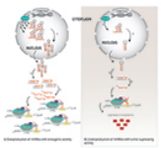
RNA Biology



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POINT OF VIEW

Temperature-dependent small RNA expression in *Drosophila melanogaster*

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ABSTRACT

Temperature has a major impact on gene expression in ectotherms. But until recently, it was not clear in which way, if any, small non-coding RNAs such as miRNAs or piRNAs contribute to thermosensitive gene regulation. We have recently shown that temperature-responsive miRNAs in *Drosophila* drive adaptation to different ambient temperatures on the transcriptome level. Moreover, we demonstrated that higher temperatures lead to a more efficient piRNA-dependent transposon silencing, possibly due to heat-induced unfolding of RNA secondary structures. In this commentary, we will dwell upon particular interesting aspects connected to our findings, hoping that our point of view may encourage other scientists to address some of the questions raised here. We will particularly focus on aspects related to climate-dependent transposon propagation in evolution and putative transgenerational epigenetic effects of altered small RNA transcriptomes. We further briefly indicate how temperature-responsive miRNAs may confound the interpretation of data obtained from experiments comprising heat-shock treatment which is a widely used technique not only in *Drosophila* genetics.

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Temperature shapes *Drosophila* phenotypes – new thoughts to an old issue

Evidence for a temperature dependency of genotype/phenotype correlation exists since the early 20th century. In 1915, Mildred Hoge reported on a peculiar mutation (*A, abnormal*) she observed in the course of a selection experiment: "The abnormalities were all connected with reduplication of leg or tarsal segments" [1]. At first sight, the manifestation of the phenotype cut across the ordinary Mendelian ratio and dogged efforts to produce pure stocks failed, as even after multigenerational selection, abnormal parents still produced normal offspring. Finally, Mildred Hoge discerned a connection between rising ambient temperatures in summer 1912 and spring 1913, and a decreasing amount of abnormal offspring in her stocks. Subsequent experiments – Hoge put the fly eggs and larvae into an ice chest – confirmed that a reduction in temperature during development results in an up to six-fold higher ratio of abnormal flies.

One hundred years later, a group headed by Christian Schlötterer applied genome wide approaches to verify global temperature-dependent effects. They showed that 10% of *Drosophila* genes are spliced in a temperature-dependent manner [2], and that the expression levels of more than 80% of *Drosophila* genes depend on ambient temperature [3]. Based on the observation that temperature-responsive genes were enriched for miRNA target sites it was further assumed that miRNAs could play a critical role in temperature-dependent gene regulation. To test this hypothesis, we have recently sequenced and analyzed ovary expressed small RNAs and mRNA from *Drosophila* cohorts kept at 18°C and 29°C. We further subjected each cohort to a temperature shift from 18-to-29°C or 29°C-to-18°C, respectively, to investigate the dynamics of putative expression changes [4]. We were able to verify profound and reversible changes in miRNA expression patterns and additionally showed that the expression levels of temperature-responsive miRNAs and their predicted target genes correlate inversely (Fig. 1). We also noticed that higher temperatures led to globally enhanced ping-pong processing of transposon transcripts suggesting a more efficient post-transcriptional silencing. During this process, alternate slicing of genomic piRNA cluster transcripts and transposon transcripts yields complementary PIWI-interacting (pi-) RNAs and results in post-transcriptional silencing of active transposons. Since this process requires sterically accessible single-stranded RNA transcripts, we argued that higher temperatures promote efficient ping-pong processing via relaxing RNA fold back structures.

Besides our main conclusion that adaptation to fluctuating ambient temperatures is at least in part driven by temperature-responsive miRNAs, our results prompted us to consider further possible consequences, which we believe are worth being discussed in greater depth. In the following, we will discuss our results with respect to

i. interpretation of experimental results after heat-shock treatment,
ii. the evolutionary success of transposons in *Drosophila* species that populate different habitats, and
iii. other more proximate transgenerational effects caused by altered small RNA repertoires in germ cells.

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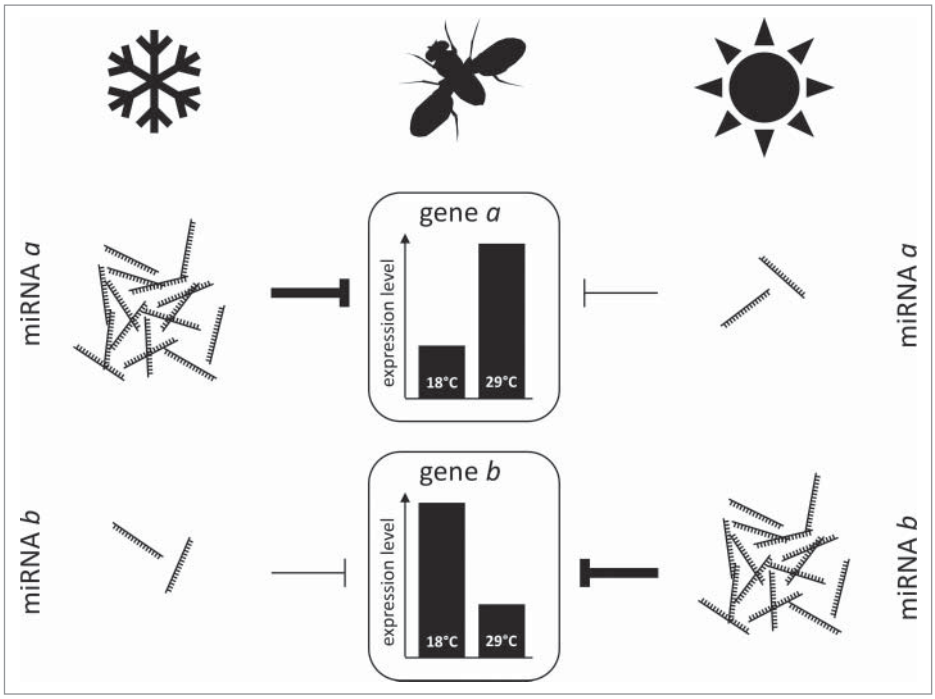

Figure 1. Model of miRNA-mediated temperature adaptation. miRNA *a* targets gene *a*, miRNA *b* targets gene *b*. miRNA *a* is upregulated at 18°C, resulting in stronger post-transcriptional repression of gene *a* at 18°C. miRNA *b* is upregulated at 29°C, resulting in stronger post-transcriptional repression of gene *b* at 29°C. Thus, expression levels of gene *a* and gene *b* behave different along thermal gradients.

We will also present additional data gained in the course of the original project that, however, was not published either for reasons of space restriction or absence of statistical significance. We want to emphasize that we do not consider this data as formal evidence and therefore, our interpretations in this respect should be regarded only as our point of view.

i) Off-target effects of heat-shock treatment

From the cold-blooded point of view, temperature is a crucial factor in all life situations and extreme temperature changes will trigger physiological stress. Therefore, species have developed mechanisms that act in response to thermal stress and ensure canalization during development [5–7]. Heat shock response (HSR) and associated genes were first discovered in *D. melanogaster* [8], where the heat shock protein 70 (HSP70) represents one of the most important factors protecting cells from thermal stress and providing thermotolerance [9,10]. For more than 30 years, genetic research utilizes HSR by using constructs in which a gene of interest is put under the control of a HSP70 promoter [11]. Combining this genetic technique with laser-induced heat shocks further allows to control gene expression in a very spatio-temporally specific manner [12,13].

Although this technique undoubtedly will remain an important part of experimental setups in genetics, evidence for a broad spectrum of off-target effects is mounting. Besides a temperature dependency of gene expression and splicing [2,3], Funikov et al [14]. showed that heat-shock treatment also affects miRNA expression in various ways. miRNAs that are organized in genomic clusters were mostly downregulated after heat-shock and pri-miRNA expression was reduced, but reached the regular expression level after a period at normal temperature. Paradoxically, some mature miRNAs were upregulated while their corresponding pri-miRNAs were downregulated. Further, strand-specific effects were observed for miR-14 with heat-shock-dependent downregulation of miR-14-5p, but unaffected or even upregulated expression of miR-14-3p. In addition, we have recently demonstrated that miRNA expression levels can greatly vary even when temperature shifts are rather modest, staying in the range of 18°C to 29°C [4], while heat-shock treatments typically involve temperatures close to 40°C. Noteworthy, although we have shown that altered miRNA expression globally affects gene expression, more specific evidence for particular temperature-dependent miRNA-target interactions is needed. With this in mind, we want to stress that interpreting results obtained from experiments based on heat-shock treatment should always involve putative off-target effects that may blur the expected signal or even produce misleading results. Further, targeted- and off-target effects may mutually influence each other, leading to unforeseen and opaque outcomes (Fig. 2). It is therefore important to include a second line of negative controls in addition to flies that carry the transgene but are not subject to heat-shock treatment. These controls should carry an irrelevant (or no) transgene while being subjected to heat-shock treatment [15]. This is the more important considering the possibility that off-target effects due to heat-shock induced ectopic miRNA expression are probably moderate but also more widespread at the same time, making them less obvious compared to possible off-target effects in CRISPR/Cas9 or other genetic background systems [16].

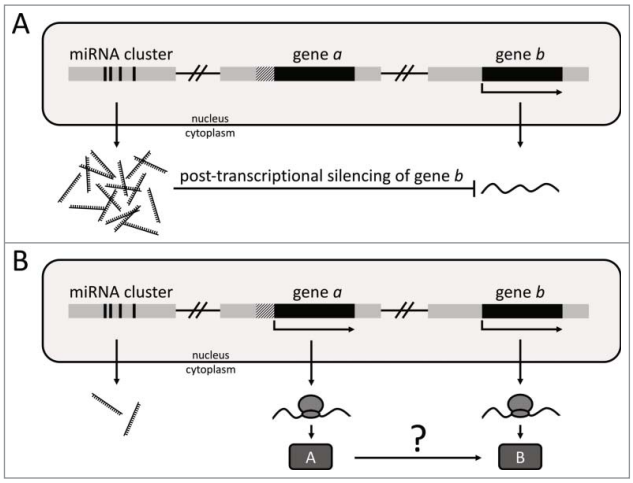

Figure 2. Differentiating targeted from off-target effects can be difficult. (A) A hypothetical project: According to the working hypothesis, expression of gene *a* affects expression of gene *b* at time point x. To verify this hypothesis, gene *a* was introduced into flies and put under control of a heat-shock promoter (dashed). (B) Same hypothetical project: Flies were subject to heat-shock treatment at timepoint x. After heat-shock, the experimentalist verifies expression of *a*/*A* on mRNA and protein level and further notes that levels of *b* and *B* are upregulated as well. Consequently, he concludes that *a* affects *b*. In fact, heat-shock treatment has led to reduced expression of miRNAs that silence *b* on the post-transcriptional level.

ii) Temperature and transposon propagation in Drosophila *evolution*

The last common ancestor of the more than 1500 today living *Drosophila* species [17] presumably existed as early as in the Paleocene more than 60 million years ago [18], a time marked by the demise of many plant and animal species including all non-avian dinosaurs [19]. Since then, *Drosophila* flies have conquered all continents of the world except Antarctica, colonizing habitats ranging from deserts via rain forests through to alpine zones [20]. And wherever they settled, they did not come alone, but brought along their inner molecular parasites: Mobile and self-replicative DNA, so-called transposable elements (TEs) [21]. As genome occupants, TEs were forced to migrate along with their hosts, encountering conditions more or less conducive for their own propagation. Although the genomic environment presumably represents the most important determinant for the success of a TE, abiotic factors connected to the ecological niche of the host might be important as well. In fact, we know that heat stress can induce TE activity in *Drosophila* [22,23]. However, no attempts have ever been made to link the evolutionary success of specific TEs in different *Drosophila* species to climatic factors such as ambient temperature.

Faced with the grave threat posed by active TEs, animals employ germline-expressed PIWI proteins and PIWI-interacting (pi-) RNAs to suppress TE activity on the transcriptional and post-transcriptional level, thus ensuring the transmission of intact genomes from one generation to another [24]. We have recently shown that shifts in temperature from 18°C to 29°C not only alter the expression level of specific TEs in *Drosophila melanogaster*, but also lead to a more pronounced PIWI/piRNA response, presumably due to reduced back folding of TE transcripts, making them more accessible for piRNA-guided PIWI attacks [4]. Given these opposing effects, it is not easy to predict in which way variation in temperature affects the propagation success of TEs in poikilothermic animals. Here we provide additional data that supports our hypothesis that ambient temperature can contribute in shaping the TE landscape of a species over evolutionary timespans.

We have compared the TE repertoire of 10 *Drosophila* species with distinct distribution areas (Fig. 3A,B), five of which are located predominantly above 15 degrees North latitude in habitats with annual mean air temperatures ranging from approximately 4°C to 20°C (northern species), while the others are located predominantly beyond 15 degrees North latitude in habitats with annual mean air temperatures ranging from approximately 20°C to 30°C (southern species). We found that from the five species with the highest fraction of TEs, four belong to southern species (Fig. 3C). Further, although there is no general correlation between annual mean temperature and genome size, two independent boosts in genome size due to massive TE expansion occurred in *D. willistoni* and *D. ananassae*, both of which live in tropical habitats (Fig. 3C). Next we analyzed particular TEs whose expression was found to be temperature-dependent in *Drosophila melanogaster* [4]. Although all of these TEs are more abundant in southern species, TEs with higher expression at 29°C are particularly successful in these species compared to TEs with higher expression at 18°C (Fig. 3D).

Needless to say, the presented data can scarcely be seen as an ultimate evidence for a connection of ambient temperature and TE propagation in evolution. While this hypothesis still remains to be proven, our main intention is to give rise to a reasonable suspicion that may motivate other researchers to conceive clever experiments to address this issue in a more comprehensive manner.

iii) Proximate transgenerational effects

When thinking about choosing a suitable model for studying temperature-dependent changes of small RNA expression levels, our choice fell on *Drosophila* ovaries since they express miRNAs, piRNAs and siRNAs in parallel [4]. Choosing this model rather unintentionally brought along another interesting aspect: Any changes of small RNA pools in oocytes potentially affect the fitness of the next generation, raising the issue of transgenerational epigenetics. Assuming that a modified small RNA repertoire within a fly's germ cell represents a physiological adaptation to parental environment, we would expect that males and females that were kept at identical temperatures should have fitter offspring compared to parents kept at different temperatures.

Although many studies have addressed the question how temperature shapes *Drosophila* phenotypes, available data on transgenerational effects is rather rare, particularly considering the question if different temperatures for maternal and paternal germ cells impair the fitness of the next generation. Huey and colleagues [26] have performed a series of experiments, which showed that housing temperature of male flies significantly affects early fecundity of the female offspring. However, no according effect for females was observed and 18°C males paired with 25°C females or vice versa produced female offspring with equal early fecundity compared to 18°C-18°C or 25°C-25°C pairs.

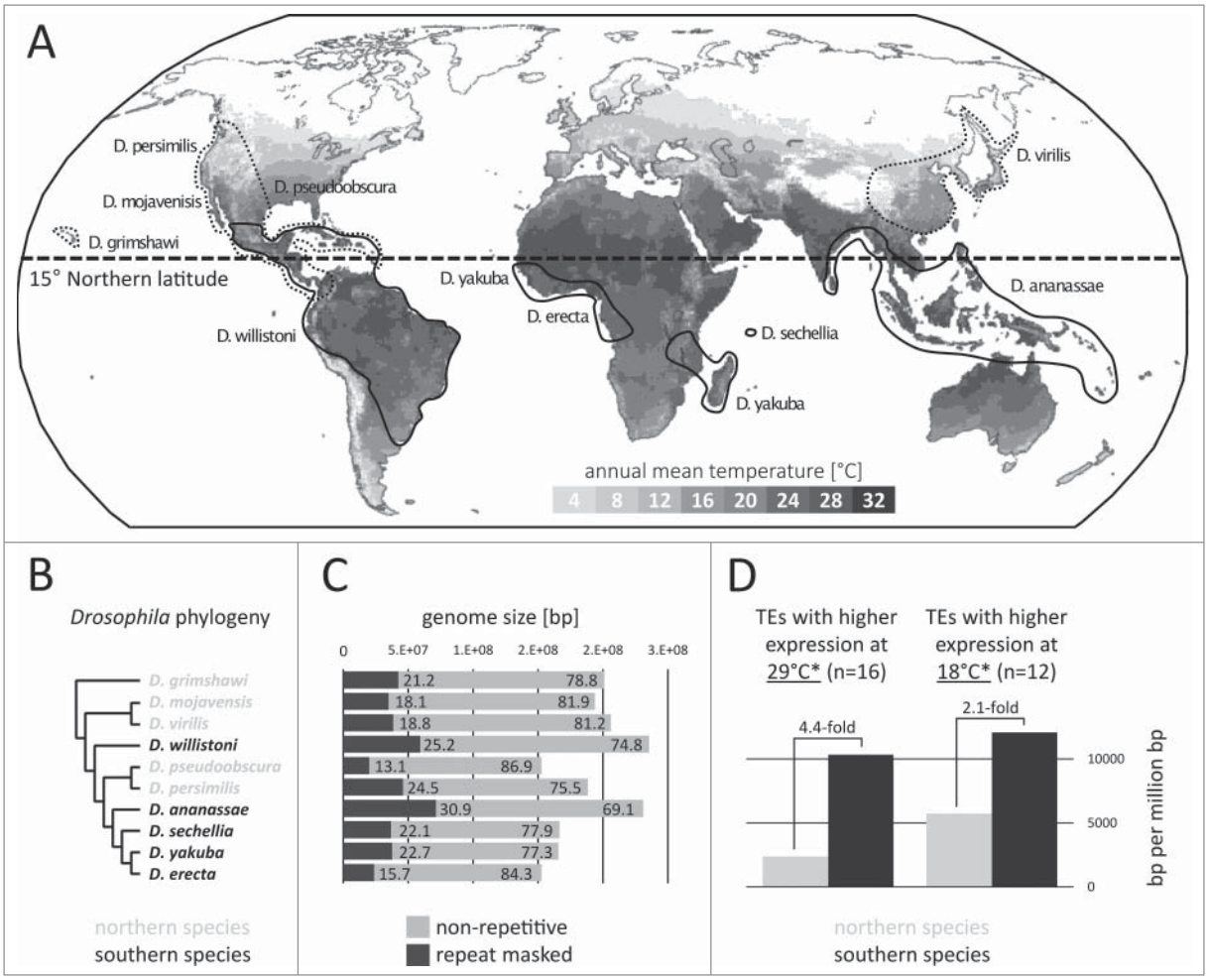

Figure 3. Transposons and ambient temperature. (A) Annual mean surface air temperature and distribution area of 10 *Drosophila* species. Dotted lines refer to habitats of species above 15° Northern latitude (northern species), solid lines refer to habitats of species below 15° Northern latitude (southern species). (B) Phylogenetic relationship of 10 *Drosophila* species [25]. (C) Genome size and fraction of repetitive sequences masked with RepeatMasker. Numbers refer to fraction of masked and unmasked bp in percent, respectively. (D) Average amount of TEs with higher expression at 29°C or 18°C, respectively, in southern and northern *Drosophila* species. *expression in Drosophila melanogaster [4].

In order to obtain a more complete picture, we have performed additional experiments in which we crossed males and females, both kept at different temperatures. Subsequently we determined the longevity of F1 individuals as well as the ratio of F1 eggs that evolved into the imago stage. Regarding longevity, we did not observe an advantage for F1 cohorts descending from parents reared at the same temperature (either 18°C or 29°C), compared to F1 cohorts descending from parents reared at different temperatures (Fig. 4A). If anything, a trend for longer-living F1 individuals from 18°C-reared females and 29°C-reared males became apparent. Since we considered it likely that putative transgenerational effects may manifest at earlier stages of ontogenesis, we further determined the ratio of eggs that evolved into imagos. In fact, we found that eggs from parents reared at the same temperature had a higher probability to give rise to adult animals compared to eggs from parents reared at different temperatures, although even the the most pronounced difference did not reach the significance level (Fig. 4B, two-tailed P value for *t* test is indicated).

However, based on the latter observation we consider it possible that different temperatures of parents impair the fitness of the resulting embryo, possibly because of less compatible germ cells. Alternatively, different rearing temperatures of parents may increase the number of unfertilized eggs due to other molecular mechanisms. Either way, clearly more experiments comprising considerably larger cohorts are needed to make credible statements.

Methods

Repeat annotation

Genomes (assembly) from *D. ananassae* (dana_caf1), *D. erecta* (dere_caf1), *D. grimshawi* (dgri_caf1), *D. mojavensis* (dmoy_caf1), *D. persimilis* (Dper_caf1), *D. pseudoobscura* (Dpse_3.0), *D. sechellia* (dsec_caf1), *D. virilis* (dvir_caf1), *D. willistoni* (dwil_caf1) and *D. yakuba* (Dyak_caf1) were downloaded from Ensembl Metazoa database (release 37) [27]. Repeat sequences were annotated with a local copy of RepeatMasker (v. 4.0.7, cross_match algorithm with option -s) using ancestral and lineage-specific *Drosophila* repeat sequences from RepBase repeat library version 20170127 [28].

Climate data

Climate data was taken from the National Center for Atmospheric Research Staff (Eds). Last modified 02 Aug 2017. "The

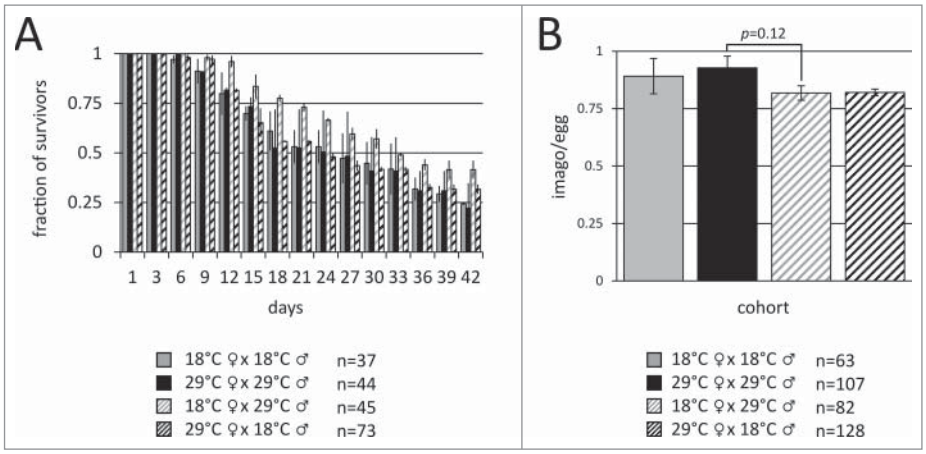

Figure 4. Effect of different parental temperatures on next generation's fitness. (A) Amount of surviving individuals as function of time. Error bars indicate standard error rates for two replicates. Data for offspring from different combinations of maternal/parental temperature are shown. (B) Eggs that developed to imago stage at 25°C. Error bars indicate standard error rates for two replicates. The two-tailed P value for *t* test comparing the most pronounced difference is indicated (not significant). Cohorts with different combinations of maternal/parental temperature are shown.

Climate Data Guide: Global (land) precipitation and temperature: Willmott & Matsuura, University of Delaware." Retrieved from https://climatedataguide.ucar.edu/climate-data/global-land-precipitation-and-temperature-willmott-matsuura-university-delaware. Data refers to the annual surface air temperature over land according to UDEL v3.0.1, averaged for the years 1951–1980.

Fly cultures

Drosophila melanogaster flies (Oregon R, wildtype) were kept at 25°C. Offspring was collected 0–5 hours after hatching and was split in four gender-separated groups, reared at 18°C for five days and at 29°C for two days, respectively, to achieve same developmental stages. We mated individuals reared at the same and different temperature (3♂ and 1♀) for five hours. Males were removed and after 24 hours the females were transferred to new culture tubes for three hours to lay eggs. Adult flies were transferred to new culture tubes in intervals of three days.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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