Decoding the genetic and chemical basis of sexual attractiveness in parasitic wasps

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Abstract

Attracting and securing potential mating partners is of fundamental importance for reproduction. Therefore, signaling sexual attractiveness is expected to be tightly coordinated in communication systems synchronizing senders and receivers. Chemical signaling has permeated through all taxa of life as the earliest and most widespread form of communication and is particularly prevalent in insects. However, it has been notoriously difficult to decipher how exactly information related to sexual signaling is encoded in complex chemical profiles. Similarly, our knowledge of the genetic basis of sexual signaling is very limited and usually restricted to a few case studies with comparably simple pheromonal communication mechanisms. The present study jointly addresses these two knowledge gaps by characterizing two fatty acid synthase genes, that most likely evolved by tandem gene duplication, simultaneously impacting sexual attractiveness and complex chemical surface profiles in parasitic wasps. Gene knock-down in female wasps dramatically reduces their sexual attractiveness coinciding with a drastic decrease in male courtship and copulation behavior. Concordantly, we found a striking shift of methyl-branching patterns in the female surface pheromonal compounds, which we subsequently demonstrate to be the main cause for the greatly reduced male response. Intriguingly, this suggests a potential coding mechanism for sexual attractiveness mediated by specific methyl-branching patterns in CHC profiles. So far, the genetic underpinnings of methyl-branched CHCs are not well understood despite their high potential for encoding information. Our study sheds light on how biologically relevant information can be encoded in complex chemical profiles and on the genetic basis of sexual attractiveness.

Main Text

Introduction

Tightly coordinated chemical signaling has repeatedly been shown to be of fundamental importance for successful reproduction in a wide range of animal species (1, 2). Particularly insects have exploited this type of signaling as their primary mode of communication (3, 4). Nevertheless, exactly how specific information such as mating status or attractiveness is encoded in the myriad of signaling molecules documented to be involved in sexual communication remains poorly understood (5, 6). Cuticular hydrocarbons (CHCs), major components on the epicuticle of insects, are capable of chemically encoding and conveying a wide variety of biologically relevant information (7, 8). Most prominently, CHCs have been shown to play pivotal roles in sexual communication as the main cues to attract and elicit courtship from conspecific mates (9, 10) to enable discrimination of cons- from heterospecific mating partners (11, 12) and to signal receptivity and mating status (13, 14). Despite such diversified CHC-encoded signals and mediated behaviors, our knowledge on exactly how CHCs encode biologically relevant information has remained surprisingly scarce. This is particularly problematic in studies considering CHC profiles in their entirety as the main signaling entities (13, 15). The exact compounds or their combinations actually encoding the relevant information within CHC profiles remain largely elusive, except for a few case studies mainly involving the dipteran model organism Drosophila melanogaster, where single unsaturated CHC compounds appear to be the main mediators in sexual communication (16, 17). In most other cases, chemical information appears to be encoded in a much more complex manner, involving several CHC compounds in different quantitative combinations, with no deeper understanding on the actual coding patterns conveying specific information (9, 18).

In addition to our limited understanding on how CHC profiles encode information, our knowledge of the genetic basis of CHC biosynthesis and its impact on sexual signaling has remained comparably restricted and biased towards the Drosophila model system as well (8, 19). In short, the CHC biosynthetic pathway consists of the elongation of fatty-acyl-Coenzyme A units to produce very long-chain fatty acids that are subsequently converted to CHCs (8, 20). An important early switch in CHC biosynthesis is either the incorporation of malonyl-CoA
or methyl-malonyl-CoA, eventually leading to the production of straight-chain (n-) or methyl-branched (MB-) alkanes, respectively (19, 20, Fig. 1). It has been hypothesized that these processes are mediated by two types of fatty-acyl-synthases (FAS), microsomal for methylmalonyl-CoA and cytosolic for malonyl-CoA (21, 22). To produce olefins (mono- and poly-unsaturated CHCs), desaturases introduce double bonds into straight-chain CHC precursors (8, 19). In Drosophila, a couple of genes have been identified that mainly affect the biosynthesis and ratios of unsaturated CHC compounds that function in sexual signaling. For instance, two desaturases (Desat1, DesatF) and one elongase (eloF) are involved in female diene production and consequently in their functionality as main sex pheromonal compounds (23, 24). Furthermore, in the Australian congeneric species D. serrata, the male-specific D. melanogaster orthologue FASN2 has been shown to affect the biosynthesis of three MB-CHCs, among them the additional female mating stimulant 2-Me-C26 (25, 26). Apart from these case studies limited to Drosophila, we know very little about the genetic basis linking CHC biosynthesis and sexual signaling in other insects (19).

The parasitoid jewel wasp Nasonia vitripennis (Hymenoptera: Pteromalidae) has emerged as a suitable model organism to combine studies on functional genetics as well as chemical communication systems in Hymenoptera (28, 29). Female CHCs serve as sexual cues capable of eliciting male courtship and copulation behavior in N. vitripennis (15, 30). The CHC profiles of N. vitripennis females exhibit a high complexity, consisting of a mixture of n-alkanes, n-alkenes and MB-alkanes in various quantities. Specifically, the latter fraction, which makes up more than 85% of the whole profile, displays a rich diversity in methyl-branch numbers, chain lengths and respective relative abundances, hinting at a considerable potential for encoding differential information (18, 30). However, the entire female CHC profile has long been regarded as encoding their sexual attractiveness, with no single compound, compound classes or particular patterns being identifiable as the main conveyers of the sexual signaling function (30, 31). Moreover, despite recent advances in unraveling the genetic architecture of CHC biosynthesis and variation in the Nasonia genus (28), the effects of individual genes on CHC profiles and, more importantly, on the encoded sexual signaling function, could not be determined as of yet.

In this study, we characterize the phenotypic effects of a two fatty acid synthase genes whose knockdown impacts the variation of several structurally related CHC components concordant with female sexual attractiveness. CHC profiles of knockdown females primarily showed significant up- and down-regulations of MB-alkane compounds with correlated branching patterns. At the same time, these knockdown females elicited significantly less courtship and copulation attempts from conspecific males. These constitute the first hymenopteran genes with a demonstrated function in governing the variation of primarily MB-alkanes as well as sexual attractiveness, hinting at a chemical coding pattern for sexual attractiveness mostly conveyed by this CHC compound class.

Results

Knockdown of fas5 significantly reduced the gene expression of fas5 and fas6

We conducted gene expression analysis in adult wasps to assess the impact of dsRNAi micro-injection specifically targeting the fas5 gene. The results revealed a significant reduction in the relative expression of our target gene fas5 compared to the control groups (Fig. 2H), whereas the other previously published and characterized fas genes (fas1–4) remained unaffected in their expression (Figure 2-figure supplement 1, Supplementary File 2). However, fas6 a previously uncharacterized fas gene was also significantly downregulated of the expression of in our fas5 dsRNAi knockdown individuals (Fig. 2H). The dsRNAi off-target analysis showed that 24% of 19-mers from fas5 dsRNA sequence matched to the fas6 transcript. Notably, fas6 is localized next to fas5 and shares high sequence similarity with the latter (90.95% at the mRNA and 88.06% at the amino acid level) (Figure 2-figure supplement 2). This indicates that both genes evolved by tandem gene duplication.

Knockdown of fas5 dramatically alters CHC profile composition
DsRNAi micro-injection into female Nasonia at the pupal stage resulted in a striking CHC profile shift in adult females, most prominently displayed in altered MB-alkane patterns (Fig. 2 A-B, Supplementary File 3), while overall CHC amounts and n-alkane quantities remain unaffected (Figure 2-figure supplement 3 A-B). More specifically, fas5 knockdown significantly increased the absolute quantities (ng) of MB-alkanes with their first methyl branches positioned on the 3rd (35.07 ± 9.47) and 5th (96.34 ± 33.22) C-atom, compared to both WT (24.08 ± 5.6 and 40.06 ± 11.79) and GFP RNAi females (24.75 ± 7.58 and 40.19 ± 12.37), respectively (Fig. 2 C-D). Conversely, MB-alkanes with their first methyl-branches on the 7th C-atom position significantly decreased in fas5 knockdowns (5.27 ± 2.02) compared to WT (34.3 ± 11.92) and GFP RNAi females (40.15 ± 17.1) (Fig. 2 E). Other MB-alkanes with their first methyl-branches mainly positioned on the 9th, 11th, 13th and 15th also significantly decreased in fas5 RNAi females (46.88 ± 11.67) as opposed to WT (94.87 ± 30.47) and GFP RNAi females (106.19 ± 40.39) (Fig. 2 F). Interestingly, overall MB-alkane, n-alkane as well as total CHC quantity remained stable in fas5 knockdown females compared to the controls, with no significant changes (Fig. 2 G, Figure 2-figure supplement 3 A&B). Lastly, n-alkene quantities, generally only occurring in negligible quantities in Nasonia females (28), also increased significantly in fas5 RNAi females (8.74 ± 3.52) compared to WT (2.09 ± 0.94) and GFP RNAi females (2.33 ± 0.85) (Figure 2-figure supplement 3 C).

Male CHC profiles were also dramatically affected by fas5 knockdowns (Figure 2-figure supplement 4). Whereas n-alkenes and MB-alkanes with their first methyl group at the 5th position are similarly up-regulated in knockdown males and females (and MB-alkanes with the first methyl group at the 7th position similarly down-regulated), overall CHC quantities as well as n-alkane quantities are significantly higher in knockdown males as opposed to females (compare Figure 2-figure supplement 3 A-B to Figure 2-figure supplement 4 B-C). Furthermore, CHCs with their first methyl groups at the 3rd and 9th (and higher) positions appear to be differentially affected as well: While clearly significantly affected in knockdown females compared to both WT and GFP controls (Fig. 2 C&F), their quantity is not significantly different in knockdown males from GFP controls, but from WT controls (Figure 2-figure supplement 4 E&H). However, as male CHCs have not been shown to function in chemical communication in N. vitripennis, those were not investigated further.

**fas5 knockdown decreases attractiveness of female CHC profiles**

Fas5 RNAi and control (WT and GFP) females elicited antennation from similar proportions of males, while a significantly reduced proportion of males performed courtship and copulation towards fas5 RNAi females (~ 50%) compared to controls (~ 90%) (Fig. 3 B). Furthermore, 70 % of the males rejected fas5 RNAi females at least once, a behavior which was not present at all towards control females (Fig. 3 C). To further minimize active female involvement in mate choice and increase male reliance on chemical cues (15, 18), we subsequently offered differentially treated female dummies (i.e., freeze-killed females) to WT males. Similar proportions of males showed antennation towards freeze killed fas5 RNAi, GFP RNAi and WT females (Fig. 3 D). However, significantly fewer males performed courtship and copulation behavior towards freeze killed fas5 RNAi females (20%) compared to both controls (> 75%, respectively) (Fig. 3 D). To further test whether this dramatic reduction in sexual attractiveness is related to the altered chemical profile, we proceeded to manipulate the CHC profile of female dummies. Female dummies either had their chemical profiles completely removed (i.e., cleared) or we reconstituted initially cleared WT female dummies with chemical profiles from fas5 RNAi, GFP RNAi, or WT females, respectively. Likewise, no significant difference was found on the proportion of males that performed antennation towards female dummies of all treatments (Fig. 3 E). However, significantly less males performed courtship towards female dummies that were cleared of their chemical profiles (0%) and fas5 RNAi re-constituted dummies (16%), compared to WT (50%) and GFP RNAI-reconstituted (75%) female dummies (Fig. 3 E). Furthermore, copulation attempts were initiated by less than 5 % of the males towards fas5 RNAI reconstituted dummies, which is similar to completely cleared dummies (0%). These numbers were in both cases significantly lower compared to control dummies reconstituted with WT and GFP RNAi profiles (~ 40%, respectively) (Fig. 3 E). This demonstrates that the dramatic reduction in female
attractiveness in fas5 knockdown females is predominantly mediated by their altered chemical profiles.

Female sexual attractiveness is mainly governed by MB-alkanes

To further pinpoint the part of the chemical profile that is responsible for encoding sexual attractiveness, we fractionated the chemical profiles of N. vitripennis females and focused on the MB-alkane fraction, which displayed the most conspicuous changes in fas5 knockdown females (Fig. 2 B). The separation process reduced both the n-alkane and n-alkene proportions to less than 1% of the whole profile, allowing to focus almost exclusively on the MB-alkane fraction (Fig. 4 A; Fig. 4-source data1). Specifically, the MB-alkane fraction of fas5 RNAi females maintained the dramatically higher proportions of alkanes with 3\textsuperscript{rd} and 5\textsuperscript{th} C-atom methyl-branch positions and lower proportions of alkanes with 7\textsuperscript{th} and 9\textsuperscript{th}, 11\textsuperscript{th}, 13\textsuperscript{th} and 15\textsuperscript{th} C-atom methyl-branch positions, compared to the respective control females' MB-alkane fractions (Fig. 4 B). We also reconstituted cleared WT female dummies with the separated MB-alkane fractions and offered them to WT males in further behavioral assays. As in our previous behavioral assays with female dummies and reconstituted whole CHC extracts, the MB-alkane fraction from fas5 RNAi females elicited significantly less courtship and copulation attempts from WT males than the MB-alkane fractions of both WT and GFP RNAi females (Fig. 4 C). Overall, these results demonstrate that sexual attractiveness appears to be mainly encoded in the MB-alkane fraction of N. vitripennis females.

Fas5 knockdown does not affect desiccation resistance

Since CHC profiles also play a pivotal role in desiccation resistance (8), we further performed survival assays to explore the impact of fas5 knockdowns on the wasps’ ability to survive under different degrees of desiccation stress (Fig. 5). Unsurprisingly, males (Fig. 5 B & D) and females from both treatments survived longer under medium desiccation stress (~ 55% humidity) than under high desiccation stress (~ 9% humidity) (compare Fig. 5 A&C with B&D, respectively). However, under both high and low desiccation stress, there were no significant differences in survival probability between fas5 and GFP dsRNAi treated males (Fig. 5 C-D) and females (Fig. 5 A-B).

Discussion

In our study, we shed light on how sexual attractiveness can be encoded by differentially branched cuticular hydrocarbons (CHCs) and unravel its genetic architecture to be based on two highly similar fatty acid synthase genes. Knocking down these two genes in Nasonia vitripennis females leads to a consistent pattern of primarily up- and down-regulated methyl-branched (MB-) alkanes with specific branching patterns. This dramatic shift is accompanied by a significant reduction of courtship and copulation behavior towards knockdown females by conspecific males which we demonstrate to be mainly determined by the altered MB-alkane fraction. This advances our understanding of how genetic information is translated into chemical information and brings us a step closer in decoding complex chemical profiles.

Most conspicuously in females, quantities of CHCs with their first methyl groups at the 7\textsuperscript{th} and 9\textsuperscript{th} (and higher) C-atom positions are dramatically down-regulated, whereas the ones with their first methyl groups at the respective 3\textsuperscript{rd} and 5\textsuperscript{th} positions appear mainly up-regulated, in most cases by several orders of magnitude (Fig. 2 and Supplementary File 3). Intriguingly, overall CHC quantities do not differ between knockdown and control individuals (Figure 2-figure supplement 3 A), suggesting a very specific regulatory function for fas5 in governing these opposing and potentially compensatory branching patterns. Concordantly, this emphasizes the pivotal role of both these particular genes and the wild-type MB-alkane branching patterns in encoding and maintaining the attractiveness of female N. vitripennis CHC profiles. Moreover, there appear to be a number of sex-specific differences in the knockdown effects on the different CHC compound classes, most notably apparent in significant upregulations of both total and n-alkane CHC quantities in knockdown males as opposed to females and partially differentially affected MB-alkane quantities (compare Fig 2 to Figure 2-figure supplement 4). Curiously, GFP dsRNAi appears to have a generally
upregulating effect as opposed to WT controls despite for n-alkene quantities in males, which has not been reported before. However, a recent study hints at potential off-target effects of GFP dsRNAi on a small subset of N. vitripennis genes mainly involved in microtubule and sperm development (32). Interestingly, this appears to be also the case for the expressions of fas1 and fas2 gene transcripts, which are significantly upregulated in the GFP dsRNAi controls, again exclusively in males (Figure 2-figure supplement 1B). Though no off-target effects on CHC profile biosynthesis and variation have been reported so far for GFP dsRNAi, we cannot exclude this possibility, particularly since a trend towards higher CHC quantities in GFP dsRNAi controls as opposed to WT controls is also discernible in some cases for the females (Fig. 2 E-G). Therefore, as Rougeot et al. already hinted at (32), we strongly suggest alternative non-target controls in future studies on the genetics of CHC biosynthesis and variation. Concerning the partially different effects of fas5 on CHC quantities in females and males, as no functionality in chemical communication could be attributed to the latters’ CHC profiles so far, these effects could not be investigated any further. However, opposing sex-specific effects of CHC biosynthesis genes on the quantity of different compound classes appear to be a rather common occurrence, particularly in the insect model system Drosophila melanogaster (19). Based mainly on research on the latter, CHC-based sexual signaling mechanisms have long been assumed to be comparatively simple, mainly mediated by two doubly unsaturated dienes in females and a mono-unsaturated n-alkene in males (16, 17). However, experimental evidence has accumulated that other CHC compounds can also complement sexual signaling in Drosophila in various ways (26, 33). When regarding CHC profiles in their entirety as opposed to single compounds, direct causal links between sexual attractiveness and CHC profiles properties have rarely been experimentally demonstrated and have most often defied clear patterns (9, 12). In the genus Nasonia and other related parasitoid wasp species, it has so far been assumed that sexual attractiveness is a trait attributed to their entire CHC profile as either present or absent depending on the studied species and also potentially reinforced by other factors such as polar cuticular compounds (18, 34). Our study clearly shows that CHC-mediated sexual attractiveness is primarily conveyed through a relatively complex chemical pattern with a comparatively simple genetic basis.

Interestingly, knockdown of fas5 also upregulated n-alkene quantities (Figure 2-figure supplement 3C), which have recently been shown to have a repellent effect on N. vitripennis males, preventing them from engaging in homosexual courtship behavior (35). However, our CHC compound class separation greatly reduced the increased proportion of n-alkenes in fas5 knockdown females to levels almost equivalent to those found in wild-type females (0.94 % and 0.91 %, respectively in the extract of fas5 and WT MB fractions, compare Fig. 4-source data 1 to Supplementary File 3). This renders the contribution of n-alkenes to the sharp reduction in sexual attractiveness in fas5 knockdown females unlikely and strongly suggests that the female sexual signaling function is mainly mediated by MB-alkanes (Fig. 4). We argue that this CHC compound class indeed possess the highest potential for encoding a wide variety of chemical information through the myriad of possible positions and numbers of methyl branches. In fact, a couple of studies have already hinted at MB-alkanes as the main carriers for chemical information in insect CHC profiles, providing evidence for the involvement of MB-alkanes in chemical communication processes (36, 37). This might be of particular importance in Hymenoptera, an insect order with CHC profiles largely dominated by MB-alkanes (38, 39) and in which both theoretical considerations as well as empirical evidence have accumulated for the increased complexity and high sophistication of their chemical communication systems (40, 41). Contrary to this, it has been argued that olefins (mainly n-alkenes and dienes) have a higher potential for encoding chemical information than MB-alkanes (42). However, this view might have been biased and mainly informed by findings from Drosophila, where unsaturated compounds appear to be, in fact, the main mediators of sexual communication (16, 43). In direct comparison, MB-alkanes constitute the dominant fraction in Nasonia CHC profiles (> 85 %) (28) as opposed to Drosophila profiles (16-24 %) (44). Since the split between Hymenoptera and other holometabolous insects including Diptera has been estimated to have occurred 327 mya (45), fundamental shifts in basic properties of both surface profile compositions as well as chemical signaling functionalities might be expected. Therefore, the promising role of MB-alkanes in conveying chemical information should be investigated more prominently in future studies. Through a wider
evolutionary lens, it will be interesting to investigate how the present findings compare to
other CHC-based communication systems in the vastly diverse insect order Hymenoptera.
Notably, whereas in solitary Hymenoptera CHCs can function as contact sex pheromones,
they are prominent and fundamental nestmate and caste recognition cues in eusocial
Hymenoptera (15,46,47). However, the main encoding mechanisms as well as the underlying
genetic basis still remain largely elusive in most taxa, and our study constitutes an important
steppingstone for investigating potential similarities in these important communication
modalities in a larger evolutionary context. Moreover, since CHC profiles can be highly
species-specific, their potential involvement in species recognition and assortative mating
warrants further investigation to elucidate the exact underlying chemical and genetic
differentiation mechanisms (15,48,49).
Concerning gene orthology, fas5 has been annotated as a homolog to FASN3 in
D. melanogaster (28, 50). Interestingly, knockdowns of FASN3 alone do not induce any
compound changes in D. melanogaster CHC profiles but increase the flies’ sensitivity to
desiccation (25). A FASN3 ortholog expressed in the kissing bug Rhodinius prolixus also
contributes to desiccation resistance, but simultaneously down-regulates MB-CHCs while up-
regulating straight-chain CHCs (51). Similarly, in the migratory locust Locusta migratoria,
silencing two FAS genes decreased insect survival under desiccation stress while altering the
amounts of both MB- and straight-chain CHCs (52). In contrast to these studies, we were not
able confirm any functionality in desiccation resistance for fas5 in both N. vitripennis males
and females (Fig. 5), nor did the previous studies report any impact on CHC-based chemical
signaling. There are two additional FAS genes characterized in D. melanogaster: FASN1,
which is responsible for overall CHC production with no specific effects on any particular
compound classes (25) and FASN2, which exclusively regulates MB-alkane production in
males (26). Although not a direct homolog, the main impact on MB-alkane variations of
FASN2 in D. melanogaster is comparable to that of fas5 in N. vitripennis, with the exception
that we were able to document effects on this compound class for both sexes (compare Fig. 2
and Figure 2-figure supplement 4). Such diversified functionalities of the FAS genes
characterized so far indicate their high versatility as early mediators in the CHC biosynthesis
pathway (Fig. 1). FAS genes have been implied as instrumental for generating the huge
diversity of different CHC profiles across insects, with high evolutionary turnover rates and
differences in the specific functional recruitments of FAS gene family members (46, 54).
However, FAS genes are far from restricted to impact CHC biosynthesis alone and have been
documented to be involved in a wide variety of other physiological processes, ranging from
lipogenesis to diapause induction (49, 54). Therefore, specifically predicting functionalities of
FAS genes and unambiguously associating them with CHC biosynthesis and variation has
been notoriously difficult (19). Our study suggests a very specific affinity of fas5 for particular
methyl branching patterns, potentially originating in an enzymatic preference for methyl-
malonyl-CoA predecessors with pre-existing branching patterns (8, 20). To clarify this, future
studies should determine whether the fas5 gene product constitutes a soluble cytosolic or
membrane-bound microsomal FAS enzyme, the latter of which has been postulated to be
specific for the incorporation of methyl-malonyl CoA subunits (21, 22, Fig. 1).
The previously uncharacterized fas gene fas6 not only shows high sequence similarity to
fas5, but is also its physical neighbor (Figure 2-figure supplement 2). Moreover, both of
these genes show similarly high expression patterns in wild-type N. vitripennis wasps (Fig. 2
H and Figure 2-figure supplement 4 I). This basically implies that these two genes
originated from a tandem gene duplication event and therefore constitute paralogs of the
D. melanogaster FASN3. Hence, we cannot state at this point whether either one of these two
genes alone or both are responsible for the observed phenotypic changes. Interestingly, this
mirrors a similar finding in N. vitripennis males concerning the biosynthesis of a lactone
functioning in a long-range pheromonal blend attractive to virgin females (29). The production
of this compound was unambiguously attributed to three short-chain
dehydrogenase/reductase genes with high sequence similarity, which could also not be
targeted independently by dsRNAi knockdowns. This demonstrates the difficulty to narrow
down specific genes responsible for the biosynthesis of pheromonal active compounds
when their sequences show particularly high degrees of similarity and simultaneously high
expression patterns.
In conclusion, our study demonstrates the considerable impact of two highly similar fatty acid synthase genes on female sexual attractiveness in a parasitoid wasp, thereby immediately suggesting how this trait can be encoded through specific ratios of MB-alkanes. To the best of our knowledge, these are the first identified hymenopteran genes with a specific effect on MB-alkane ratios that simultaneously impact sexual attractiveness. Transcending the demonstrated impact on sexual signaling and elicited mating behavior, the present findings also substantially advance our general knowledge on the so far little investigated genetic underpinnings of MB-alkane variation and production. This particular compound class dominates the surface profiles of many insects, most notably in the ecologically and economically important insect order Hymenoptera, and harbors a considerable potential for encoding chemical information, inviting a stronger emphasis on these compounds in future studies on chemical signaling and its behavioral impact.

Materials and Methods

Nasonia strain maintenance and preparation

The standard laboratory strain AsymCX of Nasonia vitripennis, originally collected in Leiden, the Netherlands, was used for all experiments. The wasps were reared under 25°C, in 55% relative humidity and a light:dark cycle of 16:8h, leading to a life cycle of ~14 days. Pupae of Calliphora vomitoria (Diptera: Calliphoridae) were used as hosts.

DsRNAi gene knockdown

For the knockdown initially targeting fas5, dsRNA was synthesized following the manual of Supplementary File 1. The Quick-RNA Tissue/Insect Kit (Zymo Research, Freiburg, Germany) was used to purify the resulting dsRNA product. GFP (Green Fluorescent Protein) dsRNA, which has no known targets in the Nasonia genome (32), was used as a control. GFP dsRNA was synthesized from the vector pOPINEneo-3C-GFP, which was kindly donated by Ray Owens (Addgene plasmid #53534; http://n2t.net/addgene:53534; RRID: Addgene_53534). Microinjections were performed with 4-5 µg/µl (diluted in nuclelease free water, Zymo Research) fas5 and GFP dsRNA on a Femtojet microinjector (Eppendorf, Hamburg, Germany) following the protocol published by Lynch et al. (55). N. vitripennis yellow pupae (7 to 8 days old after egg deposition) were gently fixed on a cover slide using double-sided tape (Deli, Zhejiang, China), with their abdomens facing up. DsRNA mixed with 10% red food dye (V2 FOODS, Niedersachsen, Germany) was injected into the abdomens of the pupae using a thin needle, which was produced in a PC-10 puller (Narishigne Group, Tokyo, Japan) by heating a glass capillary (100 mm length x 85 µm inner diameter, Hilgerberg, Malsfeld, Germany) to 100°C, and subsequently breaking the stretched capillary in two parts in the narrow middle section at 67 °C. Individual injections were performed until the red dye has spread evenly within the abdomen of each pupa as described by Wang et al. (35). The injected pupae were then stored inside a petri dish with a piece of wet tissue at the bottom to ensure saturation with sufficient humidity for the pupae to mature and eclose. After the pupae elosed as adults, they were collected at an age of 0-24 h and snap-frozen with liquid nitrogen, after which they were stored at -80 °C for further experiments.

DsRNAi efficiency analysis

DsRNAi knockdown efficiency was determined by quantitative PCR (qPCR), assessing fas5 gene expression levels between knockdown and control individuals. RNA from each individual wasp after chemical extraction (see below) was obtained using the Quick-RNA Tissue/Insect Kit (Zymo Research, Freiburg, Germany), and reversely transcribed into complementary DNA (cDNA) utilizing the cDNA Synthesis Kit (CD BioSciences, New York, USA). As controls for the qPCR procedure, we used N. vitripennis elongation factor 1a (NvEF-1a) as a housekeeping gene, as described by Wang, et al. (35). The qPCR was performed in a Lightcycler480 qPCR machine (Roche, Basel, Switzerland), with a pre-incubation of 95°C for 3 minutes, 40 amplification cycles of 15 seconds at 95°C and 60 seconds of 60°C, as well as
a final standard dissociation curve step to check the specificity of the amplification. The analysis of qPCR data was conducted using the ΔΔCt method (56). Firstly, the Ct (number of cycles required for the fluorescent signal to cross the threshold, i.e., exceeding the background level) values of the target genes were normalized to the Ct values of the housekeeping gene, yielding the respective difference between the two (ΔCt). Secondly, the ΔCt values of the knockdown treatments were normalized to the average ΔCt values of the wild-type (WT) group, which resulted in ΔΔCt values. The relative expression patterns of the target genes were then determined using the formula $2^{-\Delta\Delta C_t}$. To compare the relative expressions of $fas$ genes among the treatments, a Sequential Mann-Whitney U test was employed. Subsequently, the resulting $p$-values from these tests (18 in total, considering 6 $fas$ genes and 3 treatments) were subjected to the Benjamini-Hochberg procedure for correction of the false discovery rate (57).

**DsRNAi $fas5$ off-target effects**

The off-target effect of $fas5$ dsRNA was assessed using the dsRNAi off-target prediction tool in WaspAtlas (58), an online *N. vitripennis* genomic database. Briefly, the $fas5$ dsRNA sequence was first split into all possible 19-mers, which were further matched to the *N. vitripennis* transcriptome. The transcripts to which at least one of the 19-mers matched were identified and then for each transcript the percentage of matching 19-mers was calculated.

**Chemical analysis**

Chemical extractions of single wasps were performed by immersing them in 50 µl HPLC-grade n-hexane (Merck, KGaA, Darmstadt, Germany) in 2 ml glass vials (Agilent Technologies, Waldbronn, Germany) on an orbital shaker (IKA KS 130 Basic, Staufen, Germany) for 10 minutes. Extracts were subsequently evaporated under a constant stream of gaseous carbon dioxide and then resuspended in 10 µl of a hexane solution containing 7.5 ng/µl dodecane (C12) as an internal standard. Following this, 3 µl of the resuspended extract was injected in splitless mode with an automatic liquid sampler (ALS) (PAL RSI 120, CTC Analytics AG, Zwingen, Switzerland) into a gas-chromatograph (GC: 7890B) simultaneously coupled to a flame ionization detector (FID: G3440B) and a tandem mass spectrometer (MS/MS: 7010B, all provided by Agilent Technologies, Waldbronn, Germany). The system was equipped with a fused silica column (DB-5MS ultra inert; 30 m x 250 µm x 0.25 µm; Agilent J&W GC columns, Santa Clara, CA, USA) with helium used as a carrier gas under a constant flow of 1.8 ml/min. The FID had a temperature of 300 °C and used nitrogen with a 20 ml/min flow rate as make-up gas, and hydrogen with a 30 ml/min flow rate as fuel gas. The column was split at an auxiliary electronic pressure control (Aux EPC) module into an additional deactivated fused silica column piece (0.9 m x 150 µm) with a flow rate of 0.8 ml/min leading into the FID detector, and another deactivated fused silica column piece (1.33 m x 150 µm) at a flow rate of 1.33 ml/min leading into the mass spectrometer. The column temperature program started at 60 °C and was held for 1 min, increasing 40 °C per minute up to 200 °C and then increasing 5 °C per minute to the final temperature of 320 °C, held for 5 min. CHC peak detection, integration, quantification and identification were all carried out with Quantitative Analysis MassHunter Workstation Software (Version B.09.00 / Build 9.0.647.0, Agilent Technologies, Santa Clara, California, USA). CHCs were identified according to their retention indices, diagnostic ions, and mass spectra as provided by the total ion count (TIC) chromatograms, whereas their quantifications were achieved by the simultaneously obtained FID chromatograms, allowing for the best-suited method for hydrocarbon quantification (Agilent Technologies, Waldbronn, Germany, pers. comm.) while simultaneously retaining the capability to reliably identify each compound. Absolute CHC quantities (in ng) were obtained by calibrating each compound according to a dilution series based on the closest eluting n-alkane from a C21-40 standard series (Merck, KGaA, Darmstadt, Germany) at 0.5, 1, 2, 5, 10, 20, 40 ng/µl, respectively.

To compare the amount of each of the 54 single CHC compounds among different treatments, sequential Mann-Whitney U-tests were performed between all pairs of the two treatments (namely, $fas5$ vs GFP, $fas5$ vs WT & GFP vs WT). Subsequently, Benjamini-
Physical separation of the methyl-branched (MB-) alkane fraction from the other compound classes in *N. vitripennis* CHC profiles was performed according to an adapted protocol from Würf, Pokorny, Wittbrodt, Millar and Ruther (9) and Bello, McElfresh and Millar (59). CHC profiles of approximately 700 females were extracted in 9 mL HPLC-grade *n*-hexane (Merck, KGaA, Darmstadt, Germany) which was subsequently evaporated under a stream of gaseous carbon dioxide. The dried extract was re-suspended in 10 mL isooctane (99%, Sigma-Aldrich, Taufkirchen, Germany), and stirred overnight with a magnetic stirrer (Model: C-MAG HS4, IKA, Germany) after adding 2 g activated (*i.e.*, baked at 300°C for 2h) molecular sieves (5Å, 45-60 mesh size, Merck, KGaA, Darmstadt, Germany). The molecular sieves were filtered out by loading the extract into a glass funnel (50 mm inner diameter) with glass wool (Merck, KGaA, Darmstadt, Germany) and 0.2 g silica gel (High purity grade, pore size 60Å, 230-400 mesh particle size, Merck, KGaA, Darmstadt, Germany) containing 10% pulverized AgNO₃ (99.7%, Merck KGaA, Darmstadt, Germany). This procedure is devised to effectively filtering out *n*-alkanes and olefins, retaining only the MB-alkane fraction in the remaining extract (9, 58). The isooctane in the extract was condensed to 2 mL under a stream of gaseous carbon dioxide, from which we sampled 50 µl to estimate the quantity of the overall extract. Based on the quantity of overall MB-alkanes and single female CHC profiles, the MB-alkanes were reconstituted in hexane to a final concentration of approximately one female equivalent per 5 µl, which was saved for further behavioral assays.

### Behavioral assays

Mating behavior assays were carried out to test whether female sexual attractiveness decreased after knockdown of *fas5*. First, virgin females of three treatments (*fas5* dsRNAi, GFP dsRNAi and WT) were offered to 0-48 h old virgin WT *N. vitripennis* (AsymCX) males, following the protocol described in Wang, et al. (34). A female was transferred into a transparent plastic vial (76mm height, 10mm diameter) that contained a male. The assay was started as soon as the male was introduced and observed for 5 mins. The males’ behavior towards the females was then scored based on presence or absence of three consecutive behavioral displays: antennation (physical contact of male antennae with the female’s body surface), courtship (series of stereotypic headnods and antennal sweeps after mounting the female) and actual copulation attempts, which have been established as indicators of male mate acceptance and female sexual attractiveness (15, 18,60, Fig. 3 A). If the male did not initiate any further courtship or copulation behaviors after antennation, this was scored as a male mate rejection as described in Buellesbach, Greim & Schmitt (-60).

To increase the focus on the male mate choice behavior in relation to female chemical cues, further behavioral assays were carried out with freeze-killed females (*i.e.*, dummies) offered to 0-48 h old virgin WT males. In the first set of these experiments, male behavior was recorded and compared on female dummies of the three previously mentioned treatments (*fas5* dsRNAi, GFP RNAi and WT). In the second set, female dummies were manipulated by either soaking them individually in 50 µl hexane for 3 h, effectively removing their CHC profiles (15, 18) or reconstituting soaked WT female dummies with one female CHC profile equivalent (resuspended in 5 µl hexane as described above for chemical analysis) from *fas5* dsRNAi, GFP dsRNAi and WT females. In the third set, approximately one female equivalent of the separated methyl-branched alkane fraction, prepared directly after the CHC separation
process (see above), was reconstituted to CHC cleared female dummies, and offered to the WT males.

All behavioral assays with female dummies were performed in a mating chamber which consisted of two identical aluminium plates (53 x 41 x 5 mm). Each plate contained 12 holes (6 mm diameter) that served as observation sites. In preparation for the recording of the behavioral assays, single female dummies were placed into each hole in one plate, while single WT males were placed into each hole in the opposite plate and immediately covered with glass slides (Diagonal GmbH &Co. KG, Münster, Germany). The behavioral assays were initiated by quickly adjoining the two plates. Recordings were conducted with a Canon camera (EOS 70D, Tokyo, Japan) for 5 mins. All behavioral assays were performed in an eclosed wooden box with constant illumination (100Lm, LED light L0601, IKEA Dioder, Munich, Germany). Recordings of mating behaviors were further assessed and the frequencies of males’ mating displays were compared among treatments with Benjamini-Hochberg corrected Fisher’s exact tests (57).

Desiccation assays

High desiccation stress conditions were implemented by placing 0.6 g desiccant (DRIERITE, Merck, KGaA, Darmstadt, Germany) into transparent plastic vials (76mm height, 10mm diameter), which were air tightened with rubber plugs, resulting in low (~ 9%) relative humidity after 24 h. Inside each vial, a piece of cotton and a stainless-steel grid was placed in the middle to separate the desiccant from the remaining space of the vial (~ 35 mm height), which served as an observation site where individual wasps were placed for the duration of the desiccation assay. Control vials with moderate desiccation stress (~ 55% relative humidity) were prepared similarly but without adding the desiccant. The relative humidity in the test tubes of different humidity treatments was monitored using a humidity-temperature probe (Feuchtemesssystem HYTELOG-USB, B+B Thermo-Technik GmbH, Donaueschingen, Germany) with a measurement accuracy of ± 2% relative humidity at 25°C. Newly eclosed (0-24 h old) male and female wasps were collected, sorted in groups of 10, and fed with honey water (Bluetenhonig, dm-drogerie markt GmbH & Co. KG, Karlsruhe, Germany) for 9 hours. Afterwards, each group of wasps was randomly assigned to the previously prepared vials with either high or moderate desiccation stress. For each treatment, 10 replicates (vials) were observed. Recording was performed with a looped VLC media player (VideoLAN, Paris, France) script, initiating a 2 min recording (Logitech C920 HD PRO webcam, Logitech GmbH, München, Germany) every 2 hours, until the last wasp fell down on the grid and stop moving. The numbers of alive wasps in each vial were assessed, based on which survival curves were built and further compared among treatments. Survival analysis was conducted using the Cox Proportional Hazards Model to evaluate the survival probability, employing the R package “survival” (60). The knockdown treatment was considered as the fixed factor, while the variation among replicates was treated as a random factor. To determine the statistical significance, the log-rank test was employed. Additionally, the Benjamini-Hochberg procedure was applied to correct for multiple testing across the different humidity treatments (57).

Data availability

The datasets generated or analyzed during this study are available at the figshare data repository under 10.6084/m9.figshare.20411958

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References


**Figure and table captions**

**Figure 1.** Simplified overview of CHC biosynthesis emphasizing fatty acid synthase (FAS) catalyzed reactions. Initially, acetyl-Coenzyme A (CoA) is converted into malonyl-CoA by the enzyme Acetyl-CoA carboxylase (ACC). Then, further malonyl-CoA subunits are successively incorporated onto the acetyl-CoA primer to form long chain fatty acids (LCFAs) catalyzed by fatty acid synthase enzymes hypothesized to be subcellularly located in the cytosol (c-FAS). For the synthesis of methyl-branched (mb)-CHCs with internal methyl groups, methyl-malonyl-CoA units are incorporated at specific chain locations instead of malonyl-CoA units, catalyzed by m-FAS enzymes whose subcellular location has been hypothesized to be microsomal. The methyl-branched or straight-chain LCFAs are then further processed through a series of biosynthetic conversions catalyzed by the elongase (ELO) enzyme complex, fatty acyl-CoA reductase (FAR) enzymes and cytochrome P450 decarboxylase (CYP4G) enzymes to either methyl-branched or straight-chain, saturated CHCs, respectively. For the biosynthesis of unsaturated CHCs (olefins), desaturase (DESAT) enzymes introduce double bonds into the fatty acyl-CoA chain between elongation steps. For a detailed description of CHC biosynthesis and the involved enzymatic reactions see Blomquist and Ginzel (7) as well as Holze, Schrader and Buellesbach (19).

**Figure 2.** Fas5 knockdown primarily alters quantities and ratios of methyl-branched CHCs with specific branching patterns in females. A) Chromatogram comparison of surface extracts from single female *N. vitripennis* wasps injected with fas5 dsRNA (top) and GFP dsRNA (bottom). CHC compound peaks with significantly different quantities in fas5 knockdown vs. GFP control females are indicated in color (compare to Supplementary File 3).
Different colors are used for methyl-branched (MB) alkanes with their first methyl group at positions 3-, 5-, 7- and 9+ (also including positions 11-, 13- and 15-) as well as n-alkanes and n-alkenes; **B)** Average relative abundances (%) of different CHC compound classes (as indicated in A) compared between wildtype (WT, N=14), control knockdown (GFP, N=15) and fas5 knockdown (fas5, N=15) female wasps; **C)** Average absolute quantities (in ng) of MB alkanes with their first methyl group at the 3rd C-atom position (3-MeC) compared between wildtype (WT), control knockdown (GFP) and fas5 knockdown (fas5) female wasps, indicated by blue, green and orange violin plots, respectively, from here on, sample sizes as in B; **D)** Average absolute quantities of MB alkanes with their first methyl group at the 5th C-atom position (5-MeC), violin plot colors and group designations as in C; **E)** Average absolute quantities of methyl-branched CHCs with their first methyl group at the 9th (as well as 11th, 13th and 15th, indicated as 9+-MeC) C-atom position; **F)** Average absolute quantities of methyl-branched CHCs with their first methyl group at the 7th C-atom position (7-MeC); **G)** Average absolute quantities of total MB alkane amounts; **H)** Relative expression of fas5 (plain) and fas6 (hatched) in WT, GFP and fas5 dsRNAi females (N=15 for WT and fas5 RNAi; N=16 for GFP RNAi), indicated by blue, green and orange boxplots, respectively. Significant differences (p < 0.05) were assessed with Benjamini-Hochberg corrected Mann-Whitney U tests in **A), C) – H)** and are indicated by different letters, in **H)** significance for fas5 expression vs. controls is indicated by lower case letters, and fas6 expression vs. controls by uppercase letters.
Figure 3. Fas5 knockdown females elicit less courtship and copulation behaviors from WT males. A) Depiction of consecutively displayed mating behavior of *N. vitripennis* males towards females, consisting of initial antennation, courtship (stereotypical head-nods on the female antennae after mounting) and actual copulation (injection of the male aedeagus into the female’s genital opening) (images by Quoc Hung Le). B) The proportions of males performing antennation, courtship and copulation towards alive WT, GFP and fas5 RNAi females, which are marked by blue, green and orange bar plots, N=20 for each treatment; C) Male mate rejection rates towards alive WT, GFP and fas5 RNAi females, separated between no (light green) and one or more rejections (light orange); D) The proportions of males performing antennation, courtship and copulation towards freeze-killed WT, GFP and fas5 RNAi females. Bar plot colors and group designations as in B, N=24 for each treatment; E) The proportions of males performing antennation, courtship and copulation towards either CHC cleared (in grey) female dummies, or CHC cleared female dummies reconstituted with one female CHC profile equivalent from WT, GFP and fas5 RNAi females (treatment colors and group designations as in B and D), N=24 for each treatment. Significant differences (p<0.05) were assessed with Benjamini-Hochberg corrected Fisher’s exact tests in B), D) - E) and are indicated by different letters.

Figure 4. Methyl-branched alkane fraction from fas5 RNAi females elicits less courtship and copulation from WT males. A) Chromatogram comparison of representative fas5 RNAi female CHC profiles before (in black) and after (in red) physical separation of the methyl-branched (MB) alkane fraction from the other compound classes (n-alkanes and n-alkenes). Individual n-alkane and n-alkene compound peaks are indicated by arrows (all other peaks correspond to MB-alkanes). Note that only the part of the *Nasonia* CHC profile where these compounds do occur is shown (compare to Fig. 1 A); B) Average relative abundances (%) of different compound classes, including MB alkanes with their first methyl group at positions 3-, 5-, 7- and 9’ (also including positions 11-, 13- and 15-) as well as n-alkanes and n-alkenes, compared between wildtype (WT, N=3), control knockdown (GFP, N=3) and fas5 knockdown (fas5, N=3) individuals; C) The proportions of males performing antennation, courtship and copulation towards CHC cleared female dummies reconstituted with approximately one female equivalent of MB-alkane fractions derived from WT (in blue, N=23), GFP (in green, N=23) and fas5 RNAi (in orange, N=24) females. Significant differences (p<0.05) were assessed with Benjamini-Hochberg corrected Fisher’s exact tests in C) and are indicated by different letters.

Figure 5. Fas5 knockdown does not change survival times of male and female wasps under desiccation stress. A) Comparison of survival probabilities along the observation time under high desiccation stress between control knockdown (GFP RNAi) and fas5 knockdown (fas5 RNAi) females; B) Comparison of survival probabilities along the observation time under medium desiccation stress between GFP RNAi and fas5 RNAi females; C) Comparison of survival probabilities along the observation time under high desiccation stress between GFP RNAi and fas5 RNAi males; D) Comparison of survival probabilities along the observation time under medium desiccation stress between GFP RNAi and fas5 RNAi males. N=10 for each treatment. The high desiccation stress treatment was achieved with approximately 9% relative humidity, and the medium desiccation stress treatment with approximately 55% relative humidity as assessed by humidity-temperature probes. Survival probability was assessed with a Cox Analysis, and the colored area along the survival curve represents the 95% confident interval.
Figure 2-figure supplement 1: Fas5 knockdown does not affect the expressions of the previously published Nasonia vitripennis fas genes. A) Relative expression of fas 1-4 in WT, GFP and fas5 RNAi females (N=15 for WT and fas5 RNAi; N=16 for GFP RNAi), indicated by blue, green and orange boxplots, respectively. B) Relative expression of fas 1-4 in WT, GFP and fas5 RNAi males (N=15 for WT and fas5 RNAi; N=16 for GFP RNAi), indicated by blue, green and orange boxplots, respectively. Significant differences (p<0.05) were assessed with Benjamini-Hochberg corrected Mann-Whitney U tests.

Figure 2-figure supplement 2. Fas5 is an adjunct to fas6 in the Nasonia vitripennis genome, exhibiting high sequence similarity. A) The physical locations of fas5 and fas6 are indicated in the N. vitripennis genome, specifically in NC_045761.1, chromosome 5 of the Nvit_psr_1.1 (GCF_009193385.2) genome assembly. Fas5 is precisely located from 21808178 to 21820384, while fas6 spans from 21821083 to 21833200. The gene locations are visualized in National Center for Biotechnology Information (NCBI) webpage. B) Alignment between fas5 and fas6 transcripts. The identical sequences between fas5 transcript XM.008209455.4 and fas6 transcript XM.031931029.2 are indicated by a consistent grey bar, while single nucleotide polymorphisms are indicated by the red lines inserted in the bar. Fas5 and fas6 transcripts share 92% query coverage and 90.95% sequence identity. The alignment of sequences was performed by the Basic Local Alignment Search Tool (BLAST) and visualized in Multiple Sequence Alignment Viewer 1.22.0 from NCBI.

Figure 2-figure supplement 3.. Fas5 knockdown does not change total CHC and n-alkane quantities but increases n-alkene quantities in females. A) Average absolute quantities (ng) of total extracted CHC amounts, compared between wildtype (WT, N=14), control knockdown (GFP, N=15) and fas5 knockdown (fas5, N=15) N. vitripennis females, indicated by blue, green and orange violin plots, respectively; B) Average absolute quantities (ng) of the total amount of n-alkanes from female CHC extracts, violin plot colors and group designations as in A; C) Average absolute quantities (ng) of the total amount of n-alkenes from female CHC extracts, violin plot colors and group designations as in A. Significant differences (p<0.05) were assessed with Benjamini-Hochberg corrected Mann-Whitney U tests and are indicated by different letters.

Figure 2-figure supplement 4.. Fas5 knockdown primarily alters the ratios of methyl-branched CHCs with specific branching patterns in males. A) Average relative abundances (%) of different CHC compound classes compared between wildtype (WT, N=15), control knockdown (GFP, N=15) and fas5 knockdown (fas5, N=14) male N. vitripennis wasps. Different colors are used for methyl-branched (MB) alkanes with their first methyl group at positions 3-, 5-, 7- and 9’ (also including positions 11-, 13- and 15-) as well as n-alkanes and n-alkenes (compare to Fig. 1) B) Average absolute quantities (ng) of total CHC amounts, compared between wildtype (WT), control knockdown (GFP) and fas5 knockdown (fas5) male wasps, indicated by blue, green and orange violin plots, respectively, from here on, sample sizes as in A. C) Average absolute quantities (ng) of total n-alkane amounts in male wasps, violin plot colors and group designations as in B D) Average absolute quantities (ng) of total n-alkane amounts in male wasps E) Average absolute quantities of MB alkanes with their first methyl group at the 3rd C-atom position (3-MeC); F) Average absolute quantities of MB alkanes with their first methyl group at the 5th C-atom position (5-MeC); G) Average absolute quantities of methyl-branched CHCs with their first methyl group at the 7th C-atom position (7-MeC) H) Average absolute quantities of methyl-branched CHCs with their first methyl group at the 9th (as well as 11th, 13th and 15th, indicated as 9’-MeC) C-atom position; I) Relative expression of fas5 (plain) and fas6 (hatched) in WT, GFP and fas5 RNAi males (N=15 for WT and GFP RNAi; N=17 for fas5 RNAi treatment), indicated by blue, green and
orange boxplots, respectively. Significant differences (p<0.05) were assessed with Benjamini-Hochberg corrected Mann-Whitney U tests and are indicated by different letters (fas5 vs. controls: lower case letters, fas6 vs controls: uppercase letters).

**Figure 4-source data 1.** Methyl-branched alkane separation process dramatically decreases quantity of \textit{n}-alkane and \textit{n}-alkene. The average relative abundance (%) of overall MB-alkanes, \textit{n}-alkanes and \textit{n}-alkenes in fas5 RNAi female CHCs before and after separation of the methyl-branched (MB) alkane fraction from the other compound classes. N=3 for each treatment.

**Supplementary File 1.** List of primers used in the present study. All primers were designed using the Primer-BLAST tool from the National Center for Biotechnology Information (NCBI). Indicated are the respective primer names, their sequences, and their usage in the experimental protocol.

**Supplementary File 2.** Gene names, gene IDs and transcript IDs of all fas genes and the housekeeping gene (elongation factor 1a) from the present study. Note that there are two transcript IDs for fas6 (compare to Figure 2-figure supplement 2).

**Supplementary File 3.** Comparison of absolute quantities and relative abundances of single CHC compounds between differentially treated female wasps. Indicated are retention indices (RI), CHC compound identifications or possible configurations in case of ambiguities, their mean absolute (ng) amounts with their respective absolute standard deviations (sd) as well as their respective relative amounts (in %) compared between wildtype (WT, N=14), control knockdown (GFP RNAi, N=15) and fas5 knockdown (fas5 RNAi, N=15) female wasps. To compare the absolute quantities (ng) of 54 single CHC compounds among the different treatments, we employed a sequential Benjamin-Hochberg corrected Mann-Whitney U-test between each pair of treatments: fas5 vs GFP, fas5 vs WT, and GFP vs WT. Significant effects in fas5 knockdown (KD) females are indicated by up- (white) and downwards (black) arrows, corresponding to either up- or down-regulation of the absolute compound quantities, respectively. Where compound identifications were ambiguous due to multiple possible methyl branch positions which could be interpreted based on the detected ion pairs, all possible compound configurations are given.
A. GFP RNAi ♀

B.

WT

GFP RNAi

fas5 RNAi

Proportions of CHC compound classes (%) 

C.

3-MeC quantity (ng)

D.

5-MeC quantity (ng)

E.

7-MeC quantity (ng)

F.

9\(^{-}\)-MeC quantity (ng)

G.

MB alkane quantity (ng)

H.

Rel. gene expression (%) 

A.

B.

C.

D.

E.

F.

G.

H.

3-MeC

5-MeC

7-MeC

9\(^{-}\)-MeC

n-alkane

n-alkene

WT

GFP RNAi

fas5 RNAi

fas5 RNAi ♀
A. Relative gene expression in female

B. Relative gene expression in male
Male mating behavior on alive females

- **Antennation**: 100%
- **Courtship**: 75%
- **Copulation**: 50%

Male mate rejection rates:
- **Fas5 RNAi**: 70% (none), 30% (one or multiple times)
- **GFP RNAi**: 100%
- **WT**: 100%

Treatment:
- **WT**
- **GFP RNAi**
- **Fas5 RNAi**
- **CHC cleared**
A. x10^5

![Abundance vs. Acquisition time (min)]

- **n-C29**
- **n-C30**
- **n-C31**
- **7-C31ene**
- **9-C31ene**
- **n-C33**
- **7-C31ene**

B. Proportion (%)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GFP RNAi</th>
<th>Fas5 RNAi</th>
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<tbody>
<tr>
<td>3-MeC</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>5-MeC</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>7-MeC</td>
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<td>25</td>
<td>25</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
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<tr>
<td>n-alkene</td>
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</table>

C. Male mating behavior on MB-alkane applied females

- **Antennation**
- **Courtship**
- **Copulation**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GFP RNAi</th>
<th>Fas5 RNAi</th>
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<tbody>
<tr>
<td>0-25%</td>
<td>a</td>
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</tr>
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<td>26-50%</td>
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<td>a</td>
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</tr>
<tr>
<td>51-75%</td>
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<td>a</td>
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<tr>
<td>76-100%</td>
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</table>
A. females under high desiccation stress

B. females under medium desiccation stress

C. males under high desiccation stress

D. males under medium desiccation stress

Survival probability

Time (h)

p = 0.079

p = 0.1

p = 0.3

p = 0.098

Treatment

Fas5 RNAi

GFP RNAi