microRNAS and metamorphosis in the hemimetabolous insect *Blattella germanica* (L.) (Dictyoptera, Blattellidae)

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Abstract

Previous work carried out in the host laboratory, using the basal insect *Blattella germanica* as model, showed that microRNAs (miRNAs) are crucial to complete metamorphosis. The general goal of this thesis was to identify particular miRNAs involved in this process. As a first step, we established a general catalogue of miRNAs in *B. germanica* using high throughput Solexa sequencing. Thereafter, we prepared two miRNA libraries; one in the metamorphic stage and other one in the non-metamorphic stage, to distinguish miRNAs differentially expressed between the two stages, and to assess the influence of the main metamorphosis hormones on the expression of these miRNAs. Our experiments also showed that Broad complex transcription factors induce the expression of let-7 and miR-100, and that these miRNAs play a role in regulating the size and the vein-intervein patterning of *B. germanica* wings. Finally, we studied the role of miR-8-3p and miR-8-5p in regulating the transcript levels of atrophin, a factor involved in neuromuscular coordination, which is important to ensure a proper ecdysis in the metamorphic molt.

Resumen

Trabajos previos llevados a cabo en el laboratorio de acogida, usando como modelo el insecto basal *Blattella germanica*, mostraron que los microRNAs (miRNAs) son cruciales para completar la metamorfosis. El objetivo general de esta tesis fue identificar miRNAs que estuviesen implicados en este proceso. Como primer paso, se estableció un catálogo general de miRNAs en *B. germanica* mediante secuenciación con Solexa. A partir de ahí, se prepararon dos librerías de miRNAs, una en la etapa metamórfica y otro en la etapa no metamórfica, para distinguir miRNAs diferencialmente expresados entre las dos etapas y evaluar la influencia de las hormonas principales de la metamorfosis sobre la expresión de estos miRNAs. Nuestros experimentos también mostraron que los factores de transcripción Broad-complex inducen la expresión de let-7 y miR-100, y que estos miRNAs desempeñan un papel en la regulación del tamaño y del patrón de venas e intervenas de las alas de *B. germanica*. Por último, se estudió el papel de miR-8-3p y miR-8-5p en la regulación de los niveles de transcrito de atrofina, un factor implicado en la coordinación neuromuscular, que es importante para asegurar una adecuada ecdisis en la muda metamórfica.

Preface

The metamorphosis has been one of the key innovations in the evolution of insects, which had a decisive influence on the dramatic diversification of that class, at present having about one million described species. Today, we know the endocrine factors that regulate the metamorphic changes in insects. In short, the ecdysteroid 20-hydroxyecdysone (20E) induces successive molting, while the terpenoid, juvenile hormone (JH) determines the characteristics of the molting: juvenile when JH is present, and imaginal when it is absent (Truman & Ridiford, 2002). microRNAs (miRNAs) are small (about 22 nucleotides) non-coding RNAs that bind to the 3'-untranslated regions of the messenger RNA of the target genes by imperfect base pairing, and cause regulation of gene expression at the post-transcriptional level.

This thesis aims at studying the potential regulatory role of miRNAs in regulating insect metamorphosis, using the hemimetabolous insect *Blattella germanica* as model. Previous results obtained in our laboratory indicate that silencing of dicer-1 by RNAi in last nymphal instar, results in an imperfect transition to the adult stage. The knockdown specimens molt into creatures showing a mixture of adult and nymphal features. Given that dicer-1 mediates the transformation of miRNA precursors into mature miRNAs, the above results suggest that mature miRNAs are important to regulate the transition from nymph to adult, that is, to modulate metamorphosis. The next steps, which will constitute the objectives

of the present thesis, would be the identification of particular miRNAs involved in regulating the metamorphic change in *B. germanica*.

Given that the sequence of the genome of *B. germanica* is not available, the first objective that we had to undertake was to obtain a catalogue of miRNAs, and make expression studies of the most important miRNAs found in a small RNA library of the last instar nymph.

Then we studied the influence of JH and the 20E, the main metamorphic hormones, on the expression of these miRNAs and those found in two additional small RNA libraries of the penultimate and the last instar nymph. The difference between these two last instars nymphs is the expression of the JH (present in the transition from penultimate to last instar nymph, and absent in the transition from the last instar nymph to adult).

In a second line of objectives I focused in the miRNAs that appeared more relevant according to the previous results. These were let-7, miR-100 and miR-125, which are involved in the transition from larvae to pupae in *D. melanogaster*, and their expression is related to 20E and Broad complex, a 20E-dependent factor with different functions in the metamorphosis of *B. germanica* and *D. melanogaster*.

As a last objective I investigated the interplay between miR-8-3p and miR-8-5p regulating atrophin and their influence in metamorphosis. Previous studies have shown that miR-8 regulates in a very precise manner atrophin, a transcriptional co-repressor associated with histone deacetylase activity, which prevents neurodegeneration in *Drosophila melanogaster*. In *B. germanica*, RNAi of atrophin led to an improper ecdysis in the metamorphic molt and we concluded that the interplay between these two miRNAs is essential to assess the correct levels of atrophin before the metamorphosis.

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Abbreviations

20E	20-hydroxyecdysone
ab	Abrupt
Ago	Argonaute
atro	Atrophin
BR-C	Broad complex
bs	Blistered
CA	Corpora allata
Dcr	Dicer
DSRF	Drosophila Serum Response Factor
dsRNA	Double-strand RNA
EcR	Ecdysone receptor
Egfr	Epidermal growth factor
JH	Juvenile hormone
Kr-h1	Krüppel homolog 1
let-7-C	Let-7 complex
Met	Methoprene tolerant
miRNAs	microRNAs
MREs	miRNA recognition elements
ncRNAs	Non-coding RNAs
piRNAs	Piwi-associated RNAs
PPTH	Prothoracicotropic hormone
pre-miRNA	miRNA precursor
pri-miRNAs	miRNA primary transcript
qRT-PCR	Quantitative real-time PCR
r.h.	Relative humidity

RERE	Vertebrate arginine-glutamic acid dipeptide repeats protein gene
REST	Relative Expression Software Tool
rho	Rhomboid
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNAs	Small interfering RNAs
smRNAs	Small modulatory RNAs
snoRNAs	Small nucleolar RNAs
ssRNA	Single-strand RNA
Ubx	Ultrabithorax
USP	Ultraspiracle

1 Introduction and Aims

1.1. Introduction

Insect metamorphosis is the most fascinating postembryonic developmental process by which an insect transforms from an immature stage into an adult, involving more or less dramatic changes in the body structure through cell growth and differentiation.

Insects are the most diverse group on Earth, at present having about one million described species, and metamorphosis has been one of the most important innovations accounting for this diversification (Grimaldi & Engel, 2005). There are two types of metamorphosis: hemimetaboly, or gradual metamorphosis, and holometaboly or abrupt metamorphosis (Belles, 2011; Sehnal et al, 1996), the latter being evolutionarily the most successful (Grimaldi & Engel, 2005). At present, we know the main hormones involved in controlling insect metamorphosis, 20-hydroxyecdysone (20E) triggers molting, whereas juvenile hormone (JH) determines the type of molt, juvenile when it is present and metamorphic when it is absent. These hormones exert their respective action through a cascade of transcription factors that transduce the hormonal signal to the effector genes (Belles, 2011; Truman & Riddiford, 2002).

Moreover, a number of reports have pointed to microRNAs (miRNAs) as important players in the metamorphic transition in hemimetabolan as well as in holometabolan species (Belles et al, 2011). miRNAs are small non-coding RNAs of about 21-22 nucleotides that modulate gene expression at post-transcriptional level, often in the context of developmental and morphogenetic

processes (Ambros, 2004; Bartel, 2009; Bushati & Cohen, 2007). One of the more dramatic demonstrations that miRNAs are involved in insect metamorphosis was reported by Gomez-Orte & Belles (2009), who silenced Dicer-1 expression (a key enzyme in miRNA processing) by RNAi in the last nymphal instar of the cockroach *Blattella germanica*, and the experimental specimens molted to supernumerary nymphs after the following molt, instead of molting to adults (Gomez-Orte & Belles, 2009). These results showed that Dicer-1 and miRNAs are important factors in the mechanisms regulating metamorphosis.

a) Types of metamorphosis in insects

The most primitive insects do not undergo significant morphological changes through the life cycle and are called ametabolous. The juvenile that emerges from the egg looks like a tiny version of the adult. It will molt and grow until it reaching sexual maturity without dramatic morphological changes.

Metamorphosis arose from an ametabolous ancestor about 350 million years ago, and it evolved successively into two types: hemimetaboly and holometaboly. Hemimetabolous insects pass through a series of juvenile stages (generally called nymphs and that are morphologically similar to the adult) until reaching the adult stage. Metamorphosis takes place in the molt of the last instar nymph, when an adult with wings and genitalia is formed. In holometabolous insects there are three stages well differentiated: larva, pupa and adult. Larvae and adults are morphologically and

physiologically very different, and the transition from one to another occurs through the pupae, which can be considered a transitional stage and where all morphological changes are produced. (Figure 1.1).

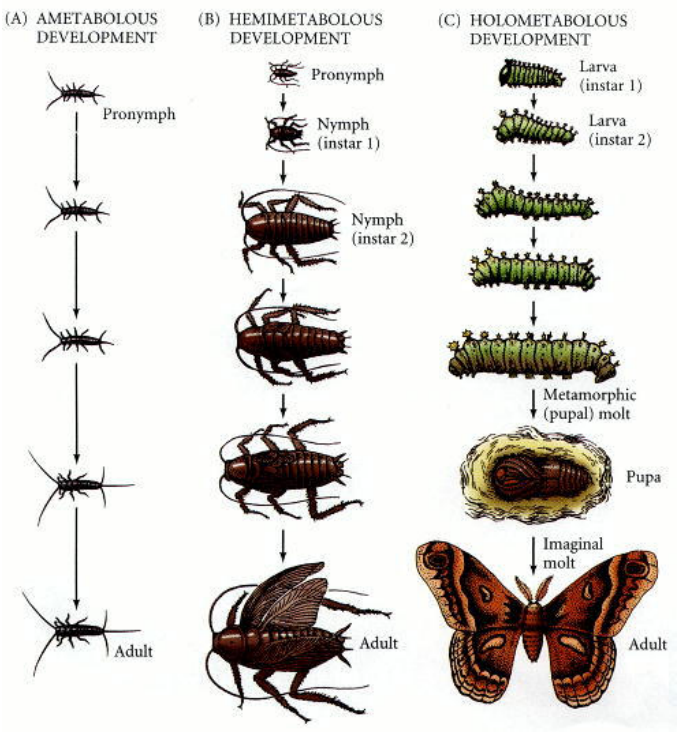

Figure 1.1. Types of postembryonic development in insects. (A) Ametabolous development (no metamorphosis) (B) Hemimetabolous development (gradual metamorphosis) (C) Holometabolous development (abrupt metamorphosis).

b) Endocrine control of molting and metamorphosis

The molting cycle is initiated and regulated by the endocrine system, and molting occurs as a result of the coordinated activity of all the epidermal cells in the insect, with hormones providing the

means by which this coordination occurs. The basic hormonal mechanism is identical whether the molt is juvenile to juvenile or juvenile to adult, and what differs during metamorphosis is the nature of the cuticle produced at each molt, and this is regulated by the presence or absence of JH.

In a number of studied model insects, the prothoracicotropic hormone (PPTH) is secreted in the brain after a large meal is ingested, and it stimulates the release of ecdysone from the prothoracic glands. Then, ecdysone is converted into 20E, the active molting hormone in target tissues. 20E circulates in the hemolymph and activates the epidermal cells to begin the cycle of epidermal cell division and the synthesis of a new cuticle.

In a juvenile to juvenile molt, the new cuticle is identical to the old one, whereas in a juvenile to adult molt (nymph to adult in hemimetabolous species, and larva to pupa, and pupa to adult in holometabolous species), the insect produces a new cuticle that has different properties and contains different proteins with respect to the previous stages. This is called the metamorphic molt, and requires the activation of the epidermal cells by 20E and the absence of the JH to reprogram the epidermal cells to produce the pupal and adult proteins.

The JH is synthesized by the corpora allata (CA), which are retrocerebral glands that are innervated to the brain. The JH is transported to the tissues through the hemolymph. (Figure 1.2).

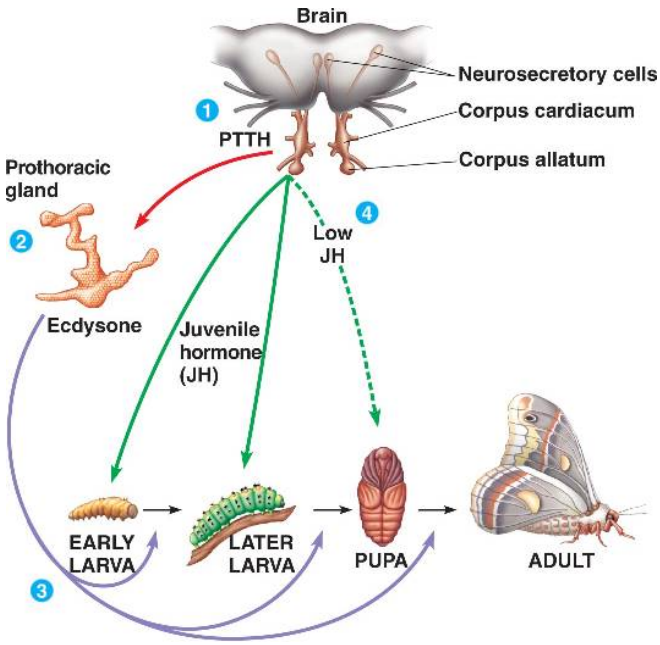

Figure 1.2. Hormonal regulation of insect metamorphosis. The prothoracicotropic hormone (PPTH) is secreted in the brain and stimulates the release of ecdysone from the prothoracic glands, and each molt is triggered by one or more pulses of 20-hydroxyecdysone (20E). Juvenile hormone (JH) is synthesized by the corpora allata (CA), and it is transported to the tissues through the hemolymph. Metamorphosis takes place when JH is absent.

c) Receptors and Transcription factors

The effect of 20E is mediated by a cascade of transcription factors that starts upon its binding to the heterodimeric receptor composed of the ecdysone receptor (EcR) and the ultraspiracle (USP), both of which belonging to the nuclear receptor superfamily. This activates expression of a hierarchy of transcription factors generally belonging to the same superfamily, like E75, E78, HR3, HR4 and FTZ-F1, which regulate the genes that underlie the

cellular changes associated to molting and metamorphosis (King-Jones & Thummel, 2005; Nakagawa & Henrich, 2009). Most of the information available on this cascade refers to *Drosophila melanogaster* (Thummel, 2002; Yin & Thummel, 2005), but there are a good deal of data from hemimetabolous species, especially from the cockroach *B. germanica*. Factors involved in 20E signaling in *B. germanica* are generally the same as in *D. melanogaster*, although the functions of some of them and their epistatic relationships may differ with respect to those observed in the fly (Cruz et al, 2003; Mane-Padros et al, 2012; Martin et al, 2006).

Among the most interesting 20E-dependent factors are the products of the Broad complex (BR-C) gene, whose functions may have radically diverged in hemimetabolous and holometabolous species. BR-C encodes a group of C2H2 zinc-finger transcription factors (Bayer et al, 1996; DiBello et al, 1991) that, in holometabolous species, like the dipteran *D. melanogaster*, the lepidopterans *Manduca sexta* and *Bombyx mori*, and the coleopteran *Tribolium castaneum*, are expressed in the final larval stage, and this transient expression is essential for the successful formation of the pupae (Konopova & Jindra, 2008; Parthasarathy et al, 2008; Suzuki et al, 2008; Uhlirova et al, 2003; Zhou & Riddiford, 2002). Experiments carried out on the hemimetabolan species *Oncopeltus fasciatus* (Erezyilmaz et al, 2006), *Pyrrhocoris apterus* (Konopova et al, 2011) and *B. germanica* (Huang et al, in press), suggested that BR-C transcription factors only regulate gradual wing bud growth.

The molecular action of JH it is still poorly understood (Jindra et al, 2012), we know that an important transducer of the JH signal is Methoprene tolerant (Met), a transcription factor that was discovered in *D. melanogaster* and that plays an important role in JH reception (Charles et al, 2011). Key functional evidence that Met is required for the repressor action of JH on metamorphosis was obtained from the beetle *T. castaneum*, a basal holometabolous insect where depletion of Met expression induced larvae to undergo precocious metamorphosis (Konopova & Jindra, 2007; Parthasarathy et al, 2008). More recently, the function of Met as an early JH transducer has been demonstrated in the hemimetabolous species *P. apterus* (Konopova et al, 2011), which established the first regularity in the signaling pathway of JH in hemimetabolous and holometabolous insects (Figure 1.3). Another important element in JH transduction in relation to metamorphosis is the transcription factor Krüppel homolog 1 (Kr-h1), whose antimetamorphic action was firstly demonstrated in *D. melanogaster* (Minakuchi et al, 2008) and *T. castaneum* (Minakuchi et al, 2009). More recently, the role of Kr-h1 as a transducer of the JH signal has been reported in three hemimetabolous insects: the cockroach *B. germanica* (Lozano & Belles, 2011) and the bugs *P. apterus* and *Rhodnius prolixus* (Konopova et al, 2011). RNAi studies in these species have shown that Kr-h1 represses metamorphosis and that it acts downstream of Met in the JH signaling pathway. Kr-h1 therefore appears to be the more distal transcription factor in the JH signaling cascade whose role as

mediator of the antimetamorphic action of JH has been conserved from cockroaches to flies.

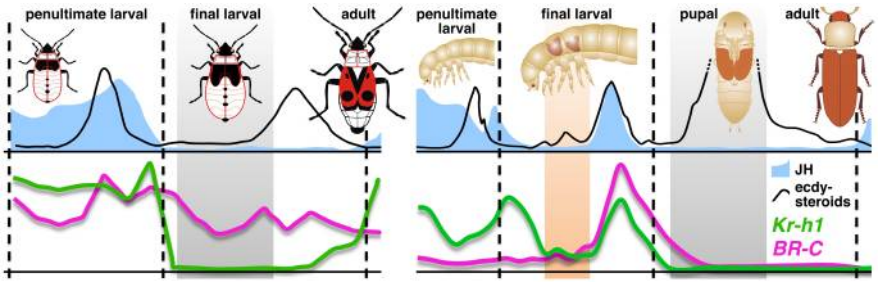

Figure 1.3. Regulation of hemimetaboly and holometaboly. *Pyrrhocoris apterus* (left) and *Tribolium castaneum* (right) cartoons signify the main innovations – postponement of wing development and the resting pupal stage in holometabolans. The absence of JH-dependent Kr-h1 expression in pupae and final instar hemimetabolous larvae (gray shaded areas) is prerequisite to adult development in both types of metamorphosis, supporting the view that these final juvenile stages of both insect types may be homologous. The orange shaded area marks a period of low Kr-h1 activity in the absence of JH, which is necessary to permit partial metamorphosis during the pupal molt, specified by the newly acquired function of BR-C in holometabolans. Figure from Konopova et al. (2011)

c) *Blattella germanica* as a model

B. germanica is a polyneopteran exopterigote insect that shows a gradual morphological transformation during the sixth nymphal instars of the life cycle, and is a good representative of the less modified hemimetaboly. Figure 1.4 shows the basal position of *B. germanica* comparing to other hemimetabolous species, like hemipterans which are very close to the holometabolous species.

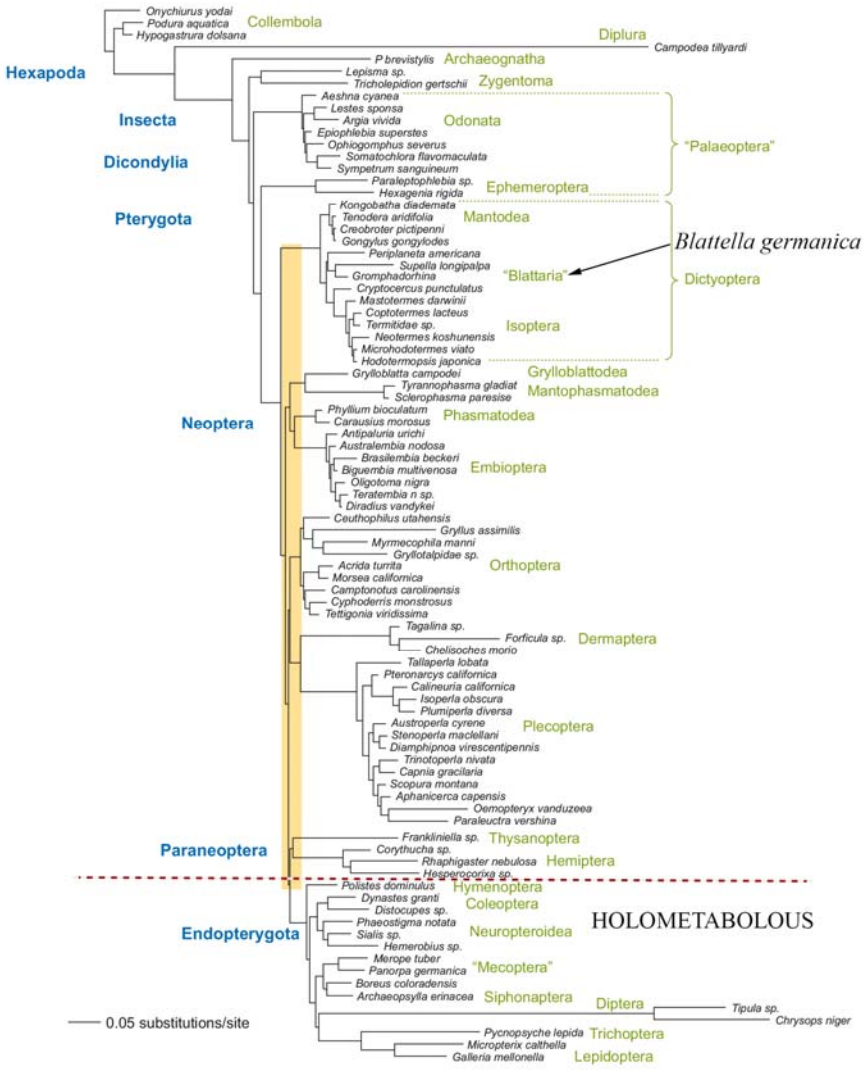

Figure 1.4. Position of *Blattella germanica* in the phylogeny of insects reconstructed by (Whitfield & Kjer, 2008).

This, and the fact that it is especially sensitive to gene silencing by RNAi (Belles, 2010), have made *B. germanica* a favorite hemimetabolan model to study metamorphosis.

Moreover, the levels of 20E and JH have been thoroughly described for pre-metamorphic and metamorphic stages, and in the adult (Cruz et al, 2003; Pascual et al, 1992; Romaña et al, 1995; Treiblmayr et al, 2006), and a number of transcription factors transducing the signal of both hormones have been also studied in this insect (Figure 1.5). However, the genome of *B. germanica* has not been sequenced yet.

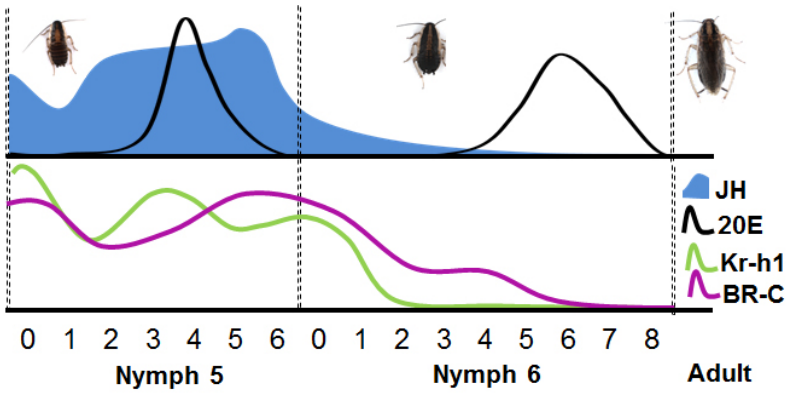

Figure 1.5. Expression patterns of hormones and important transcription factors studied in *Blattella germanica.* Data of Juvenile hormone (JH) was taken from Treiblmayr et al. (2006), ecdysone (20E) from Cruz et al. (2003), Krüppel holomog-1 (Kr-h1) from Lozano & Belles (2011) and Broad complex (BR-C) from Huang et al. (in press). JH and 20E levels represent ng/mL in hemolymph, Kr-h1 and BR-C represent mRNA copies per 1,000 copies of the reference gene BgActin-5c in whole body extracts.

d) Small non-coding RNAs

Step by step, some of the old paradigms of molecular biology have been falling away. The most significant of these is the central dogma that "one gene equals one protein". The formation of

different mRNAs by alternative splicing giving rise to different proteins coming from the same gene, the transcription factors that activate or repress transcription and finally the discover of the non-coding RNAs (ncRNAs), that can regulate gene expression at a post-transcriptional level, finally broken this myth.

ncRNAs form a heterogeneous group of RNA molecules that are classified into three categories according to their length and function: miRNAs and small-interfering RNAs (siRNAs), of about 18-25 nucleotides; Small RNAs, from 20 to 200 nucleotides and large RNAs, up to 10,000 nucleotides. Functions can be very diverse, and the most important are indicated in Figure 1.6. In the present thesis, we focused our studies on miRNAs.

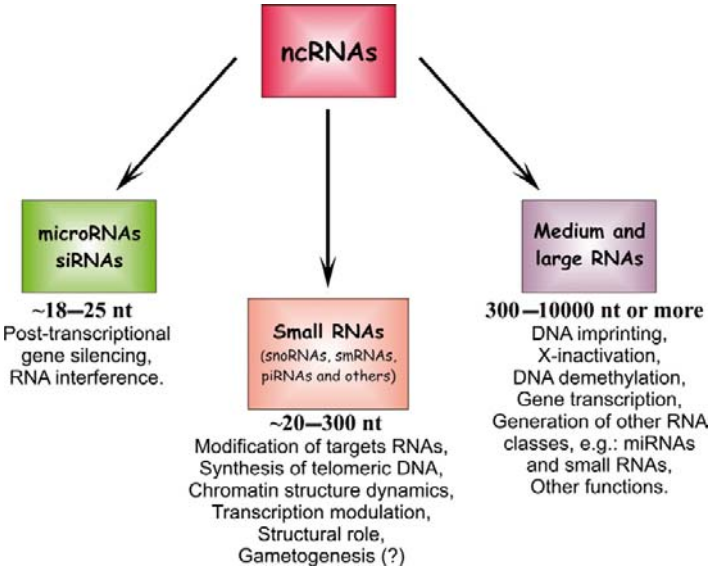

Figure 1.6. Types of non-coding RNAs (ncRNAs) classified according to their length and functions: very small RNAs – microRNAs and small interfering RNAs (siRNAs); small RNAs; and medium and large RNAs. The corresponding established functions for each type are indicated. snoRNAs, small nucleolar RNAs; smRNAs, small modulatory RNAs; piRNAs, Piwi-interacting RNAs. Data from Costa (2007)

e) miRNAs: A class of small silencing RNAs

miRNAs are small non-coding RNAs of about 20-22 nucleotides that regulate gene expression at a post-transcriptional level. The first miRNA discovered was lin-4, reported by Lee et al. (1993) in *Caenorhabditis elegans*. These authors showed that the locus lin-4 produces a 22-nucleotide RNA that binds to the 3' UTR of the lin-41 mRNA and inhibits its translation. In 2001, miRNAs were identified in humans, flies and worms by cloning and sequencing small RNAs. At present, the miRBase (Griffiths-Jones, 2004) (Release 18.0) has 21,643 mature miRNA in 168 species of plants, animals and viruses.

miRNAs undergo molecular processing before becoming mature products (Bartel, 2004; Ghildiyal & Zamore, 2009). First, miRNA-containing primary transcripts (pri-miRNAs) are transcribed in the nucleus by the RNA polymerase II or III, and these are quickly cleaved into miRNA precursors (pre-miRNA) of about 50-80 nucleotides. The pre-miRNA is recognized by the enzyme exportin 5 that transport it to the cytoplasm. In insects, the enzyme Dicer-1 and his partner Loquacious cleave the pre-miRNA into a miRNA duplex, which contains the mature form (the miRNA) and the passenger strand. The duplex is loaded into the RNA-induced silencing complex (RISC) through the key enzyme of the complex, Argonaute 1. Then, the passenger strand is released and the mature strand guides the RISC to the target mRNA provoking translational repression, degradation and, in some cases, mRNA recycling (Bartel, 2004) (Figure 1.7).

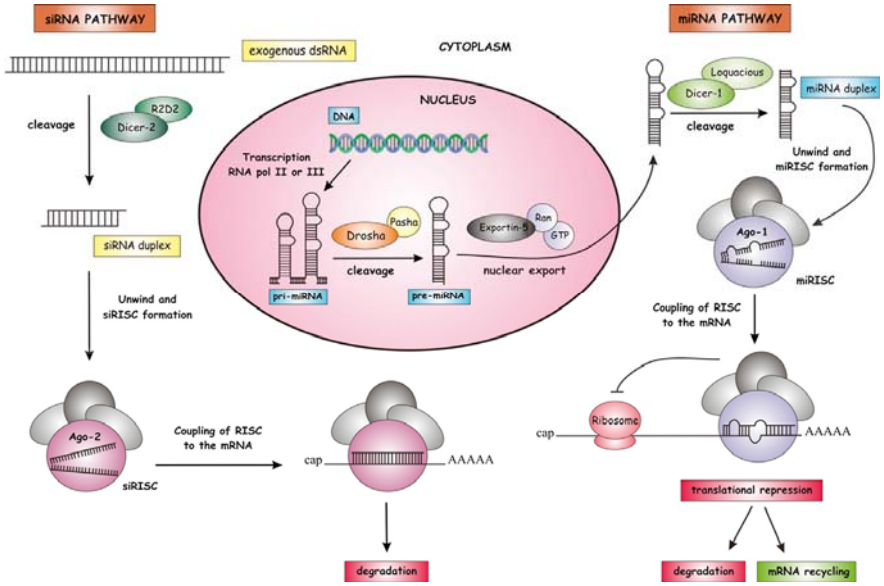

Figure 1.7. Biogenesis of miRNAs and siRNAs. The miRNA gene is transcribed by RNA Pol II/III into a primary transcript (pri-miRNA) that is processed by Drosha/Pasha and exported to the cytoplasm by Exportin-5. In the cytoplasm, the precursor (pre-miRNA) undergoes the final step of maturation being cleaved by Dicer-1/Loquacious into a miRNA duplex. After the miRNA duplex unwinds, the mature miRNA is maintained with Argonaute-1 protein (Ago-1) forming a RISC which will be coupled to the target mRNA and will degrade, destabilize, or translationally inhibit it, whereas the passenger miRNA is released and degraded. On the other hand, siRNA is formed when a long and exogenous double-strand RNA (dsRNA) is cleaved by Dicer-2/R2D2 into a siRNA duplex. Likewise with the miRNA pathway, the siRNA duplex is unwound and single-strand siRNAs are maintained with Argonaute-2 protein (Ago-2) forming a RISC which will recognize the target mRNA and degrade it.

The passenger strand released form the RISC complex is normally degraded, but in some cases it can also play the role of a miRNA and bind to the RISC (often having greater affinity binding to Argonaute 2). Structural and thermodynamic properties of the processed duplex regulate the loading of each strand, either in the

miRNA as well as in the RNAi pathway (Figure 1.8) (Czech et al, 2009; Okamura et al, 2009).

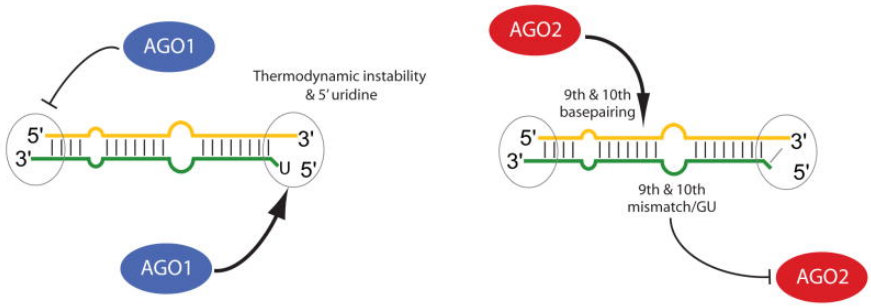

Figure 1.8. Model for differential miRNA strand selection by Ago1 and Ago2. Ago1 strand selection is influenced by 5′ uridine in addition to thermodynamic instability, while it is insensitive to the positions of central mismatches. In contrast, Ago2 strand selection is highly sensitive to 9th and 10th basepairing status. *Drosophila melanogaster* miRNA genes are generally configured to access distinct strand preferences of Ago1 and Ago2, thereby yielding the seemingly independent sorting of miRNA and miRNA* species (Okamura et al, 2009).

f) miRNAs and insect metamorphosis

Work reported by Sokol and colleagues (2008) showed that the *D. melanogaster* let-7-Complex locus (let-7-C) (which comprises let-7, miR-100 and miR-125 miRNAs) is mainly expressed in the pupal and adult neuromusculature. let-7-C knockout flies appeared morphologically normal, but displayed defects in different adult behaviors (like flight and motility) and in relation to fertility. Importantly, the neuromusculature clearly showed juvenile features, which suggests that an important function

of let-7-C is to ensure the appropriate remodeling of the abdominal neuromusculature during the larval-to-adult transition in flies. The study also showed that this function is carried out predominantly by let-7 (Sokol et al, 2008).

In a related work, Caygill and Johnston (2008) obtained a *D. melanogaster* mutant lacking let-7 and miR-125 activities, and obtained a pleiotropic phenotype arisen during metamorphosis. These authors showed that the loss of let-7 and miR-125 results in temporal delays in the terminal cell-cycle exit in the wing, and in the maturation of neuromuscular junctions of imaginal abdominal muscles. The authors focused on the latter process by identifying the abrupt (ab) gene (which encodes a nuclear protein) as a let-7 target, and by providing evidence showing that let-7 regulates the maturation rate of abdominal neuromuscular junctions during metamorphosis, by regulating ab expression (Caygill & Johnston, 2008). Wing morphogenesis has been studied by Biryukova and colleagues (2009), who described that miR-9a regulates *D. melanogaster* wing development through a functional target site in the 3′ UTR of the LIM only mRNA, a transcription cofactor, that directly inhibits the activity of Apterous, the factor required for the proper dorsal identity of the wings. Another miRNA involved in wing morphogenesis of *D. melanogaster* is iab-4. Sequence analysis suggested that iab-4 could regulate Ultrabithorax (Ubx), and expression pattern studies of iab-4 and Ubx showed that they are complementary in critical developmental stages (Ronshaugen et al, 2005).

All previous experiments were carried out in the same holometabolous insect, *D. melanogaster,* but one of the more dramatic demonstrations that miRNAs are involved in insect metamorphosis was obtained in a hemimetabolous insect. Gomez-Orte & Belles (2009), silenced Dicer-1 expression by RNAi in the last nymphal instar of the cockroach *B. germanica*, and obtained supernumerary nymphs after the following molt, instead of adults (Gomez-Orte & Belles, 2009). These results showed that Dicer-1 and miRNAs are important factors in the mechanisms regulating metamorphosis (Figure 1.9). However, which particular miRNAs were involved remained as a pending question.

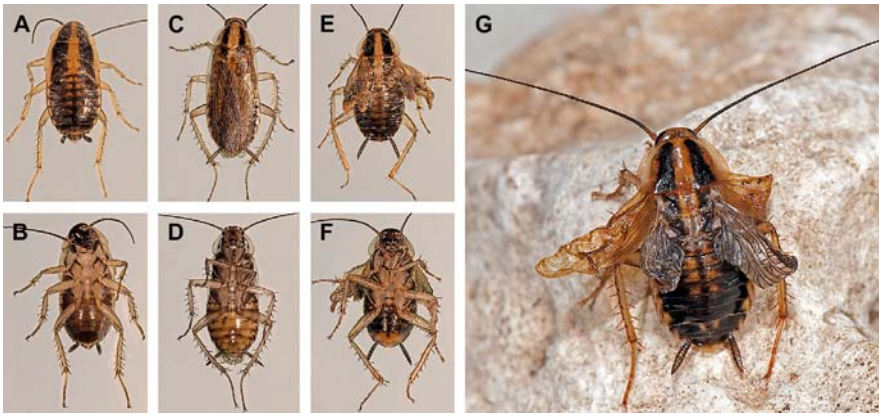

Figure 1.9. Inhibition of *Blattella germanica* metamorphosis after impairing miRNA maturation by depleting Dicer-1 expression in sixth (last) instar nymph. Dorsal and ventral view of normal sixth instar nymph (A, B), normal adult (C, D) and seventh instar supernumerary nymphoid (E, F) resulting from metamorphosis inhibition. The nymphoids resemble those obtained after treating the last instar nymph with juvenile hormone (G). Photos from Albert Masó; data from Gomez Orte & Belles (2009)

1.2. Aims of the Thesis

miRNAs are necessary for insect hemimetabolous metamorphosis (Gomez-Orte & Belles, 2009), but it is still unknown which particular miRNAs are involved in this process. Therefore, the main goal of the present thesis is to identify miRNAs that participate in the regulation of hemimetabolous metamorphosis.

To fulfill this goal, we planned the following three objectives:

1. Build a catalogue of miRNAs in *Blattella germanica*.
2. Find miRNAs differentially expressed between metamorphic and non-metamorphic transitions.
3. Study, from a functional point of view, the more promising miRNAs emerging from the two former objectives.

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A catalogue of miRNAs of *Blattella germanica*

2

Given that the genome of *B. germanica* is not sequenced, we approached the objective of obtaining a catalogue of microRNAs (miRNAs) by preparing and sequencing miRNA libraries at selected stages. The work was carried out in the context of the general needs of the laboratory, and two libraries of small RNAs were prepared in cooperation with other members of the group: One of them from adult ovaries in the first gonadotrophic cycle (Ov-A), which is not the most relevant in our context, and the other from the whole body of sixth instar nymph females (WB-6) (the last and, thus the metamorphic instar), which is the most relevant for our thesis purposes.

This work was published in the journal PLoS One, co-authored with other members of the group. The pages following reproduce the original manuscript of the paper, in order to maintain a harmonic format in the thesis memoire. In any case, the link to the original paper is provided in the cover page. Also provided are the links to access the Supporting Information, which contains a number of massive tables, and, for this reason, were not reproduced here.

Deep sequencing of Organ- and Stage-Specific microRNAs in the Evolutionarily Basal Insect *Blattella germanica* (L.) (Dictyoptera, Blattellidae)

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Cristino AS, Tanaka ED, Rubio M, Piulachs MD, Belles X. Deep sequencing of organ- and stage-specific microRNAs in the evolutionarily basal insect Blattella germanica (L.) (Dictyoptera, Blattellidae). *PLoS One* (2011) **6:** e19350

miRNAs in metamorphic and non-metamorphic transitions

3

The high throughput sequencing of the miRNA libraries described in the previous chapter resulted in approximately 11 and 8 million reads for the whole-body last instar nymph and adult ovaries, respectively. This massive sequencing effort served to have a first comprehensive catalogue of the miRNAs of *Blattella germanica*. But the essential goal of the present thesis is to identify particular miRNAs having roles in the regulation of insect metamorphosis. Thus, to gain insight in this direction, we constructed two new miRNA libraries, one of the whole body around the peak of molting hormone of the penultimate, pre-metamorphic nymphal instar, and the other equivalent but of the last, metamorphic nymphal instar. The sequencing effort could be more modest that that applied to obtain the general catalogue, but the comparison of both libraries would shed light about which miRNAs may be important to the metamorphic transition.

This work has been published in the journal BMC Genomics. The pages following reproduce the original manuscript of the paper, in order to maintain a harmonic format in the thesis memoire. In any case, the link to the original paper is provided in the cover page.

MicroRNAs in metamorphic and non-metamorphic transitions in insect hemimetabolan metamorphosis

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Rubio M, de Horna A, Belles X. MicroRNAs in metamorphic and non-metamorphic transitions in insect hemimetabolan metamorphosis. *BMC Genomics* (2012) **13:** 386

4

The microRNAs let-7, miR-100 and miR-125 play a key role in wing morphogenesis of hemimetabolan metamorphosis

In the previous chapter we have described the study of two miRNA libraries, one with RNA extracted around the peak of 20-hydroxyecdysone (20E) of the penultimate nymphal instar, and the other with RNA extracted around the peak of 20E of the last instar. From the comparison of these two libraries, four miRNAs clearly emerged as differentially expressed: miR-252-3p, which is well expressed in the penultimate nymphal instar and declines their expression in the last instar, and the trio let-7-5p, miR-100-5p and miR-125-5p, which exhibited the reverse pattern. The functional study of miR-252-3p has been reported in the previous chapter, whereas the present one reports that of let-7-5p, miR-100-5p and miR-125-5p in *B. germanica* metamorphosis.

The present chapter has been formatted to be submitted to a SCI journal.

microRNA-mediated action of Broad complex on wing morphogenesis in hemimetabolan metamorphosis

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5.1. Abstract

In most insect species, the miRNA let-7 clusters with miR-100 and miR-125 in the same primary transcript. The three miRNAs are involved in developmental timing in the nematode *Caenorhabditis elegans* and in the fly *Drosophila melanogaster*. In the cockroach *Blattella germanica*, the expression of these miRNAs increases dramatically in the wing pads around the peak of 20-hydroxyecdysone (20E) of the last instar nymph. When let-7 and miR-100 were depleted with specific anti-miRNAs in this instar, the resulting adult showed the wings reduced in size (anti-miR-100) or with malformed vein patterning (anti-let-7 and anti-miR-100). Depletion of miR-125 induced no apparent effects. In *D. melanogaster*, Broad-complex (BR-C) and 20E are required for let-7, miR-100 and miR-125 expression. Our results showed that BR-C is also needed for the expression of these miRNAs in *B. germanica*, and depletion of BR-C transcripts by RNAi in the last instar nymph lead to a reduction of let-7, miR-100 and miR-125 expression. In *D. melanogaster*, blistered (bs) (the *D. melanogaster* serum response factor homolog), plays an important role in wing morphogenesis. Towards the end of the last instar larvae, bs expression is down-regulated in the longitudinal veins by Ras-mediated epidermal growth factor (Egfr) signaling. Reduction of bs expression is necessary to reach the right proportion between wing veins and interveins. In *B. germanica* wing buds, bs is down-regulated during the last instar nymph, in parallel to the up-regulation experienced by let-7 and miR-100, and depletion of these two miRNAs leads to an

increase of bs transcript levels. Moreover, depletion of BR-C elicits a phenotype (which includes wing reduction and disorganization of wing vein patterning) that is similar to that obtained after depleting let-7 and miR-100 levels. We propose that let-7 and miR-100 expression is induced by BR-C, and that these miRNAs play a key role in regulating the size and the vein-intervein patterning of *B. germanica* by modulating bs mRNA levels.

5.2. Introduction

miRNAs are small non-coding RNAs of about 21-22 nucleotides that modulate gene expression at post-transcriptional level, frequently in the context of developmental and morphogenetic processes (Ambros, 2004; Bartel, 2009; Bushati & Cohen, 2007). miRNAs are transcribed as part of a primary transcript (pri-miRNA), which contains one or more miRNA precursors (pre-miRNAs). In the nucleus, the pri-miRNAs are processed into hairpin structured pre-miRNAs by the ribonuclease drosha, and exported to the cytoplasm, where they are cleaved by the ribonuclease dicer-1 into an imperfectly paired duplex, whose 5'- and 3'-strand can give two respective mature miRNAs or only one (Bartel, 2004; Ghildiyal & Zamore, 2009).

In insects, miRNAs are important regulators of gene expression, and have been frequently related to metamorphic processes (Belles et al, 2011). A dramatic demonstration that miRNAs are involved in insect metamorphosis was reported by Gomez-Orte & Belles (2009), who observed that knockdown of

dicer-1 with RNAi in the last nymphal instar of the cockroach *Blattella germanica*, depleted miRNAs and led to the formation of supernumerary nymphs, instead of adults, after the following moult (Gomez-Orte & Belles, 2009). To gain more information about which miRNAs may be important for *B. germanica* metamorphosis, we recently constructed two miRNA libraries, one with RNA extracted around the peak of 20-hydroxyecdysone (20E) of the penultimate nymphal instar, and the other equivalently extracted in the last instar (Rubio et al, 2012) . We identified a number of miRNAs that are differentially expressed in these two stages, and among them, let-7, miR-100 and miR-125 emerged as being clearly upregulated in the last, metamorphosing nymphal instar (Rubio et al, 2012). The present paper reports the particular role of these three miRNA in *B. germanica* metamorphosis.

Historically, lin-4 (homolog of miR-125) was the first miRNA discovered, using *Caenorhabditis elegans* as model (Lee et al, 1993). However this kind of factors were not recognized as a distinct class of biological regulators until the early 2000s, when let-7 was found to be a developmental time regulator in *C. elegans* (Reinhart et al, 2000), and it was recognized that its sequence and possibly their general functions are conserved across animal phylogeny (Pasquinelli et al, 2000). With the exception of the silkworm *Bombyx mori* and the pea aphid *Acyrthosiphon pisum*, in all studied insects, including *D. melanogaster*, let-7, miR-100 and miR-125 cluster together in the same primary transcript. In that of *B. mori*, the precursor of miR-125 is completely absent and another miRNA (miR-2795) clusters with let-7 and miR-100; in the

primary transcript of *A. pisum*, only a part of the miR-125 precursor sequence is present together with the complete precursors of let-7 and miR-100 (Legeai et al, 2010).

In *D. melanogaster*, expression of let-7, miR-100 and miR-125 is upregulated by 20E (Chawla & Sokol, 2012; Garbuzov & Tatar, 2010), and the 20E response is mediated by Broad complex transcription factors (BR-C) (Sempere et al, 2002). In *D. melanogaster*, BR-C are expressed in the last instar larvae and in the prepupae, and trigger pupal morphogenesis (Kiss et al, 1988). Moreover, expression of let-7 and miR-125 (miR-100 was not studied) follows the BR-C expression pattern (Sempere et al, 2002). In the case of *B. mori*, expression of let-7 significantly increases in late larval instars, and show maximal levels in prepupal and pupal stages (Liu et al, 2007).

Regarding functional studies, a number of metamorphic processes of *D. melanogaster* are affected by let-7 and/or miR-125 depletion, like the terminal cell-cycle exit in wing formation and the maturation of neuromuscular junctions (Caygill & Johnston, 2008). Let-7 also plays an important role in abdominal neuromusculature remodeling (Sokol et al, 2008), and in innate immunity (Garbuzov & Tatar, 2010). In contrast, there is no much functional data on miR-100, as no significant effects were observed after depleting it in *D. melanogaster* (Sokol et al, 2008). With respect to regulatory aspects, there is evidence on the indirect stimulatory effect of miR-125 on let-7 mediated by the protein lin-28 in *D. melanogaster* and in mammals (Liu et al, 2007; Moss & Tang, 2003; Nolde et al, 2007). The RNA-binding protein lin-28 blocks let-7 biogenesis by

uridylating the let-7 precursor at its 3' end (Heo et al, 2008; Thornton & Gregory, 2012). In turn, miR-125 blocks the expression of lin-28 by binding to the 3’ UTR region of its mRNA.

In summary, the scarce available data suggest that let-7, miR-100 and miR-125 play a number of roles in the transition from larvae to adult in the fruit fly *D. melanogaster* and in the silkworm *B. mori*, which are very modified holometabolan species, exhibiting complete metamorphosis. Therefore, our work in *B. germanica* is double pertinent because this cockroach is a model interesting in itself, and because gathering data on it can enable comparisons with holometabolan species and shed light to the role of miRNAs in the evolution of insect metamorphosis.

In the present work, we have first characterized the structure of the precursors of let-7, miR-100 and miR-125 in *B. germanica*, then we have established the expression patterns for them in the life cycle, and finally we have depleted specifically their respective expression, which has led to infer that at least let-7 and miR-100 are necessary to reach the correct size and vein patterning of the adult wing. In order to determine which could be the mRNA targeted by these miRNAs to exert the above functions, we identified bs as a plausible candidate because its mRNA contains putative binding sites for let-7 and miR-100, and because their functions related to wing size and vein patterning. bs is the *D. melanogaster* homolog of the mammalian Serum Response Factor (DSRF), and is required to spatially restrict the formation of veins (Montagne et al, 1996). In turn, the epidermal growth factor (Egfr) modulates the expression of bs and rhomboid (rho, also known as veinlet) (Blair, 2007; Roch et

al, 1998), and bs and rho act together to specify the vein-intervein patterning in *D. melanogaster* wings (Fristrom et al, 1994). In the lasts steps of wing development, Egfr signaling upregulates rho expression and represses bs expression in vein cells (Blair, 2007). Interestingly for our hypothesis is that fine-tuned downregulation of bs is essential for a correct vein patterning.

5.3. Results

a) The sequence of let-7, miR-100 and miR-125 precursors is conserved in *B. germanica*

Cloning of let-7, miR-100 and miR-125 precursors (pre-let-7, pre-miR-100 and pre-miR-125) was accomplished by a 3'RACE approach using as primers the mature sequences of the miRNAs, which had been obtained from a *B. germanica* small RNA library (Cristino et al, 2011). The amplifications rendered cDNAs of 69 bp for pre-let-7 and 67 bp for pre-miR-100 and pre-miR-125. Alignment of these sequences with the precursors of let-7, miR-100 and miR-125 of all insect species recorded in the miRBase (Griffiths-Jones, 2004) (Release 18) shows a high degree of conservation (Figure 4.1 and Supplementary Figure 4.1). The most conserved region is that corresponding to the canonical mature miRNA sequence in each pre-miRNA and the region corresponding to the passenger strand in the case of pre-let-7 and pre-miR-125, whereas this region is less conserved in the pre-miR-100.

The inferred folded structure of these three sequences shows the typical hairpin structure that characterizes miRNA precursors (Figure 4.1 and Supplementary Figure 4.2).

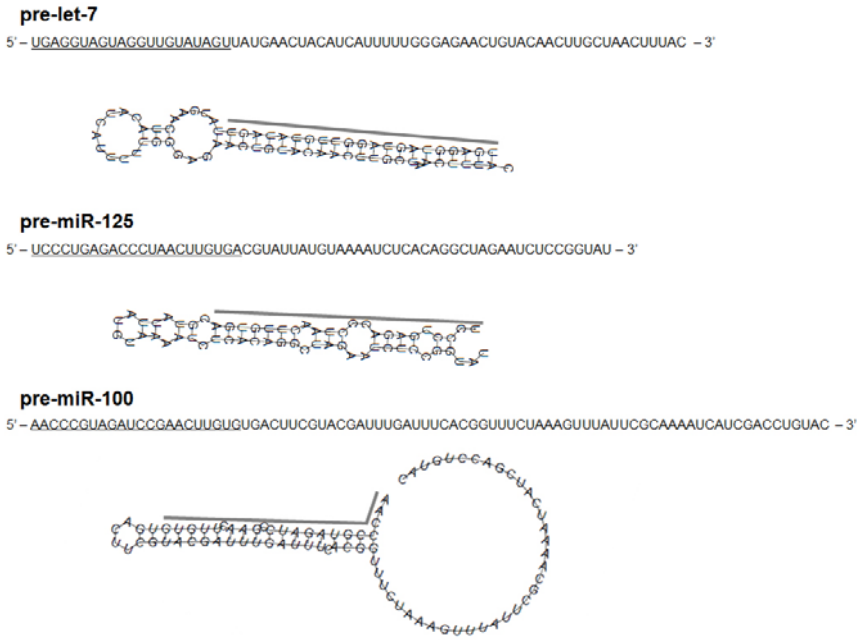

Figure 4.1. Sequences and folding structures of let-7, miR-100 and miR-125 precursors of *Blattella germanica.* Sequences were obtained by 3' RACE using RNA extracts from specimens depleted for dicer-1. The respective primers correspond to sequences of the corresponding mature miRNAs. Folding structures were obtained using the RNAfold webserver (Gruber et al, 2008). Sequences of the mature miRNAs are indicated by a grey line.

b) Expression of let-7, miR-100 and miR-125 peaks in the last nymphal instar

The expression of let-7, mir-125 and miR-100 was studied in whole body samples in penultimate (N5) and last (N6) nymphal

instars, as well as in selected days of younger nymphal instars (from N1 to N4) and in the adult. Results (Figure 4.2A) showed that the three miRNAs are detectable in all life cycle stages of *B. germanica*, although expression levels increase in N5 and N6, and peak on day 6 of the latter instar, coinciding with the 20E peak. In addition, we studied the expression of the three miRNAs in the forewing and hind wing pads in the three days encompassing the 20E peak (days 5, 6 and 7) of the last instar nymph, and results (Figure 4.2B) showed that expression of all three miRNAs increase from day 5 to day 7.

c) BR-C is required for let-7, miR-100 and miR-125 expression

In *B. germanica*, let-7, mir-100 and mir-125 are upregulated in parallel to the peak of 20E in the last instar nymph, and exogenously applied 20E increase the expression of the three miRNAs (Rubio et al, 2012). These results were in agreement with equivalent observations carried out in *D. melanogaster* (Chawla & Sokol, 2012; Garbuzov & Tatar, 2010; Sempere et al, 2002). In this insect, 20E is necessary for the expression of let-7, miR-100 and miR-125, and the response appears mediated by BR-C transcription factors (Sempere et al, 2002). At the light of these data, we tested whether expression of let-7, miR-100 and miR-125 would be also mediated by BR-C in *B. germanica*.

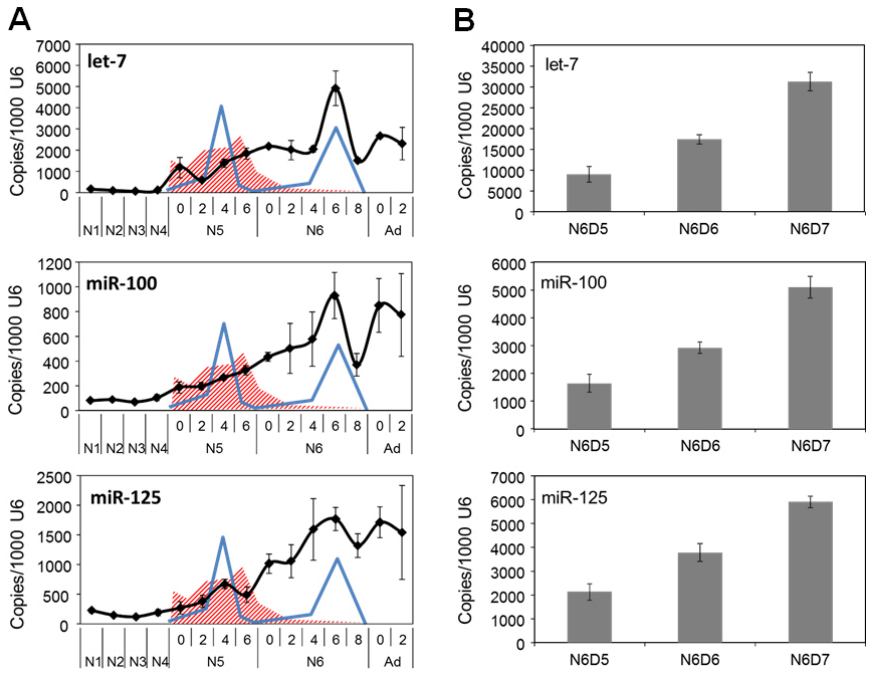

Figure 4.2. Expression patterns of let-7, miR-100 and miR-125 in *Blattella germanica*. miRNA levels were measured with qRT-PCR; **A)** Expression patterns in whole body of first nymphal instar (N1) to sixth nymphal instar (N6). Data from N5, N6 and adult are from Rubio et al. (2012); schematic patterns of juvenile hormone III and 20-hydroxyecdysone titers in N5 and N6 are superimposed in every graphic as pink and blue patterns, respectively; **B)** Expression patterns in the wing pads in days 5, 6 and 7 of the last instar nymph (N6D5, N6D6 and N6D7); data represent the mean ± SEM, and are indicated as copies of the respective miRNA per 1000 copies of U6; each point represents 3 biological replicates.

Our experiments showed that BR-C RNAi treatment on freshly emerged fifth instar nymphs led to a decrease of let-7, miR-100 and miR-125 levels in the sixth (last) instar (Figure 4.3). Specimens with depleted BR-C molted to the adult stage with the wings malformed, showing the typical defects of BR-C knockdowns described by Huang et al. (in press). These defects especially concentrate in the hind wings, which, with respect to controls, are smaller, have the CuP vein shorter, which leaves a

notch in the wing edge at the CuP end, show the vein-intervein patterning disorganized in the anterior part, and have A-veins broken, especially in the posterior part (Supplementary Figure 4.3).

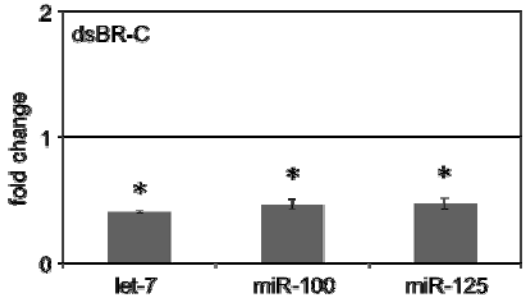

Figure 4.3. Effect of RNAi of Broad-complex on let-7, miR-100 and miR-125 expression. BR-C RNAi treatment was carried out on N5 and depleted all BR-C isoforms. miRNA levels were measured 5 on N6D6; data represent 3 biological replicates and are normalized against the control females (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool (Pfaffl et al, 2002).

d) LNA antimiR treatment effectively depletes let-7, miR-100 and miR-125 levels

To study the function of let-7, miR-100 and miR-125, we depleted the corresponding miRNA levels with specific antisense LNAs. We injected two doses of 1 µL of antisense LNA at 50 µM in the last instar nymph, one 3 days after the molt (N6D3) and another 2 days later (N6D5). The antisense LNAs were injected separately to see the specific effect of each miRNA (LNA-let-7, LNA-miR-100 and LNA-miR-125), and together, in order to see the effect of the simultaneous depletion of the three miRNAs (LNA-let-7-C).

On day 6 of the last instar nymph (N6D6) the levels of the respective miRNAs had specifically decreased in the wing pads (Figure 4.4A) according to the specific LNA treatment, and the three miRNAs simultaneously decreased after the treatment with LNA-let-7-C. LNA treatment against let-7 and miR-100 specifically depleted these miRNAs, 96% and 91% respectively; whereas the treatment against miR-125 led to a decrease of the three miRNAs, although the decrease was less dramatic for miR-100 (80%) and let-7 (50%) than for miR-125 (96%). The let-7-C treatment led to a decrease of about 90% of the three miRNAs (Figure 4.4A).

e) Functions of let-7, miR-100 and miR-125 are associated to wing development

The treatment with LNA-miR-100 affected the size of the wings. All (n=10) adult specimens that had received a treatment with this LNA showed the forewings and the hindwings smaller than controls (Figure 4.4B). Moreover, 6 out of 10 specimens had the wings wrinkled and not properly extended, a feature that we categorized as defect A. The same defect was found in 5 out of the 10 specimens treated with LNA-let-7 (Figure 4.4C).

A closer examination of the hindwings of individuals treated with LNA-let-7 or LNA-miR-100 with the defect A, led to notice a number of vein patterning defects, including disorganization of the vein-intervein pattern in the anterior part of the hindwing (defect B), and atypical A-vein bifurcations in the posterior part (defect C) (Figure 4.4D). Only those individuals that were not able to properly

extend the wings showed one or more of these defects (Supplementary Table 4.S1).

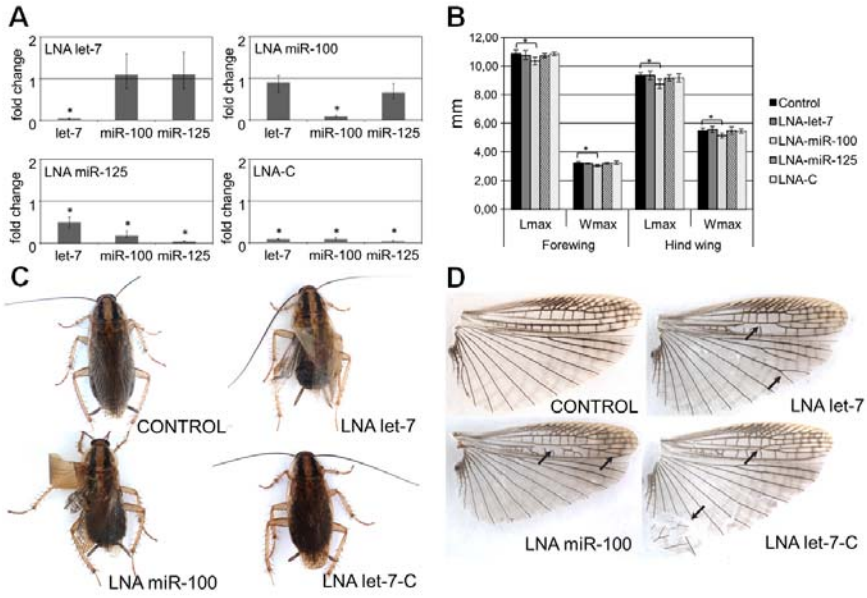

Figure 4.4. Effect of let-7, miR-100 and miR-125 depletion on metamorphosis of *Blattella germanica*. Treated females received two injections of 50 µM of LNA miRNA on day 3 and day 5 of last nymphal instar (N6D3 and N6D5, respectively); treatments were with LNA-let-7, LNA-miR-100, LNA-miR-125, or LNA-let-7-C; control females received an equivalent treatment with miRCURY LNA™ microRNA Inhibitor Negative Control. **A)** Levels of let-7, miR-100 and miR-125 measured on N6D6 following the respective LNA treatment. **B)** Forewing and hindwing biometrics in adult specimens resulting from the treatments; maximal length (Lmax) and maximal width (Wmax) were measured in the forewing (tegmina) and the hindwing. **C)** General aspect of a control specimen and specimens treated with LNA-let-7, LNA-miR-100 and LNA-let-7-C, 24 h after the adult molt. **D)** Hindwings in control specimen and specimens treated with LNA-let-7, LNA-miR-100 and LNA-let-7-C 24 h after the adult molt. qRT-PCR data in A represent 3 biological replicates and are normalized against the control females (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool (Pfaffl et al, 2002). Data in B represent 8 biological replicates; the asterisk indicates statistically significant differences with respect to controls (*t*-test, $p < 0.01$).

All adults emerging from the nymphs that were treated with the cocktail LNA-let-7-C had the wings well extended, but showing the apex of the tegmina slightly bent towards the ventral part (defect D); moreover, 3 out of 8 individuals (38%) showed the defect B, 2 (25%) the defect C, and 1 (13%) the defects B and C (Figure 4.4C and Supplementary Table 4.S1). It is worth noting that the wings of these specimens are significantly more fragile than controls, as they easily break during the process of manual extension on a slide to make the microscope observations. Interestingly, depletion of miR-125 did not elicit any defect, as the adults emerging from the treated nymphs were identical to control specimens.

f) blistered, a candidate target for let-7 and miR-100

At this point, we wondered about the let-7 and miR-100 target that could explain the phenotype observed in the hindwing. A good candidate would be a gene product that is downregulated when the expression of these two miRNAs increases, and that is involved in regulating the vein-intervein organization. In *D. melanogaster*, the expression of bs is down-regulated prior to metamorphosis in the longitudinal veins by Ras-mediated Egfr signaling. Reduction of bs expression is necessary to reach a right proportion between wing veins and interveins (Fristrom et al, 1994).

Using RNAhybrid and PITA algorithms (Kertesz et al, 2007), we predicted miRNA binding sites in the bs mRNA (Figure 4.5A). A miR-100 site in the position 23, and a let-7 site in the position 936 were predicted by both algorithms. A second let-7 site in the

position 1037, within the 3'UTR, was only predicted by the RNAhybrid algorithm, but at a high score, which made us to consider it as well.

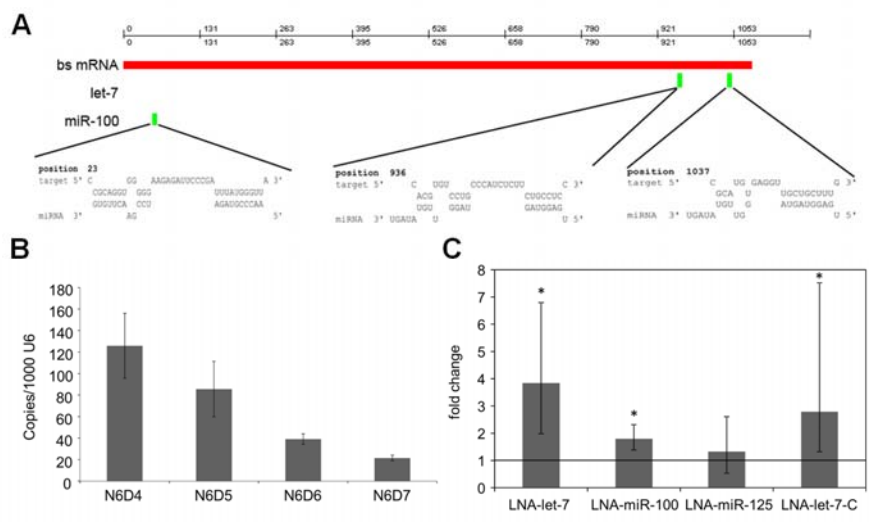

Figure 4.5. Data supporting bs as candidate target of let-7 and miR-100. A) Predicted sites for let-7 and miR-100 in bs mRNA. **B)** Expression pattern of bs in wing pads in days 4, 5, 6 and 7 of the last instar nymph (N6D4, N6D5, N6D6 and N6D7) measured by qRT-PCR; data represent the mean ± SEM, and are indicated as copies of the respective miRNA per 1000 copies of U6; each point represents 3 biological replicates. **C)** Levels of bs mRNA in wing pads measured on N6D6 following the treatment with LNA-let-7, LNA-miR-100, LNA-miR-125 and LNA-let-7-C. Treated females received two injections of 50 µM of each miRNA LNA on day 3 and day 5 of last nymphal instar; control females received an equivalent treatment with miRCURY LNA™ microRNA Inhibitor Negative Control A; data represent 3 biological replicates and are normalized against the control females (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool (Pfaffl et al, 2002).

Furthermore, we studied the expression of bs in the wing buds of *B. germanica*, and we found that the transcript is down-regulated during the last instar nymph, in parallel to the up-regulation experienced by let-7, miR-100 and miR-125 (Figure 4.5B). Finally, we studied the effect of let-7, miR-100 and miR-125 depletion upon

bs transcript levels, and we found that treatment with LNA-let-7 and LNA-miR-100 leads to an increase of bs mRNA levels, but not with miR-125. The whole data strongly suggest that let-7 and miR-100 target bs mRNA and contribute to its depletion during the formation of the wing (Figure 4.5C).

5.4. Discussion

a) Let-7, miR-100 and miR-125 of *B. germanica* are possibly encoded by the same primary transcript

Let-7, miR-100 and miR-125 are clustered in the same primary transcript in many insects, like *D. melanogaster*, *Tribolium castaneum* and *Anopheles gambiae*. There are some exceptions, however, like *B. mori* and *A. pisum*, in which miR-125 has been lost from the primary transcript (Legeai et al, 2010). Although we did a number of trials, we were not able to clone and sequence the let-7 primary transcript in *B. germanica*. Indeed, the generally very short half-life of primary transcripts, which are being cleaved by drosha almost simultaneously with transcription (Kim & Kim, 2007; Morlando et al, 2008), makes cloning them extremely difficult. Therefore we cannot confirm whether let-7, miR-100 and miR-125 precursors cluster in the same primary transcript in *B. germanica*. However, given that: 1) the let-7 cluster is generally well conserved across metazoans (Chawla & Sokol, 2012; Pasquinelli et al, 2000), 2) that, in *B. germanica*, the precursors and the sequences of mature

let-7, miR-125 and miR-100, are also very well conserved with respect to other metazoans, and 3) that, in *B. germanica*, the three miRNAs have parallel expression patterns, we presume that these three miRNAs cluster in the same primary transcript also in our model species.

b) The expression of let-7, miR-100 and miR-125 is enhanced by 20E via BR-C, and impaired by juvenile hormone

In *B. germanica*, let-7, miR-100 and miR-125 are present in the whole life cycle, from the first instar nymph until the adult stage, but maximal expression levels occur in parallel to the peak of 20E in the last instar nymph, when juvenile hormone (JH) is absent. This suggests that 20E enhances the expression of these miRNAs, whereas JH impairs it, and results of our previous experiments of 20E and JH treatments (Rubio et al, 2012) are consistent with this hypothesis. Moreover, we have shown that BR-C mediates the 20E action, as depletion of BR-C factors by RNAi led to depletion of let-7, miR-100 and miR-125 levels.

Interestingly, 20E and BR-C are required for let-7 expression in *D. melanogaster* (Chawla & Sokol, 2012; Sempere et al, 2002), which suggests that the hormonal regulation of let-7 cluster by 20E and BR-C is conserved from cockroaches to flies.

c) Let-7, miR-100 and miR-125 contribute to regulate wing size and vein patterning

The increase of expression of let-7, miR-100 and miR-125 in *B. germanica* wing pads in days 5, 6 and 7 of the last instar nymph, already suggests that these miRNAs play a role in this particular tissue during metamorphosis.

Depletion of miR-100 led to adult specimens showing the hindwings smaller that controls and with a subtle disorganization of the vein patterning. Depletion of let-7 gave a similar phenotype concerning vein patterning (Figure 4.2B, D). Depletion of miR-125 triggered a significant decrease of let-7 and miR-100 levels, but the resulting adult specimens did not show any apparent difference with respect to controls. Possibly, the decrease of miR-100 and let-7 provoked by LNA-miR-125 treatment was below the threshold necessary to give the size and vein patterning defects observed by depleting specifically let-7 and miR-100.

In *D. melanogaster*, the RNA-binding protein lin-28 degrades the let-7 precursor (Heo et al, 2008; Rybak et al, 2008; Thornton & Gregory, 2012), whereas miR-125 and let-7 target and degrade lin-28 (Liu et al, 2007; Moss & Tang, 2003; Nolde et al, 2007). Although this regulatory loop is presumed to be conserved across animal kingdom (Moss & Tang, 2003), we do not know whether it exists in *B. germanica* and whether this species has a lin-28 homologue. However, we can speculate that if there were a protein with similar properties, then the decrease of let-7 (and that of miR-100) derived from miR-125 depletion could be explained by the

interaction with this protein. Some kind of positive effect of miR-125 over let-7 and miR-100 is also suggested by the expression patterns, which show that let-7 and miR-100 form a clear peak coinciding with the 20E peak in the last instar nymph, whereas miR-125 levels start to increase early in this stage, when the levels of let-7 and miR-100 are still low (Figure 4.1A). The whole data suggest that in *B. germanica* there is an interplay between let-7 (and miR-100), miR-125 and proteins like lin-28, as occur in other species (Rybak et al, 2008).

In *D. melanogaster,* let-7 is important to ensure the appropriate remodeling of abdominal neuromusculature during metamorphosis (Sokol et al, 2008). In 2008, Caygill and Johnston reported that the absence of let-7 and miR-125 results in temporal delay of maturation of neuromuscular junctions of imaginal muscles (Caygill & Johnston, 2008), and identified abrupt (ab) as the let-7 target in this context. In the same work, these authors found that let-7 is required for terminal exit from the cell cycle in wing imaginal disc, and loss of let-7 led to adult specimens with small wings with more and smaller cells. This effect of let-7 in *D. melanogaster* is reminiscent of that that we observed after depleting miR-100 in *B. germanica*. Concerning effects on neuromuscular junctions, we did not carry out specific neuroanatomical studies, but the gradual metamorphosis that characterizes *B. germanica* do not involve extensive tissue remodeling, as in the case of *D. melanogaster*.

d) The phenotype resulting from let-7, miR-100 and miR-125 depletion is similar to that of BR-C knockdowns

Huang et al. (in press) have recently described in detail the phenotype obtained after depleting BR-C factors with RNAi in *B. germanica*. The specific features of this phenotype concentrate in the adult wings, especially in the hindwings, which, in comparison with controls, are smaller, have a CuP vein shorter, which leave a notch in the wing edge, have the vein-intervein disorganized in the anterior part, and show anomalous bifurcations of the A-veins in the posterior part. This phenotype was recovered in our experiments of BR-C RNAi, as briefly described above. Interestingly, the phenotype of BR-C knockdowns is similar to that obtained with the LNA treatments of let-7 and miR-100, except for the character of the shorter CuP vein and associated edge notch (Supplementary Figure 4.3), which was not triggered by let-7 and miR-100 depletion.

e) Let-7, miR-100 and miR-125 ensure proper wing size and vein patterning probably by regulating bs expression

In *D. melanogaster*, the let-7 cluster is upregulated by 20E and BR-C, and is inhibited by JH (Chawla & Sokol, 2012; Sempere et al, 2002). Moreover, and relevant in the context of the wing phenotype that we are observing, it is worth noting that to ensure a proper organization between veins and interveins in *D. melanogaster* wing development, Egfr inhibits bs expression by

Ras-mediated and increases rho expression (Fristrom et al, 1994). In *B. germanica*, we have shown that the regulatory network organized around 20E and JH pulses, BR-C expression, let-7, miR-100, miR-125 and bs, appears to ensure the correctness of particular aspects of vein patterning. We presume that, as occurs in *D. melanogaster*, and due to the high conservation of the mechanisms involved, Egfr, rho and lin-28 are additional players in this regulation, as depicted in Figure 4.6. Further studies to establish the structure and function of these factors in *B. germanica* would be necessary to completely assess the epistatic relationships hypothesized in the Figure 4.6.

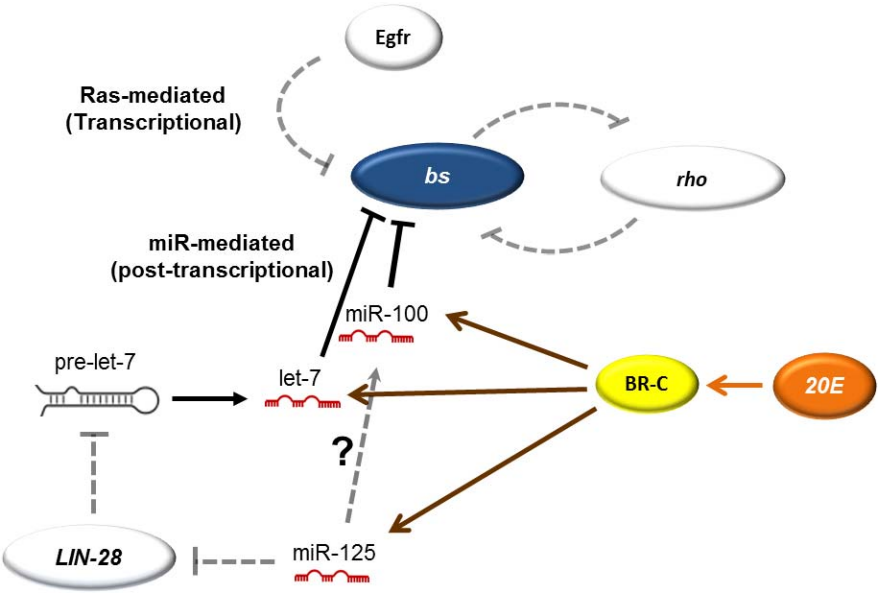

Figure 4.6. Proposed model of regulation of wing vein development in *Blattella germanica* through bs, BR-C, let-7, miR-100 and miR-125 20E acting through BR-C increases the expression of let-7, miR-100 and miR-125; let-7 and miR-100 target bs which is responsible of the proper vein wing patterning. The involvement of other regulatory players, like Egfr, rho and lin-28 (indicated in white color), which operate in *D. melanogaster*, has not been demonstrated in *B. germanica*.

5.5. Materials and Methods

a) Insects, dissections and RNA extracts

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1ºC and 60-70 % relative humidity (r.h.) Freshly ecdysed female nymphs were selected and used at the appropriate ages. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens. We performed total RNA extraction from the whole body (excluding the head and the digestive tube to avoid ocular pigments and intestine parasites) and from the wing pads, using the miRNeasy extraction kit (QIAGEN).

b) Cloning of miRNA precursors

We used RNA extracted from the last instar female nymphs treated with dsDicer-1 as described by Gomez-Orte & Belles (2009), and retrotranscribed with the NCode^{TM} miRNA first-strand synthesis and qRT-PCR kit (Invitrogen) following the manufacturer's protocol. Then, we performed a 3'-RACE using the mature sequences of let-7, miR-100 and miR-125 of *B. germanica* as forward primers. In the case of let-7 we obtained two fragments, one of 21 bp and the second one of 69 bp, for miR-100 we obtained two fragments of 21 and 67 bp, and for miR-125 we also obtained two fragments of 21 and 67 bp. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced. Folding of the

putative precursors obtained was predicted using RNAfold (Gruber et al, 2008).

c) Expression patterns

To establish the expression patterns of miRNAs, three biological replicates were used. Samples dissections and total RNA extraction was carried out with the procedure described above. Quantification of miRNA levels and bs mRNA levels was performed by qRT-PCR. Amplification reactions were carried out using IQTM SYBR Green Supermix (BioRad) and the following protocol: 95ºC for 2 min, and 40 cycles at 95ºC for 15 s and 60ºC for 30 s, in a MyIQ Real-Time PCR Detection System (BioRad). A dissociation curve was carried out to ensure that there was only one product amplified after the amplification phase. All reactions were run in triplicate. Results are given as copies of RNA per 1000 copies of U6. Primer sequences are indicated in Supplementary Table 4.S2.

d) RNAi of BR-C

The detailed procedures for RNAi of BR-C have been described by Huang et al. (in press). A dsRNA encompassing a 326-bp fragment of the BR-C core region was designed to deplete all isoforms simultaneously. The primers used to generate the fragment to prepare dsBR-C are indicated in Supplementary Table 4.S2. The fragment was amplified by PCR and cloned into the pSTBlue-1

vector. A 307 bp sequence from *Autographa californica* nucleopolyhedrosis virus (Accession number K01149, from nucleotide 370 to 676) was used as control dsRNA (dsMock). dsBR-C and dsMock were injected in freshly ecdysed fifth instar female nymphs, as described by Huang et al. (in press) at the same ages and doses. Samples were taken at day six of the last instar nymph (N6D6). Dissections, RNA extraction, retrotranscription and RNA quantification was carried out as described above.

e) miRNA depletion

To deplete let-7, miR-100 and miR-125 levels in *B. germanica*, we used miRCURY LNA™ microRNAs Power Inhibitors (Exiqon).We performed two abdominal injections of 1 μL of LNA at 50 μM, the first injection carried out in the third day, and the second in the fifth day of the last instar nymph. Samples were collected 24 h after the second injection. Controls were injected equivalently with miRCURY LNA™ microRNA Inhibitor Negative Control A (Exiqon). We used 20 specimens per experiment for phenotypic studies, and 3 biological replicates to measure miRNA depletion. RNA extraction and quantification of miRNA and mRNA levels were performed as described above. Statistical analyses between groups were tested by the REST 2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research) (Pfaffl et al, 2002). This program makes no assumptions about the distributions, evaluating the significance of the derived results by

Pair-Wise Fixed Reallocation Randomization Test tool in REST (Pfaffl et al, 2002)

f) Wing biometrics

Biometric measurements of the wings were performed in 1-day-old control and treated female adults. The wings were dissected from carbon dioxide-anaesthetized specimens and mounted in slides with Mowiol. Axiovision software was used to obtain photographs and measures. Maximal length (Lmax) and maximal width (Wmax) were measured on the forewings (tegmina) and hindwings.

g) Cloning of blistered

To clone bs, specific primers were designed based on a partial sequence obtained from a *B. germanica* transcriptome (X. Belles and M. D. Piulachs, unpublished) and used to extend the sequence by 3'- and 5'-RACE using cDNA from wing pads of the last instar nymph of *B. germanica* as template. Following this approach, we obtained a sequence of 1081 bp that encodes a 335 aa protein. Blast analysis indicated that the protein corresponds to a bs homolog (GenBank accession number in process of submission, see the sequence in the Annex 1, at the end of this chapter). Primer sequences used are indicated in Supplementary table 4.S2.

h) miRNA site prediction

To identify putative miRNA recognition elements (MREs) involved in bs mRNA regulation by let-7, miR-100 and miR-125, we used two different prediction methods, RNAhybrid (Kruger & Rehmsmeier, 2006) and PITA (Kertesz et al, 2007). We considered reliable MREs those that had the best scores in each method and which were located at a highly positional entropy site in the secondary structure of the mRNA.

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SUPPLEMENTARY MATERIAL

microRNA-mediated action of Broad complex on wing morphogenesis in hemimetabolan metamorphosis

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Additional file 1. Contents:

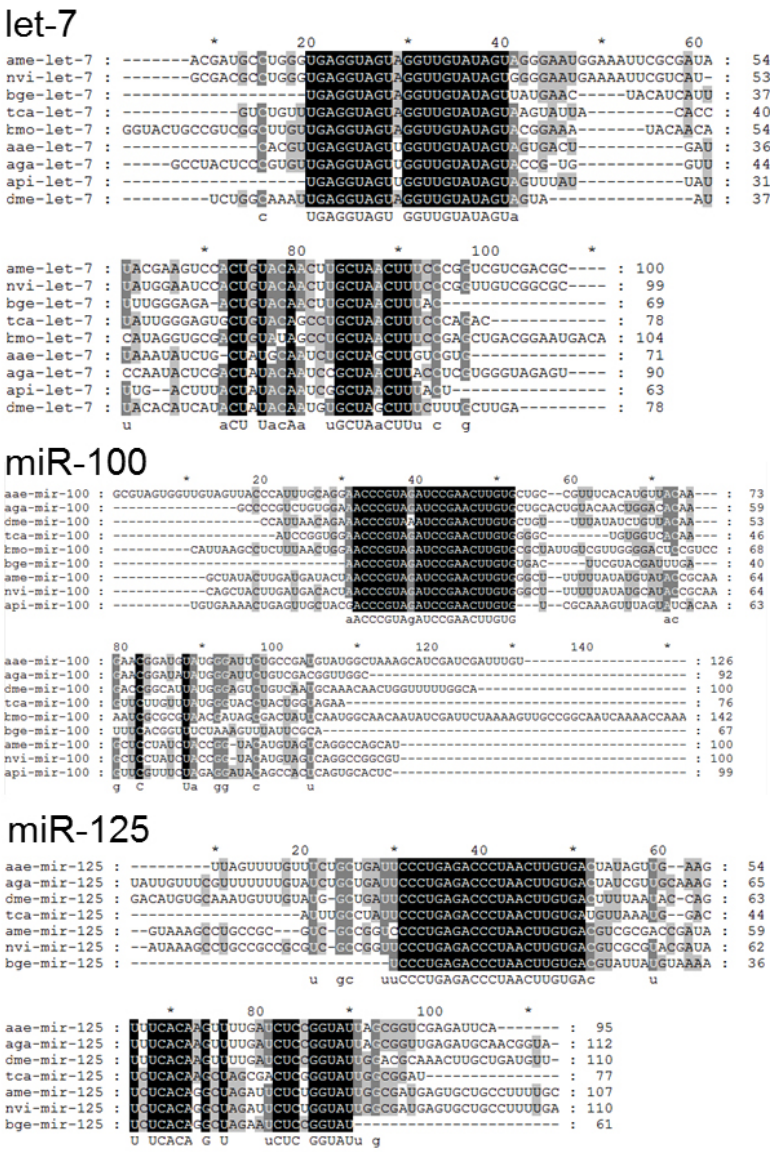

Figure 4.S1. Alignment of the different precursors of let-7, miR-100 and miR-125 in insects. Stemloop sequences from the precursors of let-7, miR-100 and miR-125 were obtained from the miRBase (aae: *Aedes aegypti*, ame: *Apis mellifera*, aga: *Anopheles gambiae*, bmo: *Bombyx mori*, bge: *Blattella germanica*, cqu: *Culex quinquefasciatus*, dme: *Drosophila melanogaster*, nvi: *Nasonia vitiprennis*, tca: *Tribolium castaneum*). Alignment was carried out using ClustalW (Larkin et al, 2007)

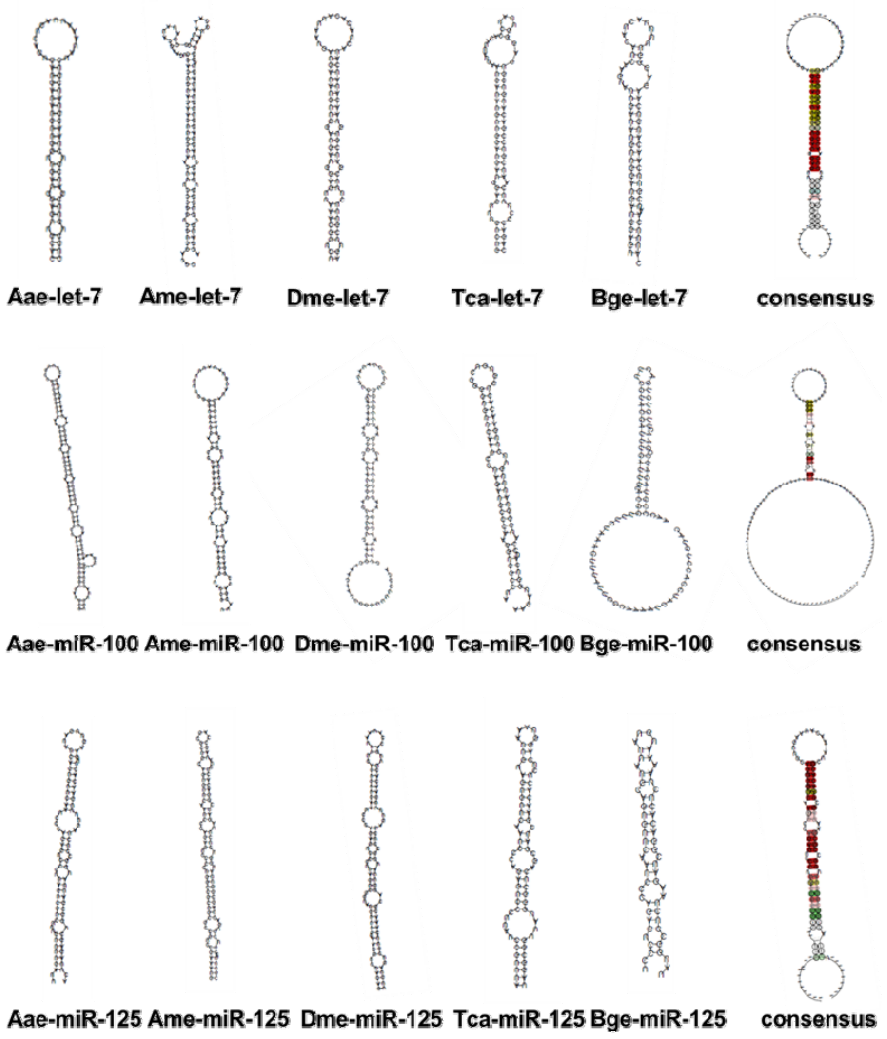

Figure 4.S2. Conservation of the folding structure of let-7, miR-100 and miR-125. Data of the conservation of the folding structure was calculated using RNAalifold (Bernhart et al, 2008; Gruber et al, 2008). Precursor showed in the figure are from *Aedes aegypti* (Aae); *Apis mellifera* (Ame); *Drosophila melanogaster* (Dme); *Tribolium castaneum* (Tca) and *Blattella germanica* (Bge); but the consensus folding structure was made using all the insect precursor sequences in the miRBase (Griffiths-Jones, 2004) (Release 18)

Figure 4.S3. Hindwing phenotype after depleting BR-C by RNAi in *Blattella germanica.* Adult hindwing phenotype studied 3 days after the imaginal molt, from a control specimen treated with dsMock (Control) and a dsBR-C-treated specimen.

Supplementary Table 4.S1. Phenotypes found with the LNA antimiR treatments. Defect A: Wings wrinkled, not properly extended; Defect B: Disorganization of the vein-intervein pattern in the anterior part of the hindwing; Defect C: A-vein bifurcations in the posterior part of the hindwing; Defect D: Tegmina apex bent towards the ventral part.

	Treatment	LNA-let-7	LNA-miR-100	LNA-miR-125	LNA-let-7-C
	Individuals	**10**	**10**	**10**	**8**
	No phenotype	5 (50%)	4 (40%)	10 (100%)	0 (0%)
Defect A	**Plus defect B**	1 (10%)	3 (30%)	0	0
	Plus defect C	1 (10%)	2 (20%)	0	0
	Defect B+C	3 (30%)	1 (10%)	0	0
Defect D	**Only D**	0	0	0	2 (25%)
	Plus defect B	0	0	0	3 (38%)
	Plus defect C	0	0	0	2 (25%)
	Plus defect B+C	0	0	0	1 (13%)

Supplementary Table 4.S2. Primers sequences. (A) Primers used to generate dsBR-C **(B)** Primers used for bs mRNA cloning. **(C)** Primers used for quantification by qRT-PCR

	Transcript or miRNA	primer name	primer sequence
A	BR-C	BRcoreF7	5'-CGCAACAAGCCGAAGACAGA-3'
		BRcoreR5	5'-GCTATTTTCCACATTTGCCG-3'
B	bs	bsFw1	5'-GATGGGCATGACAGAGTGTG -3'
		bsRv1	5'-CTGGAACAGGGAATGAGGTG-3'
		bsFw2	5'-GAGTTGAGGCACGTCTGGAT-3'
		bs_5_Outer	5'-CCGCTTCGAGAATGTTGTGTA-3'
		bs_5_Inner	5'-CACACGCCCTTTTGTCTT-3'
C	bs	RTbsFw	5'-GACGGAGCTCACGTACAACA-3'
		RTbsRv	5'CCAGCGGTCTTACTTTCTGC-3'
	let-7	let-7	5'-TGAGGTAGTAGGTTGTATAGT-3'
	miR-100	miR-100	5'-AACCCGTAAATCCGAACTTGTG-3'
	miR-125	miR-125	5'-CCCTGAGACCCTAACTTGTGA-3'

ANNEX 1

Nucleotide and protein sequence of bs of *Blattella germanica*

```
    ---------|---------|---------|---------|---------|---------|
  1 AAAAGTGCAGTGatggatgcccccgcaggtgggggaagagattcccgatttatgggttac 60
  1            M  D  A  P  A  G  G  G  R  D  S  R  F  M  G  Y   16
    ---------|---------|---------|---------|---------|---------|
 61 aatatgtcgcttattagtggagaagcgccgtccgaaatttacgccactaccttcaacctc 120
 17 N  M  S  L  I  S  G  E  A  P  S  E  I  Y  A  T  T  F  N  L   36
    ---------|---------|---------|---------|---------|---------|
121 attagtggggtggagggagtgtcaaggtcctcgatattaccaagtacttgtggtgcaggg 180
 37 I  S  G  V  E  G  V  S  R  S  S  I  L  P  S  T  C  G  A  G   56
    ---------|---------|---------|---------|---------|---------|
181 ctatcctctcgtggcataaaaagaacgtcttctgaagtgtgtttcgatcaagaagtgtca 240
 57 L  S  S  R  G  I  K  R  T  S  S  E  V  C  F  D  Q  E  V  S   76
    ---------|---------|---------|---------|---------|---------|
241 ggaggtaggccgccgggcatggcttcgcaaagtgggatgggcatgacagagtgtggttcc 300
 77 G  G  R  P  P  G  M  A  S  Q  S  G  M  G  M  T  E  C  G  S   96
    ---------|---------|---------|---------|---------|---------|
301 gatataggggatgacagtttttctcagtcgcagaaaaagactccgccgccgaatggcaaa 360
 97 D  I  G  D  D  S  F  S  Q  S  Q  K  K  T  P  P  P  N  G  K   116
    ---------|---------|---------|---------|---------|---------|
361 aagacaaaagggcgtgtgaaaattaaaatggagtacatcgacaacaaactgagaagatac 420
117 K  T  K  G  R  V  K  I  K  M  E  Y  I  D  N  K  L  R  R  Y   136
    ---------|---------|---------|---------|---------|---------|
421 acaacattctcgaagcggaaaactggaataatgaaaaaggcatatgagctgtcgacgctg 480
137 T  T  F  S  K  R  K  T  G  I  M  K  K  A  Y  E  L  S  T  L   156
    ---------|---------|---------|---------|---------|---------|
481 acgggcacgcaggttatgctcctggtggcctctgaaacgggccacgtgtacacgtttgca 540
157 T  G  T  Q  V  M  L  L  V  A  S  E  T  G  H  V  Y  T  F  A   176
    ---------|---------|---------|---------|---------|---------|
541 acgcgcaagttgcagcccatgattacatcggaggccgggaaggccttgatccagacgtgc 600
177 T  R  K  L  Q  P  M  I  T  S  E  A  G  K  A  L  I  Q  T  C   196
    ---------|---------|---------|---------|---------|---------|
601 ctcaactcgcccgatcctcctccgtccggagtgggaggcgaccagcggatgtcggcgacg 660
197 L  N  S  P  D  P  P  P  S  G  V  G  G  D  Q  R  M  S  A  T   216
    ---------|---------|---------|---------|---------|---------|
661 ggcttcgaagagacggagctcacgtacaacatcggagaggacgagcagaaagtaagaccg 720
217 G  F  E  E  T  E  L  T  Y  N  I  G  E  D  E  Q  K  V  R  P   236
    ---------|---------|---------|---------|---------|---------|
721 ctggtgtattcgccgcattcgcacactcaacacatgggggtggccttccccaacagcac 780
237 L  V  Y  S  P  H  S  H  T  Q  H  M  G  G  G  L  P  Q  Q  H   256
    ---------|---------|---------|---------|---------|---------|
781 caacacacgcaacacttccagcaacaccccgggtccccgcaccagcaccacatgatggct 840
257 Q  H  T  Q  H  F  Q  Q  H  P  G  S  P  H  Q  H  H  M  M  A   276
    ---------|---------|---------|---------|---------|---------|
841 cctccccacccgcatgctcagtcgccccttcgcgcctcattccctgttccagccccgga 900
277 P  P  H  P  H  A  Q  S  P  P  S  R  L  I  P  C  S  S  P  G   296
    ---------|---------|---------|---------|---------|---------|
901 cccatgatgggcagcggataccagcagccctctcacgtgtcctgcccatctcttctgcct 960
297 P  M  M  G  S  G  Y  Q  Q  P  S  H  V  S  C  P  S  L  L  P   316
    ---------|---------|---------|---------|---------|---------|
961 ccacacacgggctacccccccaccgcatccacacatgtcacactcacatccgcagcggTAG 1020
317 P  H  T  G  Y  P  P  P  H  P  H  M  S  H  S  H  P  Q  R      335
    ---------|---------|---------|---------|---------|---------|
1021 TTATTTTCAGTAGTCGCATGTGAGGTTGCTGCTTTGGCATTATAAAAAAAAAAAAAAAAA 1080

     -
1081 A 1081
```

Atrophin plays a key role in adult ecdysis and is regulated by mir-8-5p and mir-8-3p

5

Another factor that we identified as important in the ecdysis to the adult instar was atrophin (atro), a transcriptional co-repressor that in *Drosophila melanogaster* is involved in nuclear receptor signaling and in neuromotor coordination. Moreover, studies carried out in this fly by Stephen Cohen and co-workers showed that atro mRNA levels are regulated by miR-8-3p. Interestingly, in the miRNA libraries of *Blattella germanica* we had found that miR-8-3p (considered the mature miRNA of the miR-8 precursor) was abundant, but miR-8-5p (which should be considered the passenger strand of the miR-8 precursor) was even more abundant. With these antecedents, we wondered whether atro might be regulated post-transcriptionally by the interplay of miR-8-3p and miR-8-5p in our model species, which would represent a unique case of a transcript regulated by the two miRNA products of the same miRNA precursor.

The present chapter has been formatted to be submitted to a SCI Journal.

Super-finely tuned regulation of Atrophin expression by miR-8-3p and miR-8-5p interplaying

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5.1. Abstract

Analysis of a small RNAs library from the whole body of the last instar nymph led to the identification of a high number of copies of the microRNA (miRNA) miR-8-3p, but even more copies of its passenger strand, miR-8-5p, were found. This high number of copies and the fact that miR-8-5p is conserved across insect orders suggested to us that miR-8-5p is a functional miRNA. Previous studies have shown that miR-8 regulates in a very precise manner atrophin (atro), a transcriptional co-repressor associated with histone deacetylase activity that prevents neurodegeneration in *Drosophila melanogaster* (Karres et al, 2007). In *B. germanica*, we show that the expression patterns of miR-8-3p and miR8-5p are opposed. Using LNA specific antimiRs against miR-8-3p and miR8-5p, we observed that atro mRNA levels are affected by the two treatments, thus suggesting that atro is regulated by both, miR-8-3p and miR8-5p. Moreover, target prediction algorithms led to identify miR-8-3p and miR-8-5p binding sites in the 3'UTR region of atro mRNA. Finally we inferred that miR-8-3p is loaded mostly with Argonaute 1 (Ago1) to form the RNA-induced silencing complex (RISC), whereas miR-8-5p can equally load with Ago1 and Ago2, the latter having a higher capability for mRNA degradation than Ago1. We propose that the interplay of both miRNAs, miR-8-3p and miR-8-5p, and their interaction with Ago1 and Ago2 and with the miRNA binding sites of atro mRNA, maintain the appropriate expression levels of atro in *B. germanica*.

5.2. Introduction

microRNAs (miRNAs) are endogenous ~22 nt RNAs that couple to mRNA targets and modulate their availability for translation. Indeed, miRNAs sculpt mRNA patterns during the life cycle, thus configuring an important layer of regulation of gene expression. It has also been suggested that miRNAs might buffer target levels to prevent potentially detrimental excess expression while allowing required expression of the target. Bartel and Chen (2004) coined the term "tuning targets" to describe this relationship.

miRNA biogenesis and function is related to RNA interference (RNAi) induced by small interfering RNAs (siRNAs) (Farazi et al, 2008; Okamura & Lai, 2008; Zhou et al, 2008). Both siRNAs and miRNAs are associated with Argonaute (Ago) proteins and compose the RNA-induced silencing complex (RISC). RISC can also contain additional proteins thought to extend the functions of Ago proteins or to direct the own complex to specific sub-cellular locations. The simplest, and likely ancestral, Ago function is cleaving its RNA target at a single phosphodiester bond. The structure of Ago ensures that the cleaved bond lies between the target nucleotides paired to the tenth and eleventh nucleotides of the guide RNA (Cenik & Zamore, 2011).

Unlike other animals, insects have substantially separate molecular pathways for miRNAs and siRNAs (Belles et al, 2011). In *Drosophila melanogaster*, while miRNAs are processed by dicer-1 (Dcr-1) and mostly incorporated into Ago1 complex, siRNAs are processed by Dcr-2 and sorted with Ago2 (Tomari et al, 2007). The

miRNA incorporated into the RISC complex is called the mature sequence, and its partner, the miRNA passenger strand, is thought to be degraded, although it has been recently found that several miRNA passenger strand species are preferentially incorporated into Ago2 complex, and require canonical RNAi factors for their accumulation (Czech et al, 2009; Forstemann et al, 2007; Ghildiyal et al, 2010; Okamura et al, 2009).

High-throughput sequencing of miRNAs in the cockroach *Blattella germanica* (Cristino et al, 2011), indicated that there are a high number of copies of miR-8-5p, which is supposed to be the passenger strand of the miRNA precursor; we found even a higher number of copies than those of miR-8-3p, which is supposed to be the mature miRNA. In vertebrates (Yang et al, 2011) and *D. melanogaster* (Okamura et al, 2008), a significant fraction of miRNA passengers have been conserved along evolution, and they have a relevant role in the gene regulatory networks. We wondered, thus, whether miR-8-5p might have some function in *B. germanica*.

In *D. melanogaster*, miR-8-3p regulates atrophin (atro) mRNA levels through specific binding sites for this miRNA found in the 3' UTR of atro mRNA (Karres et al, 2007). Atro proteins are conserved transcriptional co-repressors involved in nuclear receptor signaling (Wang & Tsai, 2008). Regulation of atro by miR-8 (indeed, miR-8-3p) appears to be a conserved feature, because Karres et al. (2007) found that 3'UTR of the vertebrate arginine-glutamic acid dipeptide repeats protein gene (RERE), which is the homologue of atro in vertebrates, also contains three putative binding sites for miR-429 and miR-200b, which are vertebrate

homologues of miR-8. Therefore, a key function of miR-8 and miR-429/miR-200b may be to fine-tuning the expression levels of atro and RERE in *D. melanogaster* and in vertebrates, respectively.

Therefore, atro might be a target of miR-8-3p in *B. germanica*, and perhaps also a target of miR-8-5p. Our hypothesis is that miR-8-3p might act in concert with miR-8-5p to achieve a super-finely tuned regulation of atro. If so, then atro should be considered a unique case where a transcript is regulated by the two strands of the same miRNA precursor, both acting as functional miRNAs.

5.3. Materials and Methods

a) Insects and RNA extractions

Freshly ecdysed sixth (last) instar nymphs of the cockroach *B. germanica* were obtained from a colony reared in the dark at 30 ± 1°C and 60–70% relative humidity. The entire animal except the head (to avoid interferences with eye pigments) and the digestive tube (to avoid contamination with parasites) was used for RNA extractions. All dissections were carried out on carbon dioxide-anaesthetized specimens. Samples for RNA extraction and quantification were frozen immediately after dissection, and stored at -80ºC until use. RNA isolation was carried out with miRNeasy® Mini Kit (QIAGEN), which increases the yield of small RNAs. Reverse transcription was carried out with the NCode^{TM} miRNA First-Strand cDNA Synthesis and qRT-PCR Kits (Invitrogen),

which allows the quantification of large and small RNAs by real-time PCR.

b) Cloning the miR-8 precursor

Using the sequences of miR-8-5p and miR-8-3p as primers, and cDNA from last instar female nymphs of *B. germanica* as template, we amplified a fragment of 59 bp by PCR, which was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. Using the RNA fold algorithm (Gruber et al, 2008), we assessed that the obtained sequence folds with the typical hairpin structure of a pre-miRNA.

c) Cloning atro cDNA

A partial sequence of *B. germanica* atro, including the 3' UTR, was obtained following a RT-PCR strategy using degenerate primers designed on the basis of conserved motifs from insect atro sequences and cDNA from last instar female nymphs of *B. germanica* as template. The sequence of the amplified fragment (973 bp) was highly similar to the equivalent region in known insect atro sequences, and we called it BgAtro (GenBank accession number in process of submission, see the sequence in the Annex 1, at the end of this chapter). Then, the 3' fragment of the sequence was completed by 3' RACE (3'-RACE System Version 2.0; Invitrogen) using the same template, thus obtaining a final fragment of 1823 bp. PCR products were subcloned and sequenced as

described above. Primer sequences used are indicated in the Supplementary Table 5.S1.

d) Cloning Ago1 and Ago2 cDNAs

The *B. germanica* Ago1 and Ago2 were obtained following a RT-PCR strategy using specific primers based on sequences obtained from *B. germanica* libraries (X. Belles and M. D. Piulachs, unpublished), and cDNA from the last instar female nymphs of *B. germanica* as template. We further extended the sequence by subsequent 3' and 5' RACE using cDNA from last instar female nymphs, and we obtained a sequence of 2,834 bp length for Ago1 and of 3,361 bp length for Ago2 (GenBank accession numbers in process of submission, see the sequences in the Annexes 2 and 3, respectively, at the end of this chapter). PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced as described above. Primer sequences are indicated in the Supplementary Table 5.S1.

e) Quantification of miRNAs and mRNAs by quantitative real-time PCR

For mRNA expression studies by quantitative real-time PCR (qRT-PCR), 400 ng of total RNA were reverse transcribed using the NCodeTM First-Strand cDNA Synthesis Kit (Invitrogen), following the manufacturer's protocol. Amplification reactions were carried out using IQTM SYBR Green Supermix (BioRad) and the

following protocol: 95 ºC for 2 min, and 40 cycles of 95 ºC for 15 s and 60ºC for 30 s, in a MyIQ Real-Time PCR Detection System (BioRad). After the amplification phase, a dissociation curve was obtained in order to ensure that there was only one product amplified. All reactions were run in triplicate. Statistical analyses between groups were tested by the REST 2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research) (Pfaffl et al, 2002). This program makes no assumptions about the distributions, evaluating the significance of the derived results by Pair-Wise Fixed Reallocation Randomization Test tool in REST (Pfaffl et al, 2002). Therefore, results are given as copies of RNA per 1000 copies of U6. Primer sequences used are indicated in the Supplementary Table 5.S2.

f) mRNA depletion by RNAi

Procedures for RNAi experiments were as described previously (Gomez-Orte & Belles, 2009; Lozano & Belles, 2011). To deplete BgAtro, we prepared a dsRNA encompassing a 268 bp region located between nucleotides 1 and 268 (see the sequence in Annex 1), using the primers AtroFwF and AtroRvF (sequences indicated in the Supplementary Table 5.S3). The dsRNA was injected at a dose of 2.5 µg in *B. germanica* females in freshly emerged sixth (last) nymphal instar. Expression of BgAtro, miR-8-5p and miR-8-3p was examined on whole body extracts 48, 72 and 96 h later by qRT-PCR. Depletion of BgAgo1 was carried out with a dsRNA encompassing a 249 bp region, located between

nucleotides 400 and 648 (see the sequence in Annex 2), using the primers Ago1Fw and Ago1Rv (sequences indicated in the Supplementary Table 5.S3). To deplete BgAgo2, we used a dsRNA encompassing a 437 bp region located between nucleotides 1,498 and 1,934 (see the sequence in Annex 3), using the primers Arg2_Fw_1 and Arg2_Rv_1 (sequences indicated in the Supplementary Table 5.S3). dsBgAgo1 and dsBgAgo2 were injected at a dose of 3 µg and 2.5 µg, respectively in *B. germanica* females in freshly emerged fifth (penultimate) nymphal instar. Expression of BgAgo1, BgAgo2, miR-8-5p and miR-8-3p was examined on whole body extracts in the freshly emerged last instar nymph by qRT-PCR

A 307-bp sequence from *Autographa californica* nucleopolyhedrosis virus (Accession number K01149, from nucleotide 370 to 676) was used as control dsRNA (dsMock), injected at the same dose and days of the specific treatments.

g) Depletion of miRNAs

Depletion of miRNAs was carried out by abdominal injection of LNA specific antimiRs: miRCURY LNA™ microRNA Inhibitor (Exiqon), at a concentration of 50 µM, in freshly emerged last instar female nymphs of *B. germanica*. Controls were injected with miRCURY LNA™ microRNA Inhibitor Control A (Exiqon) at the same dose and age. Expression of BgAtro, miR-8-3p and miR-8-5p was examined by qRT-PCR on whole body extracts 2, 4, 6 and 24 h after the treatment.

h) Behaviour experiments

One day (24 h) after the injection of dsBgAtro, LNA-miR-8-3p or LNA-miR-8-5p, we anaesthetized the cockroaches during 30 sec with a constant flux of carbon dioxide, and we placed them into a glass jar of 17 cm high and 10 cm diameter. The top 10 cm of the jar were covered with a black plastic, and we placed a source of light 21 cm from the jar and 5 cm from the base. Cockroaches started to wake up 2-3 min after the anaesthesia and we measured the time that they spent climbing to the top of the jar to become hidden in the dark.

i) Prediction of miRNA binding sites

Using the complete set of miR-8 isomirs that we found in the miRNAs libraries of the penultimate and last instar nymph of *B. germanica* (Rubio et al, 2012) (Supplementary Figure 5.S1), we predicted miRNA binding sites for miR-8-3p and miR-8-5p in the 3'-UTR sequence of BgAtro. We combined the predictions of four different methods (miRanda, RNAhybrid, miRiam and PITA), and only the miRNA recognition elements (MREs) that were predicted by two or more different methods and which were located at a highly positional entropy site in the secondary structure of the mRNA, were subsequently considered.

j) Luciferase assay to validate predicted miRNA binding sites

Luciferase reporters were generated by PCR amplification of a DNA fragment corresponding to the 3'UTR of the BgAtro transcript from pSTBlue-1 vector (Novagen) using the primers Atroph-Fwd and Atroph-Rev (sequences indicated in the Supplementary Table 5.S4). They were cloned under the control of the tubulin promoter downstream of the Firefly luciferase coding region between the SpeI and XhoI sites of plasmid pJ-Luc described elsewhere (Rehwinkel et al, 2005). All PCR fragments were cloned SpeI-XhoI in the pJ-Luc vector and confirmed by sequencing. To study the expression of miR-8, a fragment of 272 bp encompassing the miR-8 hairpin was amplified by PCR with the primers miR-8-Fwd and miR-8-Rev (sequences indicated in the Supplementary Table 5.S4) from *D. melanogaster* genomic DNA and cloned in vector pAc5.1 (Actin Promoter) between the EcoRI and XhoI sites. pAc5.1 plasmid expressing Renilla luciferase served as transfection control.

S2 cells were transfected in 24-well plates with Cellfectin (Invitrogen) following the manufacturer's instructions. Transfections were performed in triplicate and contained per well: 100 ng of Firefly luciferase reporter plasmid, 0.5 μg of the Renilla luciferase transfection control plasmid; moreover, in a first set of experiments we transfected 1 μg of miRNA expression plasmid or empty vector, and in a second set of experiments we transfected single-strand RNA (ssRNA) oligo with the sequence of miR-8-3p or

miR-8-5p (SIGMA). Dual-luciferase assays (Promega) were performed 48-60 h after transfection according to the manufacture's protocol.

5.4. Results and discussion

a) The miR-8 precursor of *Blattella germanica* and the expression patterns of miR-8-3p and miR-8-5p

Cloning the precursor of miR-8 gave a 59 bp sequence that, according to the predictions of RNAfold WebServer (Gruber et al, 2008), folded into the characteristic hairpin structure of a miRNA precursor (Figure 5.1A). Alignment of the miR-8 precursor in different species show that the regions corresponding to miR-8-5p and miR-8-3p are conserved across the insect class (Figure 5.1B); the folding structure is conserved not only in insects but also in other metazoans, where the homologues of miR-8 miRNA are five miRNAs from the same family (miR-200a, miR-200b, miR-200c, miR-429 and miR-141) (Figure 5.1C).

Usually, miR-8-5p is considered the passenger strand in other insects, for example, in the miRBase (Griffiths-Jones, 2004) (Release 18: Nov. 2011). Moreover, in *D. melanogaster* this strand had 51864 reads in 50 experiments, whereas the miR-8-3p, which is considered the mature strand, had 725634 reads in the same number of experiments. In *B. germanica*, high-throughput sequencing of small RNAs in the whole-body of the last instar female nymphs

resulted in approximately 11 million reads, in which a total of 47,160 corresponded to miR-8-5p, and 4,906 to miR-8-3p (Cristino et al, 2011).

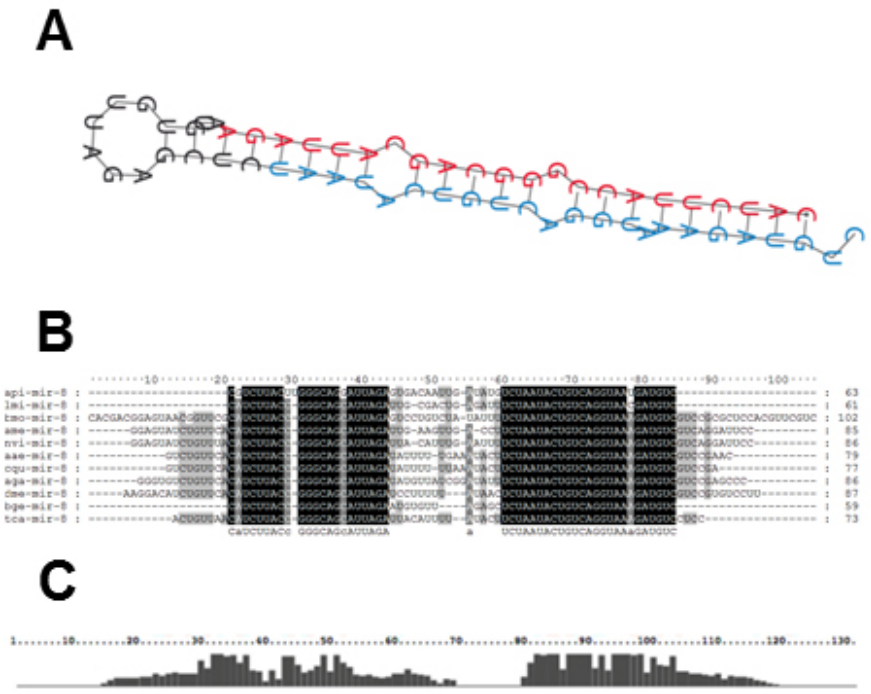

Figure 5.1. The precursor of miR-8 in *Blattella germanica*. (A) Hairpin structure of pre-miR-8 of *B. germanica*; in red: sequence corresponding to miR-8-5p, in blue: sequence corresponding to miR-8-3p. (B) Alignment of the miR-8 precursor of different insects (aae: *Aedes aegypti*, ame: *Apis mellifera*, aga: *Anopheles gambiae*, api: *Acyrthosiphon pisum*, bmo: *Bombyx mori*, bge: *Blattella germanica*, cqu: *Culex quinquefasciatus*, dme: *Drosophila melanogaster*, lmi: *Locusta migratoria*, nvi: *Nasonia vitiprennis*, tca: *Tribolium castaneum*). (C) Conservation of the folding structure among miR-8 family in other metazoas. Data from B and C were obtained of miRBase (Griffiths-Jones, 2004) (Release 18).

Expression patterns of miR-8-3p and miR-8-5p in the fifth (penultimate) and sixth (last) instar female nymphs of *B. germanica* show profiles approximately opposed, with generally higher levels of miR-8-5p except in the freshly emerged fifth instar nymph and the last day of the last instar nymph (Figure 5.2).

b) Structure and function of atro in *B. germanica*

In search of an atro ortholog in *B. germanica*, we obtained a cDNA of 1823 bp (GenBank accession number under submission; see the sequence in the Annex 1, at the end of this chapter). Database BLAST searches confirmed that it encoded an atro homologue, which we called BgAtro.

BgAtro levels fluctuate very little in the two last nymphal instars of *B.* germanica, with the exception of the last day of the last instar nymph, just before the metamorphosis, where expression dramatically peaks (Figure 5.2).

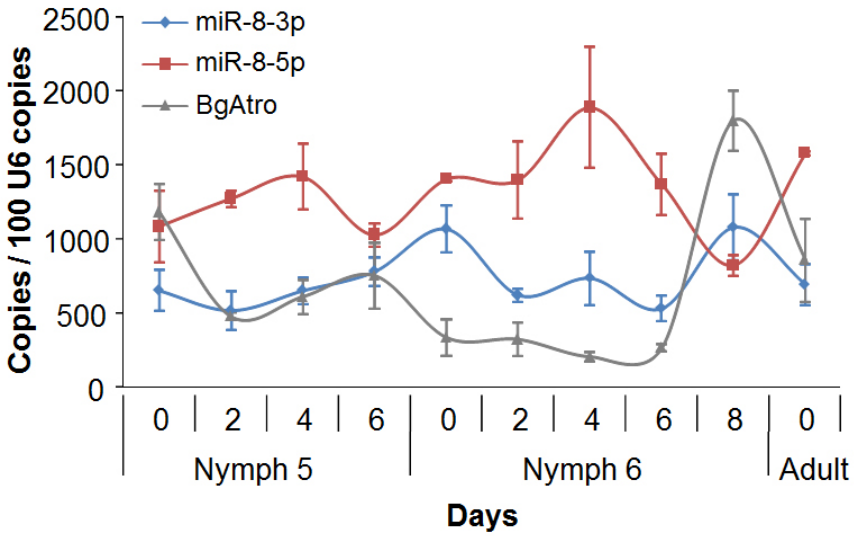

Figure 5.2. Expression patterns of miR-8-5p, miR-8-3p and BgAtro in fifth and sixth nymphal instars; and the first day of adult of *Blattella germanica*. Data show miRNA and mRNA copies per 1000 copies of U6, and are based on 3 biological replicates.

To assess the functions of BgAtro in *B. germanica*, we injected dsRNA of BgeAtro in freshly emerged sixth instar female nymphs, in order to deplete the peak occurring on day 8 of the instar. Figure 5.3A show that BgAtro mRNA levels decreased 24, 48 and 96 h after the treatment, being more pronounced after 96 h. Twenty four hours after the RNAi treatment, the specimens showed problems of mobility, which made them much slower in the behaviour tests, when climbing from the bottom to the top of the experimental jar (Figure 5.3B). Treated specimens were able to metamorphose but showed defects in the legs (being twisted and curved) and wings (which were wrinkled) (Figure 5.3C), probably resulting from mechanical problems derived from an improper ecdysis. This suggests that the peak observed in the last day of the

last instar nymph is important for ecdysis is related to the neuromuscular coordination associated to exuvia removal.

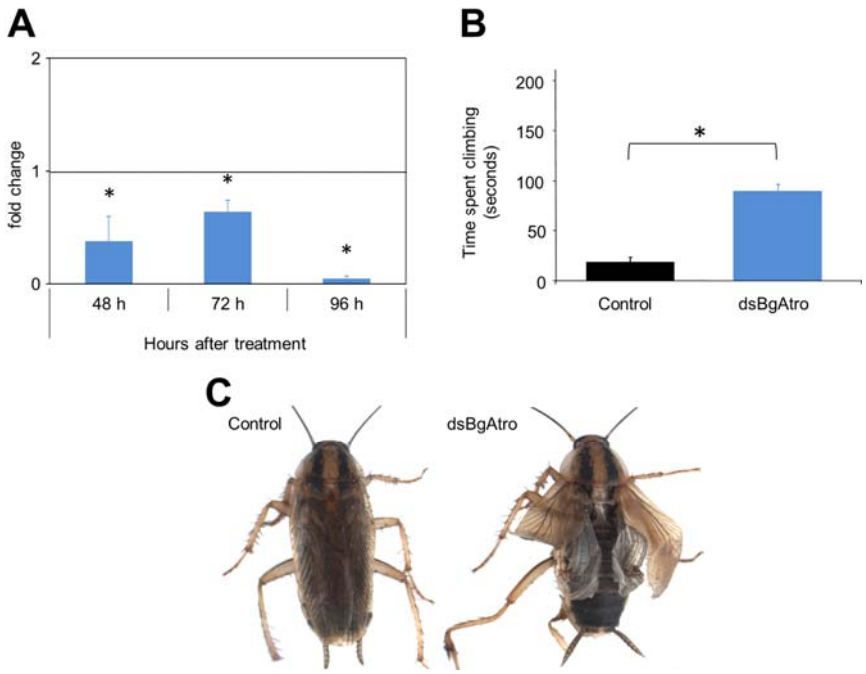

Figure 5.3: Depletion of BgAtro by RNAi in *Blattella germanica*. (A) Levels on BgAtro mRNA in specimens treated with dsBgAtro in freshly emerged last instar nymphs. Samples were studied 48, 72 and 96 h after the treatment, and mRNA levels were measured by qRT-PCR, using U6 as a reference. (B) Time spent climbing from the bottom to the top of the jar by controls and treated specimens 24 h after RNAi treatments. (C) Morphological phenotype of the adult of dsBgAtro-treated specimens showing defects in the extension of wings and legs. Data in A represent 3 biological replicates and are normalized against control females (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool (Pfaffl et al, 2002). Data in B represent 12 biological replicates; the asterisk indicates statistically significant differences with respect to controls (*t*-test, $p < 0.01$).

In *D. melanogaster*, high levels of atro led to significant apoptosis in brain tissues, which apparently affect motor coordination and provokes ecdysis problems (Karres et al, 2007). Moreover, mutations of atro in *D. melanogaster* cause leg patterning defects (Erkner et al, 2002; Wang et al, 2006) and ectopic vein formation in the wings (Charroux et al, 2006). In *B. germanica*, depletion of BgAtro appears specifically associated to altered neuromotor coordination, given that the phenotype do not include ectopic veins in the wings and defects in leg patterning. Indeed, the resulting phenotype seems rather related to mechanical problems derived from an improper ecdysis, which are reminiscent of the phenotype found in *D. melanogaster* when atro levels are overexpressed.

c) BgAtro mRNA has putative binding sites for miR-8-5p and miR-8-3p

We used the set of miR-8 isomirs that we found in the miRNAs libraries described by Rubio et al. (2012) (Supplementary figure 5.S1) to look for miR-8-3p and miR-8-5p MREs in the 3'-UTR of BgeAtro mRNA, combining the predictions of four different methods (miRanda, RNAhybrid, miRiam and PITA). Figure 5.4 indicates the MREs that were predicted by two or more different methods and that are located at a highly positional entropy site in the secondary structure of the mRNA. These were considered the most probably functional MREs.

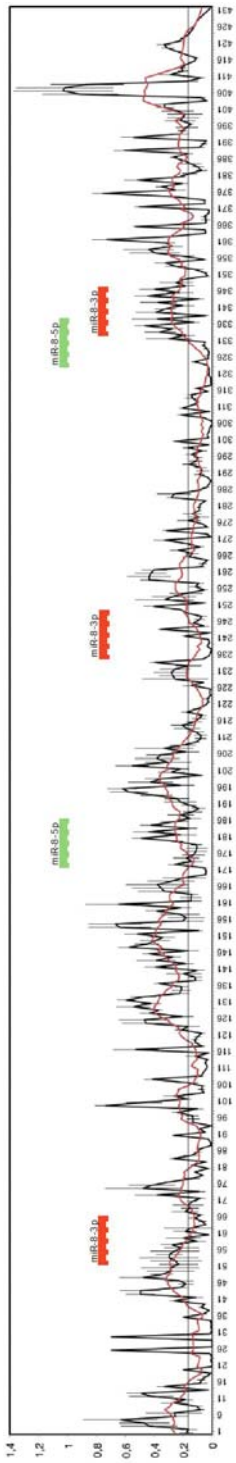

Figure 5.4. Organization of the 3'UTR region of BgAtro, indicating the miR-8-5p and miR-8-3p sites predicted. Positional entropy in the 3'-UTR of *Blattella germanica* Atrophin mRNA with entropy in the abscissae and nucleotide position in the ordinate. The grey line indicates the average positional entropy and the red line show the simple moving average of the positional entropy, with a fixed subset size of ten. The MREs for miR-8-3p are indicated in red and those for miR-8-5p are indicated in green.

To assess the functionality of these MREs, we performed a luciferase assay in S2 cells using the precursor of Dme-miR-8 (which leads to the production of miR-8-5p and miR-8-3p mature sequences identical to those of Bge-miR-8-5p and Bge-miR-8-3p), with the luciferase reporter gene linked to the complete sequence of 3'UTR of BgAtro.

Co-expression of the luciferase reporters in S2 cells with pre-miR-8 reduced (60%) luciferase activity (Figure 5.5), a reduction that is similar to that obtained in *D. melanogaster* in equivalent experiments (Karres et al, 2007). To assess which strand is responsible for the decrease in the luciferase activity we performed equivalent experiments using single strand RNA (ssRNA) oligonucleotides of miR-8-3p and miR-8-5p separately. Previous experiments reported by Martinez and colleagues (2002) showed that, although usually double strand RNA are loaded with Ago proteins, the RISC complex can be constructed with ssRNA. With this premise in mind, we transfected the luciferase reporter gene linked to the complete sequence of 3'UTR of BgAtro, and we added ssRNA of miR-8-3p and miR-8-5p. We observed 60% reduction of luciferase enzyme activity in response for each ssRNA, which is comparable to that obtained when we transfected the pre-miR-8 (Figure 5.5).

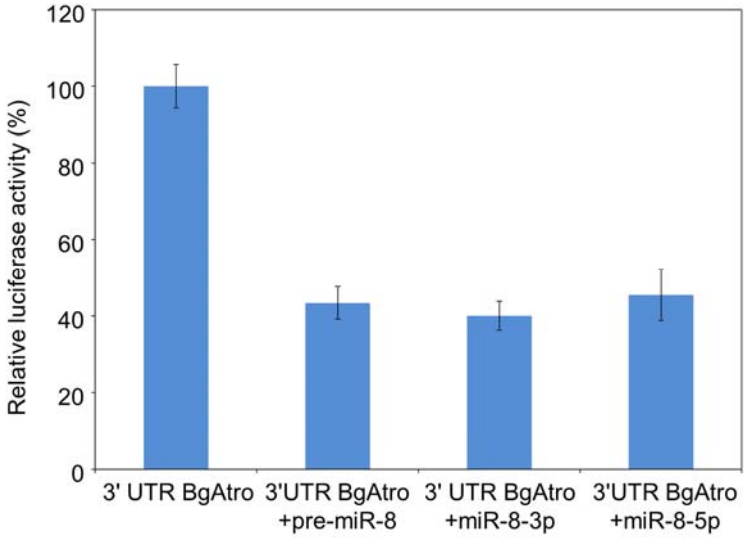

Figure 5.5. Luciferase assay of the miR-8-5p and miR-8-3p sites in the 3'UTR of BgAtro mRNA. Data presented as normalized luciferase activity for a reporter transgene containing the BgeAtro 3'UTR. The BgeAtro 3'UTR was coexpressed with pre-miR-8, with miR-8-3p ssRNA and with miR-8-5p ssRNA.

d) Depleting miR-8-5p and miR-8-3p

Depleting miR-8-3p by treating freshly emerged sixth nymphal instar with 1 µL of LNA-miR-8-3p at 50 µM, resulted in a clear depletion of the miRNA (Figure 5.6A). Levels of miR-8-5p were not affected by the treatment (Figure 5.6A), thus assessing the specificity of the depletion. We simultaneously measured the levels of BgAtro mRNA, and results (Figure 5.6A) showed that they had increased 4 h after the treatment but recovered normal levels 2 h later. Depletion of miR-8-5p under the same conditions was very efficient (more than 95%) 2 h after the treatment; then, miR-8-5p levels kept low 4 h later, but at 6 h they started to increase, though

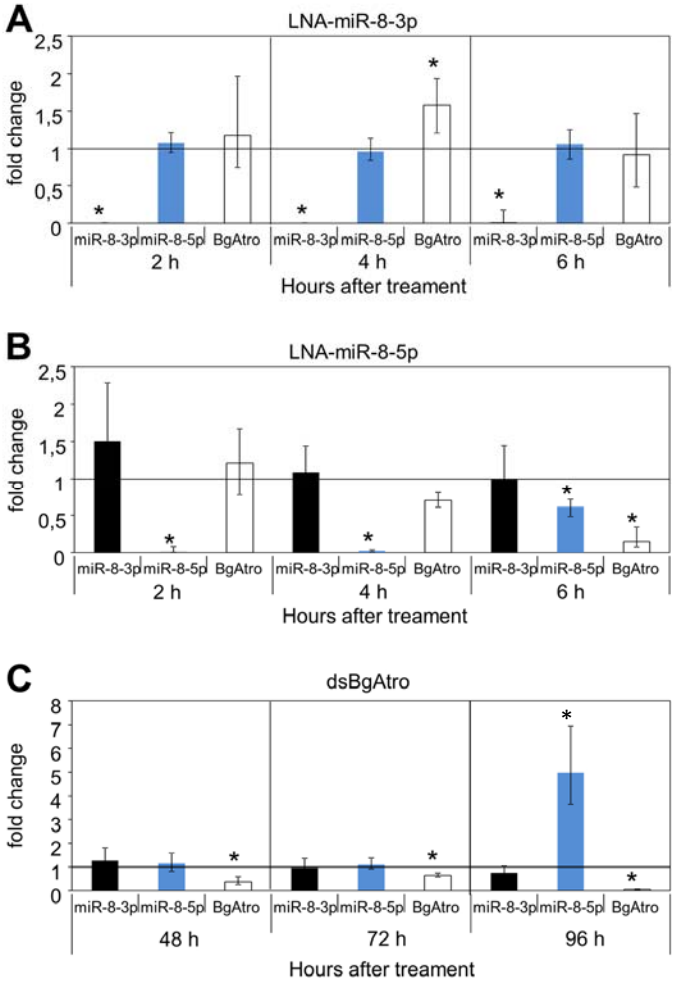

Figure 5.6. Effect of miR-8-3p, miR-8-5p and BgAtro depletion on the last instar nymph of *Blattella germanica.* (A) Effect of the depletion of miR-8-3p on miR-8-3p, miR-8-5p and BgAtro mRNA 2, 4 and 6 h after the treatment. **(B)** Effect of the depletion of miR-8-5p on miR-8-3p, miR-8-5p and BgAtro mRNA 2, 4 and 6 h after the treatment. LNA-treated females in (A) and (B) received an injection of 50 µM of each LNA-miRNA when freshly emerged to the last nymphal instar; control females received an equivalent treatment with miRCURY LNA™ microRNA Inhibitor Negative Control A. **(C)** Effect of the depletion of BgAtro by injection of 2.5 µg of dsBgAtro in freshly emerged last instar nymph, on the levels of miR-8-3p, miR-8-5p and BgAtro mRNA at the same age; control females received an equivalent treatment with dsMock. Data represent 3 biological replicates and are normalized against control females data (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool (Pfaffl et al, 2002).

still remaining significantly lower than controls. As expected, miR-8-3p levels were not affected (Figure 5.6B). In parallel, and quite unexpectedly, BgAtro mRNA levels decreased progressively and were significantly lower than control 6 h after the treatment (Figure 5.6B). Depletion of BgAtro by RNAi did not trigger apparent effects on the miRNAs, except in the case of miR-8-5p, whose levels increased 96 h after the treatment, although with a high variability (Figure 5.6C).

Concerning phenotypical effects, depletion of miR-8-3p and miR-8-5p led to a similar behavior than that observed after BgAtro depletion, that is, the experimental specimens exhibited remarkable problems of mobility, which made them much slower when climbing from the bottom to the top of the experimental jar (Figure 5.7). Karres and colleagues (2007) reported similar results after depleting atro in *D. melanogaster*. We presume that climbing deficiencies observed in *B. germanica* arose from atro deregulation problems derived from miR-8-3p and miR-8-5p depletion. However, we did not observe any apparent phenotype after the imaginal molt, 6 days later, which might be due to the short-lasting effects of LNA treatments.

The whole data points to a tight interplay between atro mRNA, miR-8-3p and miR-8-5p, as suggested by the clear response of the system when one of the three elements changes its expression.

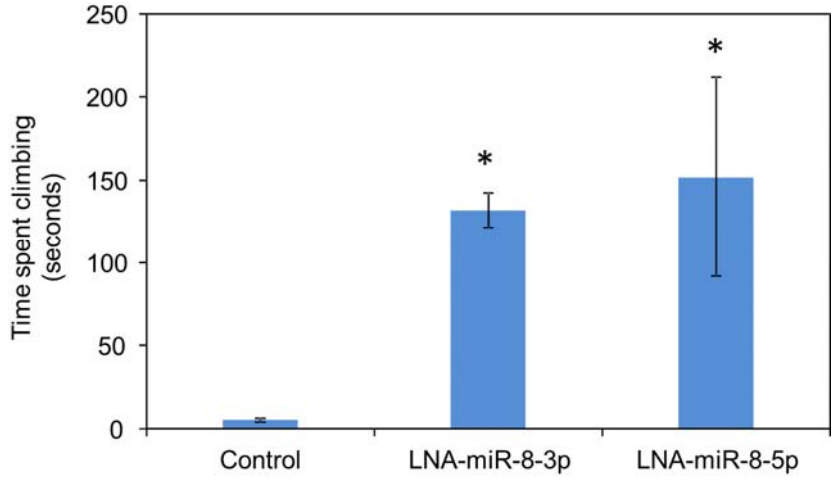

Figure 5.7. Time spent climbing by controls and LNA-treated specimens of *B. germanica* females. Data represent 6 biological replicates 24 h after treatment with LNA-anti-miR-8-3p and LNA-anti-miR-8-5p in freshly emerged last instar female nymph; the asterisk indicates statistically significant differences with respect to controls (*t*-test, $p < 0.01$).

e) The role of Argonaute proteins

In insects, mature miRNA preferentially load with Ago1 into the RISC, but a number of miRNA from the passenger strand can load with Ago2. Given that both strands of the pre-miR-8 of *B. germanica* appear to yield functional miRNAs that look related to atro mRNA regulation, an analysis of the involvement of Ago1 and Ago2 in this context appeared necessary.

Cloning of Ago1 and Ago2 in *B. germanica* was accomplished by a RT-PCR approach. The conceptual translation rendered two proteins, one of 896 amino acids for Ago1 (BgAgo1) and 965 amino acids for Ago2 (BgAgo2), which contain the two classical domains PAZ and PIWI, which are typical of Ago proteins (See Annexes 2 and 3).

Expression patterns in the two last nymphal instars and in freshly emerged adults shows a prominent peak in the 4th day of the penultimate nymphal instar for BgAgo2, and two smaller peaks in the 4th day of the last nymphal instar and the day before the imaginal moult (Figure 5.8A). Expression of BgAgo1 is much lower than that of BgAgo2, and show respective mild peaks before every molt (Figure 5.8A).

Depletion of BgAgo1 and BgAgo2 by RNAi performed on freshly emerged penultimate instar nymph resulted in reduced levels of miR-8-3p and miR-8-5p (Figure 5.8B), although the reduction was more pronounced in the case of miR-8-3-p when BgAgo1 is depleted. This suggests that miR-8-3-p preferentially loads with Ago1 in the RISC. Conversely, reduction of miR-8-5p levels is similar in Ago1- and in Ago2-depleted specimens, which suggests that this miRNA loads either in Ago1 or in Ago-2-containing RISC.

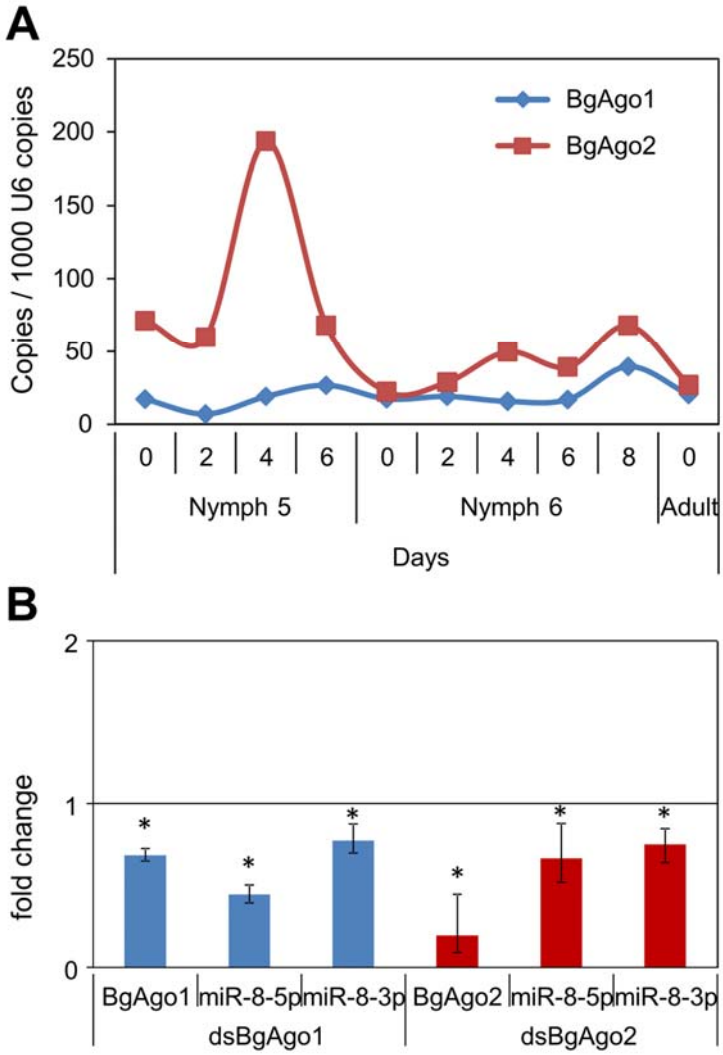

Figure 5.8. Expression of Ago1 and Ago2 mRNAs in *Blattella germanica* females. (A) Expression patterns of Ago1 and Ago2 in fifth and sixth nymphal instars and in freshly emerged adults. Data are expressed as mRNA copies per 1000 copies of U6. **(B)** Effects of RNAi of BgAgo1 and BgAgo2 (carried out in freshly emerged fifth instar nymph and evaluated in freshly emerged sixth instar nymph) on miR-8-5p and miR-8-3p expression; levels of BgAgo1 and BgAgo2 mRNAs were measured to assess that the corresponding transcripts were effectively depleted. Data represent 3 biological replicates and are normalized against the control females (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool (Pfaffl et al, 2002).

f) Regulation of BgAtro by miR-8-5p and miR-8-3p

The whole results obtained suggest a scenario where miR-8-3p and miR-8-5p are differentially sorted in the BgAgo1 and BgAgo2 respective RISC. In *D. melanogaster*, miR-8-3p is loaded in Ago1 RISC with 80% probability and miR-8-5p in Ago2 RISC with 60% probability (Czech et al, 2009). This Ago sorting depends on complex mechanisms, because each Ago protein has a different catalytic activity being the relative catalytic constant of Ago2 43 fold higher than that of Ago1. In principle, thus, miRNAs bound to Ago2 might degrade 43 times more atro mRNA than miRNAs bound to Ago1 (Forstemann et al, 2007).

Moreover, the number of putative MREs in the 3'-UTR of BgAtro transcript and the binding energy of the miRNA-mRNA duplex, determine the binding equilibrium between both miR-8 strands in competitiveness behaviour. In addition, it must be taken into account that there is a differential binding efficiency of miR-8-5p and of miR-8-3p for their corresponding sites, and that overlapping miR-8-5p and miR-8-3p sites make incompatible the simultaneous binding of both miRNAs in these sites.

Figure 5.9. Schematic representation of the proposed mechanisms of regulation of BgAtro by miR-8-5p and miR-8-3p. The model is based on: 1) the differential loading of miR-8-5p and miR-3p with BgAgo and BgAgo2; 2) that at equal concentrations of BgAgo1 and BgAgo2, BgAgo2 is more efficient in degrading atro mRNA, and 3) that there is a competition between miR-8-3p and miR-8-5p for the respective overlapping binding sites in atro mRNA.

Figure 5.9 shows a schematic representation on the proposed model of interaction of miR-3-p, miR-5p, BgAgo1, BgAgo2 and BgAtro mRNA, based on the following premises: The expression of BgAtro and miR-8 precursor is approximately constant, the miRNAs are sorted differentially between BgAgo1 and BgAgo2, miR-8-3p binds preferentially to Ago1 whereas miR-8-5p binds preferentially to Ago2, BgAgo2 is more efficient in degrading its

target , there is a differential binding efficiency of miR-8-5p and of miR-8-3p for their corresponding sites, and overlapping miR-8-5p and miR-8-3p sites make incompatible the binding of both miRNAs in these sites simultaneously.

A pertinent next step would be to build a mathematical model based on the empirical observations obtained, including the dynamics of BgAtro mRNA, BgAgo1 mRNA, BgAgo2 mRNA, miR-8-3p and miR-8-5p observed in our expression studies, the results observed from the depletion treatments, the miR-8-3p and miR-8-5p sites predicted in the mRNA of atro, and taking into account the kinetics of the miRNA regulation determined by global cell properties. Work along this line is presently in progress in our laboratory. In any case, we have provided evidence, for the first time, of the cooperation between the two strands of the same pre-miRNA and differential Ago loading to achieve a superfinely tuned regulation of atro expression, not only by reducing the mRNA levels but by ensuring the optimal protein levels at every moment, which is imperative to achieve proper neuromotor coordination.

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SUPPLEMENTARY MATERIAL

Super-finely tuned regulation of Atrophin expression by miR-8-3p and miR-8-5p interplaying

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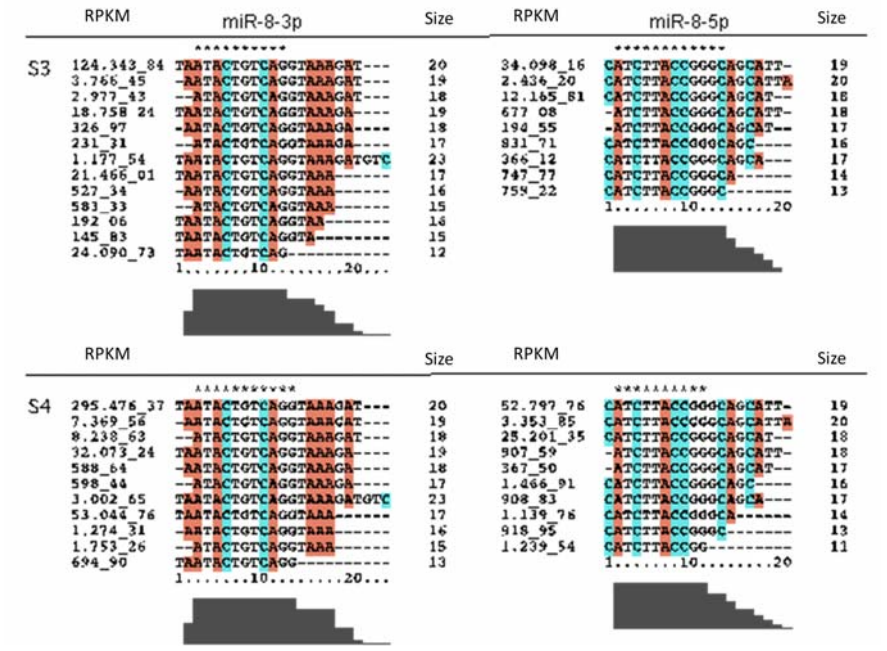
Additional file 1. Contents:



Supplementary Figure 5.S1. Alignment of the most represented miR-8-3p and miR-8-5p isoforms in both penultimate (S3) and last (S4) developmental stage of *B. germanica*. Headers show the reads per kilobase mapped (RPKM) values of each isomiR-8 according to the method by Mortazavi et al. (2008), and its size in nucleotides. Data from small RNA libraries by Rubio et al. (2012).

Supplementary Table 5.S1. Primers sequences used to obtain the mRNA sequence of BgAtro, BgAgo1 and BgAgo2.

transcript	primer name	primer sequence
BgAtro	AtroFwF	5'-ACGGAGTCAGAGTGCTATTTT-3'
	AtrophRv	5'-GCATGYGTATGNGCGTG-3'
	AtrophFw_2	5'-GAGTATGCKMGACCACATGC-3'
	BgAtroFw	5'-CAGCAGCTCAGCTAGAAGCA-3'
	BgAtroFw_2	5'-CCAGAGTACCATGCGCAC-3'
	BgAtroRv	5'-CAAGCTGACAGCAGAGTTCG-3'
BgAgo1	Ago1 Fw2	5'-ATGTCTCCCGTTGGGCAGC-3'
	Ago1Rv2	5'-ATGGTCTAACGGACTGATG-3'
	Ago1 Fw3	5'-GCCAGACTCTCACAACTTGT-3'
	Ago1 Rv3	5'-CACATGCAAGAACTGTCCTTC-3'
	Ago1 Fw4	5'-GAAGGACAGTTCTTGCATGTG-3'
	Ago1 Rv4	5'-GCTCTTGACCAACTTGAAGACA-3'
BgAgo2	Ago2 Fw	5'-GGCTACAATCAGAGCCCCTA-3'
	Ago2 Rv	5'-GCATAGTAACCCGTCCCCTA-3'
	Ago2 Fwcontig1	5'-CATTTCAGACCGCTGCTACA-3'
	Ago2 Rvcontig1	5'-GATGGGTCACATCAGCTCCT-3'
	Ago2 Fwcontig2	5'-TCAAAAATACCACCGGCTATG-3'
	Ago2 Rvcontig3	5'-AAACATGAAATAACGACTAAGGAGAAA-3'
	Ago2 5' Fw_1	5'-CACTCCCGGTGGACGTTCTC-3'
	Ago2 5’ Rv_1	5'-CTTCCATGGCATACCTGAGC-3'
	Ago2 Fw2	5'-GTCTCCAGCACCGGAAAAG-3'
	Ago2 Rv2	5'-ACAACTTTTTGTCCCAGCAAG-3'

Supplementary Table 5.S2. Primer sequences used for quantification of BgAtro, BgAgo1 and BgAgo2 by qRT-PCR.

transcript	primer name	primer sequence
BgAtro	RTAtro Fw	5'-GGTTACCTCCCCAGTGCATA-3'
	RTAtro Rv	5'-CCAAATGCTCCAATTCCAGT-3'
BgAgo1	RTAgo1 Fw	5'-CACTCACTGTGGGACCATGA-3'
	RTAgo1 Rv	5'-CTCCTCGTCACATTGCACAC-3'
BgAgo2	RTAgo2 Fw	5'-GCAAGGACCATGCAGAGAAT-3'
	RTAgo2 Rv	5'-AGACTGATGCGATGCAGTGA-3'

Supplementary Table 5.S3. Primer sequences used to prepare the dsRNA to deplete BgAtro (AtroFwF and AtroRvF), BgAgo1 (Ago1Fw and Ago1Rv) and BgAgo2 (Arg2_Fw_1 and Arg2_Rv_1) by RNAi.

dsRNAs	primer name	primer sequence
BgAtro	AtroFwF	5'-ACAGGAGTCAGAGTGCTATTTT-3'
	AtroRvF	5'-TATGGAGAAGTGATAGTTTCAATA-3'
BgAgo1	Ago1Fw	5'-CCTCTTCCCATAGGAAATGACA-3'
	Ago1Rv	5'-TCCATCAGGAGATGAGAAAAA-3'
BgAgo2	Arg2_Fw_1	5'-AAGGGCTGGCTACAATCAGA-3'
	Arg2_Rv_1	5'-AGCAGTCTTTGAACCGAGGA-3'

Supplementary Table 5.S4. Primer sequences used in the luciferase experiments to validate the predicted miR-8-3p and miR-8-5p sites in the atro mRNA.

primer name	primer sequence
Atroph-Fwd	5'-GGACTAGTATTAGACACTCGACTGCCATC-3'
Atroph-Rev	5'-CCGCTCGAGTGTCTTCTAGAACAGAAACTAAAG-3'
miR-8-Fwd	5'-GGAATTCAATGGAATACCGAATCTTGCT-3'
miR-8-Rev	5'-ATGCTCGAGTTGTCTTCGCATTATCCAC-3'

ANNEX 1

Nucleotide and protein partial sequence of BgAtro.

```
    ---------|---------|---------|---------|---------|---------|
  1 ACaggagtcagagtgctattttcttgcgccattggaaccgtggggacttcaattcctgta 60
  1   R  S  Q  S  A  I  F  L  R  H  W  N  R  G  D  F  N  S  C  T 20
    ---------|---------|---------|---------|---------|---------|
 61 ctcgtacagatctaactttcaaacctgtgcctgactccaagttggcacgtaaacgagaag 120
 21   R  T  D  L  T  F  K  P  V  P  D  S  K  L  A  R  K  R  E  E 40
    ---------|---------|---------|---------|---------|---------|
121 aaagattgcgcaagcaagctgagaaggaaagggaagacagagaaaaggcagcagctcaag 180
 41   R  L  R  K  Q  A  E  K  E  R  E  D  R  E  K  A  A  A  Q  A 60
    ---------|---------|---------|---------|---------|---------|
181 cacaagcgcgtaaaattgccactccagaaaaaccagaaggtccaaagccaccttcaagag 240
 61   Q  A  R  K  I  A  T  P  E  K  P  E  G  P  K  P  P  S  R  G 80
    ---------|---------|---------|---------|---------|---------|
241 gtcctattgaaactattacttctccatatgatcgcttcacccctcgtcaagggtatccag 300
 81   P  I  E  T  I  T  S  P  Y  D  R  F  T  P  R  Q  G  Y  P  D 100
    ---------|---------|---------|---------|---------|---------|
301 acacgccagcattacgtcaacttagtgaatatgcacgtccacacgcaggattctcaccag 360
101   T  P  A  L  R  Q  L  S  E  Y  A  R  P  H  A  G  F  S  P  G 120
    ---------|---------|---------|---------|---------|---------|
361 gtacactacctcgatcggctggtttagggttacctccccagtgcatagatcctatgcttc 420
121   T  L  P  R  S  A  G  L  G  L  P  P  Q  C  I  D  P  M  L  H 140
    ---------|---------|---------|---------|---------|---------|
421 actaccagttaaacagcatgtatggacccagcgcaagggaaagactggaattggagcatt 480
141   Y  Q  L  N  S  M  Y  G  P  S  A  R  E  R  L  E  L  E  H  L 160
    ---------|---------|---------|---------|---------|---------|
481 tggagcgtgagaagcgagaacgtgaaattcgtgagctgcgtgaacgtgaactaagcgacc 540
161   E  R  E  K  R  E  R  E  I  R  E  L  R  E  R  E  L  S  D  R 180
    ---------|---------|---------|---------|---------|---------|
541 gtttgaaggacgagttgatgaagaatgctgcaagtgctgggccacgaatgcccaatcctc 600
181   L  K  D  E  L  M  K  N  A  A  S  A  G  P  R  M  P  N  P  L 200
    ---------|---------|---------|---------|---------|---------|
601 tggatccgcattggcttgaattacacaggagatactcaacgttgggaccaggtggtccac 660
201   D  P  H  W  L  E  L  H  R  R  Y  S  T  L  G  P  G  G  P  P 220
    ---------|---------|---------|---------|---------|---------|
661 cataaggagctgctgcactkmatcmgykkgggckgcacscgtctmswrctagtggmyyss 720
  1   *  G  A  A  A  L  N  Q  R  G  R  H  P  S  T  T  S  G  P  P 19
    ---------|---------|---------|---------|---------|---------|
721 ycrggctgcastgartyaackgsacgagggaacggttggagagactcggaattcctactg 780
 20   R  L  H  *  I  N  G  T  R  E  R  L  E  R  L  G  I  P  T  G 16
    ---------|---------|---------|---------|---------|---------|
781 gacctggtggtcctcatggtccccctccagtaggccctcctggtccacatgctcaccctc 840
 17   P  G  G  P  H  G  P  P  P  V  G  P  P  G  P  H  A  H  P  H 36
    ---------|---------|---------|---------|---------|---------|
841 accctggatccgtagcagcagctcagctagaagcagccgaraggttggctctggcaacgg 900
 37   P  G  S  V  A  A  A  Q  L  E  A  A  E  R  L  A  L  A  T  D 56
    ---------|---------|---------|---------|---------|---------|
901 accctatggtrcggctgcaaatggccgggatctctccagagtaccatgcgcatacacacg 960
 57   P  M  V  R  L  Q  M  A  G  I  S  P  E  Y  H  A  H  T  H  A 76
    ---------|---------|---------|---------|---------|---------|
961 cacatacgcatgcacattctcatacacacctccacctgcatcccagtcagcaggcccagc 1020
 77   H  T  H  A  H  S  H  T  H  L  H  L  H  P  S  Q  Q  A  Q  Q 96
    ---------|---------|---------|---------|---------|---------|
1021 aagcccaagcacagcaggaagctgctgcagctgcttcaggctctgctggtttcccactgc 1080
 97   A  Q  A  Q  Q  E  A  A  A  A  A  S  G  S  A  G  F  P  L  P 116
    ---------|---------|---------|---------|---------|---------|
1081 cagcttcggccaccactggatacccacggccaagcttattgccccccagagaaggtcctc 1140
 117   A  S  A  T  T  G  Y  P  R  P  S  L  L  P  P  R  E  G  P  L 136
    ---------|---------|---------|---------|---------|---------|
1141 ttggacttcaccacccatcagacattctcggacgtggtggacctggatatgcagatcagc 1200
 137   G  L  H  H  P  S  D  I  L  G  R  G  G  P  G  Y  A  D  Q  L 156
    ---------|---------|---------|---------|---------|---------|
1201 tagcacatcagctttctgcacatgctgcggcccaagaacagttacagcgtcagatgctgt 1260
```

```
 157   A  H  Q  L  S  A  H  A  A  A  Q  E  Q  L  Q  R  Q  M  L  L 176
     ---------|---------|---------|---------|---------|---------|
1261 taragcgtgaarggtttccacatccacatccctccatagtggcccaacatgaggagtata 1320
 177   K  R  E  R  F  P  H  P  H  P  S  I  V  A  Q  H  E  E  Y  I 196
     ---------|---------|---------|---------|---------|---------|
1321 tcagacaacaacgcgagcgtgaaatgaaagttcgggcactagaggaggctgcaaggggat 1380
 197   R  Q  Q  R  E  R  E  M  K  V  R  A  L  E  E  A  A  R  G  S 216
     ---------|---------|---------|---------|---------|---------|
1381 ctcgacctTGAATTAGACACTCGACTGCCATCCTGGTGTTACCAGGAACGCAATCTTCAA 1440
 217   R  P                                                       218
     ---------|---------|---------|---------|---------|---------|
1441 TTTTGTAATCTCTGTGTTGCGTTATAATGTAGTAATATGATTTAGTACAATTTTTTAGTA 1500
     ---------|---------|---------|---------|---------|---------|
1501 CAGAGGGAAAAAAACAAAATTATTAATATAATTATTATATAATCTTAAATGTAACTTTGA 1560
     ---------|---------|---------|---------|---------|---------|
1561 AAAACTAAAARAAGATAGTGGAGAATAAAAATATTTTGTGACATTTGGTAGCAACACGGG 1620
     ---------|---------|---------|---------|---------|---------|
1621 TACTGGGTGTGCACATGTATTTGTACATCTGTAACCTCTCTAAATGTTGTTTGCGTAGTC 1680
     ---------|---------|---------|---------|---------|---------|
1681 TTCCGTGCACATCTTTCCTTCTTTTTGTCAGCTTGTGAAGGTGGAATGCATTGTATTGGT 1740
     ---------|---------|---------|---------|---------|---------|
1741 TCTGTGATAGATAAGCAAGTAATGCTTTAGTTTCTGTTCTAGAAGACATAGAAAAATTTT 1800
     ---------|---------|---
1801 AAGAAAACAAAAAAAAAAAAAAA 1823
```

ANNEX 2

Nucleotide and protein sequence of BgAgo1. PAZ and PIWI domains are highlighted in grey and blue, respectively.

```
    ---------|---------|---------|---------|---------|---------|
  1 atgtttcccgttgggcagcctcctcctcctggacccccctggtggtccaccaggacctgtg 60
  1 M  F  P  V  G  Q  P  P  P  P  G  P  P  G  G  P  P  G  P  V   20
    ---------|---------|---------|---------|---------|---------|
 61 ggcccaccaggtgctgttgctgtgccacctggtgccatgggcctggtgccccctcagcaa 120
 21 G  P  P  G  A  V  A  V  P  P  G  A  M  G  L  V  P  P  Q  Q   40
    ---------|---------|---------|---------|---------|---------|
121 ccacatcagccgcctcaaccgccagagttaccaatgttcaactgcccaagaagaccaaac 180
 41 P  H  Q  P  P  Q  P  P  E  L  P  M  F  N  C  P  R  R  P  N   60
    ---------|---------|---------|---------|---------|---------|
181 ttgggtcgggaaggccgaccaatagttttaagagcaaatcacttccagatatcaatgcca 240
 61 L  G  R  E  G  R  P  I  V  L  R  A  N  H  F  Q  I  S  M  P   80
    ---------|---------|---------|---------|---------|---------|
241 cgggggtttgtccatcattatgacatcaacattcagcctgacaaatgtcctcgaaaggtg 300
 81 R  G  F  V  H  H  Y  D  I  N  I  Q  P  D  K  C  P  R  K  V   100
    ---------|---------|---------|---------|---------|---------|
301 aacagagaaatcatcgagacaatggttcatgcttacagcaaaatcttcggaactctgaaa 360
101 N  R  E  I  I  E  T  M  V  H  A  Y  S  K  I  F  G  T  L  K   120
    ---------|---------|---------|---------|---------|---------|
361 cctgtgttcgatgggcggaataatttatacacgagagatcctcttcccataggaaatgac 420
121 P  V  F  D  G  R  N  N  L  Y  T  R  D  P  L  P  I  G  N  D   140
    ---------|---------|---------|---------|---------|---------|
421 agagtcgaattggaggtcacattacctggggaaggcaaggatcgagttttcagagtaaca 480
141 R  V  E  L  E  V  T  L  P  G  E  G  K  D  R  V  F  R  V  T   160
    ---------|---------|---------|---------|---------|---------|
481 ataaagtgggtggcgcaggtgtcgttgtttgccttagaggaagctcttgagggaagaacg 540
161 I  K  W  V  A  Q  V  S  L  F  A  L  E  E  A  L  E  G  R  T   180
    ---------|---------|---------|---------|---------|---------|
541 aggcaaattccatatgatgcaattctggccttggatgttgtaatgaggcatttaccatca 600
181 R  Q  I  P  Y  D  A  I  L  A  L  D  V  V  M  R  H  L  P  S   200
    ---------|---------|---------|---------|---------|---------|
601 atgacatacactccggtagggaggtctttttttctcatctcctgatggatattaccatccg 660
201 M  T  Y  T  P  V  G  R  S  F  F  S  S  P  D  G  Y  Y  H  P   220
    ---------|---------|---------|---------|---------|---------|
661 ttgggaggaggaagagaggtgtggtttggtttccatcagtccgttagaccatctcagtgt 720
221 L  G  G  G  R  E  V  W  F  G  F  H  Q  S  V  R  P  S  Q  C   240
    ---------|---------|---------|---------|---------|---------|
721 aaaatgatgctcaatatcgatgtttctgctactgctttctacaaggcgcagccagtgatc 780
241 K  M  M  L  N  I  D  V  S  A  T  A  F  Y  K  A  Q  P  V  I   260
    ---------|---------|---------|---------|---------|---------|
781 gaattcatgtgcgaagttctggatattagggacatcaatgagcagcgcaaaccgctgaca 840
261 E  F  M  C  E  V  L  D  I  R  D  I  N  E  Q  R  K  P  L  T   280
    ---------|---------|---------|---------|---------|---------|
841 gactcgcaaagagtaaaattcaccaaagagatcaaaggtcttaagatcgagatcactcac 900
281 D  S  Q  R  V  K  F  T  K  E  I  K  G  L  K  I  E  I  T  H   300
    ---------|---------|---------|---------|---------|---------|
901 tgtgggaccatgagacgcaagtacagggtgtgcaatgtgacgaggagaccggcacagatg 960
301 C  G  T  M  R  R  K  Y  R  V  C  N  V  T  R  R  P  A  Q  M   320
    ---------|---------|---------|---------|---------|---------|
961 cagtccttcccgctgcagctagagagtggtcaaacagtggagtgtacggtagcaaaatat 1020
321 Q  S  F  P  L  Q  L  E  S  G  Q  T  V  E  C  T  V  A  K  Y   340
    ---------|---------|---------|---------|---------|---------|
1021 tttctagacaagtacaaaatgaagctccgttacccgcacctgccctgcctccaagtggga 1080
341 F  L  D  K  Y  K  M  K  L  R  Y  P  H  L  P  C  L  Q  V  G   360
    ---------|---------|---------|---------|---------|---------|
1081 caagagcacaaacatacatatctgcctctcgaggtttgtaatattgtggctggtcagagg 1140
361 Q  E  H  K  H  T  Y  L  P  L  E  V  C  N  I  V  A  G  Q  R   380
```

```
     ---------|---------|---------|---------|---------|---------|
1141 tgtatcaagaaattgacagatatgcagacctcgacaatgattaargccactgctcgctct 1200
 381 C  I  K  K  L  T  D  M  Q  T  S  T  M  I  K  A  T  A  R  S   400
     ---------|---------|---------|---------|---------|---------|
1201 gcccccgatcgagaaagagagatcaacaacttagtgaggcgagccgacttcaataacgat 1260
 401 A  P  D  R  E  R  E  I  N  N  L  V  R  R  A  D  F  N  N  D   420
     ---------|---------|---------|---------|---------|---------|
1261 gcttatgtgcaagagtttggcctcaccatcagcaacaatatgatggaagtgcggggacgc 1320
 421 A  Y  V  Q  E  F  G  L  T  I  S  N  N  M  M  E  V  R  G  R   440
     ---------|---------|---------|---------|---------|---------|
1321 gtcctacccccacccaaactacagtacggtggcaggattagttcgctcagcggacagacg 1380
 441 V  L  P  P  P  K  L  Q  Y  G  G  R  I  S  S  L  S  G  Q  T   460
     ---------|---------|---------|---------|---------|---------|
1381 aagcagcaggccatgccaaaccaaggtgtttgggacatgaggggcaagcaattcttcaca 1440
 461 K  Q  Q  A  M  P  N  Q  G  V  W  D  M  R  G  K  Q  F  F  T   480
     ---------|---------|---------|---------|---------|---------|
1441 ggcgtggagatcagagtgagggccatcgcatgcttcgctcctcagaggacagtccgagag 1500
 481 G  V  E  I  R  V  R  A  I  A  C  F  A  P  Q  R  T  V  R  E   500
     ---------|---------|---------|---------|---------|---------|
1501 gacgctcttaggaactccacacaacaactgcagaagattagtaatgatgcgggaatgccc 1560
 501 D  A  L  R  N  S  T  Q  Q  L  Q  K  I  S  N  D  A  G  M  P   520
     ---------|---------|---------|---------|---------|---------|
1561 ataattggacagccatgtttttgcaaatatgccacaggtcctgaccaggtggaacccatg 1620
 521 I  I  G  Q  P  C  F  C  K  Y  A  T  G  P  D  Q  V  E  P  M   540
     ---------|---------|---------|---------|---------|---------|
1621 tttcgctacctcaagtcttcattcgcagctctccaacttgtagtcgtcgtcttgcccgga 1680
 541 F  R  Y  L  K  S  S  F  A  A  L  Q  L  V  V  V  V  L  P  G   560
     ---------|---------|---------|---------|---------|---------|
1681 aaaacacccgtatatgcggaagtgaagagggtgggtgacacagtgctggggatggccaca 1740
 561 K  T  P  V  Y  A  E  V  K  R  V  G  D  T  V  L  G  M  A  T   580
     ---------|---------|---------|---------|---------|---------|
1741 caatgcgttcaagcgaagaacgtcaataagacatcgccgcagacgctgtccaacctctgt 1800
 581 Q  C  V  Q  A  K  N  V  N  K  T  S  P  Q  T  L  S  N  L  C   600
     ---------|---------|---------|---------|---------|---------|
1801 cttaagatcaacgtgaagctgggaggaatcaacagtatattagtgccaagtattaggccc 1860
 601 L  K  I  N  V  K  L  G  G  I  N  S  I  L  V  P  S  I  R  P   620
     ---------|---------|---------|---------|---------|---------|
1861 aaagtgttcaatgagcctgtgatattccttggagcggacgtgacccatccgcctgctggc 1920
 621 K  V  F  N  E  P  V  I  F  L  G  A  D  V  T  H  P  P  A  G   640
     ---------|---------|---------|---------|---------|---------|
1921 gataataagaaaccttcaatagcagcagtcgtcggatcaatggacgctcatccgtcgcgc 1980
 641 D  N  K  K  P  S  I  A  A  V  V  G  S  M  D  A  H  P  S  R   660
     ---------|---------|---------|---------|---------|---------|
1981 tatgctgccaccgtgcgggtgcaacagcatcggcaggaaatcattcaggagttgagtagt 2040
 661 Y  A  A  T  V  R  V  Q  Q  H  R  Q  E  I  I  Q  E  L  S  S   680
     ---------|---------|---------|---------|---------|---------|
2041 atggccagggagcttctgatcatgttctacaaaagtacgggaggctacaaaccgcatcga 2100
 681 M  A  R  E  L  L  I  M  F  Y  K  S  T  G  G  Y  K  P  H  R   700
     ---------|---------|---------|---------|---------|---------|
2101 atcattttgtacagggatggagtgtctgaaggacagttcttgcatgtgttgcagcatgaa 2160
 701 I  I  L  Y  R  D  G  V  S  E  G  Q  F  L  H  V  L  Q  H  E   720
     ---------|---------|---------|---------|---------|---------|
2161 ctgacggccataagagaagcgtgtatcaagttggaaggtgactacaaacctggtattaca 2220
 721 L  T  A  I  R  E  A  C  I  K  L  E  G  D  Y  K  P  G  I  T   740
     ---------|---------|---------|---------|---------|---------|
2221 ttcatcgtggttcaaaaaaggcatcacacaagattgttctgtgccgacaagaaagagcag 2280
 741 F  I  V  V  Q  K  R  H  H  T  R  L  F  C  A  D  K  K  E  Q   760
     ---------|---------|---------|---------|---------|---------|
2281 tctggtaaatctgggaacatccctgcagggacgacagtcgatgtgggcatcacgcatccc 2340
 761 S  G  K  S  G  N  I  P  A  G  T  T  V  D  V  G  I  T  H  P   780
     ---------|---------|---------|---------|---------|---------|
2341 acagagttcgacttctacttatgcagtcatcggggtattcagggcacgagtcgacccagt 2400
 781 T  E  F  D  F  Y  L  C  S  H  R  G  I  Q  G  T  S  R  P  S   800
     ---------|---------|---------|---------|---------|---------|
2401 cactaccacgtgttatgggatgacaatcactttgattccgatgagctgcagtgtctcacg 2460
 801 H  Y  H  V  L  W  D  D  N  H  F  D  S  D  E  L  Q  C  L  T   820
     ---------|---------|---------|---------|---------|---------|
2461 taccagctttgccacacctacgtgaggtgcacacgctccgtgtccattcctgcgcctgcg 2520
 821 Y  Q  L  C  H  T  Y  V  R  C  T  R  S  V  S  I  P  A  P  A   840
```

```
     ---------|---------|---------|---------|---------|---------|
2521 tactacgcccacctggtcgcgttccgggcgcggtatcatttagtcgagaaggaacatgtc 2580
 841 Y  Y  A  H  L  V  A  F  R  A  R  Y  H  L  V  E  K  E  H  V   860
     ---------|---------|---------|---------|---------|---------|
2581 agtggtgaaggttctcaccagtcgggatgtagtgaggatcggacgccaggtgcaatggca 2640
 861 S  G  E  G  S  H  Q  S  G  C  S  E  D  R  T  P  G  A  M  A   880
     ---------|---------|---------|---------|---------|---------|
2641 cgtgcgataacggttcatgcagacacaaagaaggttatgtacttcgctTGAACTGTGTTC 2700
 881 R  A  I  T  V  H  A  D  T  K  K  V  M  Y  F  A              896
     ---------|---------|---------|---------|---------|---------|
2701 GCAAGAAAGAAAGTGTCTCGCCAAAAGGTCGTCGAATTTGTGTCGGTGATAAAATTTTCA 2760

     ---------|---------|---------|---------|---------|---------|
2761 CAGACAGTGACACAACGATAGTGGACAATTTTTATTTGCTTTATAGATCACTAGCCGGGG 2820

     ---------|----
2821 TAAAAAAAAAAAAA 2834
```

ANNEX 3

Nucleotide and protein sequence of BgAgo2. PAZ and PIWI domains are highlighted in grey and blue, respectively.

```
    ---------|---------|---------|---------|---------|---------|
   1 Taaacaaaacactctcccagcaccggaacagccacaagcgcctcaacagtcggcttggca 60
   1  K  Q  N  T  L  P  A  P  E  Q  P  Q  A  P  Q  Q  S  A  W  Q  20
    ---------|---------|---------|---------|---------|---------|
  61 aagaggaccacaaaagcaaccgtctccagcaccggaacagccacaagcgcctcaacagtc 120
  21  R  G  P  Q  K  Q  P  S  P  A  P  E  Q  P  Q  A  P  Q  Q  S  40
    ---------|---------|---------|---------|---------|---------|
 121 gggttcgcggaagggacctcaacaacaatttcctgcaggtgcaggtcgtggtaggggatt 180
  41  G  S  R  K  G  P  Q  Q  Q  F  P  A  G  A  G  R  G  R  G  F  60
    ---------|---------|---------|---------|---------|---------|
 181 tggggcacctgaacagcctcaacaaccgacacaacctgcaggtttcggtcgtggtagagg 240
  61  G  A  P  E  Q  P  Q  Q  P  T  Q  P  A  G  F  G  R  G  R  G  80
    ---------|---------|---------|---------|---------|---------|
 241 agcagaagcaccacagggaagacccagtgctccgcctccaggatttgcacaggtgccgcc 300
  81  A  E  A  P  Q  G  R  P  S  A  P  P  P  G  F  A  Q  V  P  P  100
    ---------|---------|---------|---------|---------|---------|
 301 cggcaaaccgtccgagccagaaggtgcagctgcaagaccggcagttgttcagcagatacc 360
 101  G  K  P  S  E  P  E  G  A  A  A  R  P  A  V  V  Q  Q  I  P  120
    ---------|---------|---------|---------|---------|---------|
 361 ggcagttgttcagcagagaccggcggttccagttcagaaaactttgacgaaatcgaccgg 420
 121  A  V  V  Q  Q  R  P  A  V  P  V  Q  K  T  L  T  K  S  T  G  140
    ---------|---------|---------|---------|---------|---------|
 421 cgctctgcaaatcccttcgagaaaaggcgtaggcaccttggggcgtaaaataacgctcga 480
 141  A  L  Q  I  P  S  R  K  G  V  G  T  L  G  R  K  I  T  L  D  160
    ---------|---------|---------|---------|---------|---------|
 481 cacaaatcacctggcattagacctgactaggctgcgcaacagatatgtggagcactatga 540
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    ---------|---------|---------|---------|---------|---------|
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    ---------|---------|---------|---------|---------|---------|
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 201  R  K  H  Y  S  N  R  F  P  A  F  D  G  R  K  N  L  F  S  S  220
    ---------|---------|---------|---------|---------|---------|
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 221  G  K  L  P  F  G  R  E  L  H  D  T  V  S  V  S  E  E  D  S  240
    ---------|---------|---------|---------|---------|---------|
 721 cagaccaaaagagttcaaggtctccatccagcacgtggcacagattgacatggacgatct 780
 241  R  P  K  E  F  K  V  S  I  Q  H  V  A  Q  I  D  M  D  D  L  260
    ---------|---------|---------|---------|---------|---------|
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    ---------|---------|---------|---------|---------|---------|
 841 ggacgtcgttctgcgggctgcgaccaccttcaggttcatccccgtgggacgttctctgtt 900
 281  D  V  V  L  R  A  A  T  T  F  R  F  I  P  V  G  R  S  L  F  300
    ---------|---------|---------|---------|---------|---------|
 901 ctacccgcctgaaggacgtgtcgttactctgggggatggcacagagttatggcatggctt 960
 301  Y  P  P  E  G  R  V  V  T  L  G  D  G  T  E  L  W  H  G  F  320
    ---------|---------|---------|---------|---------|---------|
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 321  F  Q  S  A  T  I  G  W  K  P  F  L  N  V  D  V  A  H  K  G  340
    ---------|---------|---------|---------|---------|---------|
1021 tttcccaacagaacagaatgtgatagacacaatttatgatgtttgcaatatgcggggcga 1080
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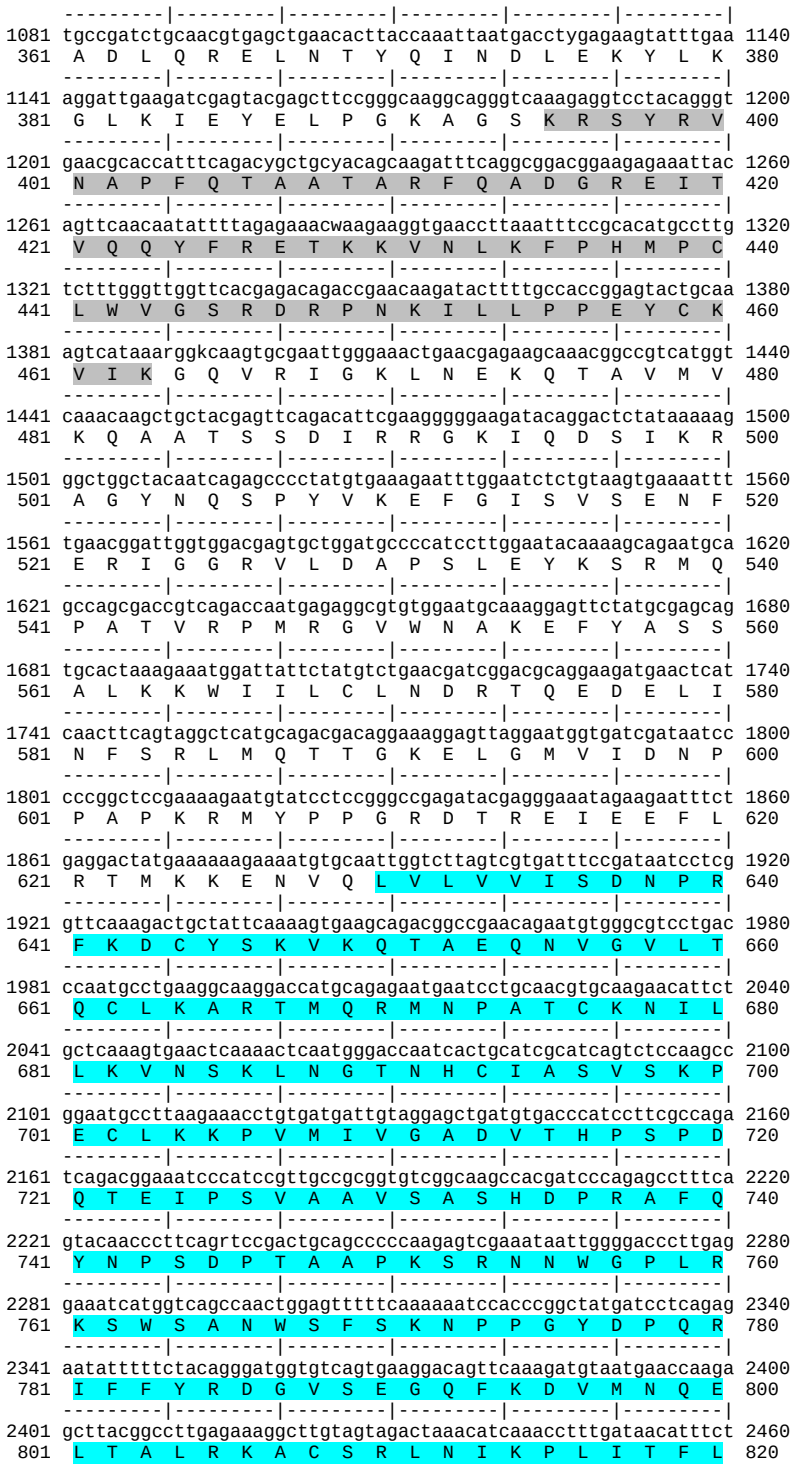

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     ---------|---------|---------|---------|---------|---------|
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     -
3361 A 3361
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General discussion

6

Cells contain a variety of non-coding RNAs (ncRNAs), including components of the machinery of gene expression, such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) and regulatory RNAs, that influence the expression of other genes. However, almost all means of gene identification assume that genes encode proteins so, even in the era of complete genome sequences, ncRNA genes have been effectively invisible.

Lately, it has become increasingly apparent that noncoding RNAs are impressively diverse, and that a significant fraction of the genes of all organisms do not encode proteins. One class of small noncoding RNAs, the microRNAs (miRNAs), has recently been recognized to be numerous and phylogenetically extensive (Lau et al, 2001; Lee & Ambros, 2001). miRNA genes produce tiny transcripts of about 22 nucleotides (~22 nt) in length that function as antisense regulators of other RNAs. They were first described in 1993 by Lee and colleagues (Lee et al, 1993), and the term microRNA was coined in 2001 (Ruvkun, 2001). Thousands of miRNAs have since been identified in various organisms through random cloning and sequencing or computational prediction. The miRBase (Griffiths-Jones, 2004), hosted by the Sanger Institute, provides miRNA nomenclature, sequence data, annotation and target prediction information.

A number of works have shown the relationships between miRNAs and metamorphic process. Some of them have related miRNA expression with the main metamorphic hormones (Chawla & Sokol, 2012; Sempere et al, 2002; Sempere et al, 2003), others showed their temporal regulation patterns before metamorphosis

(Bashirullah et al, 2003; Liu et al, 2007), or their implication by targeting genes involved in insect development (Biryukova et al, 2009; Caygill & Johnston, 2008; Ronshaugen et al, 2005; Sokol et al, 2008). Most of these reports refer to holometabolous insects, but the most dramatic demonstration that miRNAs are involved in insect metamorphosis was obtained in a hemimetabolous insect. Gomez-Orte & Belles (2009), silenced dicer-1 expression by RNAi in the last nymphal instar of the cockroach *Blattella germanica*, and obtained supernumerary nymphs after the following molt, instead of adults (Gomez-Orte & Belles, 2009). This contribution, however, did not precise which particular miRNAs were involved in the process.

Following this research line the man objective of the present thesis was to identify and to study miRNAs involved in regulating insect hemimetabolous metamorphosis, using the cockroach *B. germanica* as model.

The first step towards this goal has been to obtain a catalogue of miRNAs in this species. Given that the genome of *B. germanica* has not been sequenced, we approached this objective by obtaining miRNA libraries at selected stages. The work was carried out in the context of the general needs of the laboratory, and two libraries of small RNAs were prepared in cooperation with other members of the group: one from adult ovaries in the first gonadotrophic cycle (Ov-A), and the other from the whole body of sixth instar nymph females (WB-6) (the last and, thus the metamorphic instar). The latter was the most relevant for our purposes.

Obtaining a miRNA catalogue of *Blattella germanica*

As stated above, libraries of small RNAs of the cockroach *B. germanica* were built from the whole body of the last instar nymph, and the adult ovaries. The high throughput Solexa sequencing resulted in 10,824,998 reads (2,526,942 unique sequences) from the WB-6 library, and 8,190,720 reads (2,190,885 unique sequences) from the Ov-A. The sequence length distribution in WB-6 and Ov-A indicated that both libraries were enriched with small RNAs of 21–23 nt, which is the standard size of miRNAs. Of note, a peak at 22-nt size had been also observed in small RNA libraries of *Locusta migratoria* (Wei et al, 2009), *Aedes albopictus* and *Culex quinquefasciatus* (Skalsky et al, 2010).

Bioinformatic analyses identified 38 known miRNAs as well as 11 known miRNAs passenger strands. Some of the known miRNAs had been found as the most abundant in other insect species, such as miR-1, miR-275, miR-276 and miR-8 in *L. migratoria* (Wei et al, 2009); and miR-1, miR-8, miR-276a and miR-263a in *Bombyx mori* (Jagadeeswaran et al, 2010). In the mosquitoes *A. albopictus* and *C. quinquefasciatus*, the most highly expressed miRNA is miR-184, and these two species shared three out of ten most abundant miRNAs in *B. germanica* libraries: miR-184, miR-275, and miR-8 (Skalsky et al, 2010).

Various miRNA passenger strand sequences have been detected in high amounts in other small RNA libraries (Cai et al, 2010; Jagadeeswaran et al, 2010; Wei et al, 2009). Moreover, a

number of miRNA passenger strands detected in *B. germanica* libraries are also present in those of *L. migratoria* (miR-281-5p, miR-10-5p , miR-8-5p) (Wei et al, 2009) and *B. mori* (miR-10-5p, miR-281-5p) (Cai et al, 2010; Jagadeeswaran et al, 2010). The high number of miRNA passenger strand reads and the degree of conservation suggest that they play a miRNA function (Okamura et al, 2008; Stark et al, 2007).

We also found 70 miRNA candidates conserved in other insects that had been subjected to high throughput sequencing for small RNAs, such as *L. migratoria* (Wei et al, 2009), *Acyrthosiphon pisum* (Legeai et al, 2010), *B. mori* (Cai et al, 2010; Jagadeeswaran et al, 2010) and *C. quinquefasciatus* (Skalsky et al, 2010); these are herein called “conserved” miRNA candidates. However, most of the more abundant small RNA sequences obtained in our study are, by the moment, unique of *B. germanica* (170 sequences), and we called them “specific” miRNA candidates.

To validate miRNA candidates we used PCR and sequencing, as well as observing decreasing levels of their expression in dicer-1 RNAi knockdown specimens, which proved to be a reliable method for validation of novel miRNAs. Regarding the quantitative relationship between the number of Solexa reads and miRNA expression, the positive correlation between Solexa data and real-time PCR showed that number of reads can be used as a quantitative information, as was considered (but not demonstrated) in previous works (Jagadeeswaran et al, 2010).

Once built a comprehensive miRNA catalogue in *B. germanica*, the next step was to identify miRNAs differentially expressed in metamorphic and non-metamorphic transitions.

miRNAs that can be important in the metamorphic transition

B. germanica passes through six nymphal stages before the metamorphosis, the molts between nymph to nymph are morphologically conservative, whereas the molt from the last instar nymph (sixth, N6) to the adult is called the metamorphic molt. As in other insects, the transition between nymph to nymph is determined by 20-hydroxyecdysone (20E) plus juvenile hormone (JH), whereas the transition to the adult stage is determined by 20E alone.

In order to identify miRNAs differentially expressed in metamorphic and non-metamorphic transitions, we prepared miRNAs libraries of whole body in N5 and N6 around the peak of 20E, when the real molting process occurs. High throughput sequencing of these N5 and N6 libraries gave a total of 61 canonical miRNAs that were present in both, although in different proportions in each. In the previous work, the whole body miRNA library prepared during N6, but representing the eight days of the stage, we obtained a total of 49 canonical miRNAs (Cristino et al, 2011). Therefore, the number of miRNAs recovered using animals collected around the peak of 20E, is higher. This suggests that most miRNAs might be more efficiently expressed around the peak of 20E, and that there was a dilution effect in the former library.

Practically all canonical miRNAs reported earlier were present (except miR-1-5p and miR-184-5p), and were the most abundant in the N5 and N6 libraries. Some of the miRNAs (miR-8, miR-9a, miR-10, miR-71, miR-252, miR-276, miR-281) are represented by both strands, -3p and -5p, as was also observed in the previous, comprehensive library.

Comparison of both libraries led to the identification of three and 37 miRNAs significantly more expressed in N5 and N6 respectively. Twelve out of these 40 miRNAs were further investigated by qRT-PCR. Among the miRNAs selected to study the expression pattern, we included miR-252 and miR-276, which were represented by both, the -3p and -5p strands. In the case of miR-252, the -3p strand is expressed at much lower levels than those of the -5p strand, whereas in the case of miR-276, the situation is the reverse. These observations suggest that the expression is regulated differently in both strands, and that they play distinct miRNA functions (Belles et al, 2011). In support of this notion, Liu et al. (2010) reported that both strands of miR-276 are differentially expressed in different tissues and stages of *B. mori*, although miR-276-3p is always the more abundant strand.

Among the patterns examined, most of the expression peaks, either in N5 or N6, occur close to a peak of 20E, which suggests cause-effect relationships. Our experiments of hormone treatments indicated that 20E tend to stimulate miRNA expression, whereas JH inhibits the 20E stimulatory effect. Expression of let-7, miR-100 and miR-125 was increased by 20E, which had been also observed

in *Drosophila melanogaster* (Sempere et al, 2003). The only miRNA that was inhibited by 20E was miR-252-3p.

Conversely, the significant stimulatory effect of 20E on miR-1-3p and the tendency of 20E to stimulate miR-34-5p that we observed in *B. germanica* contrasts with the results obtained in *D. melanogaster*, where 20E did not affect miR-1-3p, and inhibited miR-34-5p (Sempere et al, 2003). We interpret these differences as reflections of different modes of regulation of these miRNAs in the two model species. The differences are also illustrated by the results obtained after JH treatment of *B. germanica*, which abolished the stimulatory effects of 20E on practically all miRNAs, which was also the case for miR-34-5p. This contrasts again with the observations of Sempere et al. (2003), which suggested that JH has a stimulatory effect on this miRNA in *D. melanogaster*.

One of the most interesting result of the experiments of hormonal treatments is that of miR-252-3p, which was the only miRNA inhibited by 20E alone. Moreover, JH did not seem to counteract the 20E effect, as compared to other miRNAs. Inhibition by 20E suggests that the decrease of miR-252-3p in N6, and the increase interrupted by a plateau around the 20E peak in N5, observed when establishing the expression patterns, might be due to the action of 20E. The other strand, classically considered the "mature" strand of miR-252, miR-252-5p, has been found in a number of insects (Griffiths-Jones, 2004), and its expression has been carefully studied in *B. mori*, where it shows a continuous high expression from the spinning larvae to pupal and adult stages (Liu et al, 2009). Conversely, miR-252-3p has never been studied, nor

has it been related with insect moulting and metamorphosis. It was for these reasons that we studied it functionally.

Depletion of miR-252-3p levels in the penultimate (N5) nymphal instar caused retarded growth and developmental delays within the instar. At the end, however, the treated insects were able to moult to N6 and to the adult normally. Observed delays in N5 reached as long as 14 days in some cases, and levels of miR-252-3p measured in specimens on N5D13, which were presumably going to moult within the next one or two days, had somewhat recovered compared with levels measured on N5D6, although they were still significantly lower than levels found in controls. Our data suggest that miR-252-3p regulates transcripts that are important for growth and development in N5, in the transition to the developmentally important N6 that precedes the imaginal molt. The fact that the treated specimens were finally able to molt to N6 and to the adult stage, even with reduced levels of miR-252-3p, suggests that these levels, although still quite low, are above the operative threshold for this miRNA.

The other three miRNAs that were highlighted in the differential expression analysis, let-7, miR-100 and miR-125, deserved an extensive study by several reasons. In a number of species, let-7, miR-100 and miR-125 cluster in the same primary transcript, as first revealed by Bashirullah et al. (2003) and Sempere et al. (2003), and later confirmed in this fly and other insects (Belles et al, 2011; Roush & Slack, 2008). Thus, the members of this miRNA cluster are expressed simultaneously, and studies in

holometabolan species, like *D. melanogaster* and *B. mori*, have shown that expression concentrates in pre-metamorphic stages. In *D. melanogaster*, expression starts in late third (last) instar larvae, around the peak of 20E that triggers puparium formation, and continues until the imaginal moult (Pasquinelli et al, 2000; Sempere et al, 2003). In *B. mori*, let-7 expression starts in the moult leading to the penultimate larval instar, and continues until the emergence of the adult (Liu et al, 2007).

let-7, miR-100 and miR-125 are involved in wing morphogenesis

We assessed that the precursors of let-7, miR-100 and miR-125 of *B. germanica* are conserved with respect to other studied species. Moreover, expression profiles indicated that these three miRNAs are present in the whole life cycle, from the first instar nymph until the adult stage, although maximal expression was found around the peak of 20E of N6.

Depletion of Broad-complex (BR-C) transcription factors, which are important components of the 20E pathway, triggered a decrease of the three miRNAs. These results were in agreement with equivalent observations carried out in *D. melanogaster* (Chawla & Sokol, 2012; Garbuzov & Tatar, 2010; Sempere et al, 2002). In the fly, 20E is necessary for the expression of let-7, miR-100 and miR-125, and the response appears mediated by Broad-complex (BR-C) transcription factors (Sempere et al, 2002).

Depletion of let-7 and miR-100 using specific antisense LNAs caused an adult phenotype showing the wings reduced in size (anti-miR-100) or with malformed vein patterning (anti-let-7 and anti-miR-100). Depletion of miR-125 induced no apparent effects. We considered that the gene blistered (bs) might be a good target of let-7 and miR-100, basically because decreasing levels of bs are necessary to reach the right proportion between wing veins and interveins in *D. melanogaster* (Montagne et al, 1996). In *B. germanica* wing buds, bs is down-regulated during the last instar nymph, in parallel to the up-regulation experienced by let-7 and miR-100, and depletion of these two miRNAs leads to an increase of bs transcript levels. Moreover, depletion of BR-C elicits a phenotype that is similar to that obtained after depleting let-7 and miR-100 levels. We concluded that let-7 and miR-100 expression is induced by BR-C, and that these miRNAs play a role in regulating the size and some subtle aspects of the vein-intervein patterning of *B. germanica* by modulating the decrease of bs mRNA levels.

BR-C has been demonstrated to be an important factor mediating wing morphogenesis by regulating miRNA expression, another factor that we identified as important in the ecdysis to the adult instar was atrophin (atro), a transcriptional co-repressor involved in nuclear receptor signaling, and also in neuromotor coordination in *D. melanogaster*. Moreover, studies carried out in this fly by Stephen Cohen and co-workers, had shown that atro mRNA levels are regulated by miR-8-3p (Karres et al, 2007). Interestingly, we had found in our miRNA libraries that miR-8-3p

(considered the mature miRNA of the miR-8 precursor) was abundant, but miR-8-5p (which should be considered the passenger strand of the miR-8 precursor) was even more abundant. With these antecedents, we wondered whether atro might be regulated post-transcriptionally by the interplay of miR-8-3p and miR-8-5p, which would represent a unique case of a transcript regulated by the two miRNA products of the same miRNA precursor.

miR-8-3p and miR-8-5p have a role in the imaginal ecdysis through modulating atrophin mRNA levels

In *D. melanogaster*, high levels of atro led to significant apoptosis in brain tissues, which appeared to impair motor coordination and provoked defects in the ecdysis (Karres et al, 2007). Moreover, mutations of atro caused leg patterning defects (Erkner et al, 2002; Wang et al, 2006) and ectopic vein formation in the wings (Charroux et al, 2006). In *B. germanica*, depletion of atro by RNAi techniques in the last instar nymph led to specimens showing problems of mobility, which made them much slower in the behaviour tests 24 h after the treatment. These individuals were able to metamorphose but exhibited the legs twisted and curved and the wings wrinkled, probably as a result of mechanical problems during the ecdysis. As we stated above, atro is regulated by miR-8-3p in *D. melanogaster*, and miR-8 precursor is well conserved among insects, not only the folding structure but also the sequences of both miRNA strands. The conservation of the miR-8-3p is a

common feature of the mature miRNAs, but the high conservation of the passenger strand, miR-8-5p, suggest not only that it is functional as a miRNA, but also that the function may be conserved.

Expression studies of miR-8-3p and miR-8-5p in the two last instars nymphs of *B. germanica*, showed that they have opposite patterns. When we studied atro mRNA expression, the most suggestive correlation observed was that atro expression is upregulated when the levels of miR-8-3p and miR-8-5p are close each other. Using LNA specific antimiRs against miR-8-3p and miR8-5p, we foundd that atro mRNA levels were affected by both treatments, thus suggesting that atro is regulated by both, miR-8-3p and miR8-5p. Moreover, target prediction algorithms led to identify miR-8-3p and miR-8-5p binding sites in the 3'UTR region of atro mRNA.

To confirm the predicted binding sites, we co-expressed in S2 cells the luciferase reporters with the 3'UTR of atro of *B. germanica*, with single strand RNA (ssRNA) oligonucleotides of miR-8-3p and miR-8-5p. Results indicated that both strands reduce luciferase activity (60%), thus suggesting that the sites predicted in the 3'UTR of atro mRNA are functional binding sites for miR-8-3p and miR-8-5p.

Another important element to take in account in this regulatory pathway, was the different loading of miR-8-3p and miR-8-5p in Ago1- and Ago2-containing RNA-induced silencing complexes (RISC). In insects, mature miRNA preferentially load with Ago1 into the RISC, but a number of miRNA from the

passenger strand can load with Ago2 (Czech et al, 2009; Ghildiyal et al, 2010; Okamura et al, 2009). In *B. germanica*, RNAi experiments of Ago1 and Ago2, measuring the resulting levels of miR-8-3p and miR-8-5p suggested that miR-8-3-p preferentially loads with Ago1 in the RISC and that miR-8-5p loads either in Ago1- or in Ago-2-containing RISC. We propose that the interplay of miR-8-3p and miR-8-5p maintain the appropriate expression levels of atro in *B. germanica* through differential interaction with Ago proteins and competition for the miRNA binding sites in the atro mRNA. This provides evidence, for the first time, of the cooperation between the two strands of the same pre-miRNA to achieve a superfinely tuned post-transcriptional regulation of a given transcript.

Concluding remarks

On the basis that miRNAs are crucial to properly complete metamorphosis in *B. germanica* (Gomez-Orte & Belles, 2009), we have identified a number of particular miRNAs involved in the process. However, we have been not able to recover the supernumerary nymph phenotype described in the RNAi experiments of dicer-1 (Gomez-Orte & Belles, 2009) by studying specific miRNAs. Rather, we have obtained subtle phenotypes related to delayed growth in premetamorphic stages (miR-252-3p), wing size and vein patterning (let-7, miR-100 and, indirectly, miR-125), and ecdysis behavior (miR-8-3p and miR-8-5p). This means

that other miRNAs, possibly having more dramatic effects on metamorphosis, wait to be discovered. We focused our work on canonical miRNAs found in our *B. germanica* libraries. But these libraries afforded also miRNA candidates (conserved in other species or specific to *B. germanica*) that were not considered here. The response to the above enigma possibly lies in these miRNA candidates.

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CONCLUSIONS 7

CONCLUSIONS

- The analysis of miRNA libraries obtained by high throughput Solexa sequencing using whole body extracts and ovarian tissue from the last instar nymph of *Blattella germanica* allowED us to identify an extensive catalogue of canonical miRNAs and novel miRNA candidates in this species. The results lead to conclude that the approach followed is suitable to obtain a comprehensive catalogue of miRNAs in a non-model species.

- The comparative analysis of a miRNA library of the penultimate nymphal instar (N5) with an equivalent library from the last instar nymph (N6) of *B. germanica* revealed three canonical miRNAs that are differentially expressed in N5, whereas 37 appeared to be more expressed in N6. These results suggest that a greater diversity of miRNAs are necessary for the metamorphic transition than for a nymphal to nymphal transition.

- Hormonal treatments and pattern expressions of 12 out of the 40 miRNA differentially expressed in N5 and N6 libraries indicated that 20E generally increase miRNA expression, whereas JH tends to inhibit them, except in the case of miR-252-3p which was the only miRNA inhibited by 20E. The whole results suggest that differential expression patterns observed in the metamorphic and non-metamorphic stages appear related to the differential levels of JH and 20E.

- Depletion of miR-252-3p in *B. germanica* caused growth and developmental delays, which suggests that this miRNA is involved in regulating these processes prior to metamorphosis.

- miRNA precursors of let-7, miR-100 and miR-125, the expression patterns of these miRNAs, and their dependence of Broad-complex and 20E for expression found in *B. germanica*, is similar to that reported for *Drosophila melanogaster*. This indicates that the regulation of these three miRNAs during insect development is conserved from cockroaches to flies.

- Depletion of let-7 and miR-100 triggers an increase of blistered mRNA levels and subtle defects in vein patterning in the hind wing. Given that blistered is involved in vein-intervein organization during wing formation in *D. melanogaster*, the above observations suggest that let-7 and miR-100 regulate the observed vein phenotype by modulating the mRNA levels of blistered.

- Expression pattern studies, luciferase assays, and depletion experiments suggest that the expression of Atrophin, which has a key role in neuromotor coordination, especially during the imaginal ecdysis, is tuned by miR-8-3p and miR-8-5p, mainly through differential loading with Argonaute 1 and Argonaute 2 in respective RNA-induced silencing complexes.