Received: 1 October 2019 | Revised: 27 December 2019 | Accepted: 8 January 2020

DOI: 10.1002/arch.21657

RESEARCH ARTICLE

Archives of INSECT BIOCHEMISTRY AND PHYSIOLOGY WILEY

Seasonal phenotype-specific expression of microRNAs during metamorphosis in the European map butterfly *Araschnia levana*

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Funding information
Hessen State Ministry of Higher Education, Research, and the Arts (HMWK)

Abstract

The European map butterfly (*Araschnia levana*) is a well-known example of seasonal polyphenism because the spring and summer imagoes exhibit distinct morphological phenotypes. The day length and temperature during larval and prepupal development determine whether spring or summer imagoes emerge after metamorphosis. Inspired by the fundamentally different transcriptomic profiles in prepupae developing from larvae exposed to long days or short days, we postulate that posttranscriptional epigenetic regulators such as microRNAs (miRNAs) may contribute to the epigenetic control of seasonal polyphenism in *A. levana*. To test this hypothesis, we used microarrays containing over 2,000 insect miRNAs to identify candidate regulators that are differentially expressed in last-instar larvae or pupae developing under long-day or short-day conditions. We used our transcriptomic database to identify potential 3′-untranslated regions of messenger RNAs to predict miRNA targets by considering both base pair complementarity and minimum free energy hybridization. This approach resulted in the identification of multiple targets of miRNAs that were differentially regulated in polyphenic morphs of *A. levana*

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including a candidate (miR-2856-3p) regulating the previously identified diapause bioclock protein gene. In conclusion, the expression profiling of miRNAs provided insights into their possible involvement in seasonal polyphenism of *A. levana* and offer an important resource for further studies.

KEYWORDS

Araschnia levana, diapause, epigenetics, metamorphosis, microRNAs, polyphenisms

1 | INTRODUCTION

Seasonal environmental changes can induce the development of distinct phenotypes in some insect species, including the European map butterfly *Araschnia levana* (Linnaeus, 1,758; Lepidoptera: Nymphalidae), which is regarded as a classic example of seasonal polyphenism (Shapiro, 1976; Simpson, Sword, & Lo, 2011). The day length and temperature during larval and prepupal development determine whether or not the pupae commit to diapause and overwintering, and thus whether the morphologically distinct spring or summer imagoes emerge (Reinhardt, 1984). It remains enigmatic how such environmental stimuli are converted via the same genome into morphologically distinct phenotypes. However, we recently found that day length during larval development leads to phenotype-specific transcriptional reprogramming in the *A. levana* prepupae (Vilcinskas & Vogel, 2016). We identified numerous genes that are differentially expressed during metamorphosis, reflecting adaptations favoring either accelerated metamorphosis and egg production or diapause and overwintering. One of the genes among those differentially expressed in prepupae according to the day length during larval development encodes an *A. levana* ortholog of the silkworm (*Bombyx mori*) diapause bioclock protein which is known to be responsible for measuring the duration of diapause (Isobe et al., 2006). Moreover, a recent investigation has shown that photoperiodic modulation of phenotypes involve changes in life-history traits of *A. levana*, featuring stronger innate immune responses upon challenge with the bacterial entomopathogen *Pseudomonas entomophila* in short-day larvae (resulting in spring imagoes) in comparison to long-day larvae (resulting in spring imagoes; Baudach, Lee, Vogel, & Vilcinskas, 2018).

Modern concepts in evolutionary biology suggest that the translation of environmental stimuli such as parasites or climate into phenotypic alterations, including polyphenism, can be mediated by epigenetic mechanisms (Burggreen, 2017; Verlinden, 2017; Vilcinskas, 2017), among which small noncoding RNAs may contribute to the transcriptional reprogramming during insect diapause (Reynolds, 2017). These so-called microRNAs (miRNAs) operate at the posttranscriptional level negatively regulating the expression of target messenger RNAs (mRNAs; Asgari, 2013; Hussain & Asgari, 2014). They have been recognized as key regulators in insect metamorphosis (Belles, 2017; Ylla, Piulachs, & Belles, 2017).

Here, we investigated whether miRNAs contribute to the regulation of transcriptional reprogramming associated with seasonal polyphenism in *A. levana* using microarrays containing more than 2,000 conserved insect miRNA sequences. These microarrays have previously been designed to study the differential expression of miRNAs in another lepidopteran species, the greater wax moth *Galleria mellonella*, during development as well as in response to infection (Mukherjee & Vilcinskas, 2014, Mukherjee et al., 2017). We analyzed RNA samples from last-instar larvae and pupae originating from *A. levana* caterpillars exposed to either short-day (short-day larvae, short-day pupae) or long-day (long-day larvae, long-day pupae) conditions to identify differentially expressed miRNAs that may regulate seasonal polyphenism (Vilcinskas & Vogel, 2016). We predicted multiple targets of miRNAs including the above-mentioned diapause bioclock protein gene using previously established techniques on base-pair complementarity and minimum free energy hybridization.

2 | MATERIALS AND METHODS

2.1 | Biological sample preparation and miRNA expression analysis

A. levana caterpillars were collected in the vicinity of Albach in Hesse either in June (long-day condition) or August (short-day condition), fed with stinging nettle cultivars and reared in captivity for RNA isolation and microarray analysis as previously described (Freitak, Knorr, Vogel, & Vilcinskas, 2012; Mukherjee & Vilcinskas, 2014; Vilcinskas & Vogel, 2016). Total RNA was isolated from eight specimens exposed to long-day conditions (18-hr daylight, collected in June) and eight specimens conditioned under short-day conditions (8-hr daylight, collected in August). Specifically, we used a set of four last-instar larvae and four 1-day-old pupae for each group (long- and short-day conditions). Microarray analysis of miRNAs, including the provision of reagents, experimental procedures, and data analysis, was carried out by LC Sciences, Houston, TX, as previously described (Mukherjee et al., 2017). RNA isolated from last-instar larvae and pupae was extended using polyadenylate polymerase and ligated to an oligonucleotide tag labeled with a fluorescent dye for subsequent fluorescence detection in dual-sample experiments. Microarray hybridization, detection, and analysis were carried out as previously described (Mukherjee & Vilcinskas, 2014).

Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to cross-validate the expression of miR-2856-3p in *A. levana* larvae and pupae exposed to either long-day or short-day conditions (Mukherjee & Vilcinskas, 2014; Mukherjee et al., 2017). For the analysis of miRNAs, complementary DNA was synthesized using the miScript II miRNA first-strand synthesis and qPCR kit (Qiagen) according to the manufacturer's instructions. Small RNA-enriched total RNA was reverse-transcribed using miScript HiSpec buffer, oligo-dT primers with 3′ degenerate anchors and a 5′ universal tag sequence for the specific synthesis of mature miRNAs. The combination of polyadenylation and the universal tag ensures that miScript primer assays do not detect genomic DNA. Primers for the selected miRNAs were designed using the miScript miRNA product-design webpage (Qiagen). Candidate miRNA expression levels were normalized against miR-2491-3p, which showed uniform expression across all samples. Real-time RT-PCR was performed using the Biorad (CFX 96) Mx3000P system, starting with a 15-min incubation at 95°C to activate the hot-start polymerase followed by 40 cycles at 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. The following sequences of miRNAs were used for primer design: miR-2491-3p: CAACAACAG-CAGCAGCAA; miR-2856-3p: ACAUUCGAGAACCGUAAGACAA.

2.2 | Target prediction of individual miRNAs

The analysis of miRNA expression and the prediction of their targets was carried out as recently reported using the *A. levana* transcriptome as a reference (Mukherjee & Vilcinskas, 2014; Vilcinskas & Vogel, 2016). We screened with the sequence alignment editor BioEdit to identify open reading frames (ORFs) in all contigs. The 3′ ends of the contig sequences beyond the assigned ORFs were considered as potential 3′-untranslated regions and screened for complementarity with the expressed miRNA sequences identified by microarray analysis. The Gene Ontology categories of the identified contigs were listed by consulting a previous report (Vilcinskas & Vogel, 2016). The molecular functions targeted by miRNAs were summarized using Cytoscape v3.2.1 (http://www.cytoscape.org). The structure of miRNA–mRNA duplexes was visualized using the RNAhybrid tool provided by the Bielefeld Bioinformatics Server (Rehmsmeier, Steffen, Hochsmann, & Giegerich, 2004; Table S1).

2.3 | Data analysis

After background subtraction and normalization using a locally-weighted regression filter, analysis of variance (ANOVA) was applied across the four sample groups to produce a miRNA expression profile overview across all

samples. Model diagnostics for the ANOVA were performed and did not reveal any evidence against the model assumptions, such as homoscedasticity and normality of errors. Subsequently, post hoc *t* tests were performed to identify significantly differentiated miRNAs among all interested combinations of two groups. In addition, *p* values were corrected for multiple testing using the false discovery rate calculated using the Benjamini–Hochberg procedure. Values were considered significantly different at $p < .01$.

3 | RESULTS

3.1 | Differential expression of miRNAs in larvae and pupae of *A. levana* following exposure to long-day and short-day conditions

The expression of miRNAs in last-instar larvae or pupae developing under long-day and short-day conditions was measured by designing a DNA oligonucleotide microarray containing 2,621 unique mature arthropod miRNA sequences from miRBase v21. The miRNA sequences were derived from diverse insect species including the fruit fly, honey bee, pea aphid, silkworm, mosquito, and red flour beetle (1,735, 259, 103, 560, 282, and 422 unique mature miRNAs, respectively). All probes representing unique mature miRNAs were printed in triplicate for signal verification (Figure S1).

We detected differential expression of 355 miRNAs between long-day larvae, long-day pupae, short-day larvae, and short-day pupae (Table S2). The expression of 140 or 154 miRNAs was significantly expressed between long-day larvae and long-day pupae, and between short-day larvae and short-day pupae respectively (Table S3). We detected upregulations/downregulations of 199 miRNAs between short-day larvae and long-day larvae, and 51 miRNAs between short-day pupae and long-day pupae, respectively. Similarly, we observed differential expression of 226 miRNAs between short-day pupae and long-day larvae, 42 miRNAs between short-day larvae and long-day pupae, and 154 miRNAs between short-day pupae and short-day larvae. After removing duplicates that were expressed between the tested samples, we selected 122 miRNAs (77 downregulated and 55 upregulated) that showed significant expression level in short-day pupae, short-day larvae, and long-day pupae relative to long-day larvae (Figure 1).

This experimental design facilitated the identification of six, five, and one miRNAs that were specifically downregulated and 11, 8, and 5 miRNAs that were specifically upregulated in short-day larvae, short-day pupae, or long-day pupae, respectively (Figure 1 and Table S2). We also identified 19, 6, and 9 miRNAs that were specifically downregulated and eight, two, and six miRNAs that were specifically upregulated between short-day larvae and short-day pupae, between long-day and short-day pupae, and between short-day larvae and long-day pupae, respectively. The maximum upregulation was observed for miR-3015c (9.1 fold) in short-day pupae while miR-6497-3p was most downregulated (1.4 fold) in the same sample (Figure 2a,b). The statistical analysis of the fold differences in expression levels of differentially expressed miRNAs is provided in Table S2. The expression of miR-2856-3p was experimentally verified by measuring its relative expression levels by RT-PCR. We confirmed the upregulation of this miRNA in long-day larvae and downregulation in short-day larvae (Figure 3).

3.2 | Target prediction of selected miRNAs

Selected miRNAs that were differentially expressed between larvae and pupae developed under long- and short-day conditions were screened against a comprehensive *A. levena* transcriptome (Vilcinskas & Vogel, 2016). The candidate miRNAs were used to identify putative targets, revealing a number of mRNAs that are already known to be regulated by day length (Vilcinskas & Vogel, 2016). We identified 65 mRNAs as targets for 19 miRNAs and analyzed their corresponding molecular functions (Table 1). For example, miR-11-3p and miR-2a, both target a heat-shock protein 70 gene, miR-2781 targets a beta-glucan recognition protein (BGRP) gene, miR-289-5p targets a gene encoding collagen alpha-2 chain along with miR-252a which also targets a zinc finger protein gene.

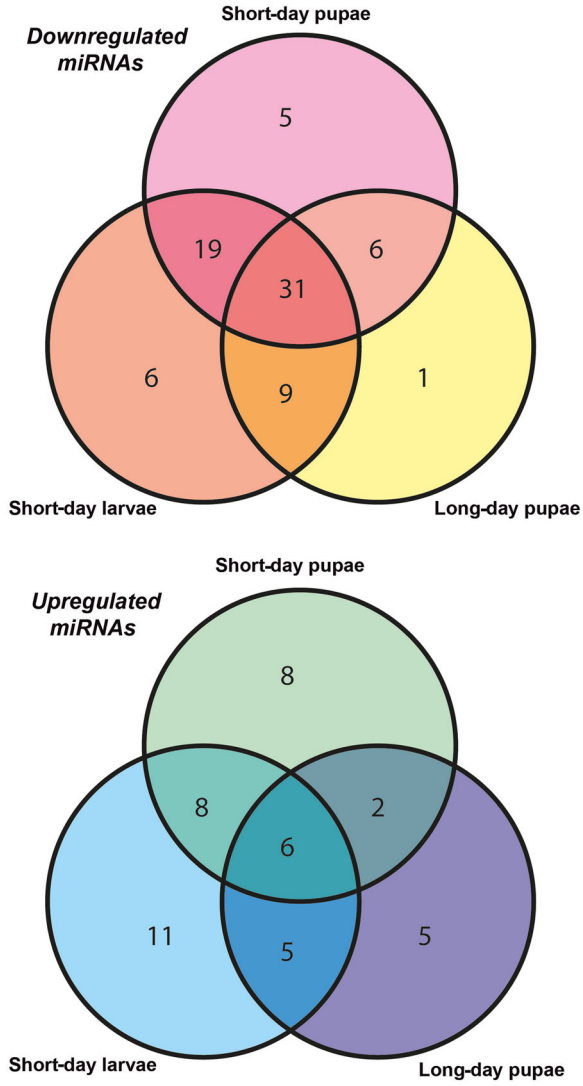

FIGURE 1 Venn diagram showing the differential expression of miRNAs in the larvae and pupae of *Araschnia levana*. The miRNA sequences were obtained from miRBase v21 and their expression profiles were determined by microarray analysis. The fold differences of downregulated and upregulated miRNAs are shown in Figure S1 and the statistical analysis of differential expression are provided in Table S2. miRNA, microRNA

Moreover, miR-92b-5p targets a gene encoding an endonuclease-reverse transcriptase. And miR-14-5p is putatively involved in lipid metabolism. Among the candidates we identified, miR-2856-3p was particularly interesting because it targets the previously identified diapause bioclock protein gene (Figure 4) which is upregulated under short-day conditions (Vilcinskas & Vogel, 2016).

4 | DISCUSSION

Environmental stimuli such as day length influence the choice between alternative phenotypes in adult *A. levana*, but the underlying molecular mechanisms are unknown. Here, we investigated whether epigenetic mechanisms contribute to the

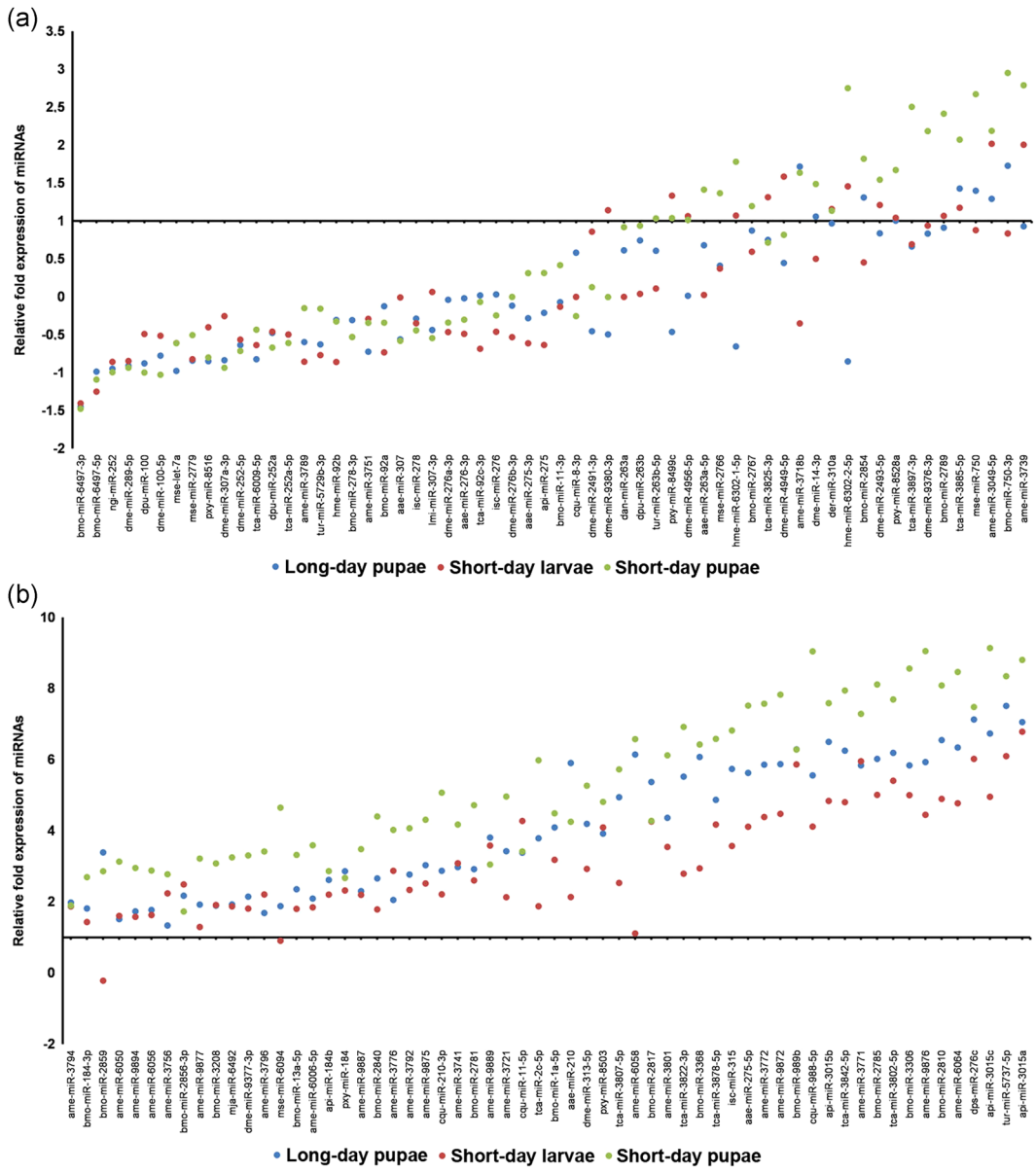

FIGURE 2 Expression of miRNAs in the larvae and pupae of *A. levana* exposed to either short-day or long-day conditions during larval development. (a,b) The miRNA sequences were obtained from miRBase v21 and their expression profiles were determined by microarray analysis. The fold differences in expression are shown relative to long-day larvae ($p < .01$). Only miRNAs expressed at significant levels in short-day larvae, and short-day and long-day pupae are shown. The fold differences are relative to expression levels in long-day larvae. miRNA, microRNA

translation of environmental stimuli into the formation of distinct seasonal phenotypes, specifically the role of miRNAs that play a key role in the posttranscriptional regulation of protein synthesis (Asgari, 2013; Burggreen, 2017). We have carried out expression analyses of miRNAs in short-day and long-day larvae and pupae of *A. levana* using microarrays containing probes representing 2621 miRNAs from model insects with available genome sequences. We identified several miRNAs that were significantly up or downregulated specifically between larvae and pupae exposed to different growth

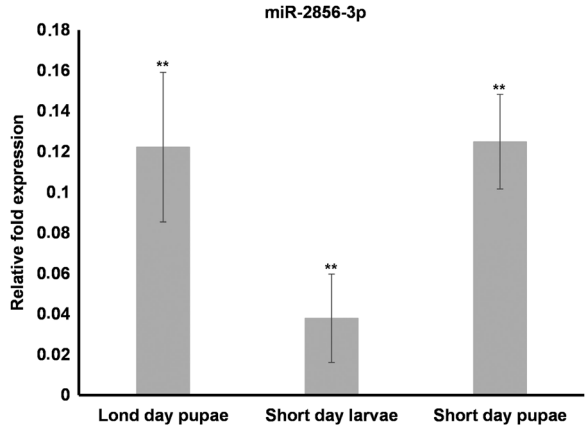

FIGURE 3 Expression of miR-2856-3p in larvae and pupae of *A. levana* developed under short-day and long-day conditions. The expression of miR-2856-3p was analyzed in *A. levana* larvae and pupae, reared under long-day or short-day conditions, by quantitative real-time RT-PCR. Basal expression was calculated as a fold change relative to long-day larvae and normalized to miR-2766, which was uniformly expressed in all samples. Data are mean $\Delta\Delta C_t$ values from three independent experiments with standard error (**$p < .001$). miRNA, microRNA; RT-PCR, reverse-transcriptase polymerase chain reaction

conditions (long-day, short-day), indicating their potential involvement in the regulation of genes mediating seasonal polyphenism. For example, let-7a and miR-275 were specifically upregulated in long-day pupae and short-day larvae, and miR-310a was downregulated in short-day pupae relative to long-day larvae, respectively. We also found the downregulation of miR-92b-5p in long-day pupae relative to short-day pupae. In a previous study, we have shown that miRNAs control transcriptional reprogramming during the metamorphosis in another lepidopteran species (Mukherjee & Vilcinskas, 2014). Our study expands the known functions of miRNAs in the regulation of gene expression in response to environmental stimuli beyond, for example, heat, starvation, and pathogens (Freitak et al., 2012; Vilcinskas, 2017) to include the conversion of seasonally changing day length into the corresponding distinct morphological phenotypes.

A. levana caterpillars kept under long-day conditions (in nature in June) are committed to rapid metamorphosis, whereas those exposed to short-day conditions (in nature in August) are prepared for diapause and overwintering. We identified a number of miRNAs that appear to target genes that are differentially expressed according to the day length during larval development, supporting our hypothesis that miRNAs contribute to the control of transcriptional reprogramming in *A. levana* in response to seasonal changes in the duration of daylight. As previously reported, short-day prepupae preferentially expressed genes related to innate immunity (Vilcinskas & Vogel, 2016), and we identified miR-2781 that targets a gene encoding a BGRP. The expression of miR-2781 was downregulated in short-day pupae relative to long-day pupae. Similarly, long-day conditions induced expression of heat-shock protein 70 in prepupae, and in the present study, we identified the downregulation of miR-2a in long-day pupae that targets an *Hsp70*. miRNAs mainly exert a negative effect on gene expression, raising the possibility that they play a role in inhibiting genes that can negatively influence the formation of polyphenic morphs in *A. levana* in response to changing environmental clues. Interestingly, Reynolds, Peyton, and Denlinger (2017) previously identified several miRNAs, which were differentially expressed in diapausing versus developing fly pupae of the flesh fly, *Sarcophaga bullata*. Among those, miR-289-5p was overexpressed in diapausing pupae compared to their nondiapausing counterparts. According to the authors, this miRNA may be responsible for silencing the expression of candidate genes during diapause in this species. In our study, miR-289-5p was significantly higher expressed in short-day larvae programmed for diapause compared to long-day larvae, but not pupae. This suggests that in *A. levana* the initiation of expression profiles responsible for developmental arrest may commence even before the pupal stage. Another example are genes encoding storage proteins which were

TABLE 1 Light-regime dependent differentially expressed miRNAs in *Araschnia levana*

MicroRNA	Target contig	Description	Function of the target contig
miR-6497-3p	ArashLevBB C305	Protein disulfide-isomerase like protein erp57	Transferase activity
miR-6497-3p	ArashLevBB C7989	Angiotensin-converting enzyme-like	Dipeptidase activity
miR-6497-3p	ArashLevBB C13987	Synaptic vesicle glycoprotein 2b-like	Transmembrane transporter activity
miR-6497-3p	ArashLevBB C15880	Endonuclease-reverse transcriptase	RNA binding
miR-252a	ArashLevBB C211	Collagen alpha-2 chain-like	Extracellular matrix structural constituent
miR-252a	ArashLevBB C7511	Zinc finger protein	Zinc ion binding
miR-289-5p	ArashLevBB C87	ADP-ribosylation factor-like protein 5b-like	GTPase activity
miR-289-5p	ArashLevBB C183	Cytochrome-C oxidase subunit partial	Cytochrome-C oxidase activity
miR-289-5p	ArashLevBB C211	Collagen alpha-2 chain-like	Extracellular matrix structural constituent
miR-289-5p	ArashLevBB C456	Reverse transcriptase	RNA binding
miR-289-5p	ArashLevBB C551	Inhibitor of apoptosis protein	Zinc ion binding
miR-289-5p	ArashLevBB C555	Probable palmitoyltransferase ZDHHC23-like	Zinc ion binding
miR-289-5p	ArashLevBB C588	Protein kinase C and casein kinase substrate in neurons	Phosphatidylserine binding
miR-289-5p	ArashLevBB C592	Eukaryotic translation initiation factor 4 gamma 2-like	Aralkylamine *N*-acetyltransferase activity
miR-289-5p	ArashLevBB C1184	Polycomb group ring finger protein 3-like	Zinc ion binding
miR-289-5p	ArashLevBB C1456	Phenol UDP-glucosyltransferase	Transferase activity
miR-289-5p	ArashLevBB C1959	Ninjurin	
miR-289-5p	ArashLevBB C2045	Signal peptidase complex subunit 3	Peptidase activity
miR-289-5p	ArashLevBB C2114	Kelch-like protein diablo-like	Actin binding
miR-289-5p	ArashLevBB C2118	F-box LRR protein	Protein binding
miR-289-5p	ArashLevBB C2178	Regulator of G-protein signaling 7-like	Signal transducer activity
miR-289-5p	ArashLevBB C2313	Serine threonine-protein phosphatase PP1-gamma catalytic subunit-like isoform 1	Phosphoprotein phosphatase activity
miR-289-5p	ArashLevBB C2452	Ring finger protein 4	Zinc ion binding
miR-289-5p	ArashLevBB C2863	Serine threonine-protein kinase HASPIN homolog	ATP binding
miR-289-5p	ArashLevBB C3401	Protein toll-like	Phosphoprotein phosphatase activity
miR-289-5p	ArashLevBB C3442	Protein split ends	Nucleic acid-binding
miR-289-5p	ArashLevBB C4290	Kinesin-like protein KIF3B	ATP binding
miR-11-3p	ArashLevBB C1327	Heat-shock protein 70	ATP binding

(Continues)

TABLE 1 (Continued)

MicroRNA	Target contig	Description	Function of the target contig
miR-11-3p	ArashLevBB C3466	Serine threonine-protein kinase ULK2-like	ATP binding
miR-11-3p	ArashLevBB C33249	Protein CBFA2T3-like	Zinc ion binding
miR-100	ArashLevBB C794	BEL12_AG transposon polyprotein	Zinc ion binding
miR-100	ArashLevBB C1074	Transcription factor dp-1	Transcription factor binding
miR-252a-5p	ArashLevBB C3712	Melanization protease 1	Serine-type endopeptidase activity
miR-252a-5p	ArashLevBB C7226	RAS-related protein M-RAS-like	Protein binding
miR-252a-5p	ArashLevBB C15083	Protein DOK-7	Insulin receptor binding
miR-2491-3p	AraschLev_BB_C434	Peptidoglycan recognition protein SA	Protein binding
miR-2856-3p	AraschLev_BB_C702	Diapause bioclock protein	Superoxide dismutase activity
miR-2781	AraschLev_BB_C4355	Beta-glucan recognition protein 3	Hydrolase activity
miR-6064	AraschLev_BB_C682	C-type lectin	Carbohydrate binding
miR-5325	AraschLev_BB_C74	Ferritin light chain	Ferric iron-binding
miR-3792	AraschLev_BB_C7205	Hermansky-Pudlak syndrome 4 isoform B	Serine-type endopeptidase activity
miR-4949-5p	AraschLev_BB_C8065	Peptidoglycan recognition protein SC2	Peptidoglycan binding
miR-2810	AraschLev_BB_C4533	Methyltransferase NSUN5-like	Methyltransferase activity
miR-2a	AraschLev_BB_C162	Elongator complex protein 1	Phosphorylase kinase regulator activity
miR-2a	AraschLev_BB_C509	RNA polymerase II second largest subunit	DNA binding
miR-2a	AraschLev_BB_C1483	Heat-shock protein	
miR-2a	AraschLev_BB_C1530	Golgin subfamily a member 4-like	ADP-ribosylation factor binding
miR-2a	AraschLev_BB_C1563	RUVB-like 2-like	
miR-2a	AraschLev_BB_C1699	Suppressor of hairless	RNA polymerase II core promoter proximal region sequence-specific DNA binding
miR-2a	AraschLev_BB_C2025	Inwardly rectifying k+ channel	Inward rectifier potassium channel activity
miR-2a	AraschLev_BB_C5506	Zinc finger cchc domain-containing protein 2	Zinc ion binding
miR-2a	AraschLev_BB_C2435	Exosome complex exonuclease rrp44-like	
miR-3818-3p	AraschLev_BB_C3837	Aryl hydrocarbon receptor nuclear translocator homolog	DNA binding
miR-3818-3p	AraschLev_BB_C32832	Dynein heavy chain axonemal	ATP binding
miR-3642-5p	AraschLev_BB_C316	CG11122 CG11122-PA	Metal ion binding
miR-3642-5p	AraschLev_BB_C362	Prominin-like protein	
miR-3642-5p	AraschLev_BB_C13785	Coiled-coil domain-containing protein 108	
miR-92b	AraschLev_BB_C1070	RAB6-interacting golgin	Protein binding

(Continues)

TABLE 1 (Continued)

MicroRNA	Target contig	Description	Function of the target contig
miR-92b	AraschLev_BB_C15880	Endonuclease-reverse transcriptase	RNA binding
miR-311-3p	AraschLev_BB_C2689	Chromodomain-helicase-DNA-binding protein mi-2 homolog	DNA binding
miR-311-3p	AraschLev_BB_C3742	Fatty acid transport protein	Catalytic activity
miR-311-3p	AraschLev_BB_C3809	SNF4 AMP-activated protein kinase gamma subunit	AMP-activated protein kinase activity
miR-311-3p	AraschLev_BB_C8080	Mitochondrial intermediate peptidase	Metalloendopeptidase activity
miR-311-3p	AraschLev_BB_C8441	Nuclease harbi1-like	Molecular function
miR-311-3p	AraschLev_BB_C19762	Potassium voltage-gated channel subfamily H member 7	Adenylyl-sulfate kinase activity

Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; miRNAs, microRNA.

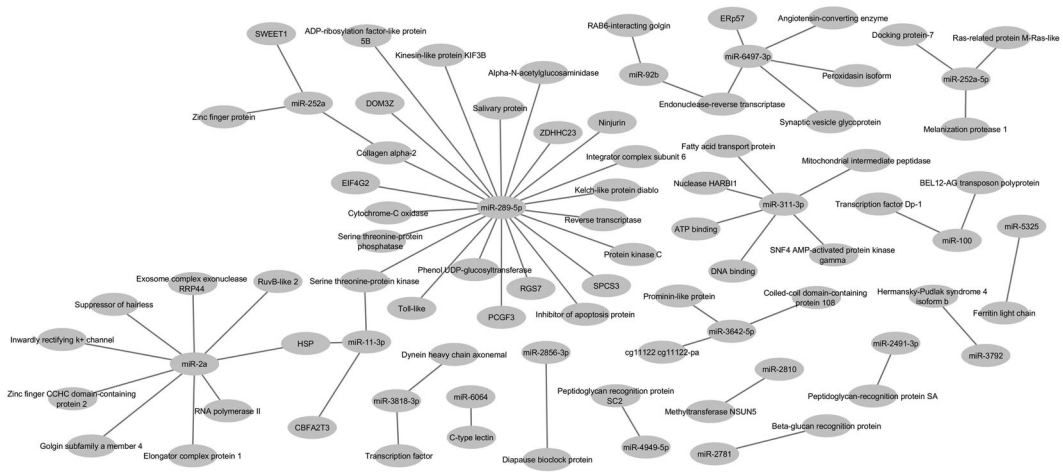

FIGURE 4 The mRNA sequences targeted by miRNAs in *A. levana*. The network diagram generated with Cytoscape shows description of mRNA sequences in *A. levana* that are targeted by differentially expressed miRNA sequences obtained from miRBase. Connecting lines (edges) are used to indicate miRNA targets identified in this study by consulting Gene Ontology terms related to *A. levana* transcriptome sequences (Vilcinskas & Vogel, 2016). mRNA, messenger RNA; miRNAs, microRNA

upregulated in long-day pupae (Vilcinskas & Vogel, 2016) and we found downregulation of a miRNA (miR-5325) that controls their expression. An interesting miRNA pertaining to the regulation of lipid metabolism is miR-14-5p. It was previously found to be downregulated during diapause in pharate larvae of *Aedes albopictus* and the knockout of its precursor in *Drosophila melanogaster* reduced lipid metabolism but increased accumulation of di- and triglycerides (Batz, Golff, & Armbruster, 2017; Xu, Vernooy, Guo, & Hay, 2003). Batz et al. (2017) concluded that the downregulation of miR-14-5p in diapausing *A. albopictus* larvae, could contribute to the accumulation of lipids. These findings are consistent with our data, as bmo-miR-14-5p was significantly differentially expressed across all four experimental groups, with the highest expression occurring in long-day larvae and the lowest in short-day larvae. The low expression of this miRNA in the latter is indicative of active fat storage in preparation of pupation and adverse seasonal conditions. This deduction is further supported by the findings of Meuti, Bautista-Jimenez,

and Reynolds (2018), who reported that miR-14-3p was underexpressed in diapausing adult female *Culex pipiens*, which correlated with rapid fat accumulation 5 days after emergence. However, it is not clear if the function of miR-14 in *D. melanogaster* and *C. pipiens* is evolutionarily conserved. Our findings will, therefore, need to be corroborated through future investigations. Our hypothesis was strengthened further by the identification of a differentially expressed miRNA targeting the *A. levana* ortholog of the diapause bioclock protein gene, which encodes an ATPase with a copper–zinc superoxide dismutase domain known as time interval measuring enzyme esterase A4 (Isobe et al., 2006). In *A. levana* prepupae, the diapause bioclock protein gene is expressed at lower levels when the larvae have been reared under long-day conditions compared to those reared under short-day conditions, plausibly because those reared under long-day conditions are not committed to diapause and, therefore, do not require a molecular clock that measures its duration (Vilcinskas & Vogel, 2016). Here, we identified a miRNA that targets the diapause clock gene and which is differentially expressed in last-instar larvae and pupae depending on the day length during larval development. Our findings also support the recent study of Batz et al. (2017) who reported that miRNAs regulate diapause in *A. albopictus*.

In conclusion, our approach resulted in the identification of miRNAs that target genes regulating metabolism, innate immunity, epigenetic mechanisms, transcription, heat-shock proteins, and formation of the extracellular matrix, all of which are important for driving seasonal polyphenism in *A. levana*.

ACKNOWLEDGMENTS

The authors thank Ruediger Lehmann for technical assistance and Dr. Richard M. Twyman for editing the manuscript. Andreas Vilcinskas acknowledges generous financial support from the Hessen State Ministry of Higher Education, Research, and the Arts (HMWK) via the LOEWE research center “Insect Biotechnology and Bioresources.” The funding source(s) had no involvement in the preparation of the manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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REFERENCES

Asgari, S. (2013). MicroRNA functions in insects. *Insect Biochemistry and Molecular Biology*, *43*, 388–397. https://doi.org/org/10.1016/j.ibmb.2012.10.005

Batz, Z. A., Golff, A. C., & Armbruster, P. A. (2017). MicroRNAs are differentially abundant during *Aedes albopictus* diapause maintenance but not diapause induction. *Insect Molecular Biology*, *26*, 721–733. https://doi.org/10.1111/imb.12332

Baudach, A., Lee, K. Z., Vogel, H., & Vilcinskas, A. (2018). Immunological larval polyphenism in the map butterfly *Araschnia levana* reveals the photoperiodic modulation of immunity. *Ecology and Evolution*, *8*, 4891–4898. https://doi.org/org/10.1002/ece3.4047

Belles, X. (2017). MicroRNAs and the evolution of insect metamorphosis. *Annual Reviews in Entomology*, *62*, 111–125. https://doi.org/10.1146/annurev-ento-031616-034925

Burggreen, W. (2017). Epigenetics in insects: Mechanisms, phenotypes and ecological and evolutionary implications. *Advances in Insect Physiology*, *53*, 1–30. https://doi.org/org/10.1016/bs.aiip.2017.04.001

Freitak, D., Knorr, E., Vogel, H., & Vilcinskas, A. (2012). Gender- and stressor-specific microRNA expression in *Tribolium castaneum*. *Biology Letters*, *8*, 860–863. https://doi.org/10.1098/rsbl.2012.0273

Hussain, M., & Asgari, S. (2014). MicroRNAs as mediators of insect–host–pathogen inter-actions and immunity. *Journal of Insect Physiology*, *70*, 151–158. https://doi.org/10.1016/j.jinsphys.2014.08.003

Isobe, M., Kai, H., Kurahashi, T., Suwan, S., Pitchayawasin-Thapphasaraphong, S., Franz, T., … Nishida, H. (2006). The molecular mechanism of the termination of insect diapause, part 1: A timer protein, TIME-EA4, in the diapause eggs of the silkworm *Bombyx mori* is a metallo-glycoprotein. *ChemBioChem*, *7*, 1590–1598. https://doi.org/10.1002/cbic.200600138

Meuti, M. E., Bautista-Jimenez, R., & Reynolds, J. A. (2018). Evidence that microRNAs are part of the molecular toolkit regulating adult reproductive diapause in the mosquito, *Culex pipiens*. *PLoS One*, *13*(11):e0203015. https://doi.org/10.1371/journal.pone.0203015

Mukherjee, K., Grizanova, E., Chertkova, E., Lehmann, R., Dubovskiy, I., & Vilcinskas, A. (2017). Experimental evolution of resistance against *Bacillus thuringiensis* in the insect model host *Galleria mellonella* results in epigenetic modifications. *Virulence*, *19*, 1–13. https://doi.org/10.1080/21505594.2017.1325975

Mukherjee, K., & Vilcinskas, A. (2014). Development and immunity-related microRNAs of the lepidopteran model host *Galleria mellonella*. *BMC Genomics*, *15*, 705. https://doi.org/10.1186/1471-2164-15-705

Rehmsmeier, M., Steffen, P., Hochsmann, M., & Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. *RNA*, *10*, 1507–1517. https://doi.org/10.1261/rna.5248604

Reinhardt, R. (1984). Die Landkärtchenfalter *Araschnia levana*, Die Neue Brehm Bücherei Vol. 458. Ziemsen, Wittenberg, Germany.

Reynolds, J. A. (2017). Epigenetic influence on diapause. *Advances in Insect Physiology*, *53*, 115–144. https://doi.org/.org/10.1016/bs.aiip.2017.03.003

Reynolds, J. A., Peyton, J. T., & Denlinger, D. L. (2017). Changes in microRNA abundance may regulate diapause in the flesh fly, *Sarcophaga bullata*. *Insect Biochemistry and Molecular Biology*, *84*, 1–14.

Shapiro, A. M. (1976). Seasonal polyphenism. *Evolutionary Biology*, *9*, 259–333. https://doi.org/org/10.1016/bs.aiip.2017.03.003

Simpson, S. J., Sword, G. A., & Lo, N. (2011). Polyphenism in insects. *Current Biology*, *21*, R738–R749. https://doi.org/org/10.1016/j.cub.2011.06.006

Verlinden, H. (2017). Epigenetics in insects: Mechanisms, phenotypes, and ecological and evolutionary implications. *Advances in Insect Physiology*, *53*, 1–30. https://doi.org/org/10.1016/bs.aiip.2017.04.001

Vilcinskas, A. (2017). The impact of parasites on host insect epigenetics. *Advances in Insect Physiology*, *53*, 145–165. https://doi.org/10.7554/eLife.25762

Vilcinskas, A., & Vogel, H. (2016). Seasonal phenotype-specific transcriptional reprogramming during metamorphosis in the European map butterfly *Araschnia levana*. *Ecology and Evolution*, *6*, 3476–3485. https://doi.org/10.1002/ece3.2120

Xu, P., Vernooy, S. Y., Guo, M., & Hay, B. A. (2003). The Drosophila microRNA mir-14 suppresses cell death and is required for normal fat metabolism. *Current Biology*, *13*, 790–795. https://doi.org/10.1016/S0960-9822(03)00250-1

Ylla, G., Piulachs, M. D., & Belles, X. (2017). Comparative analysis of miRNA expression during the development of insects of different metamorphosis modes and germ-band types. *BMC Genomics*, *18*, 774. https://doi.org/10.1186/s12864-017-4177-5

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Mukherjee K, Baudach A, Vogel H, Vilcinskas A. Seasonal phenotype-specific expression of microRNAs during metamorphosis in the European map butterfly *Araschnia levana*. *Arch. Insect Biochem. Physiol.* 2020;104:e21657. https://doi.org/10.1002/arch.21657