombyx mori* (Bombycidae) ((*E,Z*)-10-12-hexadecadienol, (E10,Z12-16:OH) (Moto et al. 2004). Two desaturases and several chain-shortening steps are involved in the biosynthesis of the pheromone components of the Masson pine moth *Dendrolimus punctatus* (Lasiocampidae) ((*Z,Z*)-5-7-dodecadienol, Z5,Z7-12:OH) (Liénard et al. 2010b) and the European grapevine moth *Lobesia botrana* (Tortricidae) ((*E,Z*)-7,9-dodecadienyl acetate, E7,Z9-12:OAc) (Ding et al. 2021).

The oriental fruit moth *Grapholita molesta* (Tortricidae)

Grapholita molesta is a brown-grey moth in the family Tortricidae, with an adult wingspan of 10-16 mm (**Figure 10**). It originates from southwest China and is now distributed all over the world in temperate regions. It is a pest on many commercial stone and pome fruits in the rose family, especially the main host peach (*Prunus persica*), causing damage by boring into fruits and shoots (Song et al. 2018).

The life cycle duration is about one month in summer temperatures, and it overwinters as larva in a cocoon, hidden in cracks in the tree bark (Il´ichev 2014). The major female sex pheromone component of *G. molesta* has been identified as (*Z*)-8-dodecenyl acetate (Z8-12:OAc), and the minor components as (*E*)-8-dodecenyl acetate (E8-12:OAc), (*Z*)-8-dodecenol (Z8-12:OH) and dodecanol

(12:OH) (Roelofs et al. 1969; Cardé et al. 1979). Peak calling time for females is at the onset of scotophase (Baker and Cardé 1979).

China is the largest producer of peaches in the world, with 15.8 million tonnes/year and a cultivated area of more than 800.000 ha in 2019, followed by Italy, Spain and Greece (FAOSTAT 2021a). Because of the hidden lifestyle of the larvae inside the plant, they can be difficult to control with insecticides, and resistance to many classes has already been observed. In 2010, 50.000 ha of peach and apple orchards worldwide was under *G. molesta* mating disruption treatment (Witzgall et al. 2010). In peach orchards in Ontario, Canada, monitoring of *G. molesta* with female sex pheromone traps to time insecticide treatments have been used since the 1970s. Resistance to various insecticide such as organophosphorus (azinphosmethyl and phosmet), carbamates (methomyl) and pyrethroids (cypermethrin) has been observed here (Pree et al. 1998; Trimble et al. 2001; Kanga et al. 2003). Production yield loss of 20-40 % was reported here in 1994 (Kanga et al. 1999). In the same region, mating disruption with pheromones in combination with chlorpyrifos (organophosphorus) treatment has been shown to be as effective as conventional treatment with insecticides such as chlorpyrifos and deltramethrin (pyrethroid) (Trimble et al. 2001). Mating disruption has also been successfully employed in many other countries, including France, Spain, Italy, China, Australia, USA, and Brazil (Cardé and Minks 1995; Kong et al. 2014). In several mating disruption studies between 1981 and 2009 in China, it was reported that the treatment reduced the percentage of *G. molesta* infested fruit by 50-82 % compared to insecticide treated orchards (Kong et al. 2014).

Z8-12:OAc immediately seems like a typical Type I lepidopteran pheromone structure, although with a less common double bond at an even-numbered position. The native biosynthesis pathways for this and similar pheromone components in the subfamily Olethreutinae with Δ^8 and Δ^{10} double bonds, have not yet been described. A pathway hypothesis can be formed by looking at biosynthesis of other pheromones with double bonds in even-numbered positions that have been described. This includes the (*Z*)-8-tetradecenyl acetate and (*Z*)-10-tetradecenyl acetate of the tortricid leafrollers *Planotortrix octo* and *Ctenopseustis obliquana* (but in subfamily Tortricinae), made by the action of a Δ^{10} desaturase with or without chain-shortening (Löfstedt and Roelofs 1985; Hao et al. 2002; Albre et al. 2012). (*E/Z*)-12-tetradecenyl acetate of the Asian corn borer *Ostrinia furnicalis* (Crambidae) is made by a Δ^{14} desaturase acting on a C_{16} substrate (Zhao et al. 1990; Roelofs et al. 2002) and (*Z,E*)-9,12-tetradecadienyl of *Spodoptera exigua* (Noctuidae) is made by a bifunctional $\Delta^{11/12}$ desaturase (Xia et al. 2019). For the codling moth *Cydia pomonella* (in the same subfamily and tribe as *G. molesta*), the pheromone (*E*,*E*)-8,10-dodecadienol (12:OH) is made by a bifunctional desaturase via one double bond in the intermediate position followed by dehydrogenation into the conjugated system (Löfstedt and Bengtsson 1988). The Δ^{11} desaturase involved in pheromone biosynthesis in the Masson pine moth *Dendrolimus punctatus* (Lasiocampidae) has also been shown to have the ability to make Δ^8 double bonds

on $C_{12/14/16}$ substrates when the native precursor (*Z*)-9-hexadecenoic acid (Z9-16:acid) of the pheromone (*Z,E*)-5,7-dodecandienol (Z5,E7-12:OH) was not present in a heterologous yeast assay (Liénard et al. 2010b).

The tomato leafminer *Tuta absoluta* (Gelechiidae)

The tomato leafminer *Tuta absoluta* is a 10 mm long, grey-brown moth in the family Gelechiidae (**Figure 10**). It has a life cycle of 24-75 days, with an optimum temperature of 30 °C (Biondi et al. 2018). It has its origin in South America where it is a pest on domestic tomato cultivars. Since 2006 it has also been an invasive pest on tomato production in Europe, and it is spreading fast in Asia and Africa, mediated by export and import of solanaceous crops (Biondi et al. 2018; Biondi and Desneux 2019).

Tomato is the tenth largest food crop in the world with a production area of 5 million ha, and in 2012 it was estimated by the Food and Agriculture Organization of the United Nations (FAO) that more than 50 % of the world production was infested by *T. absoluta* (Campos et al. 2017; FAOSTAT 2021c). Soon it might be a problem in China, the leading tomato producer in the world responsible for 35 % of total production (2019) (FAOSTAT 2021c). *Tuta absoluta* has a strong adaptation- and reproduction potential in tropical and subtropical climates, and even overwintering capacity in temperate climates (Han et al. 2019). In some non-invaded countries, such as the USA, detection- and phytosanitary measures are in place, but in other invaded countries current quarantine measures are not effective, and coordinated trans-national surveillance is required to curtail the spread (Han et al. 2019). Larvae affect production yield when they attack apical buds, flowers and fruits which makes the host plant more susceptible to pathogenic invasion (El-Shafie 2020). In the Netherlands, one of the large European tomato producers, after invasion and up until 2013 the annual cost of *T. absoluta* has been €5-25 million (Campos et al. 2017). Insecticides are most commonly used to control *T. absoluta*, even with the difficulties posed by the endophagous nature of the larvae, and resistance emerging to several classes (Desneux et al. 2010; Guedes et al. 2019; Han et al. 2019). Biological control with predators or parasitoids has been successfully tested in greenhouses, such as *Nesidiocoris tenus* (Hemiptera: Miridae), *Macrolophus pygmaeus* (Hemiptera: Miridae) and *Trichogramma achaea* (Hymenoptera: Trichogrammidae) (Zappala et al. 2013). In the neotropical region, several *Trichogramma* parasitoids are being used commercially (Salas Gervassio et al. 2019). Pheromone traps are used for early detection, and mass trapping has been shown to work in low-density populations, and mating disruption in greenhouses, but only with very high dose of the active ingredient, therefore being costly (Han et al. 2019).

The major and minor female sex pheromone components of *T. absoluta* have been identified as (*E,Z,Z*)-3,8,11-tetradecatrienyl acetate (E3,Z8,Z11-14:OAc) and (*E,Z*)-3,8-tetradecadienyl acetate (E3,Z8-14:OAc) (Attygalle et al. 1996; Griepink et al. 1996; Svatoš et al. 1996). Mating usually takes place at the onset of photophase (Attygalle et al. 1996).

There has been report of many typical Type I pheromones or attractants within Gelechiidae, similar to *T. absoluta* structures in some respects, often C_{12}-C_{14} acetates with Δ^3 or Δ^5 double bonds, often in *E*-configuration (El-Sayed 2023). (*E*)-3-tetradecenyl acetate (E3-14:OAc) structures are seen in the South American potato tuber moth *Symmetrischema tangolias* (Griepink et al. 1995), the lesser budmoth *Recurvaria nanella* (Tòth and Doolittle 1992) and *Chionodes perpetuella* (Priesner 1987). *Gelechia rhombella* has attractants with Z8 or E/Z11 double bond configurations ((*Z,E*)-8,10-dodecadienol, Z8,E10-12:OH and (*E/Z*)-11-tetradecenyl acetate, E/Z11-14:OAc) (Peltotalo and Tuovinen 1986). The potato tuberworm *Phtorimaea opercullela* has a pheromone component with a methyl-interrupted system similar to Z8,Z11, namely (*E,Z,Z*)-4,7,10-tridecatrienyl acetate (E4,Z7,Z10-13:OAc) (Yamaoka et al. 1976). No biosynthetic pathways have been described for any of these compounds in Gelechiidae, but a putative pathway for E4,Z7,Z10-13:OAc has been suggested, starting from sequestered linolenic acid, involving α-oxidation, β-oxidation, elongation and isomerisation (Roelofs and Bjostad 1984). Such a Type II pheromone pathway could be involved in *T. absoluta* pheromone biosynthesis. A Type I pathway could also be involved, where several desaturases make the double bonds in a consecutive manner, with or without intermediate chain-shortening. A putative pathway to t E3,Z8,Z11-14:OAc in *T. absoluta* could be chain-shortening of a E5,Z10-16:acid to E3,Z8-14:acid, followed by Δ^{11} desaturation. A lepidopteran Δ^5 desaturase has been described for the tortricid leafrollers *Cenopseustis* sp. (Hagström et al. 2014) and a Δ^{10} desaturase for *Ctenopseustis* sp. and *Planotortrix* sp. (Hao et al. 2002; Albre et al. 2012).

Results/summary of papers

Paper I

Holkenbrink C, Ding B-J, Wang H-L, Dam MI, Petkevicius K, Kildegaard KR, Wenning L, Sinkwitz C, Lorántfy B, Koutsoumpeli E, França L, Pires M, Bernardi C, Urrutia W, Mafra-Neto A, Ferreira BS, Raptopoulos D, Konstantopoulou M, Löfstedt C, Borodina I (2020) Production of moth sex pheromones for pest control by yeast fermentation. Metabolic Engineering 62:312-321. doi: 10.1016/j.ymben.2020.10.001

This paper describes a study where we aimed to produce moth pheromones in a yeast "cell factory", demonstrating that production can be optimised for high titres. The oleaginous yeast *Yarrowia lipolytica* was used to express insect fatty acid desaturases and reductases to produce the pheromone precursors (*Z*)-11-hexadecenol (Z11-16:OH) and (*Z*)-9-tetradecenol (Z9-14:OH). The derived compounds can be used for pheromone pest control of for example the cotton bollworm *Helicoverpa armigera* (Noctuidae) and the fall armyworm *Spodoptera frugiperda* (Noctuidae), respectively. Native yeast metabolism was also engineered to avoid degradation of pheromone precursors and to funnel necessary fatty acids toward pheromone biosynthesis instead of yeast storage lipids.

In results we show how iterative engineering of *Y. lipolytica* increases Z11-16:OH titres. First, several insect desaturases and reductases were tested for production of Z11-16:OH, revealing that a Δ^{11} desaturase from *Amyelois transitella* (Pyralidae) (*AtrΔ11*) and reductase from *H. armigera* (*HarFAR*) showed the greatest production. Second, yeast pathways degrading the pheromone product were knocked out (the fatty alcohol oxidase *FAO1*, transforming fatty alcohols to aldehydes, and fatty aldehyde dehydrogenases *HFD1* and *HFD4*, degrading aldehydes), and pathways sequestering the substrate knocked-down or knocked out (glycerol-3-phosphate acyltransferase *GPAT*, sequestering acyl-CoA for production of storage and membrane lipids, and the peroxisomal biogenesis factor *PEX10*, maintaining functioning peroxisomes that degrade acyl-CoA) (**Figure 11**). This engineered strain produced 19 times as much Z11-16:OH than the strain with just the pheromone biosynthesis pathway expressed. Third, three copies of the pathway were introduced, additionally increasing production titres 10 times. Finally, this strain was cultivated at 10 L scale in bioreactors, yielding 2.6 g/L Z11-16:OH. The yeast-produced Z11-16:OH was oxidised to the aldehyde pheromone and showed

the same efficiency in field trapping experiments with *H. armigera* as synthetic pheromone.

For Z9-14:OAc production, a *Drosophila melanogaster* (Diptera) desaturase (*DmeΔ9*), *HarFAR* and acetyl transferase 1 (*ATF1*) from the yeast *Saccharomyces cerevisiae* were expressed in *Y. lipolytica*. This strain produced a low amount of the pheromone. To increase the pool of 14:acid substrate, which is naturally low in this yeast, a specific mutation was introduced in the fatty acid synthase subunit *FAS2* (I1220F), resulting in a 15-fold increase in 14:acid products, and the pheromone.

In the discussion and conclusion of this study, we assert that we can make biologically active monounsaturated Type I pheromones at high titres in yeast, and that this production can possibly compete with chemical synthesis when it comes to both product specificity, costs and sustainability. What remains to be optimised to obtain an economically competitive platform for pheromone production in *Y. lipolytica*, is 1) yeast strains for production of shorter chain-length (12:acid and 14:acid) pheromones at high titres, as yeast mainly produces 16:acid and 18:acid; 2) production of other pheromone structures with different or multiple double bonds; and 3) down-stream processing that requires minimal efforts to extract the correct ratio of pheromone components at sufficient purity, possibly by secretion from the cells in a continuous fermentation process. Future biotechnological production of pheromones would also benefit from highly active and specific desaturases and reductases, possibly obtained by enzyme engineering.

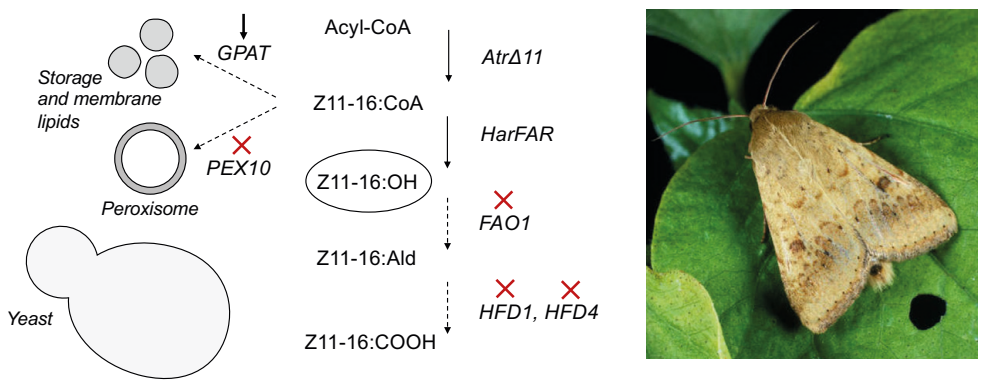


Figure 11 Overview of metabolic engineering strategies to produce (*Z*)-11-hexadecenol (Z11-16:OH) in *Yarrowia lipolytica*. Solid lines represent the pheromone production pathway, expressed by heterologous genes, dashed lines host metabolism that has been modified by knock-out (cross) or down-regulation (down arrow). *AtrΔ11*, Δ^{11} fatty acyl desaturase from *Amyelois transitella*; *HarFAR*, fatty acyl reductase from *Helicoverpa armigera*; *FAO1*, fatty alcohol oxidase in *Y. lipolytica*; *HFD1*/*HFD4*, fatty aldehyde dehydrogenases in *Y. lipolytica*; *GPAT*, glycerol-3-phosphate acyltransferase in *Y. lipolytica*; *PEX10*, peroxisome biogenesis factor in *Y. lipolytica*. Modified from Holkenbrink et al. (2020). Photo shows the cotton bollworm *Helicoverpa armigera* on a cotton leaf (Encyclopædia Britannica n.d.).

Paper II

Dam MI, Ding B-J, Svensson GP, Wang H-L, Melo DJ, Lassance J-M, Zarbin PH, Löfstedt C (2023) Sex pheromone biosynthesis in the sugarcane borer *Diatraea saccharalis*: paving the way for biotechnological production. Pest Management Science doi: 10.1002/ps.7830

This paper describes the study of sex pheromone biosynthesis in the sugarcane borer *Diatraea saccharalis* (Crambidae), a key pest on sugarcane in South America. The aim of the study was to identify and characterise pheromone biosynthesis genes in the moth, and to produce the precursor of the main pheromone component (*Z,E*)-9,11-hexadecadienal (Z9,E11-16:Ald) in a heterologous system – to pave the way for future use of pheromones for pest control in this species.

The results we present are fatty acid precursors found in the female pheromone gland, by what pathway these precursors are made into the main pheromone component, what genes are responsible for biosynthesis, and confirmation of enzyme function in a heterologous assay. We showed by GC/MS analysis that the female pheromone gland contained the pheromone fatty acid precursors 16:acid, Z9-16:acid and Z9,E11-16:acid. *In vivo* labelling experiments, applying deuterated precursors 16:acid, Z9-16:acid and E11-16:acid to the pheromone gland of live females, showed that the two first were incorporated into Z9,E11-16:Ald, whereas E11-16:acid was not. This indicates a pheromone biosynthesis pathway where first a Z9 double bond is made in 16:acid, followed by an E11 double bond in that Z9-16:acid product (**Figure 12**). The two most highly expressed and female-biased fatty acyl desaturases found by transcriptome analysis were good candidates to perform these functions. *Dsac_KPSE* and *Dsac_NPTQ* clustered phylogenetically with other lepidopteran Δ^{9} and Δ^{11} desaturases, respectively. When these genes were expressed in the yeast *Saccharomyces cerevisiae*, their expected functions were confirmed by GC/MS analysis of fatty acid products. And when the two desaturases were expressed in the heterologous system together with the most highly expressed and female-biased fatty acyl reductase gene, *Dsac_FAR3781*, the precursor Z9,E11-16:acid, made by the two desaturases, was turned into the alcohol Z9,E11-16:OH by the reductase. The reductase gene clustered phylogenetically with other lepidopteran-specific fatty acyl reductases. From the alcohol precursor there is only one step of transformation to the final pheromone component, involving a fatty alcohol oxidase (FAO). A putative FAO gene was identified from the transcriptome analysis, based on its high and female-biased expression, although we do not present functional characterisation of the gene in this study. A heterologous expression assay for such an oxidase has yet to be established, but confirmation of its role in pheromone biosynthesis could potentially be shown by gene knock-out in the insect.

Also discussed in this study were paralogs of *Dsac_NPTQ* found in a recently published *D. saccharalis* genome. We characterised one of those paralogs, differing from the transcriptome *Dsac_NPTQ* in 33 amino acid residues. This gene,

Dsac_NPAQ, showed a similar fatty acid production in heterologous expression assays, but at different ratios. Investigation of differences in the primary sequence of these desaturases and their fatty acid production profiles, could give insights about desaturase specificity and the sequence-function relationship.

To conclude, the elucidation of pheromone biosynthesis in this species, and the characterised pheromone biosynthesis genes, pave the way for biological production of the main *D. saccharalis* pheromone component and its potential use in pest management. More research has to be done for mating disruption with pheromones to work for *D. saccharalis*, but once established, this method could complement the use of parasitoids for moth control in sugarcane production and circumvent further development of resistance to conventional insecticides.

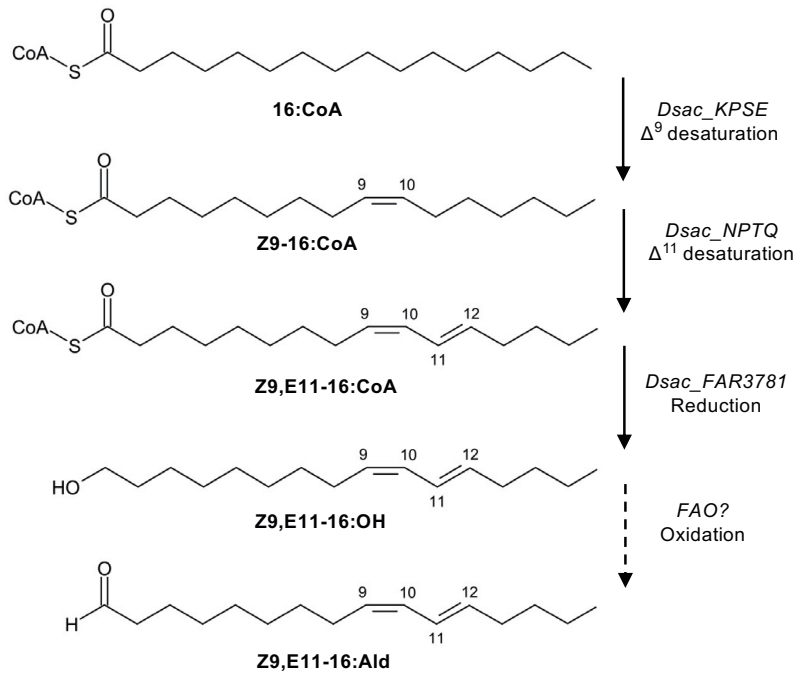


Figure 12 Pheromone biosynthesis pathway, and pathway hypothesis, in *Diatraea saccharalis*, showing the desaturases and reductase genes characterised in this study and their substrates and products. 16:CoA; hexadecanoic acid-coenzyme A; Z9-16:CoA, hexadecenoic acid-coenzyme A; Z9,E11-16:CoA, haxadecadienoic acid-coenzyme A; Z9,E11-16:OH, hexadecadienol; Z9,E11-16:Ald, hexadadienal.

Paper III

Dam MI, Ding B-J, Brauburger K, Wang H-L, Powell D, Groot AT, Heckel DG, Löfstedt C Sex pheromone biosynthesis in the oriental fruit moth *Grapholita molesta* involves unique Δ^8 desaturation. Manuscript

This manuscript describes a study of sex pheromone biosynthesis in a fruit pest, the oriental fruit moth *Grapholita molesta* (Tortricidae). The aim of the study was to elucidate the biosynthesis pathway and characterise the desaturase involved in making the uncommon Δ^8 double bond seen in the main pheromone component (*Z*)-8-dodecenyl acetate (Z8-12:OAc). Pheromones are already used for control of this species, and uncovering this desaturase is advantageous for future biological production of the pheromone.

The results presented in this study are fatty acid precursors found in the female pheromone gland, in what order particular precursors are incorporated in the pheromone component, desaturase gene candidates involved in pheromone biosynthesis, heterologous expression of genes for functional characterisation, and knock-out of one candidate desaturase to confirm its role in pheromone biosynthesis. In *in* vivo labelling experiments with deuterated precursors, subsequent GC/MS analyses of pheromone gland extracts showed that 12:acid was incorporated at a very high rate into Z8-12:OAc, whereas 14:acid and 16:acid was incorporated only at low rates. This indicates 12:acid as the substrate for the desaturation step in pheromone biosynthesis, and implicates a Δ^8 desaturase. In transcriptome analysis, the desaturase gene *Gmol_CPRQ* exhibited the highest expression of all desaturases, and only in female tissue, implying it as the main Δ^8 desaturase candidate. CRISPR/Cas9 knock-out of this gene almost completely abolished Z8-12:OAc production in the moth, confirming its key role in pheromone biosynthesis (**Figure 13**).

In the discussion in this study we speculate about how heterologous expression of *Gmol_CPRQ* in yeast produces Δ9-12:acid instead of the expected pheromone precursor Z8-12:acid. This result is inconsistent with labelling experiments that point towards a Δ^8 desaturase. We hypothesise that the enzymatic function in yeast could be different to that in the insect, showing in yeast instead an ancestral Δ^9 function because the cellular environment is sub-optimal for producing the specialised double bond structures that tortricid moth pheromone glands are evolved to make. In the knock-out strain, a trace amount of Z8-12:OAc is still seen, which could be the result of chain-shortening of Z12-16:acid, made by the Δ^{12} desaturase *Gmol_KPSQ*. We also discuss the evolutionary implications of a Δ^8 desaturase in this moth family, considering the already known Δ^9 and Δ^{10} desaturases involved in biosynthesis of pheromones with Δ8-12:acid and Δ8,Δ10-12:acid structures.

To conclude, sex pheromone biosynthesis in *G. molesta* involves a unique Δ^8 desaturase. The biotechnology and heterologous expression for production of the pheromone for pest control, remains to be developed.

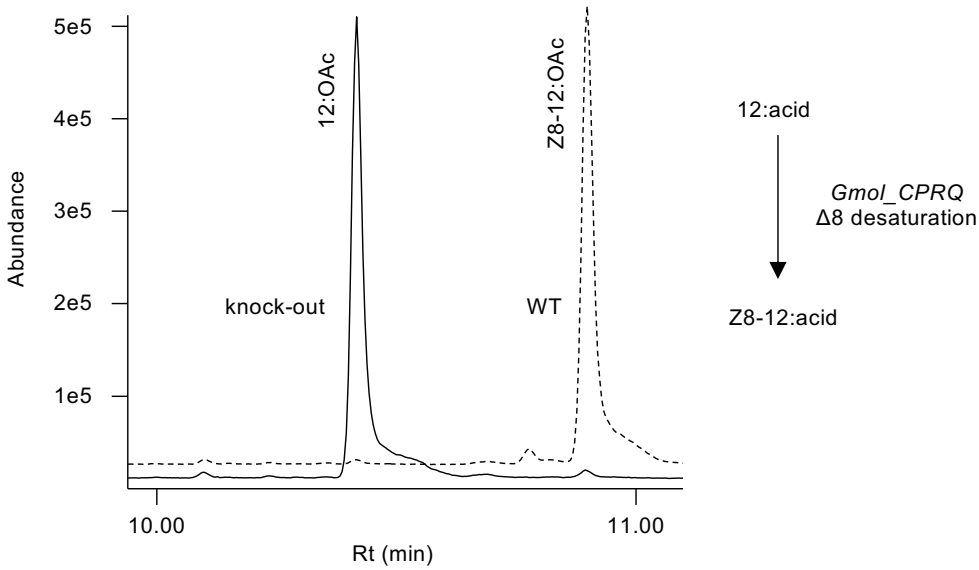


Figure 13 Chromatogram of GC/MS analysis of pheromone compounds in female G. molesta pheromone gland extracts. Extract from one wild type (WT) individual (dashed line) and from one sibling homozygous knock-out mutant individual (solid line). The knock-out showed only minimal biosynthesis of the unsaturated pheromone component Z8-12:OAc, and instead prominent biosynthesis of the saturated 12:OAc which was negligible in the WT. X-axis shows retention time (RT) and y-axis ion abundance. Also shown is the WT pheromone biosynthesis pathway with pheromone precursors, where the desaturase *Gmol_CPRQ* is involved. 12:acid, dodecanoic acid; Z8-12:acid, dodecenoic acid.

Paper IV

Dam MI, Wang H-L, Montes ZO, Konstantopoulou M, Löfstedt C Elucidation of the biosynthesis of the unusual sex pheromone of the tomato leaf miner *Tuta absoluta* requires new approaches. Manuscript

This manuscript describes experiments designed to study sex pheromone biosynthesis in the tomato leafminer *Tuta absoluta* (Gelechiidae), a key pest on tomato. Originally from South America, it has since 2006 become an invasive pest in Europe and is spreading in Africa and Asia. Pheromones are used for pest control in greenhouse tomato production. The aim of this study was to elucidate how the multiple double bonds in the pheromone structures of this species are made – with which precursors, in which order and by which enzymes. We hypothesised that biosynthesis could either follow a Type I pheromone pathway, where the pheromone components are made *de novo* from acetyl-CoA in the pheromone gland, or a Type II pheromone pathway where fatty acids eaten by the larva are used as pheromone precursors (**Figure 14**). The main pheromone component in *T. absoluta* is (*E,Z,Z*)-3,8,11-tetradecatrienyl acetate (E3,Z8,Z11-14:OAc), and the minor

component (*E,Z*)-3,8-tetradecadienyl acetate (E3,Z8-14:OAc). Insights into how these structures are biosynthesized in the moth can be useful for biotechnological production of the pheromone for pest control. This can also reveal evolutionary implications for biosynthesis of such unusual double bonds in lepidopteran pheromones.

Experiments in this study included GC/MS analyses of potential pheromone precursors in the female pheromone gland, cuticle and pupae, isotope *in vivo* labelling to follow the pathway of potential precursors as they get biosynthesised into the pheromone, and RNA-Seq and transcriptome analysis to reveal any fatty acyl desaturase genes that might be involved in biosynthesis. In results we reveal that we could not detect any hypothesised pheromone intermediates in any tissues analysed, not even the immediate precursors E3,Z8,Z11-14:acid and E3,Z8-14:acid. Detection of intermediates on the pheromone gland might have indicated a Type I pheromone pathway, detection of immediate precursors in cuticle might have indicated a Type II pheromone pathway. In the pheromone gland we did, however, detect monounsaturated acetates with E3 double bonds, in addition to that seen in the pheromone diene and triene. In experiments where pheromone biosynthesis activating neuropeptide (PBAN) was injected into the abdomen of adult females, a substance known to control Type I pheromone biosynthesis in some species, we still could not detect any precursors. Additionally, we looked for pheromone precursors in the pheromone gland of the closely related species the potato tuberworm *Phthorimaea operculella* (Gelechiidae), which has somewhat similar pheromone structures. We did not find any immediate precursors in the pheromone gland. In *T. absoluta* isotope labelling experiments with potential precursors applied to the pheromone gland (Type I pathway hypothesis), we did not see any incorporation into the pheromone. Not from 14:acid, 16:acid, 18:acid, oleic acid or possible larval plant-sequestered precursors linoleic and linolenic acids. We also did labelling experiments by injecting labelled precursors 14:acid, 16:acid and oleic acid into young pupae, to see if the precursors might be made already before ecclosion. We did not detect any label incorporation into the pheromones.

Transcriptome analysis showed a surprisingly low number of fatty acyl desaturase genes in the lepidopteran-specific lineages of that gene family, and no immediate candidates for producing the pheromone double bonds. None showed female-biased expression or clustered in the Δ11/10 lineage where many other species´ pheromone biosynthesis desaturases are found.

In the discussion we focus on what an alternative pheromone biosynthesis paradigm could look like, and how use of the canon methodology for Type I pheromone biosynthesis has been insufficient to reveal a pathway in this study. We describe how *T. absoluta* pheromone biosynthesis could be a Type II pheromone pathway, with a different involvement of fatty acid β-oxidation enzymes than what has previously been described for any Type II pheromone.

To conclude, the conventional biosynthesis pathway elucidation methodology has proved insufficient for elucidation of how double bonds are made in the unusual

T. absoluta pheromone structures. Interestingly, transcriptome analysis showed that this species does not have a fatty acyl desaturase in the lepidopteran-specific $\Delta^{11/10}$ lineage, otherwise frequently found in moth genomes. An incomplete fatty acid β-oxidation pathway for Type II structures from linolenic and linoleic acid is most likely involved in *T. absoluta* pheromone biosynthesis.

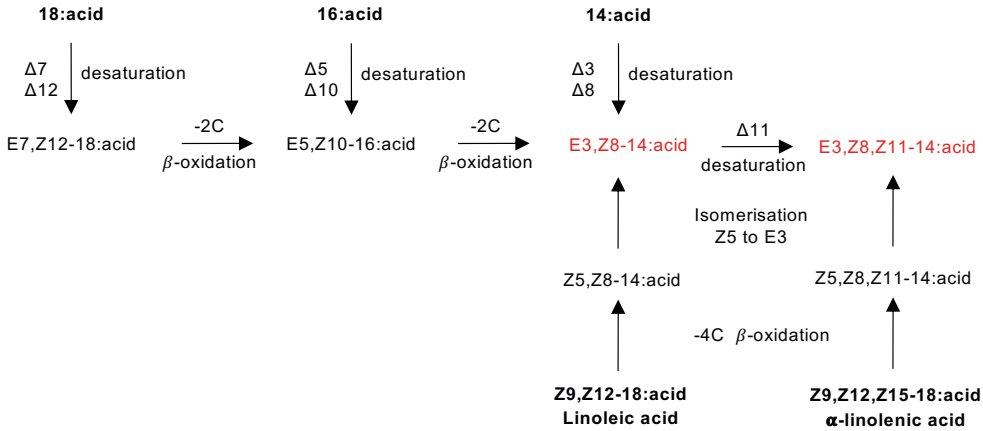


Figure 14 Pheromone biosynthesis pathway hypotheses for *Tuta absoluta*. 14:acid, tetradecenoic acid; 16:acid, hexadecanoic acid; 18:acid, octadecanoic acid. Rest of compounds abbreviated are either mono-, di- or tri-unsaturated tridecanoic (14), hexadecanoic (16), or octadecanoic (18) acids.

Conclusion and perspectives

The aim of this PhD work has been to make the journey from gene discovery in a selection of moth species, to gene application: biotechnologically produced pheromones that can be used for pest control.

In **paper I** we demonstrate that the pheromone precursor Z11-16:OH can be produced at the scale of g/L in the yeast *Y. lipolytica*, using insect enzymes. We show that the derived pheromone can successfully attract the cotton bollworm *H. armigera* in the field. The paper also demonstrates how yeast can be extensively engineered to increase production of two different pheromone precursor many-fold.

In papers II-IV we report the upstream process of discovering the pheromone biosynthesis genes that subsequently can be used in yeast or other biological systems to produce fatty acid derived pheromones. The three studied moths are economically important pest species. For the sugarcane borer *D. saccharalis* we have revealed the biosynthesis pathway and shown pheromone precursor production in yeast. Future biotechnological production of the pheromone using these results is underway to being patent protected. For the oriental fruit moth *G. molesta* we have also elucidated the biosynthesis pathway but heterologous expression for pheromone production has so far been unsuccessful. For the tomato leafminer *T. absoluta* we have shown that a Type I pheromone pathway is not involved, and a hypothesis for Type II pheromone biosynthesis is the most parsimonious.

Paper II identifies the two fatty acyl desaturases and one fatty acyl reductase involved in pheromone biosynthesis of the main pheromone precursor Z9,E11-16:OH in *D. saccharalis*. In this paper the standard methodology employed for pathway discovery was exemplary in its purpose, and the expected outcomes were realised at every step: fatty acid analysis of the pheromone gland revealed the presence of expected pheromone precursors, *in vivo* labelling experiments demonstrated a particular route from precursor to pheromone in the insect, and the female-biased candidate biosynthesis genes showed high expression in the pheromone gland and functioned to demonstrate Z9,E11-16:OH production in a heterologous yeast expression assay.

A similar approach was not as optimal a methodology for pheromone biosynthesis elucidation in *G. molesta*. In **paper III** we show that pheromone gland fatty acid analysis, *in vivo* labelling experiments and transcriptome analysis all point unequivocally toward involvement of a unique Δ8 desaturase in the biosynthesis of

the main pheromone component containing an unusual Δ8 double bond. We, however, failed to demonstrate the expected function in heterologous expression assays. A CRISPR/Cas9 gene knock-out approach was a necessary addition to the pathway discovery toolbox, to confirm the key desaturase gene in pheromone biosynthesis.

For *T. absoluta*, discovery of a pathway for the complex pheromone structure with three double bonds, including an unusual E3 double bond, did not progress as expected following the standard methodology. In **paper IV** we show that no hypothesised pheromone precursors for the pheromones E3,Z8,Z11-14:OAc and E3,Z8-14:OAc could be found in the pheromone gland or female cuticle; no pathway could be revealed with *in vivo* labelling experiments with hypothesised Type I precursors and; no fatty acyl desaturases showed high or exclusive expression in female tissues. No conclusive evidence could be found supporting a biosynthesis pathway for either Type I or Type II pheromones. We conclude that the most parsimonious hypothesis is that a pathway for Type II pheromones is used, creating the pheromone components from linolenic and linoleic acid. Possibly novel biosynthesis mechanisms are involved, not yet described for known Type II moth pheromone biosynthesis. As we employed approaches that have been successfully used to investigate mainly Type I pheromone biosynthesis, we suggest that alternative methodology may have to be used. Elucidation of an unusual Type II pathway may require feeding of hypothesised labelled precursors at the larval stage or multiple injections into adult females. Working with radioactively labelled precursors may be required to reveal any unusual metabolism leading to their incorporation into pheromone components.

My PhD thesis has implications both for understanding evolution of pheromone biosynthesis in moths, and for biotechnological production of pheromones for use in pest management:

- Our discovery of a unique Δ8 desaturase in the Tortricidae family, is of importance for further understanding of pheromone evolution in this family, and for elucidation of pheromone biosynthesis in other species with similar pheromones. It is also important for the study of desaturase function in relation to its cellular environment. We saw different activities in the insect and in the heterologous expression assays. The latter may be a remnant of an ancestral gene function, which has been modified in the specific species. The results can also be used to study the sequence-to-function relationship in desaturases, comparing closely related enzymes with diverging functions. With this comes a possibility of reaching the ultimate applicable goal; engineering of enzymes to have desired functions in biotechnological production of pheromones.
- Our failure to conclusively demonstrate the pathway for *T. absoluta* pheromone biosynthesis emphasises the need to develop and apply

alternative approaches for elucidating pathways beyond the low-hanging fruits that have already been harvested. To complement *in vivo* labelling experimental design shown to work for elucidation of specifically Type II pheromone pathways, new approaches may be required to reveal mechanisms of precursor modification that might happen using enzymatic steps not yet described to participate in pheromone biosynthesis. Auxillary enzymes from the fatty acid β-oxidation machinery (and not fatty acyl desaturases differentially expressed in the pheromone gland) may remain to be revealed and their working cellular environment to be discovered. This could involve CRISPR/Cas knock-out of candidate genes, as we successfully employed for identification of the key pheromone biosynthesis gene in *G. molesta*. Discovering the mechanism(s) behind formation of the E3 double bond in the *T. absoluta* pheromone components may prove useful for elucidation of pathways towards other pheromones with unusual double bonds in C_2-C_4 position, which could likely be intermediates in β-oxidation.

- Relating to methodology, we can acknowledge the importance of considering the differences in expression environments when characterising heterologous gene products. Dedicated research needs to be designed to reveal the specific cellular components necessary for native function of a membrane-bound protein, e.g. of a desaturase in Tortricid pheromone gland cells.

- The pheromone biosynthesis pathways for *D. saccharalis* and *G. molesta* have been identified, but work remains before their pheromones can be produced biotechnologically at large scale and used for pest management. The main pheromone components of *D. saccharalis* can be produced in yeast, but successful field application of synthetic pheromones for pest control has not yet been demonstrated in this species. The proper blend for field attraction needs to be developed, possibly with a yet unidentified pheromone component. For *G. molesta*, biotechnological production can only proceed once the Δ8 desaturase has been optimised for correct function in a heterologous production system like a yeast or a plant. Alternatively, a different pathway leading to the pheromone has to be engineered involving non-native enzymes and pathways, e.g introducing the double bond by a Δ10 or Δ12 desaturase followed by chain-shortening to produce the desired Δ8 product.

Optimisation of expression to produce significant titres at required purities remains a prominent challenge facing biotechnological pheromone production. Production costs have to be competitive with chemically synthetised pheromones. Political incentives to support sustainability and GMO-produced pheromones may facilitate and encourage a move away from conventional insecticides.

Research on moth sexual communication has come a long way since the paradigm of long-distance sexual communication was first discovered more than one hundred years ago and the first pheromone identified in 1959. We now understand a lot about pheromone evolution and the "mysteries of these mating messages" (Roelofs 2016). The research has also been driven by the possibility of application for pest management, as an alternative to the insecticides that have been discovered to inevitably cause harm. The first phase in this effort was identification of pheromones in moth pests and study of behaviour in relation to these compounds in the lab and field. A great aid in these endeavours were technical advances in methodology with development of GC/MS and EAD. The second phase in moth pheromone research was biosynthesis pathway discovery and gene discovery made possible by advances in molecular biology. The next phase is surely production of pheromones for pest control, using biology and biotechnology.

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