MicroRNAs Influence Reproductive Responses by Females to Male Sex Peptide in *Drosophila melanogaster*

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SUPPORTING INFORMATION

File S1
(A) Description of the determination of expression levels of miRNAs chosen for investigation from a preliminary small RNA sequencing screen of responses of miRNAs to SP receipt (T. Rathjen, H. Pais, C.J. Pennington, S. Moxon, T.D. & T.C., unpublished data). **(B)** qRT-PCR validation methods of the small RNA sequencing results for two of the miRNAs chosen for testing in this study.

Table S1 Normalised counts of expression of the four miRNAs chosen for investigation.

Figure S1 Summary scheme of the direction of expression in the four selected miRNAs in response to receipt of SP.

Figure S2 qRT-PCR validation of expression level changes in miRNAs following receipt of SP, for two of the miRNAs chosen for investigation in this study.

File S2
Analysis of egg to adult survival of the miRNA lines.

Figure S3 Egg-to adult survival for the miRNA-lacking females in the 24 hr intermating interval following a single mating to either a SP-lacking (SP^0) or a control male (SP^+).

Figure S4 Northern blot with probes for (A) *mir-184*, (B) *mir-278D,* (C) U6.

Figure S5 Quantitative PCR. Mean ± SE relative expression of mir-279 and mir-317 in the w^{1118}, Canton-S and the *mir-279C* and *mir-317C* hypomorph lines**.**

Supporting Information literature cited

File S1

Expression levels of miRNAs chosen for investigation – from a preliminary screen of responses of miRNAs to SP receipt (T. Rathjen, H. Pais, C.J. Pennington, S. Moxon, T.D. & T.C., unpublished data).

(A) Small RNA sequencing: Fly samples were prepared and Total RNA was extracted as in (Gioti *et al*. 2012). miRNA expression data were generated from 2 replicates each (from the total of 4 described in Gioti *et al*. (2012)) for Head+Thorax (HT) and Abdomen (Abd) samples from wild type females, at 3 hr post mating to either SP^0 null or control SP^+ males (Liu and Kubli 2003). Total RNA was isolated, using the mirVana™ kit (Ambion), following the manufacturer's protocol for total RNA (TRNA) isolation. Quality and quantity of the isolated RNA were verified using a spectrophotometer and by gel electrophoresis. miRNA assays were performed using a service provider (BaseClear). cDNA libraries of the short RNA fractions were generated as described in Szittya *et al.* (2008) and sequenced on the Illumina GA2 platform. All sRNA FASTQ files were first converted to FASTA format and adapter sequences were removed by trimming sequences with exact matches to the first eight bases of the 3' adapter (only this 3' adapter sequence is read). Any sequences without adapter matches or shorter than 16 nucleotides after processing were excluded. Processed sRNA reads were mapped to the *D. melanogaster* genome (release 5.9; Drysdale *et al*. 2008). This mapping was performed using PatMaN (Pruefer *et al*. 2008) allowing only perfect ungapped alignment of sRNAs to the reference genome. After pre-preprocessing, reads were mapped to the sequences of known miRNAs (miRbase v.13.0, Griffiths-Jones *et al*. 2008) using miRProf (Moxon *et al*. 2008). For each sample the number of reads assigned to each miRNA was normalized to the total number of reads mapping to at least one miRNA in that sample. Mapping of Illumina deep sequencing data against the database of known miRNAs (miRbase, v13.0) identified 75 expressed miRNAs. 28 Abd and 29 HT miRNAs showed evidence of signal:noise >1. Of these, the top 15 provided some basis for further investigation, with a final estimate of 5-10 miRNAs of interest in each body part. Expression levels for the four candidate miRNA loci chosen for investigation in this study are shown in Table S1.

(B) Validation of small RNA sequencing by quantitative RT-PCR: qRT-PCR was used to validate the differences in miRNA expression detected in the small RNA sequencing screen described above (C.J. Pennington & T.C. unpublished data). We used Applied Biosystems (Warrington, UK) probesets to confirm differential expression in response to SP in two of the miRNAs chosen for investigation: miR-184 and miR-279. Probes were used according to the manufacturer's instructions given in the TaqMan small RNA Assays Protocol (Applied Biosytems). 10 ng RNA was used per reaction and tests were done on all four biological replicate samples of (Gioti *et al*. 2012). Two of these replicates were subjected to the small RNA sequencing described above. Expression values for the miRNAs were determined using a standard curve method and normalized to the expression level of miR-2s. The probe target sequences used were: miR-184 (FBgn0262391, uggacggagaacugauaagggc); miR-279 (FBgn0262448, ugacuagauccacacucauuaa); miR-2S (tgcttggactacatatggttgagggttgta).

Table S1 Normalized counts of expression of the four miRNAs chosen for investigation here, from the preliminary screen of responses of miRNAs to SP receipt (T. Rathjen, H. Pais, S. Moxon, C.J. Pennington, T.D. & T.C., unpublished data). Shown are the expression levels of all four miRNAs in two replicates (rep 1, rep 2) of female Head+Thorax (HT) and Abdomen (Abd) samples, 3 hr after mating with SP^0 null or control SP^+ males. Log2 fold change values are shown (SP^+ / SP^0) along with the direction of change in expression in response to receipt of SP. Shown in grey are the body parts in which there was no consistent pattern of differential expression in response to receipt of SP.

HT	SP null 1	SP null 2	SP+ 1	SP+ 2	Fold change, rep 1 (log2 SP^+/SP^0)	Fold change, rep 2 (log2 SP^+/SP^0)	Direction of change in response to SP receipt in both replicates
miR-317	1,988	259	98	152	-4.35	-0.77	down
miR-279	24	21	5	5	-2.36	-2.07	down
miR-278	71	94	72	34	0.01	-1.45	-
miR-184	95,814	22,076	11,721	20,001	-3.03	-0.14	down

Abd	SP null 1	SP null 2	SP+ 1	SP+ 2	Fold change, rep 1 (log2 SP^+/SP^0)	Fold change, rep 2 (log2 SP^+/SP^0)	Direction of change in response to SP receipt in both replicates
miR-317	330	391	307	719	-0.10	0.88	-
miR-279	77	110	44	49	-0.80	-1.16	down
miR-278	34	79	140	104	2.06	0.40	up
miR-184	259749	130127	54091	200971	-2.26	0.63	-

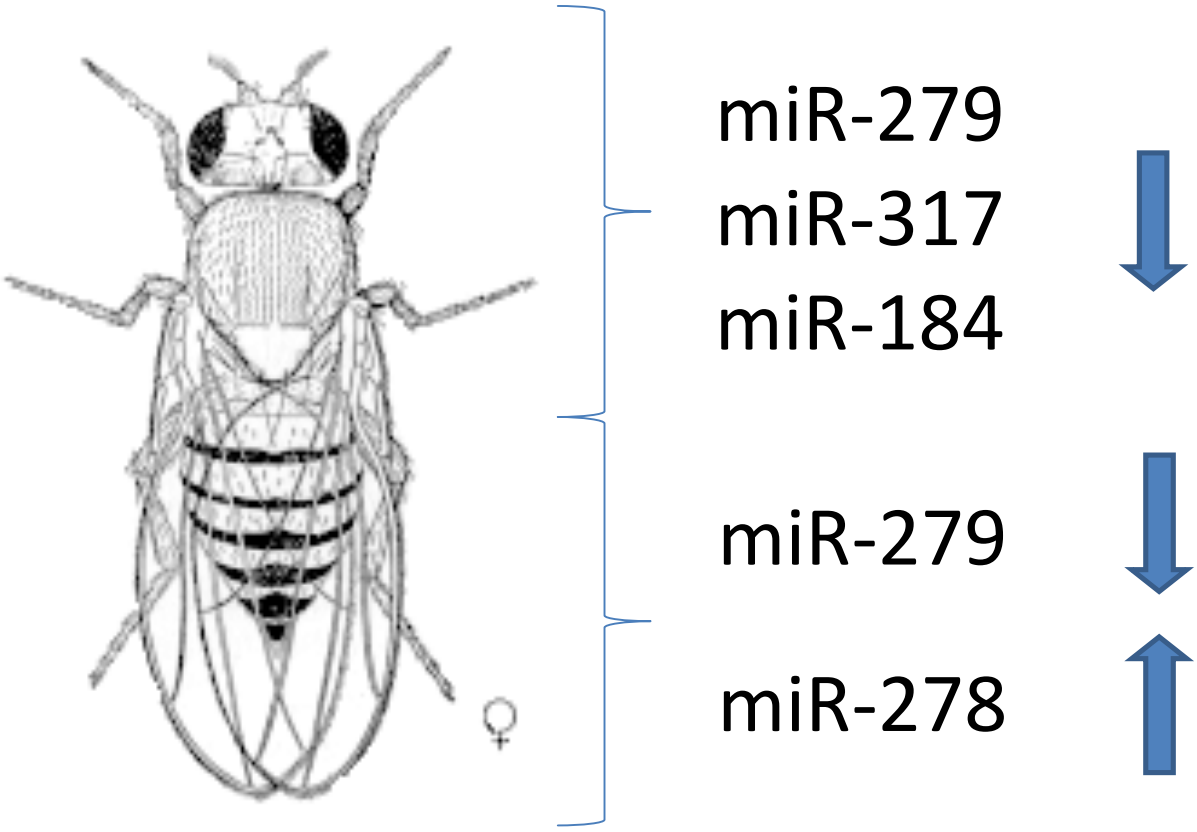


Figure S1 Summary scheme of the direction of expression change in the four selected microRNAs in female Head+Thorax and Abdomen samples 3 hr following receipt of sex peptide from males during mating.

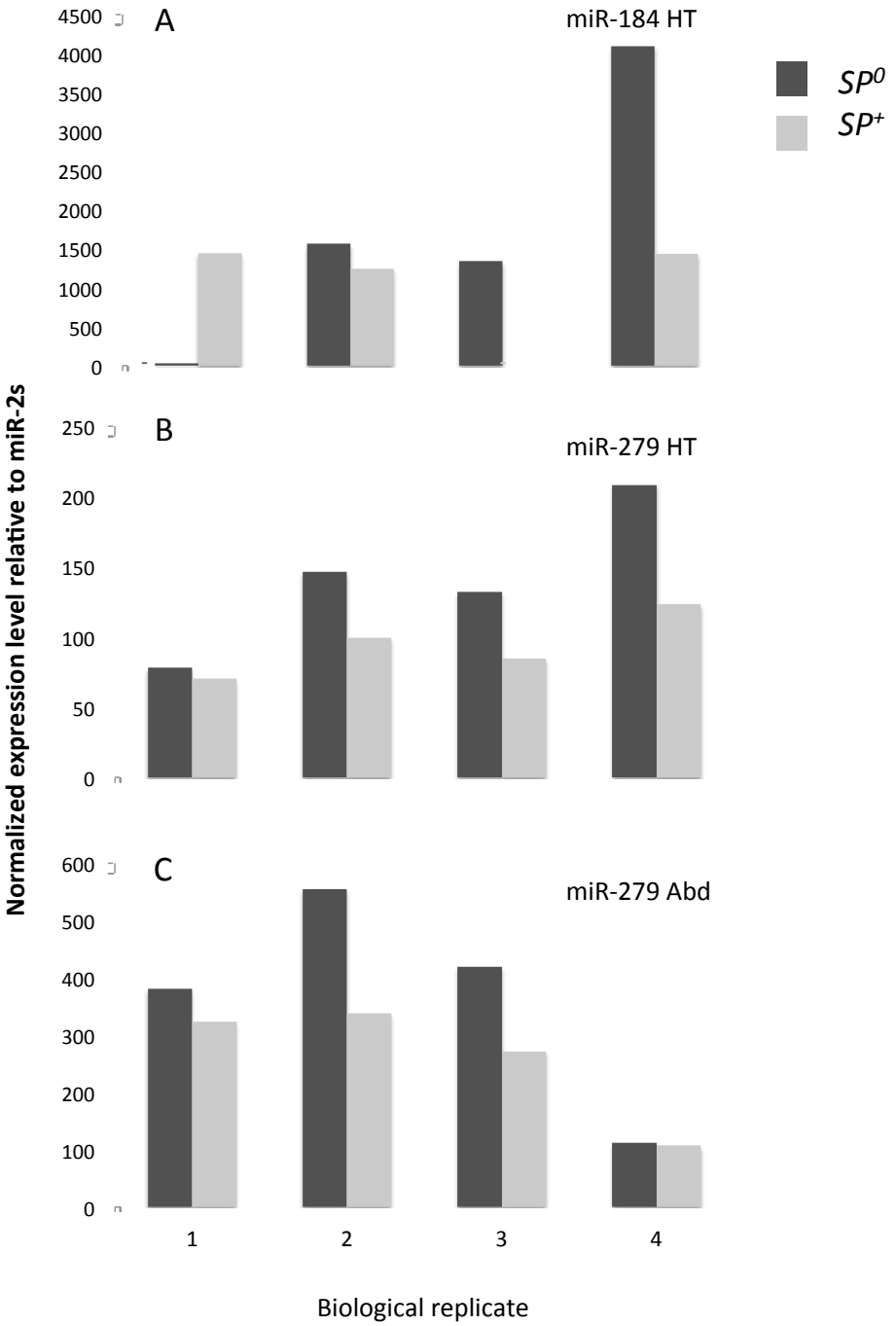

A
miR-184 HT
B
miR-279 HT
C
miR-279 Abd
SP^0
SP^+
Normalized expression level relative to miR-2s
4500
4000
3500
3000
2500
2000
1500
1000
500
0
250
200
150
100
50
600
500
400
300
1
2
3
4
Biological replicate

Figure S2 qRT-PCR validation of expression level changes in microRNAs following receipt of SP, for two of the miRNA loci investigated in this study. Shown are the normalized expression levels 3hr following SP receipt, for (A) miR-184 in the HT, (B) miR-279 in the HT, and (C) miR-279 in the Abd. Normalized expression, relative to miR-2s, derived from qRT-PCR is shown for 4 replicates of females mated to SP^0 or SP^+ males. Individual replicate data are shown to illustrate the consistent down regulation of miR-184 and miR-279 in response to SP receipt, regardless of overall miRNA expression level variation. The small RNA sequencing described in Supporting Information 1 was conducted on 2 each of the above replicate samples (replicates 3 and 4 (HT); 2 and 4 (Abd), respectively). The qRT-PCR results validated the down regulation of miR-184 (in 3/4 replicates) and miR-279 (in 8/8 replicates) observed in the small RNA sequencing data.

File S2

Analysis of egg to adult survival of the miRNA lines.

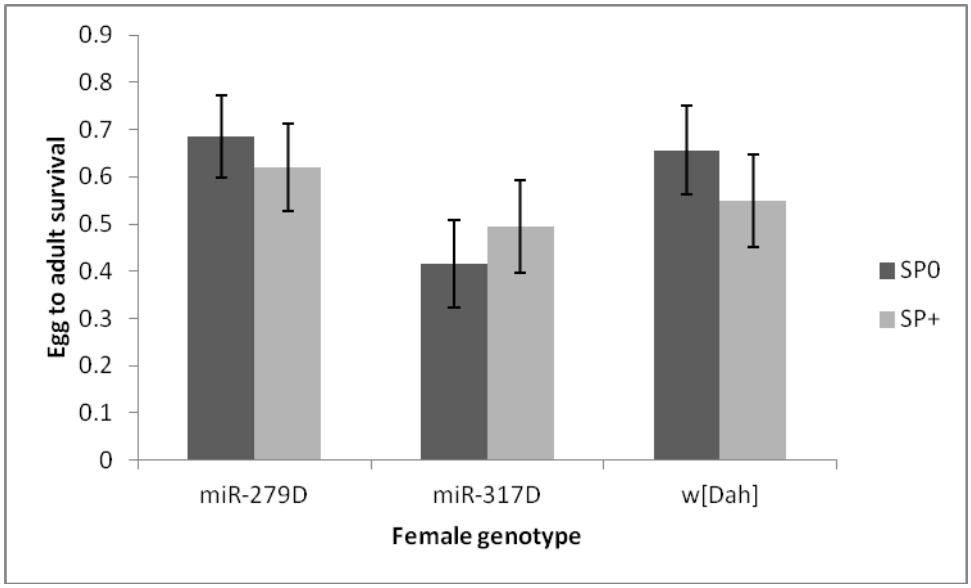
mir-279D and mir-317D hypomorphs The two Dahomey-background miRNA-hypomorph lines and their control $w^{[Dah]}$ did not differ significantly in egg to adult survival ($G^2 = 65.31$, $F_{1,157} = 2.94$, $P = 0.056$ [Dispersion parameter = 11.20]; Figure S3A).

mir-279C and mir-317C hypomorphs The two $w^{[CS]}$-background miRNA hypomorph lines differed significantly from their control in egg to adult survival ($G^2 = 137.82$, $F_{2,117} = 19.61$, $P < 0.0001$ [Dispersion parameter = 3.52]). *mir-279C* females showed the lowest egg to adult survival. Furthermore, egg-adult survival was lower in females mated to SP^+ control males compared to SP^0 males ($G^2 = 25.97$, $F_{1,116} = 7.39$, $P = 0.008$, interaction = ns; Figure S3B).

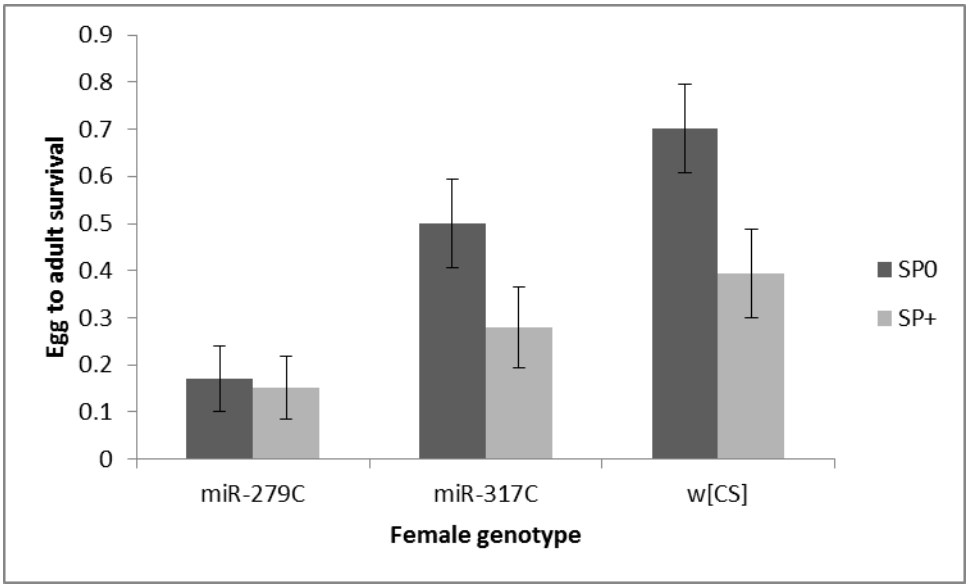
mir-278D ko *mir-278D* ko females had a tendency to exhibit lower egg to adult survival than did control females ($G^2 = 24.77$, $F_{1,114} = 3.32$, $P = 0.071$ [Dispersion parameter = 7.53]). This was independent of the male with which they mated ($G^2 = 0.54$, $F_{1,114} = 0.07$, $P = 0.789$, interaction = ns; Figure S3C).

mir-184 **ko** *mir-184* ko females had very low egg to adult survival (Female genotype: $G^2 = 1140.40$, $F_{1,75} = 82.31$, $P < 0.0001$ [Dispersion parameter = 6.85]). Control females showed no difference in egg to adult survival according to male genotype (male x female genotype: $G^2 = 99.76$, $F_{1,74} = 14.55$, $P = 0.0003$; male genotype: $G^2 = 7.48$, $F_{1,75} = 0.54$, $P = 0.465$; Figure S3D).

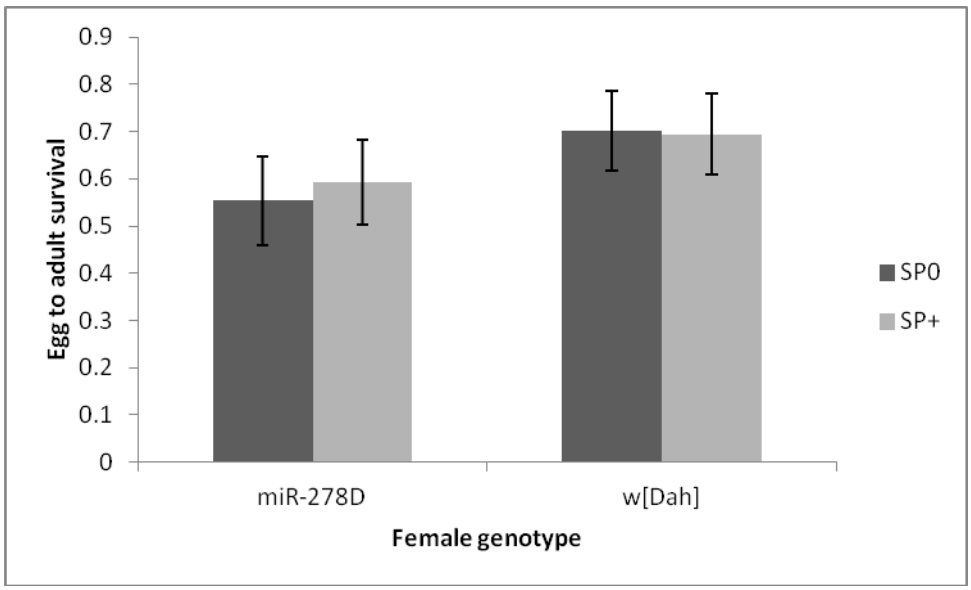
A


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Egg to adult survival
miR-279D
miR-317D
w[Dah]
Female genotype
SP0
SP+

B


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Egg to adult survival
miR-279C
miR-317C
w[CS]
Female genotype
SP0
SP+

C



D

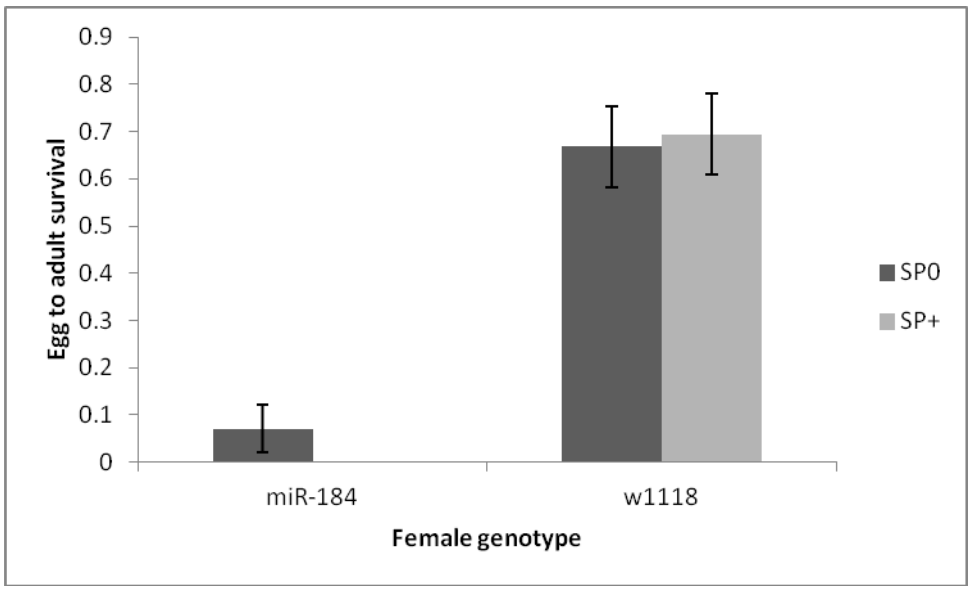


Figure S3 Egg-to adult survival (mean ± SE) for the miRNA-lacking females in the 24 hr intermating interval following a single mating to either a SP-lacking (SP^0) or a control male (SP^+): (A) hypomorphic *mir-279D* and *mir-317D* females backcrossed into the wild-type $w^{[Dah]}$ genetic background, (B) hypomorphic *mir-279C* and *mir-317C* females backcrossed into $w^{[CS]}$, (C) *mir-278D* knock out females in $w^{[Dah]}$ and (D) *mir-184* knock out females in the w^{1118} background.

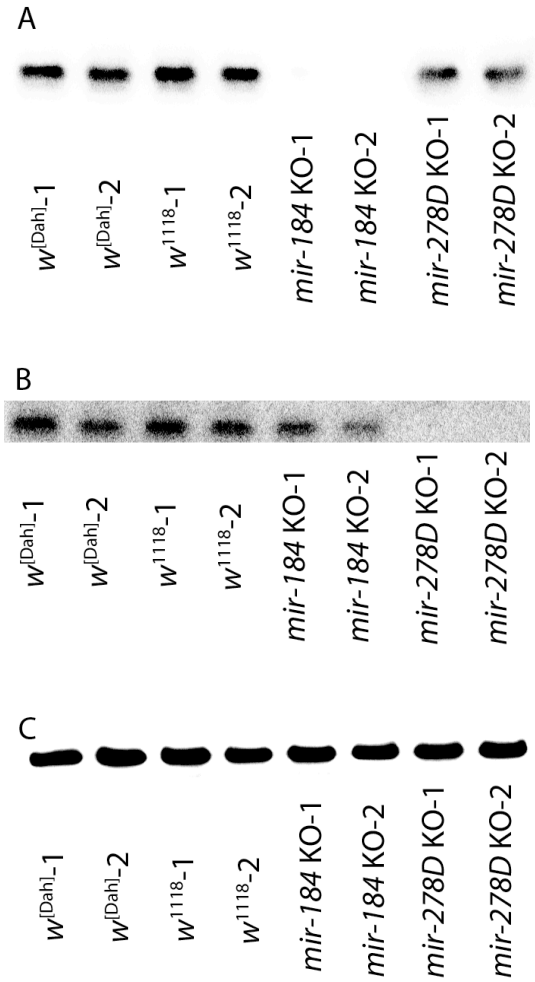


Figure S4 Northern blot with probes for (A) *mir-184*, (B) *mir-278D,* (C) U6 control. The miRNA knock out (KO) lines showed no expression of the relevant miRNA, but no difference in expression level relative to the control lines for the other miRNA and the U6 control RNA. w^{1118} is the control for the *mir-184* knock out and $w^{[Dah]}$ is the control for the *mir-278* knock out.

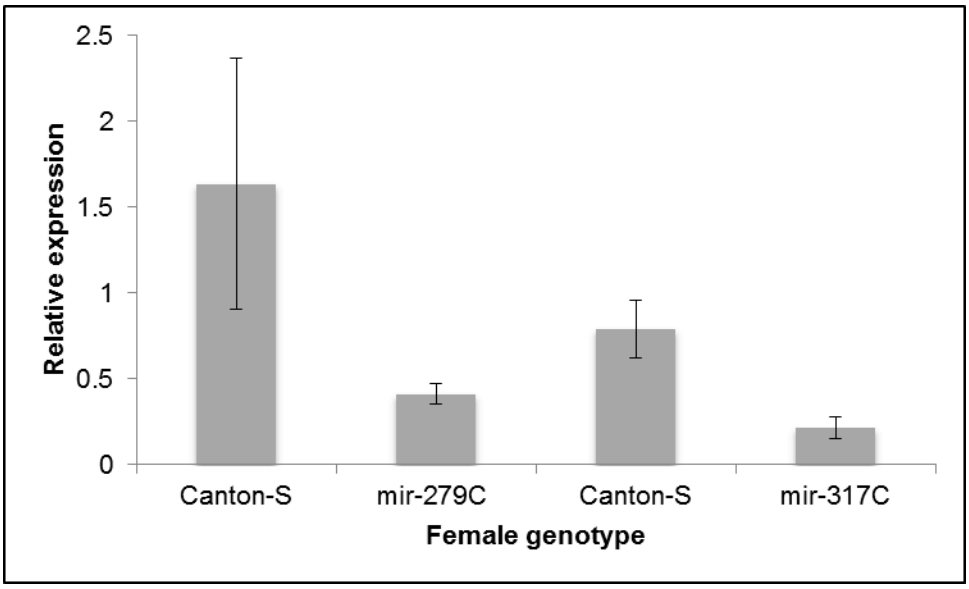


Figure S5 Mean ± SE relative expression of *mir-279* and *mir-317* in the control w^{1118}, Canton-S and the *mir-279C* and *mir-317C* hypomorph lines. The miRNA hypomorphs had significantly lower expression than the control line. We assayed the Canton-S line twice so that the qRT-PCR run for each miRNA mutant line was conducted in parallel to the control line. The miRNA hypomorph lines had significantly lower expression than their controls (Wilcox rank sum, $W = 31$, $P = 0.041$ for *mir-279* and $W = 33$, $P = 0.015$ for *mir-317*). The standard curves for the miRNA qPCR probes ran from 0.5-0.00016 ng total RNA with a 1:5 serial dilution and had the following R^2 and slope values*: miR-279*: $R^2 = 0.9866$, slope = -2.6378*; mir-317*: $R^2 = 0.9828$, slope = -2.4508*; mir-2S*: $R^2 = 0.9942$, slope = -3.2352.

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