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Proteo-transcriptomic analysis of the venom of the endoparasitoid wasp *Pimpla turionellae* and its impact on host insect epigenetic mechanisms

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I dedicate my dissertation work to my beloved family…

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List of abbreviations

AMPs	Antimicrobial peptides
Cec A, D	Cecropin A, D
COs	Carboxylesterases
CpG	Cytidine-phosphate-guanosine
Cvp1-7	Cysteine-rich peptides 1–7
DNMTs	DNA methyltransferases
ECD	Ecdysteroid hormone
ELISA	Enzyme-linked immunosorbent assay
GH1	Glycoside hydrolase family 1
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HPLC	High-performance liquid chromatography
ICK	Cysteine inhibitor knot
IMPI	Insect metalloproteinase inhibitor
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JH	Juvenile hormone
JH-Ehyd 1, 2	JH epoxide hydrolase 1, 2
JH-BP 1- 4	JH-binding protein 1–4
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
L-DOPA	L-3,4-Dihydroxyphenylalanine
MicroRNA	miRNA
mRNAs	Messenger RNAs
PAMPs	Pathogen-associated molecular patterns
P-body	Proceedings body
PDVs	Polydnaviruses
POs	Phenoloxidases
PPO-1, 2	Prophenoloxidase-1, 2
Pre-miRNAs	Precursor-miRNAs
Pri-miRNAs	Primary miRNAs
RISC	RNA-induced silencing complex
RNA-Seq	RNA sequencing

RT-PCR	Reverse transcription polymerase chain reaction
SAM	S-adenosyl-methionine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPH	Serine protease homologs
Svp2	Small venom protein 2
TETs	Ten-eleven translocation methylcytosine dioxygenase
UTRs	Untranslated regions
VLPs	Virus-like particles
Vpr1, 3	Venom protein 1, 3
5m-dCMP	2′-Deoxycytidine 5′-monophosphate

Summary

Parasitoid wasps are hymenopterans that use other insects as hosts for their offspring. Females deposit their eggs either in (endoparasitoid) or on (ectoparasitoid) the host and inject maternal venoms and viruses to modulate host physiology to ensure the survival of the wasp eggs. The endoparasitoid wasp *Pimpla turionellae* (Linnaeus) injects its venom with the egg to sabotage the host's cellular and humoral defense, arrest further host development, and paralyze the host pupa to promote their offspring's survival. In this work, the impact of parasitization was analyzed to test whether idiobiont endoparasitoids induce epigenetic reprogramming to overcome host immune defense and disturb host development, and *P. turionellae* venom components were characterized using a combined proteo-transcriptomic approach.

The impact of parasitization was tested by monitoring differentially expressed genes involved in the immunity and developmental hormone signaling pathways of parasitized and control hosts, *Galleria mellonella* (Linnaeus). My results indicate that parasitization induces the suppression of host immune responses and the modulation of host development. The changes in gene expressions were related to alterations in the host's epigenetic mechanisms. Parasitization by *P. turionellae* induced changes in the acetylation ratios of specific histones and a transient decrease in the host's global DNA methylation. Additionally, I observed strong parasitization-specific changes in the host's microRNAs pattern that regulate gene expression at the post-transcriptional level.

On a second level, the transcriptomic analysis of venom glands and the proteomic study of the crude venom of endoparasitoid wasp *P. turionellae* were combined and resulted in the identification of several enzymes, cysteine-rich peptides, and other proteins. Possible biological functions of the identified proteins were characterized with respect to other known parasitoid venoms. Interestingly, any evidence of pimplin, the previously described main paralytic factor of P*impla hypochondriaca* venom, was not identified. However, a new cysteine inhibitor knot (ICK) family (pimplin2) that is highly similar to known ICK-like neurotoxins was found as a highly expressed venom component.

In conclusion, my thesis characterizes the venom composition of *P. turionellae*, describing the known and novel venom protein families. Furthermore, the new insights reveal that parasitization reprograms the epigenetic mechanisms of the host to disrupt its development and suppress its immune system. Finally, some of the identified but functionally unknown components, such as the linear, short protein pimplin4, will be bioactivity tested to perform an in-depth assessment of their promising potential in bioinsecticidal or antimicrobial applications.

Zusammenfassung

Parasitoide sind Hymenopteren, die andere Insekten als Wirte für ihre Nachkommen nutzen. Die endoparasitoide Wespe *Pimpla turionellae* injiziert ihr Gift zusammen mit ihrem Ei, um die zelluläre und humorale Abwehr des Wirtes zu sabotieren und dessen weitere Entwicklung zu stoppen. Zudem werden die Wirtslarven gelähmt, um das Überleben der parasitoiden Nachkommen zu sichern. In dieser Arbeit wurden die Auswirkungen der Parasitierung analysiert, um zu untersuchen, ob idiobionte Endoparasitoide die Immunabwehr und die Entwicklung des Wirtes unterdrücken können, indem sie dessen epigenetische Regulationsmechanismen stören. Ferner wurden die Giftkomponenten von *P. turionellae* durch proteo-transkriptomische Ansätze charakterisiert.

Die Auswirkungen der Parasitierung auf den Wirt, die Puppe der großen Wachsmotte *Galleria mellonella*, wurden durch Analyse von differentiell exprimierten Genen untersucht. Die Ergebnisse zeigen, dass eine Parasitierung durch *P. turionellae* im verwendeten Modellwirt dessen Immunabwehr und Entwicklung hemmen. Die Veränderungen der Genexpression stehen im Zusammenhang mit Veränderungen der epigenetischen Mechanismen des Wirtes. Die Parasitierung induziert Änderungen der Acetylierungsverhältnisse spezifischer Histone, eine vorübergehende Abnahme der globalen DNA-Methylierung und eine Umprogrammierung der microRNA-Expression in *G. mellonella*.

Die proteo-transkriptomische Analyse des Giftes von *P. turionellae* führte zur Identifizierung mehrerer Enzyme, Cystein-reicher Peptide und anderer Proteine. Mögliche biologische Funktionen identifizierter Proteine wurden im Vergleich mit Giften anderer bekannter Parasitoide diskutiert. Interessanterweise wurde kein Hinweis auf Pimplin, den in der Literatur bisher beschriebenen paralytischen Hauptfaktor des Giftes von *Pimpla hypochondriaca*, gefunden. Stattdessen wurde eine neue Toxin-Familie (Pimplin2) identifiziert, die bekannten Cystein-reichen Neurotoxinen sehr ähnlich ist und das höchste Expressionsniveau aller Gift-Komponenten aufweist.

Diese Arbeit charakterisiert die Giftzusammensetzung von *P. turionellae* und beschreibt bekannte sowie neuartige Komponenten. Zu den gewonnenen Erkenntnissen gehört, dass die Parasitierung mit *P. turionellae* im befallenen Wirtsinsekt Auswirkungen auf dessen epigenetische Mechanismen hat, die zur Hemmung der Immunabwehr und zur Verzögerung der Entwicklung führen. Schließlich werden einige der identifizierten, aber funktionell unbekannten Komponenten, wie das lineare, kurze Protein Pimplin4, auf Bioaktivität getestet, um eine eingehende Bewertung ihres vielversprechenden Potenzials für bioinsektizide oder antimikrobielle Anwendungen durchführen zu können.

1. Introduction

1.1. Parasitoids

Parasitoid wasps are holometabolous insects within the order Hymenoptera that have evolved to use other arthropods as hosts for their offspring (Godfray, 1994). Such parasitoids complete their development either in (endoparasitoid) or on (ectoparasitoid) the host (Gauld, 1988; Wharton, 1993; Godfray, 1994). Parasitoid species are diverse in their life strategies, morphology, host preferences, and venoms. They can be solitary, laying a single egg per host, or gregarious, laying large clutches of eggs per host (Gauld, 1988; Godfray, 1994). Parasitoids can be further categorized according to their host regulation strategies. Parasitization (oviposition) by idiobiont parasitoids immediately arrests further host development and paralyzes their hosts (or in a very short time during oviposition) to provide a stable food source to the parasitoid offspring, while koinobiont parasitoids allow for further host development and induce only temporary paralysis (Gauld, 1988; Godfray, 1994; Strand and Pech, 1995; Pennacchio and Strand, 2006; Kathirithamby, 2009; Pennacchio et al., 2014). Depending on their developmental requirements, different parasitoid species specialize on hosts that are at different life stages and can be classified as egg, larval, pre-pupal, pupal, or adult parasitoids (Gauld, 1988; Pennacchio et al., 2014). They parasitize several taxa from Arachnida and all insect orders of Insecta, which raises interest in utilizing parasitoids as bioagents for the control of pest insects.

Parasitoids reproductive success depends on their host manipulation ability and successful parasitization requires the following of the parasitoid:

- should be able to overcome host innate immunity,
- control host development to prevent egg rejection,
- and provide food for its larvae (Pennacchio and Strand, 2006).

Parasitoids have evolved many adaptations to promote parasitization (Gauld, 1988) such as covering eggs with protective components to prevent recognition (Führer and Kilincer, 1972), producing teratocyte—a type of cell released from embryonal serosa into host insects when wasp eggs hatch—(Strand, 2014), and releasing antibacterial and antifungal anal secretions during larval development (Führer and Willers, 1986). Furthermore, female parasitoids inject maternal factors such as venom, calyx fluid, polydnaviruses (PDVs), and virus-like particles (VLPs), which are produced by ovaries and/or the venom gland (Führer, 1973; Gauld, 1988; Pennacchio and Strand, 2006; Poirié et al., 2009) to ensure offspring survival.

1.2. Biology of the endoparasitoid *Pimpla turionellae* and its host *Galleria mellonella*

1.2.1. *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae)

Pimpla turionellae Linnaeus, 1758 (Hymenoptera: Ichneumonidae) is a solitary, idiobiont, and pupal endoparasitoid with different host ranges and niches. It is a natural enemy of more than 100 species belonging to 23 families of Lepidoptera, including its laboratory host, *G. mellonella* (Meyer, 1925; Arthur and Wylie, 1959; Gauld, 1988; Uçkan, 1999; Pajač Živković and Barić, 2012).

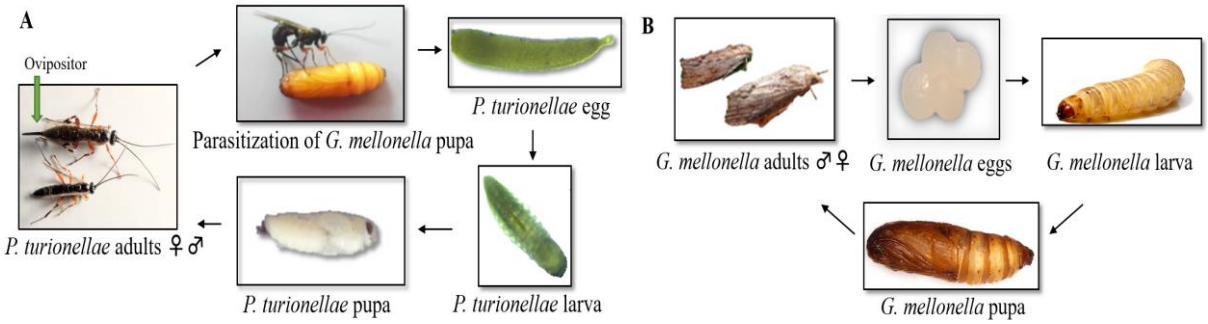

Figure 1. Life cycle of the endoparasitoid *P. turionellae* and its pupal host *G. mellonella*. The endoparasitoid *P. turionellae* (A) and pupal host *G. mellonella* (B) develop through four divergent life stages: egg, larva, pupa, and adult.

The female parasitoid is usually bigger than the male and easily recognizable with the existence of prominent ovipositor **(Figure 1A)**. After finding a suitable host, the female parasitoid, tightly holds onto the host and pierces the pupal shell with the ovipositor, injecting an egg and maternal factors, including venom and calyx fluid (Bogenschütz, 1978; Ueno and Ueno, 2007). After oviposition, with mucopolysaccharide covered *P. turionellae* egg hatches in around two days (Führer, 1973), and the first instar larva immediately migrates to the head of the host pupa to destroy the brain and surrounding organs. It then moves to the host's abdomen back and feeds on the host's hemolymph and other tissues until growing to its final larval stage (Führer and Kilincer, 1972). Toward the pupation, the emergence of female or male adults has resulted in the death of the host **(Figure 1A)**. Successful parasitization by *P. turionellae* induces paralysis, suppresses the host's immune response, modifies the host's metabolism, disrupts the host's cells and tissues, and interferes with the host's development to improve the survival of their offspring (Gauld, 1988; Pennacchio and Strand, 2006; Casewell et al., 2013; Moreau and Asgari, 2015). Their host management ability, which relies primarily on the venom function, is the reason why *P. turionellae* is an attractive resource for agricultural applications. Particularly, their host control strategies at the immunological and endocrinal levels can provide new perspectives for using this taxon as a bioagent to control a wide range of pest species.

1.2.2. *Galleria mellonella* L. (Lepidoptera: Pyralidae)

The greater wax moth, *Galleria mellonella* Linnaeus, 1758 (Lepidoptera: Pyralidae), is a holometabolous insect pest in apiculture that develops throughout four divergent life stages: egg,

larva, pupa, and adult **(Figure 1B).** The newly hatched polipod larvae undergo eight times molting until reaching the final larval stage. *G. mellonella* larvae feed on honey, wax, pollen, the cast of honeybee pupal skins, and brood and contribute to the decline of honeybee populations. The final-stage larvae spin silk cocoons, inside of which they develop first into a pupa and then into an adult wax moth (Abou-Shaara and Staron, 2019).

Although the larval stage of *G. mellonella* is quite harmful to apiculture, they resemble important model-system hosts for a variety of research topics. Owing to their easy rearing and sampling features and their reproduction tolerance at different temperatures up to 37 ˚C under laboratory conditions (Kwadha et al., 2017), *G. mellonella* has been used as a model host in many studies, such as Listeria pathogenesis (Mukherjee et al., 2010), uropathogenic *Escherichia coli* infections (Heitmueller et al., 2017; Kalsy et al., 2020), entomopathogenic fungus *Metarhizium robertsii* (Mukherjee and Vilcinskas, 2018), and *Bacillus thuringiensis* infections (Mukherjee et al., 2017). These studies provide deep knowledge into the immune reactions and epigenetic reprogramming of *G. mellonella* against microbial/fungal invasion. In addition to these applied experimental results, their sequenced genome (Lange et al., 2018) and available transcriptome (Vogel et al., 2011) and microRNA data (Mukherjee and Vilcinskas, 2014) allow them to be a suitable *in vivo* model host for studying the impact of parasitization by *P. turionellae.*

1.3. Epigenetic, immunity, and hormonal regulation of metamorphosis in insects

1.3.1. Epigenetic in insects

Epigenetic regulation is an environmentally responsive mediator between genotype and phenotype, providing heritable and reversible alterations in gene expression and cell function without inducing any changes in the primary DNA sequence (Skinner, 2011; D'Urso and Brickner, 2014). Currently, there are several epigenetic molecular mechanisms described in insects, including DNA methylation and histone modifications (Felsenfeld, 2014) and expression of microRNAs (miRNAs) (Lee, 2015; Villasenor, 2013). Insect epigenetic modifications regulate complex parameters, such as environmental adaptation, development, metamorphosis, metabolism, reproduction, and immune response to microbial invasion and parasitoid eggs (Vilcinskas, 2017; Glastad et al., 2019; Villagra and Frías-Lasserre, 2020).

Highly conserved histone proteins bind strongly to negatively charged DNA to form nucleosomes, which serve as the fundamental units of chromatin structure (Turner, 2001). Acetylation of histone proteins is one of the best-known covalent histone modifications and is tightly controlled antagonistically by histone acetyltransferases (HAT) and histone deacetylases (HDAC). Acetylation of histone proteins reduces their linkage to DNA (euchromatin) and increases the accessibility of

DNA to other proteins, such as transcriptions factors **(Figure 2)**. Therefore, the transcriptional regulation of target genes of many important processes, such as immune response, metamorphosis, diapause, and development, in insects is linked to the alterations of histone-DNA interactions. (Mukherjee et al., 2012; Reynolds et al., 2016; Roy and Palli, 2018)

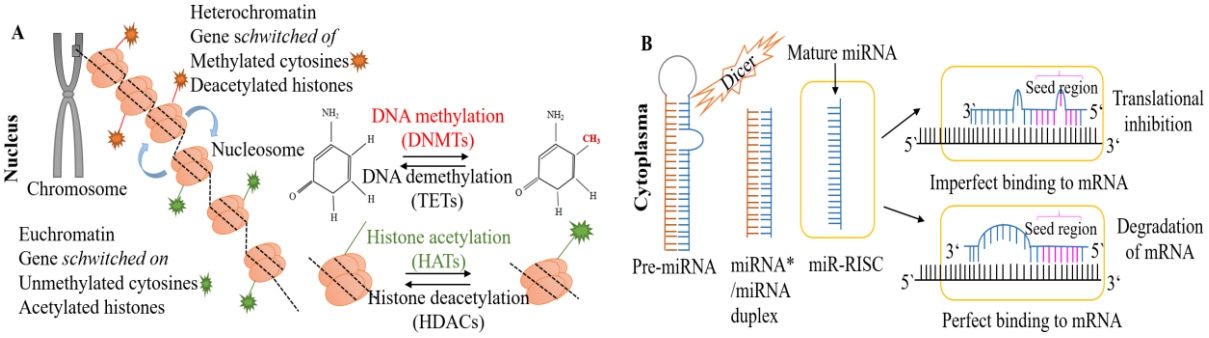

Figure 2. Major epigenetic mechanisms in eukaryotic organisms. A) The transfer of a methyl group to the cytidine residue in the CpG islands inhibits the accumulation of transcription factors and related proteins into specific promoter regions. Acetylation of histone tails affects the conformational structure of the DNA-protein complex and switches on specific genes. B) MiRNA genes are transcribed into long primary-miRNAs that are processed in the nucleus to precursor-miRNAs (pre-miRNAs) and then transferred to the cytoplasm by Exportin-5. Pre-miRNAs are further processed by Dicer to double-stranded miRNA, which are loaded later into the RISC complex. MiR-RISC leads to the translational inhibition or degradation of the target mRNA.

The only known epigenetic modification in genomic DNA is methylation. DNA methyltransferases (DNMTs) transfer a methyl group from S-adenosyl-methionine (SAM) to the C5 position of the pyrimidine ring of the cytosine residues (Foulks et al., 2012) **(Figure 2)**. DNA demethylation is proceeded actively by the action of Ten-eleven translocation methylcytosine dioxygenases (TETs) (Ross and Bogdanovic, 2019). Methylation of CpG-rich sequences (CpG island), which are located in the promotor regions of vertebrates, suppresses gene expression by affecting transcription factor binding and recruitment of the transcription initiation complex (Elango and Yi, 2008; Glastad et al., 2019). Studies in lepidopteran and hymenopteran insects have revealed that, unlike in vertebrates, DNA methylation is found in gene bodies and is related to continuously and rapidly gene expression, not to gene suppression (Borsatti et al., 2004; Feng et al., 2010; Xiang et al., 2010; Zemach et al., 2010; Lyko and Maleszka, 2011; Wang et al., 2013; Özbek et al., 2018). The DNA methylation level in insects can be altered by external factors, such as diets, photoperiod, insecticides, microbial invasion, and parasitization (Lyko and Maleszka, 2011; Kim et al., 2016; Kumar and Kim, 2017; Özbek et al., 2020).

MicroRNAs (miRNAs) are endogenous, highly conserved, with approximately 22 nucleotide, non-coding, single-stranded, small RNAs that regulate gene expression negatively at the post-transcriptional level through base pairing between 5' untranslated regions (UTRs) of miRNAs and 3' UTRs of target messenger RNAs (mRNAs) (Gilbert, 2012). The biogenesis of miRNAs is similar to non-insect taxa **(Figure 2)**. In an Argonaute protein in the RNA-induced silencing complex

(RISC), a loaded mature miRNA strand forms a miR-RISC complex and guides it to its target mRNAs (Hussain and Asgari, 2014). Upon pairing with the miR-RISC complex, the target mRNA is degraded or stored in the proceedings body (Gilbert, 2012). Insect miRNAs are associated with several functions, including cell proliferation, apoptosis, metamorphosis, and a response to biological stress. Their expression profile can be altered by external factors, such as microorganismal invasion and parasitization (Asgari, 2011; Etebari et al., 2013; Hussain and Asgari, 2014; Mukherjee and Vilcinskas, 2014; Özbek et al., 2020).

1.3.2. Insect immunity

Although insects do not have adaptive immunity as vertebrates do, they do have innate immunity, which is composed of cellular and humoral responses. Such cellular innate immune responses include encapsulation, nodulation, and phagocytosis mediated by hemocytes. Antimicrobial peptides (AMPs) (lysozyme, galiomycin, gallerimycin, gloverin, cecropin-A,-D, x-tox [from *G. mellonella*]) and immunity-related protein production (insect metalloproteinase inhibitor [IMPI] and transferrin [from *G. mellonella*]), melanin synthesis, and clotting, known together as humoral innate immunity, are mediated by fat bodies (Beckage, 2008). Both systems collaborate when non-self surfaces are encountered, such as bacteria, fungi (Lavine and Strand, 2002), artificial objects (Lavine and Strand, 2001), or parasitoid eggs (Christensen et al., 2005). Encapsulation refers to multicellular capsule formation around larger invaders, such as parasitoids. Following the encapsulation by granulocytes and plasmatocytes, the melanization occurs around the encapsulated invading organism. The melanin contributes to killing the invading organism by blocking the absorption of nutrients by encapsulated organisms and releasing toxic intermediates during melanin synthesis (Gilbert, 2012). Thus, parasitoids inhibit or avoke the host's cellular and humoral response to ensure the survival of the wasp eggs (Poirié et al., 2009).

1.3.3. Hormonal control of insect metamorphosis

In holometabolous insects, molting and metamorphosis are regulated by two effector hormones: juvenile hormone (JH) and ecdysteroid (20-hydroxyecdysone) (Nation, 2008). Juvenile hormone is secreted by the *corpora allata*, and these secretory cells are active only during larval molts (Gilbert et al., 2000). As long as JH is present in the hemolymph, the ecdysteroid-stimulated molting results in a new larval instar. In the final larval instar, JH production is inhibited and JH degradation is induced. As a result, JH levels drop below a critical threshold value and trigger the production of prothoracicotropic hormone, which promotes the synthesis of ecdysone by the prothoracic gland (Mueller et al., 2015). In the absence of JH, 20-hydroxyecdysone first inactivates larva-specific genes and then activates pupa-specific genes, resulting in pupation. During metamorphosis, the old

body of the larva is systematically destroyed by apoptosis, and new organs develop from undifferentiated nests of cells and imaginal discs, and the adult is formed within the pupa cuticle (Browder et al., 2001; Mueller et al., 2015). Therefore, it is very typical that parasitoids prevent metamorphosis of their pupal host by inducing alterations in the host's endocrine physiology to promote their offspring's survival (Edwards et al., 2006).

1.4. Parasitoid venomics: development and current perspective

Venoms are complex chemical cocktails of bioactive compounds that contain various combinations of enzymes, non-enzymatic proteins, peptides, salts, and organic molecules, which are produced in specialized glands and are generally named as toxins (Fry et al., 2009; Utkin, 2015). Toxin genes evolve from an ancestor or physiological genes, which are involved in non-toxin-related bioactivity and regulatory processes. Different mechanisms of toxin evolution include gene duplication, modification and alternative splicing of exons, domain duplication, consecutive gene duplication, and domain loss (Casewell et al., 2013). Venoms' great potency in prey has evolved over millions of years. Currently, more than 220,000 species of venomous animals are known (Holford et al., 2018) and these animals use their venom to facilitate diverse missions, including defense (Lee et al., 2013), predation (Undheim et al., 2015), blood-feeding (Kakumanu et al., 2019), and habitat creation (London, 2012).

Hymenoptera is a mega-diverse insect order containing more than 150,000 extant venomous species (Aguiar et al., 2013). Their venom has evolved for communication, defense, predation, and offspring care in accordance with their life history strategies, such as pollen-feeding, predation, and parasitoidism (Walker et al., 2018). In contrast with common venom functions, female parasitoids use venom to manipulate the development, metabolism, and behavior of other arthropods for reproductive purposes (Werren and Werren, 2015). Most hymenopteran parasitoids are small insects with limited quantities of venom, which explains why the current information on parasitoid venoms is based on a limited number of species. Accessing these low-quantity, complex mixtures requires multidimensional high-throughput techniques, including genomics, transcriptomics, and proteomics, so-called omic technologies. The term "venomics" is used to describe the study of crude venom and venom glands to unravel a venom profile of a venomous animal through the integration of omics methodes (Bernot, 2004; Paulson et al., 2016; Wilson and Daly, 2018). Earlier parasitoid venomics studies, using *Pimpla hypochondriaca, Bracon hebetor*, and *Cotesia rubecula*, focused only on crude venom proteome analysis and gained limited numbers of secreted peptides and proteins (Parkinson and Weaver, 1988; Quistad et al., 1994; Asgari et al., 2003a). Therefore, over the past decade, studies have been extended to the combination of other omics approaches to

improve venomics results, beginning with the venom analysis of the endoparasitoid *Chelonus inanitus* by linking the proteome from the crude venom and transcriptome (proteo-transcriptomics) of the venom gland; the research then continued with many other species, such as *Cotesia chilonis*, *Bracon nigricans*, *Toxoneuron nigriceps,* and *Pteromalus puparum* (Vincent et al., 2010). Additionally, the first genome data was generated for the *Nasonia* species by Werren and colleagues (2010). Afterward, the genome analysis of *Nasonia vitripennis* was combined with a proteome analysis (proteogenomics) by employing bioinformatics tools. The combination of omics approaches introduced an extraordinary richness of identified venom proteins (Danneels et al., 2010).

These previous studies revealed that parasitoid venoms are rich sources of biomolecules that contain enzymes (proteinase/peptidase, hydroxylases, esterases, and oxidases), protease inhibitors (PpS1V, LbSPNy, and Vn4.6), immune-related proteins (calreticulin, Vn50, Vpr1, Vpr3, and Vn11), recognition/binding proteins, and neurotoxin-like peptides/paralytic factors (cvp3, cvp5, and pimplin) (Asgari and Rivers, 2011; Poirié et al., 2014; Moreau and Asgari, 2015). Through a variety of omics methods, hundreds of venom components have been described from different parasitoid species; however, until today, only a few (less than 25) endo- and ectoparasitoid venom proteins have been tested and subsequently successful bioactivities (predicted or new function) published. Given their great potential as agrochemicals, the lack of more comprehensive studies on parasitoid venoms and the activity of their toxins is surprising. For instance, the venom of the ectoparasitoid *Bracon hebetor* contains neurotoxins Brh-I and Brh-V, which block the neuromuscular transmissions in Lepidopteran hosts and were patented as insecticidal toxins by Novartis AG in the 1990s (Quistad et al., 1994). Identifying endoparasitoid venom proteins is important for understanding the underlying mechanisms of parasitization and discovering new bioactive compounds for agrochemical applications.

1.5. Aim of the project

Following parasitization by *P. turionellae,* the major physiological changes in the host *G. mellonella* were mainly described; however, these manipulations have not been well characterized at the molecular level. I hypothesize that idiobiont endoparasitoids induce epigenetic reprogramming to disturb host development and overcome host immune defense, and endoaprasitoid venom proteins contribute to these host manipulations in favor of parasitoid eggs.

A- Parasitization-induced epigenetic regulation of host immunity and development

The first part of this thesis tests the hypothesis that endoparasitoid *P. turionellae* may use "*epigenetic reprogramming*" to overcome host immune defense and arrest host development to ensure the

success of parasitization. To investigate epigenetic reprogramming and its association in the suppression of immunity and the modulation of developmental hormone signaling in the parasitized host, first the global DNA methylation level and the percentage acetylation of histones, and the expression levels of conserved host miRNAs, and the genes encoding regulators of epigenetic modifications were measured. Then, the expression of genes inolved in the immunity and developmental hormone signaling pathways were monitored in parasitized and control group host individuals of *G. mellonella* **(Figure 3A)**.

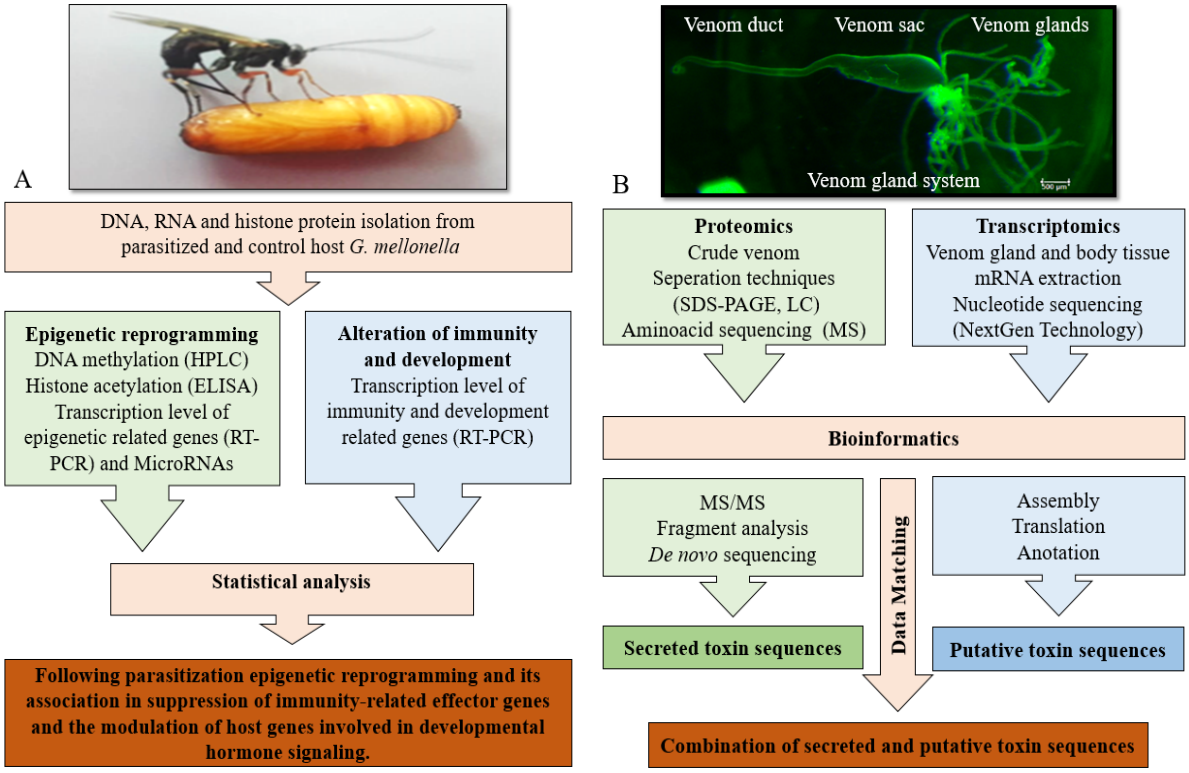

Figure 3. Workflow diagram of this Ph.D. project. A) Parasitization behavior of *P. turionellae* and workflow to test parasitization-induced epigenetic regulation of host immunity and development. B) Dissection of the venom gland system in *P. turionellae* and workflow of proteomic and transcriptomic analysis.

B- Transcriptomic and proteomic analysis of the venom gland of *P. turionellae*

To identify bioactive venom proteins of *P. turionellae* that might interfere with the host's physiology, I developed a comprehensive inventory of the venom by combining proteotomic and transcriptomic analysis. To characterize the RNA-Seq of the venom gland system and the female and male body tissue transcriptomes, multiple transcriptome assembly and annotation strategies were applied. Then, the venom-gland-specific transcripts were compared with the identified major protein components of crude venom, which were separated by SDS-PAGE and analyzed via LC-MS/MS (**Figure 3B**). To avoid false-positive hits, strict transcriptome and proteome filters were set. Only the transcripts that matched these thresholds were used for comparison with other parasitoid venom proteo-transcriptomic analyses to characterize their possible origin and biological function.

2. Role and composition of parasitoid venom

Parasitoid venoms are generally adapted to induce paralysis, regulate host development, and suppress host immune responses to overcome the physiological challenges associated with their extraordinary lifestyle (Gauld, 1988; Asgari and Rivers, 2011). In accordance with these multiple functions, parasitoid venoms are rich sources of bioactive molecules that contain different combinations of peptides and proteins (Asgari and Rivers, 2011; Poirié et al., 2014; Moreau and Asgari, 2015). Analysis of the venom function in host-parasitoid interactions and its composition allows for understanding species-specific parasitization strategies and providing ideas for applied research.

2.1. Parasitoid venom functions in host-parasitoid interactions

Parasitoids utilize a variety of methods to ensure their offspring's development (Pennacchio and Strand, 2006). *P. turionellae* inactivates the immune system and arrests the further development of *G. mellonella* pupae (Er et al., 2010; Uçkan et al., 2010; Er et al., 2011). I hypothesized that *P. turionellae* succeeds with this by reprogramming the epigenetic mechanisms that control the expressions levels of developmental and immunity-related genes of the host. To investigate this manipulation strategy of *P. turionellae* venom on host physiology, I monitored different parameters, including the host's global DNA methylation level (ng in 1 µg of genomic DNA) by using reverse-phase HPLC, the percentage acetylation of histones H3 and H4 through ELISA-based commercial colorimetric kits, and the expression levels of conserved *G. mellonella* pupa miRNAs via microarray analysis at different periods (4, 8, and 24 h) of post-parasitization. Furthermore, the fold-change of development-, immunity- and epigenetic-related gene expressions of *G. mellonella* pupa were determined by using real-time PCR at different periods (4, 8, 24, and 96 h) after parasitization (Özbek et al., 2020).

2.1.1. Immune suppressive function of *P. turionellae* venom

The reproductive success of parasitoids depends on their capability to suppress the host's cellular and humoral response (Poirié et al., 2009). Humoral immunity refers to the secretion of soluble effector molecules, including antimicrobial peptides (AMPs), which can be synthesized during the microorganismal invasion and wounding, such as through piercing by an ovipositor via parasitization (Beckage, 2008). In this thesis, I monitored the expression levels of 10 immunity-related genes in parasitized *G. mellonella* pupa and found all genes to be suppressed at most time points following oviposition. Among these genes, gloverin, cecropin D (Cec-D), phenoloxidase 2 (PO2), prophenoloxidase 1 (PPO-1), and prophenoloxidase 2 (PPO-2) were downregulated at all time points of parasitization. The other five genes were suppressed, as well, save for 24 h post

parasitization. While galiomycin, cecropin A (Cec-A), and IMPI (up to six times) were upregulated, two other genes, namely X-Tox and lysozyme, were not significantly changed at 24 h (Özbek et al., 2020).

The suppression of the main enzymes of the melanization process, namely PPOs and POs, in the parasitized *G. mellonella* pupae indicates that the parasitoids protect the eggs from melanization. IMPI is the only known defense peptide that can suppress virulence-associated metalloproteinases (Vilcinskas, 2010). The venom glands of *P. turionellae* (Laurino et al., 2016; Özbek et al., 2019) and many other parasitoids are known to synthesize metallopeptidases **(Supp. Table 1)**. The metallopeptidase of *P. turionellae* could trigger the activation of the innate immune response, and cause increase in IMPI expression in parasitized *G. mellonella* pupa as bacterial and fungal metallopeptidases (Griesch and Vilcinskas, 1998; Vilcinskas, 2019), explaining the downregulation of IMPI expression levels, in the parasitized host. Furthermore, the *P. turionellae* venom metallopeptidases, such as a metalloprotease homolog VRF1 protein from *M. mediator* (Lin et al., 2018), might impair the Toll pathway, which is responsible for AMP production (Ezzati-Tabrizi et al., 2013; Lin et al., 2018), and contribute to the strong suppression of antimicrobial peptide expression after parasitization. The upregulation of four AMP genes encoding lysozyme, X-Tox, Cec-A, galiomycin, and IMPI gene at 24 h after parasitization and again downregulation at 48 and 96 h post parasitization indicated that the related defense response is actively manipulated by the parasitoid's offspring.

2.1.2. Developmental arrest function induced by *P. turionellae* venom

Pupal endoparasitoids prevent metamorphosis of their host by injecting venom. These developmental arrests are almost always associated with alterations in the host's endocrine physiology. In this dissertation, I monitored 10 genes related to the ecdysteroid hormone (ECD) and juvenile hormone (JH) signaling pathway (Vogel et al., 2011; Lange et al., 2018). The expression profiles of the hormone-signaling pathway-related genes were quite complex but indicated the upregulation of JH and the downregulation of ECD activity (Özbek et al., 2020).

JHs are secreted by *corpora allata* and then transported via hemolymph to the target cells. During transport, JHs are bound to the carrier proteins, JH-binding protein (JH-BP) 1–4, which preserve them from JH-epoxide hydrolase (JH-Ehyd) activity (Sanburg et al., 1975; Touhara and Prestwich, 1993; Gilbert et al., 2000; Zalewska et al., 2009). The activation of JH-BP2 at all time points (up to 20-fold) and JH-BP3 until 24 h (up to 420-fold) indicated that JH signaling increases after oviposition, as already reported in other host-parasitoid systems (Edwards et al., 2006; Zhu et al., 2009). Following parasitization, the enzymes that can degrade JH (JH-esterase and JH-Ehyd1 at all

time points and JH-Ehyd2 at later time points) were downregulated. Furthermore, oviposition induces the transient upregulation of ECD-22 kinase (until the 24 h time point), which inactivates ecdysteroid hormones via phosphorylation, and the downregulation of ECD-related protein at all time points, whose expression depends on the presence of ECD. Thus, parasitization by *P. turionellae* represses the accumulation of ecdysteroid hormones and inhibits the degradation of JH, which helps to explain the developmental arrest following parasitization in *G. mellonella* pupa. Similarly, *Eulophus pennicornis* can disturb metamorphosis-associated endocrine events (Edwards et al., 2006). In addition, the JH can repress the innate immune response in *Drosophila melanogaster* by inhibiting the stimulating activity of 20-hydroxyecdysone on AMP production (Flatt et al., 2008; Schwenke and Lazzaro, 2017), explaining the suppression of AMPs after parasitization. Therefore, these data indicate that *P. turionellae* interferes with hormonal regulation to arrest host development and suppress immunity.

2.1.3. Epigenetic reprogramming induced by *P. turionellae* venom

Gene expression in insects is regulated epigenetically at the transcriptional level by DNA methylation and histone acetylation and at the post-transcriptional level by miRNAs (Glastad et al., 2019). In this dissertation, I monitored the pattern of these epigenetic modifications and expressions of their regulatory genes to test whether parasitization triggers the transcriptional reprogramming of development- and immunity-related genes by affecting major epigenetic mechanisms.

The global DNA methylation level in the non-parasitized pupa is 30 ng 5m-dCMP per µg genomic DNA (3% total methylation); however, parasitization by *P. turionellae* caused a transient decrease of up to 35% after 4 h. This reduction in global DNA methylation reduced to ~ 33% after 8 h and ~5% after 24 h. The downregulated methylation-related gene expressions (encoding DNA methyltransferase-1 associated protein and DNA cytosine 5-methyltransferase) exhibited similar recovery in downregulation after 24 h. This aligns with the transient recovery of the five immunity-related genes discussed above. Furthermore, the decrement of global DNA methylation was reported in *Plutella xylostella* larvae after oviposition by the endoparasitoid wasp *Cotesia plutellae*, in accordance with the downregulation of DNA methyltransferases 1 and 2 genes (Kumar and Kim, 2017). Therefore, my data indicate that parasitoids may interfere with global DNA methylation in the host to suppress developmental and immunity-related genes that require the existence of methylated sites for expression.

Parasitization by *P. turionellae* affected the histone acetylation pattern at different lysine residues of H3 and H4 histones (H3K9, H3K14, H4K5, and H4K12) in the pupal host *G. mellonella*. The downregulation of the percentage of histone acetylation at H3K14 and H4K12 and the upregulation

at H4K5 were significant, while alterations at H3K9 were not significant following parasitization. Among seven monitored genes encoding HATs and HDACs, all genes were suppressed in the parasitized pupa, save for the HDAC 8 isoform 2, which was strongly upregulated until 24 h after oviposition and then leveled back at 96 h (Özbek et al., 2020). The upregulation of HDACs activity then causes histone deacetylation and switches off the gene expression. The transient upregulation of HDAC 8 isoform 2 might promote the inactivation of host genes via the deacetylation of H3K9, H3K14, and H4K12. Furthermore, H4K5 acetylation regulates the fine-tuning of ecdysone biosynthesis in *D. melanogaster* (Borsos et al., 2015). The upregulation of H4K5 acetylation might be related to developmental hormone regulation in *G. mellonella*. Therefore, the rearrangement of histone acetylation patterns by the parasitoid is another key mechanism for the transcriptional regulation of development and immunity.

Finally, we identified 82 miRNAs that exhibited significantly altered expression profiles following the egg oviposition. Among these miRNAs, 24 were downregulated up to 13-fold, and 58 were upregulated in parasitized *G. mellonella* pupa. The expression profile of insect miRNAs is known to change during development and following parasitization (Yu et al., 2008; Etebari et al., 2013). For example, miR-989 in *D. melanogaster* is a regulator of border cell migration and its abcence impair oogenesis in the female ovary (Kugler et al., 2013). Furthermore, the cell migration plays role in the immune response in insects to tissue damage and infections (Moreira et al., 2010; Krautz et al., 2014). Interestingly, the strongly suppressed seven *G. mellonella* miRNAs are variants of miR-989. Therefore, *P. turionellae* venom might inactivate the expression of miRNAs to arrest cell migration as a part of host development and immunity. Furthermore, slightly upregulated *G. mellonella* miRNAs, including ame-miR-3756, mjamiR-6492, tca-miR-2c-5p, ame-miR-3796, and dme-miR-263a-5p, are known to target developmental and immunity-related genes. Another upregulated *G. mellonella* miRNA is miR-124, which involves neural plasticity in honeybees (Michely et al., 2017) and the gene expression in the sensory nervous system of *Caenorhabditis elegans* (Clark et al., 2010). *P. turionellae* might use miR-124 to control neural gene expression in the parasitized host. Moreover, parasitoids, such as *Cotesia vestalis*, produce miRNAs in their venom and teratocytes, which are injected into the host through parasitization to modulate host genes (Wang et al., 2018). Therefore, it remains ambiguous whether the upregulated miRNAs in the parasitized pupa originated from the parasitoid venom or the host.

2.2. Venom components of the endoparasitoid wasp *P. turionellae*

A proteo-transcriptomic approach was applied to identify *P. turionellae* venom proteins. The RNA-Seq data from the venom gland system and body tissue, which were assembled by a multiple

assembler strategy, were combined with the proteome analysis of the crude venom. To avoid false-positive hits, strict transcriptome and proteome filters were applied, including the higher expression in the venom gland and the presence in the proteome. In total, 399 transcripts were obtained from the combination of proteome and transcriptome analyses. However, in this work, we focused on 88 transcripts in the limit of our thresholds and relating to the venom function. These 88 transcripts belong to 12 known venom protein families, which were divided into three major groups: 1) enzymes, 2) cysteine-rich peptides and proteins, and 3) others (Özbek et al., 2020) **(Figure 4, Supp. Table 1)**. The putative functions of the identified proteins/peptides in host-parasitoid interactions were predicted with respect to other parasitoid venom proteome and/or transcriptome analysis**.** The identified *P. turionellae* venom proteins are primarily linked to paralysis, interrupting development, inducing apoptosis, releasing carbohydrates, inhibiting encapsulation, and modulating the melanization process; however, the functions of some venom proteins remain unknown **(Supp. Table 1)**.

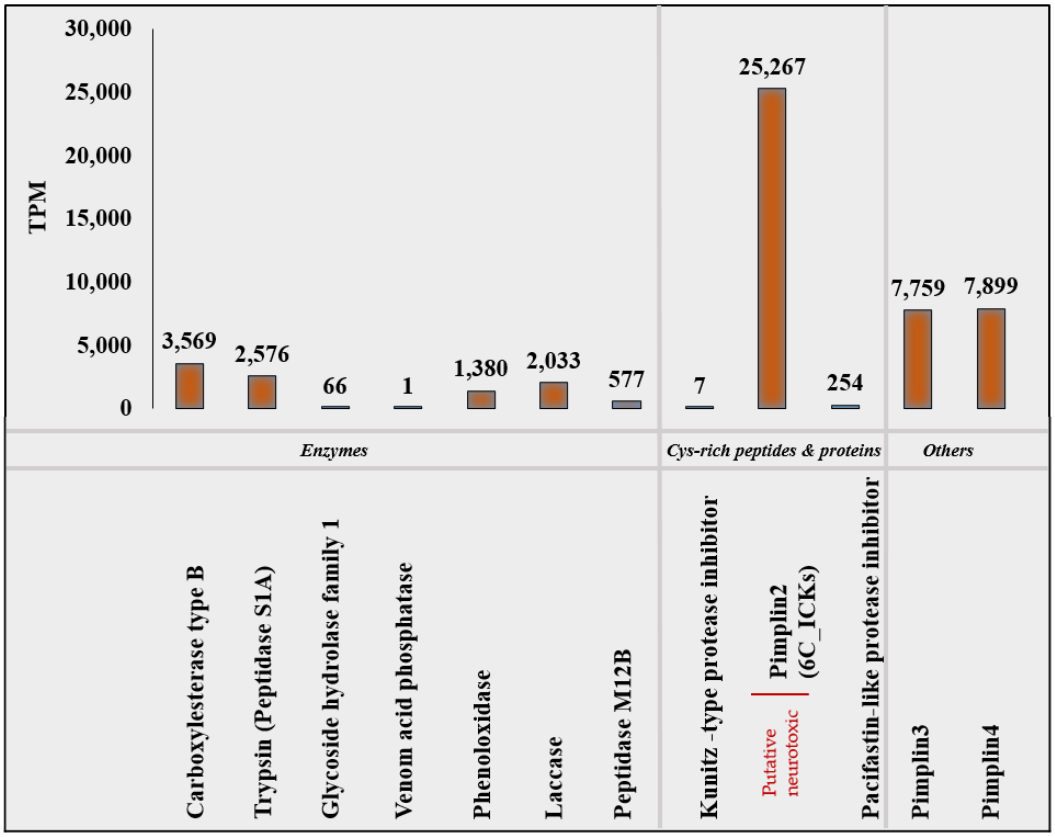

Figure 4. Identified venom protein families from *P. turionellae* venom glands. The summarized expression levels in transcript per million (TPM) per protein class are displayed for functional groups of protein families: enzymes, cyc-rich peptides and proteins, and others.

2.2.1. *P. turionellae* venom components linked to encapsulation cascade

Once the egg of the endoparasitoid *P. turionellae* reaches the hemolymph, host recognition molecules recognize the pathogen-associated molecular patterns (PAMPs) (Beckage, 2008). The

capsule formation around the parasitoid egg involves cooperation between two or more adherent hemocytes (Burke and Strand, 2014), which can be suppressed in a variety of ways, such as killing hemocytes or altering their aggregation and adhesion ability around foreign objects. In this dissertation, three protein families were characterized in *P. turionellae* venom and are related to the suppression of encapsulation cascade: metallopeptidase M12B (ADAM/reprolysin), venom protein 1 (Vpr1; renamed pimplin3), and Peptidase S1A **(Figure 4, Supp. Table 1)**.

Metalloproteases are components that are toxic to the host and play an important role in the degradation of the host's defense molecules and suppression of the host's cellular defense (Griesch and Vilcinskas, 1998; Liehl et al., 2006) and are widely distributed in parasitoid venoms **(Supp. Table 1).** It has recently been demonstrated that a metalloprotease homolog VRF1 enables the endoparasitoid wasp *Microplitis mediator* to inhibit egg encapsulation in its host, *Helicoverpa armigera*, by impairing the Toll pathway (Lin et al., 2018). Furthermore, reprolysin-like metalloproteases are involved in the manipulation of insect host development. The injection of EpMP3, a reprolysin-like metalloproteinase venom protein of ectoparasitoid *Eulophus pennicornis*, on insect host *Lacanobia oleracea* results in partial mortality or retarded development associated with failed molting process (Price et al., 2009). Microbial and entemopathogenic fungal metallopeptidases, which trigger an upregulation in the transcription fold of the IMPI of the host insect, can hydrolyze *G. mellonella* hemolymph proteins, including AMPs and proteinase inhibitors, and exhibit strong inhibitory activity on plasmatocytes (Altincicek et al., 2007; Griesch and Vilcinskas, 1998; Vilcinskas, 2019). Therefore, it can be assumed that *P. turionellae* venom M12B peptidases target host hemolymph proteins and hemocytes and impair the Toll pathway, which could lead to the inhibition of the encapsulation cascade, suppression of AMPs, and retardation of host development. This hypothesis is supported by older *in vivo* tests, which revealed hemocyte reduction, encapsulation inhibition, AMPs suppression, and metamorphose inhibitory effects of *P. turionellae* crude venom on pupal host *G. mellonella* (Er et al., 2010; Uçkan et al., 2010; Özbek et al., 2019).

Pimplin3 is an anti-hemocyte aggregation factor of *P. hypochondriaca* venom (Dani and Richards, 2010, 2009; Richards and Dani, 2008). The injection of recombinant Pimplin3 can suppress the ability for *L. oleracea* and *Mamestra brassicae* to drive cellular immune responses. Furthermore, it is known that this anti-aggregation factor increases the susceptibility of *M. brassicae* larvae to the commercially available, fungal bio-control agent *Beauveria bassiana* (Dani et al., 2004; Richards et al., 2011; García-Estrada et al., 2016). Therefore, the newly identified pimplin3 protein sequences might be applicable in integrated pest management programs to decrease the use of agrochemicals.

Peptidase S1A proteins play a vital role in blood coagulation, development, apoptosis, digestion, and immunity (Di Cera, 2009). The S1A family with trypsin domain is known to be widely distributed in parasitoid venoms **(Supp. Table 1)**. Parasitoid wasp venoms exhibit *in vitro* and *in vivo* cytotoxic activity by inducing apoptosis (Ergin et al., 2006; Er et al., 2011; Keenan et al., 2007; Rivers and Brogan, 2008; Formesyn et al., 2013), which can be decreased via serine protease inhibitor treatment (Formesyn et al., 2013). Therefore, *P. turionellae* venom peptidase S1A variants might help to suppress the host's cellular immune reactions and arrest the host's development by involving apoptotic processes.

2.2.2. *P. turionellae* venom components linked to melanization process

The process of melanization is strictly controlled by serine proteases and their inhibitors, in which phenoloxidases (POs) play an important role and produce melanin (Thomas and Asgari, 2011). Melanization of parasitoid eggs can be impaired by serine protease homologs (SPHs), serine protease inhibitors, and the inhibition of PO activity. In this dissertation, four protein families are characterized in *P. turionellae* venom that could be involved in the modulation of the melanization process: PO, Laccase, and kunitz-type and pacifastin-like protease inhibitors **(Figure 4, Supp. Table 1)**

It is known that insects have two different phenoloxidases—laccase-type and tyrosinase-like (ie., POs)—which can catalyze the oxidation of L-DOPA in melanin production (Mason, 1965). The oxidative POs and laccase (Lac1) proteins as venom constituents in endoparasitoids are reported for the first time from *P. hypochondriaca* (Parkinson et al., 2001; Parkinson et al., 2003), after which only laccase is reported from *N. vitripennis*. **POs and laccase** oxidative enzymes are expressed in *P. turionellae* venom, as well. Interestingly, the inhibition of phenoloxidase activity by using phenoloxidase inhibitor phenylthiourea in *P. hypochondriaca* (Rivers et al., 2009) and *N. vitripennis* (Rivers and Brogan, 2008) venoms results in less cytotoxicity in the host organism. Therefore, the presence of these enzymes in parasitoid venom could have two functions: They might initiate immune responses against microbes in parasitized and defenseless pupa, and/or they might contribute to the suppression of the host's cellular immune reactions by involving apoptotic processes.

POs are expressed as inactive zymogens in all insects, and their activation occurs following serine proteases, which are regulated negatively by plasma serine protease inhibitors (SPIs) (Kanost, 1999). Such SPIs have been reported in several parasitoid venoms, including *P. hypochondriaca* (Cvp2 and Cvp4) (Parkinson et al., 2004) **(Supp. Table 1). Kunitz-type and pacifastin-like serin proteases inhibitors** are expressed in *P. turionellae* venom, as well. The function of Cvp2 (cysteine-

rich kunitz-type serine protease inhibitor) and Cvp4 (cysteine-rich pacifastin-like protein) of *P. hypochondriaca* remained unclear; therefore, it remains speculative whether the newly identified *P. turionellae* serine protease inhibitors might help to suppress the host's melanization process and/or contribute to keeping venom serine proteases inactive while stored in the venom sac.

2.2.3. Venom proteins potentially linked to paralysis

Idiobiont endoparasitoids can paralyze their host permanently and suppress the host's growth and development. In this way, they prevent the mortality of parasitoid eggs due to host ecdysis and movement (Desneux et al., 2009). Several paralytic factors from parasitoid venoms have been isolated, including pimplin from *P. hypochondriaca* (Parkinson et al., 2002b). In this dissertation, I found a new peptide family, **pimplin2**, with the highest expression level, which has a cysteine-rich scaffold with an ICK motif **(Figure 4, Supp. Table 1)** (Özbek et al., 2019).

ICKs are widely disturbed in animal venoms, and they specifically act on targets in the nervous system of the recipient, particularly ion channels (Billen et al., 2010; Undheim et al., 2016; Drukewitz et al., 2018). Various cysteine-rich peptides 1-7 (Cvp1-7) have already been characterized in *P. hypochondriaca* venom, and among them, cvp3, cvp5, and cvp7 are more similar to pimplin2; however, their function remained unclear. Due to the high similarity of pimplin2 to known ICK-like neurotoxins from various animals, including robber flies, assassin bugs, and spiders, it is suggested here that pimplin2 might act as a paralytic factor in *P. turionellae* venom. Despite *P. hypochondriaca* and *P. turionellae* being close species, we could not find any evidence of pimplin in *P. turionellae* venom, which might be a species-lineage-specific toxin or a possible false-positive identification in the venom of *P. hypochondriaca* or false-negative hit in my analysis.

2.2.4. Venom proteins linked to nutritional functions

Parasitoids control the host's physiology to provide optimal conditions for their offspring to feed and develop (Pennacchio and Strand, 2006). In this dissertation, three protein families were characterized in *P. turionellae* venom and are assumed to contribute to providing the optimal nutriment for its offspring: glycoside hydrolase family 1 (GH1) and venom acid phosphatase and multifunctional serine proteases **(Figure 4, Supp. Table 1).**

GH1 proteins are widely distributed in the animal kingdom and are the most abundant venom proteins of the olive fruit fly parasitoid *Psyttalia sp.* (Mathé-Hubert et al., 2016). GH1 enzymes, which catalyze the hydrolysis of glycosidic bonds, play important roles in detoxification, defense, and carbohydrate metabolism (Eyun et al., 2014; Koudounas et al., 2015; Berlemont and Martiny, 2016; Cairo et al., 2016). Therefore, I speculate that GH1 might release host carbohydrates to feed the parasitoid larva.

Venom acid phosphatases were already characterized in several hymenopteran species **(Supp. Table 1)**. Although *P. hypochondriaca* venom reveals high acid phosphatase activity (Dani et al., 2005)**,** a low expression level of venom acid phosphatase protein in *P. turionellae* venom was found. Venom acid phosphatase enzymes catalyze the hydrolysis of phosphate esters to release inorganic phosphate and carbohydrates. In *A. mellifera* and *N. vitripennis*, it is proposed to be involved in the predigestion of prey before being eaten (Benton, 1967; Danneels et al., 2010). Thus, I presume that venom acid phosphatase proteins might play a role in providing carbohydrates from the host hemolymph and tissues for the parasitoid offspring.

A variety of potential functions of *P. turionellae* venom **serine proteases** are proposed above. However, it is known that the larvae of the ectoparasitoid *Euplectrus separatae* release saliva containing a trypsin-like enzyme to digest the host tissues (Nakamatsu and Tanaka, 2004). Therefore, in addition to their other roles in *P. turionellae* venom, serine proteases might play a role in assuring food for the development of parasitoid larva.

2.2.5. Venom components with unknown function

The venom components of parasitoids are of interest primarily due to their immunosuppressive, paralysis, and development inhibitory functions. However, the extraordinary lifestyle of parasitoids is still a mystery in many aspects. Therefore, known and novel venom proteins with unknown functions represent a very interesting group. This dissertation, on the venom composition of *P. turionellae*, reveals the presence of known proteins of unknown function: small venom protein 2 (svp2, renamed pimplin4) and Carboxylesterase type B **(Figure 4, Supp. Table 1).**

Carboxylesterases (COs) catalyze the hydrolysis of carboxylic esters to alcohols and acids (Ross et al., 2010). COs are highly diverse in insects and have already been identified in parasitoid venoms **(Supp. Table 1).** Insect COs metabolize specific juvenile hormones and degrade neurotransmitters (cholinesterase) (Oakeshott et al., 1999; Gilbert et al., 2000). However, their putative function has not yet been clarified in the context of insect venoms. It is speculated here that *P. turionellae* venom COs might contribute to the developmental processes by interfering with hormonal regulation.

Pimplin4, a small protein with a proline-rich scaffold, has already been identified as svp2 (small venom protein 2) in *P. hypochondriaca,* (Parkinson et al., 2004). Although pimplin4 is the second-highest-expressed protein in *P. turionellae* venom, its function remains unclear. As a linear, short protein, it might have antimicrobial peptide activity, and it represents an exciting candidate for *in vivo*/*in vitro* activity tests.

2.3. Conclusions and future perspectives

Parasitoids are a unique but lesser-known group among venomous organisms. In contrast with other venomous animals that use venom for defense against predators or prey capture, parasitoids utilize venom for the completion of their extraordinary lifestyle. Parasitoid venoms manipulate the host in several ways, such as altering host behavior, metabolism, and physiology, and inducing paralysis, immune suppression, developmental arrest to ensure the successful development of the wasp's larva. In this dissertation, new insights into the venom function of *P. turionellae* were revealed and serve as an important cornerstone for further studying the co-evolution of parasitoid wasps and their hosts. All three major epigenetic mechanisms—DNA methylation, histone acetylation, and miRNAs—are known to participate in the regulation of insect immunity and development (Vilcinskas, 2016; Glastad et al., 2019). My analyses support the hypothesis that parasitization by *P. turionellae* interrupts the host's epigenetic mechanisms to arrest host development and sabotage its immune system, filling in the gaps in the literature regarding the parasitization strategies of idiobiont endoparasitoid *P. turionellae* **(Figure 5)**. Understanding how genome regulations promote the development of the parasitoid in its host will help to shed new light on the molecular mechanisms of host-parasitoid interactions and their regulation.

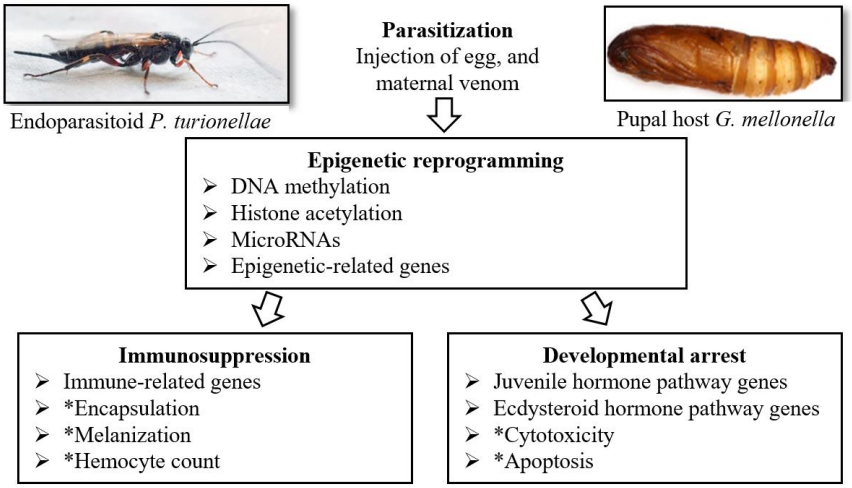

Figure 5. Schematized effects of parasitization by the endoparasitid *P. turionellae* on the pupal host *G. mellonella*. Newly tested and known impacts (marked with an asterisk) of parasitization on the host's epigenetics, development, and immunity are summarized.

I identified 12 venom peptide/protein families, including lineage-specific pimplin3-like and pimplin4-like proteins and a new protein family pimplin2, all of which are promising candidates, expecially for agrochemical applications. Pimplin2 is a promising candidate that demonstrates high similarity to ICK-like neurotoxins and might induce paralysis, likely by affecting ion channels as in many other arthropod ICKs (Drukewitz et al., 2018). Together with the second-highest-expressed pimplin4-like linear protein, these two proteins are of such interest that they were synthesized, and their activity will be tested in detail.

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4. Supplementary

Supp. Table 1. Common venom proteins identified from *P. turionellae* and other parasitoid wasps with their putative functions in host-parasitoid interactions

	Venom protein	Putative function	Other parasitoids	Reference
Enzymes	**Carboxylesterase type B**	Unknown	*Bracon hebetor* *Nasonia vitripennis* *Bracon nigricans*	Manzoor et al., 2016 Graaf et al., 2010 Becchimanzi et al., 2020
	Trypsin (Peptidase S1A)	Induction of apoptosis Inhibition of encapsulation Interrupting development Providing food	*Cotesia chilonis* *Aenasius arizonensis* *Nasonia vitripennis* *Pteromalus puparum* *Chelonus inanitus* (Ci-40a) *Bracon hebetor* *Pimpla hypochondriaca*?	Teng et al., 2017 Shaina et al., 2016 Graaf et al., 2010 Zhu et al., 2010 Vincent et al., 2010 Manzoor et al., 2016 Parkinson et al., 2002a
	Glycoside hydrolase family 1	Providing food	*Psyttalia lounsburyi* *Psyttalia concolor*	Mathé-Hubert et al., 2016
	Venom acid phosphatase	Providing food	*Nasonia vitripennis* *Pteromalus puparum* *Anisopteromalus calandrae* *Bracon hebetor*	Graaf et al., 2010 Zhu et al., 2010 Perkin et al., 2015 Manzoor et al., 2016
	Phenoloxidase	Melanization	*Pimpla hypochondriaca*	Parkinson et al., 2001
	Laccase	Melanization	*Pimpla hypochondriaca* *Nasonia vitripennis*	Parkinson et al., 2003 Graaf et al., 2010
	Peptidase M12B	Inhibition of encapsulation Interrupting development	*Pimpla hypochondriaca* *Toxoneuron nigriceps* *Cotesia chilonis* (Cc-Ven2) *Aenasius arizonensis* *Nasonia vitripennis* *Microplitis mediator(VRF1)* *Eulophus pennicornis (EpMP3)*	Parkinson et al., 2002c, Laurino et al., 2016 Teng et al., 2017 Shaina et al., 2016 Graaf et al., 2010 Lin et al., 2018 Price et al., 2009
Cys-rich peptides-proteins	**Kunitz-type serine protease inhibitor**	Inhibition of melanization	*Pimpla hypochondriaca* (Cvp2)	Parkinson et al., 2004
	Pacifastin - like protease inhibitor	Inhibition of melanization	*Pimpla hypochondriaca* (Cvp4) *Anisopteromalus calandrae* *Nasonia vitripennis* (NvSPPI)	Parkinson et al., 2004 Perkin et al., 2015 Qian et al., 2017
	Pimplin2 (6C-ICKs)	Paralysis		
Others	**Pimplin3**	Inhibition of encapsulation	*Pimpla hypochondriaca* (Vpr1)	Dani and Richards, 2010
	Pimplin4	Unknown	*Pimpla hypochondriaca* (svp2)	Parkinson et al., 2004

5. Publications

Publication 1

1 Proteo-Transcriptomic Characterization of the Venom from the Endoparasitoid Wasp *Pimpla turionellae* with Aspects on Its Biology and Evolution

Rabia Özbek, Natalie Wielsch, Heiko Vogel, Günter Lochnit, Frank Foerster, Andreas Vilcinskas and Björn Marcus von Reumont

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Article

Proteo-Transcriptomic Characterization of the Venom from the Endoparasitoid Wasp *Pimpla turionellae* with Aspects on Its Biology and Evolution

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Abstract: Within mega-diverse Hymenoptera, non-aculeate parasitic wasps represent 75% of all hymenopteran species. Their ovipositor dual-functionally injects venom and employs eggs into (endoparasitoids) or onto (ectoparasitoids) diverse host species. Few endoparasitoid wasps such as *Pimpla turionellae* paralyze the host and suppress its immune responses, such as encapsulation and melanization, to guarantee their offspring's survival. Here, the venom and its possible biology and function of *P. turionellae* are characterized in comparison to the few existing proteo-transcriptomic analyses on parasitoid wasp venoms. Multiple transcriptome assembly and custom-tailored search and annotation strategies were applied to identify parasitoid venom proteins. To avoid false-positive hits, only transcripts were finally discussed that survived strict filter settings, including the presence in the proteome and higher expression in the venom gland. *P. turionella* features a venom that is mostly composed of known, typical parasitoid enzymes, cysteine-rich peptides, and other proteins and peptides. Several venom proteins were identified and named, such as pimplin2, 3, and 4. However, the specification of many novel candidates remains difficult, and annotations ambiguous. Interestingly, we do not find pimplin, a paralytic factor in *Pimpla hypochondriaca*, but instead a new cysteine inhibitor knot (ICK) family (pimplin2), which is highly similar to known, neurotoxic asilid1 sequences from robber flies.

Keywords: hymenopteran venomics; parasitoid wasps; proteomics; transcriptomics; Pimplin2; ICK; knottins

Key Contribution: Our proteo-transcriptomic analysis sheds new light on the venom biology of the endoparasitoid *Pimpla turionealle*. Although the previously only proteomically in *P. hypochondriaca* described paralyzing factor pimplin was not found, we name a new ICK peptide family (pimplin2) that likely resembles a neurotoxic factor, and identify several novel venom proteins.

1. Introduction

Hymenoptera constitutes a mega-diverse insect order that is well known for its vast number of species (~150,000 according to reference [1], in which venom evolved for predation, defense, and communication [2–4]. They feature multiple life-style forms as solitary or social pollinators, predators, and parasitoids [4–6]. However, studied in more detail since the 1950s especially, are the venom components from a few aculeate species that occur in closer proximity to humans, such as eusocial bees and wasps (Apidae and Vespidae). For example, in aculeate hymenopterans, considered the original ovipositor, as the egg-laying structure was modified as from an apomorph character in this group to a stinger to employ venom exclusively from a connected venom gland system [4,6,7]. Nonetheless, more than 75% of known hymenopteran species are non-aculeate, i.e., are parasitoid wasps (parasitoids) that still utilize the ovipositor in its original function to lay eggs and "weaponize" it in a dual function to inject venom into host species they parasitize [8–11]. In stark contrast to aculeate venom, which is streamlined for defense to immobilize or to kill their prey [7,12–14], venom of parasitoids mainly alters the physiology and behavior of the host to keep it alive while feeding the offspring [15–19]. Despite this interesting biology, only a few parasitoid venom systems were studied in more detail.

Ectoparasitoids that lay eggs outside/on the host normally induce paralysis with their venom to ensure a successful feeding of the larvae; the time scale of the paralysis can vary [10,11,17,20–22]. The practical "zombification" of hosts that is induced by some species reflects a climax to ensure the successful development of their larvae. A prominent example is the jewel wasp *Ampulex compressa* that injects venom into the central nervous system of American cockroaches. The sting results in lethargy and hypokinesia accompanied by the suppression of any escape reflex without altering other behavior [19,23]. Proteomics analyses indicate that the neuropeptides tachykinin and corazonin induce these effects [19].

In contrast, idiobiont endoparasitoids, such as the herein studied taxon *Pimpla turionellae* (see Figure 1), induce eggs into the host, and their venoms are rather designed to interfere with the host's immune system and development [17]. Parasitoids evolved diverse strategies to attack specific stages of the hosts and either stop (idiobiont) or allow the host to continue (koinobiont) its development [24]. The specific parasitization of host stages and the ability of parasitoids to manipulate host physiology at the behavioral [23], endocrinal [25], nutritional [26], or immunological level [27] evoked a strong interest in their venom components for pharmaceutical and agrochemical research.

Figure 1. Female and male specimens of *P. turionellae*. The larger female is seen at the top. Males do not show typical female characteristics, such as the prominent ovipositor, and, therefore, also lack the venom system.

It might be reasoned by the small size and miniaturization of most parasitoids that only for few species are the venom, and its composition analyzed in more depth, despite studies on the general effects of envenomation dating back over four decades [7,17,28]. Details of specific components and single proteins derive mostly from proteome work that started more extensively in the 1980s [7,10,17,29]. Nonetheless, slowly proteo-transcriptomic data analyses that combine proteomics and transcriptomics are becoming well published for selected taxa [17,29], see Supplementary Table S1. A general picture is that the main venom components of many parasitoids are proteases and their inhibitors can involve and impair normal host physiology to guarantee the survival of the parasitoid's offspring [16,29–31]. Other biomolecules complement the venom cocktail, such as small peptides, mid-to high-molecular-weight enzymes, protease inhibitors, recognition/ binding proteins, immune-related proteins, neurotoxin-like peptides and paralytic factors [17,22,32]. The known biological function and identification source of major venom components of endoparasitoids are summarized in Supplementary Table S1.

From an evolutionary and biological perspective, ectoparasitism represents the ancestral form in parasitoid hymenopterans, which evolved the first time in orussid woodwasps, the closest relative to Apocrita [6]. The rapid radiation of parasitoid lineages in the natural group of Parasitoida [6] that comprises of primarily parasitoid wasps is besides miniaturization and a wasp waist (better maneuverability to position the sting), also linked to a diverse venom evolution and adaptation to the ecology and biology of hosts. In endoparasitoids, the paralyzing function of the venom becomes less important because the eggs are laid into the host, and the offspring no longer needs physical protection outside the host, i.e., by paralyzing it. However, by introducing the offspring into the host, parasitoid survival is jeopardized by the exposure to the host's immune system. A typical immune response to neutralize an intruding factor such as parasitoid progeny normally involves encapsulation by "enveloping" the egg with layers of hemocytes. This reaction is often associated in parallel with melanization, a process in which melanin pigments are deposited on an invading parasite or pathogen [11]. Most known and activity tested venom components of endoparasitoids manipulate the pathways and cascades that are related to these immune responses, such as the inactivation of hemocytes or melanization [17,33]. One of the endoparasitoids in which the venom and its components are better studied is *P. hypochondriaca* [17,34–44], despite the fact that many of its venom proteins remain unstudied, and thus far only proteome-derived data are available for this species. Nevertheless, to understand the complex venom evolution in parasitoids, an extended taxon sampling is essential combined with comparative, in-depth venomics studies.

2. Results

In this study, we used a proteo-transcriptomic approach to characterize the venom and the possible function of its components from *P. turionellae*, of which thus far only a few, older proteome based studies are available [45]. For characterizing the venom compositions in more detail, including expression levels of the venom components, a combination of proteomics and transcriptomics was needed. Our proteome analysis of the crude venom was combined with RNA Seq data from body tissue and venom gland system transcriptomes. In newly developed analysis pipelines and workflows, including multiple assemblies, secreted proteins in the proteome were matched with gland specific transcripts considering the higher expression levels as important thresholds as well. Several of the identified transcripts that survived our strict proteo-transcriptomic approach match were already known and described venom components of endoparasitoid venoms, such as laccase and phenoloxidase, which were mostly linked to the encapsulation or melanization processes, and several proteinase inhibitors, metalloproteinase M12B, carboxylesterase, and peptidase S1 variants. Most importantly, we identified the possibly paralyzing factor in the venom, an ICK-fold knottin peptide that we named pimplin2. ICKs are well known for their neurotoxicity in venoms from spiders and several other arthropods. Interestingly, our results did not support earlier findings of venom components of *Pimpla* that were identified via proteome-only approaches, such as apamin, melittin and pimplin. The latter has been described as a major paralyzing factor from *P. hypochondriaca*.

2.1. Proteo-Transcriptomic as a Strategy to Identify Coding Transcripts Based on Proteomics

All reads of the three generated cDNA libraries from the tissue of the female venom gland system (Vg), female body (BtF), and male body (BtM) were assembled together utilizing an in-house pipeline applying multiple assemblers (Trinity, rnaSPADES with and without error correction), see material and methods. After quality trimming, identical reads were merged, resulting in a total of 448,782 contigs. From those, 391,271 coding regions were identified by the Transdecoder and finally used in all downstream analyses. For each transcript, protein-coding regions were further annotated via Interproscan, BLASTX (e-value $\leq 10^{-6}$) against the non-redundant NCBI database, ToxProt, and 118 own hmmer profiles (min. bitscore 15) of known venom peptides and proteins. Additionally, JACKhmmer searches (min. bitscore 15) were performed for 151 known single venom protein sequences that were mined manually for parasitoid wasps (see material and methods for details).

The components of the crude venom were separated by SDS-PAGE, and the bands were observed from less than 15 kDa to more than 250 kDa (apparent molecular mass), which were separated into 24 samples, see Figure 2. The in-gel digested and trypsinized samples were analyzed via LC-MS/MS. After the fragment identification, the generated transcripts were used as a specific database to match the fragments from the MS analyses with transcripts of secreted proteins using the MASCOT software suite (see material and methods for further details). Subsequently, we only discuss protein families with transcripts for which reads map back that derive from the venom gland tissue library and that were as well identified by at least one transcript via the proteome data (Mascot value ≥ 30 and number of fragments ≥ 2, search window of 0.002 Da, see Table S2). Applying this strict and extensive filtering we avoid the possible over- or mis-interpretation of the transcriptome data as recently discussed [46–48].

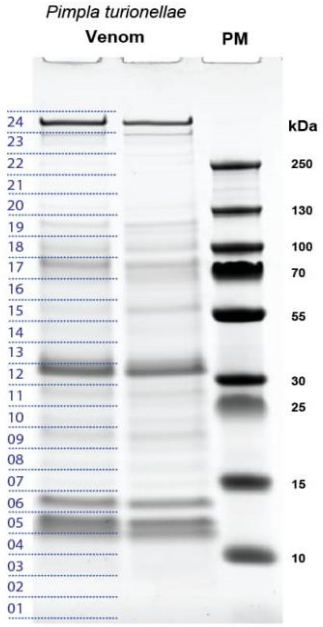

Figure 2. The SDS-PAGE analysis of *P. turionellae* venom proteins. Proteins obtained from the lumen of *P. turionellae* venom glands were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R250. PM = protein marker; numbers on the left indicate the 24 bands cut out from the gel and processed as individual samples for LC-MS/MS. Molecular mass is in kDa.

2.2. General Overview of the Transcripts that are Supported by Proteomics

In total, the 339 transcripts that remained after all filtering steps, could generally be separated into three major groups. Group 1: Non-venom related transcripts that were annotated with clear cellular functions such as ribosomal and membrane proteins (175 transcripts), which were not further analyzed.

Group 2: Transcripts with annotation similar to known venom protein classes (117 transcripts). Group 3: Transcripts with no similarity to known protein groups (18 transcripts), or with annotations at the amino acid level without conclusive information on protein domain or family (29 transcripts), see Tables S3 and S4. Groups 2 and 3 were further analyzed applying additional thresholds at the expression level to avoid misinterpretation of our data based on the hypotheses that venom proteins should be more highly expressed in the venom gland compared to non-venom-system related body tissue. Our downstream analysis here was thus focused on venom-related candidates that matched known venom proteins classes (group 2), and putative novel venom proteins with inconclusive or no annotation (group 3) that were higher expressed with a TPM (transcripts per million) value of >1 and a two-times higher expression (log2 fold change under minus one) compared to the expression levels of similar or identical transcripts in the female and male body tissue, respectively (see Tables S3–S4).

2.3. Composition and Expression of Genes from Known Venom Protein Classes

From the final 117 venom function related transcripts, 88 remained that passed our thresholds, and which belonged to 12 known venom protein families (all sequences and alignments are available in the additional data file 1). The identified protein families could be classified into three major groups: Enzymes, cysteine-rich peptides/proteins, and others (Figure 3). Among the identified enzymes in the *P. turionellae* venom, carboxylesterase constitutes the most diverse protein family with 22 transcripts, followed by laccase (14), phenoloxidase (12), S1A superfamily trypsin domain (9), glycoside hydrolase family 1 (4), metallopeptidase M12B (4) and venom acid phosphatase (1) (Figure 3A).

Identified cysteine-rich venom proteins were new variants of ICK-fold knottin peptides with a 6-C scaffold, which we named Pimplin2 (see Table 1) and kunitz-type and pacifastin-like protease inhibitors. Other components were the here renamed families pimplin3 (including venom protein1, Vpr1) and pimplin4 (including small venom protein2, svp2) that have been described in *P. hypochondriaca* (see Table 1). Most dominantly expressed in the venom were interestingly novel candidates of these three classes: Pimplin2 (TPM 25,267), pimplin4 (TPM 7899), and pimplin3 (TPM 7759). All remaining components show lower levels of expression (see Figure 3B and Table S3).

Table 1. Renamed known peptides and proteins with novel variants in *P. turionellae*. * Pimplin described in *P. hypochondriaca* was not found in *P. turionellae*, but is listed for completeness. The brackets indicate the domain structure of pimplin 2, the C's are the pattern of the cysteine scaffold for whole sequences. The lengths range is given for all sequences, including those from *P. hypochondriaca*. All sequence alignments with the named sequence-IDs and corresponding neighbor-joining networks are provided in the additional data file 1.

Name	Structural Fold	Scaffold	Length(aa)	TPM
Pimplin *	Dimeric protein	Prolin scaffold	143	NA
Pimplin2	ICK	X-CX_7-[C-X_6-C-X_{5-8}-CC-X_{2-4}-C-X_{6-9}]-X	63–115	25,267
Pimplin3	Protein	Potential P and C scaffold	167–315	7759
Pimplin4	Short protein	No cysteine scaffold, 3 P residues	70–78	7899

2.4. Novel, Uncharacterized Venom Peptides and Proteins

We also identified 12 putative novel venom proteins. However, for most of those, our search against known venom proteins (hmmer-profiles, ToxProt) and the annotation via InterProscan, remained inconclusive, see Table 2. Manual BLAST search via NCBI revealed many low-scoring matches against bacteria.

Table 2. Overview of identified putative novel venom proteins. Shown are the IDs for each transcript that were identified via proteomics, the manual BLAST/annotation results, and the expression levels (TPM). Peptides (<50 aa) and Proteins (>50 aa) are sorted according to their expression levels. Candidates that could be of interest for more detailed analyses, for example, bioactivity tests, are highlighted in light grey. All sequences and alignments of novels are available in the additional data (additional data file 2) deposited in the open access database ZENODO (see additional data).

Name	Transcript ID	Manual BLAST Match	Length(aa)	TPM	Signal Peptide
NovelP1	pitu_v1_174267	Inconclusive-non cytoplasmic domain	126	7746	Yes
NovelP2	pitu_v1_002265	Inconclusive-bacterial	50	6141	No
NovelP3	pitu_v1_377983	Inconclusive-bacterial	17	1209	No
NovelP4	pitu_v1_378290	Inconclusive-bacterial	14	1159	No
NovelP5	pitu_v1_002208	Inconclusive-non cytoplasmic domain	73	288	No
NovelP6	pitu_v1_468063	Inconclusive-non cytoplasmic domain	70	239	Yes
NovelP7	pitu_v1_094627	Inconclusive-non cytoplasmic domain	167	219	Yes
NovelP8	pitu_v1_377800	Inconclusive-non cytoplasmic domain	214	208	Yes
NovelP9	pitu_v1_473891	Inconclusive-bacterial	11	180	No
NovelP10	pitu_v1_176834	Inconclusive-Water bear-uncharacterized	43	102	No
NovelP11	pitu_v1_172572	Inconclusive-bacterial	49	36	No
NovelP12	pitu_v1_285207	Inconclusive-bacterial	19	2	No

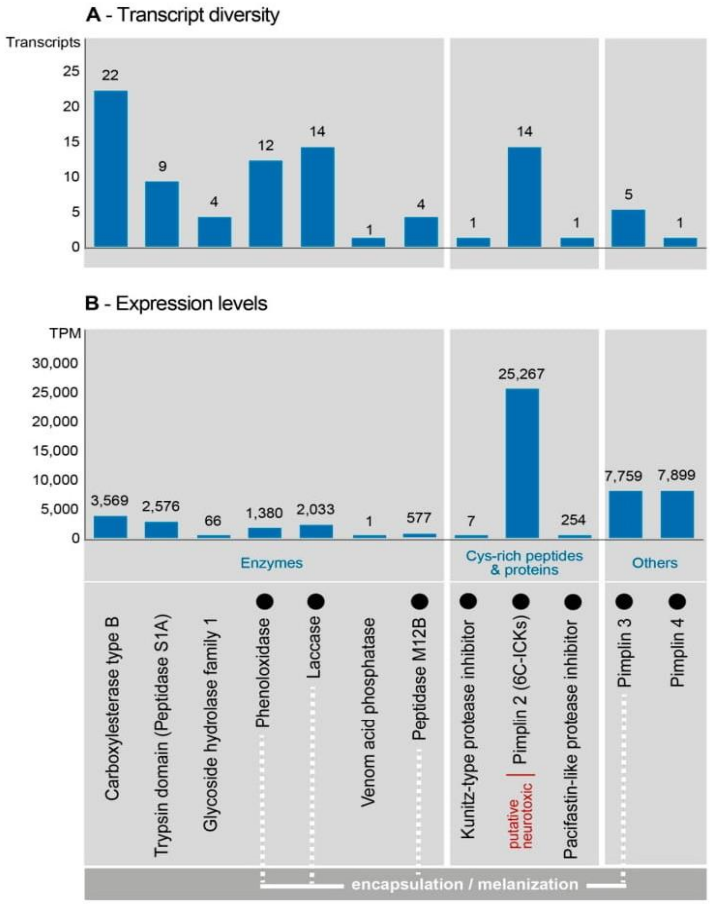

Figure 3. Transcript diversity and expression levels of identified known venom protein families from *P. turionellae* venom glands. The number of transcripts (**A**) and the summarized expression levels in transcript per million (TPM) per protein class (**B**) are shown for functional groups of protein families. The black dots highlight protein families for which sequences from *P. hypochondriaca* were described. Proteins that probably act on the encapsulation and melanization process are highlighted by the white dotted lines. All sequence alignments of known venom proteins are provided in the additional data file 1.

3. Discussion

Several studies on parasite-host interaction and envenomation effects describe that the idiobiont parasitoid, *P. turionellae*, inflicts the venom injection before oviposition—a quick, obviously permanent paralytic effect on the host followed by the suppression and alteration of the host's physiology [49–51]. The paralyzing component probably prevents, firstly, rapid action from the host to changing its habitat [24]. However, permanent paralysis of the host is reported for only a few endoparasitoids, since most species in this group adopted the koinobiont lifestyle that allows normal development of the host [17]. In contrast, ectoparasitoids generally induce paralysis of the host to prevent any harm to the offspring on/next to it [17]. Other venom components from *P. turionellae* modulate the response of the immune system, such as encapsulation after eggs are placed into the paralyzed host and possibly subsequent melanization of the encapsulated parasitoid. Using a proteo-transcriptomic approach, here we provide a more detailed picture of the two-fold envenomation process (paralysis and suppression of immune response) in *P. turionellae*. The diversity and putative biology of its venom components are discussed in comparison to the few, more in-depth venomics studies of endoparasitoid wasps.

3.1. Missing Evidence of the Paralytic Venom Component Pimplin Described for P. hypochondriaca

Until today, only three potentially paralytic or neurotoxic venom components identified in parasitoid wasps were tested for their activity (see Supplementary Table S1). Two of them are *BrhI* and *BrhV* (74 kDa), which were described for the ectoparasitoid wasp *B. hebetor* (Braconidae), and both of which showed potent effects when injected into caterpillars [52]. From the venom of the endoparasitoid *P. hypochondriaca,* a heterodimeric protein (22 kDa) consisting of two polypeptide chains (10.5) and (6.3 kDa) that are linked through a disulfide bond, was isolated and named pimplin. The paralytic effects of pimplin have been shown using adult stages of the housefly *Musca domestica*, the cockroach *Blatella germanica,* and the tomato moth *Lacanobia oleracea* [34].

Surprisingly, we found no evidence of this protein in our proteo-transcriptomic data. We can only speculate why pimplin might be missing in the venom of *P. turionellae*. One obvious possibility is that pimplin resembles a species-lineage specific toxin in *P. hypochondriaca* and is thus linked to unique host adaptations. However, given that the two species are not that distinct from each other, the recruitment of this toxin in *P. hypochondriaca* must have occurred very recently. Other reasons could be a false negative hit in our analysis or a possible false-positive identification in the venom of *P. hypochondriaca*.

3.2. Pimplin2 (a New ICK Family) Might Act as Paralytic Factor in P. turionellae

The most expressed venom component in *P. turionellae* is the new peptide family pimplin2 that features a structural cysteine inhibitor knot (ICK) motif. Generally, ICKs with varying cysteine scaffolds are widely employed in animal venoms [53], and are of particular applied interest because of their various effects on ion channels. Pimplin2 shows high similarity to known ICK-like toxins explicitly known in spiders, cone snails, assassin bugs, scorpions, and robber flies [53,54]. Interestingly, cysteine-rich venom proteins named cvp1-cvp7 (Cvp = cysteine rich venom protein) have been described already in older studies for *P. hypochondriaca* [42]. In phylogenetic analyses based on structural alignment of known ICK with a similar scaffold, it was revealed that cvp5, cvp3, and cvp7 are highly similar to the new pimplin2 sequences, see Figure 4. Consequentially, the three peptides from *P. hypochondriaca* were renamed to U-Pimplin$_2$-Phy2a (Cvp3), U-Pimplin$_2$-Phy1b (Cvp5), and U-pimplin$_2$-Ph1a (Cvp7), according to the naming scheme in reference [55]. Figure 4 illustrates that two distinct clades of pimplin2 are identified. U-Pimplin$_2$-Phy2a and several pimplin2 transcripts of *P. turionellae* are grouping in a clade that is more closely related to asilidin1, an ICK family for which paralytic activity was revealed in robber flies (Asilidae, Diptera). For a more detailed discussion on the evolution of pimplin2 a larger taxon sampling of ichneumonoid wasps would be necessary. Nevertheless, our hypothesis is that functionally, pimplin2 reflects the paralytic component in the venom of *P. turionellae* and possibly in *P. hypochondriaca* as well, which is in congruence with the findings

by Parkinson and colleagues [42]. Interestingly, no further components which could potentially have paralytic activity, similar to known parasitoid venom proteins such as arginine kinase from *B. hebetor* and *Aenasius arizonensis* [56,57], were identified in our data.

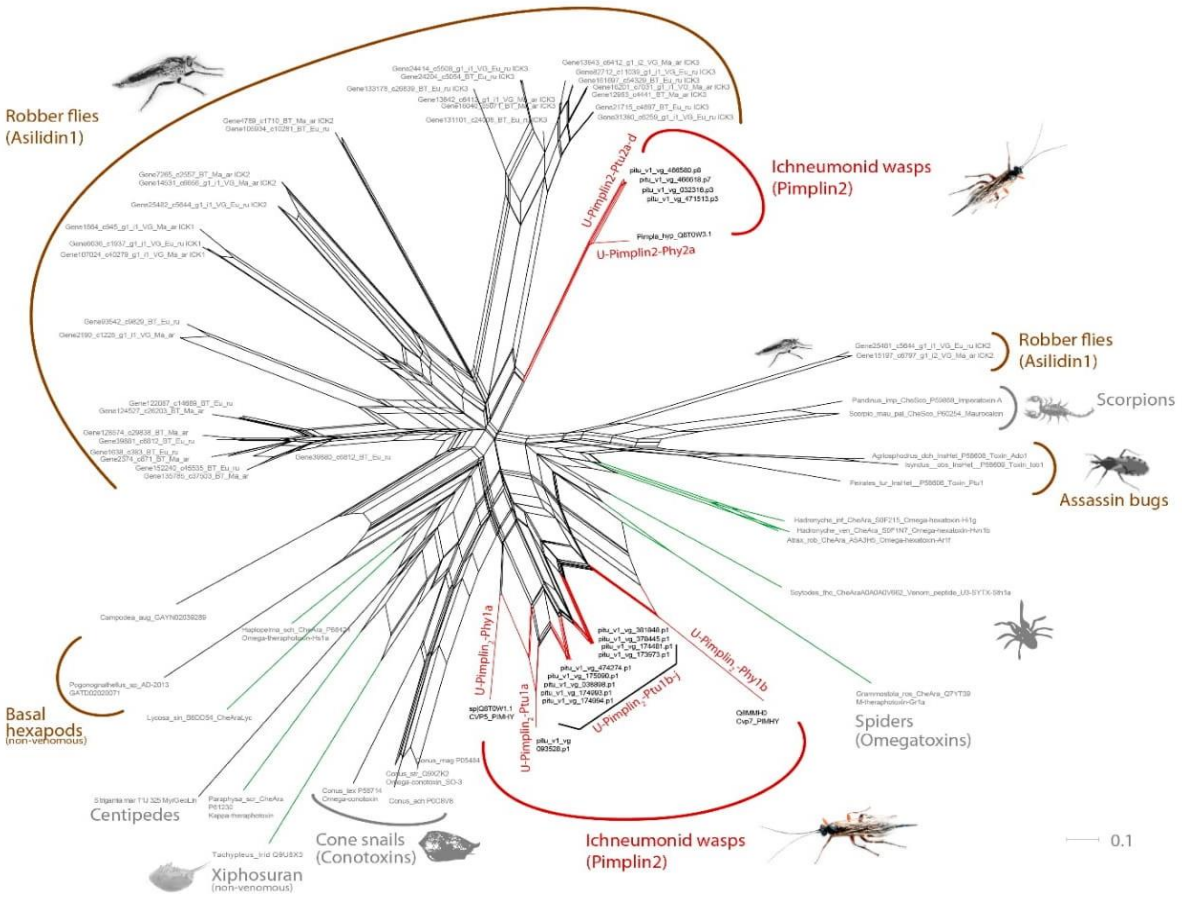

Figure 4. Diversity of known and described cysteine inhibitor knot (ICK) peptides similar to pimplin2. The neighbor-joining network reconstructed in Splitstree 4 [58] is based on protein distances that were optimized using the WAG-Gamma protein substitution model provided in Splitstree [58] and includes known sequences that share the cysteine scaffold of the identified pimplin2 ICK peptide. Known variants from other insect groups such as robber flies or assassin bugs are highlighted in brown. Pimplin2 transcripts from *Pimpla* are colored in red, the spider ICK variants (omegatoxins) are highlighted in green.

3.3. Venom Components Linked to Encapsulation

Diverse proteins that were shown or were suspected to suppress the encapsulation cascade have been previously identified in the venoms of parasitoid wasps. The protein arsenal acting on this part of the host's cellular immune response includes several protein classes. Metalloproteases were found in *M. mediator* [15] *E. pennicornis* [59], *P. hypochondriaca* [39], *Toxoneuron nigriceps* [18], *Cotesia chilonis* [29], *A. arizonensis* [57], *N. vitripennis* [16], and *M. mediator* venom (VRF1) [15]. Calretriculin is described for *B. hebetor* [56], *A. arizonensis* [57], *C. rubecula* [60], and *P. puparum* [30]. Venom of *P. hypochondriaca* was shown to contain venom protein 1 (Vpr1) and venom protein 3 (Vpr3) [35,36,43], while Vn.11 was identified in *P. puparum* [61] and the virulence protein P4 (RhoGAP protein) in *L. boulardi* [62,63].

Metallopeptidase M12B (ADAM/reprolysin) has a low expression in *P. turionellae* venom. Reprolysin-type metallopeptidases require zinc for catalysis, and the catalytic site is characterized by a consensus HEXXHXXGXXH sequence [64]. Metallopeptidases act as a general toxic component and

show a broad range of activities, including the utilization of host proteins for nutrition, the suppression of host cellular defense, and the degradation of host defense molecules [65,66]. It was shown that a metalloprotease homolog VRF1 from the endoparasitoid wasp, *M. mediator* could modulate egg encapsulation in its host, *Helicoverpa armigera*, by suppressing the Toll pathway [15]. Thus, it can be speculated that M12B peptidase present in *P. turionellae* might as well attack host hemocytes and inhibit encapsulation. This assumption is supported by older in vivo tests that revealed encapsulation inhibitory effects of *P. hypochondriaca* crude venom [39].

Serine proteinases from the peptidase S1A family with trypsin domain that occurs in the venom of *P. turionellae* show a high expression level. Peptidase S1A proteins typically exhibit a conserved catalytic triad of Asp, His, and Ser residues [67] and are known as a widely distributed venom component in hymenopterans, such as woodwasps (*Sirex noctilio*) [68], parasitoid wasps (*C. chilonis* [29]; *A. arizonensis* [57]; *N. vitripennis* [16], *P. puparum* [30]; *Chelonus inanitus* [31], and higher aculeates (*Bombus ignites* [69]; *Vespa magnifica* [70]; *Polybia occidentalis* [71]). Several studies suggest that venoms of parasitoid wasps show in vitro and in vivo cytotoxic activity in host and insect derived cell lines and probably induce apoptosis [37,51,72–75]. This indicates that the peptidase S1A variants in *P. turionellae* might help to arrest host development and suppress host cellular immune reactions by involving apoptotic processes.

Finally, variants of the venom protein 1 (Vpr1) described in earlier studies of *P. hypochondriaca* venom [35,36,43] likely play a predominant role as possible hemocyte anti-aggregation factor in *P. turionellae*, since the injection of recombinant rVPr1 suppressed the ability of *L. oleracea* and *Mamestra brassicae* to mount hemocyte-mediated immune responses. Protein sequences highly similar to Vpr1 resemble the third most expressed venom component in *P. turionellae*, and we subsequently renamed this protein family pimplin*3*. Interestingly, the recombinant pimplin3 sequence from *P. hypochondriaca* increases, if injected, the sensivity of *M. brassicae* larvae to the commercially available, fungal bio-control agent, *Beauveria bassiana* [44,76,77]. Therefore, the newly described pimplin3 proteins might play an important role as bioactive agent that suppress key immune responses in target pests and increase the efficacy and decrease the use of agrochemicals [44].

3.4. Venom Components Involved in the Modulation of Melanization

Encapsulated pathogenic objects like parasitoid eggs usually experience a second host immune response and are melanized in this humoral defense reaction [78]. The melanin capsule can block absorption of nutrients by parasites and may contribute to their killing by starvation [79]. Phenolic intermediates, which are formed during the synthesis of melanin, probably additionally help to kill invading organisms [80]. However, phenoloxidase proteins (POs) of (endo)parasitoid crude venom are thus far only reported from *P. hypochondriaca* [37]. Sequencing of complementary DNA (cDNA) of fractionated crude venom of *P. hypochondriaca* indicated the presence of POs encoded by three genes (POI, II, and III) that derived by gene duplication [38]. POs are also expressed in *P. turionellae* venom (TPM 1380). Using L-DOPA as a substrate, PO activity has been reported from venom of the ectoparasitoid *N. vitripennis* [81], but PO proteins were not identified via proteomics. Instead, a multicopper oxidase, laccase, was found [16], which can catalyze the oxidation of L-DOPA [82]. Laccase (lac1) was also described in *P. hypochondriaca* venom, and the authors suggested that in *P. hypochondriaca* venom laccase and PO proteins may orchestrate L-DOPA oxidizing activity [41]. Laccase is also represented in *P. turionellae* venom (TPM 2033) and potentially has a dual function in endoparasitoid venom based on its involvement in insect cuticle sclerotization [83]. When suppressing the melanization cascade in the host, parasitoid eggs are defenseless against microbes and POs in *P. turionellae* venom could, therefore, initiate defense reactions in case of egg rupture or attacks by microorganisms. Finally, cytotoxic action of *P. hypochondriaca* [84] *and N. vitripennis* [74] venom was inhibited in insect cells using phenoloxidase inhibitor phenylthiourea to (PTU). This indicates that laccase and POs additionally mediate cell death related to the suppression of the host immune response.

3.5. Known and Novel Venom Components with Unknown Function

The negative regulation of the serine protease-mediated melanin synthesis is carried out with different types of serine protease inhibitors (SPIs) such as serpins, kunitz-type, and pacifastin [85]. SPIs have been identified in several parasitoid venoms, including *P. puparum* (PpS1V) [33], *L. boulardi* (LbSPNy) [86], *C. chilonis* [29], *N. vitripennis* [16], *Anisopteromalus calandrae* [87], and *P. hypochondriaca* (Cvp2 and Cvp4) [42].

The cysteine-rich protein (Cvp2) was first determined in *P. hypochondriaca* venom, but its function remains unclear. These cysteine-rich kunitz-type serine protease inhibitors feature ~60 amino acids stabilized by three disulfide bridges connecting cysteins 1–6, 2–4, 3–5 [88]. In *P. turionellae*, kunitz-type peptides similar to Cvp2 are higher expressed compared to other components (TPM 2194), but we can only speculate that its function is to interfere with the melanin biosynthesis. Another proteinase inhibitor is the pacifastin-like protein (Cvp4), which was identified in *P. hypochondriaca* venom, containing a triplicated six-cysteine motif [42], while *in N. vitripennis*, this motif is repeated four times [16]. In *P. turionellae*, a pacifastin-like protein is moderately expressed, but shows only two repeats of this motif. In addition to their main function, some kunitz-type and pacifastin peptides are also capable of blocking ion channels, especially the voltage gated potassium channels, which are essential for regulating various physiological processes such as blood coagulation or host defense [88].

Venom acid phosphatases were already identified in other hymenopteran species, including *N. vitripennis* [16], *P. puparum* [30], *A. calandrae* [87], *S. noctilio* [68], *B. hebetor* [56], *Apis mellifera* [89], and *P. hypochondriaca*. However, these proteins might only play a secondary role in the venom of *P. turionellae* since they display rather low gene expression levels compared to the other major components. Although the function of venom acid phosphatases is not known yet, and first tests from *P. hypochondriaca* showed no effects on hemocytes from *L. oceracea* [32,90], therefore, a cytotoxic activity is hypothesized.

Carboxylesterase type B has been reported in ectoparasitoid venoms, including *B. hebetor* [56] and *N. vitripennis* [16], and shows a high number of transcripts (20) and high levels of expression (TPM 3569) in the venom system of *P. turionellae*. Carboxylesterases (COs) are serine hydrolases that catalyze the hydrolysis of carboxylic esters to their component alcohols and acids and are highly diverse in insects [91,92]. COs play an important role in insect metabolism, such as degrading neurotransmitters (cholinesterase) and metabolizing specific juvenile hormones [93–95]. Their function in *P. turionellae* remains unclear, but they could be involved in the developmental processes that are controlled by the parasitoid.

Glycoside hydrolase family 1 (GH1) is one of the most abundant venom components of the parasitoids *Psyttalia lounsburyi* and *Psyttalia concolor* (Hymenoptera, Braconidae), which are important bioagents against the olive fruit fly [96]. GH1 enzymes catalyze hydrolysis of glycosidic bonds between carbohydrates and breakdown polysaccharides into smaller products [97]. They are widely distributed in the animal kingdom and play important roles in carbohydrate metabolism, defense, and detoxification [96,98–102]. For *P. turionellae*, it is speculated that GH1 might release host carbohydrates in order to feed the parasitoid larva. Nevertheless, GH1 reflects in our study a rather low expressed component in *P. turionellae* venom.

The venom component expressed at the second highest level in *P. turionellae* we named pimplin4 (TPM 7899), which is a small venom protein that was already described as svp2 (small venom protein2) in *P. hypochondriaca*, [42]. The function of pimplin4 in *P. turionaellae* remains speculative. Interestingly, it shows no similarity to other known proteins and would represent an interesting candidate for activity tests. We refrain from discussing the function of pimplin4 and the potential novel candidates here in detail, because the annotations are inconclusive, and without any further information, it remains to be tested which function they have.

4. Conclusions

Hymenopterans belong to one of the most venomous species-rich groups in the animal kingdom, and a large majority of its species are parasitoid wasps. Despite many articles suggesting that hymenopteran venoms are well understood or most of their effector proteins are known, only a few species have been studied in more detail. This is particularly the case for taxa in closer proximity to humans, such as bees and wasps [17]. Parasitoids, especially endoparasitoid wasps, however became of interest rather late, mostly due to their employment in some cases of paralyzing and immune suppressive venoms. These functions are, in particular, of desire for pharmacological and agricultural applications. In this study we describe new variants of pimplin3-like proteins, which could, for example, make pest species more vulnerable to agrochemicals [44], thus increasing their effectiveness while reducing the applied quantities of these toxic substances. Nevertheless, because only few parasitoids have been analyzed using in-depth venomics studies, this leaves a huge potential for applied research untouched.

Comparative approaches that include more taxa are crucial to understanding venom evolution in general and for particular groups [3]. Especially in mega-diverse groups such as hymenopterans more species need to be studied. Of particular interest are obviously the occurrence of lineage-specific venom proteins, such as the herein named pimplin3 and 4. Some venom proteins that use a common motif like the ICK pimplin2 might be lineage-specific as well after convergent recruitment, or their evolutionary origin could be shared in *P. turionellae* with robber flies. To finally unravel the origin and to understand the mechanisms how these cysteine-rich and other venom proteins evolve the inclusion of comparative genomic data in venomics studies is of utmost importance as shown for asilidin1 in the robber fly *Dasypogon diadema* [54] and for *N. vitripennis* [103].

5. Materials and Methods

5.1. Rearing and Dissection of P. turionellae Specimens for Proteomics and Transcriptomics

To obtain sufficient specimens for proteome and transcriptomic work, colonies of *P. turionellae* (Hymenoptera, Ichneumonidae) were reared in the lab according to reference [104], using pupal stages of *Galleria mellonella* (greater wax moth) as hosts. Adult parasitoids were collected after hatching from the host pupae and held in 1 L glass jars without a host and fed on 50% (v/v) honey solution.

For proteomics work, the venom gland system of 25 mated females of *P. turionellae* was dissected after 10–20 days, see Figure 5. All specimens were anesthetized in −80 °C for 3 min, and each venom sac was carefully removed from the abdomen on ice in 60 µL sterile PBS buffer and separately transferred into a 0.2 mL microfuge tube with 20 µL of PBS buffer. Each venom sac was then punctured with sterile forceps to obtain the crude venom. All tissue remains were removed, and the crude venom stored at −80 °C for subsequent proteome analysis.

The dissection for venom glands for transcriptomics followed the same protocol, except that 50 venom sacs and ducts had to be used and were finally preserved in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA). All samples were stored at −80 °C. Complementary to the venom gland tissue samples, 3 male and 3 female body tissue samples (excluding the venom apparatus) were prepared and stored separately in TRIzol reagent at −80 °C.

5.2. RNA Isolation, Library Preparation, and Illumina Sequencing

Total RNA was isolated from pooled female venom glands (Vg), female body tissue (BtF), and male body tissue (BtM) samples using TRIzol according to the manufacturer's instructions, followed by DNase treatment (Turbo DNase, Thermo Fisher Scientific, Waltham, MA, USA) and further purification using RNA Clean and Concentrator 5 (Zymo Research, Irvine, CA, USA). RNA quantity was determined using an Implen Nanophotometer (Implen Inc., Westlake Vilalge, CA, USA), and the integrity of all RNA samples was verified using an Agilent 2100 Bioanalyzer and an RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA). Transcriptome sequencing was carried out on the

Illumina HiSeq 3000 platform by GATC Biotech (Konstanz, Germany). Poly-A containing mRNAs were isolated from 1 µg total RNA using oligo-dT attached magnetic beads, the obtained mRNA was fragmented to an average of 250 bp. Afterwards, 150 bp, paired end sequencing libraries were generated using the Illumina TruSeq RNA library preparation kit for each sample. All information and data related to RNASeq was submitted to NCBI, and the raw transcriptome data for Vg (SRR9901353), BtF (SRR9901351), and BtM (SRR9901352) are accessible via the umbrella BioProject PRJNA555750.

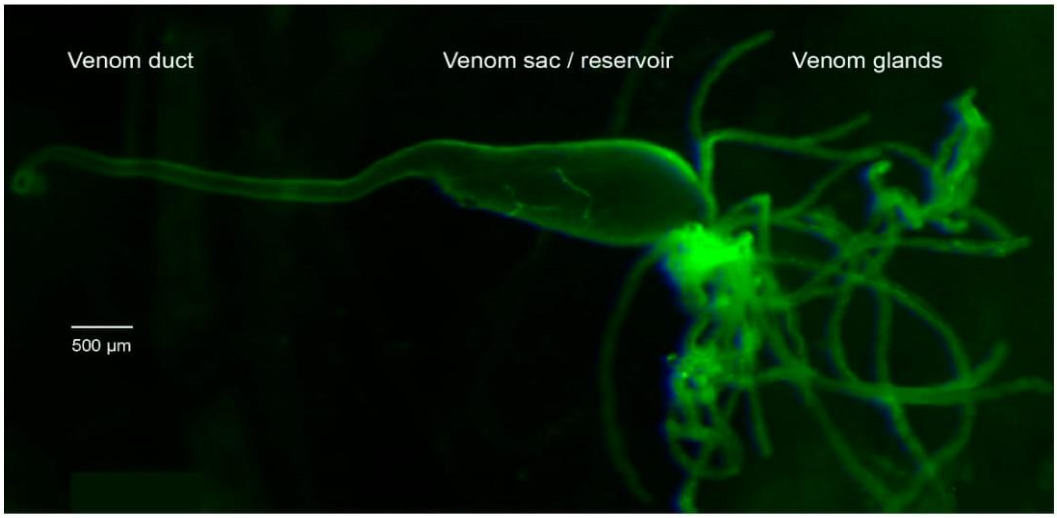

Figure 5. The dissected parts of the venom gland system in *P. turionellae*. All tissue parts (venom duct, venom sac, and venom glands) were together used for the transcriptome and proteome data generation.

5.3. Transcriptome Assembly, Read Mapping, and Identification of Venom Proteins

All reads generated by the sequencing provider were processed in an in-house assembly and annotation pipeline of the Animal Venomics group. For maximizing reproducibility, all software components were packed into docker images that can be run with all used settings easily on any Linux system. The input sequence reads were inspected using FastQC (v0.11.7) [105]. Afterwards, all reads were trimmed using Trimmomatic v0.38 [106] (docker image greatfireball/ime_trimmomatic:v0.38) and the following settings: ILLUMINACLIP:/opt/Trimmomatic/adapters/TruSeq3-PE.fa:2:30:10 LEADING:10 TRAILING:10 SLIDINGWINDOW:4:30 MINLEN:120. The resulting trimmed reads were used as input for the assembly based on multiple assemblers, currently Trinity 2.8.4 [107,108] (docker image greatfireball/ime_trinity:v2.6.6_1), and rnaSPAdes v3.12 [109] (docker image greatfireball/ime_spades:3.12.0) with and without error correction. Contigs from all assemblers were subsequently combined to establish a comprehensive assembly in which transcripts were merged that were derived from the different assembler runs and had the same length and 100% identity. The expression levels were quantified for all transcripts by re-mapping the reads to the assemblies using the mapper Hisat2 v2.1.0 [110] (docker image greatfireball/ime_hisat2:v2.1.0) and the quantification tool stringtie v1.3.5 [111,112] (docker image greatfireball/ime_stringtie:v1.3.5). Conversions between SAM and BAM files were performed by samtools v1.3.1 [113].

Open reading frames in transcripts were predicted with Transdecoder v5.0.2 [108] or https://transdecoder.github.io/; docker image greatfireball/ime_transdecoder:5.0.2) and annotated on the amino acid level performing Interproscan v5.27.66 [114] (docker image greatfireball/ime_interproscan:v5.27-66) and BLASTX searches (e-value $\leq 10^{-6}$) against the NCBI non-redundant and ToxProt databases, see Table S3. Additionally, an in-house database of 118 HMMER profiles of known venom components was utilized to distinguish and annotate possible toxins, venom proteins, and peptides in the transcriptome data (min. bitscore = 15), the used pipeline is accessible via github: (https://github.com/reumont/av_hmm_pipeline.git). Known sequences of 151 venom components from parasitoid hymenopterans

were mined from the literature and NCBI GeneBank to search these single peptides and proteins via JackHMMER in the data (min. bitscore = 15). To identify the unknown or unannotated venom components, transcripts with higher expression values were included as well. The alignments that were used to perform HMMsearches and the sequences that were the base for JackHMMER searches are available as additional data (additional file 3 and 4) in the public database ZENODO (see additional data), where the assembly and Transdecoder prediction files are also accessible (additional files 5 and 6).

To avoid overestimation of the transcriptome data, we only discuss venom gland based transcripts that were identified and supported by proteome data using the transcriptome as a species-specific database for spectral searches in MASCOT, see 5.5 and, Table S2. Additionally, an expression value of >1 TPM (transcripts per kilobase million) was used to reduce false positives transcripts and a two-fold higher expression level cut-off in venom gland samples compared to body tissue samples based on normalized expression values (see Table S3).

5.4. SDS-PAGE and LC-MS/MS Analysis of P. turionellae Venom Proteins

Venom proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12% Criterion™ XT gradient gels (BioRad, Feldkirchen, Germany) with XT MES running buffer, see Figure 2. Before loading, samples were mixed with XT sample buffer and reducing agent and heated for 5 min at 95 °C. Gels were run for 70 min at 130 V and stained using Coomassie Brilliant blue R250 (Imperial Protein stain, Thermo Scientific). Molecular weights (kDa) of separated venom proteins were assessed using a pre-stained protein marker.

Two lanes of the SDS-PAGE gel were excised into 24 molecular weight fractions each, containing nearly equal staining densities across both lanes (Figure 2). Tryptic digestion was carried out as described by reference [115]. For LC-MS analysis, samples were reconstructed in 50 µL aqueous 1% formic acid, and 1 µL of the peptide mixture was injected onto an UPLC M-class system (Waters, Eschborn, Germany) online coupled to a Synapt G2-si mass spectrometer equipped with a T-WAVE-IMS device (Waters, Eschborn, Germany). Samples were first on-line pre-concentrated and desalted using a UPLC M-Class Symmetry C18 trap column (100 Å, 180 µm x 20 mm, 5 µm particle size) at a flow rate of 15 µL min^{-1} (0.1% aqueous formic acid). Peptides were eluted onto an ACQUITY UPLC HSS T3 analytical column (100 Å, 75 µm X 200, 1.8 µm particle size) at a flow rate of 350 nL/min using an increasing acetonitrile gradient from 2% to 90% B over 65 min (Buffers: A, 0.1% formic acid in water; B, 100% acetonitrile in 0.1% formic acid). The eluted peptides were transferred into the mass spectrometer operated in V-mode with a resolving power of at least 20,000 full width at half height FWHM. All analyses were performed in a positive ESI mode. A 100 fmol/µL human Glu-Fibrinopeptide B in 0.1% formic acid/acetonitrile (1:1 v/v) was infused at a flow rate of 1 µL min^{-1} through the reference sprayer every 45 s to compensate for mass shifts in MS and MS/MS fragmentation mode.

Data were acquired using data-dependent acquisition (DDA). The acquisition cycle for DDA analysis consisted of a survey scan covering the range of m/z 400 to 1800 Da followed by MS/MS fragmentation of the 10 most intense precursor ions collected at 0.5 s intervals in the range of 50 to 2000 m/z. Dynamic exclusion was applied to minimize multiple fragmentations for the same precursor ions. MS data were collected using MassLynx v4.1 software (Waters, Eschborn, Germany). All proteome data files (including the raw data and mzML file) generated in the proteomics analysis are accessible as additional data (additional files 7–10) via the public database ZENODO (https://zenodo.org/record/3545834).

5.5. Matching Mass Spectrometry Data with Transcriptome Data

The ion spectra of peptides generated by mass spectrometry were interpreted using MASCOT (v2.6.2, Matrix Science, London, UK) and the generated transcriptome assembly as a specific database. The following searching parameters were applied: Fixed precursor ion mass tolerance of 10 ppm for survey peptide, fragment ion mass tolerance of 0.02 Da, estimated calibration error of 0.002 Da, 1

missed cleavage, fixed carbamidomethylation of cysteines and possible oxidation of methionine. After de-grouping the transcripts identified in MASCOT, only transcripts that matched a minimum MASCOT score of 30 and were identified with a minimum of 2 fragments were finally discussed, see Table S2. All further characterization of transcripts that matched fragments, which were discovered in the MS analysis was conducted based on the MS results in the MASCOT table applying the transcriptome data processing as described in 5.3.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/11/12/721/s1, Table S1: Gives an overview of studies focused on endoparasitoid venoms. The known and tested biological function of major components and the applied methods to identify the proteins/peptides are illustrated, Table S2: Summarizes the results from the mass spectrometry that were analyzed in Mascot and showed the identified protein families and corresponding expression values, Table S3: Gives an overview of the differently expressed transcripts and the TPM values of reads that map to the venom gland tissue transcriptome. Additionally, the annotation results blasting against the Toxprot, and NCBI databases are given, Table S4: Shows the detailed annotation for each transcript that maps to the venom gland tissue transcriptome based on a scan against the InterPro database. Additional Data: Additional data is provided in the open access database ZENODO (https://zenodo.org/record/3545834) including following additional data files: Additional data file 1: Alignments of known venom proteins, Additional data file 2: Alignments of novel venom proteins, Additional data file 3: Sequence alignments to train HMMsearch, Additional data file 4: Sequences to train JACHHMMERSearch, Additional data file 5: RNASeq *de novo* assembly, Additional data file 6: Coding regions predicted with Transdecoder, Additional data file 7: MassSpec mgf file, Additional data file 8: MassSpec mzML file, Additional data file 9: MassSpec pkl file, Additional data file 10: MassSpec raw data.

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Publication 2

2 Reprograming of epigenetic mechanisms controlling host insect immunity and development in response to egg-laying by a parasitoid wasp

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Reprograming of epigenetic mechanisms controlling host insect immunity and development in response to egg-laying by a parasitoid wasp

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Parasitoids are insects that use other insects as hosts. They sabotage host cellular and humoral defences to promote the survival of their offspring by injecting viruses and venoms along with their eggs. Many pathogens and parasites disrupt host epigenetic mechanisms to overcome immune system defences, and we hypothesized that parasitoids may use the same strategy. We used the ichneumon wasp *Pimpla turionellae* as a model idiobiont parasitoid to test this hypothesis, with pupae of the greater wax moth *Galleria mellonella* as the host. We found that parasitoid infestation involves the suppression of host immunity-related effector genes and the modulation of host genes involved in developmental hormone signalling. The transcriptional reprogramming of host genes following the injection of parasitoid eggs was associated with changes in host epigenetic mechanisms. The introduction of parasitoids resulted in a transient decrease in host global DNA methylation and the modulation of acetylation ratios for specific histones. Genes encoding regulators of histone acetylation and deacetylation were mostly downregulated in the parasitized pupae, suggesting that parasitoids can suppress host transcription. We also detected a strong parasitoid-specific effect on host microRNAs regulating gene expression at the post-transcriptional level. Our data therefore support the hypothesis that parasitoids may favour the survival of their offspring by interfering with host epigenetic mechanisms to suppress the immune system and disrupt development.

1. Introduction

Parasitic wasps are a polyphyletic group of insects within the order Hymenoptera that have evolved to use other insects as hosts for their offspring. Insects that adopt this so-called parasitoid lifestyle deposit their eggs either on (ectoparasitoid) or in (endoparasitoid) the host [1], and the latter often inject maternal venoms and viruses along with the eggs to suppress host immune responses such as multicellular encapsulation and melanization [2,3]. Idiobiont parasitoids not only suppress host defences but arrest host development when they deposit their eggs, whereas koinobiont parasitoids allow development to continue. Entomopathogenic bacteria and fungi influence the expression of immunity-related and developmental genes in host insects by interfering with epigenetic mechanisms [4,5]. It is plausible that idiobiont parasitoids also interfere with host epigenetic mechanisms to sabotage the immune system and arrest host development, and we therefore set out to test

this hypothesis. As a model idiobiont, we selected the pupal endoparasitoid wasp *Pimpla turionellae* (Hymenoptera, Ichneumonidae). This species parasitizes many lepidopteran insects [6,7] including the greater wax moth *Galleria mellonella* (Lepidoptera, Pyralidae), an apicultural pest and favoured laboratory model. *P. turionellae* delivers virulence factors in the form of maternal venom (injected into the host insect along with the eggs) and the anal secretions of wasp larvae. The venom inhibits the encapsulation response in *G. mellonella* pupae, as well as reducing host cell viability, haemocyte numbers and the mitotic index [8–10]. We selected *G. mellonella* as a host for *P. turionellae* because it has recently emerged as a powerful model to study the impact of entomopathogenic bacteria and fungi on host insect epigenetics [11–13].

To determine the effect of parasitoids on innate immunity, we monitored the expression of 10 immunity-related effector genes by real-time polymerase chain reaction (PCR). We selected the genes encoding cecropin A, cecropin D, X-Tox, galiomycin and gloverin because they encode antimicrobial peptides that defend *G. mellonella* against bacteria [14–17], and the gene encoding c-type lysozyme, which is active against Gram-positive bacteria and fungi [16]. We also selected the gene encoding insect metalloproteinase inhibitor (IMPI), which inhibits virulence-associated microbial metalloproteinases [18,19]. Finally, we selected the genes *PPO1* and *PPO2* (encoding prophenoloxidases 1 and 2) and *PO2* (encoding phenoloxidase 2), which mediate the production of melanin associated with multicellular defence reactions targeting parasitoid eggs [9,20,21]. To determine the effect of parasitoids on host development, we monitored the expression of 10 genes related to the juvenile hormone (JH) and edysteroid hormone signalling pathways [22,23].

To determine whether parasitoids influence epigenetic mechanisms in *G. mellonella*, we evaluated the levels of DNA methylation and histone acetylation/deacetylation, which regulate the initiation of transcription. The conversion of DNA cytidine residues to 5-methylcytidine influences the ability of DNA to interact with proteins, thus providing a mechanism for gene regulation [4,24]. The impact of parasitoids on host DNA methylation was determined by comparing the amount of 5-methylcytidine monophosphate (5 m-dCMP) released from the genomic DNA of infested pupae and controls at different time points following the injection of wasp eggs. We also measured the expression of epigenetic marker genes encoding two components of the DNA methyltransferase complex, three histone acetyltransferases (HATs) and four histone deacetylases (HDACs), whose opposing activities are tightly regulated in *G. mellonella* [25], as well as measuring the levels of histone acetylation directly. Finally, we measured the expression of microRNAs (miRNAs), which regulate target genes at the post-transcriptional level [26]. These short, noncoding RNAs, 18–24 nucleotides in length, inhibit the translation of specific mRNAs by base-pairing with the untranslated regions or occasionally the coding region, and they play a role in insect antiviral responses [27]. We recorded the expression 603 miRNAs using a microarray spotted with more than 2000 insect miRNA probe sequences [28] which have been used to study entomopathogenic bacteria and fungi in *G. mellonella* [11–13].

2. Material and methods

(a) Insect rearing

Stock cultures of *G. mellonella* and *P. turionellae* were maintained in the laboratory as previously described [29]. Last-instar *G. mellonella* larvae, each weighing 200–250 mg, were reared to the pupal stage. We presented the *G. mellonella* pupae (1–2 days after the onset of pupation) to *P. turionellae* adults for egg laying and further development into adults. Adult parasitoids were collected and held in glass jars without a host and fed on 40% (v/v) honey solution at 25 ± 2°C and 60% relative humidity, with a 12 h photoperiod.

(b) Parasitization technique

Male and female *P. turionellae* adults were reared in glass jars containing *G. mellonella* pupae. *P. turionellae* can lay eggs in 1–3 pupae per day, and we used the first parasitized pupa for further investigation. *G. mellonella* pupae were recovered from the jars after 4, 8, 24 and 96 h of parasitization for the isolation of nucleic acids and histone proteins. Non-parasitized pupae were used as negative controls, and pupae injected with Sephadex A25 beads 40–120 µm in diameter (Sigma-Aldrich, Taufkirchen, Germany) were used as positive controls because Sephadex induces encapsulation and melanization in *G. mellonella* larvae [30]. Three pupae representing each sample were pooled for further analysis.

(c) DNA isolation and global DNA methylation analysis

DNA was isolated from parasitized *G. mellonella* pupae and controls as previously described [31]. DNA integrity was determined by 1% agarose gel electrophoresis and by measuring the absorbance ratios 260/280 nm and 260/320 nm by spectrophotometry. High-quality DNA (1250 ng per reaction) was hydrolysed to nucleotides with DNA Degredase (Zymo Research, Orange, CA, USA). All samples were passed through a 0.2 µm nylon Corning Costar Spin-X centrifuge tube filter (Sigma-Aldrich) before injection into the high-performance liquid chromatography (HPLC) column. Isocratic reversed-phase HPLC analysis was carried out using a PerkinElmer (Waltham, MA, USA) P200 instrument fitted with an ACE 5 C18 (125 × 4 mm) column [32]. The mobile phase, 50 mM ammonium orthophosphate (pH 4.1), was filtered (0.2 µm) and degassed thoroughly before use. For each reaction, we injected 1000 ng of DNA in a volume of 20 µl at a flow rate of 0.75 ml min^{-1}. To measure global DNA methylation, we used 5 m-dCMP as a standard (Cayman Chemicals, Ann Arbor, MI, USA; electronic supplementary material, figure S1). The global DNA methylation level was calculated as the amount (ng) of 5 m-dCMP in 1 µg of genomic DNA, detected at 280 nm.

(d) RNA isolation and real-time polymerase chain reaction

Total RNA was isolated using TRI reagent (Sigma-Aldrich) and 1 µg of total RNA was reverse transcribed using the QuantiTect kit (Qiagen, Hilden, Germany). The cDNA was amplified by real-time PCR using a CFX96 instrument (Bio-Rad Laboratories, Hercules, CA, USA) and the Sensi Fast SYBRgreen kit (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions. The target genes were amplified using the primers listed in electronic supplementary material, table S1. Each reaction was heated to 95°C for 15 min, followed by 39 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 10 s and extension at 70°C for 30 s, then a final extension step at 65°C for 5 s. The 18*S* rRNA housekeeping gene was used for normalization [25], and the fold-change in gene expression was calculated using the $2^{\Delta\Delta CT}$ method [33]. We estimated a primer efficiency of approximately 1 for all genes, including the 18*S* rRNA gene, given the similar curves in the logarithmic PCR amplification plots recorded by CFX Manager (Bio-Rad). This method to determine primer efficiency avoids the need to calculate separate efficiencies for a large number of genes in separate experiments and is estimated during analysis [34].

(e) Histone protein isolation and acetylation analysis

Histone proteins were isolated using the Total Histone Extraction kit (Epigentek, Farmingdale, NY, USA). The protein yield was measured using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as the standard. The percentage acetylation of histones H3 and H4 (H3K9, H3K14, H4K5 and H4K12) was measured using the Epigentek colorimetric detection kit according to the manufacturer's instructions. In each experiment, 200 ng of extracted histone protein was used and the absorbance was measured at 450 nm using a Synergy H4 Hybrid microplate reader (Biotek, Winooski, VT, USA).

(f) Microarray analysis of miRNAs

The expression profiles of conserved miRNAs in parasitized *G. mellonella* pupae and controls were monitored by microarray analysis. This is a high-throughput and relatively inexpensive technique for the analysis of miRNA expression although the low signal-to-noise ratio can generate false-positive and false-negative results. Microarrays are often used to analyse miRNA expression in insects that lack annotated genome sequences [11,12]. An oligonucleotide microarray containing 2621 unique mature arthropod miRNA sequences was designed using miRBase v. 21. The miRNA sequences were obtained from the fruit fly *Drosophila melanogaster* (1735 miRNAs), the pea aphid *Acyrthosiphon pisum* (103 miRNAs), the silkworm *Bombyx mori* (560 miRNAs), the red flour beetle *Tribolium castaneum* (422 miRNAs) and the western honeybee *Apis mellifera* (259 miRNAs), with some redundancy across species [28]. To determine changes in miRNA expression profiles during parasitization, total RNA was isolated from parasitized *G. mellonella* pupae and controls 4 h after egg deposition as described above. The RNA samples were pooled from three parasitized and three non-parasitized pupae. Microarray hybridization, detection and analysis were carried out by LC Sciences (Houston, TX, USA) as previously described [28].

(g) Prediction of miRNA targets

Target mRNAs were identification *in silico* by complementarity to miRNA probes using the sequence alignment editor BioEdit v. 7.2.5 as previously described [11]. Briefly, we identified open reading frames (ORFs) in all contigs in the sequenced *G. mellonella* transcriptome using the 'Find next ORF' option in BioEdit. Nucleotide sequences at the 3′ end of individual contigs but outside confirmed ORFs were considered as potential 3′ UTRs, and were screened for complementarity (seed sequence complementarity) with the expressed miRNA sequences identified by microarray analysis. Expressed miRNAs were defined as those for which the average microarray signal was above background in at least two different pools of the same treatment group. The Gene Ontology categories of the identified contigs were listed by consulting the UniProt database and a previous report [22]. The biological processes targeted by miRNAs in the parasitized and non-parasitized pupae were summarized using Cytoscape v. 3.2.1. The identified miRNA targets were validated using the RNA hybrid tool provided by the Bielefeld Bioinformatics Server v. 32 [35].

(h) Statistics

All experiments except the microarray analysis were carried out at least three times (biological replicates) with statistical analysis using SPSS v. 18.0 (SPSS, Chicago, IL, USA). Each parameter was analysed using an independent samples test with a significance threshold of $p < 0.05$. However, for the analysis of miRNAs, log2 values were assessed with significance threshold of $p < 0.01$.

3. Results

(a) Parasitoid-dependent transcriptional reprogramming of immunity-related genes and hormonal pathway genes in *Galleria mellonella* pupae

We compared the expression levels of 10 immunity-related genes in parasitized *G. mellonella* pupae and controls by real-time PCR (figure 1). We found that all 10 genes were strongly downregulated at most time points after egg deposition (4, 8, 24 and 96 h) but that the genes naturally fell into two groups based on their expression profiles. The first group comprised those genes showing transient upregulation at 24 h (galiomycin, cecropin A and IMPI, with IMPI showing a particularly striking sixfold induction) and those following the same trend in which the suppression was transiently lifted, but not quite enough to reach basal levels (X-Tox and lysozyme). The second group comprised those genes that were downregulated throughout the period of parasitization (gloverin, cecropin D, PPO1, PPO2 and PO2).

We also compared the expression levels of 10 hormone-related genes involved in development. Eight of the genes encoded components of the JH pathway, namely JH-inducible (JH-Ind), JH-binding proteins 1–4 (JH-BP1, JH-BP2, JH-BP3 and JH-BP4), JH epoxide hydrolases 1 and 2 (JH-EHyd1 and JH-EHyd2) and JH esterase (JH-Est). The last two genes encoded ecdysteroid hormone 22-kinase (ECD-22Ki) and ecdysteroid-regulated protein (ECD-RP). As above, we compared parasitized *G. mellonella* pupae and controls at four time points (4, 8, 24 and 96 h) after egg deposition (figure 2). The expression profiles of the hormone-related genes were more complex than the immunity-related genes, with more diverse responses. JH-Ind, JH-EHyd2 and ECD-RP formed a group that showed general downregulation or minimal change but a spike of induction after 8 h, whereas JH-BP1, JH-EHyd1 and JH-Est followed the profile of the first set of immunity-related genes, with general downregulation but a spike of induction (or partial recovery from repression) after 24 h. JH-BP2 was strongly induced after egg deposition followed by a return to basal expression after 96 h. JH-BP3 was similar to JH-BP2, but the return to basal levels occurred after 24 h and was followed by strong repression. ECD-22Ki also fitted this profile, but the switchover between induction and repression occurred somewhere between the 8 h and 24 h time points. JH-BP4 showed a unique profile with strong repression at all time points and no transient recovery at 24 h.

(b) Parasitoid-dependent effects on DNA methylation in *Galleria mellonella* pupae

The global DNA methylation level of *G. mellonella* pupae was determined 4, 8 and 24 h after parasitization by measuring the quantity of 5 m-dCMP released from 1 µg of genomic DNA (figure 3). The retention time of 5 mdCMP is 14.623 min (electronic supplementary material, figure S2). The mean level of global DNA methylation in the control pupae (30 ng μg^{-1}) declined after egg deposition, reaching approximately 35% below normal levels 4 h after parasitization and then recovering to approximately 33% below normal levels after 8 h and approximately 5% below normal levels after 24 h (figure 3). In a parallel set of experiments, we measured the expression of the genes encoding DNA cytosine 5-methyltransferase and DNA methyltransferase 1-associated protein by real-time

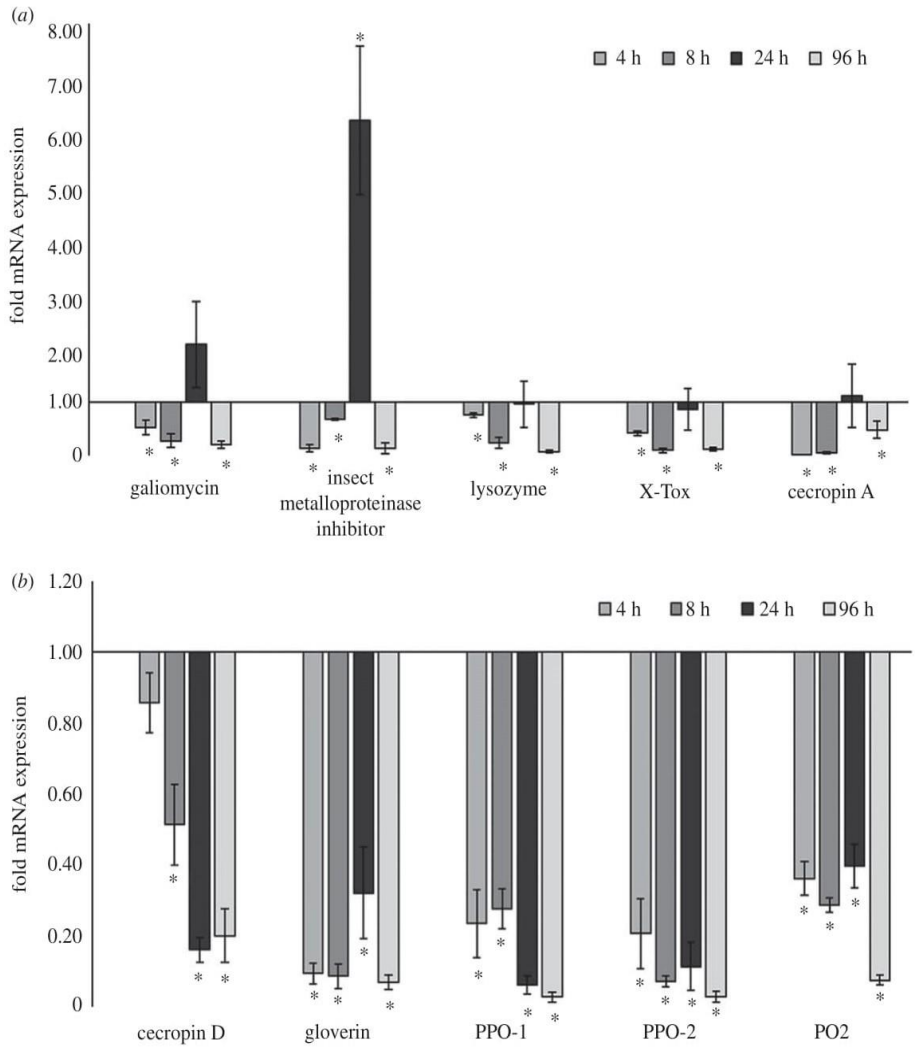

Figure 1. Fold-changes in mRNA expression for 10 immunity-related genes in *G. mellonella* pupae at four time points (4, 8, 24 and 96 h) after parasitization by *P. turionellae*. The 10 genes encode the peptides/proteins galiomycin, insect metalloproteinase inhibitor (IMPI), lysozyme, X-Tox, cecropin A, cecropin D, gloverin, prophenoloxidase 1 (PPO-1) prophenoloxidase 2 (PPO-2) and phenol oxidase 2 (PO2). Fold-changes are shown relative to the positive control. The housekeeping gene 18S rRNA was used for normalization. Fold-changes were calculated using the $2^{-\Delta\Delta CT}$ method. Data are means ± s.e. ($n = 3$, $^*p < 0.05$).

PCR. Both genes were significantly downregulated by parasitization, with the severity of repression generally increasing over time but, as observed for the first class of immunity-related genes, a transient recovery at 24 h (figure 4). Parasitization by *P. turionellae* therefore appeared to cause the suppression of two genes associated with DNA methylation but a transient decline in the levels of 5 mdCMP determined by direct measurement, with recovery at 24 h coincident with the gene expression profiles.

(c) Parasitoid-dependent effects on histone acetylation in *Galleria mellonella* pupae

Changes in histone acetylation (H3K9, H3K14, H4K5 and H4K12) in parasitized *G. mellonella* pupae were monitored by enzyme-linked immunoassay. Parasitization caused a reduction in the proportion of acetylated H3K14 and H4K12 at all time points, which was statistically significant at 8 h for H3K14 and at 24 h for H4K12 (figure 5). There was no statistically significant change in the acetylation of H3K9 after parasitization. However, there was an increase in the acetylation of H4K5 at all time points after parasitization, which was statistically significant at 24 h. We also measured the expression of three HAT and four HDAC genes by real-time PCR (figure 4). Six of the genes were downregulated in the parasitized *G. mellonella* pupae with a general trend showing greater repression over time. Interestingly, the gene encoding HDAC 8 isoform 2 showed an exceptional profile, with strong induction after 4 and 8 h, falling off but still showing weak induction after 24 h, and finally strong repression after 96 h, in line with the other six genes (figure 4).

(d) Parasitoid-dependent effects on miRNA expression in *Galleria mellonella* pupae

We investigated the expression profiles of 603 candidate miRNAs and selected 82 for further analysis based on their moderate or strong modulation. We found that 58 of these miRNAs were upregulated and 24 were downregulated in parasitized *G. mellonella* pupae compared to controls ($p < 0.01$), with many of the upregulated miRNAs showing moderate (<two-fold) induction but several of the downregulated sequences

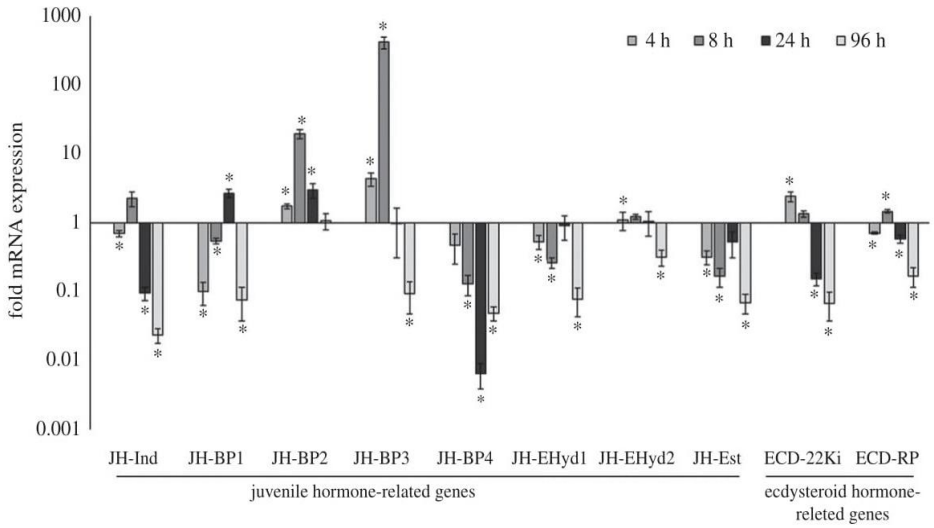

Figure 2. Fold-changes in mRNA expression for 10 genes related to the juvenile hormone (JH) and ecdysteroid (ECD) pathways in *G. mellonella* pupae at four time points (4, 8, 24 and 96 h) after parasitization by *P. turionellae*. The 10 genes encode the JH-inducible protein (JH-Ind), JH-binding proteins 1–4 (JH-BPs 1, 2, 3 and 4), JH epoxide hydrolases 1 and 2 (JH-EHyd1, JH-EHyd2) and JH esterase (JH-Est), ECD-22-kinase (ECD-22Ki) and ECD-regulated protein (ECD-RP). Fold-changes are shown relative to the positive control. The housekeeping gene 18S rRNA was used for normalization. Fold-changes were calculated using the $2^{-\Delta\Delta CT}$ method. Data are means ± s.e. ($n = 3$, $^*p < 0.05$).

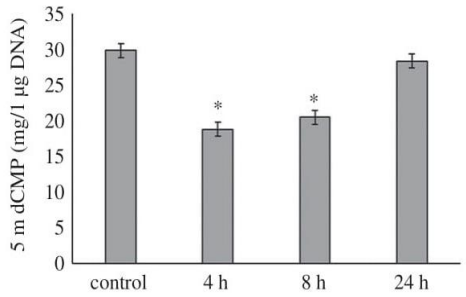

Figure 3. Analysis of DNA methylation in parasitized and non-parasitized *G. mellonella*. Absolute global DNA methylation levels (shown as ng of 5 m-dCMP released from 1 µg genomic DNA, with 5 m dCMP as a standard) in *G. mellonella* pupae at three time points (4, 8 and 24 h) after parasitization by *P. turionellae*. Levels are shown relative to negative control. Data are means ± s.e. ($n = 3$, $^*p < 0.05$).

showing much stronger effects. The most extreme was bmo-miR-3365, which was downregulated 14-fold in the parasitized pupae (electronic supplementary material, figure S3 and S4). The mRNA targets of selected miRNAs were tested against the *G. mellonella* transcriptome [22] as previously described [28]. We found that these miRNAs targeted genes with important roles in host–parasitoid interactions, such as defence responses and host development (electronic supplementary material, figure S5).

4. Discussion

Parasitoids have evolved a successful reproductive strategy in which they use the larvae or pupae of host insects to provide a source of nutrition for their offspring [1]. Koinobiont parasitoids feed on the host insect as it develops, but idiobiont parasitoids arrest host insect development and the underlying mechanisms are not yet understood. However, the host is not defenceless against parasitoid eggs and can attack them with various defence responses, including melanization and multicellular encapsulation [36–38]. The coevolution of parasitoids and their hosts has led to counterstrategies in parasitoid wasps to circumvent such host defence reactions. For example, *P. turionellae* not only arrests the development of *G. mellonella* pupae but can also suppress the host immune system [8–10]. We hypothesized that *P. turionellae* achieves this by subverting the epigenetic mechanisms of the host in order to effect the transcriptional reprogramming of immunity-related and developmental genes, thus sabotaging both host defence responses and normal development. Accordingly, we monitored the expression of 10 immunity-related genes and 10 genes involved in the hormonal control of development in *G. mellonella* pupae parasitized by *P. turionellae*.

The expression profiles of the 10 immunity-related genes confirmed their profound repression 4 and 8 h after egg deposition, but five of the genes mounted a partial or complete transient recovery at the 24 h time point before succumbing to repression thereafter. Three of these genes (encoding cecropin A, X-Tox and lysozyme) recovered to the point of restoring near normal basal expression (equivalent to the expression level in control larvae), whereas the genes encoding galliomycin and IMPI showed a strong spike of induction at 24 h, with a mean sixfold upregulation in the case of IMPI. Interestingly, IMPI is part of an immunity-related signalling cascade that does not require microbial pathogen-associated molecular patterns (PAMPs) to elicit the synthesis of antimicrobial peptides, but senses the presence of danger-associated molecular patterns (DAMPs) [39,40]. Microbial metalloproteinases can digest targets such as collagen type IV in insects [41], generating peptide fragments that operate as DAMPs [42], which in turn activate immune responses including the synthesis of IMPI, galiomycin and cecropin A [43]. IMPI is the only known defence peptide that can inhibit virulence-associated metalloproteinases [44]. Similar to parasitic fungi that produce metalloproteinases as virulence factors, leading to the activation of innate immune responses in *G. mellonella* [43], we postulate that *P. turionellae* injects metalloproteinases

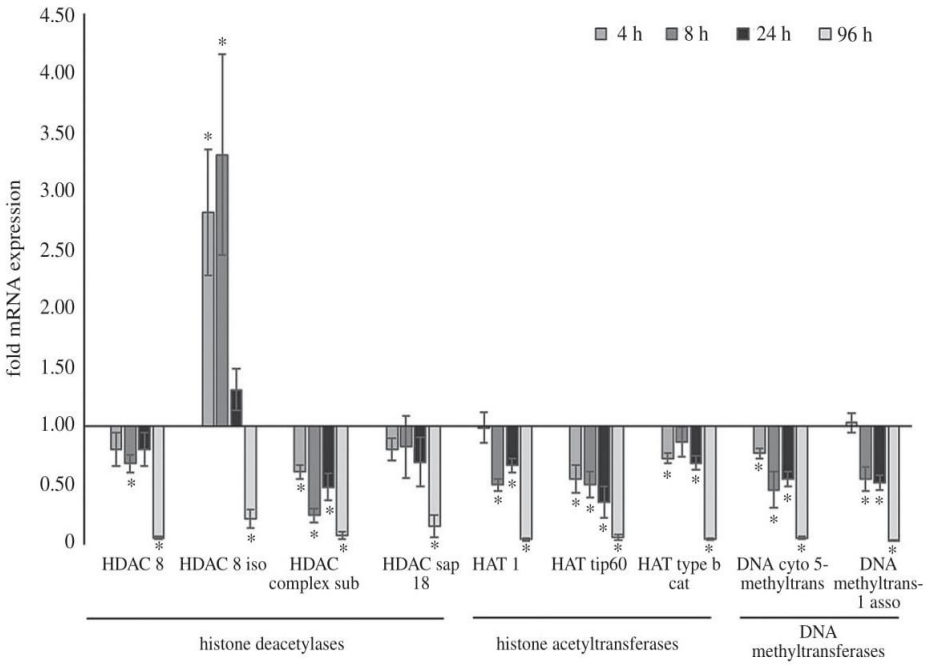

Figure 4. Fold-changes in mRNA expression for nine epigenetic regulation genes in *G. mellonella* pupae at four time points (4, 8, 24 and 96 h) after parasitization by *P. turionellae*. The nine genes encode the proteins HDAC 8, HDAC 8 isoform 2, HDAC complex subunit, HDAC complex subunit sap18, HAT 1, HAT tip60, HAT type b catalytic, DNA cytosine 5-methyltransferase and DNA methyltransferase-1 associated protein. Fold-changes are shown relative to the positive control. The housekeeping gene 18S rRNA was used for normalization. Fold-changes were calculated using the $2^{-\Delta\Delta CT}$ method. Data are means ± s.e. ($n = 3$, $^*p < 0.05$).

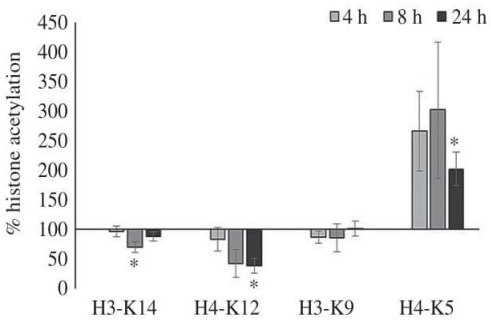

Figure 5. Changes in the percentage of histone acetylation (H3-K9, H3-K14, H4-K5 and H4-K12) in *G. mellonella* pupae at three time points (4, 8 and 24 h) after parasitization by *P. turionellae*. Levels were measured relative to negative control pupae without parasitoids, which were set to 100%. Data are means ± s.e. ($n = 3$, $^*p < 0.05$).

along with its eggs into the infected host insect, which could elicit a strong immune response if the parasitoid does not block the corresponding transcriptional reprogramming. Indeed, the venom glands of *P. turionellae* and another endoparasitoid (*Toxoneuron nigriceps*) are known to synthesize a metalloproteinase [45,46]. The transient nature of the recovery we observed for IMPI, galliomycin, cecropin A, X-Tox and lysozyme, followed by strong suppression at the 96 h time point, suggests that this defence response is actively targeted and overcome by the parasitoid eggs. Furthermore, the silencing of genes encoding PPOs and PO in the parasitized pupae indicates that the parasitoids have also evolved a counterstrategy to protect the eggs from melanization.

We also found that the deposition of eggs by *P. turionellae* into *G. mellonella* pupae caused the profound transcriptional reprogramming of host genes representing the JH and ecdysteroid hormone pathways, which play important roles in lepidopteran growth and development. In the corpora allata, secreted juvenile hormones are carried to their target tissues by JH-BPs 1–4, which protect the hormones from nonspecific esterases and JH epoxide hydrolase activity [47–50]. The upregulation of JH-BP2 at all time points (up to 20-fold) and JH-BP3 until the 24 h time point (up to 420-fold) indicated that JH signalling increases after parasitization, as already reported in other host–parasitoid systems [51,52]. The transformation of lepidopteran pupae into adults requires high levels of ecdysteroid hormones in the absence of JH [53,54]. Our data show that parasitization triggers the profound downregulation of enzymes that degrade JH (JH-Ehyd1 and JH-Est at all time points, and JH-Ehyd1 at later time points) as well as ECD-RP at all time points. Furthermore, parasitization also triggers the transient upregulation of ECD-22Ki (until the 24 h time point), which phosphorylates and thereby inactivates ecdysteroid hormones. These combined effects would help to explain the developmental arrest of parasitized *G. mellonella* pupae and agree with earlier reports showing that wasp venom can inhibit the degradation of JH, while suppressing the accumulation of ecdysteroid hormones [52]. JH can suppress the immune response in *D. melanogaster* by blocking the ability of 20hydroxy-ecdysone to stimulate the production of antimicrobial peptides [55,56]. Together, these data indicate that parasitoids interfere with hormonal regulation to arrest host development and to compromise innate immunity.

The overall goal of our study was to determine whether the parasitoid-dependent transcriptional reprogramming of immunity-related and developmental genes in the host involves epigenetic mechanisms. We therefore compared the levels of DNA methylation and histone acetylation in the parasitized pupae and controls, as well as measuring the level of selected miRNAs. The global DNA methylation

level in control pupae was 30 ng 5 m-dCMP per µg genomic DNA (3% total methylation) but the deposition of eggs by *P. turionellae* caused a transient decline, with a minimum value of approximately 35% below normal after 4 h, followed by a recovery to approximately 33% below normal after 8 h and approximately 5% below normal after 24 h. This matches the transient recovery of the five immunity-related genes discussed above as well as two genes directly related to DNA methylation (encoding DNA cytosine 5-methyltransferase and DNA methyltransferase-1 associated protein, respectively). The methylation-related genes also showed a moderate recovery or at least no significant increase in downregulation after 24 h, before the recovery was quashed and stronger suppression became evident after 96 h. The loss of DNA methylation was also observed in diamondback moth (*Plutella xylostella*) larvae following the deposition of eggs by the parasitoid wasp *Cotesia plutellae*, commensurate with the suppression of genes encoding DNA methyltransferases 1 and 2 [57]. Therefore, it appears that parasitoids may trigger global DNA demethylation in the host in order to suppress immunity-related and developmental genes that require the presence of methylated sites for full expression.

The impact of *P. turionellae* on the epigenetic mechanisms of *G. mellonella* was also evident at the level of histone acetylation. Six of seven genes encoding HATs and HDACs were silenced in pupae following the deposition of parasitoid eggs (figure 4). The exception was the gene encoding HDAC 8 isoform 2, which was initially strongly induced before falling back towards basal levels after 24 h and succumbing to strong repression (compared to control pupae) after 96 h. The transcriptional activation of HDACs leads to histone deacetylation and the suppression of gene expression. The transient transcriptional activation of HDAC 8 isoform 2 may therefore trigger the downregulation of innate immunity-related genes via the deacetylation of H3K14, H3K9 and H4K12 [5]. The sabotage of histone acetylation by the parasitoid offers another plausible mechanism for the suppression of immunity-related and developmental genes in the host insect because this epigenetic mechanism plays a key role in the regulation of transcriptional reprogramming during metamorphosis and infection in *G. mellonella* [27].

Finally, we identified 24 miRNAs that were downregulated in parasitized *G. mellonella* pupae, some strongly, and 58 that were moderately upregulated (electronic supplementary material, figure S4). The expression of miRNA genes in insects is known to change during development [58,59] and is also modulated by immune challenges [28] or parasitization [60]. We identified seven *G. mellonella* miRNAs that were strongly suppressed by parasitization, ranging from threefold to 13-fold downregulation (bmo-miR-989a, tca-miR-989-3p, ame-miR-989, dme-miR-989-3p, bmo-miR-989b, dps-miR-989, and mse-miR-989). Interestingly, all of them appear to play a role in the regulation of cell migration, which is a key developmental process as well as an important part of the innate immune response [61]. For example, miR-989 is normally expressed in somatic cells of the *D. melanogaster* ovary and its absence delays their migration and arrests oogenesis [62]. Additionally, border cell migration in *D. melanogaster* involves several conserved signalling pathways such as the JAK/STAT pathway [62], which coordinates cytokine-dependent immune responses and regulates homeostasis via multiple mechanisms [63]. The parasitization of *D. melanogaster* has been shown to suppress the secretion of JAK/STAT pathway ligands by haemocytes [63]. These data suggest that *P. turionellae* venom may suppress the expression of miRNAs to inhibit cell migration as a component of development and immunity. We identified a larger number of *G. mellonella* miRNAs that were slightly upregulated by parasitization, including mja-miR-6492, dme-miR-263a-5p, ame-miR-3796, tca-miR-2c-5p and ame-miR-3756, which are known to target immunity-related and developmental genes (electronic supplementary material, figure S4). Interestingly, the parasitoid wasp *Cotesia vestalis* is known to produce miRNAs in its venom and teratocytes, which are introduced in the host during oviposition and in order to modulate host genes [64]. Accordingly, it is unclear whether the upregulated miRNAs we detected in the parasitized *G. mellonella* pupae are derived from the host or the parasitoid. Among the miRNA induced most strongly in the parasitized pupae was api-miR-124, which controls neural plasticity and transient memory in honeybees [65]. Similarly, miR-124 controls gene expression in the sensory nervous system of the nematode worm *Caenorhabditis elegans* [66]. Therefore, *P. turionellae* may use miR-124 to modulate neural gene expression in the host.

In summary, we found that the parasitoid wasp *P. turionellae* interferes with the epigenetic mechanisms of its host, which may help to sabotage the host immune system and arrest its development. All three major epigenetic mechanisms are affected, resulting in the global depletion of DNA methylation, changes in the levels of specific acetylated histones and the modulation of miRNA expression, in line with the observed transcriptional reprogramming of immunity-related and developmental genes. Further work should focus on the components of the venom injected into the host during oviposition, which may reveal the identity of venom compounds that directly interfere with the epigenetic mechanisms in the host.

Data accessibility. The raw data is available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.jwstqjq6b [67].

Authors' contributions. R.Ö. carried out the experiments, participated in the design of the study and carried out statistical analysis; K.M. participated in the design of the study, analysed the data and helped draft the manuscript; F.U. participated in the design of the study and provided the laboratory stock colonies of *P. turionellae*; A.V. conceived and designed the study, coordinated the work and wrote the manuscript with R.Ö. All authors gave final approval for publication and agree to be held accountable for the work described therein.

Competing interests. The authors declare no conflict of interest.

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Weitere Publikationen

1. Özyılmaz, D., **Özbek, R.**, Altuntaş, H., Uçkan, F. **(2019)**: Indole-3-Acetic Acid induced oxidative stress in model host *Galleria mellonella* L. (Lepidoptera: Pyralidae) and its endoparasitoid *Pimpla turionellae* (L.) (Hymenoptera: Ichneumonidae). Invertebrate Survival Journal. 16:1, 184-188. https://doi.org/10.25431/1824-307X/isj.v0i0.184-188.
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Beitrage zu Konferenzen

1. **Özbek, R.*,** Wielsch, N., Vogel, H., Billion, A., Vilcinskas, A., Foerster, F., Reumont, B.M. Insights into the venom composition and its biological functions of the endoparasitoid wasp *Pimpla turionellae.* 112th Annual Meeting of the German Zoological Society (DZG). 10-13 September **2019**, Jena, Deutschland. (Vortrag)
2. **Özbek, R.**, Uçkan, F., Sazcı, A. Determination of CpG Dinucleotids and its Methylation Pattern of Different stage of *Galleria mellonella* by Using Methylation-Sensitive Restriction Endonucleases, ECE 2018, XI. European Congress of Entomology, 2-6 Juli **2018,** Neapel, Italien, pg: 313. (Poster)
3. Çakır, E., **Özbek, R.**, Uçkan, F., Ergin, E. Zebularine Induced Alterations in Antioxidant Enzyme Activity and Lipid Peroxidation in the Model Host *Galleria mellonella*, ECE 2018, XI. European Congress of Entomology, 2-6 Juli **2018**, Neapel, Italien, pg: 309. (Poster)
4. Özyılmaz, D., **Özbek R.***, Uçkan, F., Altuntaş, H. Indole-3-Aacetic Acid Induced Alterations in Antioxidant Enzyme Activity and Lipid Peroxidation of *Pimpla turionellae* (L.) (Hymenoptera: Ichneumonidae). International Conference on Biochemistry and Molecular Biology, 3-5 April **2017**, München, Deutschland, 52. (Vortrag)

5. **Özbek, R.***, Uçkan, F., Özlem Aksoy. Effects of Gibberellic Acid on Genomic Stability and Immune Defense of *Galleria mellonella* L. (Lepidoptera: Pyralidae), III. International Zoology and Technology Congress. 12-15 July 2017, Afyon, Türkei, 39. (Vortrag)
6. **Özbek, R.***, F. Uçkan, Ö. Aksoy, Gibberellic Acid Induced Genomic Variations in *Galleria mellonella* L. (lepidoptera: Pyralidae), International Conference on Biochemistry and Molecular Biology, Venedig, Italien 27 April **2016**, 13. (Poster)
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Erklärung zur Dissertation

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Gießen, den 24. November 2020

(Rabia Özbek)