



Identification and profiling of stable microRNAs in hemolymph of young and old *Locusta migratoria* fifth instars

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ABSTRACT

Since the discovery of the first microRNA (miRNA) in the nematode *Caenorhabditis elegans*, numerous novel miRNAs have been identified which can regulate presumably every biological process in a wide range of metazoan species. In accordance, several insect miRNAs have been identified and functionally characterized. While regulatory RNA pathways are traditionally described at an intracellular level, studies reporting on the presence and potential role of extracellular (small) sRNAs have been emerging in the last decade, mainly in mammalian systems. Interestingly, evidence in several species indicates the functional transfer of extracellular RNAs between donor and recipient cells, illustrating RNA-based intercellular communication. In insects, however, reports on extracellular small RNAs are emerging but the number of detailed studies is still very limited. Here, we demonstrate the presence of stable sRNAs in the hemolymph of the migratory locust, *Locusta migratoria*. Moreover, the levels of several extracellular miRNAs (ex-miRNAs) present in locust hemolymph differed significantly between young and old fifth nymphal instars. In addition, we performed a 'proof of principle' experiment which suggested that extracellularly delivered miRNA molecules are capable of affecting the locusts' development.

Introduction

Non-coding small (s)RNAs, also referred to as regulatory sRNAs, are not translated into proteins but function by regulating gene expression at several levels. Based on their biogenesis, length, target complementarity, associated proteins and regulatory functions, several classes of non-coding sRNAs are generally described. One main class comprises the microRNAs (miRNAs), which post-transcriptionally regulate the expression of endogenous genes (Asgari, 2013; Siomi and Siomi, 2009).

In short, miRNAs arise from longer genomic transcripts that are further processed into 18 – 24 nt RNAs by specialized machinery, including Drosha and Dicer-1 in insects. Then, when integrated in an RNA-induced silencing complex (RISC), the miRNA binds to target transcripts based on complementary basepairing. At this point, Argonaute-1, the catalytic component of the RISC, induces post-transcriptional gene silencing via translational blockage or cleavage of the mRNA (Siomi and Siomi, 2009).

Since the discovery of the first miRNA in the nematode *Caenorhabditis elegans* (Fire et al., 1998; Lee et al., 1993), numerous novel miRNAs have been reported in a wide range of metazoan species, with crucial roles in presumably every biological process. In accordance with

this, InsectBase (2.0), the integrated genome and transcriptome resource database for insects contains 112,162 miRNA entries from 807 different species, well illustrating that a vast number of insect miRNAs are already identified. In addition, evidence for miRNA-based regulation of several physiological processes in insects has been accumulating over the last decade, with clear examples concerning the involvement of miRNAs in several aspects of insect development (Asgari, 2013; Belles, 2017; Belles et al., 2012; Lucas et al., 2015).

While regulatory RNA pathways are traditionally described at an intracellular level, extracellular miRNAs (ex-miRNAs) have been extensively studied in the past decade in mammalian systems, mainly due to their potential as a new source of biomarkers (Chen et al., 2008; Javidi et al., 2014; Reid et al., 2011; Sayed et al., 2014). Moreover, at this point, ample evidence in mammals points towards the controlled secretion of ex-miRNAs by donor cells (Groot and Lee, 2020; Guduric-Fuchs et al., 2012), as well as their functional transfer to recipient cells (Abels et al., 2019; Dalvi et al., 2017; Ghamloush et al., 2019; Hergenreider et al., 2012; Ismail et al., 2013; Kogure et al., 2011; Li et al., 2013; Lucero et al., 2020; Mittelbrunn et al., 2011; Montecalvo et al., 2012; Varciana et al., 2019).

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Despite the progress in elucidating the presence and function of ex-miRNAs in mammals, the number of studies reporting on ex-miRNAs in insects is limited. In the honey bee, *Apis mellifera*, ex-miRNAs present in worker and queen jellies contribute to caste determination (Guo et al., 2013). In the fruit fly, *Drosophila melanogaster*, stable ex-miRNAs were discovered in the hemolymph and accumulated in an age-dependent manner (Dhahbi et al., 2016). Moreover, fruit fly glial cells secrete miR-274 in the circulating hemolymph, which is functionally transferred to its recipient target cells, neurons and tracheal cells (Tsai et al., 2019). In accordance, we demonstrated the presence of ex-miRNAs in the cell-free culture media of two *D. melanogaster* cell lines (Van den Brande et al., 2018) and in extracellular vesicles (EVs) isolated from the culture medium of *Tribolium castaneum* cells (Mingels et al., 2020). Furthermore, ex-miRNAs were detected in the trophallactic fluid of the Florida carpenter ant, *Camponotus floridanus* (LeBoeuf et al., 2016), and in saliva of *Aedes aegypti* and *Aedes albopictus* mosquitoes (Maharaj et al., 2015). In addition, larvae of the parasitoid wasp, *Cotesia vestalis*, secrete miRNAs to arrest the development of the host in which they are growing (Wang et al., 2018).

In this report, we demonstrate the presence of stable sRNAs in the hemolymph of the migratory locust, *Locusta migratoria*, an important swarming pest. Moreover, the migratory locust displays a robust systemic RNA interference (RNAi) response and is an important research model for several processes, including the RNAi pathways. Remarkably, the levels of several of these hemolymph ex-miRNAs were significantly altered between young and old fifth nymphal locusts. In addition, as some of these ex-miRNAs might act as intercellular communicators, we performed a 'proof of principle' experiment, which revealed that modulating the extracellular levels of specific miRNAs affected the moulting process.

Materials & methods

Locusta migratoria rearing

Gregarious migratory locusts (*L. migratoria*) were reared under controlled temperature ($30 \pm 1^\circ\text{C}$) and light conditions (a photoperiod of 14 h per day) at an ambient relative humidity between 40 to 60 %. The locusts were fed daily with fresh indoor-grown wheat grass, supplemented *ad libitum* with dry rolled oats.

Hemolymph collection and serum preparation

In general, hemolymph was taken by piercing the locusts' soft cuticle behind the base of the hind leg with a needle and transferring the hemolymph into an Eppendorf tube on ice, followed by centrifugation to remove hemocytes and apoptotic bodies. The remaining supernatant (*i.e.* hemolymph-serum, henceforth referred to as serum) was used for the experiments.

For the initial identification of extracellular sRNAs in hemolymph, hemolymph samples collected from nymphs and adults were pooled to one final sample (760 μl) and processed as described above.

For the stability experiments, hemolymph samples were collected from fifth nymphal locusts and transferred to 25 μl of *L. migratoria* Ringer solution (1L: 9.82 g NaCl; 0.32 g CaCl₂; 0.48 g KCl; 0.73 g MgCl₂; 0.25 g NaHCO₃; 0.19 g NaH₂PO₄; pH 6.5) until a final volume of 250 μl was obtained. The samples were centrifuged for 10 min at 1000 g and 10 min at 5000 g to remove hemocytes and apoptotic bodies, respectively, and the remaining supernatant was used for the experiments. For each set-up [oligoribonucleotide – 5 min and oligoribonucleotide – 60 min (Fig. 2B); miRNA mimic – 5 min and miRNA – mimic 60 min (Fig. 2C)] three replicates (n=3) were analysed. Each replicate contained the pooled hemolymph collected from 10 locusts.

For the differential sRNA sequencing experiment, 20 μl of hemolymph was collected on a daily basis from day two (D2) until day eight (D8) of N5 stage locusts. Samples derived from three animals were

pooled per replicate for each time point and processed as above. In parallel, for the assessment of the ecdysteroid levels, 5 μl of hemolymph was collected and pooled from the same individuals in 100 μl of 100 % ethanol. The samples were stored at -80°C until further processing.

Synthetic oligoribonucleotide stability experiments

Three serum samples (three replicates) of 200 μl were thawed and each was divided in two aliquots of 100 μl . An unmodified synthetic oligoribonucleotide corresponding to the *C. elegans* microRNA cel-miR-39-3p (UCACCGGGUGUAAAUCAGCUUG) (IDT, Leuven, Belgium) was added (10 fmol) to three serum samples directly and incubated for 5 min, before adding Qiazol solution ("native samples"). The other three serum aliquots were first inactivated (*i.e.* the degrading enzymes present in the sera are inactivated) with the Qiazol solution prior to adding the synthetic oligoribonucleotide and incubated for 5 min ("inactivated samples"). The samples were then subjected to RNA extraction as described below. This set-up (Fig. 2A) was repeated with 60 min incubations. In addition, a similar set-up was performed for the miRNeasy Plasma/Serum Spike-in Control (219610, Qiagen), which is a *C. elegans* microRNA cel-miR-39-3p mimic.

RNA extraction

Total RNA (including sRNA) was extracted from the serum samples using the miRNeasy Serum/Plasma kit (Qiagen) according to manufacturer's protocol. This kit is specifically designed for the purification of cell-free RNA from serum and plasma. The quality and concentration of the resulting RNA samples were determined using a Nanodrop Spectrophotometer (NanoPhotometer N60, Implen). In addition, a more detailed analysis of the sRNAs isolated from the samples used for sequencing was performed using the Bioanalyzer Small RNA kit (Agilent Technologies, Inc.).

cDNA synthesis and qRT-PCR

Equal volumes of the RNA samples (7 μl for each) were used for cDNA synthesis using the qScriptTM microRNA cDNA synthesis kit (QuantaBio) following manufacturer's protocol, as previously described (Van den Brande et al., 2018). First, a poly(A) polymerase reaction was performed for the polyadenylation of the miRNAs, followed by cDNA synthesis using an oligo-dT adapter primer. The obtained cDNA was diluted tenfold with Milli-Q water (Millipore). Amplification of cDNA samples was performed using a microRNA-specific forward and a universal poly(T) adapter reverse primer (Table S1). Based on their high abundance in hemolymph serum (highlighted in Table S2), as well as on their available characterization in literature (Belles, 2017; He et al., 2016), we selected miR-276, let-7 and bantam for qRT-PCR. Primer pairs were previously validated by designing relative standard curves with serial dilutions of appropriate hemolymph serum derived cDNA samples. All qRT-PCR reactions were performed in duplicate in 96-well plates on the QuantStudioTM 3 System (ThermoFisher). Each reaction contained 5 μl of PerfeCTa SYBR Green Fastmix, ROX (QuantaBio), 0.5 μl of each forward and reverse primer (10 μM), 1.5 μl of Milli-Q water and 2.5 μl of cDNA. The following thermal cycling profile was used: 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s.

sRNA sequencing and data analysis

For the detection of sRNAs in locust serum, nymphal and adult sera was collected and combined to one final sample. The sRNA library was prepared using the Illumina Truseq kit according to manufacturer's protocol and sequenced together with 2% Illumina PhiX internal control spike-in on an Illumina MiSeq[®] System with a single read length of 50 nt. The Illumina adapters/primers were removed from the raw reads

with Cutadapt and the processed reads were aligned to the sRNA transcriptome of *L. migratoria* (Wei et al., 2009) and to the sequences obtained from miRBase (release 21.0) (Table S2).

For the differential sRNA sequencing, 7 sRNA libraries (four replicates of sRNAs derived from hemolymph with low ecdysteroid levels, D2-D3; three replicates of sRNAs derived from hemolymph with high ecdysteroid levels, D6-D7) were prepared using the TailorMix microRNA Sample Preparation Kit V2 (SeqMatic) according to the manufacturer's protocol. Each sRNA library was sequenced on the Illumina MiSeq® System with a single read length of 50 nt and sequencing quality control was monitored via PhiX spike-ins (2%). Adapter and quality trimming of the raw reads was done using Cutadapt (v1.11) (Martin, 2011). The trimmed reads were used for identification and quantification of known miRNA, as well as prediction and quantification of novel miRNA candidates using miRPro (v1.1.4) (Shi et al., 2015) in combination with all insect data from miRBase (release 21.0), published *L. migratoria* miRNA sequences (Wang et al., 2015) and the *L. migratoria* genome (V2.4.1 LocustBase) (Wang et al., 2014). The resulting miRNA feature counts were used for differential expression analysis in R (v3.3.1) ("R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria." n.d.) using the edgeR (v3.16.5) (Robinson et al., 2009) and SARTools (v1.4.0) (Varet et al., 2016) packages (Table S3). Features not having a counts per million greater than 1 in at least 3 samples were removed prior to the analysis. The differential sequencing data have been submitted in SRA under accession code PRJNA843578.

RNA interference experiments

Double-stranded (ds)RNA constructs targeting the *L. migratoria Dicer-1* (*LmDicer-1*) and *Green Fluorescent Protein* (*GFP*, control) transcripts were prepared using Ambion's MegaScript RNAi kit following the manufacturer's protocol. The primers including T7 promoter sequences can be found in Table S4.

Newly moulted fifth nymphal locusts (N5 D0) were injected with 6 µl of dsRNA against *LmDicer-1* (*dsLmDcr-1*) (300 ng dsRNA/locust, diluted in *L. migratoria* Ringer). A second injection was given on day three of the fifth nymphal stage (N5 D3) to ensure an efficient knockdown of *LmDicer-1* during this nymphal stage. As a control condition, another group of locusts was injected with dsRNA against GFP (*dsGFP*) following the same injection scheme.

In silico microRNA target predictions

For the miRNAs bantam and miR-13b, *in silico* target predictions were performed using all available *L. migratoria* nucleotide mRNA sequences (in total 1192 non-redundant transcripts) from NCBI as possible targets. This dataset contains an unbiased list of annotated *L. migratoria* mRNA transcripts. Potential mRNA target transcripts were predicted using the miRNAconsTarget program from sRNAToolbox (Rueda et al., 2015), which combines three software prediction tools (TargetSpy (Sturm et al., 2010), miRanda (Enright et al., 2003) and PITA (Kertesz et al., 2007)), and RNAhybrid (Krüger and Rehmsmeier, 2006), using default settings. Only miRNA-mRNA interactions predicted by all four tools were retained (Table S7).

Agomir experiments

As a 'proof of principle' experiment, we increased the extracellular levels of bantam and miR-13b by agomir injections in the hemolymph. Agomirs for lmi-Bantam-3p and lmi-miR-13b-3p were synthesized by Genepharma (Table S5).

For bantam, newly moulted N5 locusts (D0) were injected with 0.1 nmol of agomir, as well as on day three (N5 D3). Control animals were injected with the negative control agomir following the same injection scheme.

For miR-13b, 4-day old fourth nymphal locusts (N4 D4) were injected with 0.1 nmol of agomir and boost injected on the day of ecdysis to the fifth nymphal stage (N5 D0), as well as three days later (N5D3). Control animals were injected with the negative control agomir following the same injection scheme. In the case of miR-13b, we already started injecting in the fourth nymphal stage as previous research in our lab demonstrated that Kr-h1 depletion during the fourth nymphal instar resulted in a delayed moult and adultoid phenotype (Gijbels et al., 2020).

Ecdysteroid measurements using an enzyme immunoassay

Ecdysteroid titers in *L. migratoria* hemolymph were measured using an EIA (enzyme-linked immunosorbent assay) modified by Porcheron et al (Porcheron et al., 1989). This protocol uses a peroxidase conjugate of 20-hydroxyecdysone (20E), the active form of ecdysone, as tracer together with rabbit L2 polyclonal antibodies against ecdysteroids. This L2 antiserum has a strong affinity for ecdysone (E), 3-deoxyecdysone and 2-deoxyecdysone; and a 6- to 8-fold lower affinity for 20E. Both serum and tracer were very kindly supplied by Prof. J.P. Delbecq (Université de Bordeaux, France). The hemolymph samples in 100% ethanol were processed as follows: samples were vortexed and centrifuged for 10 min at maximum speed (16.000 g), after which the supernatant was transferred to a fresh Eppendorf tube. The remaining pellet was extracted two more times as described above and the combined extracts were then dried in a SpeedVac concentrator and redissolved in EIA sample buffer (80 mM K₂HPO₄, 20 mM KH₂PO₄, 0.4 M NaCl, 1.25 mM EDTA, 0.1% BSA, 0.01% thimerosal, pH 7.4). A 20E dilution series with concentrations ranging from 10⁻¹² to 10⁻⁸ M was used as standard (Sigma-Aldrich). Secondary goat anti-rabbit IgG (Jackson Immuno Research) coated MaxiSorp 96 well-plates (Nunc) were washed with a polysorbate buffer and loaded with i) 50 µl tracer, ii) 50 µl sample or standard and iii) 50 µl polyclonal antibodies. Two control reactions (0%, constituting of tracer and buffer; 100%, constituting of tracer, buffer and antibody) were included. After a 3 h incubation, the plates were washed and a coloration substrate mixture (0.42 mM 3,3',5,5'-tetramethylbenzidine in dimethylsulfoxide, 165.8 mM sodium acetate, 0.85 mM urea hydrogen peroxide and 1.6 mM citric acid, pH 6) was added. Absorbance was measured at 370 nm and 37°C every five minutes for one hour in a UV spectrophotometer-microplate reader (Mithras LB 940, Berthold Technologies). The resulting absorbance values were compared to the 20E standard curve.

Data Availability Statement

The raw sequence data of the differential miRNA sequencing experiment is submitted to NCBI as described above; all experimental data may be found in the supplementary material.

Results

sRNAs are present in locust serum

To address the question whether extracellular sRNAs are present in insect hemolymph, we used hemolymph serum of locusts (*L. migratoria*). Serum from nymphal and adult locusts was collected, pooled and the purified RNA was analyzed by sRNA sequencing. The read length distribution profile is compatible with the presence of sRNAs in the size range of the three main classes, namely miRNAs, as well as small interfering (si) and PIWI-interacting (pi) RNAs (Fig. 1A). Accordingly, the presence of sRNAs belonging to these three classes was analyzed *in silico* (Table S2). A distribution profile of the read counts corresponding to a particular sRNA class is shown in Fig. 1B, indicating that most sRNAs featuring a high read count belong to the miRNA class.

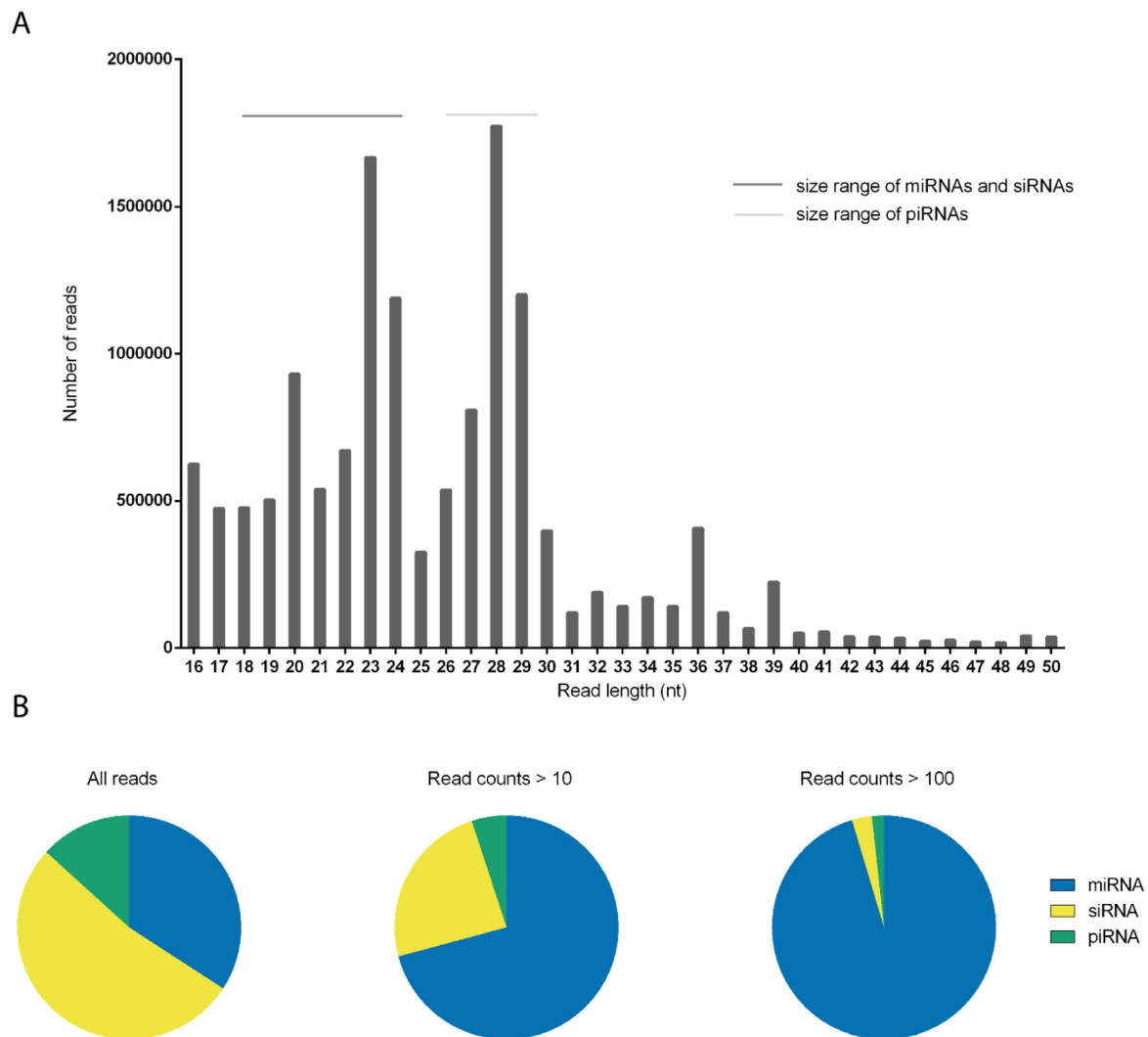


Fig. 1. Distribution profile of sRNAs from *L. migratoria* hemolymph serum. (A) Read length distribution of RNA purified from pooled sera of locust nymphs and adults analyzed by sRNA-seq. Reads from 16 to 50 nt were included. Dark and light grey lines indicate the size range of the main classes of regulatory sRNAs (mi-, si- and piRNAs). (B) The distribution profile of the read counts corresponding to a particular sRNA class compared for all reads (left), and for read count cut-offs of 10 (middle) and 100 (right).

miRNAs circulate in serum in a stable form

We continued by investigating if the identified extracellular miRNAs circulate in the hemolymph in a stable form. For this, we compared the stability of endogenous ex-miRNAs with the one of an exogenous oligoribonucleotide. Specifically, we introduced an unmodified synthetic oligoribonucleotide corresponding to a well-known *C. elegans* miRNA (cel-miR-39) in native and inactivated hemolymph serum; let it incubate for 5 min or 60 min; and performed RNA extraction (Fig. 2A). We then conducted a qPCR analysis for the cel-miR-39 and for three highly abundant endogenous extracellular miRNAs (bantam, let-7 and miR-276; highlighted in Table S2). We observed that, after 5 min, the cel-miR-39 levels were already significantly reduced in native serum compared to inactivated serum. On the other hand, the levels of the endogenous miRNAs remained stable up to 1 h in both native and inactivated sera. This is represented in Fig. 2B, where the ratio of Ct values measured in inactivated and native sera is depicted. As a negative control, a cel-miR-39 stable mimic was used in a similar approach (the miRNeasy Plasma/Serum Spike-in Control (219610, Qiagen)). In this case, degradation was not witnessed after 5 min of incubation and, even after 1 h of incubation, only a mild decrease in the Ct ratio was

observed (Fig. 2C), although not to the same extent as the unmodified oligoribonucleotide.

Temporal profile of ecdysteroids in hemolymph of fifth nymphal locusts

Next, we profiled the hemolymph ecdysteroid levels of fifth nymphal locusts over time. This was performed considering our following goal of assessing whether ex-miRNAs would be differentially present in the hemolymph at distinct developmental time points during the fifth nymphal stage. For this, the levels of ecdysteroids were measured daily, from day 2 (D2) until day 8 (D8). Increased ecdysteroid levels were observed near the end of the fifth nymphal stage, a few days before moulting to adulthood (Fig. 3).

Differential analysis of extracellular sRNAs in hemolymph of young and old fifth nymphal instar locusts

Based on the obtained ecdysteroid profile, we performed differential sRNA-seq of extracellular sRNAs from hemolymph samples of nymphal locusts collected at early (D2-D3) and late (D6-D7) time points in the fifth instar characterized by, respectively, low and high ecdysteroid

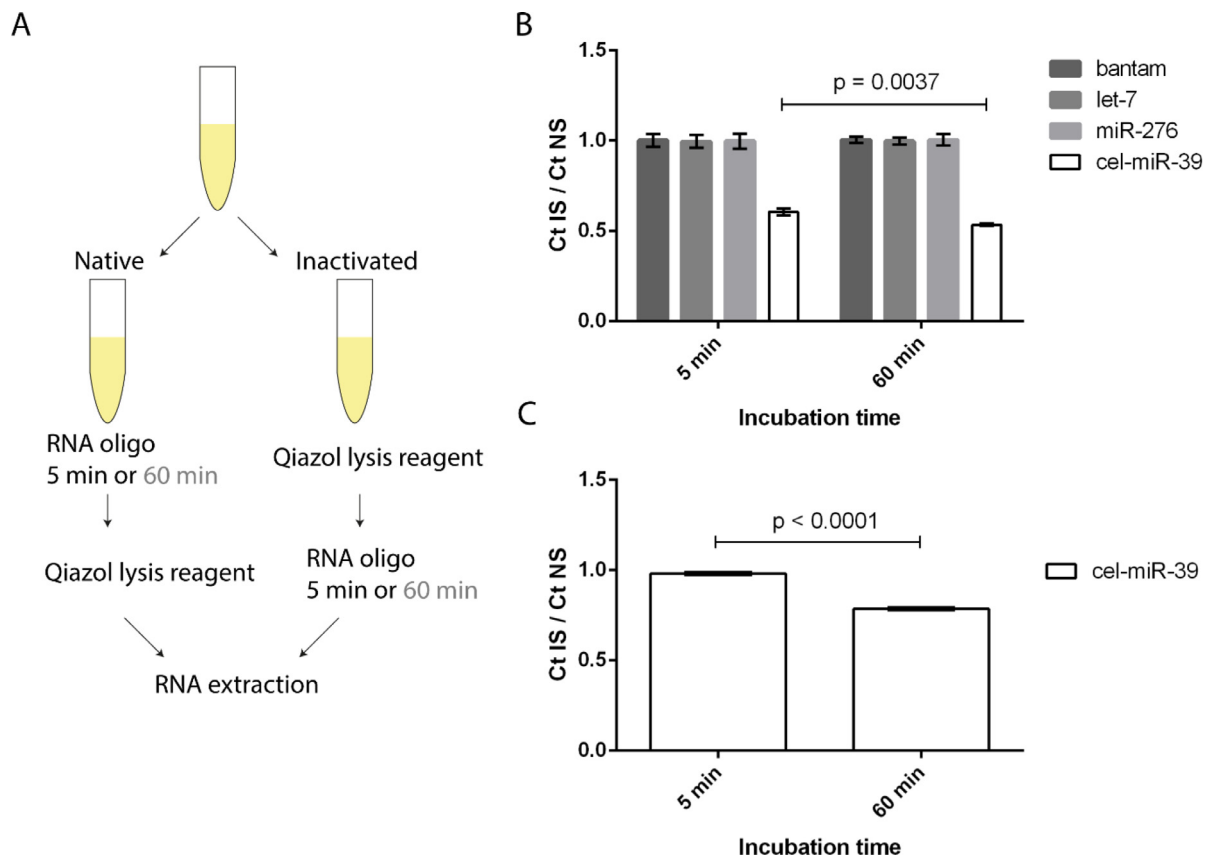


Fig. 2. Experimental set-up and characterization of miRNA stability in hemolymph serum. (A) Each hemolymph serum sample ($n=3$) was divided in two. In one half, termed native serum, the unmodified synthetic oligoribonucleotide was added and incubated for 5 min before adding the Qiazol lysis reagent. In the other half, termed inactivated serum, the Qiazol lysis reagent was added immediately before adding the unmodified synthetic oligoribonucleotide, followed by a 5 min incubation. Then, normal RNA extraction was performed. This set-up was repeated with a 1 h incubation ($n=3$), and with the cel-miR-39-3p modified miRNA mimic ($n=3$ for both the 5 min and 1 h incubations). (B) The levels of cel-miR-39 and of the endogenous miRNAs (bantam, let-7 and miR-276) were measured by qRT-PCR. Ct values of the inactivated serum (Ct IS) were divided by the Ct values of the native serum (Ct NS) and the ratios are depicted in the graph. Each bar represents the mean \pm SD of three replicates ($n=3$). (C) The levels of cel-miR-39 were measured in the samples of the set-up where we used the cel-miR-39-3P modified miRNA mimic by qRT-PCR. Ct values of the inactivated serum (Ct IS) were divided by the Ct values of the native serum (Ct NS) and the ratios are depicted in the graph. Each bar represents the mean \pm SD of three replicates ($n=3$). Two-tailed unpaired t-tests were conducted to compare the miRNA levels after 5 and 60 min incubations. No statistical differences were observed for bantam, let-7 and miR-276. A significant decrease of 0.072 (95% CI: 0.039 to 0.11) in cel-miR-39 miRNA levels was witnessed after 60 min incubation (mean = 0.53) compared to 5 min incubation (mean = 0.61) of the unmodified oligoribonucleotide; $t(4) = 6.066$, $p = 0.0037$. A significant decrease of 0.196 (95% CI: 0.18 to 0.21) in cel-miR-39 miRNA levels was witnessed after 60 min incubation (mean = 0.79) compared to 5 min incubation (mean = 0.98) of the miRNA mimic; $t(4) = 34$, $p < 0.0001$.

levels. The abundance of 43 predicted miRNAs significantly differed between both groups (Table 1 and Fig. 4; corresponding sequences are shown in Table S6). Specifically, 26 miRNAs were significantly more abundant in hemolymph derived from early N5 nymphs (D2-D3), while the remaining 17 were more abundant in hemolymph of late N5 nymphs with high ecdysteroid levels (D6-D7). Based on available literature (Wei et al., 2009) and similarity with reported miRNAs on miR-Base, 16 miRNAs were characterized as known (Table 1, "Identified" column). The remaining 27 miRNAs represent either potentially novel miRNA candidates (termed lmi-novel-miR-XXX), or miRNA candidates previously identified in the sRNA sequencing experiment performed by Wang et al (indicated with IDXXX or IMXXX) (Wang et al., 2015).

Modulating the extracellular levels of microRNAs miR-13b and bantam affects the moulting process

The miRNAs miR-13b and bantam, identified in the differential sRNA sequencing (Table 1), have previously been demonstrated to be involved in the regulation of development in some insect species (Becam et al., 2011; Boulan et al., 2013; Herranz et al., 2012, 2008; Ling et al., 2015;

Lozano et al., 2015; Marco et al., 2012; Nolo et al., 2006; Oh and Irvine, 2011). To investigate their potential mRNA target transcripts in *L. migratoria*, *in silico* target prediction analyses were performed using four different prediction tools. Both miRNAs are predicted to potentially target a broad range of transcripts, several of which are involved in hormonal signaling or chitin metabolism pathways (Table S7).

Given their differential abundance in serum during the fifth nymphal stage, their well-described regulatory functions in insects, and their *in silico* predicted targets, we selected these miRNAs for a 'proof of principle' experiment. Hence, we increased the extracellular levels of these miRNAs by agomir injections in the hemolymph and assessed the possible effect on the moulting process.

Injection of miR-13b agomir during the fourth and fifth nymphal stage significantly affected the duration of the fifth nymphal stage. While negative control animals (injected with negative control agomir) lasted in the N5 stage for 6 to 8 days, locusts injected with the miR-13b agomir were characterized with a longer N5 stage of 8 to 10 days (Fig. 5A). Nevertheless, no difference was observed in terms of moulting success, as all experimental (miR-13b treated) and control animals were able to moult into adults. By contrast, 60% of fifth nymphal locusts

Table 1

Differential miRNA analysis. Differential miRNA sequencing data were analysed using edgeR. Sequencing was performed on small RNAs of hemolymph serum samples derived from locusts at early (four replicates; N5D2r1, N5D2r2, N5D3r1 and N5D3r2) and late (three replicates; N5D6r1, N5D6r2 and N5D7) time points in the fifth nymphal stage, characterized by low and high ecdysteroid levels, respectively. Normalised read counts, Log2FoldChanges and adjusted p-values ($\alpha < 0.05$) are given per sample of the differentially abundant miRNAs. Abundant miRNAs are characterized by higher read counts, less abundant miRNAs by a lower read count. Log2FoldChange values reflect the differential abundance between early and late N5 nymphs. Positive values indicate miRNAs more abundant in hemolymph of early N5 nymphs (N5D2-3, with low ecdysteroid levels) compared to hemolymph of late N5 nymphal locusts (N5D6-7, with high ecdysteroid levels), and vice versa for negative values. MiRNAs were ranked (top to bottom) based on their adjusted p – values. The “identified” column demonstrates known *Locusta migratoria* miRNAs or miRNAs identified based on similarity with miRNA sequences in miRBase; the remaining miRNAs represent either potential novel miRNA candidates (termed lmi-novel-miR-XXX), or miRNA candidates previously identified (Wang et al., 2015).

miRNA		Early				Late			Log2FC	Padj value
ID sequence	Identified	N5D2r1	N5D2r2	N5D3r1	N5D3r2	N5D6r1	N5D6r2	N5D7		
lmi-novel-miR-47		131	146	120	195	15	31	40	2.292	1.93213E-09
lmi-novel-miR-17-19		100	97	83	103	20	16	37	1.865	2.85949E-07
lmi-novel-miR-80-82	lmi-bantam-3p	164	194	197	213	54	36	87	1.627	9.27414E-07
lmi-novel-miR-29		7	13	7	14	0	0	0	6.387	6.69714E-06
lmi-miR-8-3p	lmi-miR-8-3p	3365	3203	3348	3075	1940	1504	1792	0.896	3.6423E-05
lmi-novel-miR-27/115	lmi-miR-305-5p	51	52	80	59	22	10	20	1.727	3.67427E-05
lmi-novel-miR-125-128	lmi-miR-279-3p	100	97	90	101	32	29	48	1.341	6.02936E-05
lmi-novel-miR-87		25	27	49	42	12	5	5	2.3	0.000139452
ID50	lmi-miR-305-5p	55	51	82	65	27	13	23	1.541	0.000540895
IM18	lmi-miR-306-5p	38	53	38	46	12	16	18	1.481	0.000680117
lmi-miR-9a-5p	lmi-miR-9a-5p	430	394	480	349	219	161	264	0.925	0.002458449
lmi-novel-miR-23	lmi-miR-12-5p	18	18	20	18	7	5	5	1.669	0.004169384
lmi-novel-miR-58	lmi-miR-13b-3p	32	28	19	38	5	8	15	1.502	0.00597058
ID1142		8	6	15	2	0	0	1	3.419	0.006296376
IM59	lmi-miR-9a-5p	73	43	86	28	7	3	32	1.816	0.009873078
lmi-novel-miR-118/119		33	46	43	40	22	16	18	1.13	0.01020884
lmi-novel-miR-121		279	291	293	274	197	154	195	0.635	0.012620113
lmi-novel-miR-24	lmi-miR-12-5p	17	13	19	16	7	5	5	1.469	0.012833774
lmi-novel-miR-120		30	26	27	27	7	16	14	1.127	0.013568979
lmi-novel-miR-45		12	7	3	8	2	0	2	2.132	0.016562865
ID1281		8	8	6	4	2	3	0	2.357	0.019450101
ID1582	lmi-miR-315-5p	6	3	2	0	0	0	0	4.48	0.022719101
lmi-novel-miR-129		8	5	12	12	2	5	1	1.908	0.024229191
lmi-novel-miR-37	lmi-miR-190-5p	64	89	60	129	32	34	57	1.007	0.029746911
lmi-novel-miR-54-57		7	5	7	7	2	3	1	1.84	0.037334967
lmi-novel-miR-77		38	31	27	38	30	5	17	0.974	0.049578481
lmi-novel-miR-28	lmi-miR-275-3p	281	315	262	289	524	640	473	-0.921	4.60012E-05
lmi-novel-miR-16		1	1	1	0	12	23	0	-3.945	0.00041374
ID1794		0	1	0	0	2	8	4	-3.897	0.000526465
lmi-miR-8-5p	lmi-miR-8-5p	49	58	43	46	103	120	88	-1.049	0.000622173
ID604		30	30	20	27	74	78	48	-1.238	0.000650521
ID1454		4	3	3	2	10	5	15	-1.754	0.003746306
ID607		29	35	18	30	74	83	42	-1.166	0.004984008
lmi-novel-miR-32		0	0	0	1	2	3	5	-3.028	0.006296376
lmi-novel-miR-1/2		5	4	7	7	17	18	10	-1.328	0.019688533
lmi-novel-miR-63/64		6	9	3	6	15	34	8	-1.471	0.020664736
lmi-novel-miR-46		29	18	15	20	86	44	23	-1.27	0.020947402
ID877		0	1	1	0	7	5	1	-2.355	0.035640902
lmi-novel-miR-67-70		24	23	12	21	42	49	30	-0.931	0.035640902
lmi-novel-miR-113		30	28	24	28	62	75	29	-0.929	0.036781183
ID1684		1	0	0	1	0	8	2	-2.561	0.037334967
lmi-miR-281-5p	lmi-miR-281-5p	5	3	2	1	0	10	11	-1.492	0.042049155
lmi-novel-miR-86		7	4	2	5	22	10	7	-1.271	0.043240447

injected with bantam agomir were unable to moult into the adult stage (Fig. 5B). While attempting ecdysis, these locusts were unable to shed their old cuticle and eventually died.

Discussion

In this manuscript, we have investigated the presence of ex-miRNAs in locust hemolymph. At the intracellular level, miRNAs regulate the expression of endogenous genes and are therefore crucial for proper development, as shown by *Dicer-1* loss-of-function studies in several insects (Gomez-Orte and Belles, 2009; Lee et al., 2004; Rahimpour et al., 2019; Wu et al., 2017). In *Locusta migratoria*, loss of *Dicer-1* induced developmental defects, as multiple locusts were unable to moult to the adult stage and displayed phenotypes (Fig. S1) in accordance with the findings previously reported by Wang and colleagues (Wang et al., 2013).

Although regulatory sRNAs are well-described at an intracellular level, the presence of these molecules in the extracellular environment remains a question mark in most insects. In this study, we validated the presence of extracellular sRNAs in cell-free hemolymph of *L. migratoria*, an important insect pest and research model. The length distribution profile of the sRNAs is compatible with the size of the main sRNA classes, namely miRNAs, siRNAs and piRNAs (Fig. 1A). In addition, specific sRNAs belonging to these three classes were identified by *in silico* prediction (Fig. 1B). This finding adds to the identification of ex-miRNAs in insect and arthropod bio-fluids (Arcà et al., 2019; Dhahbi et al., 2016; Guo et al., 2013; LeBoeuf et al., 2016; Lefebvre et al., 2016; Maharaj et al., 2015; Mingels et al., 2020; Tsai et al., 2019; Van den Brande et al., 2018).

Given that hemolymph contains high levels of RNase activity (Garbutt et al., 2013; Wynant et al., 2014), we aimed to determine

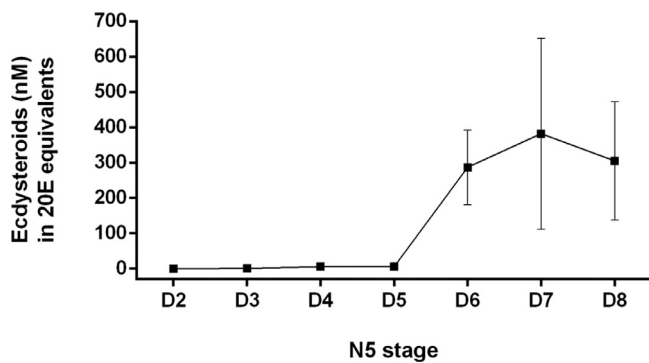


Fig. 3. Ecdysteroid levels in hemolymph of fifth nymphal locusts. Ecdysteroid levels (20E equivalents in nM) were measured in hemolymph samples collected daily from day two (D2) until day eight (D8) in the fifth nymphal stage. Data represent means \pm SD of three replicates (two replicates of D7), each containing hemolymph of three animals.

the stability of the hemolymph ex-miRNAs. In this context, we have shown that an unmodified synthetic oligoribonucleotide corresponding to cel-miR-39 was degraded to a large extent when incubated in hemolymph serum, while the endogenously present miRNAs remained stable (Fig. 2B). On the other hand, a cel-miR-39 mimic remained quite stable when incubated in hemolymph serum, albeit not to the same extent as the endogenous miRNAs (Fig. 2C). MiRNA mimics are chemically synthesized molecules that mimic mature miRNAs. They generally bear modifications that improve their stability and uptake, such as 2'-sugar modifications, phosphorothioate backbones and cholesterol additions (Jin et al., 2015; Thomson et al., 2013; Wu, 2018). It is remarkable that the levels of endogenous extracellular miRNAs remain stable in the hemolymph, similarly to (or even more stable than) a synthetic miRNA mimic that was designed to remain more stable than a synthetic unmodified single stranded oligoribonucleotide with the same sequence and

length. These observations highly suggest that the endogenously present ex-miRNAs circulate in the hemolymph in an RNase-resistant form. Of note, mammalian ex-miRNAs have been demonstrated to be protected from RNase activity due to their encapsulation in extracellular vesicles (EVs) (Lotvall and Valadi, 2007; Valadi et al., 2007) or their association with RNA-binding proteins (RBPs) such as Argonaute-1 (Ago-1) (Arroyo et al., 2011; Turchinovich et al., 2011; Turchinovich and Burwinkel, 2012) or lipoproteins (Michell and Vickers, 2016; Vickers et al., 2011). Although the mechanisms responsible for ex-miRNA stability in locust hemolymph remain to be investigated, we have previously demonstrated the presence of miRNAs in EVs and in complexes containing Ago-1 isolated from the cell-free culture media of *D. melanogaster* cells (Van den Brande et al., 2018); as well as in EVs from the cell-free culture media of *T. castaneum* cells (Mingels et al., 2020).

Together with the currently available literature, the presence of stable ex-miRNAs in locust hemolymph (Figs. 1B and 2; Table S2) raises the intriguing question whether (some of) these might regulate biological events in target tissues. In line with this, we observed that several miRNAs are differentially enriched in the hemolymph serum samples obtained at different time points (early versus late) within the 5th nymphal instar of *L. migratoria* (Figs. 3 and 4; Table 1). In fact, several of the identified miRNAs are known to be involved in the regulation of developmental events in some other insect species (Table 2). In addition, the relationship between miRNAs and ecdysone has been demonstrated for several miRNAs at the intracellular level. For example, the let-7-cluster miRNAs are induced by 20E due to the presence of EcRE (ecdysteroid response elements). As such, high 20E titers result in an increased expression of these miRNAs (Rubio et al., 2012; Sempere et al., 2003). While these miRNAs are under tight hormonal control, examples of miRNAs affecting the ecdysone pathway exist as well. In *D. melanogaster*, ecdysteroid signaling involves a feedback mechanism that is modulated by miR-14 which is able to regulate the expression of the ecdysone receptor EcR (Belles, 2017; Varghese and Cohen, 2007). In addition to these famous examples, several of the miRNAs that we identified in the differential miRNA sequencing experiment (Table 1) have

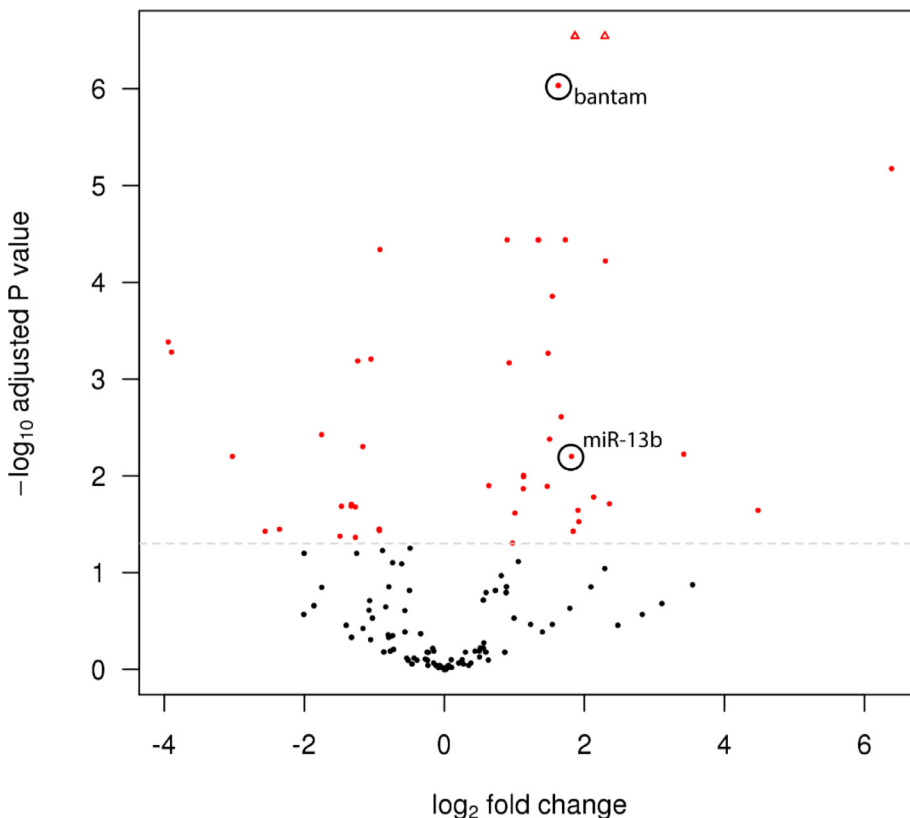


Fig. 4. Volcano plot indicating the 43 differentially enriched miRNAs (red dots and triangles). Bantam and miR-13b are highlighted on the Volcano plot. Triangles correspond to miRNAs featuring a too low adjusted P-value to be displayed on the plot.

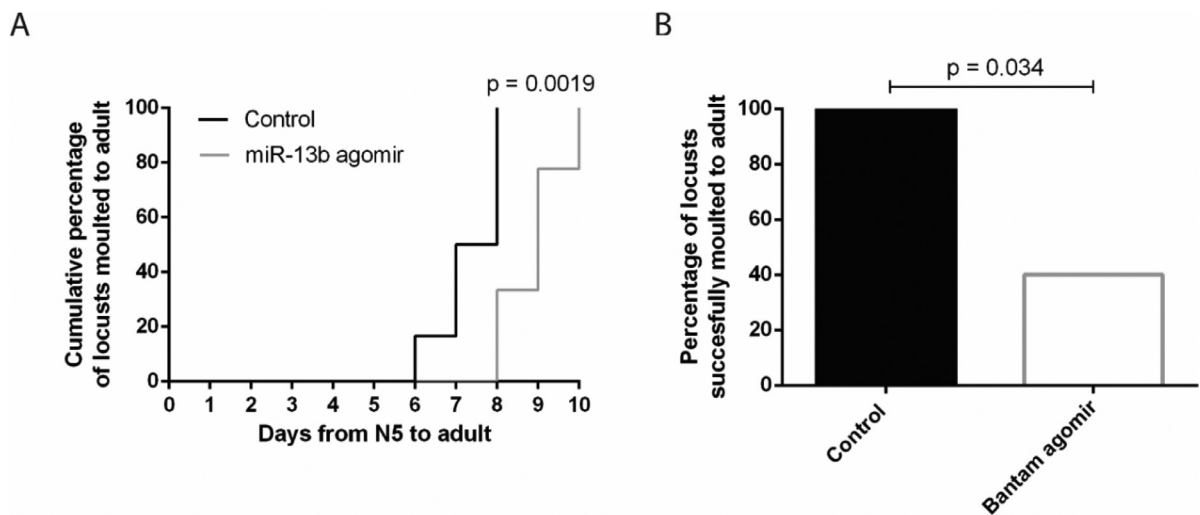


Fig. 5. Effect of miR-13b and bantam agomir treatment on locust development. (A) Cumulative percentage of miR-13b agomir ($n = 9$) and control (negative control agomir; $n = 6$) treated locusts that moulted to the adult stage. The statistically significant difference was analysed using a log-rank (Mantel-Cox) test ($p = 0.0019$). (B) Percentage of locusts that successfully moulted into the adult stage after bantam agomir treatment ($n = 10$) or control (negative control agomir; $n = 6$) treatment. The statistically significant difference was analyzed using Fisher's exact test ($p = 0.034$).

Table 2

Involvement of specific miRNAs in the regulation of developmental processes in several insects. In some studies, the specific target genes are mentioned. The miR-2 family comprises miR-2 and miR-13.

miRNA	Target	Process	Insect	Reference
bantam	<i>Hid</i>	Tissue growth by regulating cell proliferation and apoptosis	<i>Drosophila melanogaster</i>	(Brennecke et al., 2003; Hipfner et al., 2002)
		Hippo signaling pathway that controls tissue growth	<i>Drosophila melanogaster</i>	(Nolo et al., 2006; Thompson and Cohen, 2006)
	<i>Enabled</i>	Notch signaling pathway mediating boundary formation in wings	<i>Drosophila melanogaster</i>	(Becam et al., 2011; Herranz et al., 2008)
		Decapentaplegic signaling pathway controlling growth	<i>Drosophila melanogaster</i>	(Oh and Irvine, 2011)
	<i>Capicua</i>	EGFR signaling controlling growth	<i>Drosophila melanogaster</i>	(Herranz et al., 2012)
		Insulin signaling & ecdysone biosynthesis promoting growth	<i>Drosophila melanogaster</i>	(Boulan et al., 2013)
miR-8		Germline stem cell maintenance	<i>Drosophila melanogaster</i>	(Shcherbata et al., 2007)
		Ecdysteroid biosynthesis	<i>Chilo suppressalis</i>	(He et al., 2017)
	<i>Atrophin</i>	Developmental and neuronal processes	<i>Drosophila melanogaster</i>	(Karres et al., 2007)
	<i>Atrophin</i>	Developmental and neuronal processes	<i>Blatella germanica</i>	(Rubio et al., 2013)
	<i>Tre-2</i>	Chitin biosynthesis	<i>Nilaparvata lugens</i>	(Chen et al., 2013)
	miR-305	<i>InR / PI3K</i>	Notch & insulin signaling pathways	<i>Drosophila melanogaster</i>
		20E-responsive microRNA (induced by ecdysone pathway)	<i>Drosophila melanogaster</i>	(Jin et al., 2020)
		20E-responsive microRNA (induced by ecdysone pathway)	<i>Bombyx mori</i>	(Jin et al., 2020)
miR-9a	<i>dLMO</i>	Wing development	<i>Drosophila melanogaster</i>	(Biryukova et al., 2009)
	<i>SNPF1</i>	Insulin signaling and body growth	<i>Drosophila melanogaster</i>	(Suh et al., 2015)
		General growth and development	<i>Spodoptera exigua</i>	(Zhang et al., 2015)
	<i>Neverland</i>	Ecdysteroid biosynthesis	<i>Chilo suppressalis</i>	(He et al., 2017)
miR-2 family	<i>Kr-h1</i>	Metamorphosis via JH signaling	<i>Blatella germanica</i>	(Lozano et al., 2015)
	<i>awd / fng</i>	Wing morphogenesis	<i>Bombyx mori</i>	(Ling et al., 2015)
miR-315	<i>Axin / Notum</i>	Wingless signaling controlling growth and tissue specification	<i>Drosophila melanogaster</i>	(Silver et al., 2007)
miR-275		20E-responsive microRNA (induced by ecdysone pathway)	<i>Drosophila melanogaster</i>	(Jin et al., 2020)
		20E-responsive microRNA (induced by ecdysone pathway)	<i>Bombyx mori</i>	(Jin et al., 2020)
miR-281	<i>EcR</i>	Ecdysone signaling	<i>Bombyx mori</i>	(Jiang et al., 2013)
miR-306	<i>Abrupt</i>	Wing development	<i>Drosophila melanogaster</i>	(Simoes da Silva et al., 2019)

been shown to be affected by ecdysone as well. Jin *et al.* systemically investigated the genome-wide responses of miRNAs to 20E treatment in *Bombyx mori* and *D. melanogaster* cell lines. Increased expression levels were witnessed for miR-8-3p, miR-305-5p, miR-13b-3p, miR-190-5p and miR-275-3p in *B. mori* cell lines, and for miR-190-5p, miR-306-5p and miR-13b-3p in the *D. melanogaster* S2 cell line, upon 20E treatment (Jin *et al.*, 2020). In *Blattella germanica*, the intracellular expression profile of miR-190-5p and bantam-3p correlated with the 20E titers measured in the hemolymph (*i.e.* the expression of these miRNAs increased concomitantly with the 20E peak) (Rubio *et al.*, 2012). By contrast, we observed that these miRNAs (with the exception of miR-275-3p) were more abundant in hemolymph of early fifth instar locusts (characterized by relatively low ecdysteroid levels) compared to late ones (with higher ecdysteroid titers) (Table 1). Of note, since we profiled the abundance of

miRNAs in hemolymph serum (*i.e.* the extracellular environment), their levels can be affected by several factors, such as miRNA secretion and release, altered miRNA half-life and intracellular miRNA abundance.

In mammals, ex-miRNAs associated with EVs and RBPs can be functionally transferred between cells and tissues (Lotvall and Valadi, 2007; Valadi *et al.*, 2007; Vickers *et al.*, 2011). Here, we demonstrated that miRNAs are stably present in locust hemolymph (Fig. 2) and previously, we have shown that ex-miRNAs are associated with EVs and Ago-1 in insect cell culture media (Mingels *et al.*, 2020; Van den Brande *et al.*, 2018). Based on these observations, we performed a 'proof of principle' experiment in which we modulated the extracellular levels of two well-known miRNAs (bantam and miR-13b) by agomir injections in the hemolymph. These synthetic miRNA mimics are modified to enhance their uptake and therefore could mimic a potential naturally occur-

ring communication system based on the intercellular transfer of certain ex-miRNAs, similarly as reported in mammals (Ismail et al., 2013; Mittelbrunn et al., 2011; Valadi et al., 2007; Varciana et al., 2019; Vickers et al., 2011). We selected the miRNAs bantam and miR-13b for two reasons: these miRNAs were identified in the differential sRNA sequencing experiment (Table 1 and highlighted in Fig. 4) and they have been previously demonstrated to have developmental roles in insects (Table 2). The *in silico* target predictions suggested that both miRNAs can potentially target a broad range of transcripts, several of which are directly or indirectly involved in hormonal signaling or chitin metabolism pathways (highlighted in Table S7). Hence, in a 'proof of principle' experiment, we tried to verify whether modulating the extracellular levels of these miRNAs by agomir injections could induce any noticeable effect(s). Injections of miR-13b agomir in fourth and fifth instars affected the duration of the fifth nymphal instar, as the final moult was delayed in comparison to control animals (Fig. 5A). On the other hand, injection of bantam agomir clearly affected the moulting process as 60% of the treated animals did not successfully moult into adults (Fig. 5B). These data illustrate that agomir injections (which increase the extracellular levels of specific miRNAs) have indeed the capacity to elicit a functional effect. In line with our observations, agomir injections in *L. migratoria* have been shown to affect moulting (Yang et al., 2016), metamorphosis (Song et al., 2018) and phase transition (Yang et al., 2014). Taken together, these intriguing results allow us to speculate that (some of the) ex-miRNAs that stably circulate in insect hemolymph could potentially be taken up by target cells in which they may regulate gene expression by interfering with targeted transcripts.

Interestingly, some reports already demonstrated a role of ex-RNAs in intercellular communication: i) in parasitic wasps, teratocytes secrete miRNA-containing EVs that are functionally taken up by the host, inducing a developmental delay (Wang et al., 2018); ii) EV-encapsulated siRNAs spread the RNAi signal between *T. castaneum* cultured cells (Mingels et al., 2020) and mediate the systemic antiviral immune response in *D. melanogaster* (Tassetto et al., 2017); iii) EV-encapsulated miR-274 is secreted in the hemolymph of the fruit fly and functionally taken up by neurons and tracheal cells (Tsai et al., 2019), and iv) miRNAs in nurse bee secretions influence caste determination in *A. mellifera* (Guo et al., 2013). Moreover, functional RNA transfer has been suggested to occur between insects and plants (Jia et al., 2015; Sattar et al., 2012; Thompson et al., 2019; Wang et al., 2017; Zhang et al., 2019; Zhu et al., 2017). In fact, a regulatory layer based on functional transfer of sRNAs via extracellular fluids seems to be widely present in several forms of life (Hudzik et al., 2020; Lefebvre and Lécuyer, 2017; Zeng et al., 2019). Thus, although further functional research is needed, it seems relevant to speculate that some hemolymph ex-miRNAs might play a role in fine-tuning the regulation of physiological or developmental processes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Stijn Van den Brande: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization. **Marijke Gijbels:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **Niels Wynant:** Conceptualization, Methodology, Validation, Investigation. **Paulien Peeters:** Investigation, Resources. **Yannick Gansemans:** Software, Investigation, Formal analysis. **Filip Van Nieuwerburgh:** Investigation, Formal analysis, Writing – review & editing. **Dulce Santos:** Writing – review & editing, Supervision. **Jozef Vanden Broeck:** Writing – review & editing, Supervision.

Conflicts of interest and funding

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2022.100041.

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