MicroRNA-dependent metamorphosis in hemimetabolan insects

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How does a juvenile insect transform into an adult? This question, which sums up the wonder of insect metamorphosis, has fascinated mankind since ancient times. Modern physiology has established the endocrine basis regulating these transformations, which mainly depend on two hormone types: ecdysteroids, which promote molts, and juvenile hormones, which repress the transformation into the adult stage. The interplay of these two hormones regulates the genes involved in juvenile and adult programs and the shift from one to the other. microRNAs (miRNAs) are small noncoding RNAs, which participate in many biological processes, and we wondered whether they might be also involved in insect metamorphosis. In insects, Dicer-1 ribonuclease transforms miRNA precursors into mature miRNAs. Thus, using systemic RNA interference (RNAi) to silence the expression of Dicer-1 in the hemimetabolan insect *Blattella germanica*, we depleted miRNA contents in the last instar nymph. This practically inhibited metamorphosis after the next molt, as the resulting specimens showed nymphoid features and were able to molt again. The experiments show that miRNAs play a key role in hemimetabolan metamorphosis, perhaps regulating genes that are juvenile hormone targets.

Blattella | dicer | microRNAs

Research into insect metamorphosis has traditionally focused on morphological aspects by differentiating two basic modes: the hemimetabolan and the holometabolan. Hemimetabolan species grow gradually, with each successive nymphal instar increasingly resembling the adult form until the final transition from nymph to adult, which is characterized by the displaying of functional wings and the appearance of external genitalia. Holometabolan species grow gradually through larval instars, which are very different from the adult, until the transitions from larva to pupa and from pupa to adult, which are characterized by dramatic morphological and functional changes (1). Since the 1940s, and mainly thanks to the contributions of Sir Vincent B. Wigglesworth, research in this field has focused on the endocrine aspects, particularly the actions of juvenile hormones (JHs) and ecdysteroids, and the gene cascades activated or repressed by them (2). Only a few reports considered the role of miRNAs, and all of which were based on the extremely modified holometabolan species *Drosophila melanogaster* (3, 4).

miRNAs play a critical role in many biological processes, by modulating gene expression at the posttranscriptional level through binding at the 3′-untranslated region of the target mRNA (5, 6). Dicer ribonucleases are important in the biogenesis of miRNAs as they are involved in the production of mature miRNAs from miRNA precursors (premiRNAs), and of small interfering RNAs (siRNAs) in the RNA interference (RNAi) pathway (7). However, whereas a single Dicer ribonuclease is involved in both miRNA and siRNA production in the nematode *Caenorhabditis elegans* and in vertebrates, two of them, known as Dicer-1 and Dicer-2, which act in the miRNA and siRNA pathways, respectively, exist in the fruitfly *D. melanogaster* (7).

As miRNA production is Dicer-dependent, one approach to studying the function of miRNAs in developmental processes has involved studying Dicer mutants. Thus, loss of Dicer-1 in *D. melanogaster* results in embryogenesis defects, within both the somatic- and germ-lineages (7), and in impaired olfactory neuron morphogenesis (8). Dicer silencing might therefore be a useful approach when studying the possible role of miRNAs in insect metamorphosis and, to this end, hemimetabolan species can offer more convenient models than holometabolan ones because they are less modified and, in principle, the mechanisms involved should be simpler.

Results

Dicer-1 of *Blattella germanica*. We used the cockroach *Blattella germanica* as a hemimetabolan model species. In a first step, we cloned a fragment of its Dicer-1 cDNA by RT-PCR using ovarian tissue as template and degenerate primers based on conserved motifs of known insect Dicer-1 sequences. Subsequent 5′ and 3′ rapid amplification of the cDNA ends (RACE) gave a full-length sequence of 7,300 nucleotides, which encoded a protein of 2,271 amino acids with a predicted molecular mass of 259.27 kDa. BLAST analysis indicated that the protein was a Dicer ortholog. In addition, a ScanProsite search revealed that the sequence has two amino-terminal DExH-Box helicase domains, a PAZ (Piwi/Argonaute/Zwille) domain, two RNase III domains and a carboxy-terminal dsRNA binding domain (Fig. 1*A*), which is a typical organization of a Dicer protein (9). Compared with *D. melanogaster* Dicer proteins 1 and 2 (DmDcr1 and DmDcr2), the *B. germanica* sequence has two helicase domains like DmDcr2, whereas DmDcr1 has only one. However, the *B. germanica* sequence shows 45% identity with DmDcr1 and only 19% with DmDcr2, whereas the PAZ domain is much more similar to DmDcr1 (74% identity) than to DmDcr2 (10%). We thus concluded that *B. germanica* sequence corresponds to Dicer-1, and we called it BgDcr1 (GenBank accession no. FN298876).

RNAi of Dicer-1 Depletes miRNAs. To silence Dicer-1 expression in *B. germanica* by RNAi, we prepared a dsRNA encompassing a 343-bp region placed between the RNaseI and RNaseII domains of BgDcr1 (dsBgDcr1-A) (Fig. 1*A*), which was injected at a dose of 3 μg in *B. germanica* females at the freshly emerged fifth nymphal instar. As control dsRNA, we used a noncoding sequence from the pSTBlue-1 vector (dsMock) injected at a dose of 3 μg. Expression of BgDcr1 showed few variations during the sixth (last) instar nymph (Fig. 1*B*), and we chose day 4 of that stage to assess the effects of the RNAi treatment on BgDcr1 mRNA levels, as this day precedes the onset of the ecdysteroid peak that determines the imaginal molt (10). The results showed

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Author contributions: X.B. designed research; E.G.-O. performed research; X.B. analyzed data; and X.B. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The sequence reported in this paper (BgDcr1) has been deposited in the GenBank database (accession number: FN298876).

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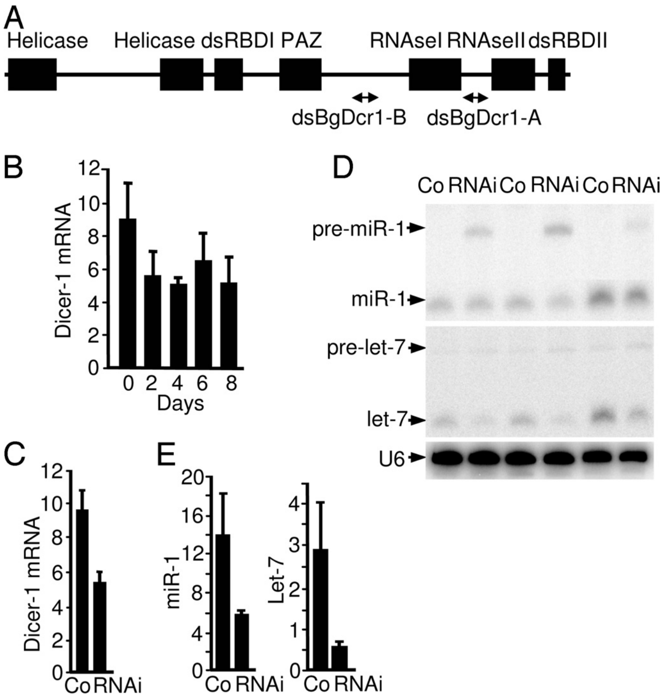

Fig. 1. *Blattella germanica* Dicer-1 (BgDcr1) and effects of RNAi. (*A*) Scheme of the organization of BgDcr1, indicating the regions targeted by dsBgDcr1-A and dsBgDcr1-B. (*B*) Expression profile of BgDcr1 during last instar nymph ($n = 3$). (*C*) BgDcr1 mRNA levels in dsBgDcr1-A-treated (RNAi) and dsMock-treated (Co) specimens ($n = 3$). In *B* and *C*, results are expressed as copies of Dicer-1 mRNA per 1,000 copies of actin-5C mRNA. In *C*, REST© statistical analysis indicates that Dicer-1 is down-regulated by a mean factor of 0.519, and that RNAi sample group is different to Co group ($P(H1) = 0.048$). (*D*) Northern blot analysis of miR-1 and let-7 and their corresponding precursors (premiR-1 and prelet-7) in dsBgDcr1-A-treated (RNAi) and dsMock-treated (Co) specimens; the small noncoding RNA U6 was used as a reference; the three lanes of RNAi and respective Co, represent three parallel experiments (the same as in *C* and *E*). (*E*) RT-PCR quantification of miR-1 and let-7 in dsBgDcr1-A-treated (RNAi) and dsMock-treated (Co) specimens ($n = 3$). In *E*, results are expressed as copies of miR-1 or let-7 per copy of U6. REST© statistical analysis indicates that miR-1 is down-regulated by a mean factor of 0.450, and let-7 by a mean factor of 0.232. Both miR-1 and let-7 sample groups (RNAi) are different to their respective control (Co) group ($P(H1) = 0.0001$).

that BgDcr1 mRNA levels had decreased significantly with respect to controls (Fig. 1*C*). We then assessed whether RNAi of BgDcr1 had impaired miRNA formation, taking miR-1 and let-7 as examples (*SI Results and Discussion*). These two miRNAs are conserved across the animal kingdom and can be used as appropriate references as miR-1 shows a practically invariant expression during the whole postembryonic development whereas let-7 is characteristically up-regulated in the transition from larval to adult stages, at least in *D. melanogaster* and *Bombyx mori* (11–13).

Total RNA enriched for small RNAs was extracted on day 4 of the last instar nymphs, which had been treated in the former instar with dsMock or dsBgDcr1-A. Northern blot analysis showed that miR-1 and let-7 levels were lower in specimens treated with dsBgDcr1-A (Fig. 1*D*) whereas those corresponding to the respective premiRNAs were higher, as expected. We then quantified miR-1 and let-7 levels by quantitative RT-PCR, and the results (Fig. 1*E*) confirmed that they were significantly lower in dsBgDcr1-A-treated specimens than in controls.

Depletion of Dicer-1 and miRNAs Impairs Metamorphosis. All females treated as freshly emerged penultimate nymphal instar with dsMock ($n = 73$), molted to last instar nymph (Fig. 2*A* and *B*) and then to adult (Fig. 2 *C* and *D*), with the proper timing and morphology. In contrast, those treated with dsBgDcr1-A ($n = 106$) molted to last instar nymph normally, but in most of them (87, i.e., 82%) the next molt gave specimens with nymphoid features; that is, nymphal general shape, black abdominal sternites, genital region with deformities (sometimes showing the genital pouch partially reversed and somewhat swollen), and wings severely shortened and twisted (Fig. 2 *E* and *F*). The remaining specimens (19, i.e., 18%) molted to adults morphologically similar to controls (adult shape, yellow abdominal sternites, and genital region well-formed) except for the morphology of the wings, which were moderately twisted (Fig. 2 *G* and *H*) (Table 1). Most of the nymphoid specimens (68 out of 87, i.e., 78%) died within the first 9–14 days after the molt, whereas the 19 specimens that survived underwent a subsequent molt (see below).

To assess the specificity of the effects observed, we repeated the experiments using an alternative dsRNA for BgDcr1, this time targeting a 469-bp region placed between the PAZ domain and the RNaseI domain, which we called dsBgDcr1-B (Fig. 1*A*). Results (Table 1) were virtually identical to those obtained with dsBgDcr1-A (*SI Results and Discussion*).

In additional experiments, we treated freshly emerged fourth nymphal instar specimens with dsBgDcr1-A, which molted correctly to the fifth and sixth nymphal instars and then to adult. The adults had a normal appearance although occasionally (31% of the specimens, Table 1) showed the wings not well-stretched. Finally, we treated freshly emerged sixth (last) instar nymphs with dsBgDcr1-A, which underwent a practically normal imaginal molt, although 62% of the adults (Table 1) showed the wings slightly twisted. In these two series of experiments, BgDcr-1 mRNA levels on day 4 of last instar nymph tended to be lower in dsBgDcr1-A-treated specimens than in controls, but differences were not statistically significant (Fig. 2 *I* and *J*). The mild phenotype obtained in the experiments with fourth instar nymphs may be explained by the recovery of Dicer-1 after the three instars following the treatment with dsBgDcr1-A. The practical absence of effects in the experiments with sixth instar nymphs may be because Dicer-1 was not depleted enough at the onset of the imaginal apolysis.

Dicer-1 Knockdowns Do Not Have Higher JH Levels. The nymphoid phenotype is reminiscent of those resulting from treatments with JH (14). Therefore, we treated freshly emerged last instar nymphs with 10 μg of JH III, which is the native JH in *B. germanica* (15), and we found that they molted to nymphoids (*SI Results and Discussion*) practically identical to those treated with dsBgDcr1-A (Fig. S1). This suggested to us that interference of Dicer-1 might have increased JH production. We therefore measured JH III synthesis in experimental specimens on day 4 of the sixth instar nymphs, and the results indicated that rates of JH synthesis in specimens that had been treated with dsBgDcr1-A were as low as in the controls (Fig. 2*K*). We also measured JH production in untreated 5-day-old fifth instar nymphs, which gave higher levels (Fig. 2*K*), as expected in a nonmetamorphic instar (15).

Nymphoids Resulting From Dicer-1 RNAi Are Able to Molt Again. All nymphoid specimens that survived beyond day 14 in the seventh stage ($n = 19$) molted again 1 or 2 days later (see below), although none of them were able to completely shed their exuviae (Fig. 3*A*). Examination of the ectodermal tissues revealed that there was a new cuticle below the remnants of the old one (Fig. 3 *B–E*), thus indicating that these insects were able to undergo apolysis but could not complete the ecdysis. They died within 48 h, but peeling off the exuviae revealed that they had adult features (adult general shape, yellow abdominal sternites, and genital region well-formed), but with the wings not well-

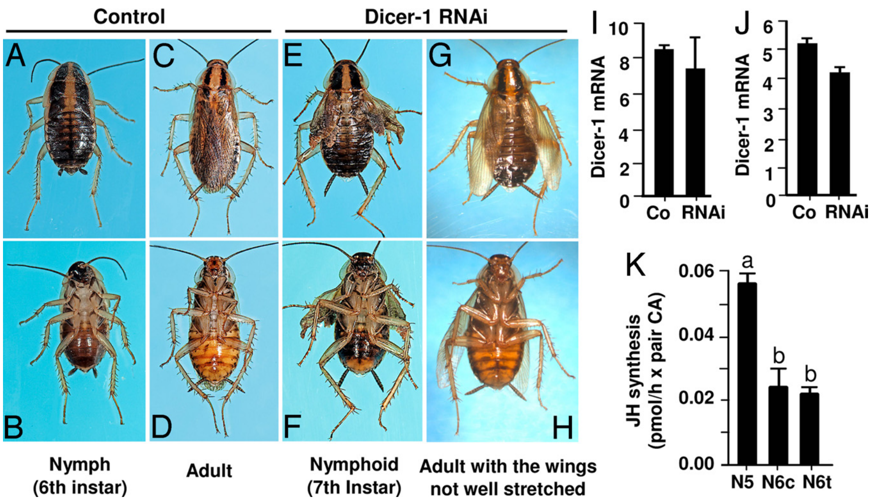

Fig. 2. Effects of dsBgDcr1-A in *Blattella germanica*. (*A*—*H*) Effects on metamorphosis in the experiments carried out in fifth instar nymphs; dorsal and ventral view of: normal sixth instar nymph (*A* and *B*), normal adult (*C* and *D*), seventh instar nymphoid (*E* and *F*) and adult with the wings not well stretched (*G* and *H*), females in all cases. (*I*—*J*) BgDcr1 mRNA levels in dsBgDcr1-A-treated (RNAi) and dsMock-treated (Co) specimens (n = 3) in the experiments carried out in fourth (*I*) and in sixth (*J*) instar nymphs; results are expressed as copies of Dicer-1 mRNA per 1,000 copies of actin-5C mRNA; both in *I* and *J*, REST© statistical analysis indicates that RNAi sample group is not different to Co group (P(H1) = 0.331 and P(H1) = 0.199, for *I* and *J*, respectively). (*K*) Rates of juvenile hormone III (JH) synthesis in control penultimate instar nymphs (N5) and in dsMock-treated (N6c) and dsBgDcr1-A-treated (N6t) last instar nymphs (day 4 of the respective stage in all experiments); results expressed as the mean ± SD (n = 7–9); different letters indicate statistically significant differences (one-way ANOVA, $P < 0.0001$).

stretched. The imperfect ecdysis carried out by seventh instar nymphoids might be explained by remnant effects of the Dicer-1 RNAi in the previous instar.

In *B. germanica* the prothoracic gland (PG), which produces the ecdysteroids necessary for molting, has a characteristic X-shape and degenerates within the first 24 h after the imaginal molt (16). The above supernumerary molt (Fig. 3*A*), however, suggested to us that the PG had not degenerated in the seventh instar nymphoids. Indeed, 10 days after the molt, the PG of these nymphoids has a turgid and lobulated aspect (Fig. 3*F*), very similar to that of fully secreting glands from untreated sixth instar nymphs (Fig. 3*G*). Observed at higher magnification, the PG from nymphoids shows the polyploid glandular cells densely packed (Fig. 3*H*), which is typical of secreting cells (16). In addition, TUNEL assays in PG from dsMock-treated specimens, indicated that cell death was actively proceeding 1 day after the imaginal molt (Fig. 3*I*), as expected (16). Conversely, the PG from seventh instar nymphoids on day 10 did not show labeled cells (Fig. 3*J*), thus indicating that they were alive.

Finally, we measured the ecdysteroid titer in seventh instar nymphoids on day 10 (75% of time elapsed in the instar) and found that it was high (Fig. 3*K*), with values that were not significantly different from those measured on day 7 (75% of time elapsed in the instar) of untreated sixth instar, and much higher than those found in 7-day-old adults that had been treated with dsMock when they were still nymphs (Fig. 3*K*). Ecdysteroids in the adult female do not come from the PG, which has degenerated, but from the ovaries (16).

Discussion

Our results indicate that depletion of Dicer-1 in the penultimate nymphal instar of the cockroach *B. germanica* results in reduced levels of mature miRNAs in the last instar nymph and in severely impaired metamorphosis after the next molt. Thus, instead of an adult, seventh instar nymphoids were obtained, which can be considered to be a supernumerary nymphal instar in light of their morphology, the persistence of their PGs and their ability to molt again. The results therefore suggest that Dicer-1 and miRNAs are crucial for modulating hemimetabolan metamorphosis and that miRNAs apparently act by disrupting translation and promoting mRNA decay of genes expressing nymphal features. The nymphoids obtained after RNAi of Dicer-1 are externally similar to those resulting from JH treatment in last instar nymph. However, RNAi of Dicer-1 does not increase JH production.

Table 1. Summary of the RNAi experiments carried out on different nymphal instars of *Blattella germanica*

			Phenotype** (number of specimens and percentage)		
Instar	dsRNA*	*n*	Nymphoid, %	Adult with twisted wings, %	Normal adult, %
5th	dsDicer1-A	106	87 (82)	19 (18)	0 (0)
5th	dsMock	73	0 (0)	0 (0)	73 (100)
5th	dsDicer1-B	23	21 (91)	2 (9)	0 (0)
5th	dsMock	14	0 (0)	0 (0)	14 (100)
4th	dsDicer1-A	36	0 (0)	11 (31)	25 (69)
4th	dsMock	18	0 (0)	0 (0)	18 (100)
6th	dsDicer1-A	42	0 (0)	26 (62)	16 (38)
6th	dsMock	16	0 (0)	0 (0)	16 (100)

*Each experiment with dsDicer-1 is accompanied by their respective control (dsMock).
**See the text for a complete description of the phenotypes.

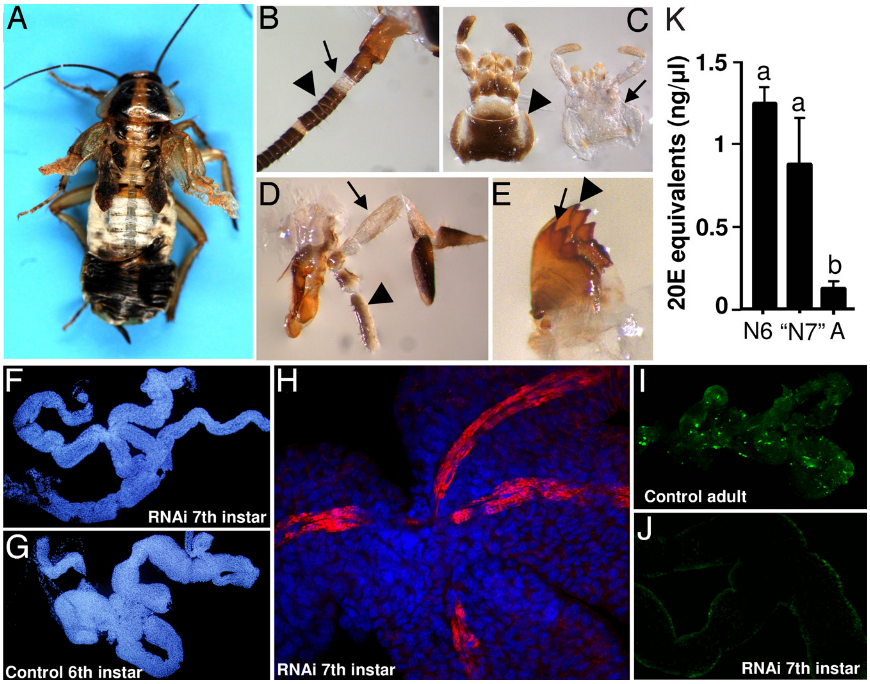

Fig. 3. Supplementary molt in *Blattella germanica* treated with dsBgDcr1-A. (*A*) Adult emerging after the molt of a seventh instar nymphoid, which resulted from Dicer-1 RNAi. (*B–E*) double cuticle structures in the same specimen, antennae (*B*), labium (*C*), maxillae (*D*), and mandible (*E*); the new and old cuticle structures are indicated by arrows and arrowheads, respectively; in *C*, the double structures have been separated by dissection. (*F* and *G*) Central portion of a PG from a 9-day-old seventh instar nymphoid (*F*) and from an untreated 7-day-old sixth instar nymph (*G*), stained with DAPI. (*H*) Detail of a PG from a 9-day-old seventh instar nymphoid stained with DAPI-rhodamine phalloidin, showing the polyploid glandular cells densely packed; the structures stained in red are the muscular axis of the gland. (*I* and *J*) Central portion of a PG from an untreated 1-day-old adult (*I*) and from a 9-day-old seventh instar nymphoid (*J*) stained with TUNEL method; the fluorescent green points (present in *I* and absent in *J*) correspond to fragmented DNA. (*K*) Ecdysteroid contents in untreated 7-day-old last instar nymph (N6), in 10-day-old seventh instar nymphoids (''N7'') and in 7-day-old adults (*A*); results expressed as the mean ± SD ($n = 6–11$); different letters indicate statistically significant differences (one-way ANOVA, $P < 0.001$).

This suggests that the miRNA pathway acts either independently or downstream of the JH signal, perhaps regulating genes that are JH targets, and whose gene products give nymphal features or have antimetamorphic properties.

The loss of Dicer-1 in *D. melanogaster* results in embryogenesis defects (7) and disruption of olfactory neuron morphogenesis (8). Moreover, developmental expression patterns of selected miRNAs in *D. melanogaster* (11, 12) and *B. mori* (13, 17) have revealed that a number of them are up-regulated in the transition from larvae to pupae, and of these let-7 is the most thoroughly studied (18, 19). In the transition to adult of *D. melanogaster*, let-7 is required for neuromusculature remodeling (4), for proper timing in wing cell cycle, and for the maturation of neuromuscular junctions (3). Let-7 knockout flies display behavioral defects (deficient flight and motility), impaired fertility, and weakened neuromusculature, although externally they appear normal (3). Interestingly, RNAi of Dicer-1 at metamorphosis of another holometabolan species, the beetle *Tribolium castaneum*, results in a mild morphological phenotype with only occasional wing expansion defects (20), which suggests that there may be important differences concerning the role of miRNAs in holometabolan and hemimetabolan metamorphosis.

It has not escaped our notice that specific miRNAs, which are generally up-regulated in the transition from immature stages to the adult, such as let-7 and others, like miR-100 and miR-125 (11, 12), could repress nymphal characters and contribute to adult differentiation. Work along this line, including the identification of miRNAs which increase their expression in the last instar nymph of *B. germanica*, the study of the effects of selective silencing of these miRNAs on metamorphosis, and the study of the action of juvenile hormone and 20-hydroxyecdysone on their expression, is currently in progress in our laboratory.

Methods

B. germanica colony was reared on dog chow and water, in the dark at 30 ± 1 °C and 60–70% r.h. Degenerate primers based on the conserved regions of insect Dicer-1 followed by 3′-RACE and 5′-RACE approaches were used to obtain a *B. germanica* homologue of Dicer-1 (21, 22). Methods for dsRNA preparation and of RNAi were as described in refs. 21 and 22; dsRNA was injected into the abdomen of newly emerged female nymphs. Quantification of mRNAs, RNA extraction, and reverse transcription were performed as in previous works (21, 22), real-time PCR was carried out as described in ref. 23, and results are given as copies of mRNA per 1,000 copies of actin-5c mRNA. For Northern blot analysis and PCR quantification of miRNAs, RNA was extracted with miRNeasy Mini kit (Qiagen); enrichment of low molecular weight RNA and blot hybridization were performed as described in ref. 24; [γ-^{32}P] ATP labeling of oligonucleotides complementary to miR-1, let-7, and the small noncoding RNA U6 of *B. germanica*, and Northern blot procedures were as reported in ref. 24. For PCR quantification of miR-1 and let-7, qtRT-PCR was carried out according to the instructions of the Ncode miRNA first-strand cDNA synthesis kit (Invitrogen); relative expression was determined with reference to U6. Quantification of JH III synthesis by corpora allata incubated in vitro was performed as described in ref. 25. Hemolymph ecdysteroids contents were quantified by ELISA, as reported in ref. 16. Current dissections and microscopical observations were carried out as described in ref. 22. To detect cell death in the prothoracic gland, TUNEL assays were performed as in previous works (22). Detailed methods are provided in *SI Methods*.

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