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PhD Thesis

Àngel Llopis Giménez

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Getting on host's nerves:

Baculovirus-associated changes in the neuronal system of *Spodoptera exigua*

Supervisors **Dr. Salvador Herrero Sendra**

Professor titular

Departament de Genètica

Institut de Biotecnologia i Biomedicina

Universitat de València

Dra. Cristina Maria Crava

Investigadora doctora

Departament de Genètica

Institut de Biotecnologia i Biomedicina

Universitat de València

VNIVERSITAT DE VALÈNCIA

El Dr. Sr. Salvador Herrero Sendra, Professor titular del Departament de Genètica de la Facultat de Ciències Biològiques de la Universitat de València, y la Dra. Sra. Maria Cristina Crava, Investigadora doctora del Departament de Genètica de la Facultat de Ciències Biològiques de la Universitat de València,

Informen:

Que el Sr. Àngel Llopis Giménez, Llicenciat en Ciències Biològiques, ha realitzat sota la seua direcció el treball d'investigació recollit en esta tesi doctoral que porta per títol: "Getting on host's nerves: baculovirus-associated changes in the neuronal system of *Spodoptera exigua*", per tal d'optar al Grau de Doctor per la Universitat de València.

I per fer constància, d'acord amb la legislació vigent, signen la present a Burjassot, a 13 de Gener de 2021.

Director: Dr. Sr. Salvador Herrero Sendra Directora: Dra. Sra. Cristina María Crava

A Lurdes

A ma mare, mon pare i el meu germà

Als companys i companyes de CBP Lab

Als meus directors, per la seua amistat i paciència.

"Somewhere, something incredible is waiting to be known"

Carl Sagan

Portada i il·lustracions a càrrec d'Ángel Rubio Laguna

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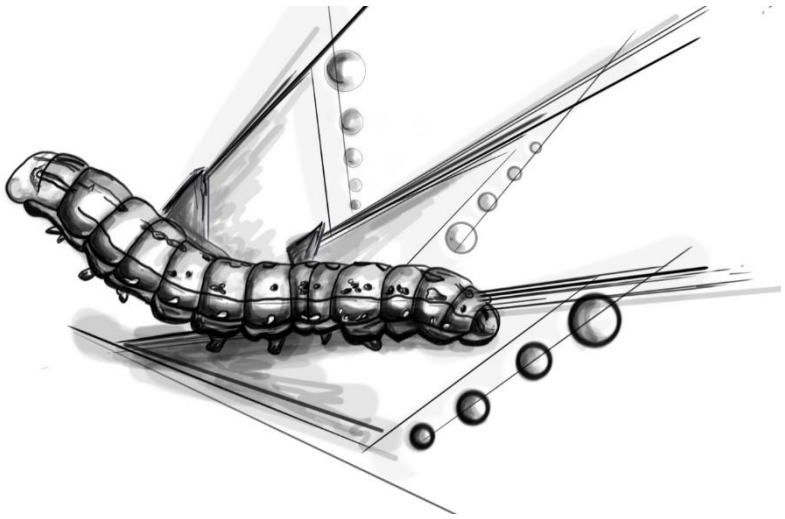


Baculoviruses constitute a diverse group of entomopathogenic viruses characterised by their high specificity and their strong field incidence. These two characteristics have facilitated their usage as bioinsecticides for controlling lepidopteran crop pests. During the baculovirus infection in the larvae, the virus spreads through the insect body, infecting and replicating in many different tissues. As a consequence of this systemic infection, baculovirus produces behavioral changes in its host to promote its viral dispersion in the field, in a clear example of evolutionary advantage. These well-known behavioral phenotypes, whose main examples are the enhanced locomotion activity and the tree-top disease, have been connected with the presence in the baculovirus genome of genes previously acquired from the host. In addition, the existence of this parasite-induced phenotypes may also imply expression changes in host genes that would play a key role in controlling behavioral patterns. Thus, we hypothesize that the already described baculovirus-associated behavioral phenotypes and others that might be discovered, could also be connected to changes in the neural systems of the insect. The nervous system of the insect is composed of the central nervous system and the peripheral nervous system. Both of them participate in the control of the insect's physiology and behavior, and also in the communication of the insect with its surrounding environment. Our hypothesis is supported by the fact that baculovirus presence in brain or antennas has been already observed.

Considering this background, the main purpose of this doctoral thesis is to study the influence of the Spodoptera exigua Multiple Nucleopolyhedrovirus (SeMNPV) in the neuronal systems of the lepidopteran crop pest *Spodoptera exigua*. Within the central nervous system, we have centred our efforts in the study of the neuropeptidergic system, formed by neuropeptides that regulate the insect's internal physiology. Within the peripheral nervous system, we have focused on the chemosensory-related genes, concretely in the odorant receptors, that are the centre piece of the insect's olfaction. For analysing the SeMNPV influence in both systems,

we have followed a common pipeline. We first have described the neuropeptide and chemosensory-related gene repertoires of *S. exigua*. Then, we have studied SeMNPV-associated changes in the expression of these gene repertoires. Finally, we have selected gene candidates for functional characterization to understand their role in the host-pathogen interaction.

In the first chapter, the neuropeptidome of *S. exigua* is annotated. That supposes the identification of 63 neuropeptide unigenes from a transcriptome assembled with samples of larval heads, larval gut and adult brains. We also build phylogenetical trees with the *S. exigua* neuropeptides and those from other related species whose neuropeptidomes are already available. Other information as the description of brain-gut neuropeptides, a comparison of the neuropeptidome expression in different tissues and developmental stages, and how external factors as light or temperature can influence it, complete the description of the neuropeptidome of *S. exigua*.

In the second chapter, we analyse how the baculovirus infection affects the expression of neuropeptide genes following different approaches. At first, we perform differential gene expression in RNA-Seq samples of SeMNPV- and mock-infected larval head samples. Some genes related with the ecdysis process appear to be up-regulated, connecting this with previously observed phenotypes produced by the baculovirus infection. More comprehensive results appear studying the SeMNPV influence in brain samples through RT-qPCR. One putative neuropeptide gene, *proctolin-like*, resembling the insect neuropeptide *proctolin*, is clearly down-regulated after the SeMNPV infection. For studying its role in the host-pathogen interaction, recombinant baculoviruses are generated expressing this gene, in a gain-of-function strategy. *Proctolin-like* overexpression resulted in a decrease of the larval locomotion activity and digestion, confirming its similar role to the neuropeptide *proctolin*. These results permit us to hypothesize about the *proctolin-like* regulation after the baculovirus infection and the consequences of its down-regulation, possibly

making larvae to become more active and bigger and thus, increasing viral fitness by releasing more viral progeny to the environment.

In the third chapter, the chemosensory-related gene repertoire of *S. exigua* is reannotated, improving the previous annotations and using a new nomenclature system that permits its comparison with that of other related species. This new description focuses on larval chemosensory genes, contrary to the previous annotations that used adult transcriptomes. We also analyse how the pre-exposition to specific odorants provokes changes in the expression of some chemosensory genes and we devise a new method for studying behaviourally active odorants against *S. exigua* larvae.

In the fourth chapter, we analyse the SeMNPV influence in the expression of the chemosensory-related genes, mainly focusing on odorant receptors. Using RNA-Seq samples of larval head, some odorant receptors appear to be up-regulated after the virus infection. Two of them, SexiOR23 and SexiOR35 are selected for their functional characterisation using heterologous expression in *Drosophila* and electrophysiological techniques. SexiOR35 result in a broad-tuned receptor able to recognise many different plant odorants. Behavioral assays with baculovirus- and mock-infected samples reveal changes in the larval perception of odorants recognised by SexiOR35 after baculovirus infection. This supposes the first description of baculovirus-associated changes in the olfaction of lepidopteran larvae.

The results obtained from this doctoral thesis discover baculovirus-associated changes in the neuronal systems of *S. exigua*. On one hand, the down-regulation of *proctolin-like*, would make the SeMNPV-infected larvae to become bigger and more active, increasing the viral fitness in the environment. On the other hand, the up-regulation of specific odorant receptors during the SeMNPV infection would produce strong changes in the odorant preferences of the larvae, producing behavioral alterations that could increase the viral incidence or unveiling a larval response to decrease the consequences of the viral infection. These results open new questions

that aim to understand if the observed changes are directly caused by the virus or are collateral effects of the infection. They also allow to hypothesize about the biological significance of these phenotypes and to discover new potential targets for pest control of the lepidopteran pest *S. exigua*.

La família *Baculoviridae* constitueix un grup divers de virus entomopatogens amb un genoma d'ADN de doble cadena circular. Generalment, els baculovirus infecten en estadis larvaris dels ordres Lepidoptera, Hymenoptera i Diptera. Dins dels baculovirus, els nucleopoliedrovirus són els més comuns, tenint els seus virions oclosos dins d'una estructura proteica anomenada cos d'inclusió (OB) amb forma de poliedre. Els baculovirus tenen dues classes de virions, els virions oclosos dins dels OBs, que s'anomenen *occlusion-derived viruses* (ODVs); i els virions no oclosos que es coneixen com *budded viruses* (BVs). El cicle d'infecció pel baculovirus comença quan les larves ingerixen els OBs després d'alimentar-se de superfícies contaminades pel virus. Quan les larves ingerixen els OBs, l'alta alcalinitat dels sucs intestinals de l'insecte, produïx la hidròlisi de l'estructura proteica dels OBs, alliberant els ODVs al lumen del intestí. Els ODVs són els causants de la infecció primària, infectant les cèl·lules columnars de l'intestí larvari. Llavors, els BVs comencen a ser produïts i s'estenen a través del cos de l'insecte, infectant i replicant en molts teixits diferents, en el que es coneix com la infecció secundària.

Com a conseqüència d'esta infecció sistèmica, el baculovirus produïx canvis de comportament en el seu hoste que promouen la dispersió vírica en el camp, en un clar exemple d'avantatge evolutiu. Un d'estos fenotips de comportament ben coneguts és l'augment de l'activitat de locomoció (*enhanced locomotor activity*, ELA) que fa que les larves esdevinguen més actives, augmentant els seus desplaçaments horitzontals, que alhora amplien la dispersió vírica al camp. En algunes espècies de lepidòpters, com ara *Bombyx mori*, este augment de la locomoció ha estat relacionada amb l'acció del gen *ptp*, present al genoma del baculovirus i que codifica per a la proteïna tirosina fosfatasa. Tot i que els mecanismes moleculars a través dels quals este gen produïx este fenotip encara no estan clars.

El *tree-top disease* és un altre dels fenotips de comportament induïts pels baculovirus que empeny les larves a escalar a posicions apicals en les plantes quan estan prop de morir a causa de la infecció. Allí liqüen i moren. Com a conseqüència,

el virus augmenta les seues possibilitats de dispersió amb ajuda dels elements naturals com ara l'aigua o l'aire. Inicialment, este fenotip ha estat vinculat a l'acció del gen *egt* present al genoma del baculovirus, com s'ha vist al baculovirus de *Lymantria dispar*. El gen *egt* codifica per a una UDP-glucosiltransferasa que catalitza la conjugació de l'hormona ecdisteroide amb sucres, esdevenint inactiva i evitant o retardant la inducció de la muda o la pupació de les larves. Per tant, les larves de lepidòpters amb estes hormones inactivades, continuen alimentant-se i creixent, augmentant la seua vida mitja i esdevenint més grans al moment de la seua mort. Com a conseqüència, la progènie vírica lliurada al medi ambient és molt major. No obstant això, l'acció d'*egt* i el *tree-top disease* necessiten de més investigació per tal de revelar el mecanisme molecular que els connectaria entre sí.

Estos dos gens que han estat relacionats amb estos fenotips específics van ser adquirits ancestralment en el genoma del baculovirus procedent dels seus hostes. A més, l'existència d'estos fenotips induïts pel paràsit també pot implicar canvis d'expressió en els gens de l'hoste que jugarien un paper clau en el control de patrons fisiològics o de comportament. Tenint en compte això, plantegem la hipòtesi que estos ja descrits fenotips conductuals i d'altres que podrien ser descoberts, podrien estar relacionats amb canvis en els sistemes neurals dels hostes.

Spodoptera exigua (Lepidoptera: Noctuidae) (Hübner, 1808) també és conegut com el cuc soldat i constituïx una plaga polífaga distribuïda a tot el món, especialment en zones càlides i temperades. Les etapes larvàries de *S. exigua* s'alimenten de més de 200 espècies de cultius i plantes ornamentals, amb enormes conseqüències econòmiques i agronòmiques. S'han utilitzat diferents estratègies per al control de *S. exigua* al camp sobre tot emprant pesticides químics, així com diferents agents de control biològic, com ara bactèries, fongs, nematodes o virus, com els baculovirus. La majoria dels baculovirus tenen un rang d'hostes estret, i això representa un avantatge per al seu ús com insecticides en el camp. Això, juntament amb la seva gran virulència i el seu baix impacte mediambiental, els convertix en

excel·lents alternatives al control químic. Els baculovirus són el producte víric més utilitzat en el control de plagues de lepidòpters.

Tenint en compte tot açò, el propòsit principal d'esta tesi doctoral és estudiar la influència del Spodoptera exigua nucleopoliedrovirus múltiple (SeMNPV) en els sistemes neuronals de *S. exigua*. El sistema nerviós de l'insecte està compost pel sistema nerviós central i el sistema nerviós perifèric. Tots dos participen en el control de la fisiologia i el comportament de l'insecte, encara que també en la comunicació de l'insecte amb el seu entorn circumdant. La nostra hipòtesi està recolzada pel fet que la presència i la replicació dels baculovirus en els òrgans neuronals, com el cervell o les antenes, ja s'ha observat.

Dins del sistema nerviós central, hem centrat els nostres esforços en l'estudi del sistema neuropeptidèrgic, format per neuropèptids que regulen la fisiologia interna de l'insecte i formen part del sistema de comunicació química entre les diferents cèl·lules de l'organisme. Els neuropèptids són xicotetes proteïnes lliurades per les neurones i cèl·lules neurosecretores que actuen com neuromoduladors, neurotransmissors i neurohormones. Els neuropèptids insecticides són sintetitzats principalment en les cèl·lules neurosecretores del protocerebrum de l'insecte. Després s'emmagatzemen en òrgans neurohemals i s'alliberen a través d'axons que s'estenen i penetren la barrera de sang a l'hemolimfa. Els neuropèptids deriven de precursors proteics anomenats prepropèptids que s'escindixen i després es modifiquen post-transcripcionalment. El resultat és el pèptid actiu que serà alliberat i enllaçat a receptors específics. Els neuropèptids també poden ser alliberats per glàndules endocrines situades en el tracte intestinal i en altres llocs perifèrics. Generalment es classifiquen segons la seua funció principal i el receptors que activen normalment són receptors acoblats a proteïnes G (GPCRs). Els neuropèptids regulen aspectes fisiològics molt diferents com ara: la alimentació i el desenvolupament, la reproducció, el moviment muscular, l'homeòstasi, la memòria i els ritmes circadians.

Encara que la majoria d'ells són considerats pleiotròpics, es a dir, intervenen en més d'una funció biològica.

Dins del sistema nerviós perifèric, ens hem centrat en els gens relacionats amb la quimiorecepció. La quimiorecepció és el procés de detecció d'estímuls químics externs. És molt important en la biologia dels insectes, ja que la informació proporcionada pels senyals químics influïx en comportaments fonamentals com ara l'aparellament, la cerca d'aliments, la ponència d'ous o la fugida de parasitoids o depredadors. La quimiorecepció inclou dues branques principals: l'olfacció i el gust. Diferents famílies de gens participen en l'olfacció i el gust com: receptors d'odorants (ORs), receptors ionotròpics (IRs), receptors gustatius (GRs), proteïnes d'unió a odorants (OBPs) o proteïnes quimiosensorials (CSPs). Cadascun d'estos repertoris gènics tenen funcions concretes en el sistema de quimiorecepció d'insectes, i permeten que la informació proporcionada per molècules químiques s'integre i es combine en el cervell. El cervell de l'insecte llavors emet respostes de comportament concretes segons el canviant entorn químic. Dins dels gens relacionats amb la quimiorecepció, ens hem centrat principalment en els receptors d'odorants, que són la peça central de l'olfacció de l'insecte. Els receptors d'odorants s'expressen a la membrana de les neurones olfactives receptores que estan situades dins de les sensilla distribuïdes al llarg de la cutícula de l'insecte, principalment a les parts bucals i a les antenes.

Per tal d'analitzar la influència de SeMNPV en tots dos sistemes, hem seguit un protocol comú. En primer lloc, hem descrit els repertoris gènics dels neuropèptids i els gens quimiosensorials de *S. exigua*. Això, ha estat possible gràcies a la realització d'estudis d'ARN-Seq realitzats amb mostres de caps de *S. exigua* infectats i no infectats, però també, utilitzant enfocaments transcripcionals com la RT-qPCR. Després, hem estudiat canvis associats a SeMNPV en l'expressió d'estos repertoris gènics. Finalment, hem seleccionat candidats gènics per a la seua caracterització

funcional i així intentar entendre el seu paper en la interacció hoste-patogen i les conseqüències de la seua regulació després de la infecció per baculovirus.

Al primer capítol de la tesi, anotem el neuropeptidoma de *S. exigua*. La identificació i la descripció dels neuropèptids ajuda a entendre millor la regulació de la fisiologia dels insectes i la seua adaptació als diferents entorns, però també proporciona nous objectius per al desenvolupament d'estratègies avançades de control de plagues d'insectes. La primera descripció del neuropeptidoma d'un insecte va ser realitzada a *Drosophila melanogaster*, encara que durant els últims anys s'ha estès a diferents espècies, especialment a plagues agrícoles o vectors per a malalties humanes.

En este context, anotem el neuropeptidoma més complet d'insectes fins el moment. S'obté a través de un transcriptoma de *S. exigua* generat a partir de mostres de caps de larva, intestí de larva i cervells d'adult. Seixanta-tres gens són identificats i anotats com a neuropèptids putatius, incloent-hi *splicing variants* per a sis gens i isoformes genètiques diferents per a dos gens específics. Es duu a terme una anàlisi filogenètica per estudiar l'homologia dels neuropèptids de *S. exigua* amb seqüències d'altres espècies d'insectes (algunes pertanyents a altres ordres d'insectes), i detectar possibles esdeveniments de duplicació gènica. Això ha permès revelar un nucli de neuropèptids format per 43 gens en lepidòpters.

Al sistema neuropeptidèrgic, els neuropèptids *gut-brain* tenen una importància fonamental en la regulació de l'alimentació, el creixement i la digestió, ja que s'expressen tant en cervell com en les cèl·lules neurosecretores de l'intestí. Si comparem l'expressió dels neuropèptids anotats en el cap de larva i en les mostres d'intestí, setze transcrits es consideren *brain-gut*. Alguns exemples són: *allatostatin*, *allatotropin*, *proctolin*, *CCHamide*, *neuropeptide F* i *short neuropeptide F*. A més, per obtenir informació sobre la presència o absència dels diferents transcrits de neuropèptids en les larves i els adults, la seua expressió es compara en les mostres de cap de larva, intestí de larva i cervell d'adult. Encara que no apareixen neuropèptids

específics de larva, sis transcrits es consideren específics d'adults, suggerint una implicació potencial en processos específics d'adults.

Per tal de completar la descripció del neuropeptidoma de *S. exigua*, s'estudia l'expressió d'alguns dels neuropèptids en condicions de fam induïda en les larves. La majoria dels transcrits seleccionats regulen l'alimentació i la digestió. Els resultats mostren que molts d'ells s'expressen de manera diferencial durant la fam induïda, la major part d'ells mostrant una sobreexpressió. Això possiblement correspon a una resposta general d'estrès. La sobreexpressió d'alguns d'ells, com ara el *short neuropeptide F*, *allatostatin* o *CCHamide*, podria estar relacionada amb modificacions olfactives provocades per la inanició, com ja s'havia observat prèviament en *D. melanogaster*.

L'expressió dels neuropèptids també es compara sota diferents condicions de llum i temperatura, ja que estos factors influïxen en el desenvolupament d'insectes. A més, alguns dels neuropèptids tenen un paper en la regulació dels ritmes circadians, que estan connectats amb els cicles de llum i foscor. Cap dels neuropèptids apareix regulat significativament després de la privació de llum, i així es descarta un efecte directe sobre la regulació d'estos gens. Considerant els resultats, la temperatura no té cap efecte clar, suggerint que l'expressió de neuropèptids no està fàcilment influenciada per factors externs.

En general, la anotació del neuropeptidoma de *S. exigua* ajudarà a identificar neuropèptids en altres espècies d'insectes proporcionant informació valuosa sobre la importància de l'acció dels neuropèptids en la regulació de la fisiologia de l'insecte. En el context d'esta tesi, permet el següent pas, dirigit a estudiar la influència de la infecció de SeMNPV en la regulació del sistema neuropeptidèrgic per tal de descobrir nous aspectes rellevants de la interacció baculovirus-eruga.

Al segon capítol de la tesi, una vegada s'ha identificat i anotat el repertori de neuropèptids de *S. exigua*, s'intenta analitzar els possibles canvis produïts per SeMNPV durant la infecció de l'hoste. L'objectiu és identificar alteracions en la

regulació dels gens del sistema nerviós central que podrien estar relacionats amb els fenotips induïts pel baculovirus.

Com a primer enfocament, els canvis d'expressió gènica en el sistema neuropeptidèrgic relacionats amb SeMNPV s'estudien en mostres d'ARN-Seq de cap de larva. No s'observa un patró clar d'expressió diferencial després de la infecció vírica. Tot i que alguns gens, la funció dels quals ja estava descrita, apareixen regulats segons l'anàlisi estadística. Este és el cas de l'*eclosion hormone*, l'*ecdysis triggering hormone* i la *prothoracicotropic hormone*, que són inductors de l'ècdisi i tenen una funció oposada a l'hormona juvenil. La regulació d'este conjunt de gens produiria un retard en el procés de muda normal, estenent la vida de l'hoste. Això està funcionalment relacionat amb la funció del gen *egt*, que ja ha estat comentat anteriorment. La regulació d'este grup de gens podria complementar l'acció del gen *egt* en la interacció baculovirus-hoste.

A causa de l'absència d'un patró general de regulació dels gens de neuropèptids després de la infecció per SeMNPV, i per centrar-se en el cervell, on s'expressen la majoria dels gens de neuropèptids, s'analitza l'expressió gènica de neuropèptids en mostres de cervell infectades per SeMNPV a través de RT-qPCR. Els resultats mostren que *proctolin-like*, un gen semblant al neuropèptid proctolin present en els ordres Coleoptera i Diptera, està clarament regulat després de la infecció vírica, mostrant una infraexpressió. A causa del seu patró d'expressió en els teixits cerebrals i intestinals, als seus llocs d'escissió predits, i la presència de la part activa del neuropèptid proctolin (RY/HLPT) en la seqüència de proctolin-like, es considera per a la seua caracterització funcional i per estudiar el seu paper en la interacció hoste-patogen.

El silenciament de gens a través de la interferència d'ARN a millorat l'estudi de la funció gènica en els insectes. No obstant això, l'ús d'estes tècniques en lepidòpters s'ha demostrat que és difícil d'aconseguir. Per això, el mètode seleccionat per a estudiar la funció de proctolin-like és l'estratègia de guany de funció. Així es

generen els baculovirus recombinants AcMNPV que expressen el fragment C-terminal de proctolin-like (Se-PLP). Posteriorment, es realitzen bioassajos per comprovar la influència de la sobreexpressió de Se-PLP en la patogenicitat del baculovirus. També s'analitza el seu efecte en el desenvolupament larvari i la locomoció larvària, que són factors que estan funcionalment relacionats amb el neuropèptid proctolin i la seua funció com a regulador de les contraccions dels músculs esquelètics i viscerals.

Les infeccions amb el baculovirus AcMNPV que sobreexpressa Se-PLP mostren un augment de la mortalitat a dosis baixes, encara que no a dosis altes. Tot i això, ens porta a concloure que Se-PLP influïx en la interacció baculovirus-eruga. A més, apareix una reducció en el creixement larval i també en la digestió de les larves en aquelles infectades amb el baculovirus AcMNPV-PLP en comparació amb aquelles infectades pel virus control AcMNPV-Con i en les no infectades. D'altra banda, l'expressió de Se-PLP durant la infecció pel baculovirus produïx una reducció en la locomoció de les larves. Per estudiar-ho, desenvolupem un mètode que ens permet mesurar l'activitat de locomoció larvària, comparant els diferents tractaments d'infecció. Els resultats obtinguts ens permeten vincular la funció de Se-PLP amb la regulació de les contraccions dels músculs esquelètics i intestinals, que afecten la digestió i la locomoció, com s'havia descrit anteriorment per al neuropèptid proctolin.

Com a conclusió d'este capítol, la infecció per SeMNPV produïx la infraexpressió de tres gens importants en el procés d'ècdisi, possiblement complementant la funció del gen *egt* en la interacció baculovirus-eruga. En el sistema nerviós central, *proctolin-like* apareix clarament regulat degut a la infecció per SeMNPV. La caracterització de Se-PLP podria connectar la seua funció amb el fenotip d'hiperactivitat induït pel baculovirus, ja que una disminució en l'expressió d'este gen podria produir un augment en l'activitat de locomoció de les larves. La disminució de expressió de Se-PLP també podria complementar els efectes gènics d'*egt* d'una manera alternativa, afectant a la regulació de les contraccions intestinals,

produint larves més grans i possiblement alliberant més virus al medi ambient. Queda per dilucidar si la regulació de l'expressió de neuropèptids després de la infecció de SeMNPV representa un efecte directe de manipulació directa pel virus en l'hoste, una resposta de l'hoste o un efecte colateral de la infecció baculovírica. Seria necessària fer recerca addicional per revelar els mecanismes de regulació de l'expressió gènica pel virus, augmentant el nostre coneixement sobre els canvis induïts pel paràsit en els seus hostes.

En el tercer capítol de la tesi, es descriu el repertori de gens relacionats amb la quimiorecepció de *S. exigua*. Les descripcions anteriorment publicades dels gens relacionats amb la quimiorecepció de *S. exigua* es van obtenir utilitzant nomes mostres d'adults i utilitzant diferents nomenclatures d'anotació que van dificultar les comparacions entre estudis. A més, hi havia una manca de coneixement dels gens quimiosensorials en estadis larvaris, que és l'etapa susceptible a la infecció per baculovirus. En este context, es reanoten els gens relacionats amb quimiosensors de *S. exigua* utilitzant mostres d'ARN-Seq d'adult i de larves, i també s'unifica la nomenclatura gènica amb l'espècie germana *Spodoptera frugiperda*, el repertori quimiosensorial de la qual ja s'havia anotat i publicat. Llavors, s'identifiquen un total de 200 gens relacionats amb la quimiorecepció en *S. exigua*, expandint el nombre de gens identificats en les anotacions publicades anteriorment.

Per tal d'obtenir informació sobre l'expressió d'este repertori de gens en les fases larvàries, combinem tècniques d'ARN-Seq i RT-qPCR, centrant-nos principalment en els ORs, la peça central de l'olfacció de l'insecte. En total, s'expressen en larves 50 dels 63 ORs, tot i que no apareixen gens específics d'estadis larvaris. A més, 14 OBPs pareixen ser específics de larves i quatre PBPs (proteïnes d'unió a la feromona) es troben expressades en caps de larva, tot i que la seua funció principal és el reconeixement de la feromona sexual adulta. Això, s'ha vist en altres espècies de lepidòpters i s'ha teoritzat que les larves podrien utilitzar la senyal de la feromona per trobar aliment o per protecció (hipòtesi *mother knows best*).

En altres estudis anteriors, s'havia observat una sobreexpressió de gens ORs i OBPs quan adults eren exposats a volàtils de plantes específiques, en un mecanisme conegut com "sensibilitat als olors". En este capítol es quantifiquen els nivells d'expressió d'un conjunt d'ORs i PBPs en les larves després de la preexposició a diferents compostos odorants. Després de 24 hores d'exposició, diversos ORs apareixen regulats per sobre de qualsevol que fos l'olor utilitzat. Especulem que l'àmplia sobreexpressió dels ORs podria ser una resposta fisiològica a l'alta concentració de volàtils. Per provar si estos canvis corresponen a una resposta d'estrès general, es duu a terme una altra anàlisi fent passar fam a les larves durant 24 hores. No s'observa el mateix efecte en l'expressió de gens quimiosensorials, així que es conclou que els canvis d'expressió observats anteriorment, no corresponen a una resposta general d'estrès.

La identificació d'olors que causen fenotips conductuals en larves de *S. exigua*, ajuda a enllaçar molècules volàtils amb l'ecologia de la larva. A causa de l'absència de protocols en les larves de *S. exigua*, es dissenya una nova configuració que permet estudiar les respostes de comportament de les larves a odorants específics. Per tal de provar el mètode, s'empren diferents odorants. Dos d'ells, 1-hexanol i benzaldehid, resulten atractius per a les larves; mentre que d'altres com indol, alcohol benzil, linalool, 3-octanona i cis-3-hexenil propionat, les repel·lixen. Els resultats obtinguts i l'atracció produïda per 1-hexanol, que coincidix amb l'anteriorment observat a *Spodoptera littoralis* i *Lobesia botrana*, valida el nostre mètode per identificar odorants que causen fenotips conductuals.

En conclusió, en este capítol es duu a terme una nova i més fiable anotació dels gens relacionats amb la quimiorecepció de *S. exigua*, centrant-se en els gens expressats en estadis larvaris. Per tal d'aprofundir en l'olfacció larvària, es realitzen experiments d'exposició a odorants a llarg termini, mostrant canvis no específics en l'expressió d'ORs. També es desenvolupa un nou mètode per identificar compostos odorants que causen fenotips conductuals per a larves de *S. exigua*. Tots estos resultats

constituïxen eines útils per a utilitzar en estudis addicionals destinats a caracteritzar l'olfacció larvària a *S. exigua*. En este context, els utilitzem per a estudiar la influència de SeMNPV en l'olfacció larvària per tal de descobrir noves idees en la interacció baculovirus-eruga.

Al quart capítol de la present tesi i amb l'objectiu de completar la descripció de la manipulació parasitària del comportament que desencadena el baculovirus en el seu hoste, s'estudien els canvis d'expressió en gens relacionats amb la quimiorecepció en *S. exigua* després de la infecció baculovírica. Ens centrem en els ORs, que són la peça central de l'olfacció de l'insecte. Utilitzant dades d'ARN-Seq a partir de mostres de cap de larva infectades o no, es duu a terme una anàlisi d'expressió diferencial per tal d'estudiar si la infecció per SeMNPV influïx en l'expressió dels gens. Alguns OR manifesten una forta regulació després de la infecció per SeMNPV i això, es confirma després per RT-qPCR, comparant la seua expressió en mostres infectades per SeMNPV i AcMNPV (Autographa californica nucleopoliedrovirus múltiple). Estes variacions en la expressió dels gens semblen estar associades a la infecció específica per SeMNPV, ja que la infecció amb el baculovirus generalista AcMNPV no produïx la mateixa regulació.

Els dos ORs més regulats, SexiOR35 i SexiOR23, són seleccionats per a la seua caracterització funcional, per tal de revelar els lligands que els activen. Per això, s'utilitza l'*empty neuron system* de *Drosophila* per tal de desorfanitzar-los. La identificació dels lligands d'ambdós receptors es realitza a través de la tècnica SSR (*single sensillum recording*). El SexiOR35 resulta ser un receptor d'ample espectre, capaç de reconèixer molts compostos odorants diferents. Mostra respostes fortes a odorants com ara: 1,4-dimetilbenzè, 3-carè, acetofenona, estragol, linalool, citral i p-cimen. El SexiOR23 no mostra respostes significatives a cap dels odorants testats i els seus lligands principals romanen desconeguts. Això ens duu a la hipòtesi que el seu lligand o lligands no es troba al panel d'odorants testats, ja que soles representa

una xicoteta fracció de tots els olors que una larva podria trobar al llarg de la seua vida diària.

Per tal de correlacionar la funció de SexiOR35 amb la resposta conductual de larves de *S. exigua*, dos dels seus lligands principals, linalool i estragol, són utilitzats en assajos de comportament amb larves infectades per SeMNPV, AcMNPV o no infectades. Estos assajos de comportament tenen l'objectiu de mostrar canvis en la percepció dels odorants quan les larves es troben infectades pel baculovirus. El linalool, un olor molt comú a les plantes, produïx un efecte dissuasiu en les larves infectades per SeMNPV que no mostren les larves infectades per AcMNPV i tampoc les no infectades. El linalool ja en estudis anteriors havia demostrat que millorava la patogenicitat de SeMNPV quan les larves estaven exposades a l'odorant, mostrant un efecte sinèrgic amb el virus. Este fenotip observat podria correspondre a una resposta de defensa de les larves per evitar l'efecte sinèrgic que la infecció de SeMNPV i l'exposició a linalool es produïx.

L'estragol, un odorant molt comú a les plantes aromàtiques, produïx un efecte similar en les larves infectades per SeMNPV i AcMNPV. Això podria representar un fenotip no específic produït com a conseqüència de la infecció pel baculovirus. El SexiOR40c és l'únic receptor regulat sota ambdós infeccions de baculovirus i podria estar associat a este comportament. Els lligands que activen SexiOR40c romanen desconeguts.

L'1-indanona no va ser reconeguda activament per SexiOR35, i l'únic fenotip significatiu de comportament observat es una atracció per les larves infectades per SeMNPV en una de les concentracions testades. Este odorant podria ser detectat per un altre receptor que seria responsable del comportament observat.

Per tal de concloure, la infecció de SeMNPV regula la expressió d'alguns ORs en *S. exigua*. Un d'ells, SexiOR35 es caracteritzat funcionalment com un receptor d'ampli espectre. Els canvis en la seua expressió estan connectats amb respostes de comportament en larves exposades a dos lligands de SexiOR35. Queda

per dilucidar si els efectes observats són una manipulació per part del baculovirus o un efecte secundari de la infecció. Això, suposa la primera descripció dels canvis olfactius produïts a l'hoste durant una infecció per baculovirus, i demarca la importància de l'ecologia química en les interaccions paràsit-hoste.

El desenvolupament d'esta tesi doctoral revela diferents influències de la infecció de SeMNPV en el sistema neuronal de *S. exigua*, incloent el sistema nerviós central i el perifèric. Centrant-se en el sistema nerviós central, la infecció per SeMNPV produïx una infraexpressió del potencial neuropèptid *proctolin-like*. Tenint en compte els resultats obtinguts durant la caracterització funcional d'este gen, la seua regulació podria afectar als processos de digestió en les larves, induint la generació de larves més grans que alliberarien més virus al final del cicle d'infecció, d'una manera similar al que fa el gen *egt* en la interacció baculovirus-eruga. Però, també la regulació de *proctolin-like* i la seua probable participació en la regulació de les contraccions del múscul esquelètic, faria que les larves augmentaren la seua activitat locomotora, ajudant a la distribució geogràfica del virus. Este podria ser un dels mecanismes que explicarien el fenotip ELA, un fenotip ja estudiat i que produïx el baculovirus en els seus hostes.

En el sistema nerviós perifèric, la infecció per SeMNPV regula l'expressió d'alguns ORs, produint canvis en la percepció d'odorants de les larves de *S. exigua*. Els canvis en els receptors d'odorants tindrien conseqüències en el comportament larvari. D'una banda estos canvis en les preferències olfactives podrien ser representatius d'una resposta larvària contra la infecció, sentint-se atretes ara a plantes que podrien ajudar les larves a augmentar les seues taxes de supervivència en una estratègia d'automedicació. Tanmateix, això també podria ser indicatiu de respostes repel·lents a fonts específiques d'olors que podrien ajudar a la infecció vírica. D'altra banda, estos fenotips de comportament serien directament o indirecta causats pel virus, evitant estratègies d'automedicació per part de les larves, o fent que es sentiren

atretes per les plantes que ajudarien al desenvolupament de la malaltia vírica en les larves.

Els resultats de la present tesi doctoral requeririen d'investigacions addicionals per tal de revelar més canvis associats al baculovirus en altres repertoris gènics dels hostes i així augmentar el coneixement sobre els mecanismes específics de manipulació de la expressió gènica que el virus podria emprar per a este propòsit, el que portaria al descobriment de més aspectes de la interacció baculovirus-eruga i l'ecologia de l'insecte. Aquesta tesi obre vies per estudiar la regulació de la fisiologia de l'insecte, nous mètodes per entendre les respostes de comportament a les larves i com els insectes es comuniquen i interactuen amb el seu entorn. Els nostres resultats llancen una mica de llum en el complex món de la interacció baculovirus-eruga amb la intenció de millorar i optimitzar les estratègies actuals i futures de control de plagues de lepidòpters, utilitzant els baculovirus com un potent biopesticida.

INTRODUCTION

1. Host-parasite interactions

Evolution has made the animal behavior to be extremely complex. Animals must choose about many aspects of their lives: where to deposit eggs or have their offspring, where and from what sources to feed, how and with whom to mate, how to avoid dangers and predators and where to find a place to live safely. The study of the animal behavior is particularly interesting when referring to parasite-host systems. Parasites are those organisms that live on the expense of others, as fungi, protozoa, bacteria, viruses or other animals, and have been interacting with animals during the whole natural evolution. In some cases, these intimate relationships have led to a wide range of mechanisms and strategies of manipulation of the host's behavior that have as a consequence an improvement of the parasite's dispersion (Lefèvre *et al*., 2009a, 2009b; Thomas *et al*., 2010).

In 1982, the evolutionary biologist Dawkins proposed the term "extended phenotype" to refer to the host-parasite interactions. Under this point of view, the phenotype that the parasite's genes cause is not limited to the biological processes in its own body, is also extended to the environment outside of the body, influencing other organisms (Dawkins, 1982). The extended phenotype describes how parasite's genes, many of them acquired from their hosts during the evolution, can alter the host's physiology and behavior, granting evolutive advantages for the parasite incidence. However, the extended phenotype phenomena only describes part of the alterations produced in the host, as the mechanisms that regulate the interaction can be really complex (Lefèvre *et al*., 2009a). For example, changes in the host behavior can also occur in a non-beneficial way for the parasite, taking place non-desirable collateral effects or adaptations of the host to the parasite's action (Moore, 2013). Other possibilities could be the use of the host's compensatory responses to the benefit of the parasite (Lefèvre and Thomas, 2008).

Higher animals have interesting examples of parasitism as the case of rabies that can affect cats, dogs and humans. The parasitic agent of rabies are viruses from *Lyssavirus* genus, which infect the central nervous system (CNS) of their host producing behavioral changes as hyper-salivation, aggressiveness, photophobia, lack of appetite or a reduced co-ordination. All these symptoms produce a general behavioral alteration that increases the virus transmission (Fu and Jackson, 2005; Rupprecht *et al*., 2002).

Parasitoids are parasitic arthropods that necessarily cause the death of their host, and many examples of behavior manipulation have been related to them. That is the case of *Hymenoepimecis argyraphaga*, an ichneumonid parasitic wasp that lays eggs in the abdomen of the spider *Plesiometa argyra*. After hatching, the ichneumonid larvae start feeding on the spider's haemolymph, and before definitely killing their host, the parasite alters the spider normal web. Instead, the spider prepares a cocoon-shape web that will be used by the parasitic larvae to moult (Eberhard, 2001, 2000). In another example of animal parasitism by other animals, the cricket *Nemobius sylvestris* when infected by the nematomorph *Paragordius triscuspidatus*, suffers a behavioral change that push them to die in water, where adult worms can emerge. The nematomorph drives the cricket to abandon its natural habitat to jump in to the water, ignoring the danger that the water represents for them (Thomas *et al*., 2003a, 2002).

Fungi can also manipulate the behavior of the parasitized host, as for example, *Ophiocordyceps unilateralis*, which parasitizes the arboreal ant *Camponotus leonardi*. When the fungus enters the ant, it starts to consume the non-essential tissues until it reaches its CNS and starts to modify the ant's behavior. Before killing the ant, the parasite forces it to scale and to fix its position onto the underside of the leaves, and then, after killing the host, grows a structure to disperse the spores from the ant's head (Andersen *et al*., 2009; Evans *et al*., 2011). Other well-known parasitic microorganisms are protozoa, and a well described example is *Toxoplasma gondii*. This protozoan does not affect the global health status of their hosts but induces rats

(*Rattus norvegicus*) to lose their natural aversion to their usual predators, the cats. The effect of this protozoa in the brain has nothing to do with the smell recognition of the cats, but rather to the loss of the danger awareness and the fear that the predator produces (Berdoy *et al*., 2000; Webster *et al*., 1994). These behavioral changes increases the protozoan transmission to the cats, their final and definitive hosts.

The expertise of parasites in manipulating host behavior and the defensive responses of the hosts are results of long-term co-evolution. It is not a matter of winners and losers. It is an eternal competition between them that helps to maintain the equilibrium in the nature under the yoke of natural selection.

2. Parasite manipulation of insect behavior

Insects constitute 70% of all known animals on the Earth. They are the most diverse animal group and can be found in nearly all the environments. With this background it is not surprising that most of the parasitized animals are insects and they represent an excellent model for studying animal-parasite interactions (Stork, 2018). Parasites can alter many behaviors in the insect hosts as locomotion, food preferences, sexual behavior or social interactions through different mechanisms (Vale *et al*., 2018). These have been started to be deciphered many decades ago, at first in a simple way, only considering the specific changes produced in a host by a parasite. One example are the first studies on the alterations produced in bumblebees by the worm *Sphaerularia bombi* (Poinar and Van der Laan, 1972) or the metabolic alterations produced by the fungus *Nosema locustae* in grasshoppers and crickets (Burges, 1981).

After years of studies, researchers realized that host-parasite interactions, and especially those implying insects, are really complex, as multiple parasites can appear in the same host or a unique parasite can produce multiple behavioral alterations in its host, acquiring a multidimensional perspective that makes difficult to classify the changes produced (Cézilly *et al*., 2013). As an example, two fungi (*Metharhizium*

anisopliae and *Beauveria bassana*) can parasite the desert locust *Schistocerca gregaria* at the same time. The behavioral fever that the locust develops to protect against the pathogens is different if caused by one fungus or the other one (or a combination of them) constituting a complex system of interactions between them (Thomas *et al*., 2003b). Consequently, co-infecting pathogens can either act synergistically or compete between them (Thomas *et al*., 2011). In any case, these complex interactions suppose a great variety of parasite strategies against their host. On the counterpart, the insect tries to avoid the parasite's effect through mechanisms like moving away from the colony, altering mating preferences, feeding avoidance, decreasing social contact, self-medication or grooming (Vale *et al*., 2018). To deepen the insect-parasite relationships, a general classification of the clearest behavioral manipulation phenotypes in insects by different parasites is described.

2.1. Types of parasite behavior manipulation

2.1.1. Host paralysation

Host paralysation is a typical phenotype triggered by parasitoid wasps, which use heterogeneous strategies for converting their hosts in a food source for their progeny (Libersat *et al*., 2009). Some wasps inject a neurotoxin or a venom affecting the central and peripheral nervous system, incapacitating the insect by blocking the synaptic transmissions. This is the case of *Liris niger* that stings its prey (normally individuals from the genus Gryllus) until it is completely paralysed (Steiner, 1986). Other example is the case of *Ampulex compressa*, a wasp that parasites cockroaches. This species does not paralyse completely its prey but makes it to become in a "zombie" state leading it to the wasp's nest where it serves as a food source for the wasp larvae (Gal and Libersat, 2010) (Figure 1A). Virus can also produce this effect in infected insects. This is the case of the *Dinocampus coccinellae* paralysis virus (DcPV), a symbiotic virus of the braconid wasp *D. coccinellae* that is transmitted to the host, *Coleomegilla maculata*. This virus replicates into the CNS inducing a severe

neuropathy that produce a paralysation phenotype from which the parasitoid takes advantage: the paralysation is used by the wasp to feed its larvae in the cocoon (Dheilly *et al*., 2015).

2.1.2. Parasite defense by the host

Other well-described strategy of behavioral manipulation used by parasitoids is the use of the prey for protecting themselves or their offspring from menaces as predators or hyperparasitoids. This behavior modification is either classified as direct, when the manipulation occurs during the period of higher vulnerability of the parasitoid (the pupation stage), or indirect, when the manipulation occurs before this period (Maure *et al*., 2013). A case of direct protection is represented by the braconid wasp *Cotesia glomerata* that forces its host, the lepidopteran *Pieris brassicae*, to make a silk web surrounding the parasitoids pupae to protect them. Moreover, aggressive behavior increases in parasitized larvae, avoiding any predator or hyperparasite to disturb the *Cotesia* individuals while pupating (Tanaka and Ohsaki, 2006). In this case, a polydnavirus transmitted by the braconid wasp during parasitization has been related with these phenotypes (Burke and Strand, 2012). Another braconid wasp, *Glyptapanteles*, makes its oviposition inside young larvae of the geometrid moth *Thyrinteina leucocerae*. Parasitized caterpillars feed and grow until the last larval stage, when the braconid larvae emerge and pupate in a leaf or a stem next to the caterpillar. During the pupation process the caterpillar would produce strong and violent head movements to prevent any agent from disturbing the pupation process of the braconid wasps (Grosman *et al*., 2008) (Figure 1B).

Examples of indirect manipulation are provided by the endoparasitoid conopid flies, which make their host *Bombus terrestris* to dig in the soil until their death. That permits the fly, that are developing inside the bumblebee to be protected against the temperatures during the hibernation process, increasing their survival rates and their adult size (Müller, 1994). Other case of indirect manipulation is the

previously described case of *H. argyraphaga*, the ichneumonid parasitic wasp that induces the spider to build a protective cocoon for the moulting of its offspring (see 1. Host-parasite interactions). A similar process seems occur to the aphid *Macrosiphum euphorbiae* when is parasitized by the braconid *Aphidius nigripes.* Parasitized aphids abandon their colony before death, being mummified in specific places where the braconid offspring can safely pupate, using the corpse of the aphid to protect themselves (Brodeur and McNeil, 1990; Laval, 1992). Additional research would give light to the molecular mechanisms underlying these behavioral alterations, although these phenotypes have been often related with presence of viruses into the CNS of the host (Dheilly *et al*., 2015; Libersat *et al*., 2018).

2.1.3. Reproduction

Changes in sexual behavior are the clearest phenotypes used by distantly related parasites. This is because these changes have a direct influence in the dispersion of the parasites. For example, *Chrysomelobia labidomerae* is an ectoparasite mite that when parasitizes the beetle *Labidomera clivicollis* reduces its survival rate. As a compensatory mechanism, the beetle increases its reproductive behavior before dying, supposing a better dissemination of the ectoparasite (Abbot and Dill, 2001).

Other strategy is the feminisation, which is the increase of females in the population of the host. This mechanism is often employed by *Wolbachia*, which is an intracellular bacterium that is often transmitted by females through vertical transmission, although horizontal transmission has also been observed (Cordaux *et al*., 2001; Werren *et al*., 1995). *Wolbachia* is able to completely turn males into females, changing their morphology, their physiology and their behavior. Other phenotype produced by *Wolbachia* is known as male killing, resulting in the death of male individuals, as well as the induction of parthenogenesis in females, as it happens in the grasshopper *Zyginidia pullula* (Asgharian *et al*., 2014). The mechanisms used

by the bacterium to cause the sexual changes are diverse and still under research, but it has been observed that *Wolbachia* promotes the condensation of the paternal chromosomes when the egg is fertilised truncating the male development (Tram *et al*., 2006). In another hand, to favour parthenogenesis in females, the bacteria prevents the first cell division after the chromosome replication, turning a haploid male zygote into a diploid female one (Serbus *et al*., 2008). In another mechanism, *Wolbachia* can disrupt a gland that produces hormones necessary for the male sex development (Kageyama *et al*., 2012).

The Leptopilina boulardi filamentous virus (LbFV) is carried by *Leptopilina boulardi*, a parasitoid wasp. This virus increases the locomotion activity of the parasitoid and modifies the function of some chemoreceptors in the ovipositor, boosting the detection of clues from previous infestations in the usual hosts. As a consequence, the virus improves the parasitization of *Drosophila* females that been already parasitized by other wasps. That supposes an increase in the horizontal transmission of the virus (Varaldi *et al*., 2009, 2006). The molecular mechanism behind this manipulation has not been fully discovered but it seems that a virus-specific gene could modulate the expression of some parasitoid genes (Lepetit *et al*., 2017) (Figure 1C).

2.1.4. Foraging and locomotion

Locomotion activity can be altered by the parasite's action in many ways: i) increasing the locomotion speed of the host, ii) changing its direction or iii) increasing its foraging activity in search of food sources (Van Houte *et al*., 2013). Increasing of the foraging activity may be due either to a direct effect of the parasite or a compensatory response of the host due to the increase in the energy requirements produced by the parasitization. In some occasions that would help the host to eliminate the parasite (self-medication) (Lefèvre *et al*., 2009a). A clear example of increased foraging activity is the lepidopteran caterpillar *Platyprepia virginalis*, during

parasitization by the tachinid fly *Thelaira americana*. The host changes its feeding preferences from lupine plant to hemlock, producing an increase in the host's survival rate after the emergence of the parasite and reaching the adult stage, that would also increase the parasite's survival. This also benefits the parasite, since the pupal mass of flies that emerge from the host is increased when this latter shifts to hemlock (English-Loeb *et al*., 1993; Karbant and English-Loeb, 1997). Another strategy used by parasites of hematophagous insects, such as *Plasmodium* when infecting mosquitoes or *Trypanosoma* when infecting hemipterans, is the increase of host probing and feeding rate, rising the probability of the parasite transmission (Garcia *et al*., 1994; Koella *et al*., 2002).

Likewise the increase in foraging activity, also an increase in locomotion activity may be either a response of the host to the parasite's action or a parasite's induced mechanism to improve its distribution. One of the clearest behavioral changes that viruses from the family *Baculoviridae* produce in lepidopteran larvae is the enhanced locomotion activity that occurs at the end of the infection process and has as result an increase in the virus dispersion (Kamita *et al*., 2005). This phenotype is widely discussed in the part 5.2. of this introduction.

Phototaxis and geotaxis are locomotor movements that appear changed upon several insect-parasite interactions. A good example is represented by acanthocephalan worms (e.g. *Pomphorhynchus laevis*) that infect gammarids as intermediate hosts. Infected aquatic hexapoda remain in the water surface where they will be more exposed to predators. This phenotype includes a negative geotaxis and a positive phototaxis produced in the host (Bakker *et al*., 2017) (Figure 1D). The positive phototaxis seems to be related with an increase in the expression of a host's protein that influences the synthesis of serotonin (Ponton *et al*., 2006) as well as with variations in the histaminergic system (Bakker *et al*., 2017). The bacteria *Wolbachia* also influences the locomotion of different hosts as *Aedes aegypti* (Dobson *et al*., 1999; Evans *et al*., 2009) or *Drosophila* spp. (Peng *et al*., 2008). Its action seems to

be related with changes in gene expression in the CNS, although it is not clearly understood (Evans *et al*., 2009).

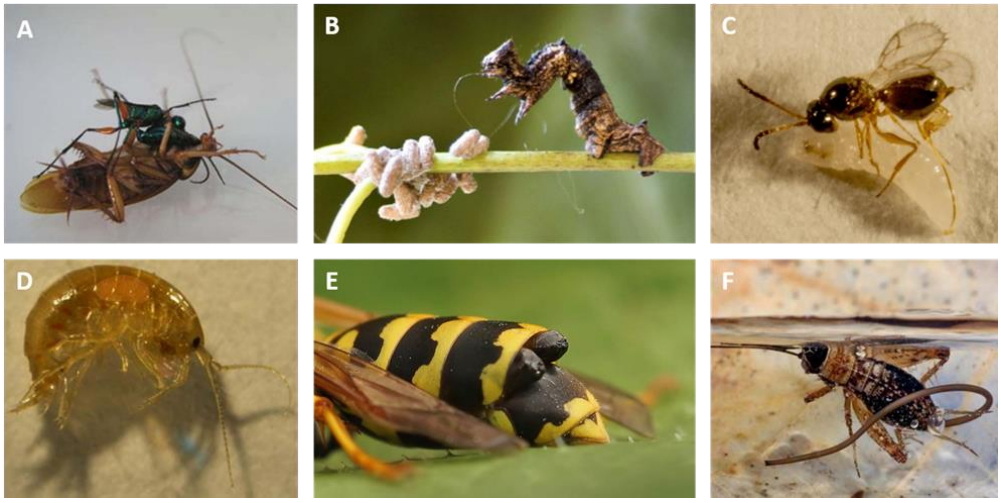


Figure 1. Examples of parasitism in insects. A) *A. compressa* parasitizes *P. americana*, inducing a "zombie" state to feed their offspring (Hughes and Libersat, 2019). B) *Glyptapanteles* makes *Thyrinteina leucocerae* to protect its offspring while pupating (José Lino-Neto, allyouneedisbiology.wordpress.com). C) A *Drosophila* larvae being parasitized by the wasp *Leptopilina boulardi* (Tomáž Doležal). D) A gammarid that has been parasitized by the acanthocephalan worm *Pomphorhynchus laevis* (Sanchez-Thirion *et al*., 2019). E) *Xenos vesparum* growing in the bottom part of the abdomen of a *Polistes dominula* individual (Henk Wallays). F) A *Nemobius sylvestris* cricket parasitized by a *Paragordius tricuspidatus* (alchetron.com/).

2.1.5. Social interactions

Parasite manipulation of insect behavior is especially interesting when it affects the social interactions among the different members of a colony. Honeybees, depending on the age of the individual, are functionally specialized in diverse functions inside the colony (Libersat *et al*., 2018). This is known as age polyethism. Microsporidia *Nosema ceranae* is a well-known parasite of the honeybees that is able to accelerate the age polyethism making young bees to behave as older ones, also

increasing their locomotion and the transfer of food (trophallaxis) among members of the colony, thus improving its transmission (Lecocq *et al*., 2016).

A textbook example of these parasitic manipulations is the case of the eusocial wasp *Polistes dominula*. Some members of this wasp "society", called gynes, have the ability to build new colonies when the spring arrives. From the original gyne, all the colony will be constructed, with reproductive (gynes) and non-reproductive castes (workers). At the end of the summer, gynes will abandon their colony and aggregate with other gynes to resist winter with the aim of forming new colonies in the spring. The parasite *Xenos vesparum*, penetrates the wasp cuticle and situates in the wasp's abdomen feeding on its haemolymph. During the infection cycle, the parasite makes the workers to become gynes and abandon their colony at the middle of the summer, close to the time of parasitoid emergence and mating. Then, parasitized wasps will join overwintering gynes, and in the next spring they start to find a new colony where they could be fed and continue growing, as well as the parasite. Using this strategy, the parasite will infect other wasps in the colony. This complex behavior manipulation seems to occur through changes in the expression of some genes in the brain of the wasp, activating genes normally related with the gyne behavior (Beani, 2006; Geffre *et al*., 2017; Hughes *et al*., 2004) (Figure 1E).

2.1.6. Circadian rhythms

General insect behavior is controlled by circadian cycles that influence several physiological functions, such as temperature regulation, search of food sources, mating and also prediction of environmental changes (Allada and Chung, 2010). A system of such importance is also susceptible of parasite-induced alterations (Van Houte *et al*., 2013; Westwood *et al*., 2019). The singing activity is used by the cricket *Teleogryllus oceanicus* to attract the opposite sex for mating but it also is a moment in which crickets are exposed to predators. When the fly *Ormia ochracea* parasitizes the cricket, it modifies the circadian clock of its host avoiding the cricket

to start singing. That is considered an advantage for the parasite, because keeps away the cricket from other enemies, avoiding competition with them (Zuk *et al*., 2006).

Another example of the importance of the circadian clock in the host-parasite interaction is the parasitization of crickets by Gordian worms that make them to suicide in water. The parasites activate a water-seeking behaviour only during night, when theoretically the search of water sources is more effective. Water permits the worm to abandon the cricket's body in search of new hosts, as it is described for the nematomorph *Paragordius tricuspidatus* when parasitizes *Nemobius sylvestris* (Ponton *et al*., 2011) (Figure 1F).

All these behavioral changes produced during insect-pathogen interaction have really complex physiological and molecular bases, and in most of the examples these effects are being investigated. In some of these cases, these phenotypes have been related with changes produced at the CNS level by altering the expression of specific genes that regulate the animal physiology and behaviour. Similarly, the peripheral nervous system may be also targeted by the parasite as it participates in the process of perception of the surrounding environment by the insect. Unveiling the molecular mechanisms underlying these alterations would not only clarify the strategies used by the parasite and the defensive responses displayed by the host, but would also explain the co-evolution of complex systems in nature. Moreover, the knowledge of neural mechanisms of parasitic manipulation in non-beneficial insects such as disease vectors or agriculture pests might offer new avenues to develop control strategies.

Table 1. Summary of behavior manipulation strategies in host-parasite interactions.

Behavior Manipulation	Host-parasite system	Parasitic phenotype	References
Host paralysation	*Gryllus-Liris niger*	Paralyses completely the host to feed the parasite's offspring	Steiner, 1986
	Blattodea-*Ampulex compressa*	Produces a "zombie" state to the host to feed the parasite's offspring	Gal and Libersat, 2010
	Coleomegilla maculata-Dinocampus coccinellae	Transmits the DcPV that paralyses the host to feed the parasite's offspring	Dheilly et al., 2015
Parasite defence	*Pieris brassicae-Cotesia glomerata*	Makes the host to prepare a protective silk web during the parasite's puppal stage	Tanaka and Ohsaki, 2006
	Thyrinteina leucocerae-Glyptapanteles spp.	Makes the host to defend actively the parasite during the parasite's puppal stage	Grosman et al. 2008
	Conopidae-*Bombus terrestris*	Makes the host to dig in soil and protect the parasite during the hibernation process	Müller, 1994
	Plesiometa argyra-Hymenoepimecis spp.	Makes the host to prepare a protective cocoon web during the parasite's moulting process	Eberhard, 2000
	Macrosiphum euphorbiae-Aphidius nigripes	Mummifies the host to protect the parasite's offspring during the puppal stage	Brodeur and McNeil, 1990; Laval, 1992
	Labidomera clivicollis-Chrysomelobia labidomerae	Increases the reproductive behavior of its host, improving the parasite's transmission	Abbot and Dill, 2001
Reproduction	*Zyginidia pullula-Wolbachia spp.*	Turn males into females, changing their morphology, physiology and behavior	Asgharian et al., 2014
	Drosophila spp.-Leptopilina boulardi	Transmits the LbFV making the host to be in contact with other parasitized hosts, improving the parasite's tranmission	Varaldi et al., 2006, 2009
	Platyprepia virginalis-Thelaira americana	Changes the feeding preferences of the host, increasing the parasite's survival rate	English-Loeb, 1993; Karband and English-Loeb, 1997
Foraging and locomotion	*Anopheles coluzzii-Plasmodium falciparum*	Increases the host's exposition to predators, improving the parasite's tranmission	Roux et al., 2015
	Culicidae-*Plasmodium spp.*	Increases the host's probing and feeding rate, improving the parasite's tranmission	Koella et al., 2002
	Hemiptera-*Trypanosoma spp.*	Increases the host's probing and feeding rate, improving the parasite's tranmission	Garcia et al., 1994
	Lepidoptera-*Baculoviridae*	Increases the host's locomotion activity, improving the parasite's tranmission	Kamita et al., 2005
	Gammaridae-*Pomphorhynchus laevis*	Increases the host's exposition to predators in water, improving the parasite's tranmission	Bakker et al., 2017
	Aedes aegypti -Wolbachia spp.	Increases the host's locomotion activity, improving the parasite's tranmission	Dobson et al., 1999; Evans et al., 2009
	Drosophila spp.-Wolbachia spp.	Increases the host's locomotion activity, improving the parasite's tranmission	Pengs et al., 2008
	Apis mellifera-Nosema ceranae	Accelerates the host's age polyethism and increases its locomotion and trophallaxis, improving the parasite's tranmission	Lecocq et al., 2016
Social interactions	*Polistes dominula-Xenos vesparum*	Makes its host to become gynes, to abandon their colony and to join other individuals, improving the parasite's tranmission	Beani, 2006; Geffre et al., 2017; Hughes et al., 2004
	Teleogryllus oceanicus-Ormia ochracea	Decreases the host's singing activity, avoiding the host to be parasitized by other parasite	Zuk et al., 2006
Circadian rythms	*Nemobius sylvestris-Paragordius tricuspidatus*	Activates in the host a water-seeking behavior, increasing the possibility of finding new hosts to parasitize	Ponton et al., 2009

3. Insect chemoreception and behavior

3.1. General perception in insects. Signal integration and transmission

Insects have multiple systems of vision adapted to their biology, have an excellent spatial navigation that permits them to recognise the geography, can detect and differentiate many different odours and flavours and can organise a whole colony of multiple castes. The machinery that permits all these abilities is a group of ganglia that form the small insect brain codified in a much smaller genome compared to the higher animals (Kinoshita and Homberg, 2017). Modulation of insect behaviour requires an integration of the information coming from very different sensors through multiple sensory channels to the brain, where the information is integrated, and memory and learning happens. How these processes are carried out in the insect brain, how the information is organised and how the responses are emitted is something that is nowadays under research and continuously under discussion (Wessnitzer and Webb, 2006).

The insect brain is formed by two main ganglia: the supraesophageal ganglion and the subesophageal ganglion that are linked by a pair of nerve trunks (Figure 2). The supraesophageal ganglion is the main one and it has been considered for long time the insect brain, whereas the classification of the subesophageal ganglion as a part of the brain is still under discussion. The supraesophageal ganglion is divided in three specific areas, each one formed by several fused ganglia: the proto-, the deuto- and the tritocerebrum. The protocerebrum is the frontmost of the brain and has the function of integrating the visual signals and to combine information of different sensory signals. It is composed by the optic lobe, the mushroom bodies and the central body. The mushroom bodies are a pair of mushroom shape neuropils that receive sensory information from the optic lobe and from the olfactory receptors, via the antennal lobe in the deutocerebrum, but also tactile and gustatory information. They are implicated in pattern recognition and learning. The central body receives inputs

from the mushroom bodies and integrate sensory inputs from different modalities, switching on appropriate locomotor activity responses. It intervenes in regulating the insect's orientation, in the higher locomotion control and in the initiation and modulation of locomotion and flight behavior (Wessnitzer and Webb, 2006). In the dorso-medial part of the protocerebrum, the so-called *pars intercerebralis* and *pars lateralis* can be found. Both act as neurosecretory centres, specially the *pars intercerebralis* is important for neuropeptide and neurohormone secretion (de Velasco *et al*., 2007) (Figure 2).

The deutocerebrum is constituted by the main area called antennal lobe and the smaller antennal mechanosensory and motor centre (AMMC). The antennal lobe integrates all the olfactory information received by the third antennal segments and sends outputs to mushroom bodies, whereas the AMMC receives inputs from mechanoreceptors situated on the first and second antennal segment, from the labial palps and from some body mechanoreceptors. The antennal lobe does not only receive inputs from the olfactory receptor neurons in the antenna, but also those ones located in the mouth parts. It is organised in a different number of neuropilar compartments called glomeruli and their number is species-specific. Along the insect's life, the number of olfactory neurons projecting into the antennal lobe glomeruli increases, according to the increasing number of olfactory sensilla (Anton and Homberg, 1999) (Figure 2).

The tritocerebrum controls the foregut and innervates the labrum, whereas the subesophageal ganglion is formed by the fusion of the ganglia from the mandibular, maxillary and labial segments. It mainly controls feeding behaviors. From the subesophageal ganglion, two nerve cords, which are called ventral nerve cords, connect the brain to the ganglia distributed along the body. Each of these ganglia functions as a local processor, receiving sensorial information from sensory receptors that are far from the brain (mainly mechanoreceptors and taste receptors) (Wessnitzer and Webb, 2006) (Figure 2).

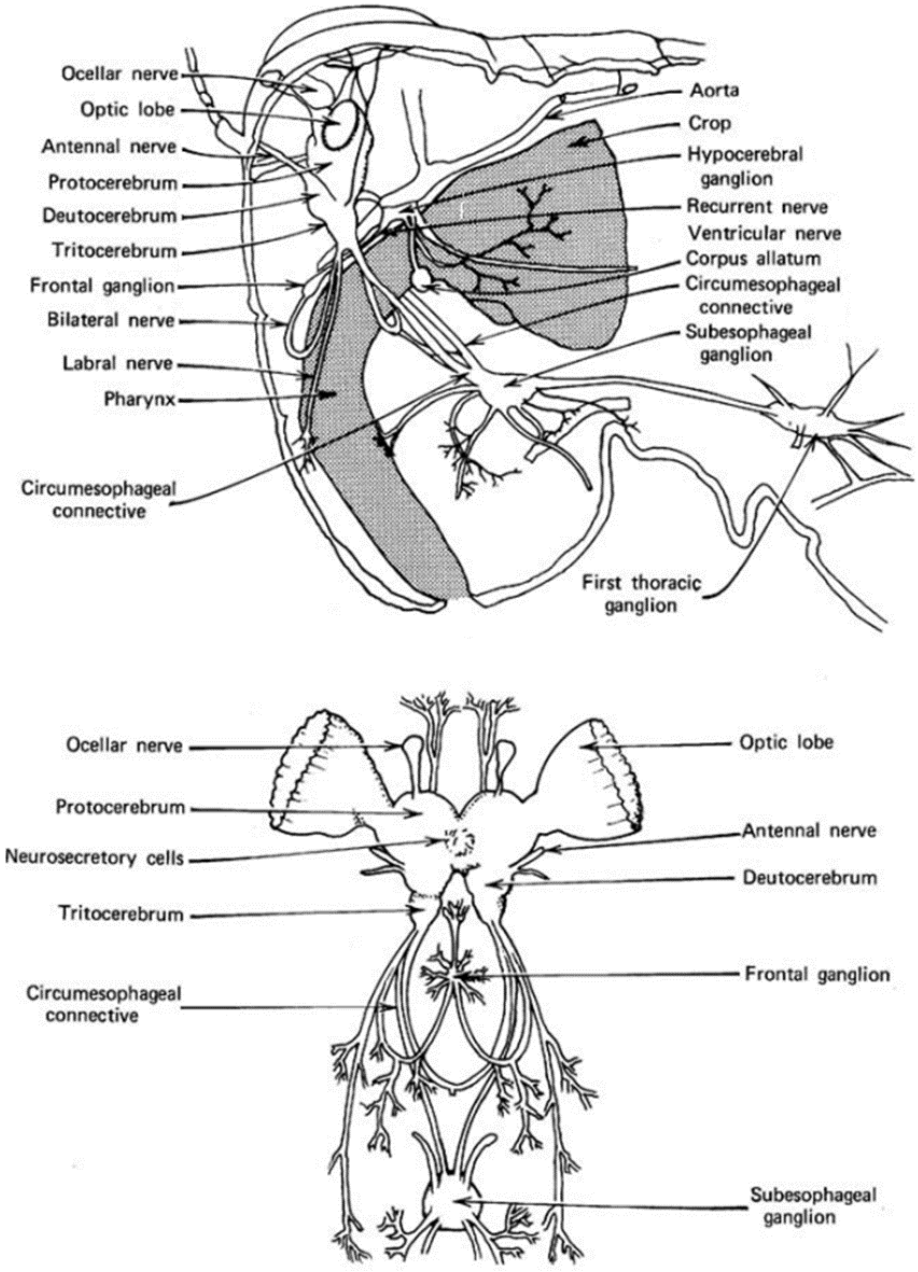


Figure 2. General insect nervous system scheme. Upper panel is a side view of the insect nervous system in the head. Lower panel is a front view of the three main regions of the insect's brain (Matthews and Matthews, 2010).

3.2. Chemoreception and behavior in insects: neuropeptides

Within the complexity of the insect's central nervous system, the neuropeptidergic system is involved in regulating different aspects of the insect's physiology and behaviour through the action of neuropeptides (Altstein and Nässel, 2009). Neuropeptides are small proteins released by neurons and neurosecretory cells that can act as neuromodulators, neurotransmitters and neurohormones (Schoofs *et al*., 2017). They are produced as precursors that are specifically cleaved and processed, becoming mature peptides that will interact with their receptors producing a wide variety of effects inside the insect's host (Nässel and Homberg, 2006).

3.2.1. General features of the neuropeptides

Insect neuropeptides are regulatory peptides mainly synthesized in the neurosecretory cells of the *pars intercerebralis* in the protocerebrum. They are 5 to 80 amino acid peptides whose residues are usually linked by different peptide bonds (Nässel, 2002). They are stored in neurohemal organs: the *corpora cardiaca* and the *corpora allata*, and released through axons that extend and penetrate the blood-barrier to the haemolymph. They derive from protein precursors called prepropeptides that are cleaved and then post-transcriptionally modified. The result is the active peptide that will be released. Enzymes called peptidases are involved in the biosynthesis of the active peptide and in the peptide inactivation once this reaches its receptor (Fónagy, 2014; Isaac *et al*., 2000) (Figure 3). Part of these peptides, the neurohormones, are released by non-neural endocrine glands as the prothoracic gland located in the prothorax of the insects (Fónagy, 2014). These endocrine glands can also be situated in the intestinal tract and in other peripheral sites (Van Hiel *et al*., 2010). The neuropeptides released by these glands, situated far from the brain, are connected with digestion, adsorption, diuresis and muscle movements (Fónagy, 2014).

Neuropeptides are usually classified according to their main function generally related with a structural pattern, although this is not always true. There are four main groups depending on the physiological aspect they regulate: growth and development, reproduction, metabolism and homeostasis, and muscle movement. Most of the already described neuropeptides in insects are classified as pleiotropic, because they elicit more than one biological action (Fónagy, 2014).

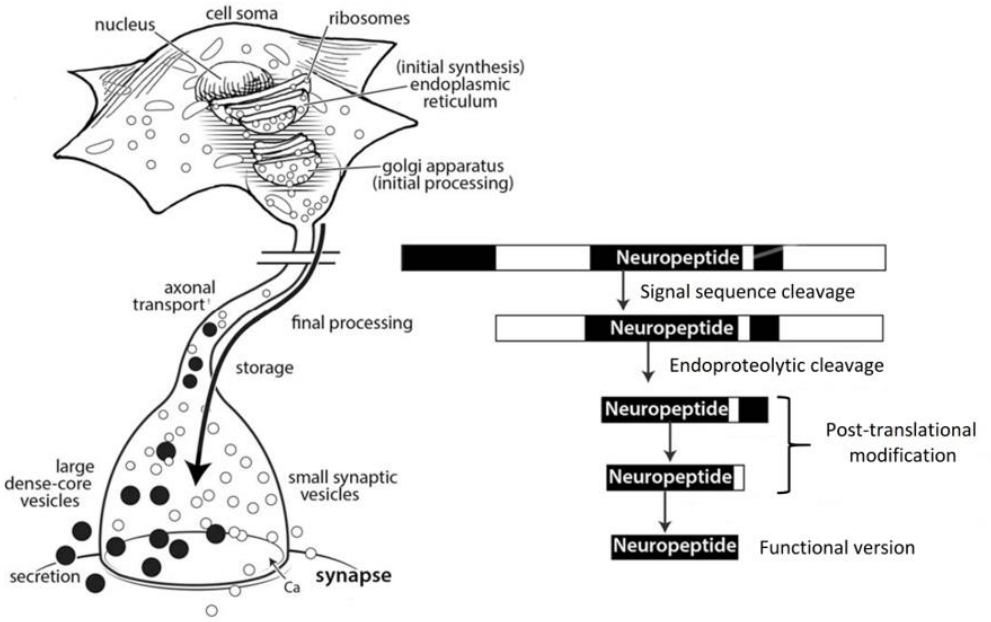


Figure 3. Schematic representation of neuropeptide synthesis and processing in a neurosecretory cell. Modified from (Russo, 2017).

Insect neuropeptide receptors can be classified mainly in two categories: single transmembrane receptors or G protein-coupled receptors (GPCRs), being the seconds that the most common (Van Hiel *et al*., 2010). A typical GPCR has seven transmembrane segments with an extracellular amino terminus and an intracellular carboxyl terminus. Once the neuropeptide has been linked to the receptor, it activates a signal transduction through a G-coupled protein (Kobilka, 2007). Studies in *D.*

melanogaster have permitted to identify GPCR receptors for different families of neuropeptides. Once the receptors are identified, it helps to connect the neuropeptide with specific functions (Claeys *et al*., 2005).

3.2.2. Main neuropeptide families and their function in insect behavior

Neuropeptides are classified according to the physiological process they regulate in the host and only the main families are described in this introduction.

3.2.2.1. Feeding behavior

Feeding behavior includes different behavioral patterns that constitute a continuous cycle. When the insect experiences a lack of energy, brain signals related to hunger make the insect to activate a foraging behavior in search of food. Once the food source has been found, the insect starts to feed, and brain signals of satiety appear in the brain, making the insect to stop feeding (Schoofs *et al*., 2017). Multiple families of neuropeptides regulate different aspects of this behavior.

Sulfakinins (SK) are a family of neuropeptides that are related with the satiety signal in the brain. They usually reduce the food consumption in different insect orders (Maestro *et al*., 2001; Meyering-Vos and Müller, 2007; Söderberg *et al*., 2012; Yu *et al*., 2012). The function of SKs is related with the short neuropeptide F (sNPF) that is a powerful orexigenic peptide and food-seeking behavior enhancer (Lee *et al*., 2004; Nässel *et al*., 2008; Root *et al*., 2011). The neuropeptide F (NPF), structurally related to the human neuropeptide Y, is another neuropeptide involved in the hunger signal and the food uptake (Wu *et al*., 2005, 2003).

Two families of neuropeptides called allatostatins (ASTs) and allatotropins (ATs) are also connected with feeding behavior in insects. Whereas their main function is the regulation of the juvenile hormone release, which controls the larval development (Edwards *et al*., 2001), they also regulate food intake in many species. In the lepidopteran *Spodoptera frugiperda*, injection of AT produces a suppression in

feeding, increasing the mortality in larvae (Oeh *et al*., 2000). In turn, in *Bombyx mori* AT promotes feeding, showing that neuropeptides can have different and even opposite functions in similar organisms (Nagata *et al*., 2012). Different ASTs have been identified in insects: type A, B, C and CC and they not always have the same effect. ASTs have been shown to be feeding reducers in the moth *Lacanobia oleracea* (Audsley *et al*., 2000), as well as in, *Blatella germanica* (Aguilar *et al*., 2003). On the contrary, in *D. melanogaster*, the AST-A peptide seems to be necessary for larval foraging (Wang *et al*., 2012).

Other remarkable neuropeptide influencing the feeding and development of the insects is tachykinin (TK) that seems to be important in the olfactory system, playing a role in the odorant recognition of *D. melanogaster* (Winther *et al*., 2006). A similar neuropeptide, leucokinin (LK) regulates the meal size and the meal frequency in fruit flies (Al-Anzi *et al*., 2010). Finally, the CCHamides are also involved in feeding behavior: CCHamide 1 is implied in regulating the olfactory behaviour of *Drosophila* to different food sources (Farhan *et al*., 2013) and CCHamide 2 seems to be an orexigenic peptide in *Drosophila* (Ida *et al*., 2012).

3.2.2.2. Reproductive behavior

Reproductive behavior involves a set of events that vary in the different insect orders starting with the courtship, followed by the mating and finishing when the eggs are deposited. Many different neuropeptides regulate this behavior (De Loof *et al*., 2001). PBAN, the pheromone biosynthesis-activating neuropeptide is the main one, as it regulates the synthesis of the pheromone from specific pheromone glands located in the abdomen (Raina *et al*., 1989). Pheromones are usually a blend of different molecules in different proportions that actuate in a species-specific way to communicate between members of the same or opposite sex (Johansson and Jones, 2007). Another neuropeptide, TK, is necessary to produce the courtship suppression

once the mating has finished, releasing an antiaphrodiasic hormone that will avoids the male to mate with the female (Shankar *et al*., 2015).

Likewise TK, NPF was described previously as a feeding regulator but it also plays a role in mating behavior as it is upregulated during mating in *D. melanogaster*, whereas its knockdown results in a reduced male courtship behaviour (Lee *et al*., 2006). It has been observed that NPF also plays a role in detecting the female sex pheromone and it is related with an increased fertility in general (Schoofs *et al*., 2001; Van Wielendaele *et al*., 2013). The pigment dispersing factor (PDF) is another neuropeptide that influences the sexual pheromone production and increases the remating frequency in males through regulations of the circadian system (Krupp *et al*., 2013). SIFamide (SIF) has been also connected with reproductive behaviour in *Drosophila*. Its knockdown produces males that are capable of courting other males. In females, it produces a hyper-receptive effect, decreasing the sexual inhibition (Terhzaz *et al*., 2007). Finally, corazonin (CRZ) affects sexual behavior as a regulator of the temporal length of the copulation (Terhzaz *et al*., 2007).

Aggressiveness is another behavior that is related with mating, since it pushes an individual to fight with other males to achieve mating with a specific female. Sexuality and aggressiveness are two behaviour aspects that are really close and neurohormonally connected (Schoofs *et al*., 2017). NPF seems to have a role in regulating and controlling the aggressive behavior (Dierick and Greenspan, 2007) and TK seems to help the insects to differ between fight and court behaviors (Yamamoto and Koganezawa, 2013).

3.2.2.3. Muscle movement

Neuropeptides regulate the contraction of the skeletal muscle and the visceral movements, affecting aspects as locomotion and digestion. This activity is described as myotropic regulation (Fónagy, 2014). Proctolin (PT) was the first identified neuropeptide in insects. It has a five amino acid cyclic structure and its function has been related with the contraction regulation of visceral and skeletal muscles in many

insect orders (Ormerod *et al*., 2016; Starratt and Brown, 1975). In some lepidopteran larvae, injection of a synthetic version of PT increases the contractions of the midgut affecting physiological aspects as digestion or absorption of nutrients (Fiandra *et al*., 2010; Orchard *et al*., 1989). PT also controls the contractions of salivary glands, oviducts and ovipositor muscles as observed in locusts (Belanger and Orchard, 1993). Other myotropic peptides are FXPRLamides, which have a common C terminus (Haddad *et al*., 2018). Neuropeptides can act in the opposite way, as a myoinhibitory peptides (*e.g.* inhibiting the action of muscles) as FMRFamide peptides (FMRF) and myossuppressin (MS) (Nachman *et al*., 1996; Orchard *et al*., 2001).

3.2.2.4. Metabolism and homeostasis

The fat body is the insect's organ that is specialized in the metabolism control. It produces the carbohydrate and lipid metabolism, actuates in the diapause process, is an energy reservoir and plays a role in detoxification and excretion (Fónagy, 2014). Adipokinetic hormones (AKHs) are peptides that regulate the lipid metabolism, increasing the concentration of energy substrates, as trehalose, in the haemolymph. Lipid metabolism usually occurs when the insect is being prepared for activities as flight. AKHs can be cardiostimulant and have antioxidant properties (Kodrík, 2008; Van Der Horst, 2003).

The other important aspect in insect's homeostasis is the maintenance of the water and ion balance through the diuresis. For that, diuretic peptides as the corticotropin-releasing factor (CRF) stimulate the urine secretion. They are helped by myotropic neuropeptides stimulating the movement of the main urine excretion organ, the malpighian tubules (Cabrero *et al*., 2002). Apart from the CRF, insect have multiple diuretic hormone (DHs), that increase the diuresis in the malpighian tubules (Zandawala, 2012). On the contrary, the ion transport peptide (ITP) is an antidiuretic peptide and increases the water reabsorption (Dircksen, 2009). Neuroparsin (NP), is a pleiotropic peptide whose main function is antidiuretic (Badisco *et al*., 2007).

3.2.2.5. Other regulatory functions

Some pleiotropic neuropeptides play a role in the memory formation in insects, which is related with the insect learning via training and storage of information in the brain for their own survival. The pleiotropic neuropeptide NPF is important in the appetitive memory formation and in the odour association process (Rohwedder *et al*., 2015). The sNPF seems to play a role also in the olfactory memory formation (Knapek *et al*., 2013).

Sleeping in insects is important for memory, learning and for general fitness (Schoofs *et al*., 2017). Generally, sleeping is controlled by the circadian rhythms that are regulated by many neuropeptides and hormones. NPF also intervenes in the regulation of the clock-pacemaker neurons that control the behavioral rhythms (Cavey *et al*., 2016). Other neuropeptides that influence the circadian rhythms are orcokinin (OK), SIF, ITP, PDF and the pleiotropic neuropeptide sNPF (Dubowy and Cavanaugh, 2014; Hermann-Luibl *et al*., 2014; Hofer, 2006; Shang *et al*., 2013).

Lastly, social behavior in insects may be regulated by many different gene families, including neuropeptides. Much more research must be done in this area but preliminary studies have demonstrated the relation between some kinins and solitary behaviors, and the connection of ITP with gregarious behaviors (Verdonck *et al*., 2016).

3.3. Chemoreception and behavior in insects: chemosensory-related proteins

Chemosensation or chemoreception is the process of detection of the external chemical stimuli. It plays a significant role in insect biology since information provided by chemical cues influence fundamental behaviors as food choice, mating, egg-laying and parasitoid or predator avoidance (Walker *et al*., 2016). Chemical recognition occurs at the peripheral level of nervous systems, in specialized structures called sensilla. It can be divided in two branches: olfaction and taste. Olfaction has to

do with the detection of hydrophobic volatile molecules transported by air whether taste is related with the detection of non-volatile hydrophilic molecules (Depetris-Chauvin *et al*., 2015). Two molecular elements detect the odorant stimuli in insects: the odorant receptors (ORs) and the antennal ionotropic receptors (aIRs), that are expressed in the olfactory sensory neurons (OSNs) in olfactory sensilla, mainly situated in the insect antennae and maxillary palps (Latorre-Estivalis *et al*., 2015). Taste is also mediated by two families of receptors: the gustatory receptors (GRs) and the divergent ionotropic receptors (dIRs) that are located in the gustatory receptor neurons (GRNs) of taste sensilla, generally found scattered along insect body (Rimal and Lee, 2018). Beside receptors, other molecules help in the detection of chemical stimuli: the odorant binding proteins (OBPs) and the chemosensory proteins (CSPs) that help the chemical stimuli to reach their respective receptors within the sensillum (Pelosi *et al*., 2005). The information provided by the chemical molecules detected by the peripheral nervous system is integrated and combined by the higher brain centres. The output in the receiver is a multiple set of behavioral responses according to the changing chemical environment.

3.3.1. Olfactory and gustatory sensilla

Odours are transformed into a neural signal by the olfactory receptor neurons (ORNs). ORNs are housed inside an olfactory sensillum, that is a sensory organ with a hair-like shape present on insect's cuticle. Generally, in insects olfactory sensilla are located in the antennae and the maxillary palps. The olfactory sensilla consist of a cuticle wall with multiple pores through which chemical odorant molecules can enter (Shanbhag *et al*., 2000). Inside the olfactory sensilla one or multiple ORNs are present, with a maximum of five neurons. These neurons have their dendrites extended into the lumen, where they interact with odorant stimuli (Figure 4). ORs or aIRs are expressed on the membranes of ORN dendrites and when they bind a volatile cue, a membrane depolarization occurs. This starts a neuron action potential that transmits the information through the axons until the glomeruli located in the antennal

lobe (Hildebrand and Shepherd, 1997). Each receptor always transmits its signal to a single glomerulus (Kaissling, 2009). In Lepidoptera, the structure of the glomeruli in the antennal lobes changes between males and females. Generally, males have their olfactory system specialized in the detection of the sex pheromone. Thus, they have a macro-glomerulus complex where ORNs specialized in pheromone detection project their signals and that is not present in females (Rössler *et al*., 1998). These in turn have other glomeruli specialized in detecting specific odours, for example, related with the egg-laying process (King *et al*., 2000). The information processed in the antennal lobe is sent to the protocerebrum where the behavioral response of the insect are coordinated (Martin *et al*., 2011).

The gustatory receptor neurons (GRNs) are situated inside sensilla distributed on the cuticle of the insect, in mouthparts, in antennae, in wings and next to the ovipositor in females, and also in the internal parts, as the internal labellum and the pharynx. Differently from olfactory sensilla, gustatory sensilla are uniporous and the pore is situated on the top of the sensilla. They are also called "taste bristles" or "taste hairs" (Isono and Morita, 2010). Normally, these sensilla contain various GRNs (generally between two and four) and one mechanosensory neuron (Agnihotri *et al*., 2016) (Figure 4). In *Drosophila*, there are four main types of neurons, the water-sensitive neurons, the sugar-sensitive neurons, the GRNs sensitive to low concentrations of salt and the neurons sensitive to bitter compounds and high concentrations of salt (Hallem *et al*., 2006). The process of receptor activation and signal transmission is very similar to that of olfactory neurons. After binding of the chemical molecule to its receptor, voltage-gated ion channels are opened producing a depolarization that triggers an action potential. Each of the neurons uses its axon to send the information to the central nervous system. The gustatory primary centres are located in the subesophageal ganglion and in the thoracic–abdominal ganglion complex of the ventral nerve chord (Wang *et al*., 2004).

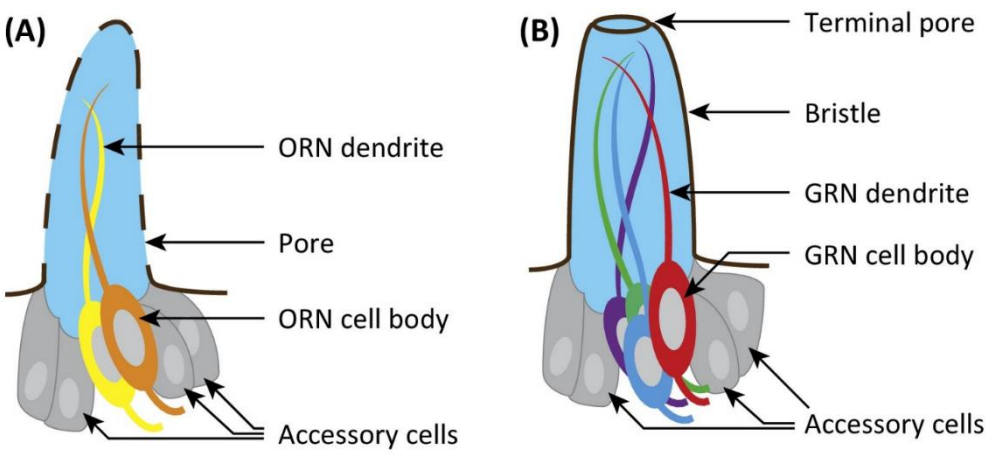


Figure 4. Chemosensory sensilla. (A) Olfactory and (B) gustatory sensilla general scheme in insects. ORN: olfactory receptor neuron. GRN: gustatory receptor neuron (Joseph and Carlson, 2015).

3.3.2. Main gene families in chemoreception

3.3.2.1. Odorant receptors

Odorant receptors (ORs) are the main pieces in the olfactory system of insects. They have been identified for the first time in *D. melanogaster* in 1999 (Clyne *et al*., 1999; Gao and Chess, 1999; Vosshall *et al*., 1999). They are specific olfactory receptors of the class Insecta, and recent research suggested that they evolved before winged flight, perhaps as an adaptation to terrestriality (Brand *et al*., 2018). The number of OR genes varies from only a few in *Pediculus humanus* to many hundreds in some ants (Montagné *et al*., 2015). *D. melanogaster* has 60 ORs, and they are enough for the detection of an enormous amount of chemical odorants (Robertson *et al*., 2003), although OR expression can vary depending on the developmental stage and the sex of the individual (Crava *et al*., 2019; Theilmann *et al*., 2007). Likewise GPCRs, insect ORs have seven transmembrane domains but they show an inverted orientation with a N-terminal in the intracellular section and the C-terminal in the extracellular part. Insect ORs form heteromeric complexes with the odorant co-receptor (Orco) that is expressed ubiquitously in the membrane of the dendrite of every ORN (Fleischer *et al*., 2018; Sato *et al*., 2008). Orco is the most conserved OR

among insect species, and it is considered necessary for a correct olfactory response in insects (Stengl and Funk, 2013). The olfactory receptor complex formed by a ligand-specific OR and Orco, when activated with an odorant, works as a ligand-gated cation channel that alters the extracellular concentration of Ca^{+2} generating an action potential that will activate the signal transmission (Bahk and Jones, 2016) (Figure 5). Generally, ORs are classified depending on the number of chemical stimuli that recognise, *i.e.* narrowly tuned ORs recognise only few odorants whereas widely tuned ORs respond to very different stimuli. In Lepidoptera, some ORs are specialized in the detection of sex pheromones, and they are named pheromone receptors (PRs) (Wang *et al*., 2018, 2011).

3.2.2.2. Ionotropic receptors

Ionotropic receptors (IRs), differently than OR, appeared before the origin of the first insects and they represent an ancestral protostome chemosensory receptor family. IRs were first discovered in the antennae of insects, in olfactory neurons that did not express any OR but responded to odorants and other molecules as ammonia and amines (Croset *et al*., 2010). IRs share from 10 to 70% homology with ionotropic glutamate receptors; however they constitute divergent lineages (Benton *et al*., 2009). They are ligand-gated ion channels with three transmembrane domains (Joseph and Carlson, 2015). Some IRs are thought to work as heteromeric channels in which a broadly expressed IR (usually IR8a, IR25a and IR76b) associates with one or more IRs (Rimal and Lee, 2018) (Figure 5). They have been classified in different families, mainly depending on their function. The antennal IRs (A-IRs) are mainly involved in olfaction. They are expressed, in a number between one and three, in OSNs of the antennae. A-IRs appear to be conserved among distant insect species (Rimal and Lee, 2018). The other type of IRs are the divergent IRs (D-IRs) that are expressed in GRNs and thought to be involved in taste. Compared to A-IRs, they are more species-specific. They can be found in sensilla of the leg and the pharynx (Koh *et al*., 2015).

The participation of the D-IRs in the taste reception is related with the detection of mainly salt and amino acids (Rimal and Lee, 2018). Some IRs have also non-chemosensory functions. For example, IR25a has been involved in temperature sensation and the regulation of the circadian rhythms (Chen *et al*., 2015), as well as in cool sensing, together with IR21a (Ni *et al*., 2016), and together in hygrosensation, with IR93a and IR40a (Knecht *et al*., 2016). In recent studies, a third family of IRs was considered specific from Lepidoptera, and they were called Lepidoptera-specific IRs (LS-IRs). More research is needed to unveil their role in olfaction and taste behaviors (Liu *et al*., 2018).

3.3.2.3. Gustatory receptors

The first insect gustatory receptors (GRs) were discovered in *D. melanogaster* (Clyne *et al*., 2000). Likewise ORs, to which are phylogenetically related, GRs have seven transmembrane domains and inverted topology respect to GPCRs. It is thought that ORs and GRs have evolved from a common ancestral lineage, and GRs are older since they are also present in basal arthropods whereas OR appeared in insects (Brand *et al*., 2018; Isono and Morita, 2010). The number of GRs is highly variable among insect lineages, ranging from few dozens to hundreds of GRs in butterflies (Suzuki *et al*., 2018; W. Xu *et al*., 2016). This variation is likely consequence of gene duplication and loss events (Engsontia *et al*., 2014). GRs mainly detect hydrophilic stimuli, commonly called tastants. However, some of them are specialized for other functions, such as the detection of volatiles like CO_2 (Jones, 2007) or thermosensing (Ni *et al*., 2013). CO_2 receptors are expressed in olfactory sensilla in the antennae, and they are the GR21a and GR63a in *D. melanogaster* (Jones *et al*., 2007), and the GR1, GR2 and GR3 in Lepidoptera species (Kumar *et al*., 2020) (Figure 5). The GRs involved in tastant detection are expressed in GRNs, which are housed in gustatory sensilla scattered through the whole insect body. Based on the phylogenical relationships with *D. melanogaster* GRs, whose function have been deorphanized, it has been identified

few GR clades conserved among insect lineages: detectors of CO_2, detectors of D-fructose, detectors of non-fructose sugars and bitter detectors (Agnihotri *et al*., 2016; Isono and Morita, 2010; Sánchez-Gracia *et al*., 2009). The differences in the number of GRs among the different species has been related to the polyphagy level since polyphagous species have a bigger repertoire of GR than monophagous species. This is mainly supported by data from Lepidoptera, where the generalist noctuid species *Helicoverpa armigera* or *Spodoptera litura* have a remarkably bigger number of GRs compared with other lepidopteran species (Cheng *et al*., 2017; Gouin *et al*., 2017; Pearce *et al*., 2017). This would suppose that the transition from specialist to generalist in terms of feeding could be accompanied by an increase in the number of GRs mainly due to gene duplication processes (Suzuki *et al*., 2018). Differently from ORs, there is an absence of a clear GR co-receptor. It seems that each GR constitutes a ligand-gated ion channel that would produce the action potential (Agnihotri *et al*., 2016). Much more investigation is required to fully understand the structure of the GRs, the ligand-receptor interaction and their functionality.

3.3.2.4. Odorant binding proteins

The first identification of an OBP was done in the silk moth *Antheraea Polyphemus* (Vogt and Riddiford, 1981); since then a high number of OBPs have been annotated in several insect genomes (Sun 2018). They are small soluble proteins involved in chemosensation. OBPs are normally classified depending on the number of conserved cysteine residues that have: classic (six cysteines), plus-C (eight cysteines), minus-C (four cysteines) and atypical (Venthur *et al*., 2014; Venthur and Zhou, 2018). OBPs are mainly expressed in antennal tissues and their function is related with the odorant transport to the specific receptors (Sun *et al*., 2018). Some studies affirm that each OBP can bind to very different volatiles (Pelosi *et al*., 2014) and others remark the specificity of each OBP (Li *et al*., 2017; Sun *et al*., 2018). Beside

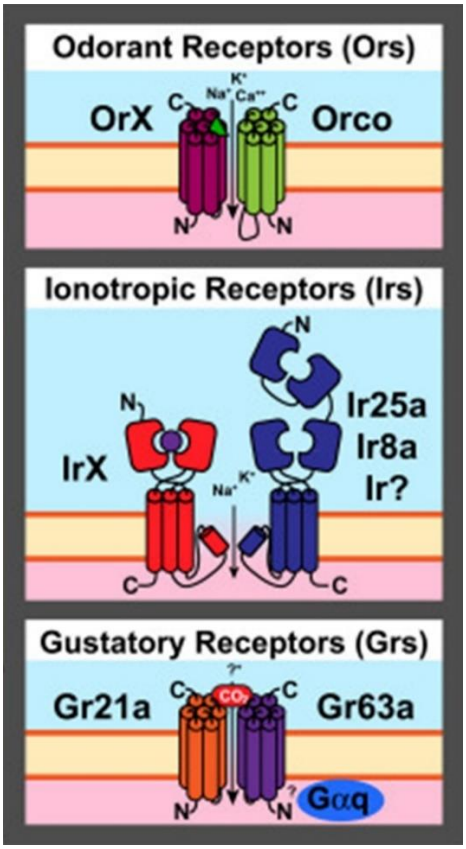


Figure 5. General structure of chemoreceptor families. OrX and IrX are generic names for *ORs* and *IRs* (Pask and Ray, 2016).

binding to odorants, other functions have been proposed for the OBPs such as odorant clearing after its linkage with the receptor, protection of odorants from degradative enzymes and odorant filtering (Sun *et al*., 2018). Nevertheless, it is very likely that they have other functions in the chemosensory system. OBPs are also expressed in gustatory organs (Matsuo *et al*., 2007), in the larval gut (Benoit *et al*., 2017) or in male reproductive organs (Sun *et al*., 2012) (Figure 6). Inside the OBP family, two main sub-families are conserved across Lepidoptera: GOBPs (general odorant binding proteins) and PBPs (pheromone binding proteins). There are generally two different GOBPs (GOBP1 and GOBP2) highly expressed in the antennae of both sexes

(Krieger *et al*., 1996). Their function was previously related with the detection of some specific host plant volatiles (Vogt *et al*., 1991) but later they have been related with the sex pheromone detection (Jacquin-Joly *et al*., 2000). PBPs (whose number ranges from three to four different genes) are believed to work as sex pheromone transporters, being indispensable for the chemical interaction between individuals (Du and Prestwich, 1995). However, nowadays new functions have been suggested for these proteins as they can bind and transport also plant volatiles (Zhu *et al*., 2016).

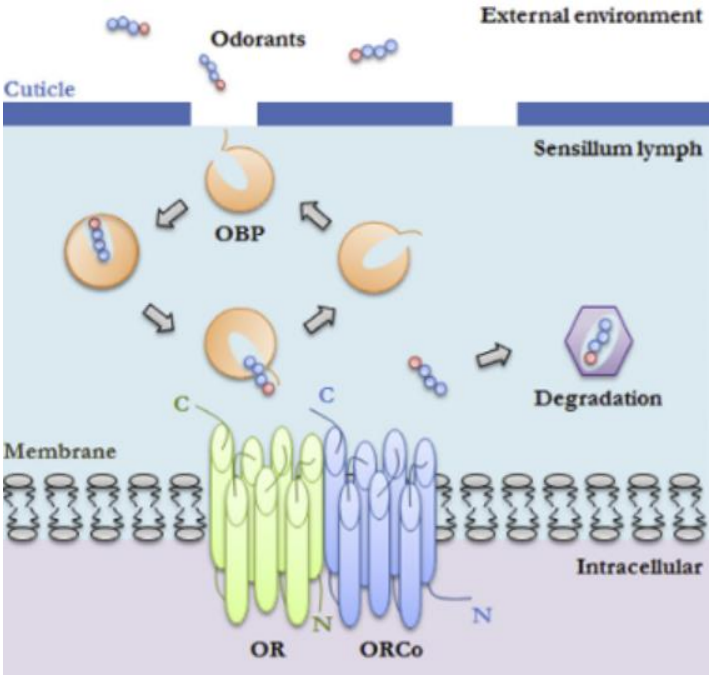


Figure 6. Schematic representation of the OBPs role in the odour perception (Brito *et al*., 2016).

3.2.2.5. Chemosensory proteins

Chemosensory proteins (CSPs) are small and soluble proteins and, likewise OBPs, possess conserved cysteine residues, generally four, which form two disulphide bonds (Younas *et al*., 2018). CSPs are highly conserved in insects and their main function is to bind external ligands in the chemosensory perception (Tunstall

and Warr, 2012). They are mainly expressed in the antennae, although they have also been observed in other non-sensory tissues (Jin *et al*., 2006). Expression of CSPs in antenna has been directly related with olfaction as it has been confirmed in binding assays, whether expression of CSPs in wings and legs may indicate gustative functions (Younas *et al*., 2018). CSPs expression has also been found in pheromone glands, where they probably collaborate to solubilise hydrophobic pheromones and release them in the environment (Pelosi *et al*., 2018). Some CSPs have been functionally related with tissue regeneration or embryo development (Forêt *et al*., 2007; Nomura *et al*., 1992). More efforts are needed to characterize the functional role of the CSPs in olfactory and gustatory detection.

3.3.3. Chemoreception and behavior

Chemoreceptors and binding proteins have undergone a quick and strong diversification among insect lineages (Robertson *et al*., 2003). Evolutive studies have contribute to hypothesize that the big diversification of the chemosensory-related genes in specific lineages has contributed to the heterogeneity of behaviours (Cande *et al*., 2013).

Nowadays, it is clear that chemoreception influences different aspects of the insect's life (Depetris-Chauvin *et al*., 2015). One of the functions that is mainly relevant to larval stages is the food seeking behavior and the food selection. Larvae must grow quickly and thus, have an adapted odour system that permits them to detect available food sources. However, larval chemosensory system is normally less diverse than that of adults, likely because they are less mobile than these latter. Some of the odours are considered as behaviourally-active and provoke an attraction or a repellent behavior to the insects, depending on their concentration and the distance in which they are found. As example, 1-hexanol that is a green leaf volatile ubiquitous in many plants, has been considered an attractant for some lepidopteran species as *Spodoptera littoralis* (de Fouchier *et al*., 2018; Rharrabe *et al*., 2014). However, insects live in a

complex chemical environment, where different stimuli and background doors are present at the same time. Thus, often only the presence of a blend of compounds is able to produce a behavior response in insects. (Depetris-Chauvin *et al*., 2015). The main food source for herbivorous insects is represented by plants, and their smells and tastes are recognised by the chemosensory system. However, plants have defence mechanisms to deter herbivory and these mechanisms alter plant's smell and taste. For example, after herbivory, they emit a blend of volatiles called HIPVs (herbivore induced plant volatiles) that attract natural enemies of the herbivores (Arimura *et al*., 2009; Dudareva *et al*., 2006). Other defence compounds are non-volatile compounds, such as the tannins, which are bitter polyphenols that cause a deterrent effect to some insects. These kind of bitter compounds, when ingested, also affect the digestion and absorbance of proteins, decreasing their nutritive power to the herbivore insects (Barbehenn and Peter Constabel, 2011). The bitter flavour has been used along the evolution as a signal of toxic metabolite presence, although then, specialist herbivores adapted to their host plants, can use that bitter metabolites as a positive feeding signal (French *et al*., 2015).

Insects not only use all the information provided by odours to identify suitable food sources but also they can sense the presence of other insects, such as parasitoids or natural enemies. As an example, *D. melanogaster*, has innate mechanisms to detect odour cues from the parasitoid *Leptopilina*, resulting in an avoidance behavior against the parasite (Ebrahim *et al*., 2015). Other species, directly detect their predators by specific odour cues and modify their behavior, avoiding them to be captured by the predator. This often happens when they meet with conspecifics that have previously detected the odour of the predator, initiating a behavior cascade between different the members of a colony (Siepielski *et al*., 2016).

Reproductive behaviors are also influenced by chemosensation. In lepidopteran species, male detection of the sexual pheromone emitted by females is crucial. Pheromone receptors have evolved to be able to detect low concentrations of

the sex pheromone and to differentiate it from the one produced by other related species, producing aphrodisiac or anti-aphrodisiac responses (Landolt, 1997). Pheromones do not only influence mating but are also involved in the communication between members of the same species (El-Ghany, 2020). Another reproductive behavior influenced by chemosensation is oviposition substrate choice, which in Lepidoptera is driven by female-biased expressed GRs in legs and antennae that are specialized in detecting suitable host plants for oviposition (Briscoe *et al*., 2013).

Overall, the role of chemoreception in insect's behavior makes its study an essential issue in applied entomology and insect's ecology. Only a throughout deciphering of the chemical world that insect face would help us to fully understand multitrophic interactions, and consequently improve the development of pest control strategies based on interspecies communication. Further studies will be necessary to increase our knowledge of insect's chemoperception and its connection with the complexity of the insect's behavior.

4. Baculovirus

4.1 *Baculoviridae* family

Baculoviruses are a diverse group of invertebrate-specific viruses with a circular supercoiled and double-stranded DNA genome whose size ranges between 80 and 200 kilobases. Their genomes encode between 90 and 180 putative proteins and have 30 genes that are considered conserved, constituting the core genes (Herniou *et al*., 2012). They encode proteins necessary for viral DNA replication, gene transcription, viral architecture, DNA packaging, virion assembly and oral infection (van Oers and Vlak, 2007).

The *Baculoviridae* family consists of two types of viruses: nucleopolyhedroviruses (NPVs) and granuloviruses (GVs). They share infection cycle, morphology and genome structure but the shape of the occlusion bodies (OBs) they produce is different. NPVs have polyhedral OBs whereas OBs from GVs have

an ovicylindrical shape, and are known as granules. Polyhedral OBs include multiple virus particles (NPV virions) ranging between 0.15 to 15 µm. In contrast, granulovirus OBs normally contain one virion and their size is between 0.3 to 0.5 µm (Rohrmann GF, 2019) (Figure 7).

Baculoviridae family is divided in four genera: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus*. *Alphabaculovirus* comprise NPVs that infect Lepidoptera, *Betabaculovirus* are NPVs infecting Hymenoptera (Jehle *et al*., 2006). *Gammabaculovirus* are NPVs that infect Diptera; and *Deltabaculovirus* are GVs of Lepidoptera. They are normally named after the insect species from which they were first isolated, as for example, Autographa californica multiple nucleopolyhedrovirus (AcMNPV). This naming system, however, does not take into account that many baculoviruses have a wide host range and can infect different species (Ikeda *et al*., 2015).

Baculoviruses have two classes of virions. When the virions are inside the proteinaceous structure of OBs, they are called occlusion-derived viruses (ODVs) and have an envelope from membranes assembled within the nucleus. Virions embedded inside an OB can be single (SNPVs) or multiple (MNPVs). When the virions are not embedded in OBs, they have an envelope surrounding the nucleocapsid that derives from the plasma membrane of previously infected cells, knowing them as budded viruses (BVs). They have the typical rod-shape morphology that gives them the name of baculovirus, resembling a *baculum* (Latin term for stick). They have a length between 250-300 nm and a diameter of 30-60 nm (Figure 7) (Ikeda *et al*., 2015).

4.2. Infection cycle

The infection cycle starts when the larvae ingest the OBs after feeding on surfaces contaminated by the virus, continues with the virus replication and dispersion inside the insect, and ends with the death of the insect, releasing new OBs that contain

infective particles to the environment (Figure 8). When the larvae ingest the OBs, the high alkalinity of the gut juices in the inset (pH 9.5 - 11), mediates the hydrolysis of

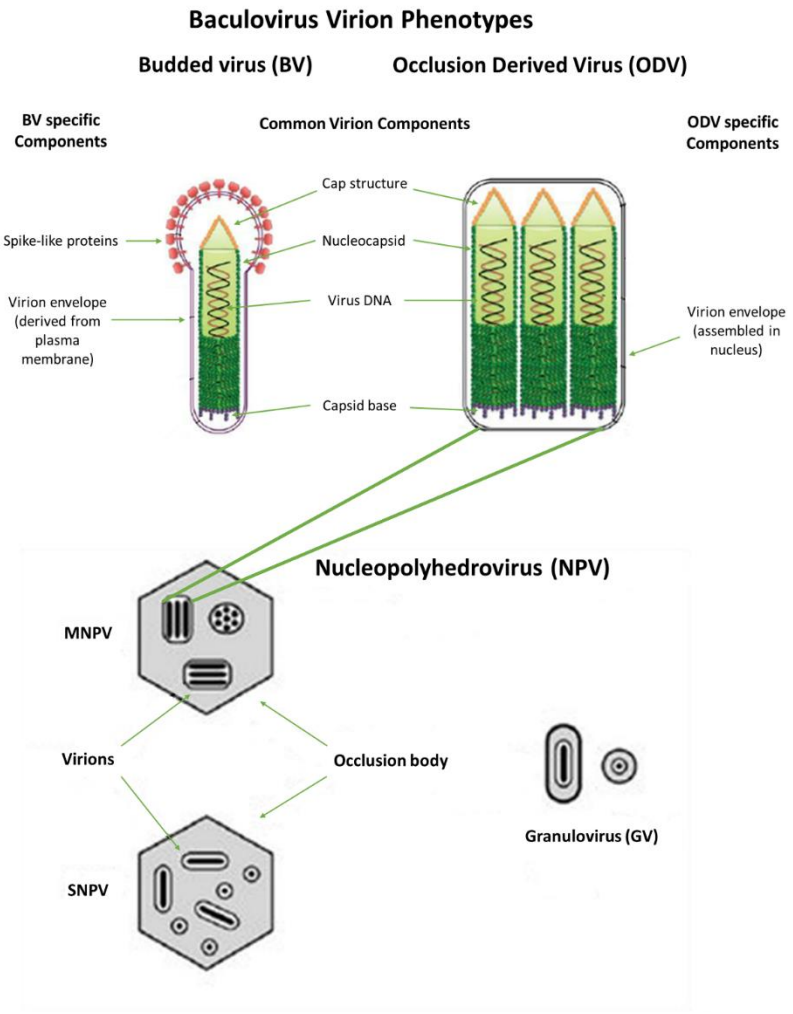


Figure 7. Structure of baculovirus virions and occlusion bodies. Modified from (Au *et al*., 2013).

the protein that forms the OBs, and the release the ODVs into the midgut lumen. Then, virions must traverse the gut peritrophic membrane and finally bind to the brush border microvilli of the columnar cells of the larval midgut. Nucleocapsids penetrate into the cells cytoplasm and migrate into the nucleus, where they release the viral

DNA through the nuclear pores (Volkman, 1997). The cell nucleus becomes hypertrophied and the transcription of the first viral genes starts, normally mediated by the *RNA polymerase II* of the host and then, transcription continues from the viral *RNA polymerase*. New viral progeny is generated and alterations in the physiological functions of the insect are produced in benefit of a better development of the disease in the host (Blissard and Rohrmann, 1990; Granados and Williams, 1986).

Baculovirus genes are classified into four classes in a temporal cascade: immediate early, delayed early, late and very late. Early genes permit the replication of the DNA, whereas late genes, which are expressed after 6-8 hours post-infection produce the viral nucleocapsid and the assembling (Friesen, 1997; Lu and Miller, 1997).

The newly produced nucleocapsids, bud out of the cell through the nuclear membrane acquiring an envelope from that membrane, emerging as BVs that will traverse the basal lamina to arrive to the haemolymph (Granados and Lawler, 1981). This first step in the viral infection is known as primary infection. When the dispersion of the virus to other tissues starts, it is considered the secondary infection (Caballero *et al*., 2001). In the secondary infection, the main mode of entrance of the virus in the cells is the clathrin-mediated adsorptive endocytosis, that permits the BV to easily infect diverse types of cells (Ikeda *et al*., 2015). During the secondary infection process, occlusion virions are also produced due to the expression of very late viral genes (as p10, polyhedrin or granulin). In a very advanced stage of infection, the insects dies, the lysis of the insect's tegument is produced, and the OBs are released in the environment, that will horizontally be transmitted to other individuals, beginning a new infection cycle (Caballero *et al*., 2001). Under sublethal infections, the vertical transmission of baculoviruses is possible by a transovarian way from adults to their offspring (Kukan, 1999) (Figure 8).

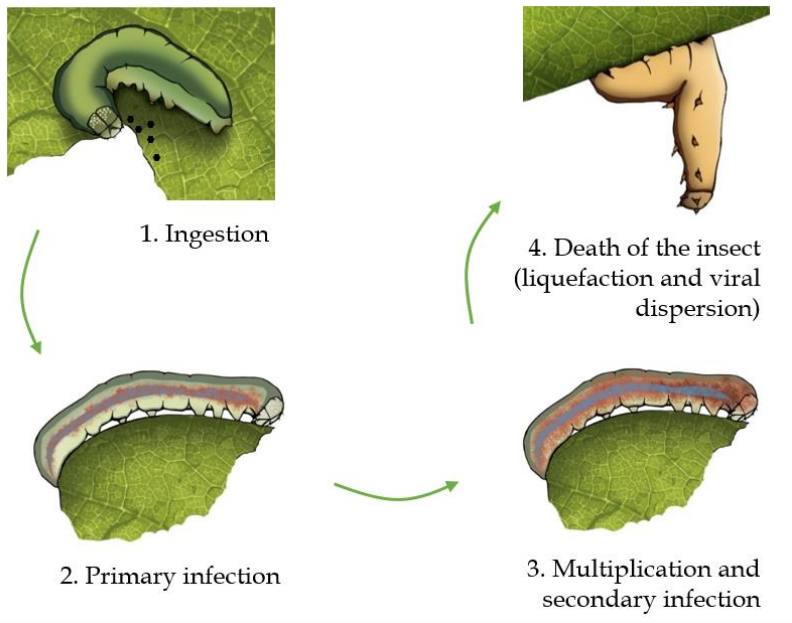


Figure 8. General baculovirus infection cycle.

4.3. Use of baculovirus in pest control

Worldwide crop production is continually threatened by different pests (virus, bacteria, fungi or animals). In the last decades large-scale monocultures and the climate change has favoured the appearance of new pests or have increased the potential damage they can cause (Bebber *et al*., 2013). Since the first part of the twentieth century, chemical pesticides have been used to control multiple pests with several collateral effects that have reduced their utilisation in the last years. Chemical insecticides can damage the agricultural land, and harm beneficial species and the soil microorganisms (Nicolopoulou-Stamati *et al*., 2016). They also affect the human health and safety, and produce an important problem: the appearance of resistance against the pesticides (Knox *et al*., 2015; Oerke, 2006). As a consequence, many chemical insecticides have been withdrawn from the market, also due to a tightening of some laws in many countries. Nowadays, integrated pest management (IPM) has

been considered an alternative to the single use of chemical pesticides, integrating different pest control strategies, as chemical and biological control, in a sustainable way to control the pests outbreaks without harming the environment and avoiding the resistance acquisition (Andra *et al*., 2012). Nowadays, baculoviruses are the only family of viruses that have been registered as insecticides and are largely used in IPM (Knox *et al*., 2015).

The majority of the baculoviruses have a narrow host range, which is an advantage for their use as insecticides. This, coupled with their high virulence and low environmental impact, make them excellent alternatives to chemical control. Baculoviruses can be produced in whole insects or in insect cell cultures, and their production can be easily performed in large-scale. To date, more than 50 baculoviral products have been commercialized to control different insect around the world (Knox *et al*., 2015). On the counterpart, baculovirus-based insecticides often present some disadvantages, since they have an expensive production, compared with chemical insecticides and a short field stability. They also have a slow speed of killing and for that, different recombinant baculoviruses have been produced expressing different toxins or hormones, to improve their insecticidal action. Nowadays, the use of recombinant baculoviruses is legally limited in many countries that ban the release of genetically modified organisms in the field (Inceoglu *et al*., 2006). These issues and other aspects as the tendency to use broad spectrum insecticides have impeded, until now, a generalized application of baculoviral-based pesticides. However, the future looks bright for these insecticides, as the development of new IPM programs and pest control strategies sets a favourable scenario for developing new baculovirus-based insecticides.

5. Host-pathogen interaction in baculovirus-lepidoptera systems

During the coevolution process between baculovirus species and the Lepidoptera hosts, the viruses and their hosts have developed a complex relationship, interacting at different levels. The virus has developed mechanisms to optimize its fitness and dispersion during the infection process. Most of those evolutionary innovations are consequences of a gene acquisition, often directly obtained from the host. Many of these acquired genes permit the alteration of the physiology and behaviour of the insect hosts for baculovirus own benefit (Drezen *et al*., 2017). On the contrary, the host has evolved to resist or overcome the baculovirus infection by developing different defence strategies (Kong *et al*., 2018).

5.1. Insect response to baculovirus

The continuous expose of insects to different pathogens and parasites as fungi, bacteria or virus, have push the development of a great variety of immune responses adjusted to any of the different types of pathogens. Insects lack the acquired immunity that is present in vertebrates but possess an efficient and powerful innate immune system that eliminates any parasite or pathogen invading them. Basically, this immune response consist of a combination between cellular and humoral immunity (Tanaka *et al*., 2008). The cellular response involves mainly the action of haemocytes that neutralize pathogens by phagocytosis or capturing them in nodules where phenoloxidases melanize the intruders, eliminating them. Generally, cellular immune response is activated by bigger parasites as parasitoids and nematodes, but it has also been observed in cells infected by baculoviruses (Dean *et al*., 2004; Washburn *et al*., 2000). Humoral immunity is led by different signaling pathways that trigger the mounting of insect defence responses. These are Toll, the immunodeficiency (Imd) and cytokine-mediated Janus kinase (JAK)/signal transduce activator of transcription (STAT) signalling pathways (Lemaitre and Hoffmann, 2007). Toll pathway responds mainly to fungi and gram-positive bacteria (Lemaitre *et al*., 1996; Rutschmann *et al*., 2000); Imd pathway is activated against gram-

negative and gram-positive bacteria (Imler, 2014) and JAK/STAT pathway has been related with the immune response against gram-negative and virus, including against baculovirus (Katsuma *et al*., 2007). The activation of these pathways imply the synthesis of antimicrobial peptide factors and lysozymes, that are small proteins with a direct antimicrobial activity with diverse mode of actions (Crava *et al*., 2015; Kong *et al*., 2018). Oxygen reactive species (ROS) are also produced together with the antimicrobial peptides with a clear anti-pathogen activity (Charroux and Royet, 2010). In the recent years, siRNA (short-interference RNA) defence pathway has been considered one of the most effective antiviral responses of the host. Nowadays, researchers are centring their efforts in unveiling the mode of action of these antiviral responses (Gammon and Craig C. Mello, 2015).

More specifically, our research group has focused on describing the antiviral responses of the animal model *Spodoptera exigua* against baculoviruses. We have identified several genes and gene families in *S. exigua* that regulate baculovirus-host interaction. Antimicrobial mechanisms have been discovered in insects as the existence of REPAT (response to pathogen genes) as a group of genes that become active against bacterial or viral (including baculovirus) intruders that seem to act as transcription factors, although their mode of action is not clearly elucidated (Herrero *et al*., 2007; Navarro-Cerrillo *et al*., 2013). In addition, there are a group of proteins called chitin deacetylases (CDA) that seem to be important in the defence against baculovirus constituting an early mechanism of protection from oral infections (Jakubowska *et al*., 2010). Also, antimicrobial peptides (AMPs), small proteins that have a direct antimicrobial activity against pathogens like fungi, virus or bacteria are part of the local and humoral responses. Between them, Cecropin D seems to play a role in the antiviral response against baculovirus (Crava *et al*., 2015). Finally, Gasmin, a protein that *S. exigua* acquired from a polydnavirus, have a detrimental effect in baculovirus production during the infectious process, helping to protect the host (Gasmi *et al*., 2016).

5.2. Baculovirus adaptation to the host

Baculoviruses use several strategies to modify the insects biology, to improve its dispersion. They can alter the insect's physiology, producing specific behavioral changes, inhibition and modulation of the insect's immune system and alterations in the normal development of the insect cells (Kong *et al*., 2018). One of the textbook examples is provided by the baculoviral *egt* gene that codifies for a UDP-glucosyltransferase that catalyses the transfer of a glucose (or galactose) from UDP-glucose to the 22-hydroxil group of ecdysteroids, a class of insect hormones (O'Reilly and Miller, 1991). Once the ecdysteroid hormone is conjugated with sugars, it becomes inactive and cannot induce molting or pupation. Thus, lepidopteran larvae with inactive hormones continue feeding and growing, providing to the virus the best environment where reproduce. The viral *egt* gene derives from ancestral Lepidoptera, and it was acquired by baculovirus during the coevolution process (Hughes, 2013). The advantage for the virus is that bigger larvae at the moment of the larval death would produce a bigger progeny increasing its horizontal transmission in nature (Ikeda *et al*., 2015). Baculoviruses with a defective *egt* gene have increased pathogenicity and produce a higher mortality, a phenomenon known as fast-killing (Simón *et al*., 2012). This phenotype has been proved by genetic engineering in different baculovirus species as AcMNPV, Helicoverpa zea nucleopolyhedrovirus (HzNPV) and B. mori nucleopolyhedrovirus (BmNPV). The susceptibility to fast killing depends also on the instar and the age of the larvae (Bianchi *et al*., 2000; Flipsen *et al*., 1995; S. Katsuma *et al*., 2012).

Baculovirus-infected larvae become more active showing an enhanced locomotion activity compared to non-infected larvae. In BmNPV, the enhanced locomotion activity was related with the baculovirus-encoded *ptp* gene that codifies for the tyrosine phosphatase protein. This protein seems to interact with another baculovirus-encoded protein called Wiskott-Aldrich syndrome protein, but the exact mechanism has not been yet deciphered (Kamita *et al*., 2005; Katsuma *et al*., 2012).

It was observed that the *ptp* gene from AcMNPV also produced the same phenotype (van Houte *et al*., 2012). The PTP protein seems to have multifunction roles during the baculovirus infection (Han, 2018). However, how it is able to produce the hyperactivity phenotype remains unclear.

Baculovirus-infected larvae also exhibit a climbing behavior tending to die at higher positions, as the top of the plants, where they liquefy. As a consequence, the virus has more possibilities of being dispersed by natural agents as water or air, increasing the horizontal transmission possibilities (Goulson, 1997). This phenotype is known as *Wipfelkranheit* or tree-top disease. Initially, the *egt* gene was found to be the responsible for the climbing behavior in larvae infected with Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV), (Hoover *et al*., 2011). Later, it was demonstrated that in the case of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) infected larvae, a light exposure from above positions at specific post-infection times was necessary to produce the climbing behavior (Van Houte *et al*., 2015). In this case, the *egt* gene encoded by SeMNPV was facilitating, as a side effect, the tree-top disease phenotype by its influence on extending the time to death of the larvae (Han *et al*., 2015). However, it was demonstrated that the *egt* gene of AcMNPV in *S. exigua* or *Trichoplusia ni* was not affecting the position at where larvae were dying, confirming that the *egt* involvement in the tree-top disease phenotype depends on many factors (Ros *et al*., 2015). In LdMNPV-infected larvae it was observed that light was not needed as in the case of SeMNPV, confirming that in some species of baculovirus light is important for tree-top disease but not in others (Han, 2018). Those studies demonstrate that additional research is still needed to unveil the molecular mechanism used by baculoviruses to induce tree-top disease (Han, 2018).

Another mechanism that is commonly used by baculovirus during the infection is both the induction and inhibition of host cell apoptosis. Apoptosis is the genetically controlled cell death procedure and is necessary for the normal development and the cell homeostasis (Elmore, 2007). Baculoviruses tend to normally

induce cell apoptosis in late infection as a viral strategy to promote the viral dissemination along the insect's body and following multiple mechanisms (Ikeda *et al*., 2013; Kelly *et al*., 2008). However, baculoviruses have also acquired during coevolution apoptosis-suppression strategies that prevent early apoptosis and ensure their own replication process inside the insect's cells (Kong *et al*., 2018). Three different apoptosis suppressors have been found in the AcMNPV baculovirus genome as *p35*, inhibitor of apoptosis (*iap*) and apoptotic suppressor (*apsup*) (Chejanovsky, 2016). These findings on the process of apoptosis clearly reflects the complexity of the baculovirus-insect interaction.

At the end of the baculovirus infection, the typical baculovirus-induced death is characterized by a liquefaction that disintegrates the insect tissues and releases OBs to the environment. This liquefaction is mediated by the action of two baculovirus-encoded proteins: cathepsin and chitinase, and also due to the anti-apoptotic protein P35 (Ikeda *et al*., 2013). Chitinase, is a host-derived protein. In insects, its main function is the degradation of the chitin during the different moulting process along the larval stages (Daimon *et al*., 2005). It was acquired by baculovirus, and it is used at the end of the infection cycle to produce the liquefaction (Daimon *et al*., 2006). Cathepsin seems to act similarly to chitinase during the larval liquefaction process (Kang *et al*., 1998; Slack *et al*., 1995). Likewise chitinase, it has been hypothesized that cathepsin was also acquired from a lepidopteran host via horizontal transmission (Daimon *et al*., 2007, 2006; Ikeda *et al*., 2015).

Recently, some mechanisms through which baculovirus can modulate the immune response of its hosts have been identified. Some conserved microRNAs (miRNAs), as BmNPV-mirR-1, were identified in the genome of BmNPV. Their functional analysis revealed that they suppress the host antiviral miRNA synthesis. The viral miRNA avoids the transporting of host miRNAs from the nucleus to the cytoplasm, avoiding them to reach their viral target and reducing their antiviral function (Singh *et al*., 2012). Additionally, it seems that baculovirus would carry

miRNAs against some proteins that constitute the immune system of lepidopteran larvae, specifically against hemolin and prophenoloxidase that are involved in the defense against viral attacks (Lu *et al*., 2014; Singh *et al*., 2010; Tang *et al*., 2019).

The detailed mechanistic behind the physiological and behavioral modifications produced by baculoviruses are nowadays still unclear and represent an interesting research fields. These changes may involve the host central and peripheral nervous system (Hughes and Libersat, 2018; Perrot-Minnot and Cézilly, 2013), as known form many parasitic interactions that directly produce changes in the host nervous system or indirectly affect the neural activity by changing the host's gene expression (Adamo, 2013, 2002). Different studies have shown that baculoviruses infect the host's brain and replicate in there, possibly altering the neurology that regulates very different physiological aspects (Torquato *et al*., 2006). Moreover, there are some evidence indicating that baculovirus infect the insect's antennas, innervated by the peripheral nervous system and where perception occurs having direct consequences on the insect's behavior (Dhungel *et al*., 2013; Naik *et al*., 2018).

6. The beet armyworm, *Spodoptera exigua*

Spodoptera exigua (Lepidoptera: Noctuidae) (Hübner, 1808) also known as the beet armyworm or the small mottled willow moth is a polyphagous pest worldwide-distributed, especially in warm and temperate areas (Che *et al*., 2013). The larval stages of *S. exigua* are capable of feeding on more than 200 species of ornamental and crop plants, producing enormous economical and agronomical losses. The most affected crops are pepper, tomato, zucchini, lettuce, cabbage, eggplant, cotton and tobacco (Soumia *et al*., 2020).

The lifecycle of *S. exigua* is divided in four stages: egg, larvae, pupa and adult. The whole lifecycle can be completed in about 24 to 30 days, depending on the environmental temperature. Its lifecycle starts when a female lays batches of 50 to

150 eggs usually on the surface of leaves. *S. exigua* egg shape is circular and they have a greenish and white colour (Capinera, 2001). Larvae have a pale green and yellow color in the field, and as they grow, some lateral stripes appear. Neonate larvae tend to have a gregarious behavior and feed on the same leaf where they have hatched. From the third instar they start to live separately (Marí and Ferragut Pérez, 2002). During the fourth and the fifth stages, the larvae produce the main crop damages feeding on leaves but also on the fruits. At the end of the fifth larval instar, larvae tend to go to the bottom parts of the plant to pupate in the soil. *S. exigua* pupae are light brown in color, and they take between six to ten days to adult emergence. Adult lifetime spans over 10 to 18 days; they have a size of 25-30 nm and a grey-brown colour. They start mating few days after emergence, and oviposition normally happens after two or three days. In nature, depending on the temperature, this species has a different number of annual generations, one in colder latitudes and from two to six in warmer places (Capinera, 2001) (Figure 9).

6.1. Field control measures

Different strategies have been used for the control of *S. exigua* in the field using chemical pesticides as well as different biological control agents. Chemical formulates used against this pest in field such as piretroids, carbamates, chlorinated and organophosphates. The most popular products are Spinosad, and growing inhibitors as Metoxifenocide and Tebufenocide (Moulton *et al*., 1999). The employment of chemical products have led to the appearance of resistant populations, and have been related to the presence of toxic remnants in the fields, which harm other insects and natural enemies (Mascarenhas *et al*., 1998; Moulton *et al*., 2009).

Natural enemies-based strategies have also been applied for controlling this pest, as parasitoids like *Chelonus insularis* (Braconidae) that parasitizes the eggs, or *Pristomerus spinator* (Ichneumonidae) that parasites the larvae. Natural predators

have also been considered, as *Orius tristicolor* (Anthocoridae) or *Hippodamia convergens* (Coccinellidae) (Ehler, 2007).

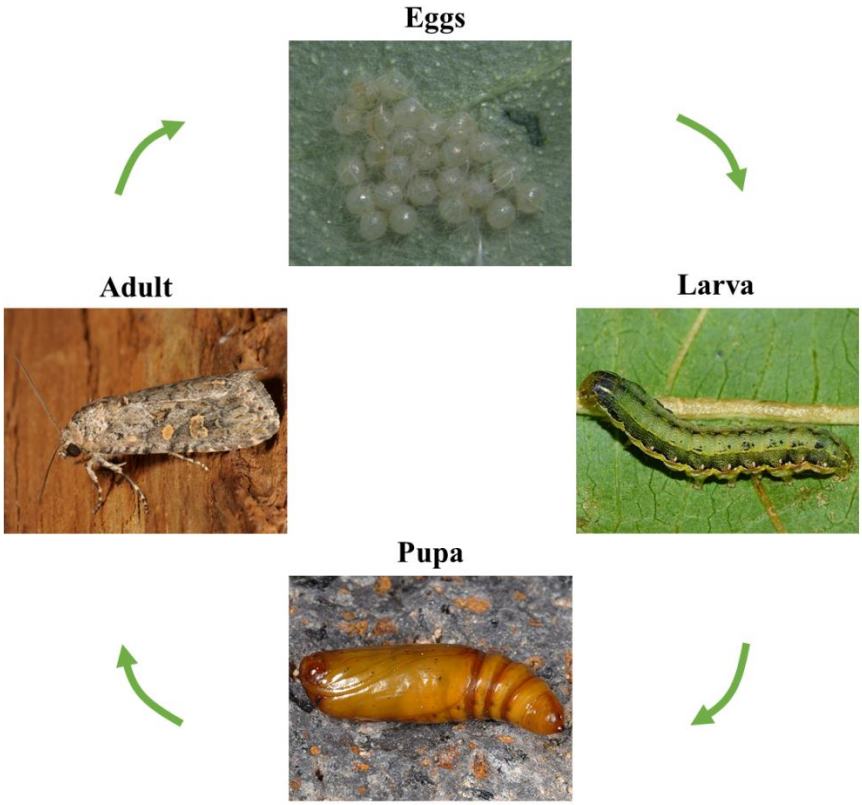
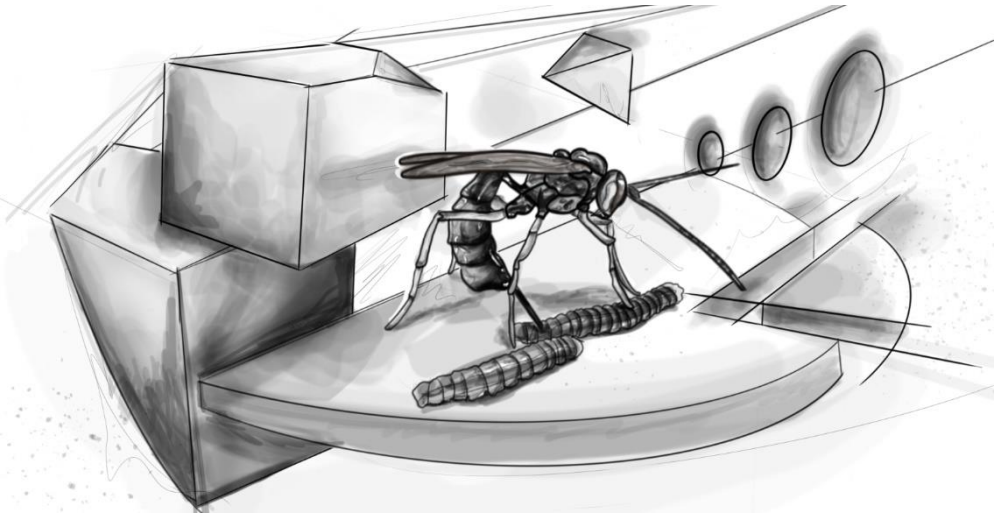


Figure 9. Schematic representation of the life cycle of *S. exigua*.

Bacterial, viral, fungal and nematode entomopathogens have been developed to control the populations of *S. exigua* and reduce the use of chemical insecticides. Many products based on the different strains of the entomopathogenic bacteria *Bacillus thuringiensis* (BT) are available, such as Dipel®, Condor®, Javelin® or Xentari® (Rowell and Bessin, 2005). Nematodes used against *S. exigua* include the species *Steinernema carpocapsae* and *Steinernema feltiae* (Hassan Askary, 2010). Baculovirus-based insecticides currently used to control *S. exigua* are developed using

S. exigua baculovirus (SeMNPV) or *A. californica* baculovirus (AcMNPV), as Spod-X® and Virex® products (Lasa *et al*., 2007; Muñoz and Caballero, 2000). Finally, entomopathogenic fungi have also been considered for controlling *S. exigua*. Some examples are *Metarhizium anisopliae*, *Nomuraea rileyi*, *Paecilomyces fumosoroseus* and some species of *Beuveria bassiana* (Hasyim *et al*., 2017).

OBJECTIVES



In the present thesis, I planned to study the effect of the SeMNPV infection in the neuronal system of *S. exigua*. The neuronal system is composed of the central nervous system, where neuropeptides are expressed and regulate the insect physiology, and the peripheral nervous system, where chemosensory-related proteins are expressed. Both peripheral and central nervous systems directly drive the insect behavior and its relationship with the surrounding environment. I focalized on the neuropeptides and the chemosensory-related genes, and how their expression was affected by SeMNPV infection. For both group of genes, the same approach has been used. First, I annotated a *S. exigua* transcriptome to obtain the corresponding sequence repertoires. Then, I studied the regulation of these genes after the baculovirus infection. Finally, I performed a functional characterisation of candidate genes in order to understand their role in the insect's physiology and behavior in the context of the host-virus interaction. This work has been performed through the accomplishment of the following objectives:

1.- **Description of the *S. exigua* neuropeptidome of larvae and adults and its regulation under different physiological conditions (chapter 1).**

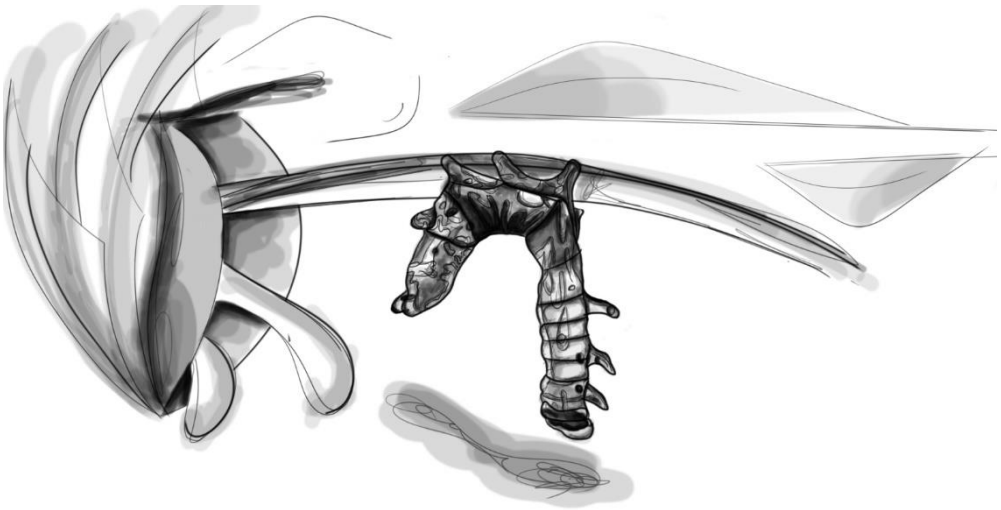
2.- **Analysis of the transcriptional regulation of the *S. exigua* neuropeptidome after the baculoviral infection and study of the physiological and behavioral changes associated with specific genes (chapter 2).**

3.- **Description of the *S. exigua* chemosensory-related genes focusing on larvae and coupling with the development of behavioral assays to study larvae olfaction (chapter 3).**

4.- **Analysis of the transcriptional regulation of the *S. exigua* chemosensory-related genes after the baculoviral infection and functional characterization of specific genes and their effects on the host-virus interaction (chapter 4).**

CHAPTER 1

DESCRIPTION OF THE *SPODOPTERA EXIGUA* NEUROPEPTIDOME OF LARVAE AND ADULTS AND ITS REGULATION UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS.



This chapter has been published as:

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1.1. Introduction

Neuropeptides are small signalling molecules produced as precursors in neurons and in neurocrine cells in multicellular organisms (Russo, 2017). These precursors are processed and modified, becoming mature peptides that will bind to their membrane receptors acting as neurohormones, neurotransmitters, and neuromodulators (Van Hiel *et al*., 2010). Neuropeptides are part of the chemical communication systems between the different cells of an organism and their importance resides in the regulation of different aspects of animal physiology and behaviour such as feeding, reproduction, development, and locomotion (Fónagy, 2014).

Invertebrates are excellent models for studying the neuropeptidergic systems, due to their simplicity and their versatility for experiments in the laboratory (Predel and Eckert, 2000). Data mining of different insect genomes, and experiments done in animal models as *Drosophila melanogaster*, have led to the discovery of novel neuropeptides with a better understanding of their structure and function (Hauser *et al*., 2018; Hewes and Taghert, 2001). Nevertheless, in recent years the study of the neuropeptidergic system has been expanded to other insect orders (Lepidoptera, Blattodea, Coleoptera, Hemiptera and Hymenoptera), with special attention to those insect species causing phytosanitary problems in crops or in human environments (Riehle *et al*., 2002; Roller *et al*., 2008). These studies contribute to our understanding of the insect's environmental plasticity and of its adaptation to different environments and adverse conditions (Hawthorne, 1997). Additionally, these studies could also provide new targets for the development of novel insecticidal agents (Van Hiel *et al*., 2010).

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a serious polyphagous insect pest occurring worldwide (Greenberg *et al*., 2006). This pest species feeds mainly on horticultural crops, although it also feeds on other

agricultural crops, producing huge economic losses in agriculture and in the ornamental plant industries each year (Greenberg *et al*., 2006).

The development of high-throughput DNA sequencing technologies during the last decade has accelerated the generation of new transcriptomic information, although in many cases detailed studies of certain gene or protein families, like neuropeptides, have been left unattended (Pascual *et al*., 2012).

In this study, we focus on the characterization of the neuropeptidome of *S. exigua.* To that aim, we have generated and mined novel transcriptomic data from larval heads and adult brains to annotate a comprehensive neuropeptidome. Additional expression analyses allowed the characterization of those genes based on their potential role in gut-brain communication as well as in their regulation in response to different physical and physiological perturbations.

1.2. Materials and methods

1.2.1. Insects

The *S. exigua* colony (SUI) used for the generation of the transcriptome and for the light deprivation and starvation experiments has been reared at University of Valencia for several years on artificial diet (Bell and Joachim, 1976). Standard rearing was carried at 25 ± 3 ºC with 70 ± 5% RH (elative humidity) and a photoperiod of 16:8 h (light:dark). For the light experiments, larvae originating from the same source colony (SUI) were reared at Wageningen University & Research on artificial diet at 27 ºC with 50% RH, using a photoperiod of 14:10 h (light:dark) (Han *et al*. 2015).

1.2.2. RNA sequencing and transcriptome assembly

S. exigua larval heads were excised from the body of fourth instar larvae using a scalpel. A total of 16 fourth instar larvae were used per treatment and the heads were stored in Trizol reagent (Roche, Basilea, Switzerland) at -80ºC. From the same batch of larvae, 32 larvae were reared until adult and once they emerged from the pupae,

the adult brains were dissected in Trizol reagent pooled and stored in 300 µl of Trizol reagent at -80 ºC. Three independent replicates were processed for each type of sample.

Total RNA was purified using TRIzol reagent following the manufacturer's instructions. A second purification step was applied using RNeasy Mini Kit (Qiagen, Hilden, Germany). Purified RNA was eluted with mQ water and RNA integrity was verified on 1% agarose gels. RNA quantity was determined in a Qubit 3.0 Fluorometer (ThermoFisher Scientific). For each of the samples, equal amounts of total RNA were used for the RNA-Seq using paired-end (PE) strategy and an Illumina HiSeq 2000 instrument (Illumina Inc., San Diego, CA, USA) in Novogen Technology Co. Ltd. (Beijing, China). PE reads were filtered, processed and assembled using Trinity software (version r2014 04 13p1) (Grabherr *et al*., 2011) using standard pipeline (Haas *et al*., 2014) and default parameters.

Samples for the light/dark experiments were obtained from a larger study (Han *et al*., 2018). The larvae used for the current study were treated as follows: newly moulted third instar larvae (moulted and starved overnight) were fed with a 10% sucrose solution using droplet feeding (van Houte *et al*., 2012) at 12 p.m. Subsequently larvae were placed individually in glass jars containing a piece of artificial diet as described in (Han *et al*., 2015). Jars were placed in incubators (27 ºC with 50% RH and a 14:10 LD photoperiod (7 a.m. lights on, 9 p.m. lights off)), with light provided from above using three luminescent tube lamps of 18 Watts each placed at a 30 cm distance above the jars. For, the 'light' treatment, larvae were kept at the normal 14:10 LD photoperiod. For the 'dark' treatment, one group of larvae were switched to complete dark conditions (0:24 LD) at nine hours post sucrose feeding (i.e. 9 p.m.). At 28 h post sucrose feeding, larvae (now late third instar) were collected from both the 'light' and 'dark' group. Larval heads were excised from the body of the larvae using a scalpel. A total of 30 larvae were used per treatment and RNA was isolated from the pooled heads using the RNeasy Micro Kit (Qiagen). Three

independent replicates were processed for each sample. One microgram of total RNA was used for library preparation following the TruSeq Stranded mRNA Sample Preparation Protocol (Illumina). Sequencing was performed on an Illumina HiSeq 2000 platform at Wageningen University & Research. Obtained PE-100 reads were filtered and used for gene expression analysis as described below.

Samples from larvae reared at different temperatures were derived from a laboratory colony. Third instar larvae were reared at the three growth temperatures with artificial diet until larvae become L5. To avoid any variation due to different developmental stages, larvae were collected at the first day (L5D1) after molting to L5. RNAs were extracted from the whole bodies of 10 larvae using Trizol reagent according to manufacturer's instructions. After DNase treatment, extracted RNA was resuspended in 40 µl diethyl pyrocarbonate-treated water. From total RNA, cDNA library was constructed following the TruSeq Stranded mRNA Sample Preparation Protocol (Illumina) and sequenced following a paired-end strategy on an Illumina HiSeq 2000 platform. Obtained PE-100 reads were filtered and used for gene expression analysis as described below.

1.2.3. Mining of the neuropeptide sequences

Unigenes encoding for neuropeptides were identified by local tblastn (BioEdit Software v.7.2.6.1) using known neuropeptide nucleotide sequences from other insects as a query, including sequences from *S. frugiperda* (Gouin *et al*., 2017), *B. mori* (Roller *et al*., 2008), *Chilo suppressalis* (G. Xu *et al*., 2016), *Drosophila melanogaster* (Hewes and Taghert, 2001), *Anopheles gambiae* (Riehle *et al*., 2002), *Tribolium castaneum* (Li *et al*., 2008), *Apis mellifera* (Hummon *et al*., 2006), *Rhodnius prolixus* (Ons *et al*., 2009), *Nilaparvata lugens* (Tanaka *et al*., 2013) and *Mastotermes darwiniensis* (Christie, 2015). Selected unigenes were manually filtered to remove partial sequences and redundant unigenes. Then, the ORFs of the resulting unigenes were predicted using the ORFfinder tool from NCBI

(https://www.ncbi.nlm.nih.gov/orffinder/) and the predicted protein sequences were used in a Blastp analysis to confirm their identity as neuropeptide. Finally, the *S. exigua* neuropeptides were classified in families using the databases NeuroPep: isyslab.info/NeuroPep (Y. Wang *et al*., 2015) and DINeR: http://neurostresspep.eu/diner/ (Yeoh *et al*., 2017).

1.2.4. Phylogenetic analyses

For each family of neuropeptides, homologs representative from different insect orders were retrieved by Blastx analysis or by name search in public databases. Predicted amino acid sequences were aligned with ClustalW software (Larkin *et al*., 2017). Phylogenetic analyses were carried out using the neighbour-joining algorithm with p-distance method and 1000 bootstrap replicates on the MEGA X software (Kumar *et al*., 2018). Phylogenetic trees were visualized with MEGA X. Sequences from other species employed in phylogenetic analyses were retrieved from the GenBank database and from the published neuropeptidomes of *B. mori* (Roller *et al*., 2008), *C. suppressalis* (G. Xu *et al*., 2016), *D. melanogaster* (Hewes and Taghert, 2001), *A. gambiae* (Riehle *et al*., 2002), *T. castaneum* (Li *et al*., 2008), *A. mellifera* (Hummon *et al*., 2006), *R. prolixus* (Ons *et al*., 2009), *N. lugens* (Tanaka *et al*., 2013) and *M. darwiniensis* (Christie, 2015). Moreover, additional sequences, not included in the published Neuropeptidome were obtained from public genome databases: SfruDB (http://bipaa.genouest.org/is/lepidodb/spodoptera_frugiperda/) in the case of *S. frugiperda*; VectorBase (https://www.vectorbase.org/) for *A. gambiae* and EnsemblMetazoa (https://metazoa.ensembl.org) for *T. castaneum* and *A. mellifera*.

1.2.5. Expression analysis

Expression levels of *S. exigua* neuropeptides in different tissues and under different conditions were compared by mapping the different RNA-Seq reads generated in this study or those available on the SRA database at NCBI (SRX1358372, SRX1358373, SRX1358374) to the unigenes from the obtained

neuropeptidome. Mapping was performed using RSEM (version 1.3.0) (Li and Dewey, 2011) and Bowtie (version 1.1.2) (Langmead *et al*., 2009). Relative abundance of each unigene is reported as FPKM (Fragments Per Kilobase of transcript per Million mapped reads). For some of the comparisons, expression data were clustered by hierarchical clustering analysis using the online Heatmapper tool (Babicki *et al*., 2016). When relevant, gene expression levels were statistical compared between tissues or conditions using a two-way ANOVA with the two-stage step-up method of Benjamini, Krieger and Yekutieli using GraphPad Prism 7.0 Software (GraphPad Software Inc., San Diego, USA).

1.2.6. Effect of starvation on neuropeptide expression

For the starvation assays, third instar *S. exigua* larvae were reared individually: larvae in the control treatment were fed for an additional 24 h with artificial diet while larvae in the starvation treatment were kept without any food for 24 h. Per treatment, 16 larvae were used. Three independent biological replicates were performed. After that, larval heads were excised as above and stored in 300 µl of TRIzol Reagent. Then, RNA extraction was carried out following manufacturer's instructions. For each sample, 500 ng of RNA was treated with DNaseI (ThermoFisher Scientific) following the manufacturer's protocol and converted into cDNA using the PrimeScript cDNA synthesis kit (Takara, Japan) following the manufacturer's protocol and using random hexamers and oligo (dT) primers.

Quantitative PCR (qPCR) was done in a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using 5x HOT FIREpol Eva Green qPCR Mix Plus (ROX) from Solis Biodyne (Tartu, Estonia) in a total reaction volume of 25 µl. Forward and reverse primers for all the genes were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA). The efficiencies for the primers were estimated to be about 100% for the neuropeptide genes. An endogenous control *ATP synthase subunit C* housekeeping gene was used in each qPCR to normalize the RNA concentration. A list of the primers used in this study is

provided in Annexed I. The differences in expression between treatments (control and starvation) were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Graphs and statistical analysis (unpaired *t*-test) were performed using the GraphPad Prism program (GraphPad Software Inc., San Diego, USA).

1.3. Results

1.3.1. Neuropeptidome annotation

Data mining of the *S. exigua* transcriptome by Blast comparison using a complete set of neuropeptides described so far in other arthropods as a query, rendered 63 unigenes coding for hypothetical neuropeptides (Table 1A, B) (Genbank accession numbers: MH028060-MH028133). In addition, splicing variants of 6 genes were identified (Table 1A, B) and different gene isoforms were detected for 2 neuropeptide unigenes. The size of annotated neuropeptide precursors is 144 amino acids on average, ranging from 35 amino acids for the diuretic hormone 34 (DH34) to 469 amino acids for the neuropeptide-like precursor 1a isoform b (NPLP1ab). All the identified neuropeptides had their closest homologue in other lepidopteran species (Table 1A, B). The obtained neuropeptidome was compared to other species of insects with published neuropeptidomes (Table 2 and Supplementary Figure 1). Supplementary figure 1 containing the phylogenetical trees is available in https://onlinelibrary.wiley.com/doi/full/10.1111/imb.12535.

1.3.2. Gut-brain neuropeptides

RNA-Seq data generated in this and previous work (Park *et al*., 2014) has been used to identify those gut-brain neuropeptides by comparing their expression in the larval gut and head as well as the adult brain (Table 3A, B). Sixteen neuropeptide and five splicing variants appear to be expressed in the larval gut as well as in the larval brain, representing the main gut-brain peptides from *S. exigua*.

Table 1A. Identified neuropeptides in the transcriptome of *S. exigua*.

Neuropeptide	Family	Precursor size (aa)	First Hit			
			Organism	E-value	Identity (%)	Accession No.
Adipokinetic Hormone 1 (AKH1)	Adipokinetic hormone/Hipertrehalosemic hormone/Red pigment-concentrating	67	*Helicoverpa armigera*	6.00E-32	82	AGH25544.1
Adipokinetic Hormone 2 (AKH2)	Adipokinetic hormone/Hipertrehalosemic hormone/Red pigment-concentrating	73	*Helicoverpa armigera*	7.00E-39	69	AGH25545.1
Adipokinetic Hormone 3 (AKH3)	Adipokinetic hormone/Hipertrehalosemic hormone/Red pigment-concentrating	76	*Spodoptera litura*	2.00E-47	82	XP_022818531.1
Allatostatin A (AstA)	Allatostatin	229	*Spodoptera litura*	5.00E-160	91	XP_022823599.1
Allatostatin C 1 (AstC1)	Allatostatin	125	*Spodoptera litura*	2.00E-85	91	XP_022814210.1
Allatostatin C 2 (AstC2)	Allatostatin	119	*Spodoptera litura*	2.00E-63	90	XP_022814210.1
Allatostatin CC (AstCC)	Allatostatin	109	*Spodoptera litura*	6.00E-72	48	XP_022814224.1
Allatotropin splicing variant a (ATa)	Allatotropin/Orexin	172	*Spodoptera litura*	8.00E-126	94	XP_022830271.1
Allatotropin splicing variant b (ATb)	Allatotropin/Orexin	134	*Spodoptera exigua*	2.00E-95	100	AEO27700.1
Bursicon subunit alpha (Burα)	Cystine knot	158	*Spodoptera litura*	1.00E-115	80	XP_022828186.1
Bursicon subunit beta (Burβ)	Cystine knot	139	*Spodoptera litura*	2.00E-87	81	XP_022828187.1
Capability/Cardioacceleratory peptide 2b/Periviscerokinin (CAPA)	Pyrokinin/Periviscerokinin/Pheromone biosynthesis activating neuropeptide	151	*Spodoptera litura*	4.00E-105	83	XP_022823728.1
CCHamide 1 splicing variant a (CCH1a)	CCHamide	149	*Spodoptera litura*	5.00E-96	81	XP_022826245.1
CCHamide 1 splicing variant b (CCH1b)	CCHamide	175	*Spodoptera litura*	2.00E-121	82	XP_022826245.1
CCHamide 2 splicing variant a (CCH2a)	CCHamide	142	*Spodoptera litura*	2.00E-99	83	XP_022825743.1
CCHamide 2 splicing variant b (CCH2b)	CCHamide	138	*Spodoptera litura*	1.00E-96	82	XP_022825740.1
Corazonin (CRZ)	Corazonin	103	*Spodoptera litura*	6.00E-68	81	XP_022827606.1
Corticotropin-releasing factor-like - diuretic hormone 44 (CRF-DH44)	Corticotropin-releasing hormone binding protein/Diuretic hormone-related	322	*Spodoptera litura*	0	98	XP_022825617.1
Crustacean cardioactive peptide (CCAP)	Crustacean cardioactive peptide	130	*Spodoptera litura*	2.00E-85	81	XP_022818878.1
Diuretic hormone 31/Calcitonin-like peptide (DH31)	Diuretic hormone	112	*Spodoptera litura*	3.00E-76	90	XP_022828300.1
Diuretic hormone 34 (DH34)	Diuretic hormone	35	*Spodoptera litura*	8.00E-15	87	XP_022829233.1
Diuretic hormone 41 (DH41)	Diuretic hormone	92	*Spodoptera litura*	5.00E-56	91	XP_022829225.1
Diuretic hormone 45 (DH45)	Diuretic hormone	80	*Spodoptera litura*	1.00E-47	99	XP_022829215.1
Ecdysis triggering hormone (ETH)	Ecdysis triggering hormone	114	*Spodoptera litura*	1.00E-76	95	XP_022817257.1
Eclosion hormone (EH)	Eclosion hormone	70	*Spodoptera litura*	6.00E-37	95	XP_022825948.1
FMRFamide (FMRF)	FMRFamide related peptide	189	*Spodoptera litura*	4.00E-131	82	XP_022823692.1
Glycoprotein hormone alpha 2 (GPα2)	Glycoprotein hormone	115	*Spodoptera litura*	3.00E-78	84	XP_022814978.1
Glycoprotein hormone beta 5 A (GPβ5A)	Glycoprotein hormone	159	*Spodoptera litura*	2.00E-111	81	XP_022814898.1
Glycoprotein hormone beta 5 B (GPβ5B)	Glycoprotein hormone	186	*Spodoptera litura*	9.00E-88	78	XP_022814443.1
IMFamide (IMF)	FMRFamide related peptide	76	*Spodoptera litura*	1.00E-48	93	XP_022816866.1
Insulin-like peptide 1 (ILP1)	Insulin/Insulin-like growth factor/Relaxin	173	*Spodoptera litura*	1.00E-43	73	XP_022823034.1
Insulin-like peptide 2 (ILP2)	Insulin/Insulin-like growth factor/Relaxin	50	*Spodoptera littoralis*	1.00E-31	100	AEE43937.1
Insulin-like precursor polypeptide 1a (ILPP1a)	Insulin/Insulin-like growth factor/Relaxin	88	*Spodoptera littoralis*	3.00E-52	85	AEE43936.1
Insulin-like precursor polypeptide 1b (ILPP1b)	Insulin/Insulin-like growth factor/Relaxin	86	*Helicoverpa armigera*	9.00E-36	77	AGH25573.1
Insulin-like precursor polypeptide 2 (ILPP2) 2	Insulin/Insulin-like growth factor/Relaxin	50	*Spodoptera littoralis*	6.00E-30	92	AEE43937.1

Table 1B. Identified neuropeptides in the transcriptome of *S. exigua*.

Neuropeptide	Family	Precursor size (aa)	First Hit			
			Organism	E-value	Identity (%)	Accession No.
Insulin-like precursor polypeptide Aa (ILPPAa)	Insulin/Insulin-like growth factor/Relaxin	89	*Spodoptera litura*	6.00E-53	73	XP_022816653.1
Insulin-like precursor polypeptide Ab (ILPPAb)	Insulin/Insulin-like growth factor/Relaxin	88	*Spodoptera litura*	2.00E-32	68	XP_022816653.1
Insulin-like precursor polypeptide B (ILPPB)	Insulin/Insulin-like growth factor/Relaxin	85	*Spodoptera litura*	5.00E-53	81	XP_022829893.1
Insulin-like precursor polypeptide D (ILPPD)	Insulin/Insulin-like growth factor/Relaxin	123	*Spodoptera litura*	2.00E-83	80	XP_022829036.1
Ion transport peptide isoform a (ITPa)	Crustacean hyperglycemic hormone family	114	*Spodoptera litura*	2.00E-79	90	XP_022814177.1
Ion transport peptide isoform b (ITPb)	Crustacean hyperglycemic hormone family	108	*Spodoptera litura*	1.00E-74	95	XP_022814187.1
Ion transport peptide isoform c (ITPc)	Crustacean hyperglycemic hormone family	106	*Operophtera brumata*	4.00E-38	95	KOB62752.1
ITG-like 1 (ITG1)	ITG-like	107	*Heliothis virescens*	6.00E-72	86	PCG77254.1
ITG-like 2 (ITG2)	ITG-like	101	*Spodoptera litura*	6.00E-67	81	XP_022817047.1
ITG-like 3 (ITG3)	ITG-like	46	*Heliothis virescens*	3.00E-21	98	PCG77254.1
Leucokinin (LK)	Kinin	339	*Spodoptera litura*	0	76	XP_022815385.1
Myosuppressin (MS)	Myosuppressin	98	*Spodoptera litura*	2.00E-64	88	XP_022815723.1
Natalisin 1 (NTL1)	Tachykinin-related peptides	197	*Spodoptera litura*	1.00E-129	73	XP_022815604.1
Natalisin 2 (NTL2)	Tachykinin-related peptides	269	*Spodoptera litura*	0.00E+00	93	XP_022815605.1
Neuroparsin (NP)	Neuroparsin/Ovary ecdysteroidogenic hormone	86	*Helicoverpa armigera*	9.00E-52	88	XP_021195927.1
Neuropeptide F 1 splicing variant a (NPF1a)	Neuropeptide Y	121	*Helicoverpa armigera*	9.00E-52	88	XP_021195927.1
Neuropeptide F 1 splicing variant b (NPF1b)	Neuropeptide Y	81	*Spodoptera litura*	6.00E-81	91	XP_022825972.1
Neuropeptide F 2 (NPF2)	Neuropeptide Y	93	*Heliothis virescens*	8.00E-52	87	PCG68789.1
Neuropeptide-like precursor 1a isoform a (NPLP1aa)	Neuropeptide-like precursor	335	*Helicoverpa armigera*	0	83	XP_021195834.1
Neuropeptide-like precursor 1a isoform b (NPLP1ab)	Neuropeptide-like precursor	469	*Spodoptera litura*	0	84	XP_022822867.1
Neuropeptide-like precursor 1a isoform c (NPLP1ac)	Neuropeptide-like precursor	395	*Spodoptera litura*	0	81	XP_022822867.1
Neuropeptide-like precursor 1b (NPLP1b)	Neuropeptide-like precursor	137	*Helicoverpa armigera*	8.00E-72	80	XP_021195833.1
Orcokinin 1 (OK1)	Orcokinin	212	*Spodoptera litura*	8.00E-127	82	XP_022813838.1
Orcokinin 2 splicing variant a (OK2a)	Orcokinin	187	*Bombyx mori*	6.00E-104	72	NP_001124366.1
Orcokinin 2 splicing variant b (OK2b)	Orcokinin	197	*Bombyx mori*	7.00E-101	69	XP_012550847.1
Pheromone biosynthesis activating	Pyrokinin/Periviscerokinin/Pheromone	191	*Spodoptera exigua*	2.00E-136	98	AAR87744.1
Pigment dispersing factor (PDF)	Pigment-dispersing hormone/Pigment-dispersing factor (PDH/PDF)	109	*Spodoptera litura*	0.00E+00	72	XP_022827090.1
Proctolin (PT)	Proctolin	286	*Heliothis virescens*	1.00E-66	68	PCG63106.1
Prothoracicostatic peptide/Myoinhibitory-like peptide/Allatostatin B1 (PTSH)	Prothoracicostatic	130	*Heliothis virescens*	1.00E-66	85	PCG63106.1
Prothoracicotropic hormone (PTTH)	Insulin/Insulin-like growth factor/Relaxin	205	*Spodoptera exigua*	4.00E-150	99	AAT64423.2
Ryamide (RY)	Luqin/Ryamide	71	*Spodoptera litura*	1.00E-43	70	XP_022817118.1
Short Neuropeptide F (sNPF)	Neuropeptide Y	182	*Spodoptera litura*	5.00E-127	87	XP_022825707.1
SIFamide (SIF)	FMRFamide related peptide	83	*Helicoverpa armigera*	2.00E-35	82	AGH25569.1
Sulfakinin (SK)	Gastrin/cholecystokinin	74	*Helicoverpa armigera*	2.00E-31	83	XP_021184067.1
Tackykinin (TK)	Tachykinin-related peptides	248	*Spodoptera litura*	4.00E-168	76	XP_022815495.1
Trissin 1 splicing variant a (TR1a)	Trissin	142	*Spodoptera litura*	1.00E-77	78	XP_022819154.1
Trissin 1 splicing variant b (TR1b)	Trissin	165	*Spodoptera litura*	8.00E-96	79	XP_022819153.1
Trissin 2 (TR2)	Trissin	101	*Spodoptera litura*	4.00E-46	72	XP_022819243.1

Table 2A. Neuropeptide gene expression in *S. exigua* and other representative insect species.

	Lepidoptera			Diptera		Coleoptera	Hymenoptera	Hemiptera		Blattodea
Neuropeptide	***S. exigua***	***B. mori***	***C. suppressalis***	***D. melanogaster***	***A. gambiae***	***T. castaneum***	***A. mellifera***	***R. prolixus***	***N. lugens***	***M. darwiniensis***
Adipokinetic Hormones (AKH)	+	+	+	+	+	+	+	+	+	+
Allatostatin A (AstA)	+	+	+	+	+	+	+	+	+	+
Allatostatin C (AstC)	+	+	+	+	+	+	nd	nd	+	+
Allatostatin CC (AstCC)	+	+	+	+	nd	+	+	nd	+	nd
Allatotropins (AT)	+	+	+	nd	+	+	nd	+	+	+
Antidiuretic factor (ADF)	nd	nd	nd	nd	nd	+	nd	nd	nd	nd
Arginine-vassopressin-like peptide (AVLP)	nd	nd	nd	nd	nd	+	nd	nd	+	nd
Bursicon α (Burα)	+	+	+	nd	+	+	+	nd	+	+
Bursicon β (Burβ)	+	+	+	+	+	+	+	nd	+	nd
Capability/Cardioacceleratory peptide 2b/Periviscerokinin (CAPA)	+	+	+	+	+	+	+	nd	nd	nd
CCHamide 1 (CCH1)	+	+	+	+	nd	+	+	nd	+	nd
CCHamide 2 (CCH2)	+	+	+	+	+	+	+	nd	+	+
CNMamide (CNM)	nd	nd	+	nd	nd	nd	nd	nd	nd	nd
Corazonin (CRZ)	+	+	+	+	+	nd	+	+	+	+
Corticotropin-releasing factor-like - diuretic hormone 44 (CRF-DH44)	+	+	+	+	+	+	+	+	+	nd
Crustacean cardioactive peptide (CCAP)	+	+	+	+	+	+	+	+	+	+
DENamide (DEN)	nd	nd	nd	nd	nd	nd	nd	nd	nd	+
Diuretic hormone 31/Calcitonin-like peptide (DH31)	+	+	+	+	+	+	+	+	+	+
Diuretic hormone 34 (DH34)	+	+	nd	+	nd	nd	nd	nd	nd	nd
Diuretic hormone 41 (DH41)	+	+	nd	+	nd	nd	nd	nd	nd	nd
Diuretic hormone 45 (DH45)	+	+	nd	+	+	nd	nd	nd	+	nd
Ecdysis triggering hormone (ETH)	+	+	+	+	+	+	+	nd	+	nd
Eclosion hormone (EH)	+	+	+	+	+	+	+	nd	+	nd
Elevenin-like peptide (ELLP)	nd	nd	nd	nd	nd	nd	nd	nd	+	nd
FMRFamide (FMRF)	+	+	+	+	+	+	+	nd	+	+
Glycoprotein hormone alpha 2 (GPα2)	+	+	+	+	+	+	nd	nd	+	nd
Glycoprotein hormone beta 5 A (GPβ5A)	+	+	+	+	+	+	nd	nd	+	nd
Glycoprotein hormone beta 5 B (GPβ5B)	+	+	+	+	+	+	nd	nd	+	nd
IMFamide (IMF)	+	+	+	+	nd	+	nd	+	+	nd

+: identified

nd: non-identified

Table 2B. Neuropeptide gene expression in *S. exigua* and other representative insect species.

Neuropeptide	Lepidoptera			Diptera		Coleoptera	Hymenoptera	Hemiptera		Blattodea
	S. exigua	*B. mori*	*C. suppressalis*	*D. melanogaster*	*A. gambiae*	*T. castaneum*	*A. mellifera*	*R. prolixus*	*N. lugens*	*M. darwiniensis*
Inotocin (Ino)	nd	nd	nd	nd	nd	nd	nd	nd	+	+
Insulin-like peptide 1 (ILP1)	+	nd	nd	nd	nd	nd	nd	nd	nd	nd
Insulin-like peptide 2 (ILP2)	+	+	nd	+	nd	+	nd	nd	nd	nd
Insulin-like polypeptide 1 (ILPP1)	+	+	nd	nd	nd	nd	nd	nd	nd	nd
Insulin-like polypeptide 2 (ILPP2)	+	nd	nd	nd	nd	nd	nd	nd	nd	+
Insulin-like polypeptide A (ILPPA)	+	+	nd	nd	nd	nd	nd	nd	nd	nd
Insulin-like polypeptide B (ILPPB)	+	+	nd	nd	nd	nd	nd	nd	nd	nd
Insulin-like polypeptide D (ILPPD)	+	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ion transport peptide (ITP)	+	+	+	+	+	+	nd	+	+	nd
ITG-like (ITG)	+	+	+	nd	+	+	+	nd	nd	nd
Leucokinin (LK)	+	+	+	+	nd	nd	nd	nd	nd	+
Myosuppressin (MS)	+	+	+	+	+	+	+	+	+	+
Natalisin (NTL)	+	+	+	+	nd	nd	nd	nd	nd	nd
Neuroparsin (NP)	+	+	+	nd	nd	+	nd	+	+	+
Neuropeptide-like precursor 1 (NPLP1)	+	+	+	+	nd	+	+	+	+	nd
Neuropeptide-like precursor 2 (NPLP2)	+	nd	nd	nd	nd	nd	nd	nd	nd	nd
Neuropeptides F (NPF)	+	+	+	+	+	nd	+	+	+	+
Orcokinin (OK)	+	+	+	+	+	+	+	+	+	+
Pheromone biosynthesis activating neuropeptide - Diapause Hormone (PBAN-DH)	+	+	+	nd	+	+	+	+	nd	nd
Pigment dispersing factor (PDF)	+	+	+	+	+	nd	+	nd	+	nd
Proctolin (PT)	+	+	+	+	nd	+	nd	nd	+	nd
Prothoracicostatic peptide (PTSH)	+	+	+	+	+	nd	nd	nd	+	nd
Prothoraticotropic hormone (PPTH)	+	+	+	+	+	+	nd	nd	+	+
Ryamide (RY)	+	nd	+	nd	nd	nd	nd	nd	+	+
Short Neuropeptide F (sNPF)	+	+	+	+	+	+	nd	+	+	+
SIFamide (SIF)	+	+	+	nd	nd	+	+	+	+	+
Sulfakinin (SK)	+	+	+	+	+	+	+	+	+	+
Tachykinin (TK)	+	+	+	+	nd	+	+	+	+	+
Trissin (TR)	+	+	+	+	+	+	nd	nd	nd	nd

+: identified
nd: non-identified

Table 3A. Neuropeptide genes expression in the larval head, larval midgut and adult brain of *S. exigua*.

Neuropeptide	Expression level		
	Larva Head	Larva Gut	Adult Brain
Adipokinetic Hormone 1 (AKH1)	++++++	-	+++++
Adipokinetic Hormone 2 (AKH2)	-	-	+++++
Adipokinetic Hormone 3 (AKH3)	+++++	-	++++
Allatostatin A (AstA)	+++++	+++++	+++++
Allatostatin C 1 (AstC1)	++++++	++++++	++++++
Allatostatin C 2 (AstC2)	-	++++++	-
Allatostatin CC (AstCC)	++++	++++++	++++
Allatotropin splicing variant a (ATa)	+++++	+++++	++++
Allatotropin splicing variant b (ATb)	+++++	+++++	+++++
Bursicon subunit alpha (Burα)	+++++	-	++++
Bursicon subunit beta (Burβ)	+++++	-	++++
Capability/Cardioacceleratory peptide 2b/Periviscerokinin (CAPA)	+++++	-	+++++
CCHamide 1 splicing variant a (CCH1a)	++++	++	++++
CCHamide 1 splicing variant b (CCH1b)	+++++	+++++	+++++
CCHamide 2 splicing variant a (CCH2a)	+++++	++++++	++++
CCHamide 2 splicing variant b (CCH2b)	+++++	+++++	+++++
Corazonin (CRZ)	+++++	-	+++++
Corticotropin-releasing factor-like - diuretic hormone 44 (CRF-DH44)	+++++	+++++	+++++
Crustacean cardioactive peptide (CCAP)	+++++	-	+++++
Diuretic hormone 31/Calcitonin-like peptide (DH31)	+++++	++++++	+++++
Diuretic hormone 34 (DH34)	-	-	-
Diuretic hormone 41 (DH41)	+++++	-	+++++
Diuretic hormone 45 (DH45)	+++++	+++++	+++++
Ecdysis triggering hormone (ETH)	+++++	-	++++
Eclosion hormone (EH)	-	-	-
FMRFamide (FMRF)	+++++	-	+++++
Glycoprotein hormone alpha 2 (GPα2)	-	-	-
Glycoprotein hormone beta 5 A (GPβ5A)	-	-	++
Glycoprotein hormone beta 5 B (GPβ5B)	+++	-	+++
IMFamide (IMF)	-	-	++++++
Insulin-like peptide 1 (ILP1)	++++++	-	++++++
Insulin-like peptide 2 (ILP2)	++++	-	++++
Insulin-like precursor polypeptide 1a (ILPP1a)	-	-	-
Insulin-like precursor polypeptide 1b (ILPP1b)	++++++	-	+++++
Insulin-like precursor polypeptide 2 (ILPP2) 2	+++++	-	+++++
Insulin-like precursor polypeptide Aa (ILPPAa)	+++++	-	+++++
Insulin-like precursor polypeptide Ab (ILPPAb)	++++++	-	+++++

Expression level by reads:

Not Expression: -
1-10 FPKM: +
10-100 FPKM: ++
100-1000 FPKM: +++
1000-10000 FPKM: ++++
10000-100000 FPKM: +++++
100000-1000000 FPKM: ++++++
1000000-10000000 FPKM: +++++++

Table 3B. Neuropeptide genes expression in the larval head, larval midgut and adult brain of *S. exigua* .

	Expression level		
Neuropeptide	**Larva Head**	**Larva Gut**	**Adult Brain**
Insulin-like precursor polypeptide B (ILPPB)	++++++	-	+++++
Insulin-like precursor polypeptide D (ILPPD)	++++++	-	+++++
Ion transport peptide isoform a (ITPa)	++++++	-	++++++
Ion transport peptide isoform b (ITPb)	+++++	-	++++++
Ion transport peptide isoform c (ITPc)	-	-	-
ITG-like 1 (ITG1)	++++++	-	++++++
ITG-like 2 (ITG2)	++++++	-	++++++
ITG-like 3 (ITG3)	-	-	-
Leucokinin (LK)	+++++	-	++++
Myosuppressin (MS)	++++++	+++++	++++++
Natalisin 1 (NTL1)	+++++	-	+++++
Natalisin 2 (NTL2)	+++++	-	+++++
Neuroparsin (NP)	+++++	-	+++++
Neuropeptide F 1 splicing variant a (NPF1a)	++++	++++++	++++
Neuropeptide F 1 splicing variant b (NPF1b)	+++++	+++++++	+++++
Neuropeptide F 2 (NPF2)	-	-	++++
Neuropeptide-like precursor 1a isoform a (NPLP1aa)	+++++	-	+++++
Neuropeptide-like precursor 1a isoform b (NPLP1ab)	+++++	-	+++++
Neuropeptide-like precursor 1a isoform c (NPLP1ac)	-	-	+++
Neuropeptide-like precursor 1b (NPLP1b)	+++++	-	+++++
Orcokinin 1 (OK1)	++++++	++++++	+++++
Orcokinin 2 splicing variant a (OK2a)	+++++	+	+++++
Orcokinin 2 splicing variant b (OK2b)	+++++	+++++	+++++
Pheromone biosynthesis activating neuropeptide - Diapause Hormone (PBAN-DH)	++++++	-	++++++
Pigment dispersing factor (PDF)	++++	-	++++
Proctolin (PT)	+++	++++	+++
Prothoracicostatic peptide 1/Myoinhibitory-like peptide 1 (PTSH1)	+++++	-	+++++
Prothoracicotropic hormone (PTTH)	+++++		+++++
Ryamide (RY)	+++++	-	++++
Short Neuropeptide F (sNPF)	+++++	++++	+++++
SIFamide (SIF)	-	-	+++
Sulfakinin (SK)	+++++	-	+++++
Tackykinin (TK)	+++++	++++++	+++++
Trissin 1 splicing variant a (TR1a)	+++++	-	+++++
Trissin 1 splicing variant b (TR1b)	++++	-	++++
Trissin 2 (TR2)	++++	-	++++

Expression level by reads:

Not Expression: -	1000-10000 FPKM: ++++
1-10 FPKM: +	10000-100000 FPKM: +++++
10-100 FPKM: ++	100000-1000000 FPKM: ++++++
100-1000 FPKM: +++	1000000-10000000 FPKM: +++++++

1.3.3. Larval versus adult neuropeptides

From the differential expression analysis, a comparison between neuropeptides expressed in the larval head and in adult brain was performed. Few neuropeptides (AKH2, IMFamide, NPLP1ac, NPF2, and SIFamide) while being expressed in the adult brain appear to be absent in the larval head (as well as the larval gut).

1.3.4. Neuropeptide regulation under starvation

One of the main aspects regulated by neuropeptides are all the processes related to feeding and digestion. Therefore, it is expected that certain neuropeptides are differentially expressed during starvation. To test this hypothesis, the expression levels of 28 previously selected neuropeptides were determined using RT-qPCR in the larval head during starvation (Figure 1). As expected, the majority of the studied

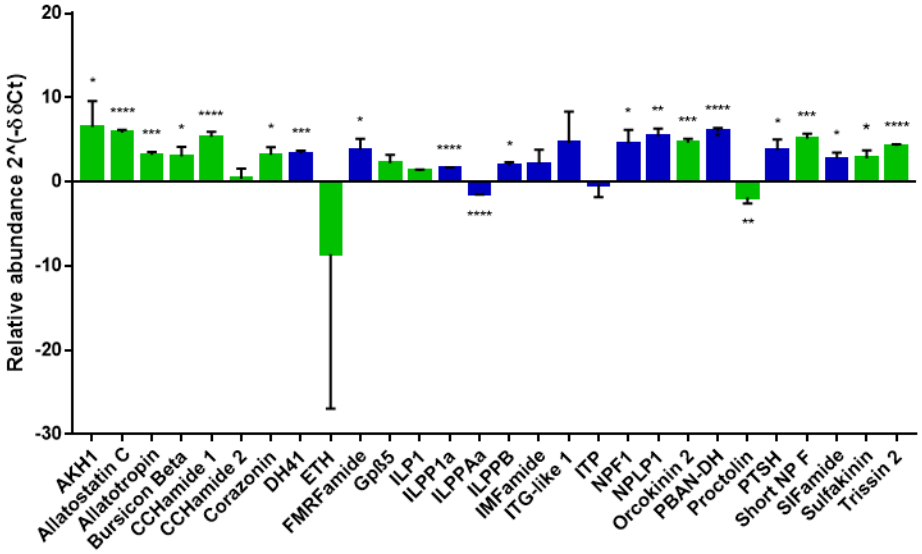

Figure 1. Effect of larval starvation on the expression of the *S. exigua* neuropeptidome. Changes in gene expression in the larval heads after starvation for 24 hours of selected unigenes from the neuropeptidome. Gene expression was measured by qPCR and each bar represents the mean of three independent experiments (± SD). Green bars represent unigenes functionally related with feeding, digestion or growing, while blue bars represent genes involved in other functions. Statistically significant changes (p-value<0.05) are indicated by an asterisk.

neuropeptides were found to be differentially expressed during starvation, most of them being upregulated. A similar pattern of regulation was found between neuropeptides linked to digestive or developmental conditions (green bars in Figure 1) and between those related to other physiological processes (blue bars in Figure 1).

1.3.5. Effect of light and temperature on neuropeptide expression

Expression levels of the annotated neuropeptides was compared between third instar larvae grown under a standard photoperiod (14:10; light:dark) and third instar larvae grown under complete dark conditions for 24 hours (0:24; light:dark) (Figure 2A and Annexed II). From the 63 neuropeptides analysed, none of them was significantly differentially expressed in response to the light deprivation (Annexed II). Clustering analysis of the values from the different replicates and conditions revealed a more similar expression profiles between light/dark treatments within each biological replicates than between the different biological replicates of the same treatment (Figure 2A).

Differential expression analysis for neuropeptides in larvae developed at different temperatures (20ºC, 25ºC and 30ºC) was also performed using available RNA-Seq data (Figure 2B). In contrast to previous data, expression of only 25 neuropeptides were identified in these RNA-Seq data. Although we could detect variation in the expression levels of certain neuropeptides in relation to the temperature at which larvae were reared, no pattern showing a general modulation in the expression of the neuropeptides was observed.

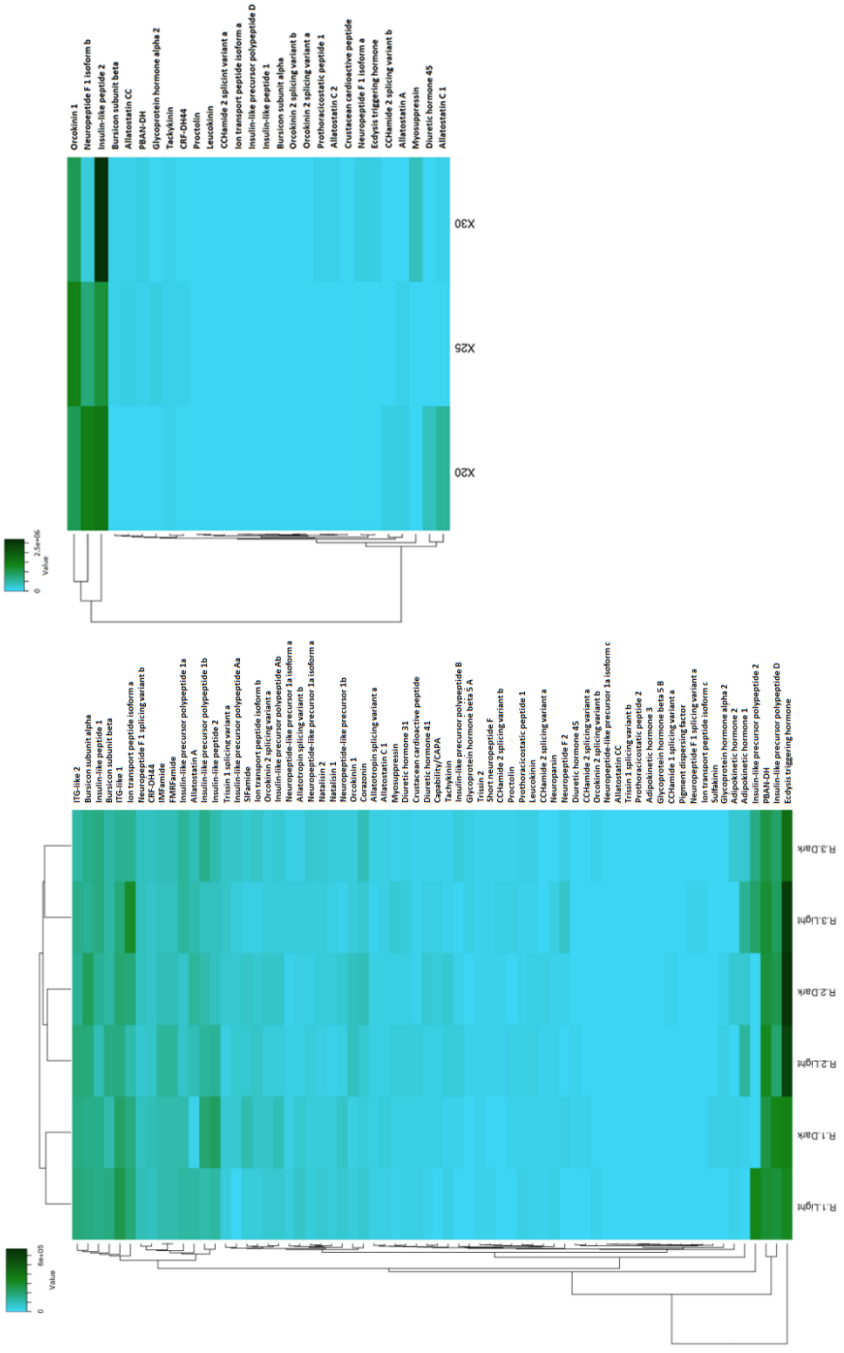

Figure 2. Effect of different physical conditions on the expression of the S. exigua neuropeptidome. Hierarchical clustering of the *S. exigua* neuropeptidome based on the gene expression measured by mapping of RNA-Seq reads from each sample and condition. A) Effect of light deprivation. R1 to R3 refers to the replicate number. B) Effect of the different rearing temperature (20, 25 and 30 ºC).

1.4. Discussion

Sixty-three neuropeptide precursor genes have been identified in the beet armyworm, *S. exigua*. Phylogenetic analysis of the 46 main families of neuropeptides showed the clustering of the *S. exigua* neuropeptides with the homologs in other species of Lepidoptera for all the studied families. The phylogenetic analysis also revealed some events of gene duplication in certain families of neuropeptides. Interestingly, for few families, more than one *S. exigua* neuropeptide was identified within the lepidopteran clusters, suggesting the presence of gene paralogs originated from a recent gene duplication within the *S. exigua* or closely related species. That was obvious for the allatostatin C (Ast C), Insulin-like Polypeptide 1 (ILPP1), Insulin-like Polypeptide A (ILPPA), and NPLP1. We identified corticotropin releasing-factor like-diuretic hormone 44 (CRF-DH44) in our *S. exigua* data, a neuropeptide that was not described in Lepidoptera before. Based on the *S. exigua* CRF-DH44 we additionally identified this neuropeptide in the recently published genome of *S. litura* (Cheng *et al*., 2017) and other Lepidoptera demonstrating the presence of this neuropeptide across different insect orders. In contrast, Insulin-like Peptide 1 (ILP-1), ILPP1, and ILPPA have only been detected in lepidopteran species, probably representing Lepidoptera-specific neuropeptides. Except for the CNMamide (CNM) (which was only detected in *C. suppressalis*), the neuropeptides not detected in the *S. exigua* neuropeptidome are also absent in the other lepidopteran species, reflecting the comprehensiveness of the obtained neuropeptidome. Overall, the comparison among insect orders reflects a core neuropeptidome in insects formed by at least 43 neuropeptides.

Gut-brain neuropeptides are generally involved in the regulation of the ingestive and digestive behaviours of the organism, but also in other processes like growth and development. They are usually identified based on their simultaneous expression in the brain and gut tissues (Duve and Thorpe, 1986). Within the 16 brain-gut neuropeptides identified in this work, the Ast family is composed by three sub-

family genes whose main function is the rapid and reversible inhibition of the juvenile hormone (JH) synthesis (Li *et al*., 2006; Stay and Tobe, 2007). Members of the AstA and AstC families appear to be present in *S. exigua*, showing expression in the larval gut (Woodhead *et al*., 1989). Interestingly, two paralogs of the AstC have been exclusively identified in *S. exigua* (Sup. Fig.1.3). AstC2, although expressed in larval gut, was not found expressed in the larval head or adult brain, suggesting an alternative role of this specific allatostatin, independent of its expression in the CNS. Allatotropin (AT) is another well stablished gut-brain neuropeptide that stimulates foregut contractions improving the digestion (Elekonich and Horodyski, 2003; Villalobos-Sambucaro *et al*., 2015). In agreement with that, both AT variants (ATa and ATb) were highly expressed in the larval gut of *S. exigua.* CCHamide 1 and 2 (CCH1 and CCH2) are described as gut-brain peptides synthesized in endocrine cells in the larval midgut and in neurons in the CNS in *D. melanogaster* (Li *et al*., 2013). In agreement with that, our expression analysis suggest a similar pattern in *S. exigua*. Similarly, other neuropeptides previously described as gut-brain neuropeptides, such as the diuretic hormone 44 (CRF-DH44) (Cannell *et al*., 2016), diuretic hormone 31/calcitonin-like peptide (DH31), and diuretic hormone 45 (DH45), appear to be also expressed in both the brain and gut of *S. exigua.* Additional gut-brain peptides identified in our transcriptomic approach are proctolin (PT) (Ormerod *et al*., 2016), myosuppressin (MS) (Vilaplana *et al*., 2008), neuropeptide F1 (NPF1), short neuropeptide F (sNPF) (Nässel and Wegener, 2011), tachykinin (TK) (Sambasivarao, 2013) and orcokinin (OK) (Sterkel *et al*., 2012). Orcokinins are described as myotropic neuropeptides, but their function seems to be related to the circadian clock. For instance, in the cockroach *Leucophaea maderae* an OK plays a neuromodulator role regulating the response to the light. This OK also has additional secondary functions like the regulation of locomotion (Hofer, 2006). In addition, OKs from *B. mori* have shown a prothoracicotropic function, stimulating the synthesis of steroid hormones (Yamanaka N *et al.*, 2016). Two splicing variants of OK2 have been

identified in *S. exigua*. While the OK2b variant is highly expressed in the larval gut, the OK2a variant is hardly expressed, suggesting a differential role or functional specificity for each variant. This is further supported by the fact that both splicing variants differ in a 10 amino acids region in the predicted mature peptide.

As neuropeptides control different aspects of an animal's growth and development (Gäde and Hoffmann, 2005), it is expected that their expression levels could change along the different larval stages or from larval to adult stages. According to our results, there was a good congruence between the expression levels of the neuropeptides among larval and adult stages, however, the results suggest the existence of neuropeptide precursors with specific function in adult tissues. Although previous studies have reported changes in the expression level of certain neuropeptides between larval and adult tissues (Abdel-latief *et al*., 2004; Li *et al.*, 2013; Sedra and Lange, 2016). To our knowledge, this is the first report of neuropeptides that are exclusively expressed in the adult brain, suggesting their potential involvement in adult-specific processes.

In agreement with our results in the neuropeptide regulation under starvation, upregulation of the short neuropeptide F, allatostatin, and CCHamide was observed in starved adults from *D. melanogaster* (Farhan *et al*., 2013), being important factors governing starvation-induced olfactory modifications. Based on these results, it would be interesting to explore the influence of these changes in neuropeptide expression on the larval ability to search for potential food.

Many of the insect neuropeptides have been reported to be related to physical alterations such as the light cycle and temperature (Schiesari *et al*., 2011; Terhzaz *et al.*, 2015). We took the advantage of available RNA-Seq data on *S. exigua* to check for the influence of those abiotic changes on neuropeptide expression. According to our results, light deprivation in the larval stage does not seem to have a major effect on the regulation of neuropeptides at the gene expression level. Neither with the temperature alterations, there was an absence of a clear pattern of regulation. ILPs

neuropeptides are known to mediate larval growth as well as control haemolymph sugar levels (Wu and Brown, 2006). Although ILP2 expression did not show a clear change in expression with the increase of temperature, this neuropeptide exhibited the highest expression level in the different samples. The ILP2 was reported to control haemolymph trehalose levels in L5 larvae of *S. exigua* (Kim and Hong, 2015). This suggests that, among the different ILPs, ILP2 could be playing a major role in larval growth and development. So far, not much information is available about the regulation of neuropeptide expression depending on physical conditions such as light and temperature fluctuation. Nevertheless, our preliminary results on these aspects do not seem to correlate these physical changes with gene expression regulation of specific neuropeptides.

The number of insect genomes and transcriptomes generated over the last few years has drastically increased due to the continuous reduction of the sequencing costs (Mardis, 2011). The *S. exigua* neuropeptidome reported here will facilitate the further annotation and phylogenetic analysis of neuropeptides in other insect species, especially from the order Lepidoptera. The neuropeptide precursors here annotated represent the biggest insect neuropeptidome described so far providing novel information that would contribute to the understanding of insects, and especially of *Spodoptera* species, to the relationship of insects with their environment.

CHAPTER 2

A PROCTOLIN-LIKE PEPTIDE IS REGULATED AFTER BACULOVIRUS INFECTION AND MEDIATES IN CATERPILLAR LOCOMOTION AND DIGESTION.

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2.1. Introduction

Baculoviruses are a large family of entomopathogenic viruses, infecting more than 700 insect species, most of these being larvae of the order *Lepidoptera*. This specification to mainly lepidopteran insects makes them a suitable biological control agent, being safe for other insects, plants and humans (Lacey *et al*., 2015; Szewczyk *et al*., 2006). In addition, the virus is highly persistent in the environment, enhancing its potential for pest control. Nowadays, baculovirus-based biological control products are in use worldwide and novel products against different pests are under development in many countries (Moscardi *et al*., 2011).

One of the distinctive properties of baculoviruses is the production of two different types of virions with different functions during the infection cycle. On one hand, the occlusion-derived viruses (ODVs) contain one or multiple nucleocapsids inside a single envelope. ODVs are embedded in a proteinaceous occlusion body (OB) that causes the primary infection of the insect host. In the alkaline midgut, OBs fall apart releasing the ODVs, which bind and fuse to the midgut epithelial cells. Subsequently, the nucleocapsids enter the midgut cells, where they replicate in the nucleus. The newly formed nucleocapsids bud out of the cells, and the second type of virion is formed, budded viruses (BVs). BVs consist of a single enveloped nucleocapsid and are responsible of the secondary infection and viral spread inside the insect body. During this secondary stage of infection, baculoviruses are able to produce a systemic infection replicating in many tissues including the fat body, hypodermis, trachea, muscle and nervous system (Passarelli, 2011; Torquato *et al*., 2006).

Baculovirus infections evoke behavioural changes in lepidopteran larvae, to the benefit of their own dispersion (Smirnoff, 1965). For example, *Bombyx mori* larvae infected with the Bombyx mori nucleopolyhedrovirus (BmNPV) were found to move three to five times further than non-infected larvae (Kamita *et al*., 2005), a behavioural change named hyperactivity. In another example of behavioural

manipulation, baculovirus-infected larvae climb to the apical parts of the plant, where they eventually die and liquefy. Viral OBs are released from the corpse, spreading to the environment in what is known as tree-top disease (Goulson, 1997; Hoover *et al*., 2011; Van Houte *et al*., 2015). These behavioural changes are mediated by virus-encoded genes (Hoover *et al*., 2011; Kamita *et al*., 2005) and could be associated to changes in the host signaling pathways in the central nervous system (CNS) where part of the physiology and behaviour of the insect is controlled (Kinoshita and Homberg, 2017).

In animals, the neuropeptidergic system is composed of different neuropeptide genes that encode small protein-like molecules used by neurons as neurohormones, neurotransmitters and neuromodulators, constituting a way of chemical communication (Van Hiel *et al*., 2010). These molecules are functionally related to some features of the animal's physiology and behaviour and could be directly modulated during baculovirus infection. The neuropeptide proctolin (RYLPT) acts as a neurohormone and a neuromodulator in insects and is considered a potent stimulator of visceral and skeletal muscles directly affecting the insect's development (Fiandra *et al*., 2010; Ormerod *et al*., 2016). Although proctolin has been identified in Coleoptera, Diptera, Blattodea and Orthoptera, there are doubts about its presence in Lepidoptera where its receptor has not been identified so far (Baines and Downer, 1991; Brown and Starratt, 1975; Ormerod *et al*., 2016). In the recent *Spodoptera exigua* neuropeptidome description, a gene containing the peptide RHLPT was firstly annotated as proctolin (Llopis-Giménez *et al*., 2019). However, due to the lack of evidence that this gene corresponds to the proctolin neuropeptide annotated in other insects, it has been redefined as proctolin-like peptide (PLP).

In this work we first analyse how a baculovirus infection may affect the neuropeptidergic system of our model organism *S. exigua* by comparative gene expression analysis in heads of infected insects. Due to the absence of major differences in global regulation of the neuropeptides expression, more specific

analyses of the changes in the larval brain were conducted, revealing a different set of regulated NPs when compared to the changes observed using the complete larval heads. Among the regulated NPs, the PLP was found to be down-regulated in the brain of caterpillar infection with Spodoptera exigua nucleopolyhedrovirus (SeMNPV) wildtype but also when infected with a mutant defective on the *ecdysteroid uridine 5'-diphosphate (UDP)-glucosyl transferase* (*egt*), a viral gene ancestrally acquired from the host and associated to physiological and behavioral changes in the larvae (Hoover *et al*., 2011; Kamita *et al*., 2005). Overexpression of this peptide and the consecutive bioassays with *S. exigua* larvae revealed its functional connection with developmental and locomotion processes, and its possible functional similarity with the neuropeptide proctolin. These results allow us to hypothesize about the direct or indirect modulation of PLP by baculoviruses and its function in the insect's physiology as a potential neuropeptide.

2.2. Material and methods

2.2.1. Insects

Batches from the same *S. exigua* colony (SUI) were used by the two laboratories involved in this study using slightly different rearing conditions. Insects were reared at Wageningen University & Research on artificial diet at 27 ºC with 50% RH and a photoperiod of 14:10 h (light:dark) (Han *et al*., 2015). At the University of Valencia insects were reared on artificial diet at 25ºC with 70 RH and a photoperiod of 16:8 h (light:dark).

2.2.2. Larval infections with SeMNPV and RNA purification

Samples for the SeMNPV infection experiments were obtained from a previous study (Han *et al*., 2018). The larvae used for the current study were treated as follows: 10 newly molted third instar larvae (molted and starved overnight) were fed, using droplet feeding, with a 10% sucrose solution containing SeMNPV at a 10^6

OBs/ml concentration, which is known to kill at least 90% of infected larvae (van Houte *et al*., 2012). As controls, 10 mock-infected larvae, droplet fed with a virus-free solution, were used per assay and none of them died due to a virus infection (assessed by visual examination of classical symptoms). Subsequently, larvae were placed individually in glass jars (120 mm high and 71 mm in diameter) containing a piece of artificial diet (aprox. 3-5 cm^3) as described in (Han *et al*., 2015). Jars were placed in incubators (27 ºC with 50% RH and a 14:10 LD photoperiod (7 a.m. lights on, 9 p.m. lights off)), with light provided from above using three luminescent tube lamps of 18 Watts each placed at a 30 cm distance above the jars. Jars were covered with transparent plastic Saran wrap containing three small holes for ventilation. Third instar mock-infected larvae develops faster than the virus-infected larvae and were collected at 28 hours post infection (hpi). To synchronize the developmental status among infected and non-infected larvae (Han, 2018), the infected larvae were collected at 54 hpi. Larval heads were excised from the body of the larvae using a scalpel and total RNA was isolated from the pooled heads using the RNeasy Micro Kit (Qiagen). Six independent biological replicates were processed. One microgram of total RNA was used for library preparation following the TruSeq Stranded mRNA Sample Preparation Protocol (Illumina). Sequencing was performed on an Illumina HiSeq 2000 platform at Wageningen University & Research. Obtained PE-100 reads were filtered and used for gene expression analysis as described below.

2.2.3. Expression analysis by RNA-Seq

Expression of *S. exigua* neuropeptides under SeMNPV infection were compared by mapping the trimmed reads from the different RNA-Seq libraries (6 replicates control and 6 replicates SeMNPV infection) to the *S. exigua* neuropeptidome (Llopis-Giménez *et al*., 2019). Read mapping was performed using RSEM (version 1.3.0) (Li and Dewey, 2011) and Bowtie 2 (version 2.3.4.3) software (Langmead and Salzberg, 2012). Relative abundance of each candidate gene is

reported as TPM (Transcript per Million). Variability among replicates and general regulation was assessed by hierarchical clustering analysis using the heatmap.2 (gplots v.3.0.1.1) package of R software. For the statistical analysis, a biological coefficient of variation (BCV) is performed with a false discovery rate (FDR) using the edgeR package (v.3.14.0) using R software.

2.2.4. Expression analysis by RT-qPCR

Eighty newly molted third instar larvae (starved overnight) were fed, using droplet feeding, with a solution containing 10% sucrose solution, 1% of blue colorant Patent Blue V Sodium Salt (Honeywell) and 10^6 OBs/ml wild type (WT) SeMNPV or a deletion mutant of SeMNPV in which a major part of the *egt* gene was removed (Δegt-SeMNPV) (Han *et al*. 2015). In both cases, concentrations are supposed to kill at least 90% of infected larvae (Han *et al*. 2015). In addition, 80 control larvae were fed with the same solution containing no virus (mock infection). Subsequently, larvae were placed individually in glass jars (120 mm high and 71 mm in diameter) containing a piece of artificial diet (approx. 3-5 cm^3) as described in (Han *et al*., 2015) and 2 larvae were used per jar and then placed as described above. Jars were covered with transparent plastic containing three small holes for ventilation. Larvae were collected at 28 hpi and 46 hpi in the case of the controls. Larvae infected with WT SeMNPV and with Δegt-SeMNPV were collected at 46 hpi (40 larvae) and 54 hpi (other 40 larvae). Six independent replicates were performed for each treatment. Larval brains were dissected in PBS and stored in 125 µl of TRIzol Reagent (Roche) at -80ºC. Total RNA was purified using TRIzol reagent following the manufacturer's instructions. One ng of each RNA sample was treated with DNAseI (ThermoFischer Scientific) following manufacturer's protocol. Then, RNA was converted into cDNA using SuperScript II Reverse Transcriptase (ThermoFischer Scientific) following manufacturer's recommendations and using random hexamers and oligo (dT) primers.

In order to aid the nucleic acid precipitation, 10 µl of Glycogene (Roche) was used per sample.

Real-time quantitative PCR (RT-qPCR) was 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. Forward and reverse primers for selected NP genes were designed using the online software tool Primer3Plus (Untergasser *et al*., 2007). The efficiencies for the primers were confirmed to be about 100% for the neuropeptide genes. As an endogenous control the *S. exigua ATP synthase subunit C* housekeeping gene was used in each qPCR to normalize the RNA concentration (Bel *et al*., 2013). A list of the used primers is 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. An 8-hour window was used for each condition to discard possible changes in gene expression due to changes in the photoperiod of the larvae. Due to the absence of differences in gene expression between the two time points (46 and 54 hpi for infected samples and 28 and 46 for mock ones), the values of the two points were used together. Graphs and statistical analysis (unpaired *t*-test with Welch's correction) were performed using GraphPad Prism software.

2.2.5. Preparation of recombinant baculoviruses

Recombinant baculoviruses were generated using the AcMNPV Bac-to-bac Baculovirus Expression system (ThermoFischer Scientific) following the manufacturer's instructions. The process started with constructing the donor vectors using the pFastBacDual as a backbone. A control donor vector had been previously generated in our laboratory, including the *polyhedrin* gene cloned under the *polyhedrin* promoter (to construct AcMNPV-Con). In order to generate the recombinant virus (AcMNPV-PLP) for the *Se-PLP* gene expression, the C-terminal part containing the predicted RHLPT peptide (position from 142 to 274 in the protein)

was amplified by PCR from cDNA derived from the *S. exigua* larval brains (Figure 1). The used primers were F: 5'-ACTGCTCGAGATGATTTCTACATTCTT GTCGGG-3' and R: 5'-CGATCCATGGTCATTCATTGAGATTACAATCT-3' and were designed to include the XhoI and NcoI restriction sites (underlined), respectively. The *Se-PLP* gene was cloned under the *p10* promoter, at the XhoI and NcoI restriction sites. A scheme of both constructions can be appreciated in Figure 1, also with the cloned sequence of PLP in the AcMNPV vectors.

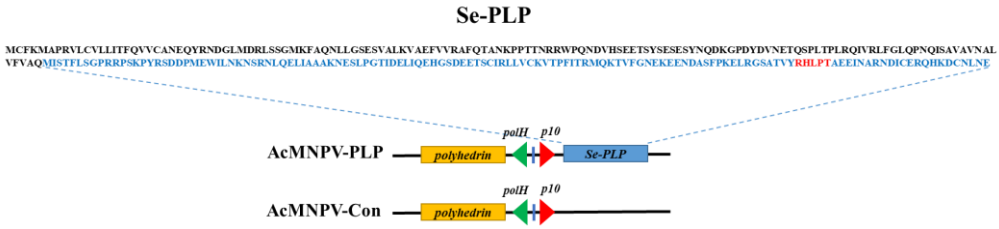


Figure 1. Predicted amino acid sequence of PLP and schematic representation of the recombinant baculoviruses. In blue appears the sequence expressed in AcMNPV viruses. In red the sequence of the hypothesized functional peptide.

The donor vectors were used to transform *Escherichia coli* DH10Bac heat-shock competent cells, containing the AcMNPV bacmid, following the instructions of the manufacturer. Bacmid DNAs were recovered from *E. coli* cells and were used to transfect Sf21 cells (in Grace's Insect medium (ThermoFischer Scientific)) using Cellfectin II reagent (ThermoFischer Scientific) following the manufacturer's recommendations. Two independent bacmid clones from each construction were used to generate the recombinant viruses (AcMNPV-Con and AcMNPV-PLP) and these two strains per recombinant virus were used as biological replicates in the subsequent bioassays. After 5 days of incubation at 27ºC, the resulting baculoviruses were collected (P0). Two more steps of baculovirus amplification were passaged in Sf21 cells in order to obtain a larger viral stock (P1 and P2). Budded virus derived from P2 passage were directly collected from the medium by centrifugation, quantified by qPCR as was previously described and used for the growth curves (Martínez-Solís *et*

al., 2017). The virus concentrations were calculated in budded virus per millilitre (BVs/ml). P2 OBs were purified using a 40% sucrose gradient protocol (Caballero *et al*., 1992). The OBs used in the bioassays were quantified in the Neubauer chamber as OBs per millilitre (OBs/ml).

The time-course replication of both types of recombinant viruses were assessed in Sf21 cells. The two independent clones of AcMNPV-Con and the two clones of AcMNPV-PLP were used to infect Sf21 cells in a 12-well plate (1 ml of cells/well). This was performed in three replicates. Each 24 hours (from 0 to 96 hours post infection (hpi)), 25 µl of each well supernatant was taken for viral DNA quantification. Sample was treated with Prepman Ultra Sample Preparation Reagent (ThermoFischer Scientific) and was directly used for qPCR, using *DNA polymerase* primers and a standard curve of known amounts of viral DNA (from 10 ng to 0.001 ng). Values were represented using GraphPad Prism software.

2.2.6. Insect bioassays: pathogenicity and virulence

Two bioassays were performed, one with a sublethal viral dose ($5 \cdot 10^5$ OBs/ml) and a second one with a lethal viral dose ($1 \cdot 10^7$ OBs/ml). Three treatments were used per bioassay: mock infection, infection with AcMNPV-Con and infection with AcMNPV-PLP. Thirty-two newly molted *S. exigua* third instar larvae were used per treatment and replicate. Infections were performed by droplet-feeding, in drops containing the virus solution or mQ water (mock controls), 10% sucrose and 10% Phenol Red as mentioned above. After that, larvae were individualized in trays with a piece of artificial diet and mortality was recorded every 12 hours until the end of the bioassay (286 hpi). At the end of the bioassay, mortality rates were calculated for each of the virus and the different doses. Graphs and statistical analysis (Gehan-Breslow-Wilcoxon survival curve test) were performed using GraphPad Prism software. Assays were done in three independent replicates involving different batches of insects and using two different viral clones for each of the recombinant viruses.

2.2.7. Insect bioassays: digestion and locomotion

In order to analyse how *PLP* could affect digestion processes, 32 newly molted *S. exigua* third instar larvae were used per treatment and per replicate and infected as described above. To discard the influence of sublethal infections on the analysed parameters, only the lethal viral dose ($1 \cdot 10^7$ OBs/ml) was used for these assays. Such concentration achieves constitutive and high PLP expression in the central nervous system of all the larvae. For these assays, larvae were not individualized. The weight of the larvae during the bioassay was monitored every 24 h from the start of the bioassay. Larvae from each treatment were weighted in groups in order to estimate average individual weight. From 72 hpi to 144 hpi, frass was taken from the bioassay trays and were weighted every 24 hours. Graphs and statistical analysis (two-way ANOVA) were performed using GraphPad Prism software. Assays were done in three independent replicates involving different batches of insects and using two independent viral clones for each of the recombinant viruses.

The possible influence of *PLP* on larval locomotion was also analysed. Ten *S. exigua* larvae were placed in one side of a 14 cm diameter Petri dish whereas a piece of artificial diet (1.5 x 0.8 x 1 cm) was placed at the opposite side. The Petri dish was placed inside a paperboard box (30 x 22 x 22 cm). A hole in the side of the box (6 cm of diameter) was made to include a 50 W halogen artificial light (at 15 cm of distance to the Petri dish) (Figure 2). For each replicate, larval mobility was scored by dividing the Petri dish in 10 areas of 1.3 cm each (Figure 2). At time 2', 5' and 10' the number of larvae in each area was recorded and then, the interval travelled by each them was averaged and converted into cm, obtaining a mean mobility index. This bioassay was performed with the three groups of larvae: mock-infected larvae, larvae infected with AcMNPV-Con and larvae infected with AcMNPV-PLP, in a total of six replicates. Graphs and statistical analysis (two-way ANOVA) were performed using GraphPad Prism software.

2.2.8. *PLP* expression in larvae heads

In order to quantify the native and recombinant PLP in bioassayed larval heads, groups of 4 heads were taken from each group of larvae at different time points: 96 hpi and 120 hpi from the mortality assay; and 120 hpi and 144 hpi from the digestion and locomotion assays. The heads were stored in 200 µl of TRIzol and RNA extraction was performed following maker's instructions. 500 ng of each RNA were treated with DNAseI and converted into cDNA as described before.

RT-qPCR analysis was performed as previously described. Different primers were used in the analysis: AcMNPV-DNApol, for quantifying viral presence in the samples; *Se*-PLP, for quantifying the native *PL*; and AcMNPV-PLP, for specific detection of the recombinant *PLP* (the reverse primer specifically hybridizes with a 3' region in the baculovirus expression vector). A list of the used primers is provided in Annexed 3. The cycle threshold (Ct) values from each sample was used to know the relative expression level of the different genes through the $2^{-Ct\text{-}Proc}/2^{-Ct\text{-}DNApol}$ method. Graphs were performed using GraphPad Prism software.

To check if recombinant *PLP* expression could affect other NPs expression, brains from the locomotion bioassay were taken at 144 hpi. The brains were stored in 200 µl of TRIzol and processed as before. A repertoire of different neuropeptide genes that are functionally related with different aspects of the insect's physiology were included in the analysis to have an overview of possible influence of PLP overexpression on NPs regulation.

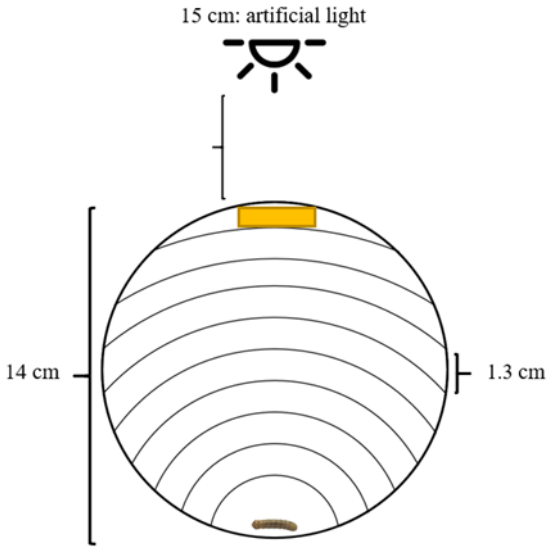


Figure 2. Scheme of the locomotion assay design. Ten larvae were put in one of the sides of the Petri dish whereas a piece of artificial diet was placed on the opposite side. A halogen artificial light was positioned at 15 cm from the Petri dish.

2.3. Results

2.3.1. Neuropeptide expression in larval heads after SeMNPV infection

Effect of viral infection on the expression of the *S. exigua* neuropeptidome was initially assessed in larval heads. The overall analysis did not reveal major difference in global regulation of neuropeptides expression between control (mock-infected) and virus-infected samples as the clustering analysis of the expression values could not group samples by their treatment (Figure 3). Although a general regulation pattern of the neuropeptidergic system after baculovirus infection was not observed, nine specific genes did show a significant regulation. Two of them were upregulated as *insulin-like peptide 1* (*ILP1*) (8.3-fold change) and *insulin-like peptide 2* (*ILP2*) (2.3-fold change). Seven neuropeptide genes were downregulated including ecdysis triggering hormone (*ETH*) (6.3-fold change), ion transport peptide (*ITP*) (6-fold

change), PBAN-DH (1,7-fold change), eclosion hormone (*EH*) (5.2-fold change), SIFamide (*SIF*) (4-fold change), leucokinin (*LK*) (2.1-fold change) and prothoracicotropic hormone (*PTTH*) (2.3-fold change).

2.3.2. Neuropeptide expression in larval brains after SeMNPV infection

Larval brain accounts for a small fraction of the total head mass and constitutes the main tissue where most of NPs are expressed (Fónagy, 2014; Nässel and Homberg, 2006). Consequently, specific changes in the larval brain could become unnoticed and a more detailed analysis of the NPs expression using more sensitive methods was needed. Differential expression analysis of selected NPs in the brain of virus-infected insects was performed through RT-qPCR. A number of 23 NPs were selected according to the previous expression results and their potential role in behaviour. Experiments were conducted with wild type (WT) SeMNPV and Δegt-SeMNPV viruses to check whether the lack of the *egt*, a gene previously associated to behavioral changes in the larvae (Hoover *et al*., 2011; Kamita *et al*., 2005), could influence the NPs regulation after viral infection. The presence of viral activity in the brain tissues, measured as the expression of the viral *DNApol* gene, was confirmed and found similar for both viruses (WT and Δegt) (Figure 4A, B). The overall transcription pattern of the selected NPs was similar for both viral infections. Independently of the viral genotype, most of the analysed genes were not showing important changes in gene expression following viral infection. From the differentially expressed genes, *PLP* appears to be significantly down-regulated for the two viral infections. *PLP* expression was 5.6- and 5.4-fold down regulated after infection with WT SeMNPV and the Δegt-SeMNPV, respectively.

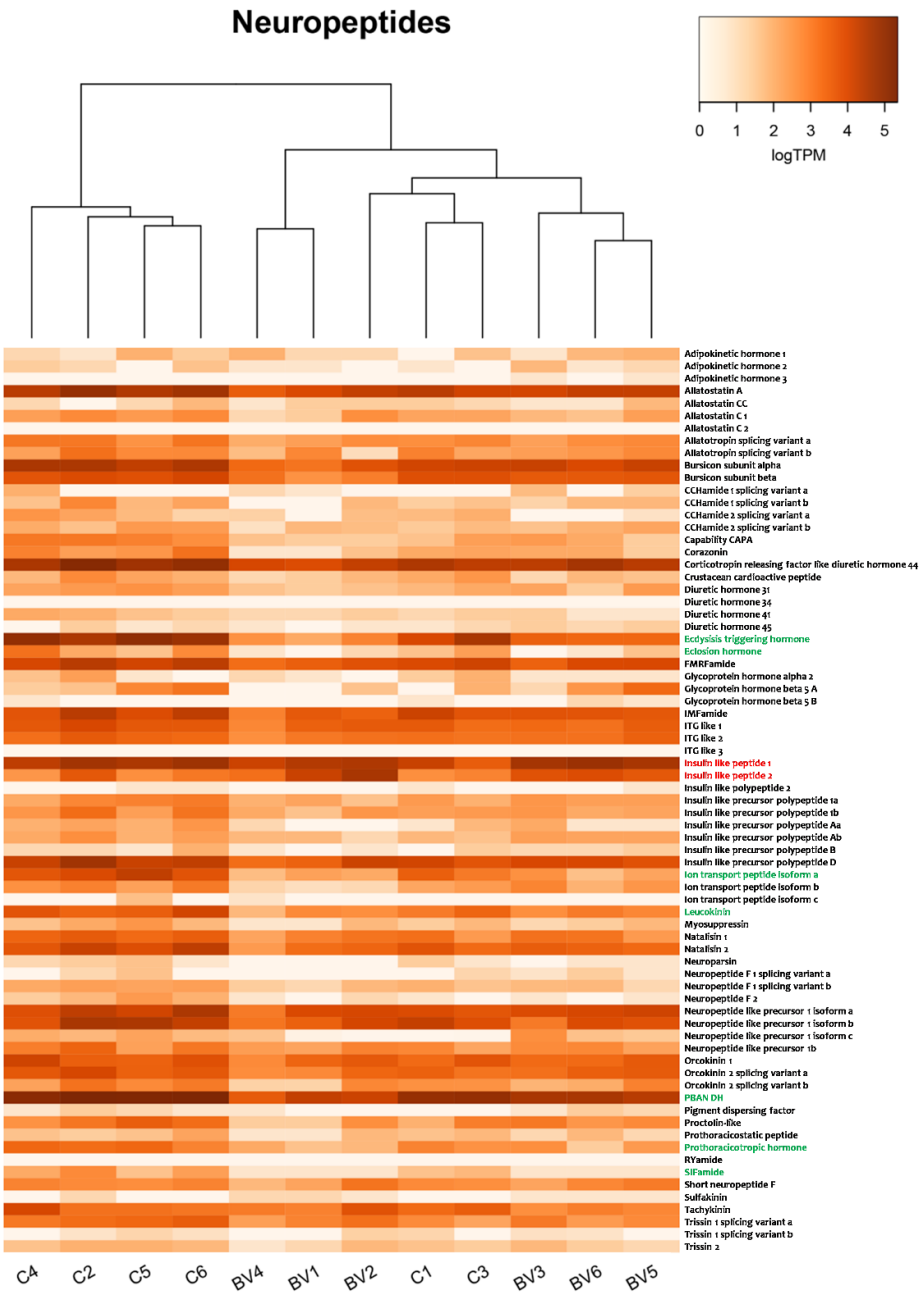


Figure 3. Heat-plot of the relative expression of neuropeptides in the head of *S. exigua* larva infected by SeMNPV. Estimation of abundance values was determined by read mapping. Colour plots

represent values of TPM logarithm. Light orange colours indicate low expression and dark orange ones indicate high expression. C: Control sample. BV: Baculovirus-infected sample. Red and green labelled genes are indicative of up-regulation or down-regulation, respectively (BCV test with FDR) (P<0.05).

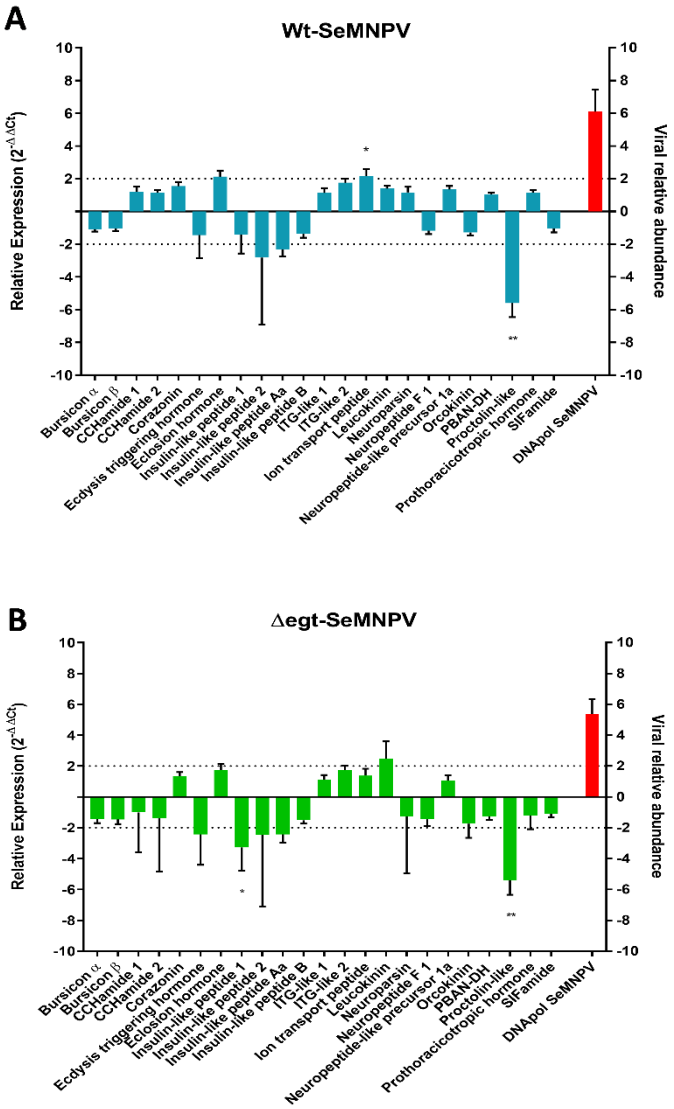
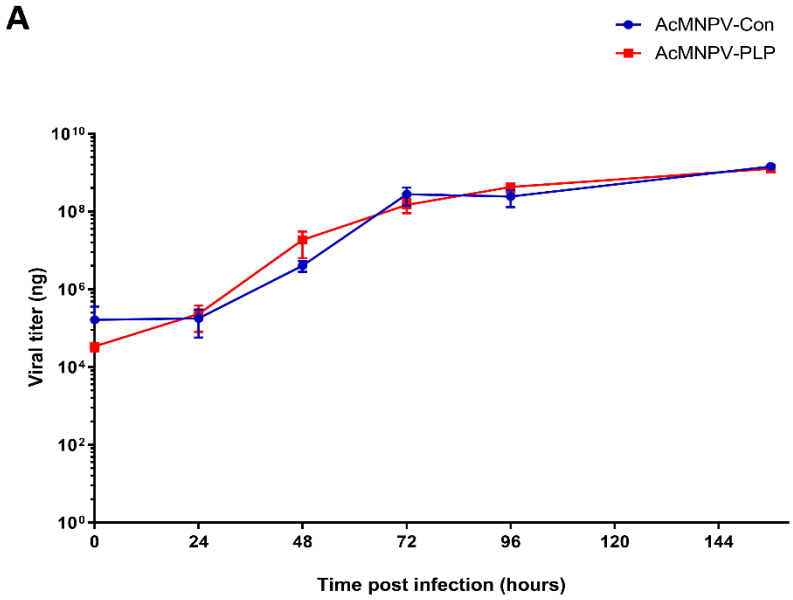


Figure 4. Differential expression analysis in *S. exigua* larval brains after infection with SeMNPV. A) WT-SeMNPV and B) Δegt-SeMNPV. RT-qPCR assays were performed using gene specific primer

pairs. Asterisks indicate statistically significant differences between non-infected and infected samples (unpaired *t*-test with Welch's correction) ($P<0.05$). The red column shows the expression of the SeMNPV DNApol gene.

2.3.3. Effect of *PLP* expression on larval physiology and behavior

Motivated by the consistent and reproducible downregulation of *PLP* in larval brains after viral infection, additional studies were conducted to dissect the influence of *PLP* on the physiology and behaviour of *S. exigua* larvae during the viral infection. For that purpose, a recombinant baculovirus constitutively expressing the *Se-PLP* fragment (AcMNPV-PLP) was constructed. AcMNPV-PLP replicates in Sf21 cells similarly to the counterpart control (AcMNPV-Con) (Figure 5A). In addition, the expression of recombinant and native *PLP* in the brain of AcMNPV-infected larvae was confirmed using specific primers (Figure 5B). Recombinant *PLP* was highly expressed in the brain of larval infected with the AcMNPV-PLP virus.



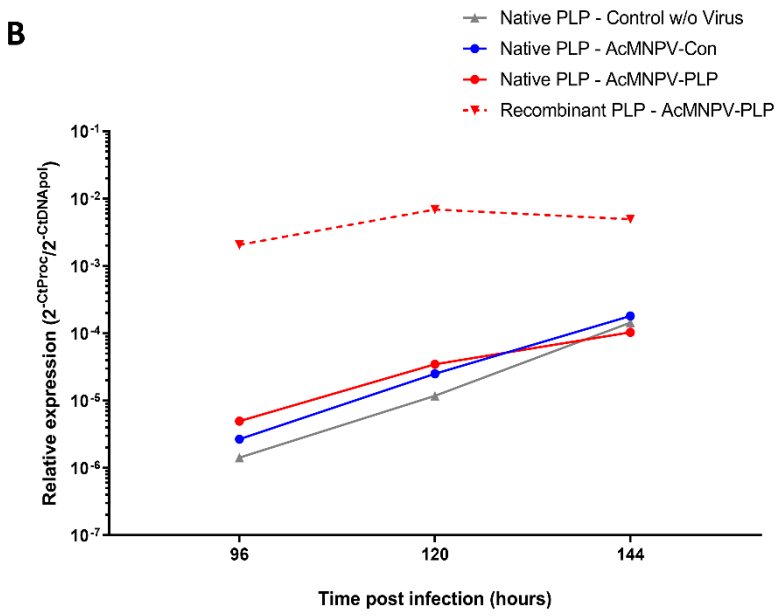


Figure 5. Recombinant AcMNPV expressing *PLP*. A) Growth curves of AcMNPV-Con and AcMNPV-PLP in Sf21 cell culture. B) Relative expression of native and recombinant SeProctolin-like in bioassayed *S. exigua* larval heads. RT-qPCR assays were performed using gene specific primer pairs. Native *PLP* in the non-infected samples appears in grey, native *PLP* in AcMNPV-Con samples appears in blue, native *PLP* in AcMNPV-PLP samples appears in continuous red and recombinant *PLP* in AcMNPV-PLP samples appears in dotted red.

Effect of the overexpression of *PLP* in the baculoviral pathogenicity and virulence was assessed by bioassaying AcMNPV constructs at sublethal ($5{\cdot}10^5$ OBs/ml) and lethal ($1{\cdot}10^7$ OBs/ml) doses. At the sublethal dose, AcMNPV-PLP was more pathogenic (45% mortality) that its control virus (25% mortality) (Figure 6A). However, at the lethal doses, no effect of *PLP* expression on viral activity was observed and similar pathogenicity and virulence was found for both viruses (Figure 6B).

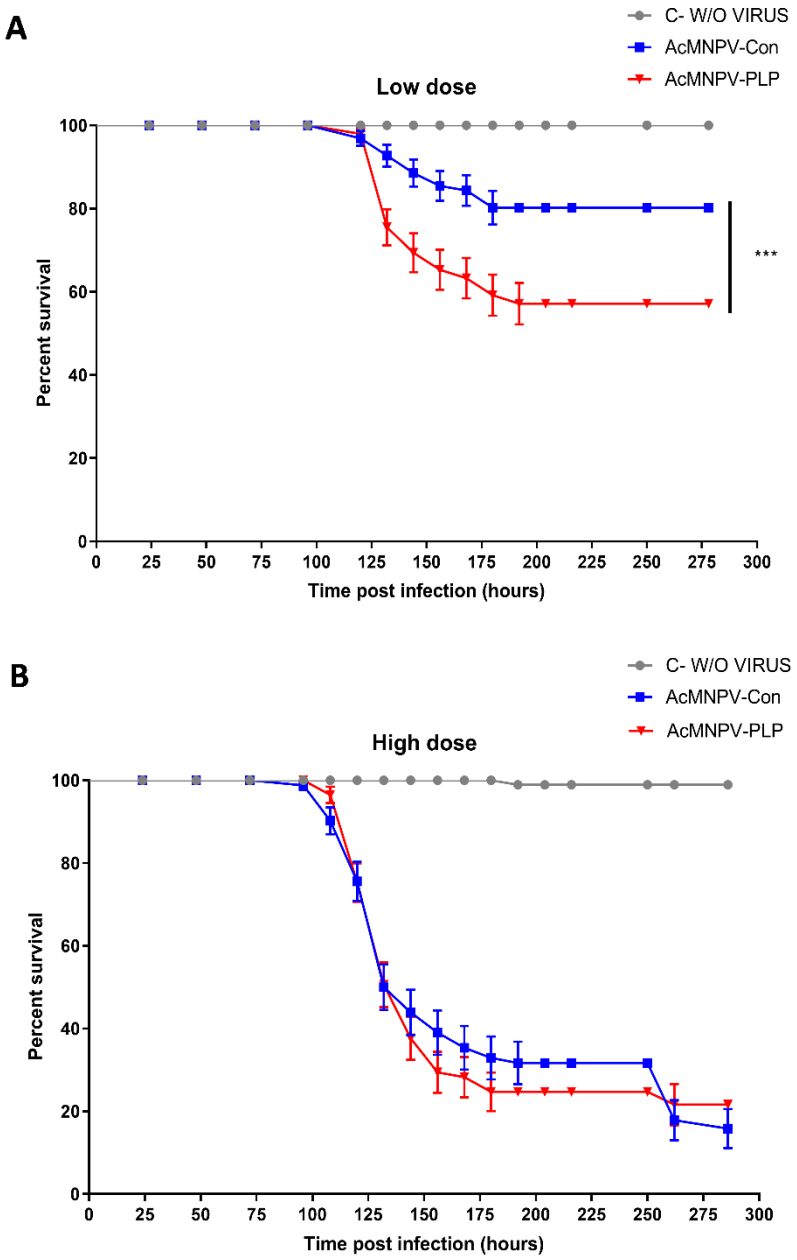


Figure 6. Pathogenicity of the Se-PLP-expressing viruses. Larval mortality was assessed at two viral concentrations. A) Mortality at sublethal viral dose. B) Mortality at lethal viral dose. Asterisks indicate

statistically significant differences between AcMNPV-Con and AcMNPV-PLP samples (Gehan-Breslow-Wilcoxon survival curve test) (P>0.05).

The lethal viral dose was then selected for further experiments to assess the effect of *PLP* on physiological parameters previously associated to the activity of the proctolin peptide in insects (Fiandra *et al*., 2010; Ormerod *et al*., 2016). Specifically, influence of virus-expressed *PLP* was studied on the larval growth, digestion rates, and locomotion.

PLP overexpression during the baculovirus infection was found to reduce the larval growth as reflected in a reduction in the larval weight and larval frass weights (Figures 7A and B). Differences between larvae infected with AcMNPV-Con and AcMNPV-PLP started to appear at 96 hpi, reaching out to a reduction of about 25% of the larval weight in AcMNPV-PLP infected larvae when compared to the non-infected or AcMNPV-Con-infected larvae at 144 hpi (p-value <0.001). Similarly, larval frass weight was also reduced in the larvae infected with the *PLP*-overexpressing virus. When compared to larvae infected with AcMNPV-Con, the frass weight of larvae infected with AcMNPV-PLP was reduced in about a 50% (p-value <0.001) at 144 hpi. These results suggest that *PLP* overexpression affects, directly or indirectly, to the insect digestion and food intake, and consequently to the larval development.

Regulation of larval locomotion has also been associated to the action of the neuropeptide proctolin (Ormerod *et al*., 2016). Our locomotion assay revealed that *PLP* overexpression reduced, directly or indirectly, the locomotion activity of the larvae. At 144 hpi larvae infected with the AcMNPV-PLP virus have less locomotion activity, compared to the controls (Figure 8). When compared to the non-infected and the AcMNPV-Con infected larvae, the infection with AcMNPV-PLP showed a significant reduction in about 70% in the locomotion value (p-value <0.001).

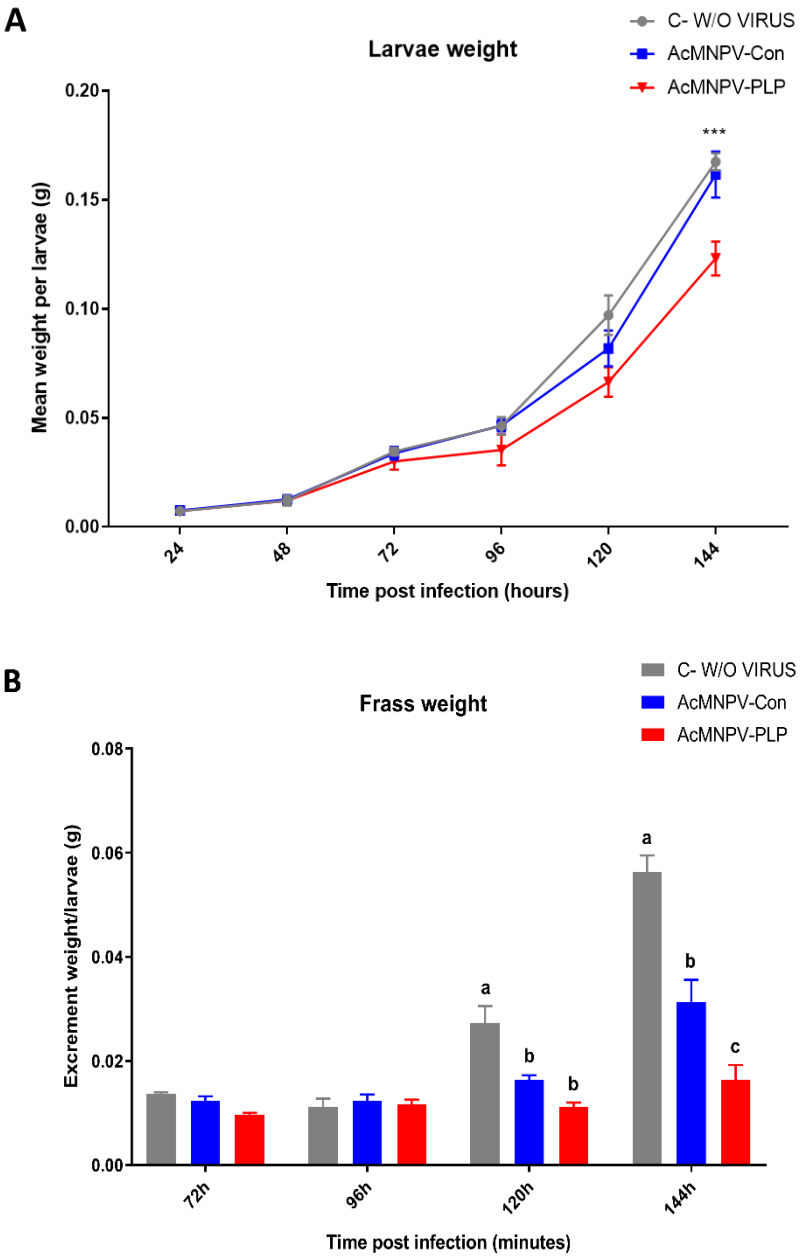


Figure 7. Effect of Se-PLP in larval growing and digestion. A) Mean larvae weight of *S. exigua* infected larvae at different time points**.** Asterisks indicate statistically significant differences between AcMNPV-PLP and AcMNPV-Con (two-way ANOVA) (P>0.05). B) Mean frass weight of *S. exigua*

infected larvae at different time points. Letters indicate statistically significant differences between the sample groups (two-way ANOVA) (P>0.05).

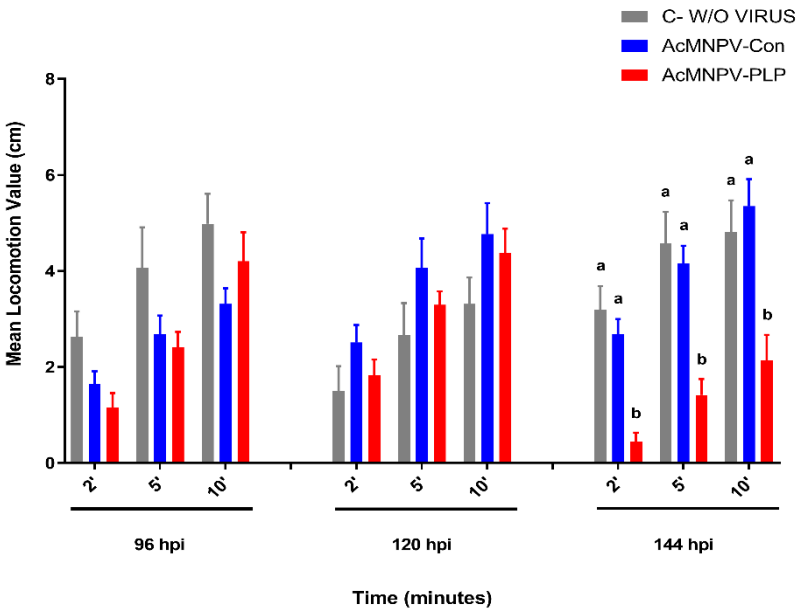


Figure 8. Effect of Se-PLP in larval locomotion. Mean locomotion value of *S. exigua* mock and infected larvae. Letters indicate statistically significant differences between the sample groups (two-way ANOVA) (P>0.05).

2.3.4. PLP effects on expression of other NPs

Effect of *plp* overexpression on the regulation of other NPs in the brains of the larvae was also studied to check if the observed effects in development and locomotion could be due to the unique action of *PLP* or to a dis-regulation of the neuropeptidergic system after *PLP* overexpression. For that, expression of 18 NPs was compared by RT-qPCR in the brain of AcMNPV-Con and AcMNPV-PLP infected larvae (144 hpi). Genes were selected because of the already described function and their role connected to developmental or locomotional aspects of the physiology. None of the analysed genes was found to be differentially regulated by

the *PLP* overexpression (Figure 9) suggesting that observed changes in digestion and locomotion were mainly attributed to the direct action of *PLP*.

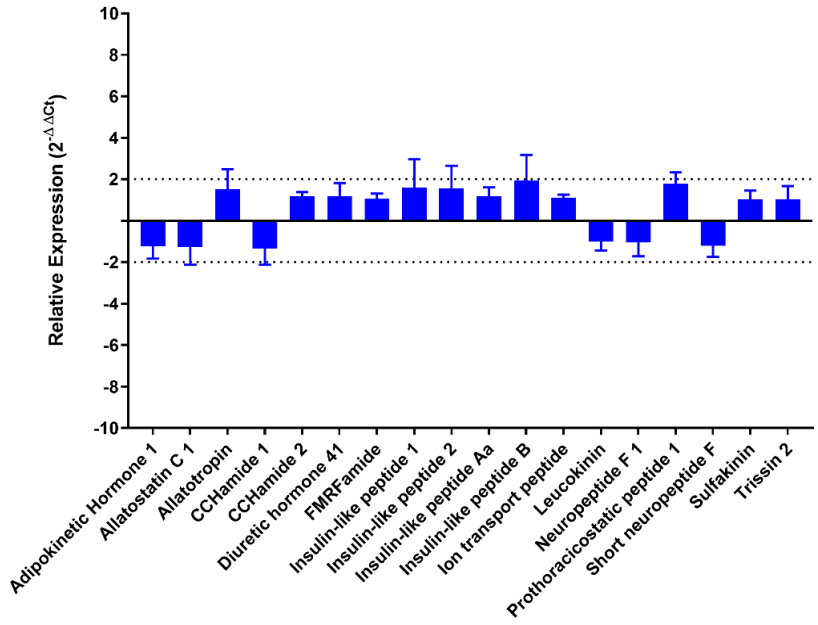


Figure 9: Differential expression analysis in *S. exigua* larva brain samples associated to PLP overexpression. Expression of selected NPs was compared in the brain of AcMNPV-Con and AcMNPV-PLP infected larvae. RT-qPCR assays were performed using gene specific primer pairs. An unpaired *t*-test with Welch's correction was applied (P>0.05).

2.4. Discussion

Baculoviruses interact with their host in many ways and have developed multiple strategies to increase their fitness and dispersion through the alteration of the physiology an behavior of the host (Cheng and Lynn, 2009; Kong *et al*., 2018). The neuropeptidergic system in insects is composed of signalling molecules playing a role in the chemical communication between cells (Elphick *et al*., 2018; Schoofs *et al*., 2017). Due to its regulatory function of the insect's physiology and behaviour (Bendena, 2010; Schoofs *et al*., 2017), understanding their regulation after

baculovirus infection would contribute to unveil some of the physiological and behavioural changes that have been associated to baculovirus infections. To study this, the expression levels of the genes encoding the *S. exigua* neuropeptidome following SeMNPV infection was initially analysed in the whole head of the larvae. Although no clear pattern of regulation of the neuropeptidergic system was detected, few NPs were found to be differentially expressed during infection. Two insulin-like peptides were the only upregulated genes. They are functionally related to the larval development and belong to a wide family of genes (Wu and Brown, 2006). Insulin-like peptides have also been linked to the innate immunity in insects (Nunes *et al*., 2020). Seven neuropeptides were downregulated although no major connection was found between them, except for the ecdysis triggering hormone, eclosion hormone, prothoracicotropic hormone and ion transport peptide that are related to the ecdysis (molting) process (Dircksen, 2009; Gammie and Truman, 1999; Mizoguchi *et al*., 2013; Zitnan, 2003). Downregulation of these four genes could produce a delay in the ecdysis, a phenotype already connected with the baculovirus infective process (O'Reilly and Miller, 1991). According to previous studies (Han, 2018; Katsuma *et al*., 2012) and corroborated by our transcriptional data (Figure 4A and B), baculovirus reach the brain of the larvae after 2 dpi (days post-infection). Thus, the lack of a clear pattern of regulation on the NPs expression, should not be consequence of early time points used in the experiment, but to the absence of a strong baculoviral influence in the neuropeptidergic system when the RNA-Seq approach is applied to a complex structure as the whole head of the larvae.

More specific analyses of the changes in the larval brain, revealed a different set of regulated NPs when compared to the changes observed using the complete larval heads. To our surprise, the observed pattern of neuropeptide expression was different between both approaches. These results show that changes in the insect brain cannot be studied through whole head analyses. The brain is the main organ in which NPs are expressed (Fónagy, 2014), representing only a small portion of the head.

RNA-seq analysis from composite tissues as larval head make it more difficult to interpret positive signals for genes with variable patterns of local expression (Johnson *et al*., 2013) and could also mask changes in the CNS. NPs such as ILP1 and ILP2 have been reported to be predominantly expressed in the brain (Belles, 2020). However, additional expression of these and other NPs in other organs (ie. neurohemal organs, etc) and head parts could be responsible of the lack of correlation between the expression data obtained with head and brain samples. For a more sensitive analysis of the gene expression regulation occurring on the larval brain we decide to use RT-qPCR methods on a set of NPs selected because of their regulation on the head (as revealed by RNA-Seq) or because their potential role on two aspects associated to the infection, larval growth and development.

Gene expression analysis of larval brain revealed a clear down-regulation of *PLP* following SeMNPV infection. Down-regulation of *PLP* was consistent among the different replicates and similar for the two viral strains used in the study. Proctolin-like peptide was firstly annotated in the neuropeptidome of *S. exigua* as proctolin (Llopis-Giménez *et al*., 2019) based on its sequence similarity to the proctolin neuropeptide annotation of *Danaeus plexippus* (Accession nº: OWR42866.1). However, a more detailed analysis revealed the lack of orthology with confirmed proctolin in *Rhodnius prolixus*, *Locusta migratoria* or *Periplaneta americana* (Baines and Downer, 1991; Brown and Starratt, 1975; Orchard *et al*., 2011), although they all contain the functional peptide RY/HLPT (Lange and Orchard, 2006). The *S. exigua* PLP, as occurs with other Lepidopteran PLP, contains 5 possible different cleavage sites (Southey *et al*., 2006) that could mediate in the generation of the active peptide/s (Bendena, 2010).

Proctolin was first discovered in *Periplaneta americana* as a five amino acid cyclic peptide that inhibited the contractions of the hindgut. The presence of proctolin and its function as a myotropic neurotransmitter and neuromodulator has been additionally confirmed in the dipteran *D. melanogaster* (Anderson *et al*., 1988; Isaac

et al., 2004), in the coleopteran *Tenebrio molitor* (Breidbach and Dircksen, 1989) and in the orthopteran *Locusta migratoria* (Clark *et al*., 2006). In lepidopteran insects, the action of a synthetic version of proctolin was first studied in *Pieris rapae* in which it was found to have a variable activity in the hindgut contractions when the five amino acids peptide was administered to the insects (Walker and Bloomquist, 1999). In *Bombyx mori*, a synthetic version of proctolin was found to increase the contractile activity in the larval hindgut at high concentrations. Moreover, when it was orally administered to *Spodoptera littoralis* larvae, growth was reduced by a 20% after proctolin treatment and a significant reduction in the conversion of ingested and digested food was observed, confirming its role in growth and development (Fiandra *et al*., 2010). Hypothesising about the functional relation between proctolin and PLP and to gain information on the role of this peptide during baculovirus infection, a gain-of-function strategy was chosen and an AcMNPV baculovirus constitutively expressing the C-terminal fragment of the *Se-PLP* was constructed. Infections with AcMNPV-PLP increase larval mortality when applied at sublethal doses, revealing an enhancing pathogenicity of SeMNPV by the expression of PLP, that could be attributed to the dysregulation of the insect physiology. Such increase in pathogenicity was not observed at lethal doses, probably because the high larval mortality at the lethal dose bioassay hide the pathogenic effects associated to the overexpression of PLP.

Infections at the lethal dose of AcMNPV-PLP showed an important reduction in larval growth and digestion when compared with the control AcMNPV (not expressing *Se-PLP*). Those experiments confirm the functionality of the recombinantly expressed precursor and reveal similar functions for this peptide in *S. exigua* larvae as proctolin neuropeptide in other insects. As reflected on the higher mortality produced by the AcMNPV-PLP virus at sublethal doses, it would be expected to find stronger effects of PLP overexpression on the larvae growth and mobility when exposed to a sublethal dose. However, the difficulty to distinguish

between infected and not infected individuals could interfere with the measurements and consequently, the growth and mobility experiments were only performed with the higher viral dose. In our approach, expression of Se-PLP was not restricted to the larval brain, and whether the observed effect with the Se-PLP overexpression was due to the direct effect of the peptide on the larval gut, as an endocrine messenger, or a combination of both remains to be elucidated.

In addition, proctolin seems to also act as a cotransmitter enhancing the neuromuscular transmission and the skeletal muscle contractions (Orchard *et al*., 1989). In fact, proctolin was suggested to be associated with a slow motor function affecting the locomotion activity of the insects although this effect was never directly showed (Lange and Orchard, 2006; O'Shea and Bishop, 1982; Witten and O'Shea, 1985). After confirming the functionally of the Se-PLP expressed in AcMNPV we have also explored the effect of the Se-PLP overexpression on the locomotion of the larvae, a parameter that has been associated to the behavioural changes that occurs during the baculovirus infections (Goulson, 1997; Kamita *et al*., 2005). To study the locomotion activity of the larvae, locomotion assay was developed. This new method resulted in an efficient set up able to detect differences between the treatments linking the PLP activity with a reduction of the larval locomotion.

Baculovirus infection has been demonstrated to produce characteristic behavior phenotypes in the host as the enhanced locomotor activity (ELA) (Kamita *et al*., 2005; van Houte *et al*., 2012; Van Houte *et al*., 2014). Another baculovirus-associated phenotype is the tree-top disease (Hoover *et al*., 2011). The underlying mechanisms of these two different phenotypes may involve modifications in the CNS, where the main behavioral aspects of the host are controlled (Gasque *et al*., 2019; Katsuma *et al*., 2012). It is tempting to hypothesize that the down-regulation of *Se-PLP* as a consequence of the baculovirus infection in the larval brain and its subsequent functional characterization could connect it to a hyperactivity phenotype, as a decrease in the expression of this neuropeptide may produce an increase in the

locomotion activity in the larvae. Nevertheless, down-regulation of *PLP* was produced under the infection of WT SeMNPV as well as the Δegt-SeMNPV. That implies that the presence of the *egt* gene (involved in the vertical climbing behaviour (Hoover *et al*., 2011)) is not associated with the regulation of the *Se-PLP* gene expression nor with locomotion changes mediated by this peptide. We hypothesize that the decrease in Se-PLP expression observed in larval brain could complement the *egt* effects, producing bigger larvae, possibly due to a decrease in the midgut contraction rates.

Whether the reduction in *PLP* expression after SeMNPV infection is the result of the direct effect of the virus on the host, a defence response of the host, or just a side effect of the physiological changes associated to the infection, remains to be elucidated. Down-regulation of host genes to increase viral fitness or evade the host's immune response by viral encoded miRNAs has been described for several baculovirus species (Kharbanda *et al*., 2015; Singh *et al*., 2010; Tang *et al*., 2019). At present, our analysis of SeMNPV-expressed miRNAs has not revealed miRNAs targeting to *Se-PLP* (data not shown).

In summary, the PLP, an uncharacterized peptide resembling the proctolin neuropeptide was downregulated as a consequence of the SeMNPV infection in *S. exigua* larval brains. Using a *Se-PLP* overexpression approach, we have confirmed its role in regulating physiological aspects as growing and locomotion, possibly relating its function with the insect neuropeptide proctolin. Although additional research will be needed to unveil the regulation mechanism of *PLP* by SeMNPV and further characterization of the PLP, these results allows us to hypothesize about the function of this peptide and its implications on the baculovirus-host interaction and the behavioral changes produced by baculoviruses.

CHAPTER 3

DESCRIPTION OF THE *SPODOPTERA EXIGUA* CHEMOSENSORY-RELATED GENES FOCUSING ON LARVAL OLFACTION.

This chapter has been published as:

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3.1. Introduction

Spodoptera exigua (Hübner, 1808) (Lepidoptera: Noctuidae), also known as the beet armyworm, is a worldwide spread Lepidoptera species. It is considered an important and aggressive horticultural pest because of its high polyphagy, which provokes important economic losses (Zheng *et al*., 2011). The larval stage is the responsible for crop damage, since caterpillars feed on both the foliage and the fruits of different host plants (Greenberg *et al*., 2006).

Chemosensation is fundamental in shaping insects' behaviours related to survival and reproduction, such as food searching, choice of oviposition substrate, mating seeking and detecting dangers like predators or parasitoids (Depetris-Chauvin *et al*., 2015; Robertson, 2015). In holometabolous insects such as Lepidoptera, the larval stage is devoted to food ingestion and growth, whereas adults are dedicated to reproductive tasks. Larvae evaluate chemical cues from their ecological niches differently than adults, and the physiological and molecular equipment required for detecting odours is different between these two life stages (Dweck *et al*., 2018; Scherer *et al*., 2003).

The peripheral chemosensory system is mainly localized in the antennae, the maxillary palps and mouthparts, and represents the first step in the detection and discrimination of environmental chemical stimuli. Several families of chemoreceptors and binding proteins are involved, such as odorant receptors (ORs), ionotropic receptors (IRs), gustatory receptors (GRs), odorant binding proteins (OBPs) and chemosensory proteins (CSPs). Volatile compounds are detected by ORs and antennal-expressed IRs (Gomez-Diaz *et al*., 2018). ORs are seven-transmembrane domain proteins that work as heteromeric ligand-gated ion channels together with the odorant coreceptor (ORco) (Joseph and Carlson, 2017). IRs are a divergent lineage of synaptic ionotropic glutamate receptors (iGluRs) (Rytz *et al*., 2013). Members of these two families are expressed in the dendrites of olfactory receptor neurons

(ORNs), which stretch inside structures called olfactory sensilla. The olfactory sensillum surface has many pores where odorants can pass and activate the receptors in the ORNs, which transmit the signal to the higher brain centres. Olfactory sensilla are mainly located on the antenna and the maxillary palps. Non-volatile chemicals are sensed by gustatory receptor neurons (GRNs), which express the GRs and a subset of IRs, the divergent IRs (Croset *et al*., 2010; Koh *et al*., 2015). GRNs are housed in gustatory sensilla, which are present in diverse body parts such as the mouth, the proboscis, the legs and even in the wings and are activated by direct contact with the chemical stimuli, which enter into the gustatory sensilla though an apical pore (Joseph and Carlson, 2017). OBPs and CSPs are two families of small soluble binding proteins secreted in the sensillar lymph of both olfactory and gustatory sensilla (Pelosi *et al*., 2005). OBPs are thought to carry the odorant molecules through the antenna lumen to the different receptors, but other functions have been proposed like odorant cleaning after its detection, protection of odorants from degradative enzymes and filtering of odorants (Sun *et al*., 2018; Zhou, 2010). Pheromone-binding proteins (PBP) are specialized OBPs that bind pheromone molecules, which are important for the insect mate recognition (Chang *et al*., 2015). CSPs are soluble proteins also secreted in the sensillar lymph and, although their function is not clearly understood, they may play a role connecting the odorant molecules with the receptors (Pelosi *et al*., 2005).

Despite the larval stage of *S. exigua* is responsible for plant damage, there is a knowledge gap of the molecular machinery underlying larval olfaction and olfactory-driven behaviour. So far, many candidate chemosensory-related genes of *S. exigua* have been identified by RNA-Seq analyses exclusively in adult tissues. Du *et al.* (2018) reported 157 candidate chemosensory genes identified in adult antennae, whereas Zhang *et al*. (2018) reported 159 ones identified in adult antennae, proboscis and labial palps. Unfortunately, both studies used a different annotation nomenclature, making difficult the comparison between sequences.

In this study, we aim to fill the gap on the knowledge of *S. exigua* larval olfaction through the analysis of a comprehensive RNA-Seq dataset that includes several larval tissues. We expand the number of putative chemosensory-related genes described in *S. exigua* and propose a unifying gene nomenclature based on reconstructed phylogenetic trees and following names used in the annotated *Spodoptera frugiperda* genome (Gouin *et al*., 2017). As previous exposure to volatile organic compounds (VOCs) has been shown to influence OR gene expression in *S. exigua* adults (Wan *et al*., 2015), thus we also analyse expression patterns of selected ORs after larvae VOC pre-exposure. Additionally, we designed a behavioural assay for *S. exigua* larvae to study the effects of VOCs in terms of attraction and repellence. Altogether, our results provide useful data and tools to set the basis for further studies of *S. exigua* larvae olfaction.

3.2. Materials and methods

3.2.1. Insects

The *S. exigua* colony (SUI) used for all the experiments has been reared at University of Valencia on artificial diet (Elvira *et al*., 2010) at 25 ± 3 ºC with 70 ± 5% RH, using a photoperiod of 16:8 h (light:dark).

3.2.2. RNA extraction, library preparation and sequencing

S. exigua fourth-instar larvae were dissected with a scalpel and heads, midgut and fat body were excised and homogenised in Trizol (Roche). Adult antennae (male and female), brains (male and female) and ovaries were excised from adults and homogenised in Trizol. Total RNA was extracted following Trizol manufacturer's instructions. A second purification step was carried out using the RNeasy Mini Kit (Qiagen). Three replicates consisting of tissues excised from 16 larvae each were done for larval head, adult antenna (male and female each) and adult brain (male and female each). Only one replicate was prepared for ovaries, larval midgut and larval fat body. Library preparation and Ilumina Hiseq 2000 sequencing were both carried out by

Novogen Technology Co. Ltd. (China). Raw reads are available at NCBI SRA database (Project number PRJNA634227).

3.2.3. *De novo* assembly and annotation of chemosensory-related genes

Paired-end (PE) raw reads were trimmed and used for *de novo* assembly using Trinity v2.3.1 (Grabherr *et al*., 2013) with --min_kmer_cov 2 parameter. Trinity-assembled contigs were further clustered with Corset (Davidson and Oshlack, 2014) and the longest transcript from each Corset cluster was selected to obtain the final assembly. *De novo* assembled transcriptome is available at NCBI TSA database with accession number PRJNA634227. Annotation of transcripts encoding odorant receptors (ORs), ionotropic receptors (IRs), gustatory receptors (GRs), odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) was performed with iterative blast searches using the amino acid sequences predicted from the *S. frugiperda* genome (Gouin *et al*., 2017) as query. Selected contigs were manually inspected, their coding sequences were predicted using BioEdit and *S. frugiperda* orthologs as a master, and 5' and 3' UTRs were removed. In few cases, when two contigs were overlapping and likely representing the same transcripts based on their alignments with *S. frugiperda* orthologous genes, they were merged together to create a consensus sequence. Redundant sequences were then identified by iterative generations of maximum-likelihood trees and removed from the dataset. Thus, the final dataset contains a non-redundant list of putatively unique transcripts, which likely correspond to unique genes, although we cannot exclude that some of them might be allelic variants of the same gene. Maximum-likelihood (ML) trees were built with protein sequences annotated from *S. frugiperda* (Gouin *et al*., 2017), *S. litura* (Zhu *et al*., 2018) and *B. mori* genomes (Forêt *et al*., 2007; Tanaka *et al*., 2009; van Schooten *et al*., 2016; Vogt *et al*., 2015; Wanner and Robertson, 2008), as well as putative proteins deduced from *S. littoralis* (Walker *et al*., 2019), *S. litura* (Gu *et al*., 2015), *Helicoverpa armigera*, *H. assulta* (Chang *et al*., 2017) and previous *S. exigua*

transcriptomes (Du *et al*., 2018; Zhang *et al*., 2018). Trees were built using RAxML (Stamatakis, 2014) based on amino acid MUSCLE alignments (Edgar, 2004) generated by MEGAX (Kumar *et al*., 2018). *S. exigua* chemosensory-related proteins were named according to the phylogenetic relationships with *S. frugiperda*, whose chemosensory-related repertoire was annotated from genome. Web-based blastx searches were then run for *S. exigua* chemosensory-related transcripts whose corresponding proteins had no one-to-one ortholog in any *Spodoptera* species, in order to verify their annotation as chemosensory-related genes.

3.2.4. RNA-Seq quantification of chemosensory-related gene expression

Expression levels of *S. exigua* chemosensory-related transcripts in larva head, female adult antenna and male adult antenna (3 replicates for each tissue) were estimated by mapping the trimmed reads to the chemosensory-related genes annotated in this study. Mapping was performed using Bowtie 2 (version 2.3.5.1) (Langmead and Salzberg, 2012) and RSEM (version 1.3.1) (Li and Dewey, 2011) with default parameters. Relative abundance of each candidate transcript is reported as TPM (Transcript per Million). For each transcript family, expression data were clustered by hierarchical clustering analysis using the heatmap.2 function from gplots v3.0.1.1 package of R software. Differential expression analysis between male and female antennae was carried out using EdgeR (Robinson *et al*., 2009). Transcripts were considered differentially expressed (DE) at false discovery rate (FDR) threshold < 0.05 and 2-fold change cut-off.

3.2.5. Expression analysis of chemosensory-related transcripts by reverse transcription (RT)-PCR

Presence of transcripts encoding ORs, PBPs, two general OBPs (GOBPs) and three GR candidates for CO_2 reception were analysed by RT-PCR in larva head and adult antenna in order to confirm their developmental expression specificity. The same RNA samples used for the RNA-Seq sequencing (Llopis-Giménez *et al*., 2019)

were employed. RNA pools were prepared for each of the two developmental stages, mixing the same amount of total RNA from each replicate (3 replicates from the larvae head, 6 replicates from the adult antenna). A total of 2 µg of RNA were treated with DNAseI (ThermoFischer Scientific) and converted into cDNA using PrimeScript cDNA synthesis kit (Takara), following the manufacturer's protocols. Amplifications were run in a StepOnePlus Real-Time PCR system (Applied Biosystems) using 5x HOT FIREpol EvaGreen qPCR Mix Plus (ROX) from Solis BioDyne. The total reaction volume was 20 µl. Forward and reverse primers for all the transcripts were designed using the online software tool Primer3Plus (Untergasser *et al*., 2007). A list of primers used in this experiment is provided in Annexed XIV. RT-PCR products were run in a 2% agarose gel to visualize the amplification of a single band of the expected size.

3.2.6. Chemicals

Chemicals used in this study were purchased from Sigma-Aldrich (95-99% purity) with the exception of methanol that was purchased from Labkem: propiophenone (CAS #: 93-55-0), cinnamaldehyde (CAS #: 104-55-2), cis-3-hexenyl propionate (CAS #: 33467-72-2), 3-octanone (CAS #: 106-68-3), trans-2-hexen-1-al (CAS #: 6728-26-3), benzaldehyde (CAS #: 100-52-7), cis-3-hexenyl acetate (CAS #: 3681-71-8), linalool (CAS #: 78-70-6), benzyl alcohol (CAS #: 100-51-6), hexyl propionate (CAS #: 2445-76-3), acetophenone (CAS #: 98-86-2), indole (CAS #: 120-72-9) and 1-hexanol (CAS #: 111-27-3).

3.2.7. Odorant exposure and tissue collection

To test the influence of VOC exposure on the transcriptional profile of selected ORs and OBPs, twenty *S. exigua* fourth-instar larvae were placed in a 9 cm Petri dish together with a perforated 1.5 ml tube containing a Whatmann paper soaked with 50 µl of the odorants (100 mg/ml). Control consisted of exposure to methanol solvent only. Petri dishes were kept at 25ºC. Ten larvae heads were dissected at 1h

and 24h of exposure and stored in 300 µl of Trizol Reagent (Invitrogen) at -80ºC for RNA extraction. Three independent replicates for each treatment were done.

3.2.8. Starvation and tissue collection

The transcript levels of ORs and OBPs were also measured under starving conditions. Sixteen *S. exigua* fourth-instar larvae were let at 25 ºC without any food for 24 h, whereas control larvae were allowed to feed on artificial diet. Larvae heads were dissected after 24 h and stored in 300 µl of Trizol Reagent (Invitrogen) at -80ºC for RNA extraction. Three independent replicates for each treatment were done.

3.2.9. RNA extraction, cDNA synthesis and quantitative real-time PCR (RT-qPCR)

Total RNA was purified using Trizol reagent following the manufacturer's instructions, and using 10 µl of glycogen (Roche) to help to the nucleic acid precipitation. 500 ng of RNA were treated with DNAseI (ThermoFischer Scientific) following manufacturer's protocol. Then, samples were converted into cDNA using SuperScript II Reverse Transcriptase (ThermoFischer Scientific) following manufacturer's recommendations and using random hexamers and oligo (dT) primers. RT-qPCR was 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. Forward and reverse primers for every gene were designed using the online software tool Primer3Plus (Untergasser *et al*., 2007). An endogenous control *ATP synthase subunit C* housekeeping gene was used in each qPCR to normalize the RNA concentration. The list of the primers is provided in Annexed XIV. The differences in expression between treatments (control and infected) were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). A one-sample *t*-test was used to search for statistical differences. Graphs and the statistical analysis were performed using GraphPad Prism software (v7.0). Heatmaps were obtained using the R packages gplots and RColorBrewer.

3.2.10. Behavioral assays

For each run, we used ten fifth-instar *S. exigua* larvae that were placed in one side of a 14 cm diameter Petri dish with a piece of artificial diet (1.5 x 0.8 x 1 cm) located at the opposite side. The Petri dish was positioned inside a paperboard box (30 x 22 x 22 cm). A hole in the side of the box (6 cm of diameter) was made to include a 50 W halogen artificial light (at 15 cm of distance to the Petri dish). Fifty µl of the odorant diluted at 100 mg/ml in methanol were added to the artificial diet. In parallel, a control with the solvent was run. Each odorant and its respective control was tested a total of nine times (three replicates with three different batches of larvae, *i.e*. larvae from different offspring). For each replicate, larval mobility was scored dividing the Petri dish in ten areas of 1.3 cm each (Figure 1). To each area, we assigned a score from 0 to 9, which increased accordingly to the distance from the starting point. After 2, 5 and 10 min, the number of larvae in each area was recorded and the mobility index was determined as the sum of the scores obtained by each of the ten larvae. The larval attraction index was calculated dividing the mobility index in presence of the odorant by that obtained in the parallel control run. Values higher than 1 meant that the larvae were more attracted to the diet + odorant source than to diet + solvent only, and values lower than 1 meant that larvae were less attracted to diet + odorant source than to diet + solvent only. Statistical analyses were conducted using a one-sample *t*-test comparing the attraction index at each time point with the theoretical value of 1. All statistical analyses were performed using GraphPad Prism software (v.7.0).

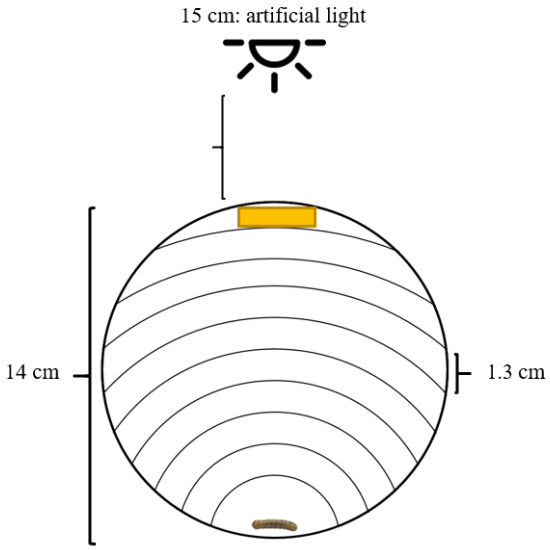


Figure 1. **Scheme of the behaviour assay design.** Ten 5th-instar larvae were put in one of the sides of the Petri dish whereas a piece of artificial diet was placed on the opposite side. A halogen artificial light was positioned at 15 cm from the Petri dish. Five mg of the odorant (or the equivalent volume of solvent) were placed on the artificial diet.

3.3. Results

3.3.1. Annotation of chemosensory-related genes

In this study, we provide an updated repertoire of 200 candidate chemosensory-related genes belonging to 5 different families: 63 ORs, 28 IRs, 38 GRs, 48 OBPs and 23 CSPs, (Table 1). These results greatly expand previous annotations of chemosensory-related genes in *S. exigua* since 51 genes appear to be newly annotated: 5 ORs, 5 IRs, 22 GRs, 16 OBPs and 3 CSPs (Figure 2, Annexed IV-VII). Moreover, our annotation efforts provide a new phylogeny-based nomenclature that follows the one in *S. frugiperda* (Gouin *et al*., 2017) and in *S. littoralis* (Walker *et al*., 2019) with the aim to aid future comparative studies among related species.

Supplementary datasets containing the annotations are available in https://link.springer.com/article/10.1007/s10886-020-01224-z.

Table 1. Summary of the candidate chemosensory-related genes in *Spodoptera exigua* annotated in this study.

Gene family	*S. exigua*				*S. frugiperda* [c]
	This study		Du's dataset[a]	Zhang's dataset[b]	
	All	Newly			
OR	63	**5**	50	64	69
IR	28	**5**	20	22	43
GR	38	**22**	7	30	233
OBP	48	**16**	45*	24	51
CSP	23	**3**	32*	19	22

[a]Du et al., (2018)

[b]Zhang et al. (2018)

[c]Gouin et al. (2017)

*These numbers include several transcripts likely mis-annotated as *S. exigua* OBPs or CSPs

We annotate 63 ORs (SexiORs), of which 44 (70 %) have a complete ORF. Five ORs have not been reported by any previous study (SexiOR46, SexiOR54, SexiOR56, SexiOR69 and SexiOr40b) (Annexed XVI). All *S. exigua* ORs (SexiORs) have a one-to-one ortholog relationship with *S. frugiperda* ORs except SexiOR40b and SexiOR40c that might represent OR40 lineage-specific duplications in *S. exigua* (Figure 2), although we cannot exclude that they are transcriptional isoforms of the same gene. Our annotation lacks three incomplete candidate SexiORs described by Zhang *et al.* (2018) (namely OR8, OR56, and OR59 according to Zhang's nomenclature).

We identify 28 IRs (SexiIRs). All of them have a one-to-one ortholog in *S. frugiperda* (Annexed IV) and 14 (50%) have a complete ORF. Compared to previous works in *S. exigua*, five IRs are new annotations (namely SexiIR7d.2, SexiIR100a, SexiIR100b, SexiIR100c and SexiIR100i) (Annexed XVII).

Thirty-eight candidate *S. exigua* GRs (SexiGRs) have been annotated from our transcriptome but only ten sequences are complete (26%) (Annexed XVIII). Among these latter ones, there are the SexiGRs orthologous to lepidopteran GR1, GR2 and GR3 (Annexed VII), which are the lepidopteran antenna-expressed CO_2

receptors (Guo *et al*., 2017). Twenty-two GRs are new annotations compared to previous studies in *S. exigua* (Annexed XVIII). Identification of orthologous genes in *S. frugiperda* is difficult due to the incomplete ORF retrieved for the majority of SexiGRs, which lead to low branch support in many cases (Annexed V); however, a tentative nomenclature is proposed (Annexed XVIII). Compared to the annotation made by Zhang *et al*. (2018), 11 of the GRs annotated in their study are not present in our transcriptome. Special mention should be made to GR24 (according to Zhang's nomenclature), also mis-annotated by Du *et al*. (2018) as the CO_2 receptor GR1. Both annotations report a sequence of a likely partial GR (136 amino acids for Zhang's GR24 and 120 for Du's GR1).

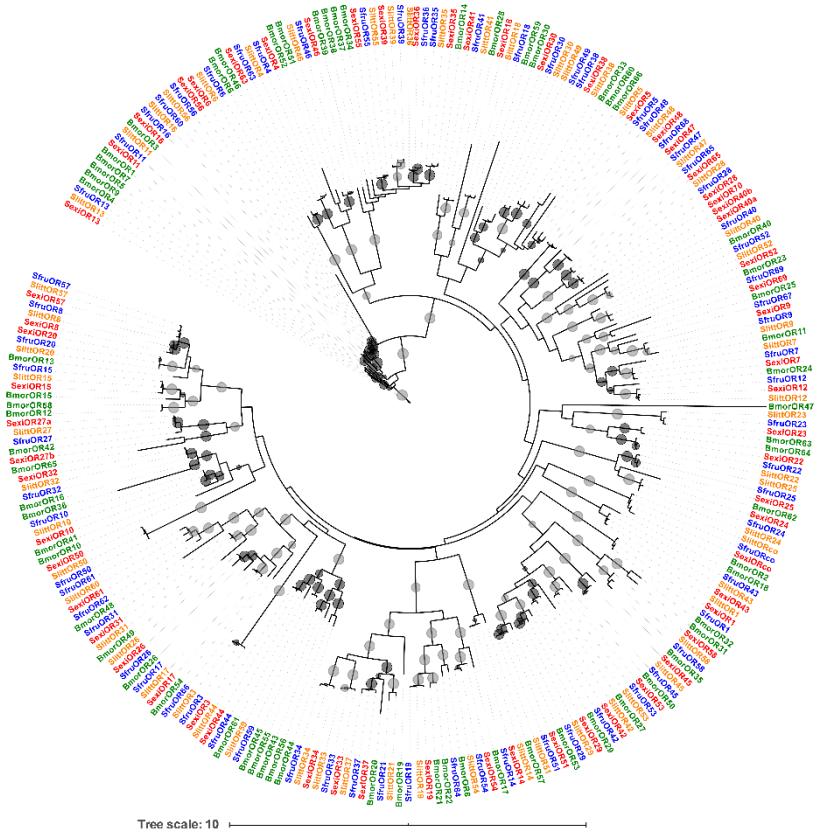


Figure 2. Phylogenetic tree of *Spodoptera exigua* odorant receptors (SexiORs). Maximum-likelihood (ML) tree built with protein sequences annotated from *S. frugiperda* (Gouin *et al*., 2017) and *B. mori* genomes (Tanaka *et al*., 2009) as well as putative proteins annotated from *S. littoralis* transcriptome (Walker *et al*., 2019).The *S. exigua* sequences are shown in red, the *S. frugiperda* ones in blue, the *S. littoralis* ones in yellow, and the *B. mori* ones in green. *S. exigua* amino acid sequences used for the tree are given in the supplementary datasets. Grey dots show a bootstrap value higher than 80.

We retrieve the same sequence in our transcriptome, but blast searches and phylogenetical analysis clearly show that it corresponds to an unrelated and uncharacterized protein of around 120 amino acids present in the genomes of multiple Lepidoptera species (Annexed VI).

Forty-eight *S. exigua* OBP candidate transcripts have been identified (SexiOBPs). Most of them are complete (69%), and only 15 have a partial ORF (Annexed XIX). Sixteen transcripts are new annotations. All SexiOBPs have a clear one-to-one orthologue in *S. frugiperda* except SexiOBP46 and SexiOBP47 (Annexed VIII). Two of the transcripts described by Zhang *et al.* (2018) (OBP25 and OBPN-3) are not present in our transcriptome. Compared to Du *et al.* (2018), our annotation lacks 19 putative OBPs. However, a closer look to these missing OBPs reveals that they do not cluster with any *Spodoptera* OBPs. Twelve of them group with *Helicoverpa* OBPs in our phylogenetic tree whereas the other seven are far distant (Annexed VII). Further analysis with blastx reveals that the best hit of missing OBPs is against sequences from distantly-related species (such as butterflies or Coleoptera species) (Annexed XXI). Consequently, we suspect that these nineteen sequences (which are neither annotated by Zhang *et al*., 2018) might arise from contamination during library preparation and sequencing, and thus not belonging to *S. exigua*.

Twenty-three candidate *S. exigua* CSP transcripts have been annotated (SexiCSPs). Of them, 21 have a complete ORF (91%). Three of the annotated sequences are not described in any prior study: SexiCSP11, SexiCSP23 and SexiCSP24 (Annexed XX). All SexiCSPs have a clear one-to-one ortholog in *S. frugiperda* except SexiCSP23 and SexiCSP24 (Annexed VIII). Our annotation retrieves all but one CSP (CSP-N3) described Zhang *et al.* (2018). Compared to

annotation reported by Du *et al* (2018) our annotation lacks fifteen tentative CSPs that are also absent in Zhang's annotation. However, likewise missing OBPs, missing CSPs likely arise from sample contamination since eight of them cluster with *Helicoverpa* sequences instead of *Spodoptera* ones and the remaining sequences have best blast hit against sequences from distantly-related Lepidoptera species or from other insect orders (Coleoptera, Diptera and Hymenoptera).

3.3.2. Chemosensory-related transcripts in *S. exigua* adults and larvae

Transcript levels were evaluated mapping RNA-Seq reads obtained from larval head, female and male antennae to our manually curated chemosensory-related gene dataset. Since data from larvae and adults are not directly comparable because they proceed from different tissues (isolated antennae in adults versus whole heads from larvae), we cannot directly relate expression levels between adults and larvae. Instead, we use these data to i) establish the larval chemosensory-related gene set by detecting the transcripts that have transcription signals in larval head samples, ii) identify genes that are differentially expressed between male and female antennae.

Mapping of reads obtained from larval head to the manually curated chemosensory-related transcript dataset only gives us a hint about expressed genes without providing any information of transcripts not expressed at all, since RNA-Seq data from composite structures (such as head) often lead to false negatives (Johnson *et al*., 2013). In total, 30 ORs, 22 IRs, 13 GRs, 35 OBPs and 20 CSPs have mapping reads in at least one of the larval head replicates (Figure 3, Annexed IX – XII). By qualitative comparison with the adult chemosensory-related gene set, we notice that the larval gene set is smaller than that of adults (120 versus 187). Only some OBPs have a larval-specific expression in our analysis: 14 OBPs have mapping reads from larval heads but not from adult antennae (Annexed XI) (8 of them are newly annotated transcripts). RNA-Seq data of ORs, CO_2 receptors and PBPs and GOBPs were confirmed using RT-PCR. Results show that 50 out of 63 ORs are actively transcribed in larval heads whereas all ORs are transcribed in adult antennae tissue; thus,

revealing that 13 ORs are likely adult-specific (*i.e.* SexiOR1, SexiOR7, SexiOR10, SexiOR17, SexiOR20, SexiOR24, SexiOR26, SexiOR34, SexiOR37, SexiOR38, SexiOR40a, SexiOR43 and SexiOR44). GOBPs and PBPs are expressed in both larval heads and adult antennal tissues. Of three candidate CO_2 receptors, our results show that only SexiGR1 and SexiGR2 are expressed in larval heads and adult antennae whereas SexiGR3 is not expressed in any sample (Annexed XIII).

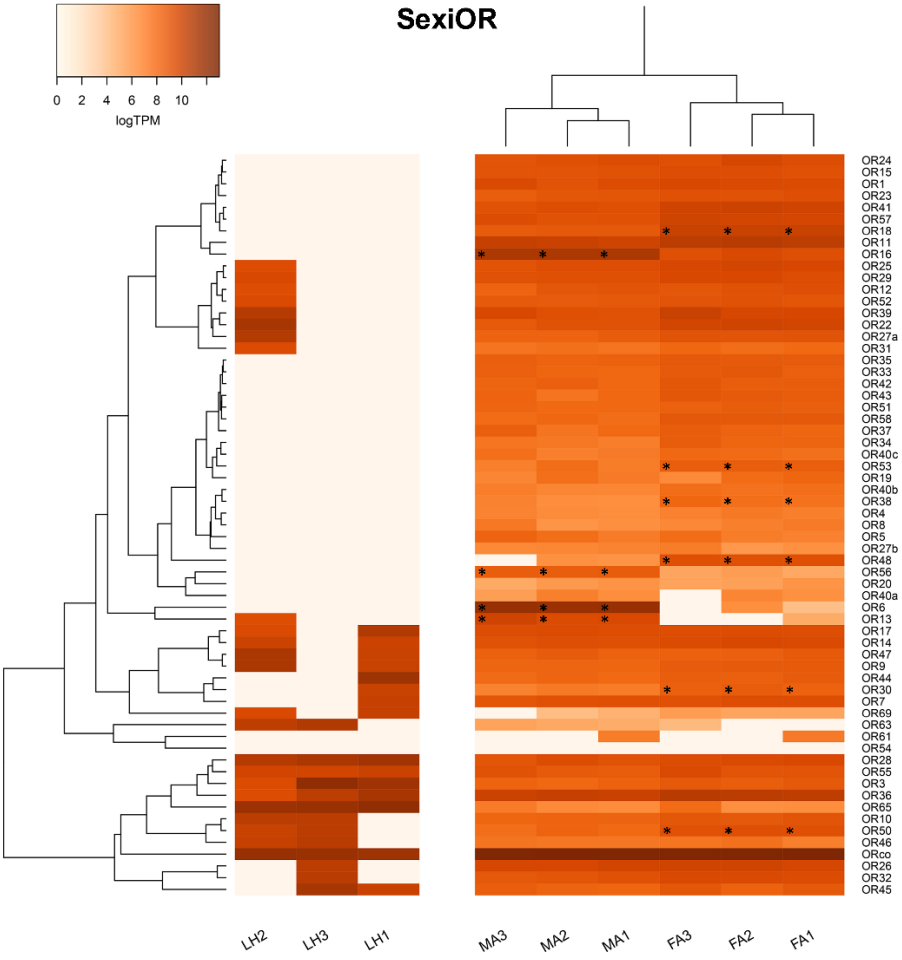


Figure 3. Heatmap of relative expression values of odorant receptors (SexiORs) in whole head of *Spodoptera exigua* larvae and adult antennae. Colour plots represent log2 of transcripts per million (TPM) values estimated by RSEM. LH: Larvae Head. MA: Male Antenna. FA: Female Antenna.

Asterisks indicate statistically significant differences between male and female antenna samples identified by EdgeR analysis (FDR<0.05).

Differentially expressed (DE) transcripts between male and female adults are sixteen (Annexed XXII). Of these, ten transcripts have significantly higher expression in male antenna, and six in female antenna. Transcripts with higher expression in males are four ORs, four OBPs, one GR and one CSP. Of them, the DE transcripts that show the highest variation in expression (more than 4-fold change) are ORs and OBPs involved in pheromone binding (SexiOR6, SexiOR13, SexiOR16 and SexiPBP1). Transcripts upregulated in females are six ORs, whose expression levels vary less than ORs upregulated in males, except for SexiOR48 that is 20-fold more expressed in female than in male antenna.

3.3.3. Regulation of larval chemosensory-related gene expression after exposure

Expression levels of selected ORs and PBPs expressed in larvae were analysed by RT-qPCR after VOC exposure. Results show that short-time exposure (1h) triggers low levels of variation of few ORs to some specific VOCs (Figure 4). Specifically, 1-hexanol exposure increases the expression of SexiOR23 and SexiORco (2.5-fold change and 2.3-fold change, respectively). Indole exposure up-regulates 20.9-fold the expression of SexiOR25, and acetophenone increases 5.8-fold the expression of SexiOR11. Benzaldehyde down-regulates 1.4-fold the expression of SexiOR65. Cis-3-hexenyl acetate exposure does not induce any significant variation.

Long-term exposure (24h) leads to wider transcriptional changes than short-time exposure. After 24h, all the odorants tested trigger expression changes in 2 to 5 genes, depending on the VOC (Figure 4). Interestingly, exposure to any of the tested odorants only triggers strong up-regulation of mRNA levels and never down-regulation. Expression of SexiOR63 and SexiOR40c is strongly up-regulated after exposure with any of the odorants tested (from 12- to 49-fold changes). The mRNA levels of SexiOR35 increases after exposure to any odorants, although only the

changes triggered by four out of the five odorants are statistically significant (fold changes vary from 7- to 33-fold). Likewise, expression of SexiOR11, SexiOR23 and SexiOR25 shows a general trend of up-regulation after exposure to any odorants, although only SexiOR11 changes after acetophenone exposure (27.7-fold) and SexiOR23 changes after cis-3-hexenyl acetate (11.7-fold) are statistically significant. This last VOC also triggers SexiPBP1 up-regulation (2.6-fold change).

Figure 4. Heat-plot of relative expression levels of odorant receptors (SexiORs) and pheromone-binding proteins (SexiPBPs) in *Spodoptera exigua* larvae after exposure to different odorants or in starving conditions. Expression values were estimated by RT-qPCR using the ΔΔCt method. Colour

plots represent Log fold change values. Dark red colours indicate a decrease in the expression and dark green ones indicate an increase in the expression. Asterisks indicate statistically significant differences (one-sample *t*-test) (P < 0.05 *, P < 0.01 **, P < 0.001 ***).

3.3.4. Regulation of larval chemosensory-related genes under starvation

The same set of genes whose expression was tested after odorant exposure was also used to analyse mRNA levels after starvation. The absence of food ingestion for 24h leads to down-regulation of only SexiOR23 (2.8-fold) and SexiOR45 (4.1-fold) (Figure 4).

3.3.5. Behavioral experiments

The response of *S. exigua* 5th instar larvae to thirteen VOCs was investigated as a proof of concept to test our behavioural assay design (Figure 1). VOCs used in this assay were chosen because: i) they have been previously tested in behavioural assays with larvae of other lepidopteran species, such as benzyl alcohol, acetophenone, benzaldehyde, indole, 1-hexanol, cis-3-hexenyl acetate, trans-2-hexen-1-al and linalool (Becher and Guerin, 2009; Carroll and Berenbaum, 2002; de Fouchier *et al*., 2018; Di *et al*., 2017; Rharrabe *et al*., 2014); ii) they are structurally similar to VOCs that triggered behavioural effects in related species *S. littoralis* (propiophenone and cinnamaldehyde) (de Fouchier *et al*., 2018); and iii) they are attractant of parasitoids of lepidopteran larvae (3-octanone) (Ramachandran *et al*., 1991).

The behavioural response of the larvae in the control run, which consists in artificial diet plus solvent, is time-dependent (Annexed XIV). After 2 min larvae move on average 2.2 (± 0.64) cm, 4.8 (± 1.4) cm after 4 min and 7 (± 1.4) cm after ten min. Larvae never reach the diet in the time allowed. Since we observe this linearity in larval movement, we use the movement of the larvae in parallel runs where an odorant is added to the artificial diet, to calculate the differences with the control and evaluate the behavioural response to a certain odorant. In this setup, 1-hexanol and benzaldehyde enhance attractancy to artificial diet whereas other five VOCs have a

deterrent effect (Figure 5). Specifically,1-hexanol evokes enhanced attraction at the three time-points whereas benzaldehyde is active at only one time point. Indole exhibits a deterrent effect at the three tested time-points, 3-octanone at both 5 min and 10 min time-points, benzyl alcohol at the 10 min time-point only, linalool and cis-3-hexenyl propionate at the 5 min time-point only. The remaining odorants (propiophenone, cinnamaldehyde, trans-2-hexen-1-al, cis-3-hexenyl acetate, hexyl propionate and acetophenone) do not show any significant effect on diet attraction at any time.

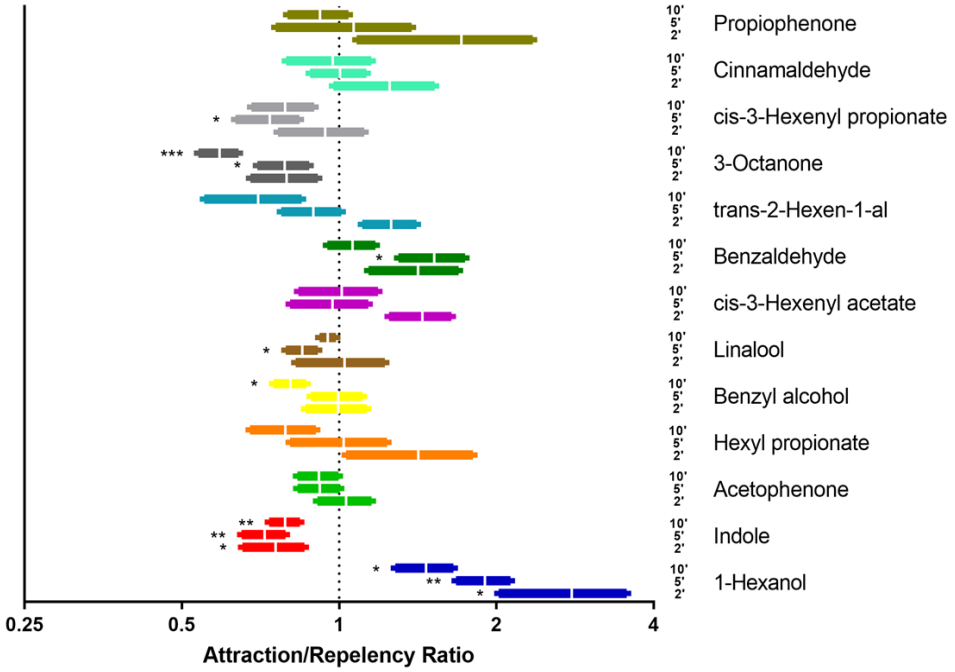


Figure 5. Larval attraction index of *Spodoptera exigua* larvae to different odorant stimuli at different times. Fifty µl diluted at 100 mg/ml of each odorant were used for the bioassay. Bars represent the mean value and the standard deviation. Values above 1 are indicative of attraction and values below 1 are indicative of repellence. Asterisks indicate statistically significant differences (one-sample *t*-test) ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***).

3.4. Discussion

In Lepidoptera, the study of olfaction has been mainly limited to adult stage, and focused on the understanding of sex-linked behaviours such as sex pheromone detection and egg-laying substrate selection (Allison and Carde, 2016; García-Robledo and Horvitz, 2012; Haverkamp *et al*., 2018). In contrast, the molecular and physiological mechanisms that underlie olfactory behaviours in larvae are poorly understood and only few reports have described the chemosensory gene set expressed at the larval stage (Chang *et al*., 2017; Di *et al*., 2017; McCormick *et al*., 2017; Poivet *et al*., 2013; Tanaka *et al*., 2009; Walker *et al*., 2016) compared to the plethora of adult transcriptomes available (Montagné *et al*., 2015). Here, we provide the first tools to study olfaction in the larvae of the pest *S. exigua*. We first identify chemosensory-related genes expressed in *S. exigua* larval heads using RNA-Seq and RT-PCR data, expanding previous datasets built from adult data (Du *et al*., 2018; Zhang *et al*., 2018). We demonstrate that exposure to high doses of VOCs drives changes in OR transcription and we additionally develop a method to analyse the behavioural response of *S. exigua* larvae to volatiles.

Previous annotation efforts of chemosensory-related genes in *S. exigua* reported 157 and 159 candidate transcripts identified in adult tissues (Du *et al*., 2018; Zhang *et al*., 2018). Our results greatly expand these previous annotations since we report 200 chemosensory-related genes annotated from RNA-Seq from multiple adult and larval tissues. Moreover, our annotation identifies mis-annotated transcripts previously reported as *S. exigua* candidate GRs, OBPs and CSPs, and provides a more reliable nomenclature of *S. exigua* chemosensory-related candidate genes based on orthologs identified in the *S. frugiperda* genome (Gouin *et al*., 2017). In total, we identify 63 ORs, 28 IRs, 38 GRs, 48 OBPs and 23 CSPs. *S. frugiperda* and *S. litura* are two species closely related to *S. exigua* whose genomes are fully sequenced (Cheng *et al*., 2017; Gouin *et al*., 2017). The number of ORs in both genomes ranges from 69 to 73, suggesting that the ORs described in *S. exigua* almost cover the full

OR repertoire in this species. OBP and CSP repertoires are likewise almost complete since these two gene families have 51 and 22 members in *S. frugiperda*, and 36 and 23 members in *S. litura*, respectively. On the contrary, the number of GRs is far lower than what is described in the *Spodoptera* spp. (231 GRs in *S. frugiperda* and 237 in *S. litura*), likely due to the sampled tissues and the low expression level of GRs (Dunipace *et al*., 2001). Many IRs members are still missing for *S. exigua* compared to the 43 IRs present in the *S. frugiperda* genome. In Lepidoptera, this gene family is divided in three subclasses: antennal IRs (aIRs), divergent IRs (dIRs) and Lepidoptera-specific IRs (lsIRs) (Liu *et al*., 2018). Of these, aIRs are highly conserved in sequence, and in Lepidoptera they cluster in 15 monophyletic groups that are largely characterized by a one-to-one orthologous relationship, although examples of lineage-specific duplications were reported (*e.g. S. frugiperda* has 17 aIRs) (Liu *et al*., 2018). Here, we describe the sequence of 17 *S. exigua* aIRs homologous to those present in *S. frugiperda* genome (Gouin *et al*., 2017; Liu *et al*., 2018). Hence, the missing *S. exigua* IRs probably belong to the dIRs and lsIRs subclasses, which are characterized by lineage-specific expansions (Liu *et al*., 2018).

Expression data for ORs and other chemosensory-related transcripts in *S. exigua* are limited to the main adult olfactory tissues, antenna and maxillary palps (Du *et al*., 2018; Liu *et al*., 2015b; Wan *et al*., 2015; Zhang *et al*., 2018). Our results provide the first comprehensive dataset of chemosensory-related transcripts expressed in the larval stage. We combine RNA-Seq and RT-PCR since RNA-Seq data from composite tissues (such as head) often delivers false negative results (Johnson *et al*., 2013), especially for genes expressed in few cells and at a low level, which is the case of chemosensory receptors. Altogether, 50 out of 63 ORs are expressed in larvae, including the four sex pheromone receptors (Liu *et al*., 2013). The number of ORs expressed in *S. exigua* larvae is higher than those observed in *S. littoralis* (22 larval-expressed ORs) (Poivet *et al*., 2013), in *H. armigera* (17 larval-expressed ORs) (Di *et al*., 2017) and *B. mori* (23 larval-expressed ORs) (Tanaka *et al*., 2008). Such

differences may be related to the different methodologies used for tissue dissection and RT-PCR. Further fluorescent hybridization studies are needed to reveal the cellular localization of each OR transcript and the real number of larval-expressed ORs. We do not observe any OR expressed in larvae but not present in adult antennae. Similarly, no larval-specific ORs were found in transcriptomic data from *S. littoralis*, *Dendrolimus punctatus* and *Lymantria dispar* larvae (Poivet *et al* 2013, Zhang *et al* 2017, McCormick *et al* 2017). On the contrary, six larval-specific ORs were identified in *Bombyx mori* (Tanaka *et al*., 2009), one in *H. armigera* (Di *et al*., 2017) and one in *C. pomonella* (Walker *et al*., 2016).

Contrary to ORs, there are 14 OBPs that are expressed in larval head but not in adult antenna. These sequences may represent larval-specific OBPs, although they can be also expressed in adult tissues other than antennae. In other Lepidoptera species, one larval-specific OBP was found in *S. littoralis* (Poivet *et al*., 2013), and ten in *L. dispar* (McCormick *et al*., 2017), suggesting that the occurrence of larval specific OBPs might be common in Lepidoptera.

We observe the expression of the four pheromone-binding proteins (PBPs) in *S. exigua* larval heads. PBPs and pheromone receptors are part of the molecular machinery used by adult moths to detect the sex pheromone, and their expression in *S. exigua* larvae is intriguing. This, however, corroborates previous studies that reported PBP and pheromone receptor expression in larvae from diverse Lepidoptera species (Jin *et al*., 2015; Poivet *et al*., 2012; Zhu *et al*., 2016; Zielonka *et al*., 2016). As proposed by Poivet *et al* (2012), larvae may use the pheromonal signal to find food.

We annotate the three putative Lepidoptera CO_2 receptors in *S. exigua*, but only two of them are expressed in larval head and adult antenna. Similarly, only two are expressed in adult antenna and proboscis in *S. littoralis* (Walker *et al*., 2019). We suggest that these two transcripts might be sufficient for CO_2 sensing in *S. exigua*, as it has been shown that only two out of the three CO_2 receptors are indispensable and

sufficient for CO_2 sensing (Ning *et al*., 2016, Xu *et al*., 2020). However, further functional experiments are needed to verify this hypothesis.

Differential expression analysis between male and female adult antennae identifies sixteen chemosensory-related transcripts with a sex-biased expression. As expected, among transcripts upregulated in males, there are three sex pheromone receptors (SexiOR6, SexiOR13 and SexiOR16) and one pheromone-binding protein (SexiPBP1), which are involved in sexual communication (Liu *et al*., 2015a, 2013). Our results support those of Liu *et al*., (2013, 2015), which showed male-biased expression of these pheromone receptors and SexiPBP1, using RT-qPCR (Liu *et al*., 2015a, 2013). A fourth *S. exigua* candidate pheromone receptor, SexiOR11, which had a male-biased expression in a previous RNA-Seq study (Du *et al*., 2018), does not exhibit differential expression between sexes in our study. This last result corroborates previous finding of Liu *et al* (2013) that showed no differences in expression between males and females for SexiOR11 and no functional response to five pheromones (Liu *et al* 2013). In addition to previous described pheromone receptors and PBPs, there is another OR with a male-biased expression, SexiOR56. This observation, together with the fact that this OR clusters in the sex pheromone receptor clade of Lepidoptera, leads us to suggest that SexiOR56 may be a fifth pheromone receptor present in *Spodoptera* spp.

Female-enriched transcripts identified from DE analysis are six ORs. We propose that they may detect volatiles involved in female important behaviours such as egg-laying substrate searches and choice. Of them, only the two ORs that show the greatest variation (SexiOR18 and SexiOR48) were also previously described as female-biased ORs (Du *et al*. 2018).

In *S. exigua* adults, previous exposition to sex pheromone or plant volatiles triggered a broad up-regulation of several ORs and OBPs, including some known pheromone-receptors (Wan *et al*., 2015). This was explained as a likely mechanism that mediates odour sensitization (Dion *et al*., 2019), a phenomenon that was observed

in *S. littoralis* (Anderson *et al*., 2013). To molecularly validate some of the annotated transcripts, we quantified the mRNA levels of a set of ORs and PBPs in fourth instar larvae after pre-exposure to VOCs. Our results highlight few and specific changes in gene expression after 1h of exposure. In contrast, 24h-exposure triggers up-regulation of several ORs, whatever VOC used. This observation is somewhat similar to what observed in *S. exigua* adults (Wan *et al*., 2015), where all the transcripts appeared up-regulated upon both pheromone and plant volatile exposure. However, in that case, also SexiORco, SexiOR3 and SexiPBP1 were up-regulated, whereas we do not observe any change in the expression of these transcripts in larvae (except for a limited but statistically significant fold-change in SexiPBP1 expression after cis-3-hexenyl acetate exposure). In mammals, exposure to a high dose of a given odorant triggers down-regulation of the expression of the responding OR, suggesting that exposure experiment could be used to identify OR-ligand pairs (Von Der Weid *et al*., 2015). The same has been suggested for *Drosophila melanogaster* (Von Der Weid *et al*., 2015) but a subsequent thorough screening revealed that changes in OR expression were observed only for few OR-ligand pair (Koerte *et al*., 2018), suggesting that this mechanism cannot be extended to insects. We speculate that the broad overexpression of ORs in *S. exigua* might be the consequence of a physiological response to the pharmacological effects produced by a 24h-long exposure of high concentration of VOCs. To test if the observed up-regulation is a general stress response that can be observed also after nonpharmacological treatments, we measured OR expression after 24h starvation. In this case, we observe a small down-regulation of two ORs, thus discarding the hypothesis that OR overexpression could be a general marker of stress.

Larvae detect odours in the environment to succeed in many ecological tasks such as selecting host plants or moving to more palatable food sources in the same plant, escaping from parasitoids, detecting harmful microbes or correct places for pupating (Becher and Guerin, 2009; Carroll *et al*., 2008, 2006; Carroll and Berenbaum, 2002; Ebrahim *et al*., 2015; Mooney *et al*., 2009; Piesik *et al*., 2008;

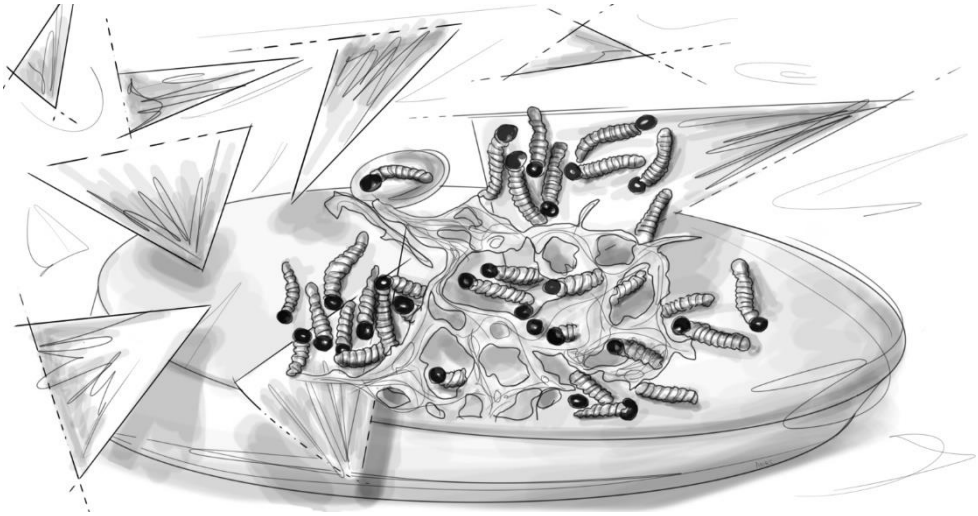
Poivet *et al*., 2012; Singh and Mullick, 2002; Stensmyr *et al*., 2012; Tanaka *et al*., 2009; Zhu *et al*., 2016). Identification of behaviourally active odorants is the first step needed to link volatile molecules to larval ecology. Yet, only data for pheromone-triggered behaviour are available for *S. exigua* larvae (Jin *et al*., 2015) and no methods for studying the behavioural effect of VOCs have been specifically developed for *S. exigua* larvae, although there are studies available for other caterpillar species (Becher and Guerin, 2009; Carroll and Berenbaum, 2002; de Fouchier *et al*., 2018; Di *et al*., 2017; Rharrabe *et al*., 2014). *S. littoralis* is the most related species whose VOC attraction/repellence was studied in larvae, using only filter papers that were used as odour source (de Fouchier *et al*., 2018). We initially used the same setup described for *S. littoralis* varying larval instars, starvation and VOC concentration, but we did not obtain no significant response to any VOC tested (data not shown), neither for 1-hexenol (a green leaf volatile) that has been observed to be attractive for several caterpillars (Rharrabe *et al*., 2014, de Fouchier *et al*., 2014; Becher and Guerin, 2009). Thus, we designed a new setup where we add the odorant on a piece of diet, to stimulate larval movement. We tested both fourth (data not shown) and fifth instar larvae and we got the best results for this latter stage since larvae were much more active and with a higher locomotion activity, likely due to the larval size and the food seeking state prior to pupation. In our experimental design, the volatile signal emitted by the VOC is mixed with the odorants emitted by the components of the diet (such as hexanol, hexanal and hexenol) (Rharrabe *et al*., 2014). Inferences on the behavioural activity of the different VOCs tested should be made with caution, as the behavioural response could be masked by the diet odours. However, this method is suitable to compare a battery of VOCs and to choose the ones among them that elicit attraction or repellency. For example, among the VOCs tested in this study, two of them enhanced attraction to the diet (1-hexanol and benzaldehyde), and five repelled larvae (indole, benzyl alcohol, linalool, 3-octanone and cis-3-hexenyl-propionate). Noticeably, we observed a clear attraction to 1-hexanol, similarly to what previously

observed in both *S. littoralis* and *L. botrana* (de Fouchier, Rharrabe and Becher & Guerin). Among other VOCs that elicited behavioural response, one of the most consistent responses was obtained for indole, that repelled larvae at any time-point. It is tempting to relate this avoidance towards indole with previous results that showed that indole enhances susceptibility of *S. exigua* larvae to two common Lepidoptera pathogens: baculovirus and *Bacillus thuringiensis* (Gasmi *et al*., 2019).

In conclusion, our work provides i) a reliable annotation of the chemosensory-related transcripts in the noctuid pest *S. exigua*, focusing on larval expressed genes; ii) the observation that long-term odorant exposure triggers broad and unspecific changes in ORs expression, iii) a new method suitable to identify behaviourally-active VOCs against *S. exigua* larvae. The data shown here represents the first step for further studies aimed to characterize receptors and their cognate ligands that are important for the ecology of *S. exigua* larvae. Identification of larval attractants or repellents, has the additional value to be of further interest in the development of olfactory-based control techniques, which might help in protect crops from larvae attack and increase the yield.

CHAPTER 4

CHANGES IN CATERPILLAR CHEMOPERCEPTION AFTER BACULOVIRUS INFECTION



This chapter is under review:

Llopis-Giménez, A., Caballero-Vidal, G., Jacquin-Joly, E., Crava, CM., Herrero, S. Baculovirus infection alters caterpillar chemoperception. *Journal of Virology* .

4.1. 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 makes 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). For example, 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 behavior that baculovirus-infected larvae show (called tree-top disease). The infected larvae climb at high positions, 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 examples of host behavioral changes are triggered by baculovirus-encoded genes that had been ancestrally acquired from Lepidoptera. The final phenotype displayed by infected larvae might be mediated through changes in the host peripheral (PNS) and central nervous systems (CNS), where the behavior and the physiology of the insect are mainly controlled (Kinoshita and Homberg, 2017).

The PNS includes sensory neurons that receive external stimuli from the environment. Chemical stimuli are detected by chemosensory 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). In insects, it is mainly carried out by members of three gene families: the odorant receptors (ORs), the ionotropic receptors (IRs) and the odorant-binding proteins (OBPs). Among these, ORs are the centrepiece (Breer *et al*., 2019). 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. Each OR is specialized in detecting specific odorants, and it normally acts together with the OR-coreceptor (Orco), forming an heteromeric complex (Benton *et al*., 2006). After the activation of the complex, a cation exchange occurs leading to the membrane depolarization that starts the consequential 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. 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. 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 can alter the neuronal circuits devoted to volatile detection.

Spodoptera exigua (Hübner, 1808) (Lepidoptera: Noctuidae) constitutes an excellent model for studying host-pathogen interactions between baculovirus and

insects (Crava *et al*., 2015; Han *et al*., 2015; Jakubowska *et al*., 2013; Van Houte *et al*., 2014) 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). In addition, repertoires of chemosensory genes have been described (Du *et al*., 2018; Liu *et al*., 2015; Llopis-Giménez *et al*., 2020; Y. Zhang *et al*., 2018). In this study, we first characterized the changes in *S. exigua* OR (SexiOR) expression in larval heads upon SeMNPV or AcMNPV infection, identifying a set of ORs specifically regulated 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 behavior analysis revealed that the species-specific SeMNPV infection induces changes in larval perception of two of the identified ligands.

4.2. Materials and methods

4.2.1. Insects

The *S. exigua* colony used for all the experiments has been maintained at the University of Valencia (Spain). Larvae have been reared on artificial diet (Elvira *et al*., 2010) at 25 ± 3 ºC with 70 ± 5 % RH, using a photoperiod of 16:8 h (light:dark).

4.2.2. Viral infections

Newly molted third-instar *S. exigua* larvae were infected with wild-type (WT) SeMNPV or WT AcMNPV using a dose 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 feed with the same solution containing no virus (mock infection). For RNA extraction, at 96 hours post-infection (hpi), larval heads were excised from the body using a scalpel, pooled and stored in 300 µl of TRIzol

(Roche) at -80 ºC. Each replicate consisted of sixteen pooled heads, and three independent replicates were processed for each treatment.

4.2.3. High-throughput sequencing

Total RNA from SeMNPV- and mock-infected larvae was purified using TRIzol reagent following the manufacturer's instructions. A second purification step was done using the RNeasy Mini Kit (Qiagen). Purified RNA was eluted with water, and RNA integrity was checked on 1 % agarose gels. Library preparation and paired-end RNA sequencing on a Ilumina Hiseq2000 machine were both carried out by Novogen Technology (China). Raw reads are available at NCBI SRA database (Project number PRJNA634227).

4.2.4. 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 transcripts repertoire annotated from *de novo* transcriptome (Llopis-Giménez *et al*., 2020). 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).

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

RNA from SeMNPV-, AcMNPV- and mock-infected larvae was purified as described above and used to prepare cDNA (500 ng RNA/sample). Samples were first treated with DNAseI (ThermoFischer Scientific) following manufacturer's protocol and then converted into cDNA using PrimeScript RT Reagent (Takara) and 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. Forward and reverse primers were designed using the online software tool Primer3Plus

(Untergasser *et al*., 2007). An endogenous control *ATP synthase subunit C* housekeeping gene was used in each RT-qPCR to normalize the RNA concentration. The sequences of the used primers are provided in the Annexed XXIV. 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).

4.2.6. Heterologous expression of SexiORs in *Drosophila*

For the construct generation, the open reading frame (ORF) fragment of *SexiOR23* was amplified and cloned in the destination vector, *pUAST.attB,* using the appropriate restriction enzymes, generating *pUAST.attB-SexiOR23*. The ORF fragment of SexiOR35 was amplified and cloned in *pUAST.attB* by Genscript Biotech (USA), generating *pUAST.attB-SexiOR35*. Both constructs were purified from liquid cultures of *Escherichia coli* DH10β using the Illustra plasmidPrep Midi Flow Kit (GE Healthcare).

For P-element transgenesis, *pUAST.attB-SexiOR23* and *pUAST.attB-SexiOR35* were separately injected into w^{1118} *Drosophila melanogaster* embryos, and fly lines harbouring a transgene insertion into the third chromosome were used for further crossings. For phiC31-targeted transgenesis, *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 genomic locus 86Fb of the third chromosome. The whole process of plasmid injection and P-element transgenesis and phiC31-targeted transgenesis was carried out by BestGene Inc (CA, USA).

The recombinant *D. melanogaster* flies used for the single-sensillum recording (SSR) experiments were maintained at the INRAE, Sorbonne Université (France). Flies were reared on standard cornmeal-yeast-agar medium (Bass *et al*., 2007) at 25 ºC, using a photoperiod of 12:12 h (light:dark). To obtain flies expressing *SexiOR23* and *SexiOR35* in the ab3 sensilla, recombinant flies carrying the *UAS-SexiOR* constructs were crossed with the GAL4-driver w;Δhalo/CyO;Or22a-GAL4 line (de Fouchier *et al*., 2017; Dobritsa *et al*., 2003).

4.2.7. Single-sensillum recordings

SSR experiments were performed to deorphanize SexiOR23 and SexiOR35 using the empty neuron system 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 between the second and the third antennal segments, also held by dental wax. 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 tested against a panel of 58 odorants listed in the Annexed XXV. 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 cartridge, which was then, inserted into a Pasteur pipette. Pipettes with filter papers containing 10 µl of solvent were used as controls. A constant flux of 1.51 min^{-1} of humidified air delivered the odorant to the antenna. The action potentials were recorded from ab3 sensilla using electrolytically sharpened tungsten electrodes. One of the electrodes was inserted inside the eye of the fly as a reference. The thinner recording electrode was inserted at the base of the sensillum of interest using a DC-3K micromanipulator (Märzhäuser). The entire

odorant panel was tested six times on six different flies. Single stimulation cartridges were used at most two times on each fly and a maximum of five times in total. Since it is not possible to distinguish ab3 sensilla only by localization and morphology, 100 ng of 2-heptanone were used as a diagnostic stimulus. 3-heptanone is one of the most potent ligands for DmelOR85b, which is expressed in the ab3B neuron (Hallem and Carlson, 2006). The absence of DmelOR22a in ab3A neuron expressing a SexiOR was verified using a stimulus cartridge containing 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 performed using the gplots (version 3.0.3) and RColorBrewer (version 1.1-2) packages of R software.

4.2.8. Behavioral assays

Behavioural assays were performed to study the effect (attraction or deterrence) of three plant volatiles to SeMNPV-, AcMNPV- and mock-infected *S. exigua* larvae in a complex background. The behavioural assay was carried out as detailed by Llopis-Gimenez *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 with a piece of artificial diet (1.5 x 0.8 x 1 cm) placed at the opposite side. The Petri dish was put inside a paperboard box (30 x 22 x 22 cm) with a hole in the side of the box (6 cm of diameter) to include a 50 W halogen artificial light (at 15 cm of distance to the Petri dish). Fifty µl of the odorant diluted at 10, 1 and 0.1 µg/µl in methanol (Labkem) were added to the artificial diet. Odorant tested were purchased from Sigma-Aldrich and were: (±)-linalool (CAS #: 78-70-6), estragole (CAS #: 140-67-0) and 1-indanone (CAS #: 83-33-0). A control run with the solvent alone was run in parallel to every run with an odorant.

Each odorant and its respective control was tested a total of nine times (three replicates with three different batches of larvae, *i.e*. larvae from different offspring). The mobility index and the larval attraction index were calculated as described by Llopis-Giménez *et al*., 2020. Values of the attraction index higher than 1 mean that the larvae are more attracted to the diet + odorant than to diet only (attraction effect), whereas values lower than 1 mean that larvae are less attracted to diet + odorant than to diet only (deterrent effect). Statistical analyses were conducted using a one-sample *t*-test comparing the attraction index at each time point 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).

4.3. Results

4.3.1. 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 heads of SeMNPV-infected and control larvae. These last only fed on a droplet solution that did not contain any virus particle (hereinafter called mock-infected larvae) larvae. 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 robust statistical significance, we used total counts to select some *SexiORs* that displayed 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 also analysed by RT-qPCR 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 (Figure 1): *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). In contrast, *SexiOR19* and *SexiOR34* showed no expression change after SeMNPV infection (Figure 1), as observed using total read counts.

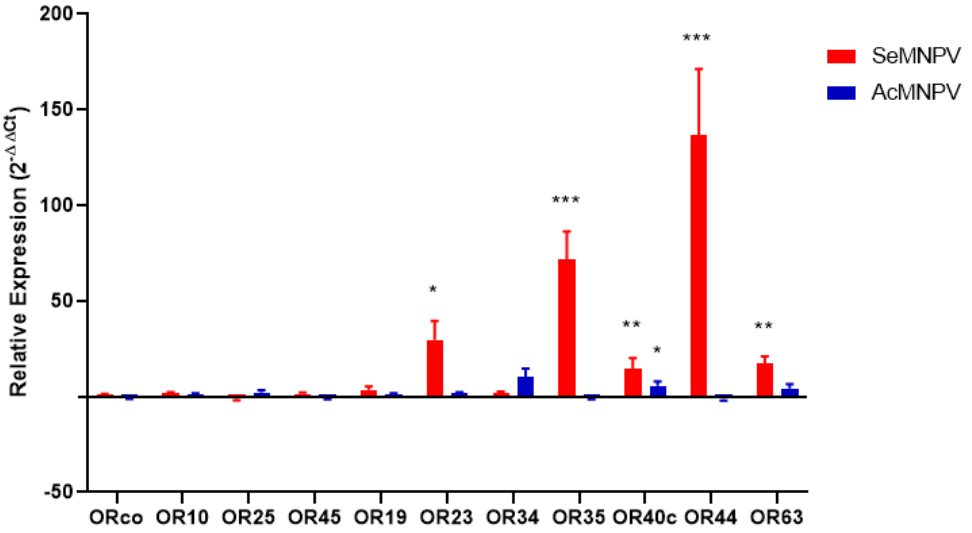


Figure 1. Differential analysis of selected odorant receptors (ORs) in *Spodoptera exigua* whole heads after infection with SeMNPV and AcMNPV. mRNA levels were quantified with real-time quantitative PCR. Red color indicates expression changes upon SeMNPV infection and blue expression changes upon AcMNPV infection. Asterisks indicate statistically significant differences between pairwise non-infected and infected samples (unpaired *t*-test with Welch's correction) ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***).

To study if the observed changes in *SexiOR* expression after SeMNPV infection were specific to this virus or were triggered also by a generalist baculovirus infection, a RT-qPCR analysis of the expression after AcMNPV infection 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) (Figure 1). These results suggest that the observed *SexiOR* expression changes are unique to the species-specific SeMNPV.

4.3.2. SexiOR35 is a broad-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 (expression in *Drosophila melanogaster* ab3A neurons devoided of their own OR, (Dobritsa *et al*., 2003)) coupled to single-sensillum recording (SSR), and using a stimulation panel of 58 odorants representative of aliphatics, aromatics and terpenes. Observation of spontaneous activity in transformed *D. melanogaster* ab3A neurons confirmed the correct expression of both SexiORs. SexiOR35 responded to 24 different volatiles out of 58 (41.4 % of the tested odorants), thus showing a broad spectrum profile. The stronger 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}) (Figure 2A). The broad spectrum of SexiOR35 was illustrated by the sparseness value of the distribution (S) (S = 0.16) (Figure 2B). SexiOR23 was not activated by any of the 58 volatile compounds tested (Figure 2A).

4.3.3. SeMNPV infection alters larval attraction to behavioral cues

To understand the effects of the observed changes in the expression of SexiORs in a behavioral context, larval response to some SexiOR35 ligands were recorded in healthy and infected larvae. Three volatile compounds were selected for the behavioral experiments in which we evaluated the mobility of larvae towards a piece of diet where the odorants were added. If the tested odorant would trigger enhanced attraction, larvae would walk longer distances than control with di*et al*one, resulting in attraction indexes > 1. Otherwise, if the tested odorants would trigger a deterrent effect, larvae would walk less than controls resulting in attraction indexes <

1. Attraction indexes were calculated for mock-, SeMNPV- and AcMNPV-infected fifth-instar *S. exigua* larvae (Figure 3). Estragole and linalool were chosen because they strongly activated SexiOR35. In contrast, 1-indanone was used since it did not activate SexiOR35. Results showed that diet + linalool at the lowest concentration (0.1 mg/ml) was more attractive than di*et al*one to mock-infected larvae at the last time-point (10 min) (Figure 3A). In the same conditions of dose and time, no significant enhanced attraction of both SeMNPV- and AcMNPV-infected larvae could be observed. At a higher dose (1 mg/ml), linalool had a significant deterrent effect on SeMNPV-infected larvae at the 5 min and 10 min time points. This was not observed for mock- and AcMNPV-infected larvae (Figure 3A). At the highest concentration (10 mg/ml) either SeMNPV- and mock-infected larvae did not display deterrence nor enhanced attraction at any time point. Estragole triggered deterrence in mock-infected larvae at both 1 and 10 mg/ml, and this effect was time-dependent. This effect was not observed in SeMNPV- nor in AcMNPV-infected larvae (Figure 3B) at any dose and any time point. 1-indanone only showed an enhanced attraction effect on *S. exigua* SeMNPV-infected larvae at 2 min time-point and a concentration of 1 mg/ml. No effect was recorded in mock-infected samples (Figure 3C).

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

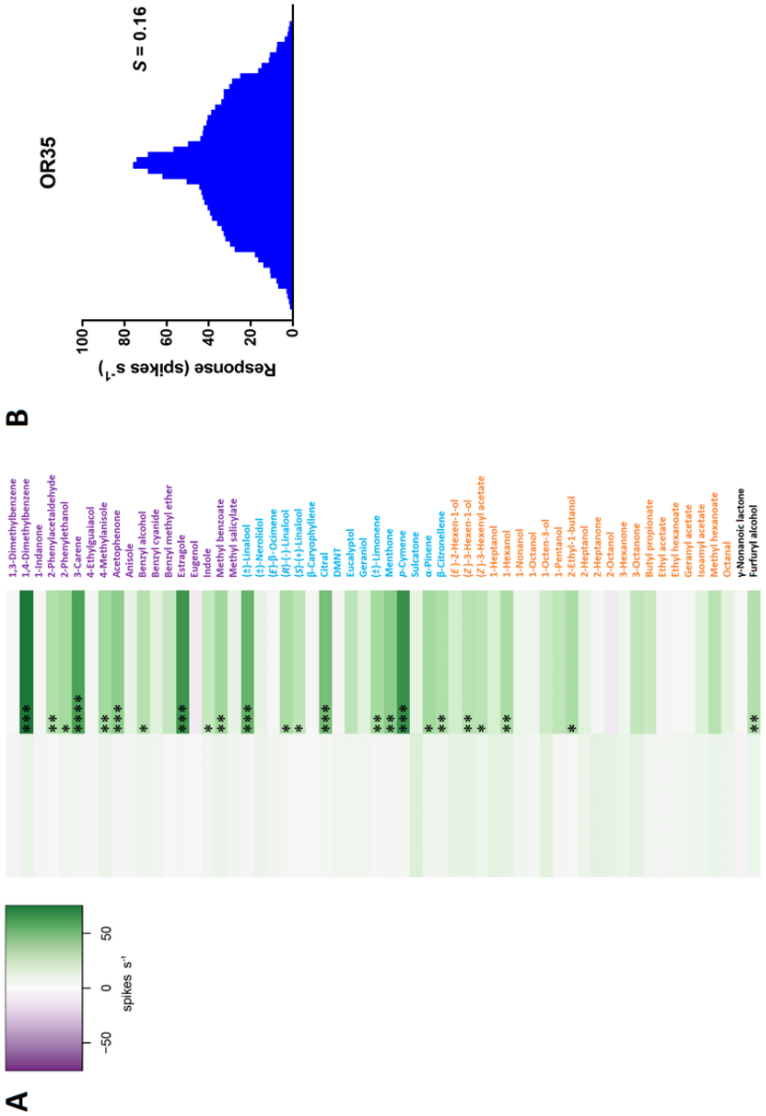


Figure 2. SexiOR35 and SexiOR23 response spectra at high-stimulus doses. A) Heat map summarizing the mean responses of both ORs to a panel of 58 odorants (100 µg) on the filter paper). Responses (firing rate in spikes s^{-1}) are coloured according to the scale on the left. Odorants are classified depending 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*) (63). A low *S* value indicates a broad tuning and a value of 1 indicates a narrow tuning of the receptor.

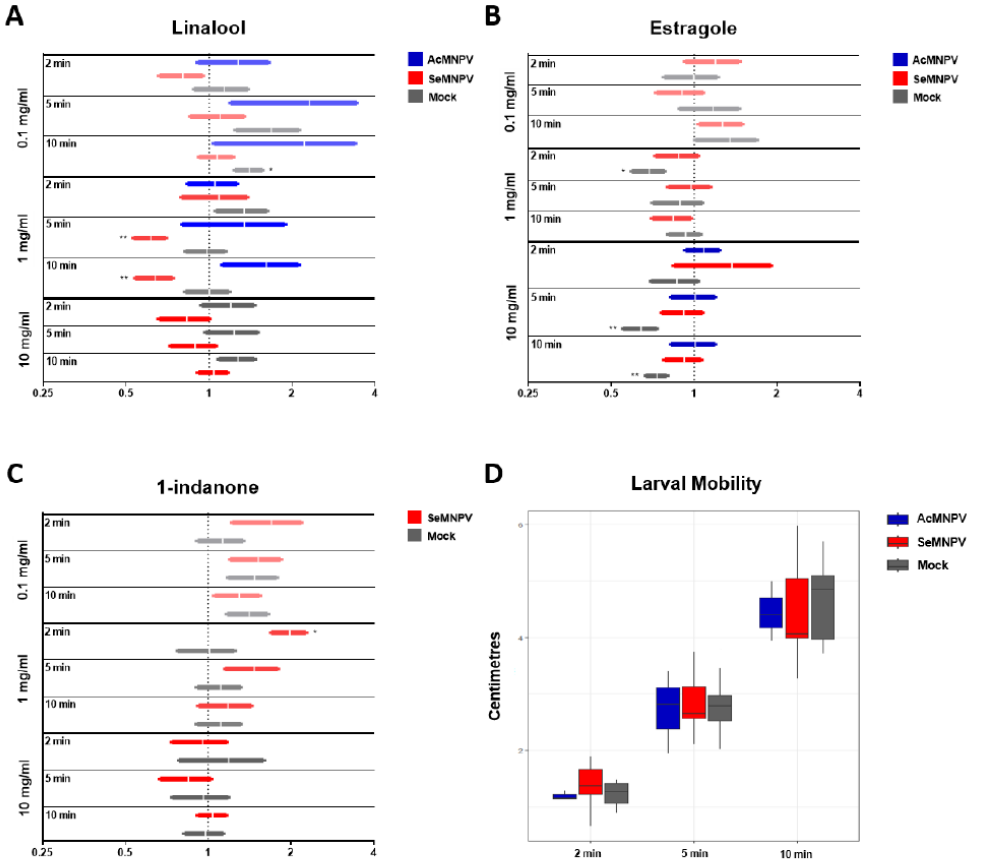


Figure 3. Larval attraction index of *Spodoptera exigua* larvae to different odorant stimuli at different times and doses. Fifty µl diluted at 0.1, 1 and 10 mg/ml of each odorant were used for the bioassay. Bars represent the mean value and the standard deviation. Values above 1 are indicative of attraction and values below 1 are indicative of deterrence. 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) Behavioral response of larvae exposed to linalool. B) Behavioral response of larvae exposed to estragole. C) Behavioral response of larvae exposed to 1-indanone. D) Behavioral response of larvae in the control run with diet + solvent. Results are shown in centimetres of larval displacement at 2 min, 5 min and 10 min time-points.

4.4. Discussion

In this work we have described the effects of the SeMNPV infection in the chemoperception of *S. exigua* larvae, combining gene expression, functional studies and behavioural experiments. According to our data, some *SexiORs* 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 and the ligands that activate them. SexiOR35 is a broadly-tuned odorant receptor that recognised multiple odorant molecules from different chemical structures, mainly aromatics and terpenes. Subsequent behavioral assays with SeMNPV-infected larvae permitted to connect the SexiOR expression changes with altered behavioural responses to specific odorants. To our knowledge, this represents the first study that shows altered host's olfaction due to baculovirus infection and allows us to hypothesize about the biological meaning of these changes in the host-pathogen interaction.

During the coevolution, baculoviruses have acquired different mechanisms that permit them to improve their fitness and dispersion during the infection process. Many of these strategies involve alterations of some physiological and behavioral aspects of their hosts (Gasque *et al*., 2019). The underlying mechanisms of baculovirus manipulation are likely to involve modifications at neuronal level, 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 potential neuropeptide *proctolin-like* is clearly down-regulated in the brain of SeMNPV-infected larvae (Llopis-Gimenez *et al*., 2020). 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. To our knowledge, this is the first report of changes in transcription of genes involved in peripheral chemoreception upon baculovirus infection, although a plethora of RNA-Seq studies aiming to detail expression changes between infected and non-infected larvae in several baculovirus-host models have been produced (Bhattarai *et al*., 2018a, 2018b; G. Wang *et al*., 2015; S. 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 (Johnson *et al*., 2013). We forecast that further studies using only antennae of SeMNPV-infected larvae might show finer OR regulation, or even, expression changes of other chemosensory-related genes as OBPs or ionotropic receptors (IRs). We must be aware that the experimental setup of our study made impossible to distinguish between direct regulation exerted by the virus or larval response against the infection. A direct regulation by the virus has been observed in 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). Further studies may shed light on the molecular mechanisms of *SexiOR* expression changes, for example the role of miRNAs, which may underlie the transcriptional variation.

We used the powerful *Drosophila* empty neuron system to deorphanize some SexiORs whose expression was altered by SeMNPV infection. It consists in 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 data on ORs from *S. exigua*, in which three ORs (SexiOR3, tuned to terpenoids, and SexiOR13 and 16, tuned to pheromones) have been deorphanized to date (Liu *et al*., 2014, 2013), laying the foundation for a full understanding of the neuroethological aspects of the chemical ecology of *S. exigua*. SexiOR35, which is upregulated upon SeMNPV infection, is a

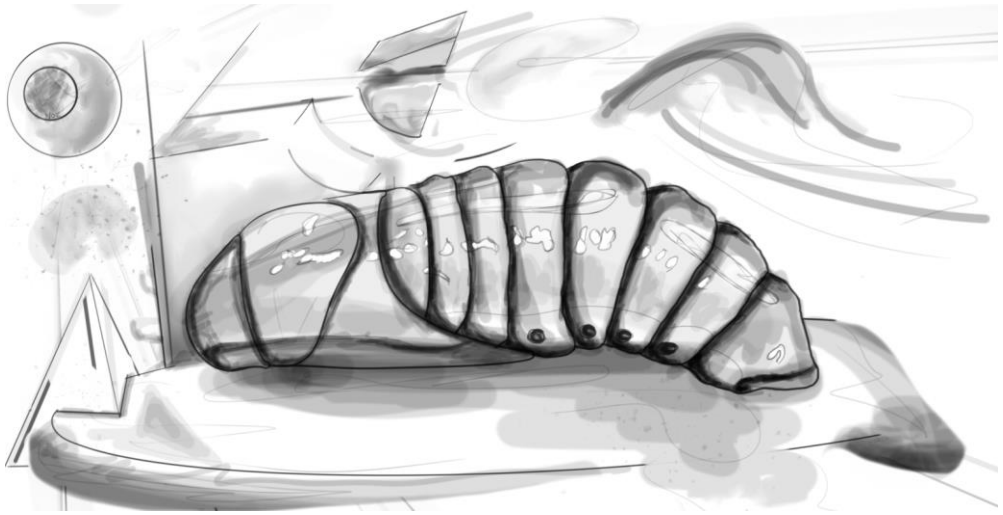
very broadly-tuned receptor, able to recognise many different odorant compounds. The strongest responses were to 1,4-dimethylbenzene, 3-carene, acetophenone, estragole, linalool, citral and p-cymene. By using the same deorphanization method, the ortholog SlitOR35 of the African cotton leafworm *S. littoralis*, demonstrated activation by similar compounds, such as estragole, 3-carene and linalool among others (de Fouchier et al., 2017). Likewise SexiOR35, SlitOR35 is a broadly-tuned receptor. However, SlitOR35 showed responses to benzyl methyl ether and sulcatone, that did not activate SexiOR35. This suggests a general conserved function for SlitOR35 and SexiOR35, whose shifts in sensitivity to some odorants may be related to species-specific behaviors (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 in our odorant panel, as it represents a minute fraction of all odours that a moth might encounter in its daily life (Hansson *et al*., 2010). Similarly, the orthologue SlitOR23 from *S. littoralis* could not be deorphanized (de Fouchier *et al*., 2017). 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 correlation of the strongest ligands of SexiOR35 with their behavioural effects on *S. exigua* larvae in the context of a baculovirus infection further provides neuroethological insights in the possible role of this receptor in the host-pathogen interactions. Two of the main odorants detected by SexiOR35, linalool and estragole, altered larval olfactory behavior upon baculovirus infection at specific doses and time points. Linalool is one of the most common volatiles produced in response to herbivory attacks by many plants as cotton, rice, maize or tomato (Elsharif *et al*., 2015). 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), likewise observed in our study for control *S. exigua* larvae (mock-infected) at low dose (0.1

mg/ml). Whereas no behavioural effect could be observed at intermediate dose for control larvae, the results revealed deterrence in species-specific SeMNPV-infected larvae but not in AcMNPV-infected larvae. In previous studies, 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 behavioral shift upon infection by SeMNPV could be interpreted as a defence response of the larvae, as the deterrence effect of linalool would lead to avoid the synergistic effect of linalool and SeMNPV, increasing larvae survival. Estragole, a volatile common to aromatic plants, in turn, produced a behavioural shift common to both SeMNPV and AcMNPV infection. This could be an unspecific phenotype produced during infection, regardless of the baculovirus species. OR40c was the only receptor that exhibited an expression changes under both AcMNPV and SeMNPV infections, so this behavioral phenotype could be associated to its mRNA levels. However, more research is needed to identify ligands for OR40c, as well as further transcriptional studies might highlights other changes in chemosensory-related genes that might be common between both baculovirus infection processes. 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 is detected by other ORs, whose regulation would be responsible for the observed behavioural shift. Alternatively, it is possible that behavioural changes are due to changes at higher brain centres, where the information coming from external sensory receptors is integrated (Kinoshita and Homberg, 2017).

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 correlate the changes in its expression with infection-related shifts in larvae behavioral responses when exposed to two of its main ligands, linalool and estragole. What remains unknown is whether the observed behavioral changes are indicative of a manipulation by the parasite or are a defence strategy of the host, which may change its feeding habits as an example of self-medication to decrease the pathogenicity of SeMNPV, and enhance its survival possibilities. Additional research will contribute to a better understanding of the consequences of the olfactory changes induced in *S. exigua* under the SeMNPV infection, that will help to clarify the importance of chemical ecology in the host-pathogen interactions.

GENERAL DISCUSSION



Baculovirus represent a clear example of parasites that induce alterations in the host behavior with the aim of enhancing their field fitness (Van Houte *et al*., 2013). Baculovirus-infected caterpillars show an increased locomotion activity that allows the virus to increase the area over which its progeny can be spread (Kamita *et al*., 2005). In addition, baculovirus-infected larvae tend to die at elevated positions in plant or trees enhancing the viral transmission to the underlying foliage and consequently, increasing the chance of transmission to other caterpillars, in what is known as tree-top disease (Hoover *et al*., 2011). These parasite-induced phenotypes, and others that could be discovered, may involve changes in the nervous system of the host where physiology and behavior is controlled (Gasque *et al*., 2019). Previous works have demonstrated baculovirus presence in brain and antennas, organs where the insect central and peripheral nervous system is mainly located (Herz *et al*., 2003; Naik *et al*., 2018).

In the present doctoral thesis we have aimed to explore gene expression changes in the central and peripheral nervous system of *S. exigua* that could explain these and other possible phenotypes produced by SeMNPV possibly impacting its field incidence. In the central nervous system, neuropeptides play an important role in regulating the insect physiology. In the peripheral nervous system, chemosensory-related proteins are in charge of insect chemoperception and clearly influence the behavioral responses that insects show to the constantly changing environment. In a first approach, identification and annotation of the selected gene repertoires was performed. Then, transcriptomic analyses were applied to unveil gene expression changes produced in the neural systems related to the virus infection. That permitted to identity candidate genes for further functional characterisation and for studying their role in the host-pathogen interaction.

Annotation and expression analysis of the *S. exigua* neuropeptidome

Among the repertoire of regulatory genes in the central nervous system, neuropeptides play an important role in the chemical communication between cells and in the regulation of different physiological and behavioral aspects such as reproduction, feeding, development or locomotion (Fónagy, 2014).

The first insect neuropeptidome description was performed in *Drosophila melanogaster*, although in the recent years it has been extended to different insect species, especially agricultural pests or vectors for human diseases (Hauser *et al*., 2018; Riehle *et al*., 2002; Roller *et al*., 2008). Neuropeptide identification and description helps to better understand the insect's physiology and their adaptation to the different environments, but also provides new targets for the development of advanced insecticidal strategies (Hawthorne, 1997; Van Hiel *et al*., 2010).

In this context, we annotated the most complete insect neuropeptidome up to date. It was obtained through data mining of a *S. exigua* transcriptome generated from larva heads, larvae gut and adult brain samples. Sixty-three genes were identified and annotated as putative neuropeptides, including splicing variants for six unigenes and different gene isoforms for two neuropeptide unigenes. Phylogenetical analysis were carried out to study the homology of *S. exigua* neuropeptides with sequences from other insect species, and detecting possible duplication events. This has allowed to reveal a neuropeptide core formed by 43 genes in Lepidoptera.

In the neuropeptidergic system, gut-brain neuropeptides have a pivotal importance in the regulation of feeding, growing and digestion, as they are expressed both in brain and in the neurosecretory cells of the gut (Duve and Thorpe, 1980). Comparing the expression of annotated transcripts in larva head and gut samples, sixteen transcripts were considered as brain-gut neuropeptides. Some examples are: *allatostatin*, *allatotropin*, *proctolin*, *CCHamide*, *neuropeptide F1* and *short neuropeptide F1*. In addition, to gain information about the presence or absence of the

different neuropeptide transcripts in larvae and adult, their expression was compared in larval head, larval gut or adult brain samples. Although no larval specific neuropeptides were found, six transcripts were considered adult-specific, suggesting a potential involvement in adult-specific processes.

To complete the neuropeptidome description of *S. exigua*, we studied the expression of some of the selected neuropeptides under starving conditions in larvae, as most of them regulate feeding and digestion. Most of the selected neuropeptides were found to be differentially expressed during starvation, the majority being up-regulated. This possibly corresponds to a general stress response. The upregulation of some of them as *short neuropeptide F*, *allatostatin* and *CCHamide* could be related with starvation-induced olfactory modifications, as it was observed in *D. melanogaster* (Farhan *et al*., 2013).

Neuropeptide expression was also compared under different light and temperature conditions, as these factors strongly influence the insect development. Moreover, some of the neuropeptides play a role in the circadian rhythms regulation, that is connected with light and dark cycles (Hofer, 2006). None of the neuropeptide transcripts was differentially regulated after light deprivation, discarding a direct effect on the regulation of these genes. Similarly, temperature did not have any clear effect, suggesting that expression of neuropeptides is not easily influenced by external factors.

Overall, the annotation of the *S. exigua* neuropeptidome will help to identify neuropeptides in other insect species providing valuable information about the importance of the neuropeptides action in the insect's physiology regulation. In the context of this thesis, it has allowed the next step, aimed to study the influence of the SeMNPV infection in the neuropeptidergic system regulation, in order to discover new relevant aspects in the baculovirus-caterpillar interaction.

SeMNPV infection regulates proctolin-like, a potential neuropeptide that modulates locomotion and digestion

Once the neuropeptide repertoire of *S. exigua* was identified and annotated, we aimed to analyse the possible changes produced by SeMNPV during the host infection. The objective was to identify alterations in the regulation of genes of the central nervous system that might be related with the baculovirus-induced phenotypes.

As a first approach, SeMNPV-related gene expression changes in the neuropeptidergic system was analysed in larval head of SeMNPV- and mock-infected RNA-Seq samples. A clear pattern of differential expression after the viral infection was not observed. Although, some genes whose function was already described were regulated according to the statistical analysis. This is the case of *eclosion hormone*, *ecdysis triggering hormone* and *prothoracicotropic hormone* that encode for ecdysis inductors and have an opposite function to the well-described juvenile hormone (Eldridge *et al*., 1992; Mizoguchi *et al*., 2013; Park *et al*., 2002). Down-regulation of this set of genes would produce a delay in the normal moulting process, extending the lifetime of the host. This is functionally related with the baculovirus-encoded *egt* gene that promotes the same effect in host larvae, increasing the time until the host dies, keeping it in an active feeding state and increasing the viral progeny that is released at the end of the infection cycle (O'Reilly and Miller, 1991). The regulation of this group of genes could complement the action of the *egt* gene in the baculovirus-host interaction.

Due to the absence of a general pattern of differential expression of the neuropeptide genes after SeMNPV infection, and to better focus on the brain, where most neuropeptide genes are expressed, we then analysed the neuropeptide genes expression in mock- and SeMNPV-infected brain samples through RT-qPCR. Results show that *proctolin-like* (*PLP*) a gene resembling the neuropeptide gene *proctolin* that

is present in order Coleoptera and Diptera but that seems absent in Lepidoptera, was clearly down-regulated after the viral infection. Due to its expression pattern in brain and gut tissues its predicted cleavage sites, and the presence of the active part of the proctolin neuropeptide (RY/HLPT) in the PLP sequence, it was considered for its functional characterization and for studying its role in the host-pathogen interaction.

Gene silencing through RNA interference has enhanced the study of the gene function in insects. However, the usage of these techniques in Lepidoptera have been proven to be difficult to achieve (Terenius *et al*., 2011). For that, the selected method for functionally characterise *Se*-PLP was the gain-of-function strategy. For that, AcMNPV baculoviruses constitutively expressing the C-terminal fragment of *Se*-PLP were generated. Bioassays were performed to check the influence of *Se*-PLP overexpression in the baculovirus pathogenicity. We also analysed its effect in the larval development and the larval locomotion, which are factors that are functionally related to the proctolin neuropeptide present in Coleoptera and Diptera as a regulator of both the skeletal and visceral muscle contractions (Breidbach and Dircksen, 1989; Clark *et al*., 2006; Fiandra *et al*., 2010; Isaac *et al*., 2004; Orchard *et al*., 1989; Walker and Bloomquist, 1999).

Infections with AcMNPV overexpressing *Se*-PLP showed an increased mortality at lower doses, what led us to conclude that *Se*-PLP influences the baculovirus-caterpillar interaction. Also, a reduction in the larval growth and digestion appeared in AcMNPV-PLP-infected larvae compared to the control virus AcMNPV- (not expressing *Se*-PLP) and the mock-infected larvae. Moreover, *Se*-PLP expression during the baculovirus infection produced a reduction in the locomotion of the larvae. To study that, we developed a method that allowed us to measure the larval locomotion activity comparing the different infection treatments. The obtained results allowed us to link *Se*-PLP function with the regulation of the skeletal and gut muscle contractions, affecting digestion and locomotion, as it was previously

described for the neuropeptide proctolin (Fiandra *et al*., 2010; Lange and Orchard, 2006; Orchard *et al*., 1989).

As a conclusion, SeMNPV infection produces the down-regulation of three important genes in the ecdysis process, possibly complementing the function of the *egt* gene in the baculovirus-caterpillar interaction. In the central nervous system, *PLP* was clearly down-regulated after SeMNPV infection. The characterization of *Se*-PLP could connect its function with the hyperactivity phenotype, as a decrease in the expression of this gene may produce an increase in the locomotion activity of the larvae. The decrease in *Se*-PLP expression could also complement the *egt* gene effects in a different way, affecting the regulation of the gut contractions, producing bigger larvae and possibly releasing more virus to the environment (O'Reilly and Miller, 1991).

Whether the regulation of the neuropeptide expression after SeMNPV infection represents a direct effect of the virus to the host, a defence response of the host or a side effect of the baculoviral infection, remains to be elucidated. Additional research will be necessary to unveil gene expression regulation mechanisms by the virus, increasing our knowledge about the parasite-induced changes in lepidopteran hosts.

Annotation of the *Spodoptera exigua* chemosensory-related genes focusing on the larval olfaction

Chemoperception defines the detection and discrimination of environmental chemical stimuli, establishing the base for the taste and smell of insects. Is a key factor when studying insect behavior, since it influences many aspects of the insect's biology as reproduction, egg laying, food source seeking and danger detection (Depetris-Chauvin *et al*., 2015; Robertson, 2015).

Previous descriptions of the chemosensory-related genes of *S. exigua* were obtained using adult samples and using different annotation nomenclatures that difficulted the comparisons among studies (Du *et al*., 2018; Y. Zhang *et al*., 2018). In addition, there was a lack of knowledge of the larval chemosensory genes, which is the stage susceptible to baculovirus infection. In this context, we reannotated the chemosensory-related genes of *S. exigua* using RNA-Seq samples of adult and larvae, also unifying the gene nomenclature with the closely related species *S. frugiperda*, whose chemosensory repertoire was already annotated from the genome (Gouin *et al*., 2017). We identified a total of 200 chemosensory-related genes in *S. exigua*, expanding the number of genes identified in the previous published annotations.

To gain information about the expression of chemosensory-related genes in larval stages, we combined RNA-Seq and RT-PCR techniques, mainly focusing in ORs, the centrepiece of the insect's olfaction. Altogether, 50 out of the 63 ORs genes were observed expressed in larvae, although no larval-specific OR was found. In addition, 14 OBPs were found to be larval-specific and 4 pheromone binding proteins (PBPs) were found expressed in larval head, even though their main function is the recognition of the adult sex pheromone (Poivet *et al*., 2012). This has been seen in other Lepidoptera species, and it has been hypothesized that larvae may use the pheromonal signal to find food (Jin *et al*., 2015; Poivet *et al*., 2013; Zhu *et al*., 2016; Zielonka *et al*., 2016).

Up-regulation of ORs and OBPs was previously observed in adults exposed to specific plant volatiles in a mechanism explained as odour sensitization (Anderson *et al*., 2013; Dion *et al*., 2019). We quantified the expression levels of a set of ORs and PBPs in larvae after pre-exposure to different odorant compounds. After 24 hours of exposure, several ORs were up-regulated whatever the odorant used. We speculated that the broad overexpression of ORs might be a physiological response to the high concentration of volatiles. To test if these changes correspond to a general stress response, another analysis was carried out starving the larvae for 24 hours. We

did not observe the same effect on chemosensory-genes expression, so we conclude that the observed expression changes do not correspond to a general stress response.

Identification of behaviourally active odorants for the *S. exigua* larvae would help to link volatile molecules with the larval ecology. Due to the absence of protocols in *S. exigua* larvae, a new setup was designed that allowed to study the behavioral responses of the larvae to specific odorants. To test the method, different odorants were employed. Two of them, 1-hexanol and benzaldehyde, resulted attractive for the larvae, whereas others as indole, benzyl alcohol, linalool, 3-octanone and cis-3-hexenyl propionate, repelled them. The obtained results and the attraction produced by 1-hexanol, that coincides with the previously observed in *S. littoralis* and *L. botrana*, validated our method for identifying behaviourally active odorants (Becher and Guerin, 2009; de Fouchier *et al*., 2018; Rharrabe *et al*., 2014).

In conclusion, a new and reliable annotation of the chemosensory-related genes of *S. exigua* was carried out, focusing on larval expressed genes. To deepen larval olfaction, long-term odorant exposure experiments were performed, showing unspecific changes in ORs expression. We also developed a new method to identify behaviourally active compounds for *S. exigua* larvae. All these results constitute a useful toolbox to be used in further studies aimed to characterize larval olfaction in *S. exigua*. In this context, we used them to study the SeMNPV influence in larval olfaction in order to discover new insights in the baculovirus-lepidopteran interaction.

SeMNPV-induced changes in caterpillar's olfaction

As part of the peripheral nervous system, chemosensory-related genes encode proteins that are located in sensory organs and that interact with chemical external stimuli from the environment. Chemoperception has a crucial importance in the behavioral responses that insects show in concordance with the changing environment that surrounds them (Walker *et al*., 2016). In the present thesis, annotation of the *S.*

exigua chemosensory-related genes repertoire was performed. To complete the description of the parasitic behavior manipulation that baculovirus triggers in their host, we characterized the changes in the chemosensory-related genes expression after baculovirus infection, focusing on SexiORs, which are the central piece of insect's olfaction.

Using RNA-Seq data from larva head samples we carried out differential expression analysis to study the effects of the SeMNPV infection in the ORs expression. Some ORs manifested a strong up-regulation after SeMNPV infection, and this was then confirmed by RT-qPCR comparing their expression in SeMNPV-, AcMNPV- and mock-infected samples. Those transcriptional variations in the ORs expression seemed to be associated with the species-specific SeMNPV infection, since the generalist AcMNPV did not produce the same up-regulation.

Two strongly up-regulated *SexiORs*, *SexiOR35* and *SexiOR23*, were selected for their functional characterization, in order to unveil the ligands that activate them. For that, we used the *Drosophila* empty neuron system to deorphanize them. Ligand identification of both receptors was performed through the SSR technique. SexiOR35 resulted in a very broad tuned receptor, as it was able to recognise many different odorant compounds. It had strong responses to odorants as 1,4-dimethylbenzene, 3-carene, acetophenone, estragole, linalool, citral and p-cymene. SexiOR23 had no significant response to any of the tested odorants and its main ligands remained unknown. This led us to hypothesize that its ligand(s) was(were) not in our odorant panel, as it represents a minute fraction of all odours that a moth might encounter in its daily life (Hansson *et al*., 2010).

To correlate the function of SexiOR35 with the behavioral response of *S. exigua* to specific odorants, two of its main ligands, linalool and estragole, were used in behavioral assays with SeMNPV-, AcMNPV- and mock-infected individuals. These behavioral tests had the aim to show changes in the perception of the odorants related with the baculovirus infection. Linalool, a very common odorant in plants,

produced a deterrent effect in SeMNPV-infected larvae that was not present in AcMNPV- and mock-infected individuals. Linalool in previous studies was shown to enhance SeMNPV pathogenicity when larvae were exposed to it, in a synergistic effect with the virus (Gasmi *et al*., 2019). This observed phenotype could correspond to a defence response of the larvae to avoid that synergistic effect that linalool and SeMNPV infection produce on them.

Estragole, a common odorant in aromatic plants, instead, produced a similar effect in SeMNPV- and AcMNPV-infected larvae. That could be representing an unspecific phenotype produced as a consequence of the baculovirus infection. SexiOR40c was the only up-regulated receptor under both baculovirus infections and could be the associated to this behavior. The ligands that activate SexiOR40c remain unknown.

1-indanone was not actively recognised by SexiOR35, and the only behavioral significant phenotype that produced was an attraction to SeMNPV-infected larvae at one of the tested concentrations. This odorant could be detected by other non-characterised OR, that will be responsible of the observed behavioral effect.

To conclude, SeMNPV infection up-regulates some ORs expression in *S. exigua* larvae. One of them, SexiOR35 was functionally characterised and resulted in a broad tuned receptor. Changes in its expression were connected with behavioral responses of the larvae when exposed to two of SexiOR35 main ligands. Whether the observed effects are a manipulation by baculovirus or a side-effect of the infection remains to be elucidated. This supposes the first description of olfactory changes produced in the host by a baculovirus infection, and demarcates the importance of chemical ecology in parasite-host interactions.

Could the discovered baculovirus-associated changes in the neuronal system of *Spodoptera exigua* increase viral fitness?

The development of this doctoral thesis has unveiled different influences of the SeMNPV infection in the neuronal system of *S. exigua*, including the central and the peripheral nervous system. Our hypothesis is that those changes associated to the baculovirus infection could be representative of an evolutionary advantage to enhance the viral fitness. Focusing on the central nervous system, SeMNPV infection produces a down-regulation of the hypothetical neuropeptide *proctolin-like*. Considering the results obtained during the functional characterization of this gene, its down-regulation may affect the larval digestion processes, inducing the generation of bigger larvae that would produce more virus at the end of the infection cycle, in a similar way that *egt* gene does in the baculovirus-caterpillar interaction. In addition, the down-regulation of *proctolin-like* and its probable participation in the skeletal muscle contractions regulation, would make larvae to enhance their locomotion activity, improving the geographical dispersion of the virus and consequently increasing its infectivity.

In the peripheral nervous system, SeMNPV infection up-regulates the expression of certain odorant receptors, producing changes in the odorant perception of the *S. exigua* larvae. Changes in the odorant receptors expression would have many consequences on the larval behavior. These behavioral phenotypes would be directly or indirectly caused by the virus, avoiding self-medication strategies by the larvae, or making them to feel attracted to plants that would contribute to the viral disease in the larvae. However, these changes in the odorant preferences could also be representative of a larval response against the infection, feeling now attracted to plants that would help the larvae to increase their survival rates in a self-medication strategy. Although, this could also be indicative of repellent responses to specific odour sources that may enhance the viral infection.

The results of this doctoral thesis would require additional research to unveil more baculovirus-associated changes in the gene repertoires of its main hosts and to increase our knowledge about specific mechanisms of gene expression manipulation that the virus could use for that purpose, leading to the discovery of further aspects of the baculovirus-caterpillar interaction and its impact on the insect's ecology. This thesis open avenues to study the insect's physiology regulation, novel methods of understanding the behavioral responses of the larvae, and how the insects communicate and interact with their environment. Our results shed some light in the complex world of the baculovirus-caterpillar interaction with the intention of improving and optimizing the current and future pest control strategies of lepidopteran pests using baculoviruses as biopesticides.

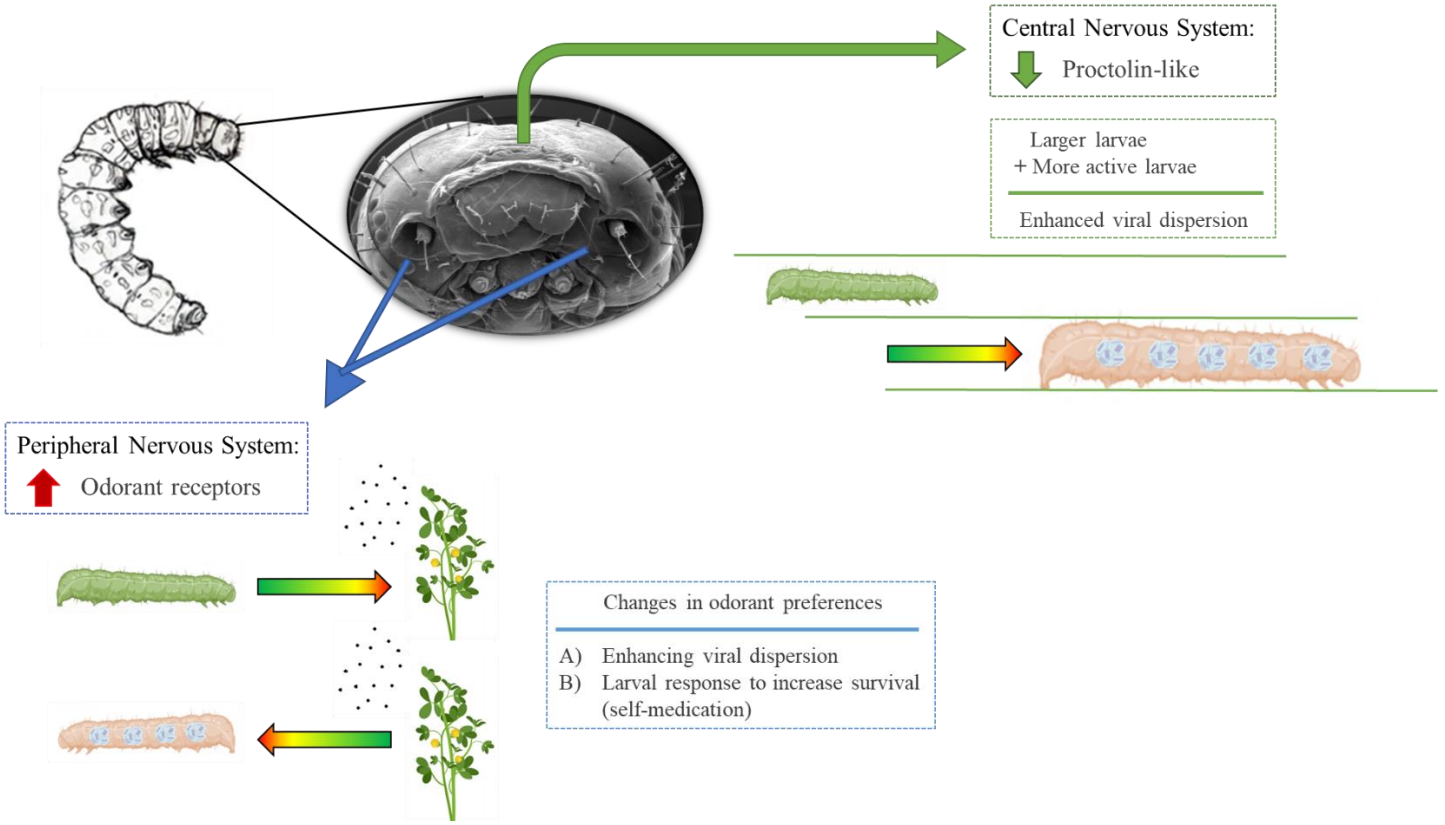
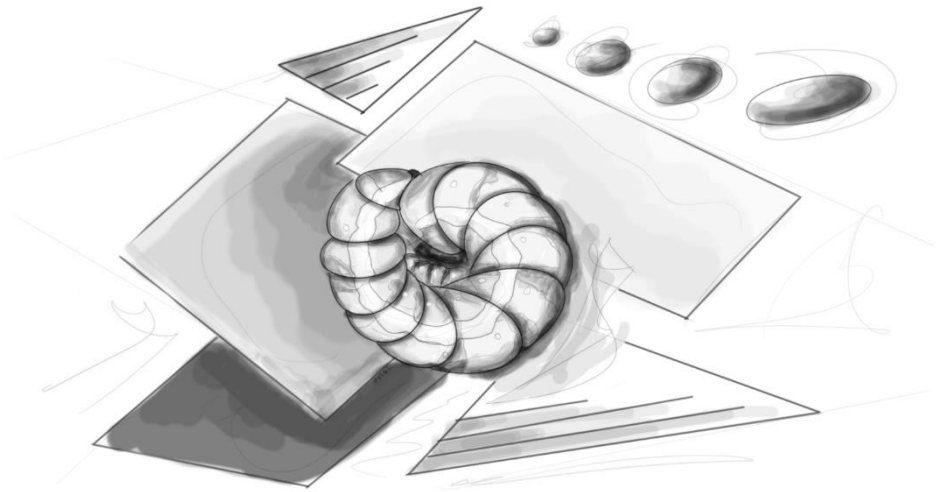


Figure 1. Graphical summary including the results of the present doctoral thesis. Represents the baculovirus-associated changes in the neuronal system of *S. exigua*. In the central nervous system there is a down-regulation after the SeMNPV infection that would make the larvae to become larger and with an increased locomotion activity that would lead to an enhanced viral dispersion. In the peripheral nervous system, the SeMNPV infection would produce changes in the odorant receptors expression, changing the odorant preferences of the host. This could be representative of a viral evolutive strategy to increase the viral dispersion or a larval response to enhance its survival against the baculovirus disease.

CONCLUSIONS



1. The *Spodoptera exigua* neuropeptidome includes at least 63 putative neuropeptide transcripts and the lepidopterans share a core of 43 neuropeptides. Sixteen of these have been identified as brain-gut neuropeptides and six of them are adult-specific.

2. SeMNPV infection induces expression changes in specific transcripts of the *S. exigua* neuropeptidergic system. Concretely, proctolin-like, a newly-described potential neuropeptide resembling the neuropeptide proctolin, is down-regulated upon infection. Gain-of-function bioassays have shown a higher larval mortality making larvae to become smaller and reducing their locomotion activity when proctolin-like is overexpressed. Something similar to what is observed for the neuropeptide proctolin in other insect species. We hypothesized that during the baculovirus-caterpillar interaction, a proctolin-like down-regulation would produce bigger and more active larvae that could help to the viral dispersion in the field.

3. A new annotation of the *S. exigua* chemosensory-related genes based on transcriptomic data has been performed focusing on larval–expressed transcripts. Phylogenetical analysis has allowed to identify 63 ORs, 28 IRs, 38 GRs, 48 OBPs and 23 CSPs that have been renamed following the *Spodoptera frugiperda* nomenclature. Fifty out of the 63 ORs are expressed in larvae.

4. RNA-Seq might not be the most appropriate technique to perform differential expression analyses of low expressed genes and/or in composite organs. RT-qPCR has demonstrated to be a much more sensitive approach to perform these kind of studies.

5. A new assay has been designed to identify ecologically relevant plant volatiles representing a useful system for identifying changes in larval behavior after infection with viral entomopathogens.

6. SeMNPV infection alters the expression of some chemosensory-related genes in *S. exigua*, concretely several ORs are up-regulated upon infection. Two of these, *SexiOR23* and *SexiOR35* have been functionally characterised using the *Drosophila* empty neuron system and single sensillum recording, which have shown that SexiOR35 is a very broad-tuned receptor able to recognise many different odorants.

7. Behavioral assays with two of the main ligands of SexiOR35 show olfactory changes upon baculovirus infection. Linalool produces a deterrent effect on SeMNPV-infected larvae, not observed in AcMNPV- or non-infected larvae. Estragole produces a deterrent effect only in non-infected larvae, loosing this effect after infection with both baculoviruses. These direct or indirect phenotypes could be representative of a larval response against the baculovirus infection or a viral strategy for increasing its field incidence.

1. El neuropeptidoma de *Spodoptera exigua* inclou com a mínim 63 transcrits putatius de neuropèptids i els lepidòpters compartixen un nucli de 43 neuropèptids. Setze d'estos han sigut identificats com a neuropèptids *brain-gut* i sis d'ells són específics d'adults.

2. La infecció per SeMNPV induïx canvis d'expressió en transcrits específics del sistema neuropeptidèrgic de *S. exigua*. Concretament, proctolin-like, un potencial neuropèptid novament descrit que s'assembla al neuropèptid proctolin, apareix infraexpressat després de la infecció. Bioassajos de guany de funció han mostrat una major mortalitat larvària produint larves més xicotetes i amb menys activitat locomotora durant la sobreexpressió de proctolin-like. Cosa similar al que s'observa per al neuropèptid proctolin en altres espècies d'insectes. Podem teoritzar que durant la interacció baculovirus-larva, una infraexpressió de proctolin-like produiria larves més grans i més actives que podrien ajudar a la dispersió viral al medi.

3. Una nova anotació dels gens quimiosensors de *S. exigua* basada en dades transcriptòmiques ha sigut realitzada enfocant-se en els transcrits expressats en larves. Anàlisis filogenètiques han permès identificar 63 ORs, 28 IRs, 38 GRs, 48 OBPs i 23 CSPs que han sigut reanomenats seguint la nomenclatura de *Spodoptera frugiperda*. Cinquanta dels 63 Ors estan expressats en larves.

4. El RNA-Seq podria no ser la tècnica més apropiada per a fer anàlisis d'expressió diferencial amb gens poc expressats i/o en òrgans complexos. La RT-qPCR ha demostrat ser una aproximació molt més sensible per a este tipus d'estudis.

5. S'ha dissenyat un nou bioassaig per a identificar volàtils de plantes ecològicament rellevants. Açò representa un sistema útil per identificar canvis en el comportament de les larves després de la infecció amb entomopatogens virals.

6. La infecció per SeMNPV altera l'expressió d'alguns gens quimiosensors en *S. exigua*, concretament, alguns ORs apareixen sobreexpressats després de la infecció. Dos d'ells, *SexiOR23* i *SexiOR35* han sigut caracteritzats funcionalment emprant l'*empty neuron system* de *Drosophila* i el *single sensillum recording*, que ha mostrat que SexiOR35 és un receptor d'ampli espectre capaç de reconéixer molts odorants diferents.

7. Assajos de comportament amb dos dels principals lligands de SexiOR35 mostren canvis en l'olfacció després de la infecció per baculovirus. El linalool produïx un efecte deterrent en larves infectades per SeMNPV, cosa que no s'observa en larves infectades per AcMNPV o no infectades. L'estragol produïx un efecte deterrent soles en larves no infectades, perdent-se este efecte en larves infectades amb ambdós baculovirus. Estos fenotips directes o indirectes podrien ser representatius de respostes de les larves contra la infecció per baculovirus o una estratègia viral per a incrementar la seua incidència al medi.

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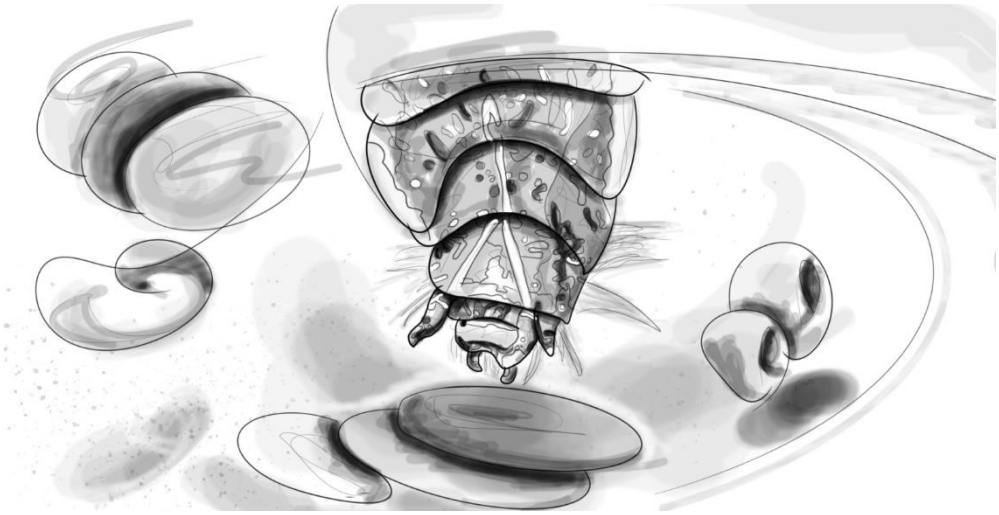

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ANNEXED

Annexed I. Sequence list of the primers for RT-qPCR (Chapter 1).

Unigene	Primer	Primer (5'-3')
CCHamide 1	Forward	AAGTGTTCGCGTTGCTTCTC
	Reverse	AAGGGTCGACGTTTTGCTAC
CCHamide 2	Forward	GGCGCAAATGTTCTTAGCTG
	Reverse	TGATCCATGGCAGAAGTGTC
ILP1	Forward	TTCGTCTGCTGAAGAGCATC
	Reverse	TGGTGATTGTGTTGGTGGTG
ILPAa	Forward	TGGTTCTTGTGGTCCTCGTAG
	Reverse	CCAGCATCACGTTTCTCTTC
ILP1a	Forward	TCTACGTCGTCTTTGCCTTG
	Reverse	GTAGCACAAATCGGCTATGG
ILPB	Forward	TGTGTTTTTCGCTGTTGTGC
	Reverse	ACCAGCTTCGCTTGTTGAG
Allatostatin C	Forward	AGAACACTCTAGTGGCGCATC
	Reverse	AAGTTGCAGCAGCAGTTTGC
Orcokinin 2	Forward	AGTCCATACGGAAGCAAACG
	Reverse	TTCTTCACGAACGTGTCCAG
Short NP F	Forward	GCCGATCGGACAACAATATG
	Reverse	AGCCGTTAGCTTTCACTTCC
DH41	Forward	ATTCACAGACTGGCAGTGGTC
	Reverse	TCGGATTAACTGGGCCTTC
Trissin 2	Forward	ACAGCTTCACGAGCTAATGG
	Reverse	TAAGGTCGGTATTCGCTTGC
Allatotropin	Forward	GACTTTGGCAAAAGGAGTGG
	Reverse	TCCCAGAAGTTGTCCAAACC
AKH1	Forward	CTATTCCTGGCTTGCCTTTG
	Reverse	CAATTGTCTCGTCGCTTCTG
Bursicon Beta	Forward	CGAAAACTACTTGCGGGAAC
	Reverse	TAGCACTTGCAATCGTCAGG
Corazonin	Forward	ATGGTAACCAACACGACCCTAC
	Reverse	ATCCGCGAGAGTATTGGAAG
ETH	Forward	TCCGTTCTATTGGCGTACTG
	Reverse	CATAGATCCTTGGTCGAACG
IMFamide	Forward	TACGATCAACGCAGCAAGAC
	Reverse	TTTCAGGAGATGGGAACCAC
ITP	Forward	TTCACCCTCGAGTGCAAAG
	Reverse	ATAGAGCTGTGGTTCCCTGAAG
ITG-like 1	Forward	ATTGGTGTGCGAGGAAGTTG
	Reverse	TCGCTTGAAGAGTTGCAGTC
PBAN-DH	Forward	AAGGATGGCGGTTCAGATAG
	Reverse	AGGATCGTTTTCCGAGTCTG
PTSH	Forward	GAGGCTGGAATGACATGAGC
	Reverse	GTTGGCCCATTTTTCAGGTC
SIFamide	Forward	TTATCCTGGCCTTGTGCTTC
	Reverse	CTCAACAACTCCCCTTTTGC
Sulfakinin	Forward	GATGTTGGTGCATTGCTACG
	Reverse	TCATCGAAGGCGTCATCAG
NPLP1	Forward	GAAGGACGAAGCGAATGAAG
	Reverse	TTTCAGACAGAGGAGCGATG
FMRFamide	Forward	GCGAGGGACCATTTCATTAG
	Reverse	ATGACACGCTTCCAAAGACC
Gpß5	Forward	GATTTCAGACTGGGCGTTTC
	Reverse	TCTTCAGCGGCAATGTAGTG
NPF1	Forward	TCCGTATGCTGCAAGAACTG
	Reverse	CGTCAGAACGCTTTCCAAAC
Proctolin	Forward	GCTAACAAACCGCCAACAAC
	Reverse	TAATCTGGGCCTTTGTCCTG

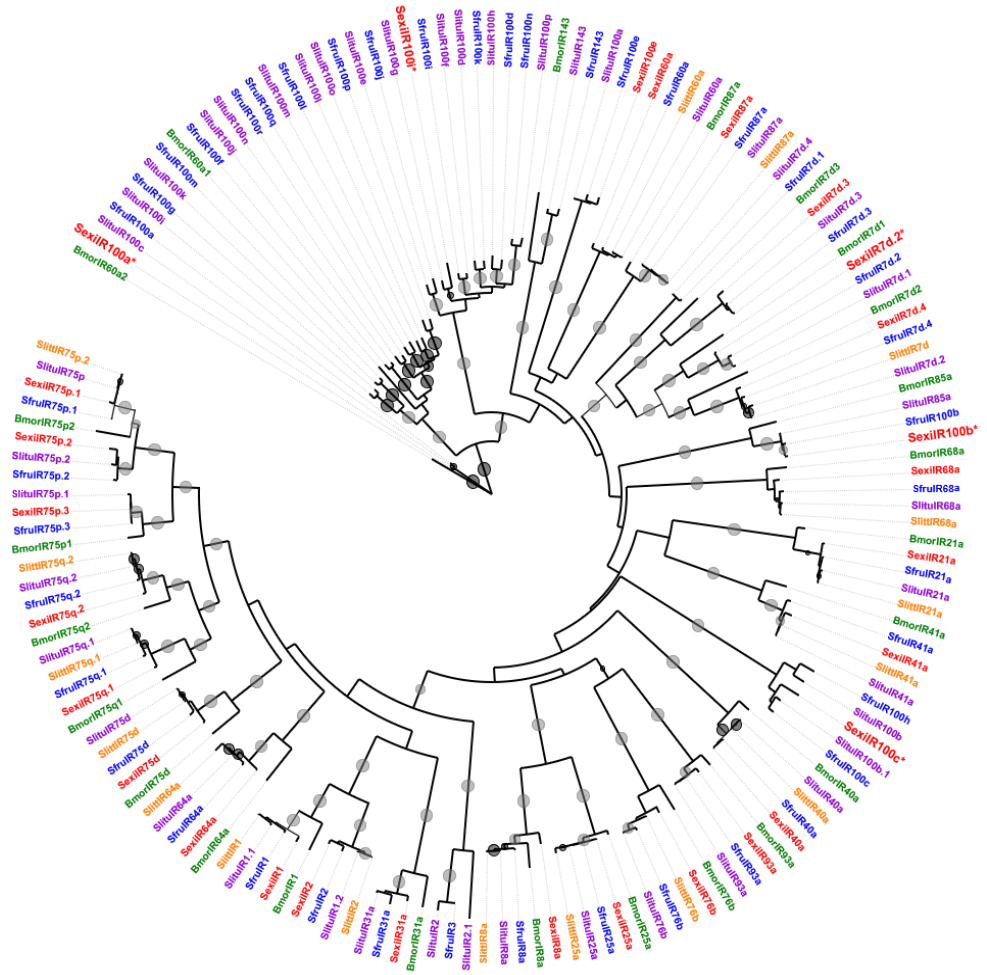
Annexed II. Gene expression changes after light deprivation for the *S. exigua* neuropeptidome unigenes (Chapter 1).

ORF	Fold Changes	SD	t-test (p-value<0.05)	ORF	Fold Changes	SD	t-test (p-value<0.05)
Adipokinetic hormone 1a	0.99	1.56	No	Insulin-like precursor polypeptide 1b	0.86	0.21	No
Adipokinetic hormone 2	0.68	1.86	No	Insulin-like precursor polypeptide 2	4.96	11.35	No
Adipokinetic hormone 3 splicing variant a	1.72	18.00	No	Insulin-like precursor polypeptide Aa	0.83	1.66	No
Allatostatin A	0.95	0.02	No	Insulin-like precursor polypeptide Ab	0.86	0.47	No
Allatostatin C 1	0.91	0.51	No	Insulin-like precursor polypeptide B	0.90	0.94	No
Allatostatin C 2	0.00	17.97	No	Insulin-like precursor polypeptide D	0.92	0.06	No
Allatostatin CC	3.80	6.08	No	Ion transport peptide isoform a	1.06	1.25	No
Allatotropin splicing variant a	0.90	0.11	No	Ion transport peptide isoform b	1.23	0.65	No
Allatotropin splicing variant b	1.46	0.31	No	Ion transport peptide isoform c	1.24	18.00	No
Bursicon subunit alpha	1.07	0.11	No	ITG-like 1	1.09	0.08	No
Bursicon subunit beta	1.02	0.16	No	ITG-like 2	1.09	0.09	No
Capability CAPA	0.90	0.41	No	ITG-like 3	0.00	0.00	No
CCHamide 1 splicing variant a	0.83	2.17	No	Leucokinin	0.93	0.28	No
CCHamide 1 splicing variant b	0.74	1.49	No	Myosuppressin A	0.95	0.79	No
CCHamide 2 splicing variant a	0.79	1.38	No	Myosuppressin B	1.29	4.65	No
CCHamide 2 splicing variant b	1.10	0.29	No	Natalisin 1	0.90	0.15	No
Corazonin	0.81	0.70	No	Natalisin 2	0.99	0.10	No
Crustacean cardioactive peptide	1.27	0.37	No	Neuroparsin	2.04	2.75	No
Diuretic hormone 31	1.30	0.18	No	Neuropeptide F 1 splicing variant a	1.55	1.96	No
Diuretic hormone 34	0.00	0.00	No	Neuropeptide F 1 splicing variant b	0.80	0.13	No
Diuretic hormone 41	0.85	0.35	No	Neuropeptide F 2	0.79	4.05	No
Diuretic hormone 45	0.90	18.00	No	Neuropeptide-like precursor 1 isoform a	1.01	0.12	No
Ecdysisis triggering hormone	1.05	1.42	No	Neuropeptide-like precursor 1 isoform b	1.05	0.12	No
Eclosion hormone	0.00	0.00	No	Neuropeptide-like precursor 1 isoform c	0.80	3.96	No
FMRFamide	0.98	0.05	No	Neuropeptide-like precursor 1b	0.66	0.20	No
Glycoprotein hormone alpha 2	0.74	2.16	No	PBAN-DH	1.17	0.10	No
Glycoprotein hormone beta 5 A	1.01	2.72	No	Proctolin	1.35	0.43	No
Glycoprotein hormone beta 5 B	0.72	3.51	No	Prothoracisotatic hormone	0.00	0.00	No
IMFamide	1.25	0.11	No	RYamide	0.00	0.00	No
Insulin-like peptide 1	0.97	0.20	No	Sulfakinin	0.71	3.57	No
Insulin-like peptide 2	0.87	0.99	No	Tachykinin	0.76	0.42	No
Insulin-like precursor polypeptide 1a	1.14	0.18	No				

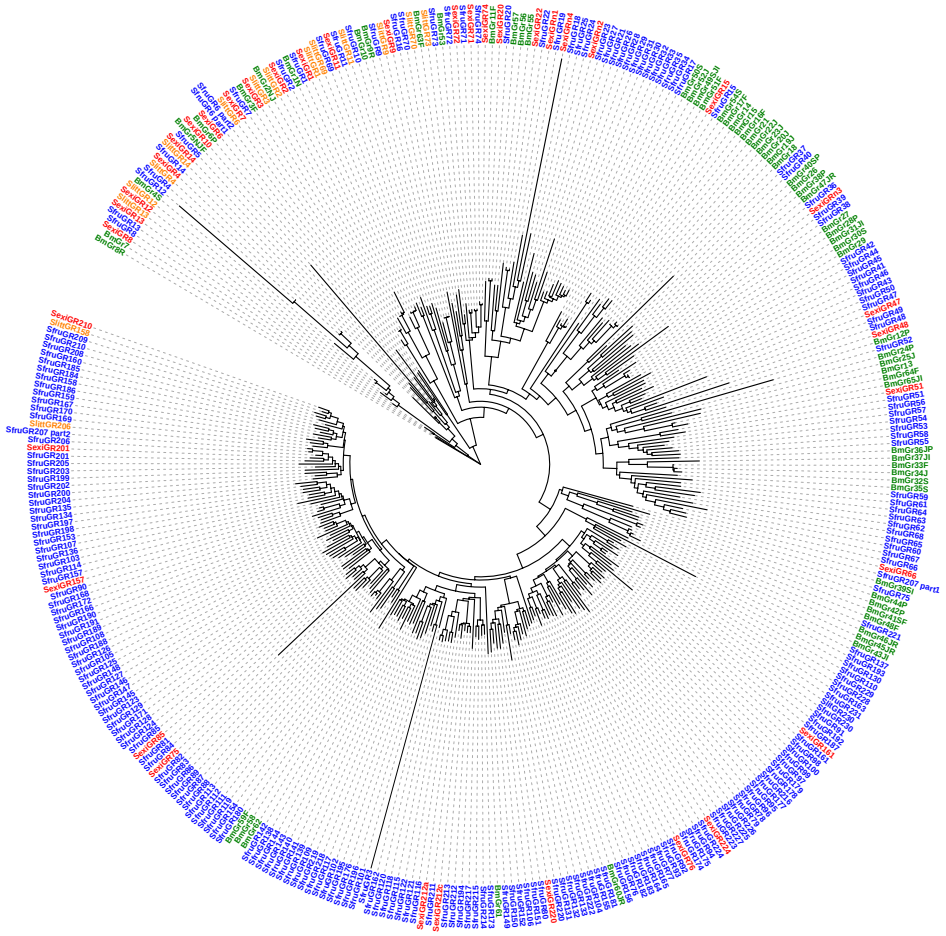
Annexed III. Sequence list of the primers used for RT-qPCR (Chapter 2).

Target	Primer	Primer (5'-3')
AcMNPV-DNApol	Forward	GGGTCAGGCTCCTCTTTGC
	Reverse	TTACGCAGCCATCACAAACAC
AcMNPV-PLP	Forward	AGAACGATGCAAGTTTTCCT
	Reverse	GATGCATAGCATGCGGTACC
Adipokinetic hormone 1	Forward	CTATTCCTGGCTTGCCTTTG
	Reverse	CAATTGTCTCGTCGCTTCTG
Allatostatin C 1	Forward	AGAACACTCTAGTGGCGCATC
	Reverse	AAGTTGCAGCAGCAGTTTGC
Allatotropin	Forward	GACTTTGGCAAAAGGAGTGG
	Reverse	TCCCAGAAGTTGTCCAAACC
ATPsynthase subunit C	Forward	TCCTGCTGTTGTTCGCTTTC
	Reverse	CCACACATTGGATTCCATGGC
Bursicon subunit α	Forward	GCGATACCTTCTTTCGCTTG
	Reverse	TACAGGTCCGTTCCATTTGC
Bursicon subunit β	Forward	CGAAAACTACTTTGGGGAAC
	Reverse	TAGCACTTGCAATGGTCAGG
CCHamide 1	Forward	AAGTGTTCGCGTTGCTTCTC
	Reverse	AAGGGTCGACGTTTTGCTAC
CCHamide 2	Forward	GGCGCAAATGTTCTTAGCTG
	Reverse	TGATCCATGGCAGAAGTGTC
Corazonin	Forward	ATGGTAACCAACACGACCCTAC
	Reverse	ATCGCGCGAGAGTATTGGAAG
Diuretic hormone 41	Forward	ATTCACACAGACTGGCAGTGGTC
	Reverse	TCGGATTAACTGGGCCTTC
Ecdysis triggering hormone	Forward	TCCGTTCTATTGGCGTACTG
	Reverse	CATAGATCCTTGGTCGAACG
FMRFamide	Forward	GCGAGGGACCATTTCATTAG
	Reverse	ATGACACGCTTCCAAAGACC
Insuline-like peptide 1	Forward	TTCGTCTCGCTGAAGAGCATC
	Reverse	TGGTGATTGTGTTGGTGGTG
Insuline-like peptide 2	Forward	CTCGCTGATGAAGACGATTCC
	Reverse	CAAGCTCGTACATAGGCTTGC
Insuline-like precursor polypeptide Aa	Forward	TGGTTCTTGTGGTCCTCGTAG
	Reverse	CCAGGATCACGTTTCTCTTC
Insuline-like precursor polypeptide B	Forward	TGTGTTTTTCGCTGTTGTGC
	Reverse	ACCAGCTTCGCTTGTTGAG
ITG-like 1	Forward	ATTGGTGTGCGAGGAAGTTG
	Reverse	TCGCTTGAAGAGTTGCAGTC
ITG-like 2	Forward	TCACCTTACCGCCATTATCC
	Reverse	GATGCGGTTACCTTCCAAAG
Ion transport peptide	Forward	TTCACCCTCGAGTGCAAAG
	Reverse	ATAGAGCTGTGGTTCCCTGAAG
Leucokinin	Forward	CCGCAGTACATCAACAATGG
	Reverse	TTGAGAGGGCAAGTTGAAG
Neuropeptide F 1	Forward	TCCGTATGCTGCAAGAACTG
	Reverse	CGTCAGAAACGCTTTCCAAAC
Neuropeptide-like precursor 1a	Forward	GAAGGACGAAGCGAATGAAG
	Reverse	TTTCAGACAGAGGAGCGATG
Orcokinin	Forward	AGTCCATACGGAAGCAAACG
	Reverse	TTCTTCACGAACGTGTCCAG
PBAN-DH	Forward	AAGGATGGCGGTTCAGATAG
	Reverse	AGGATCGTTTTCCGAGTCTG
Prothoracicostatic peptide 1	Forward	GAGGCTGGAATGACATGAGC
	Reverse	GTTGGCCCATTTTTCAGGTC
Se -PLP	Forward	GCTAACAAACCGCCAACAAC
	Reverse	TAATCTGGCCTTTGTCCTG
Short neuropeptide F	Forward	GCCGATCGGACAACAATATG
	Reverse	AGCCGTTAGCTTTCACTTCC
SIFamide	Forward	TTATCCTGGCCTTGTGCTTC
	Reverse	CTCAACAACTCCCCTTTTGC
Sulfakinin	Forward	GATGTTGGTGCATTGCTACG
	Reverse	TCATCGAAGGCGTCATCAG
Trissin 2	Forward	ACAGCTTCACGAGCTAATGG
	Reverse	TAAGGTCGGTATTCGCTTGC

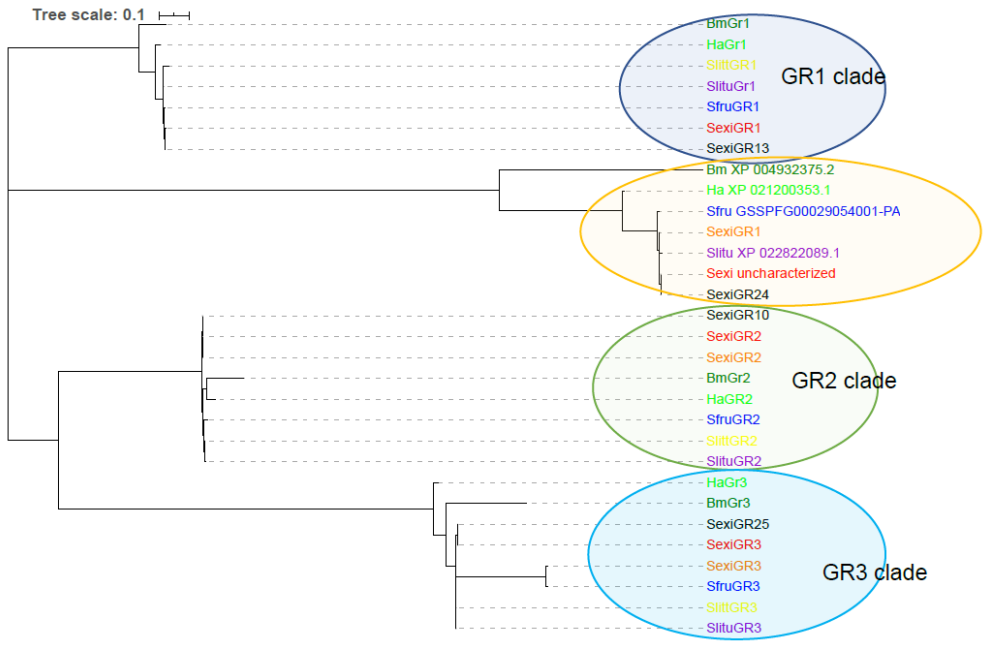
Annexed IV. Phylogenetic tree of *Spodoptera exigua* ionotropic receptors (SexiIRs) (Chapter 3). Maximum-likelihood (ML) tree built with protein sequences annotated from *S. frugiperda* (Gouin *et al*., 2017), *S. litura* (Zhu *et al*., 2018) and *B. mori* genomes (van Schooten *et al*., 2016) as well as putative proteins annotated from *S. littoralis* transcriptome (Walker *et al*., 2019). SexiIRs are shown in red, *S. frugiperda* IRs in blue, *S. litura* IRs in purple, *S. littoralis* IRs in yellow, and *B. mori* IRs in green. Grey dots show a bootstrap value higher than 80.



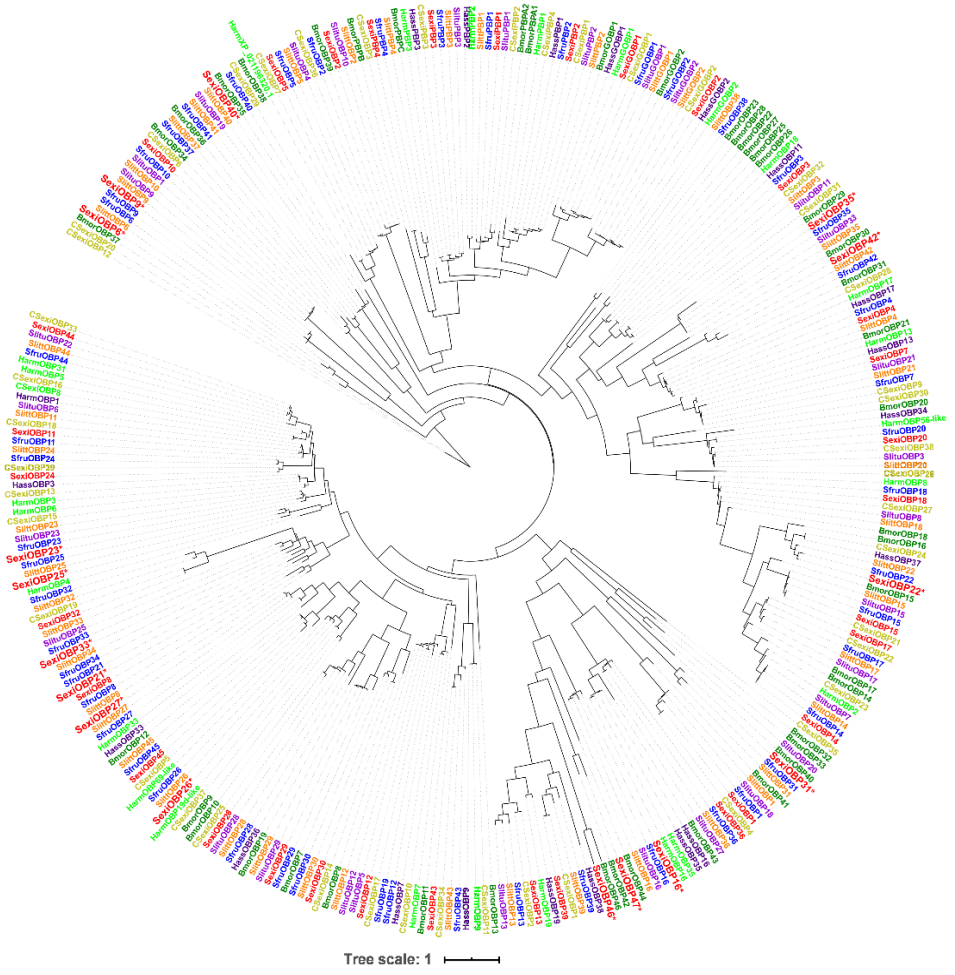
Annexed V. Phylogenetic tree of *Spodoptera exigua* gustatory receptors (SexiGRs) (Chapter 3). Maximum-likelihood (ML) tree built with protein sequences annotated from *S. frugiperda* (Gouin *et al*., 2017) and *B. mori* genomes (Wanner and Robertson, 2008) as well as putative proteins annotated from *S. littoralis* transcriptome (Walker *et al*., 2019). SexiGRs are shown in red, *S. frugiperda* GRs in blue, *S. littoralis* GRs in yellow, and *B. mori* GRs in green. Grey dots show a bootstrap value higher than 80.



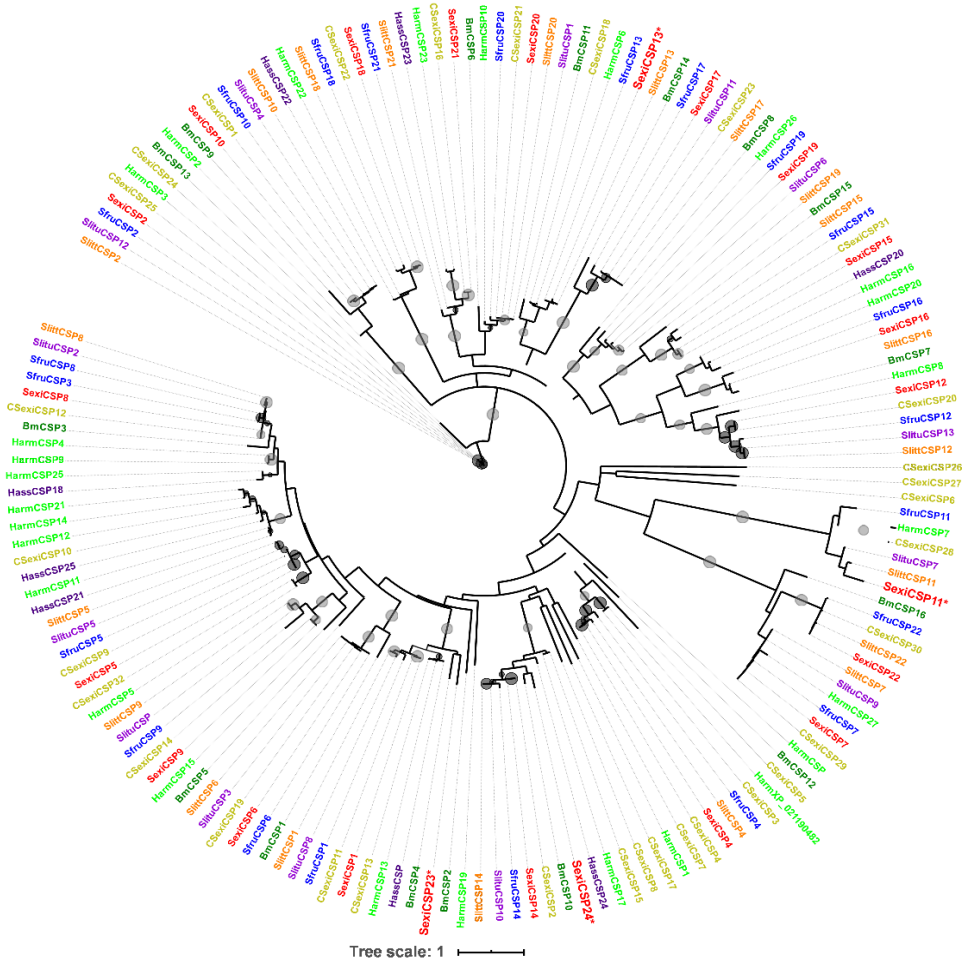
Annexed VI. Phylogenetic tree of Lepidoptera CO2 receptors (Chapter 3). Maximum-likelihood (ML) tree built with protein sequences of Lepidoptera CO2 receptors. *S. exigua* sequences described in this study are shown in red, *S. exigua* GRs annotated by Zhang *et al* (2018) are shown in black, *S. exigua* GRs annotated by Du *et al* (2018) are shown in orange, *S. littoralis* sequences are in yellow (Walker *et al*., 2019), *S. frugiperda* sequences in blue (Gouin *et al*., 2017); *S. litura* genes in purple (Cheng *et al*., 2017), *B. mori* sequences in dark green (Wanner and Robertson, 2008), *H. armigera* genes in light green (Ning *et al*., 2016). Candidate CO2 receptors clearly cluster in three clades whereas sequence mis-annotated by Du *et al*. (2018) as CO2 receptor GR1 (and by Zhang *et al*., 2018 as GR24) clusters with other Lepidopteran sequences.



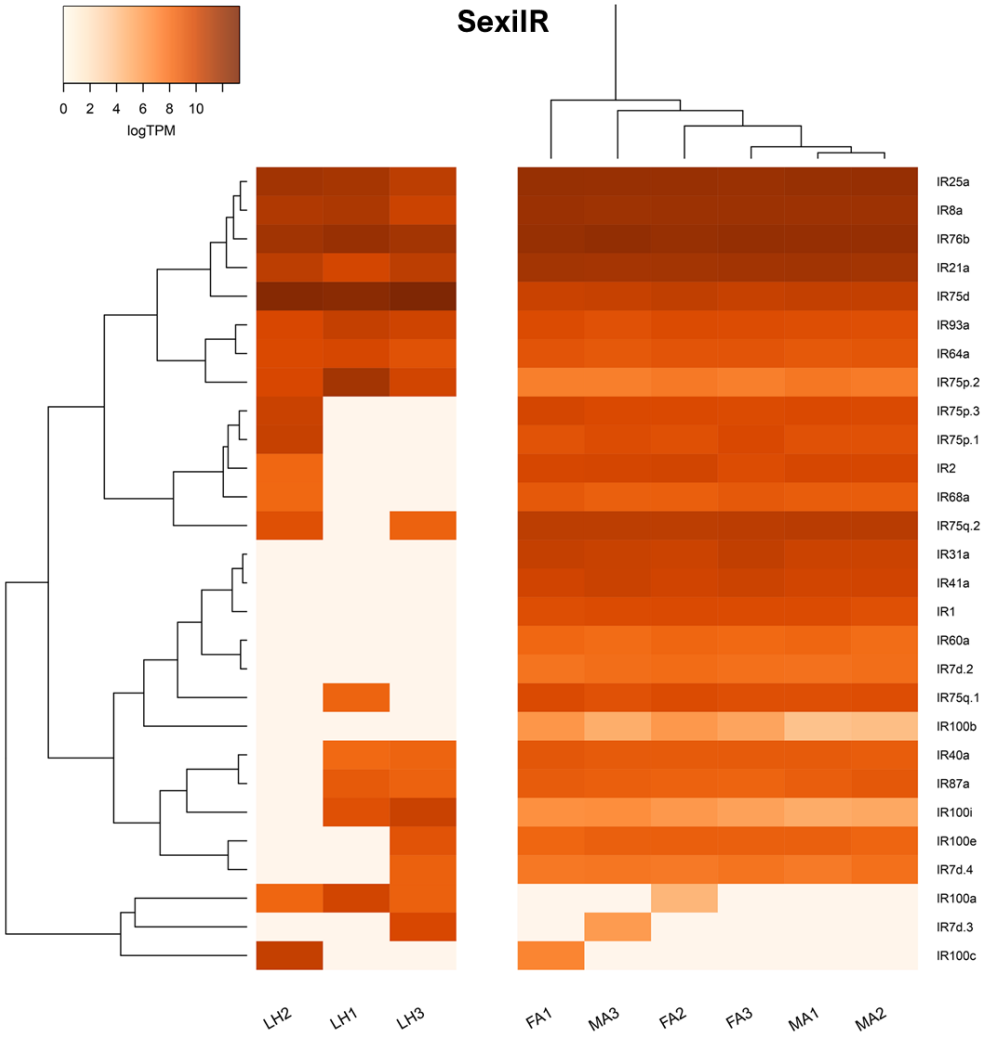
Annexed VII. Phylogenetic tree of *S. exigua* OBP proteins (Chapter 3). The *S. exigua* translated genes are shown in red; the *S. frugiperda* translated genes are shown in blue; the *S. littoralis* translated genes are shown in yellow; the *S. litura* translated genes are shown in light purple, the *B. mori* translated genes are shown in dark green, the *H. armigera* translated genes are shown in light green, the *H. assulta* translated genes are shown in dark purple and the *S. exigua* sequences obtained by Du *et al*. (2018) are shown in ocher (CSexiOBPs). This tree was constructed using RAxML based on alignment results of MEGA-X.



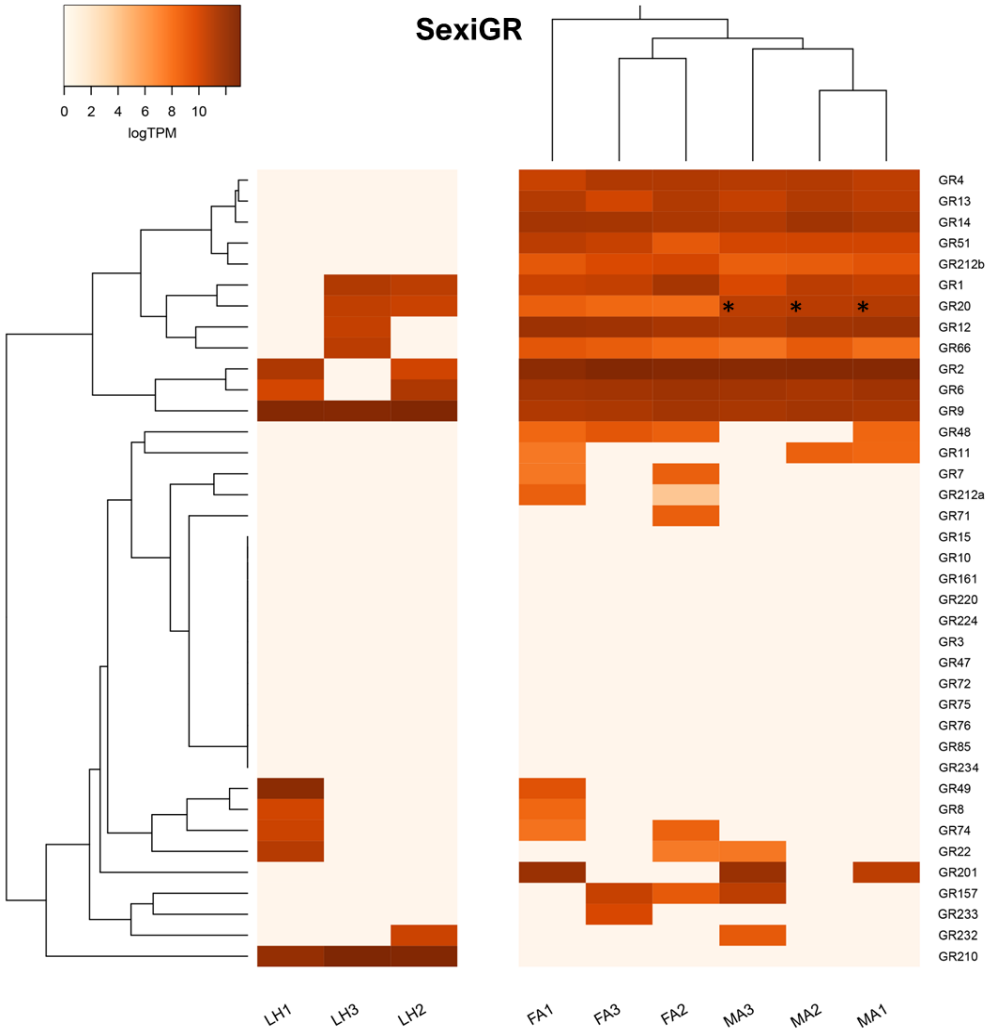
Annexed VIII. Phylogenetic tree of *S. exigua* CSP proteins (Chapter 3). The *S. exigua* translated genes are shown in red; the *S. frugiperda* translated genes are shown in blue; the *S. littoralis* translated genes are shown in yellow; the *S. litura* translated genes are shown in light purple, the *B. mori* translated genes are shown in dark green, the *H. armigera* translated genes are shown in light green, the *H. assulta* translated genes are shown in dark purple and the *S. exigua* sequences obtained by Du *et al*. (2018) are shown in ocher (CSexiCSPs). This tree was constructed using RAxML based on alignment results of MEGA-X.



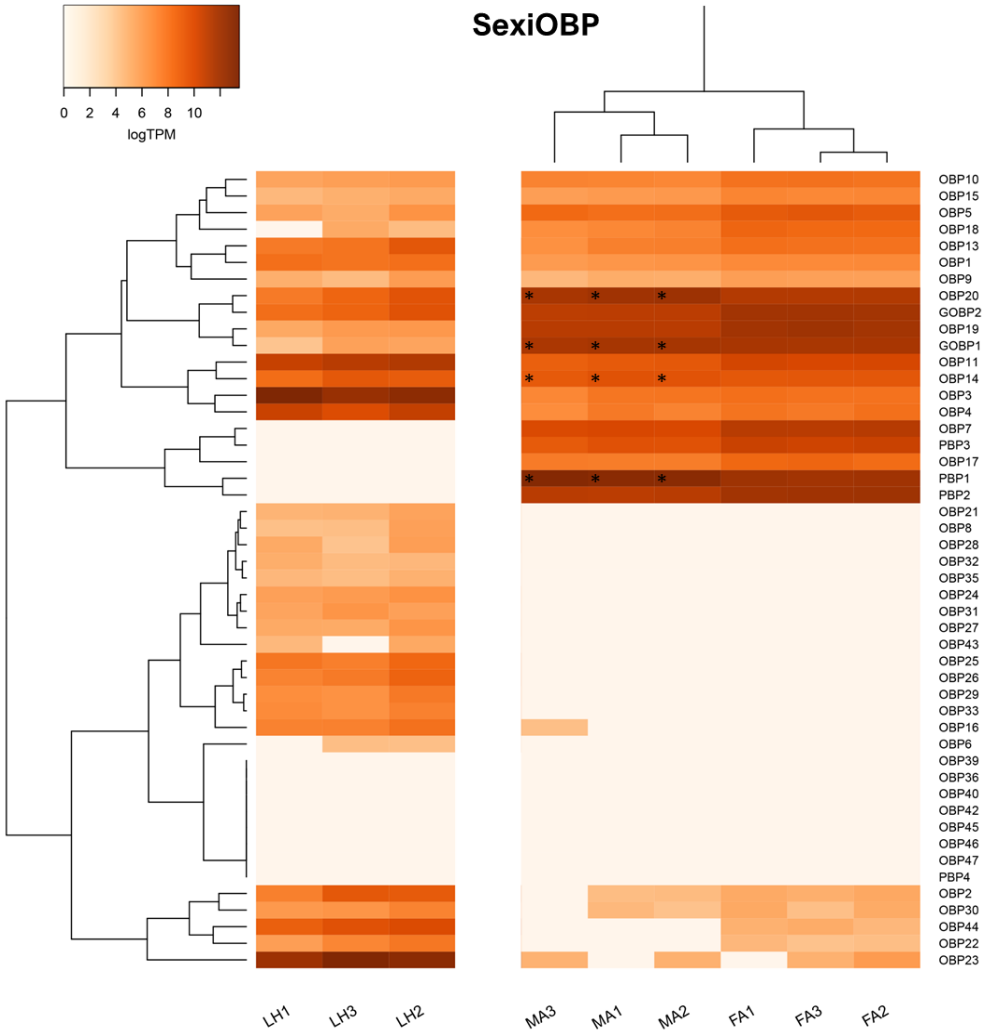
Annexed IX. Heat-plot of relative expression values of ionotropic receptors (SexiIRs) in the head of *Spodoptera exigua* larvae and adult antennae (Chapter 3). Colour plots represent Log2 of transcripts per million (TPM) values estimated by RSEM. Light orange colours indicate low expression and dark orange ones indicate high expression. LH: Larvae Head. MA: Male Antennae. FA: Female Antennae.



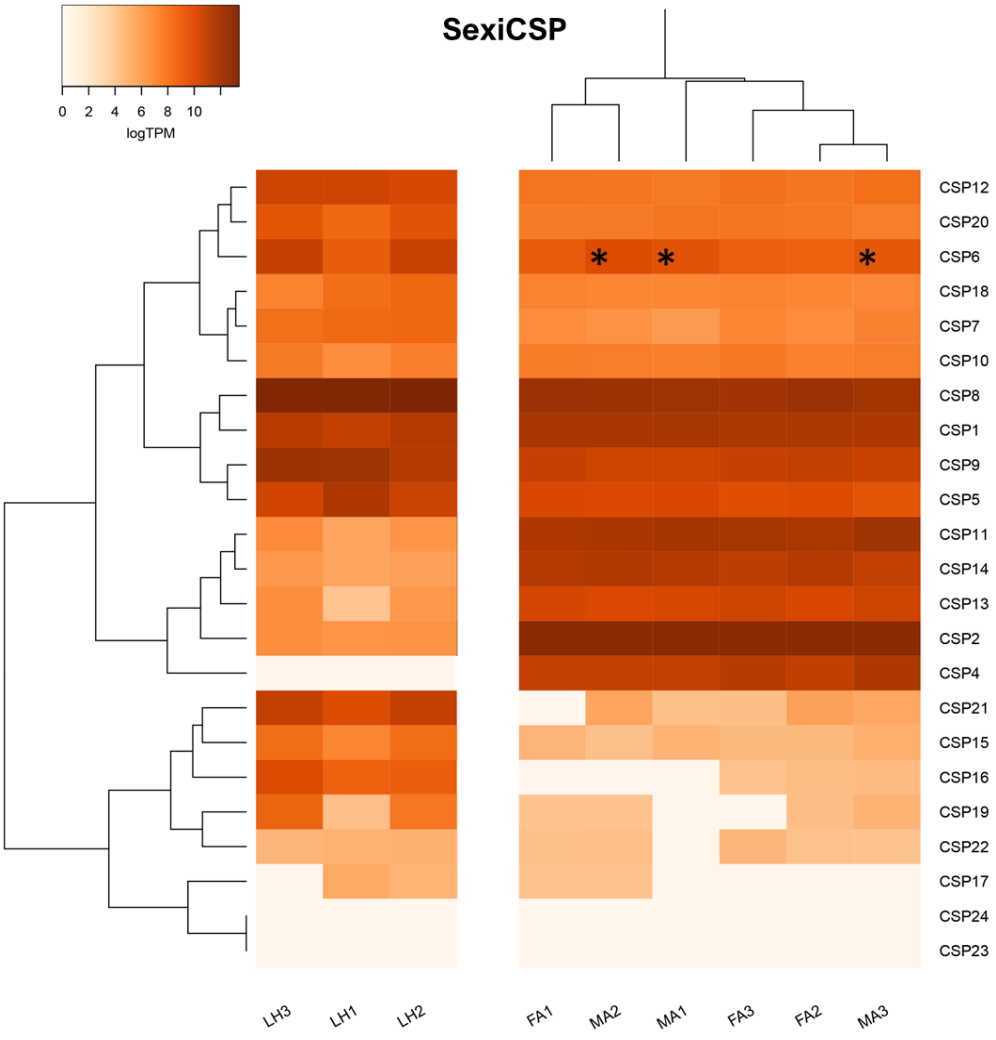
Annexed X. Heat-plot of relative expression values of gustatory receptors (SexiGRs) in the head of *Spodoptera exigua* larvae and adult antennae (Chapter 3). Colour plots represent log2 of transcripts per million (TPM) values estimated by RSEM. Light orange colours indicate low expression and dark orange ones indicate high expression. LH: Larvae Head. MA: Male Antennae. FA: Female Antennae. Asterisks indicate statistically significant differences between male and female antenna samples identified by EdgeR analysis (FDR<0.05).



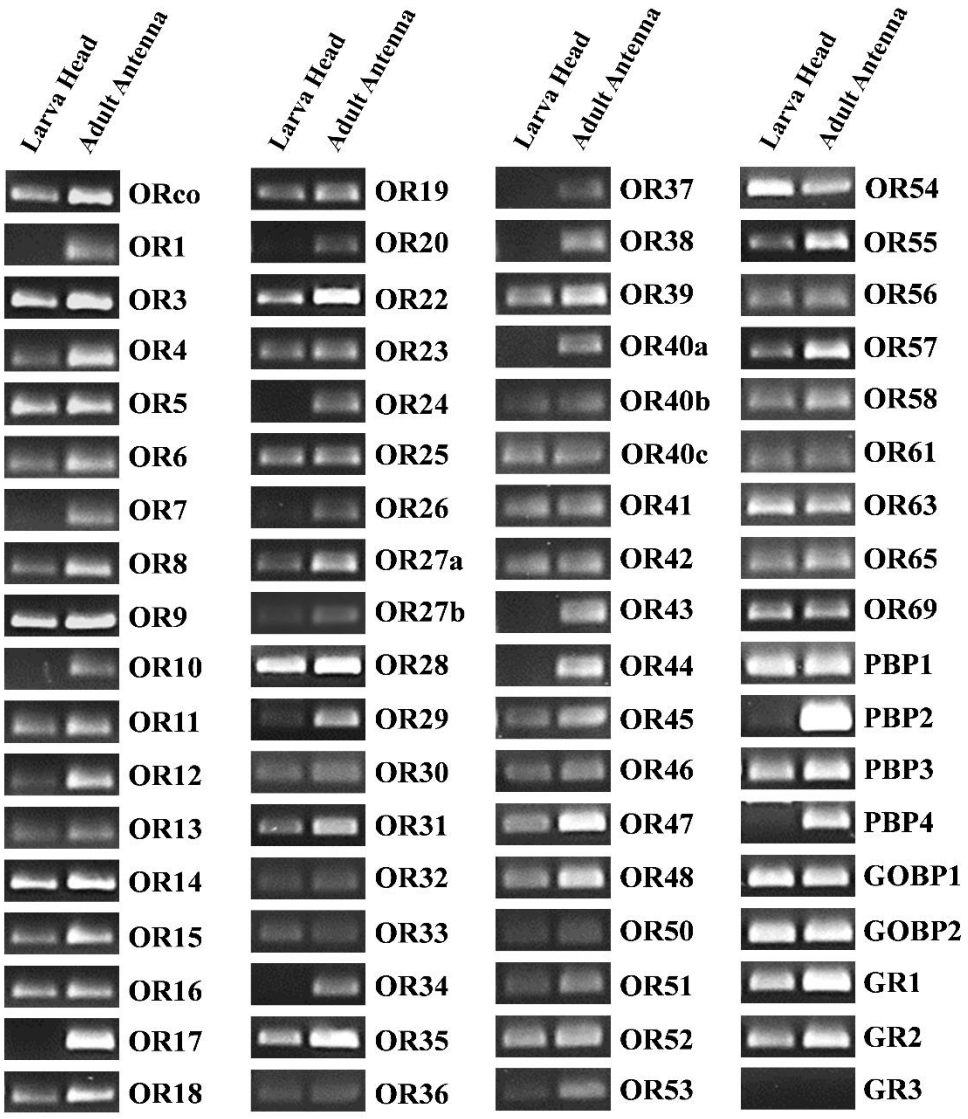
Annexed XI. Heat-plot of relative expression values of odorant binding proteins (SexiOBPs) in the head of *Spodoptera exigua* larvae and adult antennae (Chapter 3). Colour plots represent log2 of transcripts per million (TPM) values estimated by RSEM. Light orange colours indicate low expression and dark orange ones indicate high expression. LH: Larvae Head. MA: Male Antennae. FA: Female Antennae. Asterisks indicate statistically significant differences between male and female antenna samples identified by EdgeR analysis (FDR<0.05).



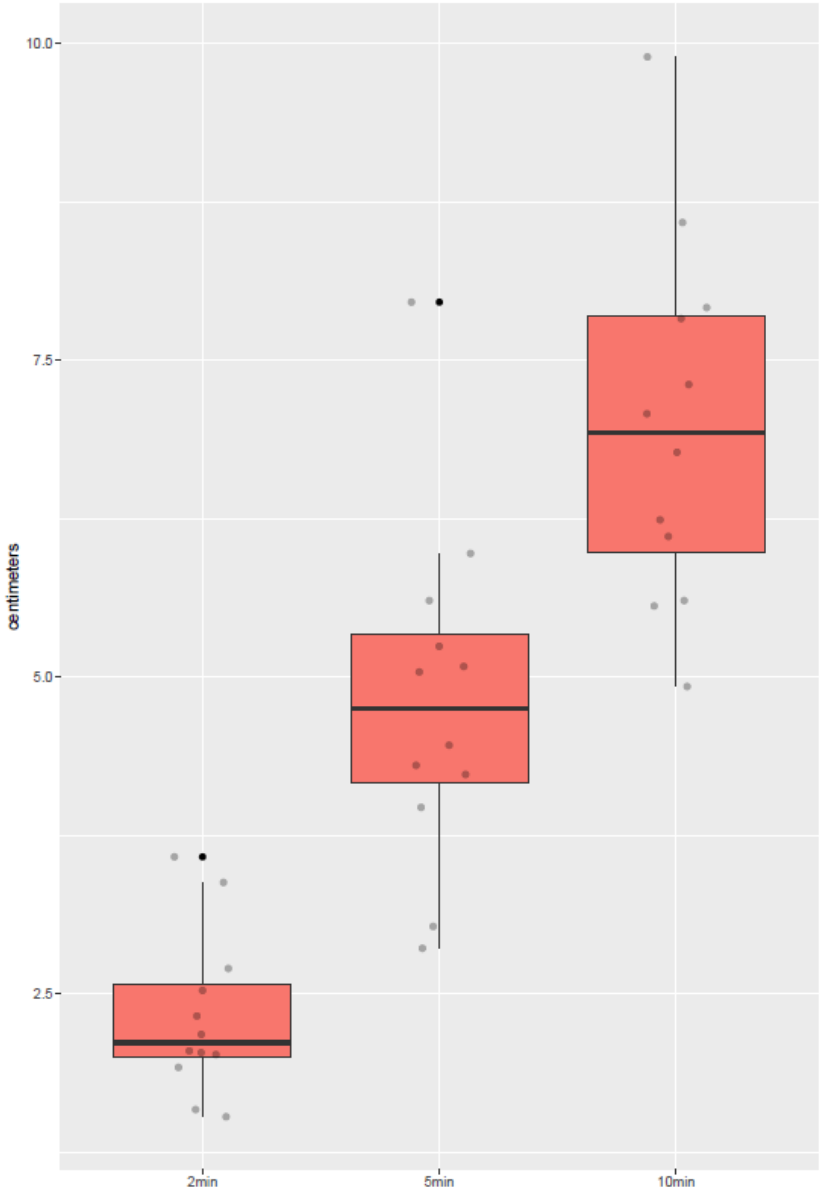
Annexed XII. Heat-plot of relative expression values of chemosensory proteins (SexiCSPs) in the head of *Spodoptera exigua* and adult antennae (Chapter 3). Colour plots represent log2 of transcripts per million (TPM) values estimated by RSEM. Light orange colours indicate low expression and dark orange ones indicate high expression. LH: Larvae Head. MA: Male Antennae. FA: Female Antennae. Asterisks indicate statistically significant differences between male and female antenna samples identified by EdgeR analysis (FDR<0.05).



Annexed XIII. Expression profiles of selected chemosensory-related genes (Chapter 3). RT-PCR assays were performed using gene specific primer pairs and cDNAs from different *Spodoptera exigua* tissues: larva head and adult antenna. PCR products were analysed on 2% agarose gels.



Annexed XIV. Behavioral response of larvae in the control run with diet + solvent only (Chapter 3). Results are shown in centimetres of larval displacement at 2 min, 5 min and 10 min time-points.



Annexed XV. Sequence of primers used for RT-qPCR (Chapter 3).

Unigene	Primer	Primer (5'-3')	Unigene	Primer	Primer (5'-3')	Unigene	Primer	Primer (5'-3')
ATPsynthase subunit C	Forward	TCCTCTCTGTTCTTCGCTTTC	SexiOR26	Forward	TCACATCCATAGCCAAGCAG	SexiOR47	Forward	CATTCTCGCATGCCTGCTATC
	Reverse	CCACACATTCGATTCCATTGCC		Reverse	TGTACAACGGGTCATTCAGC		Reverse	CGCAGACAGATTGCAATGAAC
SexiORCO	Forward	GAAAGCCTGGTACCCATTTCA	SexiOR27a	Forward	GCTTTTAGTTTGGCGTCAGC	SexiOR48	Forward	GAATGGTACAACGGCTGCCTAC
	Reverse	CCACGCAAGCACTAAGTTCC		Reverse	ATGCCCATCCAAGCTATCTG		Reverse	CCATGCAAATGTACCGAGTG
SexiOR1	Forward	GTATGATGACGCAAACGATGG	SexiOR27b	Forward	TCTCATAATGTCGGGAACG	SexiOR50	Forward	CCCACAGCAACTTTCCTTG
	Reverse	CGAAGCTCTCGAACATTTCC		Reverse	TGGTCACGCGAGTTGTGAAG		Reverse	TAATATCCGTCGGTCCTTC
SexiOR3	Forward	ACGCTGGTTACCTCTGCTGT	SexiOR28	Forward	GTGGAACGAGGTGGCATAC	SexiOR51	Forward	ATTCACGAAGCTGACTGTCG
	Reverse	TAACGTTGAGTCGCTTCGCTG		Reverse	AATCCGTGTCGTCACGGTA		Reverse	TTTGAGATTGGCTCAGCAGTG
SexiOR4	Forward	TGGGCACAGCAATATACCAG	SexiOR29	Forward	GTGTTCGCTCATGCTTTGTG	SexiOR52	Forward	AAACTACGGGCAGATCGCTTG
	Reverse	AACCACCAGCTGTCATTTCC		Reverse	TTGCTGTGCCAACAGAGAC		Reverse	AGCCGACCAGCACATTAAAC
SexiOR5	Forward	TACATGTGGTTGGGAGATGC	SexiOR30	Forward	AGGATCCCTTTGGGCATTTAC	SexiOR53	Forward	TGGCATAGCAACGAAGTGAC
	Reverse	TAGGGCGTCCAAGCATAAAG		Reverse	TTAGGATCCCGTAGGATTGG		Reverse	TGCTCTTCCACCAATCACTG
SexiOR6	Forward	TGTGTTAAGGGCATCACTGC	SexiOR31	Forward	GAGTCGATATTTGGCGAAGC	SexiOR54	Forward	GTTCATCGCTTTGTGTGTGC
	Reverse	AGAGGCGATGATACGGCAAAG		Reverse	AACTGGGCTAACATGCAACC		Reverse	AAAGTTGGCGACAAGGTAGG
SexiOR7	Forward	ATTGGCCAGTTCGCTACTTC	SexiOR32	Forward	ATTTGTCTGCTGTGGTGTG	SexiOR55	Forward	ATCTTCTGGCAGCCATCATC
	Reverse	ATAATCAGCGGCACGCCATAG		Reverse	TCATGGCGAAACACAGATCC		Reverse	TACGGAATCCGCACTCCAAAC
SexiOR8	Forward	TGCCTGCAGAAATGGGTTAC	SexiOR33	Forward	GCCTTACTGGCAAATGATCG	SexiOR56	Forward	TTGGCCTCAGAAAGAACTGG
	Reverse	TCCATAAGACGAGCCAAG		Reverse	CCACATAAAGAACCCAACG		Reverse	AAATTGGCACGCACTAGCAG
SexiOR9	Forward	TGGCGCACGTTATTTTCATTG	SexiOR34	Forward	TGCTCTCTGTCCTCATTTCTG	SexiOR57	Forward	GCTTGGCACTCGTATAATGG
	Reverse	CCTCAGTGCTTGGTGTGAGA		Reverse	TGATCACGCATCGAAGGTCTG		Reverse	TCCGCCAGTAATGGAAAGAG
SexiOR10	Forward	CTTGTACGTTGTCGGCAATG	SexiOR35	Forward	GCTGGGCAAGTATTTGAACG	SexiOR58	Forward	TGGCATACCGTCACATTCTG
	Reverse	TCAATGGGTTACTGGCATC		Reverse	TCTGCAGCATCAGCAATACC		Reverse	ATTCGCCACAACACTGTTACC
SexiOR11	Forward	ATGCTCTCAATAGCGGCTTG	SexiOR36	Forward	GCAGGTTTCCATGCAGCTGTT	SexiOR61	Forward	ATGCGGGTGTTCCTTTACTG
	Reverse	AGTTATCGAAGACGCCGAAG		Reverse	GTCATGGTCTTCTTGGGCATT		Reverse	ACTGCAGTCGGCTACAGATTCAC
SexiOR12	Forward	TGGTTGAACATGGACCTGAG	SexiOR37	Forward	TGATGACCAGCATCAAGGAG	SexiOR63	Forward	ATATTTGCCGCACTCCTG
	Reverse	AAACTCAGGGTGATGCAAGG		Reverse	CACGCAATCATCTTGCTTCTC		Reverse	GCGTAGAAGTGTCCCAGCTC
SexiOR13	Forward	TTTCCTTGATACCGGGTACG	SexiOR38	Forward	AAGCTTGCCGAAACCTAACC	SexiOR65	Forward	TCACAAACGTGGCTTCTCTG
	Reverse	ATGACCCAACTCGAAACACG		Reverse	TCCAGAATGAGAGCATGCTG		Reverse	GGGCATCTGATAATCCTTCG
SexiOR14	Forward	ATTCCTAGGTCTGGGCTGGT	SexiOR39	Forward	TTGGCGCTGTTACAAGACTG	SexiOR69	Forward	TGGGCACAGTAAAGACATGG
	Reverse	GGGTGAGGTTATGGGTGATG		Reverse	AAGCAACTTCACTGGTGCTC		Reverse	TTATCGCATCCGTCTTCTCC
SexiOR15	Forward	ACGCAAGCGAAGGTCTAAATC	SexiOR40a	Forward	GTGATTATGCGCAGCGTTTG	SexiPBP1	Forward	ATGACGAGGTTGCCAAGAAG
	Reverse	TGGTCGGGATATTCGTTCTC		Reverse	TCATACAGCACGGTTTCTGC		Reverse	TGAGCTCGTGAATCTTGGTG
SexiOR16	Forward	ATGGTCTACCATGGGAATGC	SexiOR40b	Forward	TCATGCTGTCGCTATTAGG	SexiPBP2	Forward	CGTCGCAAGATGTCATGAAG
	Reverse	ACACCCACGCGCCTTTATATG		Reverse	TGTAGCAGCTTTCTGGCTTG		Reverse	AGCCTTCCTTCCAGAAGTTG
SexiOR17	Forward	CTGCGTCGTCTGTGATAGTGT	SexiOR40c	Forward	CGCTATCCGGACAATTCTTG	SexiPBP3	Forward	ACGAGCGTATGATGATTGAG
	Reverse	CGATCGCCACTAAATTCGTT		Reverse	TCACTTTGACATCGCTCTCG		Reverse	TCACAATGACCTCGACCTTG
SexiOR18	Forward	GGAGATCAAAAGACGCATCC	SexiOR41	Forward	CGTTCAAATCTGAGCCAGTG	SexiPBP4	Forward	CGCTCTGGTCTGTGTTTTTG
	Reverse	TCTCTTCAGGCAGTTTTCC		Reverse	AATCTGGCTTCGGGTTATC		Reverse	TTGCACATCTCGAACAGGTC
SexiOR19	Forward	ACGCCTCAATATACGGCAAC	SexiOR42	Forward	TTCACAACTGACCACGGAAG	SexiOBP1	Forward	GCGTGATGACTTCAAGTTCG
	Reverse	ACGCACCACCAGTATAAAAGG		Reverse	ACAACACGTCGTTGCTATGC		Reverse	TAAACTGCTCGGTGTTGTCG
SexiOR20	Forward	TTGGTCGAGAGGCAAACAG	SexiOR43	Forward	ATCAACGCTTCCCAAGACC	SexiOBP2	Forward	GAGTGCAGGGAAGAGTCAGG
	Reverse	CGTTACGTAGCAAAGCGAAG		Reverse	CGTTGAGAACAGCGAAGATG		Reverse	AGGGAAGCGCTTGACGTAGT
SexiOR22	Forward	ATCGGCGTACACACAACAA	SexiOR44	Forward	GCCGAAAAACATCAGCTACC	SexiGR1	Forward	CCGAAACATCGCACGACAAC
	Reverse	TCTCGTCGTGTTTTCATCCA		Reverse	AGGAATGCTGCAGATTGAGAG		Reverse	ATTCGCTAAAGCGCTCTCTGC
SexiOR23	Forward	ATTGCTGTGGGAATGGTCTC	SexiOR45	Forward	TCTCACCGGTAATGTTCGTG	SexiGR2	Forward	AGGGACGTGGAACAAGAATG
	Reverse	TACTGCCTCCAACTTCTTCG		Reverse	TAGCGATGTCGTCGTGAATC		Reverse	TAAGCGTTGCCAAGTGACTG
SexiOR24	Forward	TATCCACGGTGAACGTCATC	SexiOR46	Forward	ATCTCCCTCTTCGTGAATGC	SexiGR3	Forward	CCCTGTTTAAAAGCCTGCTG
	Reverse	CCAAAGCGTTCGGCTAGTTTC		Reverse	CAAAATTCTCCCACCCACAG		Reverse	GCGCATCTTCTTCATCATCC
SexiOR25	Forward	TTGAAGGAGTGCCGCAGAAAC						
	Reverse	TGACCCACGCAGTGCTTTATG						

Annexed XIX. Comparison of the candidate OBP genes annotated in this study with previous annotations from different Spodoptera exigua transcriptomes (Chapter 3).

			S. exigua		*S. frugiperda*				*S. exigua*		*S. frugiperda*
Gene	**Size (aa)**	**Complete**	**Du *et al.*, 2018**	**Zhang *et al.*, 2018**	**Gouin *et al.*, 2017**	**Gene**	**Size (aa)**	**Complete**	**Du *et al.*, 2018**	**Zhang *et al.*, 2018**	**Gouin *et al.*, 2017**
SexiORCO	474	Yes	Orco	Orco	ORco	SexiOR33	408	No	OR24	OR35	OR33
SexiOR1	427	No	OR2	OR24	OR1	SexiOR34	410	No	OR23	OR55	OR34
SexiOR3	413	No	OR15	OR3	OR3	SexiOR35	409	Yes	OR20	OR33	OR35
SexiOR4	387	Yes	OR45	OR51	OR4	SexiOR36	419	Yes	OR9	OR21	OR36
SexiOR5	398	Yes	OR48	OR49	OR5	SexiOR37	403	Yes	OR26	OR30	OR37
SexiOR6	433	Yes	OR6	OR6	OR6	SexiOR38	391	Yes	OR49	OR46	OR38
SexiOR7	386	No	OR22	OR26	OR7	SexiOR39	419	Yes	-	OR12,OR63	OR39
SexiOR8	247	No	-	OR42	OR8	SexiOR40a	379	No	-	OR45	OR40
SexiOR9	397	No	OR28	OR9	OR9	SexiOR40b	412	Yes	OR50	OR34	-
SexiOR10	392	Yes	OR43	OR5,OR41	OR10	SexiOR40c	409	Yes	OR40	OR17	-
SexiOR11	436	Yes	OR11	OR11	OR11	SexiOR41	394	Yes	OR19	OR14	OR41
SexiOR12	408	Yes	OR21	OR48	OR12	SexiOR42	402	Yes	OR29	OR32	OR42
SexiOR13	435	Yes	OR13	OR13	OR13	SexiOR43	380	Yes	OR47	OR47	OR43
SexiOR14	443	Yes	OR3	OR7	OR14	SexiOR44	393	Yes	-	OR52	OR44
SexiOR15	390	Yes	OR44	OR19	OR15	SexiOR45	394	Yes	OR42	OR58	OR45
SexiOR16	433	Yes	OR16	OR16	OR16	SexiOR46	387	Yes	-	-	OR46
SexiOR17	392	No	OR38	OR27	OR17	SexiOR47	399	Yes	OR33	OR15,OR38	OR47
SexiOR18	399	Yes	OR34	OR62	OR18	SexiOR48	412	Yes	OR18	OR39	OR48
SexiOR19	402	Yes	OR30	OR28	OR19	SexiOR50	397	Yes	OR37	OR57	OR50
SexiOR20	401	No	OR41	OR43	OR20	SexiOR51	403	Yes	OR27	OR10	OR51
SexiOR22	423	No	OR4	OR61	OR22	SexiOR52	388	Yes	OR35	OR54	OR52
SexiOR23	423	Yes	OR8	OR50	OR23	SexiOR53	405	Yes	OR25	OR1	OR53
SexiOR24	299	No	OR10	OR44	OR24	SexiOR54	146	No	-	-	OR54
SexiOR25	416	Yes	OR12	OR2	OR25	SexiOR55	415	Yes	OR14	OR23	OR55
SexiOR26	351	Yes	OR39	OR60	OR26	SexiOR56	433	Yes	-	-	OR56
SexiOR27a	430	Yes	OR5	OR36	OR27	SexiOR57	397	Yes	OR17	OR53	OR57
SexiOR27b	404	Yes	-	-	OR27	SexiOR58	312	No	OR7	OR37	OR58
SexiOR28	454	Yes	OR1	OR31	OR28	SexiOR61	104	No	-	OR18	OR61
SexiOR29	363	No	OR36	OR29	OR29	SexiOR63	318	No	-	OR25	OR63
SexiOR30	396	Yes	OR32	OR20	OR30	SexiOR65	370	Yes	-	OR40	OR65
SexiOR31	401	No	OR31	OR4	OR31	SexiOR69	381	No	-	-	OR69
SexiOR32	386	Yes	OR46	OR22	OR32						

Annexed XVII. Comparison of the IR genes annotated in this study with previous annotations from different *Spodoptera exigua* transcriptomes of (Chapter 3).

			S. exigua		*S. frugiperda*
Gene	**Size (aa)**	**Complete**	**Du *et al*., 2018**	**Zhang *et al*., 2018**	**Gouin *et al*., 2017**
SexiIR1	365	No	IR1	IR6	IR1
SexiIR2	654	Yes	IR1.1	IR5	IR2
SexiIR7d.2	600	Yes	-	-	IR7d.2
SexiIR7d.3	220	No	-	IR8	IR7d.3
SexiIR7d.4	595	Yes	IR7d2	IR18	IR7d.4
SexiIR8a	733	No	IR8a	IR3	IR8a
SexiIR21a	551	No	IR21a	IR10	IR21a
SexiIR25a	919	Yes	IR25a	IR15	IR25a
SexiIR31a	611	Yes	-	IR16	IR31a
SexiIR40a	713	Yes	IR40a	IR22	IR40a
SexiIR41a	566	No	IR41a	IR9	IR41a
SexiIR60a	662	Yes	IR60a	IR12	IR60a
SexiIR64a	602	Yes	IR64a	IR14	IR64a
SexiIR68a	687	Yes	IR68a	IR1	IR68a
SexiIR75d	554	No	IR75d	IR20	IR75d
SexiIR75p.1	344	No	IR75p	IR13	IR75p.1
SexiIR75p.2	434	No	IR75p.2	-	IR75p.2
SexiIR75p.3	224	No	IR75p.1	IR19	IR75p.3
SexiIR75q.1	562	No	IR75q.1	IR17	IR75q.1
SexiIR75q.2	631	Yes	IR75q.2	IR11	IR75q.2
SexiIR76b	611	Yes	IR76b	IR2	IR76b
SexiIR87a	643	Yes	IR87a	IR7	IR87a
SexiIR93a	902	Yes	IR93a	IR4	IR93a
SexiIR100a	619	Yes	-	-	IR100a
SexiIR100b	612	No	-	-	IR100b
SexiIR100c	141	No	-	-	IR100c
SexiIR100e	351	No	-	IR21	IR100e
SexiIR100i	258	No	-	-	IR100i

Annexed XVIII. Comparison of the candidate GR genes annotated in the study with previous annotations from different *Spodoptera exigua* transcriptome (Chapter 3).

			S. exigua		*S. frugiperda*
Gene	**Size (aa)**	**Complete**	**Du *et al*., 2018**	**Zhang *et al*., 2018**	**Gouin *et al*., 2017**
SexiGR1	465	Yes	-	GR13	GR1
SexiGR2	434	Yes	GR2	GR10	GR2
SexiGR3	475	Yes	GR3	GR25	GR3
SexiGR4	403	Yes	-	GR16,GR17	GR4
SexiGR6	452	Yes	GR6,GR7	GR12	GR6
SexiGR7	265	No	-	GR8	GR7
SexiGR8	434	Yes	-	-	GR8
SexiGR9	465	No	GR4	GR29	GR9
SexiGR10	182	No	-	-	GR10
SexiGR11	267	No	-	-	GR11
SexiGR12	430	Yes	-	GR30	GR12
SexiGR13	428	No	-	GR4	GR13
SexiGR14	477	Yes	GR5	GR27	GR14
SexiGR15	93	No	-	GR19	GR15
SexiGR20	376	No	-	GR7	GR20
SexiGR22	389	Yes	-	-	GR22
SexiGR47	196	No	-	-	GR47
SexiGR48	260	No	-	-	GR48
SexiGR49	141	No	-	GR2	GR49
SexiGR51	208	No	-	-	GR51
SexiGR66	333	No	-	-	GR66
SexiGR71	168	No	-	GR26	GR71
SexiGR72	136	No	-	-	GR72
SexiGR74	377	No	-	GR28	GR74
SexiGR75	77	No	-	-	GR75
SexiGR76	52	No	-	-	GR76
SexiGR85	236	No	-	-	GR85
SexiGR157	146	No	-	-	GR157
SexiGR161	145	No	-	-	GR161
SexiGR201	94	No	-	-	GR201
SexiGR210	254	No	-	-	GR210
SexiGR212a	369	No	-	-	GR212
SexiGR212b	405	Yes	-	GR5, GR9	GR212
SexiGR220	115	No	-	-	GR220
SexiGR224	88	No	-	-	GR224
SexiGR232	384	Yes	-	-	-
SexiGR233	167	No	-	-	-
SexiGR234	63	No	-	-	-

Annexed XIX. Comparison of the candidate OBP genes annotated in the study with previous annotations from different *Spodoptera exigua* transcriptome (Chapter 3).

			S. exigua		*S. frugiperda*
Gene	**Size (aa)**	**Complete**	**Du *et al*., 2018**	**Zhang *et al*., 2018**	**Gouin *et al*., 2017**
SexiGOBP1	146	Yes	GOBP1	GOBP1	GOBP1
SexiGOBP2	163	Yes	GOBP2	GOBP2	GOBP2
SexiOBP1	187	Yes	OBP4	OBP18	OBP1
SexiOBP2	185	Yes	OBP36	OBP24	OBP2
SexiOBP3	129	No	OBP32	OBP2	OBP3
SexiOBP4	134	Yes	-	OBP9	OBP4
SexiOBP5	158	Yes	-	OBP7	OBP5
SexiOBP6	106	No	-	-	OBP6
SexiOBP7	139	No	OBP9	OBP8	OBP7
SexiOBP8	146	Yes	-	OBP-N2	OBP8
SexiOBP9	340	Yes	-	-	OBP9
SexiOBP10	245	Yes	OBP6	OBP11	OBP10
SexiOBP11	129	No	OBP18	OBP1	OBP11
SexiOBP13	217	Yes	OBP2	-	OBP13
SexiOBP14	143	Yes	-	OBP7.2	OBP14
SexiOBP15	146	Yes	OBP21	OBP12	OBP15
SexiOBP16	168	Yes	-	-	OBP16
SexiOBP17	142	No	OBP22	-	OBP17
SexiOBP18	140	Yes	OBP27	OBP5	OBP18
SexiOBP19	148	Yes	OBP17	ABP	OBP19
SexiOBP20	138	Yes	OBP38	OBP-N1	OBP20
SexiOBP21	154	Yes	-	-	OBP21
SexiOBP22	141	Yes	-	-	OBP22
SexiOBP23	146	Yes	-	-	OBP23
SexiOBP24	97	No	OBP39	OBP27	OBP24
SexiOBP25	148	Yes	-	-	OBP25
SexiOBP26	155	Yes	-	-	OBP26
SexiOBP27	154	Yes	-	-	OBP27
SexiOBP28	131	No	OBP25	-	OBP28
SexiOBP29	146	Yes	-	OBP4	OBP29
SexiOBP30	148	Yes	OBP14	-	OBP30
SexiOBP31	646	No	-	-	OBP31
SexiOBP32	148	Yes	OBP19	-	OBP32
SexiOBP33	152	Yes	-	-	OBP33
SexiOBP35	157	Yes	-	-	OBP35
SexiOBP36	149	Yes	-	OBP17	OBP36
SexiOBP39	186	No	OBP1	-	OBP39
SexiOBP40	145	No	-	-	OBP40
SexiOBP42	108	No	-	-	OBP42
SexiOBP43	126	No	OBP34	-	OBP43
SexiOBP44	150	No	OBP33	-	OBP44
SexiOBP45	157	Yes	OBP5	-	OBP45
SexiOBP46	137	No	-	-	-
SexiOBP47	179	No	-	-	-
SexiPBP1	165	Yes	PBP2	PBP1	PBP1
SexiPBP2	171	Yes	PBP1	PBP2	PBP2
SexiPBP3	165	Yes	PBP3	PBP3	PBP3
SexiPBP4	212	Yes	OBP3	-	PBP4

Annexed XX. Comparison of the candidate CSP genes annotated in this study with previous annotations from different *Spodoptera exigua* transcriptomes (Chapter 3).

			S. exigua		*S. frugiperda*
Gene	**Size (aa)**	**Complete**	**Du *et al*., 2018**	**Zhang *et al*., 2018**	**Gouin *et al*., 2017**
SexiCSP1	129	Yes	CSP11	CSP7	CSP1
SexiCSP2	121	Yes	CSP25	-	CSP2
SexiCSP4	152	Yes	CSP4	CSP-N1	CSP4
SexiCSP5	129	Yes	CSP9	CSP1	CSP5
SexiCSP6	124	Yes	CSP19	CSP-N2	CSP6
SexiCSP7	108	Yes	CSP29	CSP20	CSP7
SexiCSP8	129	Yes	CSP12	CSP2	CSP8
SexiCSP9	127	Yes	CSP14	CSP3	CSP9
SexiCSP10	288	Yes	CSP1	CSP14	CSP10
SexiCSP11	112	Yes	-	-	CSP11
SexiCSP12	124	Yes	CSP20	CSP4	CSP12
SexiCSP13	123	Yes	-	-	CSP13
SexiCSP14	128	Yes	CSP2	CSP6	CSP14
SexiCSP15	132	Yes	CSP31	CSP5	CSP15
SexiCSP16	125	Yes	-	CSP19	CSP16
SexiCSP17	123	Yes	CSP23	CSP10	CSP17
SexiCSP18	123	Yes	CSP22	-	CSP18
SexiCSP19	124	Yes	-	CSP13, CSP-N4	CSP19
SexiCSP20	123	Yes	CSP21	CSP11	CSP20
SexiCSP21	126	Yes	CSP16	CSP12	CSP21
SexiCSP22	88	No	CSP30	CSP8	CSP22
SexiCSP23	89	No	-	-	-
SexiCSP24	129	Yes	-	-	-

Annexed XXI. Blastx best hit against nr database of misannotated chemosensory proteins (CSPs) and odorant binding proteins (OBPs) from Du *et al*. (2018) (Chapter 3).

Sequence	Best blastp hit against nr database	Organism	Accession number	% identity	% query cover
CSP3	ejaculatory bulb-specific protein 3-like	*Helicoverpa armigera*	XP_021190482.1	99	100
CSP5	chemosensory protein 8	*Monochamus alternatus*	A1X97040.1	59	92
CSP6	ejaculatory bulb-specific protein 3-like	*Contarinia nasturtii*	XP_031618432.1	41	96
CSP7	chemosensory protein	*Cylas formicarius*	QFO46789.1	58	97
CSP8	ejaculatory bulb-specific protein 3	*Bactrocera dorsalis*	XP_011202324.1	54	92
CSP10	;jaculatory bulb-specific protein 3-like [Helicoverpa armigera	*Helicoverpa armigera*	XP_021190466.1	99	100
CSP13	chemosensory protein	*Helicoverpa assulta*	ABB91378.1	98	100
CSP15	chemosensory protein	*Helicoverpa armigera*	A1W65099.1	100	100
CSP17	PREDICTED: ejaculatory bulb-specific protein 3	*Drosophila busckii*	XP_017836987.1	54	94
CSP18	hypothetical protein B5X24_HaOG200668	*Helicoverpa armigera*	PZC77872.1	97	99
CSP24	hypothetical protein B5V51_14025	*Heliothis virescens*	PCG73971.1	95	100
CSP26	putative odorant-binding protein A10	*Melanaphis sacchari*	XP_025208687.1	89	100
CSP27	chemosensory protein 9	*Pieris rapae*	QDW65475.1	34	98
CSP28	CSP7	*Helicoverpa armigera*	AEX07268.1	93	100
CSP32	CSP5	*Helicoverpa armigera*	AEB54579.1	99	100
OBP7	uncharacterized protein LOC110380618	*Helicoverpa armigera*	XP_021196320.1	99	100
OBP8	hypothetical protein B5X24_HaOG200804	*Helicoverpa armigera*	PZC74746.1	96	96
OBP10	odorant-binding protein 7	*Helicoverpa assulta*	AGA16511.1	97	100
OBP11	odorant-binding protein 9	*Helicoverpa assulta*	ACG92789.1	99	99
OBP12	odorant-binding protein 2	*Cryptolaemus montrouzieri*	ALW95359.1	80	100
OBP13	odorant-binding protein 3	*Helicoverpa assulta*	ACG92788.1	100	100
OBP15	odorant-binding protein, partial	*Helicoverpa assulta*	AEX07270.1	99	100
OBP16	odorant-binding protein, partial	*Helicoverpa assulta*	AEX07271.1	99	100
OBP20	odorant-binding protein 2	*Cryptolaemus montrouzieri*	ALW95359.1	64	98
OBP23	OBP2	*Helicoverpa armigera*	AEB54586.1	93	100
OBP24	odorant-binding protein 37	*Helicoverpa assulta*	ASA40075.1	100	100
OBP26	odorant binding protein 33, partial	*Holotrichia parallela*	AVM18959.1	85	97
OBP28	general odorant-binding protein 56a-like	*Helicoverpa armigera*	XP_021187261.1	99	100
OBP29	Odorant binding protein	*Operophtera brumata*	KOB70699.1	32	68
OBP30	odorant binding protein 37, partial	*Holotrichia parallela*	AVM18963.1	81	91
OBP31	odorant-binding protein 6	*Dastarcus helophoroides*	A1X97052.1	50	96
OBP35	PREDICTED: general odorant-binding protein 69a-like	*Papilio machaon*	XP_014362389.1	31	60
OBP37	general odorant-binding protein 19d-like	*Helicoverpa armigera*	XP_021194665.1	96	100
PBP4	pheromone binding protein	*Helicoverpa assulta*	AAW65077.1	100	100

Annexed XXII. Differential expression analysis of chemosensory-related transcripts between male and female (Chapter 3). Transcripts were considered differentially expressed (DE) at false discovery rate (FDR) threshold <0.05 and 2-fold change cut-off. In green, transcripts up-regulated in males. In red, transcripts up-regulated in females.

Transcript _id	logFC	logCPM	LR	PValue	FDR
Sexi_OR6	-9.842	10.143	609.206	0.000	0.000
Sexi_OR13	-8.170	7.060	316.003	0.000	0.000
Sexi_PBP1	-2.817	18.587	162.028	0.000	0.000
Sexi_OR16	-2.918	9.208	153.020	0.000	0.000
Sexi_OBP20	-2.112	16.553	149.986	0.000	0.000
Sexi_OR56	-5.429	5.400	132.349	0.000	0.000
Sexi_OR48	4.431	5.866	88.893	0.000	0.000
Sexi_OBP14	-1.371	13.166	68.816	0.000	0.000
Sexi_GOBP1	-1.267	16.542	57.059	0.000	0.000
Sexi_OR18	1.560	7.610	41.489	0.000	0.000
Sexi_GR20_i	-3.668	3.051	27.035	0.000	0.000
Sexi_OBP18	0.888	10.626	26.087	0.000	0.000
Sexi_CSP6	-1.125	10.255	19.298	0.000	0.000
Sexi_OBP3_i	-0.681	10.301	10.869	0.001	0.012
Sexi_OR53	1.193	5.412	9.293	0.002	0.024
Sexi_IR25a	-0.501	11.568	9.186	0.002	0.024
Sexi_OR50	1.090	6.117	9.144	0.002	0.024
Sexi_OR38	1.280	4.324	8.390	0.004	0.035
Sexi_OR22_i	0.753	7.307	8.178	0.004	0.037
Sexi_OR30	1.343	4.927	7.771	0.005	0.043
Sexi_ORco	-0.472	12.585	7.729	0.005	0.043
Sexi_IR75q,2	-0.513	9.422	7.314	0.007	0.052
Sexi_OBP16	-1.562	5.287	7.218	0.007	0.052
Sexi_OR34_i	1.081	5.027	6.859	0.009	0.061
Sexi_IR87a	-0.701	6.485	6.675	0.010	0.065
Sexi_OBP7_i	0.430	14.400	6.581	0.010	0.066
Sexi_OR41	0.615	7.566	6.502	0.011	0.066
Sexi_IR21a_i	-0.390	10.337	5.593	0.018	0.107
Sexi_IR75d_i	-0.475	8.711	5.263	0.022	0.121
Sexi_CSP11	-0.386	13.336	5.258	0.022	0.121
Sexi_OBP39_i	1.157	4.010	5.199	0.023	0.121
Sexi_OR5	-1.130	4.802	4.802	0.028	0.148
Sexi_OR17_i	-0.508	7.208	4.543	0.033	0.164
Sexi_OR63_i	-3.108	0.952	4.486	0.034	0.164
Sexi_IR76b	-0.430	11.274	4.415	0.036	0.164
Sexi_IR2	-0.495	8.193	4.402	0.036	0.164
Sexi_OBP11_i	0.341	13.081	4.376	0.036	0.164
Sexi_OBP32	-3.347	1.788	4.162	0.041	0.173
Sexi_OR40b	0.806	4.442	4.147	0.042	0.173
Sexi_OBP8	-3.182	0.942	4.130	0.042	0.173
Sexi_PBP3	0.349	14.330	4.108	0.043	0.173
Sexi_IR41a_i	-0.445	8.094	3.218	0.073	0.288
Sexi_OBP44_i	0.594	5.644	2.976	0.084	0.320
Sexi_OBP31_i	-1.252	4.002	2.970	0.085	0.320
Sexi_GR74_i	3.512	0.668	2.862	0.091	0.333
Sexi_PBP4	0.772	3.868	2.806	0.094	0.333
Sexi_IR8a_i	-0.264	11.180	2.770	0.096	0.333
Sexi_OBP1	-0.322	9.589	2.766	0.096	0.333
Sexi_IR100b_i	1.532	2.029	2.679	0.102	0.344
Sexi_OR10	0.467	6.100	2.612	0.106	0.347
Sexi_OR57	0.392	7.492	2.603	0.107	0.347
Sexi_CSP9	0.278	12.235	2.554	0.110	0.350
Sexi_OR69_i	1.912	1.425	2.521	0.112	0.350
Sexi_OR58_i	0.615	5.133	2.502	0.114	0.350
Sexi_OR43	0.534	5.791	2.429	0.119	0.359
Sexi_OR46	-0.618	4.413	2.300	0.129	0.384

Transcript _id	logFC	logCPM	LR	PValue	FDR	Transcript _id	logFC	logCPM	LR	PValue	FDR
Sexi_OR27a	0.385	6.500	2.180	0.140	0.407	Sexi_CSP15	-0.243	3.838	0.257	0.612	0.901
Sexi_IR31a	-0.325	8.132	1.922	0.166	0.474	Sexi_OR40c	0.219	4.823	0.245	0.620	0.901
Sexi_CSP4	-0.290	13.308	1.874	0.171	0.481	Sexi_OR51	0.158	5.732	0.241	0.624	0.901
Sexi_OBP27	1.651	1.317	1.718	0.190	0.519	Sexi_OR42	0.151	5.965	0.240	0.624	0.901
Sexi_IR1_i	-0.342	6.646	1.713	0.191	0.519	Sexi_OBP23	-0.438	6.666	0.215	0.643	0.908
Sexi_OR26	-0.300	7.750	1.675	0.196	0.524	Sexi_OR45	0.147	5.916	0.212	0.645	0.908
Sexi_IR7d4	-0.548	4.698	1.613	0.204	0.538	Sexi_OBP30	0.201	6.167	0.212	0.646	0.908
Sexi_IR7d,2	-0.483	5.147	1.564	0.211	0.547	Sexi_OR25	0.112	7.402	0.173	0.677	0.945
Sexi_GR212b_i	1.160	2.101	1.511	0.219	0.551	Sexi_OBP22	0.163	4.886	0.160	0.689	0.949
Sexi_IR100e_i	-0.463	5.171	1.511	0.219	0.551	Sexi_CSP2	-0.070	15.398	0.153	0.696	0.949
Sexi_OBP2	0.341	6.991	1.474	0.225	0.557	Sexi_OR24_i	-0.118	6.598	0.151	0.697	0.949
Sexi_GR48_i	1.769	0.906	1.294	0.255	0.623	Sexi_GOBP2	-0.065	16.425	0.143	0.705	0.951
Sexi_IR40a	-0.274	6.895	1.248	0.264	0.628	Sexi_CSP5	-0.107	11.249	0.122	0.727	0.965
Sexi_OR40a_i	-0.988	2.977	1.243	0.265	0.628	Sexi_GR6	0.133	5.074	0.117	0.733	0.965
Sexi_GR1	0.605	4.080	1.200	0.273	0.639	Sexi_OR37	-0.131	5.625	0.115	0.735	0.965
Sexi_OR7_i	-0.277	6.955	1.128	0.288	0.665	Sexi_OR19	0.190	4.672	0.108	0.743	0.965
Sexi_IR75p,3_i	-0.315	5.992	1.093	0.296	0.672	Sexi_OBP13	0.065	11.528	0.094	0.759	0.965
Sexi_OR47	-0.332	6.039	1.075	0.300	0.672	Sexi_OBP5	0.065	12.369	0.086	0.769	0.965
Sexi_OR27b	-0.582	3.374	1.001	0.317	0.700	Sexi_CSP16	-0.235	2.673	0.086	0.770	0.965
Sexi_OBP4	-0.260	10.186	0.988	0.320	0.700	Sexi_OR33_i	0.092	5.998	0.085	0.770	0.965
Sexi_OR32	0.246	7.025	0.950	0.330	0.710	Sexi_GR51_i	0.268	1.846	0.078	0.780	0.965
Sexi_OR39	0.259	7.681	0.929	0.335	0.710	Sexi_OBP17_i	0.053	10.974	0.077	0.781	0.965
Sexi_OR1_i	-0.244	7.305	0.916	0.339	0.710	Sexi_OBP21	0.398	1.242	0.069	0.793	0.965
Sexi_IR75p,1_i	-0.294	6.369	0.886	0.347	0.710	Sexi_CSP13	-0.043	11.554	0.067	0.795	0.965
Sexi_OBP43_i	0.767	2.838	0.873	0.350	0.710	Sexi_GR4	-0.121	4.240	0.066	0.797	0.965
Sexi_IR75p,2_i	-0.512	3.212	0.872	0.350	0.710	Sexi_OBP10	-0.049	11.744	0.066	0.798	0.965
Sexi_OBP15	0.182	8.961	0.846	0.358	0.715	Sexi_GR201_i	-0.383	1.214	0.063	0.802	0.965
Sexi_CSP1	-0.190	13.774	0.775	0.379	0.743	Sexi_CSP12	-0.049	8.228	0.054	0.817	0.967
Sexi_GR2	-0.226	6.891	0.770	0.380	0.743	Sexi_PBP2	-0.042	16.719	0.054	0.817	0.967
Sexi_IR64a	-0.196	6.890	0.708	0.400	0.765	Sexi_OR52	-0.055	6.521	0.051	0.822	0.967
Sexi_OBP29	1.404	0.767	0.679	0.410	0.765	Sexi_OBP36	0.353	1.064	0.048	0.827	0.967
Sexi_OBP28_i	1.183	1.102	0.669	0.414	0.765	Sexi_OR9_i	0.078	5.204	0.044	0.835	0.969
Sexi_OR20_i	-0.661	2.352	0.667	0.414	0.765	Sexi_OBP45	-0.152	2.288	0.032	0.857	0.979
Sexi_OBP33	-0.688	3.017	0.666	0.415	0.765	Sexi_OR23	0.045	6.837	0.030	0.863	0.979
Sexi_IR60a	-0.251	5.718	0.630	0.427	0.772	Sexi_GR13_i	-0.083	4.189	0.029	0.865	0.979
Sexi_OBP26	0.402	4.007	0.628	0.428	0.772	Sexi_CSP18	0.041	7.017	0.027	0.870	0.979
Sexi_OR3_i	0.211	6.077	0.503	0.478	0.845	Sexi_GR157_i	-0.248	0.855	0.025	0.874	0.979
Sexi_OR36	0.143	8.777	0.502	0.479	0.845	Sexi_OR55	0.039	7.341	0.023	0.879	0.979
Sexi_OR11	0.152	8.728	0.471	0.492	0.846	Sexi_CSP10	-0.030	9.445	0.021	0.884	0.979
Sexi_OR44	0.224	5.709	0.468	0.494	0.846	Sexi_OBP9	-0.030	9.070	0.019	0.891	0.980
Sexi_CSP7	0.261	5.876	0.467	0.495	0.846	Sexi_CSP17	-0.096	2.482	0.016	0.900	0.982
Sexi_CSP21	-0.546	4.225	0.420	0.517	0.867	Sexi_GR14	-0.043	5.115	0.013	0.910	0.982
Sexi_GR9_i	-0.253	5.053	0.408	0.523	0.867	Sexi_OR12	0.032	6.701	0.011	0.916	0.982
Sexi_OBP25	-1.122	0.696	0.403	0.526	0.867	Sexi_OR14	0.025	7.435	0.010	0.919	0.982
Sexi_OBP19	-0.119	16.273	0.396	0.529	0.867	Sexi_OR29_i	0.023	7.094	0.009	0.923	0.982
Sexi_CSP22_i	0.601	1.711	0.390	0.532	0.867	Sexi_OR31_i	0.031	4.995	0.007	0.935	0.983
Sexi_OBP35	-0.677	1.368	0.357	0.550	0.887	Sexi_IR100i_i	0.084	1.487	0.006	0.937	0.983
Sexi_IR75q,1_i	-0.136	7.363	0.332	0.565	0.901	Sexi_OR8_i	-0.045	2.934	0.004	0.947	0.983
Sexi_GR66_i	0.611	1.380	0.298	0.585	0.901	Sexi_GR12	0.024	5.231	0.004	0.948	0.983
Sexi_IR93a	-0.115	8.154	0.297	0.585	0.901	Sexi_OR35	-0.013	6.127	0.002	0.962	0.984
Sexi_IR68a	-0.140	6.661	0.297	0.586	0.901	Sexi_CSP20	-0.011	7.886	0.002	0.966	0.984
Sexi_OR15	-0.131	6.916	0.296	0.586	0.901	Sexi_OR65	-0.027	3.680	0.002	0.966	0.984
Sexi_OR4	0.257	3.688	0.269	0.604	0.901	Sexi_OR61_i	-0.033	0.748	0.000	0.987	0.998
Sexi_CSP19	-0.354	2.932	0.265	0.607	0.901	Sexi_OR28	0.001	7.463	0.000	0.997	0.998
Sexi_CSP14	-0.150	13.159	0.263	0.608	0.901	Sexi_CSP8	-0.001	14.105	0.000	0.998	0.998

Annexed XXIII. Raw counts of the differential expression analysis of chemosensory-related genes between male and female adults (Chapter 3).

Transcript_id	Female rep1	Female rep2	Female rep3	Male rep1	Male rep2	Male rep3
Sexi_CSP1	44156.00	25098.00	22206.00	24380.00	18211.00	13527.00
Sexi_CSP10	2190.00	1299.00	1282.00	1024.00	877.00	737.00
Sexi_CSP11	24831.00	18562.00	17544.00	17783.00	11930.00	14635.00
Sexi_CSP12	884.00	555.00	579.00	377.00	349.00	415.00
Sexi_CSP13	8580.00	5509.00	6164.00	4479.00	2977.00	4111.00
Sexi_CSP14	29890.00	19068.00	11858.00	15766.00	13645.00	6496.00
Sexi_CSP15	39.00	23.00	22.00	25.00	9.00	21.00
Sexi_CSP16	3.00	18.00	10.00	6.00	7.00	9.00
Sexi_CSP17	19.00	5.00	7.00	5.00	8.00	4.00
Sexi_CSP18	423.00	242.00	239.00	193.00	149.00	134.00
Sexi_CSP19	18.00	16.00	6.00	5.00	7.00	16.00
Sexi_CSP2	122354.00	90312.00	75584.00	67492.00	51027.00	48074.00
Sexi_CSP20	629.00	520.00	439.00	425.00	261.00	208.00
Sexi_CSP21	3.00	73.00	13.00	12.00	37.00	30.00
Sexi_CSP22_i	7.00	4.00	6.00	1.00	3.00	2.00
Sexi_CSP23_i	2.00	0.00	0.00	0.00	0.00	0.00
Sexi_CSP24	8.00	0.00	0.00	0.00	0.00	0.00
Sexi_CSP4	22241.00	17325.00	21501.00	13422.00	10119.00	18726.00
Sexi_CSP5	8405.00	4850.00	3274.00	4562.00	3307.00	1598.00
Sexi_CSP6	2684.00	1418.00	1294.00	2352.00	2547.00	1311.00
Sexi_CSP7	182.00	116.00	130.00	40.00	46.00	111.00
Sexi_CSP8	57635.00	39723.00	25160.00	27132.00	22518.00	16387.00
Sexi_CSP9	15529.00	11832.00	8836.00	6339.00	4892.00	5186.00
Sexi_GOBP1	160893.00	111065.00	110609.00	173676.00	143078.00	191631.00
Sexi_GOBP2	246326.00	164624.00	176380.00	113960.00	91711.00	134121.00
Sexi_GR1	32.00	65.00	25.00	18.00	18.00	10.00
Sexi_GR10_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR11_i	1.00	0.00	0.00	1.00	1.00	0.00
Sexi_GR12	131.00	62.00	65.00	56.00	41.00	39.00
Sexi_GR13_i	56.00	45.00	15.00	22.00	23.00	21.00
Sexi_GR14	106.00	60.00	63.00	40.00	47.00	40.00
Sexi_GR157_i	0.00	1.00	3.00	0.00	0.00	3.00
Sexi_GR15_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR161_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR2	243.00	236.00	242.00	165.00	123.00	192.00
Sexi_GR201_i	8.00	0.00	0.00	1.00	0.00	4.00
Sexi_GR20_i	5.00	1.00	2.00	22.00	14.00	22.00
Sexi_GR210_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR212a_i	2.00	0.01	0.00	0.00	0.00	0.00
Sexi_GR212b_i	7.00	11.99	8.00	4.00	0.00	3.00
Sexi_GR22	0.00	1.00	0.00	0.00	0.00	1.00
Sexi_GR220_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR224_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR3	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR4	27.00	43.00	41.00	19.00	21.00	30.00
Sexi_GR47_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR48_i	2.00	2.00	3.00	1.00	0.00	0.00
Sexi_GR49_i	2.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR51_i	13.00	2.00	4.00	4.00	3.00	1.00
Sexi_GR6	98.00	69.00	65.00	52.00	24.00	42.00
Sexi_GR66_i	7.00	2.00	3.00	1.00	2.00	1.00
Sexi_GR71_i	0.00	1.00	0.00	0.00	0.00	0.00
Sexi_GR72_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR74_i	2.00	3.00	0.00	0.00	0.00	0.00
Sexi_GR75_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR76_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR7_i	0.00	2.00	0.00	0.00	0.00	0.00
Sexi_GR8	3.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR85_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_GR9_i	70.00	76.00	51.00	40.00	38.00	54.00
Sexi_GRn1	0.00	0.00	0.00	0.00	0.00	4.00
Sexi_GRn2_i	0.00	0.00	3.00	0.00	0.00	0.00
Sexi_GRn3_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_IR100a	0.00	2.00	0.00	0.00	0.00	1.00
Sexi_IR100b_i	15.00	9.00	4.00	1.00	1.00	3.00
Sexi_IR100c_i	4.00	0.00	0.00	0.00	0.00	0.00
Sexi_IR100e_i	70.00	64.00	64.00	56.00	35.00	64.00

Transcript_id	Female rep1	Female rep2	Female rep3	Male rep1	Male rep2	Male rep3
Sexi_IR100i_i	7.00	3.00	2.00	1.00	1.00	4.00
Sexi_IR1_i	233.00	184.00	176.00	153.00	93.00	176.00
Sexi_IR2	740.00	561.00	368.00	437.00	355.00	497.00
Sexi_IR21a_i	3297.00	2059.00	2317.00	2104.00	1492.00	1952.00
Sexi_IR25a	7899.00	4834.00	4650.00	4475.00	3897.00	5016.00
Sexi_IR31a	802.00	382.00	540.00	423.00	306.00	448.00
Sexi_IR40a	352.00	191.00	201.00	173.00	122.00	195.00
Sexi_IR41a_i	668.00	417.00	493.00	341.00	305.00	540.00
Sexi_IR60a	141.00	96.00	87.00	86.00	53.00	74.00
Sexi_IR64a	314.00	212.00	223.00	149.00	150.00	172.00
Sexi_IR68a	314.00	141.00	208.00	147.00	114.00	135.00
Sexi_IR75d_i	911.00	776.00	675.00	665.00	528.00	641.00
Sexi_IR75p.1_i	168.00	126.00	195.00	101.00	87.00	152.00
Sexi_IR75p.2_i	20.00	16.00	11.00	14.00	11.00	12.00
Sexi_IR75p.3_i	195.00	97.00	100.00	95.00	70.00	98.00
Sexi_IR75q.1_i	507.00	312.00	264.00	221.00	199.00	224.00
Sexi_IR75q.2	1624.00	1083.00	1150.00	1074.00	880.00	1082.00
Sexi_IR76b	5627.00	4037.00	4610.00	3066.00	2789.00	4874.00
Sexi_IR7d.2	67.00	70.00	56.00	45.00	44.00	63.00
Sexi_IR7d.3_i	0.00	0.00	0.00	0.00	0.00	2.00
Sexi_IR7d4	56.00	34.00	49.00	31.00	37.00	44.00
Sexi_IR87a	246.00	118.00	114.00	128.00	144.00	140.00
Sexi_IR8a_i	6322.00	4052.00	4111.00	3335.00	2707.00	3503.00
Sexi_IR93a	809.00	524.00	537.00	406.00	305.00	404.00
Sexi_OBP1	1797.00	1385.00	1436.00	1086.00	1023.00	1095.00
Sexi_OBP10	9461.00	6383.00	7116.00	4273.00	3211.00	5729.00
Sexi_OBP11_i	29441.00	18874.00	18324.00	10083.00	9410.00	9136.00
Sexi_OBP13	9115.00	5660.00	5986.00	4535.00	3795.00	2473.00
Sexi_OBP14	14062.00	10275.00	10475.00	19243.00	14490.00	16292.00
Sexi_OBP15	1603.00	1067.00	965.00	581.00	549.00	617.00
Sexi_OBP16	56.00	40.00	38.00	36.00	23.00	164.00
Sexi_OBP17_i	5685.00	3540.00	4701.00	2356.00	1946.00	3155.00
Sexi_OBP18	6716.00	3717.00	3829.00	1409.00	1404.00	1328.00
Sexi_OBP19	200146.00	139368.00	173410.00	103687.00	80029.00	128211.00
Sexi_OBP2	413.00	325.00	225.00	165.00	149.00	101.00
Sexi_OBP20	97433.00	75030.00	72249.00	212202.00	184227.00	189911.00
Sexi_OBP21	2.00	7.00	0.00	0.00	0.00	4.00
Sexi_OBP22	106.00	56.00	48.00	38.00	26.00	37.00
Sexi_OBP23	1.00	382.00	126.00	63.00	147.00	222.00
Sexi_OBP24_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_OBP25	0.00	2.00	0.00	0.00	0.00	3.00
Sexi_OBP26	34.00	55.00	22.00	17.00	13.00	18.00
Sexi_OBP27	7.00	3.00	3.00	0.00	1.00	1.00
Sexi_OBP28_i	4.00	0.00	5.00	1.00	1.00	0.00
Sexi_OBP29	0.00	2.00	3.00	0.00	0.00	1.00
Sexi_OBP30	282.00	184.00	69.00	137.00	70.00	42.00
Sexi_OBP31_i	32.00	19.00	14.00	3.00	53.00	23.00
Sexi_OBP32	0.00	1.00	2.00	0.00	19.00	0.00
Sexi_OBP33	18.00	11.00	9.00	3.00	2.00	29.00
Sexi_OBP35	3.00	2.00	3.00	1.00	5.00	1.00
Sexi_OBP36	1.00	6.00	0.00	0.00	3.00	0.00
Sexi_OBP39_i	85.00	24.00	34.00	10.00	12.00	11.00
Sexi_OBP3_i	2824.00	1831.00	1976.00	2348.00	2077.00	1381.00
Sexi_OBP4	3066.00	2524.00	1656.00	2379.00	1250.00	1198.00
Sexi_OBP40_i	2.00	0.00	0.00	0.00	0.00	0.00
Sexi_OBP42_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_OBP43_i	7.00	20.00	20.00	1.00	8.00	7.00
Sexi_OBP44_i	167.00	86.00	145.00	48.00	44.00	53.00
Sexi_OBP45	13.00	2.00	10.00	4.00	3.00	8.00
Sexi_OBP46_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_OBP47_i	18.00	0.00	0.00	0.00	0.00	0.00
Sexi_OBP5	14159.00	9865.00	12479.00	5711.00	4682.00	9182.00
Sexi_OBP6_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_OBP7_i	70746.00	52276.00	46312.00	25551.00	18906.00	25169.00
Sexi_OBP8	1.00	0.00	0.00	3.00	0.00	4.00
Sexi_OBP9	1624.00	1040.00	993.00	847.00	649.00	542.00
Sexi_OR10	265.00	144.00	135.00	82.00	64.00	66.00

Transcript_id	Female rep1	Female rep2	Female rep3	Male rep1	Male rep2	Male rep3
Sexi_OR11	1295.00	1045.00	704.00	495.00	464.00	544.00
Sexi_OR12	364.00	212.00	154.00	178.00	117.00	89.00
Sexi_OR13	5.00	1.00	0.00	335.00	247.00	437.00
Sexi_OR14	531.00	382.00	281.00	239.00	204.00	197.00
Sexi_OR15	305.00	248.00	221.00	176.00	135.00	161.00
Sexi_OR16	384.00	344.00	213.00	1427.00	1142.00	1374.00
Sexi_OR17_i	372.00	256.00	209.00	234.00	198.00	218.00
Sexi_OR18	859.00	596.00	523.00	141.00	117.00	109.00
Sexi_OR19	118.00	58.00	13.00	28.00	40.00	15.00
Sexi_OR1_i	399.00	279.00	301.00	228.00	145.00	275.00
Sexi_OR20_i	15.00	4.00	4.00	10.00	6.00	3.00
Sexi_OR22_i	560.00	446.00	335.00	173.00	156.00	107.00
Sexi_OR23	384.00	225.00	192.00	173.00	134.00	112.00
Sexi_OR24_i	261.00	237.00	129.00	173.00	111.00	93.00
Sexi_OR25	493.00	422.00	274.00	248.00	201.00	157.00
Sexi_OR26	526.00	412.00	367.00	371.00	232.00	294.00
Sexi_OR27a	312.00	198.00	186.00	131.00	75.00	87.00
Sexi_OR27b	18.00	8.00	25.00	17.00	13.00	13.00
Sexi_OR28	586.00	362.00	275.00	217.00	255.00	179.00
Sexi_OR29_i	359.00	300.00	264.00	184.00	158.00	163.00
Sexi_OR30	128.00	106.00	48.00	26.00	23.00	11.00
Sexi_OR31_i	99.00	57.00	59.00	40.00	38.00	36.00
Sexi_OR32	393.00	300.00	260.00	192.00	125.00	129.00
Sexi_OR33_i	165.00	156.00	115.00	69.00	61.00	99.00
Sexi_OR34_i	125.00	70.00	92.00	18.00	22.00	34.00
Sexi_OR35	201.00	135.00	133.00	91.00	69.00	101.00
Sexi_OR36	1293.00	948.00	886.00	561.00	492.00	508.00
Sexi_OR37	122.00	91.00	99.00	62.00	32.00	99.00
Sexi_OR38	61.00	50.00	62.00	11.00	10.00	19.00
Sexi_OR39	558.00	391.00	544.00	205.00	181.00	315.00
Sexi_OR3_i	230.00	123.00	140.00	95.00	56.00	82.00
Sexi_OR4	34.00	32.00	20.00	12.00	10.00	18.00
Sexi_OR40a_i	15.00	18.00	0.00	12.00	18.00	5.00
Sexi_OR40b	85.00	44.00	49.00	18.00	16.00	21.00
Sexi_OR40c	67.00	65.00	62.00	30.00	19.00	46.00
Sexi_OR41	695.00	479.00	391.00	203.00	186.00	167.00
Sexi_OR42	188.00	112.00	141.00	65.00	81.00	70.00
Sexi_OR43	172.00	127.00	133.00	65.00	30.00	73.00
Sexi_OR44	191.00	99.00	95.00	62.00	61.00	53.00
Sexi_OR45	190.00	93.00	145.00	64.00	62.00	85.00
Sexi_OR46	36.00	42.00	30.00	31.00	25.00	38.00
Sexi_OR47	218.00	102.00	88.00	87.00	97.00	86.00
Sexi_OR48	352.00	214.00	169.00	9.00	9.00	0.00
Sexi_OR5	36.00	27.00	51.00	26.00	39.00	77.00
Sexi_OR50	285.00	229.00	119.00	59.00	66.00	35.00
Sexi_OR51	168.00	124.00	85.00	67.00	52.00	66.00
Sexi_OR52	249.00	196.00	161.00	135.00	98.00	116.00
Sexi_OR53	165.00	119.00	103.00	24.00	43.00	24.00
Sexi_OR54_i	0.00	0.00	0.00	0.00	0.00	0.00
Sexi_OR55	428.00	302.00	372.00	182.00	158.00	259.00
Sexi_OR56	6.00	4.00	3.00	118.00	92.00	109.00
Sexi_OR57	578.00	404.00	401.00	187.00	155.00	241.00
Sexi_OR58_i	98.00	80.00	94.00	27.00	34.00	38.00
Sexi_OR6	2.00	14.00	0.00	3144.00	2288.00	3123.00
Sexi_OR61_i	4.00	0.00	0.00	2.00	0.00	0.00
Sexi_OR63_i	0.00	0.00	1.00	2.00	2.00	3.00
Sexi_OR65	17.00	11.00	47.00	12.00	10.00	23.00
Sexi_OR69_i	6.00	4.00	5.00	1.00	1.00	0.00
Sexi_OR7_i	351.00	249.00	170.00	201.00	156.00	151.00
Sexi_OR8_i	24.00	13.00	8.00	6.00	4.00	15.00
Sexi_OR9_i	136.00	64.00	59.00	40.00	42.00	48.00
Sexi_ORco	16719.00	10333.00	8728.00	9718.00	8209.00	8921.00
Sexi_PBP1	294468.00	197538.00	180700.00	806872.00	625346.00	1181649.00
Sexi_PBP2	276478.00	224303.00	215897.00	129663.00	114495.00	168254.00
Sexi_PBP3	67631.00	48793.00	41577.00	26692.00	20935.00	20547.00
Sexi_PBP4	48.00	35.00	30.00	11.00	14.00	11.00

Annexed XXIV. Sequence list of the primers used for RT-qPCR and cloning (Chapter 4).

Target	Primer	Primer (5'-3')
ATPsynthase subunit C	Forward	TCCTGCTGTTGTTCGCTTTC
	Reverse	CCACACATTCGATTCCATGGC
SexiORco	Forward	GAAAGCCTGGTACCCATTCA
	Reverse	CCAGCGAAGCACTAAGTTCC
SexiOR10	Forward	CTTGTACGTTGTGGGCAATG
	Reverse	TCAATGGGGTTACTGGCATC
SexiOR19	Forward	ACGGCTCAATATACGGCAAC
	Reverse	ACGCACCACCAGTAAAAAGG
SexiOR23	Forward	ATTGCTGTGGGAATGGTCTC
	Reverse	TACTGCCTCCAACTTCTTCG
SexiOR25	Forward	TTGAAGGAGTGCCGAGAAAC
	Reverse	TGACCCAGCAGTGCTTTATG
SexiOR34	Forward	TGCTGCTGTCCTGATTTCTG
	Reverse	TGATCAGCATCGAAGGTCTG
SexiOR35	Forward	GCTGGGGAAGTATTTGAACG
	Reverse	TCTGCAGCATCAGCAATACC
SexiOR40c	Forward	CGCTATCCGGACAATTCTTG
	Reverse	TCACTTTGACATCGCTCTCG
SexiOR44	Forward	GCCGAAAAACATCAGCTACC
	Reverse	AGGAATGCTGCAATGAGGAG
SexiOR45	Forward	TCTCACCGGTAATGTTCGTG
	Reverse	TAGCGATGTCGTCGTGAATC
SexiOR63	Forward	ATATTTGCCGACCACTCCTG
	Reverse	GCGTAGAAGTGTCCCAGCTC
SexiOR23 ORF Amplification	Forward	ATGTGGCAAAAACTAAAAGATTTC
	Reverse	CTAAATATTAGCTTGCCGCAGCAT
SexiOR35 ORF Amplification	Forward	ATGTGGGATCAAATACGTAAGTTT
	Reverse	TTACATAATAGACCTGAATGCAGT

Annexed XXV. Synthetic compounds used for electrophysiology experiments (Chapter 4)

Compound	CAS	Provider	Purity (%)	Compound	CAS	Provider	Purity (%)
1,3-Dimethylbenzene	108-38-3	Sigma-Aldrich	99	**Menthone**	10458-14-7	Sigma-Aldrich	97
1,4-Dimethylbenzene	106-42-3	Sigma-Aldrich	99	***p*-Cymene**	99-87-6	Sigma-Aldrich	99
1-Indanone	83-33-0	Sigma-Aldrich	99	**Sulcatone**	110-93-0	Sigma-Aldrich	98
2-Phenylacetaldehyde	122-78-1	Sigma-Aldrich	95	**α-Pinene**	80-56-8	Sigma-Aldrich	98
2-Phenylethanol	60-12-8	Sigma-Aldrich	99	**β-Citronellene**	10281-55-7	Sigma-Aldrich	98.5
3-Carene	13466-78-9	Sigma-Aldrich	90	**(*E*)-2-Hexen-1-ol**	928-95-0	Sigma-Aldrich	96
4-Ethylguaiacol	2785-89-9	Sigma-Aldrich	98	**(*Z*)-3-Hexen-1-ol**	928-96-1	Sigma-Aldrich	98
4-Methylanisole	104-93-8	Sigma-Aldrich	99	**(*Z*)-3-Hexenyl acetate**	3681-71-8	Sigma-Aldrich	95
Acetophenone	98-86-2	Acros organics	99	**1-Heptanol**	111-70-6	Sigma-Aldrich	99
Anisole	100-66-3	Sigma-Aldrich	99	**1-Hexanol**	111-27-3	Sigma-Aldrich	98
Benzyl alcohol	100-51-6	Sigma-Aldrich	99	**1-Nonanol**	143-08-8	Sigma-Aldrich	99.5
Benzyl cyanide	140-29-4	Sigma-Aldrich	98	**1-Octanol**	111-87-5	Sigma-Aldrich	99.5
Benzyl methyl ether	538-86-3	Sigma-Aldrich	98	**1-Octen-3-ol**	3391-86-4	Sigma-Aldrich	98
Estragole	140-67-0	Sigma-Aldrich	96	**1-Pentanol**	71-41-0	Sigma-Aldrich	99
Eugenol	97-53-0	Sigma-Aldrich	98	**2-Ethyl-1-butanol**	97-95-0	Sigma-Aldrich	98
Indole	120-72-9	Sigma-Aldrich	99	**2-Heptanol**	543-49-7	Sigma-Aldrich	98
Methyl benzoate	93-58-3	Acros organics	97	**2-Heptanone**	110-43-0	Sigma-Aldrich	99
Methyl salicylate	119-36-8	Sigma-Aldrich	99	**2-Octanol**	123-96-6	Sigma-Aldrich	97
(±)-Linalool	78-70-6	Sigma-Aldrich	97	**3-Hexanone**	589-38-8	Sigma-Aldrich	98
(±)-Nerolidol	7212-44-4	Sigma-Aldrich	98	**3-Octanone**	106-68-3	Sigma-Aldrich	98
(*E*)-β-Ocimene	3779-61-1	Sigma-Aldrich	65	**Butyl propionate**	590-01-2	Sigma-Aldrich	99
(*R*)-(-)-Linalool	126-91-0	Sigma-Aldrich	95	**Ethyl acetate**	141-78-6	Sigma-Aldrich	99.8
(S)-(+)-Linalool	126-90-9	Sigma-Aldrich	95	**Ethyl hexanoate**	123-66-0	Sigma-Aldrich	99
β-Caryophyllene	87-44-5	Sigma-Aldrich	98.5	**Geranyl acetate**	105-87-3	Sigma-Aldrich	97
Citral	5392-40-5	Sigma-Aldrich	95	**Isoamyl acetate**	123-92-2	Sigma-Aldrich	99
DMNT = (3E)-4,8-dimethylnona-1,3,7-triene	19945-61-0	†	99	**Methyl hexanoate**	106-70-7	Sigma-Aldrich	99
Eucalyptol	470-82-6	Sigma-Aldrich	99	**Octanal**	124-13-0	Sigma-Aldrich	99
Geraniol	106-24-1	¶	98	**γ-Nonanoic lactone**	104-61-0	Sigma-Aldrich	97
(±)-Limonene	5989-27-5	Sigma-Aldrich	97	**Furfuryl alcohol**	98-00-0	Sigma-Aldrich	98

† Gift from Prof. Wittko Francke, Hamburg, Germany
¶ Gift from Prof. Monika Hilker, Berlin, Germany

Annexed XXVI. Total counts for the odorant receptor transcripts in differential expression analysis after RNA-seq in Chapter 4.

Gene	C1	C2	C3	BV1	BV2	BV3
SexiORco	5	13	4	15	36	1
SexiOR1	0	0	0	0	1	0
SexiOR3	4	1	5	2	2	1
SexiOR6	0	0	0	0	1	0
SexiOR7	1	0	0	2	0	1
SexiOR9	1	5	0	1	1	0
SexiOR10	0	3	1	0	1	0
SexiOR11	0	0	0	0	1	1
SexiOR12	0	1	0	0	0	0
SexiOR13	0	1	0	2	0	0
SexiOR14	1	2	0	1	1	0
SexiOR15	0	0	0	5	0	0
SexiOR16	0	0	0	0	2	0
SexiOR17	2	1	0	0	2	1
SexiOR18	0	0	0	0	0	1
SexiOR19	0	0	0	2	2	4
SexiOR20	0	0	0	2	0	0
SexiOR22	0	6	0	0	1	3
SexiOR23	0	0	0	2	0	3
SexiOR25	0	1	0	1	1	1
SexiOR26	0	0	1	0	1	0
SexiOR27a	0	4	0	0	0	0
SexiOR27b	0	0	0	0	1	0
SexiOR28	4	4	2	0	5	5
SexiOR29	0	1	0	0	0	0
SexiOR30	1	0	0	0	0	0
SexiOR31	0	1	0	0	1	0
SexiOR32	0	0	1	2	1	1
SexiOR33	0	0	0	1	0	0
SexiOR34	0	0	0	0	2	2
SexiOR35	0	0	0	3	3	3
SexiOR36	3	1	1	1	0	1
SexiOR37	0	0	0	0	0	2
SexiOR38	0	0	0	1	0	0
SexiOR39	0	4	0	2	0	0
SexiOR40c	0	0	0	3	0	6
SexiOR41	0	0	0	0	0	1
SexiOR44	4	0	0	25	5	20
SexiOR45	1	0	2	1	0	0
SexiOR46	0	2	1	0	0	0
SexiOR47	1	5	0	0	0	0
SexiOR50	0	2	1	1	0	0
SexiOR52	0	1	0	1	0	0
SexiOR55	2	3	1	0	0	0
SexiOR57	0	0	0	1	0	0
SexiOR58	0	0	0	0	0	1
SexiOR63	0	2	1	20	8	16
SexiOR65	6	8	3	2	1	5
SexiOR69	1	1	0	0	2	1