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Semi–Selective Fatty Acyl Reductases from Four Heliothine Moths Influence the Specific Pheromone Composition

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Abstract

Background: Sex pheromones are essential in moth mate communication. Information on pheromone biosynthetic genes and enzymes is needed to comprehend the mechanisms that contribute to specificity of pheromone signals. Most heliothine moths use sex pheromones with \((Z)–11\)-hexadecenal as the major component in combination with minor fatty aldehydes and alcohols. In this study we focus on four closely related species, *Heliothis virescens*, *Heliothis subflexa*, *Helicoverpa armigera* and *Helicoverpa assulta*, which use \((Z)–11\)-hexadecenal, \((Z)–9\)-tetradeccanal, and \((Z)–9\)-hexadecenal in different ratios in their pheromone blend. The components are produced from saturated fatty acid precursors by desaturation, \(\beta\)-oxidation, reduction and oxidation.

Results: We analyzed the composition of fatty acyl pheromone precursors and correlated it to the pheromone composition. Next, we investigated whether the downstream fatty–acyl reduction step modulates the ratio of alcohol intermediates before the final oxidation step. By isolating and functionally characterizing the Fatty Acyl Reductase (pgFAR) from each species we found that the pgFARs were active on a broad set of \(C8\) to \(C16\) fatty acyl substrates including the key pheromone precursors, \(Z9–14\), \(Z9–16\) and \(Z11–16:Acyls\). When presenting the three precursors in equal ratios to yeast cultures expressing any of the four pgFARs, all reduced \((Z)–9\)-tetradeccenoate preferentially over \((Z)–11\)-hexadecenoate, and the latter over \((Z)–9\)-hexadecenoate. Finally, when manipulating the precursor ratios in vitro, we found that the pgFARs display small differences in the biochemical activity on various substrates.

Conclusions: We conclude that a pgFAR with broad specificity is involved in heliothine moth pheromone biosynthesis, functioning as a semi–selective funnel that produces species–specific alcohol product ratios depending on the fatty–acyl precursor ratio in the pheromone gland. This study further supports the key role of these in pheromone biosynthesis and emphasizes the interplay between the pheromone fatty acyl precursors and the Lepidoptera specific pgFARs in shaping the pheromone composition.

Introduction

Many life forms depend on pheromones when finding a mate for reproduction [1],[2] but the detailed picture of the interplay between the genes, the enzymes and the pheromone production is barely understood. The chemical ecology of moths (Lepidoptera) has been thoroughly studied ever since the identification of *Bombyx mori* [3]. Most moths make use of long–chain fatty acid derivatives such as alcohols, acetates and aldehydes, and the vast majority of moths use a blend of pheromone components in specific ratios [4],[5]. Moth sex pheromones are produced in the female pheromone gland by a set of enzymes including \(\beta\)-oxidases, desaturases, fatty acyl reductases (FAR), oxidases, and acetyl transferases. Stereospecific members of the desaturase gene family have been extensively studied through gene characterization and expression analysis [6]–[11]. Recently, a few members of the reductase gene family have been discovered and functional assays have been developed to assess their biochemical activities [12]–[15].

The four heliothine species *Heliothis virescens*, *Heliothis subflexa*, *Helicoverpa armigera*, and *Helicoverpa assulta* belong to a phylogenetic group of moths known as a major–pest lineage. *H. virescens* and *H. subflexa* are closely related species occurring in North and South America, while *H. armigera* and *H. assulta* are both occurring in Eurasia [16]. Like most heliothines, *H. virescens* uses a binary pheromone mixture composed of \((Z)–11\)-hexadecanal \((Z)–11:Al\) as the major pheromone component, in combination with \((Z)–9\)-tetradeccanal \((Z)–9:14:Al\) as a minor compound, along with trace amounts of other aldehydes [17],[18]. Its closely related species, *H. subflexa* uses \((Z)–11:Al\) as major component in its pheromone and
biosynthesis is not selective and thus that the modulation of the species-specific ratios must occur at an earlier point in the biosynthesis. In the present study, we investigated whether the final heliothine pheromone ratios derive from a single biosynthetic step or a combination of semi-selective steps and whether the reduction stage involves one or several active pgFARS. We report on the isolation of a pgFAR ortholog from each of the studied heliothine species and the activity of the encoded enzymes. We further tested whether the pgFARS have differences in their substrate preferences that may account for the final differences in blend composition or if they display a similar activity, which, depending on the species-specific precursor ratios, would mold the intermediate alcohol profiles, and thus the species-specific pheromone blends. We found that a single pgFAR is active in pheromone biosynthesis in each of the four Heliothines, and that these enzymes have a general selectivity for fatty acyl precursors within a range of C8–16, although with differences in substrate activity. Thus, the pgFARS are involved in molding the pheromone blends. In addition, our findings of pgFARS acting on a large variety of saturated and unsaturated fatty-acyl precursors support the idea that the functional flexibility of the pgFARS provides greater possibilities for evolving signal diversity [24].

Methods

2.1. Insects

Larvae of the four heliothine moth species, *H. virescens*, *H. subflexa* and *H. armigera* (Bayer strain) and *H. assulta* originated from laboratory cultures maintained at the Department of Entomology, Max Planck Institute for Chemical Ecology, Jena, Germany, and were fed on pinto bean, or soy bean artificial diets [28]. Male and female pupae were sexed and kept separately in a rearing chamber at 22±1°C under a 17:7–h:light:dark photoperiod. Virgin females were separated daily before the scotophase and considered to be 0-day old.

2.2. Chemicals

(9–16:ME) and (9–16:OH) were obtained from Barton Fine Chemicals (Bedford, Massachusetts, USA). All other glassware was cleaned by heating in aqueous solutions containing 10% HCl, and rinsed with deionized water. The lipid content of the pupal lipids was determined using a chloroform:methanol (2:1, v:v) extraction method. The individual FAMEs were dissolved in 96% ethanol in a 0.02 M stock solution. All chemicals were used as reference compounds originated from our collection at Lund University.

2.3. Sex pheromone gland extracts and fatty-acyl precursor analyses

In virgin *H. virescens* females, the pheromone precursor content has been shown to peak at mid-scotophase in 2–day-old individuals [29]. We examined the fatty-acyl lipid content in the four heliothine species. Pheromone glands (PGs) of 2– to 3–day-old virgin females were dissected at mid-scotophase. The gland of a female was exposed by applying gentle pressure on its last abdominal segments and removed with sharp forceps or microscissors. Each gland was extracted for 30 min at room temperature (RT) in a glass vial containing 20 μl heptane and 0.5 ng/μl of pentadecyl acetate (15:OAc) as internal standard. The individual PG extracts were stored at −20°C until GC-MS analysis. The gland was transferred into a conical glass vial and its lipid content was extracted in 25 μl chloroform:methanol (2:1, v:v) spiked with 250 ng of triheptadecenoin as internal standard. The reaction was incubated at 4°C overnight, then placed at room temperature and incubated for 1 h. The gland was removed and the extract was
concentrated to dryness under a gentle nitrogen stream. The lipid extract was subjected to base methanolysis by addition of 25 μl of KOH (0.5 M in methanol) and mixed thoroughly, then incubated for 3 h at RT. The reaction was acidified by adding 25 μl HCl (1 M in water) followed by addition of 25 μl hexane. The samples were shaken, left to separate for 2 min, and then the hexane phase was transferred to a clean glass insert contained in a 2 ml–glass vial and stored at 20°C prior to GC–MS analysis.

2.4. Gas chromatography and mass spectrometry (GC–MS) analyses

A Hewlett Packard 5890II GC system, coupled to a mass selective detector (HP 5972) and equipped with a medium-polar INNOWax column (100% polyethylene glycol, 30 m×0.25 mm I.D., film thickness 0.25 μm, Agilent Technologies) was used. The GC–MS was operated in electron impact mode (70 eV), the injector was configured in splitless mode at 220°C, and helium was used as carrier gas (velocity: 30 cm/s). The oven temperature was maintained for 2 min at 50°C and increased at a rate of 10°C/min up to 220°C, and held for 20 min. The fatty alcohols, aldehydes, and fatty acid methyl esters (FAMEs) were identified by their retention time, their mass spectra and by comparison with reference compounds. The relative ratio (%) of the three pheromone methyl esters Z9–14:ME, Z9–16:ME, and Z11–16:ME, as well as the ratio of their corresponding alcohols in the gland extracts were calculated based on manual integration of the chromatogram peak areas using the Enhanced ChemStation® software (Agilent Technologies).

2.5. RNA extraction, cDNA synthesis, and amplification of the full–length FAR sequences

Glands were excised from 1 to 4 day–old females from each of the four species and total RNA extracted according to the instructions given by the manufacturer (RNeasy mini kit, Qiagen). First strand PG cDNAs were synthesized from 1 μg of total RNA with a reverse transcriptase (Stratascript, Stratagene, AH Diagnostics, Skarholmen, Sweden) and were used as template in subsequent PCR reactions. The Heliothis virescens FAR (HvFAR) nt sequence [30] was downloaded from GenBank (accession nr. EZ407233), and by performing a BLAST search in *H. armigera* (Har) genomic DNA local 454 database (Max Planck Institute for Developmental Biology, Jena, Germany) we found a hit (e value 7e−5) to a partial FAR orthologue sequence, HarFAR contig90541. Two gene-specific primers (GSPs), pFIHVfARs and pFIHVfARas (Table S1) were designed in BioEdit [31] from the full–length *HvFAR* sequence information in order to amplify the corresponding ORF. The *HvFAR* ORF was amplified from 100 ng of PG cDNA in a 20 μl PCR reaction (containing 3.5 mM MgCl2, 0.5 μM of each GSP primer, 0.4 μl Advantage 2 polymerase mix and 0.4 μl dNTPs 10 mM), with the following cycling program: 95°C for 5 min (1 x), denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 3 min (35 x), then an additional elongation step at 72°C for 10 min. The PCR products were analyzed on a 1% TAE agarose gel with a GeneRuler 100–bp ladder (Fermentas, Helsingborg, Sweden). The GSPs designed from *HvFAR* successfully amplified the orthologous pgFAR sequence from *H. subflexa* (Hs) and *H. armigera* (Har) using Taq polymerase (Metabion) under similar PCR conditions. For *H. asulata* (Has), the combination of pFIHVfARs and HvFAR1078_710R and Has PG cDNA amplified a DNA fragment of ca 1 kb. To obtain the remaining 3’ sequence we designed degenerate primers (HFAR3URTI–III) from the consensus 3’UTR sequences from *HvFAR* and HarFAR contig90541. The PCR products were cleaned with the two enzymes Exo and SAP and sequenced in both directions with the BigDye® v.3.1 cycle sequencing kit. To ensure that the different *Hv* primers did not introduce a bias in the 3’ and 5’ ends of the *Hv*, *Har*, and *HsFAR* nucleotide sequences, we designed internal GSPs based on each species’ orthologous pgFAR sequence to confirm the entire ORF sequence. Using various primer combinations (Table S1) and 3’ and 5’ RACE ready cDNAs synthesized according to instructions provided in the SMART RACE cDNA Amplification Kit (Clontech), we amplified the 3’ and 5’ ends of all pgFARs and confirmed their ORF nt sequences.

2.6. Sequence analyses and gene tree construction

DNA sequence analyses and comparisons of amino acid sequence similarities were performed using BioEdit Sequence Alignment Editor software v.7.0.5.3 [31]. The DNA sequences were compared to nucleotide collections and public non-redundant databases of Blastn, Blastx, and Blastp [32]. Multiple sequence alignments of deduced amino acid sequences were performed with the ClustalW2 algorithm [33] followed by manual inspection. The building of the Neighbor–joining gene tree was done with MEGA v.4.0.2 (JTT model, 1500 replicates, pairwise comparisons) [34] on the deduced aa sequences of FARs from various arthropods and animals retrieved from the NCBI Protein Database or as stated in Liénard et al. [15].

2.7. Functional single substrate assay in yeast

Each full–length FAR ORF was amplified using gene–specific primers (Table S1) and cloned in the pYES2.1 expression vector downstream of the GAL1 promoter according to the instructions given by the manufacturer (Invitrogen) before confirmation by sequencing with the vector specific primers Gal1 and V5. The four pgFAR constructs and the sole pYES2.1 plasmid were transformed into the *InsSel* strain of *S. cerevisiae* (Invitrogen) and grown on SC–U plates with 0.7% YNB (w/v) aa, with ammonium sulphate, and a drop–out medium lacking uracil (ForMedium™ LTD, Norwich, England), and 2% glucose. Single autotrophic colonies were inoculated in 5 mL SC–U medium and incubated for 24 h at 30°C and 300 rpm (Innova 42, New Brunswick Scientific), then diluted to an OD600 = 0.4 to a final volume of 20 mL in SC–U medium containing 2% galactose and 0.1% glucose in 250–mL flasks, and incubated for 24 h at 30°C and 300 rpm. Then 0.5 mM alcohol–free ME precursors were added to the yeast cultures diluted to 1:10 in 2 mL SC–U 2% galactose, 1% tergitol (Nonidet P-40, Sigma) following by incubation for 24 h at 30°C and 300 rpm. Mixtures of alcohol products are found both in the yeast cell pellets and the culture medium in identical proportions [15] and similarly to previous studies, we extracted alcohols from the yeast pellet only [12],[14],[15]. Briefly, cells were collected by centrifugation at 2,000× g (Labofuge 200, Heraeus Instruments), and the cell pellets were extracted with 1 mL n–hexane including 150 ng Z11–13:OH as an internal standard followed by shaking at 200 cycles/min (Vibraxmax 100, Heidolph) for 1 h. The hexane layer was recovered and samples were stored at −20°C until gas chromatography analyses, prior to which they were concentrated under a gentle flow of N2 to around 50 μl.

2.8. FAR multiple substrate ratio assay

The FAR yeast transformants were inoculated and grown as previously mentioned. A ratio of the three precursors Z9–14:ME, Z9–16:ME, and Z11–16:ME was added in a total concentration of 0.5 mM. The pheromone methyl esters were identified by their GC–MS analyses and compared to nucleotide collections and public non-redundant databases of Blastn, Blastx, and Blastp [32]. The fatty alcohols, aldehydes, and fatty acid methyl esters (FAMEs) were identified by their retention time, their mass spectra and by comparison with reference compounds. The relative ratio (%) of the three pheromone methyl esters Z9–14:ME, Z9–16:ME, and Z11–16:ME, as well as the ratio of their corresponding alcohols in the gland extracts were calculated based on manual integration of the chromatogram peak areas using the Enhanced ChemStation® software (Agilent Technologies).
initially tested. Ratio 1 was tested to assess if the different pgFARs have an identical substrate preference on a mixture of compounds supplied in equal proportions as to when the compounds are supplemented individually. We then used two different precursor ratios to test if each orthologous pgFAR was able to modulate the alcohol production (Ratio 2–3). A final ratio assay based on the pheromone gland precursor analysis was performed (Ratio 4, Table 1). Incubation, extraction and GC-MS analysis were performed as previously described. The three peaks on the chromatogram corresponding to \(Z\text{-}14\text{-}OH\), \(Z\text{-}16\text{-}OH\), and \(Z\text{-}11\text{-}16\text{-}OH\) were manually integrated to calculate their relative ratio.

2.9. Statistics

Calculations of Standard Deviations (SD) and Standard Errors of the Mean (SEM) were made in Microsoft Office Excel. All following statistical analyses were calculated with IBM SPSS v.19.0.0. Test for normal frequency distribution was made with Q-Q plot. The values tested were the relative proportions compared to the most abundant compound (set to 1) and significant differences between the ME and alcohol precursors and their corresponding aldehydes in the glands, and for the ratio assays MEs and the alcohol products, were determined by means of a one-way Analysis of Variance, including Tukey’s test and Homogeneity of Variance Test. If there were unequal variances of the sample sets, we performed the Welch and Brown-Forsythe tests of Equality of Means, and a Games Howell-test as an alternative to Tukey’s test.

Results

3.1. Pheromone gland extracts and fatty acid precursor analyses

In all glands of the four heliothine species \(H.\text{ virens}\), \(H.\text{ subflexa}\), \(H.\text{ armigera}\), and \(H.\text{ assulta}\), we found the common FA derivatives such as palmitic acid, stearic acid, oleic acid, linolenic acid, and linoleic acid. Besides these compounds, the different species displayed unique profiles of pheromone precursor ratios. The \(H.\text{ virens}\) pheromone gland contained three aldehydes, \(Z\text{-}14\text{-}Al\), \(Z\text{-}16\text{-}Al\) and \(Z\text{-}11\text{-}16\text{-}Al\, in a 8:14:100 ratio on average (\(N\text{PG samples}=7\)) in the first and latter aldehydes constitute the attractive pheromone blend [17],[18]. The saturated 16:Al and 14:Al were also found (data not shown), similarly to previous studies [18],[29],[35]. GC-MS analysis of a methanolyzed gland lipid extract showed that the corresponding methyl ester precursors were found in a 11:50:100 ratio (Figure 1A). To test whether the pheromone and precursor ratios differ significantly in this species, we performed the Welch and Brown-Forsythe tests (due to unequal variances), in which we compared the proportions of either \(Z\text{-}9\text{-}14\) or \(Z\text{-}9\text{-}16\) aldehydes to their corresponding fatty acyl precursors. A difference between fatty-acyl precursor and pheromone component ratios would suggest that downstream biosynthetic enzymes are influencing the relative proportions in the final blend. There was no statistical difference in the proportions between \(Z\text{-}9\text{-}14\text{-}Al\) and \(Z\text{-}9\text{-}14\text{-}Acid\) relative to the \(Z\text{-}11\text{-}16\text{-}derivatives. However, the relative abundance of \(Z\text{-}16\text{-}Al\) was significantly lower than its precursor (\(P=0.002\), \(F(1,12)=20.997\), \(P=0.001\)).

The \(H.\text{ subflexa}\) pheromone glands contained the two pheromone components \(Z\text{-}16\text{-}Al\) and \(Z\text{-}11\text{-}16\text{-}Al\ (\(N\text{PG samples}=11\)), in an average ratio of 53:100 (Figure 1B). These are the attractive components in this species (together with \(Z\text{-}11\text{-}16\text{-}OH\) [19],[20]. The two fatty acyl pheromone precursors, \(Z\text{-}9\text{-}16\text{-}ME\) and \(Z\text{-}11\text{-}16\text{-}ME\ (\(N\text{PG samples}=12\)) were present in a ratio of 100:61, while the corresponding fatty acyl alcohols (\(N\text{PG samples}=10\)) were found in a 22:100 ratio. The N-values of the samples differ since the aldehydes and/or alcohols were below the limit of quantification in some of the glands. When comparing the relative proportions of \(Z\text{-}16\text{-}Acid\) to its derived \(Z\text{-}16\text{-}OH\) and \(Z\text{-}16\text{-}Al\ the precursor appeared to be significantly (\(F(2,29)=54.585\), \(P=0.001\)) more abundant than both the corresponding alcohol and aldehyde (\(P<0.001\)). The \(Z\text{-}11\text{-}16\text{-}Acid\) was present in smaller amount compared to \(Z\text{-}16\text{-}Acid\, while both the corresponding alcohol and aldehyde showed the opposite pattern, being significantly more abundant than the \(Z\text{-}16\) compound (\(F(2,29)=26.518\), \(P<0.001\)). The \(Z\text{-}16\) alcohol and aldehyde did not differ in relative abundance (\(P>0.05\)).

The \(H.\text{ armigera}\) glands contained the major pheromone component \(Z\text{-}11\text{-}16\text{-}Al\, with traces of \(Z\text{-}9\text{-}16\text{-}Al\, which is in agreement with the 100:2 to 100:7 ratio of pheromone components previously reported [21]. The precursors \(Z\text{-}11\text{-}16\text{-}ME\) and \(Z\text{-}9\text{-}16\text{-}ME\ (\(N\text{PG samples}=9\)) were present in a 100:98 ratio (Figure 1C). When comparing the relative proportion of \(Z\text{-}16\text{-}Acid\ to its corresponding aldehyde, the former was significantly more abundant than the latter (\(F(1,16)=113.98\), \(P<0.001\)). There was no significant difference between the relative proportion of \(Z\text{-}11\text{-}16\text{-}Acid\ and \(Z\text{-}11\text{-}16\text{-}Al\ (P>0.05\)).

In \(H.\text{ assulta}\), the glands contained the major pheromone component \(Z\text{-}9\text{-}16\text{-}Al\ and the minor \(Z\text{-}11\text{-}16\text{-}Al\ (\(N\text{PG samples}=7\)) in a ratio of 100:9, which agrees with previously reported ratios [21]. The corresponding alcohols were not found in all gland extractions, but in those that did have them (\(N\text{PG samples}=4\)) they were present in a 100:15 ratio, and the ME precursors (\(N\text{PG samples}=5\)) in a 100:6 ratio (Figure 1D). There was a significant difference between the ratios of the \(Z\text{-}11\text{-}16\text{-}derivates in relation to the \(Z\text{-}16\text{-}derivates (\(F(2,13)=4.708\), \(P=0.029\)), with \(Z\text{-}11\text{-}16\text{-}OH\ being present in significantly larger amounts than its corresponding acid (\(P=0.024\)).

3.2. Cloning of pheromone gland biosynthetic fatty-acyl–CoA reductases (pgFARs)

The \(H.\text{ virens}\) and \(H.\text{ subflexa}\) FARs, HvFAR and HsFAR (accession no. JF709976) respectively, displayed a 1362 nt open-reading frame (ORF) that translated into a 453 aa-protein, while the \(H.\text{ armigera}\) and \(H.\text{ assulta}\) homolog FARs, HarFAR and HasFAR (accession no. JF709978 and JF709977), encompassed an ORF of 1367 nt and 1374 nt, which corresponded to 456 and 457 aa-proteins, respectively. Comparisons between aligned sequences at the nt and the aa level revealed a high level of sequence identity,
ranging from 86.3 to 91.9, and 92.5 to 97.4% respectively as shown in Table 2 and Figure 2. All the deduced protein sequences contained the Rossmann–fold NAD(P)H–binding protein domain and the C–terminal domain of fatty–acyl–CoA reductases, as confirmed by BLASTP searches and PFAM protein domain search [36]. A gene tree was constructed using a set of FAR sequences from a wide range of non–insect and insect organisms. All newly isolated heliothine FAR candidates clustered within the lepidopteran pgFAR clade that contains FARs involved in sex pheromone biosynthesis (Figure 3).

3.3. Heterologous expression in yeast

No alcohol products were found in yeast extracts prepared from the negative control (Figure 4A). In contrast, yeast samples expressing either FAR contained various amounts of alcohols corresponding to saturated 8:Acid, 10:Acid, 12:Acid, 14:Acid, and 16:Acid occurring naturally in the yeast (Figure 4B). The heliothine pgFARs were not able to reduce substrates longer than C16 in chain length. In addition, we also found minor amounts of Z9–16:OH in all samples, indicating that all four enzymes were able to reduce the Z9–16:Acid produced by the yeast (Figure 4B). When adding either Z9–14:ME or Z11–16:ME at a same concentration (0.5 mM) to yeast cultures expressing either of the four pgFAR candidates, GC–MS analyses of yeast extracts showed that the encoded FAR enzymes were able to reduce the acyl substrates into their corresponding alcohols, namely the Z9–14:OH and Z11–16:OH. The relative amounts of Z9–14:OH were higher compared to Z11–16:OH for all FAR constructs tested, which indicates an overall substrate preference for the Z9–14:ME. When supplementing the yeast expressing either FAR with the three unsaturated biosynthetic acyl precursors together, each enzyme accordingly reduced them to their corresponding alcohols (Figure 4C) and converted proportionally less of the saturated yeast acyls. Altogether this demonstrated that the heliothine FAR candidates encode active pheromone biosynthetic pgFARs with a broad activity on various saturated and unsaturated substrates ranging from C8 to C16 in chain length.

3.4. Ratio assays

The alcohol profiles obtained from assays performed with variable amounts of Z9-14, Z9-16 and Z11-16:ME are shown in Figure 5 A–D. When supplementing a mixture of the three precursors in identical proportions (Ratio 1: 0.333:0.333:0.333 for Z9–14, Z9–16, Z11–16), all pgFARs produced significantly more
When decreasing the relative proportion of Z9–14:ME in the supplied mixture (Ratio 2, 0.04:0.48:0.48), all pgFARs produced Z11–16:OH as the major alcohol product (Figure 5 A–D). This supports our observations from the assay with ratio 1 that all FARs preferentially reduce the Z11–16 over its Z9–16:acyl homolog. Interestingly, the FARs exhibited a difference in their overall production of both Z9–14:OH and Z9–16:OH compared to Z11–16:OH: HvFAR produced higher amounts of Z9–14:OH (F(3,8) = 12.313, P = 0.002) and Z9–16:OH (F(3,8) = 103.398, P < 0.001) compared to the other three FARs (Z9–14:OH: P = 0.024 (Hs–Hv), P = 0.001 (Har–Hv), and P = 0.039 (Has–Hv); Z9–16:OH: P < 0.001 for all).

When dramatically increasing the relative proportion of the least preferred substrate (i.e. the Z9–16:ME) in the three-component mixture (Ratio 3, 0.01:0.97:0.02), the alcohol profile accordingly shifted towards high proportions of Z9–16:OH (Figure 5 A–D). Altogether, experiments with Ratio 1–3 suggest that the heliothine pgFARs have a substrate preference for Z9–14 and Z11–16 acyl precursors, and that they produce a different ratio of alcohol products depending on the supplied precursor ratio. However, the alcohol ratios measured in vitro did not entirely match the ratios of aldehyde pheromone components in vivo in the respective heliothine species.

**Discussion**

The Heliothine pgFARs are broad-acting, semi-selective enzymes involved in molding the pheromone composition. In moth pheromone glands, the fatty acid pool is derived from the biosynthetic C16 homologs. In addition, it can be noted that HarFAR produced significantly more Z9–16:OH than HsFAR (Tukey, P = 0.027), and more Z11–16:OH than HvFAR did (Tukey, P = 0.029 (Hv–Har), P = 0.036 (Hs–Har)). The overall activity of HarFAR consequently seemed to be enhanced compared to HvFAR and HsFAR. The HasFAR alcohol production was intermediate and it was not significantly different from either of the extremes (P > 0.05).

When applying the Har–ratio 4 (0:0.49:0.51) to yeast expressing the HarFAR, the enzyme produced Z9–16:OH and Z11–16:OH in a 20.2:100 ratio, which matched the alcohol ratio found in *H. subflexa* female glands, but did not fully match the corresponding aldehydes, which are found in a 53.2:100 ratio. Likewise, when assaying yeast bearing the HasFAR construct with the Has–ratio 4 (0:0.94:0.06), composed of a mixture of Z9–16 and Z11–16:acyl precursors, the corresponding alcohols were found in a 100:63.4 ratio, compared to the aldehyde ratio of 100:9. Altogether, our results from the assays confirm that the heliothine pgFARs have a substrate preference for Z9–14, Z9–16, and Z11–16:acyl precursors, and that they produce a different ratio of alcohol products depending on the supplied precursor ratio. However, the alcohol ratios measured in vitro did not entirely match the ratios of aldehyde pheromone components in vivo in the respective heliothine species.

![Multiple alignment of HvFAR, HsFAR, HarFAR, and HasFAR](doi:10.1371/journal.pone.0037230.g002)
combined action of desaturases and \( \beta \)-oxidases. The fatty-acyl moieties subsequently undergo reduction [4],[5],[23],[37]. To determine if the reduction step influences the composition of the specific pheromone blends in \( H. \) virescens, \( H. \) subflexa, \( H. \) armigera, and \( H. \) assulta, we identified and functionally characterized the pgFAR of each species. These key biosynthetic enzymes convert fatty-acyl precursors into their corresponding primary alcohols [38], and are involved in shaping pheromone ratios in other moth species [14],[15]. In all four heliothines we found that the ratios of the final compounds differ compared to the fatty acyl precursor ratios, most notably in \( H. \) armigera and \( H. \) subflexa. In vitro, the four FARs are able to reduce a broad range of C8–16 substrates. In addition to this broad specificity, the four pgFARs are selective with respect to chain length and double bond position: when the enzymes were tested on a blend of compounds, the largest amount produced was that of \( Z^9 \)-14:OH, followed by \( Z^11 \)-16:OH and \( Z^9 \)-16:OH for all four pgFARs. When varying the precursor ratios, we observed that the amount of an alcohol product depended on the supplied proportion of its precursor in relation to the other precursors. Hence, the alcohol product ratio resulting from the action of each pgFAR will differ depending on the amount of precursors present in the respective species’ female gland, due to the hierarchical preference of the enzyme. This supports that the heliothine pgFARs to some extent molds the final proportions of components in the different species’ pheromone blends.

The composition of pheromone compounds in the glands of \( H. \) virescens, \( H. \) subflexa, \( H. \) armigera, and \( H. \) assulta in our study agrees to what was previously published [17]–[21]. Interestingly, our analyses of the corresponding fatty acid precursors revealed that in all species, but \( H. \) assulta, the final pheromone blend ratios differed from the precursor ratios. This indicates that the reduction and/or oxidation step modulates the pheromone composition. However, a modulating effect at the oxidation step is unlikely, as the oxidase was found to be unspecific [26],[27], whereas pgFARs in other species, both highly specific and general specific reductase enzymes, have been shown to modulate the intermediate fatty alcohol profiles both in vivo [39] and in vitro [14],[15].

The full-length FAR ORF sequences from each species were highly conserved both at the nt and aa levels (Figure 2). These four orthologous FAR candidates clustered together with gene members of the sub-family of pheromone biosynthetic lepidopteran

| Table 2. Identity between the pgFAR nucleotide and amino acid sequences. |
|-----------------|-----------------|-----------------|
| **Nt**          | **HvFAR**       | **HsFAR**       | **HarFAR**     |
| HsFAR           | 93.8            |                 |                 |
| HarFAR          | 95.4            | 94.7            |                 |
| HasFAR          | 92.5            | 97.4            | 95.2            |
| **aa**          |                 |                 |                 |
| HsFAR           |                 | 92.5            | 97.4            |
| HarFAR          | 95.4            | 94.7            |                 |
| HasFAR          | 92.5            | 97.4            | 95.2            |
| doi:10.1371/journal.pone.0037230.t002 |
pgFARs from which no apparent ortholog FAR sequences are found in other arthropods and mammals. These findings indicate that moths have recruited and evolved a specific group of FARs for the sole purpose of pheromone biosynthesis, and that a single FAR is active in heliothines, similarly to findings from Bombyx, Ostrinia and Yponomeuta [12]–[15]. Interestingly, the heliothine pgFARs cluster in the gene tree in proximity to both the B. mori and Yponomeuta that moths have recruited and evolved a specific group of FARs for found in other arthropods and mammals. These findings indicate pgFARs from which no apparent ortholog FAR sequences are expressing the pYES2.1 control (A), the Figure 4. Functional assay and GC–MS analysis of HvFAR. Typical total ion current (TIC) chromatograms from yeast cells expressing the pYES2.1 control (A), the H. virescens pgFAR (HvFAR) (B) and HvFAR in the functional assay with a blend of the three biosynthetic precursors in equal concentrations (ratio 1) (C). The control yeast produces no fatty alcohols (A) whereas the yeast expressing HvFAR convert a series of fatty acyls into their corresponding fatty alcohols (B–C). PEA refers to phenylethyl alcohol, a natural yeast aroma compound present in the extracts. The asterisks in (C) indicate the remaining methyl ester precursors (*, Z9–14:ME; **, Z9–16:ME and *** Z11–16:ME). The internal standard (IS) corresponds to 150 ng of Z11–13:OH.

Figure 4: Functional assay and GC–MS analysis of HvFAR.

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In a single-substrate-assay, the activities of the various pgFARs were not strikingly different, and thus it is most likely an interplay of a defined precursor ratio and enzymes’ substrate preference that produces the unique alcohol profiles prior to the oxidation step. Our results from the assay using the three major pheromone precursors in identical relative proportions/concentrations (Figure 5, ratio 3) reveals the pgFARs’ significant substrate preference for the Z9–14:ME over both Z9–16:ME and Z11–16:ME.

Multi substrate assays can be used to measure enzyme/substrate specificity constants and are consistent with individual measurements [41]. As long as the assay conditions remain the same for all single experiments, multi substrate assays can be used to screen the model enzymes [42]. In addition, the main characteristics, such as biomass, dry weight, glucose flux, and mRNA levels of glycolytic enzymes, in yeast that is cultured under constant conditions, where are usually comparable [43]. This supports our ratio assay as a reliable technique for investigating activity patterns of moth biosynthetic pgFARs.

Interestingly, in our multi-substrate assays, we observed topical differences in HvFAR, HsFAR and HaFAR reductive activity, which we postulate arise when the substrates compete for the enzyme’s binding pocket, as simulated in our assays. For instance, when presenting a precursor blend with the preferred substrate Z9–14:ME in a minor proportion compared to Z9–16:ME and Z11–16:ME (Figure 5, Ratio 2), the enzymes accordingly produce more Z11–16:OH, the second preferred substrate. But differences between enzymes were emphasized, i.e., HvFAR produced more Z9–14:OH and Z9–16:OH than the other three enzymes. With high amounts of Z9–16:ME (Figure 5, Ratio 1), all enzymes produce more Z9–16:OH but still individual differences occur as HsFAR produced more Z11–16:OH than the other pgFARs. This shows that differences in the biochemistry between the four pgFARs affect the alcohol production from multi-substrate precursor blends, and that the outcome of the reductive step in the pheromone biosynthesis of the four moths is dependent on a certain precursor ratio in a complex environment. It is known that the concentration of a substrate affects an enzyme’s activity, and by extension, more substrates further adds to the complexity of the system [44],[45]. If we consider that the different pheromone precursors bind to the same active site of the FAR enzyme, they can be regarded as competing molecules. These will temporary prevent the enzyme from acting on the other substrates since the possible complexes of an enzyme, a substrate and competing molecules are “enzyme–substrate” or “enzyme–inhibitor”, but never “enzyme–substrate–inhibitor” [44]. For the heliothine pgFARs, the enzyme–substrate may either be “pgFAR–Z9–14:Acyl”, “pgFAR–Z9–16:Acyl”, or “pgFAR–Z11–16:Acyl”. Thus the complex formation varies depending on the ratio of substrates that consequently affects the resulting alcohol profiles (Figure 5), which may explain minor differences between the activity levels of the four pgFARs in some of the assays, but does not affect our main findings or the reliability of our data.
The cell environment may differ between a moth pheromone gland and the yeast, which can affect the enzyme activity in vitro versus in vivo. This is a possible explanation for the result in the experiment with ratio 4. Here the pgFARs are provisioned with the species-specific gland-derived fatty-acyl precursor ratio (Figure 5, Ratio 4), and the fatty-alcohol ratio produced in vitro became closer, albeit not identical to the final pheromone composition in each species. In addition, a yeast cell contains other competing substrates such as saturated or unsaturated C8–16 [46], which can be reduced by the pgFARs, as well as factors that may have an inhibitory effect on the enzyme's folding or activity [47]. Saturated aldehydes and alcohols were also present in the gland extracts, indicating that saturated acyls are also reduced in the insect. A certain proportion of the Z9–16:Acid in the pheromone gland samples may result from metabolic fatty acid production, and may be used only to some extent to pheromone biosynthesis [4], therefore potentially causing a relative bias in the calculated ratio among the three biosynthetic precursors. Finally, it remains unexplored to date how the precursors are transported to the pheromone enzymes in the gland and if non-pheromone precursors are subjected to the pgFARs.

A precise pheromone blend is however rarely the outcome of a single gene [14], and usually results from the combined activity of several biosynthetic enzymes including desaturases, reductases and/or oxidases or acetyl transferases [5], [24], [48]. Crossing experiments have shown that the difference between the pheromone blends in H. assulta and H. armigera, which use the Z9–16 and Z11–16:Al in almost opposite ratios may be mainly controlled at one autosomal locus [21], but several QTL associated to the pheromone production in H. virescens and H. subflexa have been found [48], [49]. As mentioned, the oxidation step is largely unspecific in these species [26], [27], but it is still possible that the oxidases give the final touch to the pheromone depending on the species-specific alcohol profiles. Identifying the oxidase gene(s) involved in moth pheromone biosynthesis together with in vitro assays and further candidate gene mapping will be important steps towards a more complete understanding of the genetic basis of sex pheromone production in heliothine moths.

Figure 5. Multi-substrate assays of the four heliothine pgFARs. The graphs represent (A) the produced amounts of Z9–14:OH, Z9–16:OH, and Z11–16:OH, illustrated as relative amounts from the functional assays of HvFAR, (B) HsFAR, (C), HarFAR and HasFAR (D). Bars represent the standard error of the mean. These results show that the heliothine pgFARs are broad range enzymes with a substrate preference for Z9–14:acyls followed by Z11–16 and then Z9–16 acyls, and that there is a trend for all pgFARs that the most abundant precursor will be converted to the major product in vitro. The used precursor ratios are referred to in Table 1. The marking a equals P<5% to Z9–14:OH, b P<5% between HsFAR–HarFAR, c P<5% HvFAR–HarFAR, and * equals a significant difference of the marked bar to all other FARs. Non-significant results are marked with n.s. doi:10.1371/journal.pone.0037230.g005
Figure 6. Modulation of the alcohol profile in a ratio-assay matching gland precursor amounts. A. Pheromone gland: The relative percentage ratio of the pheromone content of H. virescens, H. subflexa, H. armigera, and H. assulta for 29–14:Al (H. virescens only), 29–16:Al and Z11–16:Al. The standard error of the mean is shown as a line in the bars. B. Yeast cells: The relative percentage ratio of 29–14:OH (present in HvFAR only), 29–16:OH, and Z11–16:OH produced when the pgFARs are expressed heterologously in yeast with addition of the corresponding precursors in ratios derived from pheromone gland analysis. The precursor ratios can be found in Table 1 as Ratio for each species.

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References

1. Bardwell L, Cook JG, Inouye CJ, Thorner J (1994) Signal Propagation and Regulation in the Mating Pheromone Response Pathway of the Yeast Saccharomyces cerevisiae. Dev Biol 166: 363–379.

2. Wyatt TD (2003) Pheromones and animal behaviour: Communication by smell and taste. Cambridge: Cambridge University Press.

3. Butenandt A, Beckmann R, Stamm D, Hecker E (1959) Über den Sexuallockstoff der Rauten. Z Naturforsch 14b: 283–294.

4. Tillman JA, Seybold SJ, Inouye CJ, Thorner J (1994) Signal Propagation and Endocrine Regulation in the Mating Pheromone Response Pathway of the Yeast Saccharomyces cerevisiae. Dev Biol 166: 363–379.

5. Blomquist GJ, Jurenka R, Schal C, Tittiger C (2005) Biochemistry and Molecular Biology of Pheromone Production. In Gilbert LI, Larrue K, Gill S, eds. Comprehensive Molecular Insect Science. Oxford: Elsevier. pp 705–752.

6. Knipple DC, Rosenfield CL, Nielsen R, You KM, Jeong SE (2002) Evolution of the Integral Membrane Desaturase Gene Family in Moths and Flies. Genetics 162: 1737–1752.

7. Roelofs WL, Liu W, Hao G, Jiao H, Rooney AP, et al. (2002) Evolution of moth sex pheromones via ancestral genes. Proc Natl Acad Sci U S A 99: 13621–13626.

8. Moto K, Suzuki MG, Hull J, Karuta R, Takahashi S, et al. (2004) Involvement of a bifunctional fatty-acyl desaturase in the biosynthesis of the silkmoth, Bombyx mori, sex pheromone. Proc Natl Acad Sci U S A 101: 8631–8636.

9. Serra M, Pina B, Bujons J, Camps F, Fabrias G (2006) Biosynthesis of 10,12-dienoic fatty acids by a bifunctional Δ11 desaturase in Spodoptera littoralis. Insect Biochem Mol Biol 36: 634–641.

10. Lénard MA, Strandh M, Hedénström E, Johansson T, Löfstedt C (2008) Key biosynthetic gene subfamily recruited for pheromone production prior to the extensive radiation of Lepidoptera. BMC Evol Biol 8: 1–13.

11. Wang, H-L., Lénard MA, Zhao C-H, Wang C-Z, Löfstedt C (2010) Neofunctionalization in an ancestral insect desaturase lineage led to rare Δ6 pheromone signals in the Chinese tussock silkworm. Insect Biochem Mol Biol 40: 742–751.

12. Moto K, Yoshiga T, Yamamoto M, Takahashi S, Okano K, et al. (2003) Pheromone gland-specific fatty-acyl desaturase of the silkworm, Bombyx mori. Proc Natl Acad Sci U S A 100: 9156–9161.

13. Antony B, Fujii T, Moto K, Matsumoto S, Fukuwasa M, et al. (2008) Pheromone gland-specific fatty-acyl desaturase in the adzuki bean borer, Ostrinia scapulalis (Lepidoptera: Crambidae). Insect Biochem Mol Biol 39: 90–95.

14. Lérand MA, Groot AT, Lérnand MA, Anthony B, Bogwardt C, et al. (2010) Allelic variation in a fatty-acid desaturase gene causes divergence in moth sex pheromones. Nature 466: 486–491.

15. Lénard MA, Hagström ÅK, Lassance JM, Lofstedt C (2010) Evolution of multicomponent pheromone signals in small ermine moths involves a single fatty-acyl desaturase gene. Proc Natl Acad Sci U S A 107: 10953–10956.

16. Cho S, Mitchell A, Mitter C, Regier J, Matthews M, et al. (2008) Molecular phylogenetics of heliotele moth (Lepidoptera: Noctuidae: Helioteleinae), with comments on the evolution of host range and pest status. Syst Entomol 33: 581–594.

17. Teal PEA, Tumlinson JH, Heath RR (1986) Chemical and behavioral analyses of volatile sex pheromone components released by calling Heliotis virescens (F) females (Lepidoptera: Noctuidae). J Chem Ecol 12: 107–126.

18. Klun JA, Leinard BA, Lérand MA, Lérand MA, Primami M, et al. (1980) Sex pheromone chemistry of the female Tobacco budworm moth, Heliothis virescens. J Chem Ecol 6: 177–183.

19. Klun JA, Leinard BA, Lérand MA, Lérand MA, Sparks AN, Primami M, et al. (1980) Sex pheromone chemistry of the female Tobacco budworm moth, Heliothis virescens. J Chem Ecol 6: 177–183.

20. Teal PEA, Heath RR, Tumlinson JH (1985) Identification of a sex pheromone of Heliothis virescens (GN) (Lepidoptera: Noctuidae) and field trapping studies using different blends of components. J Chem Ecol 11: 1011–1022.

21. Wang H-L, Zhao C-H, Wang C-Z (2005) Comparative study of sex pheromone composition and biosynthesis in Heliotis armigera, H. assulta and their hybrid. Insect Biochem Mol Biol 35: 373–383.

22. Choi MY, Groot AT, Lérand MA (2005) Pheromone Biosynthetic Pathway in the Moths Heliothis virescens and Heliothis armigera. Arch Insect Biochem Physiol 59: 53–56.

23. Lérand MA (2004) Insect pheromone biosynthesis. TopCurr Chem 239: 97–132.

Supporting Information

Table S1 Primers used to amplify and full-length sequences of HvFAR, HsFAR, HarFAR, and HasFAR. * The primers pHvFARs, pHHsFARs, and pHHarFARs, contains an additional Kozak-sequence (small letters) to promote expression efficiency when performing the functional assay. Start codons are emphasized in bold letters. ** GSP used for ORF amplification. ** GSP primer internal for HvFAR, used to amplify the partial HasFAR sequence. ** Degenerate primers for the 3′ region of the pgFARs. ** Primers for RACE amplification. ** GSP for amplification of internal region of HasFAR. ** Primers for sequencing inserts in the pYES2.1 vector.

Table S2 Gene tree sequence abbreviations. Abbreviations for the species from which the reductase sequences used in the gene tree originate.

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Author Contributions

Conceived and designed the experiments: ÅKH MAL ATG CL. Performed the experiments: ÅKH. Analyzed the data: ÅKH MAL. Contributed reagents/materials/analysis tools: EH. Wrote the paper: ÅKH MAL. ATG EH CL.
24. Liénard MA, Löfstedt C (2010) Functional flexibility as a prelude to signal diversity? Comm Int Biol 3: 586–588.
25. Roelofs WL, Rooney AP (2003) Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. Proc Natl Acad Sci U S A 100: 9179–9184.
26. Tew PEA, Tumlinson JH (1986) Terminal steps in pheromone biosynthesis by Heliothis virescens and H. zea. J Chem Ecol 12: 353–366.
27. Wang H-L, Ming Q-L, Zhao C-H, Wang C-Z (2008) Genetic basis of sex pheromone blend difference between Helicoverpa armigera (Hubner) and Helicoverpa assulta (Guenee). J Insect Physiol 54: 813–817.
28. Ahn SJ, Choi MY, Boo KS (2002) Mating effect on sex pheromone production of the Oriental tobacco budworm, Helicoverpa armigera. J Asia Pac Entomol 5: 43–48.
29. Foster SP (2005) Lipid Analysis of the Sex Pheromone Gland of the Moth Heliothis virescens. Arch Insect Biochem 59: 80–90.
30. Vogel H, Heidel AJ, Heckel DG, Groot AT (2010) Transcriptome analysis of the sex pheromone gland of the noctuid moth Heliothis virescens. BMC Genomics 11: 1–21.
31. Hall (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp 41: 95–98.
32. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.
33. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, et al. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31: 3497–500.
34. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Ecol 24: 1596–1599.
35. Groot AT, Estock ML, Hamilton J, Santanegho RG, Schal C, et al. (2009) 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.
36. Schellenberger V, Siegel RA, Rutter WJ (1993) Analysis of enzyme specificity by multiple substrate kinetics. Biochemistry 32: 4344–44.
37. Blagović Š, Rupčić J, Mesarić M, Georgić K, Marić V (2001) Lipid Composition of Brewer’s Yeast. Food technol. biotechnol 39: 175–181.
