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

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ABSTRACT Across taxa, female behavior and physiology change significantly following the receipt of ejaculate molecules during mating. For example, receipt of sex peptide (SP) in female *Drosophila melanogaster* significantly alters female receptivity, egg production, lifespan, hormone levels, immunity, sleep, and feeding patterns. These changes are underpinned by distinct tissue- and time-specific changes in diverse sets of mRNAs. However, little is yet known about the regulation of these gene expression changes, and hence the potential role of microRNAs (miRNAs), in female postmating responses. A preliminary screen of genomic responses in females to receipt of SP suggested that there were changes in the expression of several miRNAs. Here we tested directly whether females lacking four of the candidate miRNAs highlighted (miR-279, miR-317, miR-278, and miR-184) showed altered fecundity, receptivity, and lifespan responses to receipt of SP, when mated once or continually to SP null or control males. The results showed that miRNA-lacking females mated to SP null males exhibited altered receptivity, but not reproductive output, in comparison to controls. However, these effects interacted significantly with the genetic background of the miRNA-lacking females. No significant survival effects were observed in miRNA-lacking females housed continually with SP null or control males. However, continual exposure to control males that transferred SP resulted in significantly higher variation in miRNA-lacking female lifespan than did continual exposure to SP null males. The results provide the first insight into the effects and importance of miRNAs in regulating postmating responses in females.

REPRODUCTION is a fundamental biological process and it is well established that mating itself initiates a multitude of physiological and behavioral postmating changes in females. Insights into the gene expression changes underlying mating have been gained from studies in the fruit fly *Drosophila melanogaster* (Lawniczak and Begun 2004; McGraw *et al.* 2004; Mack *et al.* 2006; Innocenti and Morrow 2009). *D. melanogaster* males transfer not only sperm in their ejaculates, but also up to ~130 different seminal fluid peptides (Sfps) (Findlay *et al.* 2008; Ayroles *et al.* 2011). Many of the striking postmating responses of females to mating are mediated by the effects of these Sfps (Chapman 2001; Gillot 2003; Ram and Wolfner 2007a; Wolfner 2009). It is therefore of significant, fundamental interest to understand the detailed mechanisms underlying the profound reprogramming in gene expression that occurs in females due to Sfp receipt. Through this understanding, it will be possible to determine (i) how the extensive changes required to effectively coordinate reproduction are regulated and (ii) to what extent these processes are shaped by sexual selection and sexual conflict.

Our knowledge of the phenotypes and functions of individual Sfps in *D. melanogaster* is rapidly increasing (Herndon and Wolfner 1995; Neubaum and Wolfner 1999; Tram and Wolfner 1999; Mueller *et al.* 2007; Ram and Wolfner 2007a; LaFlamme *et al.* 2012). One well-characterized Sfp, on which we focus in this study, is the so-called "sex peptide" (SP). SP has significant effects on a range of important fitness-related traits in females. It significantly increases egg production and decreases female receptivity to remating (Chapman *et al.* 2003b; Liu and Kubli 2003), increases food uptake (Carvalho *et al.* 2006), alters nutrient balancing (Ribeiro and Dickson

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2010), and increases the expression of antimicrobial peptides (Peng *et al.* 2005). It also inhibits female siesta sleep (Isaac *et al.* 2010), alters water balance (Cognigni *et al.* 2011), and is involved in regulating sperm release from the storage organs (Avila *et al.* 2010). The sex peptide receptor has been identified, and it is expressed in the female genital tract and central nervous system (Soller *et al.* 2006; Yapici *et al.* 2008; Rezaval *et al.* 2012).

The fitness effects of SP in males and females appear to represent hallmarks of interlocus sexual conflict (Rice 1998; Chapman *et al.* 2003a; Arnqvist and Rowe 2005), in that repeated receipt of SP exacerbates the survival cost of mating in females (Wigby and Chapman 2005), while simultaneously increasing a male's "per mating" offspring production (Fricke *et al.* 2009). It has been hypothesized that males and females are locked into a cycle of antagonistic coevolution over the phenotypic effects of SP and the female's physiological responses to it. Understanding the mechanisms underlying female genomic responses to Sfps such as SP is therefore of great interest in revealing the sophisticated chemical communication between the sexes at mating. It also represents an excellent starting point to understand whole genome responses to sexual conflict and the potential role of Sfps in driving sexually antagonistic coevolution between the sexes.

Transcriptome studies have provided significant insights into the extensive genome-wide changes in gene expression that together form a synchronized response to mating and to Sfp receipt (McGraw *et al.* 2004; Mack *et al.* 2006; Domanitskaya *et al.* 2007 Innocenti and Morrow 2009). Such studies have also sought to characterize potential genomic signatures of sexual conflict (Gioti *et al.* 2012). To date (McGraw *et al.* 2004; Mack *et al.* 2006; Gioti *et al.* 2012) expression profiles have been conducted at different levels of resolution—from whole organisms (McGraw *et al.* 2004) to tissue-specific profiles (Mack *et al.* 2006)—and from total responses to mating (Mack *et al.* 2006), to courtship, to ejaculate receipt, and to specific Sfps (McGraw *et al.* 2008; Gioti *et al.* 2012). For example, McGraw *et al.* (2004) found that many genes were differentially expressed after mating. The fold changes involved were generally quite modest, which led the authors to suggest that females are "poised" to mount rapid responses to mating and hence maintain a set of mRNAs to facilitate this. McGraw *et al.* (2004) showed that distinct subsets of genes alter their expression in response to receipt of sperm *vs.* seminal fluid proteins. However, despite this, the post-transcriptional regulatory mechanisms that modulate these gene expression changes and ultimately result in the observed phenotypic changes have not been considered in any detail. In this study, we focused on the role of such post-transcriptional gene regulation in females in mediating postmating responses.

As noted above, initial insights into the underlying genomic signatures of sexual conflict mediated by SP in particular have also been gained from gene expression studies (Gioti *et al.* 2012). Genome-wide responses in females revealed widespread tissue and time-specific changes in many categories of genes (Gioti *et al.* 2012). Gene expression changes in response to SP in the head + thorax were more varied and dynamic than in the abdomen, in which genes were mainly down-regulated. In the head + thorax, genes involved in a number of biological processes (*e.g.*, the TOR pathway regulating nutrient sensing and genes involved in phototransduction) were differentially regulated, while in the abdomen, egg and early embryo development genes were overrepresented. This study supported the idea that substantial changes to female physiology can occur after mating due to receipt of a single Sfp and that these changes are spatially and temporally dynamic (McGraw *et al.* 2008). The potential for manipulation of females by males via SP is therefore widespread and thus hard for females to sidestep or "ignore," even if the effects of SP are costly to females (Wigby and Chapman 2005).

The effective interpretation and comparison of the results of gene expression studies such as those described above requires careful consideration of biological and technical differences as well as of general biases in sequencing methodologies (Van Dijk *et al.* 2014). One important factor can be tissue specificity. For example, Mack *et al.* (2006) compared their postmating gene expression profiles in the *D. melanogaster* lower female genital tract to McGraw *et al.* (2004)'s whole organism expression patterns and found little overlap. At least part of the explanation is likely to be that signatures of local tissue-specific changes are often swamped by whole organismal responses (Chintapalli *et al.* 2007). Another factor is the timing of sampling, which can drive divergence in transcript levels either due to mRNA expression *per se* or because of differences in the level of post-transcriptional control over time. Gioti *et al.* (2012) found genes to be mainly down-regulated in the abdomen following receipt of SP, whereas Mack *et al.* (2006) observed mostly gene activation in the lower genital tract following mating. Mack *et al.* (2006)'s parallel measures of the associated protein expression changes revealed a general pattern of down-regulation in 84 proteins, and, though mRNA transcripts were found for the majority of the differentially expressed proteins, there was little correspondence between up-/down-regulation of mRNA *vs.* its protein. This discrepancy between gene and protein expression is important, as it suggests that there is significant post-transcriptional regulation in the coordination of female postmating responses. It is the existence of such post-transcriptional regulation that we investigated in this study.

Our knowledge of the nature of post-transcriptional regulation has been revolutionized over the last two decades. It has been realized that there is a huge influence of small noncoding RNAs on the regulation of transcription and translation. MicroRNAS (miRNAs) are perhaps the best studied class of noncoding RNAs to date and have been identified as post-transcriptional master regulators of gene expression, typically for at least one-third of genes (Filipowicz *et al.* 2008). miRNAs are ~22 nt in length and mediate translational repression and/or mRNA degradation. They show

deep evolutionary conservation and are involved in many, if not all, biological processes (Filipowicz *et al.* 2008). miRNAs have been identified in many insects and their importance in regulating developmental processes, cell growth, and proliferation, ageing as well as host–pathogen interactions is increasingly realized (Asgari 2013; Lucas and Raikhel 2013). miRNAs are also important in reproduction and are involved in regulating functionality of the ovaries, *e.g.*, in the maintenance and differentiation of germline stem cells (Park *et al.* 2007). This suggests they are promising candidates for involvement in the regulation of female postmating responses.

In the context of sexual conflict theory, the manipulation by one sex of gene regulatory mechanisms in the other, for example by miRNAs, could be highly significant. Male ejaculate components could alter the expression of miRNAs in females in a way that alters female physiological processes to provide maximum fitness benefits for males. In this way, males could increase female reproductive output to maximize the paternity gained before the female remates. Alternatively, variation in the expression of miRNAs might represent female responses to minimize male manipulation. miRNA expression in females could, for example, be used to dampen down potentially costly oscillations in gene expression caused by responses to Sfps (Kim *et al.* 2013). In effect, this could be a mechanism to reduce noise and stabilize expression to a more benign level in terms of female fitness outcomes. Our aim in this study was to start an investigation into the potential importance of post-transcriptional regulation in sexual conflict. We did this by experimentally testing the involvement of a set of candidate miRNAs in responses to SP.

An initial screen of miRNA expression changes following receipt of SP from males highlighted several candidate miRNAs. Among these, miR-279, miR-317, and miR-184 showed down-regulation in female head + thorax samples. In female abdomens, miR-279 was down-regulated and miR-278 up-regulated (T. Rathjen, H. Pais, S. Moxon, C. J. Pennington, T. Dalmay, and T. Chapman, unpublished data; Supporting Information, File S1; Table S1; Figure S1; and Figure S2). Here, we tested directly the SP responses of females lacking these candidate miRNAs. However, this set of four miRNAs is only a subset of the miRNAs likely to be involved in SP responses. First, the miRNA count data in this preliminary screen were obtained prior to the finding that there can be significant RNA ligase-dependent bias in small RNA cloning. Some small RNA sequences, including miRNAs, can be preferred over others due to their ability to anneal to adapter molecules used for library generation, which leads to a higher chance for ligation and therefore sequencing (Sorefan *et al.* 2012). Second, we chose this set of four miRNAs for further testing not only on the basis of their validated, altered expression in response to SP (File S1; Table S1; Figure S1; Figure S2), but also on the availability of loss-of-function mutations (*Materials and Methods*, below).

We investigated the direct influence of miRNAs on female phenotypic responses to receipt of SP using both hypomorph and knockout (ko) mutants. We tested the effect of miRNA mutants in different genetic backgrounds and recorded the reproductive output and sexual receptivity of miRNA mutant females after single matings to SP-lacking or control males. We also measured the survival of miRNA-lacking females following continual exposure to SP-lacking or control males. Our prediction was that, as SP predominantly led to down-regulation of the four candidate miRNAs, we would see SP-like phenotypic responses in miRNA mutant females mated to SP-lacking males.

Materials and Methods

Culturing methods

Stocks were maintained at 25° on a 12:12 light:dark cycle in either large overlapping cage cultures (wild-type populations) or in bottle cultures (mutant stocks). miRNA stocks were cultured in glass bottles (189 ml) containing 70 ml of standard sugar-yeast (SY) food [100 g brewer's yeast powder, 100 g sucrose, 20 g agar, 30 ml Nipagin (10% w/v solution), 3 ml propionic acid, and 1 liter of water]. All experiments were conducted at 25° and in a humidified constant temperature room (~50% relative humidity, RH), using glass vials (75-mm height × 25-mm diameter) containing 7 ml of SY food with *ad libitum* live yeast granules or live yeast paste added. To collect adults for the experiments, females were allowed to oviposit on agar–grape juice plates [50 g agar, 600 ml red grape juice, 42.5 ml nipagin (10% w/v solution), and 1.1 liters of water] with a blob of yeast paste unless stated otherwise. First instar larvae were collected the following day and groups of 100 transferred to vials with SY medium. Vials were incubated at standard conditions for 10 days. Virgin adults were ice anesthetized upon eclosion, sexed, and held in groups of 10 per sex.

Fly stocks

Wild-type flies: The Dahomey wild-type stock was used throughout these experiments to provide experimental males for remating opportunities. The Dahomey stock was collected in the 1970s in Benin, Africa and held under the above conditions since then.

Sex peptide-lacking males: *SP* gene knockout males (Liu and Kubli 2003) were used to generate males that do not transfer SP during mating. These males were produced by crossing $SP^0/TM3,Sb,ry$ males to $\Delta 130/TM3,Sb,ry$ females. The resulting $SP^0/\Delta 130$ (SP^0) males produce no SP (Liu and Kubli 2003). Control males were generated by crossing $SP^0, SP^+/TM3,Sb,ry$ males to $\Delta 130/TM3,Sb,ry$ females to generate SP producing $SP^0,SP^+/\Delta 130$ (SP^+) males. The strains were previously backcrossed into the Dahomey wild type to increase vigor. The $\Delta 130/TM3,Sb,ry$ stock was backcrossed for three generations, and chromosomes 1, 2, and 4 of the $SP^0/TM3,Sb,ry$ and $SP^0,SP^+/TM3,Sb,ry$ stocks were backcrossed for four generations. To generate SP-lacking and control males, three parental males and females for

each cross were housed together in vials and transferred onto fresh food every day. Ten days later, male offspring of the correct genotype were collected and housed in groups of 10 in vials until used in the experiments.

miR-mutant females: Lines that were hypomorphic for *miR-279* and *miR-317* were gratefully received from A. Yamamoto (North Carolina State University). These mutants carry a single autosomal *P[GT1]* transposon insertion in the respective miRNA genes in a w^{1118}; Canton-S ($w^{[CS]}$) wild-type genetic background (Yamamoto *et al.* 2008). We backcrossed both these hypomorphs four times into the white Dahomey ($w^{[Dah]}$) genetic background (this is the wild-type Dahomey genetic background into which the w^{1118} allele had previously been backcrossed multiple times) (Broughton *et al.* 2005). We performed phenotypic tests (see below) using the two hypomorphs in both the Canton-S and Dahomey genetic backgrounds, using $w^{[CS]}$ or $w^{[Dah]}$ as the appropriate controls. We refer to hypomorphs as *mir-279C* and *mir-317C* in the $w^{[CS]}$ genetic background and *mir-279D* and *mir-317D* in the $w^{[Dah]}$ genetic background.

We used two miRNA knockout lines—one lacking *miR-278* and one lacking *miR-184*. The *mir-278* knockout strain was a kind gift from S. Cohen (National University of Singapore) (Teleman and Cohen 2006). The original line had been derived in a w^{1118} background. We backcrossed this line four times into the $w^{[Dah]}$ genetic background and refer to this backcrossed line as *mir-278D*. $w^{[Dah]}$ females were therefore an appropriate control. We received the *mir-184* knockout (Δ*mir-184/Kr-GFP*, *CyO;TM2/TM6B*) as a kind gift from N. Iovino (University of Munich) (Iovino *et al.* 2009). We used females homozygous for the *mir-184* deletion in our phenotypic assays and the w^{1118} stock (no. 60000 from the Vienna *Drosophila* Stock Centre) as a control. The *mir-278* and *mir-184* knockouts are reported to span the two mir genes in question, leaving nearby genes unaffected (Teleman and Cohen 2006; Iovino *et al.* 2009). To obtain virgin females homozygous for the *mir-184* knockout, we allowed three males and three females from the parental generation to interact and oviposit in vials for up to 4 days. After removing the adult flies, vials were incubated and virgin females of the correct genotype were collected shortly after eclosion and held in groups of 10 until used in the experiments.

Verification of miR-mutant lines

Northern blots for miRNA knock out verification: Loss of miRNA expression in the knockout lines used was verified by Northern blotting using the protocol by Pall and Hamilton (2008). We extracted RNA as above from two samples and loaded 10 μg total RNA mixed with Ambion gel loading buffer II on a 15% polyacrylamide gel with urea. The gel was run at 120 V for 2 hr in 0.5× TBE. We then transferred the RNA to a Hybond-NX membrane using semidry transfer conditions at 250 mA for 45 min. We cross-linked the RNA in the membrane by adding 5 ml cross-linking solution (12 ml H_2O, 122.5 μl 12.5 M 1-methylimidazole, 10 μl 1 M hydrochloric acid pH 8, and 0.373 g of EDC) and incubating at 60° for 1 hr in saran wrap. For each probe we prehybridized the membrane with Ultra-hyb-oligo buffer (Ambion) at 37° for 1 hr. We then incubated mixture of 10 μl H_2O, 4 μl 5× polynucleotide kinase (PNK) forward buffer, 2 μl 10 μM oligo probe, 1 μl T4 PNK and 3 μl γ-ATP at 37° for 1 hr. The mixture was run through a Sephadex column to elute unbound isotope. We incubated the membrane in this buffer overnight at 37° and then washed it three times in 0.2× SSC:0.1% SDS before exposing it on a phosphorimaging screen in a radioactive cassette (Fujifilm) followed by imaging on a FX Pro Plus molecular imager (Bio-Rad). Antisense DNA oligonucleotide probe sequences used (Sigma-Aldrich) were as follows: miR-184 (5′-GCCCTTATCAGTTCTCCGTCCA-3′) and miR-278 (5′-AAAC GGACGAAAGTCCCACCGA-3′). We used U6 as a loading control for all samples.

Quantitative real-time PCR for miRNA hypomorph verification: To validate reduced or absent expression of the candidate miRNAs in the hypomorph lines used, we performed quantitative real-time PCR (qRT-PCR). We extracted total RNA using the mirVana miRNA isolation kit (Life Technologies) following the manufacturer's protocol with minor modifications to the sample homogenization stage. Ten virgin females aged for 4 days from each line were homogenized on liquid nitrogen in a 2-ml microcentrifuge tube. We used the TaqMan MicroRNA assay (Life Technologies) for the qRT-PCRs with probes for *mir-279* and *mir-317*, following the manufacturer's protocol. We used miR-2S as a reference gene. Each 20-μl reaction was placed onto a MicroAmp (Life Technologies) plate and qRT-PCR was performed on the 7500 Fast Real-Time PCR system (Life Technologies). For each probe set, we produced a standard curve using a 1:5 serial dilution of a sample independent of any of those used in our assays. All standard curves had an $R^2 > 0.98$ and slopes of between −2.40 and −3.24 (efficiency of 161.0 and 103.5%, respectively). We analyzed between five and six samples per stock tested. $w^{[CS]}$ and $w^{[Dah]}$ samples served as controls for the mutant stocks in respective genetic background and were reared in the same way.

Single mating reproductive output and receptivity assays

We used 4-day-old individuals to test reproductive output (number of offspring or estimated number of offspring) and sexual receptivity responses to receipt of SP in the *mir-279C*, *mir-317C* and *mir-279D*, *mir-317D* hypomorphs and the *mir-278D* and *mir-184* knockout lines, *vs.* their wild-type controls. miRNA mutant and control females were mated to SP-lacking or control males or kept as virgins (to control for intrinsic differences between miR-mutant females). A total of 180 mutant or control females each were divided randomly across the three treatment groups by aspirating them into individual vials the morning of the experiment. The day before the beginning of the experiment, we introduced individual males of the appropriate genotype into

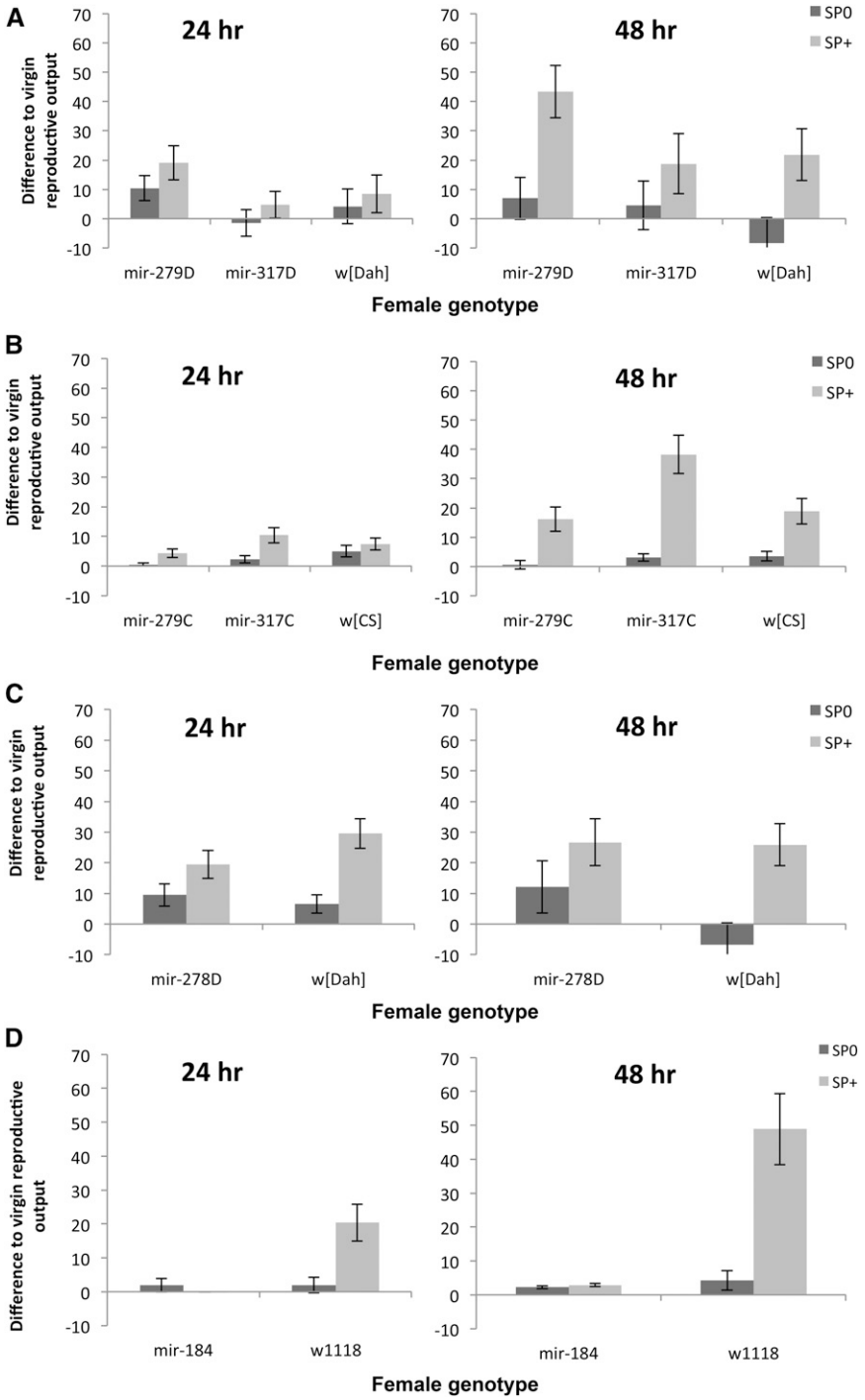

Figure 1 (A–D) Mean (±SE) reproductive output of miRNA mutant females and their controls, relative to the estimate for reproductive output of virgin females of the same genotype. Reproductive output was scored as offspring counts either 24 or 48 hr after a single mating to SP-lacking (SP^0, bars with dark shading) or SP-transferring (SP^+, bars with light shading) males. Females were either hypomorphic for *mir-279* or *mir-317* in two different genetic backgrounds (A) $w^{[Dah]}$ or (B) $w^{[CS]}$. (C) Results for knockout *mir-278* in the $w^{[Dah]}$ genetic background or (D) *mir-184* in the w^{1118} background. (E–H) Effect sizes (mean SP^+ − mean SP^0)/SD_{pooled} and 95% CI for reproductive output scored for the same female genotypes as in A–D 24 (diamonds with dark shading) or 48 hr (squares with light shading) after a single mating to either a SP^+ or a SP^0 male.

vials for the "mated" treatments. Virgin females were similarly maintained but were not given a male on this day. For the females encountering a male, we recorded the time of introduction and the start and end of mating. After a successful mating, the male was removed from the vial and the female was allowed to oviposit. Females that had not mated within 2 hr after introducing a male were discarded. We then divided females from all three treatments into two groups (n = 30 per treatment combination) and allowed half of the females to remate after 24 hr, while the other half had an opportunity to remate after 48 hr. For the remating tests, females were given a 1-hr opportunity to remate with a Dahomey wild-type male and the females from the virgin treatment were simultaneously allowed to mate for the first time. We again recorded time of introduction, the start and end of remating, as well as the overall proportion of females that remated. For females assigned to the 24-hr remating treatment, we counted the number of eggs laid by females following the initial matings to either SP-lacking or control males and the number of offspring emerging from

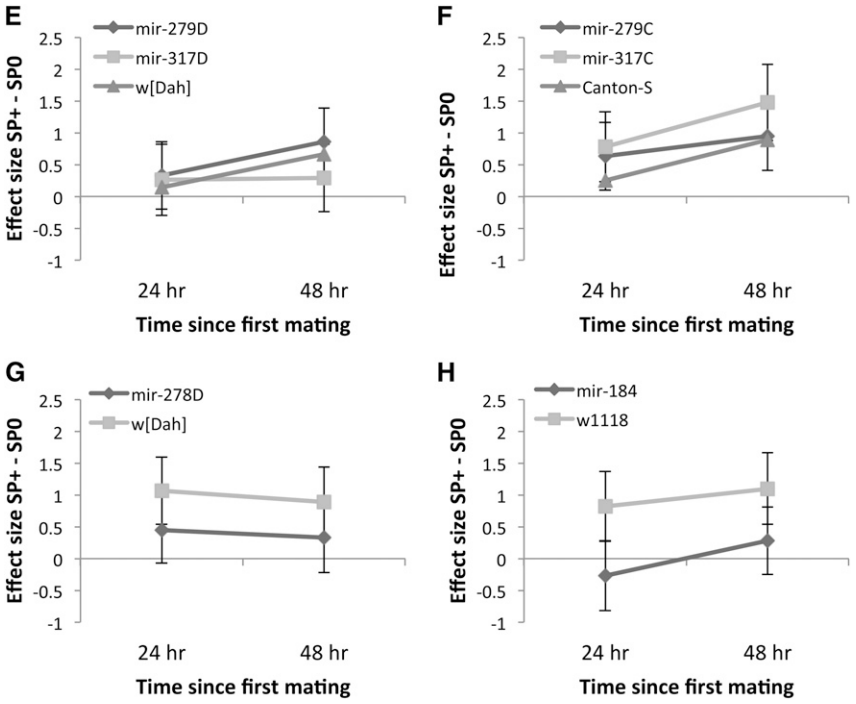

Figure 1 *Continued.*

those eggs, to calculate egg–adult survival and reproductive output. We did not directly count the number of eggs laid during the 48-hr intermating period; thus we used offspring number as our measure of reproductive output. To compare this with the reproductive output of the virgin treatment (where no offspring were produced) we multiplied the number of eggs laid by virgin females with the strain-specific egg–adult survival rate.

Throughout the experiments, reproductive output was therefore a measure of offspring number. It represented the actual number of offspring for the mated females and an estimate of offspring number for the virgin female treatments (given by the number of eggs multiplied by the relevant strain-specific egg–adult survival rate).

Female reproductive output, receptivity, and survival following continual exposure to SP-lacking or control males

We tested the reproductive output (number of offspring), receptivity, and survival of miRNA-mutant and control females when continuously exposed to SP-lacking or control males throughout life. We also maintained groups of virgin females of each genotype for comparison. The day after eclosion, females were assigned to treatments at random and held in groups of three females (virgin female treatment) or three females together with three males of the appropriate genotype (mated female treatment) in vials supplemented with live yeast granules. There were 15 vials per treatment (n = 45 females, with the exception of the *mir-278D* virgin female treatment, for which there were n = 30 females). *mir-184* knockouts were not included in this assay because we had not backcrossed them into the wild-type Dahomey genetic background. Furthermore, *mir-184* knockout females do not produce eggs, which would potentially have complicated fitness comparisons with that of other treatments, including the controls, which are fertile.

We scored female survival daily until all females were dead. Every other day, groups were transferred onto fresh food during which males and females were shuffled within treatments to form new groups of three males and three females (or three females only for the virgin treatments) to minimize vial-to-vial differences and to prevent differences in density occurring over time as the females started to die. We maintained constant sex ratio (in the mated treatments) and density by combining vials. Each vial contained at least two females or two pairs. For the mated female treatments, males were replaced each week with 2- to 4-day-old fresh males of the appropriate genotype. This minimized effects on females of any age-dependent decline in male reproductive performance.

For 10 days over the first 2 weeks of the experiments we scored mating rate in the mated female treatments by performing spot checks of behavior every 20 min for 3 hr after lights on. We counted the number of mating pairs in each treatment at each time point. Twice each week, we also scored reproductive output in the mated female treatments. We scored virgin reproductive output once per week (estimated number of offspring given by virgin female fecundity multiplied by the strain-specific egg-to-adult survival). A total of 21 randomly chosen females per treatment were allowed to oviposit in vials with 7 ml of standard SYA food with added charcoal (4 g charcoal per liter) to facilitate egg counting. Females were separated and allowed to oviposit in individual vials for 6 hr before being returned

Table 1 The results of an analysis of variance on the virgin baseline-corrected reproductive output

A Source	d.f.	MS	*F*	*P*
Female genotype (FG)	2	1660	7.28	<0.001
Male genotype (MG)	1	14,608	64.06	<0.001
Time period (time)	1	5768	25.29	<0.001
FG × MG	2	1322	5.80	0.003
FG × time	2	632	2.77	0.064
MG × time	1	6085	26.68	<0.001
Error	319	228		
B Source				
Female genotype (FG)	1	15,334	26.57	<0.0001
Male genotype (MG)	1	14,404	24.96	<0.001
Time period (time)	1	4218	7.31	0.007
FG × MG	1	13,878	24.05	<0.0001
FG × time	1	2495	4.32	0.039
MG × time	1	3075	5.33	0.022
FG × MG × time	1	1872	3.24	0.073
Error	209	577		

(A) *mir-279C* and *mir-317C* hypomorphic females in the $w^{[CS]}$ genetic background and (B) *mir-184* knockout females, either 24 or 48 hr after single matings to SP-lacking (SP^0) or control (SP^+) males.

to their experimental groups once again. We recorded reproductive output for each treatment until the time when there were <15 mated females of each genotype remaining alive.

Statistical analysis

General: Mean (±SE) values are reported throughout unless otherwise specified. We calculated standard errors for all proportion data according to the following formula: SE = square root $[p^*(1 - p)/n]$, where p is the proportion of females remating and n is the number of trials in that particular test. All analyses were conducted in Rv2.15.1 (Ihaka and Gentleman 1996). For generalized linear models, we used the appropriate error structure and conducted an analysis of deviance. In this, the significance of factors was tested by individually subtracting each factor in turn from the full model and comparing models by estimating the difference in deviance (G^2) between the two models. When using the quasiextension to correct for overdispersion of the data, we used an *F*-distribution to test for significance; otherwise the deviance values were compared against a χ^2 distribution (Crawley 2007). We removed three-way interactions from models if they did not significantly affect the fit of the model.

To display the effect of receipt of SP on mutant females, we calculated effect sizes, as the standardized mean difference (Cohen's *d*) over a pooled estimator of the standard deviation as the denominator. We calculated 95% confidence intervals (CIs) for effect sizes. In the single mating assay, we calculated the effect size for the number of offspring produced (using baseline corrected values, see below). In each case, we show the increase in reproductive output (baseline corrected value) relative to that of the relevant virgin female reproductive output (estimated number of offspring). To calculate effect sizes for the proportion of females that remated after 24 and 48 hr, we used the "failes" command in the "compute.es" package in R. This calculates Cohen's *d* from binary data using the number of nonremating females in each group (the number of "failures") relative to the total sample size. For the survival data, we calculated mean lifespan and used the associated pooled standard deviation to calculate the standardized mean difference plus 95% confidence intervals.

Statistical analysis of single mating reproductive output and receptivity assay: The different female genotypes varied in intrinsic egg-laying capacity; therefore, to compare them, we used estimates of virgin female reproductive output (virgin fecundity scaled by the appropriate egg–adult survival, calculated as described above) of the appropriate genotype in each case as a baseline for standardization (for egg-to-adult survival data and analysis see File S2; Figure S3). This allowed us to directly compare reproductive output of mated females across the different genotypes. We standardized all 24- and 48-hr offspring counts by subtracting the mean corrected virgin output. This allowed us to test by how much a mating to a SP^0 or SP^+ male elevated female offspring production above the virgin baseline. The resulting data were then analyzed using analysis of variance.

Statistical analysis of reproductive output, receptivity, and survival following continual exposure to SP-lacking or control males: Survival data were analyzed with a parametric Kaplan–Meier regression with a Weibull distribution, as described in Crawley (2007). For mating rate and reproductive output data, we used generalized linear models with appropriate error structures. The spot check data for mating rate were modeled using a binomial error distribution as the number of mating opportunities (*e.g.*, the number of females in each treatment × the number of spot checks) taken *vs.* not taken. Reproductive output checks were analyzed using a Poisson error distribution with the quasiextension to account for overdispersion in the data (Crawley 2007).

Results

Verification of lines

All miRNA knockouts and hypomorphs had significantly lower expression of the appropriate miRNA as compared to the control lines as shown by the Northern blotting and qRT-PCR (Figure S4; Figure S5). Hence all the mutant lines in the different genetic backgrounds used in the experiments described here were successfully validated.

Effect of miRNAs on female reproductive output and sexual receptivity responses to SP after a single mating

Female reproductive output responses to SP:

mir-279D and mir-317D hypomorphs: Virgin miRNA-lacking females differed significantly in fecundity [$G^2 = 209.46$, $F_{1,176} = 7.61$, $P = 0.0007$ (dispersion parameter = 13.92)]

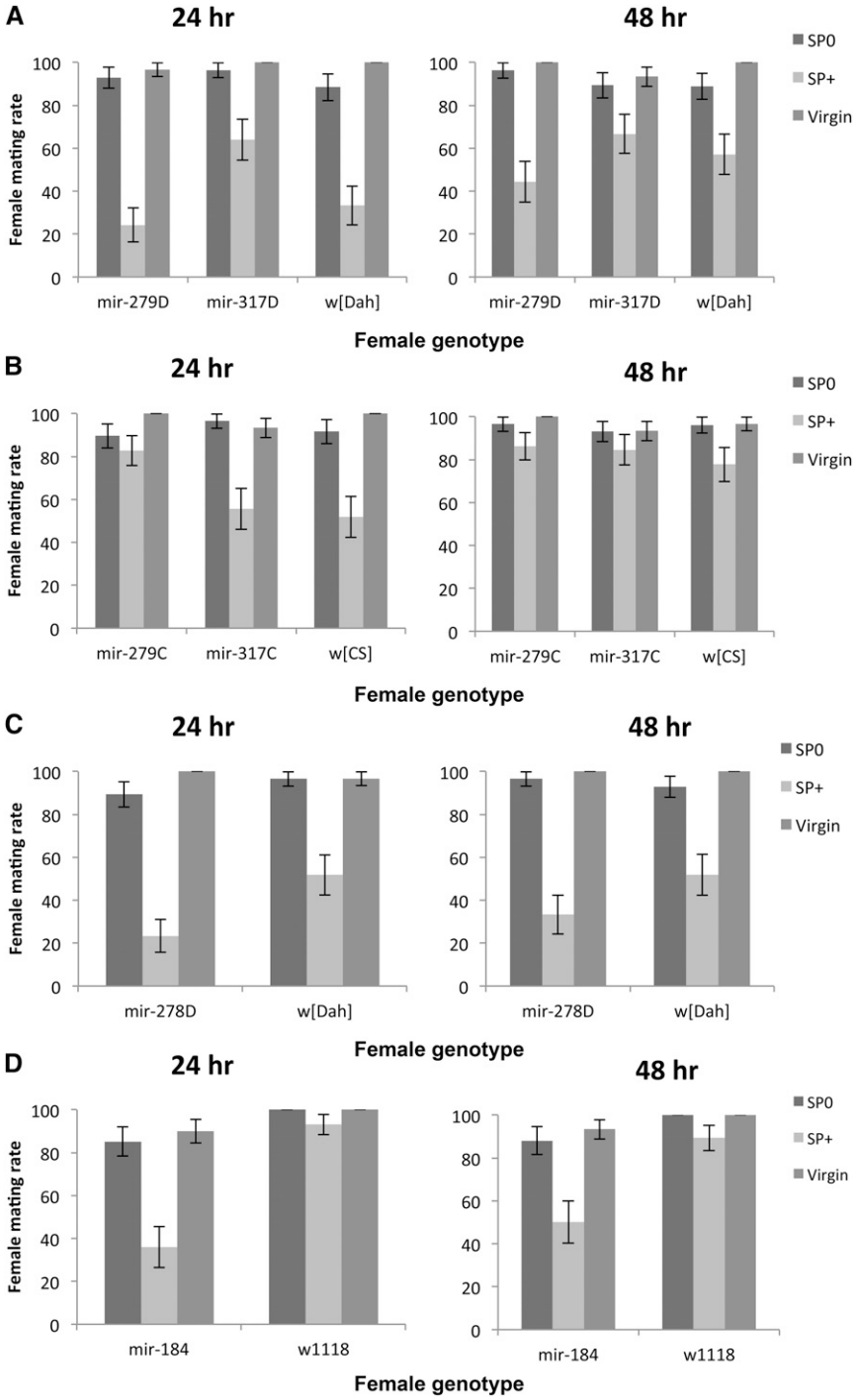

Figure 2 (A–D) Remating rate (number of females remating in 1 hr) at 24 or 48 hr following initial matings with either SP-transferring (*SP*$^{+}$, bars with light shading) or SP-lacking (*SP*^{0}, bars with dark shading) males. Simultaneously, the mating rate of age- and genotype-matched virgin females (bars with intermediate shading) was measured for comparison. (A and B) Remating in females hypomorphic for *mir-279* and *mir-317* in the *w*$^{[Dah]}$ or *w*$^{[CS]}$ genetic backgrounds, respectively. (C) Receptivity of *mir-278* knockout females in the *w*$^{[Dah]}$ genetic background and (D) receptivity of *mir-184* knockout females in the *w*^{1118} background. (E–H) Effect size (*SP*^{0} − *SP*$^{+}$ treatment) and 95% CI for female remating rate for the different female genotypes mated first to a *SP*$^{+}$ or *SP*^{0} male and provided with an opportunity to remate with a Dahomey wild-type male either 24 (diamonds with dark shading) or 48 hr (squares with light shading) after a first mating. Note that in the tests of the *mir-184* knockout, (Figure 2H) all the control *w*^{1118} females remated; therefore, Cohen's *d* could not be calculated. Hence only the effect size for *mir-184* is shown.

with *mir-279D* females laying fewer eggs than the other two groups. Overall, the number of eggs laid increased from 24 to 48 hr ($G^2 = 697.63$, $F_{1,175} = 50.72$, $P < 0.0001$) and this was similar for all female genotypes (interaction = ns). Therefore, to ascertain more clearly to what extent receipt of SP increased female reproductive output, we analyzed, as described above, the difference in reproductive output relative to the appropriate virgin female genotype, using the baseline corrected reproductive output data. This showed that reproductive output relative to the virgin level was significantly higher in females that received SP ($F_{1,318} = 16.07$, $P < 0.0001$; Figure 1A), and female genotypes also differed significantly in their reproductive output ($F_{2,318} = 4.52$, $P = 0.012$). However, there was no evidence that miRNA-lacking females differed in the extent to which they responded to SP. In *mir-279D* females, mating induced a steeper increase in reproductive output above the baseline virgin level than it did in *mir-317D* or control females (Figure 1A). There was also a nonsignificant trend for reproductive output to increase with time ($F_{1,318} = 2.97$, $P = 0.086$).

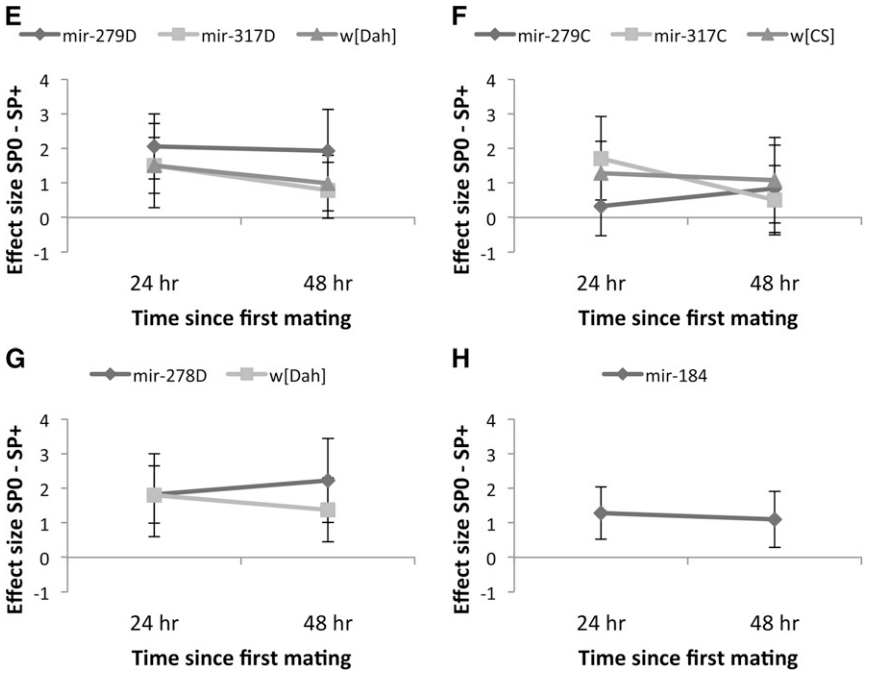

Figure 2 *Continued.*

Reproductive output increased significantly with time for all female genotypes mated to SP^+ control males ($F_{1,318} = 5.98$, $P = 0.015$, Figure 1A). This pattern was also mirrored in the effect size calculation, where the standardized mean difference for the SP^+ *vs.* SP^0 treatment was larger for the 48-hr compared to the 24-hr treatment (Figure 1E).

mir-279C and mir-317C hypomorphs: Virgin female genotypes again differed significantly in fecundity ($G^2 = 2308.2$, $F_{2,177} = 73.85$, $P < 0.0001$), but the significant increase in fecundity over time from 24 to 48 hr ($G^2 = 515.65$, $F_{1,176} = 33.00$, $P < 0.0001$) was similar across female genotypes [$G^2 = 24.19$, $F_{2,175} = 0.78$, $P = 0.461$ (dispersion parameter = 15.56)]. There were significant differences between female genotypes, however; all mated females, as expected, produced significantly more offspring when mated to a SP^+ male. There was no evidence that the female genotypes showed altered reproductive output responses to SP. Overall, *mir-317C* females had higher reproductive output after receipt of SP compared to the other two groups (Figure 1B). The magnitude of this effect increased with time, and the slope increased more steeply for females mated to SP^+ in comparison to SP^0 males (Table 1A; Figure 1B). This was also reflected in the larger effect size for all female genotypes for the 48-hr treatment (Figure 1F).

mir-278D knock out (ko): Virgin mutant and control females had similar fecundity [$G^2 = 3.66$, $F_{1,118} = 0.38$, $P = 0.541$ (dispersion parameter = 9.79)] and the fecundity of both groups increased significantly from 24 to 48 hr ($G^2 = 1601.8$, $F_{1,118} = 164.36$, $P < 0.0001$, interaction = ns). Mutant and control females did not differ in their virgin baseline-corrected reproductive output ($F_{1,223} = 0.53$, $P = 0.469$), but females mated to SP^0 males produced significantly fewer offspring ($F_{1,223} = 21.92$, $P < 0.0001$). There was, however, a nonsignificant tendency for control females to respond more strongly to receipt of SP (male × female genotype: $F_{1,223} = 3.40$, $P = 0.066$; Figure 1, C and G). This effect was stable over time (P = ns, as were all interactions).

mir-184 ko: Virgin *mir-184* ko females laid very few eggs [$G^2 = 906.61$, $F_{1,117} = 36.53$, $P < 0.0001$ (Dispersion parameter = 18.58)] as expected based on previous work (Iovino *et al.* 2009), and the low level of fecundity did not increase over time, in contrast to the observation for control females (time: $G^2 = 340.44$, $F_{1,117} = 13.72$, $P = 0.0003$; female genotype × time: $G^2 = 54.92$, $F_{1,116} = 2.96$, $P = 0.088$; File S2). *mir-184* ko females did not increase their reproductive output above the virgin level following mating to either SP^+ or SP^0 males (Figure 1D). This pattern was stable across 24–48 hr. Control females had a higher reproductive output following receipt of SP and this increased from 24 to 48 hr (Table 1B; Figure 1, D and H).

Overall the results showed no evidence that *mir-279*, *mir-317* hypomorphs in either genetic background, or *mir-184* ko females, showed altered reproductive output responses to SP in comparison to the appropriate controls. However, *mir-278D* ko females showed a nonsignificant tendency for weaker responses to SP in comparison to controls.

Sexual receptivity responses to SP:

mir-279D and mir-317D hypomorphs: Virgin mutant and control females did not differ in their willingness to mate in their first mating ($G^2_2 = 1.67$, $P = 0.434$) and this did not change over time ($G^2_1 = 2.84$, $P = 0.092$; interaction = ns). There was a marginally nonsignificant difference in mating rate among the mated females ($G^2_2 = 5.33$, $P = 0.070$) and the effect of time since the first mating on willingness to

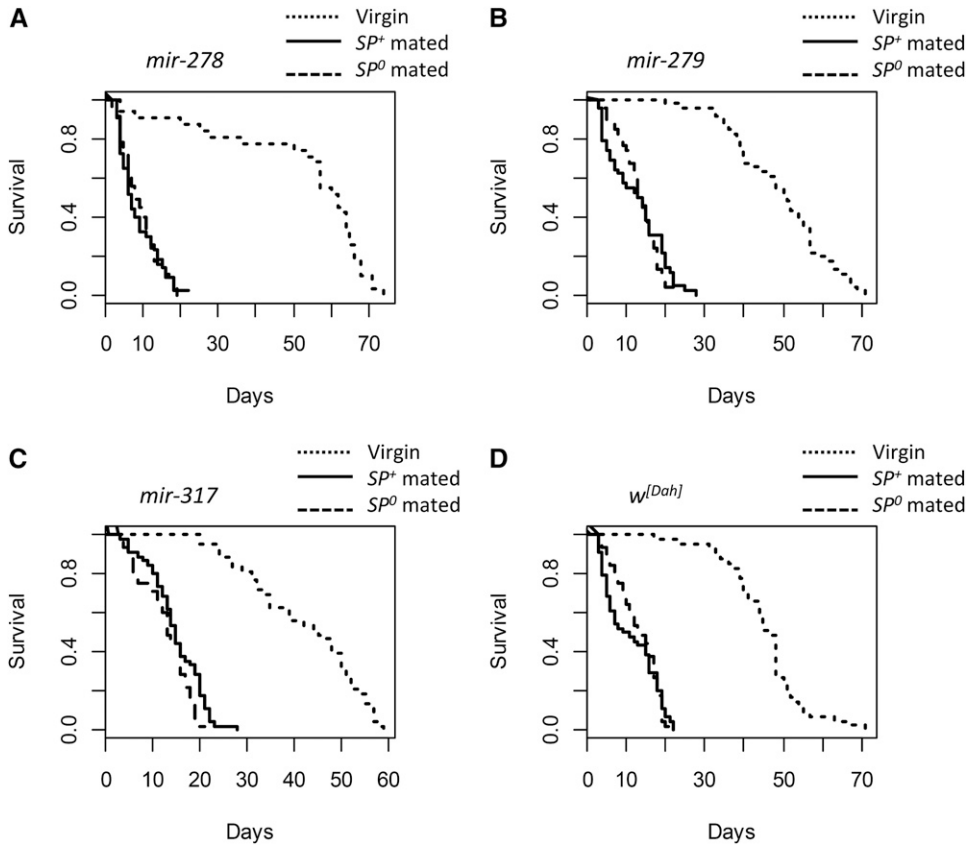

Figure 3 Survival curves for the miRNA mutant females (backcrossed into the $w^{[Dah]}$ genetic background) kept as virgins or continuously exposed to SP-lacking (SP^0) or SP-transferring (SP^+) control males throughout their lifetimes. Shown are the survival curves for the (A) *mir-278D* knockouts, (B) *mir-279D*, (C) *mir-317D* hypomorphic mutant females, and (D) $w^{[Dah]}$ controls. For each panel, the virgins are shown by the dotted lines, females mated with SP^+ males by the solid lines, and females mated with SP^0 males by the dashed lines.

remate was similarly marginally nonsignificant ($G^2_1 = 3.15$, $P = 0.076$). Receipt of SP significantly suppressed remating rate as expected ($G^2_1 = 84.26$, $P < 0.0001$). There was a marginally nonsignificant interaction between female and male genotype ($G^2_2 = 5.68$, $P = 0.059$, all other interactions = ns). This suggests that the effect of SP receipt on female sexual receptivity varied across the female genotypes tested. Across genotypes, females that received no SP remated at a rate similar to virgin females. However, SP was less efficient in suppressing remating in *mir-317D* females, whereas in *mir-279D* females, SP was equally effective in suppressing remating after 24 and 48 hr (Figure 2, A and E).

mir-279C and mir-317C hypomorphs: Virgin females differed significantly in their willingness to mate in their first mating ($G^2_2 = 6.12$, $P = 0.047$). *mir-317C* females were slightly less willing to engage in mating, but this did not change significantly over time ($G^2_1 = 0.21$, $P = 0.650$, interaction = ns). Fewer females first mated to a SP^+ control male remated ($G^2_1 = 28.00$, $P < 0.0001$), but this effect diminished with time ($G^2_1 = 7.93$, $P = 0.005$; Figure 2B). The nonsignificant interaction term indicates that females of the different genotypes showed a similar response to receipt of SP across time ($G^2_2 = 4.43$, $P = 0.109$, all interactions = ns). However, the standardized mean difference revealed that after 24 hr, SP was not efficient in suppressing remating in *mir-279C* mutant females (Figure 2F).

mir-278D ko: Virgin females of this genotype did not differ in willingness to mate from virgin control $w^{[Dah]}$ females and this effect was constant across both time periods examined (all = ns). However, SP receipt suppressed female willingness to remate more strongly in *mir-278D* ko females than in controls (female genotype: $G^2_1 = 6.24$, $P = 0.012$; male genotype: $G^2_1 = 85.29$, $P < 0.001$; Figure 2, C and G), a pattern that was constant across time ($G^2_1 = 0.48$, $P = 0.49$, all interactions = ns).

mir-184 ko: Virgin *mir-184* ko females were less willing to mate than controls ($G^2_1 = 7.05$, $P = 0.008$) and this was constant across the two time points tested ($G^2_1 = 0.22$, $P = 0.639$, interaction = ns). Receipt of SP was more efficient in suppressing remating in *mir-184* ko mutant females in comparison to controls, which showed high receptivity following their first matings independent of SP receipt (female genotype: $G^2_1 = 41.11$, $P < 0.0001$; male genotype: $G^2_1 = 28.89$, $P < 0.0001$; Figure 2, D and H). This pattern did not alter from 24 to 48 hr (time: $G^2_1 = 0.46$, $P = 0.496$, all interactions = ns).

Overall the results showed that females lacking miRNAs showed either significantly reduced or strengthened sexual receptivity responses to SP. There were also significant interactions with genetic background. *mir-317D* hypomorphs showed reduced receptivity responses to SP (Figure 2A) (but not when in the $w^{[CS]}$genetic background; Figure 2B). Similarly, *mir-279C* hypomorphs also showed reduced SP receptivity responses (Figure 2B) (but not in the $w^{[Dah]}$ genetic background; Figure 2A). SP receipt decreased female sexual receptivity more strongly in *mir-278D* ko (Figure 2C)

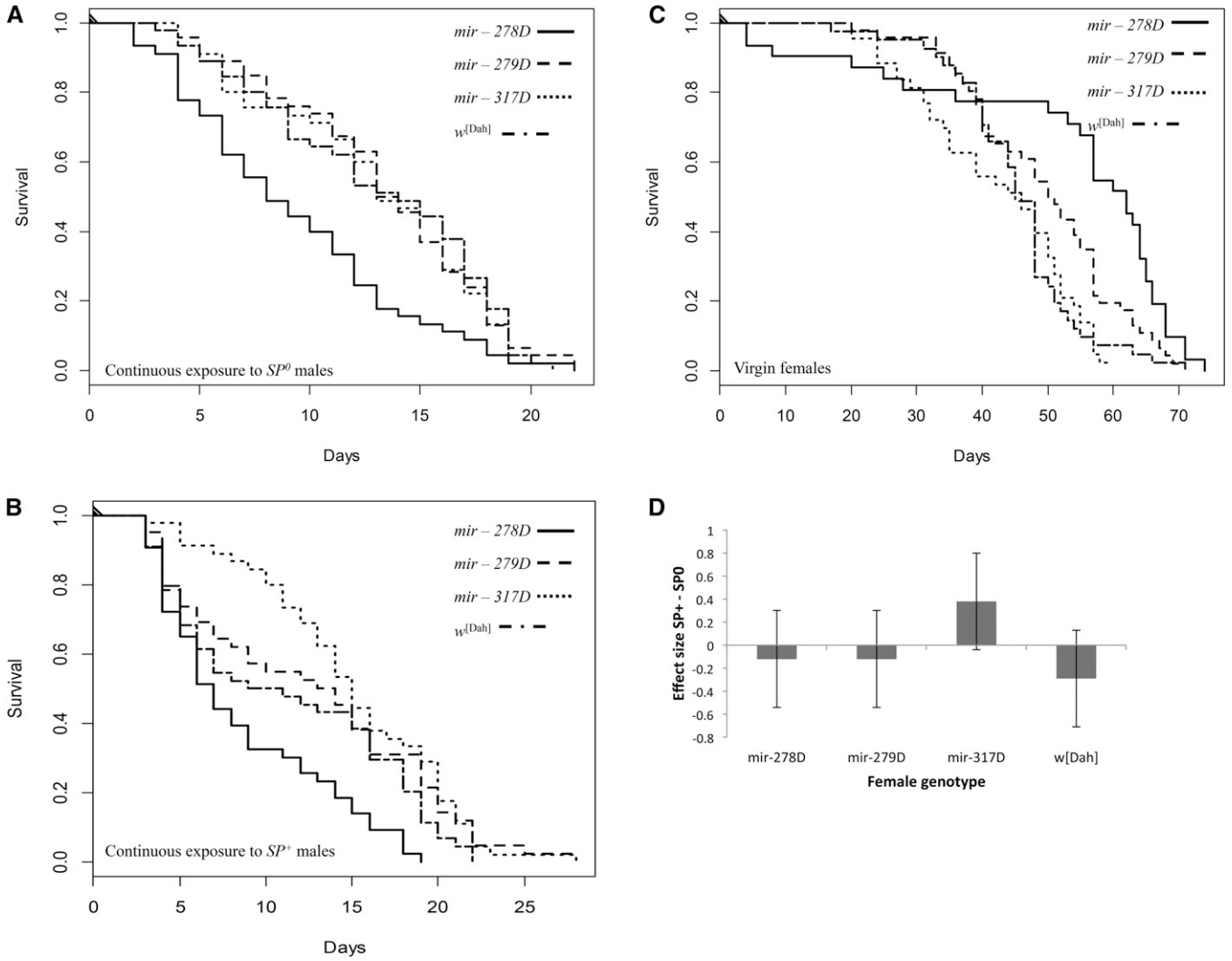

Figure 4 Survival of all the females shown in Figure 3 redrawn to illustrate survival following exposure to either (A) SP^0 or (B) SP^+ males in comparison to the survival of (C) virgin females. (D) Mean survival effect sizes (mean SP^+ − mean SP^0)/SD_{pooled} and 95% CI for the different female genotypes held continuously with SP^+ *vs.* SP^0 males.

and in *mir-184* ko females (Figure 2D) than in their respective controls.

Effect of mir-278D, mir-279D, and mir-317D mutations on female reproductive output, sexual receptivity, and survival responses to SP after continuous exposure to males

Virgin female genotypes differed significantly in survival ($G^2_3 = 23.36, P < 0.0001$; Figure 3; Figure 4). Virgin *mir-278D* ko females had low early-life but higher late-life survival (Figure 3A). Virgin *mir-317D* females showed a steady decline in survival from day 20 onward, resulting in the lowest maximum lifespan (~60 days compared to ~70 days for the other female genotypes; Figure 3C). Continuous exposure to males significantly reduced female lifespan in comparison to virgin females in all groups (mating treatment: $G^2_2 = 623.48$, $P < 0.0001$). However, this effect depended on female genotype (interaction: $G^2_6 = 38.53$, $P < 0.0001$; female genotype: $G^2_3 = 11.92$, $P = 0.008$; Table 2). Among mated females, female genotypes differed significantly in their responses to mating ($G^2_3 = 31.81$, $P < 0.0001$; Figure 4, A and B), though SP receipt had no effect on female lifespan (male genotype: $G^2_1 = 0.12$, $P = 0.730$; male × female genotype: $G^2_3 = 2.89$, $P = 0.408$). Thus females differed in their susceptibility to male exposure, with *mir-278D* ko female lifespan (Figure 3A) being markedly reduced in comparison to the other genotypes tested (Figure 3; Figure 4); however, these effects were independent of SP receipt.

Females significantly differed in the number of eggs laid within the 6-hr tests over the first 2 weeks of the assays [female genotype: $G^2 = 278.32$, $F_{3,249} = 9.42$, $P < 0.0001$ (dispersion parameter = 9.64)]. While virgin females of all four genotypes laid more eggs than mated females, females of all genotypes mated to SP^+ males laid significantly more eggs than those held with SP^0 males (mating treatment: $G^2 = 472.44$, $F_{2,248} = 23.97$, $P < 0.0001$; mating treatment × female

Table 2 Lifespan data for (A) virgin females or (B) females held continuously with SP-lacking (SP^0) or control (SP^+) males

A Female genotype	Median lifespan	Upper confidence limit	Lower confidence limit	Mean lifespan
mir - 278D	62.0	65	57	52.65
mir - 279D	50.5	57	46	49.35
mir - 317D	45.0	51	35	42.21
$w^{[Dah]}$	46.0	48	44	45.37

B	SP^0 male exposure				SP^+ male exposure			
Female genotype	Median lifespan	Upper CL	Lower CL	Mean lifespan	Median lifespan	Upper CL	Lower CL	Mean lifespan
mir-278D	8.0	12	6	9.22	7.0	11	6	8.60
mir-279D	13.5	16	13	13.28	13.5	16	8	12.57
mir-317D	13.0	16	12	12.98	15.0	19	13	15.00
$w^{[Dah]}$	14.0	17	11	13.00	10.0	16	6	11.27

Lifespan data for females hypomorphic for *mir-279D* and *mir-317D* are shown together with *mir-278D* knockout females, all backcrossed into the $w^{[Dah]}$ genetic background.

genotype: $G^2 = 110.11$, $F_{6,246} = 1.90$, $P = 0.081$; Figure 5A). As in the single mating tests, there was therefore no evidence for altered fecundity responses to SP in the miRNA-lacking females. The results remained the same even when virgin data were excluded and data for the mated females only over the first 2 weeks of the experiment were analyzed.

miRNA-mutant females in the Dahomey genetic background differed significantly in the number of mating opportunities accepted ($G^2_3 = 14.05$, $P = 0.003$; Figure 5B). However, this effect was driven by the *mir-278D* ko females accepting fewer matings, and exclusion of this line from the analysis rendered the female effect nonsignificant ($G^2_2 = 2.09$, $P = 0.351$). However, independent of the inclusion of *mir-278D*, mating rate differed significantly upon male genotype (model including *mir-278D*: $G^2_1 = 7.22$, $P = 0.007$), and the number of mating opportunities taken was also dependent upon an interaction between male × female genotype (model including *mir-278D*: $G^2_3 = 23.36$, $P < 0.0001$; Figure 5B). While SP transfer significantly reduced mating rate in the $w^{[Dah]}$ control as well as in *mir-279D* mutant females, *mir-278D* ko females were unaffected and in general showed low acceptance of matings. In contrast, *mir-317D* hypomorph females showed a reversal from the expected mating pattern and mated more frequently when held with SP^+ control as compared to SP-lacking males. Thus, while *mir-278D* ko females showed the lowest mating rate they showed the greatest reduction in lifespan due to continuous male exposure. In contrast while receipt of SP was less effective in suppressing mating rate in *mir-317D* females, these females tended to survive longer when continuously held with SP^+ control in comparison to SP^0 males (Figure 3C; Figure 4B).

Overall the results of the continual exposure experiment showed that female lifespan varied widely upon exposure to males, but this effect was independent of SP receipt. Consistent with the single mating tests we observed altered receptivity responses, measured as mating frequency, in *mir-278D* ko and *mir-317D* hypomorph females.

The results of all the phenotypic tests described above are summarized in Table 3.

Discussion

Overall, we found significant but subtle changes in miRNA-lacking females in response to SP receipt. There was little evidence that females lacking miRNAs exhibited grossly altered SP responses, such as SP-like phenotypes in the absence of SP. Instead, the results revealed a pattern of modulations to the strength of SP responses (Table 3). Females that had reduced or ablated expression for the four candidate miRNAs tested showed reduced or strengthened sexual receptivity responses following SP receipt, in comparison to relevant controls. There was little evidence, however, of consistent SP effects on reproductive output or survival. These key female responses to receipt of SP—*i.e.*, egg output and receptivity—are mediated through different neurons and are the result of the activation of different regulatory pathways (Haussmann *et al.* 2013). The four miRNAs tested may regulate different facets of known SP responses within these distinct pathways. Our data suggest that the behavioral responses to SP, specifically, may be subject to post-transcriptional regulation by miRNAs. Below we describe the known and putative functions of the different candidate miRNAs tested and highlight how they could influence female SP-mediated phenotypic responses. However, we note that a potentially complex interplay between several miRNAs might be required to regulate biological processes. As we tested only a subset of the microRNAs differentially expressed in response to SP receipt, there is the potential for others to have stronger effects than the candidates tested here or to interact with the candidate microRNAs tested to exert even stronger combined phenotypic responses in females.

Roles and functions of the miRNAs tested

The candidate miRNAs tested mainly modulated behavioral processes (see details below), which is consistent with their signatures of differential expression in response to receipt of SP, with miR-279, miR-317, and miR-184 being down-regulated in the head + thorax. miR-278 was up-regulated in the abdomen, while miR-279 was down-regulated in both body parts, perhaps suggesting some additional reproductive functions (Table S1; Figure S1; Figure S2; and see below).

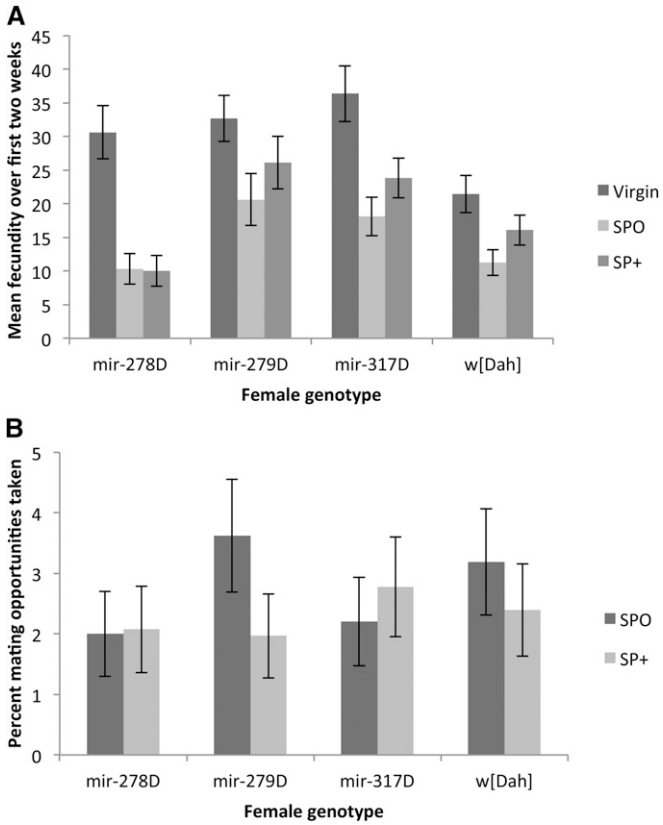

Figure 5 (A) Mean (± SE) reproductive output of miRNA mutant females shown in Figure 3. The reproductive output of 21 randomly sampled females per treatment was recorded for a period of 6 hr every week over the first 2 weeks of the survival experiment. (B) The percentage of mating opportunities taken by the females in A when continuously held with males lacking SP (SP^0, bars with dark shading) or control males (SP^+, bars with light shading).

Turning to the known functions of the miRNAs tested, little is yet known about the function of miR-317, apart from its involvement in brain development and, along with miR-279, in locomotion (Yamamoto *et al.* 2008). miR-279, in contrast, has been relatively well studied. A role for miR-279 in regulating neuron development has been shown and *nerfin-1* verified as a target (Cayirlioglu *et al.* 2008). N*erfin-1* contains several predicted seed sites for different miRNAs, and multiple miRNAs are needed to act cooperatively to regulate its spatial and temporal expression (Kuzin *et al.* 2007). In adults, miR-279 is involved in maintaining circadian rhythms and acts downstream of *period* neurons. As SP is known to inhibit female day-time rest and activity patterns (Isaac *et al.* 2010), this effect might be mediated through decreased miR-279 expression and subsequent modulation of *period* neuron signals to the downstream cascade. We found an effect of miR-279 on remating rate, suggesting that the effects of miR-279 on neurological function do influence fly mating behavior. However, the pattern was strongly dependent on genetic background (no effect in Canton-S and large effect in Dahomey genetic background). In addition to regulating neuronal processes, miR-279 is also active in the ovary. Here miR-279 is involved in a regulatory circuit to regulate expression of the *signal transducer and activator of transcription* (STAT) in follicle cells (Yoon *et al.* 2011). A subset of these cells change cell fate to become migratory border cells and form the micropyle (Montell 2003). Decreased miR-279 results in abnormal invasion of follicle cells (Yoon *et al.* 2011). However we show here that this disruption of oogenesis seems not to result in reduced fertilization success or egg-to-adult survival in mutant *mir-279* females (Figure S3, A and B).

miR-278 is reported to control energy balance, principally by regulating insulin responsiveness (Teleman and Cohen 2006). Through repression of translation of the gene *expanded*, miR-278 regulates insulin sensitivity and flies lacking miR-278 are lean and store less triglyceride (Teleman and Cohen 2006). miR-278 is also involved in regulating germline stem cell division by regulating the cyclin-dependent kinase inhibitor *dacapo* (Yu *et al.* 2009). Thus miR-278 has multiple functions, potentially including mediating links between nutrient availability and egg development. As SP receipt increases female feeding activity (Carvalho *et al.* 2006), miR-278 could be an important regulatory element between nutrient uptake, storage, and mobilization toward allocation into reproductive output. The availability of nutrition significantly affects the effect of SP on female lifespan (Fricke *et al.* 2010) and we found that *mir-278D* ko females were extremely sensitive to continuous male exposure, suffering greatly reduced lifespans (Figure 3A). SP up-regulated miR-278 expression in the abdomen, which could potentially initiate enhanced fat accumulation in the fat body to direct toward increased egg production. While SP receipt still resulted in increased reproductive output in *mir-278* knockout females, this increase was modest and smaller than in the control females (Figure 1, C and G). Elevated miR-278 expression might therefore allow males to reap the full reproductive benefits of SP transfer (Fricke *et al.* 2009, 2013).

miR-184 functions in the ovary and regulates processes during oogenesis and early embryogenesis (Iovino *et al.* 2009). Loss of miR-184 results in complete loss of egg production. These defects are so severe that we could not measure any effect of SP receipt on reproductive output in *mir-184* knockout females. However, our initial screen showed postmating expression changes in miR-184 after SP receipt in the head + thorax only, indicating that postmating regulation of miR-184 expression may alter functions in the brain/nervous system rather than the ovary. During embryogenesis, miR-184 is expressed in the developing central nervous system and in the head and eye disc in larvae (Li *et al.* 2011), consistent with accumulating evidence that miR-184 is involved in regulating neuronal processes (Greenberg *et al.* 2010; Liu *et al.* 2012). For example, miR-184 is highly expressed in honey bee *Apis mellifera* heads (Liu *et al.* 2012) and is part of the regulatory processes that confer the behavioral switch from nursing to foraging (Greenberg *et al.* 2010). In mouse, miR-184 also regulates the balance of adult neural stem cell proliferation *vs.* differentiation (Liu *et al.* 2010). Collectively these data suggest a potential role for miR-184 in neurological processes.

Table 3 Summary of the single mating and continual exposure assays

MicroRNA	Single mating assay		Continual exposure assay		
	Reproductive output	Receptivity	Survival	Reproductive output	Receptivity
mir-184	Reduced output with no response to receipt of SP	SP more effective in suppressing remating	Not tested	Not tested	Not tested
mir-278D	No altered response to SP, tendency for a weaker response ($P = 0.066$) than in control	SP more effective in suppressing remating	Strong reduction in survival with continual male exposure, independent of SP receipt	No altered response to SP	Unresponsive to SP receipt, low number of matings overall
mir-279D	No altered response to SP, though a tendency for higher output following SP receipt than in controls	No altered response to SP	Reduced survival with continual male exposure, independent of SP receipt	No altered response to SP	No altered response to SP
mir-317D	No altered response to SP	SP less efficient in suppressing remating	Reduced survival with continual male exposure, independent of SP receipt	No altered response to SP	Reversal of pattern, more matings when held with SP^+ males
mir-279C	No altered response to SP	No altered response to SP, but SP less efficient in suppressing remating after 24 hr	Not tested	Not tested	Not tested
mir-317C	No altered response to SP, though a tendency for higher output following SP receipt than in controls	No altered response to SP	Not tested	Not tested	Not tested

Females either lacked the microRNAs of interest (*mir-184* and *mir-278D* knock outs) or had reduced mir expression (*mir-279* and *mir-317* hypomorphs) in the wild-type Canton-S (*C*) or Dahomey (*D*) genetic background. Females were either mated singly to, or held continuously with, SP-lacking (SP^0) or SP-transferring males (SP^+). Female reproductive output and receptivity were recorded in both assays, and female survival was measured in the continual exposure assay.

miRNAs as buffers in the context of sexual conflict

We initiated in this study an investigation of the extent to which candidate microRNAs can regulate postmating female physiological processes in response to a male ejaculate signal subject to sexual conflict. We were interested in whether such processes have been coopted by males to potentially manipulate female physiological and behavioral responses for their own benefits. Alternatively, receipt of SP in females could result in alterations to miRNA expression to dampen down potentially deleterious oscillations in mRNAs in females (Kim *et al.* 2013). We expected that the reduced or absent levels of miR-279, miR-317, and miR-184 in the mutant females would, to some extent, mimic the down-regulation that we had previously observed following receipt of SP. Our observation of modest alterations to SP responses in the miRNA mutant females suggests that the miRNAs may function to buffer noise or stabilize responses to mating. Further work is now required to ascertain which sex is most sensitive to the relative expression level of the miRNAs involved.

miRNAs can have striking effects on the phenotype in developing individuals during ontogeny, but in adults miRNAs may have more subtle effects. The degree of protein repression by miRNAs can be modest, despite the observation that single microRNAs can suppress the translation of hundreds of proteins (Baek *et al.* 2008; Selbach *et al.* 2008). Thus it has been suggested that miRNAs might primarily perform modulatory functions, provide stability, and minimize expression noise in biological systems (Herranz and Cohen 2010). Little is known about how miRNAs confer robustness in systems characterized by gradual responses to external signals. miRNAs might act as "switches," by effectively repressing protein expression below a threshold level of the target mRNA, but might also act to fine tune gene expression when close to the threshold (Mukherji *et al.* 2011). This type of phenomenon could explain the results we obtained in the two hypomorph mutant strains, *mir-279* and *mir-317*, when expressed in two different genetic backgrounds. We found a reversal in remating behavior in response to SP in *mir-279* mutant females, and for both *mir-279* and *mir-317* mutant females the effect of SP on reproductive output was stronger/more pronounced in one genetic background than the other (Figure 1, E and F; Figure 2, E and F). This suggests the existence of epistatic effects due to different expression of either the level of target mRNA or of the regulators of expression of the miRNAs in the two genetic backgrounds. This could alter the balance of miRNA to target mRNA and, depending on closeness to threshold levels, alter the function of the miRNA from an effective suppressor to a modulator.

Our results provide an initial investigation of some of the miRNA loci potentially involved in the regulation of postmating changes in the female transcriptome. We focused on the effects of a particular male sexual antagonistic signal—the sex peptide—as this seminal fluid protein elicits diverse female phenotypic responses and is a major contributor to the

cost of mating in females. We showed that post-transcriptional regulation by miRNAs is an element of the molecular cross-talk between the sexes and mainly regulates behavioral responses in the specific miRNAs tested. Mutant female responses to receipt of SP were altered in a relatively modest manner. We suggest that females use miRNA regulators to buffer their physiological processes against transcriptional noise introduced by mating and potentially to minimize the negative fitness effects of male manipulation.

It is important to note that ours is a study of a small number of candidate miRNAs that change expression in response to receipt of a single ejaculate protein, SP. It is likely that other microRNAs are involved in mediating responses to SP and to Sfps that interact with SP. For example, even in this study there were candidate miRNAs that were not tested. In addition, the ability of SP to participate in sustained postmating responses requires the actions of a proteolytic cascade of different Sfps (Avila *et al.* 2010; LaFlamme *et al.* 2012). Therefore the actions of SP are interdependent with those of multiple other Sfps and the regulation of such networks is likely to involve as yet unknown interacting regulatory mechanisms. There are also ~130 Sfps (Findlay *et al.* 2008; Ayroles *et al.* 2011) with diverse functions in different facets of postmating responses (*e.g.*, Chapman 2001; Gillot 2003; Ram and Wolfner 2007a; Wolfner 2009; Avila *et al.* 2011). For example, postmating responses such as egg production, receptivity to remating, and sperm storage require multiple Sfps in addition to SP (*e.g.*, Ram and Wolfner 2007b; Avila *et al.* 2011). It should also be noted that many Sfps have as yet unknown functions. Future investigations of the interactions between different Sfps and their regulators in maintaining reproductive functions, buffering against perturbation, and in balancing the interests of males and females are likely to yield important results.

Acknowledgments

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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	-

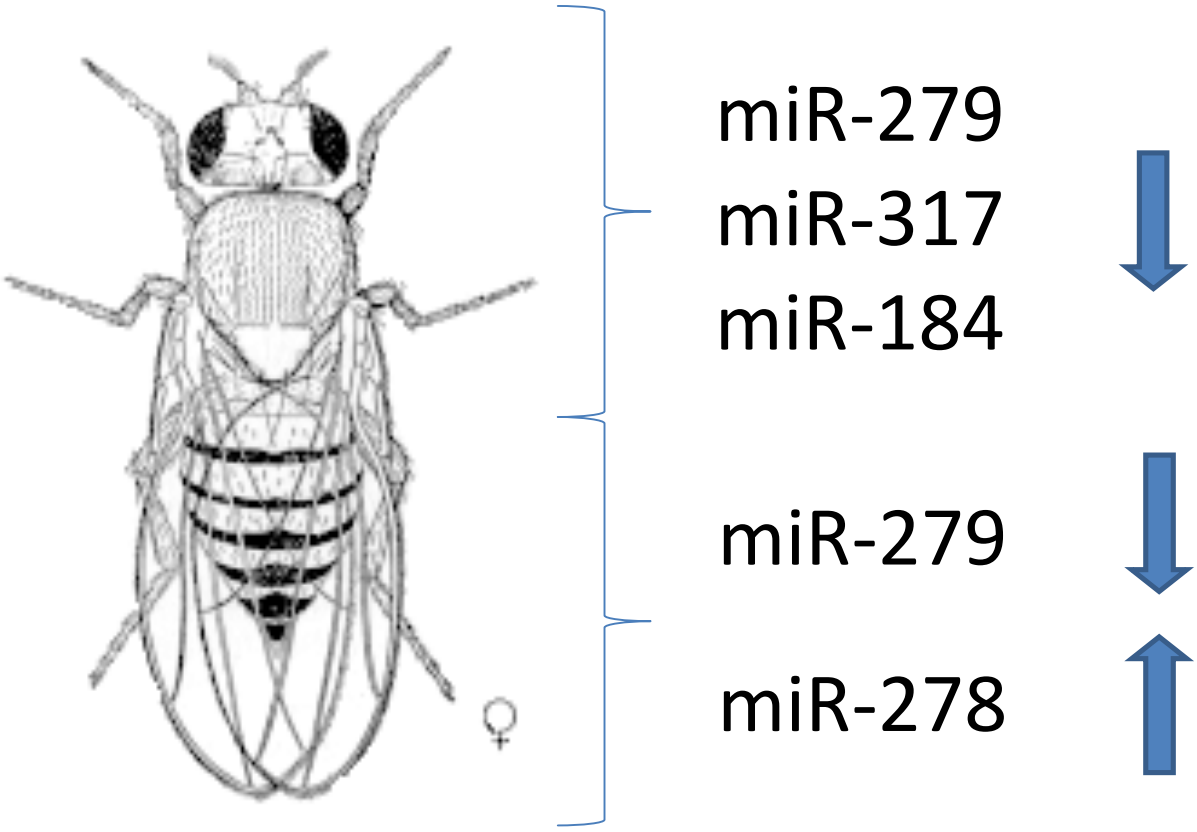


Figure S1 Summary scheme of the direction of expression change in the four selected microRNAs in female Head+Thorax and Abdomen samples 3 hr following receipt of sex peptide from males during mating.

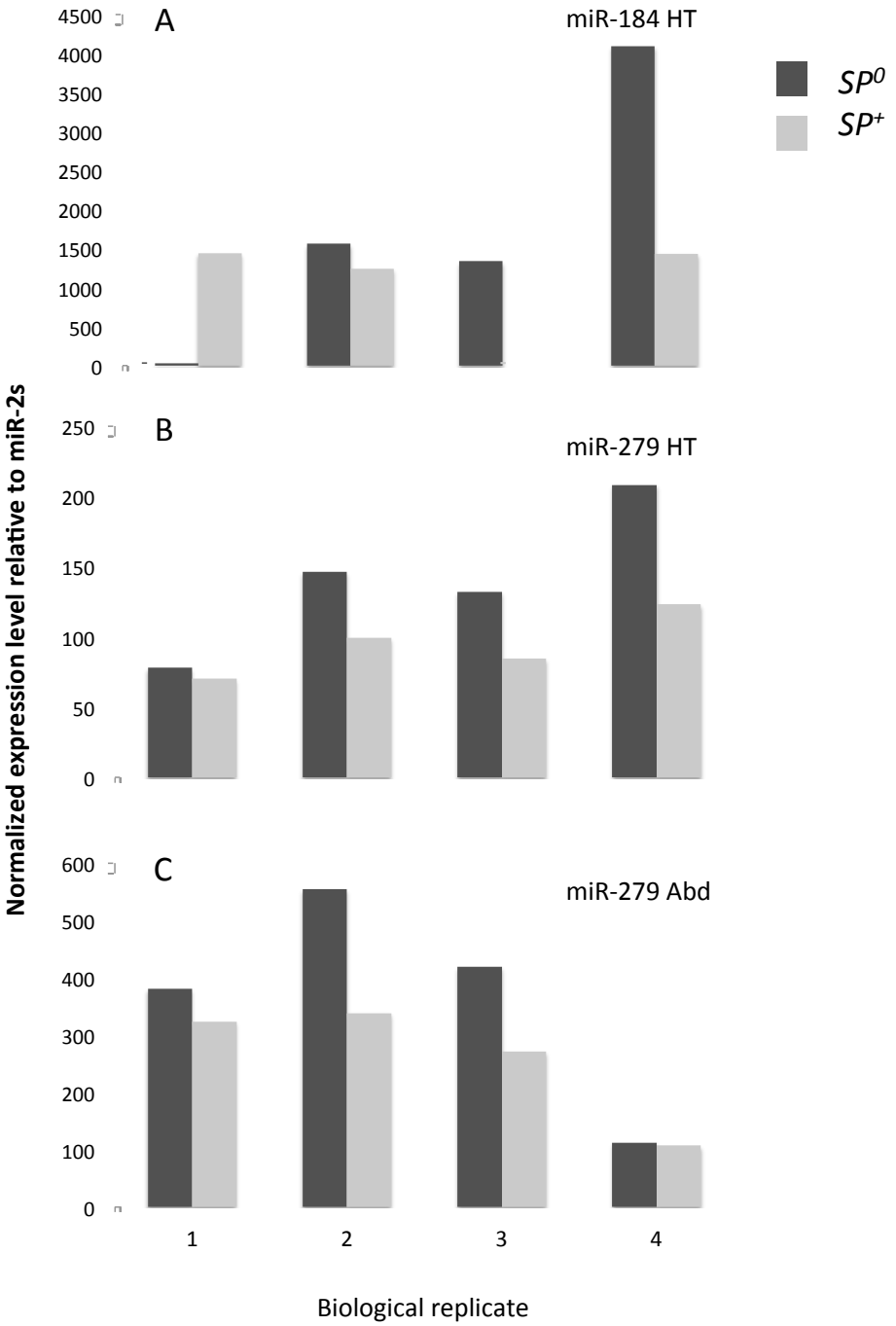

A
miR-184 HT
B
miR-279 HT
C
miR-279 Abd
SP^0
SP^+
Normalized expression level relative to miR-2s
4500
4000
3500
3000
2500
2000
1500
1000
500
0
250
200
150
100
50
600
500
400
300
1
2
3
4
Biological replicate

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.

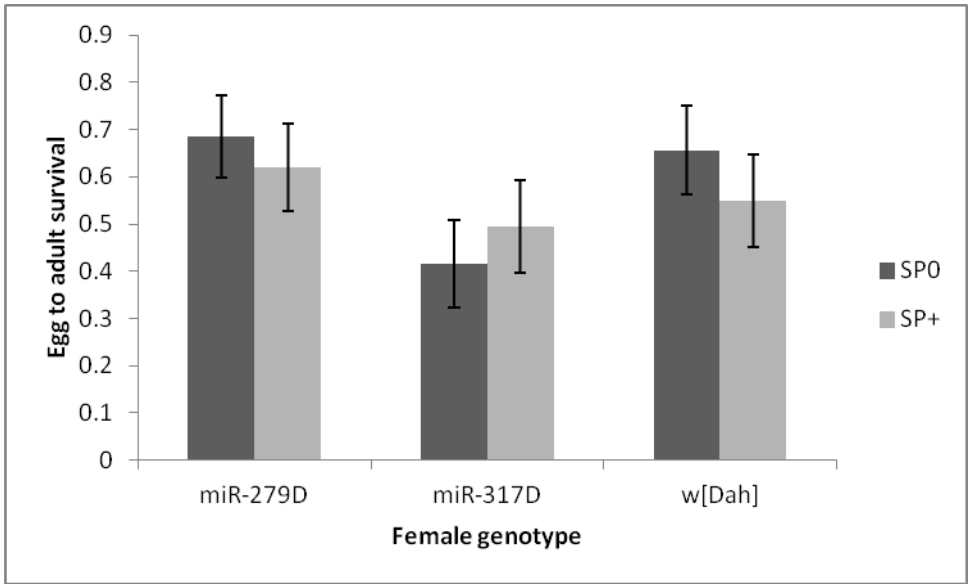
mir-279D and mir-317D hypomorphs The two Dahomey-background miRNA-hypomorph lines and their control $w^{[Dah]}$ did not differ significantly in egg to adult survival ($G^2 = 65.31$, $F_{1,157} = 2.94$, $P = 0.056$ [Dispersion parameter = 11.20]; Figure S3A).

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).

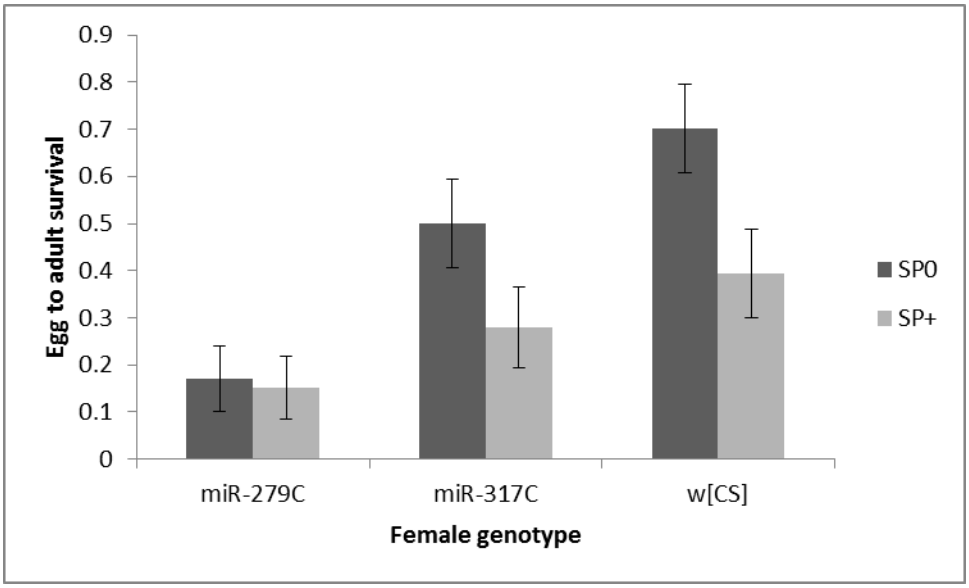
mir-278D ko *mir-278D* ko females had a tendency to exhibit lower egg to adult survival than did control females ($G^2 = 24.77$, $F_{1,114} = 3.32$, $P = 0.071$ [Dispersion parameter = 7.53]). This was independent of the male with which they mated ($G^2 = 0.54$, $F_{1,114} = 0.07$, $P = 0.789$, interaction = 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
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0.5
0.4
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0.2
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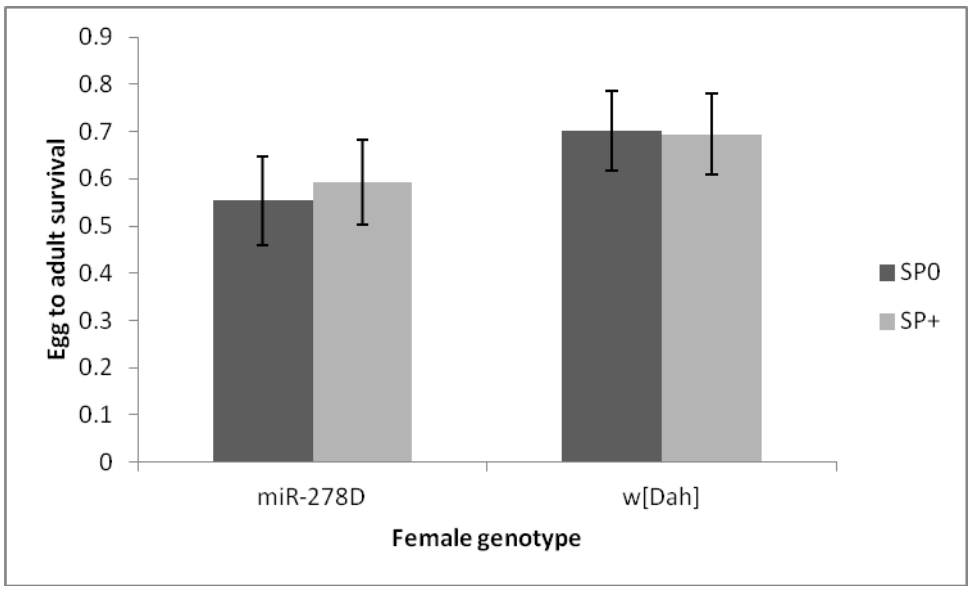
0
Egg to adult survival
miR-279C
miR-317C
w[CS]
Female genotype
SP0
SP+

C



D

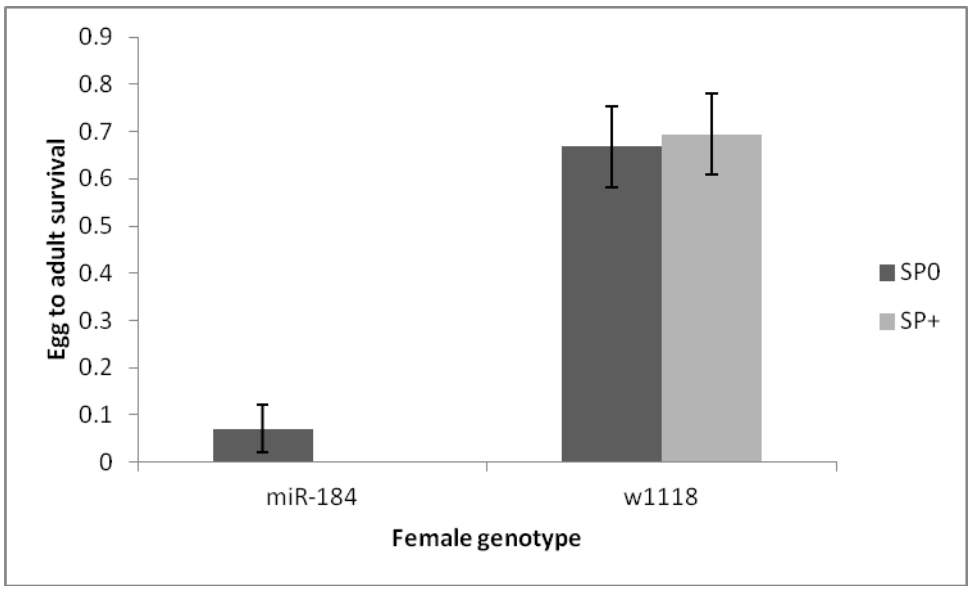


Figure S3 Egg-to adult survival (mean ± SE) for the miRNA-lacking females in the 24 hr intermating interval following a single mating to either a SP-lacking (SP^0) or a control male (SP^+): (A) hypomorphic *mir-279D* and *mir-317D* females backcrossed into the wild-type $w^{[Dah]}$ genetic background,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

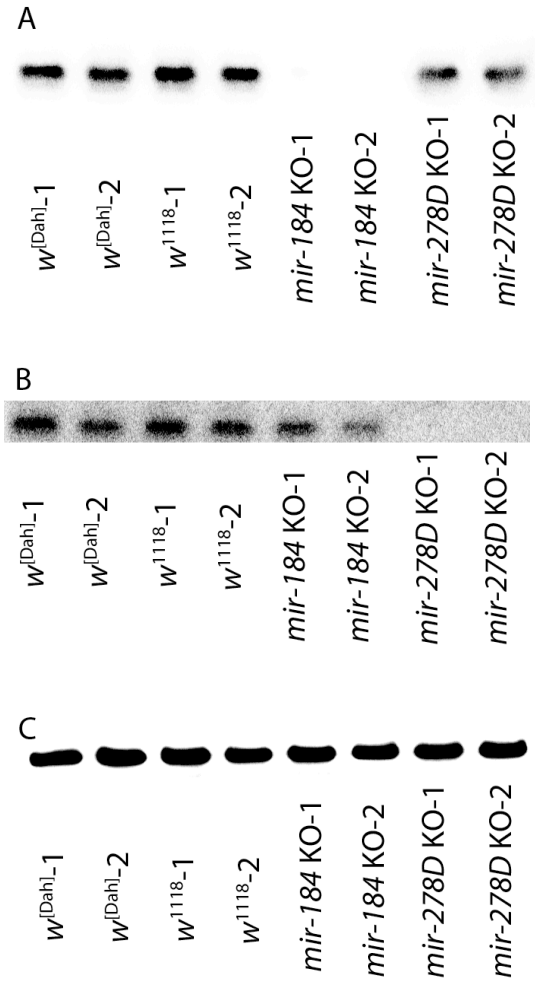


Figure S4 Northern blot with probes for (A) *mir-184*, (B) *mir-278D,* (C) U6 control. The miRNA knock out (KO) lines showed no expression of the relevant miRNA, but no difference in expression level relative to the control lines for the other miRNA and the U6 control RNA. w^{1118} is the control for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

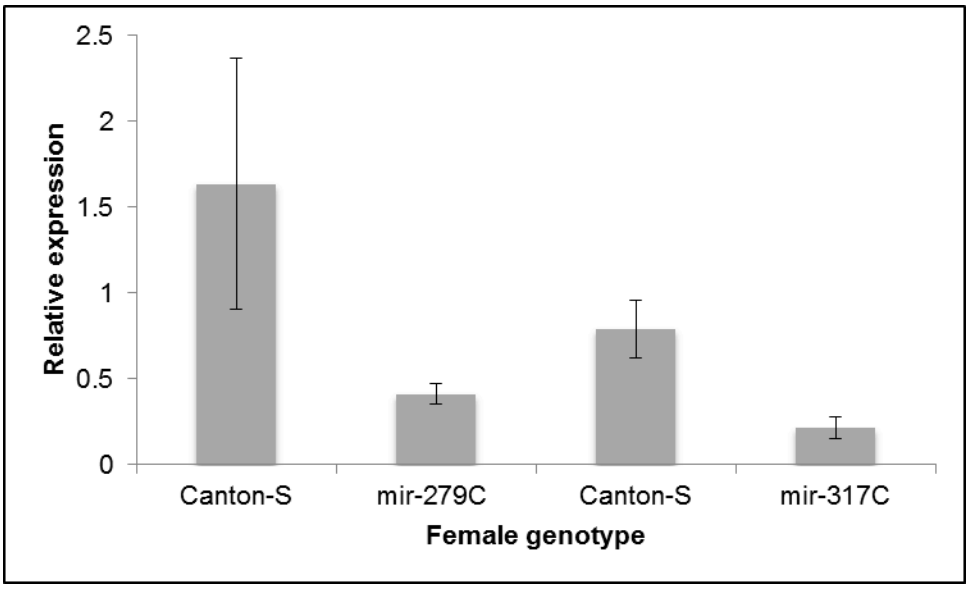


Figure S5 Mean ± SE relative expression of *mir-279* and *mir-317* in the control w^{1118}, Canton-S and the *mir-279C* and *mir-317C* hypomorph lines. The miRNA hypomorphs had significantly lower expression than the control line. We assayed the Canton-S line twice so that the qRT-PCR run for each miRNA 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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