

## RESEARCH ARTICLE

# Functional characterisation of two $\Delta 12$ -desaturases demonstrates targeted production of linoleic acid as pheromone precursor in *Nasonia*

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## ABSTRACT

Insect pheromones are often derived from fatty acid metabolism. Fatty acid desaturases, enzymes introducing double bonds into fatty acids, are crucial for the biosynthesis of these chemical signals.  $\Delta 12$ -desaturases catalyse the biosynthesis of linoleic acid by introducing a second double bond into oleic acid, but have been identified in only a few animal species. Here, we report the functional characterisation of two  $\Delta 12$ -desaturases, Nvit\_D12a and Nvit\_D12b, from the parasitic wasp *Nasonia vitripennis*. We demonstrate that Nvit\_D12a is expressed in the rectal vesicle of males where they produce a linoleic acid-derived sex pheromone to attract virgin females. <sup>13</sup>C-labelling experiments with *Urolepis rufipes*, a closely related species belonging to the '*Nasonia* group', revealed that females, but not males, are able to synthesise linoleic acid. *U. rufipes* males produce an isoprenoid sex pheromone in the same gland and do not depend on linoleic acid for pheromone production. This suggests that  $\Delta 12$ -desaturases are common in the '*Nasonia* group', but acquired a specialised function in chemical communication of those species that use linoleic acid as a pheromone precursor. Phylogenetic analysis suggests that insect  $\Delta 12$ -desaturases have evolved repeatedly from  $\Delta 9$ -desaturases in different insect taxa. Hence, insects have developed a way to produce linoleic acid independent of the omega desaturase subfamily which harbours all of the eukaryotic  $\Delta 12$ -desaturases known so far.

**KEY WORDS:** Parasitic wasp,  $\Delta 12$ -desaturase, Sex pheromone, Biosynthesis, *Nasonia vitripennis*, *Urolepis rufipes*

## INTRODUCTION

Communication by sex pheromones is crucial for mate finding in many insects (Wyatt, 2014). To fulfil their function reliably, sex pheromones need to encode their information in a species-specific

manner. Insect pheromones are often biosynthesised as variations on conserved pathways of the primary and secondary metabolism, of which fatty acid metabolism is of particular importance (Jurenka et al., 2017; Morgan, 2004; Tillman et al., 1999). For instance, more than 90% of the lepidopteran sex pheromones known to date are fatty acid derivatives (Ando et al., 2004) and the cuticular hydrocarbons used by numerous insects as contact sex pheromones are likewise derived from fatty acid metabolism (Blomquist, 2010). The number, position and configuration of double bonds are important structural features of fatty acid-derived sex pheromones and contribute to the specificity of the information they convey. Thus, fatty acid desaturases (FADs) introducing double bonds into fatty acid chains are key enzymes for the species specific communication and reproductive success of many insects (Jurenka et al., 2017; Tillman et al., 1999; Tupec et al., 2017). Animals have long been assumed to be missing a crucial type of FAD, which are the  $\Delta 12$ -FADs that can produce linoleic acid [(9Z,12Z)-octadeca-9,12-dienoic acid, C18:2 <sup>$\Delta 9,12$</sup> ] by introducing a second double bond into oleic acid [(9Z)-octadec-9-enoic acid, C18:1 <sup>$\Delta 9$</sup> ] at position 12. Hence, C18:2 <sup>$\Delta 9,12$</sup>  was considered to be an essential nutrient for animals (Cripps et al., 1986; Malcicka et al., 2018). However, studies on insects (Blaul et al., 2014; Blomquist et al., 1982; Buckner and Hagen, 2003; Cripps et al., 1986, 1990; Haritos et al., 2012; Malcicka et al., 2018; Zhou et al., 2008), springtails (Malcicka et al., 2017), nematodes (Peyou-Ndi et al., 2000), gastropods (Weinert et al., 1993), mites (Aboshi et al., 2013; Shimizu et al., 2014) and a variety of aquatic invertebrates (Kabeya et al., 2018) have shown that not all animals depend on dietary uptake of C18:2 <sup>$\Delta 9,12$</sup>  but are able to synthesise it *de novo*. Evidence for the presence of  $\Delta 12$ -FADs in insects is mostly based on stable and radioactive isotope labelling experiments (Blaul et al., 2014; Blomquist et al., 1982; Buckner and Hagen, 2003; Cripps et al., 1986; Malcicka et al., 2017). This approach, however, does not completely rule out the possibility that C18:2 <sup>$\Delta 9,12$</sup>  is produced by associated microorganisms rather than by the studied animal itself. Unequivocal evidence for C18:2 <sup>$\Delta 9,12$</sup>  biosynthetic capacity requires isolation and functional characterisation of insect-derived  $\Delta 12$ -FAD genes, which has been achieved so far only for the house cricket *Acheta domesticus* (AdD12Des, here referred to as Adom\_D12) (Zhou et al., 2008), the soldier beetle *Chauliognathus lugubris* (CL10, Clug\_D12) (Haritos et al., 2012) and the red flour beetle *Tribolium castaneum* (TcD12Des, Tcas\_D12) (Haritos et al., 2014; Zhou et al., 2008). It is unknown, however, whether the  $\Delta 12$ -FADs in these species are involved in primary fatty acid metabolism or pheromone biosynthesis.

Males of the parasitic wasp *Nasonia vitripennis* (Walker 1836), a pupal parasitoid of different fly species, produce in their rectal vesicle a sex pheromone composed of (4R,5R)- and

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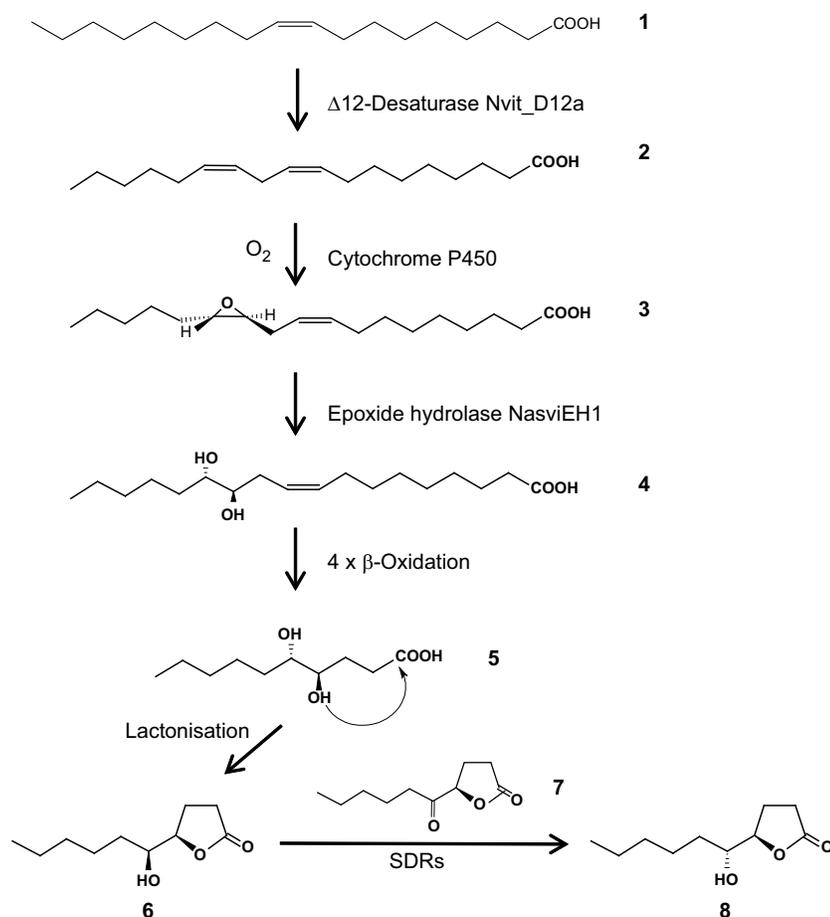
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(4*R*,5*S*)-5-hydroxy-4-decanolide and the minor component 4-methylquinazoline. After emergence, males deposit the pheromone on the host and other substrates to attract virgin females (Mair and Ruther, 2018; Ruther et al., 2007, 2008). Stable isotope labelling experiments using fully  $^{13}\text{C}$ -labelled fatty acid precursors revealed that both stereoisomers of 5-hydroxy-4-decanolide are synthesised from C18:2 $^{\Delta 9,12}$  or C18:1 $^{\Delta 9}$  (Blaul and Ruther, 2011; Blaul et al., 2014) (Fig. 1). Furthermore, *N. vitripennis* males are capable of converting C18:1 $^{\Delta 9}$  into C18:2 $^{\Delta 9,12}$ , suggesting that they possess a  $\Delta 12$ -FAD that is involved in sex pheromone biosynthesis (Blaul et al., 2014). Sixteen predicted FAD genes have been identified and mapped in the *N. vitripennis* genome (Niehuis et al., 2011), 13 of which have been studied with respect to their sex-biased expression status (Wang et al., 2015). Such gene expression analyses revealed a male-biased expression of five predicted FADs, which are thus candidate enzymes for sex pheromone biosynthesis. This is particularly true for one predicted FAD (GenBank acc. no. XP\_001599836.1), which has been found to be expressed at 800-times greater levels in males than in females (Wang et al., 2015). In contrast, a highly similar paralogue showing >90% protein sequence identity (XP\_001599873.1) was expressed unbiasedly (Wang et al., 2015).

The use of (4*R*,5*S*)-5-hydroxy-4-decanolide as a male sex pheromone component is also found in the three other species of the genus: *Nasonia giraulti*, *Nasonia longicornis* and *Nasonia oneida* (Darling and Werren, 1990; Raychoudhury et al., 2010), as well as in the closely related species *Trichomalopsis sarcophagae*

(Niehuis et al., 2013). This suggests that these species, like *N. vitripennis*, rely on the biosynthesis of C18:2 $^{\Delta 9,12}$ . The genus *Urolepis* is closely related to *Nasonia* and *Trichomalopsis* and has been suggested to form a monophyletic taxon within the Pteromalinae, the so-called ‘*Nasonia* group’ (Burks, 2009). Unlike the *Nasonia* species, males of *Urolepis rufipes* Ashmead 1896, the only *Urolepis* species studied so far, release from their rectal vesicle the sex pheromone 2,6-dimethyl-7-octene-1,6-diol, which is synthesised via the mevalonate pathway while C18:2 $^{\Delta 9,12}$  is not involved (Ruther et al., 2019). Hence, the study of C18:2 $^{\Delta 9,12}$  biosynthesis in *U. rufipes* will allow conclusions on whether  $\Delta 12$ -FADs are common in the *Nasonia* group or have evolved in the context of pheromone biosynthesis.

In the present study, we test the catalytic capacities of the two *N. vitripennis* FADs by functional expression in yeast and investigate whether they are expressed in the male sex pheromone gland (rectal vesicle) by using a proteomics approach. Finally, we investigate the ability of *U. rufipes* to synthesise C18:2 $^{\Delta 9,12}$  from C18:1 $^{\Delta 9}$  in  $^{13}\text{C}$ -labelling experiments. We demonstrate that both FADs have  $\Delta 12$ -activity and that the male-biased FAD reported by Wang et al. (2015) is expressed in the pheromone gland. A phylogenetic analysis of *N. vitripennis* FAD sequences and those of other insect  $\Delta 12$ -FADs suggests that they have evolved independently. We furthermore show that females but not males of *U. rufipes* are capable of synthesising C18:2 $^{\Delta 9,12}$ , suggesting that  $\Delta 12$ -FADs have a single evolutionary origin in the *Nasonia* group but have been adapted for pheromone biosynthesis in males of the *Nasonia* species and *T. sarcophagae*.



**Fig. 1. Biosynthetic pathway of the *N. vitripennis* sex pheromone components.** Biosynthesis starts from oleic acid (1, C18:1 $^{\Delta 9}$ ) (Blaul et al., 2014) which is converted by the  $\Delta 12$ -desaturase Nvit\_D12a to linoleic acid (2, C18:2 $^{\Delta 9,12}$ ) (this study and Blaul et al., 2014). C18:2 $^{\Delta 9,12}$  is epoxidised by a hitherto uncharacterised cytochrome P450 enzyme. The resulting 12,13-epoxy-octadec-9*Z*-enoic acid (3), is hydrolysed by the epoxide hydrolase NasviEH1 (Abdel-latif et al., 2008) to (12*R*,13*S*)-12,13-dihydroxy-octadec-9*Z*-enoic acid (4); chain shortening by four steps of  $\beta$ -oxidation leads to (4*R*,5*S*)-4,5-dihydroxydecanoic acid (5) which lactonises to (4*R*,5*S*)-5-hydroxy-4-decanolide (6). Epimerisation of (6) by the short-chain dehydrogenases/reductases (SDRs) NV10127, NV10128 and NV10129 with (4*R*)-5-oxodecanolide (7) occurring as an intermediate, finally leads to (4*R*,5*R*)-5-hydroxy-4-decanolide (8) (Niehuis et al., 2013; Ruther et al., 2016).

## MATERIALS AND METHODS

### Insects

We used *N. vitripennis* from the inbred strain Phero\_01, which originated from northern Germany and was also used in previous pheromone studies (Abdel-Latif et al., 2008; Niehuis et al., 2013; Ruther et al., 2016, 2007, 2008). The *U. rufipes* strain was kindly provided by Kevin Floate and originated from cattle feedlots in southern Alberta, Canada (Floate, 2002). Both species were reared on freeze-killed pupae of the green bottle fly *Lucilia caesar*, as reported previously (Ruther et al., 2019; Steiner et al., 2006). All studies were carried out in compliance with relevant provincial and national guidelines.

### Cloning and mutagenesis

The genes of XP\_001599836.1 (*Nvit\_D12a*) and XP\_001599873.1 (*Nvit\_D12b*) (for nucleotide and amino acid sequences see Table S1) were optimised for expression in *S. cerevisiae*, synthesised (Life Technologies GmbH, Darmstadt, Germany) and cloned into a modified YCplac111-pGAL-FLAG plasmid (gift from Philipp Milkereit, University of Regensburg, Germany) via unique *BsaI* restriction sites, as described previously (Rohweder et al., 2018). The modified YCplac111-pGAL-3FLAG plasmid was constructed such that three N-terminal FLAG-tags for protein detection and a cleavage site for TEV-protease for tag removal were located upstream of the *BsaI* cloning sites.

### Yeast transformation and enzyme activity assays

Plasmids YCplac111-pGAL-3FLAG\_XP\_001599836.1 (*Nvit\_D12a*) and YCplac111-pGAL-3FLAG\_XP\_001599873.1 (*Nvit\_D12b*) were transformed into *S. cerevisiae* BY4741 (Euroscarf/Scientific Research and Development GmbH, Oberursel, Germany) and grown on SDC-Leu agar plates at 30°C. Single colonies were transferred to SGC-Leu agar plates, grown at 30°C for 2 days and tested for target protein expression by western blot analysis.

For enzyme activity assays, overnight cultures (10 ml SGC-Leu medium) of protein-expressing clones were used to inoculate 25 ml SGC-Leu medium supplemented with 10% NP40 (Sigma-Aldrich/Merck KGaA, Darmstadt, Germany) to an OD<sub>600</sub> of 0.1. Cells were grown at 30°C to an OD<sub>600</sub> of 0.6 and then C18:1<sup>Δ9</sup> (25 mmol l<sup>-1</sup>, dissolved in ethanol) was added to a final concentration of 0.5 mmol l<sup>-1</sup>. Growth was continued for 3 days at 30°C and cells were harvested by centrifugation (2700 g, 4°C), washed first with 25 ml of 1% NP40, then with 25 ml of 0.5% NP40 and finally twice with 25 ml water. Yeast cell pellets were dried for 2.5 h in an Eppendorf Concentrator 5301 (Eppendorf AG, Hamburg, Germany) at 30°C.

### Western blot analysis

Detection of (FLAG)<sub>3</sub>-tagged proteins was performed with a monoclonal anti-FLAG antibody (cat. no.: 200474; lot: 0006262954; 1:1000; Agilent Technologies, Santa Clara, CA, USA) and a HRP-coupled anti-rat secondary antibody (cat. no.: 112035068; lot: 73749; 1:5000; Dianova, Hamburg, Germany). Protein signals were visualised using the BM Chemiluminescence Western blotting kit (Roche Diagnostics GmbH, Mannheim, Germany) in a Fluorescence Image Reader LAS3000 (Fujifilm Europe GmbH, Düsseldorf, Germany).

### Fatty acid extraction, transesterification and GC/MS-analysis

Fatty acid composition was analysed by coupled gas chromatography/mass spectrometry (GC/MS). Yeast cell pellets were cut to small pieces and extracted for 30 min in 400 μl dichloromethane in an ultrasonic bath. The extracts were transferred

to a new vial and dried under a gentle nitrogen stream. Then, 300 μl methanol and 30 μl acetyl chloride (10% dissolved in methanol) were added to the residue and transesterification was carried out for 4 h at 70°C. Subsequently, 400 ml sodium hydrogen carbonate (5%, dissolved in sterile water) was added and the FAMES were extracted with 200 μl hexane. A Shimadzu QP2010 Plus GC/MS system (Shimadzu Corporation, Kyoto, Japan) equipped with a polar BP-20 capillary column (30 m×0.25 mm inner diameter, 0.25 mm film thickness, SGE Analytical Science Europe, Milton Keynes, UK) was used for analysis. Helium served as carrier gas at a linear velocity of 40 cm s<sup>-1</sup>. The MS was operated in the electron impact ionisation (EI) mode at 70 eV, the mass to charge ratio range (*m/z*) was 35–500. Samples were injected splitless at 240°C by using an AOC 20i auto sampler. The initial oven temperature was 50°C and this was increased to 240°C at a rate of 3°C min<sup>-1</sup>, which was then held for 30 min. FAME analysis of *U. rufipes* wasps was done on a non-polar BPX5 column (60 m×0.25 mm inner diameter, film thickness 0.25 μm, SGE Analytical Science Europe, Milton Keynes, UK) with the same GC/MS parameters as described above but with 280°C as maximum oven and injector temperatures. Identification of FAMES was done by comparison of retention times and mass spectra with those of authentic reference chemicals (reference mixture of 37 FAMES, Sigma-Aldrich, Deisenhofen, Germany). Positions of double bonds of unsaturated compounds were additionally confirmed by derivatisation with dimethyl disulfide (Sigma-Aldrich, Deisenhofen, Germany) as described by Blaul et al. (2014).

### Protein analysis by LC/MS/MS

To test which of the two FADs is expressed in the pheromone glands of *N. vitripennis* males, we extracted proteins from 10 male rectal vesicles (two replicates) and analysed the in-gel digested proteins by coupled liquid chromatography/tandem mass spectrometry (LC/MS/MS) as described previously (Ruther et al., 2016).

### Sequence comparison and phylogenetic analysis

The amino acid sequences were aligned using the MAFFT alignment web server (<https://mafft.cbrc.jp/alignment/server/>) with standard parameters. Positions containing more than 50% gaps were removed by using Gblocks (Castresana, 2000) followed by the construction of a phylogenetic tree with MrBayes (version 3.2.6; Ronquist et al., 2012). Based on invariable gamma rates and the gtr model, four runs, each with six chains were executed for 1,000,000 generations. The first 25% of the generations were removed as burn-in and the option `contype=allcompat` was used to determine the consensus tree and the posterior probabilities. GenBank accession numbers for FADs used in alignments and the phylogenetic tree are: Ades\_D12, *A. domesticus* ABY26957.1; Ades\_D9, *A. domesticus* AAK25797.1; Tcas\_D12, *T. castaneum* NP\_001137206.1; Tcas\_D9, *T. castaneum* NP\_001182164.1; Tcas\_D9A, *T. castaneum* NP\_001180578.1; Tcas\_D9B, *T. castaneum* AHH30808.1; Tcas\_D9C, *T. castaneum* AHH30811.1; Tcas\_D9D, *T. castaneum* AHH30812.1; Tcas\_D5, *T. castaneum* AHH30809.1; Tcas\_D5A, *T. castaneum* AHH30810.1; Clug\_D12, *C. lugubris* AFJ66832.1; Msex\_D11, *Manduca sexta* AM076339.2; Nvit\_D12a, *N. vitripennis* XP\_001599836.1; Nvit\_D12b, *N. vitripennis* XP\_001599873.1; as well as annotated but uncharacterised sequences from *N. vitripennis*: XP\_00159579.1, XP\_003425691.1, XP\_001599665.2, XP\_001607533.2, XP\_001602540.1, XP\_001599877.2, XP\_001600683.2, XP\_001599899.2, XP\_001607893.1, XP\_008217358.1, XP\_001602565.1 was reannotated to XP\_001602565.1\_reannot, as reported previously (Blaul et al., 2014).

### <sup>13</sup>C-labelling experiment with *U. rufipes*

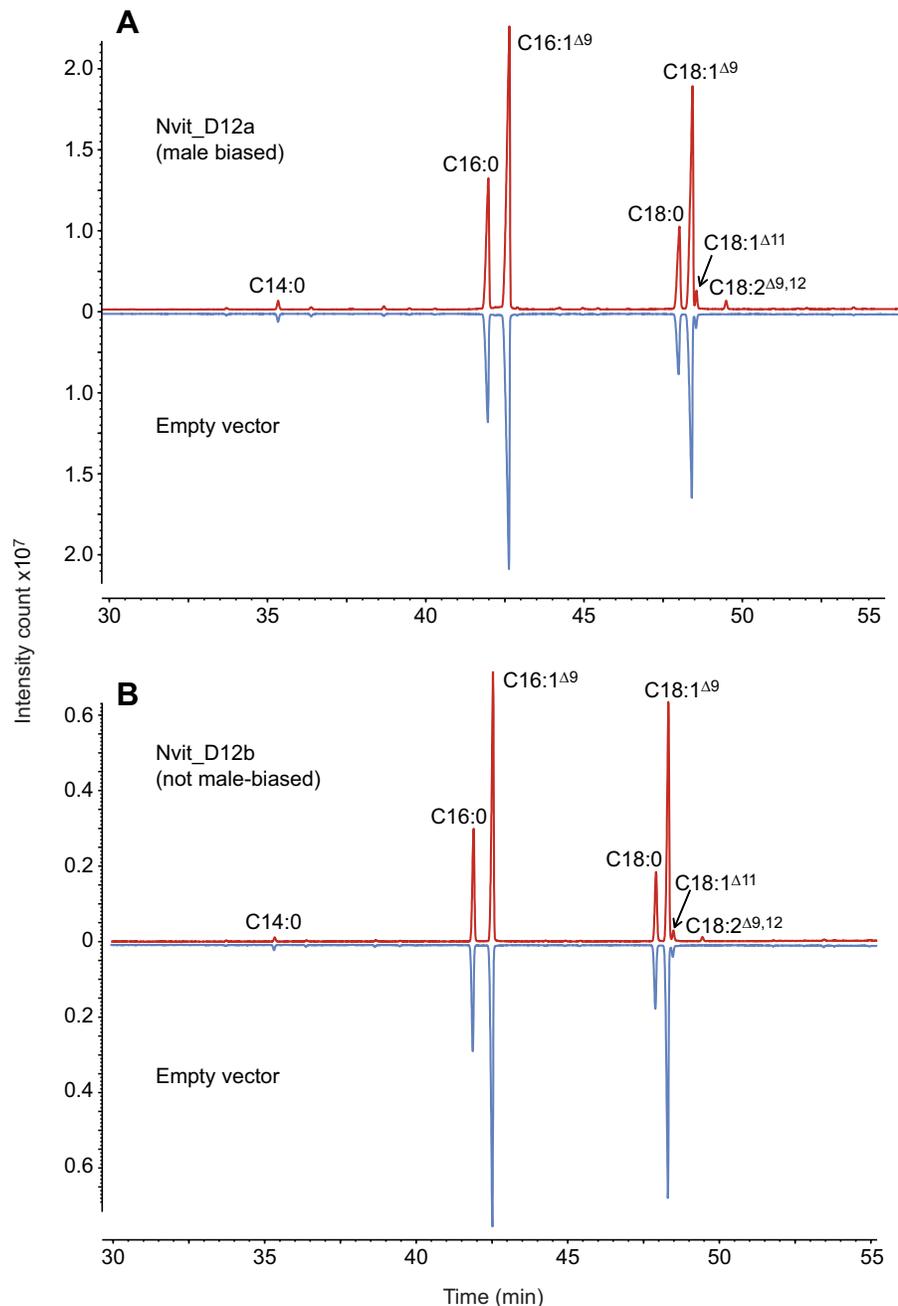
To investigate whether *U. rufipes* wasps are capable of synthesising C18:2<sup>Δ9,12</sup> from C18:1<sup>Δ9</sup>, we performed <sup>13</sup>C-labelling experiments as described previously (Blaul et al., 2014). Briefly, single randomly chosen 2-day-old parasitoids were cold-sedated in an ice bath and 0.2 μl of a solution containing 100 μg μl<sup>-1</sup> <sup>13</sup>C18:1<sup>Δ9</sup> (Campro Scientific, Berlin, Germany; *M<sub>r</sub>* 314) dissolved in acetone was applied to the abdominal tip (*n*=12 for each sex) using a 5 μl microsyringe designed for GC on-column injection (Hamilton, Bonaduz, Switzerland). The pure solvent was applied to randomly chosen control wasps of either sex (*n*=12). After 20 h, wasps were freeze-killed and lipids of homogenised wasps (3 wasps per sample resulting in 4 samples per treatment) were extracted with 200 μl dichloromethane and transesterified as described for the yeast lipids. FAME extracts were analysed by GC/MS as described above. The

incorporation of <sup>13</sup>C in C18:2<sup>Δ9,12</sup> was concluded from the occurrence of the diagnostic molecular ion *m/z*=312 at the expected retention time of C18:2<sup>Δ9,12</sup> methyl ester (Blaul et al., 2014). The percentage of <sup>13</sup>C-labelled precursor that was converted into C18:2<sup>Δ9,12</sup> was calculated by relating the peak area of the <sup>13</sup>C-labelled molecular ion of C18:2<sup>Δ9,12</sup> methyl ester (*m/z*=312) to the added peak areas of the <sup>13</sup>C-labelled molecular ions of C18:2<sup>Δ9,12</sup> methyl ester plus C18:1<sup>Δ9</sup> methyl ester (*m/z*=314).

## RESULTS

### Western blot analysis

Western blot analysis of the transformed yeast revealed protein bands at the expected molecular masses of Nvit\_D12a and Nvit\_D12b, which were absent in the respective controls expressing only the empty vector (Fig. S1).



**Fig. 2. Transformed yeast cells expressing Nvit\_D12a or Nvit\_12b synthesise C18:2<sup>Δ9,12</sup>.** GC/MS analysis on a polar BP-20 column of FAMEs extracted from transformed *S. cerevisiae* cells supplemented with C18:1<sup>Δ9</sup>. Cells were transformed with (A) Nvit\_D12a and (B) Nvit\_D12b. Cells carrying the empty vector were used as controls. The results are representative of three replicate experiments.

**Table 1. Results of LC/MS/MS analysis of Nvit\_D12a in the pooled rectal vesicles from 10 *N. vitripennis* males after SDS-PAGE and in-gel trypsin digestion**

GenBank acc. no.	Mascot score		Sequence coverage (%)		Number of peptides		emPAI value <sup>a</sup>	
	I <sup>b</sup>	II	I	II	I	II	I	II
XP_001599836.1	941.6	578.1	42.8	23.5	15	9	2.6	1.04

Protein database searching of the resulting mass spectra was performed using the Mascot search engine.

<sup>a</sup>Exponentially modified protein abundance index; <sup>b</sup>Roman numbers indicate the two individual replicates.

### Chemical analysis of transesterified yeast lipids

FAME analysis of lipid extracts from yeast clones expressing both Nvit\_D12a and Nvit\_D12b showed an additional peak of C18:2<sup>Δ9,12</sup> methyl ester, which was absent in the empty vector controls (Fig. 2). This confirms that both enzymes are Δ12-FADs, converting C18:1<sup>Δ9</sup> into C18:2<sup>Δ9,12</sup>. In contrast, the methyl ester of (9Z,12Z)-hexadeca-9,12-dienoic acid (C16:2<sup>Δ9,12</sup>) was not detected, suggesting that (9Z)-hexadec-9-enoic acid (palmitoleic acid, C16:1<sup>Δ9</sup>) is not accepted as a substrate by the two FADs.

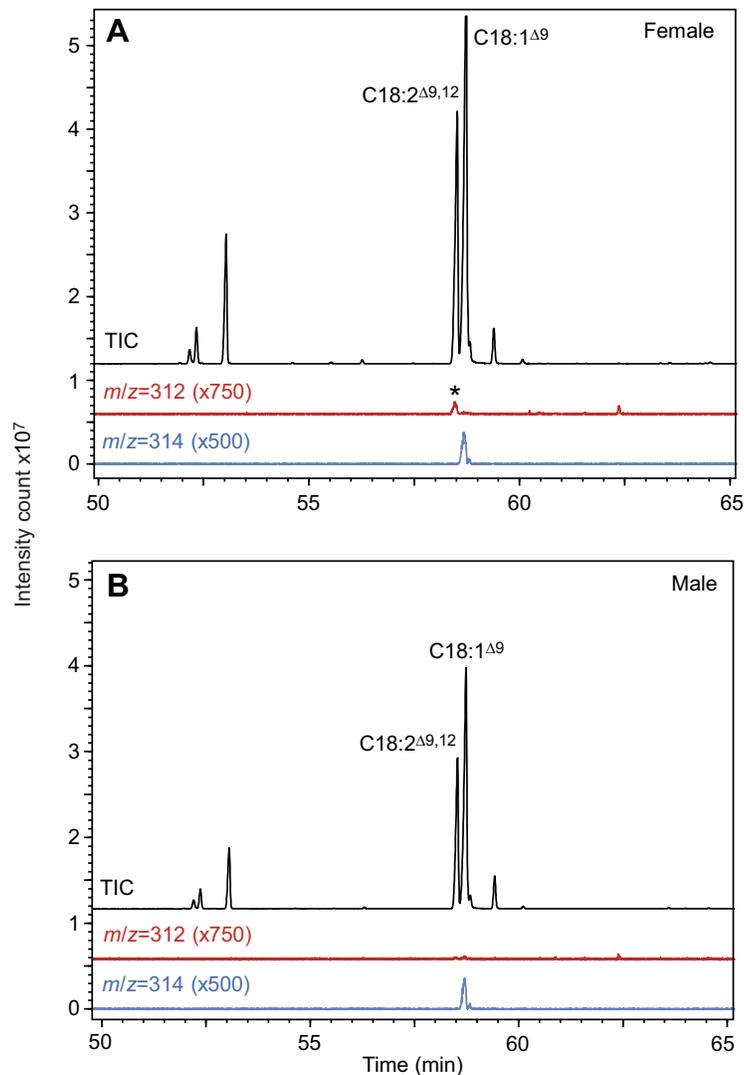
### Protein analysis by LC/MS/MS

The protein analysis by LC/MS/MS demonstrated that the highly male-biased FAD Nvit\_D12a (XP\_001599836.1) reported by Wang et al. (2015) is expressed in the male pheromone glands.

Eleven of the thirteen different peptides detected in the two replicates of the LC/MS/MS analysis were unique for Nvit\_D12a, while the sequences of two detected peptides were shared by Nvit\_D12a and its highly homologous but unbiased paralogue Nvit\_D12b (XP\_001599873.1) (Table 1, Figs S2 and S3). Unique peptides of Nvit\_D12b were not detected, indicating that exclusively Nvit\_D12a is expressed in the male pheromone gland.

### <sup>13</sup>C-labelling experiment with *U. rufipes*

Our <sup>13</sup>C-labelling experiment revealed that *U. rufipes* females are able to convert externally supplied C18:1<sup>Δ9</sup> into C18:2<sup>Δ9,12</sup> (Fig. 3). As calculated by the molecular ion peak areas of the <sup>13</sup>C-labelled FAMES, 11.1±4.6% (mean±s.e.m.) of the labelled C18:1<sup>Δ9</sup> was



**Fig. 3. Female but not male *U. rufipes* are able to synthesise C18:2<sup>Δ9,12</sup>.** GC/MS analysis on a non-polar BPX5 column of transesterified lipid extracts from (A) three female and (B) three male *U. rufipes* wasps to which fully labelled <sup>13</sup>C18:1<sup>Δ9</sup> had been applied. Shown are the total ion chromatograms (TIC) and extracted ion chromatograms (magnification factors given in brackets) of the molecular ions of fully labelled <sup>13</sup>C18:1<sup>Δ9</sup> methyl ester ( $m/z=314$ ) and <sup>13</sup>C18:2<sup>Δ9,12</sup> methyl ester ( $m/z=312$ ). Asterisk indicates peak of labelled <sup>13</sup>C18:2<sup>Δ9,12</sup> methyl ester, which is missing in males, showing that females but not males are capable of synthesising C18:2<sup>Δ9,12</sup>. Results are representative of four replicate experiments for each sex.

converted into C18:2<sup>Δ9,12</sup>. In contrast, no <sup>13</sup>C-labelled C18:2<sup>Δ9,12</sup> was detected in lipid extracts from male wasps (Table S2). Neither diagnostic ions indicative of <sup>13</sup>C-labelled C18:1<sup>Δ9</sup> nor C18:2<sup>Δ9,12</sup> were detected in lipid extracts from wasps of either sex treated with the pure solvent.

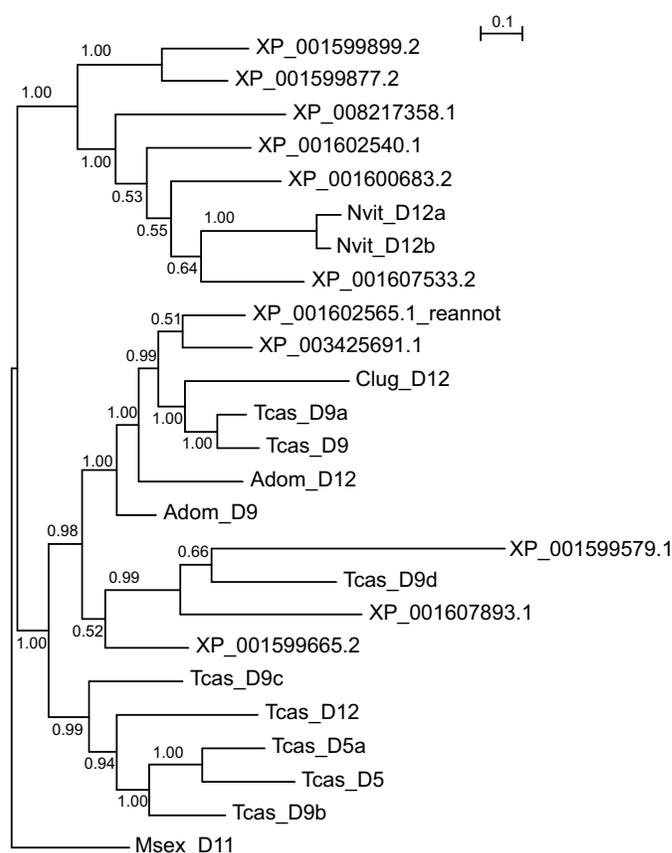
### Sequence comparison and phylogenetic analysis

Multiple sequence alignment of the *N. vitripennis* Δ12-FADs with functionally characterised insect Δ12-FADs from the house cricket *A. domesticus* (Adom\_D12) (Zhou et al., 2008), the red flour beetle *T. castaneum* (Tcas\_D12) (Zhou et al., 2008) and the soldier beetle *C. lugubris* (Clug\_D12) (Haritos et al., 2012), revealed moderate sequence identities of 32–42% and sequence similarities of 46–58% (Fig. S4, Table S3). All insect Δ12-FADs exhibit the typical sequence motifs of the ‘first’ desaturase subfamily described by Hashimoto et al. (2008). Members of this subfamily are supposed to introduce the first double bond in the saturated acyl chain of fatty acids and are characterised by three histidine boxes, the first of which has four amino acids between the histidines (Hashimoto et al., 2008). The phylogenetic analysis of Nvit\_D12a and Nvit\_D12b revealed that the insect Δ12-FADs characterised so far do not cluster together in one clade (Fig. 4). Rather, *N. vitripennis* Δ12-FADs cluster with other predicted, hitherto uncharacterised FADs from the *Nasonia* genome. Likewise, Tcas\_12 occurs in a clade with Δ9- and Δ5-FADs of the same species and Adom\_D12 is more closely related to Adom\_D9 than to the other Δ12-FADs from insects. Finally, the two coleopteran Δ12-FADs Clug\_D12 and Tcas\_D12 are located in different clades with Clug\_D12 clustering with two Δ9-FADs from *T. castaneum*.

### DISCUSSION

Here, we report the functional characterisation of two Δ12-FADs from *N. vitripennis*. Nvit\_D12a and Nvit\_D12b are the fourth and fifth functionally assigned Δ12-FADs in insects and yet the first in the Hymenoptera. The two Δ12-FADs may supply the demand for C18:2<sup>Δ9,12</sup> in *N. vitripennis* to perform numerous functions, including growth, reproduction and brain development (Wathes et al., 2007). As an abundant constituent of phospholipids C18:2<sup>Δ9,12</sup> ensures the fluidity of cell membranes and the mobility of embedded proteins (Cripps et al., 1986; Malcicka et al., 2018; Opekarova and Tanner, 2003). Furthermore, C18:2<sup>Δ9,12</sup> is involved in the biosynthesis of prostaglandins and other eicosanoids, which have various hormone functions in insects (Stanley, 2006). Our data and previous work (Blaul et al., 2014; Wang et al., 2015) strongly suggest that, in addition to these functions, Nvit\_D12a is involved in the biosynthesis of the male sex pheromone. While Nvit\_D12b is expressed in both males and females, Nvit\_D12a is expressed in an extremely male-biased manner (Wang et al., 2015) and our mass spectrometric analyses revealed the presence of Nvit\_D12a in the male pheromone gland. These results demonstrate that Nvit\_D12a enables *N. vitripennis* males to convert C18:1<sup>Δ9</sup> to C18:2<sup>Δ9,12</sup> exactly where this crucial pheromone precursor is needed. This makes pheromone production in *N. vitripennis* males widely independent of C18:2<sup>Δ9,12</sup> derived from dietary or endosymbiotic sources even though a diet rich in C18:2<sup>Δ9,12</sup> may further increase male pheromone titres (Brandstetter and Ruther, 2016).

An important question arising from the present study is whether the ability to synthesise C18:2<sup>Δ9,12</sup> is restricted to *N. vitripennis* or is shared by related species or parasitic wasps in general. All *Nasonia* species, as well as *T. sarcophagae* share C18:2<sup>Δ9,12</sup>-derived (4*R*,5*S*)-5-hydroxy-4-decanolide as the major pheromone



**Fig. 4. Phylogenetic analysis of *N. vitripennis* Δ12-FADs.** The tree shows the relationship of Nvit\_D12a and Nvit\_D12b to other FADs. These are predicted FADs from the *N. vitripennis* genome (indicated by GenBank accession numbers starting with ‘XP’) (Blaul et al., 2014; Niehuis et al., 2011; Wang et al., 2015), insect Δ12-FADs from red flour beetle *T. castaneum* (Tcas\_D12, Zhou et al., 2008), house cricket *A. domesticus* (Adom\_D12, Zhou et al., 2008) and soldier beetle *C. lugubris* (Clug\_D12, Haritos et al., 2012), as well as functionally characterised FADs from the same species (all accession numbers given in the Materials and Methods). Numbers following ‘D’ indicate position of the introduced double bond. Δ11-FAD from tobacco hornworm *M. sexta* (Msex\_D11) functioned as outgroup. Posterior probabilities are given at the branching points. The length of the bar corresponds to 0.1 mutations per site.

component (Niehuis et al., 2013). Furthermore, the homologue of Nvit\_D12a in *N. giraulti* is, as in *N. vitripennis*, expressed in a strongly male-biased manner (Wang et al., 2015). These findings suggest that homologues of Nvit\_D12a have the same function in pheromone biosynthesis in these species. Our <sup>13</sup>C-labelling experiments revealed that females but not males of *U. rufipes* are capable of synthesising C18:2<sup>Δ9,12</sup>. The abdominal sex pheromone of *U. rufipes* males is used in a similar manner as it is in *Nasonia* but is synthesised independently of C18:2<sup>Δ9,12</sup> via the mevalonate pathway (Ruther et al., 2019). Hence, Δ12-FADs appear to be common in species of the *Nasonia* group, and have acquired an additional function in the context of sexual communication in species using fatty acid-derived male sex pheromones. This adaptation might have been achieved by male-biased gene expression and/or sequence-related modifications.

Eukaryotic FADs show the characteristic features of membrane-bound, oxygen-dependent di-iron-containing enzymes sharing three conserved histidine-rich motifs (histidine boxes) coordinating two iron ions in the active site (Shanklin et al., 1994; Tupec et al., 2017). A

phylogenetic and motif analysis of 275 FAD sequences from 56 eukaryotic genomes revealed that these formed four functionally distinct subfamilies, which can be distinguished by the sequences of the histidine boxes (Hashimoto et al., 2008). Enzymes of the 'first' subfamily are proposed to introduce the first double bond, typically at position 9, into a saturated acyl chain, while members of the 'omega' subfamily introduce a double bond between an existing double bond and the acyl end. Hence,  $\Delta 12$ -FADs can be expected to show the structural motifs of the omega subfamily. While predicted  $\Delta 12$ -FADs from the omega subfamily have indeed been found in the genomes of whitefly and locust (Kabeya et al., 2018), Nvit\_D12a and Nvit\_12b as well as all other functionally characterised  $\Delta 12$ -FADs from insects, match the structural motifs of the first subfamily desaturases. This suggests that the  $\Delta 12$ -FADs from *N. vitripennis*, *T. castaneum*, *C. lugubris* and *A. domesticus* have evolved independently from ancestral  $\Delta 9$ -FADs in the respective genomes. This hypothesis is supported by our phylogenetic analysis showing that the insect  $\Delta 12$ -FADs do not cluster together but are more closely related to  $\Delta 9$ -FADs and other first-type desaturases. Hence, apart from using classical omega-type  $\Delta 12$ -FADs, insects have developed an alternative route to synthesise C18:2 <sup>$\Delta 9,12$</sup>  by modification of first-type desaturases.

The present study sheds light on the poorly understood selection pressures driving the evolution of  $\Delta 12$ -activity in desaturases. Pheromone quantity is an important factor for the sexual attractiveness of *N. vitripennis* males. Virgin females prefer higher pheromone doses over lower ones and discriminate against pheromone markings of multiply mated, pheromone-depleted males (Ruther et al., 2009). Thus, the production of the pheromone precursor C18:2 <sup>$\Delta 9,12$</sup>  was likely a key innovation for their reproductive success.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: F.S., N.K., O.M., R.S., J.R., J.E.; Methodology: F.S., N.K., A.B., J.R.; Formal analysis: F.S., K.S., R.M.; Investigation: F.S., N.K., M.M., A.B., B.B., J.R.; Writing - original draft: F.S., J.R.; Writing - review & editing: N.K., M.M., R.M., O.M., R.S., J.E.

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#### Supplementary information

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