The role of epigenetics in polyphenisms and transgenerational immune priming in Lepidoptera

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Per aspera ad astra

To my mother and late father
who have always supported me, no matter what.

Thank you!

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5mdC	5-methyl-2'-deoxycytidine
20E	20-hydroxyecdysone
AGO	Argonaute (protein)
AMP	Antimicrobial peptide
ATPase	Adenosinetriphosphatase
cDNA	Complementary DNA
CpG	5'-cytosine-phosphate-guanine-3' dinucleotide
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9
DBP	Diapause bioclock protein
dC	2'-deoxycytidine
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
Dscam	Down syndrome cell adhesion molecule
Ea4	TIME-EA4 gene
EcR	Ecdysone receptor
f.	Form (phenotype)
GFP	Green fluorescent protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
Imd	Immune deficiency (pathway)
Jak/Stat	Janus kinase/ signal transducer and activator of transcription (pathway)
JH	Juvenile hormone
JAK	C-Jun N-terminal kinase (pathway)
LC–MS	Liquid chromatography–mass spectrometry
LPS	Lipopolysaccharide
MBD	Methyl-CpG-binding domain protein
mRNA	Messenger RNA
miRNA (miR)	Micro-RNA
MRM	Multiple reaction monitoring
Nt	Nucleotide
ORF	Open reading frame
p-value	Probability-value
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PGN	Peptidoglycan
PIWI	P-element induced wimpy testes (protein)
PO	Phenoloxidase
poly(A)	3′-polyadenylate tail
PRR	Pattern recognition receptor
PTTH	Prothoracicotropic hormone
RNA	Ribonucleic acid
RNAi	RNA interference
ROX	6-carboxy-X-rhodamine (dye)
RPL3	Ribosomal protein L3 gene
RPL10	Ribosomal protein L10 gene
RT-qPCR	Reverse-transcriptase quantitative polymerase chain reaction
sRNA	Small-RNA
siRNA	Small interfering RNA
TIME-EA4	Time interval measuring enzyme-esterase A4
TGIP	Trans-generational immune priming
TGP	Transgenerational plasticity
tRNA	transfer-RNA
UTR	Untranslated region
vDNA	viral DNA
Vg	Vitellogenin

Abstract

Examples of phenotypic plasticity, referring to the capacity to express multiple phenotypes from a single genotype in response to environmental conditions, are abundant in lepidopterans. However, the exact extent and mechanistic foundations of the phenomenon are unresolved. For example, the bivoltine European map butterfly (*Araschnia levana*, L. 1758, Nymphalidae) displays a seasonal polyphenism characterized by the formation of two remarkably distinct dorsal wing phenotypes in the spring (pupal diapause) and summer (direct development) generations respectively, which depends on larval photoperiod and temperature. We investigated, if the polyphenism extends to the larval stage and found that, following the injection of a bacterial entomopathogen (*Pseudomonas entomophila*), larvae of the spring generation succumbed to infection later, displayed higher antibacterial activity in the haemolymph, and expressed higher levels of antimicrobial peptides than larvae of the summer generation. These findings likely point towards a bolstered immune phenotype in preparation of overwintering. Furthermore, from physiological experiments by Koch and Bückmann (1987), it was known, that the expression of the seasonal polyphenism depends on the photoperiodically controlled timing of the release of 20-hydroxyecdysone in pupae. Encouraged by profoundly different transcriptomic profiles in spring- and summer-primed prepupae, we hypothesised that post-transcriptional epigenetic regulators such as microRNAs (miRNAs) might be involved in controlling the seasonal polyphenism in this species. Using microarrays containing over 2,000 conserved insect miRNAs, we detected several hundred miRNAs differentially expressed between larvae (and pupae) primed for either developmental trajectory. Also, by employing *in silico* target prediction, we identified numerous targets of miRNAs that were differentially regulated in the two generations including one miRNA (miR-2856-3p) that potentially controls the expression of a diapause bioclock protein, which is vital to determine the moment when diapause should be broken in the Silkworm (*Bombyx mori*, L. 1758, Lepidoptera: Bombycidae). These results suggest a significant involvement of epigenetic regulatory systems in the expression of seasonal phenotypes in *A. levana*. Moreover, for many insect orders, including the Lepidoptera, it was known that parents invest in their offspring by relaying the experience of their own microbial environment to the offspring generation, thus conferring a (plastic) degree of protection. This phenomenon is known as trans-generational immune priming (TGIP) and had, *inter alia*, been observed in the Tobacco hornworm, *Manduca sexta* (L. 1763, Sphingidae). However, it was unknown if, in line with Bateman's principle, the transgenerational immune phenotype was sex-specifically expressed. Moreover, the mechanistic underpinnings of the previously observed effects were unclear. We used this model species to investigate these questions by feeding larvae of the parental generation with fluorescently labelled bacterial fragments or non-pathogenic *Escherichia coli* or the entomopathogen *Serratia entomophila*. We found that maternal TGIP depended on the translocation of bacterial structures from the gut lumen to the eggs. Also, we observed sex- and/or microbe-specific differences in the expression profiles of immunity-related genes as well as genes encoding enzymes involved in the regulation of histone acetylation and DNA methylation in larvae of the offspring generation. These larvae further displayed shifts in both DNA methylation and histone acetylation pointing towards the existence of multiple routes for TGIP in *M. sexta*. Moreover, the entomopathogen *S. entomophila* appeared to be capable of interfering with TGIP in the host. In summary, we contributed to the body of knowledge of lepidopteran phenotypic plasticity by widening the extent of known intra- and intergenerational phenotypes and demonstrating the involvement of the epigenetics in the regulation of polyphenisms and TGIP in *A. levana* and *M. sexta* respectively.

Abstract - Deutsch

Beispiele für phänotypische Plastizität, also die Fähigkeit, als Reaktion auf bestimmte Umweltbedingungen aus einem einzigen Genotyp mehrere Phänotypen auszubilden, gibt es bei Lepidopteren viele. Das genaue Ausmaß und die mechanistischen Grundlagen des Phänomens sind jedoch nicht abschließend geklärt. So zeigt beispielsweise der bivoltine Europäische Landkärtchenfalter (*Araschnia levana*, L. 1758, Nymphalidae) einen saisonalen Polyphänismus, der durch die Bildung von zwei bemerkenswert unterschiedlichen Flügel-Phänotypen in der Frühjahrs- (Puppendiapause) bzw. Sommergeneration (direkte Entwicklung) gekennzeichnet ist. Der *Araschnia*- Polyphänismus hängt von Photoperiode und Temperatur während der Larvalphase ab. Im Rahmen unserer Untersuchungen wurde festgestellt, dass Larven der Frühjahrsgeneration nach Injektion eines bakteriellen Entomopathogens (*Pseudomonas entomophila*) der Infektion später erlagen, eine höhere antibakterielle Aktivität in der Hämolymphe aufwiesen und größere Mengen antimikrobieller Peptide exprimierten als die Larven der Sommergeneration. Diese Ergebnisse deuten vermutlich auf einen gestärkten Immunphänotyp in Vorbereitung auf die Überwinterung hin. Außerdem war aus physiologischen Experimenten von Koch und Bückmann (1987) bekannt, dass der saisonale Polyphänismus vom photoperiodisch gesteuerten Zeitpunkt der Freisetzung von 20-Hydroxyecdyson in den Puppen abhängt. Angeregt durch deutlich unterschiedliche Transkriptome in frühlings- und sommer-geprimten Präpuppen stellten wir die Hypothese auf, dass posttranskriptionelle epigenetische Regulatoren wie microRNAs (miRNAs) an der Kontrolle des saisonalen Polyphänismus bei dieser Art beteiligt sein könnten. Mithilfe von Microarrays, die über 2.000 konservierte Insekten-miRNAs enthalten, konnten wir zwischen Larven (und Puppen) beider Entwicklungspfade mehrere hundert differentiell exprimierte miRNAs nachweisen. Darüber hinaus konnten wir durch „in-silico-target-prediction“ zahlreiche Ziele von miRNAs identifizieren, die in den beiden Generationen unterschiedlich reguliert werden. Darunter war eine miRNA (miR-2856-3p), die möglicherweise die Expression eines Diapause-Bioclock-Proteins steuert, das beim Seidenspinner (*Bombyx mori*, L. 1758, Lepidoptera: Bombycidae) entscheidend dafür ist, wann die Diapause beendet wird. Diese Ergebnisse lassen vermuten, dass epigenetische Regulationssysteme maßgeblich an der Ausprägung saisonaler Phänotypen bei *A. levana* beteiligt sind. Darüber hinaus war für viele Insektenordnungen, einschließlich der Lepidoptera, bekannt, dass Eltern in ihre Nachkommen investieren, indem sie die Erfahrungen ihrer eigenen mikrobiellen Umgebung an Folgegenerationen weitergeben. So kann die Eltern- der Tochtergeneration ein plastisches Maß an Schutz gewähren. Dieses Phänomen ist als transgenerationales Immunpriming (TGIP) bekannt und wurde unter anderem beim Tabakschwärmer, *Manduca sexta* (L. 1763, Sphingidae), beobachtet. Nicht bekannt war, ob der generationsübergreifende Immunphänotyp im Einklang mit dem Bateman-Prinzip geschlechtsspezifisch ausgebildet ist. Unklar war auch, welche Mechanismen den zuvor beobachteten Effekten zugrunde liegen. Wir nutzten *M. sexta*, um diese Fragen zu beantworten, und fütterten Larven der Elterngeneration mit fluoreszenzmarkierten Bakterienfragmenten, nicht-pathogenen *Escherichia coli* oder dem Entomopathogen *Serratia entomophila*. Maternales TGIP hing von der Translokation bakterieller Strukturen aus dem Darmlumen in die Eier ab. Außerdem beobachteten wir in der Tochtergeneration geschlechts- und/oder mikrobenspezifische Unterschiede in den Expressionsprofilen von immunbezogenen Genen sowie von Genen, die für Enzyme kodieren, die an der Regulation der Histonacetylierung und DNA-Methylierung beteiligt sind. Diese Larven wiesen außerdem Veränderungen sowohl bei der DNA-Methylierung als auch bei der Histon-Acetylierung auf. In der Gesamtschau deutet dies darauf hin, dass es in *M. sexta* verschiedene TGIP-Routen gibt. Darüber hinaus schien das Entomopathogen *S. entomophila* in der Lage zu sein, TGIP im Wirt zu beeinflussen. Zusammenfassend lässt sich sagen, dass wir zum Wissensstand phänotypischer Plastizität von Lepidopteren beigetragen haben, indem wir das Ausmaß der bekannten intra- und intergenerationellen Phänotypen erweitert und die Beteiligung epigenetischer Prozesse an der Regulation von Polyphänismen und TGIP in *A. levana* bzw. *M. sexta* nachgewiesen haben.

1. Introduction and scope of the dissertation

Environments change. This is a universal constant that all life has to accommodate. The process by which such accommodation in response to an environmental stimulus occurs is called phenotypic plasticity. It denotes the capacity to express multiple (discrete or continuous) phenotypes from a single genotype in response to altered conditions in the environment [1]. It is ubiquitous across both the plant and animal kingdoms. For example, plants respond to light variations by changing the ratio between leaf area and leaf dry mass [2]. Reptilian vertebrates, such as lizards and turtles, display a trait called temperature-dependent sex determination [3] and some teleost fish, like the bluehead wrasse (*Thalassoma bifasciatum*), have a socially controlled sex change [4]. Invertebrates also show considerable plasticity. Cladocerans, such as *Daphnia cucullata*, respond to predation by developing longer helmets and tail spines [5].

In insects, inducible plasticity is both common and thought to be – by and large – highly adaptive. It can range from morphological or physiological adaptations to behavioural or life history changes. Environmental variations that necessitate these adaptations can be temporal, spatial, biotic or abiotic and have one thing in common: They all have the potential to create a mismatch between a given phenotype and the environment. If the mismatch persists, it can lower or even negate survival and reproductive success [1]. Thus, the adaptivity of a given plastic response depends on the degree of match between phenotype and environment [6]. To counter this “mismatch threat”, organisms adapt their phenotype.

While phenotypic plasticity encompasses both discrete and continuous adaptations, the term polyphenism describes the special case where two or more distinct phenotypes are realised in response environmental triggers [7]. Famous examples in insects include caste polyphenisms in hymenopterans like bees and ants [8, 9], or dispersal polyphenisms (winged versus wingless) in aphids [10]. In both cases a stimulus during a critical period in development (e. g. chemicals, larval nutrition, pheromones, temperature) leads to the expression of an adult phenotype that is markedly different from the alternative phenotype(s) which would be realized if the trigger were absent or of a different quality. Notably, in Indian jumping ants (*Harpegnathos saltator*), if the original queen dies, workers can turn into functional reproductive queens (gamergates) even after their phenotypic fate has been decided. As highlighted by Yang and Pospisilik (2019) this means that substantial plasticity can be present after developmental switches have been activated. Furthermore, some polyphenisms need nonstop reinforcement and polyphenisms may also be controlled by inter-individual or population-dependent effects [7].

In this context, a noteworthy complementary concept to plasticity is robustness or “canalisation”. This describes the ability of an organism to maintain a constant phenotype despite environmental perturbations. Specifically, developmental programs that guide plasticity (switch networks) require robustness in order to generate reproducible responses to the plethora of environmental inputs. At the same time, the output programs (execution networks) have to produce robust phenotypic outcomes along a constrained range of potential phenotypes [7, 11].

Another type of plasticity is that which is ruled by the annual cycle of predictably varying precipitation – or lack thereof – in the tropics but also changing photoperiod and temperature in temperate regions. Lepidopterans are among the textbook examples for such seasonal polyphenisms.

Members of the nymphalid genus *Bicyclus*, who live throughout the dry and wet seasons in Africa, have large exposed ventral eyespots during the hot wet season, which help to deflect attacks of naïve vertebrate predators towards the wing margins. In the cool dry season, however, reduced eyespot size also facilitates disguise against predation from vertebrates [12]. Among pierid butterflies, many species respond to cool temperatures and/or short days during the late larval instars, by heavily melanising the ventral hindwing surface for

improved thermoregulation, relative to the warm, long-day phenotype [13]. The European Map butterfly *Araschnia levana* (Nymphalidae) is also a well-known example of seasonal polyphenism. Spring and summer imagoes exhibit distinct morphological phenotypes and key environmental factors responsible for the expression of different morphs are, as with pierids, day length and temperature [14].

However, phenotypic plasticity is not necessarily restricted to short term adaptations which transpire within the lifespan of an individual; it can also be carried over to subsequent generations [1]. For example, in the silkworm *Bombyx mori*, there are bivoltine populations in which embryonic diapause is induced across generations via the mother. If maternal embryonic development occurs at 25 °C, the resulting female imagos deposit eggs which will enter diapause. Conversely, when eggs develop at 15 °C in darkness, the resulting butterfly will lay directly developing eggs [15]. Moreover, in many insects, including lepidopterans, parents devote resources to the offspring generation by readying them for a pathogen or parasite environment that only the mother and/or father have faced [16]. This process has been termed trans-generational immune priming (TGIP) and clearly represents a case of physiological plasticity across generations.

Mechanistically, a lot of the plastic responses described above can be attributed to the action of the neuroendocrine system. For example, the plasticity of eyespot size in the Squinting bush brown *B. anynana* is largely temperature controlled, which, in the final phase of larval development, leads to different levels of the hormone 20-hydroxyecdysone (20E). When 20E signalling is tampered with at that time, the size of the eyespots is easily modified. This is due to the central cells of the eye spots expressing the ecdysone receptor (EcR), and following 20E signalling, the active 20E-EcR complex interacts with as yet unknown downstream genes which trigger these central cells to divide. These in turn create a larger group of central signalling cells, ultimately causing a larger eyespot [12]. It has become clear however, that an additional layer of regulation, which likely is intimately connected to the endocrine system, is at play in many instances when it comes to phenotypic plasticity, namely epigenetics [17–19]. While the neuroendocrine components of phenotypic control are in many cases fairly well understood, the integration of their actions on the level of DNA methylation, histone (de)acetylation and post-transcriptional modifications via small noncoding RNAs, is far less clear [20].

1.1 Hypotheses

It often remains elusive, what the specific proximate and ultimate functions of the alternative phenotypes are. However, before answering the “why”, it is important knowing the “how” and likewise the exact extent of disparity of distinct phenotypes.

In *A. levana* for example, it was unknown if the polyphenism was restricted to the wing colour and morphology of imagos, nor if epigenetic mechanisms were involved in the regulation of alternative phenotype expression. Hence, we tested the following hypotheses:

1. Given the differences in gene expression in prepupae described by Vilcinskas and Vogel (2016) [21], we proposed that transcriptomic differences in *A. levana* larvae can translate into photoperiod-dependent differences in innate immunity when challenged by an entomopathogen [22].
    - See section 2.3 Photoperiod-Specific Plastic Responses: The Extent of Phenotypic Disparity, specifically **2.3.3 Immunity**

2. We further proposed that micro RNAs (miRNAs) contribute to the regulation of transcriptional reprogramming associated with the seasonal polyphenism in *A. levana* [23].
    - See section 2.4 Regulation of phenotype expression, specifically **2.4.2 Circadian Clocks and Epigenetics**

Moreover, in the tobacco hornworm *Manduca sexta* (Sphingidae), phenotypically plastic responses of the immune system had been demonstrated to occur between generations [24–26], but it was unknown if both offspring sexes responded in kind, how the information was passed down to the offspring or if epigenetic mechanisms contributed to the observed adaptations. We therefore investigated the following hypotheses [27]:

3. Given the often-reported differences – e. g. in immunocompetence – between male and female insects [28, 29], we predicted that we would find differential gene expression in *M. sexta* male and female larvae whose parents had experienced microbial challenges and that the observed responses would also be treatment-specific.
    - See section **3.2 Trans-Generational Immune Priming**, specifically **3.2.2 Sexual dimorphism in TGIP**

4. Bacterial structures added to the maternal diet can be transferred from the gut lumen to the eggs and thereby contribute to (the specificity of) the trans-generational phenotype during embryogenesis and beyond.
    - See section **3.2 Trans-Generational Immune Priming**, specifically **3.2.3 TGIP mechanisms**

5. There is an epigenetic component of (sex-specific) transgenerational plasticity in *M. sexta* consisting of differential DNA methylation and/ or histone acetylation/ deacetylation
    - See section **3.2 Trans-Generational Immune Priming**, specifically **3.2.3 TGIP mechanisms**

2. Phenotypic plasticity in *A. levana*

This section, including most figures, is an adaptation of publication 4: Baudach, A.; Vilcinskas, A. The European Map Butterfly *Araschnia levana* as a Model to Study the Molecular Basis and Evolutionary Ecology of Seasonal Polyphenism. *Insects* 2021, 12, 325. https://doi.org/10.3390/insects12040325

2.1 The Seasonal Polyphenism

In 1758, Carl Linnaeus described two apparently distinct butterfly species, which he named *Papilio levana* and *P. prorsa*, but subsequent field observations and breeding experiments revealed them to be seasonal variants of the same bivoltine species: The European map butterfly *A. levana* [30, 31]. The dorsal wing of the spring generation (*A. levana* f. *levana*) is orange to reddish-brown (basic coloration) with black spots, some white dots, and a bluish dotted rim on the posterior end of the hindwing (Fig. 1 D). Conversely, the dorsal wing of the summer generation (*A. levana* f. *prorsa*) is brownish to bluish-black with a prominent white band (featuring varying degrees of melanisation) located basally with respect to 1–3 apical orange bands (Figure 1 E). Many variations between these two phenotypes have been reported, and they are best described as a spectrum. Some of these occur naturally as *A. levana* f. *porima*, with patterning and coloration appearing intermediate between *levana* and *prorsa*. However, most are the result of experimental manipulation [31, 32].

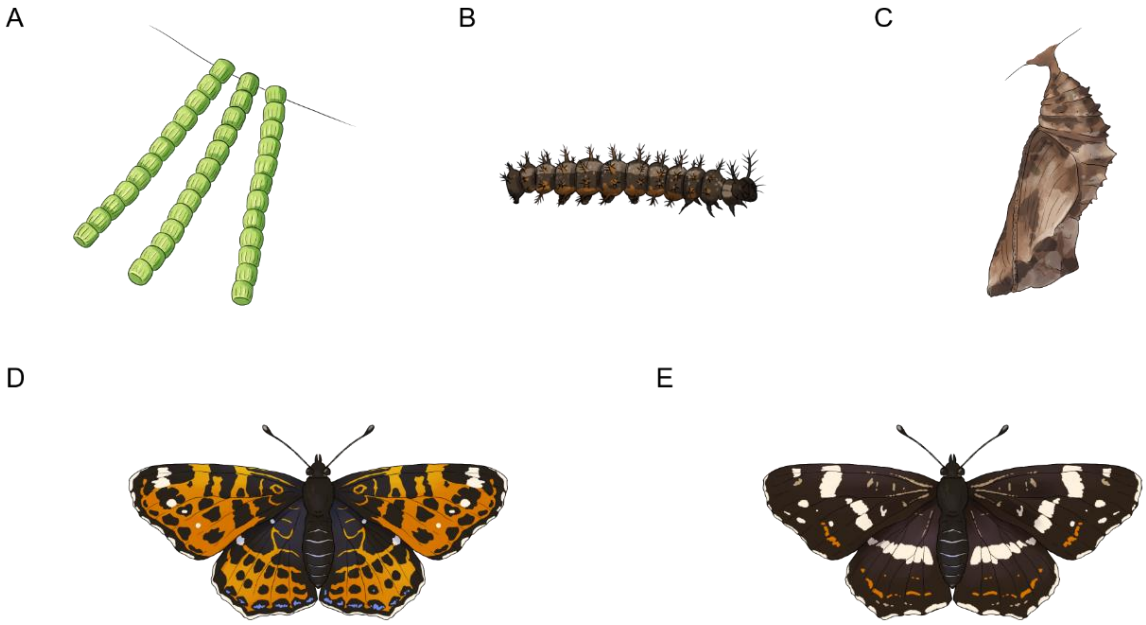

Figure 1. Life cycle stages of *A. levana*. A) Egg towers, B) Larva (5th instar), C) Pupa, D) Spring morph imago (f. *levana*), E) Summer morph imago (f. *prorsa*). Drawings are a courtesy of Mona Luo.

The elements of the wing underside are mostly consistent between morphs and gave rise to the genus name *Araschnia*. The basic coloration is a darkish brown with another prominent whitish band separating the apical and basal parts of the wings. The veins have whitish scales and, with their fine-crossed connections, form a grid that is reminiscent of a spider web or map, hence the names *Araschnia* (spelling mistake from Greek “arachne” meaning spider) or Map butterfly [33] (Fig. 2).

Figure 2. Adult *A. levana* mud-puddling on a moist forest floor with the wings in resting position. Picture is a courtesy of Norbert Schenk).

2.2 Photoperiodism and Temperature

The longstanding assumption that temperature was the abiotic factor responsible for the phenotypic switch in *A. levana* was elegantly refuted by Müller (1955). Before presenting his findings, he remarked on the matter of temperature: “*In the field, it cannot be the cause, as it is on average about the same during the crucial developmental phases of the two generations*” [34]. In his experiment, he assigned offspring from both generations into four treatment groups. He then reared one group from each parental generation under two separate light regimes but otherwise identical conditions, in particular, at the same temperature. The two groups exposed to more than 16 h of light per day developed exclusively into subitaneous (i. e. without diapause; butterflies emerge from the pupa after approximately 14 – 18 days) pupae and thus into the *prorsa* form, whereas the two groups reared under short-day conditions (8 h of light per day) invariably developed into diapause pupae and thus into the *levana* form, regardless of the parental generation [34].

Because the potentially modifying influence of temperature was still unclear at this point, Müller subsequently investigated the effects of the distinct light regimes at two different temperatures: 20 and 30 °C [35]. At 20 °C, all larvae developed into the *levana* form if exposed to fixed light regimes of 4–15 h per day, whereas there was an inverse relationship between light duration and the proportion of *prorsa* individuals in the same photoperiodic range when larvae were reared at 30 °C. Specifically, longer photoperiods led to a steady decline in the proportion of *prorsa* individuals. When the day length was 6 h, the proportions of *prorsa* and levana adults were approximately equal, but when the day length was 12 h, the ratio was 3% *prorsa* to 97% *levana*. However, at both temperatures, a switch occurred between day lengths of 15.5 and 16.5 h. More than 16.5 h resulted in the complete inhibition of *levana* development, yielding 100% *prorsa* adults. When the photoperiod falls below 15–16 h (daylight lasts for 15.5 h between the middle of May and late July, in this study at app. 51° 8’ N 11° 10’ E), temperature must, therefore, be used as an additional cue to determine whether direct development or diapause is preferred.

Later work showed that the critical photoperiod is longer at lower temperatures, with a temperature regime of 15 °C shifting the photoperiod needed for direct development towards longer days [36]. Exposure to 16 h of daylight at this temperature still committed little more than half of all larvae to direct development. These findings indicate that longer photoperiods are required to induce direct development at lower temperatures, whereas shorter photoperiods are sufficient at higher temperatures, although the latter only applies to

photoperiods of less than 12 h. In an ecological context, this means that warmer temperatures tip the risk–benefit ratio in favour of direct development (betting on continued beneficial conditions), whereas cooler temperatures have the opposite effect (betting on an overwintering strategy). The increase in critical day length is more likely to prevent direct development and consequently the formation of a potential third butterfly generation. In Central Europe, this reflects conditions in the wild, where *prorsa* larvae develop from mid-May to mid-July at day lengths of at least 16.5 h and mean temperatures of 15–18 °C [34–36] (Figure 3). In contrast, *levana* larvae develop in August and September, when the mean temperature is initially ~19 °C but quickly declines to ~11 °C by the beginning of October [34]. Day length during the same period declines from 15.5 to 11.5 h (Fig. 3). This suggests that the photoperiod takes precedence as the key climate predictor with the ultimate decision-making role, but it can be modified and fine-tuned by prevailing temperatures.

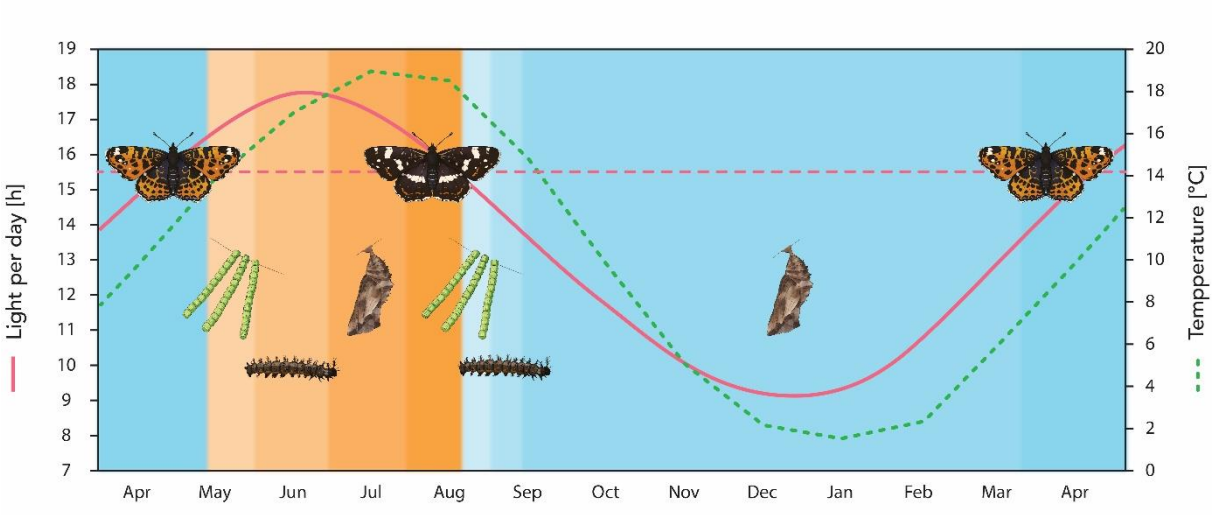

Figure 3. Annual life cycle and phenotype succession of *A. levana* depending on photoperiod and temperature. Average values for day length (solid pink line) and temperature (dotted green line) correspond to the administrative district of Giessen, Hesse, Germany. Long day lengths (>15.5 h) and high temperatures (larval development from spring to midsummer) result in pupal subitaneous development and the expression of the adult *prorsa* form, emerging in summer. Conversely, short day lengths (<15.5 h) and low temperatures (larval development from late summer to early autumn) result in pupal diapause and the development of the adult *levana* form, emerging in spring. The brown colour gradient represents *prorsa* development, blue colour gradient represents *levana* development. The dotted red pink corresponds to the threshold day length of >15.5 h per day, below which *levana* development becomes increasingly likely. The developmental trajectory is not affected by environmental cues during embryonic development (green egg towers). For details, see text. Reproduced from [14].

The development of subitaneous or diapause pupae depends on the day length in the mid (but not early or late) larval stages, with a critical photoperiod of ~15.5 h [35, 37]. These findings have been modified by a more recent study [38], although direct comparisons are not possible because the data provided in the original studies are not precise. Larvae were reared at 23 °C under short-day conditions (12 h photoperiod) or long-day conditions (20 h photoperiod), and subsets were transferred between conditions in both directions in each of the five instars. Transfer from long-day to short-day conditions during the first three larval stages invariably led to diapause development, whereas the transferred fourth instar larvae yielded ~40% *prorsa* adults and transferred final-instar larvae yielded 100% *prorsa* adults [38]. A fixed number of long days (18 h photoperiod) is necessary for direct development, representing up to half of the entire larval development period (~23 days) at 20 °C [36]. This indicates that there is a point of no return during the fourth-instar stage beyond which diapause development is no longer possible; an advantageous strategy given the additional preparations needed to survive winter, such as general physiological changes, the formation of denser tissues, and the thickening of the cuticle [38]. It also implies that natural selection favours a decision made late in larval development, when larvae have the most current information about their position in the season. Friberg and colleagues [38] also showed that transfer from short-day to long-day

conditions during the first four larval stages led reproducibly to 100% *prorsa* adults, and even when switched during the final larval stage, there was still a 50% likelihood of *prorsa* development. In nature, longer day lengths correspond to spring and early summer (Fig. 3). In years with early-season high temperatures, imagoes may emerge ahead of time, as reported in 1990 in the south-west of Germany [39]. After mating and oviposition, larval development may thus start when day lengths are below the critical photoperiod for *prorsa* development of >15.5 h. This threshold is likely to be even higher, given that the mean early-season temperatures are still comparatively low even in unusually warm years and low temperatures require longer day lengths in order to achieve direct development (contrast with the modification of critical day length by low temperatures, as discussed above). Early emerging larvae may therefore benefit from identifying and responding to a "switch from short days to long days" and accordingly favour the subitaneous pathway over diapause throughout larval development.

Temperature can also modify photoperiod effects at later stages of development. As with many nymphalids and other butterflies, the influence of higher or lower temperatures during early pupal development can lead to a brightening or darkening of wing colour patterns in both *Araschnia* generations. However, a complete change from *levana* to *prorsa* or vice versa is not possible [40]. For subsequent development, only the temperature is relevant because both pupae and imagoes are profoundly insensitive to day length. During diapause, pupae must undergo a cool period (0–10 °C) lasting at least three months before eclosion can be induced by spring temperatures of 12–24 °C [37].

2.3 Photoperiod-Specific Plastic Responses: The Extent of Phenotypic Disparity

2.3.1 Wing Pattern and Colour

The most obvious differences between the seasonal phenotypes are the wing pattern and the colour displayed by the spring and summer imagoes of *A. levana*, raising the question of why it is beneficial to be orange in spring and blackish in summer? The two predominant hypotheses regarding seasonal polyphenisms in lepidopteran wing patterns/ colours are that they are a thermoregulation response (temperate-zone regions) or a defence mechanism against predation (tropical regions) [41]. Regarding the first hypothesis, in temperate Pierid butterflies, that are mainly white with blackish melanized patterns, melanisation differences between generations relate to better thermoregulation according to the season [42]. Such seasonal phenotypic plasticity allows individuals to produce the level of melanin necessary to maintain activity at the temperatures encountered when they emerge. However, this widely accepted hypothesis does not readily apply to *A. levana* imagoes because their darker summer phenotype lives at elevated environmental temperatures when compared with the orange spring phenotype (Fig. 3). A possible explanation may be derived from the different life histories of spring and summer phenotypes. The *prorsa* form has been suggested to be a "dispersal phenotype" due to a comparatively better flight capacity (but see below, section 2.3.2) [43], and the species is known to have actively expanded its range especially during recent decades where both westward and northward expansions have been recorded [44]. However, butterflies are sensitive to convective cooling during flights and frequently need to interrupt flights by bouts of basking. *A. levana*, like most butterflies, does this by dorsal basking to absorb solar radiation and consequently heat their thorax [41]. Perhaps the *prorsa* form, in addition to differences in body design (flight capacity), boosts its range expansion capabilities through a more efficient heat absorbance phenotype. Another possible explanation for seasonal polyphenism in *A. levana* butterflies could be differing seasonal predation by insectivores like birds or dragonflies, resulting in adaptations related to camouflage, aposematism or mimicry. The evidence here is contradictory though, and to date only two studies have investigated predation in *A. levana*. One study evaluated attack rates by great tits (*Parus major*) on artificial specimens of the two forms in a laboratory setting with two substrates imitating spring (dry,

brown tree leaves resembling a forest floor in the spring) and summer (fresh, green nettle leaves resembling forest clearings in summer) environments. The *levana* form was much better protected on the dead leaves substrate than the *prorsa* form, but no such a difference was observed on the green substrate of fresh nettle leaves, pointing towards a camouflage benefit for the spring phenotype under spring conditions [41]. However, another study using blue tits *Cyanistes caeruleus* as the predator, found no evidence that *A. levana* is either warning-coloured or that its seasonal polyphenism is an adaptation to avian predation [42]. Besides being a product of direct selection pressures like thermoregulation or predation, seasonal phenotypes might also be a (non-adaptive) consequence of other evolutionary drivers that are developmentally coupled to pattern or pigment formation.

2.3.2 Morphology

In addition to the visually striking wing polyphenism, the *A. levana* adult phenotype is affected by the photoperiod in other, more subtle ways, including biomechanical design. The *prorsa* imago is larger than the *levana* imago in absolute terms (larger, longer, less pointed wings, heavier thorax) and has a higher thorax muscle ratio, whereas the *levana* imago has a higher wing loading (i. e., fresh body mass divided by wing area) and a higher relative abdomen mass [43]. This is likely advantageous because *A. levana* tends to remain in the native habitat during the spring and invests in reproduction, whereas *prorsa* disperses and expands its range in search of new habitats over the summer [43].

2.3.3 Immunity

Immunological polyphenisms are known from coleopterans, orthopterans, odonatans and hymenopterans in particular [45–50]. For lepidopterans, transcriptomic analyses suggested that the seasonal forms of *A. levana* in the larval stage might display differential immunocompetence [21]. Hence, we investigated if these transcriptomic variances could indeed translate into photoperiod-dependent differences in innate immunity when challenged by an entomopathogen [22].

2.3.3.1 METHODS

Two groups of larvae were reared on stinging nettle plants (*Urtica dioica*) under long- (20-h photoperiod) and short-day (8-h photoperiod) conditions respectively. In the fifth instar, larvae were injected with Gram-negative *Pseudomonas entomophila* (Green fluorescent protein (GFP) strain) or phosphate buffered saline (PBS) as a control. We tracked survival until pupation, and observed pupal development for 3 weeks to ensure the process was normal. We also measured bacterial clearance after administering a sublethal dose of *P. entomophila* by sampling haemolymph between 3 and 48 h post injection from separate sets of larvae. The haemolymph was transferred into pre-cooled Eppendorf tubes containing PBS. Samples were stored on ice and serially diluted in PBS before pipetting onto lysogeny broth agar plates supplemented with rifampicin for selection. The first dilution field with clearly distinct and GFP-labelled colonies was then used to count clones under a fluorescence stereomicroscope allowing us to calculate the number of freely circulating *P. entomophila* cells (per microliter haemolymph). After haemolymph extraction, larvae were flash frozen in liquid nitrogen and stored at −80 °C for subsequent gene expression analysis.

The following genes encoding members of the following four lepidopteran-specific antimicrobial peptide (AMP) families were assessed: *lebocin*, *attacin*, *hemolin*, and *gloverin*. The expression levels of these genes were measured at the six sampling time points (3, 6, 9, 12, 24 and 48 hrs) by a two-step reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). We isolated total RNA and assessed quantity and purity of the samples using a NanoDrop spectrophotometer followed by first-strand complementary DNA (cDNA) synthesis. Primers for the RT-qPCR were designed using the Primer3 software and available primer pairs were selected based on the lowest number of potential self-annealing structures and primer loops. Gene-specific primers were designed based on nucleotide sequences derived from

previously published transcriptomic data. The ribosomal protein L10 gene (RPL10) was used for normalization and PBS controls served as calibrator. We conducted the RT-qPCR using an Applied Biosystems® StepOnePlus™ Real-Time PCR System on 96-well plates with SensiMix™ SYBR® from the No-ROX Kit as the reporter mix. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method modified for amplification efficiencies as per the manufacturer's protocol.

2.3.3.2 Results and Discussion

We found a difference in immunocompetence between larvae photoperiodically primed for subitaneous development (*prorsa* form) and diapause development (*levana* form), which confirmed our first hypothesis. Specifically, following infection with the entomopathogenic bacterium *P. entomophila,* the *levana* larvae survived significantly longer than *prorsa* larvae, the antibacterial activity in the *levana* haemolymph was more potent, and genes encoding AMPs (*lebocin*, *attacin*, *hemolin*, and *gloverin*) were expressed at higher levels in *levana*. Another study subsequently found that final-instar *levana* larvae also produce higher levels of phenoloxidase (PO) and experienced greater lytic activity than *prorsa* larvae [51]. These results suggest a trade-off between immunity and other energetically costly traits. The seasonal adaptations of the distinct phenotypes of *A. levana* in terms of immunology can be explained plausibly by selective advantages. A more robust immune system in larvae committed to diapause as shown by us and Freitak *et al.* may benefit the pupae that are exposed to pathogens and parasites for extended periods as they over winter.

2.4 Regulation of Phenotype Expression

2.4.1 Hormones

Having determined the role of photoperiod and temperature on the polyphenism in *A. levana*, researchers turned their attention to the translation of these signals at the physiological level. In other lepidopterans, adult development in both subitaneous and diapausing pupae is known to be triggered by the release of the prothoracicotropic hormone (PTTH) from the brain, followed by the release of ecdysone from the prothoracic glands [52]. However, preliminary experiments in *A. levana* and the closely related species *A. burejana* indicated that morph determination depended exclusively on the timing of ecdysteroid release [53, 54].

In *A. levana*, subitaneous development is characterized by the accumulation of ecdysteroids in the fourth- and final-instar, leading to an earlier *prorsa* pupal molt [55]. In subitaneous *prorsa* pupae, ecdysteroid levels peak at the mid-stage and decrease towards the imaginal molt, whereas the ecdysteroid content of diapausing *levana* pupae is low at the time when *prorsa* pupae develop into adults. In both morphs, the titre of juvenile hormone (JH) is high in the middle of the fourth-instar stage but declines thereafter, falling to undetectable levels two days into the final larval stage. In *levana*, JH remains undetectable for the rest of larval development, but in *prorsa* larvae there are two JH peaks before the pupal molt [55]. It seems plausible that these high JH titres trigger ecdysteroid release from the prothoracic gland during the pupal stage in subitaneous *prorsa* individuals (Fig. 4).

Koch (1987) showed that when adult development was initiated by 20-hydroxyecdysone injection 3 days after pupation, the *prorsa* phenotype was produced. Conversely, when adult development was induced after 5 days or even post diapause, the *levana* form emerged [56]. In consecutive studies, adult *prorsa* development was shown to begin as early as 1 day after pupation, followed by a transitional period of another 2 or 3 days during which, if triggered, intermediate wing coloration would develop, followed thereafter solely by *levana* development. In the latter case, the ecdysteroid level remains low for a cold period lasting more than 3 months, then rises to induce the development of *levana* adults [37, 55, 57] (Fig. 3). These findings suggest that the determination of wing colour pattern changes gradually during the

first week of pupal life, beginning with the summer morph and then changing into the spring morph [56].

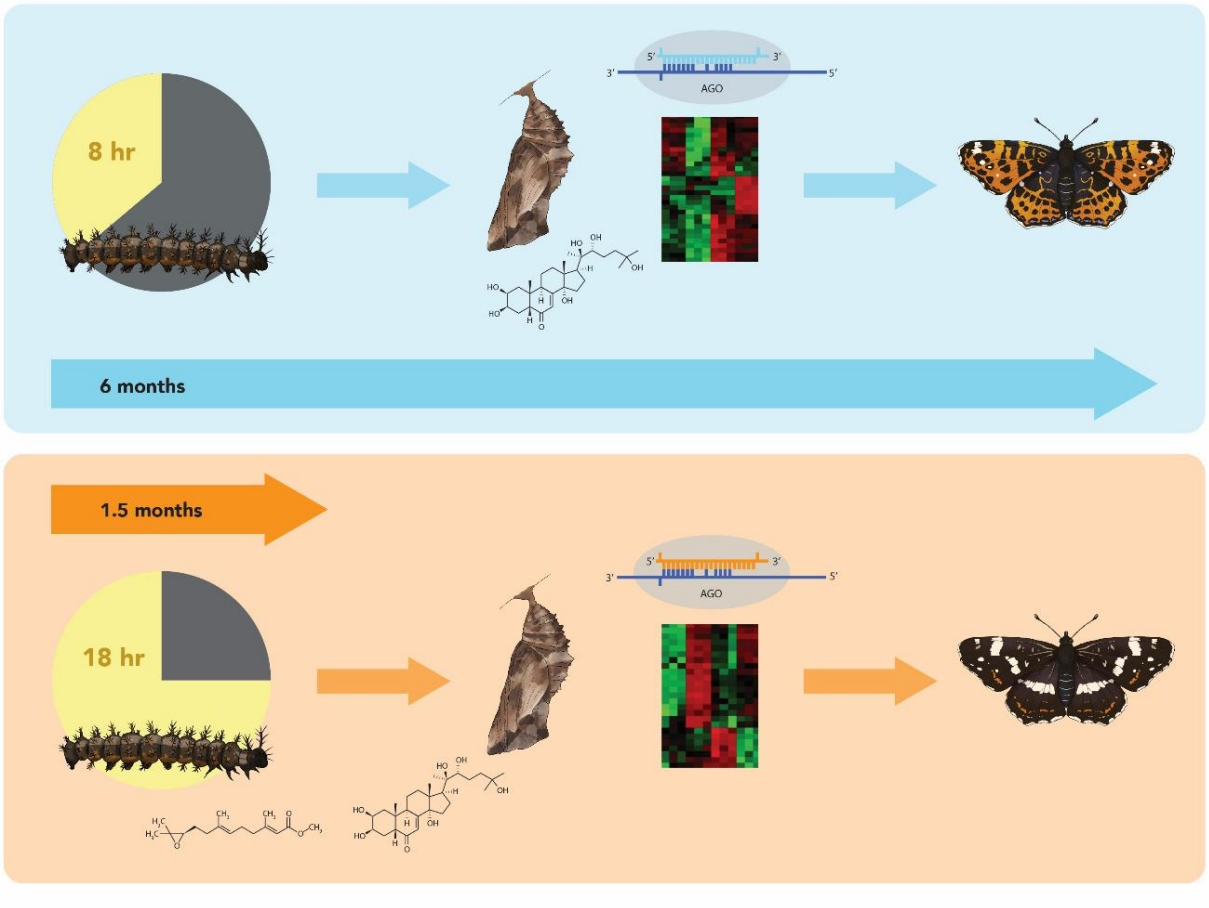

Figure 4. Model of development modes of *A. levana* according to photoperiod and temperature. At 20 °C *A. levana* develops directly (*prorsa* summer phenotype) if larval development occurs under long-day conditions (18 h photoperiod, lower half, life cycle lasting app. 1.5 months [thick orange arrow]) but switches to diapause development (*levana* spring phenotype, upper half, life cycle lasting app. 6 months [thick blue arrow]), if short-day conditions (8 h photoperiod) prevail. In the *prorsa* development path, juvenile hormone peaks just prior to pupation, followed by 20-hydroxyecdysone release within three days after the pupal molt. This initiates a gene regulation (e.g., by micro-RNAs, which are short non-coding RNAs of app. 22 nucleotides in length, that mediate gene silencing by guiding Argonaute (AGO) proteins to target sites in the 3' untranslated region (UTR) of mRNAs [58]) and expression profile (symbolized by microarray) that results in direct metamorphosis onset and development of the adult *prorsa* phenotype. In the *levana* development path, the juvenile hormone signal is absent in the last instar and 20-hydroxyecdysone is not released until a cold period lasting at least 3 months has passed. Then gene expression results in the initiation of the imaginal molt and the adult *levana* form emerges. Reproduced from [14].

In other words, the ultimate phenotype depends on the time at which adult development is initiated by the release of 20E. The authors also found that both phenotypes could form even in pupae from which the brain-corpora cardiaca-allata complex had been surgically removed, as long as ecdysteroids were injected at the appropriate time. Accordingly, no brain-derived factors like PTTH are required, and the polyphenism in *A. levana* is exclusively regulated by the timing of the 20E release. Koch and Bückmann (1987) also showed that both seasonal wing phenotypes, as well as intermediary forms, can (at least experimentally) be produced by pupae that have experienced either long or short larval photoperiods. They concluded that seasonal wing coloration is not immediately affected by the action of day length and that photoperiod only governs pupal diapause or subitaneous development. They postulated a common regulatory mechanism based on the timing of ecdysteroid secretion, which thereby specifies the duration of the pupal stage as well as the adult wing phenotype.

2.4.2 Circadian Clocks and Epigenetics

In physiological terms, *A. levana* larvae must quantify the light they receive in some manner in order to realize one of the two seasonal phenotypes. It is unclear precisely how this photoperiodic information is perceived, stored, and acted upon during the development of insects [59], although a theoretical framework has been proposed [60]. The first step is light perception by photoreceptors, which probably involves stemmata or extraretinal photoreception, followed by signal transduction [61]. In the brain, a photoperiodic clock responsible for timekeeping measures the hours of darkness in the diurnal cycle, and this mechanism appears to be directly or indirectly sensitive to temperature. Third, a counter keeps track of the number of times the "long-day threshold" has been crossed to control which developmental pathway should be initiated [57, 61, 62]. Eventually, the initial photoperiodic signal is converted into a neuroendocrine signal, consistent with the presence or absence of JH peaks in the final-instar larvae. At the onset of the pupal stage, the JH signal is then relayed to target tissues such as the developing wings via the release (presence or absence) of ecdysone. If ecdysone is released within the first couple of days post-pupation, it potentially inhibits a default "*levana* gene expression profile" that leads to morph-specific activities like the production of more red pigment ommatins in the wings. Chromatin regulation by epigenetic mechanisms such as histone modification, DNA methylation, and the expression of non-coding RNAs may subsequently fix the developmental program on the molecular level, leading to a determined phenotypic outcome [60]. A recent genome-wide study in the model lepidopteran *M. sexta* elucidated for example, that complete metamorphosis is associated with profound transcriptional reprogramming mediated by epigenetic modifications like DNA methylation, involving approximately half of all the genes in this species [63].

Hence, in insects, epigenetic mechanisms, such as the ones outlined above, potentially enable fast and flexible changes in the expression of transcriptional programs associated with the production of alternative phenotypes (but see [64]). miRNAs in particular, are involved in practically all cellular processes from cell differentiation to homeostasis to development through the action of the miRNA-induced silencing complex (miRISC), which promotes repression at the transcriptional level by degradation of targeted mRNAs [58]. In *A. levana,* pre-pupae destined either for diapause or subitaneous development were shown to possess unique transcriptomic profiles consistent with season-specific adaptations [65]. Hence, we investigated whether miRNAs contribute to the regulation of transcriptional reprogramming associated with the seasonal polyphenism in *A. levana* using microarrays containing more than 2,000 conserved insect miRNA sequences [23].

2.4.2.1 METHODS

Two groups of larvae were reared on stinging nettle (*Urtica dioica*) cultivars under long- (18-h photoperiod) and short-day (8-h photoperiod) conditions respectively. Total RNA for microarray analysis was then isolated from four last-instar larvae and four 1-day-old pupae for each group and the miRNA microarray analysis was subsequently performed by LC Sciences, Houston, TX, USA, as previously described [66]. Briefly, RNA samples of long- and short-day larvae and pupae were size fractionated using a YM-100 Microcon centrifugal filter and the small RNAs (< 300 nt) isolated were extended with a 3′-polyadenylate (poly(A)) tail using polyadenylate polymerase. Subsequently, the poly-A tail was ligated to an oligonucleotide tag labelled with one of two fluorescent dyes for later fluorescence detection in dual-sample experiments (i. e. simultaneous hybridization of two samples on a single array chip). Hybridization of microarrays was conducted overnight on a µParaflo microfluidic chip equipped with a micro-circulation pump [67, 68]. Detection probes (generated by in situ synthesis using photogenerated reagent chemistry) on the chip were chemically-modified oligonucleotides complementary to a target miRNA or a control RNA. Probes also contained a polyethylene glycol spacer segment, which separated the coding segment from the substrate. Detection probes were chemically-modified to balance the melting temperatures for hybridization, which was carried out in sodium-chloride-sodium-phosphate ethylenediaminetetraacetic acid buffer containing 25% formamide at 34 °C. Following this, Cy3 and Cy5 tags were introduced into the microfluidic chip for dye

staining. Fluorescence images were collected using a laser scanner and digitized using an image analysis software. Data were processed by first subtracting the background and then normalizing the signals using a locally-weighted regression approach (LOWESS filter) [69]. For the two-colour experiments, the ratio of the two sets of signals (log2 transformed and balanced) and the p-values of the Student's t-test were calculated. Differential expression was judged to be significant at $p < 0.01$.

To cross-validate the expression of miRNAs of special interest (miR- 2856-3p), we used a two-step RT-qPCR adapted for miRNAs. According to the manufacturer's protocol, we synthesized cDNA with the miScript II RT kit (Qiagen). Small RNA-enriched total RNA was then 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. Combining polyadenylation with the universal tag prevents the detection of genomic DNA by the miScript primer assays. Primer design for the selected miRNAs was conducted using the miScript miRNA product-design webpage (Qiagen). We normalized miRNA expression levels against miR-2491-3p, since it was uniformly expressed across all samples. For RT-qPCR we used a Biorad (CFX 96) Mx3000P system and the following protocol: 15-min incubation step 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. We used the following sequences for miRNA primer design: miR-2491-3p: CAACAACAGCAGCAGCAA; miR-2856-3p: ACAUUCGAGAACCGUAAGACAA.

MiRNA analysis and respective target prediction was performed by screening our transcriptomic database [65] with the sequence alignment editor BioEdit to identify open reading frames (ORFs) in all contigs. The contig sequences' 3′ ends outside of the identified ORFs were considered to be potential 3′ UTRs and were searched for complementarity with differentially expressed miRNA sequences detected in the microarray analysis. We defined miRNAs as "expressed" when the average signal in the microarray was above background in at least two different pools of the same treatment group. Gene Ontology categories of identified contigs were listed by consulting a previous report [21]. The structure of miRNA–mRNA duplexes was confirmed using the RNAhybrid tool provided by the Bielefeld Bioinformatics Server [70].

2.4.2.2 Results and Discussion

We identified multiple miRNAs and their targets which were differentially expressed in final-instar larvae and 1-day-old pupae according to the photoperiodic rearing regime. This indicated the potential involvement of these miRNAs in the regulation of genes mediating the seasonal polyphenism [23], which confirmed our second hypothesis. Because miRNAs silence gene expression, they may inhibit genes required to generate the *A. levana* "standard morph" in response to environmental cues. Phylogenetic analysis suggests the *levana* morph is most likely the plesiomorphic (primitive) one, whereas the *prorsa* morph is apomorphic (derived) [71]. JH release in the final-instar larvae and/or early ecdysone release in the subitaneous pupae may, therefore, modify the expression of miRNAs that regulate the expression of genes necessary for both diapause and the formation of the *levana* phenotype (Fig. 4). The examples below indicate how this may proceed at the epigenetic level. For diapause maintenance, miR-289-5p (among others) is thought to silence the expression of genes related to metabolic processes during diapause in the flesh fly *Sarcophaga bullata* [72]. In *A. levana*, this miRNA was upregulated in larvae destined for diapause compared to those primed for subitaneous development, although there was no differential expression in the pupae according to our analysis. If we assume that the properties of miR-289-5p as a diapause regulator are evolutionarily conserved, the initiation of a gene expression profile responsible for metabolic arrest begins prior to pupation at the very end of the larval stage in *A. levana*. A link to the JH signal in subitaneous final-instar larvae is also likely because its presence coincides with the significantly reduced expression of miR-289-5p at this stage. If true, JH may inhibit the expression of this candidate metabolic suppressor, which then ultimately leads to subitaneous development and vice versa.

The regulatory mechanisms that control the duration of diapause and the determination of the adult phenotype remains unclear, but preliminary and yet inconclusive experimental evidence points towards involvement of a diapause duration clock protein. In *B. mori*, the time interval measuring enzyme-esterase A4 (TIME-EA4) belonging to the adenosinetriphosphatase (ATPase) family, measures time intervals and functions as a clock in diapausing eggs. It is crucial to determine the moment when diapause should be broken [73]. In *A. levana*, an ortholog of TIME-EA4 – named diapause bioclock protein (DBP) – was found to be strongly induced in *levana* pre-pupae destined for diapause [21]. Our subsequent *in silico* target prediction provided evidence that *DBP* expression is regulated by miR-2856-3p [23]. However, this miRNA was also strongly upregulated in final-instar *levana* larvae when compared to subitaneous *prorsa* larvae, and the highest levels were reached in pupae representing both developmental pathways. These results suggest that DBP regulation via miR-2856-3p in the diapause termination context is probably less important. Also, it was found that the temporal expression of the *EA4* gene coding for TIME-EA4 in *B. mori* was unrelated to embryonic diapause termination [74]. Moreover, a transient ATPase activity of the enzyme coincides with the termination of diapause approximately 13 days after a conformational change in its protein structure is caused by the required cold period at 5 °C [75]. Thus, the timer ATPase activity of TIME-EA4 is probably caused by a structural modification under cold conditions, rather than a change in gene expression. Moreover, miRNAs can have hundreds of targets in insects [76] and further regulatory roles for miR-2856-3p cannot be ruled out. Interestingly, when injected with an inhibitor of miR-2856-3p, final-instar subitaneous *prorsa* larvae displayed an intermediate *prorsa-levana* adult phenotype in 5% of tested specimens (Fig. 5). Polyphenism-determining miRNAs have been reported in both hemimetabolous and holometabolous insects [77, 78]. Thus, it is conceivable that polyphenism in *A. levana* may also be controlled by a single miRNA master switch located directly upstream of the phenotype effector pathways, in which miR-2856-3p plays a key role but cannot be a sole determinant. Most likely, other epigenetic regulators are also involved.

In conclusion, these findings suggest a complex regulatory system controls diapause initiation, maintenance and duration, as well as the determination of the adult phenotype, with miRNAs such as miR-289-5p and miR-2856-3p as important regulators. However, the effects are not always consistent with a straightforward mechanism based on the inhibition of target effectors. Future research should focus on the functional analysis of miRNAs and regulatory proteins, which will be facilitated by the availability of genomic and transcriptomic data as well as miRNA target predictions for both spring and summer generations [23, 65, 79]. Moreover, CRISPR/Cas9 genome editing is now feasible in lepidopteran species, making gene knockout studies in *A. levana* a promising approach [80]. Even though we still lack a complete picture of the regulatory network, the examples discussed above strongly suggest that both hormones and epigenetic mechanisms control the integration of environmental signals in *A. levana* to generate specific seasonal phenotypes.

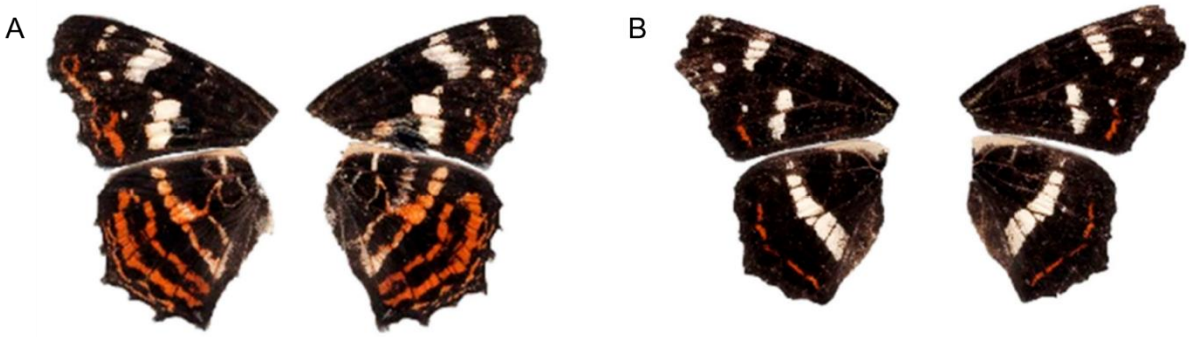

Figure 5. Intermediate and regular *prorsa* wing phenotypes of *A. levana*. The intermediate phenotype (A) was generated by injecting an inhibitor of bmo-miR-2856-3p (*B. mori*), whereas the normal phenotype (B) developed following the injection of PBS (control). Reproduced from [23].

3. Trans-Generational Plasticity in Lepidopterans

3.1 The Transgenerational Phenotype

Transgenerational plasticity (TGP) was previously put forward as a possible mechanism which might facilitate fast adaptation to environmental alterations. Briefly, the parental generation is thought to precondition offspring to a certain environment, thus creating substantial deviations in the reaction norms of the subsequent generation(s). Adaptive TGP should evolve when environmental conditions in the parental generation consistently predict the environmental conditions in the offspring generation. Various ecological factors can trigger TGP responses in animals; for example, when predation risk in the maternal environment is high, this can result in the production of improved offspring defenses. Correspondingly, temperature fluctuations may entail transgenerational adaptation of growth, swimming, and foraging performance. If access to nutritional resources changes, it can alter life-history traits in offspring such as age at maturity or reproductive success [81].

3.2 Trans-Generational Immune Priming

Invertebrate immune priming, a special case of TGP, refers to improved protection of the host when a specific non-lethal pathogenic or parasitic challenge occurs for a second time, which would otherwise have been lethal. This phenomenon resembles adaptive immunity in vertebrates [82]. However, invertebrates lack the machinery of adaptive immunity in the narrow – classical – sense, namely lymphocytes and antibodies [83]. Instead, their innate immunity rests on receptors encoded in the germ-line that recognize generic conserved pathogen epitopes like peptidoglycan (PGN) or lipopolysaccharides (LPS) [16, 84]. Nonetheless, there is increasing evidence that the invertebrate innate immune system can generate immune responses involving non-specific or even specific memory (i. e. priming or acquired immunity), both within and across generations [16, 83, 85].

Invertebrate immunity can be illustrated by considering the immune system of insects. Briefly, they fight pathogenic insults by deploying effective immune responses, which are brought about, e. g. by the midgut, fat body, salivary glands and haemocytes. Pathogens invading the insect are identified by the insect immune system when pathogen-associated molecular patterns (PAMPs) bind to pattern recognition receptors (PRRs) of the host. Consequently, immune signalling pathways are activated and boost the immune response, e. g. by inducing the production of AMPs and activating other effector pathways. The most prominent immune signalling pathways are the Toll, Imd, Jak/Stat, JNK, and insulin pathways, and all respond differentially to particular pathogens. Once activated, immune pathways entail the eradication of pathogens through lysis, phagocytosis, melanisation, cellular encapsulation, nodulation, RNAi-mediated virus destruction, autophagy and apoptosis. Notably, these effector mechanisms are not mutually exclusive [86].

As pointed out, formerly it was believed that only lymphocytes and antibodies of jawed vertebrates could confer both memory and specificity – i. e., were adaptive. However, there is no reason why these traits should be exclusively coupled to the machinery of the vertebrate's immune system. Rather, immune memory should be defined as an immune system's ability to store or use the information of a previously encountered antigen or parasite upon secondary exposure, regardless of the involved agents [83]. Specificity can be defined as the degree to which the immune system differentiates between (recognizes) different antigens [83]. Generally, it is believed that adaptive immunity uses somatic diversification processes (e. g. alternative splicing of the antibody and lymphocyte receptor genes) to increase the receptor range beyond the limitations established by the fixed number of germ line genes [83]. Thus, a prime candidate for invertebrate adaptive immunity is the Down syndrome cell adhesion molecule (Dscam), which is speculated to be a hypervariable PRR in both crustacean and

insect immunity. As a membrane-bound member of the Immunoglobulin superfamily, it can trigger phagocytosis. However, a haemolymph-soluble Dscam has also been implicated in opsonisation. It has been reported that alternative splicing of *Dscam-hyper variable* could lead to the expression of 38,016 potential isoforms in *Drosophila melanogaster* and 15,390 potential isoforms in *B. mori*, thus conceivably enabling specific pathogen recognition and memory. Notably, these ideas still need to be empirically validated [87].

Conceptually therefore, it is perhaps not so astounding that, even without antibodies – which in vertebrates can be transferred from mothers to offspring – both maternal and paternal effects on the immunity of offspring occur in invertebrates. This concept has been termed as trans-generational immune priming (TGIP). It denotes the vertical transfer of the parental immunological experience to the offspring [16]. Many studies have scrutinized this phenomenon and have produced quite a diverse picture of TGIP. It occurs in various invertebrate taxa such as bivalves, nematodes, crustaceans and insects. In the latter it has been demonstrated in orthopterans, coleopterans, hymenopterans, dipterans and lepidopterans, but not in hemipterans [16].

Evolutionary drivers behind TGIP have not been conclusively established but theoretical work has put forth life-span, dispersal capacity and pathogen virulence as reasonable predictors of parental investment in offspring immunity [88]. Firstly, if the host is short-lived, it is not likely to encounter the same pathogen more than once and should consequently refrain from investing in memory. Secondly, significant investment in parental immunological transfer is only likely to evolve in philopatric species or, should migration occur between populations, in species with considerable cross–immunity. Thirdly, pathogens with very low or very high virulence should select against TGIP. Under highly virulent conditions, investment in a resistance mechanism that will never be expressed is evolutionarily pointless, since infected individuals are most likely to die before they can reproduce. On the other hand, avirulent pathogens do not create the selective pressure necessary to evolve a transgenerational resistance mechanism. Thus, high levels of investment in TGIP are only expected to be selected for when pathogens induce an intermediate reduction in longevity (i. e. medium virulence) [88].

If TGIP is present in a given species, observed protection typically ranges from elevated effector levels (e. g. increased antimicrobial activity via PRRs and AMPs, (pro)-PO activity or haemocyte concentrations) to improved resistance manifested as decreased infection susceptibility or increased survival against homologous or even heterologous pathogenic challenges [89–93]. Notably, seldom are all of these traits expressed together – especially increased levels of AMPs and PO often seem to be mutually exclusive [89, 90, 94].

3.2.1 The cost of priming

Generally speaking, immunity is frequently traded-off against other life history traits. According to Stearns, 1992, “the evolutionary cost of immune defence relies on negative genetic covariance between a component of the immune system and another fitness-relevant trait of the organism or even another component of the immune system” [95]. The immunological cost is thought to result from antagonistic pleiotropy. Briefly, a gene with a positive influence on one component of fitness, like immune defence, negatively influences another fitness component like development time [96]. While TGIP does confer protection in many cases, it has also been demonstrated to be costly in specific settings. For example, when coleopteran *Tribolium castaneum* mothers were exposed to heat-killed *Escherichia coli*, offspring were shown to have an increased development time. Moreover, when fathers were infected with either *E. coli* or *Bacillus thuringiensis*, offspring fecundity was reduced regardless of the priming agent [91]. In lepidopterans, survival into the adult stage was decreased when both *Trichoplusia ni* parents or just the father was orally co-infected with *E. coli* and *Micrococcus luteus*, and in *M. sexta*, unchallenged offspring from *M. luteus*-derived PGN-challenged parents also displayed decreased fecundity [24, 97]. These costs are likely to significantly reduce the fitness and reproductive success of primed individuals in conditions that do not match the parental

pathogen environment. Thus, strong selection for cues that reliably predict offspring exposure risk (matched conditions) is expected.

3.2.2 Sexual diphenism in TGIP

The notion that females gain fitness through increased longevity, whilst males gain fitness by increasing mating rates, is called Bateman's principle [28, 98]. Following this line of thought, it was predicted that fitness maximization through prolonged lifespan would require, and thus select for, higher immunocompetence in females [28]. Indeed, for insects a female bias in PO activity plus a weak sexual dimorphism in haemocyte counts in the direction of female superiority was demonstrated in a meta-analysis containing 43 studies [29]. A qualitative discrepancy in male versus female transgenerational immune phenotypes had previously been established, given that priming through the paternal route can lead to different outcomes than maternally induced TGIP (see paragraph above). However, not just the parental sex matters, but also that of the offspring. For example, when Australian field crickets (*Teleogryllus oceanius*) were injected with Gram-negative *Serratia marcascens*, nutritionally stressed nymph stage male but not female offspring displayed an increased antibacterial activity against Gram-positive *M. luteus* [99]. Moreover, LPS-injection in *Bombus terrestris* mothers led to increased antibacterial activity against Gram-positive *Arthrobacter globiformis* in offspring workers but not drones, and also decreased PO activity and encapsulation response in workers but increased it in drones [100]. However, for lepidopterans, sex-specific TGIP had not been demonstrated and so we tested the hypothesis, that – following a parental microbial challenge – there would be a differential immune phenotype (gene expression) in *M. sexta* male and female offspring.

3.2.2.1 Methods

Three groups of *M. sexta* larvae were reared on a standard artificial diet [101] drenched in sublethal doses of overnight bacterial cultures of *E. coli*, *Serratia entomophila* (both Gram-negative) or without bacteria for control purposes. For each group, development time was recorded. On a portion of larvae from each group, we sex-specifically analysed the expression of various effector genes involved in immunity, histone (de)acetylation and DNA methylation (see section 3.2.3) in the third larval stage, as a reference point for the parental generation via RT-qPCR. Once the other specimen had reached the adult stage, imagoes were group-specifically mated and the resulting offspring reared on the same – but uncontaminated – diet. Larvae were group- and sex-specifically analysed once more for the same parameters as in the parental generation, once they had reached the third instar (see [27] for details).

For the gene expression analysis, total RNA from dissected midguts was isolated and purified followed by first-strand cDNA synthesis. Primers for the RT-qPCR were designed using Primer3 and available primer pairs were selected based on the lowest number of potential self-annealing structures and primer loops. We used the ribosomal protein L3 gene (*RPL3*) for normalization and conducted the RT-qPCR using an Applied Biosystems® StepOnePlus™ Real-Time PCR System on 96-well plates with the SensiMix™ SYBR® No-ROX Kit as the reporter mix. Each assay was repeated using three biological replicates (each representing pooled RNA from five third-instar larval midguts per sex) and two technical replicates. Fold changes in gene expression were calculated for treatment groups exposed to bacteria against the corresponding control group using the $2^{-\Delta\Delta Ct}$ method [102].

3.2.2.2 Results and Discussion

As expected, both diets supplemented with bacteria delayed development in the parental generation, demonstrating the "classical" trade-off between immune defence and development speed. Notably, the effect was more pronounced in the entomopathogen *S. entomophila*, compared to non-pathogenic *E. coli*. In the offspring generation, we found sex- and treatment-specific gene expression in two out of four immune effector genes, which confirmed our third hypothesis [27]. Specifically, *gloverin* expression was moderately upregulated in female (eigth-

fold, $p < 0.001$) and highly upregulated in male larvae (23-fold, $p < 0.001$) in the *E. coli* group. This finding is consistent with a study reporting a mid-gut-specific, high *gloverin* expression in *M. sexta* fifth instar larvae following an injection with heat-killed *E. coli* [103]. In the greater wax moth *Galleria mellonella*, a diet supplemented with a mix of non-pathogenic *M. luteus* (Gram-positive) and *E. coli* elicited a *gloverin* expression in egg stage offspring of exposed parents comparable to that found in our study [104]. However, in *M. sexta*, egg stage offspring of parents injected with *M. luteus*-derived PGN showed no differential expression of immune-related genes – including *gloverin* – unless eggs were parasitized by the wasp *Trichogramma evanescens* [26]. In the latter case, *peptidoglycan recognition protein 1*, *dorsal*, *pro-plasmatocyte-spreading peptide* and *pro-PO* were upregulated four to 14-fold, and *gloverin* even displayed a 107-fold expression when compared to non-parasitised eggs of both naïve and immune-challenged parents. Certainly, differences in study design, like the type (PGN injection vs. feeding viable bacteria) or timing of priming (pupal vs. larval stage) and sampling points (egg stage vs. third instar) may explain the observed discrepancies. Alternatively, these findings may suggest that Gram-positive and Gram-negative induced TGIP may influence different arms of the transgenerational defense phenotype in lepidopterans. Gram-positive induced TGIP may set the stage for an on-demand immune response that is executed only after an immune insult like a parasitoid attack. A potential sex-specificity of this effect has not been investigated so far. Gram-negative induced TGIP, on the other hand, may result in an obligate expression of antimicrobial effectors, which, at least in *M. sexta*, seems to be more pronounced in male than in female offspring. Although not significant across sexes, our study found a clear trend that male offspring of parents challenged with *E. coli* displayed higher expression levels of immunity-related genes than females. Although apparently at odds with Bateman's principle, which states that males should give preference to reproductive success over immunity, they would need to become sexually mature in the first place. If they succumb to infections before becoming adult, the trade-off becomes irrelevant. Additionally, mothers may actively provide male offspring with high(er) levels of immune gene transcripts, in order to specifically counter a trade-off dependent disadvantage in male offspring. However, the exact mechanism(s) underlying the observed TGIP effect/s is/are still unresolved, and therefore this assumption cannot yet be conclusively answered (but see section 3.2.3).

Another study recently reported a transcriptome-wide, transgenerational differential gene expression analysis on *M. sexta* mothers and their egg stage offspring after maternal exposure (injection) to live or heat-killed Gram-negative *Serratia marcescens* [105]. The authors found a stronger upregulation of immune-related genes in embryos from mothers exposed to heat-killed compared to live bacteria. Specifically, the TGIP responses in embryos of mothers who experienced heat-killed *S. marcescens*-injections involved strong upregulation of the AMP *cecropin*, a *peptidoglycan recognition protein*, *toll-like receptor 9*, and the serine protease inhibitor *plasminogen activator inhibitor 1*. Conversely, embryos from mothers exposed to live bacteria displayed an overall downregulation across reads. The stark discrepancy between heat-killed and live injected bacteria TGIP was speculated to be due to immunosuppressive effects of *S. marcescens,* which could have precluded TGIP if the mother's immune system was compromised by the immunosuppressive compounds synthesized by *S. marcescens*. This assumption is in congruence with our own study. We found no upregulation of immune genes in *S. entomophila* group offspring. For two of the lysozyme isoforms, we even detected a male-specific downregulation. This points towards an interference of the pathogen with the transgenerational phenotype, which may impact male offspring more strongly than female offspring.

3.2.3 TGIP mechanisms

In theory, the parental immunological experience could be transmitted to (the) offspring generation(s) via different routes or mechanisms that do not need to be mutually exclusive. In fact, the specific mechanisms may vary or even complement each other depending, for example, on the host and pathogen species, the sex of the infected parent or the sex of the offspring, the developmental stage during which the infection occurred or priming was

measured, the route of infection (oral, septic wounding, injection), the pathogen type (e. g. Gram-positive vs. Gram-negative bacteria) or the existence of other stressors during infection or in the offspring environment (e. g. food availability, temperatures, toxins), to give but a few examples [16, 106, 107]. Parents could transmit signals which stimulate the offspring immune system or directly provide immune elicitors or effectors to the next generation (see Fig. 6, Scs. 1 & 3). They could also directly provide offspring with effector gene mRNAs or implement an expression profile that primes the immune system to anticipate and react more effectively to a particular pathogen exposure via epigenetic mechanisms (see Fig. 6, Scs. 2 & 4). In *T. castaneum* but also in *G. mellonella*, the maternal transfer of bacterial components to egg stage offspring had previously been established [104, 108] and we tested, if this TGIP mechanism was common – at least in lepidopterans – by assaying *M. sexta* with a similar experimental procedure. Moreover, we investigated the possible involvement of histone acetylation and DNA methylation as an additional layer in lepidopteran TGIP.

3.2.3.1 METHODS

In addition to the groups reared for the gene expression analysis (see section 3.2.2.1), a group of larvae received a diet supplemented with fluorescent particles (chemically and heat-killed *E. coli* strain K-12 labelled with Texas Red®) allowing us to visually monitor the uptake and fate of ingested non-viable *E. coli*. In the offspring generation, the fate of the fluorescent particles was traced until the egg stage.

To elucidate the potential epigenetic dimension of TGIP in *M. sexta*, gene expression levels of enzymes involved in histone (de)acetylation as well as DNA methylation were determined (see section 3.2.2.1). Moreover, levels of global histone acetylation via an enzyme-linked immunosorbent assay (lysine-specific histone H3 acetylation assay, Epigentek Group Inc.) and global DNA methylation via liquid chromatography–mass spectrometry (LC–MS) were determined for the groups outlined in section 3.2.2.1.

To estimate the histone H3 lysine acetylation levels, midgut tissues were homogenized, cells lysed and histones extracted using an extraction buffer and multiple wash and centrifugation cycles were carried out. The protein concentration was adjusted in each well on a 96-well plate (except for blanks and the provided controls), followed by drying. Subsequently, capture- and high affinity H3 detection antibodies were added, incubated, and the amount of acetylated histone H3 then quantified by reading the absorbance on a microplate reader at 450 nm. Each assay was repeated using three biological replicates (each representing histones from five third-instar larval midguts per sex) and two technical replicates. Fold changes of relative histone acetylation were calculated for treatment groups exposed to bacteria against the corresponding control group.

For the analysis of global DNA methylation, we homogenized midgut tissues and then isolated DNA by sodium acetate precipitation, incubation and centrifugation. The pellet was washed and dried at room temperature and then dissolved in nuclease-free water, followed by measuring the DNA concentration (and purification, if necessary). DNA samples were then digested and diluted. Calibration curves were prepared by using 2'-deoxycytidine (dC) and 5-methyl-2'-deoxycytidine (^{5m}dC) dissolved in nuclease-free water. The nucleoside stock solutions were diluted in ultrapure water to yield standard solutions from 1 to 2000 pg/µl dC/^{5m}dC. We analysed the methylation state of genomic DNA by injecting digested DNA samples and standard solutions into an UltiMate 3000 HPLC system (Dionex) followed by quantification in an amaZon electron transfer dissociation ion trap mass spectrometer (Bruker Daltonics). Components were separated on a reversed phase column under isocratic conditions at a flow rate of 150 µl/min and 30°C. Cytidine residues were quantified by multiple reaction monitoring (MRM) after positive electrospray ionization using the following ion source parameters: 1.0 bar nebulizer pressure, 8 l/min drying gas, 200°C drying temperature, 4500 V capillary power and 500 V end-plate offset. Ionization and MRM conditions were optimized for fragmentation reactions for mass/charge ratios 228.1 → 112.0 (for dC) and 242.1 → 126.1 (for

^{5m}dC). Each assay was repeated using two biological replicates (each representing DNA samples from five third-instar larval midguts per sex). The data were analyzed using Compass Data Analysis v4.2 (Bruker Daltonics). Fold changes in relative global DNA methylation levels were calculated for treatment groups exposed to bacteria against the corresponding control group.

3.2.3.2 RESULTS AND DISCUSSION

Signals We demonstrated the direct transmission of maternally ingested PAMPs to the offspring generation, which confirmed our fourth hypothesis. Specifically, we determined that labelled bacteria translocate from the midgut lumen into the hemocoel, where they attach to the fat body. Subsequently, they are deposited in the ovaries and are taken up into the developing eggs (cf. Fig. 6, Sc. 1). The labelled bacteria were associated with the follicle epithelium, the ovariole wall and the vitelline membrane, and ultimately detected in oviposited egg stage offspring among yolk proteins and lipids [27]. Here they could potentially trigger an immune response that is tailored to specifically match the parental pathogen environment. It was previously shown in the Italian bee *Apis mellifera ligustica*, that maternal uptake of live *E. coli* was realised via an egg-yolk protein binding to immune elicitors which are then carried to the eggs. This yolk protein, called vitellogenin (Vg), is able to bind to different (Gram-positive and -negative) bacteria and PAMPs [109]. In the majority of insects, precursor Vg is synthesized extraovarially in the fat body and then internalized by competent oocytes through membrane-bound Vg-receptors belonging to the low-density lipoprotein receptor gene superfamily [110]. Hence, a Vg-dependent uptake of pathogen-derived signals may pose a common mechanism for many insect species to achieve potentially highly specific TGIP. Of note, another study investigated whether live *S. marcescens* injected in the late pupal stage of *M. sexta* mothers were incorporated into embryos by staining freshly sectioned eggs with a *S. marcescens*-specific fluorescent monoclonal antibody. They detected no signal in any region of the embryo, which could be due to technical or biological reasons. Firstly, they used 20 µm thick cryosections which may have obscured fluorescence. In in our study, we used 10 µm slides. Secondly, they stored sections for an unspecified period of time, which may have increasingly led to signal fading. Thirdly, in *M. sexta*, this TGIP mechanism may depend on the oral route of infection through a yet unknown intermediate step. Perhaps bacteria first need to be lysed, digested and processed followed by Vg binding to fragments or specific PAMPs, rather than whole bacteria.

Additionally, small-RNA (sRNA)-guided gene regulation may also act as a transgenerational signal (see Fig. 6, Sc. 1). Briefly, the term RNA interference (RNAi) refers to various RNA silencing pathways that use sRNAs, like small interfering RNAs (siRNA) or miRNAs, plus a member of the conserved Argonaute (AGO) and P-element induced wimpy testes (PIWI) proteins, to inactivate genes at the post-transcriptional or transcriptional level [111]. For example, siRNA-mediated RNAi pathways are known to play crucial roles in the detection and inhibition of RNA virus replication in insects [112]. And miRNAs function as switches in the regulation of innate immunity in *G. mellonella* larvae to discriminate between pathogenic and commensal strains of *E. coli* [113]. However, to date only one study has investigated the potential involvement of RNAi-mediated TGIP in insects [114]. The authors reported that antiviral TGIP in *D. melanogaster* and the yellow fever mosquito *Aedes aegypti* occurred after parental priming with different single stranded RNA viruses. The offspring were protected from infection and the response was viral-RNA-dependent and sequence specific but RNAi-independent. Instead, offspring inherited a viral DNA (vDNA) that was a partial copy of the RNA virus genome which initially infected the parental generation. Possibly, this vDNA-dependent transgenerational immune phenotype might represent a new TGIP mechanism. It could fall into a potential whole class of nucleotide-mediated TGIP (comprising short to medium sized DNA and RNA sequences), which could also explain paternal priming like that observed in *T. castaneum* [91, 115].

Another conceivable priming agent that could fall into this class are mRNAs (see Fig. 6, Sc. 2); mRNAs coding for PRRs, AMPs and other immune effectors could be transferred from the maternal ovary to the developing offspring eggs and then be translated by the embryo itself or the surrounding serosa. It is known from insects with telotrophic (e. g. Hemiptera and Coleoptera) and polytrophic meroistic (e. g. Hymenoptera, Diptera and Lepidoptera) ovaries that maternal mRNAs are synthetized by nurse cells which are then transferred to the oocytes via the trophic cord. Such mRNAs are generally involved in developmental control, but this process may equally well be used to achieve TGIP in embryos and early larval stages [16].

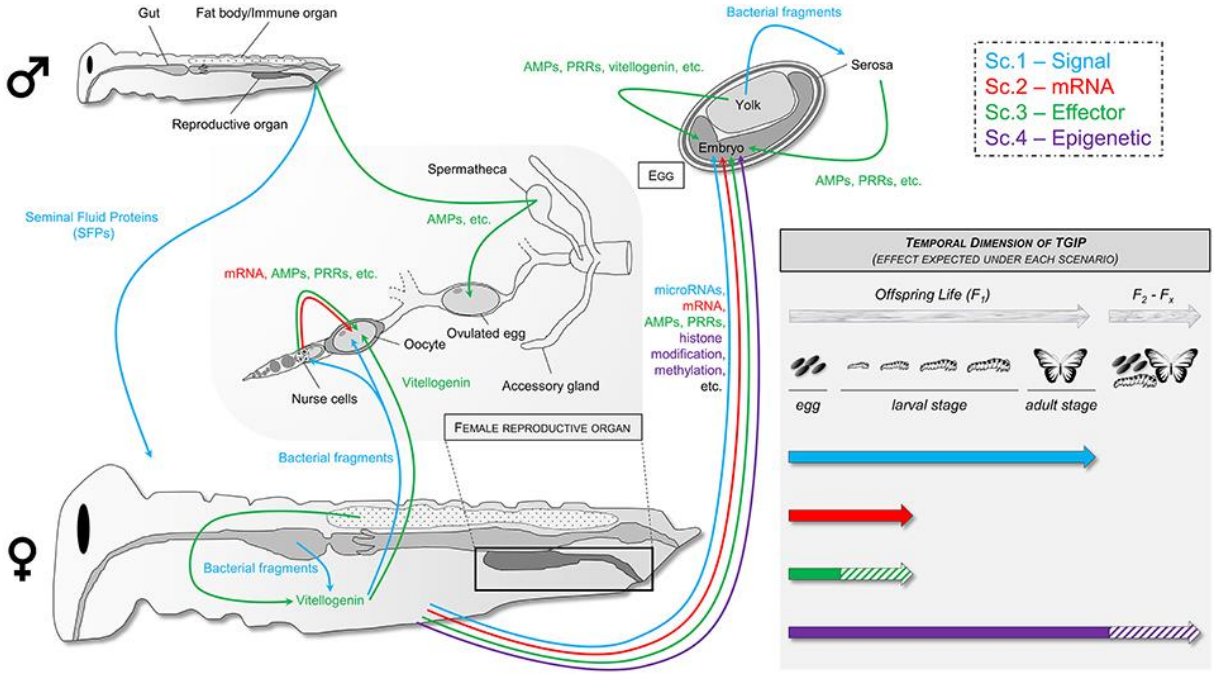

Figure 6. Theoretical mechanisms of TGIP in insects. The four displayed routes (blue, red, green, and purple) correspond to scenarios 1, 2, 3, and 4. Reproduced from [16].

Effectors Like mRNAs coding for effectors, the actual compounds themselves (PRRs, AMPs, Lysozymes, PO, Serpins etc. [116]) could equally be transferred to the offspring (see Fig. 6, Sc. 3) [16]. Increased antimicrobial activity has been demonstrated many times over (e. g. [100, 117–120]), but no study has yet shown if the involved peptides and proteins were parentally derived or produced by the offspring. Tetreau and colleagues (2019) speculated that maternal provisioning might be a passive process, i. e. diffusion or sequestration of haemolymph-derived maternal compounds into the developing eggs. Alternatively, akin to the mRNA scenario, dedicated cells, like the nurse cells, could actively provision immune compounds to the eggs [16]. The priming effect would then be expected to fade rather quickly due to dilution as offspring development progresses.

Epigenetic inheritance Beside the transfer of sRNAs or vDNA discussed above, epigenetics in the almost canonical sense – namely histone modifications and DNA methylation – may be at the heart of TGIP, especially when it comes to transgenerational phenotypes spanning multiple generations (see Fig. 6, Sc. 4). In short, post-translationally adding acetyl groups to lysines of the N-terminal histone tail can drastically reduce the electrostatic attraction between histones and the negatively charged DNA. Consequently, DNA accessibility is increased or nucleosome movement along the DNA facilitated. This promotes the binding of DNA-binding factors, such as transcription factors. Hence, enriched acetylation at numerous lysines on histone tails in gene promoters and active distal enhancer elements are often observed in actively expressed genes [121]. Two antagonistic enzyme classes are responsible for writing and erasing this post-translational histone modification: histone acetyltransferases (HATs) and

histone deacetylases (HDACs) [122]. By contrast, transcriptional regulation through DNA methylation depends on genomic context, and methylated cytosines can either promote or reduce the binding of a specific transcription factor. DNA methylation in animals happens mainly by the addition of a methyl (CH3) group at the 5′-carbon of the pyrimidine ring of cytosines residing in a 5'-cytosine-phosphate-guanine-3' dinucleotide (CpG) context [123]. The symmetrical nature of CpGs provides the basis to recreate methylation patterns after semiconservative DNA replication (maintenance), and DNA methyltransferase 1 (DNMT1) specifically restores symmetrical methylation by targeting hemimethylated CpGs [121]. In insects, CpG methylation is predominantly found in transcribed regions of genes [124] but it is unclear if *de novo* methylation generally occurs since the enzyme typically associated with it, DNA methyltransferase 3 (DNMT3), has been lost in all insect orders except for the Blattodea, Hemiptera, Hymenoptera and Coleoptera [124].

In the offspring generation, we found both differential global histone acetylation as well as DNA methylation. Both were sex- and treatment-specific, and were also partially accompanied by the differential expression of *HAT*s and *HDAC*s as well as *DNMT*s and a *methyl-CpG-binding domain protein* (MBD) [27]. These findings confirmed our fifth hypothesis. Specifically, we detected a twofold increase in global histone H3 lysine acetylation in male offspring of *S. entomophila* fed parents. By comparison, their female offspring displayed a clear acetylation decrease. No such effect was observed in the *E. coli* group offspring. In line with the male-specific acetylation response, a study experimentally observing the evolution of resistance in *G. mellonella* against *B. thuringiensis* over 30 generations found higher levels of histone H3 and H4 acetylation in the midgut, and higher levels of H4 acetylation in the fat body of resistant larvae, compared with susceptible larvae (no sex differentiation) [125]. They also reported a pattern of upregulation of *HATs* and *HDACs* in uninfected larvae that was not visible in our results. If anything, these enzymes were slightly downregulated or not differentially regulated at all. Our findings suggest that major transgenerational histone acetylation rearrangement occurs before the third instar in *M. sexta* – at least in response to a true entomopathogen – or other enzymes are involved its realization. However, since our method only quantified relative levels of global histone acetylation, gene-specific rearrangements cannot be ruled out. The higher expression in *G. mellonella* may also have been a consequence of genetic selection, usually mitigated in TGIP experiments by administering sublethal doses to parent generations to avoid mortality selection. With respect to DNA methylation, we found a universal hypomethylation in offspring of both *E. coli* and *S. entomophila* fed parents. This is again in contrast to Mukherjee *et al*. 2017, who found slightly increased total DNA methylation in the midgut and fat body of uninfected resistant larvae. In the latter organ, it was associated with a twofold increase in the expression of *DNMT1*. The general pattern in our study seemed to be a sex-specific upregulation of *DNMT1*, *DNMT2* and *MBD* in offspring from the *E. coli* group, with the higher expression in males, and a downregulation of these enzymes in the *S. entomophila* group. At this point we can only speculate why, despite unchanged or increased *DNMT1* levels, there is less DNA methylation. Perhaps, as the phenotype becomes adapted to the parental microbial environment, not only is the DNA methylation status of some loci maintained, but also in other regions 5mC is actively demethylated. Moreover, DNMT2 is mainly an RNA methyl-transferase that methylates tRNAs, which seems to protect tRNAs from stress-induced ribonuclease cleavage [126], and has been implicated in paternal TGIP in *T. castaneum* [127]. These results clearly warrant future research, for example by chromatin immunoprecipitation sequencing and bisulfite sequencing as well as transcriptomic approaches to get a global perspective of the complex dynamics of the epigenetic dimension of TGIP.

4. Conclusions

Lepidopteran phenotypic plasticity is widespread to the point where it amounts to an inherent trait of this insect order, underpinning its tremendous evolutionary success. Even though plastic responses were known from many butterfly species, we extended the knowledge of this phenomenon to encompass seasonally determined larval immunocompetence. Further, we demonstrated that mechanistically, the story of differentially expressed phenotypes does not stop at the neuroendocrine level. Instead, a new layer, namely epigenetics – at least in the form of miRNAs – seems to be intricately involved in the regulation of the photoperiodically controlled polyphenism in *A. levana*.

However, intragenerational plasticity is only one facet of the remarkable lepidopteran capacity to accommodate for adverse abiotic and biotic conditions. In *M. sexta*, we showed that the microbial experience of the parental generation is transmitted to the offspring via the transfer of bacterial structures, and is complemented by differential histone modifications and DNA methylation. Also, the observed TGIP effects, from the expression of immune effectors to enzymes of the epigenetic tool-kit, partly depended on offspring sex and the specific parental microbial environment.

However, many questions still remain unanswered. For example, in *A. levana*, how are environmental cues processed, relayed and integrated? How do epigenetic mechanisms relate to endocrine signals? Do they regulate each other or is there a hierarchy to them? And is there more plasticity at play than just larval immunocompetence, adult body-plan and wing phenotype? What about embryonic development and the pupal stage? Also, what benefits do the specific plastic responses have in their respective environments? Regarding the two wing phenotypes for example, predation does not seem explain the full story – if at all (see section 2.3.1). Future studies should thus investigate the ecological implications of the various polyphenisms in *A. levana* and also in its six sister taxa in the genus *Araschnia,* at least three of which are also polyphenic, to elucidate the adaptive value of plasticity in this lepidopteran clade.

Regarding TGIP, we have seen many revelations due to increasingly well-designed studies over the past two decades. However, the universality of the phenomenon is still unresolved and more insect orders need to be probed, especially the more basal ones such as the Archaeognatha, Zygentoma and Odonata. TGIP research in lepidopterans should focus on shedding light on the still unclear mechanisms. Do all of the suggested routes actually occur and, if so, do they arise simultaneously? Also, do they influence each other, and under which conditions? The latter questions are not only important from a basic research perspective, but also because they may have significant implications for pest control strategies. Moreover, TGIP has been primarily investigated in the laboratory setting. Given that models for the evolution of the phenomenon have been proposed (see section 3.2), these should now be tested in the field. Does TGIP occur frequently in naturally occurring populations and under which conditions? If so, what impact does this have on population dynamics and inter-species relationships? What does this sort of selection pressure mean for the evolution of parasites and pathogens?

In conclusion, we have revealed new and important aspects of lepidopteran phenotypic plasticity. However, a lot of questions remain to be answered regarding various proximate and ultimate aspects of this fascinating phenomenon.

Per aspera ad astra.

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Appendix

Publication 1: Immunological larval polyphenism in the map butterfly *Araschnia levana* reveals the photoperiodic modulation of immunity

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ORIGINAL RESEARCH

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Immunological larval polyphenism in the map butterfly *Araschnia levana* reveals the photoperiodic modulation of immunity

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Abstract

The bivoltine European map butterfly (*Araschnia levana*) displays seasonal polyphenism characterized by the formation of two remarkably distinct dorsal wing phenotypes: The spring generation (*A. levana levana*) is predominantly orange with black spots and develops from diapause pupae, whereas the summer generation (*A. levana prorsa*) has black, white, and orange bands and develops from subitaneous pupae. The choice between spring or summer imagoes is regulated by the photoperiod during larval and prepupal development, but polyphenism in the larvae has not been investigated before. Recently, it has been found that the prepupae of *A. levana* display differences in immunity-related gene expression, so we tested whether larvae destined to become spring (short-day) or summer (long-day) morphs also display differences in innate immunity. We measured larval survival following the injection of a bacterial entomopathogen (*Pseudomonas entomophila*), the antimicrobial activity in their hemolymph and the induced expression of selected genes encoding antimicrobial peptides (AMPs). Larvae of the short-day generation died significantly later, exhibited higher antibacterial activity in the hemolymph, and displayed higher induced expression levels of AMPs than those of the long-day generation. Our study expands the seasonal polyphenism of *A. levana* beyond the morphologically distinct spring and summer imagoes to include immunological larval polyphenism that reveals the photoperiodic modulation of immunity. This may reflect life-history traits that manifest as trade-offs between immunity and fecundity.

KEYWORDS

antimicrobial peptides, *Araschnia levana*, immunity, metamorphosis, phenotypic plasticity, polyphenism

1 | INTRODUCTION

Polyphenism is defined as the environmentally induced development of distinct alternative morphs encoded by the same genome (Shapiro, 1976) and plays an important role in evolutionary theory. Seasonally changing environmental parameters can in some species result in seasonally occurring phenotypes. This so-called seasonal polyphenism is widely distributed among plants and animals (Nijhout, 2003) and has been postulated to produce phenotypes which are better adapted to a forthcoming environment,

but the adaptive significance of seasonal morphs is still under debate (Simpson, Sword, & Lo, 2011). The mechanisms behind this phenomenon are poorly understood. However, its significance for sexual selection, adaptive evolution, and speciation represents as research focus in the emerging field called EcoEvoDevo (Beldade, Mateus, & Keller, 2011). Immunity represents a complex trait which has not been considered in this context. Innate immunity of insects lacks the antibodies-based memory known from vertebrates and relies mainly on cellular mechanisms such as phagocytosis or multicellular encapsulation of invading pathogens or parasites and humoral defenses among which antimicrobial peptides play a predominant role (Mylonakis, Podsiadlowski, Muhammed, & Vilcinskas, 2016).

The European map butterfly *Araschnia levana* (Linnaeus, 1758) (Nymphalidae: Nymphalinae) is a bivoltine, diphenistic species that has been studied extensively for more than a century as an example of seasonally induced phenotypic plasticity with respect to both its proximate and ultimate causes (Fischer, 1907; Fric, Konvička, & Zrzavy, 2004; Kratochwil, 1980; Müller, 1960; Vilcinskas & Vogel, 2016). Seasonal polyphenism manifests in the formation of two remarkably distinct dorsal wing phenotypes: the spring generation (*A. levana levana*), predominantly orange with black spots, which develops from diapause pupae, and the summer generation (*A. levana prorsa*), with black, white and orange bands, which develops from subitaneous pupae. The proximate cause of this polyphenism and its physiological foundation have been thoroughly investigated. The developmental trajectory (diapause/nondiapause) depends on the photoperiod (Müller, 1955, 1956). If larvae receive less than 8 hr of daylight, they exclusively develop into diapause pupae and consequently the spring *levana* form, whereas more than 16 hr of light triggers obligate subitaneous development which, following a pupal stage lasting ~2 weeks, results in the summer *prorsa* form. The molecular switch between the two routes is hormonally controlled by the timing of ecdysteroid release, which in turn is determined by day length (Koch, 1992, 1996; Koch & Bückmann, 1987). When adult development is triggered by 20-hydroxyecdysone 2 days after pupation, the pupa is said to be summer-primed (derived from a long-day larva) and the summer *prorsa* form develops, whereas if the 20-hydroxyecdysone peak occurs 5 days after pupation, the pupa is said to be spring-primed (derived from a short-day larva) and the spring *levana* form develops. In addition to the obvious color polyphenism, several other more subtle characteristics differ among the two variants. The summer form has a larger wing area, more rounded wing tips and a lower wing load, but also a heavier thorax, a lower abdomen-to-body-mass ratio, higher mobility, and a more open population structure (Fric & Konvička, 2000; Fric & Konvička, 2002). There are various hypotheses regarding the adaptive significance of the seasonal variations in this species, but few studies have provided experimental validation. Generally, lepidopteran wing color polyphenisms have been attributed to seasonally varying climatic conditions, such as wet or dry seasons in the tropics, or temperature shifts in temperate regions (Brakefield, 1996; Brakefield & Larsen, 1984; Watt, 1969 Windig, Brakefield, Reitsma, & Wilson, 1994). An alternative or complementary theory links predator avoidance to plastic color patterns, and the validity of this concept has been demonstrated in some species, but not in *A. levana* (Stevens, 2005). A phylogenetic analysis of polyphenism in *A. levana* largely rejected the thermoregulation hypothesis because the origin of the two phenotypes likely precedes the dispersal of the species into Palearctic regions (Fric et al., 2004). Predator avoidance is also unlikely to be the major selective pressure explaining the two wing color phenotypes because both forms were attacked at the same frequency in a field study (Ihalainen & Lindstedt, 2012). It seems likely that color polyphenism in insects reflects the interaction of multiple selection pressures given that coloration has various functions and is linked to morphology, life history, and development in *A. levana*. Interestingly, a recent transcriptomic analysis indicated that the phenotypic differences in the map butterfly correlate with strong differential gene expression in a number of different gene families (Vilcinskas & Vogel, 2016). Among these were genes related to cuticle formation (overwintering) and nutrient reservoir activity (accelerated metamorphosis) which seems intuitive, but also genes that regulate innate immunity—a finding which is more difficult to interpret. The immune system has a profound beneficial impact on the fitness of a species but constant activity causes the depletion of resources (Schwenke, Lazzaro, & Wolfner, 2016). Innate immunity is therefore a trait that is likely to be under constant selective pressure, the manifestation of which can potentially have unforeseeable pleiotropic effects. Neither the map butterfly's immune system nor its juvenile stages have yet received much attention, and there have been no studies focusing on both aspects under a realistic attack situation. In addition, it is difficult to determine the adaptive value of a trait when only studying a single life stage. Given the differences in gene expression in the prepupae as described by Vilcinskas and Vogel (2016), we therefore set out to test the novel hypothesis that transcriptomic differences in *A. levana* larvae can translate into photoperiod-dependent differences in innate immunity when challenged by an entomopathogen. We measured the survival of long-day and short-day larvae when attacked by *Pseudomonas entomophila*, examined the bacterial clearance rates in the hemolymph, and surveyed the expression levels of four genes encoding antimicrobial peptides (AMPs). We selected three members of AMP families which are specific for Lepidoptera (attacin, lebocin, and gloverin) as revealed in a recent comparative analysis of insect genomes and transcriptomes (Mylonakis et al., 2016).

We report, for the first time, polyphenic innate immunity in *A. levana* larvae and also characterize the different immunity-related phenotypes induced by two priming regimes representing summer and autumn day lengths. We discuss our findings in the context of life-history theory and in relation to the well-studied adult polyphenism.

2 | MATERIALS AND METHODS

2.1 | Rearing

Prior to rearing under controlled laboratory conditions, adult *A. levana prorsa* butterflies were collected in forests around Albach and Lich, Hesse, Germany (approximately 50°32′30.2″N 8°49′12.2″E) during the summer of 2016. The butterflies were transferred into indoor gauze cages (0.95 × 0.6 × 0.6 m) fitted with multiple potted stinging nettle plants (*Urtica dioica*) at 22°C and ~60% relative humidity. If the humidity fell below 50%, the cages were manually sprayed with water. The butterflies were given ad libitum access to 20% sugar solution provided in sponges attached to the cage walls, and the sponges were sprinkled daily with the solution to ensure a constant food supply. To induce subitaneous development in 100% of the offspring, the maximum amount of natural summer daylight (~17 hr at the summer solstice) was exceeded by imposing a 20-h photoperiod using a Bright Sun ULTRA Desert 150 W gas-discharge lamp (Exo Terra) complemented with a standard 40 W infrared to provide an additional mating stimulus (long-day conditions). To induce diapause development in 100% of the offspring, an 8-h photoperiod was imposed (short-day conditions). After females had mated and oviposited their eggs as typical vertical strings (egg towers) on the underside of nettle leaves, the larvae were allowed to hatch on the plants before they were transferred to smaller rearing cages (0.5 × 0.5 × 0.5 m) containing multiple *U. dioica* plants. The larvae were fed ad libitum on the nettles, which were replaced when most of the leaves had been consumed. After the completion of all five larval stages and the pupal phase, the adult butterflies were transferred back to the flight cages for reproduction.

2.2 | Treatment

On the first day after the fourth larval molt (fifth instar, L5D1), larvae from both the long-day and short-day groups (total sample size was n = 240, n = 30 per photoperiod regime and assay) were injected with 4 μl *P. entomophila* GFP strain (10^8 cells/μl) kindly provided by Prof. Dr. Bruno Lemaitre (École Polytechnique Fédérale de Lausanne) (Mulet, Gomila, Lemaitre, Lalucat, & García-Valdés, 2012) or 4 μl PBS as a control. Survival was recorded until pupation, and pupal development was tracked for 3 weeks to ensure the process was normal. All resulting adults developed according to their photoperiodic priming. We also measured bacterial clearance after administering a sublethal dose of *P. entomophila* (10^7 cells/μl) by sampling 5 μl of hemolymph 3, 6, 9, 12, 24 and 48 hr postinjection from separate sets (n = 5) of larvae. Briefly, larvae were dorsolaterally punctured with a micro-injector capillary containing 2 μl of mineral oil to prevent contact between the hemolymph and the air, which would induce the melanization reaction. The hemolymph was then withdrawn from the hemocoel and transferred into pre-cooled Eppendorf tubes containing 35 μl PBS. The samples were stored on ice and serially diluted in PBS before pipetting onto LB agar plates (supplemented with 100 μg/ml rifampicin for selection). The first dilution field with clearly distinct and GFP-labeled colonies was then used to count clones under a Leica MZ16 F fluorescence stereomicroscope allowing us to calculate the number of freely circulating *P. entomophila* cells (per microliter hemolymph). After the hemolymph was extracted, the larvae were flash frozen in liquid nitrogen and stored at −80°C for subsequent gene expression analysis. The following genes encoding members of the following four lepidopteran-specific AMP families (Mylonakis et al., 2016) were assessed: lebocin, attacin, hemolin, and gloverin.

2.3 | Gene expression analysis

The expression levels of relevant AMP genes were measured at the six sampling time points by quantitative real-time PCR. Briefly, total RNA was isolated using the PeqLab peqGOLD MicroSpin total RNA Kit, and the quantity and purity of the samples were assessed using a NanoDrop spectrophotometer (PeqLab). First-strand cDNA was synthesized using the First-Strand cDNA Synthesis Kit according to the manufacturer's protocol (Thermo Fisher Scientific). Primers for real-time PCR were designed using Primer3 software (Untergasser et al., 2012), and available primer pairs were selected based on the lowest number of potential self-annealing structures and primer loops. Gene-specific primers were designed based on nucleotide sequences derived from previously published RNA-Seq data (Vilcinskas & Vogel, 2016) (Table S1). The ribosomal protein L10 gene (*RPL10*) was used for normalization and PBS controls served as calibrator. Quantitative real-time PCR was conducted using an Applied Biosystems® StepOnePlus™ Real-Time PCR System on 96-well plates with SensiMix™ SYBR® from the No-ROX Kit as the reporter mix. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008) modified for amplification efficiencies as per the manufacturer's protocol.

2.4 | Statistical analysis

Statistical computations were carried out using the data analysis package in SigmaPlot version 12.5, from Systat Software, Inc., San Jose California USA. Survival data were analyzed using the method of Kaplan and Meier (log-rank test), and differences in mean survival times were analyzed using one-way analysis of variance (ANOVA). Two-way ANOVA with interaction effects was used to test for differences in bacterial clearance rates and gene expression levels between photoperiod and treatment groups, and their combinations. Model diagnostics for the ANOVA were also performed and did not reveal any evidence against the model assumptions, such as homoscedasticity and normality of errors.

Holm-Sidak's method for multiple comparisons was used for post hoc tests of all pairwise differences between combinations of photoperiod and treatment with the control.

3 | RESULTS

The survival analysis of all four groups of injected larvae demonstrated that control injections had no effect on development or

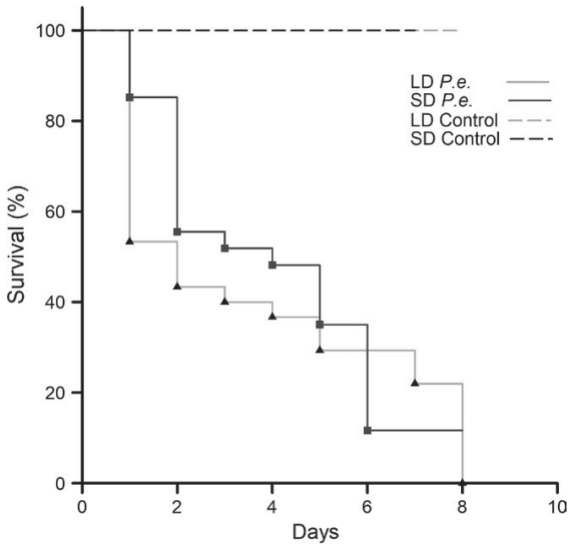

FIGURE 1 Impact of photoperiod and immune challenge on the mortality of larvae. Dashed lines indicate survivals of long-day primed (light gray) and short-day primed (black) controls. Continuous lines indicate survivals of long-day primed (light gray) and short-day primed (black) larvae injected with *P. entomophila*. Triangles and squares represent event points in the Kaplan-Meier diagram

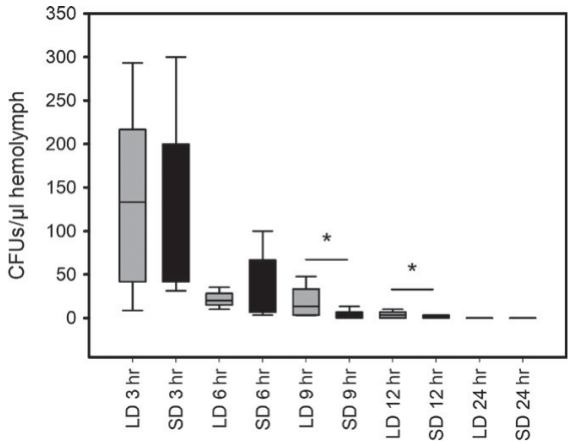

FIGURE 2 Impact of photoperiod on the bacterial loads of *P. entomophila* in the hemolymph over a one-day period. Long-day primed (LD) and short-day primed (SD) samples are displayed in juxtaposition for individual time points. Black cross lines in bars represent the median values. Error bars represent standard deviations. Asterisks above bars indicate significant differences according to pairwise multiple comparison procedures (Holm-Sidak method)

survival given that close to 100% of the control larvae—regardless of the photoperiod regime—reached the pupal stage after 5 days on average. LD control survival was 93.3%, and SD control survival was 100%.

On the other hand, only ~23% of the larvae injected with *P. entomophila* (both the long-day and short-day generations) survived until pupation, although these also reached the pupal stage 5–6 days postinjection. Notably, larvae representing the long-day generation died significantly faster (p = .008, F = 4.03, df = 50) than those representing the short-day generation, that is, after only 2.5 days compared to 5 days, a trend which also reflected in the corresponding survival curves (Figure 1). Notwithstanding the treatments, if larvae had not pupated by 9 days postinjection, they invariably died (data not shown).

The bacterial clearance rates in the larval hemolymph (Figure 2) also differed between the long-day and short-day generations at the sampling points 9 and 12 hr postinjection. However, after 1 day, GFP-marked bacterial pathogens were undetectable in either of the groups. Specifically, by 3 and 6 hr postinjection, both groups had comparably high levels of freely circulating bacteria in the hemolymph which then declined continually over the following sampling points, albeit at different rates. Nine hours after the bacterial challenge, long-day larvae still had significantly more (p < .005, F = 9.4, df = 63) circulating bacteria than short-day larvae (5:1 ratio), and after 12 hr, the difference was still significant (p = .053, F = 3.9, df = 72) with over three times as many bacteria per microliter hemolymph in the long-day larvae.

The expression levels of examined AMP genes were higher in the short-day larvae than the long-day larvae across all sampling points (Figure 3). AMP genes were selected as markers based on their relative expression levels in a recent transcriptome analysis (Vilcinskas & Vogel, 2016) but also the ability of the corresponding AMPs to kill Gram-negative bacteria such as *P. entomophila*. Interestingly, in short-day larvae, the four tested AMP genes clustered into two distinct response phases—namely early and delayed expression (Figure 3a,c vs b,d). Specifically, the expression of the lebocin and hemolin genes peaked only 3 hr after the challenge and then returned to medium (lebocin) or very low levels (hemolin) for the remaining sampling points (Figure 3a,c). In contrast, attacin and gloverin gene expression continuously increased from very low levels 3, 6, and 9 hr postinjection to peak strongly after 12 hr and then drop back to even lower levels over the remaining sampling points (Figure 3b,c). In long-day larvae, only the lebocin and attacin genes showed a comparable—but markedly less pronounced—expression trajectory (Figure 3a,b), whereas hemolin and gloverin were expressed at negligible levels (Figure 3c,d).

4 | DISCUSSION

The map butterfly *A. levana* is a textbook example of morphological polyphenism due to the strikingly different dorsal wing patterns and more subtle differences in wing size and shape between the spring and summer morphs. Accordingly, most previous studies have focused on morphological polyphenism in the adult, with little attention paid to nonmorphological traits and no reports looking at the larval and pupal stages of development. Here we report for

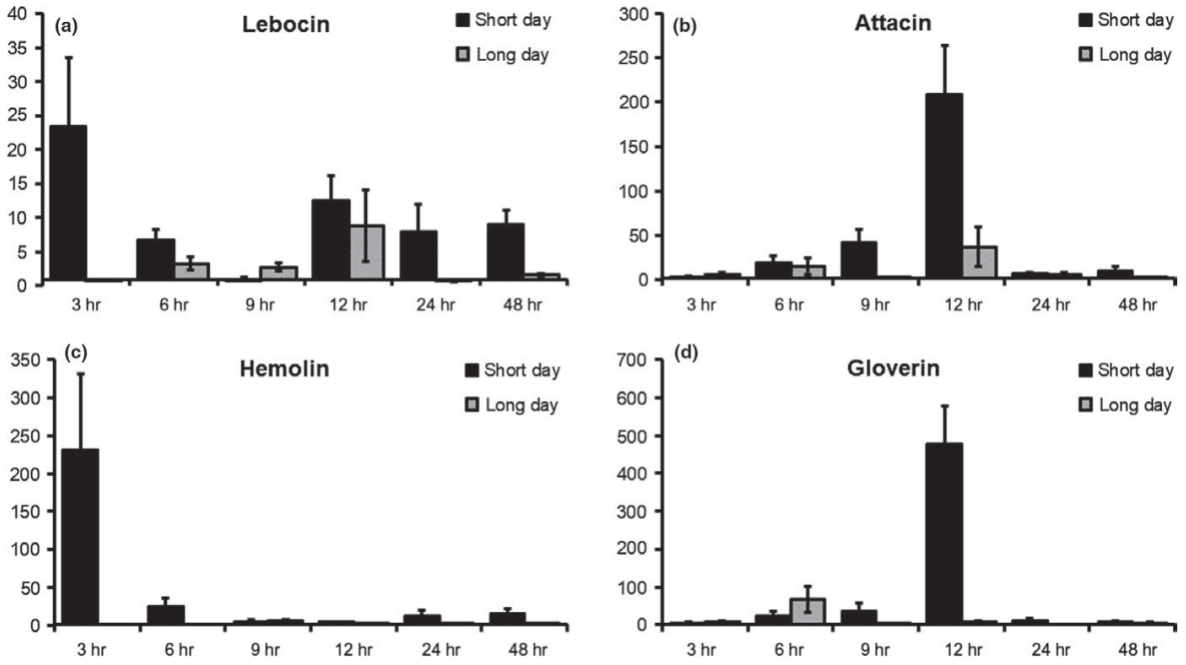

FIGURE 3 Impact of photoperiod on the expression of immunity-related genes after an immune challenge over a two-day period. Long-day primed (light gray) and short-day primed (black) treatments are displayed in juxtaposition for individual timepoints. Relative fold changes for each gene were set to 1 for the control treatment and normalized against the Ribosomal protein L10 housekeeping gene. Error bars represent standard deviations. (a) Lebocin, (b) Attacin, (c) Hemolin, (d) Gloverin

the first time a nonmorphological polyphenism manifesting at the larval stage, reflecting a difference in immunocompetence between larvae photoperiodically primed for subitaneous development (summer generation) and diapause development (spring generation). Specifically, following infection with the entomopathogenic bacterium *P. entomophila*, long-day larvae died much earlier than their short-day counterparts (Figure 1), were less efficient at reducing the pathogenic load in the hemolymph (Figure 2), and struggled to mount an efficient AMP-mediated immune response (Figure 3). In Lepidopterans, all four tested AMPs are known to be active against (at least) Gram-negative bacteria like *P. entomophila*. An exact mode of action is often unknown but it has been suggested that many AMPs promote the permeability of the bacterial plasma membrane or inhibition of outer-membrane protein synthesis (Jiang, Vilcinskas, & Kanost, 2010).

These findings agree with studies reporting photoperiodic immunological changes in small mammals, which generally indicated that shorter day lengths are associated with more potent immune responses (Nelson & Demas, 1996). Similarly, Fedorka, Copeland, and Winterhalter (2013) demonstrated that rearing the cricket *Allonemobius socius* under fall-like conditions resulted in greater resistance to the entomopathogenic bacterium *Serratia marcescens* than summer-like conditions (Fedorka et al., 2013). However, the authors of the latter study co-varied the temperature and photoperiod, making it more difficult to determine which of the two cues was the main variable responsible for the observed results.

The explanation for such marked differences in immunocompetence is unclear. Many insects are relatively short lived, so it is advantageous for temporally isolated cohorts to adapt to seasonal changes in the climate and in the available resources. Seasonal polyphenism can therefore be considered an adaptation to consistent and predictable variations in the environment such that each seasonal phenotype achieves the greatest fitness in the environmental condition under which the phenotype evolved (Brakefield, 1996; Nijhout, 2003). The wing polyphenism observed in many lepidopteran species may reflect two major ultimate causes: first, thermoregulation and climate adaptations influence seasonally varying color patterns (Hazel, 2002; Kemp & Jones, 2001); and second, predator avoidance as has been shown in many other lepidopteran species (Marsh & Rothschild, 1974, Wiklund & Tullberg 2004). In *A. levana*, the wing polyphenism probably did not evolve as a thermoregulatory response because the origin of the polyphenism predates the dispersal of the species toward Palearctic temperate regions (Fric et al., 2004). Neither is the predation-avoidance hypothesis truly compelling (Ihalainen & Lindstedt, 2012). An alternative explanation would be an induction of higher metabolism due to a longer photoperiod, distributing costs to a more elaborate wing color and morphology, thus limiting resources for immunity. On the other hand, a more effective immune system in short-day morph pupae (cold period) would be beneficial to survive exposition to litter with a high microbial titer. However, currently there is no indication of a functional link between the larval immunity-related polyphenism

reported in this study and the diphenistic wing coloration encountered in adult butterflies. A possible explanation for the difference in juvenile immunocompetence is that immunocompetence is traded off against dispersal and fecundity in the summer generation. Adult members of the summer generation seem to be better adapted to long-distance flights (through larger thoracic flight musculature), are more active than the spring generation, and invest more resources into reproduction (Friberg & Karlsson, 2010; Fric & Konvička, 2002; Morehouse et al., 2013). All these traits (especially reproduction) are energetically costly, but so is a powerful and active immune system. Life-history theory therefore predicts that the fitness value of one trait may lower the fitness value of another and a trade-off between reproduction and immunity has been demonstrated in various insect taxa under many different experimental settings (Schwenke et al., 2016; Siva-Jothy, Moret, & Rolff, 2005; Vilcinskas, 2013). In line with these findings, in the hemipteran *Acyrthosiphon pisum*, a positive relationship between fecundity and susceptibility to parasitoid attack was found (Gwynn, Callaghan, Gorham, Walters, & Fellowes, 2005). In the same aphid species, it was shown that melanization and fecundity are under control of molecules displaying pleiotropic roles in embryogenesis, longevity, fecundity, and melanization (Will, Schmidtberg, Skaljac, & Vilcinskas, 2017).

The observed Immunological larval polyphenism may result from the crosstalk between pathways regulating transcriptional reprogramming. Particularly epigenetic mechanisms such as histone acetylation and microRNAs link complex traits such as immunity and development in insects (Freitak, Knorr, Vogel, & Vilcinskas, 2012; Mukherjee, Fischer, & Vilcinskas, 2012). It is worth mentioning here, that if we assume an antagonistic pleiotropy for the life-history traits immunity and melanization, the reduced immunity may be a necessary consequence of the darker summer morph (and vice versa). In fact, both melanin-based pigmentation and the component of the insects' immune system known as the melanization cascade rely on, and thus compete for the same precursor resource, namely the amino acid tyrosine. Melanization is known to be costly in insects and for example negatively impacts reproduction investment in the sand cricket, *Gryllus firmus* (Roff & Fairbairn, 2013). Arguably, the immunity versus melanization case represents a classical physiological trade-off (Dubovskiy et al., 2013). An additional explanation for it may be that the increased pigmentation of the derived summer morph evolved due to increased day lengths in higher latitudes which correspond to an increase in UV light exposure. In that case, the darker summer morph may be a genoprotective response to this new hazard.

Although firmly grounded in the literature, here we admittedly rely on speculation and thus further research is needed to determine whether the immunity-related polyphenism is maintained in adult *A. levana*, if the species indeed displays behavioral thermoregulatory responses to an array of natural pathogens/parasites, and if there is a difference in warm-up speeds and absolute body temperature through solar radiation absorbance guided by the phenotype. Moreover, it would be interesting to test the nature and relative extent of pathogen/parasite loads in wild spring and summer generations to see if the immunity-related polyphenism we have discovered is manifested under natural conditions. Future studies should be complemented by an infection assay where both phenotypes are subsequently assessed in parallel for their reproductive outputs.

Our study provides the first indication that wing color morphology in adult *A. levana* is not the only phenotype with a plastic response to day length. We show that the juvenile stages display an immunity-related polyphenism that may at least partially explain the derived darker-wing phenotype of the summer generation: Long-day larvae have a weaker immune response than short-day larvae, which they potentially compensate through behavioral fever. We acknowledge that immunity is an aspect that is clearly underrepresented in studies concerned with lepidopteran polyphenisms and their ultimate causes, which we have begun to address in this study.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

AB performed the experiments and reared *A. levana*. HV extracted the AMP-encoding sequences from the transcriptomic data base. KL and AV designed the study. AB, KL, and AV drafted the manuscript. AV provided funding.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Publication 2: Seasonal phenotype-specific expression of microRNAs during metamorphosis in the European map butterfly *Araschnia levana*

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RESEARCH ARTICLE

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

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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.

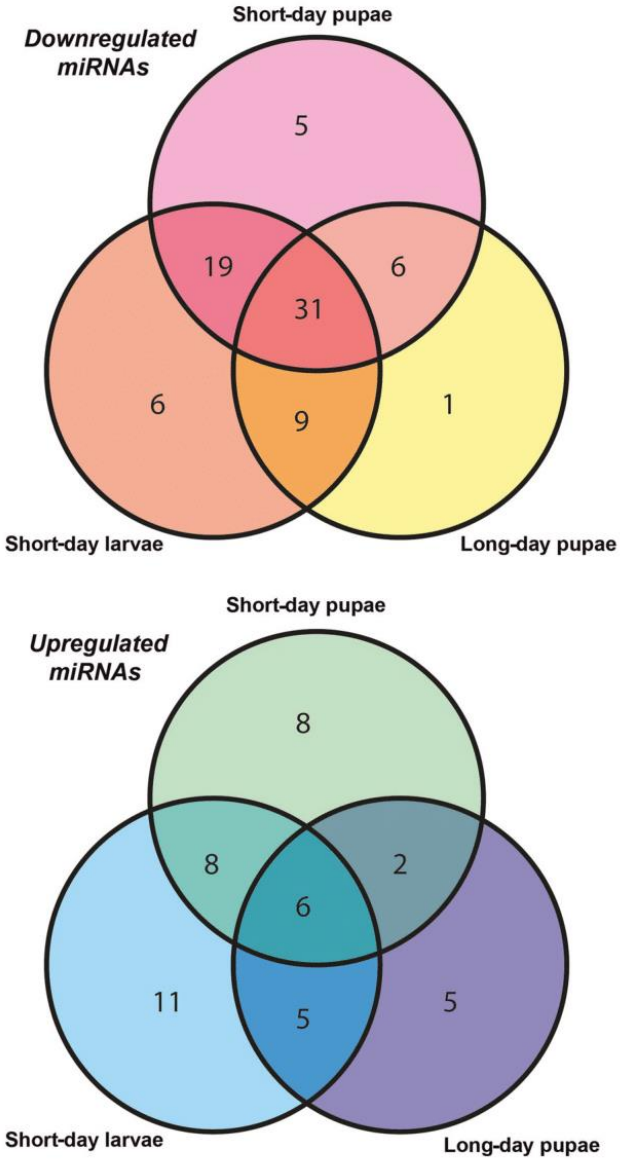

FIGURE 1 Venn diagram showing the differential expression of miRNAs in the larvae and pupae of *Araschnia levana*. The miRNA sequences were obtained from miRBase v21 and their expression profiles were determined by microarray analysis. The fold differences of downregulated and upregulated miRNAs are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

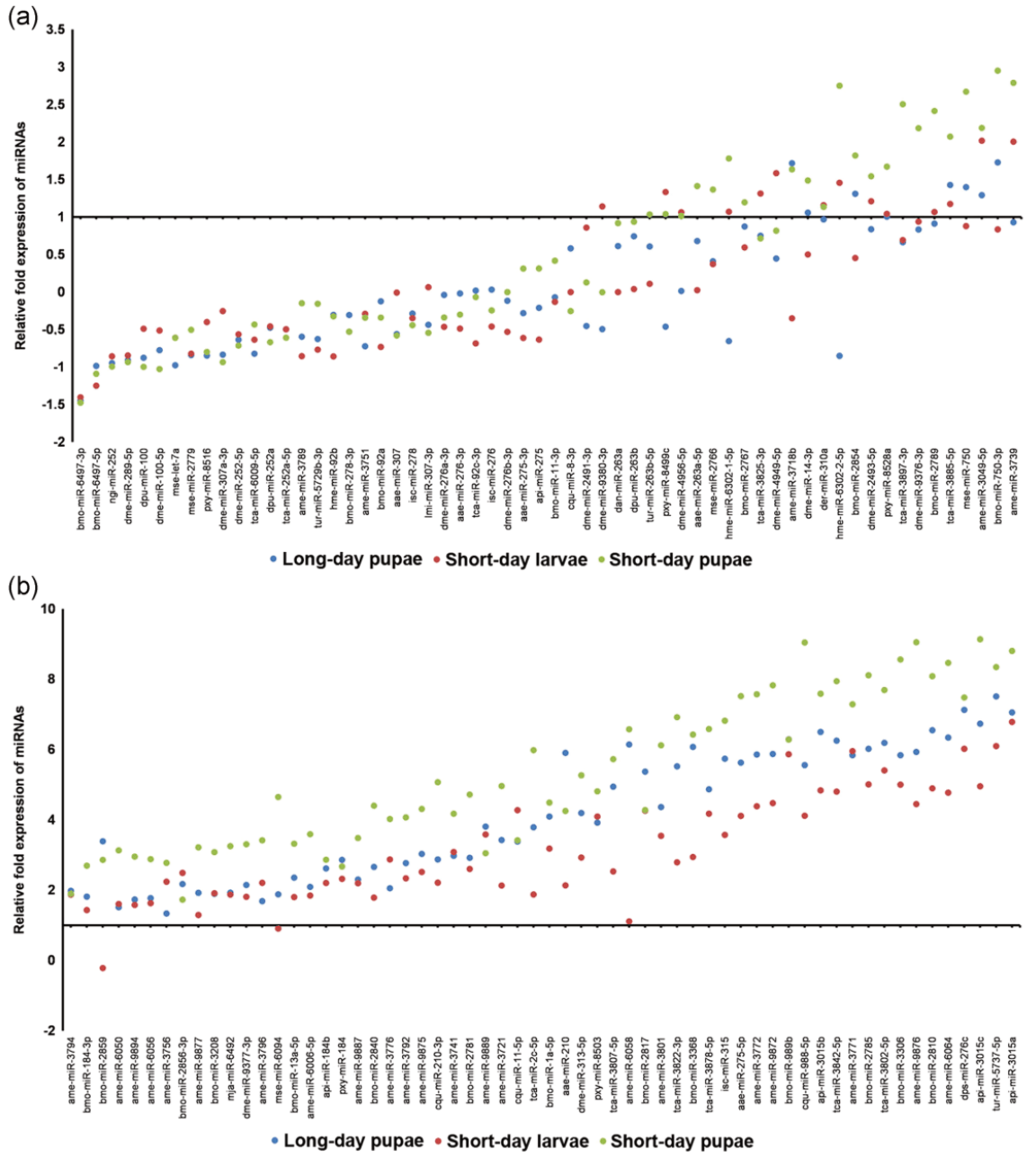

FIGURE 2 Expression of miRNAs in the larvae and pupae of *A. levana* exposed to either short-day or long-day conditions during larval development. (a,b) The miRNA sequences were obtained from miRBase v21 and their expression profiles were determined by microarray analysis. The fold differences in expression 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

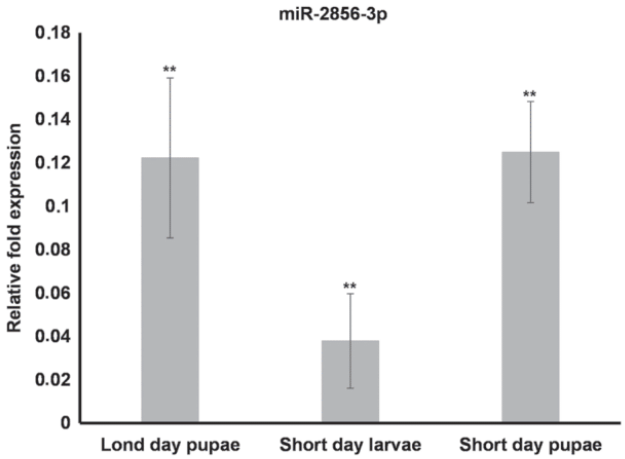

FIGURE 3 Expression of miR-2856-3p in larvae and pupae of *A. levana* developed under short-day and long-day conditions. The expression of miR-2856-3p was analyzed in *A. levana* larvae and pupae, reared under long-day or short-day conditions, by quantitative real-time RT-PCR. Basal expression was calculated 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.

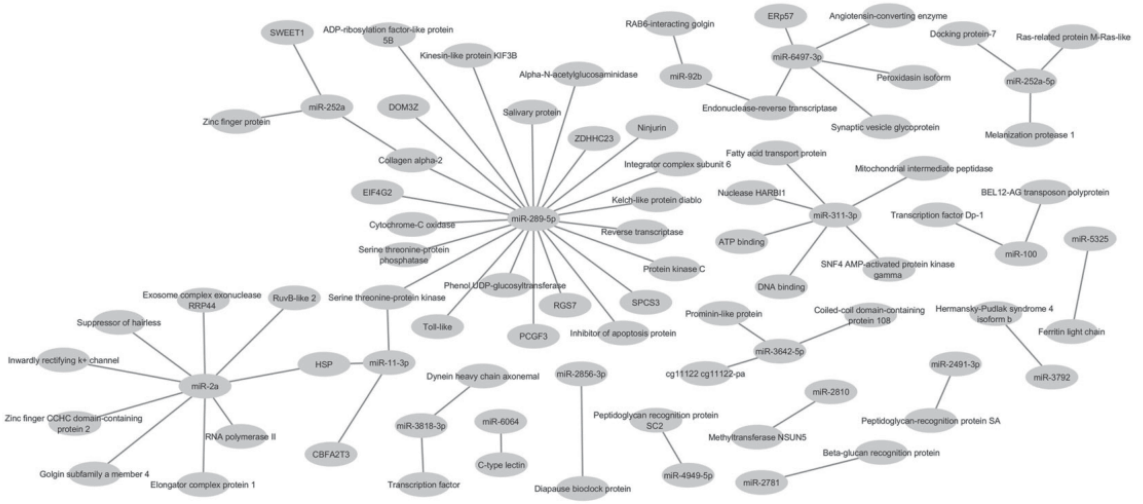

FIGURE 4 The mRNA sequences targeted by miRNAs in *A. levana*. The network diagram generated with Cytoscape shows description of mRNA sequences in *A. levana* that are targeted by differentially expressed miRNA sequences obtained from miRBase. Connecting lines (edges) are used to indicate miRNA targets 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*.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

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

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Publication 3: Epigenetic Mechanisms Are Involved in Sex-Specific Trans-Generational Immune Priming in the Lepidopteran Model Host *Manduca sexta*

Authors: Jasmin Gegner | Arne Baudach | Krishnendu Mukherjee | Rayko Halitschke | Heiko Vogel | Andreas Vilcinskas

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Epigenetic Mechanisms Are Involved in Sex-Specific *Trans*-Generational Immune Priming in the Lepidopteran Model Host *Manduca sexta*

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Parents invest in their offspring by transmitting acquired resistance against pathogens that only the parents have encountered, a phenomenon known as *trans*-generational immune priming (TGIP). Examples of TGIP are widespread in the animal kingdom. Female vertebrates achieve TGIP by passing antibodies to their offspring, but the mechanisms of sex-specific TGIP in invertebrates are unclear despite increasing evidence suggesting that both male-specific and female-specific TGIP occurs in insects. We used the tobacco hornworm (*Manduca sexta*) to investigate sex-specific TGIP in insects because it is a model host for the analysis of insect immunity and the complete genome sequence is available. We found that feeding larvae with non-pathogenic *Escherichia coli* or the entomopathogen *Serratia entomophila* triggered immune responses in the infected host associated with shifts in both DNA methylation and histone acetylation. Maternal TGIP was mediated by the translocation of bacterial structures from the gut lumen to the eggs, resulting in the microbe-specific transcriptional reprogramming of genes encoding immunity-related effector molecules and enzymes involved in the regulation of histone acetylation as well as DNA methylation in larvae of the F1 generation. The third-instar F1 larvae displayed sex-specific differences in the expression profiles of immunity-related genes and DNA methylation. We observed crosstalk between histone acetylation and DNA methylation, which mediated sex-specific immune responses in the F1 generation derived from parents exposed to a bacterial challenge. Multiple routes for TGIP seem to exist in *M. sexta* and – partially sex-specific – effects in the offspring depend on the microbial exposure history of their parents. Crucially, the entomopathogen *S. entomophila* appears to be capable of interfering with TGIP in the host.

Keywords: epigenetics, innate immunity, *trans*-generational immune priming, pathogens, *Manduca sexta*, *Serratia entomophila*

INTRODUCTION

Parents can invest in their offspring by preparing them to cope with pathogens or parasites that only the parents have encountered. The transfer of immunity from parents to offspring is known as *trans*-generational immune priming (TGIP) and has been reported in a wide range of animals, including arthropods (Little et al., 2003; Sadd et al., 2005; Dubuffet et al., 2015; Milutinović et al., 2016). The mechanisms underlying TGIP and the specificity of the resulting immune responses have been investigated in insects such as the bumblebee (*Bombus* spp.), the mealworm beetle (*Tenebrio molitor*), the red flour beetle (*Tribolium castaneum*), and lepidopterans such as the greater wax moth (*Galleria mellonella*) and the tobacco hornworm (*Manduca sexta*) (Dubuffet et al., 2015; Trauer-Kizilelma and Hilker, 2015b; Milutinović et al., 2016; Vilcinskas, 2016; Castro-Vargas et al., 2017; Rosengaus et al., 2017).

Mechanisms of parental investment in the form of TGIP differ between vertebrates and invertebrates and between sexes (Hasselquist and Nilsson, 2009; Roth et al., 2010; Herren et al., 2013; Eggert et al., 2014; Freitak et al., 2014; Knorr et al., 2015; Salmela et al., 2015), supporting Bateman's principle that males gain fitness by increasing their mating success whereas females increase fitness through longevity because their reproductive effort is much higher (Rolff, 2002). The maternal transfer of immunity in vertebrates is realized by antibodies, which are provided by the mother during gestation and (in mammals) during lactation (Hasselquist and Nilsson, 2009). However, the mechanisms of maternal TGIP in insects were unclear until a recent report revealed that bacteria taken up with the diet can translocate from the larval gut to the hemocoel and are ultimately deposited in the developing eggs (Freitak et al., 2014), apparently by binding to egg-yolk proteins (Salmela et al., 2015). The *trans*-generational transmission of bacteria via yolk proteins has also been observed in *Drosophila melanogaster* (Herren et al., 2013). The transfer of bacteria or fragments thereof from mothers to eggs explains at least in part the specificity of maternal TGIP in *G. mellonella* and *T. castaneum* (Freitak et al., 2014; Knorr et al., 2015).

Paternal TGIP is also observed in insects, but the immunological protection is less specific than that conferred by maternal TGIP and the mechanism is unclear (Roth et al., 2010; Eggert et al., 2014). Current concepts in evolutionary biology postulate that environmental stimuli such as stress and pathogens can be translated into heritable phenotypic alterations by epigenetic mechanisms (Nestler, 2016). Therefore, epigenetic mechanisms may explain how fathers can also translate information about the pathogens they have encountered (environmental stimuli) into heritable adaptations of the offspring immune system (phenotypic alteration) without any genetic changes (Vilcinskas, 2016). There is also a large body of evidence indicating that pathogens influence epigenetic gene regulation in their insect hosts (Mukherjee et al., 2017; Vilcinskas, 2017).

We investigated the potential epigenetic basis of TGIP in insects using the tobacco hornworm *Manduca sexta* because male and female larvae are easy to distinguish morphologically, it is widely used as a lepidopteran model to study innate immunity (Jiang et al., 2010), and the complete genome sequence was published recently (Kanost et al., 2016). To mimic natural oral infections and to determine the pathogen-specificity of any TGIP we observed, we supplemented the larval diet with either non-pathogenic *Escherichia coli* or with the entomopathogen *Serratia entomophila*, both of which are known to translocate from the midgut into the hemocoel in *G. mellonella* (Freitak et al., 2014). We tracked bacteria added to the diet to determine whether they were transferred from the gut lumen to the eggs. We also monitored the infected larvae for evidence of an immune response in the host by looking for shifts in complex parameters, specifically developmental timing. We analyzed the expression profiles of selected immunity-related effector genes in F0 and F1 male and female larvae. To determine whether epigenetic mechanisms were suitable to analyze sex-specific TGIP effects we observed, we compared total DNA methylation and histone acetylation in the same cohorts. DNA methylation involves the addition of a methyl group to cytidine residues in the dinucleotide sequence CpG to form 5-methylcytidine, which retains the base-pairing capacity of the unmodified nucleotide but modifies its interaction with regulatory proteins (Vilcinskas, 2017) and seems to be associated with stably expressed genes, related to basic housekeeping in lepidopterans (Jones et al., 2018). *De novo* methylation is established by DNA methyltransferase 3 (DNMT3) and is maintained by the maintenance methyltransferase DNMT1. However, some insect taxa including the Lepidoptera have lost DNMT3 (Bewick et al., 2017). We therefore focused our analysis on DNMT1 and 2 and the methyl-CpG-binding domain protein (MBD). Similarly, the core histone proteins that combine with DNA to form chromatin can be modified to control the density of packing, with the removal of acetyl groups by histone deacetylases (HDACs) causing transcriptional repression due to the tighter packing and lack of access to the DNA and the addition of acetyl groups by histone acetyltransferases (HATs) having the opposite effect (Marks et al., 2003). Accordingly, we also looked at the relationship between histone modification and the expression of HATs and HDACs in both generations.

MATERIALS AND METHODS

Insect Rearing and Diets

Manduca sexta eggs were collected from the in-house stock population for hatching and the larvae were maintained at 26°C, with 30% humidity and a 16-h photoperiod. We separated male larvae from female according to their dark spot in the posterior portion (Stewart et al., 1970). The larvae were reared on a standard artificial *M. sexta* diet (Bell and Joachim, 1976) drenched in overnight bacterial cultures of *E. coli* (9.5×10^7 cfu/meal) or *S. entomophila* (1.5×10^8 cfu/meal), or without bacteria as a control. Additionally, a group of larvae was reared on artificial diet drenched with 100 µl fluorescent BioParticles® consisting of a mixture 1 mg/ml of chemically and heat-killed *E. coli* strain K-12 labeled with Texas Red® (Molecular Probes) per 1 g of diet (Freitak et al., 2014). Larvae were fed *ad libitum*

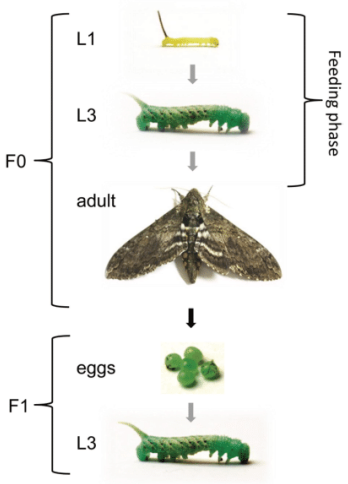

FIGURE 1 | Experimental design to investigate TGIP in *M. sexta*. Larvae were fed on diets supplemented with *E. coli*, *S. entomophila* or fluorescence-labeled bacteria (Bioparticles) starting at the first instar and continuing until the first sampling point at the third instar. Except for the first instar, all displayed stages correspond to sampling points. Gray arrows indicate stage progression, the black arrow indicates generational progression, and the white double arrow indicates the duration of the feeding regimes. The life cycle proceeds as follows: five larval molts, one pupal molt, one imaginal molt, (embryonic) egg stage.

and food was replaced when needed or at least three times per week to ensure a steady supply of bacteria. Feeding was continued until the F0 larvae were removed for dissection at the third-instar stage, or throughout development in the case of specimens that were used to provide offspring for TGIP analysis. Parental development was monitored daily. After pupation, male and female specimens (2:1 ratio) were transferred to flight cages so they could begin mating after eclosion and wing maturation (one cage per treatment). Oviposited eggs were then counted daily for 10 consecutive days after initial oviposition on the provided substrates, i.e., tobacco plants (*Nicotiana tabacum*) and laboratory paper lining the cage walls. Eggs were allowed to hatch and F1 larvae were reared on an uncontaminated artificial diet until they were 1-day-old third-instars for the analysis of gene expression, histone H3 acetylation and DNA methylation (**Figure 1**). This experiment was repeated twice.

Maternal Transfer of Bacteria

Manduca sexta third-instar larvae reared on a diet drenched with fluorescent BioParticles® were embedded in Tissue-Tek® OCT compound (Plano), flash-frozen in liquid nitrogen and stored at −80°C prior to cryostat sectioning. Abdomens, dissected ovaries and oviposited eggs from adult females were treated in a similar fashion. We prepared 10-μm cross-sections on a Leica CM 1850 Cryostat and viewed them under a Leica DM 5000 B fluorescence microscope with the N3 filter for Texas Red and the A4, L5 and Y5 filters as the negative control. Differential interference contrast (DIC) and bright field (BF) images were also captured to provide structural information. Fluorescent photomicrographs were acquired by overlaying the N3 and L5 filter cube images onto the DIC or BF images using the Leica LAS AF Lite image processing platform to optimize the fluorescence visualization.

Identification of Sequences of Putative Epigenetic Regulatory Genes and Immunity-Related Genes

To identify putative *M. sexta* epigenetic regulators (e.g., HATs, HDACs and DNMTs), as well as immunity-related genes (e.g., encoding gloverin and lysozymes), we identified predicted and annotated *M. sexta* proteins based on the published genome sequence (Kanost et al., 2016). To confirm the annotated protein identities, the predicted amino acid sequences were used as queries for BLAST searches (using BLASTp with default parameters) against the NCBI nr database. Sequences with existing annotations matching the *M. sexta* sequences and with more than 55% amino acid sequence similarity to queries were collected for further analysis. All protein sequences were aligned in Geneious (vR10, Biomatters Ltd.) using MUSCLE with default settings, inspected for regions of high-quality alignment and refined manually. During this step, candidates were also scrutinized for the presence of conserved amino acid patterns.

RNA Isolation and Quantitative Real-Time PCR

Midguts dissected from third-instar larvae (F0, F1) were homogenized in liquid nitrogen and total RNA was isolated using the PeqLab peqGOLD MicroSpin total RNA Kit. Sample quantity and purity were assessed using a NanoDrop spectrophotometer (PeqLab). If appropriate, samples were purified using RNeasy MinElute columns (Qiagen). First-strand cDNA was synthesized using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with 500 ng of DNA-free total RNA as the template and a 3:1 mixture of random hexamers and oligo-dT18 primers. Primers for real-time PCR were designed using Primer3 and available primer pairs were selected based on the lowest number of potential self-annealing structures and primer loops. Gene-specific primers are listed in **Supplementary Table 1**. The ribosomal protein L3 gene (*RPL3*) (Koenig et al., 2015) was used for normalization. Quantitative real-time PCR was conducted using an Applied Biosystems® StepOnePlus™ Real-Time PCR System on 96-well plates with the SensiMix™ SYBR® No-ROX Kit as the reporter mix. Each assay was repeated using three biological replicates (each representing pooled RNA from five third-instar larval midguts per sex) and two technical replicates. Fold changes in gene expression were calculated out using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Analysis of Histone Acetylation

Midguts were dissected from male and female third-instar larvae (F0, F1), flash frozen in liquid nitrogen, homogenized and stored at −80°C. Global levels of lysine-specific histone H3 acetylation were determined using the EpiQuik Global Histone H3 Acetylation Assay Kit (Epigentek Group Inc.) according to

the manufacturer's protocol. Fold changes of relative histone acetylation were calculated for treatment groups exposed to bacteria against the corresponding control groups.

Preparation of DNA for Methylation Analysis

DNA was extracted from two replicates of five F0/F1 third-instar female/male larvae representing the control, *E. coli* and *S. entomophila* treatment groups using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). For each sample, the larvae were homogenized in liquid nitrogen and 150–200 μg of the resulting powder was used for DNA isolation, with a final elution volume of 100 μl. The DNA was precipitated by adding 10 μl 3 M sodium acetate (Carl Roth) and 200 μl ice-cold 100% (v/v) ethanol (Carl Roth), incubating at −20°C for at least for 2 h, and centrifuging at 4°C at 13,000 × *g* in a microfuge for 15 min (Sambrook and Russell, 2000). The pellet was washed with 20 μl ice-cold 70% (v/v) ethanol in Ambion nuclease-free water (Thermo Fisher Scientific) and dried at room temperature for 15 min before dissolving in 50 μl nuclease-free water on ice for 30 min. The DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). If the A_{260}/A_{230} ratio was less than 1.5, the DNA was purified using the NucleoSpin® gDNA Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions.

Global Analysis of DNA Methylation by LC-MS

DNA samples (1 or 2 μg) were digested with Degradase Plus (Zymo Research) at a ratio of 5 U/μg in a final volume of 25 μl overnight at 37°C, then diluted to 100 μl by adding 75 μl 0.1% (v/v) formic acid (ROTIPURAN®, Carl Roth) in ultrapure water (Milli-Q® Advantage A10 water purification system, Merck Millipore) (Capuano et al., 2014). Calibration curves were prepared by dissolving 2′-deoxycytidine (dC, Sigma-Aldrich) and 5-methyl-2′-deoxycytidine (5mdC, Cayman Chemical) in nuclease-free water on ice, each to a final concentration of 1 mg/ml. The nucleoside stock solutions were diluted with 0.5% (v/v) formic acid in ultrapure water to yield 1, 2.5, 5, 10, 100, 250, 500, 1000, and 2000 pg/μl dC/^{5m}dC-standard solutions. The analysis of genomic DNA was carried out by injecting 5-μl digested DNA samples and standard solutions into an UltiMate 3000 HPLC system (Dionex) followed by quantification in an amazon EDT ion trap mass spectrometer (Bruker Daltonics). Components were separated on a reversed-phase column (Kinetex C18, 2.6 μm, 50 × 2.1 mm, 100 Å, Phenomenex) under isocratic conditions [0.1% (v/v) formic acid (ROTIPURAN) and 5% (v/v) acetonitrile (ROTISOLV, Carl Roth) in ultrapure water] at a flow rate of 150 μl/min and 30°C. Cytidine residues were quantified by multiple reaction monitoring (MRM) after positive electrospray ionization using the following ion source parameters: 1.0 bar nebulizer pressure, 8 l/min drying gas, 200°C drying temperature, 4500 V capillary power and 500 V end-plate offset. Ionization and MRM conditions were optimized for fragmentation reactions for mass/charge ratios 228.1→112.0 (for dC) and 242.1→126.1 (for 5mdC). The data were analyzed using Compass Data Analysis v4.2 (Bruker Daltonics). Fold changes in relative global DNA methylation levels were calculated for treatment groups exposed to bacteria against the corresponding control groups.

Statistical Analysis

We used the mean value for the parental and filial generations for every biological sample for males and females, as a control for natural cross-generational effects. We used the function *Summarize* of the R package FSA v0.8.17 (Ogle, 2016) to calculate means, medians, standard deviations, and standard errors of the mean for all experiments. The quantitative PCR results, global histone acetylation, and global DNA methylation data were analyzed for differences between sexes, treatments, and generations using R v3.2 as previously described (Gegner et al., 2018) (R script, **Supplementary File 1**). To test for differences in developmental times between treatment groups and controls, a Kruskal–Wallis multiple comparison test was applied with Bonferroni adjustment of *p*-values by using the function *dunnTest* in the package FSA v0.8.17.

RESULTS

To assess the potential molecular basis of TGIP in the parental generation, larvae were reared on a diet supplemented with microbes and we analyzed the expression of immunity-related genes as well as the levels of histone acetylation and DNA methylation. We also looked at developmental characteristics to determine the impact of TGIP on life history traits. In parallel, a group of larvae received a diet supplemented with fluorescent particles allowing us to visually monitor the uptake and fate of ingested microbes. In the F1 generation, the fate of the fluorescent particles was traced until the egg stage. In addition, gene expression, histone acetylation and DNA methylation were analyzed in the third larval instar (**Figure 1**).

Diets Supplemented With Bacteria Affect Development

We supplemented larval diets with either the pathogen *S. entomophila* or the non-pathogenic bacterial species *E. coli* and monitored development compared to a control group fed on an uncontaminated diet. As shown before for other species, bacterial exposure delayed larval development significantly, i.e., by approximately 2 days in larvae exposed to *E. coli* and 4 days in larvae exposed to *S. entomophila* compared to untreated controls ($p < 0.001$). The larvae infected with pathogenic bacteria took 2 days longer to pupate than larvae exposed to the non-pathogenic bacteria ($p < 0.001$) (**Figure 2** and **Supplementary Tables 2**, **3**).

Bacteria Can Be Transferred From Mothers to Offspring

We monitored the transfer of bacteria from mothers to their offspring using non-viable *E. coli* labeled with the fluorescent dye Texas Red. These bacteria were added to the larval diet

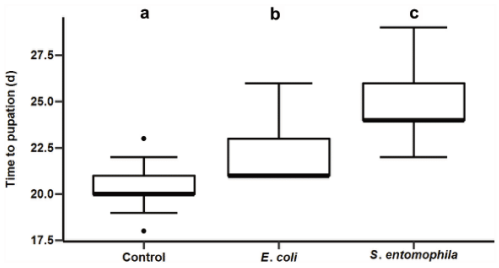

FIGURE 2 | Impact of orally administered bacteria on larval development. The developmental time until pupation of 49 uninfected control larvae, 49 larvae infected with *E. coli*, and 41 larvae infected with *S. entomophila*. Boxplots show median, interquartile, and total regions. Significant differences between groups are depicted by different letters (a–c).

and visualized by fluorescence microscopy in cryosections of third-instar F0 larvae (**Figures 3A–D**), ovaries of adult females derived from these larvae (**Figure 3E**), and oviposited F1 eggs (**Figure 3F**). Using this approach, we determined that the labeled bacteria can translocate from the midgut lumen into the hemocoel, where they attach to the fat body (**Figure 3**). The translocated bacteria are then deposited in the ovaries and taken up into the developing eggs. The labeled bacteria were associated with the follicle epithelium, the ovariole wall and the vitelline membrane. The translocated gut-derived bacteria were ultimately detected in the laid F1 eggs among yolk proteins and lipids (**Figure 3**).

TGIP Affects the Expression of Several Immunity-Related Genes in a Sex-Specific Manner

To determine whether TGIP influences the expression of immunity-related effector genes, namely those encoding gloverin, lysozyme isoforms 1 and 3, and pro-phenoloxidase 2 (PPO2), F0 third-instar larvae were fed on diets supplemented with either *S. entomophila* or *E. coli* (**Figure 4** and **Supplementary Table 4**). In both groups challenged with bacteria, gloverin gene expression was unaffected in the parental generation (**Figure 4A**). However, the lysozyme 3 gene was significantly upregulated in fathers ($p < 0.001$) and mothers ($p < 0.05$) fed on diets supplemented with *S. entomophila*, but downregulated in mothers fed on diets supplemented with *E. coli* ($p < 0.01$) (**Figure 4C**). In the latter group, *PPO2* was also significantly upregulated ($p < 0.001$) (**Figure 4D**). Remarkably, we observed sex-specific changes in gloverin expression ($p < 0.05$) in third-instar F1 larvae from parents challenged with *E. coli*, whereas there was no sex-specific difference in third-instar F1 larvae from parents challenged with *S. entomophila* or in larvae from parents fed on the uncontaminated control diet. Gloverin was upregulated in both sexes ($p < 0.001$) but this was more pronounced in males ($p < 0.05$). Both lysozyme isoforms were also specifically upregulated in F1 males (lysozyme 1, $p < 0.05$; lysozyme 3, $p < 0.001$) (**Figures 4B,C**) and so was *PPO2* ($p < 0.05$). For all of these transcripts, male larvae

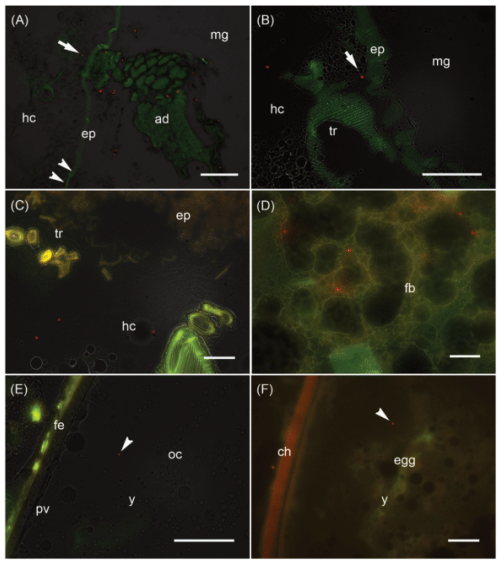

FIGURE 3 | Analysis of the maternal transfer of bacteria in *M. sexta* by fluorescence microscopy Cryosection of third-instar larva **(A–D)**, adult ovary **(E)** and oviposited egg **(F)**. **(A)** Cross-sections of midgut region (md) after the uptake of artificial diet (ad) supplemented with *E. coli* Bioparticles labeled with Texas Red (red spots). Translocated Bioparticles are also detectable in the midgut epithelium (ep, arrowheads) and in the hemocoel (hc, arrow). Scale bar = 100 μm. **(B)** Magnification of the midgut region with adjacent tracheole (tr) shows Bioparticles immediately beneath the midgut epithelium (arrow). Scale bar = 100 μm. **(C)** Cross-sections of the midgut region reveal numerous Bioparticles distributed in the hemocoel. Scale bar = 50 μm. **(D)** Fluorescent bacteria are also abundant in the fat body tissue (fb). Scale bar = 50 μm. **(E)** Female reproductive system dissected from adults reared on artificial diet containing Bioparticles during earlier larval stages. Fluorescent bacteria (arrowhead) are located in the yolk mass (y) of a developing oocyte (oc) which is surrounded by a thin perivitelline membrane (pv) and the follicle epithelium (fe). Scale bar = 100 μm. **(F)** Ovipositioned egg (egg) with an enclosing chorion (ch) contains bacterial probes (arrowhead) in the yolk mass (y). Scale bar = 50 μm.

originating from parents challenged with *E. coli* showed stronger upregulation than males stemming from parents challenged with *S. entomophila* (gloverin, $p < 0.01$; lysozyme 1, $p < 0.01$; lysozyme 3, $p < 0.001$; PPO2, $p < 0.01$). The latter group either showed no differences compared to the control cohort or the genes were slightly downregulated (lysozyme 1, $p < 0.05$; lysozyme 3, $p < 0.01$). In female offspring of parents challenged with *E. coli*, gloverin and lysozyme 1 were significantly upregulated compared to the control cohort (gloverin, $p < 0.001$; lysozyme 1, $p < 0.05$) but the other genes were not. Furthermore, there was no difference in expression between female offspring of parents exposed to the two different species of bacteria.

TGIP Also Influences Histone Acetylation/Deacetylation

The potential epigenetic basis of TGIP was investigated by monitoring the larvae fed on contaminated and uncontaminated

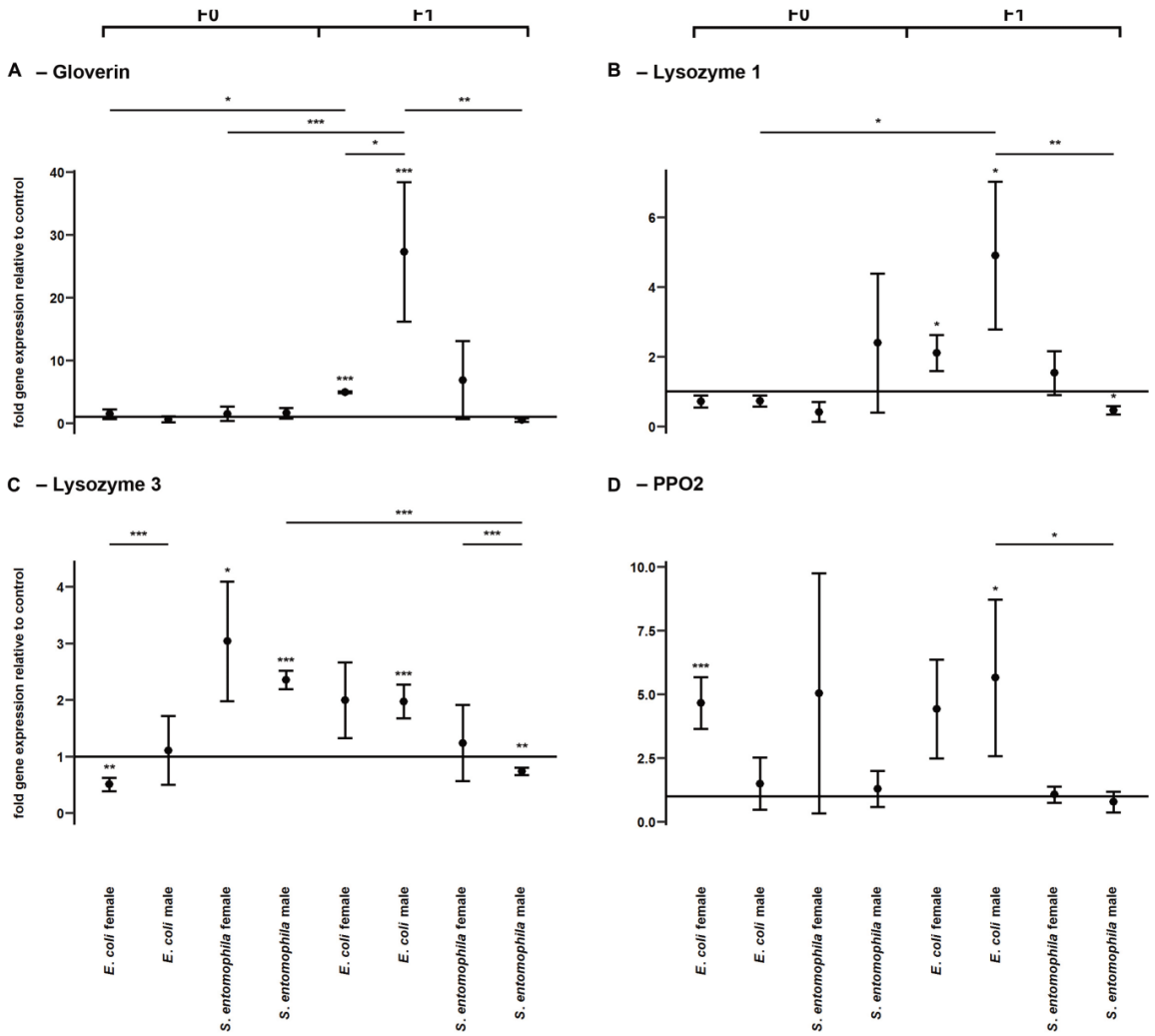

FIGURE 4 | Differential expression of immunity-related genes. Relative expression levels of immunity-related genes in the parental generation reared on bacterial diets (pathogenic and non-pathogenic) or uncontaminated control diets (F0) and unchallenged offspring (F1). The target genes were: **(A)** gloverin, **(B)** lysozyme 1, **(C)** lysozyme 3, and **(D)** pro-phenoloxidase 2. Relative fold changes for each gene were set to 1 for the control treatment and normalized against the 60S RNA housekeeping gene. Results represent the mean values of three independent biological replicates and error bars indicate the standard error of the mean. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

diets for the expression profiles of representative genes encoding either HATs (HAT enoki and HAT chameau) or HDACs (HDAC4, HDAC6, SAP18, and SAP130), and comparing the expression profiles of the same genes in the F1 larvae (**Figure 5** and **Supplementary Table 6**). Accordingly, we found that HAT enoki was upregulated in fathers challenged with *E. coli* ($p < 0.01$) and HAT chameau was downregulated in female offspring of parents challenged with *S. entomophila* ($p < 0.001$) (**Figures 5A,B**). For SAP18, we detected a significant treatment-specific difference between the mothers in the different treatment groups, with higher expression in the *E. coli* group ($p < 0.05$) (**Figure 5E**). Fathers challenged with *E. coli* displayed a reduced capacity for deacetylation. We observed the significant downregulation of HDAC6 ($p < 0.05$) and SAP130 ($p < 0.001$) (**Figures 5D,F**). In the F1 generation, female offspring of parents challenged with *S. entomophila* showed a reduced capacity for deacetylation, with a significant downregulation of HDAC4 ($p < 0.05$) and SAP130 ($p < 0.001$) (**Figures 5C,F**). SAP130 was also significantly downregulated in female larvae whose parents were exposed to *E. coli* ($p < 0.001$), but there was a less significant reduction compared with female larvae whose parents were exposed to *S. entomophila* ($p < 0.001$).

Surprisingly, these differences in HATs and HDACs were only partially reflected by the actual relative histone acetylation levels. Mothers exposed to *S. entomophila* displayed a slight but significant reduction in global histone H3 acetylation compared to the control ($p < 0.05$). Male F1 larvae from these mothers displayed significantly higher levels histone H3 acetylation than their counterparts whose parents were not exposed to bacteria ($p < 0.001$) and also when compared to their female peers ($p < 0.001$) (**Figure 5G** and **Supplementary Table 6**).

Impact of TGIP on DNA Methylation

DNA methylation was assessed by monitoring the larvae fed on contaminated and uncontaminated diets for the expression profiles of representative genes encoding DNMT1, DNMT2, and MBD (**Figure 6** and **Supplementary Table 9**). The expression profiles of the same genes were determined in F1 larvae for comparison. There was a sex-specific difference for both *DNMT1* ($p < 0.01$) and *MBD* ($p < 0.05$) expression in the *E. coli* group. These genes were significantly upregulated in mothers compared to fathers (**Figures 6A,C**). Interestingly, *DNMT1* also was significantly upregulated in mothers exposed to *E. coli* relative to those challenged with *S. entomophila* ($p < 0.05$). There also was a sex-specific difference in the expression of *DNMT1* ($p < 0.001$) and *DNMT2* ($p < 0.01$) in F1 larvae of parents challenged with *E. coli*. These enzymes were significantly more upregulated in males than in females, and also with respect to F1 male larvae from mothers challenged with *S. entomophila* (*DNMT1*, $p < 0.001$; *DNMT2*, $p < 0.05$) (**Figures 6A,B**). This treatment-specific difference in gene expression was also observed for MBD across both sexes (males, $p < 0.01$; females, $p < 0.01$).

The relative global DNA methylation levels ranged between $0.09 \pm 0.13\%$ and $1.26 \pm 0.08\%$ (**Supplementary Table 11**). For the *E. coli* treatment group, we observed significantly reduced levels of DNA methylation in both sexes (males, $p < 0.001$; females, $p < 0.01$) but no such effect was found in the *S. entomophila* treatment group. Interestingly, DNA methylation also was significantly lower in fathers challenged exposed to *E. coli* compared to those treated with *S. entomophila* ($p < 0.001$). On the other hand, in the F1 generation, male and female offspring of both parents in both treatment groups displayed significantly reduced DNA methylation levels compared to controls (*E. coli* group males and females, $p < 0.001$; *S. entomophila* group males, $p < 0.001$; *S. entomophila* group females, $p < 0.01$). In larvae stemming from parents challenged with *S. entomophila* there also was a sex-specific difference ($p < 0.05$), with females displaying significantly lower methylation levels than males. Additionally, the latter showed significantly higher DNA methylation levels than the male offspring of parents challenged with *E. coli* ($p < 0.001$) (**Figure 6D** and **Supplementary Table 9**).

DISCUSSION

Theory predicts that immune responses will be sex-specific because the reproductive effort of females is higher than that of males. According to Bateman's principle, males improve their fitness by increasing their mating success whereas females increase fitness through longevity (Rolff, 2002). Current evidence suggests that these different investment strategies and life-history traits translate into the sex-specific expression of immunity-related genes in insects, which is in turn reflected by the sex-specific expression of regulatory microRNAs (Jacobs et al., 2016). Several studies have shown that parental investment into their offspring is achieved via TGIP which is also sex-dependent (Roth et al., 2010; Eggert et al., 2014). The higher specificity of maternal TGIP in insects can be explained by – but may not be limited to – the transfer of specific bacterial or fungal cells from the mother to the offspring (Freitak et al., 2014; Fisher and Hajek, 2015). However, it is unclear how male insects can transmit information about the pathogens they have encountered, and we have previously hypothesized that epigenetic mechanisms could explain this phenomenon (Vilcinskas, 2016; Vilcinskas, 2017). We selected the tobacco hornworm (*M. sexta*) because it is a widely used model of insect physiology and immunity and previous studies have demonstrated that TGIP occurs in this species (Trauer and Hilker, 2013; Trauer-Kizilelma and Hilker, 2015a,b; Rosengaus et al., 2017). Although speculating on the potential epigenetic dimension of their findings, these earlier studies did not address this topic by way of design. To the best of our knowledge the work of Castro-Vargas et al. (2017) is the only study which investigated the influence of an epigenetic regulator in TGIP. In this work the researchers found that RNA methylation is related to immune priming within but not across generations in *T. molitor* while DNA methylation was not detected (Castro-Vargas et al., 2017). As histone acetylation assays were previously established in our group for *G. mellonella* (Mukherjee et al., 2012), and DNA methylation was detected in closely related species, for example *B. mori* and *M. brassica* (Bewick et al., 2017), these two epigenetic mechnisms provided a promissing system to investigate the potential link between epigenetics and TGIP in our model organism *M. sexta*.

Given that pathogenic and non-pathogenic bacteria can influence TGIP in different ways, we fed hornworm larvae on diets supplemented with either the entomopathogenic species *S. entomophila* or the common gut inhabitant *E. coli* to mimic natural oral infections. The supplemented diets confirmed that *S. entomophila* and *E. coli* cause different developmental effects. Similar findings in *G. mellonella* have been attributed to the ability of pathogens, but not non-pathogenic species, to interfere with epigenetic mechanisms in the infected host (Mukherjee et al., 2015; Vilcinskas, 2017).

Next, we demonstrated that bacteria added to the diet of female larvae can translocate from the gut into the hemocoel and are ultimately deposited in the eggs, where they have the potential to elicit an immune response that may be sufficient to protect offspring from pathogens as they hatch (**Figure 3**). We also found that orally delivered *E. coli* and *S. entomophila* modulated the expression of selected immunity-related genes (encoding gloverin, two lysozyme isoforms, and a pro-phenoloxidase) in the gut of the infected F0 larvae, and also in their offspring.

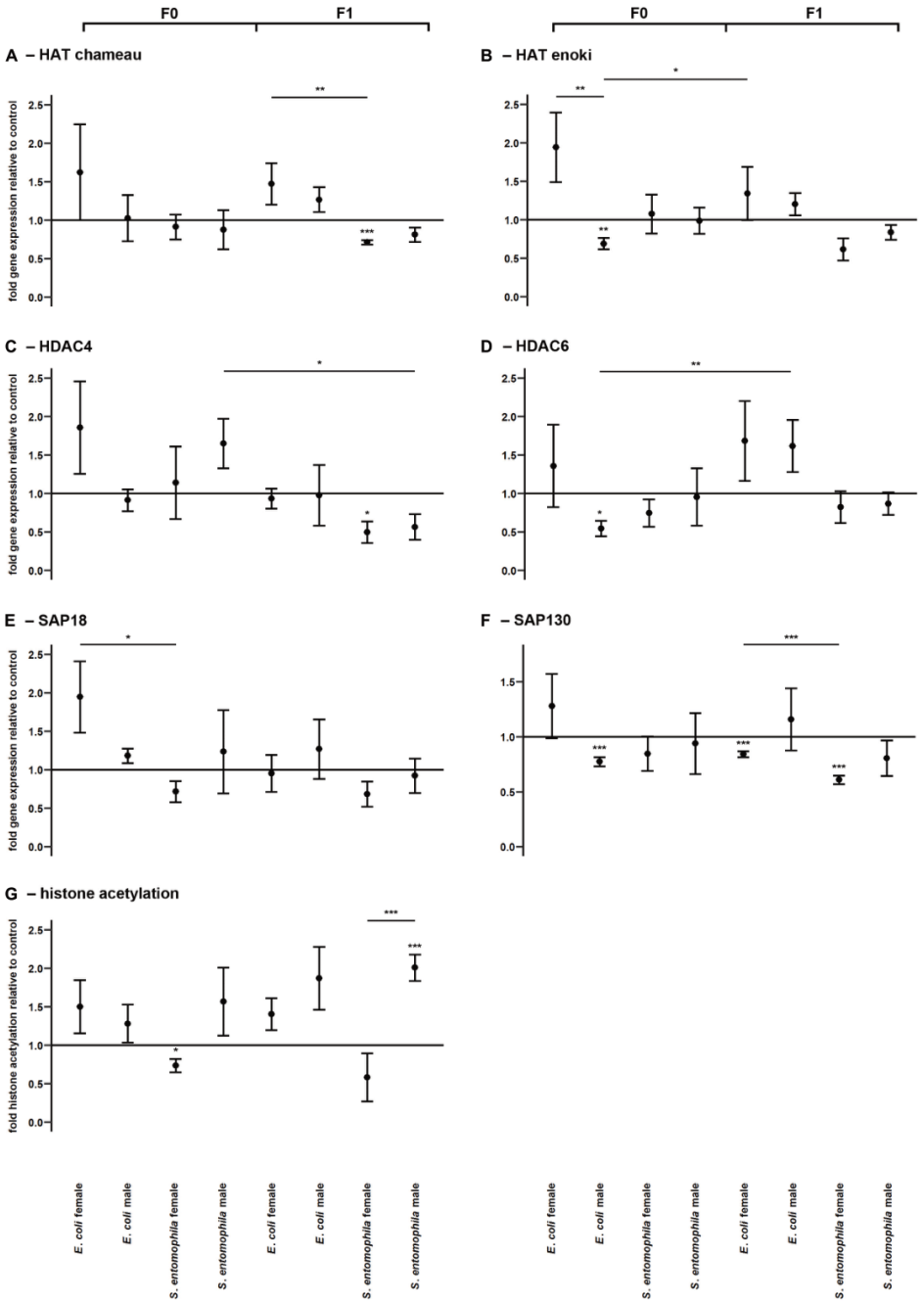

FIGURE 5 | Differential expression of genes encoding histone modifying enzymes and corresponding levels of histone acetylation. Relative expression levels of genes associated with histone modification in the parental generation reared on bacterial diets (pathogenic and non-pathogenic) or uncontaminated control diets (F0) and unchallenged offspring (F1). The target genes were: **(A)** HAT chameau, **(B)** HAT enoki, **(C)** HDAC4, **(D)** HDAC6, **(E)** SAP18, and **(F)** SAP130. **(G)** Corresponding levels of relative histone H3 acetylation. Relative fold changes for each gene were set to 1 for the control treatment and normalized against the RPL3 housekeeping gene. Results represent the mean values of three independent biological replicates for relative gene expression and four replicates for relative histone acetylation. Error bars indicate the standard error of the mean. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

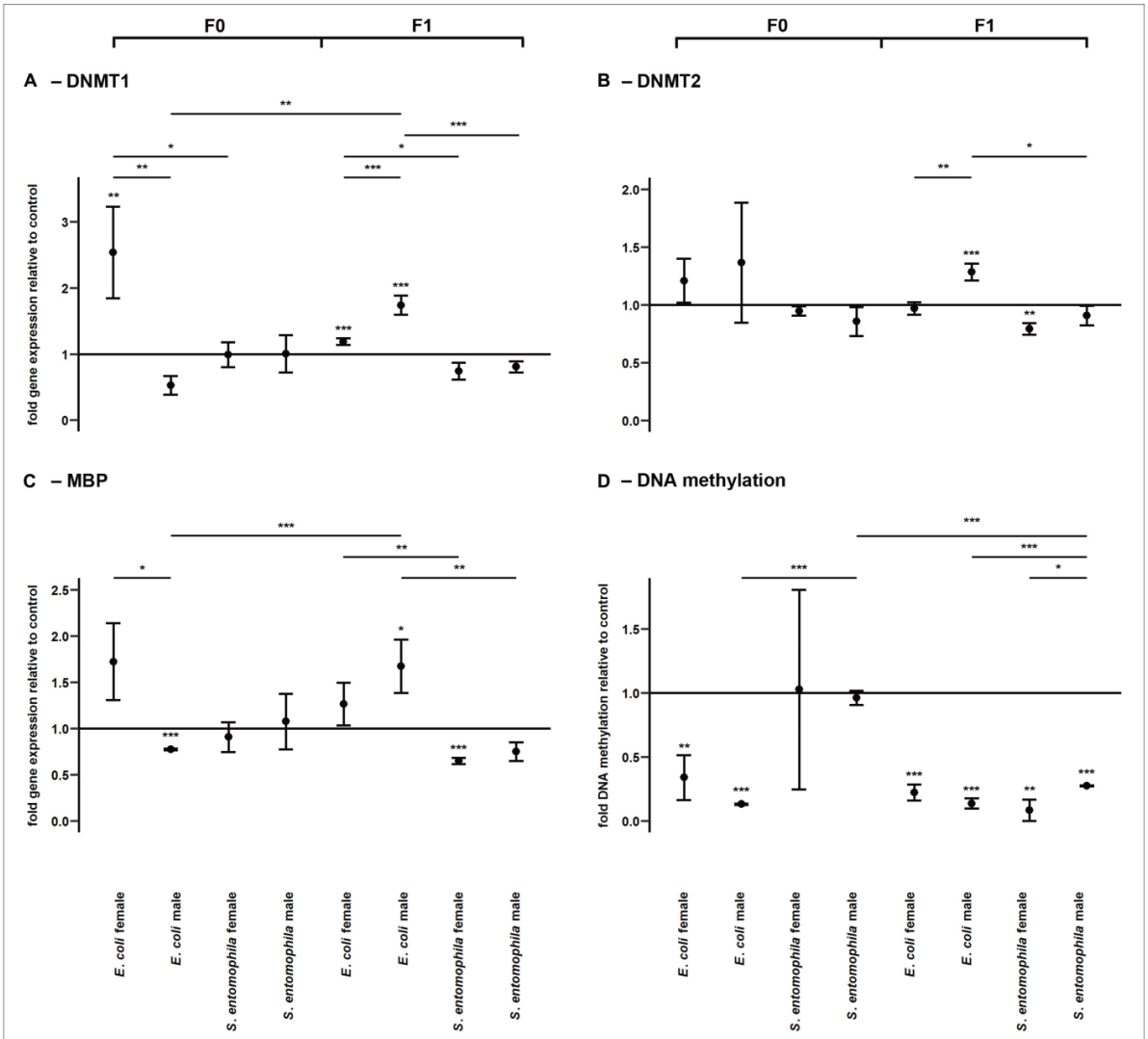

FIGURE 6 | Differential expression of genes encoding DNA modifying enzymes and corresponding levels of DNA methylation. Relative expression levels of genes associated with DNA methylation in the parental generation reared on bacterial diets (pathogenic and non-pathogenic) or uncontaminated control diets (F0) and unchallenged offspring (F1). The target genes were: **(A)** DNMT1, **(B)** DNMT2, and **(C)** MBD. **(D)** Corresponding levels of relative global DNA methylation. Relative fold changes for each gene were set to 1 for the control treatment and normalized against the *RPL3* housekeeping gene. Results represent the mean values of three independent biological replicates for relative gene expression and two replicates for the relative DNA methylation. Error bars indicate the standard error of the mean. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Interestingly, we observed significant sex-specific differences in the expression of gloverin in third-instar F1 larvae whose parents were exposed to *E. coli*, providing evidence for sex-specific TGIP in *M. sexta*. The near universal pattern was that male offspring of parents challenged with *E. coli* displayed the highest expression levels of immunity-related genes among all tested subgroups (**Figures 4A,C,D**). This agrees with an earlier report showing that *M. sexta* gloverin was induced to significantly higher levels in the hemocytes and midgut by Gram-negative *E. coli* than by other microorganisms (Xu et al., 2012). Male larvae therefore express antimicrobial peptides at higher levels than females, but the reason for this is unclear because the phenomenon does not seem to fit Bateman's principle. However, the activation of innate immunity constitutes fitness costs as well as benefits, so organisms are expected to optimize (but not necessarily maximize) their immune responses according to the circumstances. Optimality models predict that when pathogens are particularly detrimental to male mating

success but have a less severe effect on female fecundity or longevity, a superior male immune response will evolve (Stoehr and Kokko, 2006). For example, freshly eclosed *Pieris rapae* adult males display a stronger encapsulation response than females, but the female response becomes stronger as the individuals age (Stoehr, 2007). In our study, we analyzed gene expression only during the third larval stage. It is possible that the immune system dynamics are switched in favor of females during later larval stages, the pupal stage or at some point during the adult phase. However, for males to give preference to reproductive success over immunity, they would need to become sexually mature in the first place. If they succumb to pathogens during the larval stage, this trade-off becomes irrelevant. For example, in the case of *PPO2* (which is needed for the encapsulation response) we detected a near one-to-one transmission of the expression profile from parents challenged with *E. coli* to their male offspring (**Figure 4D**). Perhaps mothers specifically provide their male offspring with a form of immune competence that they would otherwise not exhibit due to a built-in trade-off. Moreover, in contrast to the results of an earlier investigation of transgenerational immune gene expression in *M. sexta* (Trauer-Kizilelma and Hilker, 2015b), we detected induced gloverin expression even though offspring remained unchallenged. This may reflect the different routes of infection and the different priming agents used in each study, and the different offspring stages assessed to investigate TGIP. The authors of this earlier study injected their parental generation with a peptidoglycan isolated from the Gram-positive bacterium *Micrococcus luteus* and sampled F1 eggs and ovaries of F1 females (Trauer-Kizilelma and Hilker, 2015b), whereas we used oral infection with Gram-negative *E. coli* and sampled male and female third-instar larvae. Similar findings to those reported herein were presented following the oral infection of *G. mellonella* with a mixture of Gram-negative *E. coli* and Gram-positive *M. luteus*, or with the entomopathogenic species *Pseudomonas entomophila* or *S. entomophila* (Freitak et al., 2014). The gloverin gene was upregulated in the eggs of challenged females and gloverin levels in their *E. coli*/+*M. luteus* challenge group were similar to the levels we observed in *M. sexta*.

Interestingly the bona fide pathogen we tested (*S. entomophila*) did not elicit any transgenerational immune responses, regardless of the offspring sex. If anything, we observed a slight downregulation of transcripts encoding immune effectors in this group (**Figure 4**). This may reflect the pathogen-induced circumvention of host efforts to protect their offspring against previously encountered microbes. If true, this would be consistent with the classical paradigm of host–parasite co-evolution and could represent a case of reciprocal epigenetic adaptations, as previously suggested (Vilcinskas, 2016). Accordingly, in the offspring generation, we found that the expression of HAT chameau was significantly downregulated in females whose parents had been exposed to *S. entomophila*, whereas the same gene was upregulated in females whose parents had been exposed to *E. coli*. We did not observe any differential regulation of HAT enoki in the *S. entomophila* treatment group but sex-specific regulation was evident in the *E. coli* treatment group (elevated in mothers but repressed in fathers) indicating that histone acetylation may underlie the sex-specific TGIP we observed (**Figures 5A,B**). Our interpretation relies on the general notion that the acetylation of histones H3 and H4 is highly correlated with gene expression, which seems to be conserved across higher eukaryotes (Zhang et al., 2015). At the same time, the expression of the HDAC SAP18 was significantly elevated in *E. coli*-fed mothers, which indicates ongoing differential gene regulation across the genome, consistent with adaptive processes that might contribute to TGIP. In line with this interpretation, HDAC6 and SAP130 were significantly downregulated in *E. coli*-fed fathers. Interestingly, the relative levels of histone H3 acetylation were lower only in the mothers challenged with *S. entomophila*, which again indicates pathogen-derived epigenetic interference that results in overall transcriptional repression (**Figure 5G**). This agrees with an earlier study showing that pathogenic bacteria can interfere with the regulation of HDACs and HATs in insects and can manipulate host immunity in *G. mellonella* (Mukherjee et al., 2012).

In the F1 offspring of parents challenged with *S. entomophila*, HDAC4 and SAP130 were significantly downregulated in female F1 larvae, which surprisingly did not correlate with the higher relative histone H3 acetylation levels. On the other hand, there was no differential regulation of histone acetylation modifiers in F1 male larvae, but the level of histone H3 acetylation was significantly higher. These results may indicate that the causal relationship between histone modifiers and marks is not as straightforward in lepidopteran species as previously assumed. Perhaps crosstalk with other histone marks such as methylation and ubiquitinylation have a dominant regulatory effect over the state of histone acetylation than the enzymes responsible for the actual addition and removal of acetyl groups. Alternatively, these results may represent prolonged sex-specific interference by the entomopathogen to disrupt the epigenetic machinery of the host well into the larval stage of the next generation, perhaps making males more susceptible to attack through the deregulation of immunity-related gene expression. Given that histone modifications occur locally due to the DNA sequence-dependent binding of transcription factors that recruit the HATs and HDACs (Zhang et al., 2015), the abundance of specific enzymes does not necessarily mean that global acetylation levels must change in the same manner.

In addition to histone acetylation, we also observed a sex-specific difference in the expression of genes related to DNA methylation when the parents were exposed to *E. coli*. DNMT1 and MBD were significantly upregulated in mothers but significantly downregulated in fathers (**Figures 6A,C**). Surprisingly, global DNA methylation levels were reduced in parents fed on diets containing *E. coli* but there was no significant difference between sexes. It is unclear why upregulation of the maintenance methyltransferase DNMT1 did not prevent the observed demethylation in *E. coli*-fed mothers. DNMT1 may perform additional functions other than maintaining the DNA methylation status across cell cycles

in *M. sexta*, or the oral challenge with *E. coli* may have triggered the major reprogramming of the methylome. It is conceivable that new genomic regions were methylated *de novo* while a larger, previously methylated portion of the genome became demethylated as a response to the infection, explaining the (partially sex-specific) gene expression we observed in their offspring. Ten-eleven translocation dioxygenases have been implicated in active DNA demethylation in vertebrates, but the demethylation apparatus in insects is unknown (Provataris et al., 2018).

Interestingly, in parents exposed to *S. entomophila*, there was no change in the expression of methylation-related enzymes or in the global DNA methylation status (**Figure 6D**). This further supports our hypothesis that the pathogen was able to interfere with or even subdue the epigenetic machinery of the host, as has recently been demonstrated in the diamondback moth (*Plutella xylostella*) during infections with the koinobiotic endoparasitic wasp *Cotesia plutellae* (Kumar and Kim, 2017). DNA methylation was reduced in parasitized larvae relative to non-parasitized controls, especially at late parasitic stages, along with reduced expression levels of DNMT1, DNMT2 and MBD. The mechanisms of epigenetic interference used by parasitic wasps and pathogenic bacteria are likely to differ substantially, given the phylogenetic distance between the invaders. Infection with *S. entomophila* did not alter DNA methylation in the parental generation but methylation levels were lower in the F1 offspring, particularly in females. Furthermore, and as observed in *P. xylostella*, the expression of DNMT1, DNMT2 and MBD in F1 larvae of both sexes was much lower when the parents had been challenged with *S. entomophila* compared to *E. coli* (**Figures 6A–C**). *S. entomophila* therefore appears to suppress TGIP by interfering with gene expression in the offspring. In contrast, in the female offspring of *E. coli*-fed parents there was no change or limited upregulation of the enzymes involved in DNA methylation whereas there was consistent upregulation in males, as observed for the expression of immunity-related genes. In both sexes, DNA methylation levels were lower than in the control treatment group. This configuration of enzymes versus methylation status again points toward an active and ongoing restructuring of epigenetically mediated gene regulation. Even though at first glance the reduced DNA methylation in both offspring groups seems to be analogous, this evidently does not translate into similar transcriptional profiles, as explained above. Interestingly, in the cotton bollworm *Helicoverpa armigera*, DNA methylation is tightly associated with stably expressed genes with basic housekeeping roles, such as transcription and translation (Jones et al., 2018). Even minute differences in such basic but diverse biological functions are likely to result in profoundly different transcriptional outcomes, depending on the methylation state of the corresponding genomic region.

CONCLUSION

We have shown that TGIP in *M. sexta* is associated with changes in both histone acetylation and DNA methylation. Effects in the offspring depended on the species of bacteria encountered by the parents, and were sex-specific for certain genes as well as for histone acetylation. We have also demonstrated that infection with non-pathogenic *E. coli* resulted in the differential expression of immunity-related genes and DNA methylation-modifying enzymes in the offspring generation, with the highest expression levels observed in males. The entomopathogen *S. entomophila* appears to influence most of the parameters we tested, consistent with counteracting the TGIP efforts of the host. The latter hypothesis warrants more research to determine the extent to which the observed effects reflect an epigenetic dimension of host–parasite coevolution. Our study shows that epigenetic mechanisms are promising tools to get further insight in the molecular mechanisms behind TGIP.

DATA AVAILABILITY

The datasets for this study can be found in the **Supplementary Material**.

AUTHOR CONTRIBUTIONS

AB and JG carried out the laboratory work related to the TGIP experiments. JG contributed to data analysis. RH and JG analyzed DNA methylation. KM analyzed histone acetylation. HV designed primers for qPCR and identification of epigenetic markers and immune genes. AV designed the study, provided funding, and supervised AB and JG. All authors drafted parts of the manuscript, gave approval for publication and agree to be accountable for the content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00137/full#supplementary-material

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Publication 4: The European map butterfly *Araschnia levana* as a model to study the molecular basis and evolution of seasonal polyphenism

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Review

The European Map Butterfly *Araschnia levana* as a Model to Study the Molecular Basis and Evolutionary Ecology of Seasonal Polyphenism

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Simple Summary: The European map butterfly looks different in spring and summer due to day length and temperature. If the butterfly's caterpillars receive 16 h of light per day, the resulting butterfly hatches a few weeks later with blackish wings. However, if caterpillars receive less than 15.5 h of daylight, many overwinter as pupae. In the following spring, butterflies have orange wings. Overwintering and wing color are decided by hormones. If a certain hormone is released in the first days after the caterpillar has become a pupa, no overwintering takes place, and the wings are black. If this hormone is released later, overwintering occurs, and the wings are orange. Different genes are activated to make either of those two options happen. They guide what happens during overwintering and how long it lasts but also how the butterfly looks once it hatches. We do not yet fully understand how the caterpillars count the amount of light they receive and how this information leads to the differences described above. In addition, the butterfly's whole body and its immune system are different in the two color types. Here we discuss how the butterfly probably makes these changes happen and which role the environment plays.

Abstract: The European map butterfly *Araschnia levana* is a well-known example of seasonal polyphenism. Spring and summer imagoes exhibit distinct morphological phenotypes. Key environmental factors responsible for the expression of different morphs are day length and temperature. Larval exposure to light for more than 16 h per day entails direct development and results in the adult f. *prorsa* summer phenotype. Less than 15.5 h per day increasingly promotes diapause and the adult f. *levana* spring phenotype. The phenotype depends on the timing of the release of 20-hydroxyecdysone in pupae. Release within the first days after pupation potentially inhibits the default "*levana*-gene-expression-profile" because pre-pupae destined for diapause or subitaneous development have unique transcriptomic programs. Moreover, multiple microRNAs and their targets are differentially regulated during the larval and pupal stages, and candidates for diapause maintenance, duration, and phenotype determination have been identified. However, the complete pathway from photoreception to timekeeping and diapause or subitaneous development remains unclear. Beside the wing polyphenism, the hormonal and epigenetic modifications of the two phenotypes also include differences in biomechanical design and immunocompetence. Here, we discuss research on the physiological and molecular basis of polyphenism in *A. levana*, including hormonal control, epigenetic regulation, and the effect of ecological parameters on developmental fate.

Keywords: evolutionary ecology; hormones; epigenetics; polyphenism; phenotypic plasticity; microRNAs; metamorphosis; diapause; *Araschnia levana*

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1. Introduction

The phenotype of an organism is dependent on the genome and its epigenetic regulation, based on a combination of cellular memory and interactions with the environment [1]. The term phenotypic plasticity thus refers to the ability of an organism to generate different phenotypes from the same genotype under different environmental conditions [2]. Phenotypic plasticity often facilitates adaptive changes by increasing phenotypic diversity in response to environmental challenges. Polyphenism is a special case of phenotypic plasticity in which the outputs are discrete and discontinuous, resulting in multiple distinct phenotypes from the same genetic background [3]. Some of the most striking examples of polyphenism in animals include sex determination in reptiles and fish regulated by temperature and social factors [4,5], the defense polyphenism in cladocerans [6], the sexual and wing polyphenism in aphids [7], and seasonal polyphenism in butterflies [8].

In 1758, Carl Linnaeus described two apparently distinct butterfly species, which he named *Papilio levana* and *P. prorsa*, but subsequent field observations and breeding experiments revealed them to be seasonal variants of the same bivoltine species: The European map butterfly *Araschnia levana* [9,10]. The dorsal wing of the spring generation (*A. levana* f. *levana*) is orange to reddish-brown (basic coloration) with black spots, some white dots, and a bluish dotted rim on the posterior of the hindwing (Figure 1). Conversely, the dorsal wing of the summer generation (*A. levana* f. *prorsa*) is brownish to bluish-black with a prominent white band (featuring varying degrees of melanization) located basally with respect to 1–3 apical orange bands (Figure 1). Many variations between these two phenotypes have been reported, and they are best described as a spectrum. Some of these occur naturally as *A. levana* f. *porima*, with patterning and coloration appearing intermediate between *levana* and *prorsa*. However, most are the result of experimental manipulation [10,11]. The elements of the wing underside are consistent between morphs and gave rise to the genus name *Araschnia*. The basic coloration is a darkish brown with another prominent whitish band separating the apical and basal parts of the wings. The veins have whitish scales and, with their fine-crossed connections, form a grid that is reminiscent of a spider web or map.

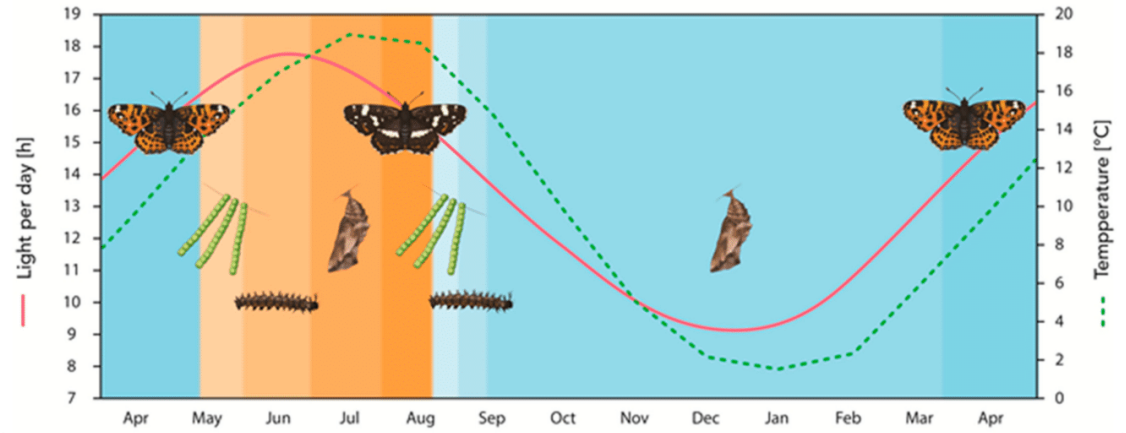

Figure 1. Annual life cycle and phenotype succession of *A. levana* depending on photoperiod and temperature. Values for day length and temperature correspond to the administrative district of Giessen, Hesse, Germany. Long day lengths (>15.5 h) and high temperatures (larval development from spring to midsummer) result in pupal subitaneous development and the expression of the adult *prorsa* form, emerging in summer. Conversely, short day lengths (<15.5 h) and low temperatures (larval development from late summer to early autumn) result in pupal diapause and the development of the adult *levana* form, emerging in spring. The brown color gradient represents *prorsa* development, blue color gradient represents *levana* development. The dotted line corresponds to the threshold day length of >15.5 h per day, below which *levana* development becomes increasingly likely. The developmental trajectory is not affected by environmental cues during embryonic development (green egg towers). For details, see text.

The remarkable change from vernal to estival phenotypes has attracted much interest from scientists. Naturalists initially tested the effect of temperature to determine the biophysical basis of the phenomenon [12–14], but their findings were also contested by contemporaries [15]. Alternatively, strict cyclic heritability was proposed to explain the rhythmic alternation of spring and summer phenotypes [16]. The first studies on seasonal changes in insects were conducted by Marcovitch in the 1920s, who revealed a connection between the appearance of sexual forms in relation to day length in aphids [17]. However, it was not until the 1950s that the true ecological parameters responsible for the polyphenic shift in *A. levana* were revealed. Müller (1955) demonstrated that larvae of either (parental) generation developed directly to become subitaneous pupae and then displayed the adult *prorsa* (long-day or summer) phenotype if they were exposed to light for more than 16 h per day, whereas all larvae became diapause pupae and thus displayed the adult *levana* (short-day or spring) phenotype if they were exposed to light for less than 8 h per day [18]. Following this breakthrough discovery, the physiological basis of polyphenism was the next problem to be addressed [19]. Another species, the Satyrid *Bicyclus anynana*, seasonally exhibits striking differences in the ventral wing pattern too [20]. In this model species, the plasticity of the eyespot size is mostly regulated by temperature, which—in the wandering stage—leads to changing titers of the hormone 20-hydroxyecdysone (20E) [21]. If the 20E signaling is manipulated at that specific time in development, eyespot size can easily be modified. However, for *A. levana*, it remained unclear for over 60 years how the physiological switches were likely regulated at the molecular level to orchestrate the manifestation of two discrete phenotypes [22,23]. In this review, we discuss research conducted to address these issues, including the role of different ecological parameters and their effect on phenotype, where known. We consider the hormones that mediate the switch from *levana* to *prorsa* morphs, and the epigenetic framework in which they operate. Finally, we briefly discuss phenotypic distinctions other than wing coloration (such as differential immunocompetence), draw some conclusions based on our current knowledge, and identify potential directions for future research.

2. Environment and Phenotype

2.1. Photoperiodism and Temperature

The longstanding assumption that temperature was the abiotic factor responsible for the phenotypic switch in *A. levana* was elegantly refuted by Müller (1955). Before presenting his findings, he remarked on the matter of temperature: "*In the field, it cannot be the cause, as it is on average about the same during the crucial developmental phases of the two generations*" [18]. In his experiment, he assigned offspring from both generations into four treatment groups. He then reared one group from each parental generation under two separate light regimes but otherwise identical conditions, in particular, at the same temperature. The two groups exposed to more than 16 h of light per day developed exclusively into subitaneous pupae and thus into the *prorsa* form, whereas the two groups reared under short-day conditions (8 h of light per day) invariably developed into diapause pupae and thus into the *levana* form, regardless of the parental generation [18].

Because the potentially modifying influence of temperature was still unclear at this point, Müller subsequently investigated the effects of the distinct light regimes at two different temperatures: 20 and 30 °C [24]. At 20 °C, all larvae developed into the *levana* form if exposed to fixed light regimes of 4–15 h per day, whereas there was an inverse relationship between light duration and the proportion of *prorsa* individuals in the same photoperiodic range when larvae were reared at 30 °C. Specifically, longer photoperiods led to a steady decline in the proportion of *prorsa* individuals. When the day length was 6 h, the proportions of *prorsa* and *levana* adults were approximately equal, but when the day length was 12 h the ratio was 3% *prorsa* to 97% *levana*. However, at both temperatures, a switch occurred between day lengths of 15.5 and 16.5 h. More than 16.5 h resulted in the complete inhibition of *levana* development, yielding 100% *prorsa* adults. When the photoperiod falls below 15–16 h (daylight lasts for 15.5 h between the middle of May and

late July, in this study at app. 51°8′ N 11°1′ E), the temperature is, therefore, used as an additional cue to determine whether direct development or diapause is preferred.

Later work showed that the critical photoperiod is longer at lower temperatures, with a temperature regime of 15 °C shifting the photoperiod needed for direct development towards longer days [25]. Exposure to 16 h of daylight at this temperature still committed little more than half of all larvae to direct development. These findings indicate that longer photoperiods are required to induce direct development at lower temperatures, whereas shorter photoperiods are sufficient at higher temperatures, although the latter only applies to photoperiods of less than 12 h. In an ecological context, this means that warmer temperatures tip the risk–benefit ratio in favor of direct development (betting on continued beneficial conditions), whereas cooler temperatures have the opposite effect (betting on an overwintering strategy). The increase in critical day length is more likely to prevent direct development and consequently the formation of a potential third butterfly generation. In Central Europe, this reflects conditions in the wild, where *prorsa* larvae develop from mid-May to mid-July at day lengths of at least 16.5 h and mean temperatures of 15–18 °C [18,24,25] (Figure 1). In contrast, *levana* larvae develop in August and September, when the mean temperature is initially ~19 °C but quickly declines to ~11 °C by the beginning of October [18]. Day length during the same period declines from 15.5 to 11.5 h (Figure 1). This suggests that the photoperiod takes precedence as the key climate predictor with the ultimate decision-making role, but it can be modified and fine-tuned by prevailing temperatures.

The development of subitaneous or diapause pupae depends on the day length in the mid (but not early or late) larval stages, with a critical photoperiod of ~15.5 h [24,26]. These findings have been modified by a more recent study [27], although direct comparisons are not possible because the data provided in the original studies are not precise. Larvae were reared at 23 °C under short-day conditions (12 h photoperiod) or long-day conditions (20 h photoperiod), and subsets were transferred between conditions in both directions in each of the five instars. Transfer from long-day to short-day conditions during the first three larval stages invariably led to diapause development, whereas the transferred fourth-instar larvae yielded ~40% *prorsa* adults and transferred final-instar larvae yielded 100% *prorsa* adults [27]. A fixed number of long days (18 h photoperiod) is necessary for direct development, representing up to half of the entire larval development period (~23 days) at 20 °C [25]. This indicates that there is a point of commitment during the fourth-instar stage beyond which diapause development is no longer possible, an advantageous strategy given the additional preparations needed to survive winter, such as general physiological changes, the formation of denser tissues, and the thickening of the cuticle [27]. On the other hand, it also implies that natural selection favors a decision made late in larval development, when larvae have the most current information about their position in the season. Friberg and colleagues also showed that transfer from short-day to long-day conditions during the first four larval stages led reproducibly to 100% *prorsa* adults, and even when switched during the final larval stage, there was still a 50% likelihood of *prorsa* development. In nature, longer day lengths correspond to spring and early summer (Figure 1). In years with early-season high temperatures, imagoes may emerge ahead of time, as reported in 1990 in the south-west of Germany [28]. After mating and oviposition, larval development may, therefore, start when day lengths are below the critical photoperiod for *prorsa* development of >15.5 h. This threshold is likely to be even higher, given that the mean early-season temperatures are still comparatively low even in unusually warm years and low temperatures require longer day lengths in order to achieve direct development (contrast with the modification of critical day length by low temperatures, as discussed above). In such cases, it would, therefore, be beneficial if larvae were able to identify and respond to a "switch from short days to long days" and accordingly favor the subitaneous pathway over diapause throughout larval development.

Temperature can also modify photoperiod effects at later stages of development. As with many nymphalids and other butterflies, the influence of higher or lower temperatures

during early pupal development can lead to a brightening or darkening of wing color patterns in both *Araschnia* generations. However, a complete change from *levana* to *prorsa* or vice versa is not possible [29]. For subsequent development, only the temperature is relevant because both pupae and imagoes are profoundly insensitive to day length. During diapause, pupae must undergo a cool period (0–10 °C) lasting at least 3 months before eclosion can be induced by spring temperatures of 12–24 °C [26].

2.2. Food Quality

Not only day length and temperature change seasonally but also availability, composition, and quality of food sources. These may, therefore, have an impact on the phenotype in their own right. In both generations, *A. levana* larvae are strictly monophagous and feed exclusively on leaves of the stinging nettle *Urtica dioica*. However, the nutritional quality of this plant deteriorates as the nettle matures and thus also varies seasonally [30–32]. Seasonal variations in adult food sources are also conceivable, suggesting that food quality may influence the fitness of *A. levana*, which may contribute to polyphenism. Access to carbohydrates, nitrogen, and amino acids across juvenile and adult stages is known to influence the adult size, longevity, and fecundity in many lepidopteran species [30,33]. Adult female *A. levana* f. *prorsa* reared on a low-quality larval diet preferred a high-quality nectar mimic containing both essential and non-essential amino acids [33]. The authors proposed that such a preference implies that adult resources are more important when larval reserves are poor and that butterflies may compensate for adverse larval conditions by selective adult feeding. They further demonstrated a negative relationship between emergence mass and amino acid preference regardless of the larval diet, with amino acid preference diminishing as female mass increased. Interestingly, male larvae did not display any food preference regardless of the larval feeding state or emergence mass, suggesting this trait is linked to female fecundity or sex determination.

A follow-up study tested whether the use of nectar amino acids by adult female *A. levana* f. *prorsa* increased fecundity [30]. The authors evaluated the effect of low-quality and high-quality larval diets (based on the *U. dioica* leaf nitrogen content) combined with adult high-quality or low-quality nectar mimics (with or without amino acids). They compared the effects of these four diets on multiple fecundity parameters, including the number of eggs laid, egg mass, longevity, and hatching rate. Female emergence mass (reflecting larval food quality), adult nectar diet, and the amount of nectar consumed all had significant effects on the number of eggs laid. Individuals produced fewer eggs only if they were reared on a low-quality larval diet and, as adults, nectar lacking amino acids. The egg number was on par in the three other groups, including individuals reared on a low-quality larval diet but switched to the amino acid-rich high-quality nectar as adults. However, the egg mass, carbon to nitrogen (C/N) ratio, and hatching rate stayed the same even under adverse conditions, indicating that the fitness cost is purely quantitative. Furthermore, there was no significant effect on longevity in any treatment group, and the C/N ratio of abdomens from female specimens did not vary. The authors reported a positive correlation between butterfly emergence mass and the total number of eggs laid. Emergence mass depends on larval nutrition, and poor-quality foliage is typical for wild *A. levana* larvae feeding later in the year. This supports the presence of polyphenism in addition to compensatory adult feeding.

Differences have been reported in the body composition of pupae and imagoes depending on sex and/or light regime applied during the rearing of larvae [32]. Groups were reared with a 16 h photoperiod representing the spring and early summer conditions of the subitaneous *prorsa* generation and a 12 h photoperiod representing the late summer and early autumn conditions of the diapausing *levana* generation [32]. The water content of male and female pupae was lower in *levana* than *prorsa* individuals, and the male *levana* pupae also featured lower concentrations of lipids, but there were no significant differences in sugar or protein content and dry weight. Interestingly, the differences were not apparent in the adults. Indeed, the water content was higher in *levana* than *prorsa* imagoes at eclosion,

and there was no seasonal difference in lipid content. These findings were put forward as evidence for phenotypic traits associated with pupal diapause and overwintering [32] For example, diapausing *levana* pupae may be adapted specifically to prevent further dehydration, allowing them to enter pupation with a lower water content, protecting them against freezing. The authors did not report an increase in sugar content, but suggested that more subtle changes in sugars related to freeze tolerance in *levana* pupae may have been overlooked. This is because their methods were insensitive to variations in sugars associated with regular metabolism (such as glucose) and sugars that act as cryoprotectants (such as trehalose) but also polyols such as glycerol with a similar role.

The *levana* adults weighed less than their *prorsa* counterparts, which may reflect the metabolic cost of diapause and overwintering [32]. Notably, the lower body mass primarily affected the adult head, thorax, and wings. Because *levana* adults also emerged with lower protein concentrations, the flight musculature (and, by extension, flight capacity) is likely to be affected, consistent with findings in field-caught butterflies [32,34,35]. Both morphs in the study were fed on the same larval diet of freshly-picked *U. dioica* collected between July and August, representing the mediocre to poor food quality typically encountered by *levana* larvae. The study design is, therefore, likely to have masked any polyphenic effects related to differences in food quality.

Recently, a study by Esperk and Tammaru (2021) reported comparisons of various parameters of larval growth schedules in a 2 × 2 × 2 crossed design with photoperiod, temperature, and host plant quality as the varied factors [36]. Specifically (among other findings), they showed that *levana* larvae spent more time in both final and penultimate larval instars. Lower (but not higher) temperatures, also promoted lower *levana* pupal masses. In contrast, *prorsa* larvae displayed higher growth rates—a pattern that was consistent across different rearing conditions, sexes, and larval instars. These authors concluded that their findings demonstrated that the between-generation differences in development have a significant element of anticipatory plasticity and thus should be considered adaptive.

The latter study elegantly demonstrated how the approaches of the other studies described above could be combined to disentangle further the responses to food regimes as well as polyphenic adaptations to predictable seasonal variations in nutrition.

3. Hormonal and Epigenetic Control of the Phenotype

3.1. Hormones

Having determined the role of photoperiod, temperature, and (to a certain extent) other environmental factors on polyphenism in *A. levana*, researchers turned their attention to the translation of these signals at the physiological level. In other lepidopterans, adult development in both subitaneous and diapausing pupae is known to be triggered by the release of the prothoracicotropic hormone (PTTH) from the brain, followed by the release of ecdysone from the prothoracic glands [37]. However, preliminary experiments in *A. levana* and the closely related species *A. burejana* indicated that morph determination depended exclusively on the timing of ecdysteroid release [38,39].

In *A. levana*, subitaneous development is characterized by the accumulation of ecdysteroids in the fourth-instar and final-instar larvae, leading to an earlier *prorsa* pupal molt [40]. In subitaneous *prorsa* pupae, ecdysteroid levels peak at the mid-stage and decrease towards the imaginal molt, whereas the ecdysteroid content of diapausing *levana* pupae is low at the time when *prorsa* pupae develop into adults. In both morphs, the titer of juvenile hormone (JH) is high in the middle of the fourth-instar stage but declines thereafter, falling to undetectable levels two days into the final larval stage. In *levana*, JH remains undetectable for the rest of larval development, but in *prorsa* larvae there are two JH peaks before the pupal molt [40]. It seems plausible that these high JH titers trigger ecdysteroid release from the prothoracic gland during the pupal stage in subitaneous *prorsa* individuals (Figure 2).

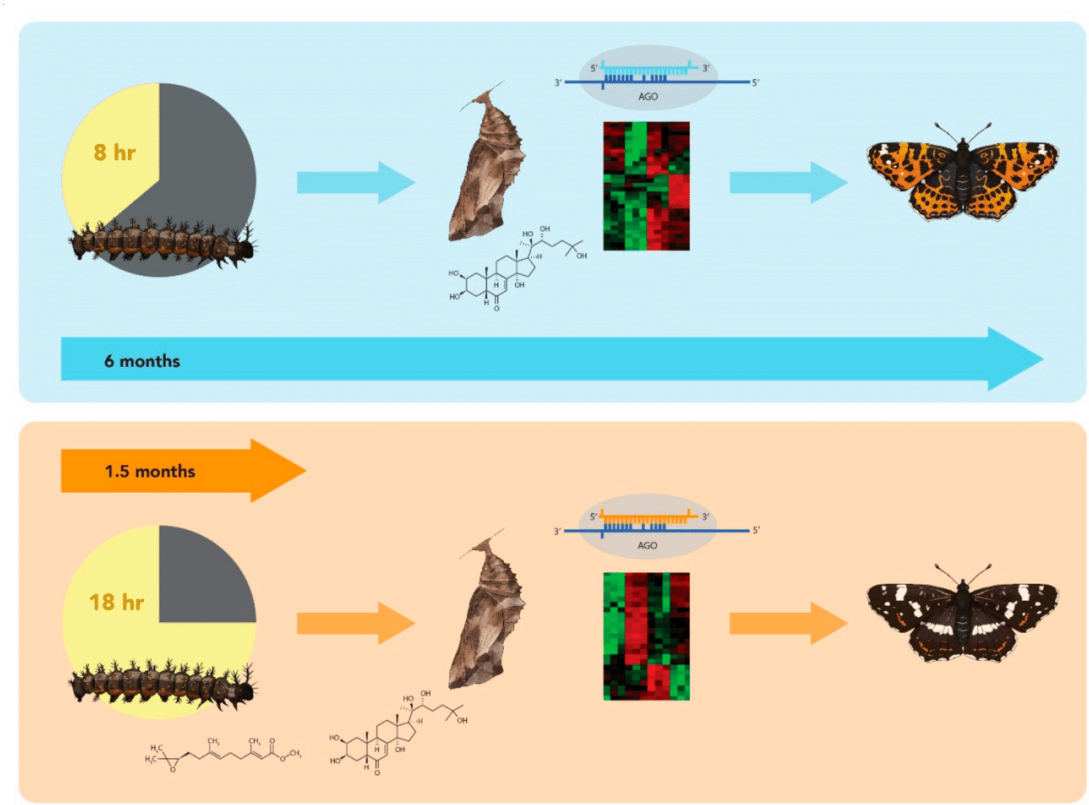

Figure 2. Model of development modes of *A. levana* according to photoperiod and temperature. At 20 °C *A. levana* develops directly (prorsa summer phenotype) if larval development occurs under long-day conditions (18 h photoperiod, lower half, life cycle lasting app. 1.5 months [thick orange arrow]) but switches to diapause development (levana spring phenotype, upper half, life cycle lasting app. 6 months [thick blue arrow]) if short-day conditions (8 h photoperiod) prevail. In the *prorsa* development path, juvenile hormone peaks just prior to pupation, followed by 20-hydroxyecdysone release within three days after the pupal molt. This initiates a gene regulation (e.g., by micro-RNAs, which are short non-coding RNAs of app. 22 nucleotides in length, that mediate gene silencing by guiding Argonaute (AGO) proteins to target sites in the 3′ untranslated region (UTR) of mRNAs [41]) and expression (symbolized by microarray) profile that results in direct metamorphosis onset and development of the adult *prorsa* phenotype. In the *levana* development path, the juvenile hormone signal is absent in the last instar and 20-hydroxyecdysone is not released until a cold period lasting at least 3 months has passed. Then gene expression results in the initiation of the imaginal molt and the adult levana form emerges.

Koch (1987) showed that when adult development was initiated by 20-hydroxyecdysone injection 3 days after pupation, the *prorsa* phenotype was produced. Conversely, when adult development was induced after 5 days or even post diapause, the *levana* form emerged [42]. In consecutive studies, adult *prorsa* development was shown to begin as early as 1 day after pupation, followed by a transitional period of another 2 or 3 days during which, if triggered, intermediate wing coloration would develop, followed thereafter solely by *levana* development. In the latter case, the ecdysteroid level remains low for a cold period lasting more than 3 months, then rises to induce the development of *levana* adults [19,26,40] (Figure 2). These findings suggest that the mination of wing color pattern changes gradually during the first week of pupal life, beginning with the summer morph and then changing into the spring morph [42].

In other words, the ultimate phenotype depends on the time at which adult development is initiated by the release of 20-hydroxyecdysone. The authors also found that both phenotypes could form even in pupae from which the brain-corpora cardiaca-allata

complex had been surgically removed, as long as ecdysteroids were injected at the appropriate time. Accordingly, no brain-derived factors such as PTTH are required, and the polyphenism in *A. levana* is exclusively regulated by the timing of the 20-hydroxyecdysone release. Koch and Bückmann (1987) also showed that both seasonal wing phenotypes, as well as intermediary forms, can (at least experimentally) be produced by pupae that have experienced either long or short larval photoperiods. They concluded that seasonal wing coloration is not immediately affected by the action of day length and that photoperiod only governs pupal diapause or subitaneous development. They postulated a common regulatory mechanism based on the timing of ecdysteroid secretion, which thereby specifies the duration of the pupal stage as well as the adult wing phenotype.

3.2. Circadian Clocks and Epigenetics

In physiological terms, *A. levana* larvae must quantify the light they receive in some manner in order to realize one of the two seasonal phenotypes. It is unclear precisely how this photoperiodic information is perceived, stored, and acted upon during the development of insects [43], although a theoretical framework has been proposed [44]. The first step is light perception by photoreceptors, which probably involves stemmata or extraretinal photoreception, followed by signal transduction [45]. In the brain, a photoperiodic clock responsible for timekeeping measures the hours of darkness in the diurnal cycle, and this mechanism appears to be directly or indirectly sensitive to the temperature. Third, a counter keeps track of the number of times the "long-day threshold" has been crossed to control which developmental pathway should be initiated [19,25,45]. Eventually, the initial photoperiodic signal is converted into a neuroendocrine signal, consistent with the presence or absence of JH peaks in the final-instar larvae. At the onset of the pupal stage, the JH signal is then relayed to target tissues such as the developing wings via the release (presence or absence) of ecdysone. If ecdysone is released within the first couple of days post-pupation, it potentially inhibits a default "*levana* gene expression profile" that leads to morph-specific activities such as the production of more red pigment ommatins in the wings. Chromatin regulation by epigenetic mechanisms such as histone modification, DNA methylation, and the expression of non-coding RNAs may subsequently fix the developmental program, leading to a determined phenotypic outcome [44]. A recent genome-wide study in the model lepidopteran *Manduca sexta* elucidated that complete metamorphosis is associated with profound transcriptional reprogramming mediated by epigenetic modifications such as DNA-methylation, involving approximately half of all the genes in this species [46].

In agreement with the above hypothesis, *A. levana* pre-pupae destined either for diapause or subitaneous development were shown to possess unique transcriptomic profiles consistent with season-specific adaptations [22]. These authors also identified a putative diapause duration clock gene expressed preferentially in diapause pupae, and several differentially expressed genes thought to play roles in the choice between seasonal phenotypes. More recent work has identified microRNAs (miRNAs) that contribute to the epigenetic control of polyphenism in *A. levana* [23]. Multiple miRNAs and their targets were shown to be differentially expressed in final-instar larvae and 1-day-old pupae. Because miRNAs predominantly have a negative effect on gene expression, they may inhibit genes required to generate the *A. levana* "standard morph" in response to environmental cues. Phylogenetic analysis suggests the *levana* morph is most likely the plesiomorphic (primitive) one, whereas the *prorsa* morph is apomorphic (derived) [32]. JH release in the final-instar larvae and/or early ecdysone release in the subitaneous pupae may, therefore, modify the expression of miRNAs that regulate the expression of genes necessary for both diapause and the formation of the *levana* phenotype (Figure 2). The examples below indicate how this may proceed at the epigenetic level.

For diapause maintenance, miR-289-5p (among others) is thought to silence the expression of genes related to metabolic processes during diapause in the flesh fly *Sarcophaga bullata* [47]. In *A. levana*, this miRNA was upregulated in larvae destined for diapause

compared to those primed for subitaneous development, although there was no differential expression in the pupae [23]. If we assume that the properties of miR-289-5p as a diapause regulator are evolutionarily conserved, the following deduction would be reasonable. In *A. levana*, the initiation of gene expression responsible for metabolic arrest begins at the very end of the larval stage. A link to the JH signal in subitaneous final-instar larvae is also likely because its presence coincides with the significantly reduced expression of miR-289-5p at this stage. If true, JH may inhibit the expression of this candidate metabolic suppressor, which then ultimately leads to subitaneous development.

The regulatory mechanism that controls the duration of diapause and the determination of the adult phenotype is unclear, but preliminary and yet inconclusive experimental evidence points towards a diapause duration clock protein. In *Bombyx mori*, the ATPase TIME-EA4 measures time intervals and functions as a clock in diapausing eggs. It is important to determine the moment when diapause should be broken [48]. In *A. levana*, an ortholog of TIME-EA4—named the diapause bioclock protein (DBP)—was found to be strongly induced in *levana* pre-pupae destined for diapause [22]. Subsequent in silico target prediction provided evidence that DBP expression is regulated by miR-2856-3p [23] However, this miRNA was also strongly upregulated in final-instar *levana* larvae when compared to subitaneous *prorsa* larvae, and the highest levels were reached in pupae representing both developmental pathways. This expression profile shows that DBP cannot be controlled by miR-2856-3p alone, but other epigenetic mechanisms such as histone acetylation or DNA methylation may well contribute to its regulation. Moreover, miRNAs can have hundreds of targets in insects [49] and further regulatory roles for miR-2856-3p, therefore, cannot be ruled out. Interestingly, when injected with an inhibitor of miR-2856-3p, final-instar subitaneous *prorsa* larvae displayed an intermediate *prorsa/levana* adult phenotype in 5% of tested specimens (Figure 3). Polyphenism-determining miRNAs have been reported in both hemimetabolous and holometabolous insects [50,51]. It is, therefore, conceivable that polyphenism in *A. levana* may also be controlled by a single miRNA master switch located directly upstream of the phenotype effector pathways, in which miR-2856-3p plays a key role but cannot be a sole determinant. Most likely, other epigenetic regulators are also involved.

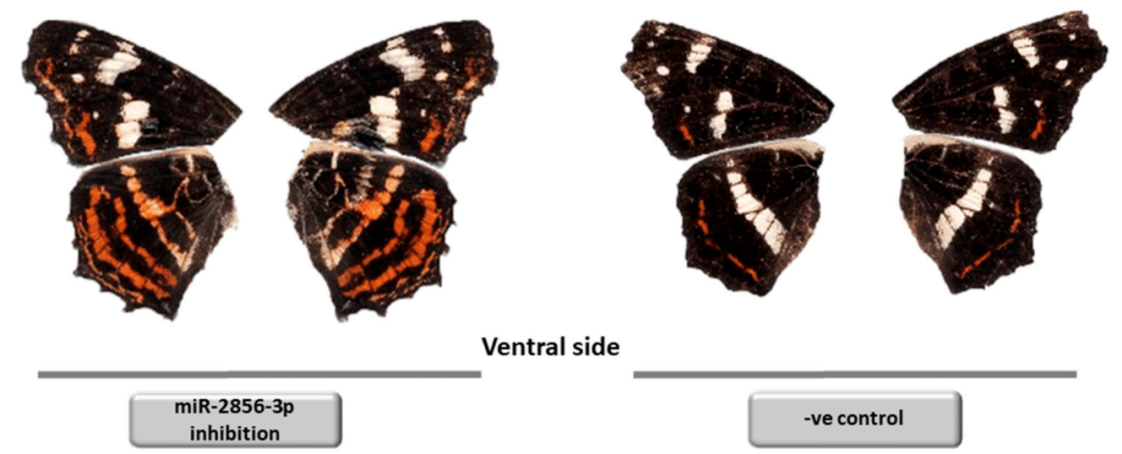

Figure 3. Intermediate and regular *prorsa* phenotypes of *A. levana*. The intermediate phenotype (**left**) was generated by injecting an inhibitor of *Bombyx mori* (bmo)-miR-2856-3p, whereas the normal phenotype (**right**) developed following the injection of PBS (control). The intermediate phenotype was observed in 5% of specimens [23].

In conclusion, these findings suggest a complex regulatory system controls diapause initiation, maintenance, and duration as well as the determination of the adult phenotype, with miRNAs such as miR-289-5p and miR-2856-3p as key components. However, the effects are not always consistent with a straightforward mechanism based on the inhibition

of target effectors. Future research should, therefore, focus on the functional analysis of miRNAs and regulatory proteins, which will be facilitated by the availability of genomic and transcriptomic data as well as miRNA target predictions for both spring and summer generations [22,23,52]. Moreover, CRISPR/Cas9 genome editing is now feasible in lepidopteran species, making gene knockout studies in *A. levana* a promising approach [53]. Even though we still lack a complete picture of the regulatory network, the examples discussed above strongly suggest that both hormones and epigenetic mechanisms control the integration of environmental signals in *A. levana* to generate specific seasonal phenotypes.

4. Photoperiod-Specific Polyphenic Responses

4.1. Morphology

In addition to the visually striking wing polyphenism, the *A. levana* adult phenotype is affected by the photoperiod in other, more subtle ways, including biomechanical design. The *prorsa* imago is larger than the *levana* imago in absolute terms (larger, longer, less pointed wings, heavier thorax) and has a higher thorax muscle ratio, whereas the *levana* imago has a higher wing loading (i.e., fresh body mass divided by wing area) and a higher relative abdomen mass [35]. This is likely advantageous because *A. levana* tends to remain in the native habitat during the spring and invests in reproduction, whereas it disperses and expands its range in search of new habitats over the summer [35].

4.2. Immunity

The two *A. levana* morphs also show differences in larval immunocompetence, based on survival analysis following infection with the bacterial entomopathogen *Pseudomonas entomophila*, the antibacterial activity analysis in the hemolymph, and the expression of selected genes encoding antimicrobial peptides [54]. The *levana* larvae survived significantly longer than *prorsa* larvae, the antibacterial activity in the hemolymph was more potent, and genes encoding antimicrobial peptides were expressed at higher levels. Further analysis revealed that final-instar *levana* larvae also produce higher levels of phenoloxidase and lytic activity than *prorsa* larvae [55]. These results suggest a trade-off between immunity and other traits, such as reproduction. The seasonal adaptations of the distinct phenotypes of *A. levana* in terms of immunology can be explained plausibly by selective advantages. A more robust immune system in larvae committed to diapause [54,55] may benefit the pupae that are longer exposed to pathogens and parasites over winter.

4.3. Wing Pattern and Color

The most obvious differences between the seasonal phenotypes are the wing pattern and the color displayed by the spring and summer imagoes of *A. levana*, raising the question of why is it beneficial to be orange in spring and blackish in summer? The degree of melanization in insects has been attributed to environmental temperature adaptations. For example, the harlequin ladybird *Harmonia axyridis* (also known as the multicolored Asian ladybird) is a textbook example of polymorphism and polyphenism. It displays a number of distinct morphs differing in overall color and the number of the spots on the elytra. The black (melanic) forms (axyridis, conspicua, and spectabilis) are postulated to be advantageous in cold climates because dark surfaces absorb heat more quickly during exposure to sunlight. Such seasonal phenotypic plasticity allows individuals to produce the level of melanin necessary to maintain activity at the temperatures encountered when they emerge. In line with this hypothesis, it has been reported that cold temperature favors the darker morphs, and exposure of last instar larvae or pupae to an elevated temperature decreases the melanization of the *H. axyridis* beetles [56,57]. However, this widely accepted hypothesis does not apply to *A. levana* imagoes because their darker summer phenotype lives at elevated environmental temperatures when compared with the brown spring phenotype (Figure 1). A stronger melanization of the summer morph may result in a higher protection against pathogens and parasitoids, but the latter instead attack larvae and pupae of *A. levana*, which do not display differences in body color. Another possibility explaining

seasonal polyphenism in *A. levana* butterflies could be distinct seasonal predation by insectivorous birds [58], resulting in adaptations related to camouflage, but there are no empirical data supporting this hypothesis.

5. Concluding Remarks

The development of *A. levana* is influenced by various environmental factors, including photoperiod, temperature, and seasonal changes in food quality [32,33,59]. However, the extent to which changes in body composition or life history traits are polyphenic adaptions to this largely predictable nutritional variation is unclear because most studies tested only the *prorsa* morph. The observed effects could also result directly from the diet quality, and this should be tested in future studies by co-varying light regimes and diets. In fact, a recent study by Esperk and Tammaru (2021) demonstrated that in terms of growth parameters, many between-generation differences have an adaptive nature and can, therefore, be considered part of the phenotypic response to seasonality. Similar study designs should be employed to scrutinize changes in body composition or life history traits reported in earlier studies.

The photoperiod takes overall precedence as the key climate signal that guides the decision between subitaneous and diapause development, but the outcome can be modified and fine-tuned by prevailing temperatures [24–26]. It will be interesting to see how *A. levana* responds to climate change, and this species could emerge as a useful bioindicator to track the increasing impact of rising temperatures on local ecosystems.

The photoperiodic signal is translated into physiological responses via hormonal regulation. If JH is released in the final-instar larvae, this is followed by early ecdysone release during the first few days of pupation, leading to subitaneous *prorsa* development. In contrast, the absence of JH delays ecdysone release such that the levels peak only after months of diapause, thus triggering *levana* development [19,40]. In addition to the striking differences in wing color and patterning, additional polyphenic distinctions include the details of biomechanical design and immunocompetence.

The perception of light signals (or their absence) and the subsequent storage and transmission of this information to the endocrine system and epigenome are not clearly understood. Despite considerable advances in the study of insect photoperiodism, the full pathway leading from photoreception to diapause and altered phenotypes is not known [43]. The mechanism of timekeeping and its integration should be prioritized when designing novel studies in this species. Epigenetic mechanisms such as the expression of miRNAs appear to play a key role in the transcriptional reprogramming that accompanies developmental commitment, with the *levana* morph seen as the default pathway and the *prorsa* morph as the alternative, but their position within the "environmental signal integration network" remains to be clarified [23].

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ in carrying out the investigations described in the dissertation.

Friedberg, April 2022

X _____

Arne Baudach