Sex pheromone biosynthetic pathways are conserved between moths and the butterfly *Bicyclus anynana*

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Although phylogenetically nested within the moths, butterflies have diverged extensively in a number of life history traits. Whereas moths rely greatly on chemical signals, visual advertisement is the hallmark of mate finding in butterflies. In the context of courtship, however, male chemical signals are widespread in both groups although they likely have multiple evolutionary origins. Here, we report that in males of the butterfly *Bicyclus anynana*, courtship scents are produced *de novo* via biosynthetic pathways shared with females of many moth species. We show that two of the pheromone components that play a major role in mate choice, namely the \((Z)-9\)-tetradecenol and hexadecanal, are produced through the activity of a fatty acyl \(\Delta^{11}\)-desaturase and two specialized alcohol-forming fatty acyl reductases. Our study provides the first evidence of conservation and sharing of ancestral genetic modules for the production of FA-derived pheromones over a long evolutionary timeframe thereby reconciling mate communication in moths and butterflies.
The order Lepidoptera comprises an estimated 160,000 species and is thought to have arisen in concert with flowering plants about 140 Myr ago. Traditionally, it has been divided into two major subgroups comprising the moths (suborder Heterocera) and butterflies (suborder Rhopalocera), the latter having diverged from their common moth ancestors in the Early Cretaceous about 110 to 100 Myr ago, with subsequent lineage-specific bursts of radiations occurring about 70 Myr ago. The ca 18,000 described species of extant butterflies (Papilionoidea and Hesperiidae) have diverged extensively from their moth relatives in a number of life history traits including among others a diurnal lifestyle, bright appearances, body structures, antennal shapes and mate-finding behaviours.

The switch from nocturnal to diurnal behaviour and the corresponding increased dependence on visual communication for food and mate finding have led to the widespread assumption that this group of insects has undergone a global decrease in their olfactory capabilities. Indeed, female butterflies seem to have lost the long-distance pheromones on which their moth relatives rely almost exclusively, and mating is typically performed by patrolling males in stereotyped and visually oriented search behaviours. Nevertheless, recent studies on the genomic architecture of the olfactory protein and receptor repertoires, the neuroanatomy and the behavioural responses to plant and conspecific odours have begun to reveal remarkable commonalities in the olfactory system of butterflies and their nocturnal cousins, the moths.

Also, chemical signalling in the form of scent bouquets disseminated by courting males at close range has long been known to complement visual patterns in many butterfly species, which may play a decisive role in species recognition and female mate choice. The extant butterfly diversity is paralleled by a great chemical diversity in volatile male pheromone components, which include alkald derivatives, terpenoids, aromatics or carboxylic acids that are in many cases suggested or proven to be plant derived. The great variety in male scent suggests, however, that the butterfly signalling traits and underlying biosynthetic machineries have evolved multiple times independently during the course of evolution. Scent-releasing structures can be found on virtually any part of the body (for example, abdominal hair pencils, wing androconia and brushes), which argues further in favour of the multiple origins of the associated odours.

*Heliconius* males produce a pheromone blend comprising a phytol-derived compound, the (2R, 6R, 10R)-6,10,14-trimethylpentadecan-2-ol together with the fatty acid (FA) derivatives (Z)-9-tetradecenol (Z9-14:OH) and hexadecanal (16:Ald). This pheromone bouquet is released during a ritualized courtship display from two sets of modified scale secretions, utilized within the butterflies.

**Results**

The *Bicyclus* pheromone pathway follows moth biosynthesis. Our first aim was to experimentally test putative biosynthetic routes that could lead to the production of adult male pheromone components in *B. anynana*, the Z9-14:OH and 16:Ald, two compounds likely produced *de novo* as part of the insect FA metabolism. The third presumptive pheromone component, trimethylpentadecan-2-ol, was not part of the study as it is a phytol-derived compound expected to originate from the diet and not related to FA biochemistry.

First, we prepared wing extracts from 4-day-old male *B. anynana* individuals. The first male-specific component, Z9-14:OH is equally distributed in the forewing and the hindwing (Fig. 1a). The second male component, 16:Ald is found almost exclusively in the hindwing, more specifically in the androconia.
The postulated biosynthesis of the alcohol and aldehyde represented over 96% of the C14-C16 fatty acyl components corresponding chromatogram traces from negative labelling with D3-14:acid and DMSO controls in each wing, respectively. The identified FA precursors distribute across both wings. The identified FA precursors distribute across both wings. (b) Proportions of FA precursors in each wing relative to the total wing FA content (100%). (c–e) Analysis of pheromone production in male butterfly wings via biochemical in vivo labelling. Panels represent typical gas chromatograms from SIM, which records diagnostic ions according to the number of deuterium atoms in the precursor applied onto the wing androconia (FW: forewing, left columns; HW: hindwing, right columns) and shows incorporation of labelled FA precursors into pheromone components (16:Ald and Z9-14:OH). The upper panels show incorporation in FW and HW androconia from D3-16:acid, D3-14:acid, D9-Z11-16:acid and D9-Z9-14:acid as single-compound solutions onto the respective fore- and hindwing androconial areas. We applied D3-16:acid, D3-14:acid, D9-Z11-16:acid and D9-Z9-14:acid as single-compound solutions onto the androconial region of each wing. Our analyses of wing extracts revealed that deuterium atoms from D3-16:acid, D9-Z11-16:acid and D9-Z9-14:acid were incorporated into Z9-14:OH in both fore- and hindwings (Fig. 1c,d, Supplementary Fig. 1). In contrast, there was no label incorporation from D3-14:acid into Z9-14:OH (Fig. 1c,d). Deuterium atoms from D3-16:acid were incorporated into the 16:Ald in hindwings (Fig. 1e), whereas diagnostic ions indicative of label incorporation were absent in DMSO controls in all analyses (Fig. 1c–e).

Figure 1 | Bicyclus male wing pheromone: FA precursors and in vivo labelling. (a) Wing FA composition in 4-day-old males B. anynana. Distribution of the FA-derived pheromone components (Z9-14:OH and 16:Ald (n = 10) and the potential immediate FA biosynthetic precursors (n = 5) in forewings and hindwings. Bars represent s.e.m. Hexadecanal is almost exclusively present in hindwings, whereas the Z9-tetradecanol distributes equally across the two wings. The identified FA precursors distribute across both wings. (b) Proportions of FA precursors in each wing relative to the total wing FA content (100%). (c–e) Analysis of pheromone production in male butterfly wings via biochemical in vivo labelling. Panels represent typical gas chromatograms from SIM, which records diagnostic ions according to the number of deuterium atoms in the precursor applied onto the wing androconia (FW: forewing, left columns; HW: hindwing, right columns) and shows incorporation of labelled FA precursors into pheromone components (16:Ald and Z9-14:OH). (c,d) The Z9-14:OH pheromone pathway. The native Z9-14:OH was monitored via its molecular ion at m/z 212 and the diagnostic ions at m/z 194 [M-18]+ or 31 [CH2OH]+. The upper panels show deuterium incorporation in FW and HW androconia from D3-16:acid (c) and D9-Z11-16:acid (d) into Z9-14:OH, which was monitored by ions showing 3 or 9 a.m.u. more, respectively, than the native ion at m/z 194. Middle and lower panels display the corresponding chromatogram traces from negative labelling with D3-14:acid and DMSO controls in each wing, respectively. (e) The 16:Ald pheromone pathway. The native 16:Ald was monitored by the diagnostic ions at m/z 222 [M-18]+ and 196 [M-44]+. The upper panels show incorporation in FW and HW androconia of the labelled D3-16:acid into 16:Ald, monitored by a diagnostic ion showing 3 a.m.u. more than the native ion at m/z 196, whereas there is no deuterium incorporation when solutions containing D3-14:acid or DMSO only are applied onto the wings. (Fig. 1a). We confirmed that its immediate precursor, 16:OH, is undetectable, which is most likely the consequence of its rapid conversion into the final aldehyde form.

In order to reveal FA that could represent potential pheromone precursors, we subjected the fore- and hindwing tissues to total lipid extraction. Both forewings and hindwings contained myristic acid (14:acid) and palmitic acid (16:acid), as well as the monounsaturated (Z)-9-tetradecenoic acid (Z9-14:acid), (Z)-9-hexadecenoic acid (Z9-16:acid) and (Z)-11-hexadecenoic acid (Z11-16:acid). The high abundance of palmitic acid, which represented over 96% of the C14-C16 fatty acyl components isolated from the wings (Fig. 1b), together with the presence of unsaturated Z9-14:acid and Z11-16:acid, are in agreement with the postulated biosynthesis of the alcohol and aldehyde components from palmitic acid in B. anynana (Fig. 2).
Butterfly genome mining for FAD and FAR gene orthologues. We analysed the available genomes of two Nymphalidae butterflies, the monarch Danaus plexippus and the postman butterfly Heliconius melpomene, which we find to harbour diversified fatty acyl-CoA desaturase (FAD) and FAR gene families (Figs 3 and 4). Both butterflies possess FAD orthologues to all known major dipteran and lepidopteran FAD subfamilies (Fig. 3, Supplementary Fig. 2), including orthologues of the metabolic ancestral Δ9-desaturases (16C > 18C) and also of two key pheromone clades so far only identified in moths: the ‘variable’ Δ11-FAD subfamily to which Hmel-ctg0, Hmel-ctg1 and Dpl-ctg1 belong, and the Δ9-FAD (18C > 16C) to which belong Hmel-ctg2 and Dpl-ctg2 (Fig. 3, Supplementary Tables 1 and 2). Nucleic acid and amino-acid sequences corresponding to butterfly in silico FAD predictions are provided in Supplementary Data 1.

Whereas Drosophila melanogaster harbours seven FAD loci of which DesatF, Desat1 and Desat2 encode Δ9-desaturases functioning in female and male long-chain cuticular hydrocarbon pheromone synthesis, the domesticated silkworm Bombyx mori genome contains at least 16 FAD paralogues (Fig. 3). Of these, four have orthologues in the fruit fly (Bmo-1 is orthologous to Desat1 and Desat2, whereas Bmo-5, Bmo-6 and Bmo-7, respectively, are orthologous to Dmel/CG15531, Dmel/CG9743 and Dmel/CG9747). Of the twelve predicted B. mori FAD genes absent in flies, five paralogues derive from an expansion of the Lepidoptera-specific Δ11-like gene subfamily (Fig. 3). With 10 and 11 predicted paralogues, the butterfly desaturase subfamilies thus appear well conserved with the B. mori gene (Fig. 3) and each pair of butterfly desaturase orthologues displays a high level of identity at the amino-acid level and a conserved gene structure (Supplementary Fig. 2, Supplementary Table 1). Also, the evolution of the distinct FAD subfamilies in butterflies was likely accompanied by a successive loss of introns (Supplementary Fig. 2), which corroborates earlier investigations of FAD evolution in moths.

Twenty-nine FAR genes were predicted in the Postman and 25 in the Monarch genome, respectively, most of which contain intact open reading frames (Fig. 4, Supplementary Tables 3, 4, Supplementary Data 2, 3). These findings are in line with the 22 FAR paralogues uncovered in B. mori (Fig. 4) Interestingly, whereas B. mori and D. plexippus harbour three gene copies of the moth-specific pheromone production subfamily (pgFAR) that is, the pheromone production enzyme Bmo-pgFAR plus two other genes named Bmo-swdb1 and Bmo-swdb2 for the silkworm, and Dpl-ctg2, Dpl-ctg3 and Dpl-ctg8 for the Monarch, respectively, H. melpomene seems to have undergone a burst of lineage-specific duplications. It harbours eight pgFAR-like orthologues, of which only one gene Hmel-ctg8 may be non-functional as a consequence of a frame shift in exon 1. All other predicted pgFAR-like genes (Hmel-ctg1 to Hmel-ctg7) are contained in two distinct scaffolds presumably as a result from tandem duplications and possess the characteristics of functional FARs.

Characterization of a male B. anynana biosynthetic Δ11-FAD. All FADs including our in silico gene predictions of butterfly orthologues share conserved protein motifs (Supplementary Fig. 2). These structural features allowed us to screen the male Bicyclus anynana wing transcriptome using oligonucleotide primers targeting histidine-rich regions (HIS1 and HIS3) of desaturase genes in a PCR-based approach. We characterized two full-length cDNAs, one of which shared high sequence conservation with moth Δ9-desaturases (C18 > C16) and corresponded to a previously identified Δ9-like EST clone (see methods). In light of our in vivo data, this gene is unlikely involved in pheromone biosynthesis. The second cDNA shared high sequence conservation with the predicted Δ11-like genes from Heliconius (71 and 67% to Hmel-ctg0 and Hmel-ctg1, respectively) and Danaus (68% to Dpl-ctg1) and other previously characterized moth Δ11-desaturases (Fig. 3, Supplementary Fig. 2). Our maximum-likelihood phylogenetic reconstruction (Fig. 3) based on representative members of the different lepidopteran desaturase subfamilies further supports the notion that the B. anynana candidate FAD, hereafter named Ban-Δ11, is orthologous to members of the pheromone-producing Δ11-desaturase lineage (Fig. 3).

Characterization of male B. anynana pgFAR-like cDNAs. We used the predicted candidate biosynthetic butterfly FAR gene sequences to probe existing EST libraries from B. anynana in search of orthologues of the pheromone FAR family alongside a RACE-PCR cDNA amplification approach followed by Sanger sequencing from male hindwing tissue. We were able to characterize two full-length FAR cDNA candidates, hereafter named B. anynana wing FARs 1 and 2 (Ban-wFAR1 and Ban-wFAR2). These share 76% identity between their coding regions. Both deduced protein sequences display high amino-acid identity with predicted FAR genes from Danaus and Heliconius (77 and 75% with Dpl-ctg3 and 76 and 64% with Hmel-ctg1, respectively) and belong to the moth pheromone gland biosynthetic FAR subfamily (pgFARs, Fig. 4).

Heterologous expression of a butterfly Δ11-FAD ortholog. To test for a biochemical role in male signal production, that is,
Oza and Oze correspond to other D encodes a functional Ban produce higher amounts of Z11-16:acid. No additional mono-

yeast the ability to catalyse the desaturation of palmitic acid and 

¼

¼ with or without the addition of copper (15:Me, P

95 ng) that results from elongation of Z9-14:acid 

16:acid (1,948 wet 2014 

Ban delta9-like) are highlighted in red. Sequence abbreviations correspond to 
species names as follows: Ape, Antheraea pernyi; Ase, Agrotis segetum; Ave, Argyrotaenia velutinana; Bma, Bombyx mori; Che, Charistoneura herana; Cpa, Charistoneura parallela; Dpu, Dendrolimus punctatus; Epo, Epiphyas postvittana; Has, Helioverpa armigera; Hvi, Heliothis virescens; Hsub, Helicoverpa subflexa; Lca, Lampronia capitella; Mbr, Mamestra brassicae; Obr, Odespertha brunata; Onu, Ostrinia nubilalis (Ofu, Ola, Onr.za, Opa, Osa, Oza and Oze correspond to other Ostrinia species45); Pex, Planotortrix excisa; Poc, Planotortrix octa; Tni, Thricoplasia ni; Tpi, Thaumetopoea pityocampa; Yev, Yponomeuta evonymella (Yro and Ypa correspond to other Yponomeuta species45). Numbers at the nodes indicate bootstrap values for 100 replicates.

Figure 3 | Phylogeny of moths and butterflies FADs. A representative set of moth and butterfly desaturases were used to reconstruct the maximum-

likelihood phylogeny. The predicted Danaus plexippus (Dpl) and Heliconius melpomene (Hme) butterfly FAD orthologues are labelled in blue and green whereas the desaturase cDNAs characterized from B. anynana (Ban-Z11 and Ban-delta9-like) are highlighted in red. Sequence abbreviations correspond to 

biosynthetic FAR cDNA candidates, their respective 
determine the putative biological role of the two characterized 

Ban-Z11 and Ban-delta9-like) are highlighted in red. Sequence abbreviations correspond to 
species names as follows: Ape, Antheraea pernyi; Ase, Agrotis segetum; Ave, Argyrotaenia velutinana; Bma, Bombyx mori; Che, Charistoneura herana; Cpa, Charistoneura parallela; Dpu, Dendrolimus punctatus; Epo, Epiphyas postvittana; Has, Helioverpa armigera; Hvi, Heliothis virescens; Hsub, Helicoverpa subflexa; Lca, Lampronia capitella; Mbr, Mamestra brassicae; Obr, Odespertha brunata; Onu, Ostrinia nubilalis (Ofu, Ola, Onr.za, Opa, Osa, Oza and Oze correspond to other Ostrinia species45); Pex, Planotortrix excisa; Poc, Planotortrix octa; Tni, Thricoplasia ni; Tpi, Thaumetopoea pityocampa; Yev, Yponomeuta evonymella (Yro and Ypa correspond to other Yponomeuta species45). Numbers at the nodes indicate bootstrap values for 100 replicates.

the ability of Ban-D11 to catalyse the desaturation of palmitic acid, we placed its ORF under the control of a copper-dependent 

CUP1 promoter then transformed the resulting pYEX-CHT-

InvSc1 yeast strain and 

ORFs were cloned under the control of a GAL1 promoter in the 

determine the putative biological role of the two characterized 

Ban-Z11 and Ban-delta9-like) are highlighted in red. Sequence abbreviations correspond to 
species names as follows: Ape, Antheraea pernyi; Ase, Agrotis segetum; Ave, Argyrotaenia velutinana; Bma, Bombyx mori; Che, Charistoneura herana; Cpa, Charistoneura parallela; Dpu, Dendrolimus punctatus; Epo, Epiphyas postvittana; Has, Helioverpa armigera; Hvi, Heliothis virescens; Hsub, Helicoverpa subflexa; Lca, Lampronia capitella; Mbr, Mamestra brassicae; Obr, Odespertha brunata; Onu, Ostrinia nubilalis (Ofu, Ola, Onr.za, Opa, Osa, Oza and Oze correspond to other Ostrinia species45); Pex, Planotortrix excisa; Poc, Planotortrix octa; Tni, Thricoplasia ni; Tpi, Thaumetopoea pityocampa; Yev, Yponomeuta evonymella (Yro and Ypa correspond to other Yponomeuta species45). Numbers at the nodes indicate bootstrap values for 100 replicates.

Heterologous expression of the butterfly FAR orthologues. To determine the putative biological role of the two characterized 

B. anynana biosynthetic FAR cDNA candidates, their respective 

ORFs were cloned under the control of a GAL1 promoter in the 

PYES2.1 vector and assayed in InvSc1 yeast cells. We established 

determine the putative biological role of the two characterized 

Ban-Z11 and Ban-delta9-like) are highlighted in red. Sequence abbreviations correspond to 
species names as follows: Ape, Antheraea pernyi; Ase, Agrotis segetum; Ave, Argyrotaenia velutinana; Bma, Bombyx mori; Che, Charistoneura herana; Cpa, Charistoneura parallela; Dpu, Dendrolimus punctatus; Epo, Epiphyas postvittana; Has, Helioverpa armigera; Hvi, Heliothis virescens; Hsub, Helicoverpa subflexa; Lca, Lampronia capitella; Mbr, Mamestra brassicae; Obr, Odespertha brunata; Onu, Ostrinia nubilalis (Ofu, Ola, Onr.za, Opa, Osa, Oza and Oze correspond to other Ostrinia species45); Pex, Planotortrix excisa; Poc, Planotortrix octa; Tni, Thricoplasia ni; Tpi, Thaumetopoea pityocampa; Yev, Yponomeuta evonymella (Yro and Ypa correspond to other Yponomeuta species45). Numbers at the nodes indicate bootstrap values for 100 replicates.
whereas Ban-wFAR2 specifically reduced the Z9-14:Acyl towards Z9-14:OH (Fig. 6b,d). Ban-wFAR1 produces eight times more 16:Ald than Ban-wFAR2 produces Z9-14:OH (Supplementary Fig. 3), indicative not only of substrate selectivity but also distinct relative affinity for their respective substrate. Ban-wFAR1 is capable of reducing not only exogenous but endogenous 16:acid naturally occurring in yeast (Fig. 6c), and in a fraction of samples, we also detected minor amounts of Z9-16:OH (≤ 2% of the total enzyme alcohol production, Fig. 6c, Supplementary Fig. 3), resulting from conversion of Z9-16:Me naturally occurring in yeast. Finally, both enzymes display a similar minor activity on myristic acid (Fig. 6, Supplementary Fig. 3), which also naturally occurs in yeast. However, in the insect wings myristic acid and Z9-16:acid represent less than 3.5% of the C14-C16 wing lipid content and are not biosynthetic precursors (Figs 1 and 2).

In addition, semi-quantitative RT–PCR analyses indicated that in contrast to female moths, whose Δ11 and pgFARs implicated in pheromone production typically exhibit pheromone gland-specific expression patterns\cite{13, 53, 54}, the functional butterfly FAD and FAR transcripts show a broader expression pattern (Fig. 7, Supplementary Fig. 4).

Discussion

We first investigated the biochemical basis of production of the FA derivatives Z9-14:OH and 16:Ald, the two key pheromone components in Bicyclus butterfly males, and demonstrate that they are produced de novo along pheromone biosynthetic routes similar to female moths. Our labelling data support that the production of Z9-14:OH in male wings proceeds in a three-step pathway from palmitic acid. Palmitic acid, which is also known as an essential precursor for female moth pheromone biosynthesis\cite{29}, undergoes a desaturation towards Z11-16:acid, followed by one cycle of chain shortening by β-oxidation towards Z9-14:acid and a final reduction to the corresponding alcohol (Fig. 2a). The alternative hypothesis involving a Δ9-desaturase (Fig. 2a) can readily be excluded based on the absence of incorporation from labelled myristic acid. Second, the 16:Ald is synthesized from palmitic acid, via a two-step pathway including reduction to hexadecanol (16:OH), presumably followed by oxidation to the final aldehyde (Fig. 2b). The biochemical pathways identified here clearly suggest that the Bicyclus male pheromone components are produced via de novo moth-like biosynthetic routes, involving both a FAD and at least one fatty acyl-CoA reductase (FAR).
In order to find genomic evidence of conserved pheromone production pathways and associated biosynthetic genes in butterflies, we mined the available genomes of two Nymphalidae, the monarch and postman butterflies. We show that both of the butterfly genomes harbour diversified FAD and FAR gene families, and that 9-desaturation despite the conservation of metabolic 9-FAD gene members in butterflies, and that Δ11-desaturation is of key importance in the genus Bicyclus. Δ11-desaturation has previously been recognized as an important innovation of the Lepidoptera that is widely used in sex pheromone biosynthetic pathways in extant ditrysian female moths27,29,36,57. This finding thus provides the very first demonstration of the sharing of conserved pheromone-producing genes and biosynthetic modules between female moths and male butterflies across the Lepidoptera.

We next characterize two duplicate genes of the Lepidoptera-specific pgFAR lineage in B. anynana (Fig. 4) and demonstrate that they encode specialized pheromone biosynthetic reductases (Fig. 6). The discovery of substrate-specific FAR gene functions may contrast with previously identified broad-range alcohol-producing FARs from the silkworm42, small ermine moths43, the majority of corn borer species44,45 and heliothine moth species46, adding to the degree of specialization and range of FAR biosynthetic activities found among lepidopteran species and Distinct from cuticular hydrocarbon synthesis in the Diptera23, and similar to pheromone biosynthesis in many moth species, we demonstrate that B. anynana pheromone production does not proceed through Δ9-desaturation despite the conservation of metabolic 9-FAD gene members in butterflies, and that Δ11-desaturation is of key importance in the genus Bicyclus. Δ11-desaturation has previously been recognized as an important innovation of the Lepidoptera that is widely used in sex pheromone biosynthetic pathways in extant ditrysian female moths27,29,36,57. This finding thus provides the very first demonstration of the sharing of conserved pheromone-producing genes and biosynthetic modules between female moths and male butterflies across the Lepidoptera.
FA-derived compounds including saturated and unsaturated butterflies have also been shown to contain a large variety of B. anynana moth-biosynthetic genes are active in a butterfly, specifically (Fig. 1). The presence of Z9-14:OH throughout both types of wings restricted to the androconia (Fig. 7), again in agreement with expressed in both male forewings and hindwings but not component. Finally, Ban likely to confer the tissue specificity of the aldehyde pheromone producing oxidase accounting for the final biosynthetic step is producing the 16:OH, one could speculate that the aldehyde-16:acid (**). The Z11-13:OH) added alongside hexane extraction, and excess of exogenous present in yeast. Ban-wFAR2 reduces essentially the Z9-14:acid and minor components provide an opportunity for uncoupling the male pheromone signals and may contribute to pheromone variation in Bicyclus. Age-dependent modulation of male Bicyclus signals could take place through changes in FAR enzyme activities and provides the exciting opportunity to further investigate the mechanistic link between male condition, pheromone composition and female choice. The Bicyclus male pheromone has been shown to play a determinant role in female mate choice by guiding their acceptance or rejection of courting males18,19. Individual males have also been shown to differ in their absolute pheromone titre and ratio at different ages, making the male pheromone composition both a reliable predictor of age and individuality13 and a honest signal of an individual male’s characteristics and associated fitness to prospective mates13,18, Nieberding et al.13 further demonstrated that the overall increase in the titre of 16:Ald is critical for females to discriminate between young and old males whereas an overall increase in total amounts of Z9-14:OH seems to participate in how females can differentiate young (3 days old) from middle-aged males (14-day to 21 day-old individuals). The two pheromone biosynthetic FARs involved in the pathways towards the production of these two key pheromone components provide an opportunity for uncoupling the male pheromone signals and may contribute to pheromone variation in Bicyclus. Age-dependent modulation of male Bicyclus signals could take place through changes in FAR enzyme activities and provides the exciting opportunity to further investigate the mechanistic link between male condition, pheromone composition and female choice. We show that the forewing and hindwing FARs are capable of producing 14:OH as a byproduct of the reduction step and arose following a recent gene duplication event (Fig. 4), suggesting that the ability to reduce 14:Acyl was acquired independently or more...
parsimoniously that the ancestral copy was less specialized. Hence, FARs characterized from moths so far are often capable of simultaneously reducing C14 and C16 substrates (except in Ostrinia85), which could imply that the ancestral state in Lepidoptera was in many instances a broad-range FAR that underwent further specialization including this butterfly system. To test whether the ability to produce 16:OH and Z9-14:OH hence follows this subfunctionalization scenario or arose subsequently by neofunctionalization in one or both duplicates will, however, require further comparative molecular and functional analyses. Genetic reshuffling, that is, gene duplication, can provide a mechanism for new pheromones to arise and pheromone variation has been suggested to be positively correlated with the number of genetic elements involved in biosynthetic pathways82. Regardless of whether this pattern of specialization at the reduction step is unique to Bicyclus or widespread among other butterfly species, gene duplication in this species likely provided the necessary raw material that allowed the dissociation of biosynthetic gene functions. Decreasing functional constraints on gene regulatory elements60,61 can promote independent variation of gene expression whereas relaxed constraints on the involved gene products could lead to the rapid evolution of new enzymatic functions, respectively, in turn facilitating intraspecific variation in the male signal62,63.

In conclusion, our central finding, that moth and butterfly lepidopteran lineages share a conserved pheromone production genetic network provides important insights into the evolutionary origin of DA-derived butterfly pheromones, and opens the door to address whether the evolution of dissociated biosynthetic pathways may have contributed to facilitate modulation in the released courtship signals.

Methods

Insect rearing. A butterfly population of B. anynana (Satyrinae) was established in Lund from hundreds of eggs originating from a lab colony stock originally derived from 80 gravid females collected in Malawi in 1988, and maintained at Leiden and at Yale University since then at a size of about 200–300 breeding individuals each generation. Larvae were raised in a climate chamber with controlled environmental conditions including a 12L-12D light-dark cycle with 70% relative humidity and 27 ± 1 °C degree Celsius. They fed on regular supplies of fresh young maize plants until pupation. For experiments, newly emerged adults were separated on the day of eclosion (day 0) and held in single-sex cohorts with fresh banana slices as food source.

Chemicals used for in vivo labelling. Deuterium-labelled (16,16,16-D3)-hexadecanoic acid (D16:16:acid) and (14,14,14-D3)-tetradecanoic acid (D14:14:acid) were purchased from Larodan Fine Chemicals, Malmo, Sweden. The (Z)-13,13,14,14,15,15,16,16-D8:11-hexadecanoic acid (D12:11:acid) was available in our chemical database and the (Z)-11,11,12,12,13,13,14,14,14,14-D9:9-tetradecenoic acid (D9:9:acid) was provided by courtesy of Dr J. Millar (UCR, USA).

Biochemical labelling and biosynthetic pathway. In order to determine the relative wing composition of Z9-14:OH and 16:Al in the fore- and hindwings, we excised individual forewings or hindwings of 4-day-old individuals and extracted them for 30 min in 1.5 ml glass vials containing n-hexane followed by gas chromatography (GC) analysis as described under the GC–mass spectrometry (GC–MS) analyses section below.

The aldehyde and alcohol biosynthetic pathways were probed using topical wing application of deuterium-labelled FAs. The D16:16:acid, D14:14:acid, D12:11:acid and D9:9:acid were dissolved individually in dimethylsulfoxide (DMSO) at a concentration of 20 µg µl⁻¹. Natural butterfly movements can cause cross-wing contamination during incubation with the labelled chemicals and, therefore, we precisely removed either the fore- or hindwings before topical application on the remaining pair of wings. Insects were anesthetized with carbon dioxide and 1 µl of each single-compound solution was applied to the androconial region, which for forewings constitutes a spot of differentiated scales located on the anal vein on the ventral side13.

For hindwing labelling, the labelled compound was applied onto the dorsal side of each hindwing in the region comprised between the subcostal and radial veins13. The labelling was carried out 4 h before the onset of scotophase and males were thereafter kept individually for a 24 h incubation period. Labelled androconia from the two hindwings or the two forewings, respectively, were excised and extracted in 200 µl hexane for 30 min. The extracts were analysed by GC–MS using selected ion monitoring (SIM) along with wing extracts from control individuals treated with DMSO only.

To analyse the biosynthetic fatty acyl precursors, wings tissues recovered after the hexane extraction for volatile content analysis were subsequently extracted with 100 µl chlorform:methanol (2:1 v:v) for 24 h at room temperature. The extract was dried under a stream of nitrogen, and the residues were subjected to base methanolation to convert fatty acyl moieties to the corresponding methyl esters64 and subsequently analysed by GC–MS and SIM as described in the next paragraph.

GC-MS analysis of male wing extracts. Fore- and hindwing hexane extracts and the corresponding FA methyl esters (FAME) were analysed on a Hewlett Packard HP 6890 GC system (Agilent, Palo Alto CA, USA) coupled to a mass selective (MS) detector (HP-5975) equipped with a HP-5MS capillary column (30 m × 0.25 mm, Agilent technologies) with helium as carrier gas and an average velocity of 30 cm s⁻¹.

The oven temperature was set at 80 °C, held for 1 min, then increased to 210 °C at a rate of 10 °C min⁻¹, held for 12 min and finally increased to 250 °C at a rate of 10 °C min⁻¹, held for 5 min. SIM was used to detect the native pheromone components and the corresponding deuterium-labelled compounds. Z9-14:OH was detected using the characteristic ions at m/z 31, 194 and 212; ions at m/z 197 and 215 were used to monitor the corresponding D9-14:OH, respectively. 16:Al was monitored with the characteristic ions at m/z 196 and 222 and the incorporation of D12:16:Al was monitored with ion at m/z 199. The identity of all components was confirmed by comparing retention times and mass spectra with those of reference standards.

Butterfly FAD and FAR gene annotation and phylogeny. The Danaus and Heliconius butterfly genomes were searched against iBLASTx databases at NCBI65 using the deduced protein sequences of active pheromone biosynthetic moth genes as queries. All scaffolds predicted to harbour desaturase and FAR-like genes were retrieved and annotated in Geneious Pro 5.6.4 (ref. 66); intron–exon boundaries were predicted using iBLASTx, Softberry FGENSEH and Expasy prediction tools, manually curated and verified. A number of automated predictions for Danaus and Heliconius FARs and proteins and for Danaus FAR proteins were made available through NCBI in the course of this study. For Danaus FARs, some automated gene predictions contained verifiable inaccuracies mainly in exon 1, for which we provide the corrected gene structures and associated nt and aa sequences in Supplementary Table 1 and Supplementary Data 1, respectively. For Danaus FARs, only a small number of genes were predicted compared with the number retrieved in our extensive in silico analysis. Scaffold and structural information for Heliconius and Danaus FAR genes are listed in Supplementary Tables 3,4, alongside accession numbers corresponding to automated predictions when available, and the associated nt and aa sequences are provided in Supplementary Data 2 and 3, respectively.

Amidation numbers for moth FAD and FAR sequences as well as ESTs, used for the phylogenetic reconstructions are listed in Supplementary Tables 2 and 5 or were given elsewhere66,67 and are available upon request. For each set of amino-acid sequences, multiple sequence alignments were generated using MAFFT v7 with E-INS-i algorithm and the BLOSUM45 scoring matrix68. Maximum-likelihood inference was carried out using the standalone version of PhyML68 and the WAG + I + G model as determined by performing model selection in Topali v2.5 (ref. 69). Clade support was evaluated using 100 bootstrap replicates. The cladograms were visualized and prepared using the online tool Evolvew70.

Molecular characterization of FAD and FAR cDNA candidates. First-strand cDNA was synthesized using a Stratascript reverse transcriptase (Stratagene) from 1 µg total RNA extracted from the portion of 6-day-old male hindwings containing the androconial region and hair pencils for screening of this tissue based on the rationale that all candidate biosynthetic genes were to be transcribed in the adult hindwing since it produces both pheromone components. For the desaturase cDNA screening, B. anynana EST libraries69,71 contained information for a predicted Δ9-desaturase desaturase transcript (EST GenBank Acc. nr GE680557, GenBank accession like, Fig. 3). Therefore, we performed a complete PCR-based screen for candidate desaturase genes using male hindwing androconial cDNA as template in PCR reactions with oligonucleotide primers designed against conserved desaturase motifs72. PCR thermal cycling conditions consisted of 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 50 °C for 45 s, 72 °C for 90 s and 72 °C for 10 min. An amplification with expected size of 560-bp was gel purified (Promega), ligated into the pCR2.1-TOPO TA cloning vector system (Invitrogen) and amplified in DH5α Escherichia coli cells (New England Biolabs). Purified plasmids were sequenced using the Big Dye Terminator cycle sequencing kit v1.1 followed by analysis on a capillary ABI 3100 sequencer instrument (Applied Biosystems). Double-stranded DNA sequence information encompassing the central fragment of desaturase genes was curtailed using BioEdit followed by BLAST searches to verify the desaturase gene identity65. Clones were identified corresponding to the
above-mentioned EST A9-desaturase and a A11-like desaturase for which 3'- and 5'-cDNA end termini were amplified using the SMART RACE Kit (Clontech) and gene-specific RACE primers (Supplementary Table 6). For the reductase cDNA screening, BLASTP and TBLASTN searches were used to screen B. anynana EST databases using the Dananue and Heliconius orthologues of biosynthetic FARs in queries. Candidate ESTs under A成本 numbers GE680761 and GE680760 (adult head EST library; DOE Joint Genome Institute project ID 16936) were assembled in a partial B. anynana contig. The assembled contig clustered with moth-biosynthetic pgFARs and was used as sequence template to design 5'- and 3'-RACE primers in NTI Vector (Invitrogen) (Supplementary Table 6), which served to obtain the full-length cDNA sequence. The B. anynana EST A9 desaturase PRIME primer was used to amplify a 2 kb fragment matching perfectly with the partial GST 5' region and the corresponding full-length cDNA was named Ban-wFAR1. Twenty-one of the 25 DNA bases from the 3'-RACE primer were conserved enough to amplify a distinct 1 kb long 3'-RACE DNA amplicon, which we found to encode the 3'-cDNA end of a distinct pgFAR-like transcript as confirmed by phylogenetic analyses. A set of gene-specific 5'-RACE primers was designed to amplify the second clone 5'-cDNA-end, and the compiled full-length cDNA was named Ban-wFAR2. The sequence integrity and distinctness of the two pgFAR-like gene candidates, which share 76% identity at the nt level, was confirmed by amplifying each ORF with gene-specific primers followed by Sanger DNA sequencing.

Tissue distribution of biosynthetic transcripts. Adult butterfly tissues from fifteen CO2-anesthetized 6-day-old male individuals (head, antenna, thorax, legs, abdomen, forewing androconia, hindwing androconia, fore- and hindwing (minus androconia)) were dissected using microscissors and collected in RNA later. Tissues were also collected from whole fore- and hind wing tissue from fifteen 6-day-old females. Total RNA was isolated using the RNeasy Isolation kit and a DNase I treatment step (Qiagen). RT-PCR reactions were carried out using the SuperScript III One Step RT–PCR System with Platinum Taq (Invitrogen) in a 25 μl reaction containing 30 ng RNA and 0.8 μM GSP (Supplementary Table 6) and cycling conditions as follows: 55 °C for 30 min, 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C for 45 s and 68 °C for 2 min. PCR products were analysed on a 2% agarose gel. Parallel RT–PCR reactions were Exo-Sap purified and sequenced to confirm gene-specific amplification.

Functional assay of Ban-wFAR1 and Ban-wFAR2. Gene-specific primers (Supplementary Table 6) were designed to amplify the desaturase and FAR candidate ORFs using male hind wing androconia cDNA as template in combination with the Advantage2 PCR system (Clontech). The desaturase ORF was ligated in the pYEX-CHT expression vector at the BamHI and EcoRI restriction sites and each FAR ORF was cloned in the pYES2.1 T 3 expression vector (Invitrogen) downstream the GAL1 promoter. All constructs were verified by sequencing. The empty pYEX-CHT and pYES2.1 control plasmids were verified by sequencing. The empty pYEX-CHT and pYES2.1 control plasmids were transformed into the E. coli host strain S. cerevisiae (MATα his3D1 leu2 trp1-289 ura3-52). Prior to analysis, hexane extracts from under a gentle flow of pure nitrogen to a final volume of 25 μl and transferred into 1.5-ml vials containing glass insert. One microtitre was injected on a Hewlett Packard HP 6890 GC system coupled to an automatic injector (HP-7683) and a mass selective (MS) detector (HP-5975). The GC was equipped with a polar HP-INNOWax column (100% polyethylene glycol, 30 m × 0.25 mm × 0.25 μm). Agilent Technologies) with helium as carrier gas and an average velocity of 30 cm s⁻¹. The MS was operated in electron impact mode (70 eV), the GC oven temperature was set at 50 °C for 2 min and then rose at a rate of 10 °C min⁻¹ up to 220 °C, held for 20 min. For analysis of hexane extracts from InvSc1 yeast cultures expressing the pYEX-only, the pYES-BanFAR1 or pYES-BanFAR2 construct, extracts were concentrated to 50 μl. Two microtubes were manually injected on a gas chromatograph (Hewlett Packard HP 5890 II GC system) coupled to a mass selective detector (HP 5972) and equipped with a polar INNOWax column (100% polyethylene glycol, 30 m × 0.25 mm × 0.25 μm. Agilent Technologies). The GC–MS was operated in electron impact mode (70 eV) and the GC injector was configured in splitless mode at 220 °C with helium used as carrier gas (average velocity: 20 cm s⁻¹). The oven temperature was set at 50 °C for 2 min and then rose at a rate of 10 °C min⁻¹ up to 220 °C, with a final hold at 220 °C for 20 min.

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