ORIGINAL ARTICLE

Evolutionary history of sexual selection affects microRNA profiles in *Drosophila* sperm

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The presence of small RNAs in sperm is a relatively recent discovery and little is currently known about their importance and functions. Environmental changes including social conditions and dietary manipulations are known to affect the composition and expression of some small RNAs in sperm and may elicit a physiological stress response resulting in an associated change in gamete miRNA profiles. Here, we tested how microRNA profiles in sperm are affected by variation in both sexual selection and dietary regimes in *Drosophila melanogaster* selection lines. The selection lines were exposed to standard versus low yeast diet treatments and three different population sex ratios (male-biased, female-biased, or equal sex) in a full-factorial design. After 38 generations of selection, all males were maintained on their selected diet and in a common garden male-only environment prior to sperm sampling. We performed transcriptome analyses on miRNAs in purified sperm samples. We found 11 differentially expressed miRNAs with the majority showing differences between male- and female-biased lines. Dietary treatment only had a significant effect on miRNA expression levels in interaction with sex ratio. Our findings suggest that long-term adaptation may affect miRNA profiles in sperm and that these may show varied interactions with short-term environmental changes.

KEY WORDS: Diet, epigenetics, nongenetic inheritance, nutrition, paternal effects, small RNAs, sperm competition, sperm RNA.

Sperm RNAs were first discovered in the late 1980s in sperm of the rat *Rattus norvegicus* and in humans (Pessot et al. 1989). In the rat, a range of RNA sizes were described from short transfer RNAs to high-molecular-weight components. However, the overall RNA content in human and rat sperm is only about 0.1 pg (Pessot et al. 1989), which may explain why the significance of sperm RNAs has been overlooked until relatively recently. RNAs found in sperm include small RNAs (sRNAs), such as PIWI-interacting and micro (miRNA) RNAs, as well as short fragments of transfer RNAs and a small number of messenger RNAs (Rando 2016; Immler 2018). The interest in sperm RNA profiles has rapidly grown over the past few years due to the discovery of their important roles in male fertility (Papaioannou and Nef 2010; Salas-Huetos et al. 2020) as well as their putative role in nongenetic inheritance and their effects on offspring development

and fitness (Immler 2018; Baxter and Drake 2019). It is currently still unclear what are the key drivers that affect the RNA content in sperm. Comparisons of the possible effects of environmental conditions with long-term evolutionary histories on sperm RNA profiles promise to provide new answers.

In our study, we focus on miRNAs because they play an important role in many physiological processes. For example, they regulate gene expression and hence, a disruption of miRNA pathways may affect processes anywhere from spermatogenesis to early embryo development (Boerke et al 2007; Salas-Huetos et al. 2020). miRNAs have been associated with fertility issues and sterility due to defects in the maintenance of germ stem cells (Park et al 2007; Papaioannou and Nef 2010; Saxe and Lin 2011; Salas-Huetos et al. 2020). Male condition is known to affect sperm miRNAs in a range of species (Rando 2016) and sperm

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miRNAs are suggested to have a putative function in mediating inter- and transgenerational inheritance (Miller et al. 2005; Dadoune 2009; Bourc'his and Voinnet 2010; Hosken and Hodgson 2014; Yan 2014; Holman and Price 2014). A common denominator across studies investigating changes in sperm miRNA profiles is to expose males to environmental stressors, such as social interactions and physical stress but also to nutritional and chemical variation (Rodgers et al 2013; Gapp et al. 2014; Fullston et al. 2016; Claycombe-Larson et al. 2020). Changes in environmental conditions may trigger a physiological stress response, which in turn may affect the germline and result in an upregulation of DNA maintenance and repair mechanisms to protect the germ cells (Jones-Rhoades and Bartel 2004; Cinalli et al 2008). As a consequence, changes in miRNA expression in the germ cells may result in associated changes in miRNA profiles in mature sperm that can affect sperm performance as well as the development of any resulting embryos.

Whether changes in miRNA profiles in response to environmental changes are adaptive is an important and unanswered question. What is clear is that variation in the miRNA content in sperm may have effects on the next generation(s) (Miller et al. 2005; Dadoune 2009; Bourc'his and Voinnet 2010; Hosken and Hodgson 2014; Yan 2014; Holman and Price 2014). miRNAs are also correlated with among- as well as within-male differences in sperm motility (see Abu-Halima et al. 2013 for an example in humans). If miRNAs contribute to the sperm phenotype, they could influence the ability of sperm to compete against rival sperm and fertilize an egg. It has been suggested that sperm miRNA profiles may carry signatures of the selection pressures acting on sperm. Hosken and Hodgson (2014) argued that if sperm RNAs affect sperm competitiveness, we could learn more about sperm RNA function by comparing sperm RNA profiles in species or selection lines that experience different intensities of sexual selection (Hosken and Hodgson 2014). Studying sperm miRNA profiles under a variety of environmental conditions over many generations may enable us to identify the evolutionary selection pressures shaping sperm miRNA content.

In our study, we investigated the selection pressures that are acting on sperm miRNA content by comparing the sperm miRNA profiles of *Drosophila melanogaster* selection lines subjected to three different sexual selection treatments (male-biased sex ratio, equal sex ratio, and female-biased sex ratio) and two different diet treatments (100% [100 g/L] yeast and 20% [20 g/L] yeast). The selection lines were derived from an experimental evolution study combining diet and sexual selection regimes in a 2 × 3 full-factorial design resulting in six different combinations. For each of the six combinations, we had three replicate lines, resulting in a total of 18 selection treatment lines. After 38 generations of selection, we separated males from females immediately after hatching and allowed them to mate for 24 h with control females before separating them for 5 days to standardize their sperm production across regimes. During this time, the males were maintained on their selected standard or low yeast diet regimes. We collected two pooled sperm samples from 20 males each per selection line and sequenced the miRNA profiles of all samples.

Material and Methods
EXPERIMENTAL EVOLUTION

All *D. melanogaster* flies used in this study came from the large, outbred Dahomey stock population with overlapping generations. The flies were kept at 25°C, ∼60% humidity, and a diurnal cycle of 12 h light:12 h darkness. The flies for the selection lines were obtained from eggs collected from four replicate Dahomey stock cages. The eggs were obtained by placing a 9-cm-diameter Petri dish containing red grape juice medium (grape juice medium for 30 Petri dishes contained 550 mL distilled water, 25 g agar, 300 mL red grape juice, and 21 mL Nipagin solution) smeared with live yeast as oviposition substrate into the cages. When the eggs had hatched, the first instar larvae were picked from the Petri dishes and placed into glass vials (75 mm height × 25 mm diameter; 100 larvae per vial) containing standard sugar-yeast medium (100 g brewer's yeast, 50 g sugar, 15 g agar, 30 mL Nipagin [10% w/v solution], and 3 mL propionic acid, per liter of medium). One day after all flies had eclosed, the adults were mixed and randomly allocated to one of six selection regimes; that is, the flies were allocated a specific combination of a sexual selection treatment and diet. Each selection line consisted of 100 flies and the selection regimes comprised the following treatments: the sexual selection regime contained three different levels of sexual selection by manipulating the sex ratios: a male-biased treatment (MB: 70 males:30 females), equal sex ratio (EQ: 50 males:50 females), and female-biased (FB: 25 males:75 females). The dietary regime consisted of two treatments: a standard yeast treatment (100% yeast) and a low yeast treatment (20% yeast content of standard diet). The two regimes were combined in a full factorial 2 × 3 design resulting in six selection regimes. For each treatment combination, we had three replicate lines resulting in a total of 18 selection lines, each of which was maintained in a plastic cage (18 × 12 × 8 cm). The flies had access to ad libitum water from two water-filled glass vials with cotton wool bungs. Every 2–3 days, the flies were fed with two food vials each containing 10 mL of the diet corresponding to the selection regime. The eggs of the flies were collected 9–10 days after the initial set up from grape plates smeared with yeast paste. These eggs were allowed to hatch and 300 first instar larvae per line were collected and raised to adulthood in vials containing 7 mL of standard diet (100 larvae per vial). The adults were allowed to enclose over 2 days and were then allocated to the same sex ratio, food treatment, and replicate number as their parents. All subsequent

generations were maintained as described above. Generation time in the experiment was 3 weeks. The selection lines were kept under these conditions for 38 generations at the School of Biological Sciences, University of East Anglia (UEA), Norwich, UK. Samples of the eggs from these lines were then collected and shipped to the Department of Evolutionary Biology (EBC), University of Uppsala, Sweden for the RNA work.

PREPARATION OF MALES FOR SPERM RNA ANALYSIS

Upon their arrival at EBC, the larvae (and later the adult flies) were kept under the same conditions as at UEA (25°C, 60% humidity, 12 h light:12 h dark, selection regime diet). The sex ratio of all regimes was equalized upon hatching by separating males and females in Uppsala to control for mating opportunities prior to sperm sampling (i.e., the number of sperm produced). During the eclosion period, the vials were scanned daily to collect freshly emerged flies (once per day, around 1200 h). Freshly emerged flies were anesthetized on ice and sorted by sex using a small soft brush. From the freshly emerged flies of the selection lines, only the males were kept, and the females were discarded. The males were transferred into plastic vials containing the same diet as in their selection regime and were then provided with the same number of stock females for 24 h (10 males in one vial were provided with 10 females). These females all came from an outbred Dahomey stock population that had been reared under standard conditions (standard food and natural sex ratio) for many generations. After eclosion, the virgin females were kept on standard food (100% yeast diet) until they were transferred to the males at 2–6 days old. The selection line vials contained on average 10 males and 10 females (range between 8 and 12 males and 8 and 12 females but in a few cases only few males hatched on the same day so they were kept at lower densities).

After 24 h with females, the males were transferred into fresh vials containing the diet corresponding to their selection regime and kept in the incubator for another 5 days. When the males were 7 days old, they were flash frozen in liquid nitrogen at 1009 h. We froze all of them at the same day time to ensure their sperm RNA profile would not differ due to diurnal differences in gene expression. For the flash freezing, we transferred the males into RNAse-free microcentrifuge tubes and placed them in liquid nitrogen. The microcentrifuge tubes containing the frozen males were then quickly transferred into a precooled cardboard box in a styrofoam box filled with dry ice and then stored at –80°C freezer until dissection of the males for sperm collection. This sampling design allows the detection of signatures of an evolutionary history of variation in adult sex ratio on sperm miRNA profiles, as the sampled males experienced a common garden sex ratio environment prior to sampling. However, as males were maintained on their selected diets during this time, any differences due to dietary treatment could be due to both short- or long-term (evolutionary) effects.

For each of the 18 selection lines, we collected two pooled samples with sperm from 20 males each (eight dissection sessions per selection line and the sperm of four to six males per session) and stored them at –80°C until RNA extraction. The flies and the sperm samples were either frozen or cooled during the whole sampling process to minimize RNA degradation. We developed a protocol to ensure extremely careful male dissection and sperm collection to avoid contamination with somatic cells. This was important because even one or two somatic cells in a sperm sample may strongly affect the RNA profiles of the samples because RNA abundance in somatic cells is several orders of magnitudes higher compared to sperm. The detailed steps of the protocol and the verification steps are described in detail in the Supporting Information.

SPERM RNA EXTRACTION, DNA LIBRARY PREPARATION, AND RNA SEQUENCING

RNA was extracted from the 36 pooled samples in six batches of six samples each, using a custom protocol (see Supporting Information for protocol and batch layout). One-microliter small RNA spike-ins (Exiqon) were added after lysis and before the extraction. RNA was extracted in 8-μL nuclease-free water. NGS libraries were prepared from 6-μL RNA using NEB small RNA library prep kit (E7330) protocol. All 36 samples were processed in a single batch, with an end library volume of 100 μL. Library quality and quantity was estimated on a TapeStation (Agilent) using RNA HS screentape (Agilent). Library quantification was estimated by qPCR using a library quantification kit (KAPA). Mean of qPCR and TapeStation estimates were considered as the final estimates for library DNA concentration. Library volume was lowered from 100 to 20 μL by freeze-drying. The standard protocol was followed for library preparation and PCR amplification was run for 15 cycles. Size selection was performed on E-gel EX 4% agarose gel (Invitrogen). A total of 20 μL of library was loaded to an E-gel cassette and run for 20–25 min. Four gels were run with nine samples each. Bands in the range 140–245 bp were cut out with a sterile scalpel into a 1.5-mL vial. Gel extraction was carried out using MinElute gel extraction kit (Qiagen). A total of 10–30 μL of excess 3 M pH 5.2 sodium acetate was added to adjust pH. Purified library was eluted in 13 μL nuclease-free water. Purified libraries were quantified using qPCR library quantification kit (KAPA) and Qubit RNA HS reagents. Mean of qPCR and Qubit estimates were considered as the final estimates. For sequencing, nine samples were pooled together per pool. Each pool was sequenced on one lane of Illumina HiSeq 2500 (50 bp single-end reads) to a depth of 20 million reads per sample.

RNA-DATA ANALYSES

Reads were mapped to spike-in sequences and to *Drosophila* mature miRNA sequences downloaded from miRBase (release 22.0; Kozomara et al. 2019) by exact matching with PatMaN (Prüfer et al. 2008). Count tables were imported into R and miRNA counts in each sample were normalized by spike-in reads. Normalization was carried out using RUVSeq's (Risso et al. 2014) RUVg function. Differential expression between treatments was determined using DESeq2 (Love et al. 2014), which uses the Benjamini and Hochberg method for multiple testing correction. The counts were first normalized to library size in DESeq2 using the "estimateSizeFactors" function and then unwanted variation was removed based on the spike in read counts per sample calculated using the RUVg function in RUVSeq.

Results

sRNA SEQUENCING QC

FASTQ files generated from the sequencing were checked using FastQC and adaptors were trimmed based on perfect matches to the first eight bases of the adaptor sequence (AGATCGGA). Small RNA reads had an average of 91.9% alignment rate to the genome (min = 87.19%, max = 94.06%). Size distribution profiles of sRNA read length showed a dominant peak at 30 nt and smaller neighboring peaks at 29 and 31 nt. Reads of this length were dominated by a 2S rRNA sequence—a 30-nt rRNA found in *Drosophila* that migrates along with the sRNA fraction and thus is not excluded through size selection (Fowler et al 2018). Other smaller peaks were evident at 19, 21, 27, and 35 nt. The average percentage of reads mapping to mature miRNAs was 0.34% across all libraries. The average percentage of rRNA mapping reads was 44.5% (Fig. S1). Small RNAs were derived from several other classes of ncRNA: tRNA (9.1%), snoRNA (2.7%), snRNA (0.8%) as well as from annotated coding genes (3.5%); 38.9% of reads were mapped to unannotated intergenic and intronic regions.

SEQUENCED miRNAs

Reads annotated as miRNAs represented a very small fraction of total sequenced reads (<1%). It is likely that other reads may correspond to other noncoding RNAs such as piRNAs. In other studies of mammal and bird sperm, large (∼32 nt) non-miRNA types of sRNA dominated the sequencing output, and this pattern was also observed here.

Across all libraries, reads mapped to a total of 91 miRNAs, including 22 where both the 5p and 3p strands were detected, resulting in 69 unique miRNAs (Table 1). Of the 91 miRNAs, 41 had >100 reads in at least one library. There was low variation across libraries in terms of the number of mapped miRNAs

(Table S1), with no interaction with sex ratio regime of individual lines, that is, equal numbers of miRNAs were sequenced in the FB lines, MB lines, and EQ lines. Every library had between nine and 12 miRNAs with >1000 reads. The identities of the most abundantly expressed miRNAs varied little across libraries, and let-7-5p was the most abundant miRNA in all libraries.

When all libraries were combined, the top 12 most abundant miRNAs were as follows: let-7-5p, miR-263a-5p, miR-14-3p, miR-8-5p, miR-14-5p, miR-1-3p, miR-276a-3p, miR-34-5p, miR-9a-5p, miR-184-3p, miR-8-3p, and miR-31b-5p.
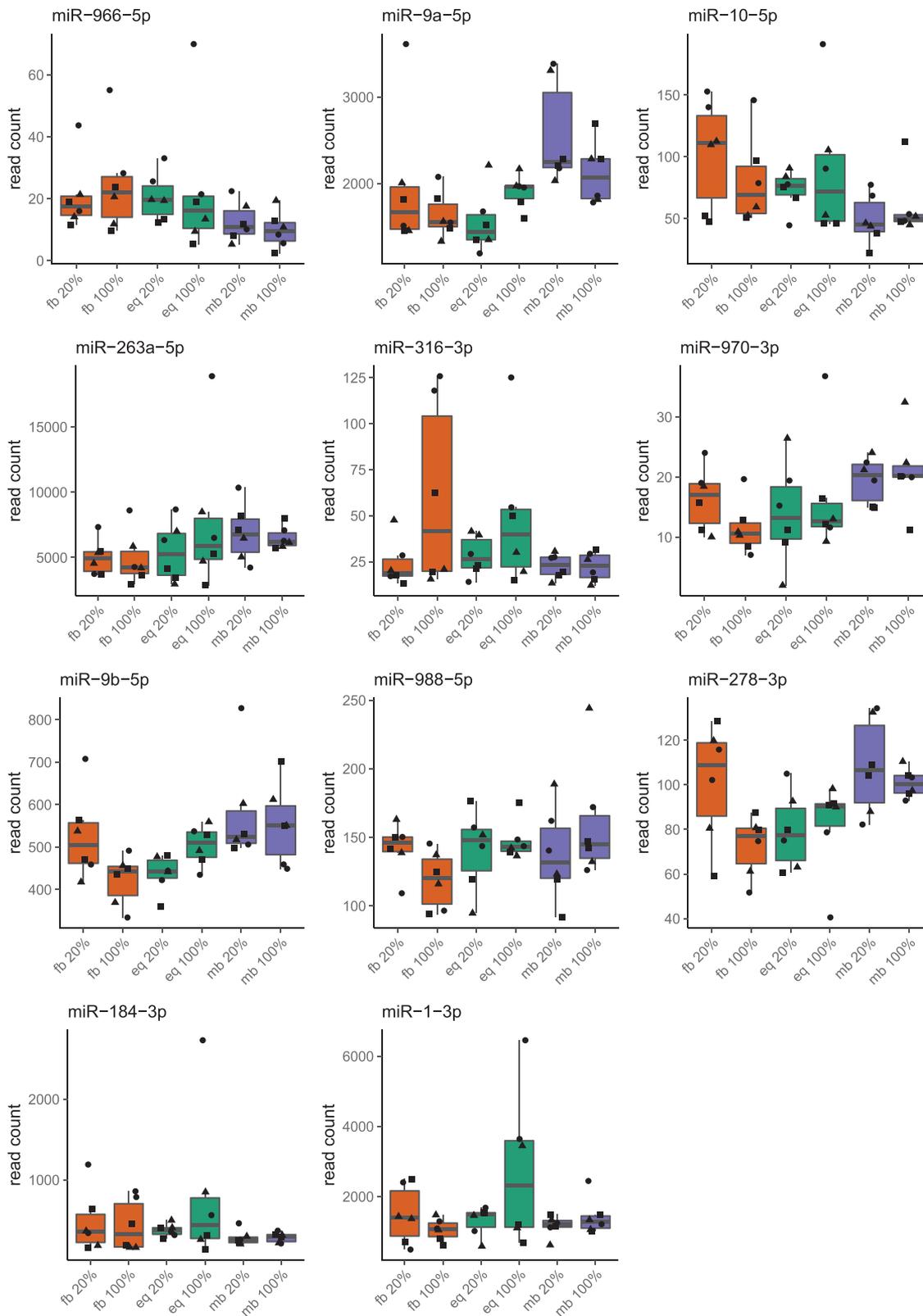
DIFFERENTIALLY EXPRESSED miRNAs

Across all selection regime comparisons, we identified 11 differentially expressed (DE) miRNAs meeting a significance threshold of p.adj < 0.1 (Table 2; Fig. 1). Of these, miR-9a-5p and miR-966-5p were DE in multiple comparisons. The most striking differences were between the female-biased (FB) and male-biased lines (MB) in the 100% yeast diet. In contrast, for the 20% yeast diet there were no DE miRNAs between FB and MB lines, although when data from across the 20% and 100% diet lines were combined, four miRNAs met the significance threshold. There were fewer differences between EQ and FB or MB lines: one miRNA was DE between EQ and FB lines, and three miRNAs were DE between EQ and MB lines. A comparison of the dietary treatments across all sex ratio lines yielded no DE miRNAs. In summary, we found most significant DE miRNAs in comparisons between treatments of different sex ratios, whereas diet per se did not seem to have a major impact, only in interaction with sex ratio.

Discussion

The main result of our study was the finding that variation in the evolutionary history of sexual selection affected miRNA profiles in sperm. We found 11 DE miRNAs between selection lines maintained for 36 generations under varying adult sex ratios. The majority of miRNAs showing differential expression were found in comparisons between male-biased and female-biased lines. In contrast, dietary variation had minimal effect on miRNA profiles and only in interaction with sex ratio treatments. We observed lower expression for three miRNAs (miR-9b-5p, miR-278-3p, and miR-970-3p) in lines with female-biased sex ratio on 100% yeast compared to lines with female-biased sex ratio on 20% yeast and lines with male-biased sex ratios on both diets (Fig. 1). There was no main effect of dietary treatment per se on miRNA expression. The number of miRNAs sequenced in each library ranged from 74 to 91, but there was no relationship between the number or composition of the different miRNAs expressed and population sex ratio or diet.

Table 1. miRNAs detected in the RNA sequencing and their chromosomal location (CL). Shaded miRNAs are clustered together in the same genomic region. Where both strands were detected, the strand with more reads (an average across all libraries) is indicated in **bold** and *italics*.

miRNA	CL	Reads	miRNA	CL	Reads	miRNA	CL	Reads
miR-10404-5p	rDNA	3	miR-184-3p	2R	444	miR-284-5p	3R	689
miR-13b-3p	X/3R	13	miR-278-***5p***, 3p	2R	502, 91	miR-995-5p	3R	1
miR-2b-3p	2L	9	miR-8-***5p***, 3p	2R	5237, 1332	miR-4951-5p	3R	17
miR-6-3p	2R	3	miR-31a-5p	2R	113	miR-11-5p, ***3p***	3R	102, 523
miR-263a-***5p***, 3p	2L	6099, 1	miR-5-5p	2R	2	miR-999-3p	3R	442
miR-9c-5p	2L	45	miR-310-3p	2R	4	miR-1010-3p	3R	10
miR-306-5p, ***3p***	2L	2, 8	miR-311-3p	2R	16	miR-1003-3p	3R	4
miR-9b-***5p***, 3p	2L	504, 2	miR-312-3p	2R	9	miR-92b-3p	3R	7
miR-1006-3p	2L	9	miR-991-3p	2R	10	miR-279-3p	3R	140
miR-100-***5p***, 3p	2L	550, 2	miR-7-5p	2R	140	miR-996-5p	3R	4
Let-7-***5p***, 3p	2L	23825, 46	miR-987-5p	2R	2	miR-1013-3p	3R	1
miR-125-***5p***, 3p	2L	122, 32	miR-14-5p, ***3p***	2R	3264, 7653	miR-10-***5p***, 3p	3R	76, 13
miR-2b-2-5p, 3p	2L	12	miR-276b-3p	3L	5	miR-954-5p	4	7
miR-1-3p	2L	1529	miR-276a-3p	3L	2403	miR-9370-5p	X	4
miR-965-5p	2L	148	miR-263b-5p	3L	4	miR-970-***5p***, 3p	X	44, 16
miR-960-5p	2L	1	miR-9a-5p	3L	1960	miR-283-***5p***, 3p	X	9, 8
miR-964-5p	2L	1	miR-33-5p	3L	32	miR-304-***5p***, 3p	X	91, 6
miR-966-5p	2L	19	miR-316-3p	3L	34	miR-12-5p	X	276
miR-275-5p, ***3p***	2L	132, 257	miR-bantam-5p, ***3p***	3L	100, 393	miR-2535b-3p	X	5
miR-305-5p, ***3p***	2L	24, 625	miR-190-5p	3L	12	miR-982-5p	X	18
miR-375-5p, ***3p***	2L	2, 33	miR-317-***5p***, 3p	3R	326, 14	miR-983-5p	X	3
miR-988-***5p***, *3p*	2R	141, 18	miR-277-5p, ***3p***	3R	46, 317	miR-984-5p	X	43
miR-281-2-5p	2R	5	miR-34-***5p***, 3p	3R	2506, 266	miR-31b-5p	X	679

Figure 1. Normalized read counts for the 11 miRNAs that were differentially expressed among selection lines (female biased [fb, orange boxes], equal sex ratio [eq, green boxes], male biased [mb, purple boxes], 20% yeast diet [20%], or 100% yeast diet [100%]). Identity of miRNA is at the top of each panel. Boxes show interquartile range (IQR) and median of technical and biological replicates of each selection line ($n = 6$), and whiskers represent the largest and smallest values within 1.5 times the IQR above and below the 75th and 25th percentiles, respectively. Individual data points are plotted with jitter. Different symbols represent two samples from each selection line.

Table 2. Identities of differentially expressed miRNAs between selection line adult diets (20% or 100% yeast), and/or sex ratio (equal [EQ], female biased [FB], male biased [MB]), for two significance thresholds—least stringent (p.adj = 0.05–0.1) to most stringent (p.adj < 0.05). A dash indicates no DE miRNAs were found for that comparison.

Comparison		DE miRNAs	
Sex ratio line	Dietary line	p.adj = 0.05–0.1	p.adj < 0.05
Combined	20% vs. 100%	–	–
FB vs. MB	20%	–	–
FB vs. MB	100%	miR-316-3pmiR-970-3pmiR-9b-5pmiR-966-5pmiR-988-5pmiR-278-3p	–
FB vs. MB	Combined	miR-9a-5p miR-10-5p miR-966-5pmiR-263a-5p	–
EQ vs. FB	20%	–	–
EQ vs. FB	100%	–	miR-1-3p
EQ vs. FB	Combined	–	–
EQ vs. MB	20%	–	miR-9a-5p
EQ vs. MB	100%	miR-184-3p	–
EQ vs. MB	Combined	–	miR-9a-5p

Differential expression of miRNAs between the sex ratio treatments is likely to have resulted from the evolutionary history of sex ratio variation in these lines. The males from all regimes were kept in a common garden environment prior to sampling (male-only environment for 5 days following a 24-h window of mating on day 1). This design was selected because males held under the varying regimes during selection have different mating opportunities that could affect spermatogenesis and sperm age (both may be correlated with sperm miRNAs) at sampling. By providing a common garden prior to sampling, by standardizing mating rate and timing since last mating at sampling, we both reduced variation in age of sperm in the samples and facilitated the detection of any evolved signatures in the miRNA profiles. However, a change in conditions in comparison to those normally experienced during selection may induce elevated or altered physiological stress responses (Lopez-Maury et al 2008). This has the potential to affect miRNA profiles in the germ cells and sperm (e.g., Rodgers et al 2013). Hence any differences in the miRNA profiles we observed was seen over and above any such stress responses, although we cannot rule out the sex ratio regimes interacting with such stress in different ways in different ways. As we did not adopt a common garden setup for two generations prior to sampling, as is usually recommended (Kawecki et al 2012), we cannot rule out the influence of strong parental effects, particularly for responses to diet, as the flies were maintained on their diet regimes prior to sampling. However, we note that recent work in model organisms is revealing that the trans-

mission of nongenetic information can in fact occur across many generations (e.g., Vastenhouw et al. 2006; Rechavi et al. 2011; Houri-Ze'evi et al. 2016) and thus would not be eliminated by the standard common garden practice. Overall, our finding that there was no direct effect of diet on miRNAs (a factor that is known to affect miRNAs upon short-term exposure; Claycombe-Larson et al 2020) but a significant effect of sex ratio regime suggests that the differences we observed in differential expression of the miRNAs in sperm were indeed likely to be attributable to the evolutionary history of sex ratio variation in these lines.

Below, we discuss the general functions of the DE miRNAs as well as the other miRNAs expressed in sperm of all males across the different lines in more detail. We also discuss the wider implications of our findings and possible roles of the miRNAs identified in our study in embryo development and nongenetic inheritance.

miRNA FUNCTIONS

Not surprisingly, the overall miRNA yield in all our samples was low as is typical for sperm in general. But despite the low yield, a total of 91 miRNAs were detected, representing approximately a quarter of all known miRNAs in *Drosophila*. In other words, many miRNAs are actually found in sperm and will be transferred into the egg. The number of detected miRNAs varied slightly with sample (74–91), but this variation did not interact with the sex bias or diet treatment of the selection lines. In every sample, the most abundant miRNA was *let-7* and the top 12 most

abundant miRNAs were generally the same across all samples. It is notable that a previous study showed that of the top 12 most abundant miRNAs identified in our study, a knock-out in *let-7* caused sterility in *Drosophila* males, but not females (Chen et al. 2014). The same study showed through knock out that none of the other abundant miRNAs tested had fertility phenotypes but that seven of the top 12 abundant miRNAs in our study affected larval to adult survival and/or male survival.

Previous studies comparing miRNA expression in male and female *Drosophila* (Marco 2014; Fowler et al. 2019) identified a robust sex-bias in the expression of certain miRNAs. This sex-bias was particularly strong in the abdomen/reproductive tissues of flies. Fowler et al. (2019) found male-biased expression in 26% of sequenced miRNAs from the abdomen, whereas 16% of miRNAs were female biased (of a total 401 miRNAs). In the current study, 41% of the sperm miRNAs detected were from the male-biased subset in the abdomen, and 27% were female biased. The proportions of sex-biased miRNAs were similar among the 11 DE miRNAs, with 55% of DE miRNAs having male-biased expression and 18% female biased. This suggests that although the subset of miRNAs detected in sperm are more likely to exhibit a sex-bias than the entire miRNA population, sex-biased miRNAs are not more likely to be DE between selection lines than miRNAs with neutral expression. This opens up the possibility for these RNAs to mediate possible sexual conflict, and particularly for males to manipulate processes in the very early zygote. This idea deserves more investigations in the future.

Information on function was available for six of the 11 miRNAs that were DE between lines varying in sex ratio (Chen et al. 2014). None of the DE miRNAs showed any fertility phenotypes. However, miR-9a that we found to be more abundant in lines with male-biased sex ratio on both diets compared to lines with female-biased sex ratio on 20% yeast is required for germline maintenance and spermatogenesis (Epstein et al. 2017). In addition, miR-9a knock-out males have been shown to exhibit reduced fertility as they age (Epstein et al. 2017). In contrast, miR-184 that we found to be more abundant in lines with female-biased sex ratio on both diets compared to all other lines is required for normal female germline development and embryogenesis (Iovino et al. 2009).

WIDER IMPLICATIONS

The transmission of nongenetic information from fathers to offspring has seen a surge of interest (Bonduriansky and Day 2009, 2020). Evidence that sperm may contribute to inheritance of paternal conditions through the transmission of nongenetic factors is mounting and paternal effects are increasingly accepted (e.g., Gapp et al. 2014; Zhang et al. 2019). Sperm may carry a range of nongenetic markers and molecules including methylomes, chromatin structure modifications, small RNAs, and prions (Rando

2016; Immler 2018). One of the key contributors to paternal effects are thought to be small RNAs and miRNAs in particular. Recent studies in the house mouse *Mus musculus*, for example, suggest that piRNAs, miRNAs, and tsRNAs may be involved in environment-specific inheritance or in the transmission of paternally acquired traits to the offspring (Chen et al. 2016a,b; Villota-Salazar et al. 2016). For example, zygotic injection of sperm tsRNA fragments from males fed with a high-fat diet triggered metabolic disorders in the offspring (Chen et al. 2016a). Furthermore, the expression levels of several sperm RNA families differed between male mice exposed to traumatic stress during their juvenile stage and control males (Gapp et al. 2014). Offspring of these male mice exposed to traumatic stress also showed elevated stress response and the role of sperm RNAs in transmitting the paternal condition into the offspring was confirmed by the experimental injection of purified sperm RNAs into zygotes and subsequent testing and verification of the stress response in the adult offspring. In addition, sperm RNAs may also be important for embryo development as shown in the house mouse (Scone et al. 2005; Kawano et al. 2012; Liu et al. 2012).

The effects of both short-term and long-term variations in sexual selection including male-male competition and sperm competition experienced by the male on sperm quality and offspring fitness have been described in a range of taxa. Short-term exposure to male-male competition resulted in the production of more, longer, or faster sperm in a wide range of species (see Snook 2005 for review). Furthermore, in zebrafish *Danio rerio*, males exposed to high male-male competition produced faster sperm and sired faster hatching offspring (Zajitschek et al. 2014; Zajitschek et al. 2017). Long-term variation in sex ratio and hence sexual selection is also known to affect the evolution of sperm traits as shown in selection lines of the nematode *Caenorhabditis elegans*, house mice, and flour beetles *Tribolium castaneum* (LaMunyon and Ward 1999; Firman and Simmons 2011; Godwin et al. 2017). Our findings of effects of sexual selection on the miRNA content of sperm are in line with these previous studies and may explain some of the inter- and transgenerational effects observed in previous studies. The exact regulatory pathways linking the miRNAs in sperm with the more general fitness effects observed in the offspring still need to be studied in more detail.

Conclusions

Although we found no variation in the composition of miRNAs expressed between the different selection regimes, we found that some miRNAs were significantly DE between lines with an evolutionary history of varying sex ratios—most notably between male- and female-biased lines. Whether such differential expression is adaptive or simply associated with a more

general stress response is currently difficult to say. However, experimental evolution under varying sexual selection may affect a number of sperm traits and their ability to win in sperm competition (LaMunyon and Ward 1999; Firman and Simmons 2011; Godwin et al. 2017), and hence at least some of the miRNAs identified in our study might be linked to the selective pressures acting on sperm directly. Which sperm traits are linked with the DE miRNAs identified in our study is not known, nor do we fully understand the possible inter- and transgenerational effects these miRNAs may have. It will be interesting to continue this line of research by artificially manipulating some of these miRNAs directly in the eggs and assess their effects on offspring development and fitness.

AUTHOR CONTRIBUTIONS

CH conceived and designed the study, conducted the sperm sampling, was involved in the sperm RNA extraction pilots, and drafted the manuscript. BK and RF extracted the sperm RNA and prepared the DNA libraries. SM and EF performed the data analyses. JM and WR created and maintained the selection lines. TC supervised and coordinated the experimental evolution part of the study. SI supervised and coordinated the transcriptomics part of the study. All authors contributed to the writing of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

Raw data files of all RNAseq data can be accessed on NCBI SRA under project accession: PRJNA778608.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Combined size distribution of reads across all libraries.
Table S1. Total number of miRNAs in each library with >0, 1, 10, 100 or 1000 mapped reads.
Table S2. Phenotypes of the most abundant sperm miRNAs (from Chen et al. 2014).