# EXPLORING HONEYBEE-MITE IMMUNE INTERACTIONS FOR DEVELOPING RNAi BASED CONTROL TECHNOLOGIES OF *VARROA DESTRUCTOR*

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*A tutta quella gente che soffre*
*e combatte quotidianamente*
*per la liberta'*

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## RIASSUNTO

Il valore economico del settore apistico, considerando non solo i vari prodotti dell'alveare ma anche il valore dell'impollinazione, è difficile da determinare. Secondo la Federazione Italiana Apicoltori (FAI), in Italia questo valore è stimato intorno a 1,5 miliardi di euro l'anno, generati da circa un milione di alveari. Negli USA si presenta uno scenario diverso, in cui la stragrande maggioranza degli apicoltori gestisce più di 500 alveari, per un totale di circa 2,6 milioni di alveari totali, utilizzati soprattutto per erogare servizi d'impollinazione, e un giro di affari che supera i 14 miliardi di dollari. L'impollinazione non favorisce solo l'aumento della produzione, ma anche quello della qualità, della *shelf-life* e, quindi, del valore commerciale delle colture impollinate. Questo aumento di valore influisce positivamente sui ricavi degli agricoltori e promuove lo sviluppo economico del settore apistico. Allo scopo di comprendere il reale valore dell'impollinazione, è necessario considerare che più del 75% delle principali specie vegetali coltivate sfrutta l'impollinazione entomofila, la quale è responsabile di circa un terzo della produzione mondiale di frutta, ortaggi, noci e semi. Le colture che beneficiano dell'impollinazione entomofila sono tra le più ricche in micronutrienti e, quindi, un improvviso declino degli insetti impollinatori potrebbe causare carenze nutrizionali, così come altri problemi per la salute umana.
É chiaro, quindi, che al di là degli aspetti economici ed ecologici su scala locale, l'impollinazione ad opera degli insetti è un servizio d'importanza globale minacciato dal progressivo cambio d'uso del suolo e dall'agricoltura intensiva. L'ape mellifera (*Apis mellifera*) è la specie impollinatrice per eccellenza e la sua popolazione mondiale è aumentata negli ultimi decenni. Ciononostante, dati della FAO mostrano che la crescita della popolazione mondiale di *A. mellifera* durante l'ultimo mezzo secolo (~45%) non ha assecondato il rapido aumento (>300%) della diffusione di colture dipendenti dall'impollinazione entomofila. Questo disequilibrio tra domanda e offerta di servizi d'impollinazione suggerisce che esiste un urgente bisogno di sviluppare un'agricoltura più sostenibile, in grado di ottimizzare impollinazione e produzione, salvaguardando la biodiversità e la salute delle api.
Sebbene le colonie d'ape mellifera siano aumentate su scala globale, significative morie sono state riportate in alcune aree negli ultimi anni. In particolare, durante l'inverno 2006/2007, negli USA è stata registrata una mortalità delle colonie inspiegabilmente elevata (~30%). Una parte di queste perdite è stata caratterizzata da una serie di particolari sintomi (la rapida perdita di api operaie adulte unita all'assenza di api morte all'interno o nei dintorni dell'alveare) che, in seguito, hanno portato a definire questa sindrome come Sindrome dello Spopolamento degli Alveari (il cui acronimo in inglese è CCD, Colony Collapse Disroder). Allo stesso tempo, in Europa, la 4ª Conferenza COLOSS (Prevention of honeybee COny LOSSes) riportava una mortalità media di circa il 20% delle colonie, con variazioni dal 1.8 al 53% a seconda del paese considerato. La dimensione drammatica di queste recenti perdite ha attratto l'attenzione dell'opinione pubblica e il crescente interesse della comunità scientifica. Dopo anni d'intense ricerche, una causa specifica di questo fenomeno non è stata individuata, bensì è stata proposta un'origine multifattoriale per la CCD. Oggi è largamente condivisa l'opinione che il degrado ambientale e l'utilizzo di pesticidi siano concause più o meno dirette di questa sindrome. Ad ogni modo, numerosi studi di monitoraggio hanno evidenziato che la perdita delle colonie è costantemente associata ad una forte presenza di parassiti e patogeni e, in particolar modo, alla compresenza dell'acaro *Varroa destructor* e del Virus delle Ali Deformi (Deformed Wing Virus, DWV).

L'acaro *V. destructor* è un ectoparassita obbligato dell'ape che si alimenta creando una ferita nella cuticola, attraverso la quale si nutre dell'emolinfa e del corpo grasso dell'ospite. Questo parassita indebolisce l'ape e agisce da vettore di numerosi virus, tra i quali il più studiato è il DWV. Quest'ultimo, così come gli altri virus, era considerato un problema minore per la salute delle api prima della diffusione, relativamente recente, dell'acaro. In colonie severamente infestate da *V. destructor*, il DWV, che normalmente è presente a livelli subletali e asintomatici, si manifesta con infezioni potenzialmente distruttive, attraverso la replicazione incontrollata mediata dall'attività trofica dell'acaro e dalla conseguente immunosoppressione dell'ape. In seguito all'arrivo di *V. destructor* negli anni '70-80, in Europa e Nord America le perdite di colonie di ape mellifera sono quasi triplicate. In assenza di misure efficaci di controllo del parassita, una colonia infestata non può sopravvivere più di 2-3 anni.
Nonostante i numerosi studi circa l'immunodepressione causata dal virus, gli effetti dell'attività trofica di *V. destructor*, mediati dall'azione delle secrezioni salivari, sono ancora poco noti. Come riportato in un recente articolo sul sialoma di *V. destructor*, le proteine salivari dell'acaro sono probabilmente coinvolte nella manipolazione e nello sfruttamento delle risorse nutrizionali offerte dall'ospite, sebbene esistano poche evidenze sperimentali a riguardo. Dai pochi studi effettuati, emerge che la saliva dell'acaro è in grado di favorire la replicazione del virus nell'ospite e di interferire con la capacità degli emociti (le cellule immunitarie degli insetti) di formare aggregati. L'approfondimento delle conoscenze circa l'attività biologica del sialoma di *V. destructor* rappresenta un passo importante per comprendere i meccanismi molecolari alla base delle interazioni ospite-parassita e per sviluppare nuove strategie di controllo.
Nel presente lavoro di tesi è descritta una pipeline di genomica funzionale impiegata per caratterizzare il secretoma di *V. destructor* e generare una lista di probabili proteine salivari. All'interno di questa lista sono stati selezionati alcuni geni, successivamente studiati tramite qPCR e *in situ* hybridization, allo scopo di valutarne l'espressione nelle ghiandole salivari dell'acaro. Questo approccio ha portato all'identificazione di una chitinasi (Vd-CHI) specificamente espressa nelle ghiandole salivari di *V. destructor.* La successiva caratterizzazione funzionale mediante interferenza dell'RNA, ha dimostrato che Vd-CHI è in grado di favorire la sopravvivenza dell'acaro e di alterare la risposta immunitaria dell'ape.
Per la caratterizzazione del secretoma sono state utilizzate le 30221 proteine predette in seguito alla più recente annotazione del genoma di *V. destructor* (Ottobre, 2017). Queste sono state filtrate per la presenza del peptide segnale, allo scopo di considerare quelle più probabilmente secrete. L'annotazione delle 2882 proteine con peptide segnale, oltre a consentire una preliminare caratterizzazione funzionale del secretoma, ha evidenziato una notevole presenza di omologie tra le componenti della saliva di acari e zecche e quelle del veleno degli Imenotteri. In particolare, circa il 60 % delle proteine del secretoma di *V. destructor* annotate è risultato condiviso da questi due taxa. Ciò è in accordo con quanto osservato da alcuni autori circa la convergenza evolutiva delle secrezioni nel regno Animale. Infatti, vari studi riportano che zecche e Imenotteri parassitoidi, così come altri animali velenosi, utilizzano le stesse famiglie di proteine, da veicolare attraverso l'iniezione di saliva e veleno, allo scopo di alterare la fisiologia dell'ospite e sfruttarlo da un punto di vista nutrizionale. Esempi di questa convergenza sono rappresentati da fosfolipasi A2, inibitori delle serin proteasi e chitinasi, proteine reclutate tra quelle partecipanti a funzioni biochimiche fondamentali, altamente conservate nel regno Animale. Queste ultime

esibiscono funzioni simili a quelle endogene quando vengono iniettate nell'ospite, rappresentando un particolare esempio di elementi attivi a livello intergenomico.
Al contrario, altre proteine coinvolte nei fenomeni parassitari possono essere molto specifiche ed esibire una bassa identità di sequenza con proteine di specie evolutivamente vicine. La nostra analisi ha riportato la presenza di 863 proteine che non mostrano alcuna similarità di sequenza con i database presi in esame. Non è da escludere che ci possano essere fattori di parassitizzazione tra queste proteine non annotate, che, ovviamente, risultano di difficile caratterizzazione funzionale. Abbiamo, quindi, deciso di prendere in considerazione per il presente lavoro unicamente candidati dotati di una funzione putativa nota.
Dalla lista dei candidati identificati in seguito all'annotazione del secretoma, in accordo con dati di letteratura, abbiamo selezionato cinque geni, per valutarne l'espressione nelle ghiandole salivari dell'acaro, mediante qPCR. Tra i geni analizzati, solo Vd-CHI è risultato sovraespresso nelle ghiandole salivari. Sebbene gli altri geni valutati (α-macroglobulina, protease aspartica, q-carboxypeptidase, serine protease 42) sono probabilmente espressi anche in altri tessuti, non è da escludere che la loro espressione nelle ghiandole salivari possa avere un ruolo nella regolazione dell'ospite.
Vd-CHI è stato, quindi, selezionato per un successivo esperimento di ibridazione *in situ,* che ne ha rilevato l'espressione specifica nelle ghiandole salivari. Questo risultato ha contribuito a indirizzare i successivi biosaggi funzionali verso la caratterizzazione di questo putativo fattore di regolazione dell'ospite.
Le chitinasi sono glicosil idrolasi responsabili della degradazione della chitina, il secondo più abbondante biopolimero sul pianeta, che è una componente strutturale di matrici biologiche protettive come, ad esempio, l'esoscheletro degli artropodi o la parete cellulare dei funghi. Negli artropodi le chitinasi hanno un ruolo fondamentale come enzimi degradativi durante la muta e un ruolo difensivo contro funghi e nematodi parassiti. La presenza di chitinasi enzimaticamente attive, ma anche di tutti gli enzimi necessari alla completa degradazione della chitina, nelle femmine adulte di *V. destructor* è già stata descritta in precedenza. Gli autori degli studi in questione hanno ipotizzato che questi enzimi possano avere un ruolo nell'idrolisi della chitina dell'ape e nel mantenimento del sito di alimentazione, attraverso il quale la femmina fondatrice e la progenie di *V. destructor* si nutrono.
Allo scopo di valutare un eventuale ruolo nel favorire l'alimentazione dell'acaro, abbiamo effettuato delle infestazioni artificiali utilizzando acari in cui è stato silenziato il gene Vd-CHI. La sopravvivenza dell'acaro è risultata ridotta del 50% in seguito al silenziamento di Vd-CHI, a 72 ore dal trattamento con dsRNA. Considerando che la maggior riduzione della sopravvivenza avviene intorno alle 24 ore dall'inizio del silenziamento (48 ore dal trattamento) e che *V. destructor* non può sopravvivere più di 24-36 ore senza alimentarsi, è ragionevole assumere che il silenziamento di Vd-CHI determina una riduzione della capacità dell'acaro di nutrirsi.
Sebbene l'apertura del sito di alimentazione sia principalmente dovuta alla rottura meccanica della cuticola da parte dell'acaro, è possibile che questo processo possa essere facilitato dalla presenza di Vd-CHI. Questa ipotesi è anche supportata dal fatto che questa chitinasi, specificamente espressa nelle ghiandole salivari di *V. destructor*, è stata rilevata da recenti studi di proteomica solo nelle femmine adulte dell'acaro, le uniche in grado di creare e mantenere aperto il sito di alimentazione. Comunque il meccanismo molecolare alla base dell'effetto positivo di Vd-CHI sull'alimentazione dell'acaro resta poco chiaro. La sequenza aminoacidica di Vd-CHI presenta i motivi conservati delle chitinasi GH18 (DXXDXDXE, dove D = acido

aspartico, E = acido glutammico, e X = qualsiasi aminoacido), che includono il sito attivo dell'enzima. É interessante notare che Vd-CHI manca del domino putativo di legame con la chitina, nonostante quest'ultimo sembri non essenziale per l'attività chitinolitica delle chitinasi degli artropodi. Comunque, l'attività chitinolitica di Vd-CHI dovrebbe essere valutata attraverso appropriati saggi enzimatici utilizzando estratti delle ghiandole salivari e/o la proteina ricombinante.

Allo scopo di indagare circa un probabile coinvolgimento nella formazione del sito di alimentazione, l'espressione di Vd-CHI è stata misurata in due diverse fasi del ciclo biologico di *V. destructor.* Abbiamo ipotizzato che femmine foretiche e riproduttive, alimentandosi principalmente attraverso le morbide membrane intersegmentali e la parte centrale del secondo sternite addominale, rispettivamente, potessero avere diverse esigenze in termini di un fattore parassitario in grado di agevolare la perforazione della cuticola.

Vd-CHI è risultato più espresso nelle femmine riproduttive estratte da cellette opercolate, supportando l'ipotesi dell'importanza di questo fattore per la formazione e il mantenimento dei siti di alimentazione, processi che possono influenzarne il livello di espressione durante il ciclo biologico di *V. destructor*.

Allo scopo di studiare l'impatto di Vd-CHI sulla presenza, la dimensione e il numero delle ferite di alimentazione su pupe artificialmente infestate, abbiamo effettuato delle prove di colorazione con Trypan Blue, che colora selettivamente le cellule morte. Queste prove hanno però dato esito negativo nelle nostre condizioni sperimentali, che prevedevano solo 24 ore di infestazione artificiale delle pupe (occhi marroni), all'interno di capsule di gelatina.

Abbiamo quindi valutato l'effetto di Vd-CHI sull'ospite determinando il profilo di espressione di alcuni geni immunitari dell'ape.

La presenza di Vd-CHI ha determinato la sovraespressione di *acidic-mammalian chitinase* e la sottoespressione di *sgabd8,* due geni che non sono differenzialmente espressi in pupe infestate con acari silenziati rispetto alle pupe-controllo non infestate. Questo risultato è in linea con recenti studi di proteomica quantitativa in cui un'altra chitinasi (XP_623995.1) e la stessa proteina *sgabd8* sono, rispettivamente, sovra- e sottorappresentate in pupe parassitizzate.

Utilizzando un approccio simile a quello descritto nel presente lavoro (silenziamento del fattore di parassitizzazione seguito da RNA-seq dell'ospite parassitizzato), è stata studiata la funzione di una chitinasi GH19 identificata nel veleno di *Nasonia vitripennis,* Imenottero parassitoide. La presenza di questa proteina nel veleno dell'imenottero parassitoide causa la sovraregolazione di una chitinasi nell'ospite *Sarcophaga bullata*. Le chitinasi endogene degli insetti sono coinvolte nella risposta immunitaria all'invasione di funghi patogeni. Sembra quindi ragionevole ipotizzare che la sovraregolazione del gene *acidic-mammalian chitinase* nelle pupe di *A. mellifera* infestate da *V. destructor* possa essere indotta dall'attività chitinolitica di Vd-CHI, in grado di stimolare una risposta immunitaria anti-fungina allo scopo di prevenire eventuali infezioni della ferita di alimentazione.

Questa ipotesi è anche supportata dal fatto che Vd-CHI determina la sovraregolazione di *spaetzle,* che attiva il pathway di Toll in *Drosophila* e la successiva produzione di peptidi antimicrobici, come osservato anche per la chitinasi GH19 di *N. vitripennis.*

L'unico trascritto risultato sottoregolato codifica per una glicoproteina strutturale dell'endocuticola dell'ape, *sgabd8*, associata alla resistenza all'acaro Varroa e probabile indicatore una compromessa capacità della cuticola danneggiata di curarsi in presenza di Vd-CHI.

Inoltre, la sovraregolazione di *β-1,3-glucan binding* avviene solo in pupe infestate con acari silenziati per la Vd-CHI. Questa proteina è coinvolta nel rilevamento delle infezioni nell'ospite e stimola la melanizzazzione, processo alla base della cura delle ferite e dell'incapsulamento negli artropodi. É quindi ragionevole considerare la sovraregolazione di questo trascritto come un indicatore di infezioni batteriche causate dall'assenza di Vd-CHI a livello delle ferite di alimentazione. Infatti, Vd-CHI potrebbe essere coinvolta nella prevenzione della proliferazione di batteri attraverso (1) la produzione di chito-oligosaccaridi antimicrobici derivanti dalla degradazione della chitina dell'ospite e (2) l'attività anti-biofilm, come osservato per altre chitinasi. Inoltre, a supporto dell'ipotesi di infezioni batteriche in atto, il peptide antimicrobico *hymenoptaecin* risulta sovraregolato in pupe parassitizzate da acari privi di Vd-CHI, così come già osservato in seguito a iniezione di buffer o di omogenato di *V. destructor*.
Nel presente lavoro viene riportata la sovraregolazione del peptide antimicrobico *defensin-1*in pupe infestate con acari silenziati e non silenziati, rispetto alle pupe controllo non infestate. Questo risultato conferma quanto già riportato in altri lavori, ovvero che la sovraespressione di *defensin-1*viene indotta dalla parassitizzazione.
Questo approccio sperimentale soffre della mancanza di informazioni circa il titolo virale delle pupe e degli acari impiegati negli esperimenti. L'effetto del virus DWV sul sistema immunitario dell'ape potrebbe, infatti, mascherare l'impatto della sola infestazione da *Varroa*. Inoltre, come già menzionato in precedenza, è improbabile che l'infestazione artificiale all'interno di capsule di gelatina possa riprodurre la stessa comunità microbica e l'effetto dei composti antimicrobici caratteristici della cera naturale (in sinergia con propoli e miele) che costituisce i favi dell'alveare. Nonostante questi limiti, il presente studio di espressione differenziale dei geni immunitari suggerisce che le proteine salivari di *V. destructor* sono in grado di stimolare la risposta immunitaria dell'ape, probabilmente allo scopo di limitare le infezioni a carico delle ferite di alimentazione.
Prolungate infestazioni artificiali di pupe di *A. mellifera* con acari silenziati, seguite da RNA-seq degli ospiti, potranno rivelare i pathway biologici sollecitati dall'azione di Vd-CHI, contribuendo alla caratterizzazione funzionale di questa proteina salivare.
Insieme, questi risultati fanno luce sull'interazione Varroa-ape, confermando che il sialoma dell'acaro riveste un ruolo importante nello sfruttamento delle risorse alimentari offerte dall'ospite. In particolare, la capacità di alimentarsi di *V. destructor* è mediata dall'azione di Vd-CHI, che probabilmente facilita l'apertura e la pervietà delle ferite di alimentazione sulla cuticola dell'ape. É ragionevole ipotizzare che questa chitinasi possa interferire con il normale processo di cura della cuticola danneggiata, garantendone l'apertura e al contempo limitandone l'invasione da parte di possibili patogeni.
La presenza di funghi e batteri a livello dell'interfaccia ospite-parassita rende questa interazione anche più complessa, suggerendo di adottare un approccio ologenomico per simili studi. L'utilizzo di particolari tecniche di microscopia, esperimenti di RNA-seq e studi di metagenomica delle ferite di alimentazione contribuiranno a caratterizzare i patogeni opportunisti e l'impatto che essi hanno sui processi curativi della cuticola, allo scopo di chiarire il ruolo di Vd-CHI nell'intricata interazione tra parassita, ospite e microrganismi.
Il presente lavoro contribuisce ad una conoscenza più profonda circa il ruolo delle proteine salivari di *V. destructor* e dimostra, inoltre, che l'utilizzo di dsRNA allo scopo di silenziare geni espressi nelle ghiandole salivari costituisce un'interessante

strumento per il controllo dell'acaro, sebbene sia necessario lo sviluppo di nuovi metodi di rilascio nell'ambiente di tali molecole.

## SUMMARY

The economic impact of beekeeping, including crop pollination, is very difficult to assess, but, according to the Italian Federation of Beekeepers (FAI), it can be estimated around 1,500 millions of euros annually, generated by about one million of hives. Honeybee colony losses have been a major problem since the beginning of modern apiculture; however, in 2006, the dramatic dimension of this phenomenon attracted the attention of the public opinion and the increasing interest of the scientific community. After a few years of intense investigation, a specific causal agent for the widespread colony losses was not found, but rather a multifactorial origin was proposed for this syndrome. It is now largely accepted that both landscape deterioration and agrochemicals can be directly or indirectly responsible for colony losses. However, several monitoring programs indicate that the large majority of losses are associated with the co-presence of the mite *Varroa destructor* and the Deformed Wing Virus (DWV). *V. destructor* is an ectoparasitic mite that creates a wound in the host's cuticle through which it feeds on haemolymph and fat body, representing an important stress factor that weakens honeybee colonies and promotes the spreading of viral diseases. In order to facilitate feeding, *V. destructor* delivers a complex of factors, including proteins, through its salivary secretions. The scarcity of information about the sialome of the mite limits the functional analysis of the host regulation process and, thus, the opportunity to impair the mite's fitness using biotechnological approaches. Here, we have used a functional genomics pipeline to identify *V. destructor* candidate salivary proteins, along with *in situ* hybridization detection to assess their expression in salivary glands. Using this approach, we identified a chitinase (Vd-CHI), specifically expressed in salivary glands, which affects mite survival and causes alterations of honeybee immune response. In particular, gene knockdown experiments revealed that Vd-CHI deficient mites tend to show a reduced survival as a likely consequence of reduced feeding capacity on honeybee pupae, due to the lack of chitinoloytic activity that favours the patency of the feeding wound. The importance of Vd-CHI for the feeding success of *Varroa destructor* is also supported by the upregulation of this gene during the reproductive phase of the mite, when the adult female creates large communal feeding site for her and the offspring, usually in the middle of the 2$^{nd}$ abdominal sternite of honeybee pupae. Vd-CHI has also an impact on honeybee's immune response, determining the upregulation of an endogenous chitinase and the downregulation of an endocuticle structural glycoprotein, associated with wound healing and *Varroa*-resistance. On the other hand, infestation by Vd-CHI deficient mites led to upregulation of *β-1,3-glucan binding protein* and *hymenoptaecin*, as a likely consequence of bacterial infections, suggesting that Vd-CHI could also prevent detrimental bacterial proliferation.
Collectively, these results shed light on *Varroa*-honeybee interaction, confirming that *V. destructor* sialome plays an important role in host exploitation. In particular, *V. destructor* feeding success on *A. mellifera* is mediated by the action of Vd-CHI, which

probably facilitates the opening and patency of the feeding wound in the honeybee's cuticle. Indeed, this chitinase is likely involved both in maintaining the feeding site open, by interfering with the regular healing process of cuticle, and in limiting opportunistic infections, by priming antimicrobial defenses. The present work contributes to a better knowledge of salivary repertoire of *V. destructor* and also demonstrates that dsRNA targeting of genes expressed in salivary glands can offer a promising new tool for controlling the mite.

# INTRODUCTION

## *1.1 Economic and ecological importance of honeybees*

The interaction between humans and honeybees had its origins with honey-hunting: the opportunistic stealing of honey from wild bees. The first Neolithic evidence of this activity comes from beeswax residues in Anatolian pottery nearly 9000 years old (Kritsky, 2017). However, the origins of true beekeeping, where bees are provided with artificial cavities in which the comb can be built, can be found in the ancient Egypt and date back to 2450 BC (Kritsky, 2015). In the ancient Egypt honey and the other products of apiculture were important commodities for trade, food, medicinal ingredients, cosmetics and religious rituals (Cilliers and Retief, 2008).
Nowadays the economic impact of beekeeping, including crop pollination, is very difficult to assess. In Italy, according to the Italian Federation of Beekeepers (FAI), this value can be estimated around 1.5 billion € annually, generated by about one million of hives. In the US, a different scenario can be found, where professional beekeepers manages more than 500 hives, for a rough total of 2.6 million honey bee colonies, largely used for crop pollination, and a value of over 14 billion $ (Ellis, 2012). Indeed, crop pollination increases not only yield but also quality, shelf life and commercial value of crops (Klatt et al., 2014) and, then, by significantly influencing farmers' income, promotes the economic development of the beekeeping industry (Geslin et al., 2017).
More than 75% of all globally important crops depend to some degree on animal pollination, that contributes to about one-third of global crop production (Klein et al., 2007). Assuming complete removal of pollinators, global fruit supplies could be reduced of 22.9%, vegetables by 16.3% and nuts and seed by 22.1% (Smith et al., 2015). Furthermore, animal-pollinated crops are among the richest in micronutrients (Daily and Karp, 2015) and pollinator declines could thus cause micronutrient deficiencies as well as other human health concerns (Chaplin-Kramer et al., 2014; Ellis et al., 2015). It thus become clear that, beyond the ecologic-economic aspects at a local scale, pollination is a service of global and strategical importance threatened by land-use change and agricultural intensification (Lautenbach et al., 2012). Wild bee species, central to the agro-ecosystem service of pollination (Garibaldi et al., 2011, 2013), have been declining in many parts of the world (Goulson et al., 2015; Potts et al., 2010a). Moreover, the western honeybee, *Apis mellifera,* represents the most important species for crop pollination, showing a rapid global growth in managed colony numbers over the past decades (Geldmann and

González-Varo, 2018). However, available data from FAO reveal that during the last half century the growth of the global population of managed honey-bee hives (~45%) is not matching the rapid (>300%) increase in the fraction of agriculture that depends on animal pollination (Aizen and Harder, 2009). This mismatch between demand and supply of pollination services (Breeze et al., 2014; Schulp et al., 2014) suggests that there is an urgent need to develop a more sustainable agriculture by optimizing pollination and agricultural production, while conserving biodiversity (Garibaldi et al., 2014; Geslin et al., 2017) and health of wild and managed bees.
The biology of the western honeybee *A. mellifera* will be briefly described in the next section, with special emphasis on the aspects that are relevant to the present work.

### *1.2 Biology of the western honeybee Apis mellifera*

The honeybee *Apis mellifera* (Hymenoptera: Apidae) is an eusocial insect that lives in colonies where a caste division occurs and only some individuals are capable of reproducing (Winston, 1991). A typical *A. mellifera* colony consists of a reproductive queen (a fertile female), 20,000-60,000 adult workers (non-reproductive females), 10,000-30,000 worker brood (eggs, larvae and pupae) and several hundred drones (reproductive males) (Bailey and Ball, 1991). The comb, made of beeswax produced by worker bees, is the substrate for all colony interactions and consists of cells with different dimension: smaller cells that are used for brood rearing and food storage, larger cells used to rear drones and queen cells, acorn-shaped, built along the lower edge of the combs (Contessi, 2016). The three castes can be easily distinguished due to some morphological differences (Fig.1).

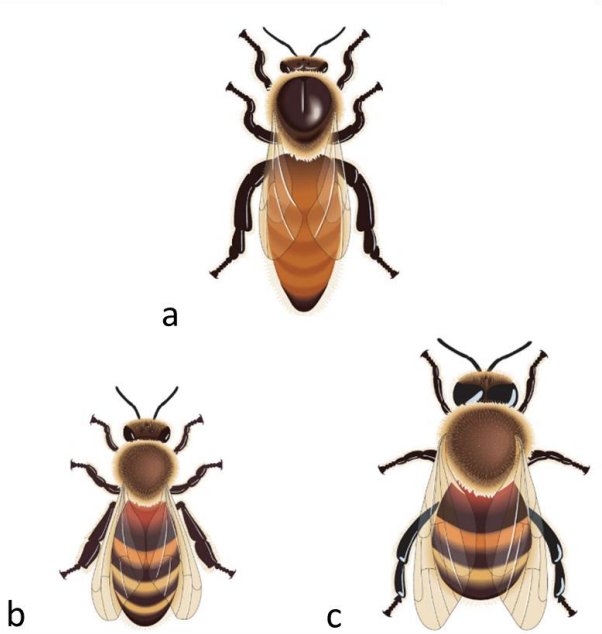



**Fig.1 – Honeybee castes**
a) queen; b) worker; c) drone
(The Visual Dictionary of Animal Kingdom, 2009)

Sex-determination in honey bees is controlled by haplodiploidy: fertilized diploid eggs develop into females, while unfertilized haploid eggs develop into males (Rinderer, 2013). The drones are present only during the early summer in Europe and their sole function is to mate with queens. The queen only leaves the colony to mate, during swarming (colony division) or when a colony deserts a nest site. Her main task is to lay eggs (Denholm, 1999). The queen can control egg fertilization that occurs during oviposition and depends by the type of cell. A fertilized egg has the potential to develop in a fertile queen or a sterile worker, two castes genetically identical, differing only for the type and amount of food received after the first three days of larval development (Contessi, 2016). Nurse workers feed larvae with a protein-rich food secreted by the hypopharyngeal and mandibular glands: the royal jelly (Snodgrass, 1984). If a larva receives a large amount of this brood food throughout the developmental stages it will become a queen; if the same larva receives smaller quantity of royal jelly plus pollen and honey during the latter part of the development it will become a worker (Contessi, 2016). All individuals show the same four developmental stages: egg, larvae, pupae and adult. Eggs are laid by the queen at the bottom of the brood's cells. After three days from oviposition, a small larva emerges and worker bees start immediately to take care of it. The larva develops very quickly and undergoes five molts; during the last larval stage, workers cap the cell with wax and the larva starts spinning a silk cocoon. Into the sealed cell, the larva develops into a pupa, starts the metamorphosis and, 12 days after sealing, it emerges from the cell. The total developmental time from egg to adult is 16 days for queens, 21 for workers and 24 for drones. Life expectation is very different among the different castes: usually a worker lives 4-6 weeks during the summer but can lives 4-5 months during the winter, the drones survive for 3-5 weeks while the queen typically lives 1-3 years (Contessi, 2016).
Among the workers, there is an age-correlated behavioural division of labour, referred to as temporal polyethism (Winston, 1991). Younger workers perform brood-nest associated tasks such as brood-cell cleaning; when they are 4 day old, they become nurses and start feeding older larvae; then later, according to the hypopharyngeal glands' development, they feed the younger larvae. Middle-aged bees typically perform food processing, nest construction, and guarding. Finally, from about 20 days of age, older bees progress to foraging outside the nest for nectar and pollen (Johnson, 2008; Seeley, 1982; Seeley and Kolmes, 1991; Winston, 1991). This polyethism schedule is not perfectly rigid, as bees can engage in other duties, if necessary, according to the colony's needs (Johnson, 2010). Juvenile hormones, food and pheromones act in concert to ensure the reproductive dominance of the queen, the workers' behavioural maturation, the coordination of colony reproduction through swarming and many other functions (Bortolotti and Costa, 2014; Grozinger et al., 2003; Hoover et al., 2003; Moritz and Southwick, 2012; Robinson, 1985).

### *1.3 Honeybee colony losses: the role of Varroa destructor*

Although global stocks of managed honeybee colonies appear to be increasing (Aizen and Harder, 2009), substantial regional losses have been documented in the last decade (Pettis and Delaplane, 2010; Potts et al., 2010b; Stokstad, 2007). In particular, during the 2006/2007 winter an apparently unexplainable high colony

mortality (~30%) affected managed honeybees in the United States (van Engelsdorp et al., 2007). A portion of those losses were characterized by a common set of specific symptoms (mainly the rapid loss of adult worker bees and the lack of dead workers within or surrounding the hives) that subsequently led to define this syndrome as Colony Collapse Disorder (CCD) (van Engelsdorp et al., 2009). Meanwhile the proceedings of the 4th COLOSS Conference (Zagreb, Croatia, 3-4 March 2009) reported a colony mortality of about 20% in Europe, ranging from 1.8 to 53% among countries (Williams et al., 2010). Extensive colony losses are not unusual and have occurred repeatedly over many centuries and locations during the long-lasting history of apiculture (van Engelsdorp and Meixner, 2010; van Engelsdorp et al., 2008; Oldroyd, 2007; Underwood and van Engelsdorp, 2007), but the dramatic dimension of these recent declines attracted the attention of the public opinion and the increasing interest of the scientific community (Stokstad, 2007). After a few years of intense investigation, a specific causal agent for the widespread colony losses was not found, but rather a multifactorial origin was proposed for this syndrome (Neumann and Carreck, 2010).

It is now largely accepted that both landscape deterioration and agrochemicals can be directly or indirectly responsible for colony losses (Goulson et al., 2015). However, several monitoring programs (van der Zee et al., 2012, 2015) indicate that high loads of parasites and pathogens are constantly associated with this problem (Neumann and Carreck, 2010), and, in particular, the symbiotic association between *Varroa destructor* and Deformed Wing Virus (DWV) (Di Prisco et al., 2016) account for the large majority of losses (Kielmanowicz et al., 2015). The mite *V. destructor* is an obligate ectoparasite of bees which feeds on host's haemolymph and fat body (Ramsey and van Engelsdorp, 2017; Ramsey et al., 2018; Rosenkranz et al., 2010).

This parasite has a severe impact on honeybee physiology, causing the reduction of weight at the emergence and shortening the life span of the host (Annoscia et al., 2012; Daly et al., 1988; Jong et al., 1982). Furthermore, *V. destructor* acts as vector of viral pathogens such as deformed wing virus (DWV), sacbrood virus (SBV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV), black queen cell virus (BQCV) and Kashmir bee virus (KBV) (Tentcheva et al., 2004). The best known is DWV infection, which causes the symptoms of crippled wings and shortened abdomens in individuals from heavily infested colonies (Boecking and Genersch, 2008). DWV, as the other viruses, has been considered a minor problem to honeybee health before the occurrence of *Varroa* mites (Bowen-walker et al., 1999). The vectoring activity and the immune stress caused by the mite *Varroa destructor*, a relatively novel parasite for *Apis mellifera*, promoted the spread and the uncontrolled replication of this virus, triggering the transition of common harmless covert infections to devastating overt infections (Nazzi and Le Conte, 2016; Nazzi et al., 2012; Wilfert et al., 2016). After the arrival of *Varroa* in the 1970s and 1980s, the rate of colony loss in Europe and North America nearly tripled (Ellis et al., 2010). In absence of effective control measures of this parasite, an infested colony cannot survive longer than 2-3 years (Fries et al., 2006; Rademacher and Harz, 2006).

In order to have a better understanding of the mechanisms underlying this dramatic effect on honeybee colonies, in the next two sections the biology of the mite *V. destructor* (Section 1.4) and the immunity of honeybees (Section 1.5) will be discussed.

### *1.4 Biology of Varroa destructor*

The mite responsible for the abovementioned clinical symptoms in *A. mellifera* belongs to the species *V. destructor* (Acari: Varroidae), which was erroneously considered as *Varroa jacobsoni* until the year 2000 (Anderson and Trueman, 2000). Therefore, every *Varroa* publications of the last century refer to *V. jacobsoni* although in nearly all cases the research subject was *V. destructor,* the only mite of economic importance (Rosenkranz et al., 2010). *V. destructor* successfully shifted from its original host, the Asiatic honeybee *Apis cerana,* to the western honeybee *A. mellifera* when this latter were transported to the Far East in the first half of the past century (Oldroyd, 1999). Rapidly spread by beekeeping activities, *V. destructor* were found on *A. mellifera* in the eastern coastal region of the USSR (1952), in Pakistan (1955), Japan (1958), China (1959), Bulgaria and ex Jugoslavia (1967), South-America (Paraguay, 1971), Germany (1977), Italy (1981), USA (1987), UK (1992), New Zealand (2000), Hawaii (2007) (Fontana, 2017; Rosenkranz et al., 2010). Today the mite is cosmopolitan, except for Australia, where on some occasions has been detected in hives on docking ships, but without consequences (Fontana, 2017; Rooth, 2018). In this section morphology, life-cycle and behaviour of *V. destructor* will be discussed, emphasizing the aspects that are relevant to mite feeding and the linked effects on honeybees.

#### *1.4.1 Morphology*

*V. destructor* displays a marked sexual dimorphism (Ifantidis, 1983): females are flattened, oval-shaped (1.6 x 1 mm); males are pear shaped and show only weak sclerotisation (Fig.2).
Males are clearly smaller than females in all developmental stages (Rosenkranz et al., 2010). In the adult phase they are easily distinguishable by colour: males are light-yellow while females have a reddish-brown colouration.

**Fig. 2 – Varroa destructor mature and immature stages**
Clockwise from top left: mature daughter mite, mother mite, two mature males and an immature (deutonymph) daughter (Huang, 2012).

The body of *Varroa* mites, like in other species belonging to the Acari group, is subdivided in two well defined parts: a complex of mouthparts called gnathosoma (Greek: *gnathos* = jaws, *soma* = body) and the unsegmented idiosoma (Greek: *idio* = belonging to one's self). The gnathosoma (Fig. 3) consists of:

- the hypostome, which ventrally delimits the gnathosoma and dorsally forms a furrow for food and saliva;
- the pair of chelicerae, situated above the hypostome, enclosed in a protrusion of the exoskeleton from which they can be protruded or retracted;
- the labrum, between the two chelicerae;
- two sensory pedipalps (Bautz and Coggins, 1992; Lucius et al., 2017).

The distal part of the chelicera is the movable digit (*digitus mobilis*), straight and spearshaped, provided with a sharp ventral edge and two thin dorsal hook-like teeth. Together the movable digits constitute a well-adapted saw-like structure to pierce the honeybee integument (Griffiths et al., 1988; de Lillo et al., 2001).

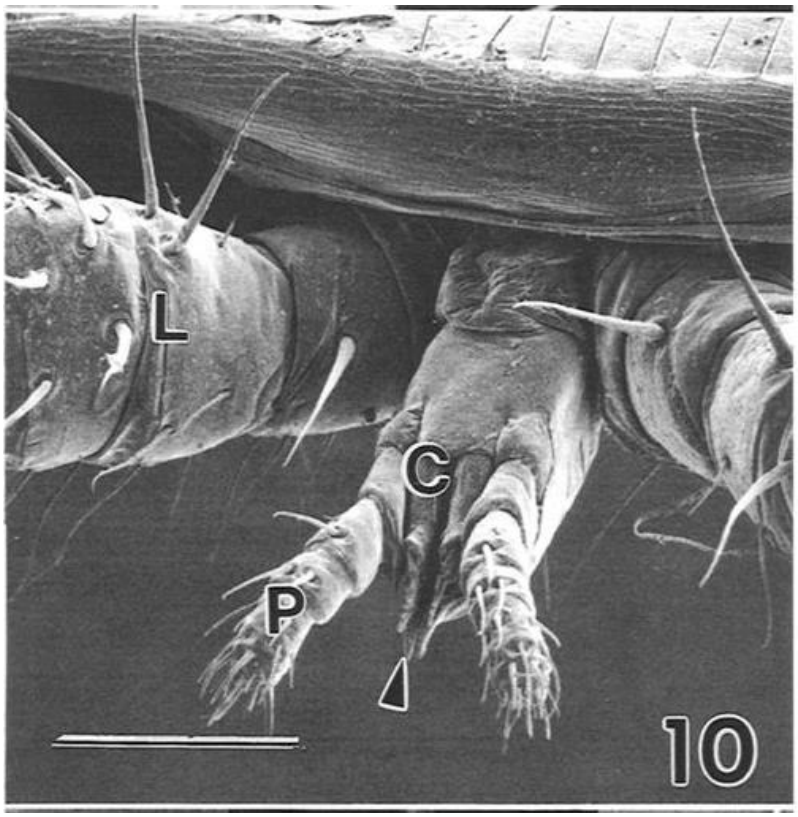



**Fig. 3 – Anterior gnathosoma**
The gnathosoma consists of a pair of lateral pedipalps (P) and a pair of modified chelicerae (C). Note the pointed tip of the hypostome legs (arrow). The first pair of legs (L) are visible. Scale bar 150 µm (Bautz and Coggins, 1992).

The idiosoma comprises one dorsal shield and different ventral shields (Lucius et al., 2017). Thin and flexible membranes between the shields enable the mite to dilate during feeding and egg formation (Rosenkranz et al., 2010). Four pair of legs, short and strong, are attached to the idiosoma and show specialized structures that facilitate the adherence to the host, the apoteles (De Ruijter and Kaas, 1983). The body and extremities, legs included, have an enormous range of bristles, the *setae*, which act as mechano- or chemoreceptors that help the mite to be oriented in the darkness of the hive (Lucius et al., 2017; Rosenkranz et al., 2010).

### *1.4.2 Life-cycle*

The life cycle of *Varroa* mites involves two distinct stages: the phoretic phase, spent on the adult bee and the reproductive phase, spent inside a bee brood cell (Fig. 4). Carried by nurse bees and guided by chemical cues, *Varroa* mated female enters a brood cell containing a 5$^{th}$ instar bee larva 15 to 20 h before worker brood cells are sealed and 40 to 50 h before drone brood cells are sealed (Boot et al., 1992; Nazzi and Le Conte, 2016). The mite uses bee brood pheromone as a kairomonal cue to choose the cell to invade, showing a strong preference for drone brood (Le Conte et al., 1990; Nazzi and Le Conte, 2016; Zuk and Kolluru, 1998). The invasion of the brood cell represents the beginning of the reproductive phase. After entering the cell, the mite reaches the bottom, where becomes stuck within the larval food, probably to avoid detection and removal by hygienic bees (Ifantidis, 1988; Rosenkranz et al., 2010). When, about 5 hours after cell capping, the honeybee larva has consumed all the food and starts to spin the cocoon against the cell wall, then the *Varroa* female climbs on the host and feeds itself for the first time (Donzé and Guerin, 1994; Rosenkranz et al., 2010). Oogenesis begins rapidly and the first egg is laid on the surface of the cell wall about 70 hours after the capping (Ifantidis, 1983; Steiner et al., 1994). This first egg is normally unfertilized and, due to the haplo-diploid sex determination system, it develops into a haploid male, while subsequent eggs are fertilized and laid every 30 hours to give rise female progeny (Martin, 1994; Rehm and Ritter, 1989). Reproductive phase ends when the adult bee hatches, thus usually a *Varroa* female can lay up to five eggs in worker brood (12 days of capping) and up to six eggs in drone brood (14 days of capping) (Garrido and Rosenkranz, 2003). In the reproductive phase, a “foundress” mite (the mite invading the cell) will produce only one male and an average of 1.30–1.45 mature females when infesting worker cells, but close to double this amount when infesting drone cells (Martin, 1994).
When the prepupa moults into a pupa a major reduction in the free space available for *Varroa* occurs. Immediately after pupation the foundress engages in “leg-pushing”, resulting in the displacement of the pupa’s third legs, to enlarge the space around the so called “fecal accumulation site” (where the mites aggregate in the cell and defecate) (Donze et al., 1998). Then, near this site, the mother mite creates a hole in the cuticle of the pupa, generally on the 2$^{nd}$ sternite, for her offspring to feed trough (Donzé and Guerin, 1994).
Preimaginal development of *Varroa* mites consists of egg, proto- and deutonymph stages (Donzé and Guerin, 1994; Rehm and Ritter, 1989). *Varroa* mites become sexually mature immediately after the last molt, about 6 days after oviposition (Donzé et al., 1996). The offspring mate with each other within the sealed brood cell, at the fecal accumulation site. The foundress and the mated daughters leave the cell when

the newly adult bee emerges (Nazzi and Le Conte, 2016). They then transfer onto a nurse bee, where they spend the phoretic phase, before entering a brood cell to reproduce again (Le Conte and Arnold, 1987). Male mites cannot survive outside the cell, and die. Female mites can go through two or three cycles over the course of their life span (Fries and Rosenkranz, 1996).

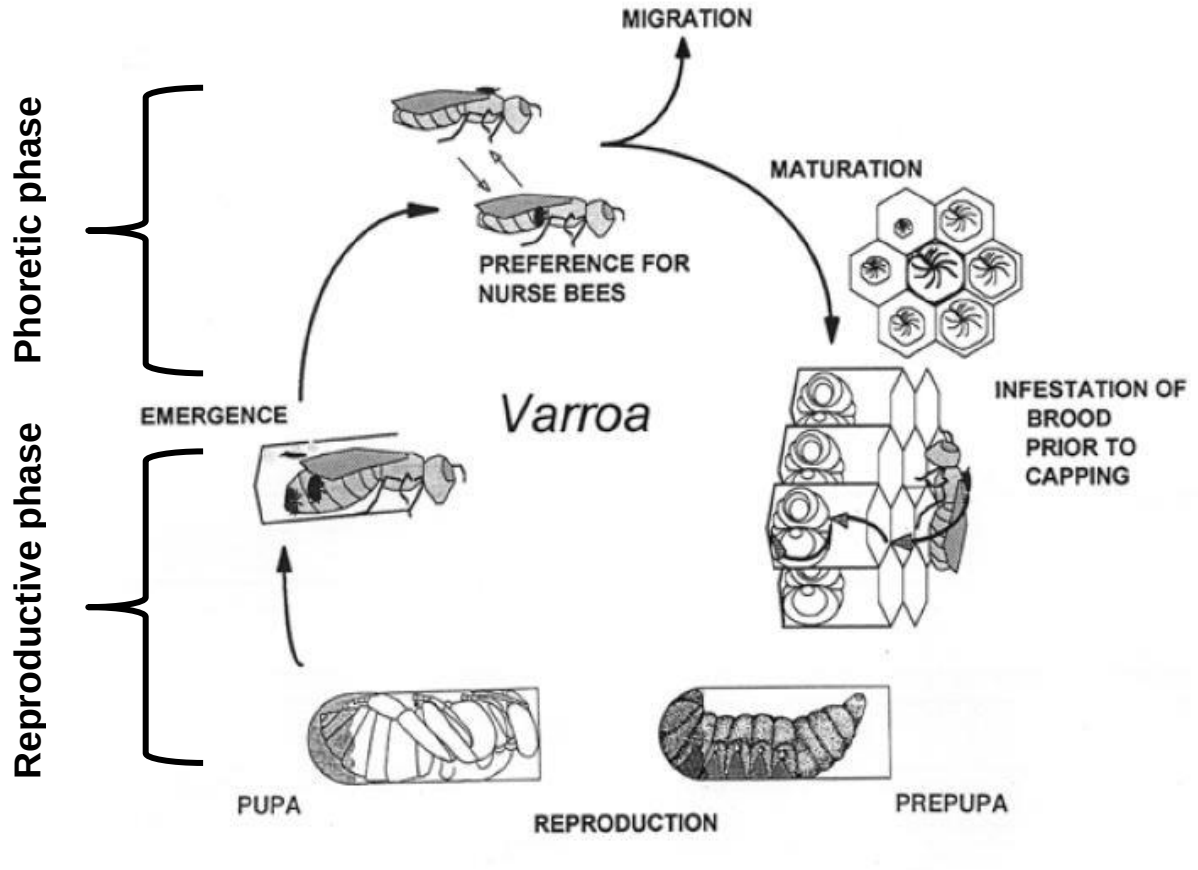



**Fig. 4 – *Varroa* life cycle**
After a phoretic phase on the adult bee, mite adult females invade the brood cells immediately before capping, start sucking haemolymph from the larva and reproduce (Donzè et al., 1998).

### *1.4.3 Feeding*

Feeding behaviour is difficult to observe and is an aspect of the mite's biology which is still relatively poorly understood. Despite a lack of experimental evidence, for the past 5 decades the conclusion that *Varroa* feed exclusively on the haemolymph (the "blood") of host bees has been the accepted view among researchers and beekeepers. Recent work, however, has shown that *Varroa* is not consuming haemolymph, but damages host adult bees by consuming fat body (Ramsey and van Engelsdorp, 2017; Ramsey et al., 2018). The fat body is analogous to mammalian liver and adipose tissue, and is abundant in insects' abdomen where it is localized subjacent to the epidermis (Keeley, 1985). During larval and pupal stage of

honeybee fat cells are detached to each other and are floating free in the haemolymph (Snodgrass, 1984). In the mature worker fat body consists of a thin layer of cells spread against the dorsal and ventral body wall, more developed in nest bees compared to foragers (Chan et al., 2011; Snodgrass, 1984). Fat body is the central storage depot of nutrients and energy reserves (Arrese and Soulages, 2010). This tissue is integral to proper immune function, pesticide detoxification, overwinter survival and several other essential processes in healthy adult and immature bees (Lemaitre and Hoffmann, 2007).

During the phoretic phase, mites are frequently found hiding between the abdominal sternites of the bee in a position that is difficult for other bees to reach (Nazzi and Le Conte, 2016). From this favourable position the phoretic mite is able to feed herself piercing the membrane between the overlapping abdominal plates (Ramsey et al., 2018). The importance of these meals for the mite is probably negligible for the reproductive success, but has relevant consequences on the viral level of the mite. Indeed, *V*. *destructor* induces more wing deformity in emerging honeybees when it has experienced a longer phoretic phase, because of the Deformed Wing Virus transmission (Piou et al., 2016).

During the host's pupal stage, adult females spend nearly 60 minutes to perforate the integument of bee pupae in such a way that they and their progeny can feed (Donzé and Guerin, 1994; Rosenkranz et al., 2010). The mother restricts her feeding to one site that is usually localized on the 2$^{nd}$ abdominal segment and never on head or torax, thus avoiding possible damage to the developing appendages of the host bee, essential for the cell opening at the emergence (Donzé and Guerin, 1994; Kanbar and Engels, 2004a). Following penetration of the intersegmental membrane by the bidentate chelicerae, the contact chemosensillae, chemosensitive receptors located distally on the movable digits, are immersed into the wound, providing a sensory feedback on the quality of the food before and during extra-oral digestion (de Lillo et al., 2001). Strick & Madel (1988) state that *Varroa* uses its chelicerae to 'rasp' a wound, first by inserting the tip of the dentate chelicerae into the membrane and then by making 'alternate sawing movements of the chelicerae (Strick and Madel, 1988). Then the mite can insert its hypostome to inject saliva and aspirate liquid tissues of the pupa (Bautz and Coggins, 1992; Nuzzaci and de Lillo, 1995), with a mechanism similar to blood sucker arthropods (Griffiths et al., 1988). However, *Varroa* is phylogenetically more closely related to predatory mites (i.e. *Gamasiphis*, *Macrocheles*, *Lasioseius*) (Klompen et al., 2007) that feed through a different feeding mechanism: the extra-oral digestion. Recent findings by Ramsey et al. (2018) on parasitized adult bees show degradation of fat body cells beneath the pierced intersegmental membrane, likely due to the extra-oral digestion of the mite at the feeding site.

Very little is known about *Varroa* mite saliva, but, due to the abovementioned findings, mite's salivary secretions are likely involved into host manipulation and exploitation. In the first study on *V. destructor* saliva bioactivity, Richards et al. reported that mite's salivary secretions damage haemocytes (invertebrate immune system cells) from the caterpillar *Lacanobia oleracea* and disrupt their ability to form aggregates (Richards et al., 2011). A recent work identified a *Varroa* toxic protein (VTP), in the saliva of the mite, which exhibits toxic activity toward *A*. *cerana* preimaginal stages and elevates DWV titer in *A. mellifera* (Zhang and Han, 2018a). Investigating the poorly known feeding behaviour and the role of salivary effectors represents an important step towards understanding the molecular

mechanisms of the host-parasite interaction and for developing new effective control strategies.

### *1.5 Pathology of Varroa destructor parasitization*

Feeding activity of the mite has profound implications for the honeybee, both at individual and colony scale. At the individual level, honeybee is damaged by *Varroa destructor* in a variety of ways, ranging from physiological to behavioural alterations. Parasitization affects longevity of honeybees (Dainat et al., 2011; Yang and Cox-Foster, 2007). De Jong reported that uninfested control bees live an average of 27.6 days, while infested ones only 13.6. The number of mites per bee is significantly negatively correlated with both longevity and weight at emergence (De Jong et al., 1983).

The loss of haemolymph and fat body during ontogenetic development decreases the weight of worker at the emergence of 7% (Jong et al., 1982). Drones are even more susceptible, varying from 11% to 19% of weight loss, depending on the infestation rate (Duay et al., 2003). The weight loss seems to be related, not just to direct feeding of the mites, but also to the alteration of cuticular hydrocarbons and the consequent water loss through cuticular transpiration (Annoscia et al., 2012; Salvy et al., 2001).

*Varroa* parasitization impairs the over-wintering capacity of honeyebees, through the reduction in protein, mainly vitellogenin, and carbohydrate content of haemolymph (Amdam et al., 2004; Bowen-Walker and Gunn, 2001). The yolk protein vitellogenin is produced in the fat body as a storage protein and have evolved alternative functions in honeybees, regulating immunity and longevity (Amdam et al., 2003; Tufail and Takeda, 2008). *Varroa* affects also juvenile hormone titre, hypopharingeal glands development, learning ability and attraction to brood (Zanni et al., 2018). Together these alterations contribute to premature transition to foraging in worker bees parasitized during development (Alphen et al., 1996; Amdam et al., 2004; Jong et al., 1982). When honeybees switch from the hive bee to the forager stage, their cellular defence machinery is down-regulated by a dramatic reduction in the number of functioning haemocytes (Amdam et al., 2003). But in emerging parasitized bees a stronger reduction of the functioning haemocyte proportion in haemolymph is observed (Amdam et al., 2004).

Furthermore, several authors reported alterations of behaviour in *Varroa* infested honeybees. Parasitized foragers show a decreased capability of non-associated learning, prolonged absences from the colony and a lower rate of return to the colony (Kralj and Fuchs, 2006; Kralj et al., 2007).

Considering the colony as a “superorganism”, *V. destructor* impacts the infested honeybee fitness by lowering the drones chance to mate (Duay et al., 2002) and reducing the capacity of parasitized colonies to produce swarms (Fries et al., 2003; Villa et al., 2008).

The final breakdown of a honey bee colony is associated with the typical “parasitic mite syndrome” (see Section 2) such as scattered brood, crippled bees and unexplainable reduction of the adult bee population (Rosenkranz et al., 2010). It is thus clear that *V. destructor* has a dramatic impact on physiology, immunity and behaviour of honeybees. However, it is difficult to distinguish between the direct effects of *Varroa* parasitization and indirect effects related to other parasites, mainly viral pathogens, vectored through feeding.

### *1.5.1 Varroa destructor as a vector*

The feeding activity of *Varroa* mites on honeybess not only subtracts nutrients but also promotes proliferation and transmission of several pathogens. Observing ultrastructural features of the feeding hole made by the foundress on honeybee pupa, Kanbar and Engels (2003) reported that the repeated and communal use of the wound, especially in multiple infested cells, is often associated with delayed healing. The remaining opening allow pathogens to grow at the feeding site and enter the host's body. Large colonies of *Melissococcus plutons*, the European foulbrood agent, were found in wounds on worker and drone pupae (Kanbar and Engels, 2003; Kanbar et al., 2004). Furthermore, manipulative experiments have demonstrated that *Varroa* can transmits bacteria, leading to septicemia in infested larvae (Gliński and Jarosz, 1992). Honeybee's haemocele invasion and subsequent septicemia it happens only for some harmful bacteria, but represents just one of the possible deleterious effects of bacteria vectored by the mite (Hubert et al., 2016). Indeed, Hubert et al. (2017) reported that regular microbial community, integral of many nutritional and immune functions, is altered in parasitized bees. The author suggests that this disruption of immune homeostasis in the gut is triggered by the immune response of the host toward bacteria vectored by *Varroa* (Hubert et al., 2017). Different routes of bacterial transmission have been proposed, such as external contamination of mouthparts (*dirty needle*) and acquisition through infected host fluids ingurgitation (*brush*). However, the mode of transport of bacteria via mites seems to be passive, because there is no evidence of bacterial replication inside the body of the mite, which is the criterion for active transport and vectoring (Hubert et al., 2016).

*Varroa* can also be responsible of fungal spores (conidia) dispersion throughout the bee colony. Soil saprophytes are normally associated with bees, their combs and provisions. The most abundant species found in this context are (in order of abundance): *Aspergillus, Penicillium, Fusarium, Trichoderma, Alternaria, Rhizopus* and *Mucor* (Benoit et al., 2004). Some fungi have been shown to be beneficial to the colony by preserving stored pollen and bee bread, whereas others are pathogenic and contribute to colony losses (Gilliam, 1997; Morse and Nowogrodzki, 1990). *Aspergillus flavus*, the agent of stonebrood disease in *A. mellifera* larvae, known to attack and damage the brood by producing a neuropathic aflatoxin (Batra et al., 1973; Glinski and Buczek, 2003), has been isolated from the external surfaces of *V. destructor* (Benoit et al., 2004). Although stonebrood is a rare disease considered to be of minor importance (Morse and Nowogrodzki, 1990), the immune stress caused by the facultative parasite *A. flavus* has been poorly investigated.

The aforementioned pathogens are passively transported by *Varroa destructor* and became harmful only in some cases. Indeed, transmission routes, dynamics of infection and effects at colony level are considerably different for viruses, in particular for DWV, which can replicate within the mite's body (Di Prisco et al., 2011; Gisder et al., 2009; Sumpter and Martin, 2004; Yue and Genersch, 2005).

Honeybees can be infected by 15-20 different viruses and many of them can be vectored by *Varroa* mites (Chen and Siede, 2007), including Acute Paralysis Virus (Ball, 1985), Slow Bee Paralysis Virus (SBPV) (Denholm, 1999) and Deformed Wing Virus (Bowen-walker et al., 1999). Much of the pathology and mortality observed in severely mite-infested bee colonies is linked to the mite-mediated transmission of viruses (Martin, 1998). Transmission occurs during feeding, which consists in a continuous interchange of fluids between the *Varroa* mite and the haemocele of the

host. As a result of this process, acquiring and injecting virus particles, the mite boosts the viral load of individual bees and promotes the spread of viral diseases at the apiary scale (Rosenkranz et al., 2010).
The major cause of widespread bee colony losses is DWV, which is normally present in managed honey bee populations as covert asymptomatic infections, supported by horizontal and vertical transmission (Boecking and Genersch, 2008). Acting in synergy with other stressors, *V. destructor* disrupts the delicate balance between the bee and the virus, promoting the transition of common sub-lethal infections to devastating overt infections (Nazzi and Pennacchio, 2018).
The mechanism of this switch will be discussed more in detail in the next section, after a brief description of the immune system and the defense strategies of the honeybee toward pathogens.

### *1.5.2 Honeybee immunity and viruses: a matter of stress*

A deeper knowledge on the bees' immune traits is central in the comprehension of how Varroa-DWV interaction, in synergy with several immune stressors, can lead to colony collapse (Nazzi et al., 2012).
The in-hive environment, with high population density, low genetic diversity, a constant temperature of about 34 °C, a high humidity and the presence of stored food, represents an ideal habitat for the proliferation of bacteria, fungi and other pathogens (Baracchi et al., 2011; Cremer et al., 2007). Nevertheless, honeybees are predicted to express only two-thirds as many immunity genes as solitary insects, suggesting a major role of social immunity to contrast parasites and pathogens (Barribeau et al., 2015; Chan et al., 2009). In fact, honeybees, as other social insects and primates, have evolved social defences, based on collective action or altruistic behaviours of infected individuals that benefit the colony (Cremer et al., 2007).
These super-organism defences can be prophylactic, such as the nest coating with antimicrobial material as propolis, to prevent fungal and bacterial growth (Simone et al., 2009). Other defences are activated on demand, for example, the removal of infected larvae or the social fever, whereby many bees simultaneously raise their body temperature to heat-kill bacteria in their hive (Starks et al., 2000).
Regarding the individual defence, likewise other insects, honeybees adopt several strategies to combat infections and infestations. These strategies involve the use of physical barriers against the external invaders, such as the cuticle covering the insect body, or the perithrophic membrane which protects the gut (Boucias and Pendland, 2012). If these barriers are crossed, honeybees can rely on very efficient cellular and humoral responses. Upon injury, the immediate onset of two proteolytic cascades occurs, leading to localized blood clotting and to melanization. Bacteria are engulfed by haemocytes through phagocytosis, while these immune cells, circulating in the haemolymph encapsulate and kill larger parasites. The fat body synthesizes potent antimicrobial peptides which are secreted in the haemolymph, where they act synergistically to kill the invading microorganisms (Hoffmann, 1995). This innate immune response shows an architecture and specific orthologs components that are shared with vertebrates (Beutler, 2004). Evans and collegues reported canonical immunity pathways in honeybees, such as Toll, Imd, Janus kinase (JAK) STAT and JNK (Evans et al., 2006).

Recent studies indicate that the Toll pathway contributes to antiviral defences against DWV infection, which at low viral loads is asymptomatic and occurs in the nearly totality of hives (Brutscher et al., 2015; Nazzi and Pennacchio, 2014).
DWV covert infections are not due to a low pathogenicity of the virus but rather to the fact that antiviral barriers are able to contain viral infection in honey bees when immuno-competence is not impaired by external stress agents (Nazzi and Pennacchio, 2014). The main stressors affecting bees are the decline in abundance and variety of flowers, the chronic exposure to aghrochemicals and the novel parasites spread by human activities (Goulson et al., 2015). These factors can interfere with the equilibrium underpinning DWV covert infections, and eventually result in uncontrolled viral replication (Nazzi and Pennacchio, 2014).
In particular, any stress factor competing for metabolic resources or negatively acting on NF-kB signaling, integral of the immune barriers against viruses under the Toll pathway, can trigger unbound replication (Di Prisco et al., 2013; Nazzi et al., 2012). The increasing viral infection is a self-boosted process, as the growing DWV titer generates an escalating immunosuppression, by targeting NF-kB signaling through an as yet unknown mechanism, which could be further complemented by poorly explored immunosuppressive effects mediated by *Varroa* feeding and saliva injection (Richards et al., 2011; Yang and Cox-Foster, 2007). Understanding the mechanisms underlying the impact of *Varroa* on honeybee immunocompetence is of key-importance and will provide new background information to develop targeted strategies of colony losses management.

### *1.6 Varroa mite control*

The mite *Varroa destructor* made apiculture a difficult activity, due to the great efforts focused on controlling the mite population (Fontana, 2017). Without any doubt, most of the colonies of *A. mellifera* in temperate climates will be damaged or even collapse within a few years if no control or inappropriate control methods are used (Boecking and Genersch, 2008). The first compounds used against *V. destructor* were synthetic acaricides, such as the organophosphate coumaphos and the pyrethroids tau-flavulinate (Milani and Barbattini, 1988; Ritter and Schneider-Ritter, 1988). Although some of these products are easy to apply and economically convenient, they have important disadvantages:

- they accumulate in wax, honey and other bee products (Chauzat et al., 2009; Johnson et al., 2009);
- they can harm bees continuously exposed to multiple compounds stored in wax (Lodesani et al., 2008; Martel et al., 2007);
- they can cause acaricide resistance in mites exposed to low levels of the compounds stored in wax (Fries et al., 1998).

Due to these drawbacks, it became necessary to include alternative methods within the often chemical biased *Varroa* control strategies (Lodesani and Milani, 2004; Milani, 2001). Organic acids and essential oils, such as formic acid, oxalic acid, lactic acid and thymol, represent the natural compounds used for the control of *Varroa* mite (Rosenkranz et al., 2010). These compounds are sufficiently efficient against *V. destructor* (Fries, 1991), without persisting as residue in wax (Bogdanov, 2006) and without eliciting resistance in the mite population (Rosenkranz et al., 2010). Thus, nowadays, especially in Italy, the methods to control *Varroa* mainly rely on the use of organic compounds (acids and essential oils) combined with beekeeping techniques

aiming at increasing the phoretic population of the mite, easier to reach by such treatments (Fontana, 2017). However, some of these natural compounds can be applied only under certain climatic or colony conditions (i.e. broodless colonies for oxalic acid) (Emsen and Dodologlu, 2009; Higes et al., 1999) and their usage has to be carefully tuned for optimal effect, because the range between efficacy on the parasite and toxicity for the host is not very large (Bolli et al., 1993; Mattila et al., 2000). In general this means that the effects from organic acids and essential oils often are more variable, compared to registered acaricides (Rosenkranz et al., 2010).
Thus, alternative measures for *Varroa* control are needed in order to be integrated in the management of the mite. In this sense research efforts are directed toward (1) the selection of *Varroa* resistance traits suitable for breeding programs (Büchler et al., 2010), (2) the disruption of semiochemicals cues used by the mite for host finding and mating (Iovinella et al., 2018), (3) the interference with vital functions of *Varroa destructor* through gene knockdown (RNA interference – RNAi), mediated by dsRNA administration (Garbian et al., 2012).
Several studies have demonstrated that RNAi approaches can successfully reduce parasite and pathogen loads in bees. These tools are very promising, since they can specifically target genetic sequences in pathogens and parasites, thereby reducing off-target effects (Grozinger and Robinson, 2015). In the case of *Varroa*, it has been reported a bidirectional transfer of the dsRNA: when dsRNA was fed to the bee it passed from bee gut to the haemolymph, and from the haemolymph to the *Varroa*, and then back to the bee (Garbian et al., 2012). However, there has been indication that off-target effects can occur, and thus additional testing should be performed to ensure that dsRNA treatments do not cause unintended effects on bees, in particular on their immunocompetence (Grozinger and Robinson, 2015). It is, therefore, mandatory to select adequate target genes and to consider the impact of their silencing on honeybee physiology and development. Furthermore, the dsRNA delivery should be always optimized, taking in account the details of *Varroa* biology and behaviour, which are still partially unexplored.

## AIM OF THE WORK AND EXPERIMENTAL APPROACH

*V. destructor* is a stress factor of crucial importance in the induction of honey bee health decline and colony losses. Together with the worldwide decline of wild pollinators, this mite could severely affect the ecological services underlying plant and crop pollination (De la Rúa et al., 2009). The lack of knowledge on some aspects of the host-parasite interaction represents an obstacle to the development of new control strategies of *Varroa*. In particular, the absence of information about the sialome of the mite limits the functional analysis of the host regulation process and the opportunity to impair the mite's fitness using biotechnological approaches.
We hypothesized that proteins injected by *V. destructor* during feeding (i.e. salivary proteins) can positively affect mite fitness and their suppression through RNAi could offer the opportunity to develop new control strategies.
The aim of the first part of the present work was to identify salivary proteins by a dual approach, based upon both in silico and wet lab techniques. Due to the lack of accessible experimental data regarding the mite's saliva, the sialome of *V. destructor* was predicted through a dedicated in-house pipeline based on the detection of the

signal peptide and the subsequent annotation of the putatively secreted proteins. Several candidates were selected and validated by qPCR and, then, by in-situ hybridization. This process allowed the identification of a putative virulence factor specifically expressed in the salivary glands of *V. destructor*, which was subsequently studied from a functional point of view.
The aim of the second part of the present work was to functionally characterize the identified salivary component, assessing the effects of gene knockdown on mites and parasitized bees. *In vivo* studies were based on silencing the identified coding gene in the mite by dsRNA administration through a non-invasive approach. The effectiveness and the time course of the silencing were then assessed by qRT-PCR and associated with the observed level of mortality on targeted *Varroa* mites. We finally scored the impact of this salivary effector on honeybee immune response by determining the expression profile of a set of immune genes in parasitized hosts.

## MATERIALS AND METHODS

### *2.1 Secretome prediction of Varroa destructor*

The *Varroa* mites secretome prediction was carried out filtering the *V. destructor* protein sequences for the presence of the signal peptide. Protein sequences in fasta format were downloaded from the last *Varroa* genome annotation (Vdes_3.0, October 2017) available on the NCBI site and used as input file for SignalP 4.0 (Petersen et al., 2011). The resulting subset of proteins containing the signal peptide was then annotated following two different approaches:
1) By sequence identity, using the Blast software (Altschul et al., 1990) through the Trinotate pipeline (Haas et al., 2013), on the following databases: venom proteins of Hymenoptera species available at NCBI (using keyword 'venom AND Hymenoptera [organism]'); "saliva-related" tick's and mite's reference proteins available at NCBI (using keyword 'saliva AND Acarina [organism]'); Uniprot/Swiss-Prot database.
2) By predicting the presence of domains and important sites (signatures), using Interproscan (Jones et al., 2014).
Trinotate also provided KEGG db annotation and transmembrane domain prediction using TMHMM (Bryant et al., 2017; Krogh et al., 2001). Venn diagram of annotated protein was plotted using the online tool Venny 2.1 (Oliveros). Gene ontologies obtained from Uniprot/Swiss-Prot and Interproscan annotation were plotted using WEGO software (Ashburner et al., 2000; Ye et al., 2006).

### *2.2 Biological material*

*Varroa* mites and honey bees used in this study were collected from brood combs of *A. mellifera* colonies maintained at experimental apiaries based in Portici (Napoli, Italy) and in Newburgh (Aberdeenshire, UK). Brood frames were collected between June and September and stored at 32°C ± 1°C, 40% ± 2% relative humidity, under dark for 24-48 hours or processed immediately. Sealed brood cells were uncapped

and checked for worker pupae (brown-eyes stage) and adult females of *V. destructor*.

### 2.3 *Salivary glands dissection*

*Varroa* adult females were collected and placed on double-side tape in a dissection plaque. Each mite was incised in the posterior and lateral region, and submerged in 30 µL of PBS 1x on a microscopy slide. Using fine tweezers and dissection needles, the mite's carapace was gently lifted-up and the salivary glands were isolated from other tissues. Pools of 5-10 mites were processed as above and the dissected tissues were stored in 200 uL of TRIzol Reagent (Thermo Fisher) at – 80°C.

### *2.4 RNA isolation*

The RNA extraction was performed, after freeze-thawing the samples, using TRIzol Reagent (Thermo Fisher), according to the manufacturer's instruction. Final pellet was resuspended in 16 µL nuclease-free $H_2O$ and quality and quantity of the isolated total RNA were evaluated measuring the absorbance with Varioskan Flash (Thermoscientific). For mite tissues, extraction methods resulted in retention of significant amounts of contaminating DNA; therefore RQ1 RNase-Free DNase (Promega) digestion after RNA isolation was performed at 37°C for 30 minutes, followed by the addition of 1 µl 25 mM EDTA (pH 8.0) and a 10 minute incubation at 65°C.

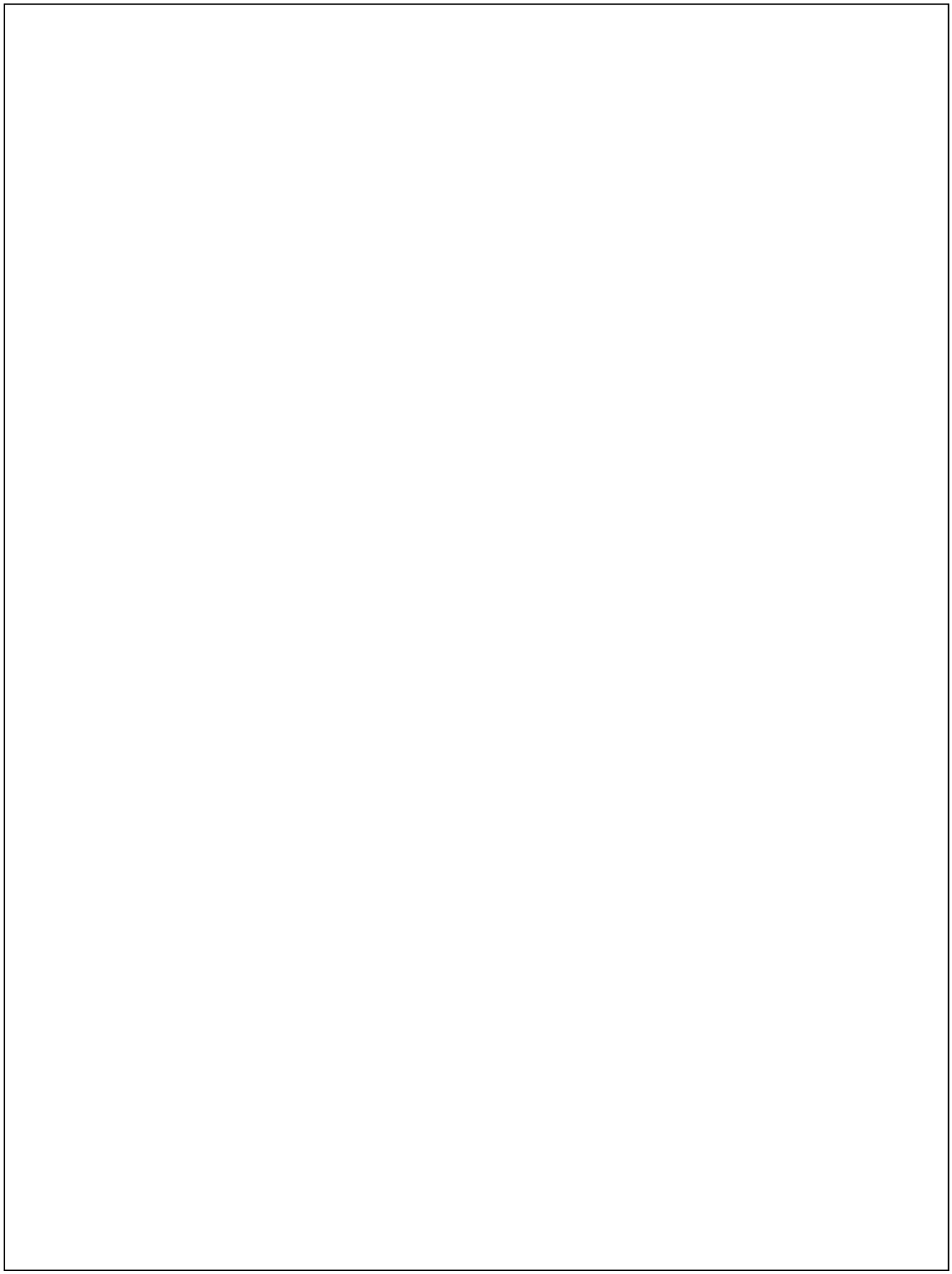
### *2.5 Quantitative real time PCR (qPCR) analysis*

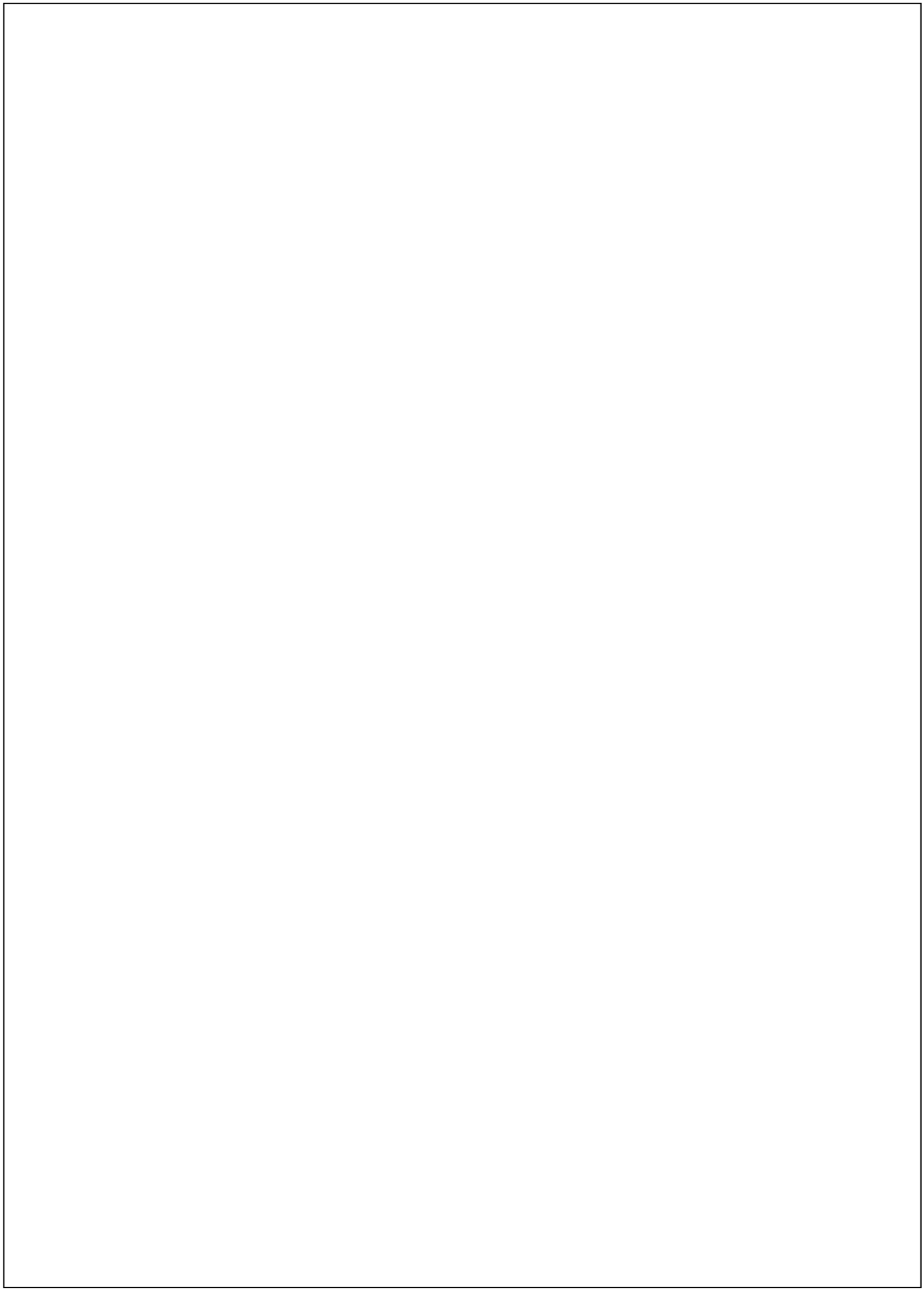
According to literature and to in-house proteomic data, five targets among the protein families identified by the previous in silico analysis were evaluated with qPCR to assess their specific expression in *V. destructor* salivary glands. The criteria for the selection of candidates were (1) the homology with known effector in parasite- and parasitoid-host studies; (2) the presence of peptides in a LC-MS/MS study on *V. destructor* saliva (Campbell, unpublished); (3) the lack of transmembrane domains detected by TMHMM (included in the Trinotate annotation step)(Krogh et al., 2001).
Differential relative expression of genes putatively expressed in salivary glands was measured by one-step qRT-PCR, using the Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems, Carlsbad, CA,USA), according to the manufacturer's instructions. Each reaction was prepared in 20 µL and contained 10 µL RT-PCR mix 2x, 100 nM of forward and reverse primer, 0.16 µL of RT enzyme, DEPC treated water and 2 µL of RNA (25 ng/ µL). For the experimental run the following cycle profile was used: 48 °C for 30 min (RT); 95°C for 10 min; 40 cycles at 95°C for 15 s, 1 min at 58°C; a last cycle for 15 s at 95°C, 60s at 58°C and 15 s at 95°C for dissociation curve. Each sample was analyzed in triplicate on a Step One Real Time PCR System (Applied Biosystems). The 18S gene of *V. destructor* (Accession Number: XM_022831401.1) was used as endogenous control for RNA loading (Campbell et al., 2016). All primers for putative salivary genes were designed using Primer Express version 1.0 software (Applied Biosystems) (Table 1). Relative gene expression data were analyzed using the $2^{\Delta\Delta CT}$ method (Livak et al., 2001, Pfaffl et

seconds, 53°C for 30 seconds and 72°C for 1 minute; final extension at 72°C for 10 minutes. The amplified products were observed under UV on a 1% agarose gel and inserted into a pCRII vector containing two promoter sequences, Sp6 and T7, using the TA Cloning kit from Thermo Fisher. OneShot TOP10 chemically competent *E. coli* were transformed with the vector and positive clones were selected by PCR on plasmids DNA purified using the Pure Link HQ Mini Plasmid kit (Thermo Fisher). Plasmid DNA was linearized by restriction endonuclease digestion and used as template for in in vitro synthesis of "run off" sense and antisense transcripts labeled with DIG-11-UTP (Roche). After treatment with DNAse I, labeled RNA molecules were precipitated with lithium chloride and resuspended in hybridization solution. Labeling efficiency was evaluated by a spot test, where serial dilutions of the labeled RNA samples were applied to a positively charged membrane, along with known dilutions of a labeled control RNA serving as standards, and processed for immunological detection.

### *2.7 In situ hybridization*

*Varroa* adults were collected and placed on double-side tape in a dissection plaque. Their bodies were incised in the posterior and lateral region, taking care that during the incision the anterior region of the body was not touched, and immediately placed in fixative (4% paraformaldehyde, 0,1% TRiTON X-100 in PBS, pH 7.2) overnight at 4°C. Mites were rinsed in PBS1x three times, and rinsed in nuclease-free water two times. Tissue dehydratation was carried out at room temperature by passing through a series of graded ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 100%); dehydrated samples were placed in a solution of 50% ethanol: 50% xylol, subsequently replaced by a 75% ethanol: 25% xylol and 100% xylol three times, to remove completely the ethanol from the tissues. Paraplast chips were added gradually in the glass containers containing the samples and finally placed in an oven at 60°C overnight. *Varroa* adults were oriented into embedding molds containing paraffin and they were left for solidification around 2 hours at room temperature. Paraffin blocks were stored at 4°C until use. Polymerised samples were removed from embedding molds and sectioned to 3-5 um on a microtome Reichert Jung 2030. Tissue sections were placed on slides, dried on a hot plate at 37°C to promote an optimal tissue adhesion to the slide and subsequently stored in dry boxes until use. To remove paraffin from the samples, the slides were inserted into slide racks and then placed into staining dishes containing 100% xilol and gently stirred twice for 15 min each one. Slides were removed from xilol and rinsed twice in 100% ethanol. Slides were then rehydrated into ethanol series (100% to 30%), rinsed in distilled water three times, treated with NonidetP-40 (1%) for 10 min and acetylated with 0.33% (vol/vol)acetic anhydride in 0.1 M triethanolamine-HCl (pH 8) for 15 min prior to hybridization. The sections were prehybridized in prehybridization solution (50% formamide, 5x SSC, 40 µg/mL salmon sperm DNA) at 65°C for 1 hour and incubated in hybridization buffer with DIG-11-UTP (Roche) probe solution to a concentration of 100 ng/mL probe in hybridization solution at 60°C overnight. After hybridization, the sections were washed twice in 50% formamide, 2x SSC, 0.1% Tween-20 at 55°C for 1 hour. The hybridization signals were detected with alkaline phosphatase (AP)-labeled sheep anti-DIG antibody conjugate (Roche Applied Science). The conjugate solution was added to sections and incubated in a humid chamber at 4°C overnight. The slides were rinsed three times with washing buffers. The colour development





### *2.12 Effectiveness and time course of Vd-CHI knockdown*

In order to evaluate the effect of the dsRNA treatment on targeted genes transcription and to measure the time window of the gene silencing, pools of 2-3 mites were sampled from each treated group at 12, 24, 48 and 72 hours after-soaking and stored at -80°C. The experiment was repeated three times. Total RNA was isolated from each mite according to the procedure provided with TRIzol (Thermo Fisher). The amount of RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher). Primers for VdASP and VdCHI (Table 1) were designed outside the dsRNA fragment to test the relative expression of targeted genes by qPCR. Relative gene expression data were analyzed using the $2^{\Delta\Delta CT}$ method as described in section 2.3. Each time point was analyzed separately and the control soaked sample was chosen as calibrator. The results are presented as mean fold changes of independent biological replicates. ΔCTs were compared using Student's t-test with statistical significance set at $p < 0.05$. All statistical analyses were performed using the Statistical Analysis Systems software (Sigma Stat Statistical Software, SPSS Science, Chicago, IL, USA).

### *2.12 Survival of V. destructor after Vd-CHI gene knockdown*

In order to measure the effects of the gene silencing on *Varroa* survival, each soaked mite was introduced in a gel capsule containing one worker pupae, as described above. Then, two different protocols were followed. For the first, mites were let feeding on the same single pupa for the entire duration of the bioassay. For the second, every 24 hours the honeybee pupa was replaced with a non-infested one. In both assays, mite survival was monitored every 24 hours. The assay was repeated three times and the data collected were plotted in a Kaplan-Meier curve using the software GraphPad Prism 7. The log rank (Mantel-Cox test) was used to compare the survival distributions of the observed groups. Statistical significance was set at $p<0.05$ and adjusted at $p<0.008$ using Bonferroni method for multiple comparison.

**Fig. 5 - Gel capsules containing bee pupae and *Varroa* mites.**

### *2.13 Expression profile of honeybee immune response to Vd-CHI dsRNA treated mite exposure*

To assess the effects of Vd-CHI on honeybee immune response worker pupae were artificially infested with treated mites 48 hours after soaking, following the protocol already described in Section 2.11. Along with infested pupae, non-parasitized controls were maintained in gelatin capsules for two days under the same conditions of relative humidity and temperature. Honeybee pupae were sampled at 24 hours from the beginning of infestation and stored at -80°C. RNA was extracted individually from each pupa with TRIzol, quantified and DNAse-treated as described in 2.4. Approximately 2 µg of RNA was used for cDNA synthesis with iScript (BioRad), following manufacturer's instructions. Final product was opportunely diluted to 50 ng/µL ng and used as input for qPCR reactions. Differential relative expression of genes involved in honeybee immune response was measured by qPCR, using the iTaq Universal SYBR Green Supermix (BioRad), according to the manufacturer's instructions. Each reaction was prepared in 10 µL and contained 5 µL PCR mix 2x, 250 nM of forward and reverse primer, DEPC treated water and 1 µL of cDNA. For the experimental run the following cycle profile was used: 95°C for 30 sec; 40 cycles at 95°C for 5 s, 30 sec at 60°C; a melt curve analysis of 65-95 °C with an increment of 0.5°C, 5 sec/step. Each sample was analyzed in duplicate on a CFX96 Touch Real-Time PCR Detection System (BioRad). Two reference genes, *β-actin* and *rps5*, were used as endogenous control for RNA loading according to prevous studies (Lourenço et al., 2008; Tesovnik et al., 2017). Primers for amplifying the major part of immune genes were taken from the literature as shown in Table 2. Primers for *sgabd-8* were designed using Primer Express version 1.0 software (Applied Biosystems). Relative quantification of gene expression was performed using the $2^{\Delta\Delta CT}$ method (Livak et al., 2001, Pfaffl et al., 2001, 2002). Heatmap was created plotting $\log_2$-transformed fold-change values with the online tool Heatmapper (http://heatmapper.ca). For validation of the ΔΔCt method the difference between the Ct value of the target genes and the Ct value of reference transcripts [ΔCt = Ct(*target*)-Ct (*mean of the references*)] was plotted versus the log of five-fold serial dilutions (100, 20, 4, 0.8 and 0.16 ng) of the purified RNA samples. The plot of log total RNA input versus ΔCt displayed a slope less than 0.1, indicating that the efficiencies of the two amplicons were approximately equal. The results are presented as ΔCt comparisons plotted in boxplots.

### 2.14 Statistical analysis

Statistical analysis mainly involved differential expression studies. The assumption of normal distribution of data was tested and met via Shapiro-Wilk test. Each dataset was checked for homoscedasticity using Leven's test. For differential expression in salivary glands, different life stages and for Vd-CHI knockdown validation in *Varroa* mites ΔCts were compared using Student's t-test. For differential expression of immune response in honeybees, we used one-way analysis of variance (ANOVA) and Tukey's test. In all cases statistical significance was set at $p<0.05$. All statistical analyses were performed using the Statistical Analysis Systems software (Sigma Stat Statistical Software, SPSS Science, Chicago, IL, USA).

**Table 2 – qPCR primers used for studying the expression profile of honeybee immune response**

| Transcript | Sequence | Category | Gene description |
|---|---|---|---|
| Dorsal-1* (GB19537) | F: AGAGATGGAACGCAGGAAAC<br>R: TGACAGGATATAGGACGAGGTAA | Immune | NF-kB transcription factor orthologue |
| *Spaetzle** (GB15688) | F: TGCACAAATTGTTTTTCCTGA<br>R: GTCGTCCATGAAATCGATCC | Immune | Toll-binding cytokine-like molecule |
| SgAbd8 (GB43670) | F: ACCGCAGGGTGCTCACTTAC<br>R: CTCGGATGTGCAGCTATCCA | Chitin-binding | Endocuticle structural glycoprotein SgAbd-8 |
| AmCht**** (GB46749) | F: GTGGTGGCAAACAAGCTGAT<br>R: CGCTGCAAAATTGTTCCACGA | Immune | Acidic mammalian chitinase, anti-fungal activity |
| β-1,3-GBP*** (GB42981) | F: TGGATGGCAAAGAATTTGGT<br>R: ATTGCAACATTGCCTTAGCC | Immune | β-1,3-glucan binding protein |
| Defensin-1 * (GB19392) | F: TGCGCTGCTAACTGTCTCAG<br>R: AATGGCACTTAACCGAAACG | Immune | Defensin, antimicrobial peptide |
| Hymenoptaecin* (GB17538) | F: CTCTTCTGTGCCGTTGCATA<br>R: GCGTCTCCTGTCATTCCATT | Immune | Hymenoptaecin, antimicrobial peptide |
| Abaecin* (GB18323) | F: CAGCATTCGCATACGTACCA<br>R: GACCAGGAAACGTTGGAAAC | Immune | Abaecin, antimicrobial peptide |
| β-Actin** (AB023025.1) | F: TGCCAACACTGTCCTTTCTG<br>R: AGAATTGACCCACCAATCCA | House-keeping | Cytoskeletal structural protein |
| RPS5* (GB11132) | F: AATTATTTGGTCGCTGGAATTG<br>R: TAACGTCCAGCAGAATGTGGTA | House-keeping | Ribosomal protein S5 |

*(Evans, 2006)
** (Lourenço et al., 2008)
*** (Zanni et al., 2017)
**** (Aronstein et al., 2010)

# RESULTS

## 3.1 In silico predicted *Varroa destructor* secretome

We utilized a broadly used functional genomics approach (Bos et al., 2010; Villarroel et al., 2016) to identify candidate effectors from *V. destructor* using publicly available protein databases (Fig 6). We hypothesized that mite's effectors are most likely secreted proteins that are delivered into the saliva through the classical eukaryotic endoplasmic reticulum (ER)-Golgi pathway of the salivary glands. A feature of proteins secreted through this pathway is the presence of an N-terminal signal peptide. Therefore, we used the SignalP v4.0 program to predict the presence of signal peptides in the amino acid sequences of *V. destructor* retrieved from the last genome annotation available on NCBI site. Out of 30221 amino acid sequences corresponding to predicted proteins, we identified 2882 sequences with signal peptide.

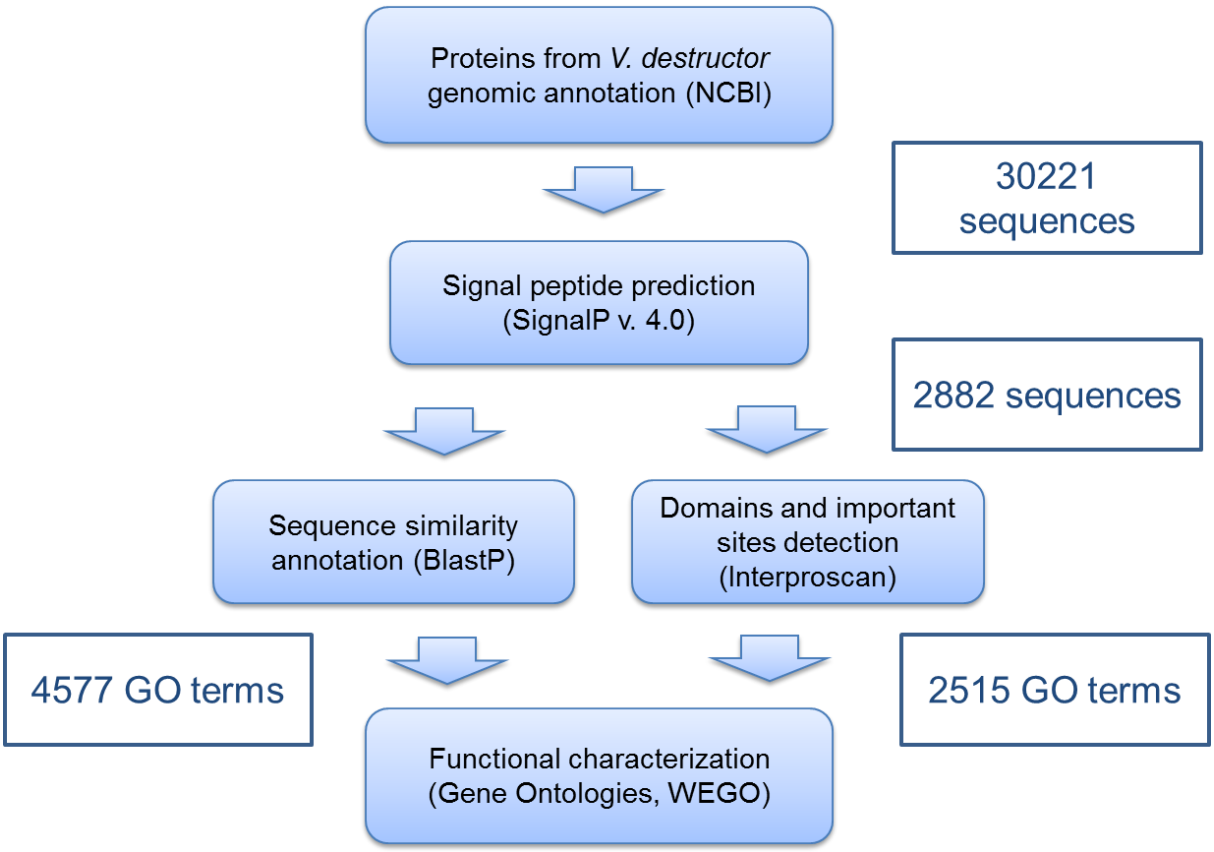



**Fig. 6 - Bioinformatic pipeline used to predict and functionally characterize the secretome of *V. destructor***
Main results are shown in the white boxes.

These proteins were subsequently annotated following two different approaches in parallel: by sequence similarity and by domains prediction. For the first approach, we used BlastP with Swiss-Prot db and two databases containing 4463 and 19894 sequences downloaded from NCBI using 'saliva AND Acarina [organism]' and 'venom AND Hymenoptera [organism]', respectively.
Swiss-Prot database provided a high level of annotation, included Gene Ontology (GO) terms, while the other databases enriched the information about the analysed proteins, representing two well-studied secreted cocktails of effectors: venoms of hymenoptera species (including parasitoid wasps) and saliva of ticks and mites. BlastP searches resulted in the annotation of 2019 proteins matching in the 3 different databases as shown in Fig. 7. Swiss-Prot alone supported 87.4% of the whole annotated subset and provided 4577 GO terms from 1760 proteins.

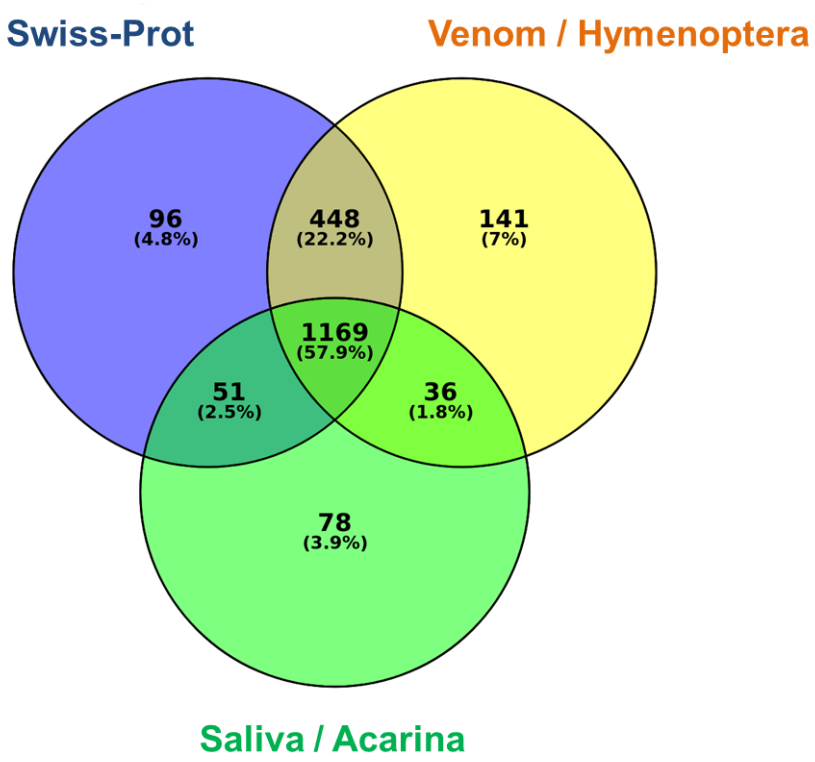



**Fig. 7 - Venn diagram showing number and percentage of secretome sequences annotated in three different databases**
Swiss-prot (blue), venom proteins of Hymenoptera (yellow) and salivary proteins of Acarina (green).

Although annotation by sequence similarity resulted in the match of a good percentage of the query sequences, this approach could poorly estimate the relevance of functional domains when predicting protein function. In order to scan the sequences for functional domains and important sites, Interproscan was launched on the same subset previously analysed with Trinotate/BlastP. Notably, Interproscan

resulted in the annotation of 1413 proteins, but led to 2515 GO terms identified, about 50% less compared to BlastP search on Swiss-Prot. The resulting GOs from domains detection by Interproscan analysis were reported in Fig. 8 and Fig. 9, together with the GOs obtained from sequence similarity annotation by Blast. Gene Ontologies Level 2 histogram shows that “catalytic activity”, “binding” and “metabolic process” are GO terms well supported by both the approaches used for annotation (Fig.8). A more in depth insight into the identified GOs reveals that both annotations well supported GO terms such as “hydrolase activity”, “catalytic activity on a protein”, “protein binding”, “ion binding”, “nitrogen compounds metabolic process” and “organic substance metabolic process”, as displayed by Level 3 histograms (Fig. 9).

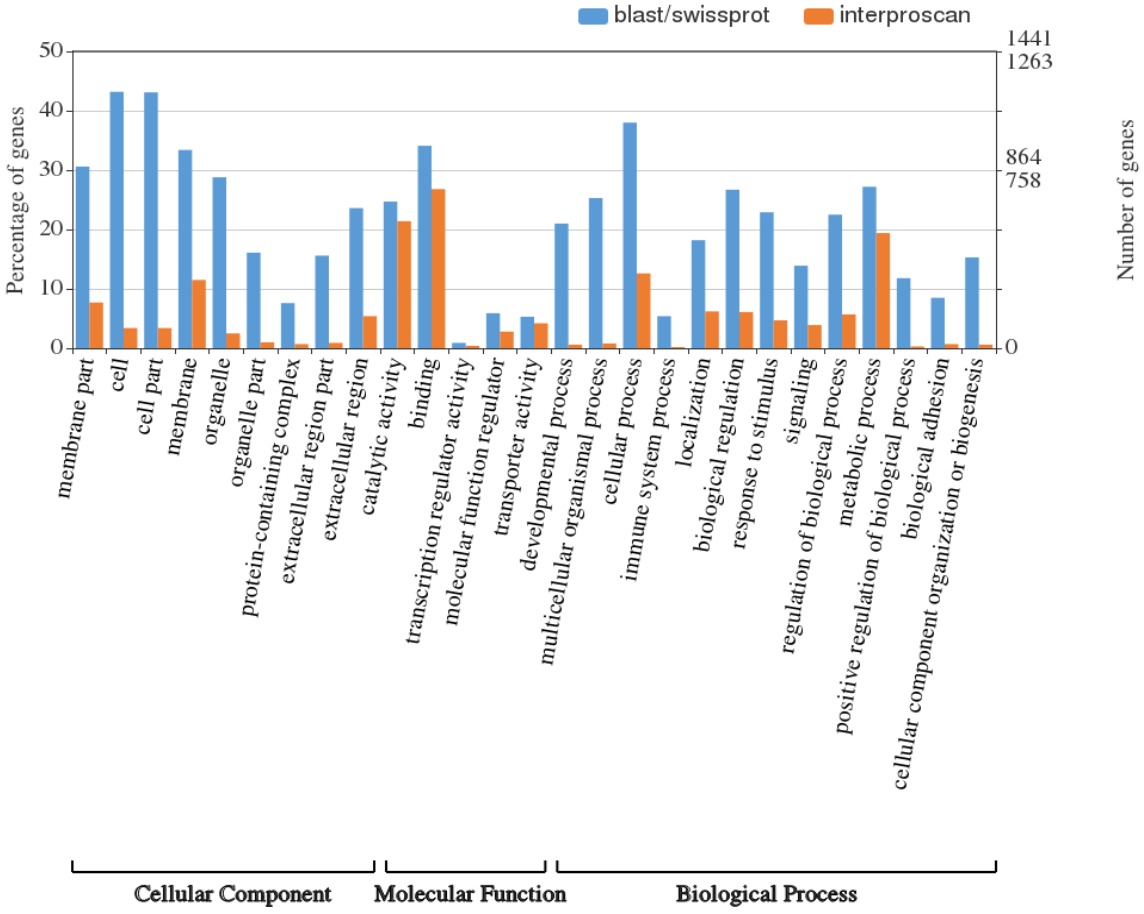



**Fig. 8 - Histograms reporting Gene Ontologies Level 2**
The abundance of GO terms Level 2 from Blast/Swiss-Prot db annotation (blue) and Interproscan analysis (orange) plotted using WEGO online tool. Percentage and number of genes are reported on left and right Y-axes, respectively.

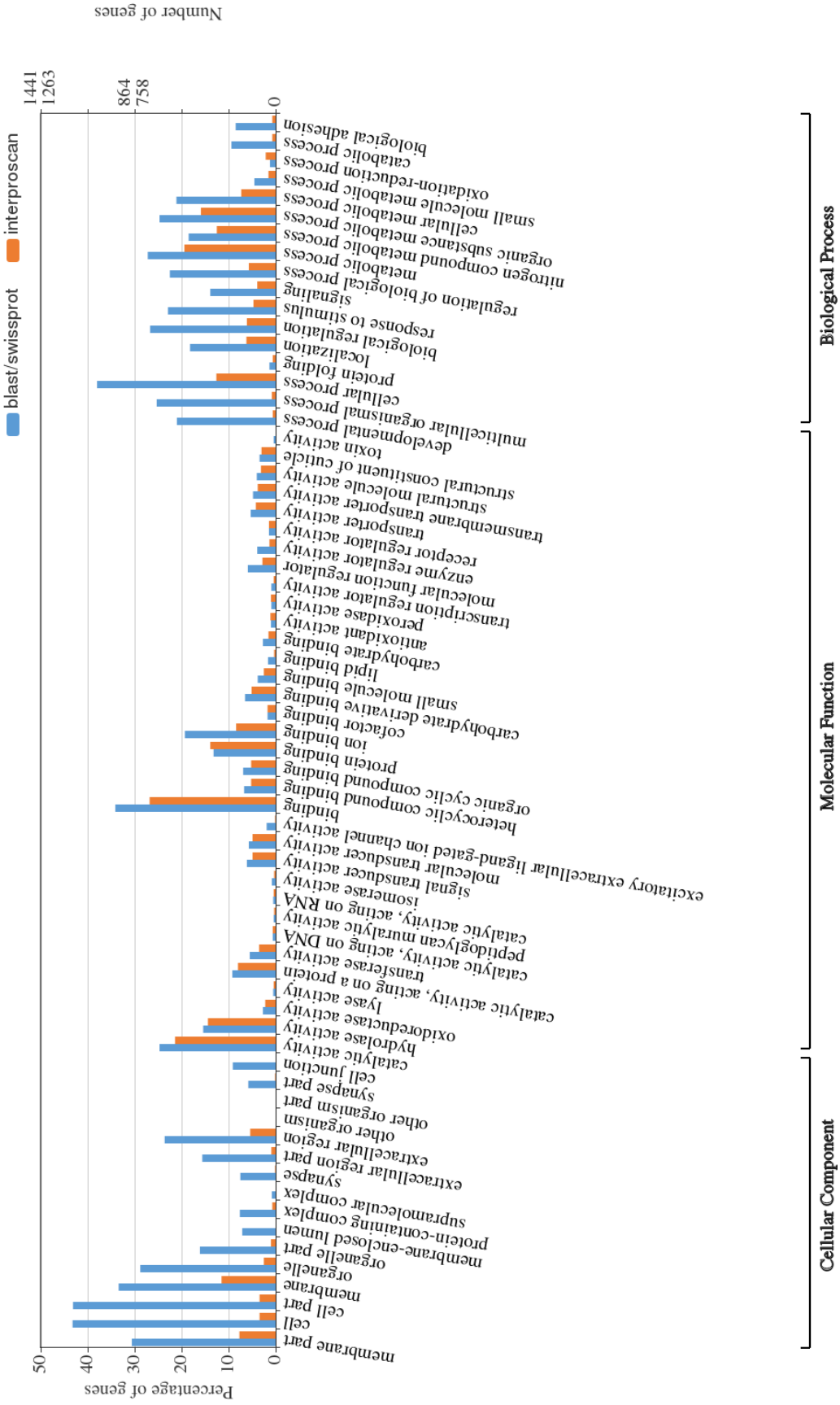



**Fig. 9 - Histogram reporting Gene Ontologies Level 3**
The abundance of GO terms Level 3 from Blast/Swiss-Prot db annotation (blue) and Interproscan analysis (orange) plotted using WEGO online tool. Percentage and number of genes are reported on left and right Y-axes, respectively.

### 3.2 Differential expression study of putative salivary glands transcripts

The selected transcripts (see Materials and Methods, Section 2.5) were homologues of different protein families: α-Macroglobulin, Aspartic Protease, Chitinase, q-Carboxypeptidase and Serine Protease.
qPCR results (Fig. 10) showed that the only significantly overexpressed transcript in the salivary glands (Student's t-test: $P < 0.005$) is Chitinase (Vd-CHI), with ~337-fold compared to the rest of the body. The other candidates resulted less overexpressed (between 2- and 5-fold), however not significantly (Student's t-test: $P = 0.24$-$0.67$).

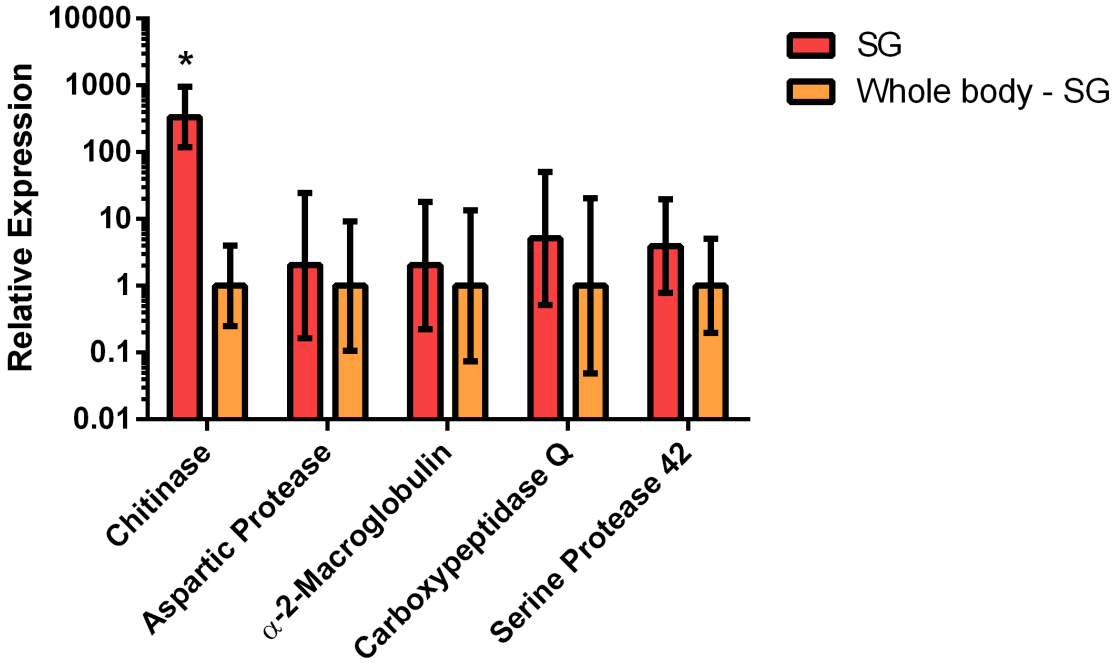



**Fig. 10 - Relative expression of candidate effectors in salivary glands of *V. destructor***
Results are presented as mean fold changes of three independent biological replicates. Each replicate consisted in a pool of 5-10 mites processed in order to isolate salivary glands (SG) from the rest of the body (Whole Body – SG). Values on Y axis are reported in $Log_{10}$ scale. Error bars represent standard deviations. Significance of Student's t-test was set at $p<0.05$ and indicated by an asterisk.

### *3.3 Localization of selected transcripts in V. destructor sections*

To further localize the expression of selected genes in *V. destructor* tissues, we developed a protocol for in-situ hybridization on two candidates, Chitinase (Vd-CHI) and Aspartic protease (Vd-ASP). Hybridization of DIG-labeled antisense RNA probe on sectioned *Varroa* mites led to a clear and specific staining in salivary glands for Vd-CHI (Fig. 11). We did not observe any staining for Vd-CHI sense probe (negative control), nor for Vd-ASP probes (sense and antisense).

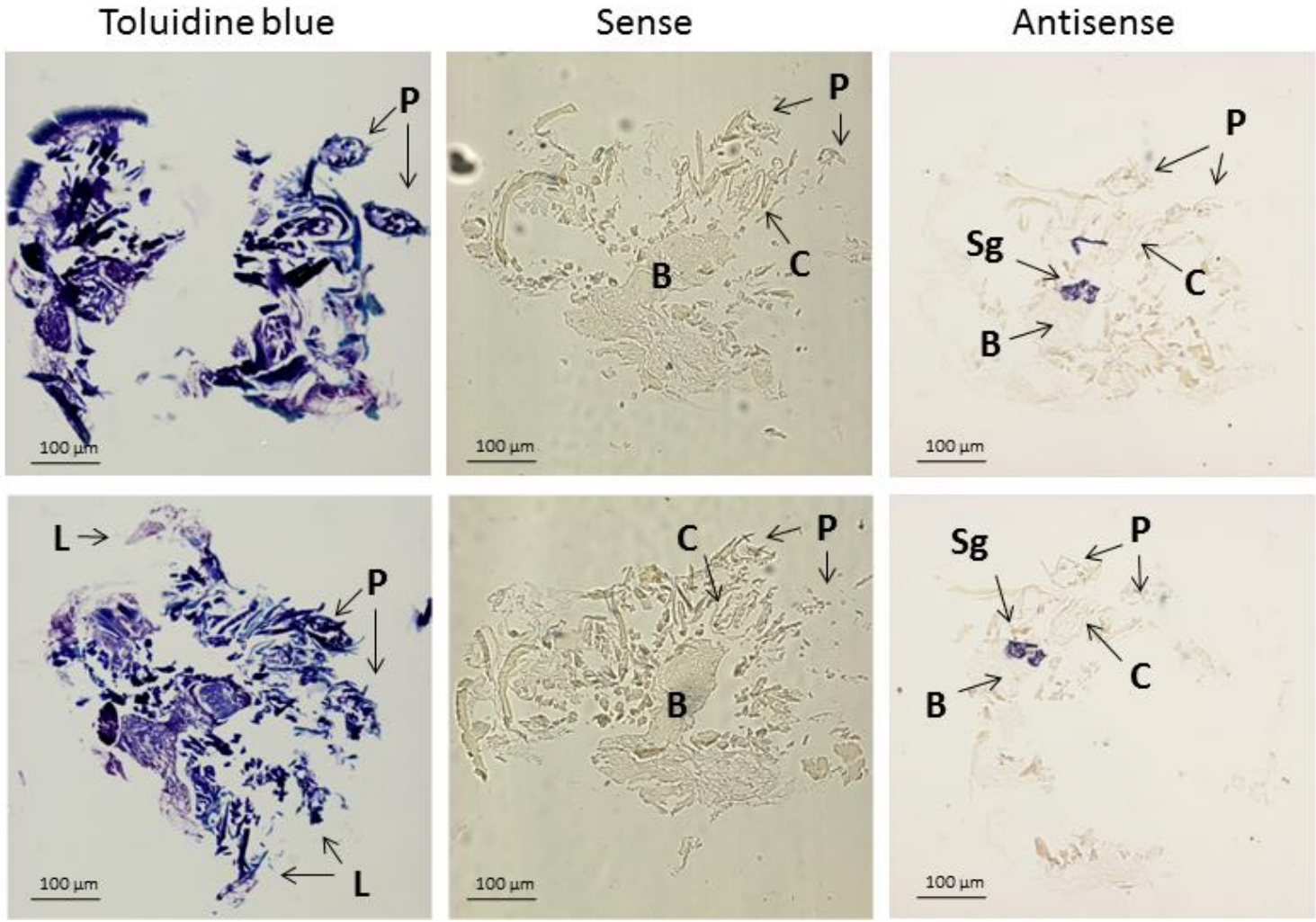
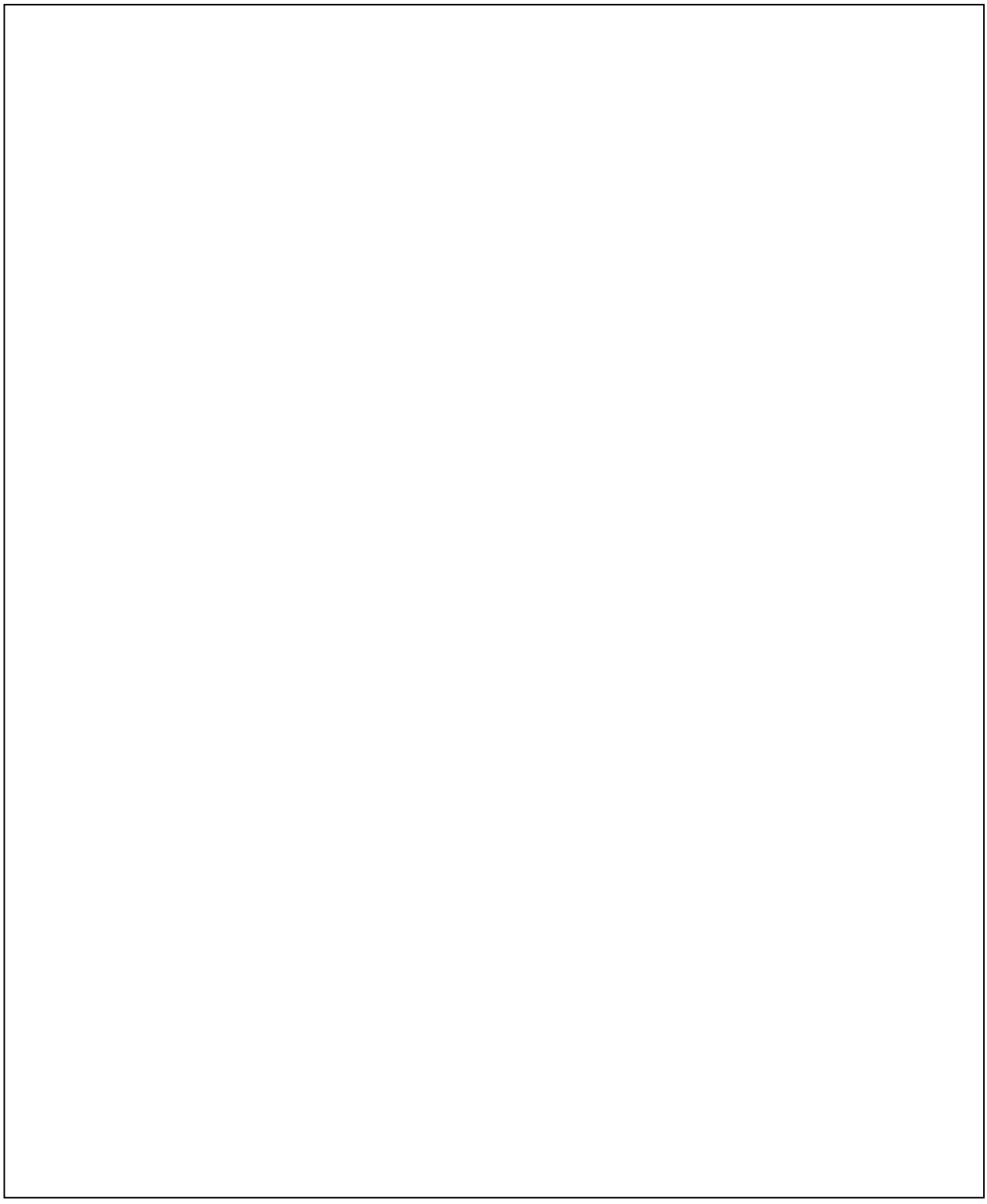



**Fig. 11 - In-situ hybridization of DIG-labeled RNA probe for Vd-CHI**
Two serial sections of 3 µm are shown. Salivary gland expressing Vd-CHI are stained in blue in the sections hybridized with the antisense probe. No background nor unspecific signal was observed for sense probe (negative control). B: Brain; C: Chelicera; L: Leg; P: Pedipalps; Sg: Salivary glands.



### *3.6 Effectiveness and time course of Vd-CHI knockdown in Varroa mites*

In order to study Vd-CHI from a functional point of view, a non-invasive approach of gene knockdown based on soaking the mites in a dsRNA solution was followed (Campbell et al., 2010). The results of qPCR on soaked mites demonstrated that gene knockdown of Vd-CHI begins around 48 hours and continues at 72 hours after treatment (Fig. 15). Vd-CHI expression in dsRNA soaked mites resulted reduced by 84% and 96% at 48 and 72 hours after soaking, respectively, compared to control mites soaked in saline solution.

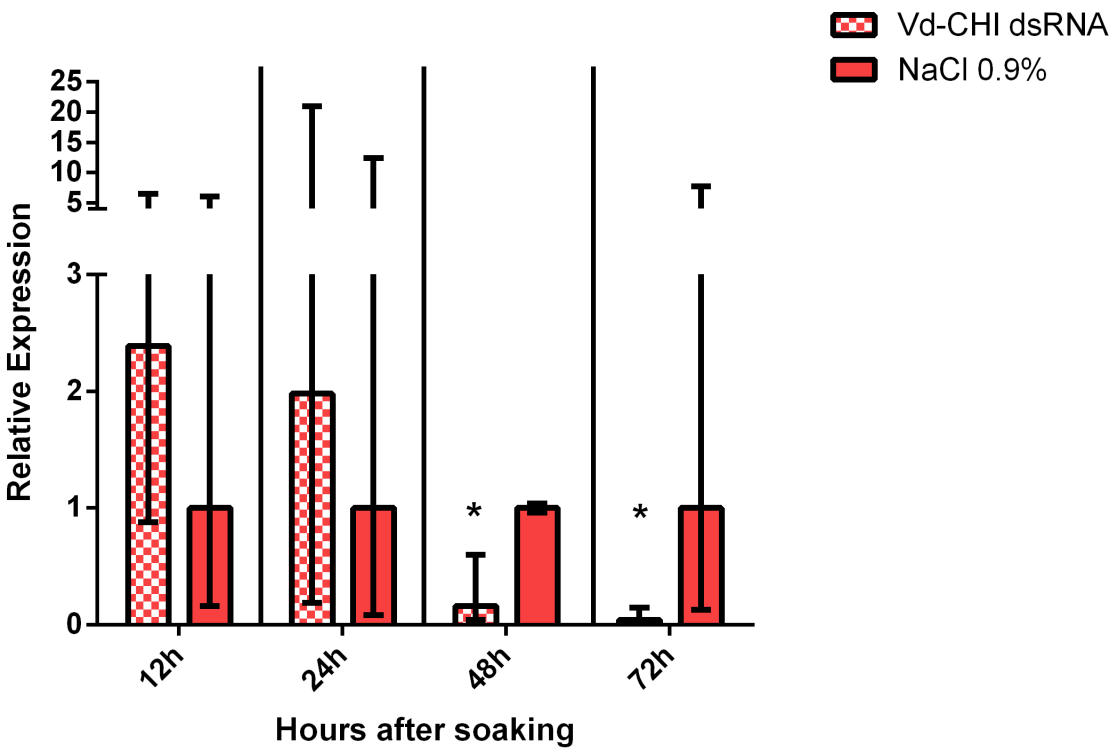



**Fig. 15 - Relative expression of Vd-CHI after soaking treatment**
Results of qPCR are presented as mean fold changes of three independent biological replicates. Each replicate consisted in a pool of 2-3 mites. Each time point was analyzed separately with Student's t-test. Error bars represent standard deviations. Significance ($p<0.05$) is indicated by an asterisk.

### *3.7 Effects of Vd-CHI knockdown on mites' survival*

The survival curves of mites soaked in dsRNA solution for Vd-CHI resulted significantly different from the curve of mites soaked in saline solution, only when the pupa is replaced ($X_2(1, N=83) = 14.96$; $p<0.001$) (Fig. 16). When pupa is non-

replaced the curves of the different soakings slightly differ, however not significantly ($X_2$(1, N=98) = 3.86; p=0.049). The higher mortality observed in dsRNA treated mites occurs earlier, around 48 hours after soaking, when honeybee pupa is replaced every 24 hours (p=0.006). After 72 hours the survival rate of dsRNA treated mites is set around 50-60%. No further alteration of mites' behaviour was observed.

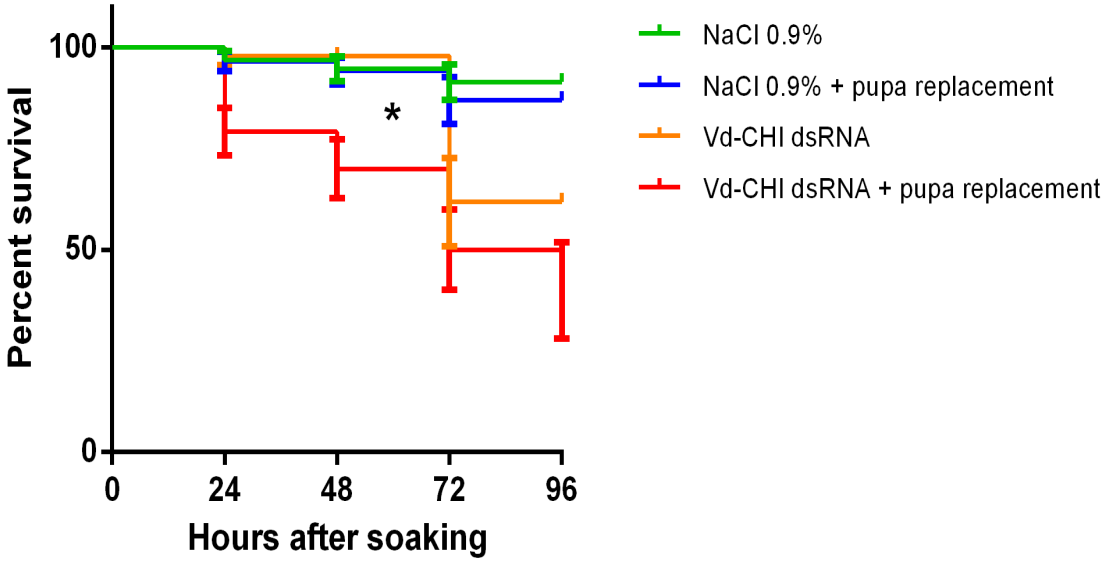



**Fig. 16 – Kaplan-meier survival curve of mites soaked in dsRNA solutions**
Replacement of host pupae occurred every 24 hours. Significativity of differences in mortality was assessed with Log rank (Mantel-Cox) test. Treshold was adjusted at p<0.008 using Bonferroni method. *p<0.001.

### *3.8 Effects of Vd-CHI knockdown on A. mellifera immune response*

To investigate the effects of Vd-CHI on honeybee immune response, we generated Vd-CHI deficient saliva via RNAi knockdown in *V. destructor* to compare to whole saliva control (soaked in NaCl 0.9% solution) and non-infested controls (WT). Brown-eyed worker pupae were exposed to treated mites 48 hours after soaking and collected 24 hours later (see Materials and Methods). Pupae artificially infested with a Vd-CHI deficient mites did not display any macroscopic physical differences from pupae parasitized by mites with a whole salivary repertoire or non-infested controls.
One-way ANOVA of qPCR results (Table 3) showed that there was a statistically significant difference between groups for six of the eight examined genes (Fig. 17). Two genes, *abaecin* and *dorsal-1* resulted not differentially expressed in any condition compared to controls. Five immune-related genes were upregulated in at least one condition of parasitization, while only one gene, *sgabd8*, was significantly downregulated (Fig. 17). A Tukey post hoc test revealed that infestation with *Varroa* mites soaked in saline solution significantly upregulated genes *defensin-1* (18.74-fold, p<0.001), *hymenoptaecin* (3.83-fold, p=0.015), *spaetzle* (10.83-fold, p<0.001) and *acidic mammalian chitinase-like* (22.05-fold, p<0.001). The antimicrobial

peptides *defensin-1* and *hymenptaecin* resulted upregulated also in pupae infested with Vd-CHI deficient mites, with 22- ($p<0.001$) and 18.70-fold ($p<0.001$), respectively. However, upregulation of *hymenoptaecin* was significantly higher ($p=0.004$) in pupae infested with Vd-CHI dsRNA soaked mites compared to pupae infested with NaCl 0.9% soaked mites (Fig. 18). *Spaetzle* resulted upregulated also in pupae infested with Vd-CHI deficient mites but to a lesser extent (3.01-fold, $p<0.001$). A*cidic mammalian chitinase-like* resulted significantly upregulated only in the presence of Vd-CHI (22.05-fold, $p<0.001$). The gene GBNP-1, which encodes a glucan recognition protein, was significantly upregulated only in pupae infested with Vd-CHI deficient mites (13.07-fold, $p<0.001$). The gene encoding an endocuticle structural glycoprotein, *sgabd8*, exhibited a significant reduction of expression only in pupae infested with control mites (0.07-fold, $p<0.001$), while downregulation in pupae infested with dsRNA treated mites was not significant ($p=0.064$).

**Table 3 - ANOVA results of ΔCt comparison between groups**

| Gene name | Df* | F | Significativity |
|---|---|---|---|
| ***acidic mammalian chitinase-like*** | 2,26 | 18.28 | p<0.001 |
| ***abaecin*** | 2,22 | 1.64 | p=0.217 |
| ***spaetzle*** | 2,22 | 48.00 | p<0.001 |
| ***hymenoptaecin*** | 2,24 | 22.08 | p<0.001 |
| ***β-1,3-glucan binding protein*** | 2,31 | 11.06 | p<0.001 |
| ***defensin-1*** | 2,25 | 15.02 | p<0.001 |
| ***sgabd8*** | 2,19 | 60.70 | p<0.001 |
| ***dorsal-1*** | 2,28 | 1.29 | p=0.29 |

*Degrees of freedom. The first value indicates degrees of freedom between the groups, while the second value, after the comma, indicates degrees of freedom within the groups.

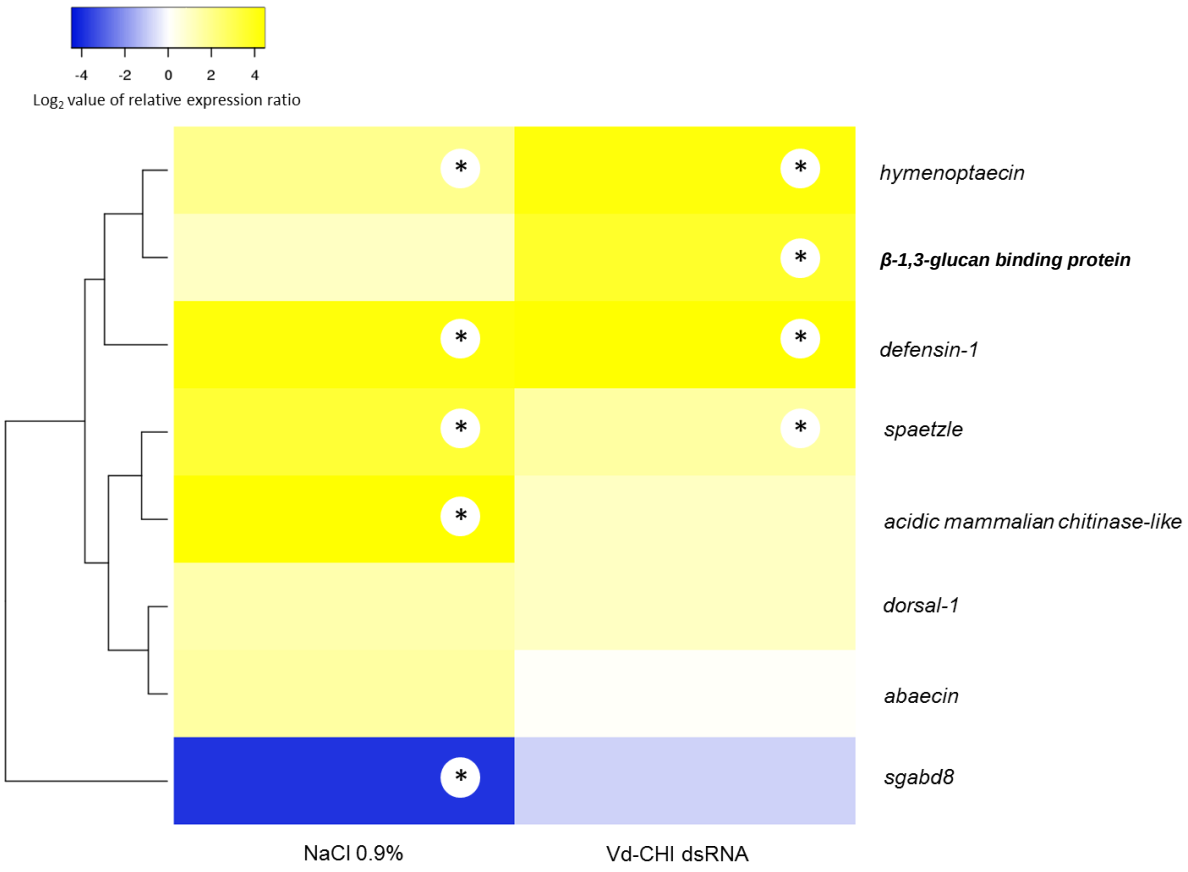



**Fig. 17 - Heatmap of differential expression of immune-related genes in honeybee pupae infested with *V. destructor***

The colors indicate the average mRNA levels compared to average levels of mRNA in non-infested pupae (WT): blue indicates lower and yellow higher levels. Range $log_2$ value of relative expression ratio (fold-change) is indicated in the legend on the upper-left. Each column corresponds to the expression profile of one treatment (exposure to *Varroa* mite soaked in saline solution or Vd-CHI dsRNA) and each row to one gene transcript. The immune-related gene names are indicated on the right. Boxes marked with the asterisk (*) shows statistically significant effect of treatment on gene expression ($p<0.05$).

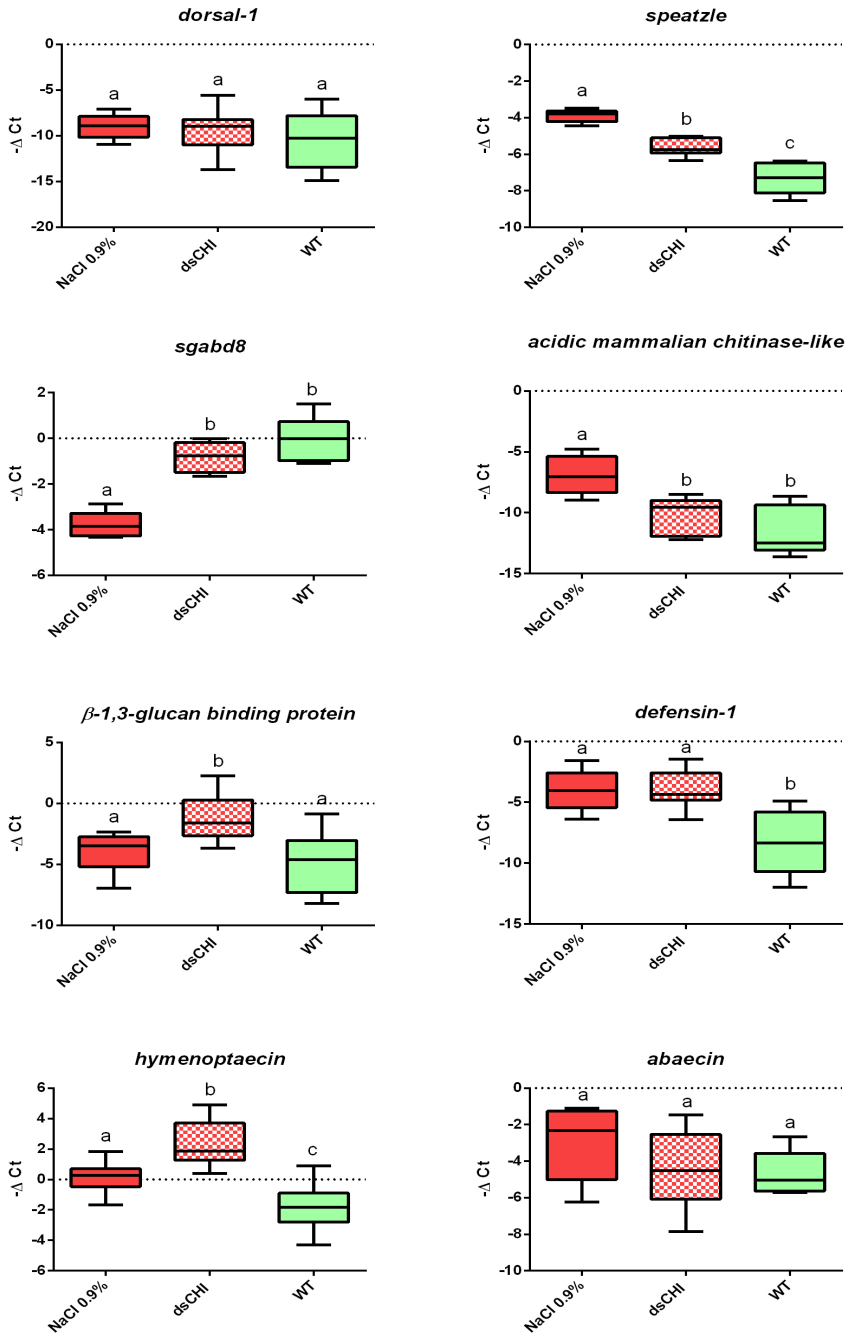



**Fig. 18 - Box plot representing the ΔCt values measured for biological replicates and selected treatment**

Each box extends from the 25th to 75th percentiles.The line at the middle of the box is plotted at the median. The whiskers go down to the smallest value and up to the largest. Treatments sharing the same letter are not significantly different from one another. Treatments are indicated in the scale at the bottom of the plots. WT: non-infested controls; NaCl 0.9%: pupae infested with mites soaked in saline solution; dsCHI pupae infested with mites soaked in Vd-CHI dsRNA solution. One-way ANOVA and Tukey post hoc test were undertaken with SPSS software.

## DISCUSSION

Parasites deliver repertoires of proteins inside their hosts to interfere with physiological processes and facilitate feeding. According to Richards et al. (2011), and to more recent studies (Zhang and Han, 2018a), the mite *V. destructor* secretes proteins into its saliva, which are injected into honeybees while feeding. The identification and characterization of these proteins will offer new insights into the molecular basis of *Varroa*-honeybee interactions, on which to develop new sustainable strategies of mite control.

Here, we have used a functional genomics pipeline to identify *V. destructor* candidate salivary proteins, along with various assays to assess their expression in salivary glands. Using this approach, we identified a salivary Chitinase (Vd-CHI) that affects mite survival and causes alterations of honeybee immune response.

We utilized the backbone of a broadly used effector-mining strategy (Bos et al., 2010; Villarroel et al., 2016) to characterize the mite secretome and generate a list of putative salivary proteins. We expected that *V. destructor* salivary proteins are likely to be secreted proteins which have homologues in the well-studied ticks' saliva, due to the evolutionary proximity of the two taxa. We also hypothesized the presence of homologues of *V. destructor* salivary proteins in the venom of parasitoid wasps (Hymenoptera), because the target of parasitism factors in both cases are insects. Around 60% of the *V. destructor* secretome proteins annotated by Blast resulted shared between the databases of salivary proteins of Acarina and venom proteins of Hymenoptera (Fig. 7). In the case of Vd-CHI, specifically expressed in salivary glands (Fig. 10 and 11), further Blast searches in nr NCBI db and phylogenetic reconstruction of the amino acid sequence identified a close relationship with chitinases found in the venoms of parasitoid wasps (Fig. 14). These results well fit in the picture of the convergent recruitment of proteins in venom and feeding secretions across multiple animal lineages (Fry et al., 2009). Indeed, several studies report that ticks and parasitoid wasps, as well as other venomous animals, use common parasitism factors for host exploitation through their secretions. Examples of this convergence are represented by phospholipases A2 (Laurino et al., 2016; Zhu et al., 1998), serine protease inhibitors (Meekins et al., 2017) and chitinases (Kim et al., 2014; Vincent et al., 2010). Many parasitism factors are, in fact, recruited body proteins that participate in fundamental biochemical functions, well conserved in the animal kingdom, and exhibit similar functions when introduced in a different organism trough injection, representing a particular example of intergenome active elements (Fry et al., 2009). On the contrary, other parasitism factors can be very specific, displaying poor similarity among related evolutionary lineages (Jonckheere et al., 2016; Villarroel et al., 2016; Zhao et al., 2015). Our analysis reported 863 secreted proteins showing no similarity with sequences in the databases searched. We may likely have parasitism factors among these unannotated proteins, which are obviously more difficult to characterize from a functional point of view.

From the list of candidates identified by the secretome annotation, according to literature and to in-house proteomic data, we selected five targets to evaluate their expression in *V. destructor* salivary glands (see Results - 3.2). Among the tested targets only Vd-CHI resulted overexpressed in salivary glands of *Varroa* mites (300-fold) compared to the rest of the whole body (Fig. 10), suggesting a tissue-specific expression. Indeed a similar pattern of expression (i.e. values ranging from 10- to more than 1000-fold) is reported for transcripts specifically expressed in salivary and

venom glands (Teng et al., 2017; Zhang et al., 2017). This result is apparently not consistent with the recently published *Varroa destructor* sialome, where not Vd-CHI, but another chitinase (with no signal peptide) and α-Macroglobulin are reported (Zhang and Han, 2018b). However, the abovementioned study is based on an *in vitro* feeding system that consisted in cotton wool soaked in insect cell culture media. We speculate that under these experimental conditions, poorly mimicking the saliva injection in the real host while feeding, the resulting sialome can be somewhat altered. Although α-Macroglobulin, Aspartic Protease, q-Carboxypeptidase and Serine Protease 42 are likely to be expressed also in other tissues, we can't exclude that their expression in salivary glands can have a role in the host exploitation.

Due to the differential expression results, we expected to observe Aspartic Protease expression not only in salivary glands but also in other tissues (e.g. gut). Indeed this protein, annotated by NCBI as "Cathepsin-D", lacks paralogues in *V. destructor* genome, while orthologues have been found in ticks' midgut, where they are involved in the host blood meal digestion (Boldbaatar et al., 2006). Instead, for Vd-CHI we expected to observe a more localized expression, namely in the salivary glands, and then harder to detect by *in situ* hybridization on sections of the mite body, compared with Aspartic Protease. DIG-labeled probe hybridization for Vd-CHI resulted in an intense and clear staining, clearly evident also at low magnification as "two dark-blue spots", localized in the salivary glands (Fig. 11). No staining was observed for Aspartic Protease, probably due to the stringency conditions of *in situ* hybridization, the same used for Vd-CHI (see Materials and Methods). This result confirmed the specific and strong expression of Vd-CHI in the salivary glands of the mite and paved the way for subsequent functional assays aimed at characterize this putative host regulation factor.

Chitinases are glycosyl hydrolases responsible for the degradation of chitin, the second most abundant biopolymer on the planet, which is a structural component of protective biological matrices, such as arthropod exoskeleton and fungal cell wall (Shahidi and Abuzaytoun, 2005). In arthropods, chitinases play a fundamental role as degradative enzymes during molting and a defensive role against parasites, such as fungi and nematodes (Arakane and Muthukrishnan, 2010). Presence of chitinase in the adult *V. destructor* has already been described by (Fraczek et al., 2009) and Colin et al. (2001). In these two studies the authors examined enzymatic activities in whole crushed mite extract using a semi-quantitative method and reported that chitinase, but also all the enzymes needed for the complete digestion of chitin, are very active (Colin et al., 2001; Fraczek et al., 2009). Our results of the secretome annotation are in line with these findings, reporting "hydrolase activity" (GO:0016787), which includes "chitinase activity" (GO:0004568), among the most abundant GO terms identified. Interestingly, Colin and colleagues hypothesized that the very high activity of chitinase in extracts of *V. destructor* can have a role in the hydrolysis of the host chitin and in keeping open the wound that serves for feeding the female-mother and the offspring (Colin et al., 2001).

To assess if Vd-CHI has a role in the mite feeding capacity, we generated Vd-CHI deficient mites through dsRNA delivery and performed artificial infestations of honeybee pupae. Mite survival resulted 50% decreased by Vd-CHI knockdown, 72 hours after the dsRNA treatment (Fig. 16), when 80-95% Vd-CHI silencing is detected by qPCR (Fig. 15). Although different delivery methods need to be developed (Garbian et al., 2012), this result suggest that dsRNA targeting genes expressed in salivary glands of *V. destructor* can interfere with feeding, representing a promising tool for controlling the mite. Indeed, considering that the stronger

reduction of *V. destructor* survival occurs around 24 hours after the beginning of Vd-CHI knockdown (48 hours post treatment) and that usually 50% of mites can't survive more than 18 hours without feeding (Garedew et al., 2004), it is reasonable to assume that Vd-CHI deficient mites died as a consequence of reduced feeding capacity on honeybee pupae. The negative impact of Vd-CHI deficiency on *V. destructor* survival is enhanced when the pupa is replaced every 24 hours (Fig. 16). This suggests that feeding disruption is enhanced when the mite cannot use the feeding wound opened at the beginning of the assay, when Vd-CHI expression level is still poorly affected by gene silencing. Piercing the honeybee pupa cuticle seems to be time-consuming and energetically expensive, according to the feeding pattern of *Varroa* foundress, which shows a first feed of 1-2 hours followed by feeding bouts of 5-10 minutes (Donze et al., 1998; Donzé and Guerin, 1994). Although the feeding site opening is likely to be mainly due to the mechanical rupture of cuticle by the mite's chelicera (de Lillo et al., 2001), we speculate that this process can be facilitated and the opening can be maintained by Vd-CHI, a chitinase specifically expressed in the salivary glands. This hypothesis is also supported by a protein atlas of *V. destructor*, which compared different developmental stages by proteomic approach and detected Vd-CHI only in the adult female mite (McAfee et al., 2017), which is the only one who can effectively create the feeding site for her and the offspring (Donzé and Guerin, 1994).

However, the molecular mechanism underlying the Vd-CHI positive effect on feeding success of *V. destructor* is still unclear. Vd-CHI exhibits the conserved sequence motifs of GH18 chitinases (DXXDXDXE, where D = aspartic acid, E = glutamic acid, and X = any amino acid), which includes the active site of the enzyme (Hamid et al., 2013). Site-directed mutagenesis studies on chitinase A1 from *Bacillus circulans* indicate that the glutamic acid residue at position 204 and aspartic acid residue at position 200 are essential for chitinase activity (Watanabe et al., 1994). The Glu and Asp residues corresponding to these 2 residues in the other chitinases are invariant and are conserved in Vd-CHI too (Fig. 13). It is also interesting to note that Vd-CHI do not have a putative chitin-binding domain, which binds to chitin prior to its degradation. However, this does not appear to be essential for the chitinolytic activity of chitinases in arthropods (Girard and Jouanin, 1999; Han et al., 2005; Tjoelker et al., 2000). A similar domain loss is also observed for a chitinase found in the venom of the parasitoid wasp *Chelonus* inanitus (Fig. 13), orthologue of an enzymatically active chitinase found in the venom of *Chelonus* sp. near *curvimaculatus* (Krishnan et al., 1994; Vincent et al., 2010). On the other hand, although enzymatically inactive, two chitinases lacking chitin-binding domain were suggested to be involved in the maintenance of a stable feeding site in the tick *Amblylomma americanum* (Kim et al., 2014). Despite these preliminary considerations, the chitinolytic activity of Vd-CHI should be evaluated through a proper enzymatic assay on salivary gland extract and/or using the purified recombinant protein.

In order to elucidate a putative involvement in the feeding site formation, we measured Vd-CHI expression in two different phases of the life-cycle of the mite. Indeed, we hypothesized that phoretic mites, feeding through the soft unsclerotized intersegmental membrane of adult bees (Ramsey et al., 2018), and reproductive females, usually creating and maintaining communal feeding sites in the middle of the 2$^{nd}$ abdominal sternite of pupae (Kanbar and Engels, 2005), have different needs in terms of a parasitic factor with a putative role in the host cuticle perforation. Vd-CHI resulted upregulated in reproductive mites in naturally infested cells (Fig. 12), supporting the hypothesis of the importance of this factor for the feeding site

formation on pupae, which may drive its changes in the expression level during *Varroa* life cycle. Upregulation of Vd-CHI during reproductive phase is not in line with a transcriptomic study, which reported the upregulation of putative salivary transcripts during the phoretic phase. However, Vd-CHI is not included in the set of putative salivary transcripts analyzed by the authors of the study (Mondet et al., 2018).

According to several studies reporting that phenotypic effect of parasitic factors can often be revealed only when they are introduced in the host (Becchimanzi et al., 2017; Digilio et al., 2000; Falabella et al., 2007), we were interested in the study of the impact by Vd-CHI on artificially infested honeybee pupae. Kanbar and Engels (2004) developed a method of visualization by staining with trypan blue of wounds produced by *Varroa* mites in honeybee pupae (Kanbar and Engels, 2004b). Unfortunately, this protocol didn't work in our conditions of artificial infestation lasting only 24 hours (data not shown). Indeed, our assays were based on Vd-CHI knockdown in mites, assessed only from 48 to 72 hours after the soaking in dsRNA solution (Fig. 15). Thus, it is reasonable to assume that this reduced time of infestation negatively affected wound staining by trypan blue, which selectively stain dead cells. We hypothesized that trypan blue staining can also be negatively affected by the alteration of natural feeding behaviour in response to parasite-host asynchronous development (Kirrane et al., 2011). Indeed, in our assays mites started feeding on brown-eyed pupae, while in natural conditions *V. destructor* foundress usually starts feeding on 5$^{th}$ instar bee larva, after the cocoon spinning (Boot et al., 1992; Donzé and Guerin, 1994; Nazzi and Le Conte, 2016). Notably, in both conditions trypan blue staining failed (Kanbar and Engels, 2004b). The lack of details on feeding site position, dimension and structure in the context of the present study represents a limit that can be stretched with a more accurate determination of the time-window of Vd-CHI knockdown and using conditions of artificial infestation closer to the natural ones.

Finally, we scored the impact of Vd-CHI on honeybee immune response by determining the expression profile of a set of immune genes in artificially infested hosts. Vd-CHI presence resulted in the upregulation of *acidic-mammalian chitinase* (22.05-fold) and in the downregulation of *sgabd8* (0.07-fold), two genes which are not differentially expressed in honeybee infested with Vd-CHI deficient mites compared to non-infested controls (Fig. 17). This result ties well with a recent quantitative proteomics study on infested worker pupae wherein another chitinase (XP_623995.1) and the same *sgabd8* are 4-fold upregulated and 6.5-fold downregulated, respectively (Surlis et al., 2018). Using a similar approach (effector knockdown followed by RNA-seq of the parasitized host), Martinson et al. (2016) studied the function of a GH19 chitinase found in the venom of *Nasonia vitripennis,* a pupal parasitoid wasp. Interestingly, presence of GH19 chitinase in the venom of the wasp caused the upregulation of a chitinase (Chitinase 8) in the host *Sarcophaga bullata* (Martinson et al., 2016). Endogenous chitinases of insects are involved in the immune response towards fungal pathogens (Arakane and Muthukrishnan, 2010). The upregulation of *acidic-mammalian chitinase* in infested *A. mellifera* pupae can potentially be triggered by Vd-CHI cleaving host chitin, that could elicit an anti-fungal response preventing infection through the feeding wound (Martinson et al., 2016; Saltykova et al., 2016). Then, Vd-CHI could be involved both in keeping open the feeding wound and in preventing any fungal infection by triggering an antifungal immune reaction by the host. This hypothesis seems to be supported also by the Vd-CHI induced upregulation of *spaetzle* (10.83-fold), which activates the toll pathway in *Drosophila* and the downstream production of antifungal and antimicrobial peptides

(Lemaitre et al., 1996; Weber et al., 2003), as similarly reported for GH19 chitinase of *Nasonia vitripennis* (Martinson et al., 2016).
The only transcript which resulted downregulated encodes an endocuticle structural glycoprotein, *sgabd8*. This latter belongs to a family of proteins that, together with chitin, are major components of the epidermis and are associated with *Varroa*-resistance in honeybees (Ji et al., 2015). According to Surlis et al. (2018), downregulation of *sgabd8* induced by mite feeding, could indicate a compromised healing of the cuticle due to Vd-CHI presence.
On the other hand, the upregulation of *β-1,3-glucan binding protein* (*β-1,3-GBP*) occurred only in pupae infested with Vd-CHI deficient mites. This protein is involved in the detection of infections in the host and triggers melanization, which is important for wound healing and encapsulation (Gottar et al., 2006; Lee et al., 1996; Söderhäll and Cerenius, 1998). Thus, it is reasonable to consider the upregulation of this transcript as an indicator of bacterial infections caused by Vd-CHI deficient mites feeding. Indeed we speculate that Vd-CHI could also prevent detrimental bacterial proliferation at the feeding site through (1) production of antimicrobial chito-oligosaccharides derived from host's chitin degradation (Benhabiles et al., 2012; Lin et al., 2009; Saltykova et al., 2016) and (2) anti-biofilm activity, as observed for other chitinases (Chung et al., 2014; Olofsson et al., 2016). Moreover, the antimicrobial peptide *hymenoptaecin* resulted more strongly upregulated in pupae infested with Vd-CHI deficient mites, supporting the hypothesis of ongoing bacterial infections (Fig. 18). This result ties well with a previous study wherein only buffer and whole *Varroa* homogenate injection, but not natural parasitization, caused *hymenoptaecin* upregulation after 24 hours (Koleoglu et al., 2017).
We also reported upregulation of the antimicrobial peptide *defensin-1* in pupae artificially infested with both Vd-CHI deficient and non-deficient mites. These findings are consistent with several previous studies showing that *defensin-1* is upregulated in *A. mellifera* following *Varroa* infestation (Aronstein et al., 2012; Gregorc et al., 2012; Khongphinitbunjong et al., 2015; Tesovnik et al., 2017).
One limitation of the present findings is the lack of information about the viral load of honeybees and mites used in this study, which can mask the effect of *V. destructor* parasitization alone. Moreover, the artificial infestation approach utilized suffers from the use of gelatin capsules, that can't reproduce the same microbial community and antimicrobial compounds found in natural wax comb (in synergy with propolis and honey) (Fratini et al., 2016).
However, the present immune gene survey suggests that *V. destructor* salivary repertoire can alter honeybee immune response, priming antimicrobial defenses in order to limit opportunistic infections. Further and prolonged infestations of *A. mellifera* pupae using Vd-CHI deficient mites, followed by RNA-seq of the hosts, will reveal the biological pathways specifically targeted by Vd-CHI, helping the functional characterization of this salivary protein.

## CONCLUSIONS AND FUTURE PERSPECTIVES

*Varroa destructor* infestation affects honeybee health, representing a major threat for beekeeping and agriculture, strongly dependent on pollination. Indeed, this ectoparasitic mite creates a wound in the host's cuticle through which it feeds on haemolymph and fat body, representing an important stress factor that weakens honeybee colonies and promotes the spreading of diseases. In order to facilitate feeding, *V. destructor* delivers a complex of factors, including proteins and viruses, through its salivary secretions. The characterization of these factors is still largely elusive and any progress in this area will offer new insights into the molecular basis of *Varroa*-honeybee interactions, on which to develop new sustainable strategies of mite control.

We utilized a functional genomics pipeline to characterize the mite secretome and generate a list of putative salivary proteins. From this list, according to literature and to in-house proteomic data, we selected five targets to evaluate their expression in *V. destructor* salivary glands. This led to the identification of a chitinase (Vd-CHI) specifically expressed in salivary glands of adult *V. destructor* females that positively affects mite survival. In particular, gene knockdown experiments revealed that Vd-CHI deficient mites show a reduced survival, likely as a consequence of reduced feeding capacity on honeybee pupae, due to the lack of chitinoloytic activity that favours the patency of the feeding wound. However, although the aminoacidic sequence of Vd-CHI exhibits all the features of active chitinases, its chitinolytic activity needs to be evaluated with a proper enzymatic assay on salivary gland extract and/or using the purified recombinant protein.

The importance of Vd-CHI for the feeding success of *Varroa destructor* is also supported by the upregulation of this gene during the reproductive phase of the mite. This finding suggests that Vd-CHI can be involved in the formation and maintaining of large communal feeding site in the middle of the 2$^{nd}$ abdominal sternite of pupae, while its function is probably less relevant during the phoretic phase, when *V. destructor* female is feeding through the soft unsclerotized intersegmental membranes of adult bees.

Vd-CHI also affects honeybee immune response, determining the upregulation of an endogenous chitinase, putatively involved in the host protection from opportunistic fungal pathogens, and in the downregulation of an endocuticle structural glycoprotein, associated with wound healing and *Varroa*-resistance. Furthermore, infestation by Vd-CHI deficient mites led to upregulation of *β-1,3-glucan binding protein* and *hymenoptaecin*, associated with detection and reaction against bacterial infections, suggesting that Vd-CHI could also prevent detrimental bacterial proliferation.

Collectively, these results shed light on *Varroa*-honeybee interaction, confirming that *V. destructor* sialome plays an important role in host exploitation. In particular, *Varroa destructor* feeding success on *A. mellifera* is mediated by the action of Vd-CHI, which probably facilitates the opening and patency of the feeding wound in the honeybee's cuticle. Indeed, this chitinase is likely involved both in maintaining the feeding site open, by interfering with the regular healing process of cuticle, and in limiting opportunistic infections, by priming antimicrobial defenses. The presence of fungi and bacteria at the parasite-host interface makes this interaction even more complex and indicates the need of adopting a hologenomic approach for the study of *Varroa*-honeybee interaction, taking into account the intricate scenario generated by

the composite mite-bee-microbiota genomes. Ultrastructural, RNA-seq and metagenomic studies of feeding sites will help characterize opportunistic pathogens and their impact on wound healing process, in order to elucidate the role of Vd-CHI in the parasite-host-microorganisms interactions. The present work contributes to a better knowledge of salivary repertoire of *V. destructor* and also demonstrates that dsRNA targeting of genes expressed in salivary glands can offer a promising new tool for controlling the mite. However, different dsRNA delivery methods need to be developed and adapted to be used in the hive system.

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# APPENDIX

### *Abroad experience*

During my PhD course, I spent a total of 5 months at the University of Aberdeen (Scotland, UK) under the guidance of Dr. Alan S. Bowman, to perform gene knockdown in *Varroa* mites and functional characterization studies.

### *Papers and communications*

In these 3 years, I had the opportunity to work in the entomology lab "Ermenegildo Tremblay" (Dipartimento di Agraria, Università degli Studi di Napoli "Federico II", Portici), under the guidance of Prof. Francesco Pennacchio. In this ideal context my research work has been focused on host regulation in arthropod interactions, which represented the main theme of published (and forthcoming) papers, posters and presentations.

- Papers:
  - Becchimanzi, A., Avolio, M., Di Lelio, I., Marinelli, A., Varricchio, P., Grimaldi, A., de Eguileor, M., Pennacchio, F., and Caccia, S. (2017). Host regulation by the ectophagous parasitoid wasp Bracon nigricans. J. Insect Physiol. 101, 7381.
  - Zimowska, B., Viggiani, G., Nicoletti, R., Furmaczyk, A., Becchimanzi, A., and Kot, I. (2017). First report of the gall midge Asphondylia serpylli on thyme (Thymus vulgaris), and identification of the associated fungal symbiont. Ann. Appl. Biol. 171, 8994.

- Posters
  - "Caratterizzazione molecolare e funzionale del veleno di *Bracon nigricans* (Hymenoptera: Braconidae)", XXXV Congresso Nazionale di Entomologia, Padova, June 2016

- Presentations
  - "Functional and molecular characterization of venom from the ectoparasitoid *Bracon nigricans* (Hymenoptera: Braconidae)", European PhD Network "Insect Science", La Colle sur Loup, November 2016
  - "RNA interference of *Varroa destructor* salivary genes: a tool to disclose honeybee-mite immune interactions", European PhD Network "Insect Science", Napoli, November 2017
  - "A *Varroa destructor* salivary chitinase is involved in the host regulation of infested honeybee pupae", European PhD Network "Insect Science", Firenze, November 2018

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# Host regulation by the ectophagous parasitoid wasp *Bracon nigricans*

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ABSTRACT

The host regulation process has been widely investigated in endophagous parasitoid wasps, which in most cases finely interact with living hosts (i.e. koinobiont parasitoids). In contrast, only very limited information is available for ectophagous parasitoids that permanently paralyze and rapidly suppress their victims (i.e. idiobiont parasitoids). Here we try to fill this research gap by investigating the host regulation by *Bracon nigricans*, an ectophagous idiobiont wasp species. Parasitism, mainly by venom action, is able to redirect host metabolism in order to enhance its nutritional suitability for the developing parasitoid larvae and to provide the required metabolic support to host tissues. The observed alterations of the host titers of haemolymph proteins, carbohydrates and acylglycerols are associated with a parasitoid-induced mobilization of nutrients stored in the fat body. This tissue undergoes a controlled degradation mediated by a close surface interaction with haemocytes, where a cathepsin L activity is localized, as demonstrated by immunolocalization, biochemical and transcriptional data. *B. nigricans* parasitism does not markedly influence the survival of haemocytes, even though a persistent suppression of the immune competence is observed in parasitized hosts, which show a reduced capacity to encapsulate and melanize non-self objects. These immune alterations likely allow a more efficient food uptake and use by the ectophagous larvae. The obtained results indicate that the host regulation process in basal lineages of parasitic Hymenoptera is more complex than expected and shares functional similarities with adaptive strategies occurring in derived koinobiont species.

## 1. Introduction

The Hymenoptera show an astonishing diversity of adaptive strategies to parasitic life, which range from the dramatic induction of rapid host paralysis and developmental arrest, occurring in the basal lineages of ectoparasitic idiobionts, to the more subtle manipulation of surviving hosts, which continue to grow, observed in derived lineages of endoparasitic koinobionts, arisen multiple times during evolution (Gauld, 1988; Pennacchio and Strand, 2006; Quicke, 1997). The host regulation strategy is extremely complex and finely modulated in koinobionts, which interact with living hosts for a long time during the development of their juvenile stages (Pennacchio and Strand, 2006). In contrast, idiobiont species are thought to have a much less intimate interaction with their hosts, which are often permanently paralyzed and rapidly suppressed (Pennacchio and Strand, 2006). While for the first category of parasitic wasps there are a number of studies addressing the physiological mechanisms underpinning the host regulation strategy and the role played by the parasitic factors involved, for idiobionts we have a very limited number of species investigated so far, and the available information is quite scattered, with the exception of the pupal parasitoid *Nasonia vitripennis* (Danneels et al., 2013, 2010; Martinson et al., 2014; Mrinalini et al., 2015; Rivers et al., 2002a, 2002b; Rivers and Denlinger, 1994, 1995) that is able to alter metabolism, development and immune response of the host fly.

The large number of studies on endophagous koinobionts has shed light on how the immune system of the host is suppressed, in order to allow the survival of the progeny into the hostile microenvironment of the body cavity, where the access to abundant nutritional resources in a protected niche is severely limited by the immune defense barriers. The venom is the most common source of host regulation factors, including those involved in the suppression of the immune response (Moreau and Asgari, 2015). The complex blend of biomolecules injected at the oviposition is responsible for a number of host alterations, essential for the survival of the wasp's progeny, which, in some cases, is complemented by the action of microbial symbionts (White et al., 2014). Among these latter, there are potent immunosuppressive viruses, which are members of the family Polydnaviridae (PDV) (Herniou et al., 2013; Strand and Burke, 2013). The expression of PDV-encoded virulence factors in

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infected host tissues allows a fine modulation over time of host physiology. This dynamic host regulation process is further modulated by molecular factors both of embryonic (i.e. teratocytes, which are cells deriving from the extraembryonic membrane of the parasitoid) and larval origin (Pennacchio and Strand, 2006). Unlike koinobionts, for idiobionts only limited information on host immunosuppression is available, and its functional role is generally thought to be related to food uptake and use by the wasp larvae (Pennacchio and Strand, 2006; Pennacchio et al., 2014), even though direct evidence of immune alterations that may play a role in this context, such as melanization, encapsulation and clotting, is very limited.

The koinobiont parasitoids do not normally induce host paralysis (Shaw, 1981; Pennacchio and Strand, 2006), which can be only transient in some cases, but they trigger a very diverse set of developmental and reproductive alterations, modulated by complex endocrine changes and resulting into an enhanced nutritional suitability of parasitized hosts (Pennacchio et al., 2014; Pennacchio and Strand, 2006). Indeed, these host changes allow a metabolic and nutritional reprogramming of the host, by disabling its energetically demanding functions and redirecting the resources left unused in favour of the developing parasitoid larvae (Pennacchio et al., 2014). If and how similar processes are in place for idiobiont parasitoids has been only limitedly investigated so far for *N. vitripennis* (Danneels et al., 2013, 2010; Martinson et al., 2014; Mrinalini et al., 2015; Rivers et al., 2002a, 2002b; Rivers and Denlinger, 1994, 1995) and a few other species (Altuntaş et al., 2010; Zhuo et al., 2016).

Here we address this research gap by focusing on the physiological regulation of the laboratory host *Spodoptera littoralis* by the generalist idiobiont wasp *Bracon nigricans*, an ectophagous larval parasitoid of lepidopteran species, among which is present the invasive South American tomato leaf miner, *Tuta absoluta* (Biondi et al., 2013; Zappalà et al., 2013). The larvae of this moth species are permissive for *B. nigricans*, and their fairly large size allows an easy collection and manipulation of samples for biochemical and molecular analyses. The experiments performed aim at unravelling the major changes induced by parasitism and natural envenomation on metabolism and immunity of the host, and their impact on its nutritional suitability for the developing parasitoid larvae.

## 2. Material and methods

### 2.1. Insect rearing

*S. littoralis* larvae were reared in groups, from egg hatching to 3rd instar, on artificial diet offered in aerated plastic boxes (25 h × 10 l × 7 w cm) at 25 ± 1 °C and 70 ± 5% relative humidity (RH), with 16:8 h light-dark period; then, older larvae were individually isolated in plastic vials (6 cm high × 2 cm in diameter) plugged with cotton wool, and kept under the same environmental conditions as above. The composition of the artificial diet used was as follows: 41.4 g/l wheat germ, 59.2 g/l brewer's yeast, 165 g/l corn meal, 5.9 g/l ascorbic acid, 1.53 g/l benzoic acid, 1.8 g/l methy4-hydroxybenzoate and 29.6 g/l agar. The six larval stages were identified on the basis of the morphological characters, according to Mochida (1973). Adults of *B. nigricans* were kept in Petri dishes (6 cm in diameter) at 27 ± 1 °C, 70 ± 5% RH, 16:8 h light-dark, and alimented with a water solution of honey (50% v/v). *S. littoralis* larvae were singly exposed to parasitoids as newly moulted 5th instars, in Petri dishes containing 4–5 female wasps; egg presence was checked every 30 min to assess parasitization occurrence. Parasitized host larvae were maintained in a climatic chamber (25 ± 2 °C, 70 ± 5% RH, 18:6 h light-dark) until the completion of parasitoid development.

### 2.2. Host metabolism

The metabolism of the host *S. littoralis*, measured as $CO_2$ produced, was assessed on parasitized larvae, naturally envenomated larvae (larvae parasitized as described in Section 2.1 from which the eggs were removed by 1 h after oviposition) and healthy larvae as a control. The measurement of $CO_2$ produced by experimental larvae was performed on groups of 5 larvae, which were incubated at 25 °C, 50% RH, 16:8 h light-dark in a hermetically sealed glass jar (9 l × 9 w × 13 h cm), along with a vial with 25 ml of distilled water and a vial with 10 ml of 0.5 M NaOH, which reacts with $CO_2$ released by the larvae, to produce $Na_2CO_3$. Control incubations were carried out in absence of *S. littoralis* larvae.

At the end of the incubation, 5 ml of 20% (w/v) $BaCl_2$ were added in the vial containing the NaOH to precipitate the carbonate, along with a few drops of phenolphthalein, as an acid-base indicator for the titration of formed $Na_2CO_3$ with 0.5 M HCl. A control titration was carried out by using 10 ml of 0.5 M NaOH. $CO_2$ measurements (mg/larva) were performed at 24 h intervals, over 3 days. The vial containing NaOH was replaced at each sampling occasion.

The calculation of the $CO_2$ released by each experimental larva was made according to the following formula: $mgCO_2/larva/h = (V_c - V_s) \times M \times 22/h \times n$ where $V_c$ = ml of HCl solution used for controls, $V_s$ = ml of HCl solution used for the sample, M = molarity of HCl solution used to titrate the NaOH at known concentration, 22 = equivalent weight of C in $CO_2$, n = number of experimental larvae, h = hours. For each treatment and each sampling time 3 replicates were performed.

### 2.3. Haemolymph biochemical profile

The nutritional suitability of the haemolymph of *S. littoralis* larvae, as affected by parasitism and envenomation, was characterized by measuring the changes over time of the titers of haemolymph proteins, acylglycerols and carbohydrates.

The quantification of proteins was performed as described by Bradford (1976), using the reagent Coomassie Protein Assay Reagent (Thermo Scientific) and bovine serum albumin (BSA) as standard. Samples of larval haemolymph were collected from a cut proleg at 0, 12, 24 and 48 h post-parasitization, transferred with a micropipette into an Eppendorf tube and added with an equal volume of anticoagulant MEAD buffer (98 mM NaOH, 145 mM NaCl, 17 mM EDTA, 41 mM citric acid, pH 4.5). The haemolymph was centrifuged at 300 × *g* for 10 min at 4 °C to remove haemocytes. The supernatant (plasma) was transferred to a new Eppendorf tube, and 5 µl of plasma were used for protein quantification after dilution with distilled water (1:20 v/v). Each sampling point was obtained from at least 6 larvae.

The titer of carbohydrates in the plasma was measured with a colorimetric method, as described by Salvador and Cônsoli (2008), using the anthrone method with some modifications. A volume of 800 µl of anthrone reagent (Sigma-Aldrich) (freshly prepared, 0.2% (w/v) in 96% $H_2SO_4$) was added to each plasma sample (400 µl of plasma diluted 1:40 with distilled water) and the mixture was placed at 100 °C for 5 min in a thermomixer. From each incubation mixture, 100 µl were collected and transferred into a 96 well-plate to measure the absorbance at 620 nm, using a Varioskan™ Flash Multimode Reader (Thermo Scientific). The calibration curve was prepared with the same protocol, by using standard solutions of D-Glucose. For both standards and samples the analysis was carried out in triplicate.

The titer of acylglycerols was determined by using the Triglyceride Quantification Colorimetric/Fluorimetric kit (BioVision), following manufacturer's instructions.

### 2.4. Host immune competence

A piece of nylon thread (0.08 mm of diameter) was inserted into the haemocoel of healthy 5th instar *S. littoralis* larvae and of synchronous parasitized larvae, soon after observing the oviposition by *B. nigricans* adult females and the subsequent host paralysis. The nylon thread was

inserted through the basal membrane of the last pair of abdominal legs, under a stereomicroscope, operated at a 20× magnification. After 24 h and 48 h, the implants were removed and photographed under a light microscope (400×). Image analysis was carried out using GIMP v. 2.8 (GNU Image Manipulation Program, www.gimp.org); the degree of encapsulation was scored as the percentage of non-white pixels (i.e. covered by haemocytes), while melanization index is a percentage calculated by the formula (1 − X/255) × 100, where X represents the mean degree of grey intensity (a numerical reading ranging from 0 for black to 255 for white) of the pixels in the area covered by haemocytes.

### 2.5. Host haemocyte counts

Haemolymph samples were collected as described above, transferred into Eppendorf tubes and added with an equal volume of MEAD buffer. Then, 10 µl of the sample were transferred into a new Eppendorf tube containing 5 µl of trypan blue dye (0.4% w/v).

After gentle pipetting, the suspension was loaded into a Bürker chamber. The counting of haemocytes (cells/ml) and cell viability (dead cells/ml) was carried out by transmission light microscopy (Zeiss Axioskop) at a 40× magnification.

### 2.6. Host tissue collection and cathepsin L enzymatic assay

Fat body and adhering haemocytes were dissected out from *S. littoralis* 5th instar larvae in PBS (Phosphate Buffered Saline: 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4), 48 h after parasitism by *B. nigricans,* and from synchronous non-parasitized controls. Tissue pieces were gently transferred into an Eppendorf tube, immediately frozen in liquid nitrogen and stored at −80 °C until use. Each sample, consisting of fat body pooled from 3 separate larvae, was replicated 6 times for parasitized larvae and 4 times for controls. This experimental set-up was adopted in 2 independent experiments. These experimental samples were homogenized in PBS with a pestle fitting Eppendorf tubes and the protein concentration of crude homogenate was measured by Bradford assay, using BSA as standard. Cathepsin L activity was measured in 100 µg of crude homogenates using a commercially available kit (Cathepsin L Activity Assay Kit, Abcam, Cambridge, MA, USA) according to manufacturer's instructions.

### 2.7. qRT-PCR

Larval haemolymph, containing fat body fragments and adhering haemocytes, as well as free haemocytes, was collected from a cut of the abdominal proleg and transferred into ice-cold MEAD anticoagulant buffer, 48 h after parasitism by *B. nigricans,* and from synchronous non-parasitized controls. Tissue pieces and cells were separated from plasma by 5 min centrifugation at 300×*g*, at 4 °C. Total RNA was extracted from samples using TRIzol® reagent (Life Technologies, Carlsbad, CA, USA), according to manufacturer's instructions. Differential relative expression of *cathepsin L* was measured by one-step qRT-PCR, using the SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA,USA), according to the manufacturer's instructions. *S. littoralis α-tubulin* gene (Accession Number EZ982121) was identified in available EST collections by blasting its homologue annotated for *Spodoptera exigua* (Accession Number GU983915; 86% sequence identity), and then used as endogenous control for RNA loading (TUB *Fw*2: CGTGACGACGTGTCTGCGGTT; TUB *Rev*2: GCGTGAGTTCGGGTACGGTG). All primers were designed using Primer Express, version 1.0 software (Applied Biosystems). Relative gene expression data were analyzed using the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001; Pfaffl et al., 2002).

Analysis by qRT-PCR of *cathepsin L* expression was carried out by using specific primers (qCAT106 *Fw*: ACGACGTCGGCTTCGTAGAC; qCAT106 *Rev*: GTCTCCTGTGAGGCGTCGA) designed on the sequence of *S. littoralis* gene (Accession Number EZ982213) identified in EST collections by blasting its homologue annotated for *S. exigua* (Accession Number EF068256; 91% sequence identity). For validation of the ΔΔCt method the difference between the Ct value of *cathepsin L* and the Ct value of *α-tubulin* transcripts [ΔCt = Ct(*cathepsin L*)-Ct (*α-tubulin*)] was plotted versus the log of ten-fold serial dilutions (100, 10, 1, 0.1 and 0.01 ng) of the purified RNA samples. The plot of log total RNA input versus ΔCt displayed a slope less than 0.1 (y = −0.0983x + 4.9083), indicating that the efficiencies of the two amplicons were approximately equal.

### 2.8. Microscopy

#### 2.8.1. Sample preparation

In order to keep unaltered as much as possible interacting host tissues, tissue samples were collected from *S. littoralis* 5th instar larvae that received an injection of fixative solution, 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2), and were maintained at 4 °C for 2 h. Tissue samples obtained upon dissection were washed in 0.1 M Na-cacodylate buffer (pH 7.2) and post-fixed at 4 °C for 2 h, with 1% osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in ethanol series, samples were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany). Semithin sections were stained by crystal violet and basic fuchsin and were observed with a light microscope (Olympus, Tokyo, Japan). Images were acquired with a Nikon DS-SM camera. Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

In order to prepare cryostat samples, the abdominal region of the larva was excised, immediately embedded in polyfreeze cryostat embedding medium (Polyscience Europe, Eppelheim, Germany), and stored in liquid nitrogen. Cryosections (8 µm thick) were obtained on a Leica CM 1850, and slides were stored at −20 °C or immediately used for amyloid fibrils characterization and immunocytochemical experiments.

#### 2.8.2. Amyloid fibril detection

Amyloid fibrils were identified according to Le Vine III (Le Vine III, 1999), by staining samples with thioflavine T and visualizing the amyloid-specific green/yellow fluorescence with an Olympus BH2 microscope (excitation wavelength = 465 nm). Images were acquired with a DS-5M-L1 Nikon digital camera system.

#### 2.8.3. Indirect immunofluorescence staining

Samples were incubated with PBS containing 2% BSA for 30 min before the primary antibody incubation (4 °C, over night). The presence of cathepsin L was assessed using anti-cathepsin L (1:200 dilution, Sigma-Aldrich) polyclonal antibody. Incubation with suitable secondary antibody conjugated with tetramethylrhodamine (TRITC) (1:200 dilution) (Jackson, Immuno Research Laboratories, West Grove, PA, USA) was performed for 1 h at room temperature in a dark moist chamber. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). All washings and dilutions were performed with PBS and 2% BSA. In negative control samples, primary antibodies were omitted. Coverslips were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA. USA); slides were observed on Olympus BH2 microscope (Olympus). Data were recorded with a DS-5M-L1 digital camera system (Nikon). Images were combined with Adobe Photoshop (Adobe Systems, Inc.).

### 2.9. Statistical analysis

Statistical analyses were performed using the Statistical Analysis Systems software (Sigma Stat Statistical Software, SPSS Science, Chicago, IL, USA). Normal distribution and homoscedasticity of all data were determined by the Kolmogorow-Smirnov's test and Levene's test, respectively. Emitted $CO_2$, total protein, acylglycerols and carbohydrate titers, as affected by experimental treatments and time were analyzed

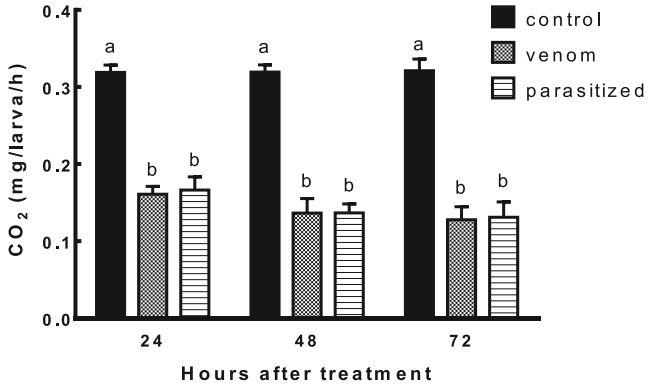


**Fig. 1.** Emission of $CO_2$ (mg/larvae/h) by parasitized, naturally envenomated and non-parasitized (control) *Spodoptera littoralis* larvae at 24, 48, 72 h after parasitization or natural envenomation (two-way ANOVA and LSD's post hoc analysis for each time point, different letters denote statistically significant differences $P < 0.001$).

using two-way ANOVA with General Liner Model (GLM), and mean values compared by least significant difference (LSD) test. Encapsulation and melanization indexes, as well as haemocyte counts, cathepsin L activity and transcription levels were analyzed by Student's *t*-test.

## 3. Results

### 3.1. Host metabolism and haemolymph biochemical profile

The impact of parasitism by *B. nigricans* on metabolism of *S. littoralis* larvae was highly significant ($F_{3,55} = 144.385$, $P < 0.001$), already evident by 24 h after parasitism, with a nearly 50% reduction of $CO_2$ production, which remained stable over time (Fig. 1). This effect was reproduced by venom, as clearly supported by the experimental data obtained for naturally envenomated host larvae (Fig. 1). These results demonstrate that parasitized host larvae undergoing paralysis, due to venom injection, have a reduced but still active metabolism, which supports tissue survival.

It is generally accepted that host regulation is more pronounced in koinobiont species, even though the level of knowledge for idiobionts, such as *B. nigricans*, is very low, due to the limited number of available studies (Pennacchio and Strand, 2006). To understand the impact of *B. nigricans* on host biochemical profile, which meets the nutritional needs of the developing parasitoid progeny and of the surviving host tissues, we investigated the changes over time of the major nutrients in the haemolymph, as affected by parasitism and venom. Parasitism and natural envenomation both resulted into an altered host biochemical profile. Indeed, the plasma titer of proteins, both for parasitized and naturally envenomated host larvae, was significantly higher than in non-parasitized controls ($F_{3,11} = 40.605$, $P < 0.001$), and increased over time ($F_{3,11} = 19.437$, $P < 0.001$) (Fig. 2A).

For what concerns the acylglycerol titer, a divergence of the curve slopes, starting at 24 h, led to significantly lower titers than in controls at 48 h after parasitism, both for parasitized and envenomated host larvae ($F_{3,25} = 13.600$, $P < 0.001$) (Fig. 2B). These changes were significant over time ($F_{3,25} = 19.880$, $P < 0.001$).

The carbohydrate titer was significantly enhanced by both parasitism and envenomation ($F_{4,25} = 67.459$, $P < 0.001$), with much higher values recorded at all sampling times considered (Fig. 2C). No significant differences were observed over time ($F_{4,25} = 1.888$, $P = 0.175$).

### 3.2. Host immune competence and haemocyte counts

The immune response by the host can have a negative impact on feeding activity of ectoparasitic wasps, which have to overcome this barrier (Pennacchio and Strand, 2006). The impact of parasitism on host immune response was assessed by scoring the degree of reaction against a nylon thread implanted in the haemocoel. Both encapsulation (Fig. 3A) and melanization (Fig. 3B) were significantly reduced (Student's *t*-test: encapsulation index at 24 h, $P < 0.001$, $t = 4.012$, df = 18; at 48 h, $P < 0.01$, $t = 3.893$, df = 18; melanization index at

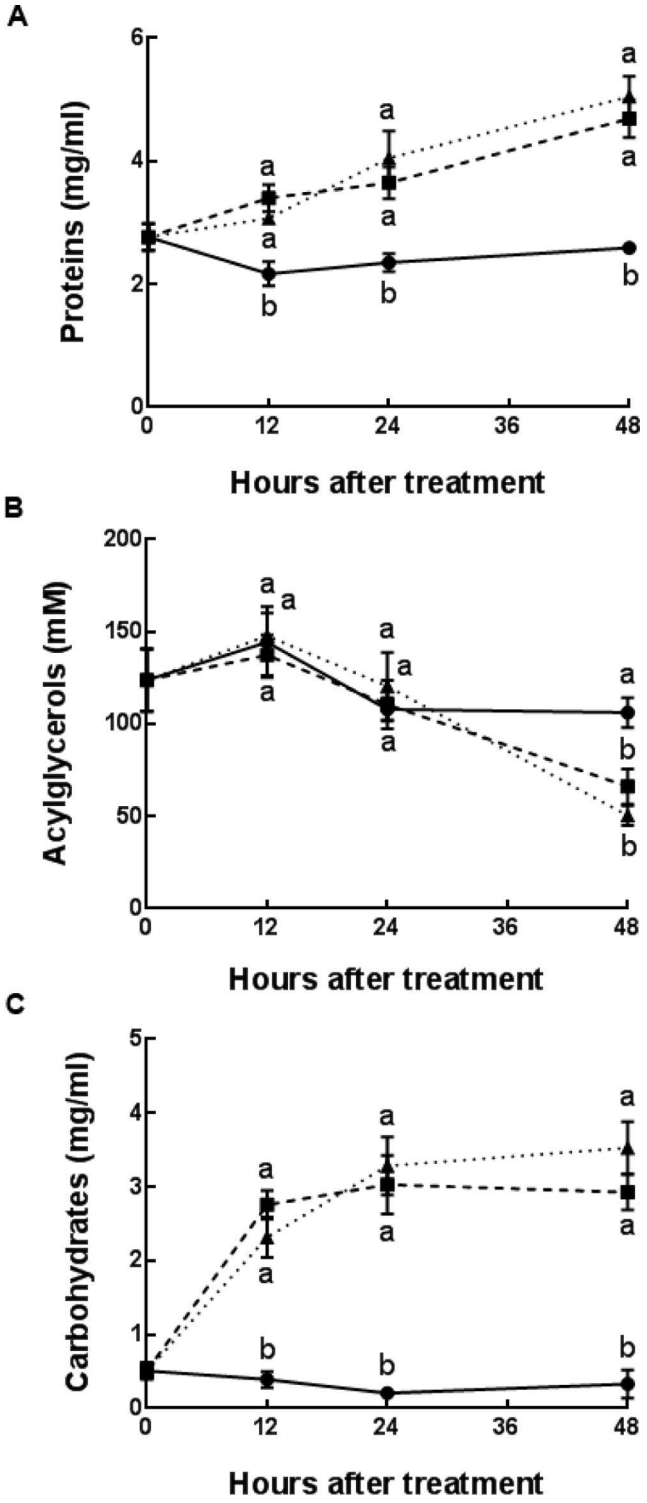


**Fig. 2.** Titer of total proteins (A), acylglycerols (B) and carbohydrates (C) in the plasma of *Spodoptera littoralis* larvae, as affected by parasitism and natural envenomation (two-way ANOVA and LSD's post hoc analysis for each time point, different letters denote statistically significant differences $P < 0.001$). Legend: control (—●—), envenomated (- -■- -), parasitized (···▲···).

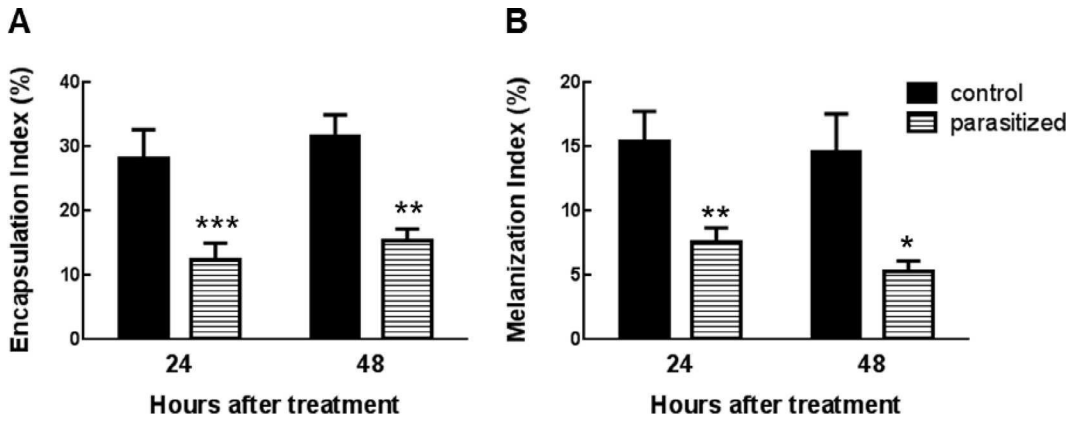


**Fig. 3.** Encapsulation and melanization index of nylon threads implanted in parasitized and non-parasitized (control) *Spodoptera littoralis* larvae at 24 and 48 h after their implant in the haemocoel. Student's *t*-test: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

24 h, $P < 0.01$, $t = 3.654$, df = 18; at 48 h, $P < 0.05$, $t = 2.433$, df = 18) by *B. nigricans* parasitism, both at 24 and 48 h after oviposition.

In order to assess if parasitism had any effect on haemocyte number and viability, total counts were carried out and mortality rates assessed. The total haemocyte counts in *S. littoralis* larvae were not markedly influenced by parasitism or natural envenomation (Table 1); indeed, the mortality rate of haemocytes was only slightly, but significantly (Student's *t*-test: haemocytes mortality rate at 24 h, $P < 0.05$, $t = 4.758$, df = 17; at 48 h, $P < 0.001$, $t = 10.59$, df = 6), higher in parasitized host larvae than in controls (Table 1). However, 33.2 ± 2.5 % of the haemocytes circulating in the haemolymph of parasitized and envenomated larvae were morphologically very similar to macrogranulocytes described by Zhai and Zhao (2012), which are involved in fat body degradation of *Helicoverpa armigera* larvae when they attain the pupal stage.

### 3.3. Histological changes

The fat body of newly moulted 5th instar larvae of *S. littoralis* showed an evident presence of cytoplasmic vacuoles and stores of lipid and glycogen (Fig. 4A). After 24 h from parasitization, the fat body was surrounded by adhering haemocytes (Fig. 4B), which were included in a matrix of thioflavin-positive amyloid material (Fig. 4C). This layer of amyloid fibrils, lining the fat body, was made of interwoven fibrillar material in which were included haemocytes with different phenotypes (Fig. 4D–F). In particular, granulocytes showed a polymorphic appearance. There were granulocytes with the cytoplasm rich of electrondense granules (Fig. 4D, E) and larger granulocytes (macrogranulocytes) with a central nucleus surrounded by dilated cisternae of the rough endoplasmic reticulum filled with fibrillar material, which is released by degranulation (Fig. 4D, F)

Transmission electron microscopy (TEM) observations of fat body cells from non-parasitized larvae showed the cytoplasm occupied by organelles surrounded by large lipid droplets and glycogen stores (Fig. 4G, H). By 24 h after parasitization, fat body cells showed a general progressive nutrient mobilization, large empty vacuoles, along with reduced stores of lipid and glycogen (Fig. 4I, J). By 48 h after parasitization, the structural integrity of the fat body cells was largely altered, showing a nearly emptied cytoplasm with organelles distributed close to the nucleus, in central position and/or lining the plasma membrane (Fig. 4K, L).

### 3.4. Fat body degradation

The presence in parasitized larvae of macrogranulocytes, which are involved in the degradation of the fat body in *H. armigera* larvae during metamorphosis, by releasing cathepsin L (Zhai and Zhao, 2012), and the observed intense surface interaction with fat body prompted us to measure any change of this enzymatic activity induced by *B. nigricans* parasitism.

To assess if an increase of cathepsin L activity was localized on the surface of degrading fat body, we performed immunofluorescence experiments. The layer of haemocytes embedded in the amyloid matrix coating the fat body of parasitized host larvae (Fig. 5A, B) was associated with an intense signal of cathepsin L (Fig. 5C); synchronous non-parasitized controls did not show the presence of this haemocyte aggregation on the surface of fat body and of associated immunofluorescence signal for cathepsin L (data not shown).

We thus measured the cathepsin L enzymatic activity of tissue homogenates obtained from host larvae 48 h after parasitism and from synchronous non-parasitized controls. The enzymatic activity associated with fat body/haemocyte complex isolated from parasitized *S. littoralis* larvae was significantly higher than that measured on fat body obtained from non-parasitized controls (Student's *t*-test: $^{*}P < 0.001$, t = 9.3, df = 20, n = 18) (Fig. 5D, black bars). The change in enzymatic activity is supported by transcriptional data, as the relative expression of *cathepsin L* was significantly higher in fat body/haemocytes complex from parasitized *S. littoralis* larvae compared to non-parasitized controls (Student's *t*-test: $^{*}P < 0.001$, t = 9.5, df = 6, n = 4) (Fig. 5D, white bars).

## 4. Discussion

Host paralysis and/or killing by idiobiont parasitic Hymenoptera is often considered to be associated with the provision to the developing

**Table 1**
Cell counts and mortality of haemocytes from parasitized and non-parasitized (control) *Spodoptera littoralis* 5th instar larvae, at 24 and 48 h after parasitization.

| | 24 h | | 48 h | |
|---|---|---|---|---|
| | cells/µl | mortality (%) | cells/µl | mortality (%) |
| Control | 7325 ± 611 (9) | 0.7 ± 0.1 (9) | 8081 ± 607 (4) | 0.4 ± 0.1 (4) |
| Parasitized | 7505 ± 1140 (10) | 2.0 ± 0.4 (10)$^{*}$ | 11718 ± 572 (4)$^{*}$ | 1.4 ± 0.1 (4)$^{**}$ |

Values are means ± s.e.; the number of replicates is indicated in parenthesis. Student's *t* test: $^{**}P < 0.001$, $^{*}P < 0.01$.

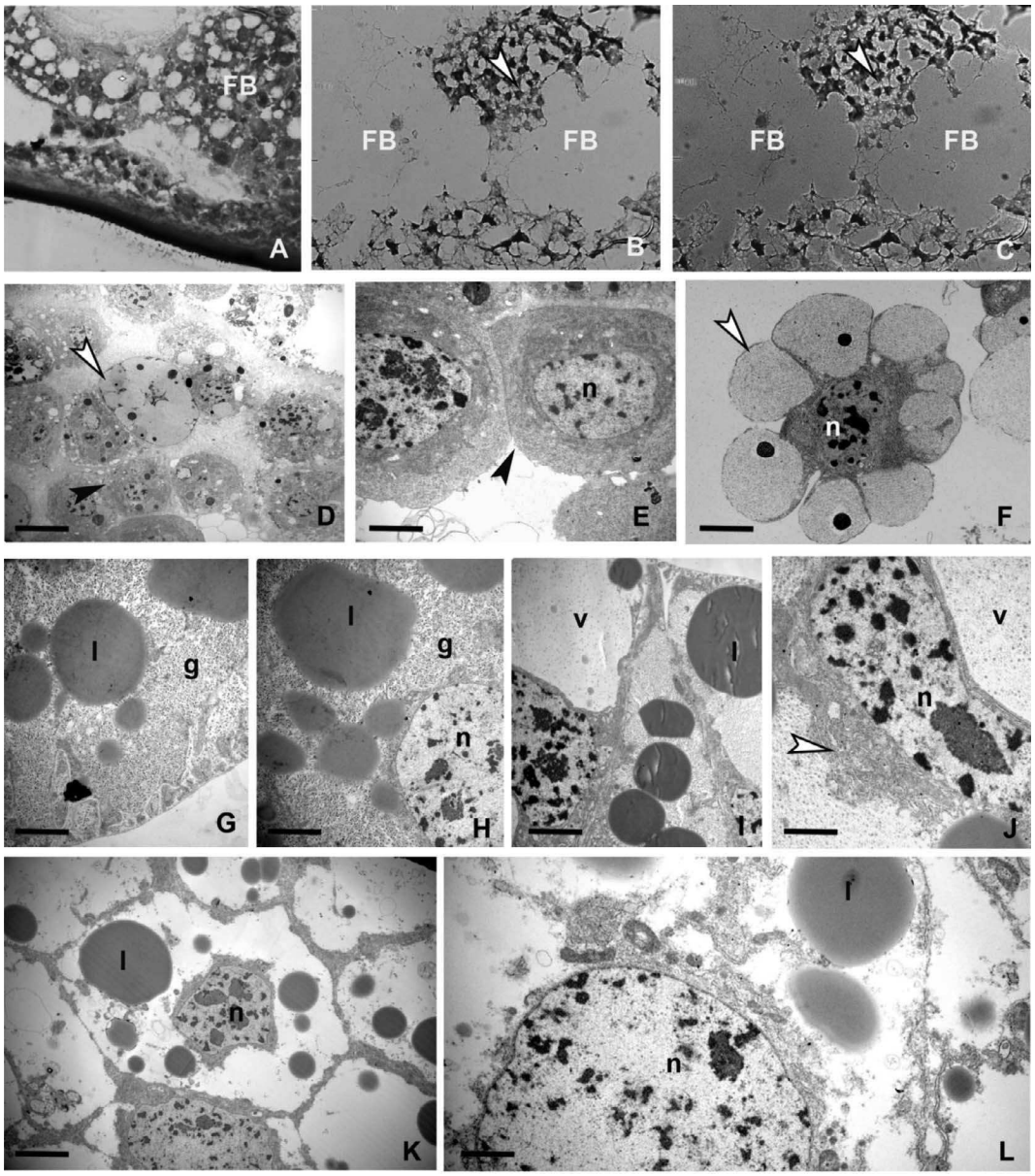


**Fig. 4.** Cryosections (A–C) and thin sections (D–L) of tissues from parasitized and non- parasitized *Spodoptera littoralis* larvae. The peripheral fat body (FB), next to the body wall, showed large storage vacuoles (A). After 24 h following parasitization, the FB was surrounded by haemocytes (white arrowheads) (B) interspersed in an amyloid fibril network, thioflavin positive (green signal) (C). Haemocytes close to fat body cells showed different phenotypes (D): cells smaller in size (D-black arrowhead, E) with the cytoplasm showing granular appearance, and larger macrogranulocytes (D-white arrowhead), with a central nucleus (n) surrounded by rough endoplasmic reticulum (RER) dilated cisternae filled with fibrillar material (F-white arrowhead). In non-parasitized larvae, FB cells were characterized by cytoplasm filled with lipid droplets (l) and glycogen (g) (G, H). 24 h after the parasitization, FB cells showed large empty vacuoles (v) and RER close to the nucleus (n) (I, J). 48 h after parasitization, FB cells, deprived of glycogen and lipid (l) stores, showed large vacuolization (K, L). Scale bars: D, K 5 μm; E, F, L 2 μm; G, H, 3 μm; I 2.5 μm; J, 1.3 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

progeny of a fixed amount of food, as a predefined and static nutritional resource (Gauld, 1988; Quicke, 1997). However, this simplistic view has been challenged by the detailed studies on *N. vitripennis* (Danneels et al., 2010, 2013; De Graaf et al., 2010; Martinson et al., 2014; Mrinalini et al., 2015; Rivers et al., 2002a, 2002b; Rivers and Denlinger, 1995, 1994), a pupal parasitoid, which have demonstrated the occurrence of significant host physiological changes largely mediated by the venom. Indeed, whole venom alone is able to arrest host development and is responsible for major changes in metabolic and immune functions of the host (Rivers and Denlinger, 1994, 1995; Rivers et al., 2002a, 2002b).

The large body of experimental evidence available on host regulation, basically relative to koinobiont wasp species, clearly supports the concept that host alterations mostly aim at suppressing the immune reaction against parasitoid eggs and at meeting the nutritional needs of developing juvenile stages (Pennacchio and Strand, 2006; Pennacchio et al., 2014). These aspects have been largely overlooked for ectoparasitic idiobiont species, for which studies on host physiological changes are quite scattered and limited (Pennacchio and Strand, 2006).

Here we have addressed this research gap by taking into consideration the multifaceted regulation of host physiology by an idiobiont parasitoid, *B. nigricans*, which induces a rapid paralysis and developmental block by venom injection at the oviposition. These dramatic changes are associated with a nearly 50% reduction of the metabolic rate of host tissues, which, however, remain quite active, over 72 h after parasitization, in spite of the total developmental block observed. This evidence is in agreement with the reported effects induced by *N. vitripennis* venom, which strongly alters host physiology and induces a reduction of its oxygen consumption (Rivers and Denlinger, 1994). It is interesting to note that when parasitoid larvae

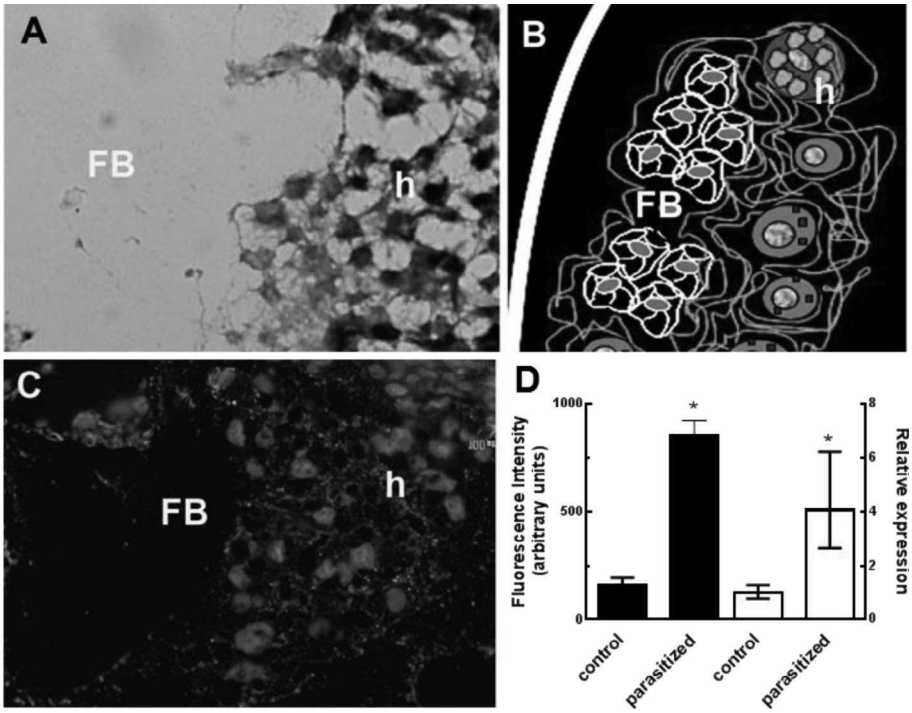


**Fig. 5.** Cryosections of parasitized *Spodoptera littoralis* larvae (A) showed haemocytes (h) adhering to the surface of fat body (FB) cells, interspersed in a matrix of amyloid fibrils, as schematically represented in panel (B), associated with a strong immunopositivity signal for cathepsin L (red) (C). Cathepsin L activity and relative expression of the coding gene, measured by qRT-PCR, was significantly higher in tissues from parasitized *S. littoralis* larvae compared to non-parasitized controls (D). Student's *t*-test: $^{*}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hatch and start to feed, the haemolymphatic concentration of protein and carbohydrates is significantly higher both in parasitized and naturally envenomated host larvae, while an opposite trend is observed for acylglycerols at 48 h after parasitization. Because a paralyzed larva is a closed system, without any external nutritional input, the biochemical profile of the haemolymph reflects the balance between the mobilization of stored nutritional resources and their use, mostly by host tissues, which is a biomass by far more abundant than newly hatched parasitoid larvae. Then, it is reasonable to assume that the decreased concentration of acylglycerols in the host haemolymph can be the final result of a selective use of these nutrients by host tissues, that remain metabolically active, even though at a reduced rate. The higher concentrations of proteins and carbohydrates significantly enhance the host nutritional suitability for feeding parasitoid larvae, which may rely on a much richer haemolymphatic substrate, as often observed in endophagous koinobiont species (Pennacchio and Strand, 2006; Pennacchio et al., 2014). Therefore, the concept that the host of an idiobiont larval parasitoid is a static food source is not correct. The observed regulation of the metabolism and biochemical profile of *S. littoralis* larvae parasitized by *B. nigricans* and the similar host changes induced by *N. vitripennis* (Rivers and Denlinger, 1994, 1995) clearly corroborate the hypothesis that in the basal lineages of ectoparasitic idiobiont Hymenoptera, as observed in more derived koinobionts, there are regulation mechanisms which enhance and dynamically modulate host nutritional suitability.

Because the parasitized host is a closed system, we wanted to assess how *B. nigricans* is able to mobilize the stored nutritional resources, in order to make them available for the feeding parasitoid larvae. TEM observations clearly support the hypothesis that the fat body is the major host tissue that releases its content, through a gradual process of cell vacuolization and subsequent cytoplasmic reabsorption. Indeed, the temporal profile of these ultrastructural changes inversely matches the time-related fluctuations of the host biochemical profile.

This process is mediated by a close surface interaction between the fat body and circulating haemocytes, which include a parasitism-induced peculiar morphotype denoted as macrogranulocytes, rich of fibrillar vacuole inclusions released by cell degranulation. These haemocytes are very much similar to macrogranulocytes actively involved in cathepsin L release and fat body degradation in *H. armigera* larvae attaining the pupal stage (Wang et al., 2010; Zhai and Zhao, 2012). This enzyme plays a more general key-role in tissue remodelling during insect development (Kuleesha et al., 2016; Zhang et al., 2013). Here we have observed that *B. nigricans* parasitism and its venom are able to induce the formation of macrogranulocytes, which trigger a rapid histolysis of the fat body and the mobilization of the stored nutrients. This process is associated with the release of cathepsin L, which is present around the haemocyte clumps interacting with the fat body surface. Moreover, the expression of *cathepsin L* is significantly higher in parasitized larvae, and the enzymatic activity is detected in a network of amyloid fibrils produced by haemocytes. It is reasonable to assume that amyloids provide a molecular scaffold that allows a strict localization of cathepsin L activity only where needed, preventing a systemic diffusion and a generalized poorly controlled degradation process of host tissues. This is in line with the functional role of amyloid fibrils in the immune response, where they are involved in the regulation of the humoral and cellular components of the defense reaction in lepidopteran larvae (Caccia et al., 2016; Di Lelio et al., 2014; Falabella et al., 2012; Pascale et al., 2014). In this latter case the amyloid scaffold, produced by the protein P102, is responsible for the induction of encapsulation/nodulation responses and for the strict localization of the melanization reaction on the surface of the foreign invader to be suppressed, thus preventing the dangerous diffusion of melanin and its quinone precursors, which can be lethal for an insect mounting a defense reaction. The involvement of P102 or of another amyloidogenic protein further corroborates the importance of this type of molecular strategy in the modulation of physiological processes that must be localized to prevent systemic undesired, or even lethal, effects. Therefore, amyloids can be functional in insects (Di Lelio et al., 2014; Falabella et al., 2012; Grimaldi et al., 2012; Pascale et al., 2014), like more generally reported for other organisms (Fowler et al., 2007; Homa et al., 2016; Ng et al., 2013).

The host regulation by *B. nigricans* has also an influence on host immune response. In spite of the fact that there are no big differences in the number of circulating haemocytes, a significant down-regulation of the encapsulation and melanization responses has been observed, as in the case of fly hosts envenomated by *N. vitripennis* females, for which, in contrast, a drop of immune cell number was recorded (Rivers et al., 2002b). Then, like in other ectoparasitic wasps, the immunosuppression

of the host is part of a physiological redirection, which is essential to facilitate food uptake and use when feeding from outside (Pennacchio and Strand, 2006). Indeed, the induction of clotting by feeding wounding, which shares molecular pathways with cellular immune responses (i.e. encapsulation and nodulation) (Lemaitre and Hoffmann, 2007), if not prevented, can interfere with feeding of ectophagous larvae. This seems to be a conserved strategy adopted by ectoparasitic arthropods, such as, for example, the mite *Varroa destuctor*, which is able to exert an immunosuppressive action on its host *Apis mellifera*, further reinforced by the honey bee viral pathogen DWV (Deformed Wing Virus) (Di Prisco et al., 2016). Indeed, this mite-virus symbiotic association enhances *Varroa* fitness and provides a nice example on how viral pathogens of the host can be used by parasites of the host to better overcome the immune barriers, in order to get a safer access to nutritional resources (Di Prisco et al., 2016; White et al., 2014). This evolutionary pattern is even more evident in the case of the association between the endophagous braconoid wasps, parasitoids of lepidopteran larvae, and symbiotic viruses in the family Polydnaviridae (Strand and Burke, 2013; White et al., 2014); the ancestor of these viral symbionts in the genus *Bracovirus,* was, indeed, a viral pathogen of the host in the Nudivirus group, which was domesticated and used by the wasp as a potent immunosuppressive agent allowing the survival of its progeny (Herniou et al., 2013).

In conclusion, the host regulation process by *B. nigricans*, an ectoparasitic idiobiont species, which determines a rapid host developmental arrest by envenomation, is by far more subtle and relevant than expected on the basis of the unsubstantiated idea that idiobiont parasitoids poorly interact with their hosts. The results here presented clearly show how parasitism, mainly by venom action, is able to redirect host metabolism in order to enhance its nutritional suitability for the developing parasitoid larvae, and provide the required metabolic support to host tissues. This is achieved through a finely tuned mobilization of nutrient stored in the host fat body, which is degraded though a very targeted and localized cathepsin L activity, with the direct involvement of the haemocytes. The survival of these cells is not markedly influenced by parasitization, even though a persistent immunosuppression is observed in parasitized hosts, likely to allow a more efficient uptake and use of the haemolymph by the ectoparasitic larvae. Collectively, these results shed light on the host regulation mechanisms in basal lineages of parasitic Hymenoptera, very poorly investigated so far, and provide interesting new information which can be used to develop on a rational basis new artificial diets for *in vitro* rearing.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2017.07.002.

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RESEARCH ARTICLE

# First report of the gall midge *Asphondylia serpylli* on thyme (*Thymus vulgaris*), and identification of the associated fungal symbiont

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## Abstract

*Asphondylia* spp. (Diptera: Cecidomyiidae) are known for inducing gall formation on many diverse plants in both wild and agricultural contexts. The species *Asphondylia serpylli* is herewith reported for the first time on thyme (*Thymus vulgaris*) cropped in Poland. The associated fungus has been identified as *Botryosphaeria dothidea*, representing its first record from cecidomyiid galls on a species of Lamiaceae. Moreover, a short account is given on the parasitoid species active in this particular ecological context. These findings point out the basic role of *B. dothidea* in the organization of these three-component biotic systems regardless of the varied assortments between the midge species and their host plant.

## Introduction

Thyme (*Thymus vulgaris*) is a medicinal plant belonging to the Lamiaceae which is cropped at a limited extent for pharmaceutical applications and culinary usage. Cultivation in south eastern Poland reached 530 hectares in 2016. Crops are usually harvested by mowing before blossoming, which takes place in the second year after plantation. However, at the end of the cropping cycle plants in a few lines are not mown in view of seed production. Observations carried out on these plants in summer 2016 evidenced the presence of swollen buds, distinctively bigger than the normal ones, scattered on all blooming shoots. When dissected in the laboratory, the presence of gall midges in larval or pupal instars was observed, together with a white cottony mycelium bordering the inner edge of the galls. At an advanced stage these galls were hard and darkened.

Midges in the genus *Asphondylia* and a few allied genera (Diptera: Cecidomyiidae) are associated to the formation of galls on different organs of many plant species worldwide, which also harbour a mycelium developing on the inner wall. According to some authors this symbiosis seems to be mutualistic, based on the ability of the midges to develop by feeding on the associated fungus, and to inoculate it into the flower buds during oviposition (Rohfritsch, 2008; Heath & Stireman, 2010). Several *Asphondylia* spp. have been reported to induce the formation of galls in flower buds of Lamiaceae, but most reports only concern the incidental finding of the insect biont and merely classify it with reference to the host plant (Urso-Guimarães & de Souza Amorim, 2002; Skuhravá *et al.*, 2006).

In this paper we report on the finding of a species of *Asphondylia* for the first time on *T. vulgaris*, together with preliminary data concerning its phenology and parasitoids. A thorough account is also provided of the associated fungal symbiont.

## Materials and methods

The plant material was collected in three locations near Lublin, where crops were in their second year of cultivation. Plants in the lines left for seed production started flowering in early June, after the rest of the crop had been harvested. In order to assess the extent of the infestation, 100 blooming shoots were collected from each of the

three sites, and placed in zip-lock bags for transportation to the laboratory. Galls were detached from shoots, and dissected to determine the developmental stage of the midge and the fungal symbiont.

### Identification of the gall inhabitants

Galls were longitudinally cut for inspection of their content, disclosing the presence of midges and their parasitoids in different instars. Pupae and adults were mounted on slides using Canada balsam as medium. The taxonomic characters were observed under a Zeiss Axiophot microscope. For identification of the parasitoids, single galls were kept in small vials at room temperature. The emerged parasitoids were mounted on card, and some of them were dissected and mounted on slides using Canada balsam as medium. Both materials were used for species identification. In order to evaluate the extent of the parasitoid activity on the gall midge, 100 galls collected in October 2016 were dissected, recording the numbers and relative ratios of empty galls, galls harbouring *Asphondylia*, and galls with parasitic activity. The dissection was also used to understand the actual host–parasitoid relationship.

### Isolation and identification of the associated fungi

Isolations from the mycelial mat developing inside the galls, and from the body surface of larvae and pupae of the insects dwelling inside the galls were carried out on potato-dextrose agar (PDA, Oxoid) amended with streptomycin sulphate (200 mg $L^{-1}$). Pins previously dipped into 96% ethanol were used to excise gall fragments to be placed on the agar medium. Larvae and pupae were transferred to the isolation plates without previous dissection. Hyphal tips from emerging fungal colonies were transferred into fresh PDA plates so as to obtain pure cultures for morphological identification and storage. Preliminary identification based on the production of conidia was carried out under a MBL-N 120 light microscope. Pycnidial production by botryosphaeriaceous isolates was stimulated by placing PDA plates under near-UV light at room temperature (Crous *et al.*, 2006).

Identification was also performed through rDNA-ITS sequencing. To this purpose, total genomic DNA was extracted from fresh mycelium taken from pure cultures using CTAB method following manufacturer's protocol (Sambrook & Russell, 2001). The PCR amplification and sequencing was performed according to reported methodology (Groenewald *et al.*, 2013; Phillips *et al.*, 2013). The original DNA sequences obtained in this study have been deposited in GenBank.

## Results

Inspection of the galls collected in the field under the dissecting microscope disclosed the dominant presence of cecidomyiid midges in larval and pupal instars (Fig. 1A and Fig. 1B), with an estimated degree of infestation of 90% of the flower buds present on the sampled shoots. Pupal and adult characters shown by the *Asphondylia* specimens emerged from galls on *T. vulgaris* were similar to those reported by Skuhravá (2011) in her redescription of the species *Asphondylia serpylli* Kieffer; in particular, the frontal horns (Fig. 1C), the spines on the abdominal tergite (Fig. 1D), and the shape of the male genitalia (Fig. 1E and Fig. 1F). Adults (Fig. 1G) continued emerging from the material kept in the plastic bags up to 10 days following collection, evidencing a likely generation overlapping. However, no more midges emerged since mid-October. According to Skuhravá (2011) *A. serpylli* develops two generations per year. Our data show that the gall midges have a long period for reproduction from late spring to autumn, probably developing more than two generations per year, and that the species overwinters as pupa.

The occurrence of parasitoids was also evident in 40% of the dissected galls. The dominant species was *Sigmophora brevicornis* (Panzer) (Hymenoptera: Eulophidae), an ectophagous, larval, solitary or gregarious parasitoid of many species of *Asphondylia* (Graham, 1987). Rather rare were the solitary parasitoids *Pseudocatolaccus nitescens* (Walker) (Hymenoptera: Pteromalidae) and *Aprostocetus westwoodii* (Fonscolombe) (Hymenoptera: Eulophidae). These species are recorded as natural enemies of *Asphondylia* spp. on host plants of several botanical families (Noyes, 2017).

When galls containing larvae at any instar were cut in view of being inspected for their content, the presence of a white cottony mycelium coating the inner wall was invariably observed (Fig. 1A). In older galls containing pupae, the walls were darkened, and the mycelium was no more cottony, but visible as a residual layer adpressed onto the gall wall (Fig. 1B). The presence of globose structures corresponding to pycnidia was occasionally observed (Fig. 1H). However, the outcome of isolations carried out on PDA in the aim to identify the fungal inquiline of the galls was not univocal. Besides *Cladosporium* and *Alternaria*-like fungi, and a few occasional miscellaneous species, the majority of the isolation attempts yielded a fungus producing a dark mycelium resembling species of Botryosphaeriaceae. Isolates corresponding to this morphotype produced aerial and highly dense mycelium on PDA. Cultures were grey to olivaceous-grey, darkening to black with ageing. When exposed to near-UV light, pycnidial conidiomata developed after 12 days (Fig. 1I). These fruit bodies were

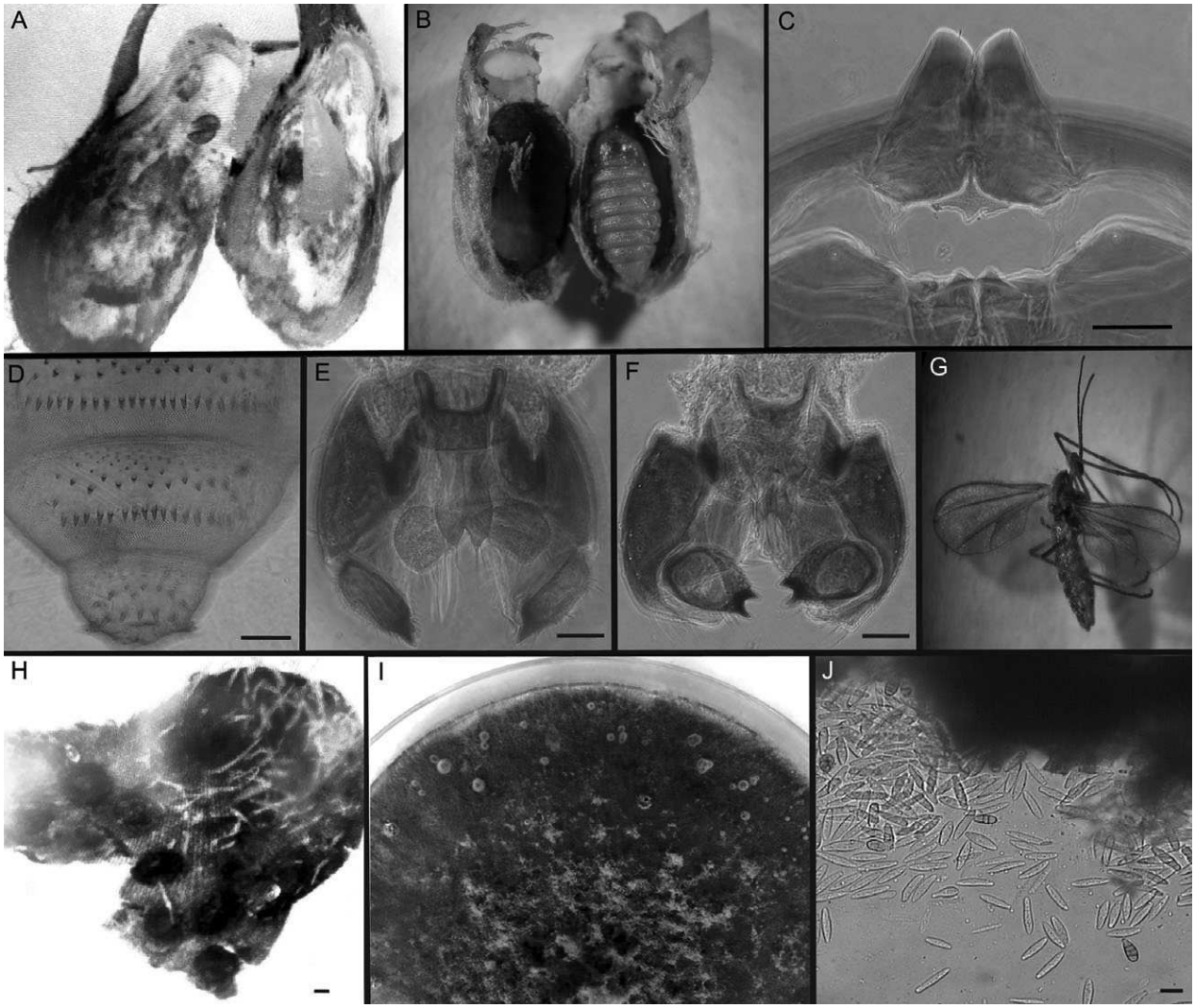


**Figure 1** (A) Flower galls induced on thyme by *Asphondylia serpylli*, with larva and cottony mycelium developing on the inner wall. (B) An opened flower gall of the same with an overwintering pupa. (C) Pupal head horns. (D) Terminal pupal segment in dorsal view showing the spine arrangement. (E) Male genitalia view showing the general shape and those of the gonocoxite, its lobe and cerci. (F) Male genitalia showing the shape of the gonostylus. (G) Adult female. (H) Pycnidia of *Botryosphaeria dothidea* on the gall wall. (I) Pycnidia formed in PDA culture. (J) Conidia of *Fusicoccum* and *Dichomera* stages produced by the same isolate. Bars = 10 μm.

solitary to aggregated, black and globose bearing conidia of two different morphological types (Fig. 1J). More in detail, *Fusicoccum*-like conidia were hyaline, unicellular, narrowly fusiform or irregularly fusiform, size (17.5–) 19.5–25.5(–29.5) × 4–8.5 μm (average of 40 conidia 22.4 × 5.6), and muriform conidia, representing the *Dichomera* synanamorph, were dark coloured, with both horizontal and vertical septa, quite variable in shape and size (13.5–)15.5–21.5(–27) × (5.5–)7.5–9.5 (average of 40 conidia 18.2 × 7.4). These characters correspond to the morphological description of the species *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not. (anamorph *Fusicoccum aesculi*, synanamorph *Dichomera* sp.) (Barber *et al.* 2005).

Identification of the fungal symbiont was confirmed by analysis of rDNA-ITS (ITS1 – 5.8S – ITS2) sequence homology. In fact 100% similarity resulted in alignments between sequences of two isolates (Th.L/125.09 and Th.143.09) recovered from galls harbouring pupae of *A. serpylli* collected in Fajsławice (51.0957N, 22.9634E), and a type strain of *B. dothidea* (GenBank accession no. GU119957.1). Sequences of the above strains have been deposited in GenBank, respectively with the reference numbers KY368175 and KY368176.

**Table 1** Plant species reported for the occurrence of *B. dothidea* in association to gall midges

| Host Plant Species/Family | Cecidomyiid Species | Country | References |
|---|---|---|---|
| Amaranthaceae:<br>*Sarcocornia quinqueflora*,<br>*Tecticornia arbuscula* | *Asphondylia floriformis*, *A. peelei*,<br>*A. sarcocorniae*, *A. tecticorniae* | Australia | Lebel *et al.* (2012) |
| Asteraceae:<br>*Solidago altissima, Solidago* spp. | *Asteromyia carbonifera* | USA, Canada | Heath & Stireman (2010)<br>and Janson *et al.* (2010) |
| Ebenaceae:<br>*Diospyros glabra* | *Asphondylia* sp. | South Africa | Adair *et al.* (2009) |
| Fabaceae:<br>*Prosopis* spp.<br>*Psoralea oligophylla, Virgilia oroboides* | *Asphondylia prosopidis*<br>*Asphondylia* sp. | South Africa<br>South Africa | Zachariades *et al.* (2011)<br>Adair *et al.* (2009) |
| Lauraceae:<br>*Machilus thunbergii* | *Daphnephila* spp. | Taiwan | Chao & Liao (2013) |
| Mesembrianthemaceae:<br>*Psilocaulon articulatum* | *Asphondylia* sp. | South Africa | Adair *et al.* (2009) |
| Mimosaceae:<br>*Acacia cyclops, A. irrorata, A. littorea, A. mearnsii* | *Asphondylia bursicola, A. seminis,*<br>*A. glabrigerminis, A. pilogerminis* | Australia | Adair *et al.* (2009) |
| *Acacia karroo* | *Asphondylia* sp. | South Africa | |
| Pittosporaceae:<br>*Billardiera heterophylla, B. variifolia* | *Asphondylia* sp. | Australia | Adair *et al.* (2009) |
| Santalaceae:<br>*Osyris compressa* | *Asphondylia* sp. | South Africa | Adair *et al.* (2009) |
| Sapindaceae:<br>*Dodonaea viscosa* | *Asphondylia dodoneae* | Australia | Adair *et al.* (2009) |
| Schisandraceae:<br>*Illicium anisatum* | *Illiciomyia yukawai* | Japan | Kobune *et al.* (2012) |
| Solanaceae:<br>*Anthocercis littorea* | *Asphondylia anthocercidis* | Australia | Adair *et al.* (2009) |
| Tiliaceae:<br>*Grewia occidentalis* | *Asphondylia* sp. | South Africa | Adair *et al.* (2009) |
| Violaceae:<br>*Hybanthus floribundus* | *Asphondylia* sp. | Australia | Adair *et al.* (2009) |
| Vitaceae:<br>*Rhoicissus digitata* | *Asphondylia* sp. | South Africa | Adair *et al.* (2009) |

## Discussion

The genus *Asphondylia* Loew (Diptera: Cecidomyiidae) belongs to the tribe Asphondyliini, a wide group of gall inducers on host plants belonging to many botanical families, and includes 289 nominal species (Gagné, 2010). At present the characterization of *Asphondylia* spp. is mostly based on the host plant and on very slight morphological differences, which makes it quite difficult to discriminate adults. In some cases larval and pupal morphological characters even among species associated with a certain host plant genus or species are used (Gagné & Waring, 1990), but genomic analyses based on DNA sequencing are more and more considered in combination with ecological, distributional and morphological information for a more effective species discrimination (Yukawa *et al.*, 2003). Although the species of *Asphondylia* reported from Lamiaceae are poorly known, in the present study the correspondence of morphological characters clearly indicated that gall midges from *T. vulgaris* and *T. serpyllum* are conspecific.

The finding of *A. serpylli* for the first time on *T. vulgaris* suggests a revisionary work to be carried out on the populations of *Asphondylia* associated with *Thymus* spp. With reference to the last updated survey concerning local distribution of gall midges (Skuhravá *et al.*, 2008), this is the first documented finding of *A. serpylli* in Poland. More recently this species has been

redescribed, with neotype designation based on specimens collected from *T. serpyllum* in Bohemia, in a paper reporting on the finding of the new species *Asphondylia coridothymi* Skuhravá on the botanically related *Coridothymus capitatus* (Skuhravá, 2011). In the same paper the epithet *Asphondylia thymi*, uncircumstantially associated by Kieffer to another sampling from *T. serpyllum*, has been reported in synonymy with *A. serpylli*, and the geographical distribution has been updated based on the available reports, indicating a widespread occurrence in Europe throughout an area ranging from the Mediterranean countries to Great Britain and Scandinavia.

No records concerning parasitoids of *A. serpylli* were previously available in the literature. Our preliminary data introduce the opportunity for further investigations in this respect, particularly on the larval behaviour of *S. brevicornis* and its presumed aptitude to also feed on the fungus within the galls (Parnell, 1964).

*B. dothidea* is a cosmopolitan endophytic and plant pathogenic fungus mainly known from forest plants (Slippers & Wingfield, 2007). However, in the past few years its occurrence as the symbiotic fungus associated to *Asphondylia* and allied cecidomyiids has been reported on an increasing number of unrelated plant species (Table 1). In a few cases its isolation in pure culture was somehow problematic, or even unattainable, and the species identification accomplished by means of biomolecular methods (Kobune *et al.*, 2012; Lebel *et al.*, 2012). The possibility that these *B. dothidea* strains may belong to a distinct phylogenetic lineage specifically adapted to the gall symbiotic condition has been considered, but no evidence resulted in this sense (Adair *et al.*, 2009; Janson *et al.*, 2010). It is also possible that a number of older reports mentioning *Macrophoma* sp. as a fungal inquiline of galls (Neger, 1913; Batra & Lichtwardt, 1963; Bissett & Borkent, 1988) may actually concern *B. dothidea*, considering that nomenclatural confusion between these two genera occurred until more recent taxonomic revisions evidenced cases of synonymy (Phillips & Lucas, 1997; Phillips *et al.*, 2013). Based on our observations, *B. dothidea* is confirmed to represent the symbiotic fungus associated to *Asphondylia* galls on *T. vulgaris* too.

So far, the only documented report concerning the fungal symbiont associated to *Asphondylia* on plant species in the Lamiaceae concerns the above-mentioned *C. capitatus* (Malagaris, 2011). In that case, the fungus was not identified at the species level, and provisionally reported as *Aureobasidium* sp. Considering the quite common epiphytic occurrence of *Aureobasidium* in several ecological contexts (Andrews *et al.*, 2002; Osono, 2008), in our opinion this finding deserves to be re-evaluated by additional investigations, in order to ascertain if in that case the possible occurrence of *B. dothidea* was again concealed due to difficulties in its isolation.

On the other hand, the fact that *Cladosporium* has been previously recovered from galls and/or the body surface of *Asphondylia* and allied midges (Docters van Leeuwen, 1929; Adair *et al.*, 2009; Kobune *et al.*, 2012; Lebel *et al.*, 2012) may be indicative of the possibility that it is also more than occasionally associated to these cecidomyiids. However, in our case it is worth considering that, unlike *B. dothidea*, *Cladosporium* isolates were also consistently obtained from achenes and from ovaries of normal flowers. These latter findings may be assumed as an evidence that at least the presence of *Cladosporium* in the galls does not depend on inoculation during oviposition by *Asphondylia* and its parasitoids. Based on morphological and biometric features almost all *Cladosporium* isolates were identified as *Cladosporium cladosporioides*. This widespread fungus has been recently typified as a species complex with an intricate taxonomy, including many cryptic species yet to be named (Bensch *et al.*, 2010); a few of our isolates will be deposited in order that specimens from such an unusual source be available for consideration in future revisions of the species assortment within this grouping.

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