Finding the phenotypic and genetic basis of intraspecific pheromone variation in the moth *Heliothis subflexa* (Lepidoptera, Noctuidae)

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1 Summary

The sex pheromone of *Heliothis subflexa* females shows geographic variation between populations in North Carolina (NC) and Western Mexico (MX), as well as temporal variation between different years. A phenotype is influenced by its underlying genotype as well as environmental factors. I therefore took a genetic approach to characterize the genetic basis of the geographic variation, as well as a bioassay approach to test the influence of the sex pheromone of a closely related species *Heliothis virescens* on the sex pheromone composition of *H. subflexa* females and the male choice of *H. subflexa* females.

To understand what may have caused the geographic differences between NC and MX populations it is important to study the genetic basis of the diverging trait. Based on crosses between *H. subflexa* from NC and MX we created a genetic map using amplified fragment length polymorphism (AFLP) markers with backcross females. We were able to identify all 30 autosomes with 4 to 23 AFLP markers per chromosome. To determine which of the 30 autosomes explained parts of the variance in the relative amount of the sex pheromone differences between *H. subflexa* from NC and MX, we conducted a quantitative trait locus (QTL) analysis. We found a total of 14 QTL that accounted for 4-46 % of the variance in the sex pheromone components. This supports the idea that there are many genes involved in sex pheromone production. One chromosome (19) was a QTL for 4 components; it explained 46 % of the variance in Z9-16:OAc, 39 % of the variance in Z11-16:OAc, 24 % of the variance in Z11-16:Ald and 13 % of the variance in Z9-16:Ald. I discuss possible candidate genes for loci on this chromosome, such as delta-11-desaturase and acetyl transferase.

Exploring the causes of variation I examined if *H. subflexa* female sex pheromone and *H. subflexa* male choice showed phenotypic plasticity when the moths were reared under the influence of the sex pheromone of the closely related species *H. virescens*. I could not find phenotypic plasticity in the female sex pheromone composition in response to *H. virescens* sex pheromone after one generation. However, my results of an assortative mating experiment indicate that the male response could be plastic in response to *H. virescens* sex pheromone rearing. Males that were reared under the influence of *H. virescens* sex pheromone mated preferentially with females that had a higher relative amount of Z11-16:OAc. Such a difference was not found with males that grew up in the absence of *H. virescens* sex pheromone.

Zusammenfassung

Das Sexualpheromon von *Heliothis subflexa* variiert zwischen geographisch getrennten Populationen aus North Carolina und Mexico. Darüber hinaus zeigt es innerhalb einer Population in North Carolina zeitliche Variation im Vergleich zwischen zwei Jahren.

Ein Phänotyp wird durch den zugrundeliegenden Genotyp und Umweltfaktoren geprägt. In der vorliegenden Diplomarbeit wurde daher zum einen versucht die genetische Basis der geographischen Variation zu ermitteln, zum anderen wurde der Einfluss des Sexualpheromons der nah verwandten Art *Heliothis virescens* auf die Sexualpheromonkomposition des *H. subflexa* Weibchens und die Partnerwahl des Männchens getestet.

Um zu verstehen welche Faktoren einer geographischen Variation zugrunde liegen, ist es wichtig die genetische Basis des variierenden Merkmals zu kennen. Mit Hilfe von amplified fragment length polymorphsim (AFLP) Markern wurde auf der Grundlage von Kreuzungen zwischen NC und MX Individuen eine genetische Karte erstellt. Es gelang uns jedes der 30 Autosomen mit 4 bis 23 Markern pro Autosom zu identifizieren. Durch eine anschließende quantitative trait locus (QTL)-Analyse konnte teilweise ermittelt werden, welches Autosom die geographische Variation der einzelnen Komponenten im Sexualpheromon von *H. subflexa* bestimmt. Insgesamt wurden 14 QTL ermittelt, die 4–46% der Varianz im Sexualpheromon erklärten. Dies bestätigt, dass an der Produktion des Sexualpheromons viele Gene beteiligt sind. Herausragende Bedeutung kommt dabei einem Chromosom (19) zu, erklärt es doch 46 % der Z9-16:OAc-, 39 % der Z11-16:OAc-, 24 % der Z11-16:Ald- und 13 % der Z9-16:Ald-Varianz. Möglicherweise auf diesem Chromosom lokalisierte Gene, wie z. B. das Gen für eine Delta-11-Desaturase und eine Acetyltransferase, werden diskutiert.

Im zweiten Teil der Diplomarbeit wurde untersucht, ob auch phänotypische Plastizität als mögliche Ursache für Variation in Betracht gezogen werden kann. Getestet wurde, ob sich das Sexualpheromon von *H. subflexa* Weibchen bzw. die Partnerwahl konspezifischer Männchen verändert, sobald die Tiere unter dem Einfluss des Sexualpheromons einer nah verwandten Art (*H. virescens*) aufwachsen. Im Sexualpheromon der Weibchen konnte diese Plastizität nicht nachgewiesen werden. Allerdings weisen die Ergebnisse eines Paarungsexperimentes darauf hin, dass Männchen, die unter dem Einfluss von *H. virescens* aufgezogen wurden, vorrangig solche Weibchen zur Paarung wählen, deren Sexualpheromon eine höhere relative Menge an Z11-16:OAc aufwies. Männchen, die ohne den Einfluss des artfremden Sexualpheromons aufgezogen wurden, zeigen diese Präferenz dagegen nicht.

2 Introduction

2.1 Sex pheromones

Chemicals play an import role in the orientation of organisms towards potential food sources, to locate predators or to mediate social interactions. Substances that are involved in the chemical interaction of organisms are called semiochemicals (Nordlund and Lewis, 1976). The term comprises allelochemicals and pheromones. While allelochemicals mediate an interaction between two individuals that belong to different species (Dicke and Sabelis, 1988), the term pheromones refers to 'substances that are secreted by an animal to the outside and cause a specific reaction in a receiving individual of the same species, for example, a definite behaviour [releaser pheromones] or a developmental process [primer pheromones]' (Karlson and Lüscher, 1959). Sex pheromones form one subdivision of releaser pheromones. In moths they play a major role in premating communication and are thus major factors for sexual selection. It is mostly the female moths which produce sex pheromones to attract conspecific males over long distances (Lintner, 1882; Raina and Menn, 1987). They consist of at least two compounds (Cardé and Haynes, 2004) and often form a bouquet of several, mostly linear fatty acid-derived compounds with a chain length of 12-18 carbons (Tamaki, 1985). The carbon chains contain zero to three double bonds and an oxygenated functional group at the end which can be an alcohol, an aldehyde or an acetate ester (e.g. Witzgall, 2004; El-Sayed, 2008). Quality, quantity and function of the compounds in a blend are species-specific. Even though several species can use the same compound(s) (e.g. Klun et al., 1980; Teal et al., 1981; Tamaki, 1985; Löfstedt et al., 1991; Groot et al., 2009a), their relative amount and function differs from species to species (e.g. Löfstedt, 1991; 1993). The major component is the most abundant component of the blend (e.g. Teal et al., 1981; Klun et al., 1980b) and essential for male attraction (Tamaki, 1985). It is often supplemented with one or more secondary critical compounds that are necessary for male attraction as well (e.g. Vickers, 2002). Other compounds of the blend can enhance conspecific male attraction, inhibit the attraction of heterospecific males (e.g. Klun et al. 1980; Vickers and Baker, 1997) or may be merely byproducts of pheromone biosynthesis (Groot et al., 2009b).

2.1.1 Sex pheromone of *Heliothis subflexa* (Lepidoptera; Noctuidae)

The sex pheromone blend of female *H. subflexa* was identified by Teal et al. (1981) and Klun et al. (1982). The major sex pheromone component of *H. subflexa* is (*Z*)-11-hexadecenal (Z11–16:Ald) (Teal et al., 1981; Klun et al., 1982; Teal and Tumlinson, 1997). (*Z*)-9-hexadecenal (Z9-16:Ald) and (*Z*)-11-hexadecenol (Z11-16:OH) serve as secondary critical compounds (Heath et al., 1990; Vickers, 2002, 2006; Groot et al., 2007), while the role of the minor compounds (*Z*)-9-tetradecenal (Z9-14:Ald), tetradecanal (14:Ald), (*Z*)-7-hexadecenal (Z7–16:Ald), and (Z)-9-16 hexadecen-1-ol (Z9-16:OH) is not clear yet. Additionally, *H. subflexa* females produce three acetate esters, (*Z*)-7-hexadecenyl acetate (Z7–16:OAc), (*Z*)-9-hexadecenyl acetate (Z9–16:OAc), and (*Z*)-11-hexadecenyl acetate (Z11–16:OAc) (Teal et al., 1981). Z7-16:OAc and Z11-16:OAc have a positive effect on conspecific male attraction (Groot et al., 2007).

2.1.2 Where and how are sex pheromones produced?

Pheromone biosynthesis in moths takes place in specialized glands that are located between the 8th and 9th abdominal segment of the females (Percy-Cunningham and MacDonald, 1987; Raina et al., 2001).

Pheromone production in many moths is induced by a pheromone biosynthesis activating neuropeptide (PBAN) (Raina et al., 1989) that is released into the hemolymph from the corpora cardiaca (e.g. Raina, 1993). This neuropeptide interacts directly with receptors on the pheromone gland membrane and thus induces key enzymes of the sex pheromone biosynthetic pathway (e.g. Tsafadia et al., 2008). Sex pheromone biosynthesis in moths starts with the production of saturated C-18 fatty acids. The synthesis is carried out by fatty acid synthetases and acetyl-CoA carboxylases (Jurenka, 2003). Special desaturases introduce double bonds into the chains. Specifically, delta-11-desaturases (Bjostad and Roelofs, 1983), but also delta-9-desaturases (Roelofs and Wolf, 1988) play an important role in many moth species (Knipple et al., 2002). The fatty acid chains are chain-shortened to 16 or less carbons by chain-shortening enzymes (Bjostad and Roelofs, 1983) and subsequently reduced to alcohols or aldehydes via fatty acid reductases (Morse and Meighen, 1987a). The following enzymes form the functional oxygenated groups (Tillman et al., 1999) of the pheromone compounds: The alcohol precursors can be converted to aldehydes via alcohol oxidases (Teal and Tumlinson, 1988) or to acetate esters via fatty alcohol acetyltransferases (Morse and Meighen, 1987b). The aldehydes can be converted to alcohols via aldehyde reductases

(Morse and Meighen, 1986). Acetates can be transformed to alcohols via acetate esterases (Teal and Tumlinson, 1987; Roelofs and Wolf, 1988; Jurenka, 2003; Gould et al., 2009).

2.1.3 Species-specificity of sex pheromones

Sender and receiver systems of one species are often thought to be specifically tuned to each other. Thus, sex pheromone specificity can be the result of intraspecific interactions of sender and receiver of a mate signaling system (Butlin and Tricket, 1997; Endler, 1992). A deviation from the standard in either one will not or with a diminished probability lead to a mating success and is therefore hypothesized to be selected against (Butlin and Tricket, 1997; Löfstedt, 1993; Cardé and Haynes, 2004). The system is thus hypothesized to be under stabilizing selection. However, there are many examples of geographic variation in the sex pheromone between populations of one species (e.g. Löfstedt, 1986, 1993; McElfresh and Millar, 1999; Kawazu et al., 2000; Groot et al., 2009a). This is often referred to as dialects (Löfstedt et al., 1986).

2.2 Geographic variation in the sex pheromone of *H. subflexa*

Significant differences in the sex pheromone composition of 4 *Heliothis subflexa* populations have been found in 2005 (Groot et al., 2009a). The most significant differences were those between populations in Clayton, North Carolina (NC) and Chamela, Western Mexico (MX). All sex pheromone compounds of *H. subflexa* except for the alcohols were significantly different between the two regions. The most significant differences were found in the relative amount of acetates: The levels of all three acetates were significantly higher in North Carolina females than in Mexican females (Fig. 1).

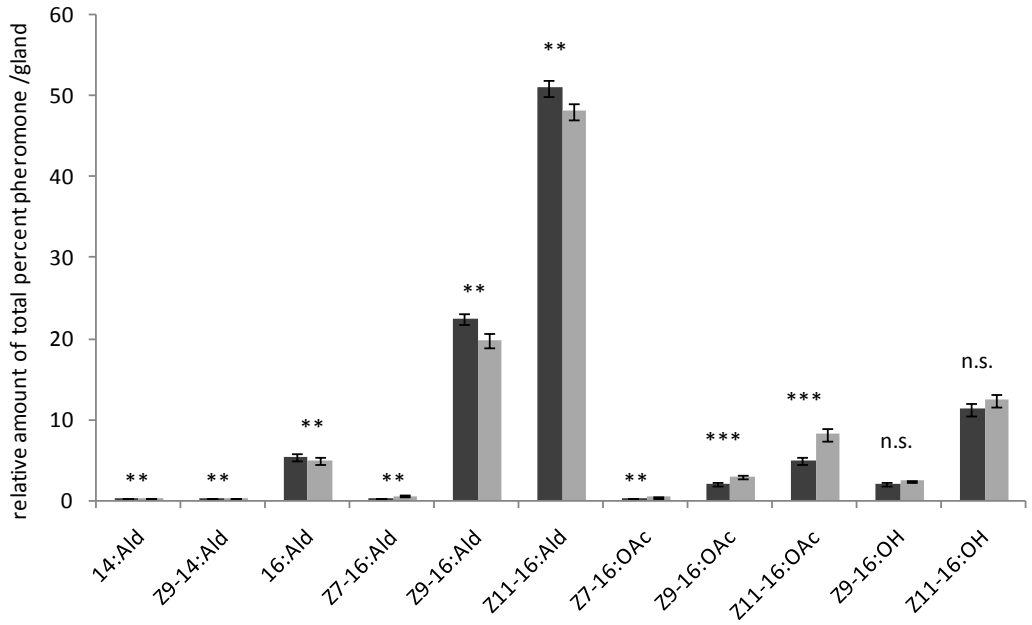

Fig. 1. Sex pheromone composition of NC females (black bars ± SE; N = 30) and MX females (grey bars ± SE; N = 31) (Groot et al., 2009a). Individual compounds tested via one-tailed ANOVA.*P < 0.05; **P < 0.01; ***P < 0.001

What may have caused the variation in the sex pheromone blend of female *H. subflexa*?

2.3 Phenotype: interactions of genotype and environment

The genotype-specific relation between phenotypes and the environment is a continuous function that has long been described as “norm of reaction” (Pigliucci, 2001). The norm of reaction is a property of the genotype and defines the extent and direction of the plasticity in the expression of a phenotype (Nager et al., 2000). Geographic variation in a trait can be due to different norms of reaction between populations, i.e. the differences have a genetic basis and this basis defines the possible extent and direction of plasticity of the trait. Individuals of the same genotype can express different phenotypes in different environments (Pigliucci, 2001).

This study therefore focuses on two main questions to gain understanding in the cause of variation in *H. subflexa* sex pheromone:

2.3A What is the genetic basis of the geographic variation between *H. subflexa* NC and MX populations?

2.3B Is there phenotypic plasticity in the sex pheromone of *H. subflexa*, i.e. does the chemical environment of *H. subflexa* affect the sex pheromone composition of a female?

2.4 Possible factors that may influence the variation in the *H. subflexa* sex pheromone

Mate signaling systems are said to be under strong stabilizing selection as mentioned above. Stabilizing selection may be counteracted if other species such as predators and parasitoids use the signal to find a potential prey (Ryan et al., 1982; Cardé and Haynes, 2004), or host (e.g. Zuk et al., 2006; Bailey and Zuk, 2008) or if closely related species are attracted to the emitted signal (Löfsted et al., 1991; McElfresh and Millar, 1999; Gries, 2001; Groot et al., 2006). All three examples of species interaction can affect the fitness of individuals of the species. The result might be directional selection that favors individuals at one end of a distribution (Löfstedt, 1993; Groot et al., 2006). The consequence could be a stable shift in a mate signaling system. If the degree of species interaction varies among populations their means of characters can become different due to differential selection between the two populations. There has been some indication that the presence of closely related species may be the cause for the variation in sex pheromone of moth species (Löfstedt, 1986; Löfstedt, 1991; Groot et al., 2006). In the case of *H. subflexa*, the closely related *H. virescens* may cause communication interference (Groot et al., 2006, 2009a).

2.5 Communication interference

The sex pheromone of closely related species often overlaps in several components (Linn and Roelofs, 1989; Löfstedt et al., 1991; Groot, 2007). Although these species can be sympatric and synchronic and even use the same major critical component, cross-attraction is rare in nature (Roelofs and Brown, 1982; Löfstedt, 1993) but can occur sporadically (Chapin et al., 1997). This cross-attraction can lead to hybridization which has been observed in the lab (Löfstedt, 1991; Laster, 1972). There may be fitness costs to both, cross-attraction and hybridization: Males that are attracted to a heterospecific sex pheromone blend spend more time and energy on searching for conspecific females which means a possible loss of mating opportunities (Cardé and Haynes, 2004). Also, hybrid offspring is often sterile or has reduced general fitness (Hendrikse, 1988; Laster, 1972). In the case of hybrid matings between *H. virescens* and *H. subflexa* the female offspring is fertile but the males are sterile (Laster, 1972). Because of these fitness costs the presence of other species that may interfere in the communication channel may cause directional selection in a pheromone blend in regions of sympatry (Groot et al., 2006). Two populations may diverge in their sex pheromone compositions if one occurs in sympatry and the other in allopatry with an interfering species (reproductive character displacement) (e.g. Gries et al., 2001; McElfresh and Millar, 1999).

2.5.1 Communication interference between *H. subflexa* and *H. virescens*

Several Heliothine species that are found in North America overlap in one or more compounds of their sex pheromone blend (e.g. Klun et al., 1980; Teal et al., 1981; Vetter and Baker, 1984; Heath et al., 1991; Chapin et al., 1997; Baker et al., 2004; Groot et al., 2007). Most Heliothines utilize the same major component (Klun et al., 1980, 1982; Teal et al., 1981; Heath et al., 1991; Vickers, 2006). Communication interference could therefore occur between synchronic species in areas of sympatry.

Of the Northern American Heliothines the phylogenetically most closely related species to *H. subflexa* is *Heliothis virescens* (Fabricius, 1777) even though they are no sister species (Mitter, 1993). The sex pheromone blends of two species overlap in several components. They produce the same aldehydes and alcohols but in different ratios (Teal et al., 1981; Klun et al, 1982; Heath et al., 1991), both use Z11–16:Ald as major component (Teal et al., 1981; Klun et al., 1982; Heath et al., 1991; Baker et al., 2004; Vickers, 2006). The secondary critical component in *H. virescens* is Z9-14:Ald (Vetter and Baker, 1983), instead of Z9-16:Ald and Z11-16:OH in *H. subflexa* (see above). In contrast to *H. subflexa*, *H. virescens* does not produce any acetates (Klun et al., 1980). However, *H. virescens* males do have receptors for

Z11-16:OAc (Berg et al., 1995). This compound that has a positive effect on *H. subflexa* male attraction (Groot et al., 2007) was found to inhibit *H. virescens* male attraction in wind-tunnel experiments (Vickers and Baker, 1997). Also, Groot et al. (2006) showed in field experiments conducted in NC with backcross females (the tested *H. subflexa* backcross females contained one *H. virescens* chromosome that contained a major QTL that is responsible for a decreased acetate production) that *H. virescens* males were significantly attracted to *H. subflexa* females with acetate levels that were lower than 5 % of the total amount of pheromone per gland. Such attraction did not occur with wild-type *H. subflexa* that contain between 10 and 30 % acetates. Thus, the acetates were found to have an important role to avoid cross-attraction between the two species and the high abundance of presence *H. virescens* could thus well be a factor that has caused an increase in the acetate levels in *H. subflexa*, by directional selection (Groot et al., 2006). The distribution of the two species in correlation to *H. subflexa* acetate levels supports this idea.

Heliothis subflexa and *H. virescens* co-occur in many regions throughout North America (Teal and Tumlinson, 1997; Groot et al., 2006). However, the relative abundance of *H. subflexa* and *H. virescens* varies geographically (Groot et al., 2007). In Western Mexico *H. subflexa* is much more abundant than *H. virescens*, while *H. virescens* is more numerous than *H. subflexa* in the Eastern US. The relative abundance of both species can be explained by their host plant distributions: *H. subflexa* is a specialist whose larvae feed exclusively on fruits of plants in the genus *Physalis* (Solanaceae) (McElvare, 1941). *Physalis* are ruderal plant species that grow in disturbed habitats along roadsides or in disturbed fields, and are scattered scarcely throughout the US. In many regions of Mexico one *Physalis* species, the tomatillo (*Physalis philadelphica* Lam.) is a major commercial crop and thus highly abundant. As a consequence, the occurrence of *H. subflexa* in the US is rather patchy, while it is very numerous in Western Mexico (Groot et al., 2007). *Heliothis virescens* is a generalist, feeding on plants from at least 14 different families, and a major pest on various crops (Laster, 1972; Sheck and Gould, 1993). As such it is highly abundant throughout the US. Trap catches conducted in 2005 on field sites in the Eastern US showed that *H. virescens* is more numerous than *H. subflexa* in the North East of the US. The traps caught 540 *H. virescens* males and 455 *H. subflexa* males in the same year (Groot et al., 2007). As mentioned above, the acetate levels of *H. subflexa* females in this region are relatively high (Groot et al., 2009a). While *H. subflexa* is a specialist on *Physalis* plants, larvae of *H. virescens* show reduced survival and less larval weight on plants of that genus (Sheck and Gould, 1993) compared to other plants. This was reflected in the number of *H. virescens* males caught in pheromone traps in Western Mexico, with 3 *H.*

virescens males and 785 *H. subflexa* males. Interestingly the acetate levels in this area are much lower (Fig. 1) (Groot et al., 2007, 2009a).

2.6A What is the genetic basis for the geographic variation in the female sex pheromone of *H. subflexa*

When larvae from NC and MX were brought into the laboratory at NCSU in 2005 and reared separately in the lab for 6 generations, the significant geographic differences found in the field populations remained in the lab over the generations (A. T. Groot, unpubl. res.). This indicates a genetic basis for the geographic variation in the sex pheromone blends of female *H. subflexa*. The relative amount of a sex pheromone compound in a multi-component blend is a quantitative trait (Sheck et al., 2006; Groot et al., 2009b) i.e. the phenotypic variation of the trait is 'continuous', so that the phenotypes cannot be classed into discrete groups (Via and Hawthorne, 1998). The genetic basis of such a trait can be studied with the means of a quantitative trait locus (QTL) analysis. This requires a genetic map that can be created by identifying markers with the amplified fragment length polymorphism (AFLP) method (Remington et al., 1999; Vos et al., 1995). The first steps in creating a map and performing a QTL analysis are crosses between parents that differ in a large number of neutral makers and are phenotypically divergent (Via and Hawthorne, 1998). Markers that are present in one of the parents and absent in the other, and that show a 1 : 1 segregation in the backcross individuals, are used to construct a genetic map. Markers that show the same segregation pattern among the individuals can be combined to linkage groups (Remington et al., 1999). Heckel (1993) found that there is no crossing-over in female moths. Consequently, the identified groups can be considered chromosomes.

With a QTL analysis one can determine a) which loci and b) how many loci are involved in producing a certain phenotype, c) the magnitude of the effect of particular loci on a phenotype and d) if there are any interactions among the loci, e.g. epistatic or additive effects (Via and Hawthorn, 1998; Groot et al., 2009b).

We hypothesize that we can characterize a genetic basis for the sex pheromone compounds of *H. subflexa* females that were found to be significantly different in their relative amounts between NC and MX populations by Groot et al. (2009a) by the means of a QTL analysis.

The distribution pattern of QTL on the map for the compounds together with phenotypic correlations among the compounds and a given knowledge of the biosynthetic pathway can

point towards candidate genes that bring about a certain phenotype. It is therefore helpful to understand the biosynthetic pathway of *H. subflexa*. (Fig. 2)

2.6.1 Biosynthesis of *H. subflexa* female sex pheromone

Figure 2 summarizes the likely biosynthetic steps of the *H. subflexa* female sex pheromone compounds (Jurenka, 2003; Choi et al., 2002, 2005; Groot et al., 2009). It is important to notice that the compounds are interrelated via biosynthesis as they often use the same precursors. As a consequence their relative amounts are not independent of each other, they are phenotypically correlated. Figure 2 shows that two groups of compounds, Z9-16- and Z11-16:Acids, can be synthesized via a delta-11-desaturase (Choi et al., 2002, 2005). Another group, Z7-16:Acids, can only be synthesized via a delta-9-desaturase, as was shown for *Helicoverpa zea* (Choi et al., 2002). Z9-14:Ald can be synthesized via both desaturases. The alternative way of Z9-16:Acids synthesis is via delta-9-desaturation of 16:Acids. However, this way was shown to play a minor role in *H. subflexa* (Choi et al., 2005).

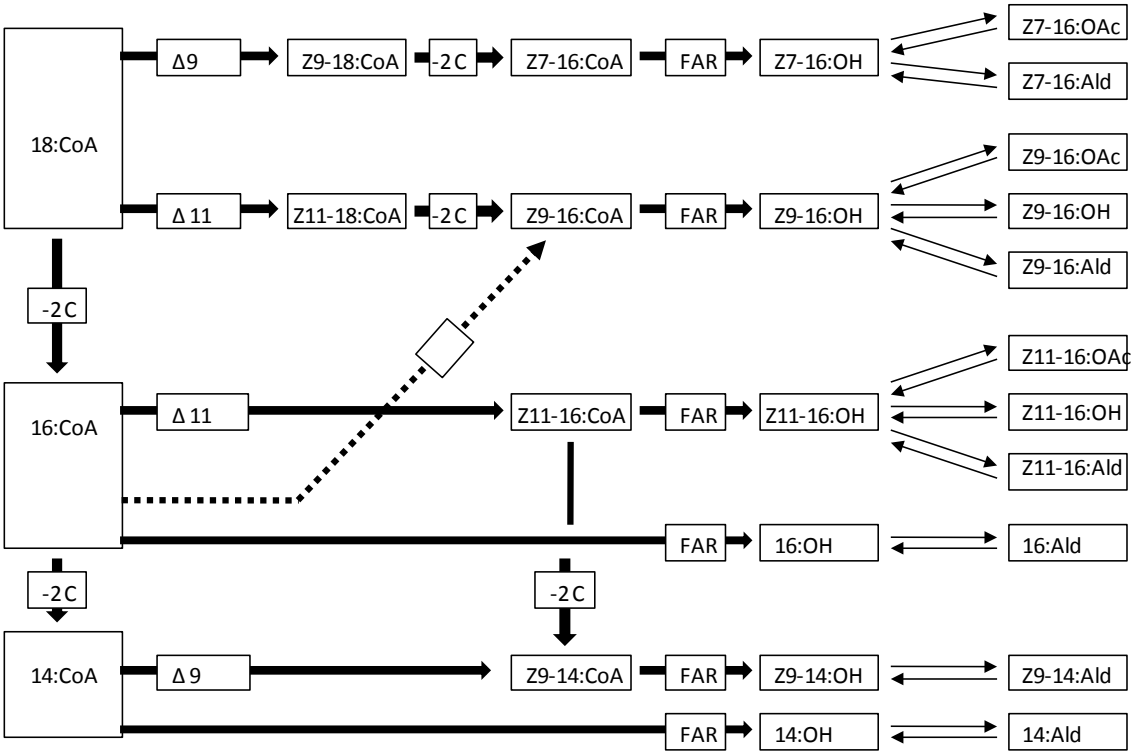

Fig. 2. biosynthetic pathway of pheromone compounds in *H. subflexa* adapted from Groot et al., 2009b, partly based on Jurenka, 2003; Choi et al., 2002, 2005; Groot et al., 2009b) FAR: Fatty acyl reductase.;Δ11: delta-11-desaturase; Δ9: delta 9-desaturase.

2.6B Is there phenotypic plasticity in the sex pheromone of *H. subflexa*: When reared under different environmental conditions, do females differ in their sex pheromone?

The term phenotypic plasticity means the ability of an organism to adjust its phenotype to the biotic or abiotic environment (Agrawal, 2001). The phenomenon can be seen on many traits in various species. A classic example is the heterophylly in the aquatic plant *Ranunculus aquatilis* (e.g. Cook, 1969). Leaves of the plant that develop underneath the water surface are feathered and leaves that develop above the water surface have laminar shape. Another famous example is the phenotypic plasticity in daphnids. *Daphnia pulex* for example develops sharp spine structures on the neck as a reaction to chemical cues of predacious midge larvae (Dodson, 1990). These structures are an effective defense against the predators. The spine structures are reduced in the absence of the predator. The reason for plasticity in this trait might be that there is a fitness cost in producing the spine structures. Reduced reproduction rates have been observed for *D. pulex* when spine structures are expressed (Dodson, 1989).

The term phenotypic plasticity is not synonymous to “norm of reaction” that was mentioned earlier. The norm of reaction comprises all the possibilities of plastic responses to environmental cues. A trait has a norm of reaction even if it is not plastic (Schlichting and Pigliucci, 1998).

There are many reports in which phenotypic plasticity and selection on a trait can interact. Waddington (1952) showed that an initially plastic trait can be stabilized by natural selection. He applied heat shocks to *Drosophila melanogaster* that induced the expression of specific wing patterns in some of the flies. Selection for this phenotype generated flies with that specific wing pattern without the application of a heat shock. Thus, the norm of reaction had changed such that a formerly extreme phenotype had become the “normal” phenotype (Schlichting and Pigliucci, 1998). Another example is polyphaenism in the eyespot pattern of the satyrine butterfly *Bicyclus anynana* (Brakefield and Reitsma, 1991). The early summer pattern of this butterfly differs from the late summer pattern. The pattern expression depends largely on temperature and is thought to camouflage the butterfly in a changing environment (Brakefield et al., 1996). Brakefield and co-workers succeeded in selecting for the two distinct phenotypes in the absence of temperature differences. This shows that plasticity itself could lead to genetic differentiation among populations of one species with selection influencing a trait depending on the environment. If an environment evokes high plasticity, plasticity might even delay or prevent natural selection (Prize et al., 2003). As described by Agrawal (2001) plasticity can facilitate the successful colonization of a new

habitat. The colonization of a new habitat by the plastic individuals might finally lead to allopatry with the original population which supports genetic differentiation. Finally plasticity can be lost if there is a cost to plasticity that exceeds the advantage of a plastic response (Agrawal, 2001).

2.7.B.1 Plasticity in the sender: is there plasticity in the female sex pheromone composition?

Sex pheromones are thought to be under strong stabilizing selection (e.g. Butlin and Tricket, 1997). The variability that we expect from a system under stabilizing selection is very low (e.g. Löfstedt, 1993). Despite that expectation we find high variability in the relative amounts of sex pheromone compounds in various species (Löfstedt, 1991; McElfresh and Millar, 1999; Groot et al., 2009a). As outlined before, a possible explanation is provided by some reports that indicate directional selection on the female sex pheromone (Groot et al., 2006; Löfstedt, 1991; Löfstedt, 1993; McElfresh and Millar, 1999). However, directional selection may not explain all of the variation. The concept of plasticity in moth female sex pheromone composition, although entirely new, could be a possibility that explains more of the variation that can be found in natural *H. subflexa* populations. A number of characteristics support the idea of plasticity in the female sex pheromone composition in moths: First of all, females produce their pheromone *de novo* every night (e.g. Rafaeli, 2002; Jurenka, 2003). In many moth species females can perceive their own species' female pheromone compounds (Den Otter et al., 1978; Schneider et al., 1998; Groot et al., 2005). Consequently, females are likely to perceive heterospecific sex pheromone, at least if their own pheromone blend overlaps with that of the other species. Recently pheromone receptors have even been found on the ovipositor sensilla of female *H. virescens* (Widmayer et al., 2009). The authors suggest that these receptors might allow a direct feedback mechanism onto the gland.

Plasticity may be due to a number of factors, abiotic as well as biotic. As outlined above there is indication of communication interference between *H. subflexa* and *H. virescens*. Temporal variation in the *H. subflexa* sex pheromone in correlation to *H. virescens* abundance (Groot et al., 2009a) makes phenotypic plasticity in *H. subflexa* females as a response to *H. virescens* pheromone plausible: Trap catches conducted in two consecutive years resulted in two different ratios of *H. subflexa* /*H. virescens*: In the year 2004 a ratio of 411 *H. virescens* to 103 *H. subflexa* males was caught. *H. virescens* was thus more numerous than *H. subflexa* in 2004. The acetate levels in *H. subflexa* females were high in that year (Groot et al., 2009a). In 2005 in the same areas and time of the year a ratio of 104 *H. virescens* to 216 *H. subflexa*

males was caught in the traps, suggesting that *H. subflexa* was more numerous than *H. virescens* in that year. Interestingly, the acetate levels were significantly lower in 2005 than in 2004 (Groot et al., 2009a). This variation may thus at least partly be due to the relative abundance of *H. virescens* at a certain time.

There are different stages possible at which the odor environment could influence the pheromone composition in adult females. For example, odor experience of early larval stages could influence the adult females' pheromone composition which would be similar to Hopkins' principle for oviposition (Hopkins, 1916). On the other hand, late-instar larvae have been shown to have a sensitive phase for some traits that are plastic in Lepidoptera (Poulton, 1887; Brakefield et al., 1996). Learning an odor at a larval stage can persist through metamorphosis. This was shown for the fruit fly *Drosophila* (Tully et al., 1994), the fly *Musca domestica* (Ray, 1999) and the moth *Manduca sexta* (Blackinston et al., 2008). The latter study proved associative learning of an odor in a caterpillar possible. The memory of the odor persisted through metamorphosis. The sensitive phase for pheromone determination could also be the late pupal stages because that is the time when glands for the pheromone production develop (e.g. Tang et al., 1991) Another possible sensitive phase of the female could be the emergence of the adult moth from the pupal stage and early adult stages, similar to the neo-Hopkins selection principle (Jaenike, 1983, 1988). Since this is the first study to explore whether there might be phenotypic plasticity in response to the presence of *H. virescens*, I exposed *H. subflexa* larvae and adults to *H. virescens* pheromone at all stages assess if there is plasticity.

I hypothesize that *H. subflexa* females that were reared at all stages under the influence of synthetic *H. virescens* sex pheromone for one generation will have a higher relative amount of acetates in their sex pheromone blend than those *H. subflexa* females that were reared in the absence of synthetic *H. virescens* sex pheromone.

2.7.B.2 Phenotypic plasticity in the male response

In contrast to phenotypic plasticity in female sex pheromone, which likely requires a change in enzymatic activity of (at least) one of the enzymes in the biosynthetic pathway of pheromone production, there are many examples of phenotypic plasticity in behavioral responses. Among insects there are various cases of associative learning that influences the behavior of the animal later in its life. For example, parasitoids are well known for their ability to learn and associate odors of a potential host (Vet and Groenewold, 1990; Steidle and Schöller, 1997). Also in sexual communication the receiver has been shown to be plastic in its response to a signal. Female crickets on Kauai of the species *Teleogryllus oceanicus* were found to be more or less "choosy" regarding the male song depending on sound experience in their early adult lives (Bailey and Zuk, 2008).

There have also been some reports on the plastic response of male moths. Linn et al. (1987) found that the oriental fruit moths *Grapholita molesta* reacts to different blends and doses of pheromone depending on temperature. In another study pre-exposure of *G. molesta* males to one compound of the pheromone modified their blend discrimination ability. The results were dependent on duration and dosage of the pre-exposure (Linn et al., 1981). Recent studies on *Spodoptera littoralis* showed that males alter their response to sex pheromone as a reaction to the pre-exposure to conspecific sex pheromone (Anderson et al., 2003, 2007).

Geographic variation was also found in the response of *H. subflexa* males. When Groot et al. (2007) tested pheromone lures with different acetate compositions in both NC and MX, they found that the three acetates as a whole were significantly more attractive in NC than in MX. Specifically, the addition of Z7-16:OAc increased male attraction in NC but did not have an effect in MX.

I hypothesize that rearing *H. subflexa* males under the influence of synthetic *H. virescens* sex pheromone will influence the male choice with regard to the *H. subflexa* female sex pheromone composition.

3 Material and Methods

3A. What is the genetic basis of sex pheromone differences between *H. subflexa* females from NC and MX?

3A.1 Crosses (Fig. 3)

The crosses to create a genetic map based on the geographic variation of two *H. subflexa* populations were performed in 2008 at North Carolina State University (NCSU). In the parental generation a NC female was crossed with a MX male. One hybrid female of the F1 generation was backcrossed to a male from Western Mexico (Fig. 3). Only female backcross individuals were used for further steps. Sex pheromone and DNA from each female were extracted by Olive Inglis at NCSU. The pheromone was extracted as described below using PBAN to induce pheromone production and analyzed at the NCSU. The extracted DNA of the parental generation, the F1 female and the backcross male as well as the DNA of the female backcross offspring was shipped to MPI, Jena, which allowed me to a) construct a map based on AFLP markers and b) perform the QTL analysis. To assess phenotypic correlations among the sex pheromone compounds a Pearson's correlation matrix was generated from the sex pheromone compositions of the backcross females. Pearson's correlation was calculated via Microsoft Office Exel (Version 2007).

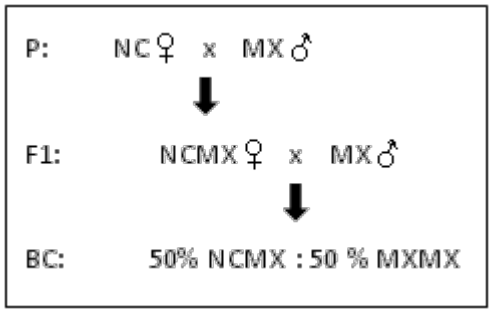

Fig. 3. Crosses to create an AFLP map and perform a QTL analysis. NC: *H. subflexa* from North Carolina, MX: *H. subflexa* from Mexico. NCMX: heterozygous for North Carolina and Mexico. MXMX: homozygous for Mexico.

3A.2 Constructing a genetic map using AFLP markers

To find AFLP markers and group them to 30 autosomal groups, an AFLP protocol was carried out with the DNA of the two parental moths, as well as the F1 female, the backcross male and 96 female backcross individuals. The AFLP protocol was adapted from Vos et al. (1995) and Remington et al. (1999). The protocol includes a restriction digest, an adapter ligation, a preamplification and selective amplifications. These reactions were conducted on Mastercycler epgradientS thermo cyclers (Eppendorf). The reactions were carried out in

96-well plates and covered with foil (nerbe plus, Winsen/Luhe). Before each reaction step the samples were vortexed (vortex genie 2, scientific industries) and centrifuged down (5810 R, Eppendorf).

For the restriction digest an initial amount of 200 ng/µL (a volume of 10.55 µL) of genomic DNA was used. The DNA was digested with 0.4 µL of the restriction enzyme *Eco*RI (5 U) and 0.3 µL of *Mse*I (3 U) (both NEB (New England Biolabs)). The reaction was buffered with 1.25 µL 10x NEB buffer #2 that contained 100 µg/mL BSA (bovine serum albumin). The mixture was complemented with 1.75 µL of ddH_2O to a volume of 12.5 µL and incubated for 2 h at 37 °C in a thermo cycler.

As a next step *Eco* and *Mse* adapters were ligated to the fragments. Therefore 1 µL of *Eco*-(5 pmol/ µL) (*Eco*RI top strand adapter 5'-CTCGTAGACTGCGTACC-3', *Eco*RI bottom strand adapter 5'-AATTGGTACGCAGTCTAC-3') and 1 µL of *Mse*-adapters (*Mse*I top strand adapter 5'-GACGATGAGTCCTGAG-3', *Mse*I bottom strand adapter 5'-TACTCAGGACTCAT-3')(both Metabion) (50 pmol/µL) were added to the complete restriction digest. 0.5 µL of T4 DNA Ligase (200 U) (NEB) served as a ligase and 2.5 µL of the respective 10x T4 DNA Ligase buffer (NEB) (1x 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM Dithiothreitol, pH 7.5 at 25 °C) were used as a buffer. To achieve a total volume of 25 µL, 7.5 µL of ddH_2O were added. This reaction mix was incubated for 2 h at 16 °C and afterwards diluted 1 : 10 by combining 10 µL of the ligation product with 90 µL of $TE_{0.1}$-Buffer (10 mM Tris-(Trishydroxymethyl)-aminomethan) HCl, pH 8.0, 0.1 mM EDTA (EthyleneDiamineTetraacetic Acid)).

2 µL of this diluted sample were preamplified using 1.0 µL of *Mse-0* (5 pmol/µL) and 1.0 µL of *Eco-0* (5 pmol/µL) (Metabion) primers, 1.0 µL dNTPs (2.5 mM) (Metabion), 1 µL of 10x *Taq* optimized PCR-buffer (contained 15 mM $MgCl_2$) (Metabion) and 1 µL of *Taq* polymerase (1 U) (Metabion). 3.8 µL of ddH_2O were added for a total reaction volume of 10 µL. The PCR program included 20 cycles of 30 s at 94 °C; 60 s at 56 °C; 60 s at 72 °C. For a 1 : 50 dilution 2 µL of the preamplification were transferred to 98 µL of $TE_{0.1}$ buffer.

Several selective amplifications were conducted with the diluted preamplification using *Mse*I and *Eco*RI primers (both Metabion) that matched the adapters. The core of the *Eco*RI primers had the sequence 5'-GACTGCGTACCAATTC the *Mse*I core sequence was 5'-GATGAGTCCTGAGTAAC. In addition to this core sequence the primers contained three "selective" basepairs that differed for each selective amplification (for all utilized combinations see table I, II and III (appendix); the tables show the primer combinations that were run by Olive Inglis (indicated with an asterisk), and me).

In order to visualize the amplified fragments on a polyacrylamide gel in the Li-Cor machine, I used *Eco*RI primers that were labeled with an infrared dye (IRD) of 700 or 800 nm respectively. The fragments were amplified in a multiplex PCR that included IRD700 as well as IRD800 labeled *Eco*RI primers that differed in the selective base pairs.

For a single sample, 0.4 µL of a 1 : 1 mixture of labeled IRD700 (0.5 pmol) and unlabeled (0.5 pmol) *Eco*RI primers was used. Since the IRD800 *Eco*RI primer is less visible I used 0.6 µL of a 1 : 1 mixture of labeled IRD800 (0.5 pmol) and unlabeled (0.5 pmol) *Eco*RI primers. The reaction mix further included 1.1 µL ddH_2O, 1.1 µL 10x *Taq* optimized PCR-buffer (15 mM $MgCl_2$), 3.0 µL *Mse*I-primer (1 pmol) (Metabion), 0.9 µL dNTPs (2.5 mM) (Metabion) and 0.1 µL of *Taq* polymerase (5 U/µL). The thermo cycler was programmed to 12 cycles of 10 s at 94 °C; 30 s at 65 °C (-0.7 °C per cycle); 60 s at 72 °C and 23 cycles of 10 s at 94 °C; 30 s at 56 °C; 60 s at 72 °C.

Before loading the samples on a gel, 15 µL of Stop solution (95 % foramide, 10 mM EDTA, 0.25 % bromophenol blue) and 10 µL of water were added to each sample. The AFLP fragments were denatured on a thermo cyler at 94 °C for 5 minutes then loaded on a polyacrylamide gel (20 mL 6.5 % gel matrix (Li-Cor), 200 µL Ammoniumpersulfat, 20 µL Ultra Pure Temed (Invitrogen)). The fragments were separated according to size. About 4 µL of the denatured samples were loaded with a 6 channel Gastight #1701 syringe. Two slots on each side flanking the samples were filled with 3 µL of a 1 : 1 mixture of IRD700 and IRD800 labeled standard size marker (Li-Cor STR marker 50-700 bp). The AFLP fragments were run for 2.5 h at 1500 V and 45 °C on a Li-Cor 4300 DNA Analyzer. A prerun of 25 minutes at the same conditions was performed before a gel was used. The Li-Cor machine includes a laser scanner that detects infrared labeled DNA fragments of 700 nm and 800 nm simultaneously. The images were recorded in a computer file.

3A.2.1 How many primer combinations were run by whom and with how many individuals?

Initially Olive Inglis ran 70 primer combinations (table I, appendix, marked with an asterisk) on 44 individuals. I ran 22 more primer combinations with these 44 individuals starting with Olive Inglis' preamplification (table I, appendix). Based on these gels we were able to construct a map of 29 linkage groups (see below).

In order to increase the sample size I repeated the whole AFLP procedure starting from the DNA with 52 additional individuals from the same family. For each chromosome that we

identified I ran at least one primer combination with the 52 extra individuals in order to find the same markers again and match the new individuals to the map (24 primer combinations, see table II, appendix). To ascertain that the same markers were identified in the new and the old gels I included 8 of the "old" individuals into the new gel layout. (These gels also contained 4 bulks of DNA, the purpose of which is described in paragraph 3A.5).
After finding at least one marker of each of the 29 groups for the 52 additional individuals, so that these 52 individuals were now included into the map, I chose 88 of all 96 individuals and ran 17 more selective amplifications and gels to find the 30^{th} chromosome. Primer combinations see appendix (table III, appendix).

With the AFLP-markers that could be found on the basis of these gels we tried to find 30 autosomal groups that make up a complete autosomal map for Lepidoptera, as follows.

3A.3 Mapping

I scored all the gels including those of Olive Inglis with the program SAGA generation 2 (Li-Cor) by hand, i.e. i.e. we visually checked each marker and scored a line in all the backcross individuals when the band was present in one of the parental individuals and in the F1 female and absent in the backcross male. The presence of a band in the backcross individuals meant that the individual was heterozygous for this locus with one copy from NC and one from MX. An absence meant that the individual was homozygous MX/MX for this locus. A presence was recorded to a Microsoft Office Exel file as 1, an absence as 0. After scoring the markers in all backcross individuals, markers that deviated significantly from a 1 : 1 segregation pattern in the backcross individuals were identified via a Chi-square test and deleted.
The markers were grouped with the mapping program Mapmaker 3.0. The group analysis was carried out with logarithm (base 10) of odds (LOD) scores from 3 to 8. The lowest LOD score that gave stable groups was decided for. Since the presence-absence marker patterns did marginally deviate from each other for one chromosome we performed a SAS analysis (proc GLM) to find a consensus pattern of presence and absence for each chromosome.
The final map was created in several steps:
3A.3.1 Construction of a preliminary map, using 207 markers from the gels run by Olive Inglis and 89 from my gels (total of 296 markers). These markers could be grouped into 29 linkage groups, which can be considered chromosomes as there is no crossing over in Lepidoptera (Heckel, 1993). The initial map was based on 44 individuals, 52 extra individuals were

matched to that map as described above. Since *H. subflexa* has 30 autosomes, we knew that one linkage group was still missing.

3A.3.2 Finding the 30th chromosome

The final map was based on 44 individuals and 356 markers that were part of all the gels. To this map we matched the 52 extra individuals again. In case of the markers that we found for 88 individuals (60 markers) we matched the 44 extra individuals respectively.

3A.4 Quantitative trait locus (QTL)-Analysis

As mentioned above, Lepidoptera have 30 autosomes, and there is no crossing-over in female Lepidoptera (Heckel, 1993). It is thus possible to localize a QTL on a specific chromosome that should include on average of 3% of the moth's DNA. This means a similar or even finer level of resolution compared to many QTL analyses where recombination is present (e.g. Gleason and Ritchie, 2004; Hawthorne and Via, 2001). For each chromosome we tested if the heterozygous individuals (NC/MX) individuals significantly differed (at a level of significance of $P < 0.05$) from the homozygous (MX/MX) individuals with regard to the relative percentage of every pheromone compound. This was tested via an analysis of variance (ANOVA) (proc GLM in SAS, Version 9.1., 2002-2003). The R^2 values from these ANOVA approximate the amount of phenotypic variation in the pheromone compounds that can be explained by a particular chromosome. If more than one chromosome affected the relative amount of one compound we tested the two chromosomes for interaction effects via a 3-factor ANOVA (SAS, Version 9.1.).

3A.5 Comparison of the intra- and interspecific QTL

In interspecific crosses between *H. subflexa* and *H. virescens* (Sheck et al., 2006; Groot et al*.,* 2009b) the acetates were mapped to 2 chromosomes. These chromosomes were given the arbitrary numbers 4 and 22. To determine whether the same linkage groups explained the intraspecifically variance in the acetate production, we did a bulk segregate analysis. The DNA of the bulks came from introgressed lines that were created by Fred Gould (NCSU). The same lines had been used by Sheck et al. (2006) and Groot et al. (2009b). Each bulk consisted of 12 individuals (females and males) that had none, one, or both chromosomes of *H. subflexa* (*Hs*) that could be correlated with the acetate production in the interspecific QTL analysis and were introgressed into *H. virescens* (*Hv*) (i.e. chr. 4 and chr. 22). The first bulk was homozygous *H. virescens*, i.e. no chromosomes came from *Hs* (00). In the second bulk chromosome 22 was heterozygous *Hv*/*Hs*, chromosome 4 was homozygous *Hv* (01). In the

third bulk chromosome 4 was heterozygous *Hv*/*Hs* and chromosome 22 homozygous *Hv* (10). The forth bulk contained individuals that were heterozygous (*Hv*/*Hs*) for both chromosomes 4 and 22 (11). The 4 different bulks of DNA were placed in this sequence on the gel together with the additional 52 individuals (see above). When a marker line was identified and belonged to a certain chromosome and the 4 bulks showed one of the following patterns the chromosomes of the intra- and interspecific crosses were considered the same. (Again "1" means a band is present "0" means that the band is absent). The pattern 0101 thus meant that the chromosome to which the marker belonged was the same as chromosome 22 of the interspecific crosses. If the bulk pattern was 0011 the group of that respective marker was the same as chromosome 4 of the interspecific crosses.

3B Is there phenotypic plasticity in the sexual communication of *H. subflexa*?

3B.1 Insect rearing in two different odor environments

Eggs were obtained from the rearing of the North Carolina State University (NCSU). The moths have been in the lab for about 110 generations. First instar larvae were separated to two different environments, an *H. virescens* Pheromone (P) environment and a Non-Pheromone (NP) environment. These environments were created in two identical climate chambers (Snijder Scientific BV) that were programmed to have a photo-reversed 10L : 14D cycle, a temperature of 24°C in the dark phase and 27°C in the light phase and a relative humidity of 75 %. In addition, the pheromone chamber P contained 10 rubber septa lures with *H. virescens* pheromone that were evenly distributed throughout the chamber. Five of them were exchanged for new ones on every 7th day. The lures contained the *H. virescens* pheromone compounds in a composition shown in table 1. The composition of the lures was based on that of Teal et al. (1986) and Heath et al. (1991). The lures were made by Coby Schal at the NCSU. There was no air exchange between outside and inside the chamber when the door was closed. The non-pheromone chamber (NP) was in a different room from the P chamber to ensure that pheromone from the P chamber could not reach the NP chamber. Moths that grew up under P conditions will in the following be referred to as the "P males/females" those which grew up in NP conditions will be called the "NP males/females".

Table 1. Composition of the *H. virescens* pheromone lures. The percentage of each compound is relative to the major component Z11-16:Ald [0.3 mg/ septum]. Rel. am. %: relative amount in percent

Compound	14:Ald	Z9–14:Ald	16:Ald	Z7–16:Ald	Z9–16:Ald	Z11–16:Ald	Z11–16:OH
Rel. am. %	5	5	10	2	2	100	1

All stages of the moths were kept under P or NP conditions respectively. After hatching the animals were individualized to 37 mL plastic cups (solo cup company, Illinois) containing artificial diet with the use of a fine pencil. For ingredients and recipe of the larval diet see appendix (Table IV). The adult moths were fed with 10 % honey water on a Dental Cotton Roll (Lohmann & Rauscher international GmbH & Co. KG). To ensure that air and thus pheromone would reach the animal, the lid was a combination of gauze with a plastic frame that closed the cups. The pupae and adults were kept in cups with gauze tops as well.

3B.2 Detection of the *H. virescens* pheromone in the climate chamber

I used a GC-(gas chromatography)-EAD (Electroantennogram Detection) system with combined mass-spectrometer (MS) to detect the *H. virescens* pheromone in the P chamber. I chose this technique because it was impossible to detect the sampled sex pheromone (described below) with a GC analysis alone. Male antennae are much more sensitive to pheromone than a GC.

To determine the retention time on the GC (6890 N, Agilent Technologies), the response of the antenna in the EAD and the mass-spectrum of the *H. virescens* major component, synthetic Z11-16:Ald (bought from Pherobank, Wageningen) was injected into the GC.

To detect the *H. virescens* pheromone in the pheromone climate chamber, the air was sampled using a solid phase micro extraction (SPME)–needle (Supelco) (fiber assembly: polydimethylsiloxane, 100 µm coating) for 24 h in both chambers. The needle was then injected into a combined GC-EAD system. For the EAD I used antenna from naïve 2 day old *H. subflexa* males. To ensure the function of the antenna an air puff with synthetic major component Z11-16:Ald was given before and after each run.

The GC was programmed as described below, but had a DB-5 column integrated. The software ChemStation (Version B.02.01-SR-1) (Agilent) was used for data analysis.

3B.3 Experiment 1. Phenotypic plasticity in the sex pheromone of female *H. subflexa*:

Is there a change in pheromone composition when females are reared in the P environment compared to the NP environment for one generation?

A first batch of eggs was obtained from the rearing of the NCSU (in April 2009). First instar larvae were separated to the two different environments. The lights in the climate chambers were on from 9 pm to 9 am. All larval stages as well as the pupae were kept in the two different environments as described above. The adult moths emerged in the two different environments. The sex pheromone glands of the adult females were all extracted three days after eclosion, four hours into the scotophase to guarantee that the pheromone was produced (Heath et al., 1991) and exclude variance that is due to age or to fluctuation in the female sex pheromone production and composition in the course of 24 h. Pheromone glands were extracted and analyzed as described below.

The females were not injected with PBAN (see below) in order to exclude a reduction of variation that is due to PBAN injection (Groot et al., 2005).

3B.3 Experiment 2. Phenotypic plasticity in the *H. subflexa* male response:

Is there assortative mating between males and females from the P environment and/or from the NP environment?

A new batch of eggs was obtained from the rearing of the NCSU (in May 2009). First instar larvae were separated into the two different environments as described above. The light cycle in the climate chambers was shifted for two hours (lights on from 11 pm to 11am). The adults all emerged from the pupal stage under P or NP conditions. They were at least 2 days old before they were used for the assortative mating experiment to ensure that the P individuals were under P influence for 2 days in their adult stage.

The experiments took place in a dark room with reversed light cycle (lights on from 11pm to 11 am) with a mean temperature of 26 °C and a relative humidity of 30 %. For the observations square cages (30 x 30 x 30 cm) (Mega View Science Education Services Co., Ltd.) were used that consisted of white gauze and one transparent plastic side. They were placed next to each other on three rows of shelves above each other. One male and two females were placed into each cage. The male was either from the P or from the NP environmental chamber, one of the females was from the P chamber and the other female was from the NP chamber. One cage with one male and two females is referred to as a mating unit (MU). Thirty of the mating units were set up with P males and thirty-six units were set up with an NP male. To exclude a location effect the cages containing a P or an NP male were alternated.

The females in one cage were in 63 out of 72 mating units of the same age. In seven mating units (6 N male MUs, 1P male MU) the females had an age difference of 1 day, in two MUs they had a difference of two days (2P male MUs). (Age of the females in N male MUs: 2days: 48; 3days: 16; 4days: 4; 5days: 4; age of the females in P male MUs: 2days: 43; 3 days: 17; 5 days: 2; age of the males: 2 days: 68, 3 days: 4 males (3N, 1P) (the day of eclosion was counted as day zero)). To distinguish between the two females in one mating unit, one of them was marked with a black whiteboard pen (Staedler). To exclude an effect of the marking on the male choice the same number of NP and P females were marked.

I used a red light MasterLED torch (Mellert) of 644 nm to observe the moths in the dark room. The moths were set into the cages in their light phase one hour before their scotophase began. One cup with honey soaked wad was placed in each cage. The experiment started with the beginning of the scotophase (11 am). I observed the animals in an interval of 30 minutes. The experiment for a single cage ended when two animals mated for the first time. Because about 95 % of the males had mated for the first time after 6-7 h, the animals were taken out of the cages after 7 h and put into single cups to prevent another mating. At the start of their following scotophase they were injected with PBAN and after another two hours extracted as described below to obtain the pheromone composition of each female. The experiment took place on four consecutive days. 72 matings were completed in total. 21 matings were conducted on the first, 32 on the second, 17 on the third and 2 on the fourth day.

Females that were used for the assortative mating experiment were injected with 7.5 pmol of *Hez*-PBAN (Pheromone Biosynthesis-Activating Neuropeptide from *Heliothis zea*, (Phoenix Europe GmbH, Karlsruhe) to exclude possible variation effects that may result from being mated compared to not mated (Groot et al., 2005). 3 µL of a stock solution of *Hez*-PBAN (200 pmol/µl in 50 % methanol and 1 N HCl) were diluted in 157 µL of 1 percent saline to a concentration of 3.75 pmol/µL. The females were injected with 2 µL of this solution (i.e. 7.5 pmol) between the 8^{th} and 9^{th} abdominal segment using a 10 µL syringe (31 gauge needle, Hamilton, Reno, NV) and incubated at room temperature for 1-2 hours before the extraction.

3B.4 Gland extraction

Extraction of the sex pheromone glands was performed in the same way for both experiments. The glands were extruded by pressing the abdomen with forceps and cut off with microdissection scissors. Each gland was incubated in a glass vial filled with 50 µL of n-hexane (Carl Roth GmbH+ Co.KG, Karlsruhe) containing 25 ng of the internal standard pentadecane. The glass vial was placed in a 4 mL Screw Neck Vial (GRACE Alltech) containing 100-200 µL hexane and capped with a solid top polypropylene cap with a TFE (Tetrafluoroethylene)/silicone bonded interseal (GRACE Alltech). After 30-40 minutes the gland was taken out and the extract stored at -20 °C until GC analysis.

3B.5 GC analysis

The extracts were blown down with a soft stream of nitrogen to a volume of 2-3 µL and taken up with a 10 µL syringe (701SN 26S GA 2 needle, Hamilton, Reno, NV) together with 1 µL of octane (Fluka, St. Louis, MO) to avoid evaporation. The whole volume of 3-4 µL was transferred to a 0.05 mL Micro-insert (GRACE Alltech) that was placed in a spring in 1.5 mL Crimp Neck Vials (GRACE Alltech) and capped with 11 mm alucrimp lids which contained a 1 mm clear silicon/clear PTFE (polytetrafluoroethylene) septum (GRACE Alltech). The sample was injected into the HP7890 gas chromatograph (GC) (Agilent Technologies) by a 7683 automatic injector. The GC contained a splitless inlet, a high resolution polar capillary column (DB-WAXetr [extended temperature range]; 30 m × 0.25 mm × 0.5 µm) and a flame ionization detector (FID). It was programmed to 60 °C for 2 minutes, then heated up to 180 °C at a rate of 30 °C per minute, after that to 230 °C at a rate of 5 °C per minute. To clean the column, after each run the temperature was increased to 245 °C at a rate of 20 °C per minute. This temperature was held for 15 minutes. The FID was held at 250 °C. To identify the particular compounds a multi-component blend (MCB) (compounds from Pherobank, Wageningen) containing all the compounds of *H. subflexa* was made and injected into the GC before or after each series of injections per day. By overlaying the signal and comparing the retention times of the sample with the MCB the compounds could be identified. For quantitative analysis the GC signal was integrated with the software ChemStation (Agilent).

3B.6 Statistical analysis

For the analysis of both experiments I used the relative percent of the total amount of pheromone per blend, i.e. the total amount of pheromone was set to 100 %, the single compounds constitute a certain percentage of the total amount.

3B.6.1 Statistical Analysis of differences in the female sex pheromone composition when reared under the two different conditions (P and NP) for one generation

The statistical analyses was conducted with the computer program SAS (Statistical Analysis Software), Version 9.1 (SAS Institute, 2002-2003). A multivariate analysis of variance (MANOVA) was performed to find differences in the sex pheromone compositions between the NP and the P females.

3B.6.2 Statistical analysis of the assortative mating experiment

Two approaches were taken.

1) Overall analysis: the females were not compared per mating unit but in groups 1 and 2:

Did P and NP males have the same choice conditions with regard to the female pheromone compositions? We performed a MANOVA analysis in SAS, Version 9.1 to identify differences between the females in NP male mating units and P male mating units (ignoring their mating status). Group 1: females in NP male MUs x females in P male MUs

To identify differences in the sex pheromone compositions between mated and unmated females we performed a MANOVA analysis. We did the analysis separately for the females in NP male mating units and P male mating units

Group 2a: mated females in P male MUs x unmated females in P male MUs

2b: mated females in NP male MUs x unmated females in NP male MUs

2) Single mating units

The mating units were examined in two different ways:

a) Male choice depending on the female rearing background:

Did P or NP males choose significantly more for P or NP females?

The number of P males that chose for NP or P females was counted and a two sided sign test performed subsequently. The same was done for the NP-male mating units. To determine whether the choice of the P males varied significantly from that of the NP males at a level of significance of $P < 0.05$ I performed an Exact Fisher test.

b) Male choice depending on the female pheromone composition

I subtracted the relative amount of pheromone per gland of the unmated female from the relative amount of pheromone of the mated female for every mating unit and every compound. If the difference was positive the male had mated with the female that had a “higher (H)” relative amount of that particular compound, if the difference was negative the male had mated with the female that had a “lower (L)” relative amount of that compound. Without taking the value of the difference into account I counted the cases “mated with higher” and “mated with lower”. To assess whether the P or NP males mated significantly more for a certain pheromone composition than NP males, sign tests were performed. To determine whether the choice of the P males varied significantly from that of the NP males at a level of significance of $P < 0.05$, an Exact Fisher was conducted.

The two females in one cage varied in their sex pheromone composition by coincidence. Since selection lines did not exist, there was no way to determine the sex pheromone composition before the experiment.

4 Results

4A Genetic basis of sex pheromone differences

4A.1 Map

Preliminary maps were created with 44 individuals. The first map that consisted of 29 linkage groups was constructed on the basis of 70 primer combinations. We found a total of 207 markers, 160 of which were unique and informative at a LOD level of 6. Another 22 primer combinations yielded 296 markers, 218 of those were informative at a LOD level of 8. The map still consisted of 29 groups. I was able to increase the number of individuals to 96 individuals for each of these groups.

The 30^{th} chromosome:

With a total of 109 primer combinations I was able to find and score a total of 356 markers. 303 of those were unique informative markers, 53 markers could not be matched to a group and were thus unlinked. The gels produced 0 to 16 markers with an average of 3 informative markers per gel. With the informative markers we were able to identify 30 linkage groups at a LOD level of 6. Each linkage group consisted of at least 4 markers of different primer pairs and contained up to 23 markers. The map of 30 chromosomes was created based on 44 individuals. It was possible to add additional 52 individuals to each of the groups. The final map of 30 chromosomes was thus based on 96 individuals.

4A.2 QTL analysis

Fourteen of the linkage groups (chromosomes 3, 4, 6, 7, 10, 12, 14, 16, 18, 19, 20, 21, 23, 26 and 28) could be correlated with the relative amount of particular pheromone compounds at a level of significance of $P < 0.05$ with the means of ANOVA analyses. Eight chromosomes were correlated with variances in the relative amounts of acetates.

For a detailed overview see table 2.

For a tabular overview of the QTL analysis performed when the minor compounds of the sex pheromone blend is set to 100 % see table V (appendix).

4A.2.1 QTL for the acetates

Figure 4 shows the effects of the four groups, chromosomes 16, 19, 21 and 4, that explained at least 5 % of the variance in the acetate levels. NC/MX heterozygotes for chromosome 16, 19 and 21 had higher acetate levels than MX/MX homozygotes. This result could be expected since Groot et al. (2009a) found higher acetate levels in North Carolina females than in Mexico females (Fig. 1). Contrary to expectation, NC/MX heterozygous individuals for chromosome 4 had lower relative acetate amounts ($r^2 = 0.05$; $P = 0.034$) than the MX/MX homozygotes (Fig. 4).

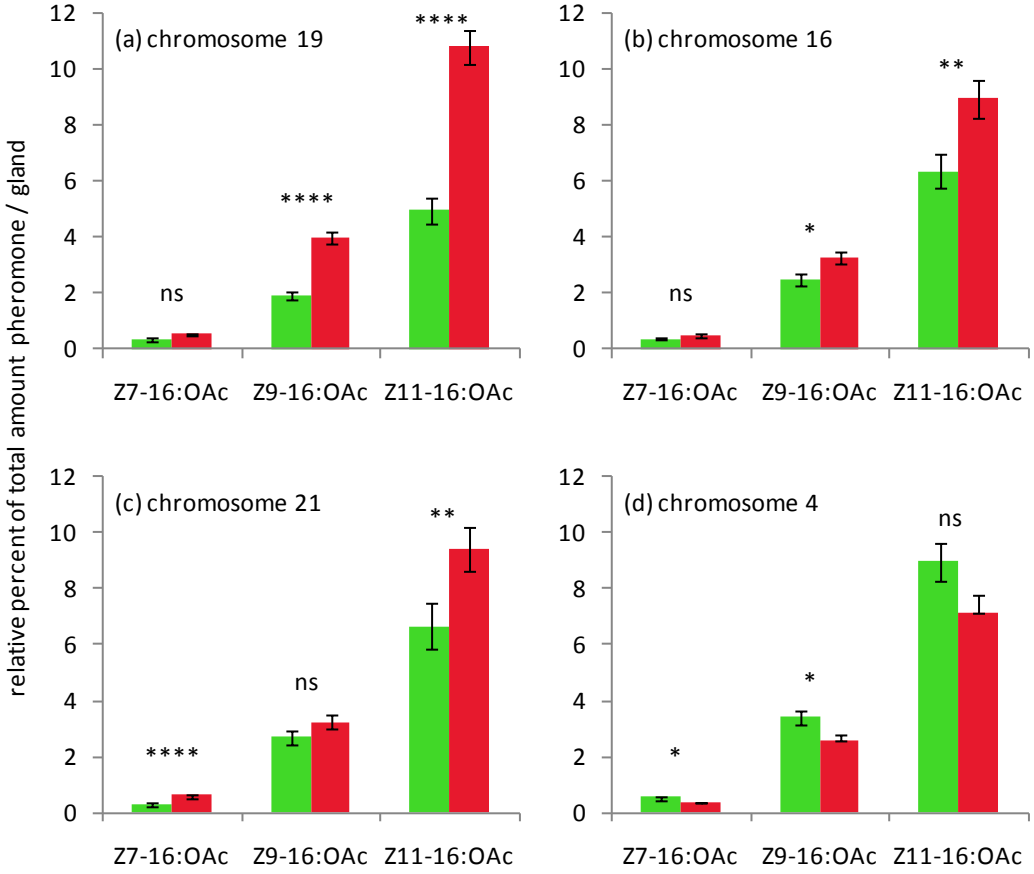

Fig. 4. Correlation between the presence (red bars ± SE (N for Chr. 19 = 47; Chr. 16 = 52; Chr. 21 = 44; Chr. 4 = 49)) or absence (green bars ± SE (N for Chr. 19 = 46; Chr. 16 = 40; Chr. 21 = 50; Chr. 4 = 43)) of an NC copy and the relative amount of acetates; shown for four chromosomes. (a-c): Individuals that were heterozygous NC/MX for the chromosomes 19, 16and 21 had higher levels for all of the three acetates than homozygous MX/MX individuals. (b): Individuals that were heterozygous NC/MX for chromosome 4 had lower relative acetate amounts compared to homozygous MX/MX individuals Significances were calculated via an ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

The strongest QTL were found for chromosome 19 with major effects on the Z9-16:OAc (r^2 = 0.46*; P* < 0,0001) and the Z11-16:OAc (r^2 = 0.39*; P* < 0,0001). An increase in the relative amount of those two compounds was further explained by chromosome 16 (Z9-16:OAc: r^2 = 0.063; *P* = 0.023; Z11-16:OAc: r^2 = 0.076; *P* = 0.008). Chromosomes 19 and 16 had no significant effect on the Z7-16:OAc. The strongest QTL for the latter was located on chromosome 21 (r^2= 0.157*; P* < 0.0001). This chromosome also effected Z11-16:OAc levels (r^2 = 0.085*; P* = 0.0044) but was not significant for Z9-16:OAc levels (Fig. 4).

4A.2.2 QTL for the aldehydes

Chromosome 19 showed strong QTL for three of the aldehydes as well. Heterozygous NC/MX individuals for chromosome 19 had lower relative amounts of the major critical component Z11-16:Ald (r^2 = 0.24; *P* > 0.0001), Z9-16:Ald (r^2 = 0.129; *P* = 0.0004) and Z7-16:Ald (r^2 = 0.099; *P* = 0.0021) than individuals that were homozygous MX/MX. There was thus an inverse effect for the aldehydes and the acetates: heterozygous females (NC/MX) had high relative amounts of acetates and low relative amounts of the three above mentioned aldehydes than homozygous (MX/MX) females (Fig. 5).

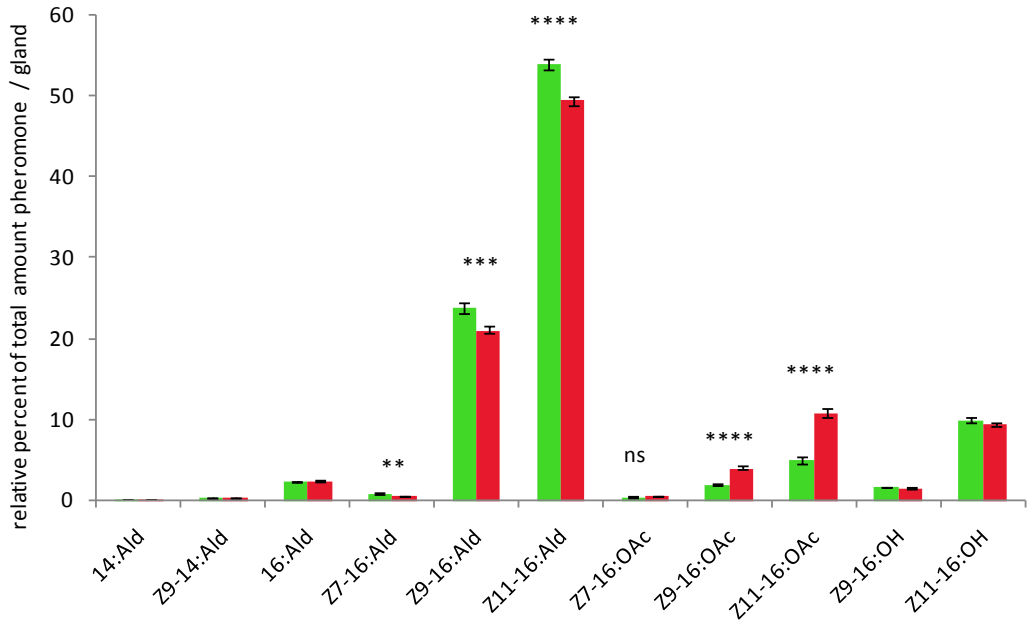

Fig. 5. Chromosome 19: relative amounts of pheromone compounds in heterozygotes (NC/MX) (red bars ± SE; N = 47) and homozygotes (MX/MX) (green bars ± SE; N = 46).

**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

Additionally to the QTL on chromosome 19 two strong QTL were found for Z9-16:Ald: one on chromosome 3 (r^2 = 0.179; P > 0.0001), individuals that were heterozygous (NC/MX) for chromosome 3 had higher relative amounts of Z9-16:ALd than homozygous individuals. Another strong QTL for this compound was found on chromosome 21 (r^2 = 0.142; P = 0.0002).

Besides QTL for two acetates and Z9-16:Ald chromosome 21 contained the strongest QTL for 16:Ald (r^2 = 0.15; P = 0.0001). We found an inverse correlation of chromosome 21 for Z9-16:Ald and 16:Ald: heterozygous (NC/MX) individuals for chromosome 21 had higher relative amounts of 16:Ald but lower relative amounts of Z9-16:Ald than homozygous (MX/MX) individuals.

The two saturated aldehydes 14:Ald and 16:Ald had QTL on the same three chromosomes: 20, 21 and 28. The strongest QTL for 14:Ald was located on chromosome 28 (r^2 = 0.126; P = 0.0006). Z7-16:Ald and Z7-16:OAc both had small QTL ((r^2 = 0.046; P = 0.038) and (r^2 = 0.043; P = 0.046)) on chromosome 6.

4A.2.3 Chromosome interaction effects

The chromosomes involved in the acetate production were tested using a 3-factor ANOVA for interaction effects with regard to the levels of the three acetates. Only chromosome 16 and 19 showed an interaction effect: Individuals that were heterozygous NC/MX for both chromosome 19 and 16 had significantly higher relative amounts of Z7-16:OAc (P = 0.0175) and Z11-16:OAc (P = 0.0142) compared to the individuals that were heterozygous for only one of the two chromosomes (Table 3).

Table 3. Interaction effects of chromosome 16 and 19 in determining the relative amounts of acetates per gland, *P*-Values.

	Z7-16:OAc (*P*)	Z9-16-OAc (*P*)	Z11-16:OAc (*P*)	SUM:OAc (*P*)
r^2	0.1	0.53	0.5	0.5
Chr16	0.16	0.0013	0.0006	0.0007
Chr19	0.08	<.0001	<.0001	<.0001
Chr16*Chr19	0.0217	0.16	0.014	0.02

No other interaction effects were found.

4A.3 Correlations between single pheromone compounds

The single pheromone compounds are interrelated through biosynthetic pathways (Fig. 2) Pearson's correlation coefficient (Table 4) may indicate groups of compounds that are produced via the same biosynthetic route. Compounds that show positive correlations could be synthesized via the same pathway. (Groot et al., 2009b) I concentrated on correlations that were highly significant ($P < 0.0001$). The three acetates were positively correlated ($P < 0.0001$) with each other, i.e. if one of the acetates was present in a high relative amount the other two were present in high relative amounts as well. The Z9-16:Ald and Z11-16:Ald showed a high negative correlation with all of the three acetates ($P < 0.0001$). High relative amounts of acetates were correlated with low amounts of the two aldehydes. Significant positive correlations were also found between the Z7-16:OAc and Z7-16:Ald ($P < 0.0001$), the 14:Ald and the 16:Ald ($P < 0.0001$) and the two alcohols ($P < 0.0001$) (Table 4).

Table 4. Pearson's correlation coefficient for the pheromone compounds, the sum of all compounds is set to 100%, one compounds has a relative percentage of the total amount pheromone/ gland

	14:Ald	16:Ald	Z7-16:Ald	Z9-16:Ald	Z11-16:Ald	Z7-16:OAc	Z9-16:OAc	Z9-16:OH
14:Ald	1							
16:Ald	0,47****	1						
Z7-16:Ald	0,08	-0,17	1					
Z9-16:Ald	-0,32**	-0,29**	-0,21*	1				
Z11-16:Ald	-0,05	0,04	0,09	0,14	1			
Z7-16:OAc	0,08	-0,02	0,46****	-0,61****	-0,46****	1		
Z9-16:OAc	-0,05	-0,07	-0,11	-0,43****	-0,80****	0,54****	1	
Z11-16O:Ac	0,07	0,02	0,00	-0,67****	-0,69****	0,70****	0,91****	
Z9-16:OH	0,12	-0,14	-0,03	0,33**	-0,30**	-0,23*	-0,15	1
Z11-16:OH	0,35***	0,25*	0,00	-0,12	-0,15	-0,07	-0,26*	0,69****

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

4A.4 Comparison between intra- and interspecific QTL

The bulk segregate analysis indicated that chromosome 21 that we found for the intraspecific map is the same as chromosome 4 of the interspecific analysis of Sheck et al. (2006) and Groot et al. (2009b). The marker that showed the 0011 pattern indicative of chromosome 4 was found with the primer combination *Mse*-CAT-*Eco*-AGG and had a size of 199 basepairs

(Fig. I, appendix). The pattern 0101 that would indicate to chromosome 22 of the interspecific QTL-analysis has not been found so far.

4B Phenotypic plasticity

4B.1 Detection of *H. virescens* sex pheromone in the pheromone chamber

It was possible to absorb the *H. virescens*/*H. subflexa* major component Z11-16:Ald to the SPME needle. With the GC-EAD technique I could detect the major component in the pheromone chamber. The control, pure synthetic Z11-16:Ald, injected into the GC produced a GC peak at 11.82 min. A reaction of the male antenna was recorded at the same time (Fig. 6).

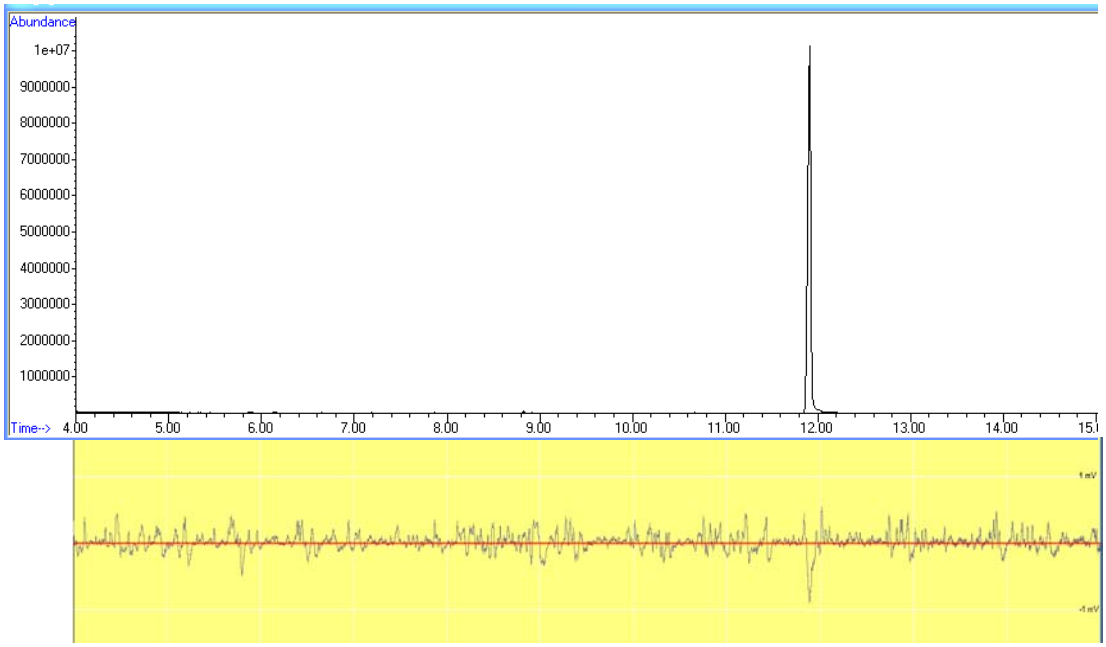

Fig. 6. GC-EAD analysis of the pure major component of *H. virescens* pheromone. The upper part shows the GC analysis, the lower part shows the reaction of a male *H. subflexa* antenna.

The SPME sample of the pheromone chamber produced more than one peak. Mass spectrometry showed that none of the clearly visible peaks (Fig. 7) was part of the pheromone blend. Not even the peak at a retention time of 11.82 min which is the time when the Z11-16:Ald was detected when the substance was injected purely. However, it was possible to find the mass-spectrum (molecular weight of 238) of Z11-16:Ald (Fig. 8), at the same time that the huge peak was visible, indicating that it was hidden under a dirt peak on exactly that spot. The EAD showed the reaction of the antenna at the same time (Fig. 7).

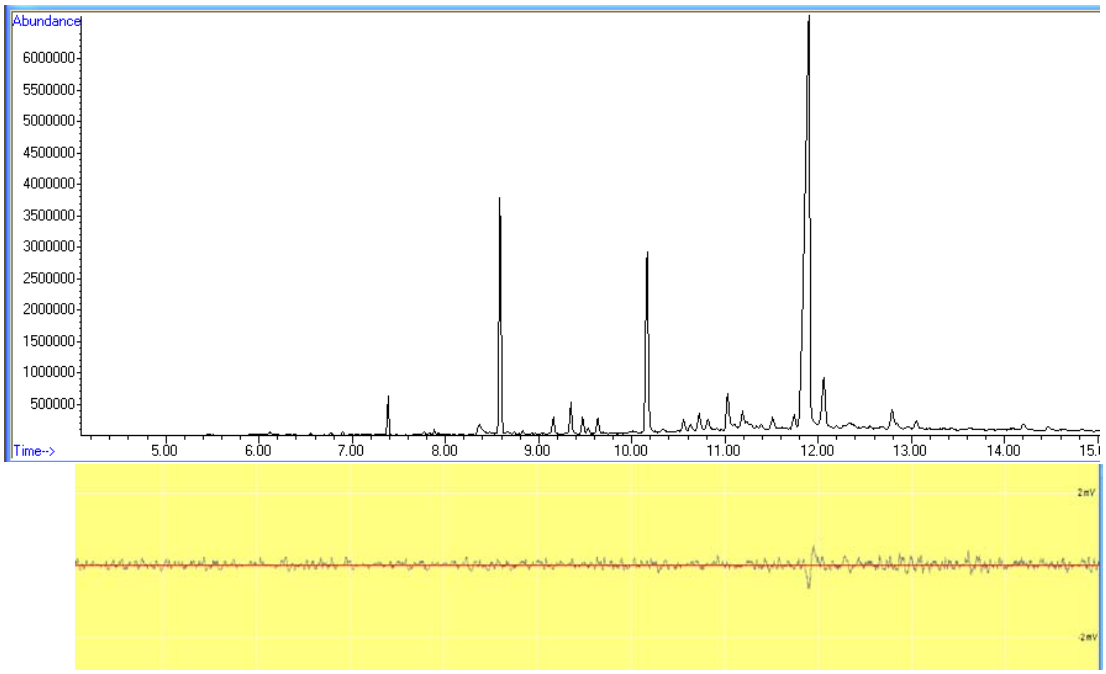

Fig. 7. GC-EAD analysis of the SPME sample that was in the **pheromone** chamber for 24 hours. The upper part shows the GC analysis, the lower part shows the reaction of a male *H. subflexa* antenna.

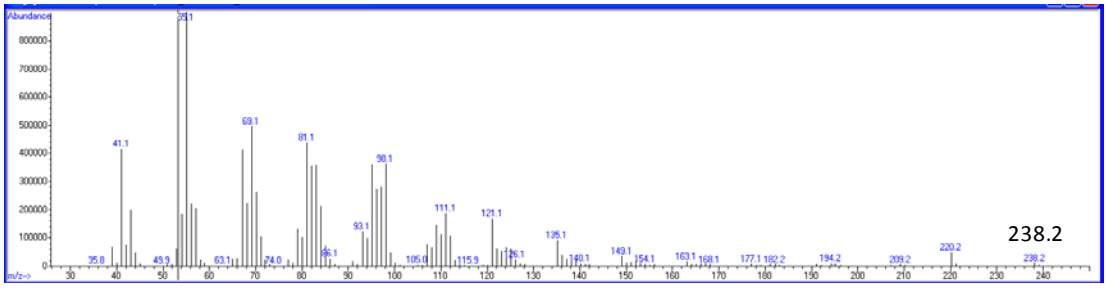

Fig. 8. Mass-spectrum of Z11-16:Ald, identified in the SPME sample from the pheromone chamber.

With the SPME sample of the NP-chamber we did find the same dirt peak at 11.82 min, but neither did we find the mass-spectrum of the major component nor did the antenna show a reaction at the respective time (Fig. 9). The antenna used for the present data were shown to be intact before and after the measurements (data not shown).

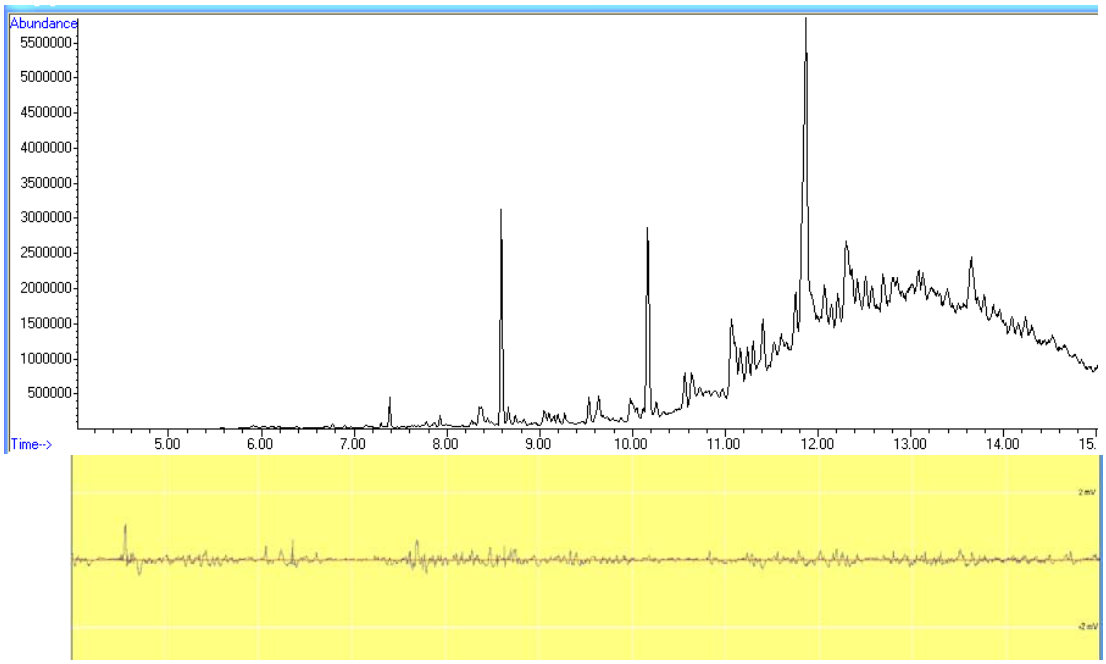

Fig. 9. GC-EAD analysis of the SPME sample that was in the **non-pheromone** chamber for 24 hours. The upper part shows the GC analysis, the lower part shows the reaction of a male *H. subflexa* antenna.

4B.2 Phenotypic plasticity in the female sex pheromone composition

A MANOVA revealed that after one generation in different odor environments the P and NP *H. subflexa* females did not show significant differences in their pheromone composition (Fig. 10) The hypothesis that the values for Z7-16:OAc or Z11-16:OAc would be significantly increased after one generation in *H. virescens* pheromone environment must be declined as tested in a one-tailed ANOVA.

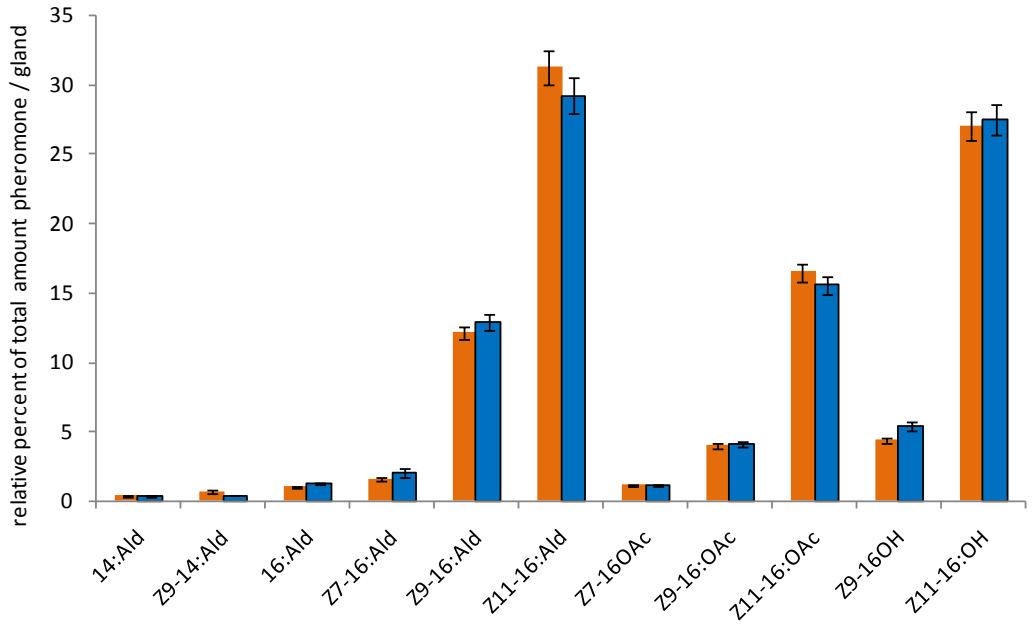

Fig. 10. Influence of synthetic *H. virescens* sex pheromone on the sex pheromone composition in *H. subflexa* females after one generation. One group of *H. subflexa* females were reared and emerged under *H. virescens* pheromone conditions (blue bars ± SE; N = 67) the other group of *H. subflexa* females were reared and emerged under non-pheromone conditions (orange bars ± SE; N = 66). MANOVA: n.s. Individual compounds were tested via a one-tailed ANOVA.

4B.3 Phenotypic plasticity in the male response: assortative mating experiments

4B.3.1 Male rearing background x female rearing background

Did P or NP male rearing background influence their choice with regard to female rearing background (P/ NP)?

The NP males had a non-significant tendency to choose more for the NP females then for the P females. 22 NP females were chosen compared to 14 chosen P females. A sign-test classified the ratio at a level of significance of $P \leq 0.5$ (n.s.). P males did not show a tendency to vary their choice depending on the female rearing background. 15 of the P males chose a NP female, 16 chose a P female. The exact Fisher test rated the choice behavior of the NP males not significantly different from the behavior of P males $P = 0.33$ (n.s.) with regard to the female rearing background. The hypothesis that the P or NP males would vary their choice depending on the female rearing background (P / NP) must be declined.

4B.3.2 Male rearing background x pheromone composition of the female

Did P males choose significantly different from NP males with regard to female pheromone composition?

4B.3.2.1 Overall analysis

Did the pheromone composition of the mated females differ significantly from the pheromone composition of the unmated females?

a) For the mating units with P males

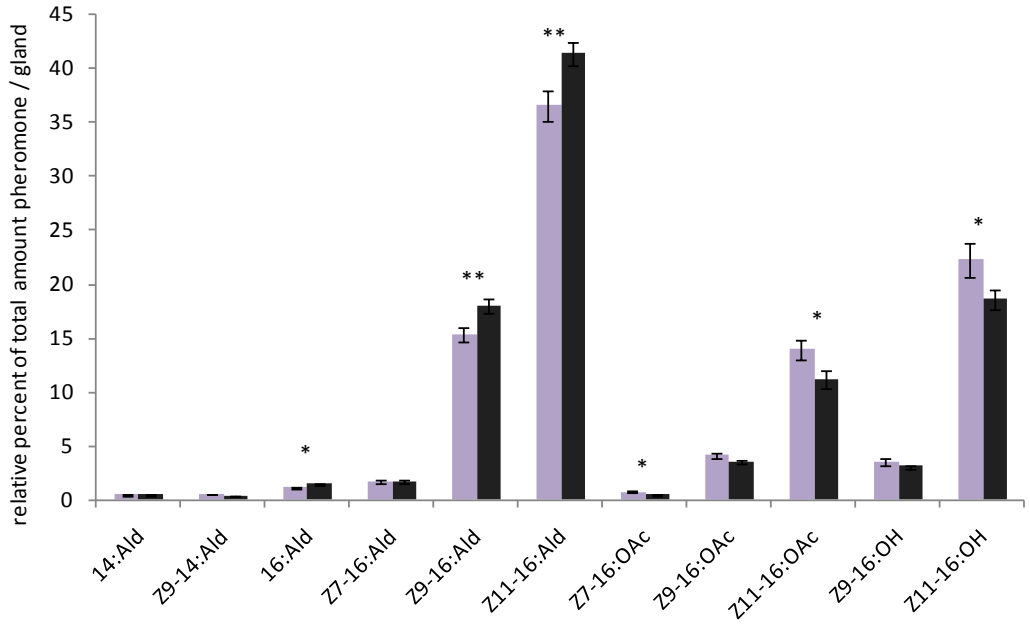

Fig. 11. P male mating units: Comparison of the relative amounts of pheromone compounds of mated females (purple bars ± SE; N = 31) and unmated females (black bars ± SE; N = 31). MANOVA: P = 0.0179; Individual compounds were tested via a one-tailed ANOVA. *P < 0.05, **P < 0.01

A MANOVA analysis revealed an overall effect (P = 0.0179). ANOVA analyses of the single compounds brought the following results: The relative amounts of 16:Ald, Z9-16:Ald and Z11-16:Ald were significantly lower in the mated females than in the unmated females (16:Ald: P = 0.021; Z9-16:Ald: P = 0.0066; Z11-16:Ald: P = 0.0097). The relative amounts of Z7-16:OAc, Z11-16:OAc and Z11-16:OH were significantly higher in the females that were mated by the P males (Z7-16:OAc: P = 0.0173; Z11-16:OAc: P = 0.0266; Z11-16:OH: P = 0.0493) than in those that were not mated (Fig. 11).

b) For the mating units with NP males

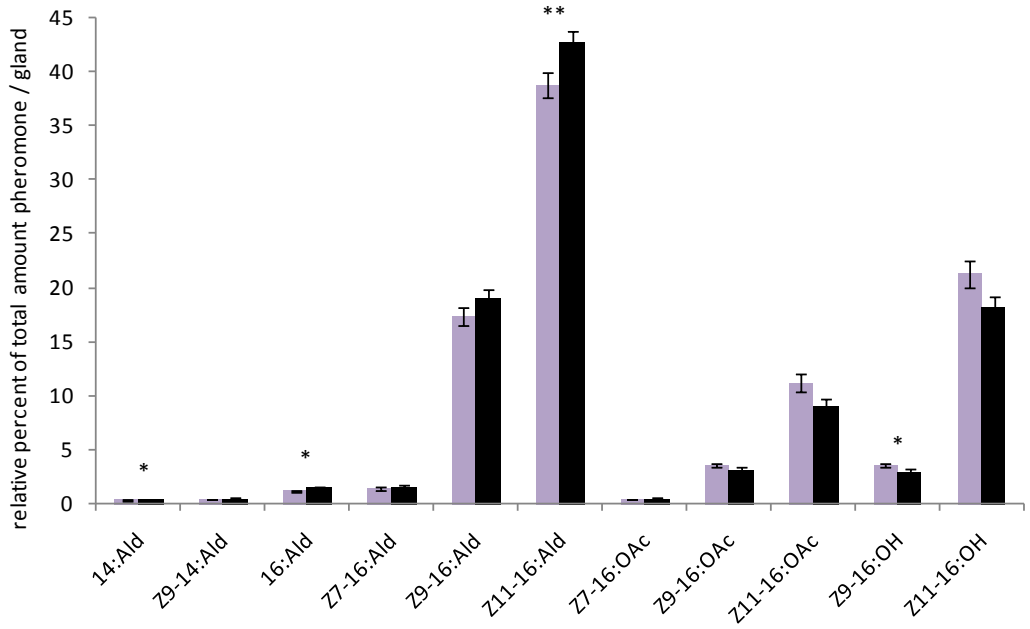

Fig. 12. NP male mating units: Comparison of the relative amounts of pheromone compounds of mated females (purple bars ± SE; N = 36) and unmated females (black bars ± SE; N = 36). MANOVA: n.s.; Individual compounds were tested via a one-tailed ANOVA *$P < 0.05$, **$P < 0.01$

A MANOVA graded the overall differences of mated and unmated females as not significant. ANOVA analyses of the single components brought the following results: The females that were mated by the NP males had significantly lower relative amounts of 14:Ald ($P = 0.0223$), Z9-14:Ald ($P = 0.0144$) and Z11-16:Ald ($P = 0.0081$) than the unmated females. The mated females had a higher relative amount of Z9-16:OH ($P = 0.0499$) than the unmated females (Fig. 12).

The two groups showed the same tendencies for all the components except for 14:Ald, Z9-14:Ald and Z7-16:OAc. Except for those compounds it accounted for both groups that the mated females had in tendency lower aldehydes and a higher relative amount of acetates and alcohols than the unmated females. Mated and unmated females of NP male MUs and P male MUs showed the same significant differences for only one compound: Mated females in NP male MUs and P male MUs had significantly lower relative amount of the major component than unmated females.

c) Did P males and NP males have the same overall choice conditions?

Did the sex pheromone composition of the females in NP mating units vary significantly from the composition of the females in P male mating units?

A MANOVA graded the differences between females in the two different groups of mating units as not significant. However, the single components revealed significant differences: Females in the P male mating units had significantly lower relative amounts of Z9-16:Ald (P = 0.017), significantly higher relative amounts of Z11-16:OAc (P = 0.0083) and marginally higher relative amount of Z7-16:OAc (P = 0.0865) than the females in the NP mating units.

4B.3.2.2 Single mating units: Male choice

The mated and unmated females of one cage were compared with regard to their pheromone composition. How did the males choose when given a choice between a "higher (H)" and a "lower (L)" female (higher and lower for one compound)? Did this choice vary between P and NP males at a level of significance of $P < 0.05$? The result of the single mating unit analysis is shown for those components that indicated significance in the overall analysis (see above) (Table 5).

Table 5. Single mating unit analysis: the value for a pheromone compound of the unmated female in one MU was subtracted from the value of the mated female of the MU. The numbers of "mated with higher" and "mated with lower" were counted and analyzed with a two sided sign test. Differences in the ratios of NP male MUs and P male MUs were analyzed with the means of the Exact Fisher test.
*$P < 0.05$

		Number of males that mated with „higher“	Number of males that mated with „lower“	sign test (two sided)	NP x P Exact Fisher Test
14:Ald	N	17	19	ns	ns
	P	22	9	*	ns
Z9-14:Ald	N	15	18	ns	ns
	P	22	9	*	ns
16:Ald	N	14	22	ns	ns
	P	10	20	ns	ns
Z9-16:Ald	N	18	17	ns	ns
	P	13	18	ns	ns
Z11-16:Ald	N	14	20	ns	ns
	P	10	21	ns	ns
Z7-16:OAc	N	22	14	ns	ns
	P	18	13	ns	ns
Z11-16:OAc	N	20	16	ns	ns
	P	22	9	*	ns
Z9-16:OH	N	24	12	ns	ns
	P	17	14	ns	ns
Z11-16:OH	N	24	12	ns	ns

Z7-16:OAc

When given a choice between a higher and a lower female both P and NP males showed a trend to prefer females with higher Z7-16:OAc. Both ratios were not significant as tested with a sign test (two sided). The P males chose for 18 high and 13 low females. The NP males chose 22 high and 14 low females. P and NP males did not choose significantly different from each other.

Z11-16:OAc

A trend in the same direction was found for the Z11-16:OAc. P and NP males both had the tendency to prefer females with higher Z11-16:OAc. P males chose significantly more for females with a higher relative amount of Z11-16:OAc (22H : 9L; sign-test $P \leq 0.05$). The NP males did not make a significant different choice between the females (20H : 16L; *P* = n.s.). The exact fisher test did not grade the choice of P males significantly different from the choice of the NP males.

Z9-16:Ald

P males showed a not significant trend to choose the females with a lower Z9-16:Ald female (13H : 18L). NP males did not make a difference between females with higher or lower relative amounts of Z9-16:Ald (18H : 17L).

14:Ald

P males chose significantly more for females with a higher relative amount of 14:Ald (22H : 9L; sign-test $P \leq 0.05$). The NP males did not make a significant choice between the females (17H : 19L; *P* = n.s.).

Z9-14:Ald

P and NP males both had the tendency to prefer females with higher Z9-14:Ald. P males chose significantly more for females with a higher relative amount of Z9-14:Ald (22H : 9L; sign-test $P \leq 0.05$). The NP males did not make a significant choice between the females (15H : 18L; *P* = n.s.).

No significances were found for the other compounds.

5 Discussion

5A AFLP mapping and QTL

Sheck et al. (2006) and Groot et al. (2009b) have conducted QTL analyses on a multi-component blend with an AFLP map that was based on interspecific crosses between *H. subflexa* and the closely related species *H. virescens*. The present study is the first QTL analysis on a multi-component blend that is based on intraspecific crosses. The comparison between inter- and intraspecific crosses will allow us to combine interpretations about biosynthetic pathways and possible candidate genes that underlie a trait.

We could identify all of the 30 autosomal chromosomes for 96 individuals. 109 primer combinations were necessary to find 303 informative markers, .i.e. an average of 3 informative markers per gel. That is about 5 times less makers per gel than interspecific crosses between *H. subflexa* and *H. virescens* have produced (Sheck et al., 2006), who found 532 markers with 33 primer combinations. As two reproductively isolated species should have diverged to a greater degree than two populations of one species it is much easier to find more neutral and phenotypically relevant markers in two species than in two populations of one species. Since our backcross individuals were all females we could not include the sex chromosome into our analysis. Hybridization studies between *H. subflexa* and *H. virescens* indicate that female pheromone production is not controlled by genes that are located on the sex chromosome (Teal and Tumlinson, 1997). Identification of the 30 autosomes is therefore sufficient to map all the loci that are involved in the different pheromone ratios between NC and MX females.

Our results provide information to the complicated biosynthetic pathways and a basis for further speculation about the way in which a trait may diverge between two populations. Since we found QTL for several pheromone compounds in the intraspecific cross, there is in fact a genetic basis to the intraspecific pheromone differences that occur in nature between the populations of NC and MX (Groot et al., 2009a). The compounds for which we found QTL that explained more than 10 % of the variances between the NC and MX populations were Z9-16:Ald, Z11-16:Ald, the three acetates, 14:Ald and 16:Ald, i.e. all the pheromone compounds except the two alcohols (Z9-16:OH and Z11-16:OH) and Z9-14:Ald. Thus, there is a genetic basis for the geographic variation of all the compounds except for the Z9-14:Ald that were found to be significantly different between the females of the two regions (Groot et al., 2009a).

5A.1 Correlations between the different pheromone compounds

The different pheromone compounds are not independent of each other. They are connected because they are produced via the same overall biosynthetic pathway (Fig. 2) (see Groot et al., 2009b) The Pearson's correlation coefficient indicates which compounds are positively correlated with each other, which are negatively correlated and which compounds do not affect each others' relative amounts. A problem with the Pearson's correlation in our analysis is that the compounds are also correlated for mathematical reasons: Factors that represent a percentage of the same total amount cannot be independent from each other. The Pearson's correlation coefficient is therefore not perfectly reliable in studying the relation of the different compounds in a pheromone blend and has to be regarded with caution.

Previously, the three acetates have been found to be strongly positively correlated, i.e. when the relative amount of one increased the other two would increase as well (Sheck et al., 2006; Groot et al., 2009b). We could confirm the strong positive correlation of the acetates in the present study. However, despite the strong positive Pearson's correlation between the three acetates and the previous finding of Sheck et al. (2006) of two QTL for all three acetates, our current intraspecific QTL analysis separates Z9-16:OAc and Z11-16:OAc from Z7-16:OAc. Chromosome 19 has a strong impact on the first two acetates but not on the latter. The same was shown by Groot et al. (2009b) with a chromosome that explained the variance in the relative amount of Z9-16:OAc and Z11-16:OAc, but not Z7-16:OAc. The differences in the QTL pattern could result from their different biosynthetic pathways using two different desaturases (Fig. 2). Z7-16:Acids can only be synthesized via a delta-9-desaturase (Choi et al., 2002), Z11-16:Acids are produced via a delta-11-desaturase. Although Z9-16:Acids could be produced via a delta-9-desaturase it has been shown to be preferentially synthesized via a delta-11-desaturase in *H. subflexa* (Choi et al., 2005). Thus, chromosome 19 could encode a delta-11-desaturase that may be differentially active in NC and MX females.

Z7-16:OAc was positively correlated to Z7-16:Ald, which was the only positive correlation between aldehydes and acetates. It is likely that these compounds are produced via the same biosynthetic pathway because they use the same alcohol precursor (Jurenka, 2003) both are produced by a delta-9-desaturase. We found a small QTL on chromosome 6 that explained about 4 % of the variance for both compounds.

As in the former study of Groot et al. (2009b), we found a negative correlation between Z9-16:Ald and 16:Ald. They both had QTL on chromosome 21 that explained 14 or 15 %of the

variance of the two compounds. Heterozygous (NC/MX) individuals for chromosome 21 had higher relative amounts of 16:Ald but lower relative amounts of Z9-16:Ald than homozygous (MX/MX) individuals. Groot et al. (2009b) proposed that the loci correlated to the QTL could encode a delta-11-desaturase that introduces a double bond into 18:Acid to form Z11-18:Acid and then be chain-shortened to Z9-16:Acid. The desaturase would compete with a chain-shortening enzyme to shorten 18:Acid directly to 16:Acid that would then be converted to 16:Ald. Preliminary mapping experiments with a delta-11-desaturase supported this hypothesis (A. T. Groot, personal communication). An alternative explanation is that both compounds compete for 16:Acids so that the QTL could point to a delta-9-desaturase. This alternative is opposed by the above mentioned results of studies by Choi et al. (2005).

The Pearson's coefficient showed a positive correlation between the two saturated compounds, 14:Ald and 16:Ald . Three QTL were found for both compounds. One of these chromosomes could possibly contain a gene encoding a FAR that prefers saturated acids as a substrate.

The gene(s) on chromosome 19 seem to play a major role for the overall pheromone blend composition, because aside from influencing the acetate levels it also significantly influenced the relative amounts of three of the aldehydes. It explained 9 % of the variance of Z7-16:Ald, 13 % of Z9-16:Ald and 24 % of the variance of Z11-16:Ald. The relative amount of these three aldehydes were positively correlated to each other but negatively correlated to the acetates. This could be due to one gene encoding an enzyme that is responsible for converting acetates to alcohols (Teal and Tumlinson, 1987) or *vice versa*. The current putative biosynthetic pathway (Fig.2) points rather towards a gene that affects the conversion of the alcohol precursors to the respective oxygenated group. Acetates and aldehydes probably use the same desaturated alcohols as a precursor (e.g. Jurenka, 2003). Z11-16:Ald and Z11-16:OAc may compete for the same Z11-16:OH precursor. Accordingly, Z9-16:Ald and Z9-16:OAc could compete for the same Z9-16:OH. The result of such competition would be a negative phenotypic correlation between the two, which is what we found. The QTL on chromosome 19 could point towards an acetyltransferase (Morse and Meighen, 1987b) or alternatively to an alcohol oxidase (Teal and Tumlinson, 1988) that is unspecific in terms of using Z9-16:OH or Z11-16:OH as a substrate. Alternatively, there might be more than one gene involved in the production of these different chromosomes that are all located on the same chromosome.

5A.2 Evolutionary scenarios

The correlation between the three acetates and the Z9-16:Ald and Z11-16:Ald could give an indication towards possible evolutionary scenario's of how pheromone changes between NC and MX females could have evolved (provided that communication interference does/did play a role between *H. subflexa* and *H. virescens*): Have MX populations decreased (1) or have NC populations increased their acetate levels (2)?

1) High acetate levels are the ancestral trait and MX individuals have decreased their acetate levels.

If there is a cost for the production of acetates, and high acetates are unnecessary (i.e. there are no interfering species in the area, the attraction of which should be inhibited), they should be reduced via natural selection.

The acetates, specifically Z11-16:OAc, have an inhibiting effect on *H. virescens* male attraction (Vickers and Baker, 1997) and thus play a role in avoiding cross-attraction. As in the Mexican populations studied by Groot et al. (2007) virtually no *H. virescens* were present, high acetate levels to avoid cross-attraction seem to be unnecessary in that region. Our results indicate that high acetate levels are correlated with low relative amounts of Z9-16:Ald and Z11-16:Ald because they compete for the same precursor (Jurenka, 2003). The production of high amounts of acetates could thus have a cost: less amounts of critical components that are essential for male attraction (Vickers, 2002). A gene responsible for the increase of acetate would indirectly lead to a decrease in two critical components. Such a pleiotropic effect that decreases the relative amounts of critical components should be selected against (Sheck et al., 2006). It is possible that *H. subflexa* and *H. virescens* have co-occurred everywhere before tomatillo monocultures in Mexico have driven away *H. virescens,* as they show reduced survival and larval weight on *Physalis* species (Sheck and Gould, 1993). The absence of communication interference made the production of acetates unnecessary, which were reduced due to their cost. As a consequence *H. subflexa* would have a higher relative amount of critical compounds and could more effectively attract conspecific males.

2) Low acetates is the ancestral trait and NC females have increased their acetate levels.

As outlined above, an increase in the acetate levels should be selected against because their increase is correlated with a decrease in two of the critical components, and thus seem costly. What makes it probable anyways that *H. subflexa* population in NC have increased their acetate levels in the course of evolution?

Mitter (1993) reported that the *virescens* group to which *H. subflexa* belongs has its greatest diversity in Brazil. *H. subflexa* is phylogenetically placed among South American species. It is therefore probable that the origin of the group lies in South America. *H. subflexa* and *H. virescens* both occur in North America as well (Teal and Tumlinson, 1997). It cannot be stated with security which of the species immigrated to North America first since they were not recognized as different species in North America until 1941 (McElvare, 1941). The two species could have immigrated to North America at the same time. The start of industrial growth of tobacco and cotton was probably the time point when *H. virescens* populations increased significantly and that the relative abundance of *H. virescens* exceeded *H. subflexa*. The increased number of *H. virescens* may have lead to a decrease of the signal to noise ratio for *H. subflexa* which could have brought about a change in the sex pheromone composition of *H. subflexa* females to ensure intraspecific premating communication. As mentioned in the introduction the acetates do help to avoid cross-attraction between *H. subflexa* and *H. virescens* (Vickers and Baker, 1997; Groot et al., 2006) The augmented presence of *H. virescens* could have caused directional selection towards higher acetates, as was proposed by Groot et al., (2006).

It has been speculated that changes in critical components will occur in small steps (Sheck et al., 2006), because huge changes in the pheromone composition would not attract any males and thus be selected against. Huge and fast changes could be possible for less critical compounds, because their change has less effect on premating communication. Small steps are correlated with many small QTL (i.e. many genes) huge steps are associated with a few strong QTL (one or a few genes) that explain much of the variance. Accordingly, in the study of Sheck et al. (2006) and Groot et al. (2009b), small QTL that were spread on many chromosomes were found for the secondary critical components. Rather large QTL were found for the less critical compounds. This tendency seems to hold in this study, where one large QTL was found for two of the acetates that explained more than 39 % of the variance. Nevertheless, we found a rather strong QTL for the major component Z11-16:Ald that explained more than 24% the compounds' variance. Since one gene could therefore account for a high percentage of variance of the major component it is possible that sudden shifts in the relative amount of critical compounds happen as well. A hypothesis for the mechanism of such a sudden shift was proposed by Roelofs et al. (2002) for *Ostrinia* species.

5A.3 Comparison of the chromosomes in intra- and interspecific QTL analyses

To assess whether the same gene(s) that are involved in the intraspecific variation are involved in the interspecific variation for the acetate production as well, I determined whether the chromosomes that we found in the present study are homologous to the chromosomes that were found by Sheck et al. (2006) and Groot et al. (2009b). Such a homology may point to a signature of selection that may have caused a divergence in pheromone production.

Chromosome 21 of the intraspecific study could be chromosome 4 of the two interspecific studies as indicated via the bulk segregate analysis. It explained a high percentage of the variance in all three studies: 12 % in the study of Groot et al. (2009b), 17% in the study of Sheck et al. (2006) and 16 % in the present study. Additionally this chromosome contains a QTL for Z11-16:OAc in all three studies. Further analyses are necessary to verify the homology of chromosome 4 (interspecific studies) and chromosome 21 (intraspecific study).

It is tempting to speculate that chromosome 22 of the interspecific studies is the same as chromosome 19 of the present study since most of the variation in the Z9-16: OAcs and the Z11-16:OAc is explained by chromosome 22 (interspecific studies) and chromosome 19 (intraspecific studies).

5B Phenotypic plasticity

5B.1 Phenotypic plasticity in the sex pheromone composition of *H. subflexa* females?

We found a genetic basis for the geographic differences in the sex pheromone of *H. subflexa*. This genetic basis does in no case explain 100% of the phenotypic variance of a compound. The question that I investigated was whether there is phenotypic plasticity in the female pheromone composition due to *H. virescens* pheromone. I could not find plasticity in the sex pheromone of *H. subflexa* females due to *H. virescens* pheromone, i.e. the sex pheromone blend of *H. subflexa* females reared in the constant presence of *H. virescens* pheromone was not significantly different from the blend of *H. subflexa* females reared in the absence of *H. virescens* pheromone. Males in the assortative mating experiment did also not significantly distinguish between P and NP females. Various reasons can have lead to this negative result. First of all, lab populations might not be the best choice for testing phenotypic plasticity, because it is not unlikely that plasticity was lost over the generations in the lab. The *H. subflexa* animals that I used in my study have been kept in the lab for 110 generations and allele frequency might have changed in favor for less plastic females. Lab rearing was shown to significantly affect the composition of the sex pheromone in *H.*

subflexa in only five generations (A. T. Groot, unpubl. res.). This has also been shown for *Spodoptera frugiperda* (Marr, unpublished data).

I could not detect the whole *H. virescens* pheromone blend in the pheromone chamber, because it was difficult to collect pheromone in the gas phase. However, I could detect the major component with the help of the GC-EAD technique. I thus conclude that *H. virescens* sex pheromone was present in the climate chamber. A total of 10 lures filled with a total of 300 mg pheromone compounds, with an exchange of 5 every seven days, should ensure the presence of *H. virescens* pheromone and generate a relatively high concentration. Under field conditions lures like the ones I used attract conspecific males for two to four weeks (Groot et al., 2007). The GC-analysis of the pheromone detection experiment showed that there were a lot of other odors present in the climate chambers. This suggests that there was a low signal-to- noise ratio in the climate chambers.

Lastly, the female sex pheromone composition may not react plastically to the presence of *H. virescens* pheromone. That does not mean that the sex pheromone production cannot be plastic. Other factors remain to be tested. Possible factors are temperature, humidity or host plant odor.

5B.2 Phenotypic plasticity in the male response of *H. subflexa*?

My results indicate that growing up under *H. virescens* pheromone influence could cause phenotypic plasticity in the male response. The overall analysis showed significant effects on various compounds for the P males that were not shown for the NP males. Interestingly, the mated females in P male mating units had higher relative amounts of Z11-16:OAc and Z7-16:OAc. The latter compound has been shown to increase male attraction in NC where *H. virescens* males are abundant but had no such an effect in MX (Groot et al., 2007). Z11-16:OAc has long been shown to inhibit *H. virescens* male attraction (Vickers and Baker, 1997). Astonishingly, the mated females also showed a lower amount of Z9-16:Ald and Z11-16:Ald. Considering the interpretation of the Pearson's correlation coefficient and the QTL analysis could provide an explanation: Since the relative amounts of acetates are negatively correlated to the relative amounts of Z9-16:Ald and Z11-16:Ald a choice for high acetate levels would inevitably lead to a choice for low Z9-16:Ald and Z11-16:Ald (and *vice versa*).

It is important to note that the females in P male mating units had by coincidence significantly different relative amount of Z9-16:Ald and Z11-16:OAc and marginally higher levels of Z7-16:OAc than females in NP male mating units. It cannot be decided whether the

NP males would have chosen in the same way or in another way compared to the P males if these differences would not have been there.

When looking at the male choice in single mating units, the compounds 14:Ald, Z9-14:Ald and Z11-16OAc showed significant assortative mating effects for the P males but not for the NP males. Since the biological role of the first two compounds is not clear yet it is hard to assess the biological relevance of these results. The finding for the Z11-16:OAc is interesting because of its intra- and interspecific roles (Groot et al., 2007; Groot et al., 2006; Vickers and Baker, 1997).

The distribution of the differences between the two females of one mating unit was not the same in the NP and the P male mating units for Z11-16:OAc with a tendency for higher differences in the P male mating units (data not shown). It is important to notice that when analyzed on a single mating unit levels the P males do not choose for "higher" Z7-16:OAc or "lower" Z9-16:Ald as could have been speculated from the overall analysis. The only compound for which an effect could be shown in both analysis approaches was the Z11-16:OAc. A general problem of this experiment was that the differences between the females of one mating unit were very variable among the mating units, i.e. females of some mating units had big differences in one pheromone compound, while the differences between two females in other mating units were very small for the same compounds.

Because of the sometimes unequal choice conditions for P and NP males the experiment must be repeated to verify the data. If the results can be repeated, the question if the effect can be attributed to *H. virescens* pheromone is still not answered. It is possible that the assortative mating effect I observed in P male mating units was actually due to the influence of the major component of the *H. virescens* pheromone. As *H. subflexa* and *H. virescens* female sex pheromone blends overlap in several compounds and they even use the same major component (Teal et al., 1981; Klun et al., 1982; Heath et al., 1991; Baker et al., 2004; Vickers, 2006), if my results are repeatable it is necessary to test if conspecific sex pheromone produces the same assortative mating results. In earlier studies repeated pre-exposure or long pre-exposure times (15-60 min) to conspecific sex pheromone (e.g. Daly and Figuerendo, 2000; Figuerendo and Baker, 1992; Stelinski, 2003) resulted in habituation of the male response, i.e. the pre-exposed males were less responsive to conspecific sex pheromone. It could be speculated that habituation has played a role in my experiments as well. Habituation of *H. subflexa* males could have happened for those compounds that are shared by the two species. When confronted with a conspecific female (with a conspecific sex pheromone) in the assortative mating situation the habituated males were more

"choosy" with regard to the acetate levels, since the acetates distinguish the *H. subflexa* from the *H. virescens* blend in quality.

It has been shown that the injection of PBAN reduces variation in female moth pheromone that is due to mating (Groot et al., 2005). However, the possibility that the mated females could have a different pheromone composition than the unmated females cannot be completely disregarded.

With these somewhat ambiguous results it is hard to judge if there was phenotypic plasticity in the male response.

6 Conclusions

Exploring the genetic basis of the geographic variation in the *H. subflexa* sex pheromone I found QTL that explain from 4 % up to 46 % of the variance for the compounds that were found to be significantly different between NC and MX in earlier studies (Groot et al., 2009a). The relative amount the particular compounds seem to be under the control of many genes. Nevertheless, we found some strong QTL for several compounds that explained a high proportion of their variance. Many of these QTL were located on chromosome 19 which thus seems to play a major role in controlling the compound ratios of the sex pheromone blend. Some correlations that were found between compounds point towards candidate genes, i.e. a delta-11-desaturase, an acetyl transferase or an alcohol oxidase that could be located on that chromosome.

The next steps in investigating the genetic basis of the geographic variance could be further bulk segregate analyses to find homologous chromosomes between inter- and intraspecific analyses. To determine which genes underlie the QTL that we found fine-scale mapping could be conducted. One way of finding the gene that underlies a QTL is to run primer combinations that were used to identify a chromosome, cut out the bands and sequence them. Since the map is based on genomic DNA and a marker does not have to be part of a gene it is improbable but possible to find a candidate gene via that approach. We found indications for candidate genes that could underlie particular QTL. It could be tried to map these genes to specific linkage groups, i.e. by designing primers for a candidate gene and test them on all individuals that were heterozygous (NC/MX) for a chromosome versus all that were homozygous (MX/MX) for the chromosome.

The role of phenotypic plasticity as a cause of variation in the sexual communication of *H. subflexa* remains very speculative. My results indicate that the sex pheromone composition of female *H. subflexa* is not plastic in response to *H. virescens* sex pheromone, other factors

like temperature, humidity or host plant odors that might yield a plastic response remain to be tested. The indication that there might be plasticity in the male response as a consequence of being reared under the influence of *H. virescens* sex pheromone must be verified in order to speculate about its role in causing variation.

In order to produce more unambiguous results it might be helpful to perform a future assortative mating experiment for NP and P male choice with females from selection lines. The selection focus could for example be on high and low acetates. Another possibility may be an assortative mating experiment in the presence of *H. virescens* pheromone, to assess which effect a signal that is interfering in a communication channel of a species might have on male choice. An alternative to assortative mating experiments might be windtunnel experiments with P and NP males.

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8 Literature

Agrawal, A. A. 2001. Phenotypic plasiticy in the interactions and evolution of species. *Science* 294:321-326.

Anderson, P., Sadek, M. M, and Hansson, B. S. 2003. Pre-exposure modulates attraction to sex pheromone in a moth. *Chem. Senses* 28:285-291.

Anderson, P., Hansson, B. S., Nilsson, U., Han, Q., Sjöholm, M., Skals, N., and Anton, S. 2007. Increased behavioral and neuronal sensitivity to sex pheromone after a brief odor experience in a moth. *Chem. Senses* 32:483-491.

Bailey, W. N. and Zuk, M. 2008. Acoustic experience shapes female mate choice in field crickets. *Proc. R. Soc. B* 275:2645-2650.

Baker, T. C., Ochieng, S. A., Cossé, A. A., Lee, S. G., Todd, J. L., Quero, C., and Vickers, N. J. 2004. A comparison of responses from olfactory receptor neurons of *Heliothis subflexa* and *Heliothis virescens* to components of their sex pheormone. *J. Comp. Physiol.* 190:155-165.

Berg, B. G., Tumlinson, J. H., and Mustaparta, H. 2004. Chemical communication in heliothine moths. *J. Comp. Physiol. A* 177:527-534.

Bjostad, L. B. and Roelofs, W. L. 1983. Sex pheromone biosynthesis in *Trichoplusia ni*: key steps involve delta-11 desaturation and chain-shortening. *Science* 220:1387-1389.

Blackinston, D. J., Silva Casey, E., and Weiss, M. R. 2008. Retention of memory through metamorphosis: can a moth remember what it learned as a caterpillar? *PLoS ONE* 3:1-7.

Brakefield, P. M. and Reitsma, N. 1991. Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. *Ecol. Entomol.* 16:291-304.

Brakefield, P. M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P. J., Monteiro, A., French, V., and Carroll, S. B. 1996. Development, plasticity and evolution of butterfly eyspot patterns. *Nature* 384:236-242.

Butlin, R. K. and Trickett, A. J. 1997. Can population genetic simulations help to interpret pheromone evolution? pp. 548-562, *in* R. T. Cardé and A. K. Minks (eds.). Insect Pheromone Research: New Directions. Chapman and Hall, New York.

Cardé, R. T. and Haynes, K. F. 2004. Structure of the pheromone communication channel in moths,pp. 283-332, *in* R. T. Cardé and J. G. Millar (eds.). Advances in Insect Chemical Ecology. Cambridg. University Press, Cambrigde.

Chapin, J. B., Ganaway, D. R., Leonard, B. R., Micinski, S., Burris, E., Graves, J. B. 1997. Species composition of Heliothinae captured in cone traps baited with synthetic bollworm or tobacco budworm pheromones. *Southwest. Entomol.* 22:223-231.

Choi, M.-Y., Han, K. S., Boo, K. S., and Jurenka, R. A. 2002. Pheromone biosynthetic pathway in the moths *Helicoverpa zea* and *Helicoverpa assulta*. *Insect Biochem. Mol. Biol.*32:1353-1359.

Choi, M.-Y., Groot, A. T., and Jurenka, R. A. 2005. Pheromone biosythetic pathway in the moths *Heliothis subflexa* and *Heliothis virescens*. *Arch. Insect Biochem.* 59:53-58.

Cook, C. D. K. 1969. On the determination of leaf form in *Ranunculus aquatilis. New Phytol.* 68:469-480.

Daly, K. C. and Figueredo, A. J. 2000. Habituation of sexual response in male *Heliothis* moths. *Physiol. Entomol.* 25:180-190.

Den Otter, C. J., Schuil, H. A., and Sander-Van Oosten, A. 1978. Reception of host-plant odours and female sex pheromone in *Adoxophyes orana* (Lepidoptera: Tortricidae): electrophysiology and morphology. *Entomol. Exp. Appl.* 24:570-578.

Dicke, M. and Sabelis, M. W. 1988. Infochemical terminology: based on cost-benefit analysis rather than origin of compounds? *Funct. Ecol.* 2:131-139.

Dodson, S. 1989. Predator-induced reaction norms. *BioScience* 39:447-452.

El-Sayed, A. M. 2008. The Pherobase: Database of Insect Pheromones and Semiochemicals. http://www.pherobase.com.

Endler, J. A. 1992. Signals, Signal Conditions, and the Direction of Evolution. *Am. Nat.* 139:125-153.

Figueredo, A. J. and Baker, T. C. 1992. Reduction of the response to the sex pheormone in the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae) following successive pheromonal exposures. *J. Insect Behav.* 5:347-363.

Gleason, J. M. and Ritchie, M. G. 2004. Do quantitative trait loci (QTL) for courtship song difference between *Drosophila simulans* and *D. sechellia* coincide with candidate genes and intraspecific QTL? *Genetics* 166:1303-1311.

Gould, F., Groot, A. T., Vasquez, G. M., and Schal, C. 2009. Sexual communication in Lepidoptera: a need for wedding genetics, biochemistry, and molecular biology, *in* M. B. Goldsmith and F. Marec (eds.). Molecular Biology and Genetics of the Lepidoptera. Taylor and Francis, London.

Gries, G., Schaefer, P. W., Gries, R., Liska, J., and Gotoh, T. 2001. Reproductive character displacement in *Lymantria monacha* from Northern Japan? *J. Chem. Ecol.* 27:1163-1176.

Groot, A. T., Gemeno, C., Brownie, C., Gould, F., and Schal, C. 2005a. Male and female antennal responses in *Heliothis virescens* and *H. subflexa* to conspecific and heterospecific sex pheromone compounds. *Chem. Ecol*. 34:256-263.

Groot, A. T., Yongliang, F., Brownie, C., Jurenka, R. A., Gould, F., and Schal, C. 2005b. Effect of PBAN on pheromone production by mated *Heliothis virescens* and *Heliothis subflexa* females. *J. Chem. Ecol.* 31:15-28.

Groot, A. T., Horovitz, J. L., Hamilton, J., Santangelo, R. G., Schal, C., and Gould, F. 2006. Experimental evidence for interspecific directional selection on moth pheromone communication. *Proc. Natl. Acad. Sci. USA* 103:5858-5863.

Groot, A. T., Santangelo, R. G., Ricci, E., Brownie, C., Gould, F., and Schal, C. 2007. Differential attraction of *Heliothis subflexa* males to synthetic pheromone lures in eastern US and western Mexico. *J. Chem. Ecol.* 33:353-368.

Groot, A. T., Inglis, O., Bowdridge, S., Santangelo, R. G., Blanco, C., López Jr., J. D., Vargas, T. A., Gould, F., and Schal, C. 2009a. Geographic and temporal variation in moth chemical communication. *Evolution* 63:1987-2003.

Groot, A. T., Estock, M. L., Horovitz, J. L., Hamilton, J., Santangelo, R. G., Schal, C., and Gould, F. 2009b. QTL analysis of sex pheromone blend differences between two closely related moths: Insights into divergence in biosynthetic pathways. *Insect Biochem. Mol. Biol.* 39:568-577.

Hawthorne, D. J. and Via, S. 2001. Genetic linkage of ecological specialization and reproductive isolation in pea aphids. *Nature* 412:904-907.

Heath, R. R., Mitchell, E. R., and Cibrian Tovar, J. 1990. Effect of release rate and ratio of (*Z*)-11-hexadecen-1-ol from synthetic pheromone blends on trap capture of *Heliothis subflexa* (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 16:1259-1268.

Heath, R. R., McLaughlin, J. R., Proshold, F., and Teal, P. E. A. 1991. Periodicity of female sex pheromone titer and release in *Heliothis subflexa* and *H. virescens* (Lepidoptera: Noctuidae).*Ann. Entomol. Soc. Am.* 84:182-189.

Heckel, D. G. 1993. Comparative genetic linkage mapping in insects. *Annu. Rev. Entomol*. 38:381-408.

Hendrikse, A. 1988. Hybridization and sex-pheromone responses among members of the *Yponomeuta padellus*-complex. *Entomol. Exp. Appl.* 48:213-223.

Hopkins, A. D. 1917. Economic investigations of the scolytid bark and timber beetles of North America. U.S. Department of agriculture Program of Work for 1917, Oregan.

Jaenike, J. 1983. Induction of host plant preference in *Drosophila melanogaster*. Oecologia 58:320-325.

Jaenike, J. 1988. Effects of early adult experience on host selection on insects: some experimental and theoretical results. *J. Insect Behav.* 1:3-15.

Jurenka, R. A. 2003. Biochemistry of female moth sex pheromones, pp. 53-80, *in* G. J. Blomquist and R. Voigt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, Amsterdam.

Karlson, P. and Lüscher, M. 1959. 'Pheromones': a new term for a class of biologically active substances. *Nature* 183:55-56.

Kawazu, K., Hasegawa, J., Honda, H., Ishikawa, Y., Wakamura, S., Sugie, H., Kamiwada, H., Kamimuro, T., Yoshiyasu, Y., and Tatsuki, S. 2000. Geographical variation in female sex pheromones of the rice leaffolder moth, *Cnaphalocrocis medinalis*: identification of pheromone components in Japan. *Entomol. Exp. Appl.* 96:103-109.

Klun, J. A., Bierle-Leonhardt, B. A., Plimmer, J. R., Sparks, A. N., Primiani, M., Chapman, O. L., Lepone, G., and Lee, G. H. 1980. Sex pheromone chemistry of the female tobacco budworm moth, *Heliothis virescens*. *J. Chem. Ecol.* 6:177-183.

Klun, J. A., Bierle-Leonhardt, B. A., Lopez Jr., J. D., and LeChance, L. E. 1982. Female *Heliothis subflexa* (Lepidopetera: Noctuidae) sex pheormone: Chemistry and congeneric comparisons. *Environ. Entomol.* 11:1084-1090.

Knipple, D. C., Rosenfield, C.-L., Nielsen, R., You, K. M., and Jeong, S. E. 2002. Evolution of the integral membrane desaturase gene family in moths and flies. *Genetics* 162:1737-1752.

Laster, M. L. 1972. Interspecific hybridization of *Heliothis virescens* and *H. subflexa*. *Environ. Entomol.* 1:682-687.

Lim, H., Park, K. C., Baker, T. C., and Greenfield, M. D. 2007. Perception of conspecific female pheromone stimulates female calling in an arctiid moth, *Utetheisa ornatrix*. *J. Chem. Ecol.* 33:1257-1271.

Linn, Jr., C. E. and Roelofs, W. L. 1981. Modification of sex pheromone blend discrimination in male oriental fruit moths by pre-exposure to (E)-8-dodecenyl acetate. *Physiol. Entomol.* 6:421-429.

Linn Jr., C. E., Campell, M. G., and Roelofs, W. L. 1987. Temperature modulation of behavioural thresholds controlling male moth sex pheromone response specificity. *Physiol. Entomol*. 13(1):59-67.

Linn Jr., C. E. and Roelofs, W. L. 1989. Response specificity of male moths to multicomponent pheromones. *Chem. Senses* 14:421-437.

Lintner, J. A. 1882. A new principle in protection from insect attack. *West. N.Y. Hort. Soc. Proc*. 27:52-66.

Löfstedt, C., Löfqvist, J., Lanne, B. S., Van Der Pers, J. N. C., Hansson B. S. 1986. Pheromone dialects in European turnip moths *Agrotis segetum*. *Oikos* 46:250-257.

Löfstedt, C., Herrebout, W. M., and Menken, S. B. J. 1991. Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecol.* 2:20-28.

Löfstedt, C. 1993. Moth pheromone genetics and evolution. *Philos. T. Roy. Soc. B* 340:167-177.

McElfresh, J. S. and Millar, J. G. 1999. Geographic variation in sex pheromone blend of *Hemileuca electra* from Southern California. *J. Chem. Ecol.* 25:2505-2525.

McElvare, R. R. 1941. Validity of the species *Heliothis subflexa* (Gn.) (Lepidoptera). *Bull. Brooklyn Entomol. Soc.* 36:29-30.

Mitter, C., Poole, R. W., and Matthews, M. 1993. Biosystematics of the Heliothinae (Lepidoptera: Noctuidae). *Annu. Rev. Entomol.* 38:207-225.

Morse, D. and Meighen, E. 1986. Pheromone biosynthesis and role of functional groups in pheromone specificity. *J. Chem. Ecol.* 12(2):1573-1561.

Morse, D. and Meighen, E. 1987a. Pheromone biosynthesis: enzymatic studies in Lepidoptera, pp. 212-215, *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, New York.

Morse, D. and Meighen, E. 1987b. Biosynthesis of the acetate ester precursor of the spruce budworm sex pheromone by an acetyl CoA: fatty alcohol acetyltransferase. *Insect Biochem.* 17(1):53-59.

Nager, R. G., Keller, L. F., and van Noordwijk, A. J. 2000. Understanding natural selection on traits that are influenced by environmental conditions, pp. 95-115, *in* T. A. Mousseau, B. Sinervo and J. Endler (eds.). Adaptive Genetic Variation in the Wild. Oxford University Press, New York.

Nordlund, D. A. and Lewis, W. J. 1976. Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *J. Chem. Ecol.* 2:211-220.

Percy-Cunningham, J. E. and MacDonald, J. A. 1987. Biology and ultrastructure of sex pheromone-producing glands, pp. 27-69, *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Inc., Orlando, Fl.

Pigliucci, M. 2001. Phenotypic Plasticity: Beyond Nature and Nurture. The John Hopkins University Press, Baltimore.

Poulton, E. B. 1887. An inquiry into the cause and extent of a special colour-relation between certain exposed lepidopterous pupae and the surfaces which immediately surround them. *Proc. R. Soc. London* 42:94-108.

Rafaeli, A. 2002. Neuroendocrine control of pheormone biosynthesis in moths. *Int. Rev. Cytol.* 213:49-92.

Raina, A. K. and Menn, J. J. 1987. Endocrine regulation of pheromone production in Lepidoptera, pp. 157-174, in G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Inc., Orlando

Raina, A. K., Jaffe, H., Kempe, T. G., Keim, P., Blacher, R. W., Fales, H. M., Riley, C. T., Klun, J. A., Ridgway, R. L., Hayes, D. K. 1989. Identification of a neuropeptide hormone that regulates sex pheromone production in female moths. *Science* 244:796-798.

Raina, A. K., Kingan, T. G., and Mattoo, A. K. 1992. Chemical signals from host plant and sexual behavior in a moth. *Science* 255:592-594.

Raina, A. K. 1993. Neuroendocrine control of sex pheromone biosynthesis in Lepidoptera. *Annu. Rev. Entomol.* 38:329-349.

Raina, A. K., Wergin, W. P., Murphy, C. A., and Erbe, F. E. 2001. Structural organization of the sex pheromone gland in *Helicoverpa zea* in relation to pheromone production and release. *Arthro. Struct. Dev.* 29(4):343-353.

Ray, S. 1999. Survival of olfactory memory through metamorphosis in the fly *Musca domestica*. *Neurosc. letters* 259:37-40.

Ryan, M. J., Tuttle, M. D., and Rand, A. S. 1982. Bat predation and sexual advertisement in a neotropical anuran. *Am. Nat.* 119:136-139.

Remington, D. L., Whetten, R. W., Liu, B. H., O'Malley, D. M. 1999. Construction of an AFLP genetic map with nearly complete genome coverage in *Pinus taeda*. *Theor. Appl. Genet.* 98:1279-1292.

Roelofs, W. L. and Comeau, A. 1969. Sex pheromone specificity: taxonomic and evolutionary aspects in Lepidoptera. *Science* 165:398-400.

Roelofs, W. L. and Brown, R. L. 1982. Pheromones and evolutionary relationships of Tortricidae. *Annu. Rev. Ecol. Syst.* 13:395-422.

Roelofs, W. L. and Wolf, W. A. 1988. Pheromone biosynthesis in Lepidoptera. *J. Chem. Ecol.* 14:2019-2031.

Roelofs, W. L., Liu, W., Hao, G., Jiao, H., Rooney, P., and Linn, Jr., C. E. 2002. Evolution of moth sex pheromone via ancestral genes. *P. Natl. Acad. Sci. USA* 99:13621-13626.

Schlichting, C. D. and Pigliucci, M. 1998. Phenotypic Evolution: A Reaction Norm Perspective. Sinauer Associaties, Inc., Sunderland.

Schneider, D., Schulz, S., Priesner, E., Ziesmann, J., and Francke, W. 1998. Autodetection and chemistry of female and male pheromone in both sexes of the tiger moth *Panaxia quadripunctaria*. *J. Comp. Physiol. A* 182:153-161.

Sheck, A. L. and Gould, F. 1993. The genetic basis of host range in *Heliothis virescens*: larval survival and growth. *Entomol. Exp. Appl.* 69:157-172.

Sheck, A. L., Groot, A. T., Ward, C. M., Gemeno, C., Wang, J., Brownie, C., Schal, C., and Gould, F. 2006. Genetics of sex pheromone blend differences between *Heliothis virescens* and *Heliothis subflexa*: a chromosome mapping approach. *J. Evol. Biol.* 19:600-617.

Steidle, J. L. M. and Schöller, M. 1997. Olfactory host location and learning in the granary weevil parasitoid *Lariophagus distinguendus* (Hymenoptera: Pteromalidae). *J. Insect Behav.* 10:331-342.

Stelinski, L. L., Miller, J. R., and Gut, L. J. 2003. Presence of long-lasting peripheral adaptation in oblique-banded leafroller, *Choristoneura rosaceana* and absence of such adaptation in redbanded leafroller, Agryrotaenia velutiana. *J. Chem. Ecol.* 29:405-423.

Tamaki, Y. 1985. Sex pheromones, pp. 145-191, *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry, and Pharmacology. Pergamon Press, Oxford.

Tang, J. D., Wolf, W. A., Roelofs, W. L., and Knipple, D. C. 1991. Development of functionally competent cabbage looper moth sex pheromone glands. *Insect Biochem. (USA)* 21(6):573-581.

Teal, P. E. A., Heath, R. R., Tumlinson, J. H., and McLaughlin, J. R. 1981. Identification of a sex pheromone of *Heliothis subflexa* (GN.) (Lepidoptera: Noctuidae) and field trapping studies using different blends of components. *J. Chem. Ecol.* 7:1011-1022.

Teal, P. E. A., Tumlinson, J. H., and Heath, R. R. 1986. Chemical and behavioral analysis of volatile sex pheromone components released by calling *Heliothis virescens* (F.) females (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 12:107-125.

Teal, P. E. A. and Tumlinson, J. H. 1987. The role of alcohols in pheromone biosynthesis by two noctuid moths that use acetate pheromone components. *Arch. Insect Biochem.* 4:261-269.

Teal, P. E. A. and Tumlinson, J. H. 1988. Properties of cuticular oxidases used for sex pheromone biosynthesis by *Heliothis zea. J. Chem. Ecol.* 14:2131-2145.

Teal, P. E. A. and Tumlinson, J. H. 1997. Effects of interspecific hybridization between *Heliothis virescens* and *Heliothis subflexa* on the sex pheromone communication system, pp. 535-547, *in* R. T. Cardé and A. K. Minks (eds.). Insect Pheromone Research: New Directions. Chapman and Hall, New York.

Tillman, J. A., Seybold, S. J., Jurenka, R. A., and Blomquist, G. J. 1999. Insect pheromones-an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol.* 29(6):481-514.

Tsfadia, O., Azrielli, A., Falach, L., Zada, A., Roelofs, W. L., and Rafaeli, A. 2008. Pheromone biosynthetic pathways: PBAN-regulated rate-limiting steps and differential expression of desaturase genes in moths. *Insect Biochem. Mol. Biol.* 38:552-567.

Tully, T., Cambiazo, V., and Kruse, L. 1994. Memory through metamorphosis in normal and mutant *Drosophila. J. Neurosci.* 14:68-74.

Vet, L. E. M. and Groenewold, A. W. 1990. Semiochemicals and learning in parasitoids. *J. Chem. Ecol.* 16:3319-3135.

Vetter, R. S. and Baker, T. C. 1983. Behavioral responses of male *Heliothis virescens* in a sustained-flight tunnel to combinations of seven compounds identified from female sex pheromone glands. *J. Chem. Ecol.* 9:747-759.

Vetter, R. S. and Baker, T. C. 1984. Behavioral responses of male *Heliothis zea* in sustained-flight tunnel to combinations of 4 compounds indentified from female sex pheromone gland. *J. Chem. Ecol.* 10:193-202.

Via, S. and Hawthorne, D. J. 1998. The genetics of speciation, pp.352-365, *in* D. J. Howard and S. H. Berlocher (eds.). Endless Forms: Species and Speciation. Oxford University Press, New York.

Vickers, N. J. and Baker, T. C. 1997. Chemical communication in heliothine moths. VII. Correlation between diminished responses to point-source plumes and single filaments similarly tainted with a behavioral antagonist. *J. Comp. Physiol.* 180:523-536.

Vickers, N. J. 2002. Defining a synthetic pheromone blend attractive to male *Heliothis subflexa* under wind tunnel conditions. *J. Chem. Ecol.* 28:1255-1267.

Vickers, N. J. 2006. Inheritance of olfactory preferences III. Processing of pheromonal signals in the antennal lobe of *Heliothis subflexa* x *Heliothis virescens* hybrid male moths. *Brain Behav. Evolut.* 68:90-108.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23:4407-4414.

Waddington, C. H. 1952. Genetic assimilation of an acquired character. *Evolution* 7:118-126.

Widmayer, P., Heifetz, Y., and Breer, H. 2009. Expression of a pheromone receptor in ovipositor sensilla of the female moth (*Heliothis virescens*). *Insect Mol. Biol.* 18(4):541-547.

Witzgall, P., Lindblom, T., Bengtsson, M., Tóth, M. 2004. The Pherolist. www-pherolist.slu.se

Zuk, M., Rotenberry, J. T., and Tinghitella, R. M. 2006. Silent night: adaptive disappearance of a sexual signal in a parasitized population of field crickets. *Biol. Lett.* 2:521-524.

9 Appendix

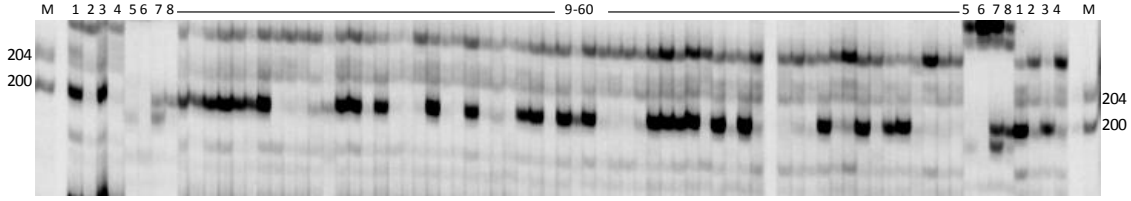

Fig.I. Creating a genetic AFLP map based on crosses between NC and MX population and bulk segregate analysis. 1: NC female; 2: MX male (parental generation). 3: F1 female; 4: backcross male (MX); 5-8: bulk DNA (12 individuals per bulk) from introgressed lines (*Hv* x *Hs* for chromosome 22 or chromosome 4): 5: homozygous *H. virescens*, i.e. no chromosomes came from *H. subflexa* (00). 6: chromosome 22 was heterozygous *Hv*/*Hs*, chromosome 4 was homozygous *Hv* (01). 7: chromosome 4 was heterozygous *Hv*/*Hs* and chromosome 22 homozygous *Hv* (10). 8: heterozygous (*Hv*/*Hs*) for both chromosomes 4 and 22 (11).9-60: female backcross individuals. M: marker.

Table I: Primer combinations run with 44 individuals; *: combinations run by Olive Inglis

Mse	Eco
AAG*	AAG
AAG*	ACG
AAG*	ACC
AAG*	ACT
AAG*	CGA
AAG*	CGC
AAG	AAC
AAG	ACA
AAG	ATG
AAG	AGG
ACA*	AAG
ACA*	ACG
ACA*	ACC
ACA*	ACT
ACA	AAC
ACA	ACA
ACA	ATG
ACA	AGG
ACG*	AAG
ACG*	ACG
ACG*	ACC
ACG*	ACT
ACG*	CGA
ACG	AAC
ACG	ACA
ACG	ATG
ACG	AGG
AGG*	AAG
AGG*	ACG
AGG*	ACC
AGG*	ACT
AGG*	CGA
AGG*	CGC
AGG	AAC
AGG	ACA
AGG	ATG
AGG	AGG
CAA*	AAG
CAA*	ACG
CAA*	ACC
CAA*	ACT
CAA*	CGA
CAA*	CGC

Mse	Eco
CAA	AAC
CAA	ACA
CAC*	AAG
CAC*	ACG
CAC*	ACC
CAC*	ACT
CAC*	CGA
CAC*	CGC
CAT*	AAG
CAT*	ACG
CAT*	ACC
CAT*	ACT
CAT*	CGA
CAT*	CGC
CAT	AAC
CAT	ACA
CAT	ATG
CAT	AGG
CCT*	AAG
CCT*	ACG
CCT*	ACC
CCT*	ACT
CCT*	CGA
CCT*	CGC
CGA*	AAG
CGA*	ACG
CGA*	ACC
CGA*	ACT
CTA*	ACC
CTA*	ACT
CTA*	CGA
CTA*	CGC
CTC*	AAG
CTC*	ACG
CTC*	ACC
CTC*	ACT
CTC*	AAC
CTC*	ACA
CTG*	AAG
CTG*	ACG
CTG*	ACC
CTG*	ACT
CTG*	CGA

Mse	Eco
CTG*	CGC
CTT*	AAG
CTT*	ACG
CTT*	ACC
CTT*	CGA
CTT*	CGC

Table II. Primer combinations to find at least one marker for the 29 identified groups for additional 52 individuals

	Mse	Eco
1	CCT	CGA
2	CCT	ACG
3	AAG	AAC
4	AAG	ACA
5	CAA	AAC
6	CAA	ACA
7	ACG	AAC
8	ACG	AGG
9	CAT	AAC
10	CAT	AGG
11	CAA	AAG
12	CAA	ACT
13	ACA	ATG
14	ACA	ACA
15	CAT	ACC
16	CAT	ACT
17	CTC	ACC
18	CTC	ACT
19	CAC	CGA
20	CAC	ACG
21	CGA	CGA
22	CGA	ACG
23	AAG	ACT
24	AAG	ATG

Table III. Primer combinations run with 88 individuals to find the 30th chromosome

	Mse	Eco
1	CAA	ATG
2	CAA	AGG
3	CAC	AAC
4	CAC	ACA
5	CAC	ATG
6	CAC	AGG
7	CGA	AAC
8	CGA	ACA
9	CTA	AAC
10	CTA	ACA
11	CTA	ATG
12	CTA	AGG
13	CTC	AAC
14	CTC	ACA
15	CTT	AGA
16	CTT	AGC
17	CAT	AGA

Table IV Ingredients of the larval diet

Ingredient	Amount To produce 1.0L	Manufacturer
NutriSoy R flour	41.2 g	Archer Daniels Midland Co., Decatur, IL
Wheat germ	35.1 g	Anacon Foods, Atkinson, KS
Wesson salt	9.5 g	MP Biomedicals, Inc., Solon, OH
Table sugar	41.2 g	Different local sources
Vitamin mix	9.5 g	DSM Nutritional Products, Alberta, Canada
Agar	11.2 g	AEP Colloids, Saratoga Springs, NY
Methyl hydroxybenzoate	1.0 g	MP Biomedicals, Inc., Solon, OH
Sorbic acid	1.0 g	MP Biomedicals, Inc., Solon, OH
Aureomycin	1.0 g	Fort Dodge Animal Health, Fort Dodge, IA
Propionic acid	1.1mL	
Phosphoric acid	0.1mL	

Table V. Chromosomes that were correlated with variance in pheromone compound levels. It was tested for each chromosome if the relative amounts of a particular pheromone compound differed significantly between the heterozygous individuals (NC/MX) and the homozygous (MX/MX) individuals. Analysis with minor compounds set to 100%.

		C3	C4	C7	C9	C10	C12	C16	C18	C19	C20	C21	C23	C26	C28
14:Ald	*r2*	0.11													0.103
	P	0.0011													0.0022
Z9-14:Ald	*r2*			0.06	0.054	0.05				0.076					
	P			0.015	0.025	0.029				0.0076					
16:Ald	*r2*										0.0576	0.095		0.046	
	P										0.0205	0.0026		0.0385	
Z7-16:Ald	*r2*									0.13					
	P									0.0004					
Z9-16:Ald	*r2*	0.097	0.045	0.048		0.048				0.28		0.135			
	P	0.0022	0.041	0.035		0.033				>.0001		0.0003			
Z7-16:OAc	*r2*											0.165		0.05	
	P											>.0001		0.029	
Z9-16:OAc	*r2*		0.057					0.06	0.05	0.487					
	P		0.022					0.017	0.03	>.0001					
Z11-16:OAc	*r2*						0.059	0.074		0.387		0.092			
	P						0.019	0.0087		>.0001		0.003			
SUM:OAc	*r2*						0.052	0.072		0.405		0.086			
	P						0.0277	0.0097		>0.0001		0.0042			
Z9-16:OH	*r2*									0.083					
	P									0.0051					
Z11-16:OH	*r2*	0.045						0.061		0.106			0.054		
	P	0.02						0.0172		0.0015			0.023		

10 Statutory declaration

I hereby declare that the thesis has been written by myself without any external unauthorized help. It has not been previously presented to any university for evaluation. The only sources used were the ones referred to. All parts which have been adopted either literally or in a general manner from these sources have been referred to accordingly. Any parts, words or ideas which are based on other sources, have been acknowledged as such.

Date Heike Staudacher