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Author for correspondence:
Andreas Vilcinskas
e-mail: andreas.vilcinskas@agrar.uni-giessen.de

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

Rabia Özbek[1], Krishnendu Mukherjee[1], Fevzi Uçkan[2] and Andreas Vilcinskas[1,3,4]

[1]Branch of Bioresources, Fraunhofer Institute for Molecular Biology and Applied Ecology, Ohlebergsweg 12, 35392 Giessen, Germany
[2]Department of Biology, Faculty of Science and Literature, Kocaeli University, 41380 Kocaeli, Turkey
[3]Institute for Insect Biotechnology, Justus Liebig University Giessen, Heinrich Buff Ring 26-32, 35392 Giessen, Germany
[4]LOEWE Centre for Translational Biodiversity Genomics (LOEWE-TBG), Senckenberganlage 25, 60325 Frankfurt, Germany

AV, 0000-0001-8276-4968

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

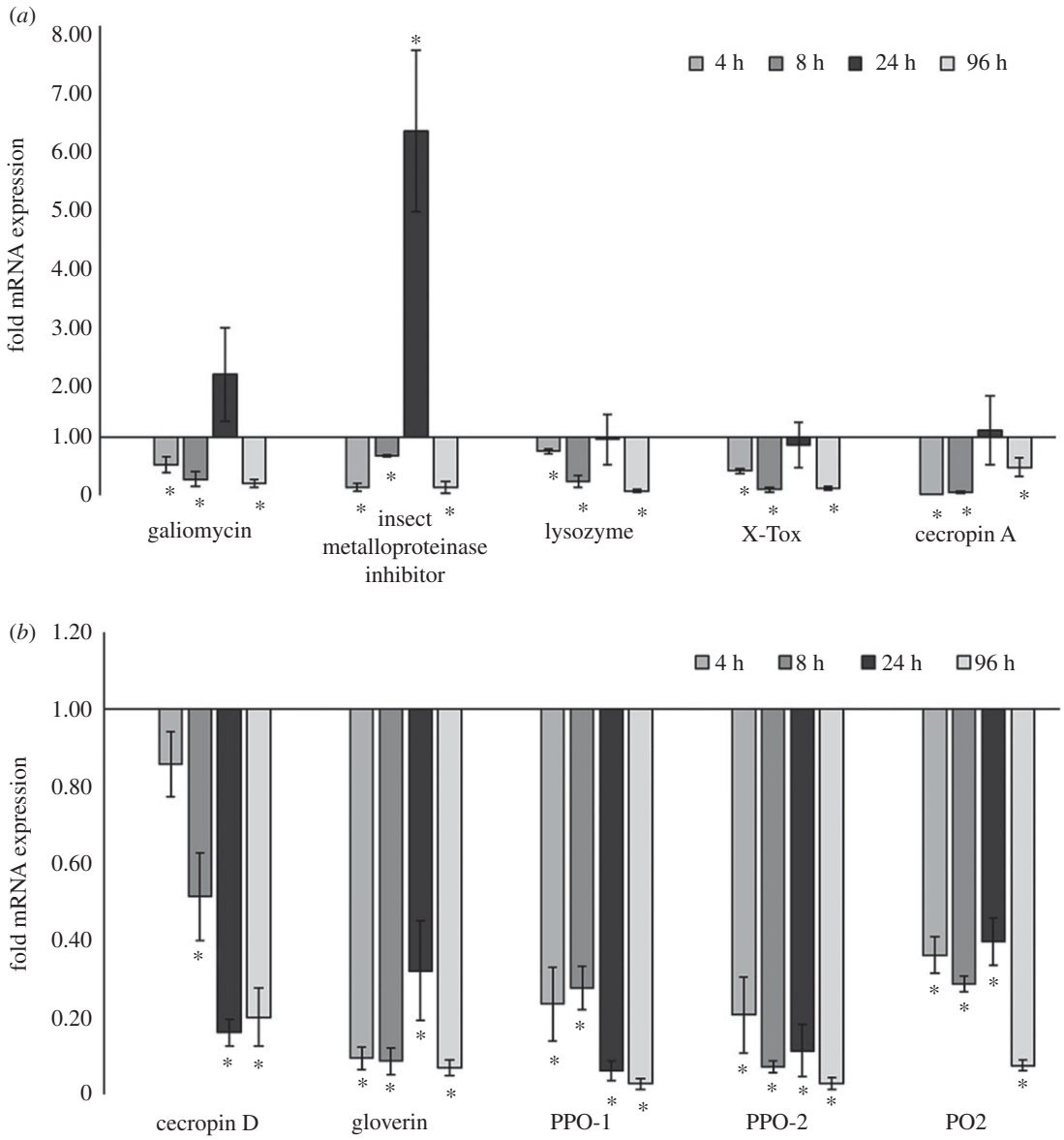

Figure 1. Fold-changes in mRNA expression for 10 immunity-related genes in *G. mellonella* pupae at four time points (4, 8, 24 and 96 h) after parasitization by *P. turionellae*. The 10 genes encode the peptides/proteins galiomycin, insect metalloproteinase inhibitor (IMPI), lysozyme, X-Tox, cecropin 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

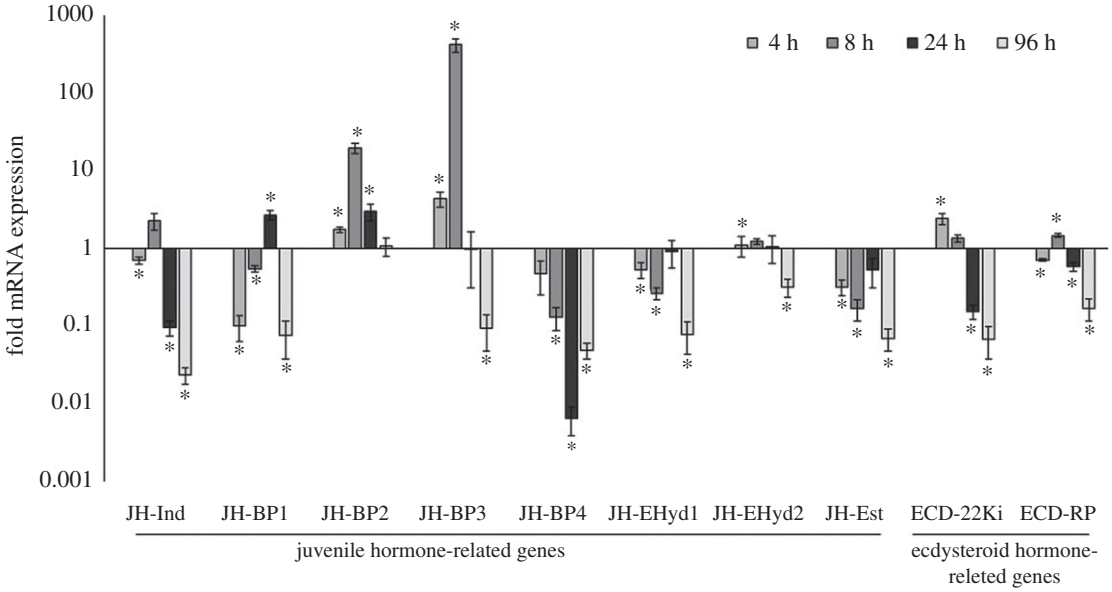

Figure 2. Fold-changes in mRNA expression for 10 genes related to the juvenile hormone (JH) and ecdysteroid (ECD) pathways in *G. mellonella* pupae at four time points (4, 8, 24 and 96 h) after parasitization by *P. turionellae*. The 10 genes encode the JH-inducible protein (JH-Ind), JH-binding proteins 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. ($n = 3$, $^*p < 0.05$).

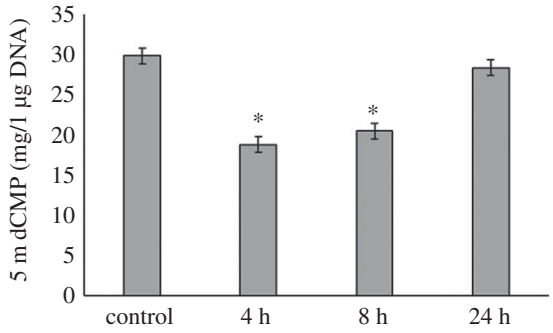

Figure 3. Analysis of DNA methylation in parasitized and non-parasitized *G. mellonella*. Absolute global DNA methylation levels (shown as ng of 5 m-dCMP released from 1 µg genomic DNA, with 5 m dCMP as a standard) in *G. mellonella* pupae at three time points (4, 8 and 24 h) after parasitization by *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

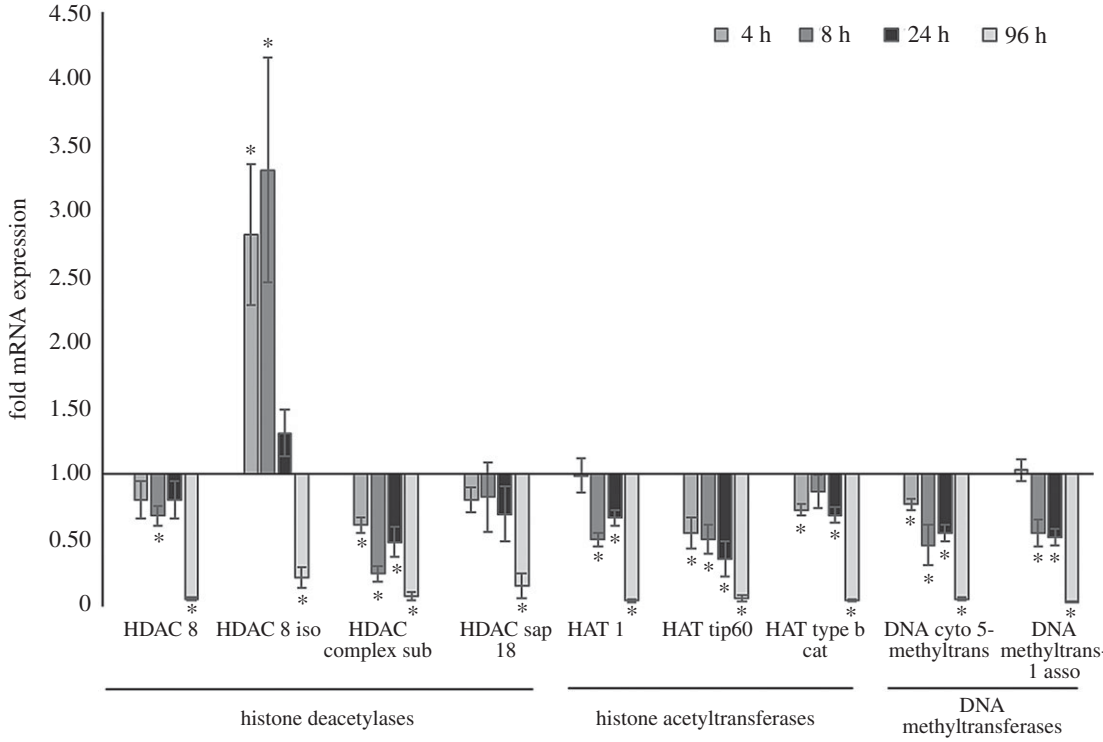

Figure 4. Fold-changes in mRNA expression for nine epigenetic regulation genes in *G. mellonella* pupae at four time points (4, 8, 24 and 96 h) after parasitization by *P. turionellae*. The nine genes encode the proteins HDAC 8, HDAC 8 isoform 2, HDAC complex subunit, HDAC complex subunit sap18, HAT 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. ($n = 3$, $^*p < 0.05$).

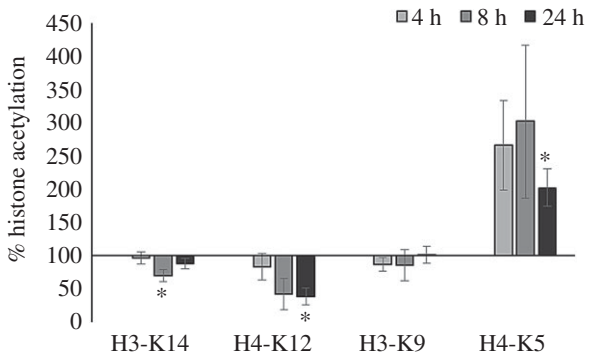

Figure 5. Changes in the percentage of histone acetylation (H3-K9, H3-K14, H4-K5 and H4-K12) in *G. mellonella* pupae at three time points (4, 8 and 24 h) after parasitization by *P. turionellae*. Levels were measured relative to negative control pupae without parasitoids, which were set to 100%. Data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

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