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Distinct microRNA and mRNA responses elicited by ecdysone, diapause hormone and a diapause hormone analog at diapause termination in pupae of the corn earworm, *Helicoverpa zea*

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A B S T R A C T

Ecdysone, diapause hormone and a diapause hormone analog are all capable of breaking pupal diapause and prompting initiation of adult development in the cotton earworm, *Helicoverpa zea*. In this study we asked whether these three chemically-distinct diapause terminators elicit the same effect on expression of a collection of microRNAs and transcripts encoding components of the ecdysone signaling pathway. Injection of all three endocrine agents resulted in downregulation of one miRNA, miR-277-3p, a miRNA previously linked to the insulin/FOXO signaling pathway, and all three agents promoted upregulation of *spook*, a member of the ecdysone biosynthesis pathway, and *iswi*, an ecdysone-responsive transcript. Other miRNA and mRNA responses varied depending on the agent used to terminate diapause, thus suggesting that different endocrine pathways and mechanisms can lead to the same final developmental response.

1. Introduction

Diapause is an alternative developmental pathway that provides insects, and other animals, a means to survive seasons of unfavorable environmental conditions by becoming dormant and to synchronize periods of development, growth, and reproduction with seasons of abundant resources (Tauber et al., 1986; Danks, 1987). This developmental arrest is coordinated by the brain and components of the insect's neuroendocrine system (Denlinger et al., 2012), and the specific hormones involved depend on the stage of the life cycle used for diapause. In pupal diapause of the corn earworm, *Helicoverpa* zea, two hormones, the steroid hormone ecdysone and the neuropeptide diapause hormone (DH), are of particular interest because they both can prompt the termination of diapause and appear to function as critical diapause regulators in members of this important complex of agricultural pests (Xu and Denlinger, 2004). Yet, some differences are apparent. Ecdysteroids can terminate diapause at any temperature, while DH can do so only at permissive temperatures at, or above, 21° C (Zhang et al., 2008). The goal of this study is to further understand the molecular mechanisms underpinning the roles of ecdysone and DH signaling in the regulation of diapause in pupae of *H. zea* by examining changes in the abundance of microRNAs and genes related to ecdysone and DH signaling that may, not only terminate diapause, but also promote post-diapause development.

Downstream mechanisms triggered by ecdysone and DH and that lead to diapause termination are not well understood, but one hypothesis is that DH stimulates the ecdysone signaling pathway. To test this hypothesis and to further decipher the functions of ecdysone and DH in diapause termination and post-diapause development, we evaluated changes in abundance of candidate microRNAs (miRNAs) and their putative mRNA targets in pupae that were injected with synthetic DH, ecdysone, or a diapause hormone analog to terminate diapause.

MicroRNAs are small, noncoding RNAs that post-transcriptionally regulate gene expression and are involved in multiple diapause-relevant processes including developmental timing, cell-cycle progression, metabolism, and environmental stress-resistance (Abbott et al., 2005; Barrio et al., 2014; Brennecke et al., 2003; De Lella Ezcurra et al., 2016; Hammell et al., 2009; Ramírez et al., 2013; Teleman et al., 2006; Zhao et al., 2015). Typically, miRNAs silence the genes they target by preventing translation or degrading the transcript, although there are exceptions (Vasudevan et al., 2007). Accumulating evidence suggests that miRNAs regulate gene expression during diapause in flesh flies (Reynolds et al., 2013, 2017), mosquitoes (Batz et al., 2017) and killifish (Romney and Podrabsky, 2017) and are predicted to be part of a conserved molecular "toolkit" of mechanisms that regulate diapause in evolutionarily diverse species (Poelchau et al., 2013; Reynolds, 2017). It is likely that miRNAs regulate diapause in *H. zea* as well. The seven miRNAs we selected to evaluate in this study are either known to be associated with ecdysone signaling in other insects, or they are differentially regulated during and/or after diapause in pupae of the flesh

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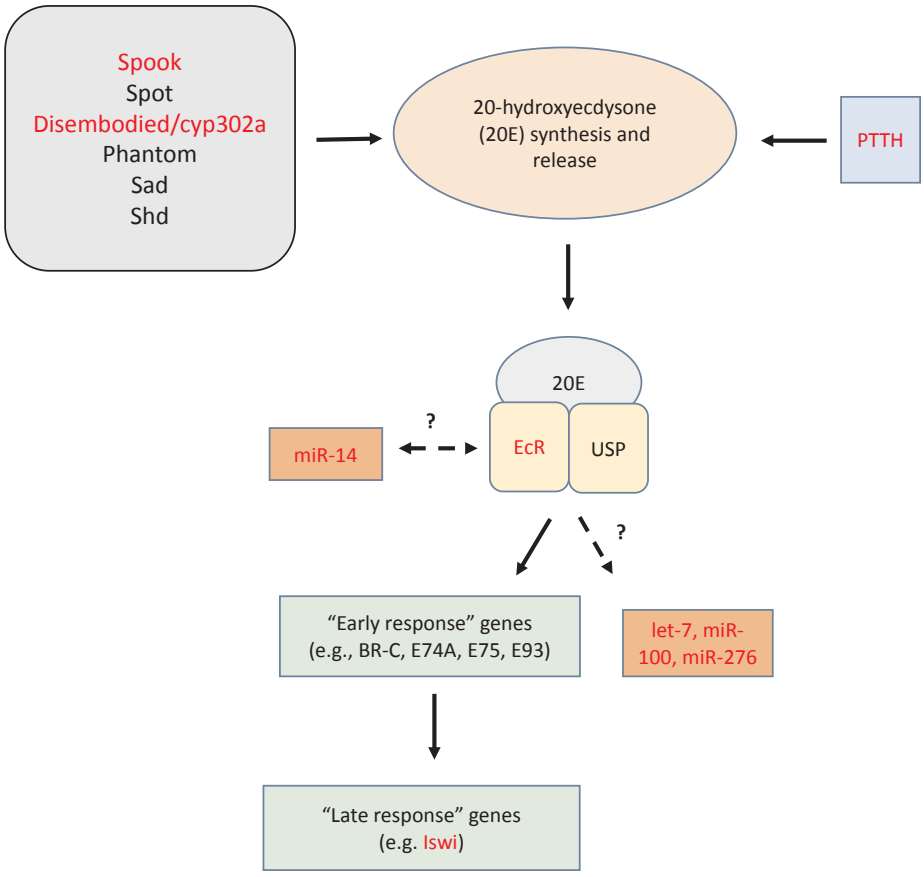

Fig. 1. Generalized summary of ecdysone biosynthesis and signaling in insects. Figure was adapted from Vellichirammal et al. (2017) to illustrate the putative relationships between candidate miRNAs and mRNAs evaluated in this study (see text for additional references). Genes and miRNAs evaluated in this study are in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fly, *Sarcophaga bullata*. MiR-14, let-7, and miR-100 regulate, or are regulated by, ecdysone signaling in *Drosophila melanogaster* (Chawla and Sokol, 2012; Chen et al., 2014; Jayachandran et al., 2013; Vargese and Cohen, 2007) or *Helicoverpa armigera*, a species closely related to *H. zea* (Jayachandran et al., 2013; Pearce et al., 2017). MiR-275, miR-277, miR-289, and miR-305-5p are differentially regulated in diapausing pupae of *S. bullata* (Reynolds et al., 2017).

We also evaluated changes in transcript abundance of the gene encoding DH and five additional genes that are related to ecdysone signaling including *ptth*, *cyp302A*, *spook*, *ecdysone receptor* (*EcR*), and *iswi*. *Cyp302A*, *spook*, *EcR*, and *iswi* are predicted targets of miRNAs evaluated in this study (Fig. 1). *Cyp302A* and *EcR* are putative targets of miR-14, and *spook* and *iswi* are putative targets of miR-289-5p (Jayachandran et al., 2013; Vargese and Cohen, 2007; Vlachos et al., 2015). Evaluating both miRNAs and their putative targets following diapause termination with ecdysone or DH provides a new tool for evaluating mechanisms involved in diapause regulation and its termination in *H. zea*, thereby increasing our understanding of miRNA regulation of gene expression in diapausing insects.

2. Materials and methods

2.1. Rearing and diapause induction

Helicoverpa zea larvae, purchased from Frontier Agricultural Sciences (Newark, Delaware USA), were reared as previously described (Zhang et al., 2008). Briefly, larvae were fed General Purpose Lepidoptera Diet (Frontier Sciences) and kept in individual cells to prevent cannibalism. The colony was maintained at 25 °C under a long day photoperiod (16 h light: 8 h dark). Adults were kept in 1 gallon plastic buckets with unlimited access to a 10% sucrose solution. Unbleached muslin was provided for oviposition, and first instar larvae were placed on diet within 24 h of hatching.

To induce diapause, third instar larvae were transferred to 18 °C and a short day photoperiod (8 h light: 16 h dark). Diapause incidence was assessed fourteen days after pupation as previously described (Zhang et al., 2008; Phillips and Newsome, 1966). Briefly, pupae were transferred from 18 °C to 25 °C for five days at which time we checked for eye spot placement, a diagnostic tool for evaluating diapause (Phillips and Newsome, 1966). Diapausing pupae were then kept at 18 °C until used in diapause termination experiments.

2.2. Termination of diapause with ecdysone, diapause hormone, or diapause hormone analog '1963'

20-hydroxyecdysone was purchased from Sigma (St Louis MO USA). Synthetic diapause hormone (DH, NDVKDGAASGAHSDRLGLWFG-PRL-NH_2) was synthesized by Genemed Synthesis, Inc (San Antonio TX). DH analog '1963', synthesized by Ron Nachman, is a fragment-analog of DH that has a ten-carbon, aliphatic hydrocarbon chain appended to the N-terminus via a succinyl linker (Decyl-NH-Suc-FTPRL-NH_2). It features enhanced biostability and bioavailability characteristics and can elicit termination of pupal diapause via injection in *H. zea* with an EC_{50} of 1.5 pmols/pupa. Unlike DH, the enhanced bioavailability/biostability characteristics of '1963' allow it to elicit termination of diapause by topical application to pupae of *H. zea* (Zhang et al., 2015). One nmole of either 20-hydroxyecdysone or DH, or 0.5 nmole of Decyl- '1963', a DH analog, in 5 µl of water was injected into each pupa as described (Zhang et al., 2008). The amount of hormone or analog applied was selected based on published diapause termination activity assays (Zhang et al., 2011; Zhang et al., 2015). A dose near the higher end of each effective range was used to insure a high incidence of diapause termination. Pupae were allowed to recover at 25 °C for 1 h, then maintained at 21 °C under a 12 h light: 12 h dark photoperiod. Survivorship was assessed beginning 1 d post-injection, and diapause status was assessed every 1–2 d beginning 2 d post-injection for up to

Table 1
Genes related to ecdysone signaling predicted with mirPath to be targets of candidate miRNAs.

GO Term	mirPath Predictions		
	Gene	miRNA	P-value
ecdysone receptor-mediated signaling pathway|GO:0035076	*iswi*	miR-289-5p	1.310421e−4
ecdysone biosynthetic process|GO:0006697	*disembodied/cyp302a*	miR-14-3p	7.371302e−5
	spook	miR-289-5p	7.371302e−5
ecdysone metabolic process|GO:0008205	*scully*	miR-277-3p	8.190536e−6

Table 2
Identification of ecdysone-related genes in *Helicoverpa spp.*

D. melanogaster Gene Name	*H. armigera* Ortholog	Sequence ID	Similarity to *D. melanogaster* protein sequence	
			E-value	% ID
disembodied/cyp302a	cytochrome P450 302a1	XP_021200922.1	5e−138	44
scully	3-hydroxyacyl-CoA dehydrogenase type-2	XP_021186997.1	5e−121	69
spook	cytochrome P450 CYP307A1	XP_021199680.1	0	53
iswi	chromatin-remodeling complex ATPase chain Iswi isoform X2	XP_021184577.1	0	88
ecdysone receptor, isoform A	ecdysone receptor isoform X7	XP_021181323.1	0	66

14 d.

2.3. Selection of candidate miRNAs and mRNAs

Candidate miRNAs were selected because published studies suggest they have a role in ecdysone signaling (Jayachandran et al., 2013; Vargese and Cohen, 2007; Vlachos et al., 2015) or they are differentially regulated following termination of pupal diapause in another insect (Reynolds, et al., 2017). Sequences used for evaluating miRNA abundance were taken from miRBase release 21 (Supplementary Table S1) (Kozomara and Griffiths-Jones, 2013).

Genes (mRNAs) were selected because published studies indicate they are targets of the miRNAs we evaluated or because they are identified as putative targets using DIANA mirPath v.3.0 (Table 1) (Vlachos et al., 2015). mirPath algorithms identify miRNA targets in *D. melanogaster* genes. The *H. zea* or *H. armigera* orthologs of predicted gene targets were found using *D. melanogaster* nucleotide sequences identified from Flybase (http://flybase.org) to perform blastx searches (Table 2) (Altschul et al., 1990).

2.4. RNA extraction and quantitative-reverse transcription PCR

RNA was isolated from treated and control individuals 24 h after injection, in the case of DH treatment, and on the first day that it was apparent diapause was successfully terminated, based on the position of the eye spots, as described above. For comparison, RNA was isolated from control individuals that were still in diapause 14 d after treatment. Total RNA was isolated from whole pupae using the mirVana kit (Life Technologies, Grand Island, NY USA) according to the manufacturer's directions. Individuals were pulverized in liquid nitrogen, and 50 mg of powdered tissue from a single individual was used for each replicate sample. RNA amount and purity (*i.e.* 260:280 ratio) were evaluated

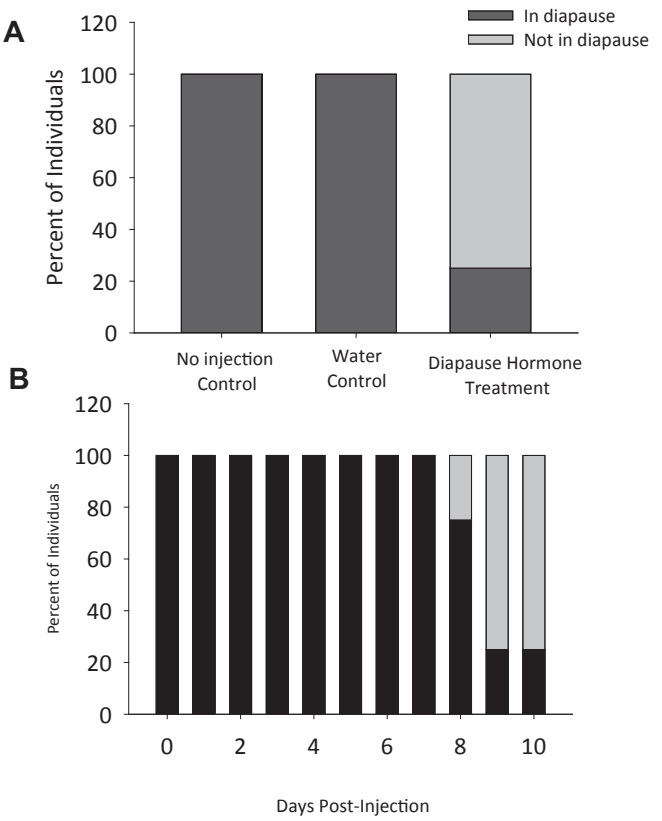

Fig. 2. Diapause termination in response to Diapause Hormone. A) Changes in diapause incidence in DH injected pupae 1 nmole compared to controls. B) Timing of diapause termination following injection. N = 10 individuals per group.

using a Nanodrop spectrophotometer (ThermoFisher Grand Island, NY). Reverse transcription was performed using a miScript Reverse Transcription kit (Qiagen Valencia, CA, USA) according to the manufacturer's directions for the HiFlex buffer, which allowed us to measure both mature miRNAs and mRNAs in the same sample. One microgram of total RNA was used for each replicate reaction. We did not include a no-RT control in this study because the mirVana kit uses uses an acid-phenol extraction method that removes the DNA, a feature that reduces/eliminates the need for DNase treatment or no-RT controls

Quantitative Reverse Transcript PCR (qRT-PCR) was used to measure abundance of miRNAs and mRNAs as previously described (Reynolds et al., 2013, 2017). MicroRNA abundance was measured using miScript primer assays (Qiagen) which use one miRNA specific primer and one universal primer, and miScript SYBR. mRNA abundance was measured using two gene specific primers that were designed using PrimerQuest software (Integrated DNA Technology, Skokie, Illinois USA) and LUNA Universal qPCR Master Mix (New England BioLabs, Inc. Ipswich, MA USA). Gel electrophoresis and melt curve analyses confirmed that all primer pairs used amplified single product. Standard curves using cDNA, diluted 5-fold, confirmed all primers conformed to MIQE standards for efficiency and performance (Bustin et al., 2010; see Supplementary Table S1). Relative abundance of each miRNA or mRNA was calculated using a modified $2^{-\Delta Ct}$ as previously described (Reynolds et al., 2013). Geometric means of RpS7 and 5s rRNA were used to normalize the Cts of miRNAs and mRNAs of interest. These RNAs were selected *a priori* from published qRT-PCR studies with *H. armigera* and *H. zea* (Yang et al., 2017; Zhang and Denlinger, 2012). Their suitability as reference genes for this study was evaluated based on the range of Ct values of all samples used for each study (*i.e.,* DH, Ecdysone, or '1963') and was confirmed using Pearson's Coefficient and One-way ANOVA (DH study) or Student's *t*-test (Ecdysone and '1963'

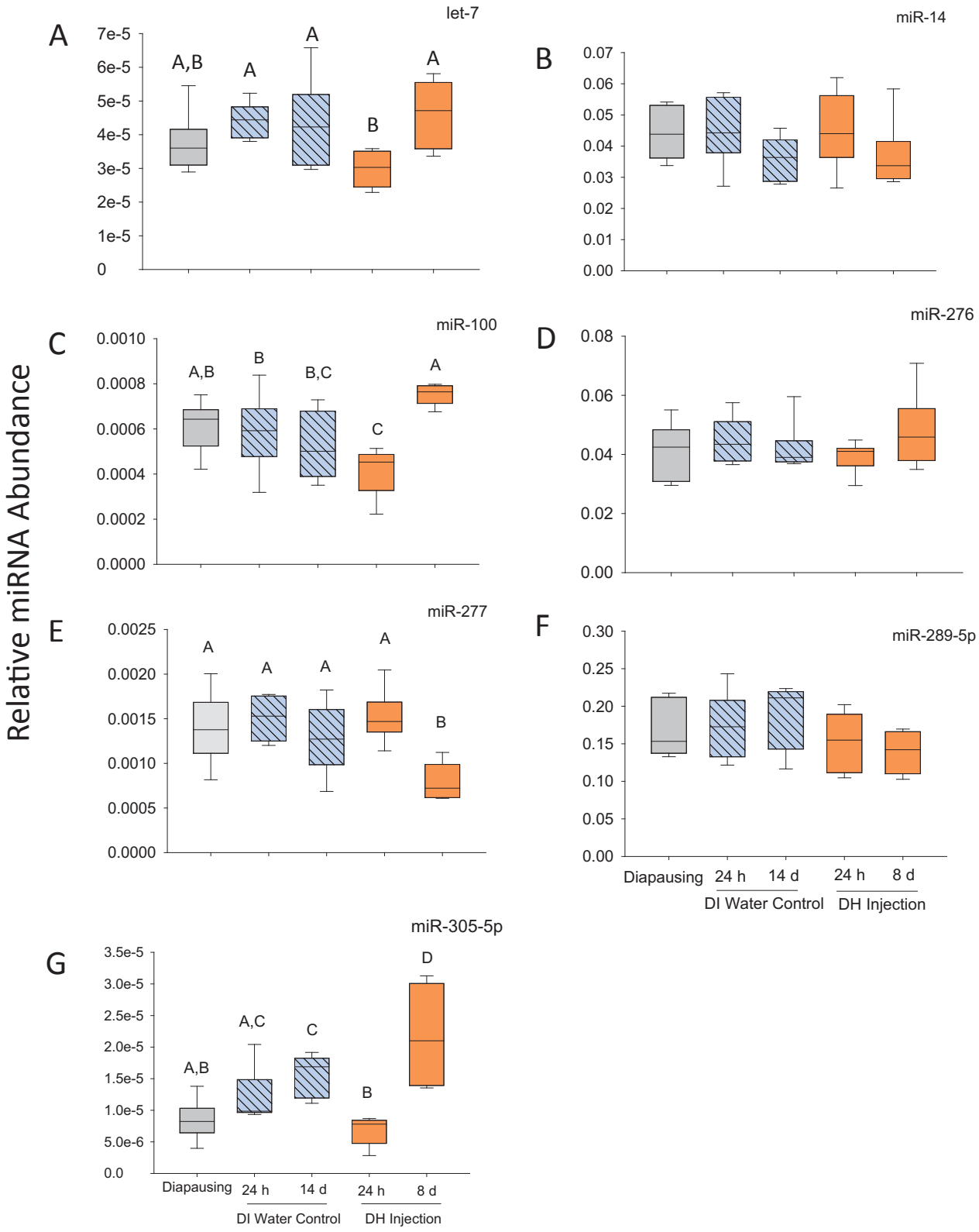

Fig. 3. Changes in miRNA abundance following diapause termination with diapause hormone. N = 6–8 individuals. Boxes show the 25th and 75th percentiles, error bars indicate the 10th and 90th percentiles, and the median is indicated with a line across the box. Boxes labeled with the same letter are not significantly different from each other (Fishers LSD $P < 0.05$).

studies). The stability of RpS7 and 5 s rRNA between samples is illustrated in Supplementary Fig. S1 and descriptive statistics are provided in Supplementary Tables S3–S5. In all studies, the geometric mean of RpS7 and 5s rRNA was considered an appropriate reference value if they met 2 of the three criteria: 1) the range of Ct values was less than 1; 2) the Pearson's coefficient was ≥ 0.05; and 3) the ANOVA or Student's *t*-test results were not significant.

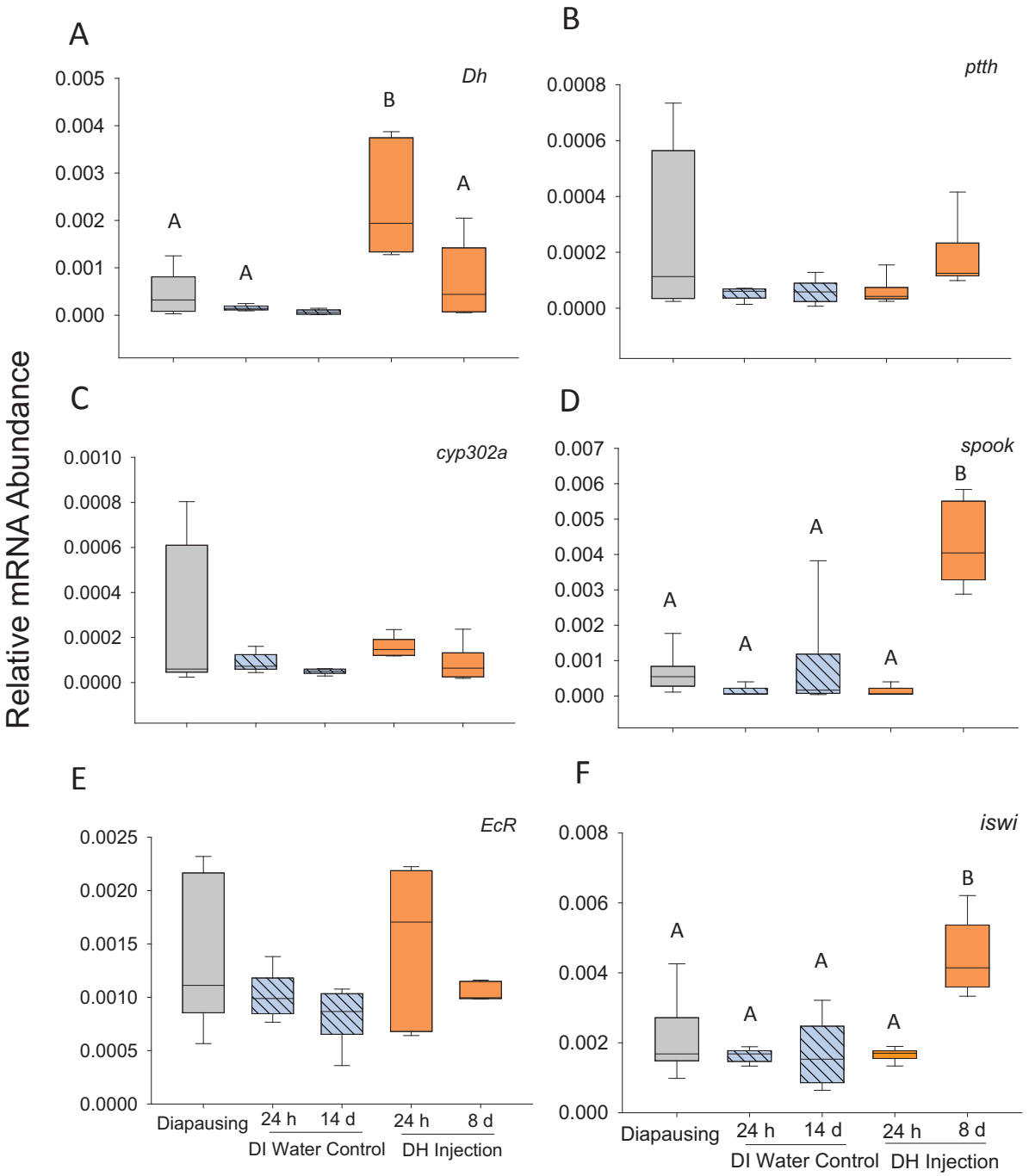

Fig. 4. Changes in mRNA abundance following diapause termination with diapause hormone. N = 6–8 individuals. Boxes show the 25th and 75th percentiles, error bars indicate the 10th and 90th percentiles, and the median is indicated by the line across the box. Boxes labeled with the same letter are not significantly different from each other (Fisher's LSD $P < 0.05$).

2.5. Statistical analysis

Statistical analysis was performed with Minitab 17 (State College, PA USA). One-way ANOVA, followed by Fisher's LSD test, was used to evaluate differences between groups in the study evaluating the response to DH injections. Student's *t*-test was used to evaluate the response to 20-hydroxyecdysone or '1963' injections. P-values were adjusted using False Discovery Rate (FDR) calculations (Benjamini and Hochberg. 1995) to prevent type-1 errors (*i.e.* rejection of a true null hypothesis). Mood's Median test was used to evaluate differences as needed to further evaluate ambiguous Student's *t*-test results.

3. Results

3.1. Changes in miRNA and mRNA abundance following diapause termination with synthetic diapause hormone

Injecting synthetic diapause hormone (1 nmole) into diapausing pupae terminated diapause in 75% of individuals compared to 0% in pupae injected with water or non-injected controls (Fig. 2A). Diapause termination, as indicated by migration of the eye spots, was first apparent 8 d following injection, with the majority terminating diapause 9 d post-injection (Fig. 2B).

Four of seven miRNAs evaluated were differentially regulated

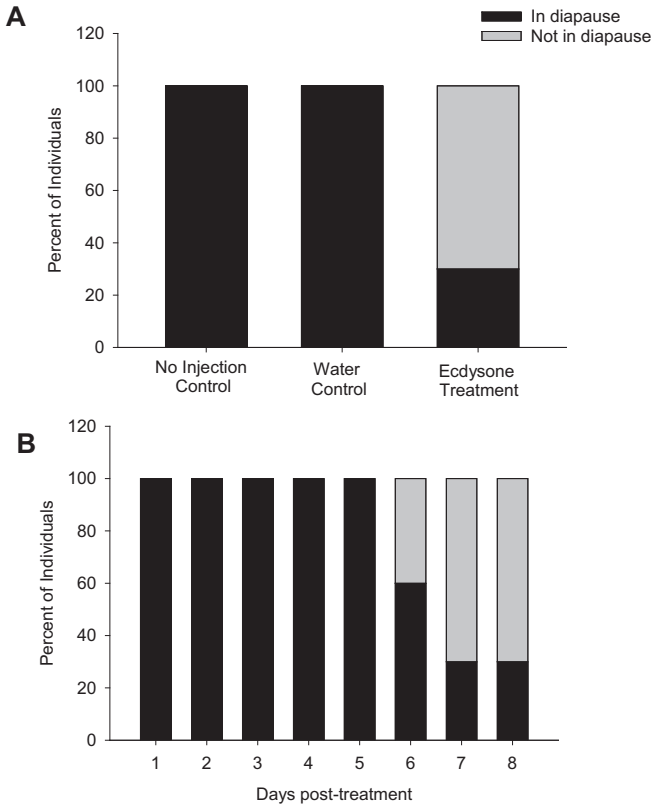

Fig. 5. Diapause termination in response to 20-hydroxyecdysone. A) Changes in diapause incidence following injection of 1 nmole, 20-hydroxyecdysone compared to controls. B) Timing of diapause termination following injection. N = 10 individuals for each treatment or control.

following DH injections to terminate diapause (Fig. 3). Let-7 was downregulated 24 h after DH-injection (P = 0.002, Fig. 3A). The abundance of miR-100-5p (P = 0.005, Fig. 3B) appeared higher 8 d after DH injection, but the difference was not significant (Fisher's LSD α = 0.05). MiR-277-3p (P = 0.003; Fig. 3E) was downregulated ~50% 8 d post-DH injection, and miR-305-5p was significantly upregulated 8 d post-injection (P < 0.001, Fig. 3G). There was no change in miR-14 (P = 0.266, Fig. 3C), miR-276 (P = 0.546, Fig. 3D), or miR-289-5p (P = 0.286, Fig. 3F).

Transcript abundance of the gene encoding diapause hormone (*dh*) as well as genes related to ecdysone production (*ptth, spook, and cyp302a*) or ecdysone function (*EcR* and *iswi*) were also measured following treatment with synthetic diapause hormone (Fig. 4). Three of these were differentially regulated following diapause termination. *Dh* was upregulated 24 h following DH injection (P < 0.001, Fig. 4A). *Spook* and *iswi* were upregulated 4-fold (P = 0.019, Fig. 4D) and 2-fold (P < 0.001, Fig. 4F), respectively. There was no significant change in *ptth* (P = 0.062; Fig. 4B), *cyp302a* (P = 0.131, Fig. 4C), or *EcR* (P = 0.193; Fig. 4E).

3.2. Changes in miRNA and mRNA abundance following diapause termination with ecdysone

Injecting 20-hydroxyecdysone (1 nmole) into diapausing pupae terminated diapause in 70% of treated individuals compared to 0% in non-injected controls and pupae injected with water (Fig. 5A). In response to 20-hydroxyecdysone, diapause termination was first apparent 6 d post-injection, with additional individuals terminating diapause on day 7 (Fig. 5B). Thus, diapause termination in response to 20-hydroxyecdysone was noted several days earlier than diapause termination in response to DH.

Because non-injected and water-injected controls showed no difference in our previous experiments (Figs. 1 and 2), we limited our evaluations in this case to comparisons of miRNA abundance in non-injected controls (*i.e.*, diapausing pupae) and post-diapause pupae 7 d post-treatment. This is the day when diapause termination was first apparent, based on eye-spot migration. Only miR-289-5p was differentially regulated following ecdysone injection (P = 0.042, Fig. 6E). There was no change in the abundance of miR-14 (P = 0.976, Fig. 6A), miR-100 (P = 0.976, Fig. 6B), miR-276 (P = 0.976, Fig. 6C), miR-305-5p (P = 0.976, Fig. 6F). There appeared to be a 2-fold downregulation in miR-277-3p (Fig. 6D), but the difference was not significant when evaluated using a Student's *t*-test with FDR correction for multiple comparisons (P = 0.198, Fig. 6D). There was however, a significant difference when the same data was evaluated with Mood's Median Test (P = 0.005). Considering the nearly 2-fold difference in the average abundance of miR-277-3p between treated and control groups, we think it likely that this is a type-II error, and there is a biologically meaningful change in miR-277-3p abundance in spite of the non-significant P-value.

Four of six genes related to ecdysone production were upregulated following injection of 20-hydroxyecdysone (Fig. 7). *Dh* was upregulated 2-fold (P = 0.036), *EcR* increased ~3.5-fold (P = 0.024), *spook* increased 5.5-fold (P = 0.036), and *iswi* increased 3-fold (P = 0.027). There was not a significant change in *ptth* (P = 0.117) or *cyp302a* (P = 0.064).

3.3. Changes in miRNA and mRNA abundance following termination with DH analog, '1963'

DH analog '1963' is a fragment-analog of DH that features an appended hydrophobic moiety at its N-terminus, leading to enhanced bioavailability and biostability characteristics that allow it to elicit termination of diapause in *H. zea* pupae either via injection or topical application (Zhang et al., 2015). Injecting 0.5 nmole into diapausing pupae terminated diapause in 83% of the pupae, compared to 0% in non-injected controls (Fig. 8A). In response to '1963', diapause was first apparent 6 d post-injection, with additional individuals terminating diapause over the next four days (Fig. 8B). Thus, the timeline for the response was, in some ways more similar to ecdysone than to DH, but the total time required for all individuals to terminate diapause was longer for '1963' than for either ecdysone or DH

As with experiments using 20-hydroxyecdysone, we limited our evaluation to comparisons between diapausing (*i.e.* non-injected) individuals and individuals that had clearly terminated diapause based on eye-spot migration. Following diapause termination with '1963' there were significant decreases in miR-14-3p (P = 0.007), miR-276-3p (P = 0.046), and miR-277-3p (P = 0.007). There were not significant changes for let-7 (P = 1), miR-100 (P = 1), miR-289-5p (P = 0.068) or miR-305-5p (P = 1) (Fig. 9).

Only one gene related to ecdysone signaling was differentially regulated following injection of '1963' (Fig. 10). *Iswi* was upregulated ~2-fold (P = 0.012). There was no significant change in *ptth* (P = 0.271), *dh* (P = 0.233), *EcR* (P = 0.093); or *cyp302a* (P = 0.578). *Spook*, which appeared to be upregulated by 2.5-fold was not significantly different when evaluated using an FDR corrected Student's *t*-test (P = 0.116), but was significantly different when median values were compared using Mood's Median test (P = 0.016). We think it likely that this represents a type-II statistical error, and the increase in *spook* is biologically relevant.

4. Discussion

The complex phenotype of diapause is regulated by networks that include neuropeptides, steroid hormones such as 20-hydroxyecdysone, and in the case of adult diapause, the isoprenoid juvenile hormones (Denlinger et al., 2012). Pupal diapause of the corn earworm,

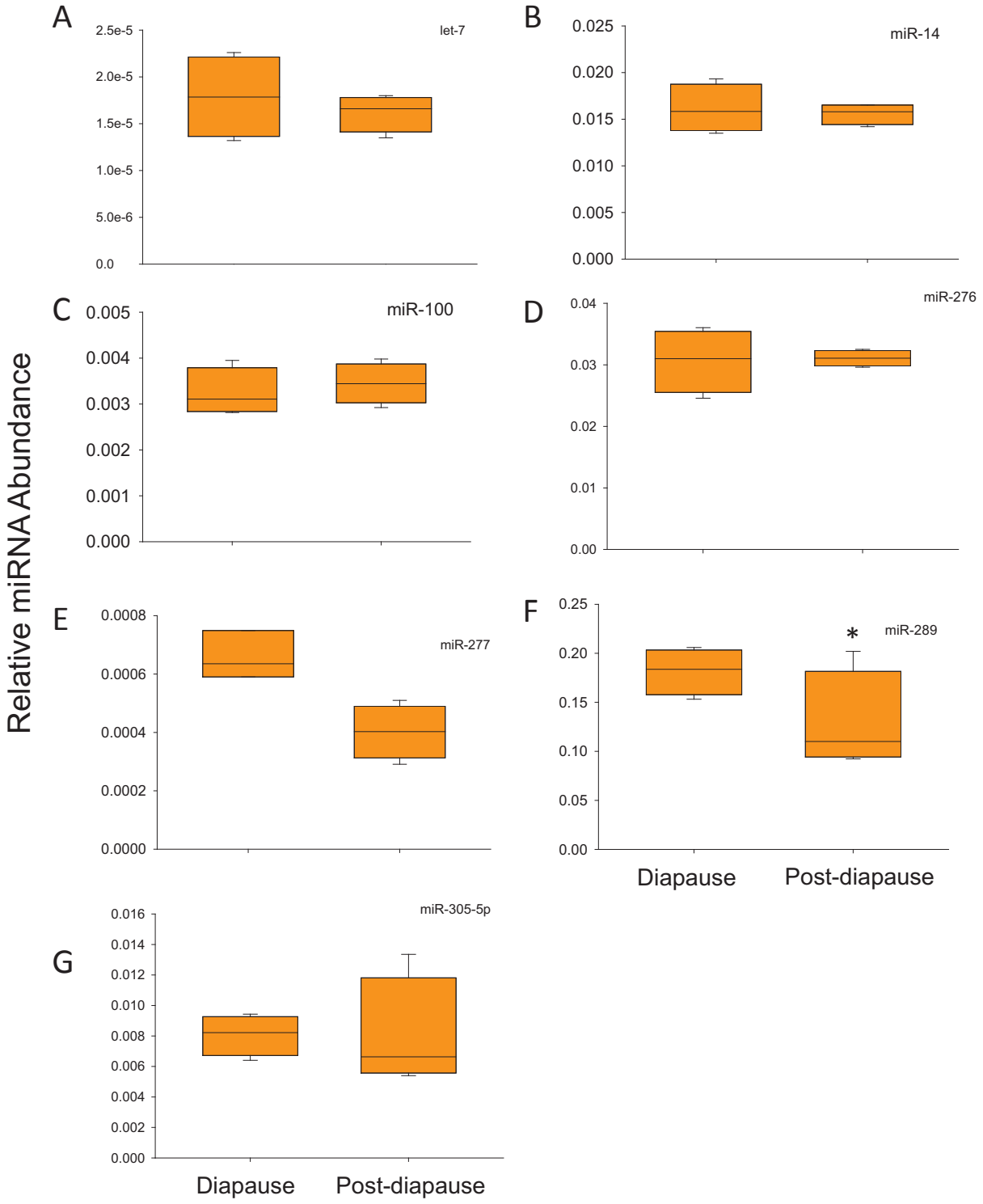

Fig. 6. Changes in miRNA abundance following diapause termination with 1 nmole of 20-hydroxyecdysone. N = 4. Boxes show the 25th and 75th percentiles, error bars indicate the 10th and 90th percentiles, and the median is indicated with a line across the box. Bars that are significantly different from each other are marked with "*".

Helicoverpa zea, can be terminated with injections of ecdysone, synthetic diapause hormone, or diapause hormone analogs, but whether all of these agents operate through the same molecular pathways remains unknown. Our current experiments asked whether all three of these diapause terminators elicit a common miRNA response. Our results show that abundance of one of the miRNAs, miR-277-3p, is decreased in response to all three diapause terminating agents, while the response of other candidate miRNAs varies depending on the agent used to terminate diapause. MiR-14-3p and miR-276a-3p were downregulated following diapause termination with the DH analog '1963' but not with ecdysone or DH. MiR-100 and miR-305-5p were downregulated only after diapause termination with DH, but not with ecdysone or '1963'. MiR-289-5p was downregulated when diapause was terminated with ecdysone or '1963', but not with DH. We also monitored expression of genes known to be in the ecdysone signaling pathway to determine if all three diapause terminators elicited the same response. Again, we found

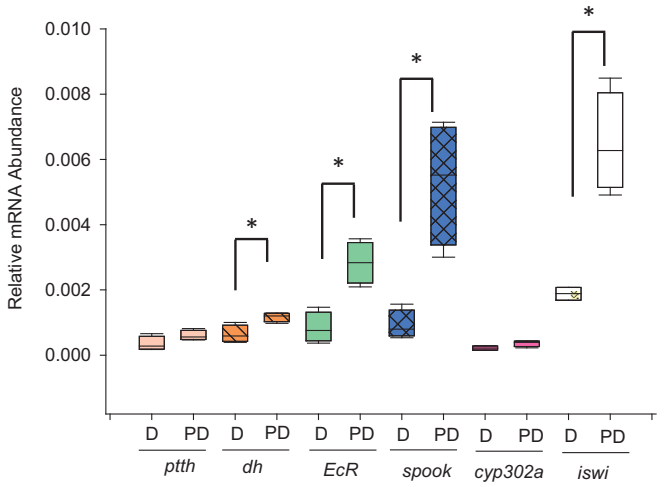

Fig. 7. Changes in the mRNA abundance following diapause termination with 20-hydroxyecdysone. Transcript abundance was evaluated in diapausing (D) and post-diapause (PD) pupae of *H. zea* 7 d following ecdysone injections. N = 4.

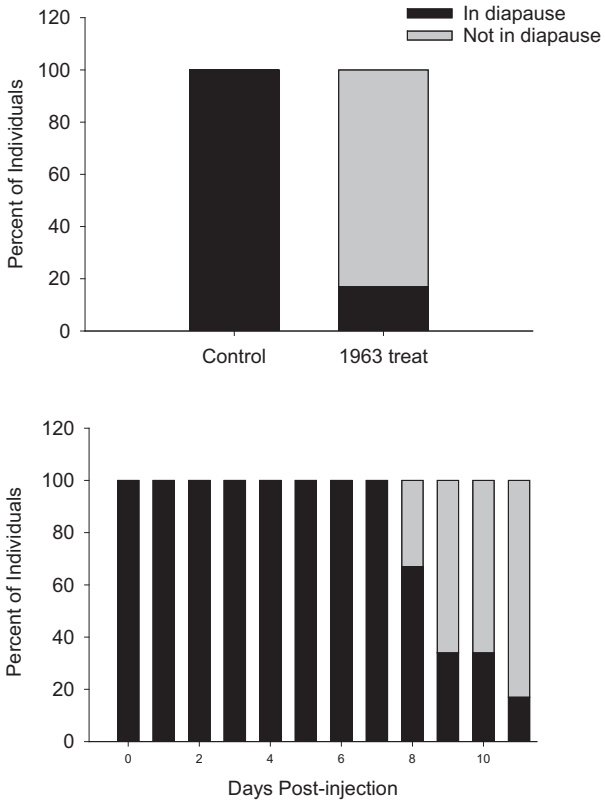

Fig. 8. Diapause termination with Diapause Hormone analog, '1963'. A) Changes in diapause incidence following injection of 0.5 nmol of '1963'. B) Timing of diapause termination. N = 6 individuals per treatment or control.

treatment-specific changes in transcription of candidate genes in the ecdysone signaling pathway. Only *iswi* was upregulated following all three treatments. The *dh* transcript was significantly upregulated when ecdysone and DH were used to terminate diapause, but not in response to '1963'. *Spook*, which was upregulated 4 to 5-fold in response to ecdysone or DH, was upregulated ~2-fold following treatment with '1963'. We are uncertain whether this increase is statistically, or biologically, meaningful, but it is clear that the upregulation of this gene in the ecdysone biosynthesis pathway is less pronounced when '1963' was used to terminate diapause. *EcR* was upregulated following diapause termination with ecdysone, but not with DH or '1963'. There were no changes in expression of either *ptth* or *cyp302a1* in response to any of the diapause terminators.

The significance of these changes in miRNA and mRNA abundance and their contribution to diapause termination and/or post-diapause development has yet to be experimentally validated, but we can infer function based on published studies examining *H. zea* and *H. armigera, D. melanogaster*, and other insects.

In *H. armigera*, a close relative of *H. zea* (Pearce et al., 2017), miR-14-3p regulates the gene encoding Ecdysone receptor (*EcR*) (Jayachandran et al., 2013), and in *D. melanogaster,* miR-14-3p and EcR form an autoregulatory feedback loop (Vargese and Cohen, 2007). Thus, we expected to find significant changes in both miR-14-3p and *EcR* following diapause termination. Surprisingly, we saw a significant decrease in miR-14-3p only when '1963' was used to terminate diapause, and *EcR* was upregulated only when ecdysone was used to terminate diapause. As a result, it is difficult to conclude whether miR-14-3p regulates *EcR* in *H. zea* in the context of diapause termination. Additional studies, especially sequencing the 3′ UTR of the *EcR* gene from *H. zea* will be needed to more adequately interpret these puzzling results.

We were also surprised to find there was little, or no, change in abundance of miRNAs in the let-7 complex (*i.e.* let-7, miR-100, and miR-125) following diapause termination in *H. zea* because let-7 is upregulated following diapause termination in pupae of *S. bullata* (Reynolds et al., 2017) and is regulated by ecdysone in multiple insect species, including *D. melanogaster* and *Bombyx mori* where it is required for the transition from larval to adult life stages (Chen et al., 2014; Ling et al., 2017; Thummel 2001). In *B. mori*, let-7 abundance is highest following a peak of ecdysone, and inhibiting let-7 leads to developmental arrest (Ling et al., 2014). In *D. melanogaster*, let-7 is required for neuromuscular remodeling during metamorphosis, and inhibiting let-7 causes neuromuscular and behavioral defects (Chawla and Sokol, 2012). Considering the importance of let-7 to developmental timing and metamorphosis, and its association with ecdysone, we expected to find upregulation of let-7 following diapause termination. Possibly we failed to see a significant change in let-7 abundance due to the timing of our sampling, but an alternative explanation is that let-7-5p, which was evaluated in this study, does not have analogous functions in *D. melanogaster* and *H. zea*. Chawla and Sokol (2012) reported that let-7 was highly expressed during pupal development in *D. melanogaster*, but let-7-5p abundance was quite low in both diapausing and nondiapausing pupae of *H. zea* (*i.e.* the raw Ct value was ~ 28). Possibly the functional, mature miRNA in *H. zea* is derived from the 3′ arm of the miRNA precursor rather than the 5′ arm as it is in *D. melanogaster*, a phenomena known as "arm switching" (Griffiths-Jones et al., 2011) and a feature that can have significant implications for the evolution of miRNA function.

Though most changes in miRNAs abundance that we noted depended on the agent used to terminate diapause, miR-277-3p was substantially downregulated in all three groups, thus offering one common theme observed with all of the diapause terminators. The functional significance of miR-277-3p downregulation during diapause termination/post-diapause development is unclear because of a general lack of published studies on this miRNA. We are not aware of experimental evidence suggesting that miR-277-3p is linked to the ecdysone signaling pathway, but *scully*, a 3-hydroxyacyl-CoA dehydrogenase that has a role in ecdysone metabolic processing (Shafqat et al., 2003), may be targeted by miR-277-3p, based on the mirPath target prediction program (Vlachos et al., 2015). We did see a change in abundance of *scully* following diapause termination with DH (see Supplementary Fig. S2), but those changes were not correlated with changes in miR-277-3p abundance, suggesting that upregulation of this gene may be a response to the injection rather than to DH, or miRNA-277 abundance, *per se*.

A likely role for miR-277-3p in diapause termination of *H. zea* is

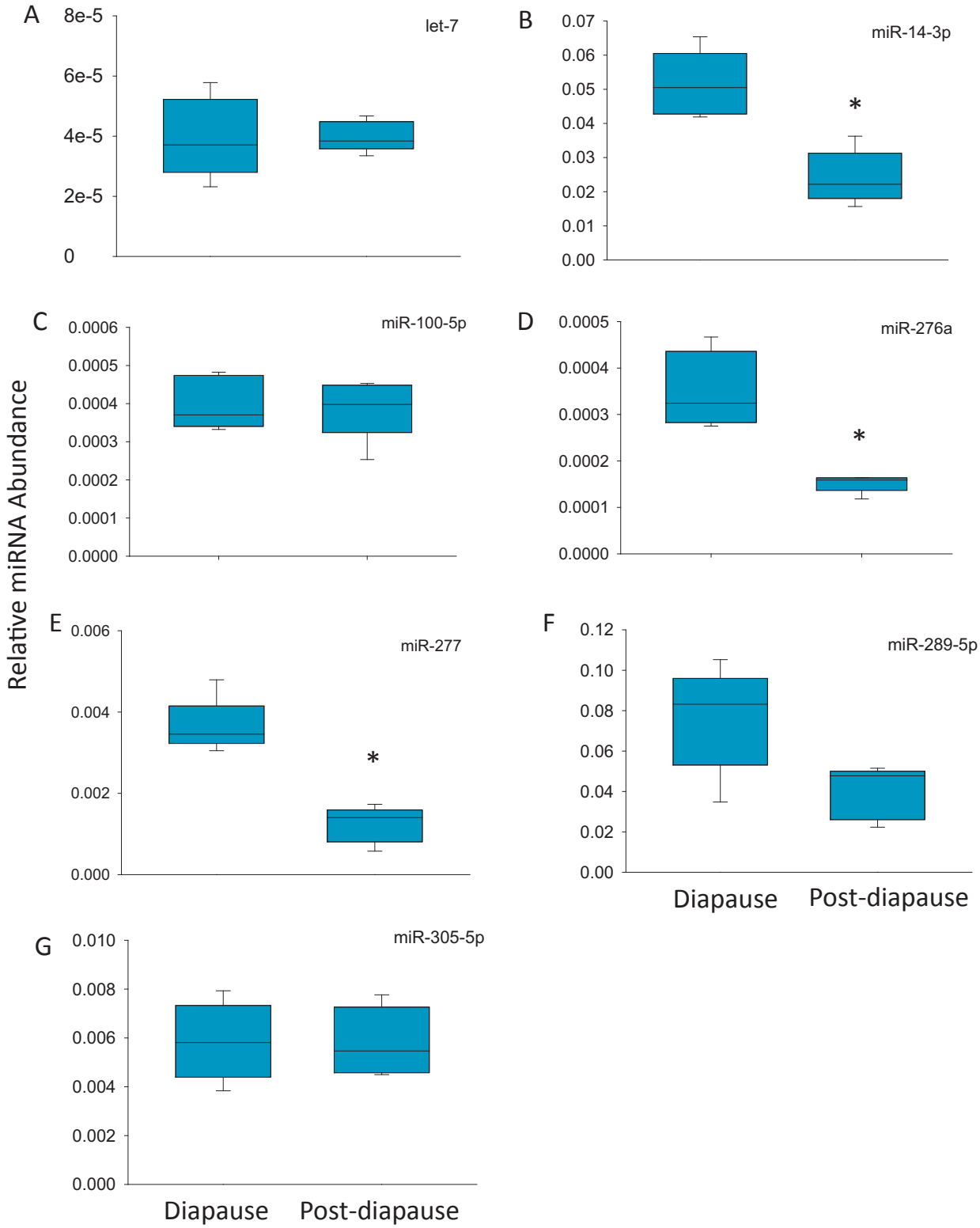

Fig. 9. Changes in miRNA abundance following diapause termination with DH analog '1963'. N = 5. Boxes show the 25th and 75th percentiles, error bars indicate the 10th and 90th percentiles, and the median is indicated with a line across the box. Bars that are significantly different from each other are marked with "*".

regulation of insulin signaling and activity of the FOXO transcription factor. Changes in insulin signaling are an integral part of diapause in multiple insect species (Ragland and Keep; 2017; Sim and Denlinger, 2013), and diapause in *H. armigera* is characterized by a reduction in insulin-like peptides (Ilps) (Zhang et al., 2017). In the mosquito, *Aedes aegypti*, miR-277-3p directly targets two genes encoding Ilps, and knockdown of miR-277-3p upregulates insulin signaling, increases the nuclear export of FOXO, and reduces lipid storage in the fat body (Ling et al., 2017). We expect to find that miR-277-3p has a similar role in *H. zea*, but experimental evidence is needed to confirm that this function of miR-277-3p is conserved.

As with miRNAs, changes in the transcription of genes related to

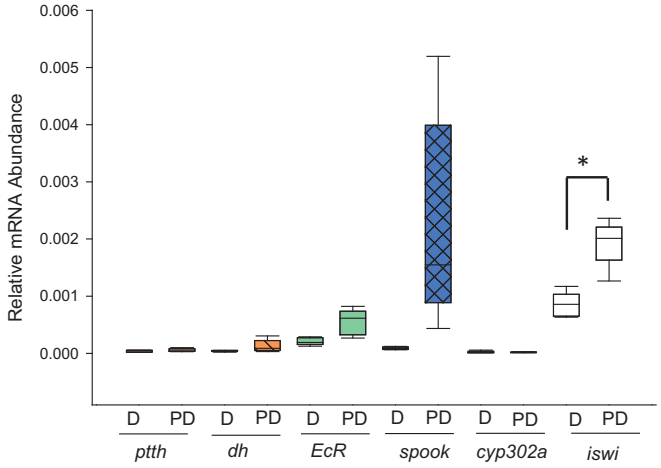

Fig. 10. Changes in mRNA abundance following diapause termination with DH analog '1963'. N = 5 Boxes show the 25th and 75th percentiles, error bars indicate the 10th and 90th percentiles, and the median is indicated with a line across the box. Bars that are significantly different from each other are marked with "*".

ecdysone signaling depended on the agent used to terminate diapause. With the exception of *ptth*, all genes evaluated were upregulated in at least one group. Notably, *spook,* a Halloween gene in the ecdysone biosynthesis pathway (Ono et al., 2006; Vellichirammal et al., 2017; Zheng et al., 2017) was upregulated up to 5-fold following treatment with ecdysone and DH, but only increased ~2-fold with '1963'. This suggests that, as we would expect for pupal diapause (Denlinger et al., 2012), increased ecdysone is critical for diapause termination and post-diapause development, at least for the two naturally-occurring diapause terminators, ecdysone and DH.

Iswi was the only gene upregulated following diapause termination in response to all three diapause terminators. *Iswi* is a "late-response" gene that both depends on ecdysone (Ables and Drummond-Barbosa, 2010) and is also a putative target of miR-289-5p. Thus, upregulation in expression of this gene post-diapause is likely the result of increased ecdysone, downregulation of miR-289-5p, or a combination of the two. *Iswi* encodes an ATPase component of the ISWI/NURF chromatin remodeling complex in *D. melanogaster* and other insects and is an important regulator of chromatin structure and gene expression in both males and females (Corona et al., 2007; Deuring et al., 2000). ISWI is required for oogenesis in *D. melanogaster,* a role that is conserved in evolutionarily diverse insects including Lepidoptera (Ables and Drummond-Barbosa, 2010; Carter et al., 2013; Fishilevich et al., 2016). Thus, upregulation of *iswi* in *H. zea* is likely important for post-diapause development of germline stem cells in both males and females.

We were surprised to observe discrepancies between the response to DH peptide and '1963' since these likely target the same PK-like receptors. One possible explanation is that differences in miRNA and mRNA abundance following injection with DH and '1963' are not directly related to diapause termination but are, instead, off-target effects that result from these peptides binding to other PK-like, or even, PRXamide receptors. Whether '1963' can bind to, and activate, FXPRLamide receptors in *H. zea* is currently unknown. However, in the red flour beetle, *Tribolium castaneum*, PK-like analogs, such as '1963' do show cross-activity with PRXamide receptors (Jiang et al., 2015). Possibly PK-like receptors can give rise to changes in miRNA abundances that are not directly related to diapause termination, a question deserving future examination. For now, we postulate that miRNAs and mRNAs activated by DH, '1963', and ecdysone reflect the minimum RNA activity required for diapause termination.

A final objective of this study was to compare changes in miRNA abundance following diapause termination in *H. zea* with what is known in other diapausing species. When pupal diapause is terminated with hexane in the flesh fly, *S. bullata*, there is a significant increase in let-7, a decrease in miR-289-5p, and no change in miR-305-5p (Reynolds et al., 2017). We saw similar changes in miR-289-5p and miR-305-5p in *H. zea*, but not with let-7 abundance. These results suggest some similarities in miRNA regulation of diapause and post-diapause development across species, at least for moths and flies that have a pupal diapause. As more insect studies are completed, it will be interesting to see if these same miRNA responses are linked to diapause in evolutionarily diverse species.

Taken together, the results we report here indicate that changes in miRNA and related mRNA abundance occur in response to diapause termination and post-diapause development. Although certain miRNA responses were noted in multiple species and in response to different hormonal signals, it is also abundantly clear from these results that distinct miRNA responses can be elicited by both natural and synthetic agents that are all capable of generating the same end point, the breaking diapause. Such results underscore the fact that diverse endocrine pathways can lead to diapause termination, a challenge that currently makes it difficult to create a simple and unified molecular model for diapause termination that can accommodate the diverse observations noted in *H. zea*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.09.013.

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