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ABSTRACT

Baculoviruses are double-stranded DNA entomopathogenic viruses that infect predominantly insects of the order Lepidoptera. Research in the last decade has started to disentangle the mechanisms underlying the insect-virus interaction, particularly focusing on the effects of the baculovirus infection in the host's physiology. Among crucial physiological functions, olfaction has a key role in reproductive tasks, food source detection and enemy avoidance. In this work, we describe that Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) induces expression changes in some odorant receptors (ORs) - the centrepiece of insect's olfaction - when infecting larvae from its natural host *Spodoptera exigua* (Lepidoptera: Noctuidae). Different ORs are up-regulated in larvae after SeMNPV infection, and two of them, SexiOR35 and SexiOR23, were selected for further functional characterization by heterologous expression in empty neurons of *Drosophila melanogaster* coupled to single-sensillum recordings. SexiOR35 appears to be a broadly tuned receptor able to recognise multiple and different chemical compounds. SexiOR23, although correctly expressed in *Drosophila* neurons, did not display any significant response to a panel of 58 stimuli. Behavioural experiments revealed that larvae infected by SeMNPV exhibit altered olfactory-driven behaviour to diet when it is supplemented with the plant volatiles linalool or estragole, two of the main SexiOR35 ligands, supporting the hypothesis that viral infection triggers changes in host perception through changes in the expression level of specific ORs.

1. Introduction

Baculoviruses are a large family of viruses with double stranded DNA genomes able to infect more than 700 insect species belonging to the orders Lepidoptera, Diptera and Hymenoptera (Slack and Arif, 2006; van Oers and Vlak, 2007). Due to their specificity that make them harmless to humans and non-target insects, coupled with a high persistence in the environment, baculoviruses are widely used in pest control (Moscardi et al., 2011). They have a strong pathogenic activity in larvae, producing a systemic infection in their host through replication in different tissues such as fat body, trachea, midgut, muscles and nervous system (Passarelli, 2011; Torquato et al., 2006).

During the evolutionary arm race between baculoviruses and Lepidoptera, the pathogens have developed strategies that alter the host's physiology and behaviour to finally improve virus incidence in the environment (Gasque et al., 2019; Kong et al., 2018; Rebolledo et al., 2015). For instance, baculovirus-infected *Bombyx mori* larvae have an enhanced locomotion activity that supposes a better viral dispersion when the death of the insects occur (Kamita et al., 2005). Another example is the climbing behaviour that baculovirus-infected larvae show (called tree-top disease). These, climb at higher positions than not infected counterparts, where they liquefy after death and efficiently spread the viral particles in the environment (Goulson, 1997; Hoover et al., 2011; Van Houte et al., 2015). Both manipulations of hosts' phenotype are triggered by baculovirus-encoded genes that had been ancestrally acquired from Lepidoptera (Han et al., 2015; Kamita et al., 2005).

Behaviour strongly depends on the nervous system, thus, the altered phenotypes displayed by infected larvae might be mediated through changes in the host peripheral (PNS) and central nervous systems (CNS), where the insect body's functions are mainly controlled (Gasque et al., 2019; Kinoshita and Homberg, 2017; Llopis-Giménez et al., 2021). The PNS includes sensory neurons that receive external stimuli from the environment. In insects, chemical stimuli are detected by chemosensory

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neurons encapsulated within cuticular structures called chemosensory sensilla. Detection of volatile and water-soluble chemical cues is mediated by the actions of several receptors and binding proteins that interact with the stimuli, allowing the insects to taste and smell. Olfaction is of pivotal importance to the insect's biology, since it influences many fundamental aspects as mating, egg-laying, food choice and predator avoidance (Jacobson, 1966). It is mainly carried out by receptors belonging to two gene families: the odorant receptors (ORs) and the ionotropic receptors (IRs), which have been initially discovered in *Drosophila melanogaster* in the first decade of 2000 (Abuin et al., 2012; Benton et al., 2009; Clyne et al., 1999; Pelosi et al., 2005; Vosshall et al., 1999) and subsequently characterized in many other insect species. In particular, ORs, which have been considered the olfaction centrepiece (Breer et al., 2019), are present in almost every insect lineage and they likely evolved during adaptation to terrestriality (Brand et al., 2018). They are seven-transmembrane receptors situated in the membrane of the olfactory receptor neurons, which are housed in sensilla located in the antennae and the maxillary palps. Functional studies in *Drosophila* genus and in an handful of other insect species, have shown that each OR is specialized in detecting specific odorants, and it normally acts together with the OR-coreceptor (Orco), forming an heteromeric complex (Larsson et al., 2004). After the activation of the complex, a cation exchange occurs leading to the membrane depolarization that starts the consequent signal transmission. ORs display a varying degree of specificity: some of them have a high degree of selectivity and recognise only few odorants, while others respond to a broad spectrum of different stimuli (De Bruyne and Baker, 2008). In Lepidoptera, the most well-known narrow-spectrum ORs are those involved in sex pheromone recognition, which are even able to discriminate between enantiomeric forms in some species (Wang et al., 2018).

Baculoviruses enter the insect's body through the oral cavity. In Lepidoptera, the primary infection occurs when the virus attacks midgut epithelial cells and in the secondary infection, the budded forms of baculovirus spread to within insect body through a clathrin-mediated adsorptive endocytosis, reaching almost all tissues, including brain (Clem and Passarelli, 2013; Ikeda et al., 2015). Indirect evidence showed that infection also reaches the antennae of the larvae (Dhungel et al., 2013; Naik et al., 2018). This, coupled with the parasitic manipulation of host behaviour triggered by baculovirus, let us hypothesize that baculovirus infection could alter the expression of some key genes in the peripheral nervous system of the lepidopteran hosts.

Spodoptera exigua (Hübner, 1808) (Lepidoptera: Noctuidae) constitutes an excellent model for studying host-pathogen interactions since its larvae are susceptible to a species-specific (Spodoptera exigua multiple nucleopolyhedrovirus, SeMNPV) as well as to a generalist baculovirus (Autographa californica multiple nucleopolyhedrovirus, AcMNPV) (Crava et al., 2015; Han et al., 2015; Jakubowska et al., 2013; Van Houte et al., 2014). In addition, the *S. exigua* repertoire of larval-expressed chemosensory genes has been recently described by our group (Llopis-Giménez et al., 2020). Here we describe on expression changes of *S. exigua OR* (*SexiOR*) in whole larval heads upon SeMNPV or AcMNPV infection, identifying a set of ORs whose transcript levels specifically varied after SeMNPV infection. Two SexiORs whose expression was up-regulated after SeMNPV infection were then functionally characterized (deorphanized) using the *Drosophila* empty neuron system (Dobritsa et al., 2003) to identify their ligands. Further behaviour analysis revealed that the species-specific SeMNPV infection is correlated with changes in larval perception of two of the identified ligands.

2. Materials and methods

2.1. Insects

The *S. exigua* colony used for all the experiments was originally provided by Andermatt Biocontrol AG (Switzerland) and has been maintained at the University of Valencia (Spain) for more than 100 generations. Larvae have been reared on artificial diet (Elvira et al., 2010) at 25 ± 3 °C with 70 ± 5% relative humidity, using a photoperiod of 16:8 h (light:dark).

2.2. Viral infections

Newly molted third-instar *S. exigua* larvae were infected with wild-type (WT) SeMNPV SP2 strain (Murillo et al., 2006) or WT AcMNPV C6 strain (Martin et al., 1994) using a concentration that killed about 90% of the larvae (10^6 and 10^7 OBs/ml for SeMNPV and AcMNPV, respectively). Virus was delivered using the droplet feeding method, in a 10% sucrose solution stained with 0.5% of Phenol red dye (Sigma). Control larvae were fed with the same solution containing no virus (mock infection). For RNA extraction, at 96 h post-infection (hpi), larval heads were excised from the body using a scalpel, pooled and stored in 300 μl of TRIzol (Roche) at −80 °C. Each replicate consisted of sixteen pooled heads, and three independent replicates were processed for each treatment.

2.3. High-throughput sequencing

Total RNAs from SeMNPV- and mock-infected larval heads was purified using TRIzol reagent following the manufacturer's instructions. A second purification step was done using the RNeasy Mini Kit (Qiagen). Purified RNAs were eluted with water, and RNA integrity was quality checked with an Agilent Bioanalyzer. Quality check, library preparation and paired-end (PE) RNA sequencing on an Ilumina Hiseq2000 machine were carried out by Novogen Technology (China). The PE-150 bp long reads obtained were used for gene expression analysis as described later. Raw reads are available at NCBI SRA database (Project number PRJNA634227).

2.4. RNA-seq transcript quantification

Raw reads were trimmed with Trimmomatic v0.39 (Bolger et al., 2014). Expression of *SexiORs* upon SeMNPV infection was analysed by mapping the trimmed reads (three replicates for each condition) to the *SexiOR* repertoire annotated from *de novo* transcriptome in *S. exigua* (Llopis-Giménez et al., 2020). In addition, the infection of head tissues was confirmed by mapping reads to SeMNPV genes (*DNApol* and *ie-l*). Mapping was performed with Bowtie2 (version 2.3.4.3) (Langmead and Salzberg, 2012) and raw counts were estimated using RSEM (version 1.3.0).

2.5. Expression analysis by real time quantitative PCR (RT-qPCR)

RNAs from SeMNPV-, AcMNPV- and mock-infected larval heads were purified as described above and used to prepare cDNAs (500 ng RNA/sample). Samples were first treated with DNaseI (ThermoFischer Scientific) following manufacturer's protocol and then converted into cDNAs using PrimeScript RT Reagent (Takara), random hexamers and oligo (dT) primers. RT-qPCRs were performed in a StepOnePlus Real-time PCR system (Applied Biosystems) using 5x HOT FIREpol Eva Green qPCR Mix Plus (ROX) (Solis Biodyne) in a total reaction volume of 20 μl. An endogenous control consisting of *ATP synthase subunit C* housekeeping gene was used in each RT-qPCR to normalize the RNA concentration (Bel et al., 2013). Forward and reverse primers were designed using the online software tool Primer3Plus (Untergasser et al., 2007) and sequences are provided in the Supplementary Table 1. The differences in expression between treatments (control and infected) were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Only expression changes statistically significant and greater than 2-fold were considered. Graphs and statistical analysis (unpaired *t*-test with Welch's correction) were performed using GraphPad Prism software (version 8.0.1).

2.6. Heterologous expression of SexiORs in Drosophila

Selected *SexiORs* were functionally characterized by expression in the so-called empty-neuron system, in which heterologous ORs are expressed in *D. melanogaster* ab3A neurons devoid of its own OR22a (Dobritsa et al., 2003). For the construct generation, the open reading frame (ORF) of *SexiOR23* was amplified and cloned in the destination vector, *pUAST.attB*, using the *Not*I and *Xba*I sites, generating *pUAST.attB-SexiOR23*. The ORF of *SexiOR35* was synthetized and cloned in *pUAST.attB* by Genscript Biotech (USA), generating *pUAST.attB-SexiOR35*. Afterwards, presence of the correct ORF in both constructs was confirmed by Sanger sequencing. Both plasmids were purified from liquid cultures of *Escherichia coli* DH10β using the Illustra plasmidPrep Midi Flow Kit (GE Healthcare).

Transformant *D. melanogaster* lines were generated by BestGene Inc. (Chino Hills, CA, USA), using the PhiC31 integrase system. *pUAST.attB-SexiOR23* and *pUAST.attB-SexiOR35* plasmids were injected separately into *D. melanogaster* embryos with the genotype y^1 *M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH*-86Fb, leading to the insertion of the *UAS-SexiOR* constructs into the third chromosome. The *UAS-SexiOR* lines were then crossed with the *Drosophila* Gal4-driver w; Δhalo/CyO; Or22a-Gal4 line (de Fouchier et al., 2017; Dobritsa et al., 2003) to generate flies which express one of the *SexiOR* transgenes in ab3A neurons. The presence of *UAS-SexiOR* transgenes was verified by PCR on genomic DNA extracted from two flies, and the expression of the correct *SexiOR* in *Drosophila* antenna was verified by RT-PCR on total RNA extracted from a pool of antennae. The recombinant *D. melanogaster* flies were reared on standard cornmeal-yeast-agar medium (Bass et al., 2007) at 25 °C, using a photoperiod of 12:12 h (light:dark) and used for SSR experiments.

2.7. Single-sensillum recordings

SSR experiments were performed on flies expressing *SexiOR23* and *SexiOR35* using a stimulation panel of multiple odorants representative of aliphatic, aromatics and terpenes, all of which are widely occurring odorants emitted by numerous flowering plants (de Fouchier et al., 2017). This was all performed according to the experimental procedure described by de Fouchier et al. (de Fouchier et al., 2017). Briefly, randomly chosen recombinant flies (females from 2 to 6 days old) were restrained in a plastic pipette tip with only the head exiting from the end. Pipette tip with the fly was fixed with dental wax on a microscope glass side, with the ventral part of the fly facing up. The antenna was fixed placing a glass capillary held by dental wax between the second and the third antennal segments, since in *Drosophila* olfactory sensilla are only present in the third segment. The slides were put under a light microscope (BX51WI, Olympus) equipped with a magnification objective (LMPLFLN 50X, Olympus). Response spectra of ab3A neurons expressing *SexiORs* were stimulated with a panel of 58 odorants listed in the Supplementary Table 2 to establish SexiOR response spectrum. The compounds were used at a 10 μg/μl in paraffine oil, except indole, which was diluted in hexane. Ten μl of each dilution were deposited on a filter paper, which was then inserted into a Pasteur pipette. Pipettes with filter papers containing 10 μl of solvent were used as controls. Odorant stimulations consisted of a 500 ms air pulse (0.6 l min^{-1}) into a constant flux of humidified air (1.5 l min^{-1}) directed to the antenna. The action potentials were recorded using electrolytically sharpened tungsten electrodes, one inserted inside the eye of the fly as a reference and the other inserted at the base of the sensillum of interest. The entire odorant panel was tested six times on six different flies. Each stimulation cartridge was used at most two times on each fly and a maximum of five times in total. Responses of ab3A neurons (in spikes.s^{-1}) were calculated by subtracting the spontaneous firing rate from the firing rate during the odorant stimulation (time windows of 500 ms) (Dweck et al., 2016). To ensure we recorded ab3 sensilla, 100 ng of the diagnostic stimulus 2-heptanone were used, as it is known to activate the ab3B neuron also housed in ab3 sensilla (Hallem and Carlson, 2006). The absence of DmelOR22a in ab3A neurons expressing a SexiOR was verified using 100 ng of ethyl hexanoate, a strong ligand for DmelOR22a (Hallem and Carlson, 2006). Odorants were considered active if the response they elicited was statistically different from the response elicited by the solvent alone (Kruskal-Wallis ANOVA followed by a Dunn's *post hoc* test $p < 0.001$). Statistical analysis and tuning breadth graphs were performed with GraphPad Prism Software. Heatmaps were generated using the gplots (version 3.0.3) and RColorBrewer (version 1.1–2) packages of R software.

2.8. Behavioural assays

Behavioural assays were performed to study the effect of three volatile organic compounds (VOCs) to SeMNPV-, AcMNPV- and mock-infected *S. exigua* larvae in a complex background. *S. exigua* third-instar larvae were infected following the same procedure and viral concentrations as described above. Behavioural experiments were carried out at 96 hpi in order to assure that baculovirus infection had already reached the larval head (Gasque et al., 2019; Van Houte et al., 2014). After the end of each assays, the larvae were kept at 25 °C to confirm the absence of mortality in the non-infected larvae (mock--infected) and 90% mortality by baculovirus in the infected insects.

The behavioural assay was carried out as detailed by Llopis-Giménez et al. (2020). Briefly, every experimental run consisted of ten fifth-instar *S. exigua* larvae placed in one side of a 14 cm diameter Petri dish (including the lid) with a piece of artificial diet (1.5 × 0.8 × 1 cm) placed at the opposite side. To score movement of each larva, a template with curved lines (numbered 1–9 from the starting point to the other side) was placed under the Petri dish. The Petri dish was put inside a paperboard box (30 × 22 × 22 cm) with a hole in the side of box (6 cm of diameter) to include a 50 W halogen artificial light (at 15 cm of distance to the Petri dish). The piece of artificial diet, the light and the temperature emitted by the light serves as attractants for the larvae, stimulating their movement to the other side of the dish. In each treatment run, fifty μl of the odorant diluted in methanol (Labkem) were added to the piece of artificial diet whereas in a parallel control run 50 μl of the solvent alone were added. Walked distances (Supplementary Movie 1) were measured and used to define the mobility index (sum of the scores −1 to 9 according to the larval position at the defined time point (2, 5 and 10 min) - obtained by each of the 10 larvae) for both treatment and control run. Side-by-side recorded mobility indexes for the control and the treatment were used to calculate the attraction index by dividing the mobility index in presence of the odorant by that obtained in the parallel control run. Side-by-side runs (treatment + control run) were replicated a total of nine times (three replicates with three different batches of larvae, *i.e.* larvae from different offspring) for each odorant concentration and condition (AcMNPV-infected, SeMNPV-infected and mock-infected larvae). Values of the attraction index higher than 1 mean that the larvae walked closest (*i.e.* are likely more attracted) to the diet + odorant than to diet only (attraction effect). Values lower than 1 mean that larvae walked less to the diet + odorant than to diet only (*i.e.* deterrent effect). Values close to 1 mean that larvae walked the same distance as in the control, thus not displaying any attraction nor deterrence.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ibmb.2021.103648

Three VOCs were selected for the behavioural experiments accordingly to deorphanization results: estragole and linalool that strongly activated SexiOR35; and indanone that did not activate SexiOR35. Doses (5, 50 and 500 μg) were selected in a fashion similar to doses used in previous behavioural assays with the close related species *Spodoptera littoralis* (de Fouchier et al., 2018). Statistical analyses were conducted using a one-sample *t*-test comparing the attraction index with the theoretical value of 1. Graphs and statistical analyses were performed using GraphPad Prism software. Boxplot graph was obtained using the

car (version 3.0–8) and tidyverse (version 1.3.0) packages of R software (R Core Team).

3. Results

3.1. SeMNPV specifically drives expression changes of selected SexiORs

The effect of SeMNPV infection on the expression of the *SexiORs* was initially assessed using RNA-Seq data obtained from whole heads of SeMNPV-infected and control larvae. These latter samples only fed on a droplet solution that did not contain any virus particle. SeMNPV presence in infected larval head was confirmed by mapping reads against two viral genes (*DNApol* and *ie-1*) (Supplementary Table 3). Total counts profiled by mapping trimmed reads to the repertoire of 63 *SexiORs* were low (from 0 to 6), except for *SexiOrco, SexiOR44 and SexiOR63* (Supplementary Table 3). Since the count number was inadequate to achieve statistical significance, we used the total counts to select some *SexiORs* that displayed potential differences between SeMNPV- and mock-infected samples to verify their expression by RT-qPCR (*SexiOR19, SexiOR23, SexiOR34, SexiOR35, SexiOR40c, SexiOR44 and SexiOR63*). As a control, we included in the RT-qPCR analysis some other *SexiORs* whose read counts did not seem different between the treatments (*SexiORco, SexiOR10, SexiOR25 and SexiOR45*). RT-qPCRs were performed using cDNA retro-transcribed from the same RNA samples used for RNA-Seq. Results showed that five genes were upregulated in larval heads after the SeMNPV infection: *SexiOR23* (29.3-fold change), *SexiOR35* (71.5-fold change), *SexiOR40c* (14.3-fold change), *SexiOR44* (136.7-fold change) and *SexiOR63* (17.3-fold change) (Fig. 1).

To study if the observed changes in *SexiOR* expression after SeMNPV infection were also triggered by infection by a generalist baculovirus, a similar RT-qPCR expression analysis was performed in AcMNPV- and mock-infected larval heads. In contrast to the results observed with SeMNPV, no *SexiOR* showed a significant regulation except *SexiOR40c* that appeared to be upregulated upon AcMNPV infection (5.6-fold change) (Fig. 1). These results suggest that the observed *SexiOR* expression changes are in most cases unique to the specialist SeMNPV.

3.2. SexiOR35 is a broadly-tuned odorant receptor

Among *SexiORs* that were differently expressed upon SeMNPV infection, two of the most up-regulated ORs, *SexiOR23* and *SexiOR35*, were selected for functional characterization by expression in the so-called empty-neuron system (Dobritsa et al., 2003) and SSR against a panel of 58 odorants (see material and methods section). *SexiORs* chosen for deorphanization were selected based on their expression pattern but also because they had been previously studied in a closely related specie, *S. littoralis* (de Fouchier et al., 2017). RT-PCR confirmed the expression of both *SexiOR* transcripts in *D. melanogaster* antennae and the observation of spontaneous activity in transformed ab3A neurons suggested the correct expression of both proteins. SexiOR35 responded to 24 different volatiles out of 58 (41.4% of the tested odorants), thus showing a broad spectrum profile. The strongest responses were to aromatics and terpenes: 1,4 dimethylbenzene (85.6 spikes.s^{-1}), 3-carene (68.3 spikes.s^{-1}), acetophenone (49.11 spikes.s^{-1}), estragole (68.3 spikes.s^{-1}), (±)-linalool (32 spikes.s^{-1}), citral (56 spikes.s^{-1}) and *p*-cymene (75.3 spikes.s^{-1}) (Fig. 2A). The broad spectrum of SexiOR35 was illustrated by the sparseness value of the distribution (S) ($S = 0.16$) (Fig. 2B). SexiOR23 was not activated by any of the 58 volatile compounds tested (Fig. 2A).

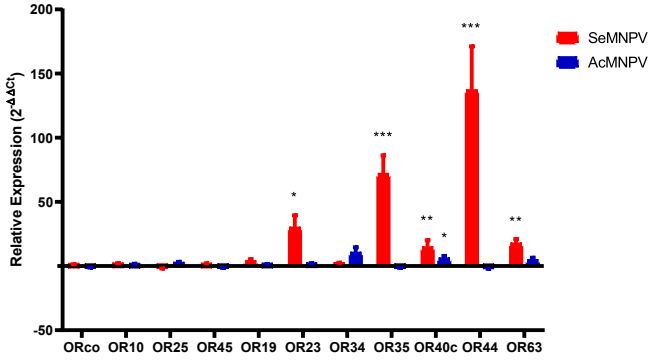

Fig. 1. Differential analysis of selected odorant receptors (ORs) in *Spodoptera exigua* whole heads after infection with SeMNPV and AcMNPV. mRNA levels were quantified with real-time quantitative PCR and data were obtained through the $2^{-\Delta\Delta Ct}$ method. Red color indicates expression changes upon SeMNPV infection and blue expression changes upon AcMNPV infection, compared to the expression in mock-infected samples. Asterisks indicate statistically significant differences between pairwise non-infected and infected samples (unpaired *t*-test with Welch's correction) ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. SeMNPV infection alters larval behaviour to odorant cues

To understand the effects of the observed changes in the expression of SexiORs in a behavioural context, larval response to some SexiOR35 ligands was recorded in mock and infected larvae. Results showed that diet + linalool was more attractive than diet alone to mock-infected larvae at the lowest dose (5 μg) and at the last time point (10 min) (Fig. 3A). In contrast, SeMNPV- and AcMNPV-infected larvae did not display any significant response. At an intermediate dose (50 μg), linalool lost its attraction to mock-infected larvae but had a significant deterrent effect specifically on SeMNPV-infected larvae at 5 and 10 min (Fig. 3A). At the highest dose (500 μg), significant responses disappeared. Estragole had a deterrent effect to mock-infected larvae exposed to both low and intermediate doses. This effect was not observed in SeMNPV and AcMNPV-infected samples at any dose (Fig. 3B). Lastly, 1-indanone only produced attraction to SeMNPV-infected larvae at one time point (2 min) and at a single dose (50 μg) (Fig. 3C).

Since SeMNPV infection might affect the movement mobility of *S. exigua* larvae, we compared the travelled distances of all mock-, AcMNPV- and SeMNPV-infected larvae across all control runs (diet + solvent) in our experimental setup. No differences were observed (Fig. 3D), indicating that, under our experimental arena, infection itself was not promoting or decreasing larval mobility.

4. Discussion

In this work we have started to describe the changes occurring during the SeMNPV infection in the chemoperception of *S. exigua* larvae, combining gene expression analysis, functional studies and behavioural experiments. According to our data, some *SexiOR* transcripts were strongly up-regulated after infection with SeMNPV. Two of these were selected for functional characterisation in order to unveil their function in the insect's olfaction. Result showed that SexiOR35 was a broadly-tuned odorant receptor that recognised multiple odorant molecules with different chemical structures, mainly aromatics and terpenes. Subsequent behavioural assays with SeMNPV-infected larvae established a link between the *SexiOR35* expression changes and olfactory-driven behaviours. To our knowledge, this represents the first study that shows altered host's olfaction in relation to baculovirus infection and allows us to hypothesize about the biological meaning of these changes in the host-pathogen interaction.

During the coevolution between baculoviruses and hosts, baculoviruses have evolved different mechanisms that permit them to improve fitness and dispersion during the infection process. Many of these strategies involve alterations of some physiological and behavioural aspects of hosts (Gasque et al., 2019). The underlying mechanisms of baculovirus manipulation are likely to involve modifications at neuronal level,

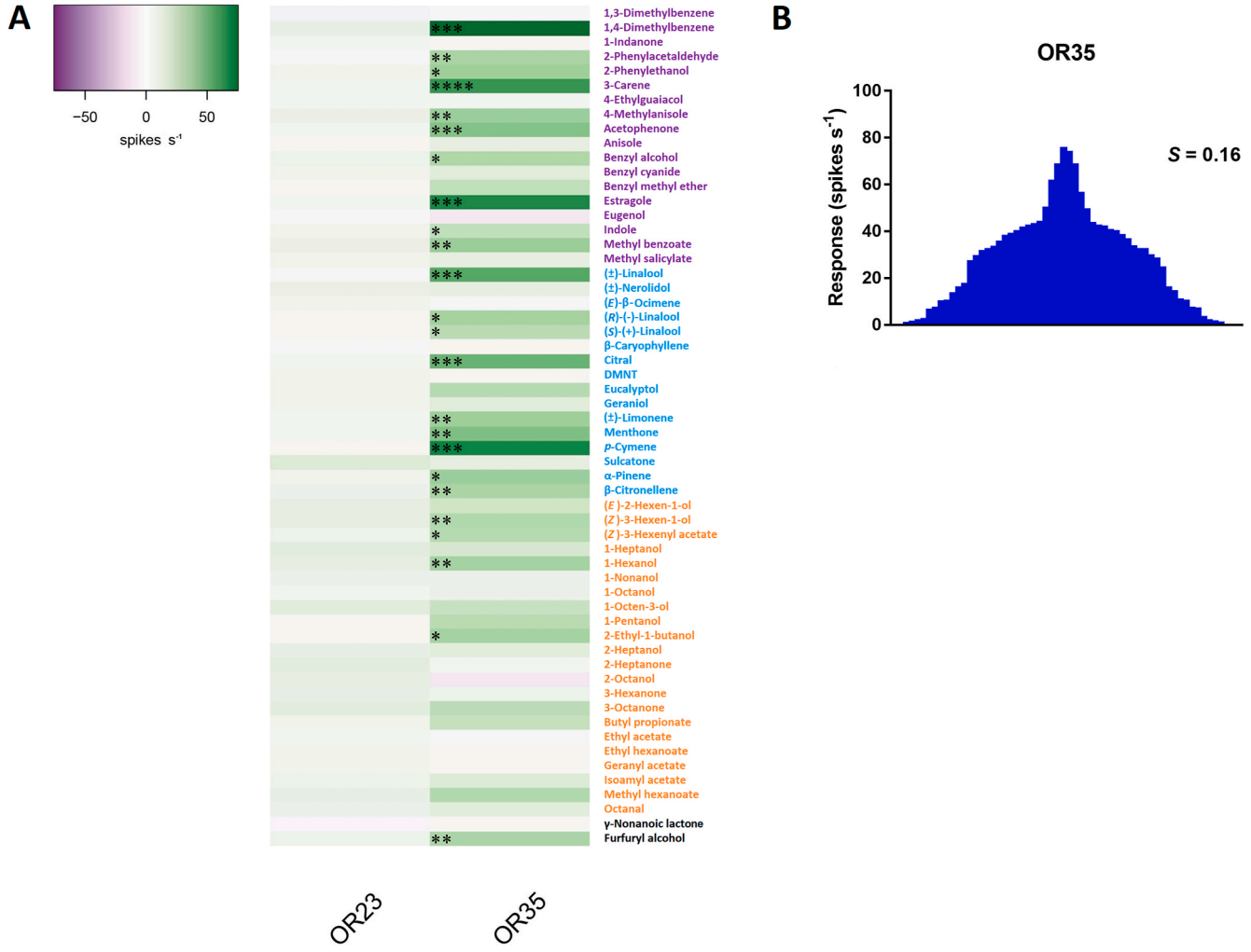

Fig. 2. Response spectra at high-stimulus doses of SexiOR35 and SexiOR23 expressed in *Drosophila melanogaster* empty neuron system. A) Heat map summarizing the mean responses of both ORs to a panel of 58 odorants (100 μg) on the filter paper. Responses (firing rate in spikes s^{-1}) are coloured according to the scale on the left. Odorants are classified depending on their chemical class (magenta, aromatics; cyan, terpenes; orange, aliphatics; black, unclassified). B) Tuning curve of SexiOR35, showing the distribution of mean responses to the panel of 58 odorants. The tunning breath of the receptor is represented by the spareness value of the distribution (*S*) (Rolls and Tovee, 1995). A low *S* value indicates a broad tuning and a value of 1 indicates a narrow tuning of the receptor. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

as observed in behavioural manipulations induced by parasites other than viruses (Hughes and Libersat, 2018; Perrot-Minnot and Cézilly, 2013). Baculoviruses reach the brain of infected larvae (Herz et al., 2003; Katsuma et al., 2012; Knebel-Mörsdorf et al., 1996; Torquato et al., 2006), and likely the antennae (Dhungel et al., 2013; Naik et al., 2018), where the neuronal activity at both CNS and PNS level can be manipulated. We recently showed that a transcript coding for a potential neuropeptide, *proctolin-like*, is clearly down-regulated in the brain of SeMNPV-infected larvae (Llopis-Giménez et al., 2021). Here we show the up-regulation of five *SexiORs*. Such transcriptional variations seem to be associated with the species-specific SeMNPV infection since the generalist AcMNPV did not promote the same effect. We must be aware that the experimental setup of our study made impossible to distinguish between direct regulation exerted by the virus, larval response against the infection or side effect of the pathological processes. In some baculovirus-host systems, a direct regulation by baculovirus-encoded miRNA has been observed targeting host's immune system genes, such as that of hemolin or prophenoloxidase that are involved in the host's defense against viruses (Singh et al., 2010; Tang et al., 2019), as well as in preventing early apoptosis and consequently enhancing virus replication in the insect's cells (Chejanovsky, 2016; Kong et al., 2018). Changes in transcription of genes involved in peripheral chemoreception upon baculovirus infection have been overlooked by previous RNA-Seq studies detailing expression changes between infected and non-infected larvae in several baculovirus-host models (Bhattarai et al., 2018a, 2018b; Wang et al., 2015; Zhang et al., 2018). This may be due to the use of composite tissues that diluted the transcription signal of mRNAs expressed in few cells, such as chemosensory receptors (Johnson et al., 2013). We forecast that further gene expression analysis using only antennae or specific larval head tissues of SeMNPV-infected larvae, although challenging for the small size of the samples, might show finer OR regulation, as well as expression changes of other genes regulating the complex behavioural changes affected by baculovirus infection.

We used the powerful *Drosophila* empty neuron system to deorphanize two SexiORs whose expression was altered by SeMNPV infection. This technique consists of generating recombinant *D. melanogaster* flies that express the heterologous SexiOR in "empty" ab3A neurons, whose ligands can be identified through SSR (Peterlin et al., 2014). Our results complement previous functional studies on ORs from *S. exigua*, in which three ORs (SexiOR3, tuned to terpenoids, and SexiOR13 and 16, tuned to pheromones) were deorphanized (Liu et al, 2013, 2014), laying the foundation towards the full understanding of the

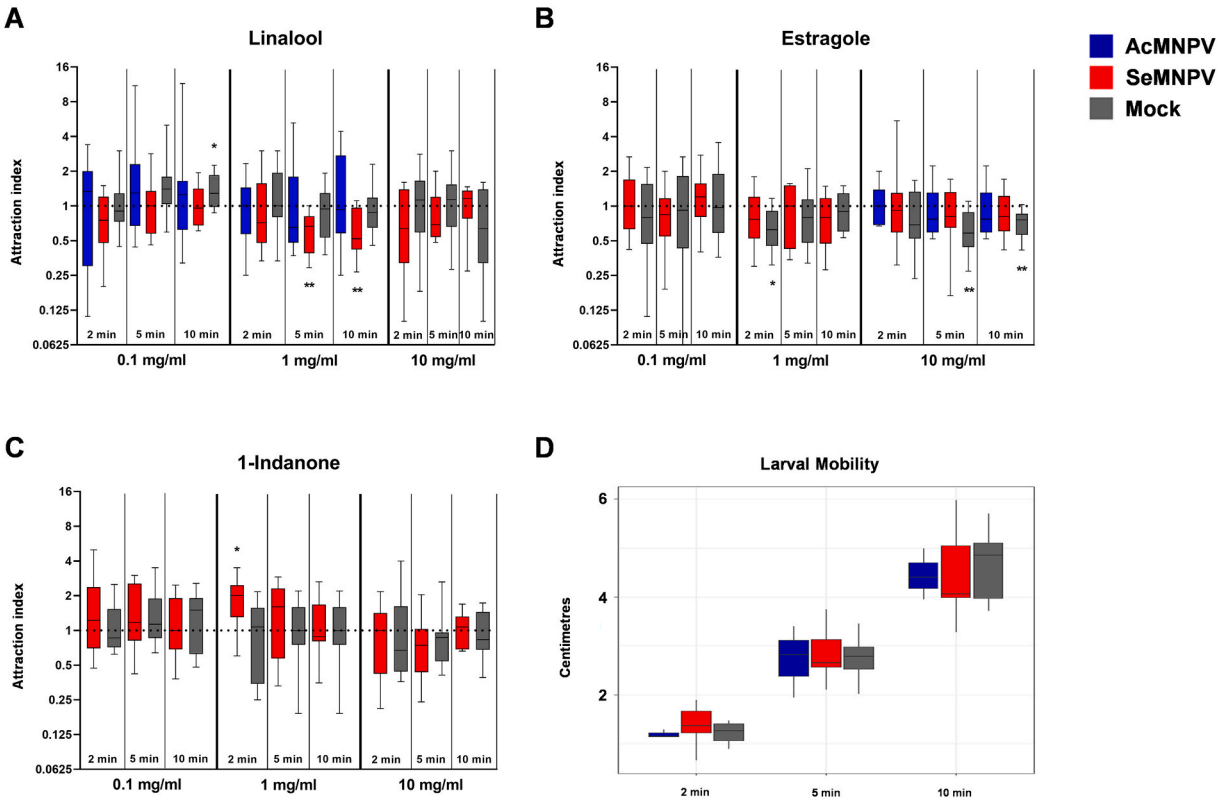

Fig. 3. Attraction index of *Spodoptera exigua* larvae to different odorant stimuli at different times and doses. Behavioural set-up and index calculation are as described in (Llopis-Giménez et al., 2020). Fifty μl of 0.1, 1 and 10 mg/ml dilutions of each odorant were used for the bioassay. Results are shown as attraction indexes, obtained by dividing the mobility indexes (score from 0 to 9 representative of the walked distance from the starting point) in presence of the odorant by that in the parallel control run. Indexes were calculated at 2 min, 5 min and 10 min time points. Values above 1 are indicative of attraction and values below 1 are indicative of deterrence. Bars represent the mean value; boxes, the minimum and maximum values; and error bars, the standard deviation. Asterisks indicate statistically significant differences (one-sample *t*-test for odorant exposure runs and Shapiro-Wilk test for the control run) ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***). A) Behavioural response of larvae exposed to linalool. B) Behavioural response of larvae exposed to estragole. C) Behavioral response of larvae exposed to 1-indanone. D) Behavioural response of larvae in the control run with diet + solvent. AcMNPV: AcMNPV-infected larvae; SeMNPV: SeMNPV-infected larvae; Mock: Mock-infected larvae.

neuroethological aspects of the chemical ecology of *S. exigua*. SexiOR35, which is upregulated upon SeMNPV infection, is a very broadly-tuned receptor, able to recognise many different odorant compounds. The strongest responses were to 1,4-dimethylbenzene, 3-carene, acetophenone, estragole, linalool, citral and p-cymene. By using the same deorphanization method, the ortholog SlitOR35 of the African cotton leafworm *S. littoralis* demonstrated activation by similar compounds, such as estragole, 3-carene and linalool, among others (de Fouchier et al., 2017). Hence, similar to SexiOR35, SlitOR35 is a broadly-tuned receptor. However, SlitOR35 showed responses also to benzyl methyl ether and sulcatone that in turn did not activate SexiOR35. This suggests a general conserved function for SlitOR35 and SexiOR35, whose slight shifts in sensitivity to some odorants may be related to species-specific behaviours (de Fouchier et al., 2017; Ray et al., 2014). SexiOR23 had no significant response to any of the 58 tested odorants although observation of spontaneous activity in transformed neurons suggests correct expression. This led us to hypothesize that its ligand(s) was (were) not present in our odorant panel, as this represents a minute fraction of all odours that a moth might encounter in its daily life (Hansson et al., 2010). Nevertheless, although the expression of *SexiOR23* in *Drosophila's* antennae was verified by RT-PCR, we cannot fully discard the lack of proper SexiOR23 membrane location or folding in the transformed *Drosophila* lines. Similarly, the ortholog SlitOR23 from *S. littoralis* could not be deorphanized (de Fouchier et al., 2017) using a similar panel of volatiles. Based on the integration of SlitOR phylogeny and ligand data, SlitOR23 (and its ortholog SexiOR23) cluster in an OR clade mainly responding to terpenes and short-chain acetates (de Fouchier et al., 2017).

The link between the strongest ligands of SexiOR35 and the *S. exigua* larvae behavioural response in the context of baculovirus infection may provide neuroethological insights on the possible role of this receptor in the host-pathogen interactions. Two of the main odorants detected by SexiOR35, linalool and estragole, shifted larval olfactory-driven behaviours upon baculovirus infection at specific doses and time points, compared to behaviour of mock-infected larvae. Linalool is a monoterpene alcohol which occurs naturally in many plants as cotton, rice, maize or tomato (Elsharif et al., 2015) as well as it may be produced by microorganisms (Reddy et al., 2020). This compound has been reported as attractive for larvae of the related species *S. littoralis* and *S. frugiperda* (Carlsson et al., 1999; Carroll et al., 2006), similar to what observed for mock-infected *S. exigua* larvae at low dose (5 μg). Whereas no significant behavioural effects were observed at intermediate dose (50 μg) in absence of infection, deterrence was observed in SeMNPV-infected but not in AcMNPV-infected larvae. In a previous study, linalool was shown to enhance SeMNPV pathogenicity, producing a synergistic effect with the virus, when infected larvae were exposed to it (Gasmi et al., 2019). Hence, the observed behavioural shift upon infection by SeMNPV may represent a larval response aimed to avoid the synergistic effects of linalool and SeMNPV. This may imply that SeMNPV-infected larvae would move to other plant parts that produce less linalool. Estragole, an alkylbenzene common to aromatic plants, in turn, produced a behavioural shift common to both SeMNPV and AcMNPV infection. This may be a phenotype related to general baculovirus infection, regardless of the viral species. SexiOR40c was the only receptor that exhibited an

expression change under both AcMNPV and SeMNPV infections, so this behavioural phenotype could be associated to its mRNA levels. To falsify this hypothesis, more research is needed to identify ligands for SexiOR40c. Lastly, 1-indanone, which was used as a control because it did not activate SexiOR35, induced larvae attraction upon infection with SeMNPV at only one time point at the intermediate dose, whereas control *S. exigua* larvae did not show any attraction, as previously observed for *S. littoralis* larvae (de Fouchier et al., 2018). In this last species, 1-indanone is recognised by SlitOR19 and SlitOR25 (de Fouchier et al., 2017; Gonzalez et al., 2015; Llopis-Giménez et al., 2020). The *S. exigua* orthologs, SexiOR19 and SexiOR25, did not show any significant expression changes after SeMNPV infection. However, it is possible that this odorant may be detected by other ORs that were not included in our transcriptional study or that behavioural alterations resulted from changes at higher neural levels. The interpretation of behavioural studies related to volatiles activating a broad-tuned receptor, which is activated by several dozens of different compounds, is not straightforward and it is more complicated by the possibility that the specific compound can activate other ORs expressed in other neurons. Olfactory-driven decision making in Lepidoptera are often achieved through combinatorial coding (Haverkamp et al., 2018). It is not just the activation of a single OR that likely dictates the larva behaviour but rather the combination of different activated ORs that tell the larvae whether an odour is meaningful or not. Thus, our results represent only a first scratch on olfactory-driven behaviours altered by SeMNPV infection. More comprehensive studies analysing both transcription and function of a bigger set of ORs, as well as targeted knockout of genes of interests, will hopefully reveal the whole effect of baculovirus infection on larval olfaction.

In summary, SeMNPV infection produces expression changes of some *SexiORs* in *S. exigua* larvae. Functional characterization of SexiOR35, a broadly tuned receptor expressed during larval stage, has permitted to establish a link between transcriptional changes and infection-related shifts in larvae behavioural responses when exposed to two of its main ligands, linalool and estragole. What remains unknown is whether the observed behavioural changes are indicative of specific effects of the infection, such as a direct manipulation by the parasite or a defence strategy of the host, which may change its feeding habits as an example of self-medication to decrease the pathogenicity of SeMNPV, or they are just side effect associated to pathogenesis. Further research focused on the understanding of the ecological consequences of the olfactory shifts induced in *S. exigua* upon the SeMNPV infection will help to clarify the importance of chemical ecology in host-pathogen interactions.

Authors' contributions

ALG participated in the conceptualization, investigation, data curation, formal analysis and in the original draft preparation. GCV participated in the investigation. EJJ participated in the conceptualization, supervision and in the review and edition of the writing. SH participated in the conceptualization, funding acquisition, investigation, review, edition of the writing and supervision. CC participated in the conceptualization, formal analysis, supervision and edition of the writing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2021.103648.

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