Developmental Biology

An important role for triglyceride in regulating spermatogenesis

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Abstract

Drosophila is a powerful model to study how lipids affect spermatogenesis. Yet, the contribution of neutral lipids, a major lipid group which resides in organelles called lipid droplets (LD), to sperm development is largely unknown. Emerging evidence suggests LD are present in the testis and that loss of neutral lipid- and LD-associated genes causes subfertility; however, key regulators of testis neutral lipids and LD remain unclear. Here, we show LD are present in early-stage somatic and germline cells within the *Drosophila* testis. We identified a role for triglyceride lipase *brummer* (*bmm*) in regulating testis LD, and found that whole-body loss of *bmm* leads to defects in sperm development. Importantly, these represent cell-autonomous roles for *bmm* in regulating testis LD and spermatogenesis. Because lipidomic analysis of *bmm* mutants revealed excess triglyceride accumulation, and spermatogenic defects in *bmm* mutants were rescued by genetically blocking triglyceride synthesis, our data suggest that *bmm*- mediated regulation of triglyceride influences sperm development. This identifies triglyceride as an important neutral lipid that contributes to *Drosophila* sperm development, and reveals a key role for *bmm* in regulating testis triglyceride levels during spermatogenesis.

eLife assessment

This **important** study identifies a role for triglycerides and lipid droplets in spermatogenesis, with data supporting relevance of this finding across phyla. The work shows with **convincing** data that a triglyceride lipase is required cell-autonomously for germline differentiation into meiotic stages and haploid spermatids and that an increase in triglycerides is detrimental to spermatogenesis. This paper would be of interest to developmental and cell biologists working on gametogenesis.

Introduction

Lipids play an essential role in regulating spermatogenesis across animals [1–4]. Studies in *Drosophila* have illuminated key roles for multiple lipid species in regulating sperm development [5–7]. For example, phosphatidylinositol and its phosphorylated derivatives participate in diverse aspects of *Drosophila* spermatogenesis including meiotic cytokinesis [1,8–11], somatic cell differentiation [12], germline and somatic cell polarity maintenance [13–16], and germline stem cell (GSC) maintenance and proliferation [17]. Membrane lipids also influence sperm development [18,19], whereas fatty acids play a role in processes such as meiotic cytokinesis [20] and sperm individualization [21,22]. While these studies suggest key roles for membrane lipids and fatty acids during *Drosophila* spermatogenesis, some of which are conserved in mammals [23–25], much less is known about how neutral lipids contribute to spermatogenesis.

Neutral lipids are a major lipid group that includes triglyceride and cholesterol ester, and reside within specialized organelles called lipid droplets (LD) [26]. LD are found in diverse cell types (*e.g.* adipocytes, muscle, liver, glia, neurons) [27,28,26], and play key roles in maintaining cellular lipid homeostasis. In nongonadal cell types, correct regulation of LD contributes to cellular energy production [29–31], sequestration and redistribution of lipid precursors [32–36], and regulation of lipid toxicity [37–39]. The importance of LD to normal cellular function in nongonadal cell types is shown by the fact that dysregulation of LD causes defects in cell differentiation, survival, and energy production [26,37,40,41]. In the testis, much less is known about the regulation and function of neutral lipids and LD, and how this regulation affects sperm development.

Multiple lines of evidence suggest a potential role for neutral lipids and LD during spermatogenesis. First, genes that encode proteins associated with neutral lipid metabolism and LD are expressed in the testis across multiple species [42–44]. Second, testis LD have been identified in mammals and flies under both normal physiological conditions [27,44–48] and after mitochondrial stress [49]. Third, loss of genes associated with neutral lipid metabolism and LD cause subfertility phenotypes in both flies and mammals [27,50–52]. While studies suggest that mammalian testis LD contribute to steroidogenesis [53,54], the spatial, temporal, and cell-type specific requirements for neutral lipids and LD in the testis have not been explored in detail in any animal. It remains similarly unclear which genes are responsible for regulating neutral lipids and LD during spermatogenesis.

To address these knowledge gaps, we used *Drosophila* to investigate the regulation and function of neutral lipids and LD during sperm development. Our detailed analysis of spermatogenesis under normal physiological conditions revealed the presence of LD in early-stage somatic and germline cells in the testis. We identified triglyceride lipase *brummer* (*bmm*) as a regulator of testis LD, and showed that this represents a cell-autonomous role for *bmm* in the germline. Importantly, we found that *bmm*-mediated regulation of testis LD was significant for spermatogenesis, as both whole-body and cell-autonomous loss of *bmm* caused defects in sperm development.

Given that our lipidomic analysis revealed an excess accumulation of triglyceride in animals lacking *bmm*, and that genetically blocking triglyceride synthesis rescued many spermatogenic defects associated with *bmm* loss, our data suggests that *bmm*-mediated regulation of triglyceride is important for normal *Drosophila* sperm development. This reveals previously unrecognized roles for neutral lipids such as triglyceride in regulating spermatogenesis, and for *bmm* in regulating sperm development under normal physiological conditions. Together, these findings advance knowledge of the regulation and function of neutral lipids during spermatogenesis.

Results

Lipid droplets are present in early-stage somatic and germline cells

We previously reported the presence of small LD (<1 μm) at the apical tip of the testis [27], a finding we reproduced in w^{1118}males using neutral lipid stain BODIPY (**Figure 1A**). These LD were present in the region that contains stem cells, early-stage somatic cells, and germline cells (**Figure 1A-A**', arrows). LD were also present in the hub, an organizing center and stem cell niche in the *Drosophila* testis (**Figure 1A**"-A"', arrows) [55], but largely absent within the area occupied by spermatocytes (**Figure 1A and A**', arrowheads). This LD distribution was reproduced in two independent genetic backgrounds and at two additional ages (**Figure 1B**, 1C). While LD may contain multiple neutral lipid species [56], cholesterol-binding fluorescent polyene antibiotic filipin III did not detect cholesterol within testis LD (**Figure S1A**), suggesting triglyceride is the main neutral lipid in *Drosophila* testis LD.

Drosophila spermatogenesis requires the co-development and differentiation of the germline and the somatic lineages [57]. To identify LD in each lineage, we used the GAL4/UAS system to overexpress a GFP transgene fused to the LD-targeting motif of motor protein *Klarsicht* (*UAS-GFP-LD*) [58]. Somatic overexpression of *UAS-GFP-LD* using *Traffic jam (Tj)-GAL4* revealed that the majority of somatic LD in 0-day-old males were located <30 μm from the hub (**Figure 1D**, 1E). Because the somatic LD distribution coincided with a marker for somatic stem cells and their immediate daughter cells (Zinc finger homeodomain 1, Zfh-1) (**Figure 1F**; two-sample Kolmogorov-Smirnov test) [59], but not with a marker for late somatic cells (Eyes absent, Eya) [12,60], our data suggest LD are present in early somatic cells. Germline overexpression of *UAS-GFP-LD* using *nanos (nos)-GAL4* demonstrated the presence of LD within germline cells near the apical tip of the testis in 0-day-old males (**Figure 1G**, 1H). Specifically, the disappearance of germline LD coincided with peak expression of a GFP reporter that reflects the expression of Bag-of-marbles (Bam) protein in the testis (Bam-GFP) [61] (**Figure 1I**, 1J). Because peak Bam expression signals the last round of transient amplifying mitotic cell cycle prior to the germline's transition into the meiotic cell cycle [62–64], our data suggests that germline LD, like somatic LD, are present in cells at early stages of development.

brummer plays a cell-autonomous role in regulating testis lipid droplets

Adipose triglyceride lipase (*ATGL*) is a critical regulator of neutral lipid metabolism and LD [65–74]. Loss of *ATGL* in many cell types triggers LD accumulation, and *ATGL* overexpression decreases LD number [30,67,68,71,73,75,76]. Given that the *Drosophila ATGL* homolog *brummer* (*bmm*) regulates testis LD induced by mitochondrial stress [49], we explored whether *bmm* regulates testis LD under normal physiological conditions. We first examined *bmm* expression in the testis by isolating this organ from flies carrying a *bmm* promoter-driven *GFP* transgene (*bmm-GFP*) that recapitulates many aspects of *bmm* mRNA regulation [77]. GFP expression was present in the germline of *bmm-GFP* testes, and we found germline GFP levels were higher in spermatocytes than at earlier stages of sperm development (**Figure 2A**, 2B; one-way ANOVA with Tukey multiple comparison test). In further support of this observation, we analyzed a publicly available single-cell RNA sequencing dataset from the male reproductive organ [78]. Using pseudotime analysis, we arranged the germline (**Figure S2A**) and somatic cells (**Figure S2B**) based on their annotated developmental trajectory. The expression pattern of *bmm* in the germline matched our observation with the *bmm-GFP* reporter (**Figure S2C**). While levels of the *bmm-GFP* reporter were low in somatic cells, single-cell RNA sequencing data identified *bmm* expression in the somatic lineage, with higher expression in cells at later stages of development (**Figure S2D**).

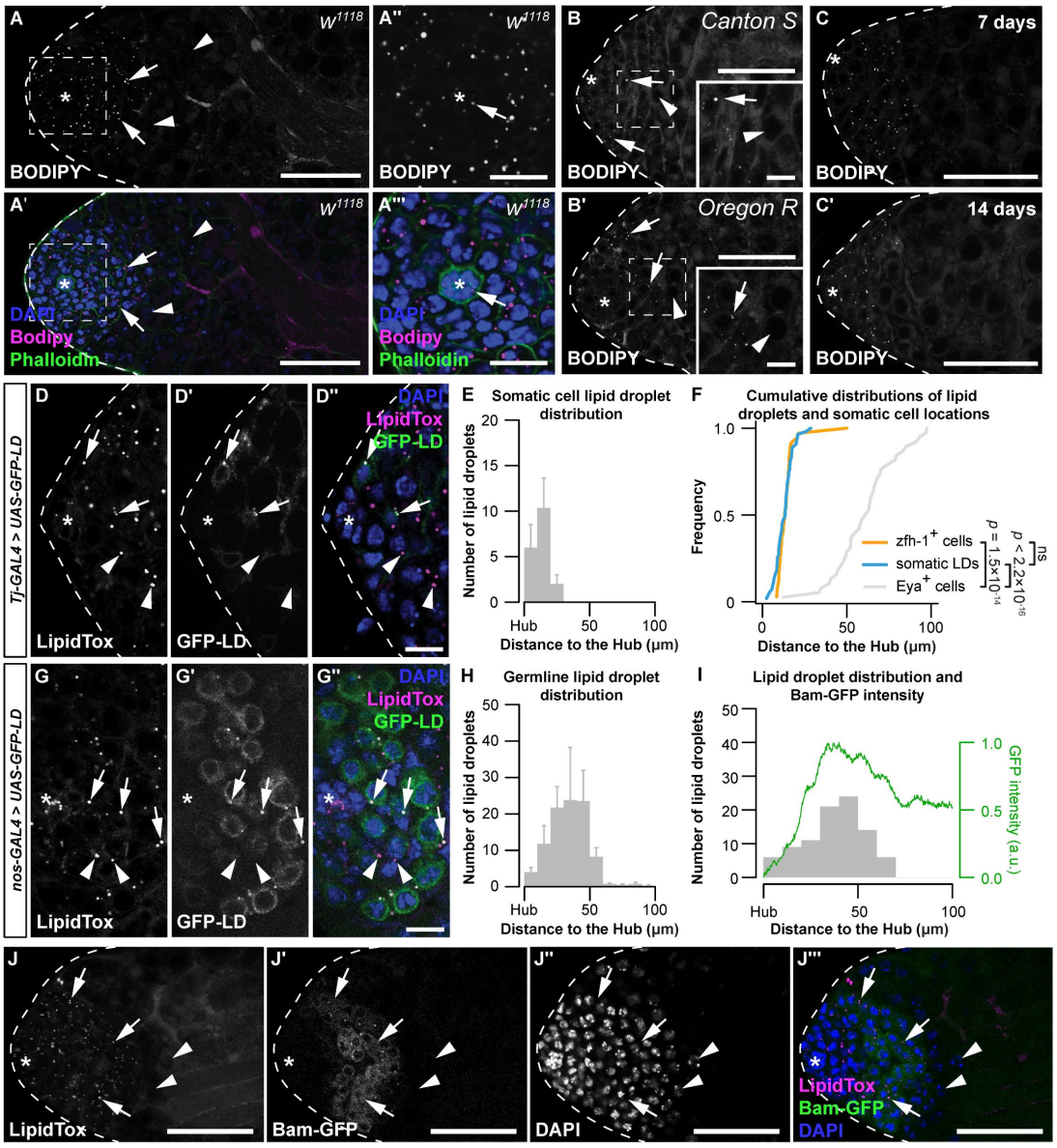


Figure 1

Lipid droplets are present in early-stage somatic and germ cells.

(A) Testis lipid droplets (LD) in w^{1118} animals visualized with neutral lipid dye BODIPY. (A,A') Scale bar=50 μm; (A'',A''') scale bar=15 μm. Asterisk indicates hub in all images. Arrows point to LD; arrowheads point to spermatocytes in A,B. Spermatocytes were identified as described in methods section. (B) Testis LD visualized with BODIPY in newly-eclosed males from two wild-type genotypes. Scale bars: main image=50 μm; inset image=10 μm. (C) Testis LD from w^{1118}animals at different times post-eclosion. Scale bars=50 μm. (D) Testis LD visualized with LipidTox Red in animals with somatic cell overexpression of GFP-LD (*Tj-GAL4>UAS-GFP-LD*). GFP- and LipidTox Red-positive punctae are somatic LD (D–D'' arrows); LipidTox punctae without GFP indicate germline LD (D–D'' arrowheads). Scale bars=10 μm. (E) Histogram showing the spatial distribution of somatic cell LD; error bars represent standard error of the mean (SEM). (F) Cumulative frequency distributions of somatic LD (blue line, data reproduced from E), zfh-1-positive somatic cells (zfh-1^+ cells, orange line), and Eya-positive somatic cells (Eya^+ cells, grey line). (G) Testis LD visualized with LipidTox Red in males with germline overexpression of GFP-LD (*nos-GAL4>UAS-GFP-LD*). GFP- and LipidTox Red-positive punctae indicate germline LD (arrows); LipidTox punctae without GFP indicate non-germline LD (arrowheads). Scale bars=10 μm. (H) Histogram representing the spatial distribution of LD within the germline; error bars represent SEM. (I) Histogram representing the spatial distribution of LD and GFP fluorescence (green line) (arbitrary units, a.u.) in a representative testis of a *bam-GFP* animal (panel J). (J) Testis LD in a *bam-GFP* animal; arrows point to LD and arrowheads point to spermatocytes. Scale bar=50 μm. See also Supplemental Figure 1.

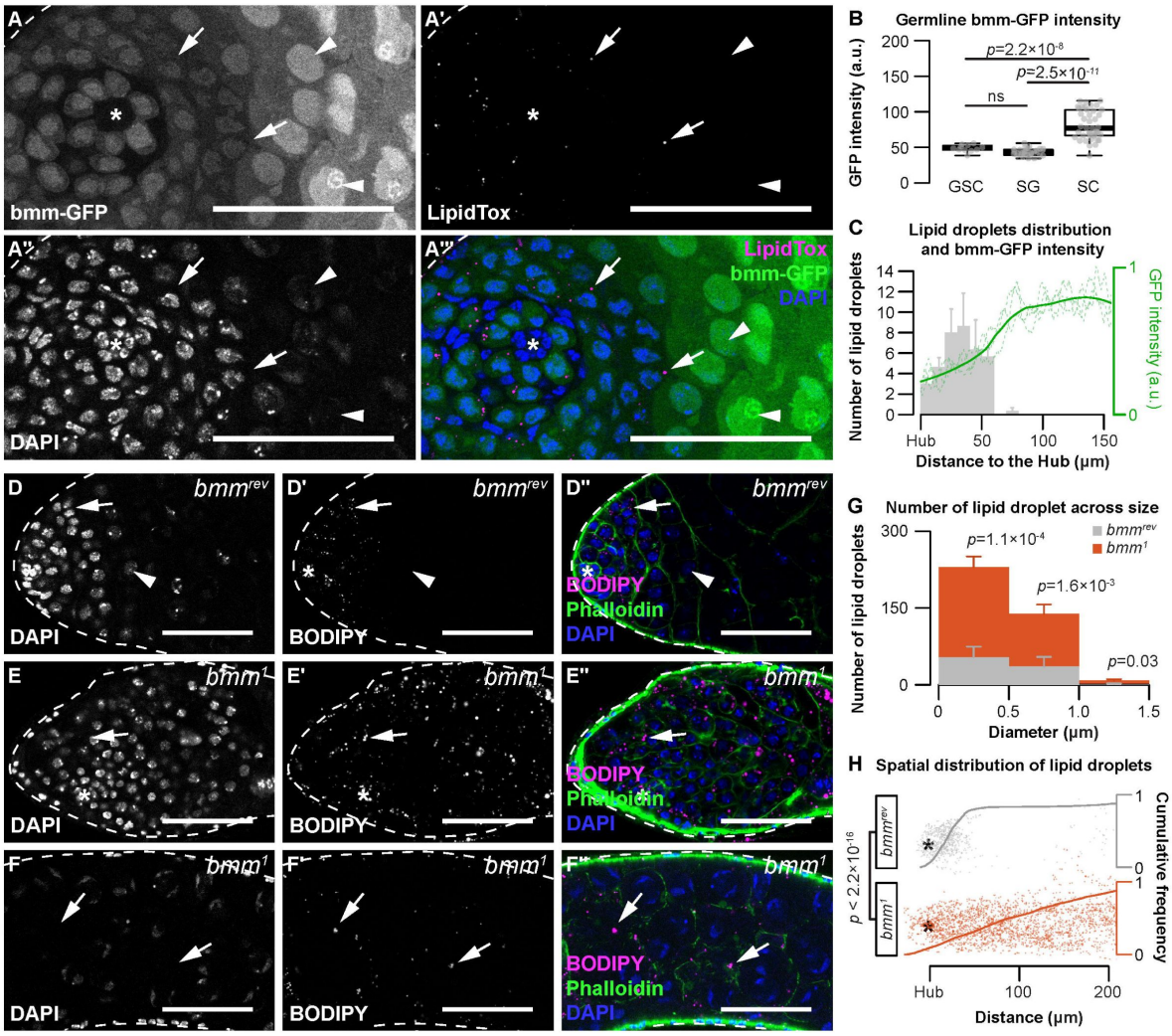


Figure 2

brummer regulates lipid droplets in both germline and somatic cells of the testis.

(A-A'''') Testis lipid droplets (LD) indicated by LipidTox Red in *bmm-GFP* animals. Arrows point to LD in all images. Arrowheads point to spermatocytes. Scale bars=50 μm. Asterisks indicate the hub in all images. (B) Quantification of nuclear GFP intensity in testes isolated from *bmm-GFP* animals (n=3). Germline stem cell (GSC), spermatogonia (SG), spermatocyte (SC). (C) Spatial distribution of LD (grey histogram) and GFP expression (green line) in testes from *bmm-GFP* animals as a function of distance from the hub (n=3). (D,E) LD near the apical region of the testis in bmm^{rev} (D) or bmm^{1} (E) animals. (F) LD further away from the apical tip in bmm^{1} animals. (D–F) Scale bars=50 μm. (G) Histogram representing testis LD size distribution in bmm^{rev} (grey) and bmm^{1} (orange). (H) Apical tip of the testes is at the left of the graph; individual dots represent a single LD and its relative position to the hub marked by an asterisk. Cumulative frequency distribution of the distance between LDs and the apical tip of the testes are drawn as solid lines. See also Supplemental Figure 2 **and 3**.

Additional neutral lipid- and lipid droplet-associated genes such as *lipid storage droplet-2*, *Seipin*, *Lipin*, and *midway* also showed differential regulation during differentiation in the testis (**Figure S2C**, **S2D**). Combined with our data on the location of testis LD, these gene expression data suggest that *bmm* upregulation in both somatic and germline cells during differentiation corresponds to the downregulation of testis LD. Supporting this, germline GFP levels were negatively correlated with testis LD in *bmm-GFP* flies (**Figure 2A**, 2C), suggesting regions with higher *bmm* expression had fewer LD.

To test whether *bmm* regulates testis LD, we compared LD in testes from 0-day-old males carrying a loss-of-function mutation in *bmm* (bmm^{1}) to control male testes (bmm^{rev}) [67]. bmm^{1} males had significantly more LD across all LD sizes compared with control males at the apical tip of the testis (**Figure 2D**–**2G**; Welch two-sample t-test with Bonferroni correction) and showed a significantly expanded LD distribution (**Figure 2D**– 2F, 2H; two-sample Kolmogorov-Smirnov test). This suggests *bmm* normally restricts LD to the apical tip of the testis, an observation we confirmed in both somatic and germline lineages using lineage-specific expression of GFP-LD (**Figure S3A**–**S3D**). Importantly, after inducing homozygous bmm^{rev}or bmm^{1} clones in the testes using the *FLP-FRT* system (**Figure 3A**, 3B) [79], we found bmm^{1} spermatocyte clones had significantly more LD at 3 days post-clone induction (**Figure 3C**; Welch two-sample t-test), a stage at which LD were absent from bmm^{rev} clones. Because we observed no significant effect of cell-autonomous *bmm* loss on LD at any other stage of germline development (**Figure 3C**), this suggests *bmm* does not regulate LD at early stages of germ cell development. Instead, our data suggest *bmm* plays a role in regulating LD at the spermatogonia-spermatocyte transition. While we were unable to assess LD in bmm^{1} somatic clones, our data reveals a previously unrecognized cell-autonomous role for *bmm* as a key regulator of LD in germline cells.

brummer plays a cell-autonomous role in regulating germline development

To determine the physiological significance of *bmm*-mediated regulation of testis LD, we investigated testis and sperm development in males without *bmm* function. In 0-day-old bmm^{1} males reared at 25°C, testis size was significantly smaller than in age-matched bmm^{rev} controls (**Figure 4A**, 4B; Welch two-sample t-test), and the number of spermatid bundles was significantly lower (**Figure 4C**; Kruskal-Wallis rank sum test). When the animals were reared at 29°C, a temperature that exacerbates spermatogenesis defects associated with changes in lipid metabolism [21], bmm^{1} phenotypes were more pronounced (**Figure S3A**, **S3B**; Welch two-sample t-test, Kruskal-Wallis rank sum test). Defects in testis size were also observed at 14 days post-eclosion; suggesting testis size defects persist later into the life course (**Figure S4C**; Welch two-sample t-test). In contrast, the number of spermatid bundles per testis was not significantly different between bmm^{1} and bmm^{rev}males at this age (**Figure S4D**; Welch two-sample t-test), potentially due to a large decrease in the number of spermatid bundles in 14-day-old bmm^{rev} males (**Figure 4C**, **S4D**). Together, these data suggest loss of *bmm* affects testis development and spermatogenesis. Similar phenotypes are observed in male mice without ATGL [52], and supplementing the diet of bmm^{1}males with medium-chain triglycerides (MCT) partially rescues the testis and spermatogenic defects we observed in flies (**Figure S4E**, **S4F**; one-way ANOVA with Tukey multiple comparison test), as it does in mice [52,80]. This identifies similarities between flies and mice in fertility-related phenotypes associated with whole-body loss of *bmm*/*ATGL*.

To explore spermatogenesis in bmm^{1} animals, we used an antibody against germ cell-specific marker Vasa to visualize the germline in the testes of bmm^{1} and bmm^{rev}males (**Figure 4D**, 4E) [81]. We observed a significant increase in the number of germline stem cells (GSC) (**Figure 4F**; Kruskal-Wallis rank sum test) and higher variability in GSC number in bmm^{1} males (p=5.7×10^{-12} by F-test). Given that GSC number is affected by hub size and GSC proliferation

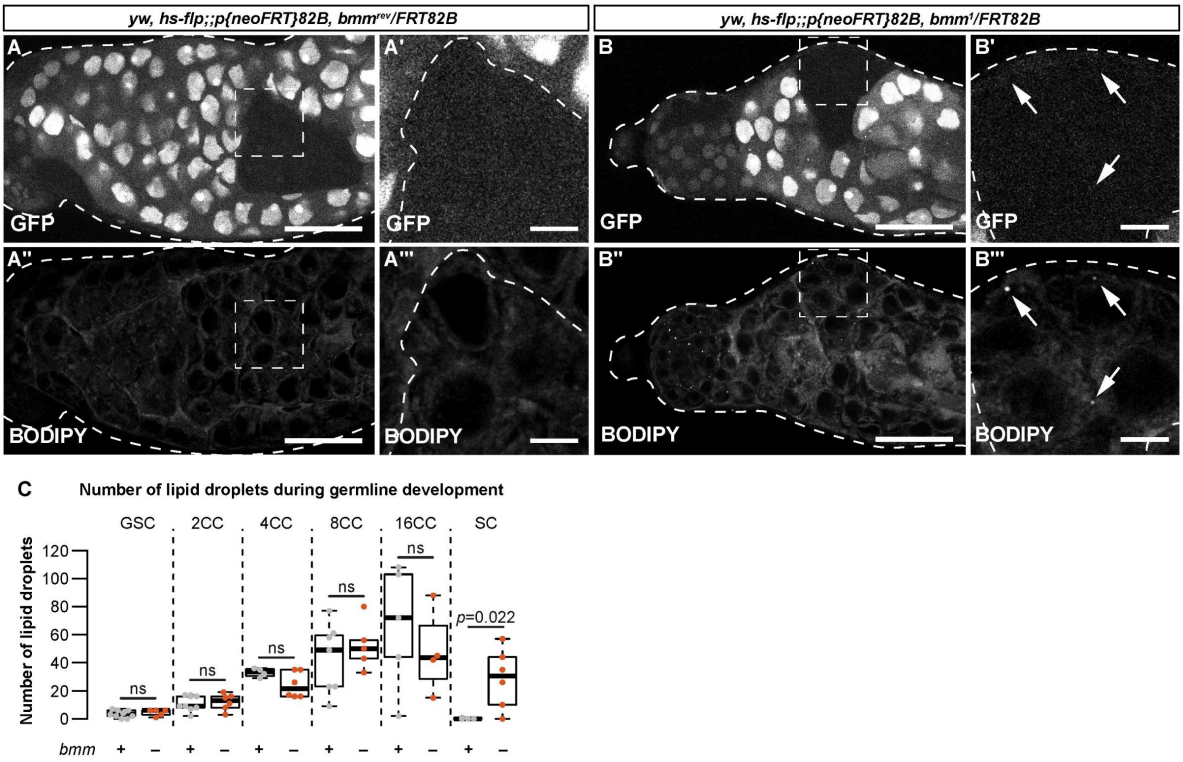


Figure 3

bmm regulates germline lipid droplets in a cell-autonomous manner.

(A–B) Single confocal slices through a representative testis isolated from an individual carrying clones induced using the FLP-FRT system at 3 days post-clone induction. Clones are homozygous for an allele that encodes a functional *bmm* protein product (bmm^{rev}; A–A‘‘‘) or a loss-of-function *bmm* allele (bmm^{1}, B–B‘‘‘). GFP negative areas mark homozygous clones in panel A and B; the boxed areas in A, A‘‘ and B, B‘‘ are shown in A‘, A‘‘‘ and B‘, B‘‘‘, respectively. In homozygous bmm^{rev} spermatocyte clones we detected no LD using neutral lipid dye BODIPY (A‘‘ and A‘‘‘). In contrast, spermatocyte clones homozygous for bmm^{1} have detectable LD (B‘‘ and B‘‘‘, arrows). Scale bars=50 μm in A,A‘‘ and B,B‘‘; scale bars=10 μm in A‘,A‘‘‘ and B‘,B‘‘‘. (C) Number of testis LD in bmm^{rev} (grey) or bmm^{1} (orange) in FLP-FRT clones 3 days post-clone induction; dots represent measurements from a single clone. The number of cells in each cyst (CC) counted is indicated. There were significantly more LD in bmm^{1} spermatocyte (SC) clones (p = 0.026; Welch two-sample t-test) but not at other stages of development.

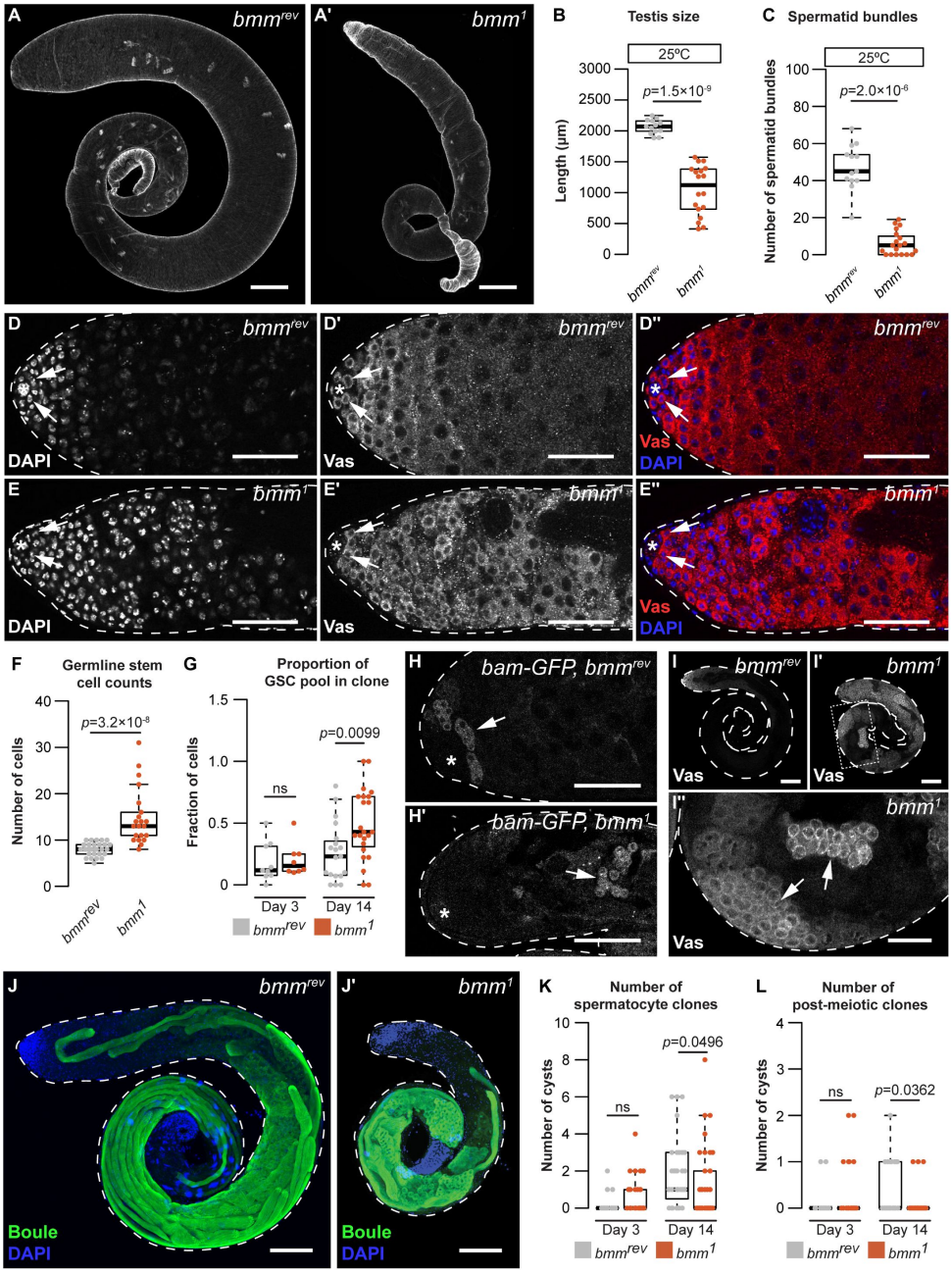


Figure 4

A cell-autonomous role for *bmm* in regulating spermatogenesis.

Testes isolated from *bmm*^{rev} (A) and *bmm*^{1}(A′) animals raised at 25°C stained with phalloidin. Scale bars=100 µm. (B) Testis size in *bmm*^{1} and *bmm*^{rev} animals raised at 25°C. (C) Spermatid bundle number in *bmm*^{1} and *bmm*^{rev} testes from animals reared at 25°C. (D,E) Representative images of *bmm*^{rev} (D) or *bmm*^{1} (E) testes stained with DAPI and anti-Vasa antibody. Arrows indicate germline stem cells (GSC). Scale bar=50 µm. The hub is marked by an asterisk in all images. (F) GSC number in *bmm*^{1} and *bmm*^{rev} testes. (G) Proportion of GSCs that were either *bmm*^{1} or *bmm*^{rev}clones at 3 and 14 days post-clone induction. (H) Representative images of *bmm*^{rev}(H) and *bmm*^{1} (H′) testes carrying *bam-GFP*; data quantified in **Figure S4J**. Arrows indicate regions with high Bam-GFP. Scale bars=50 µm. (I) Representative images of *bmm*^{rev} (I) or *bmm*^{1}(I′,I′′) testes stained with anti-Vasa antibody. Arrows indicate Vasa-positive cysts in *bmm*^{1} testis. Panel I′′ is magnified from the boxed region in I′. (I,I′) Scale bars=100 µm; (I′′) scale bar=50 µm. (J) Maximum projection of *bmm*^{rev} (J) or *bmm*^{1} (J′) testes stained with anti-Boule antibody (green) and DAPI (blue). Scale bars=100 µm. Number of *bmm*^{1}and *bmm*^{rev} spermatocyte clones (K) or post-meiotic clones (L) at 3- and 14-days post-clone induction. See also Supplemental Figure 4.

[82,83], we monitored both parameters in bmm^{1} and bmm^{rev} controls. While hub size in bmm^{1} testes was significantly larger than in testes from bmm^{rev} controls (**Figure S4G**, **S4H**; Welch two-sample t-test), the number of phosphohistone H3-positive GSCs, which indicates proliferating GSCs, was unchanged in bmm^{1} animals (**Figure S4I**; Kruskal-Wallis rank sum test). While this indicates a larger hub may partly explain *bmm*'s effect on GSC number, *bmm* also plays a cell-autonomous role in regulating GSCs, as we recovered a higher proportion of bmm^{1} clones in the GSC pool compared with bmm^{rev}clones at 14 days after clone induction (**Figure 4G**; Welch two-sample t-test). Given that we detected no effect of cell-autonomous *bmm* loss on the number of GSC LD (**Figure 3C**), more work will be needed to understand how *bmm* regulates GSCs at a stage prior to its effects on LD number. Future studies will also need to confirm whether bmm^{1} mutant GSCs show an increased ability to occupy space at the hub.

Beyond GSCs, we uncovered additional spermatogenesis defects in bmm^{1}testes. Peak Bam-GFP expression in germline cells of the testes from 0-day-old bmm^{1} and bmm^{rev} males showed GFP-positive cysts were significantly further away from the hub in bmm^{1}testes (**Figure 4H**, **S4J**; Welch two-sample t-test). Indeed, 15/18 bmm^{1} testes contained Vasa-positive cysts with large nuclei in the distal half of the testis (**Figure 4I**, arrows), a phenotype not present in bmm^{rev} testes (0/8) (p=0.0005 by Pearson's Chi-square test). Because these phenotypes are also seen in testes with differentiation defects [13,84], we recorded the stage of sperm development reached by the germline in bmm^{1} testes. Most bmm^{1} testes contained post-meiotic cells in males raised at 25°C (**Figure S4K**); however, germline development did not progress past the spermatocyte stage in most bmm^{1} testes from animals raised at 29°C (**Figure S4K**).

Testes from bmm^{1} males reared at 25°C also had a smaller Boule-positive area (**Figure 4J**, **S4L**; Welch two-sample t-test), and fewer individualization complexes and waste bags (**Figure S4M**, **S4N**; Kruskal-Wallis rank sum test). Because Boule-positive area, individualization complexes, and waste bags indicate germline cells at later stages in development, these data suggest the loss of *bmm* causes a reduction in differentiated cell types. Because we observed significantly fewer bmm^{1} spermatocyte and spermatid clones at 14 days after clone induction (**Figure 4K**, 4L; p = 0.0496, Kruskal-Wallis rank sum test), these effects on germline development may represent a cell-autonomous role for *bmm* in regulating spermatogenesis in this cell type. Given that the statistical significance of this finding was not as strong as for our other data, future studies should repeat this experiment with more samples. We also reveal a potential non-cell-autonomous role for somatic *bmm*. While there was no difference in the ratio of Zfh-1-positive cells between homozygous clones and heterozygous clones in animals carrying the bmm^{1} or bmm^{rev}alleles at 14 days post clone induction (**Figure S4O**; Kruskal-Wallis rank sum test), the distance from the hub to the Zfh-1 positive clones reside was significantly decreased in bmm^{1} homozygous clones (**Figure S4P**; Kruskal-Wallis rank sum test). Together, these data indicate *bmm* plays a cell-autonomous role in germline cells, and potentially a non-cell-autonomous role in somatic cells, to regulate spermatogenesis.

brummer-dependent regulation of testis triglyceride levels affects spermatogenesis

ATGL catalyzes the first and rate-limiting step of triglyceride hydrolysis [73,85,86]. Loss of this enzyme or its homologs leads to excess triglyceride accumulation [27,30,67,73,75] and shifts in multiple lipid classes [66,87–89]. To determine how loss of *bmm* affects spermatogenesis, we carried out whole-body mass spectrometry (MS)-based untargeted lipidomic profiling of bmm^{1} and bmm^{rev} males. Hierarchical clustering of lipid species suggests that bmm^{1} and bmm^{rev} males show distinct lipidomic profiles (**Figure 5A**). Overall, we detected 2464 and 1144 lipid features with high quantitative confidence in positive and negative ion modes, respectively. By matching experimental *m/z*, isotopic ratio, and tandem MS spectra to lipid libraries, we confirmed 293 unique lipid species (Supplemental table 1). We found 107 lipids had a significant change in abundance between bmm^{1} and bmm^{rev} males

(padj<0.05): 85 species were upregulated in bmm^1 males and 22 lipid species were downregulated. Among differentially regulated species from different lipid classes, triglyceride had the largest residual above expected proportion (p=5.00×10^{-4} by Pearson's Chi-squared test). This suggests triglyceride is the lipid class most affected by loss of *bmm* (**Figure 5B**, 5C).

In bmm^1 males, the majority of triglyceride species (55/97) were significantly higher in abundance compared with bmm^{rev}control males. Because we observed a positive correlation between the fold increase in triglyceride abundance with both the number of double bonds (p=7.52×10^{-8} by Kendall's rank correlation test; **Figure S5A**) and the number of carbons (p=2.77×10^{-10} by Kendall's rank correlation test; **Figure S5B**), our data align well with *bmm*/*ATGL's* known role in regulating triglyceride levels [67,68,73] and its substrate preference of long-chain polyunsaturated fatty acids [85]. While we also detected changes in species such as fatty acids, acylcarnitine, and membrane lipids (**Figure S5C**–**S5H**), in line with recent *Drosophila* lipidomic data [90,91], the striking accumulation of triglyceride in bmm^1 males suggested that excess testis triglyceride in bmm^1 males may contribute to their spermatogenic defects. To test this, we examined spermatogenesis in bmm^1 males carrying loss-of-function mutations in *midway* (*mdy*). *mdy* is the *Drosophila* homolog of *diacylglycerol O-acyltransferase 1* (*DGAT1*), and whole-body loss of *mdy* reduces whole-body triglyceride levels [92–94]. Importantly, testes isolated from males with global loss of both *bmm* and *mdy* ($mdy^{QX25/k03902}$;bmm^1) had fewer LD than testes dissected from bmm^1 males (**Figures 5D**, **S5I**; one-way ANOVA with Tukey multiple comparison test).

We found that testes isolated from $mdy^{QX25/k03902}$;bmm^1males were significantly larger and had more spermatid bundles than testes from bmm^1 males (**Figure 5E–G**; one-way ANOVA with Tukey multiple comparison test). The elevated number of GSCs in bmm^1 male testes was similarly rescued in $mdy^{QX25/k03902}$;bmm^1 males (**Figure 5H**; one-way ANOVA with Tukey multiple comparison test). These data suggest that defective spermatogenesis in bmm^1 males can be partly attributed to excess triglyceride accumulation. Notably, at least some of the effects of global *mdy* loss on bmm^1 males can be attributed to the germline: RNAi-mediated knockdown of *mdy* in the germline of bmm^1 males partially rescued the defects in testis size (**Figure 5I**; Kruskal-Wallis rank sum test with Dunn's multiple comparison test) and GSC variance (**Figure S5J**; p=4.5 x 10^{-5} and 8.2 x 10^{-3} by F-test from the GAL4- and UAS-only crosses, respectively). While future studies will need to test whether germline-specific loss of *mdy* also rescues spermatid number defects in bmm^1 males, our data suggest *bmm*-mediated regulation of testis triglyceride plays a previously unrecognized role in regulating sperm development.

Discussion

In this study, we used *Drosophila* to gain insight into how the neutral lipids, a major lipid class, contribute to sperm development. We describe the distribution of LD under normal physiological conditions in the *Drosophila* testis, and show that LD are present at the early stages of development in both somatic and germline cells. While many factors are known to regulate LD in nongonadal cell types, we reveal a cell-autonomous role for triglyceride lipase *bmm* in regulating testis LD during spermatogenesis. In particular, we identified a requirement for *bmm* in mediating the decrease in LD at the spermatogonia-spermatocyte transition. This regulation is important for sperm development, as our data indicates that loss of *bmm* causes a decrease in the number of differentiated cell types in the testis. This phenotype may be attributed to a delay in differentiation, a block in differentiation, or to a loss of differentiated cells through cell death. Future studies will therefore be essential to resolve why *bmm* loss causes a reduction in differentiated cell types. Nevertheless, these defects in the number of differentiated cell types can be partially explained by the excess accumulation of triglyceride in flies lacking *bmm*, as global and cell type-specific inhibition of triglyceride synthesis rescues multiple spermatogenic defects in

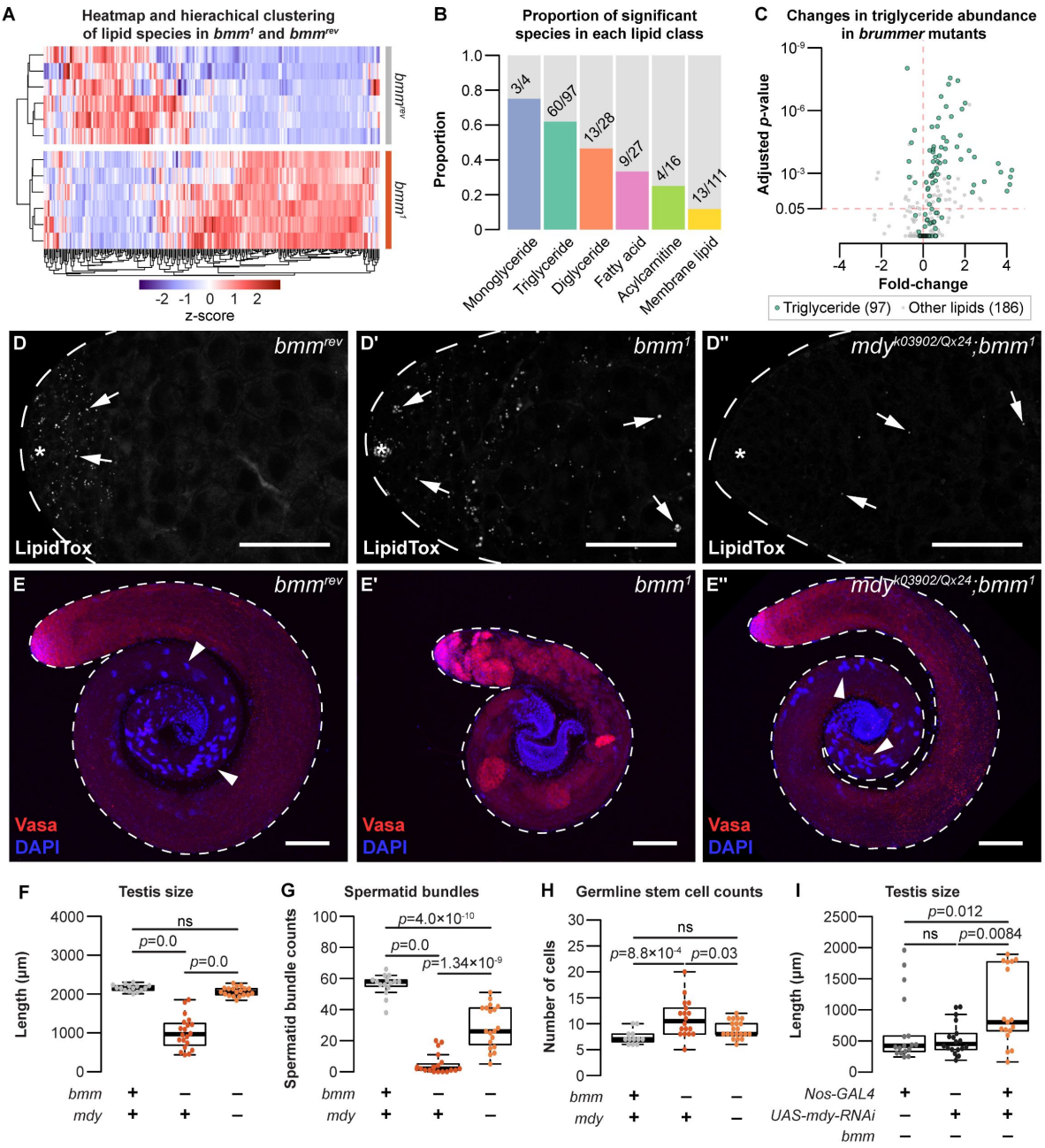


Figure 5

Loss of *bmm* disrupts triglyceride homeostasis and leads to spermatogenic defects.

(A) Hierarchical clustering of lipid species detected in bmm^{rev} and bmm^{1} animals. (B) Histograms showing the proportion of species in each lipid class with different levels between bmm^{1} and bmm^{rev}. Numbers on histograms indicate the number of species with differences in abundance. (C) Volcano plot showing fold change in abundance of triglycerides (green; 97 species) and non-triglyceride lipids (grey; 186 species) in our dataset. (D) Arrows indicate testis LD stained with LipidTox Red in bmm^{rev} (D), bmm^{1} (D'), or $mdy^{QX25/k03902}$; bmm^{1} (D'') animals. (E) Whole testes isolated from bmm^{rev}(E), bmm^{1} (E'), or $mdy^{QX25/k03902}$;bmm^{1}(E'') animals stained with anti-Vasa antibody (red) and DAPI (blue). Arrowheads indicate spermatid bundles. Scale bars=100 µm. (F) Testis size in bmm^{rev}, bmm^{1}, and $mdy^{QX25/k03902}$;bmm^{1}animals. Spermatid bundles (G) and number of germline stem cells (H) in bmm^{rev}, bmm^{1}, and $mdy^{QX25/k03902}$;bmm^{1} animals. (I) Testis size in animals with germline-specific *mdy* knockdown (*nos-GAL4>mdy RNAi*; bmm^{1}) compared with controls (*nos-GAL4>+*; bmm^{1} and *+>mdy RNAi*; bmm^{1}). See also Supplemental Figure 5.

bmm mutants. Together, our data reveals previously unrecognized roles for LD and triglycerides during spermatogenesis, and for *bmm* as an important regulator of testis LD and germline development under normal physiological conditions.

One key outcome of our study was increased knowledge of LD regulation and function in the testis. Despite rapidly expanding knowledge of LD in cell types such as adipocytes or skeletal muscle, less is known about how LD influence spermatogenesis under normal physiological conditions. In mammals, testis LD contain cholesterol and play a role in promoting steroidogenesis [95,96]. In flies, we show that LD are present in the testis, and that excess accumulation of these LD affects sperm development. In nongonadal cell types, triglycerides provide a rich source of fatty acids for cellular ATP production, lipid building blocks to support membrane homeostasis and growth, and metabolites that can act as signaling molecules [26]. Because ATP production, lipid precursors, and lipid signaling all play roles in supporting normal sperm development [97,98], future studies will need to determine how each of these processes is affected when excess triglyceride accumulates in testis LD (**Figure 6**). It will also be important to determine whether it is the loss of metabolites produced by *bmm*'s enzymatic action, or an increase in triglycerides, that leads to the reduction in differentiated cell types during spermatogenesis. Together, these experiments will provide critical insight into how triglyceride stored within testis LD contributes to overall cellular lipid metabolism during spermatogenesis. Because of the parallel spermatogenic defects we observed in *bmm* mutants and *ATGL*-deficient mice, we expect these mechanisms will also operate in other species.

A more comprehensive understanding of neutral lipid metabolism during sperm development will also emerge from studies on the upstream signaling networks that regulate testis LD and triglyceride. Given that we show an important and cell-autonomous role for *bmm* in regulating testis LD and triglyceride, future studies will need to identify factors that regulate *bmm* in the testis. Based on public single-cell RNAseq data and the *bmm-GFP* reporter strain, our data suggest *bmm* mRNA levels are differentially regulated between early and later stages of sperm development.

Candidates for mediating this regulation include the insulin/insulin-like growth factor signaling pathway (IIS), Target of rapamycin (TOR) pathway, and nuclear factor κB/Relish pathway (NFκB), as all of these pathways influence *bmm* mRNA levels in nongonadal cell types [99–105]. Beyond mRNA levels, Bmm protein levels and post-translational modifications may also be differentially regulating during spermatogenesis.

For example, studies show that the proteins encoded by *bmm* homologs in other animals are regulated by phosphorylation [106], mediated by kinases such as adenosine monophosphate-activated protein kinase (AMPK) and protein kinase A (PKA) [107–109]. Importantly, many of these pathways, including IIS, TOR, AMPK, NFκB and possibly PKA influence *Drosophila* sperm development [110–115]. Identifying the signaling networks that influence *bmm* regulation during sperm development will therefore lead to a deeper understanding of how testis LD and triglyceride are coordinated with physiological factors to promote normal spermatogenesis. Because pathways such as IIS and AMPK, and others, regulate sperm development in other species [116–118], these insights may reveal conserved mechanisms that govern the regulation of cellular neutral lipid metabolism during sperm development.

Acknowledgements

We thank Dr. Ronald Kühnlein for $bmm^{1}$and bmm^{rev} lines [67], Dr. Michael Welte for *UAS-GFP-LD* [58], and Dr. Kaeko Kamei for *bmm-GFP* [77]. We used stocks from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) and Vienna *Drosophila* Resource Center (VDRC). We acknowledge critical resources and information provided by FlyBase [119] (supported by the National Human Genome Research Institute at the U.S. National Institutes of Health (U41

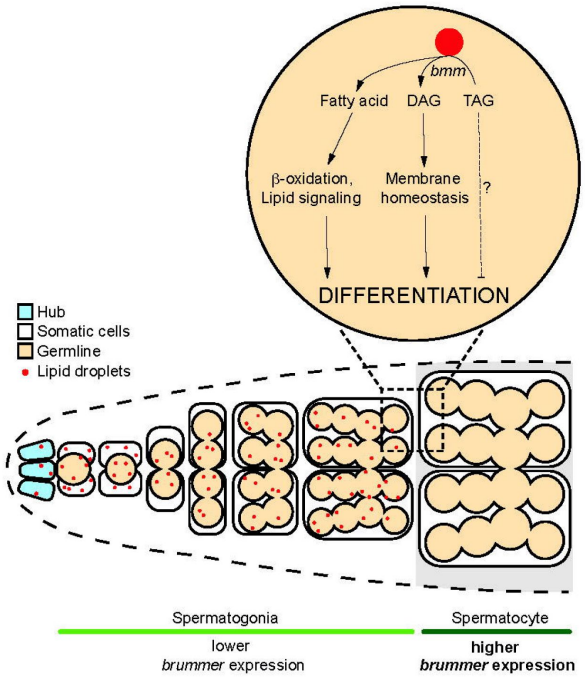


Figure 6

Model of *bmm*-mediated lipid droplet regulation in the *Drosophila* testis.

Schematic representation summarizing *bmm*-mediated lipid droplet regulation in the testis during development.

HG000739) and the British Medical Research Council (MR/N030117/1)). This work was supported by the Life Sciences Institutes Imaging Core, supported by the UBC GREx Biological Resilience Initiative. Funding for this study was provided by grants to EJR from the Canadian Institutes for Health Research (PJT-153072), Michael Smith Foundation for Health Research (16876), and the Canadian Foundation for Innovation (JELF-34879). GT was supported by a grant from the Natural Sciences and Engineering Research Council (NSERC; 2018-04648), TH/HY/CW were supported by NSERC (2020-04895), MA was supported by the Jacob's foundation. We would like to acknowledge that our research takes place on the traditional, ancestral, and unceded territory of the Musqueam people; a privilege for which we are grateful.

Author contributions

Conceptualization, C.C. and E.J.R.; Methodology, C.C. and Y.Y.P.; Software, C.C.; Investigation, C.C., H.Y., and Y.Y.P.; Lipidomics, M.A., H.Y., C.W., T.H.; Writing – Original Draft, C.C. and E.J.R.; Writing – Review and Editing, C.C., E.J.R., and Y.Y.P.; Supervision, E.J.R., G.T., and T.H.; Project administration, E.J.R.; Funding Acquisition, E.J.R., G.T., and T.H.

Declaration of interests

The authors declare no competing interests.

Materials and methods

Materials and Resource availability

Drosophila strains and their source are listed in a Key Resources table. Further information and requests for resources and reagents should be directed to, and will be fulfilled by, lead contact Dr. Elizabeth J. Rideout (elizabeth.rideout@ubc.ca).

Data and Code availability

All raw data and results of statistical tests reported in this paper are located in Supplementary files 1-4. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Fly husbandry

Fly stocks were maintained at room temperature in 12:12 hour light:dark cycle. Unless otherwise indicated, all flies were raised at 25°C with a density of 50 larvae per 10 mL fly media. Because this project examines sperm development, we used male flies in all experiments. Fly media contained 20.5 g sucrose (SU10, Snow Cap), 70.9 g Dextrose (SUG8, Snow Cap), 48.5 g cornmeal (AO18006, Snow Cap), 30.3 g baker's yeast (NB10, Snow Cap), 4.55 g agar (DR-820-25F, SciMart), 0.5 g calcium chloride dihydrate (CCL302.1, BioShop Canada), 0.5 g magnesium sulfate heptahydrate (MAG511.1, BioShop Canada), 4.9 mL propionic acids (P1386, Sigma-Aldrich), and 488 µL phosphoric acid (P5811, Sigma-Aldrich) per 1L of media. For diets with medium- or long-chain triglyceride, 4 g of coconut oil (medium chain triglyceride) or olive oil (long chain triglyceride) was added per 100 mL of media described above prior to cooling. Males were collected and dissected within 24 hours of eclosion unless otherwise indicated. Fixations were performed at room temperature with 4% paraformaldehyde (CA11021-168, VWR) in PBS for 20 minutes on a rotating platform followed by washing in PBS twice before staining. Fly strains used in our study are listed in a Key Resources table.

Testis cell stage classification and measurements

Cells at an early stage of development (stem cells and early-stage somatic and germline cells) were located in the apical region of the testis, and were identified by their small and dense nuclei[120]. GSCs were defined as Vasa-positive cells in direct contact with the hub; proliferating GSCs were identified as Vasa-positive cells in direct contact with the hub that were also phospho-H3 positive. Cells in the testis region occupied by primary spermatocytes were identified by their large cell size and decondensed chromosome staining occupying three nuclear domains [120]. Spermatid bundles were identified by their condensed and needle-shaped nuclei, which roughly corresponds to nuclei with protamine-based chromatin [121]. Hub size was measured by quantifying FasIII-positive area of the testis. Testis size was measured by quantifying the length of a line drawn down the middle of a testis image; starting from the apical tip of the testis and ending where the testis meets the seminal vesicle.

FLP-FRT clone induction

Adult males were collected at 3-5 days post-eclosion and heat-shocked three times at 37°C for 30 min followed by a 10 min rest period at room temperature between heat shocks. After heat-shock, the flies were incubated at room temperature until dissection.

Immunohistochemistry

Fixed samples were rinsed three times with blocking solution containing 0.2% bovine serum albumin (A4503, Sigma-Aldrich), 0.3% Triton-X in PBS, then blocked for 1 hr on a rotating platform at room temperature. During the incubation, the blocking solution was changed every 15 minutes. After blocking, the sample were resuspended in blocking solution with the appropriate concentration of primary antibody (see Key Resources table), and incubated overnight at 4°C. Samples were rinsed three times with blocking solution after removing primary antibody, and blocked for one hour on a rotating platform in blocking solution. Secondary antibody was applied in blocking solution and left on the rotating platform at room temperature for 40 min. The sample was rinsed with blocking solution three more times, and washed four times for 15 min per wash in blocking solution. Testis samples were resuspended in Vectashield mounting media with DAPI (H-1200-10, Vector Laboratory) or SlowFade Diamond mounting media (S36972, Thermo Fisher Scientific) prior to mounting.

Lipid droplet staining

Fixed testes were briefly permeabilized with 0.1% Triton-X in PBS for 5 min prior to applying phalloidin. For BODIPY (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-*s*-Indacene) staining, samples were suspended in PBS containing 10 µg/mL DAPI (2879083-5mg, PeproTech), 1:500 BODIPY 495/503 (Thermo Fisher Scientific D3922), and 1:1000 phalloidin iFluor647 (ab176759, Abcam) or 1:40 phalloidin TexasRed (T7471, Thermo Fisher Scientific). For staining with

LipidTox Red, samples were suspended in PBS containing 10 µg/mL DAPI (2879083-5mg, PeproTech), 1:200 LipidTox Red (H34476, Thermo Fisher Scientific), and 1:1000 phalloidin iFluor647 (ab176759, Abcam). For staining free sterols, samples were prepared as for BODIPY staining with 50 µg/mL filipin in place of BODIPY for 30 min. Samples were incubated on a rotating platform for 40 minutes at room temperature.

After incubation, samples were washed twice with PBS, then resuspended in SlowFade Diamond mounting media (Thermo Fisher Scientific S36972) prior to mounting.

Image acquisition and processing

All images were acquired on a Leica SP5 confocal microscope system with 20X or 40X objectives and quantified with Fiji image analysis software[122].

Drosophila lipidomics

Drosophila extracts were prepared following the previously reported protocol[123]. Briefly, 10 *Drosophila* males (~10 mg) were weighed, 300 µL of ice-cold methanol/water mixture (9:1, v:v) was added to these males, and the samples were homogenized with glass beads using a bead beater (mini-beadbeater-16, BioSpec, Bartlesville, Ok, USA). Sample weight was used for sample normalization. Fly lysate was kept at -20°C for 4 hours for protein precipitation. Then, 900 µL of methyl tert-butyl ether was added and the solution was shaken for 5 min to extract lipids. To induce phase separation 285 µL of water was added, followed by centrifugation. The upper layer was separated, dried, and reconstituted in isopropanol/acetonitrile (1:1, v:v) for liquid chromatography-mass spectrometry (LC-MS) analysis. The volume of reconstitution solution was proportional to sample weight for normalization. Quality control (QC) samples were prepared by pooling 20 µL aliquot from each sample. The method blank sample was prepared using an identical workflow but without adding *Drosophila*.

Drosophila extracts were analyzed on an UHR-QqTOF (Ultra-High Resolution Qq-Time-Of-Flight) mass spectrometry Impact II (Bruker Daltonics, Bremen, Germany) interfaced with an Agilent 1290 Infinity II LC Systems (Agilent Technologies, Santa Clara, CA, USA). LC separation was performed using a Waters reversed-phase (RP) UPLC Acquity BEH C18 Column (1.7 µm, 1.0 mm ×100 mm, 130 Å) (Milford, MA, USA) maintained at 30°C. For positive ion mode, the mobile phase A was 60% acetonitrile in water and the mobile phase B was 90% isopropanol in acetonitrile, both containing 5 mM ammonium formate (pH = 4.8, adjusted by formic acid). For negative ion mode, the mobile phase A was 60% acetonitrile in water and the mobile phase B was 90% isopropanol in acetonitrile, both containing 5 mM NH4FA (pH = 9.8, adjusted by ammonium hydroxide). The LC gradient for positive and negative ion modes was set as follows: 0 min, 5% B; 8 min, 40% B; 14 min, 70% B; 20 min, 95% B; 23 min, 95% B; 24 min, 5% B; 33 min, 5% B. The flow rate was 0.1 mL/min. The injection volume was optimized to 2 µL in positive mode and 5 µL in negative mode using QC sample. The ESI source conditions were set as follows: dry gas temperature, 220 °C; dry gas flow, 7 L/min; nebulizer gas pressure, 1.6 bar; capillary voltage, 4500 V for positive mode and 3000 V for negative mode. The MS1 analysis was conducted using following parameters: mass range, 70-1000 m/z; spectrum type: centroid, calculated using maximum intensity; absolute intensity threshold: 250. Data-dependent MS/MS analysis parameters: collision energy: 16-30 eV; cycle time, 3 s; spectra rate: 4 Hz when intensity < 10^4 and 12 Hz when intensity > 10^5, linearly increased from 10^4 to 10^5. External calibration was applied using sodium formate to ensure the m/z accuracy before sample analysis.

The raw LC-MS data were processed using MS-DIAL (ver. 4.38)[124]. The detailed MS-DIAL parameters are: MS1 tolerance, 0.01 Da; MS/MS tolerance, 0.05; mass slice width, 0.05 Da; smoothing method, linear weighted moving average; smoothing level, 3 scans; minimum peak width, 5 scans. Lipid features with high quantitative confidence were selected by the following criteria: retention time was within the gradient elution time (< 23 min); average intensity in QC samples is larger than 5-fold of the intensity in method blank sample. Lipid identification was performed by matching experimental precursor m/z, isotopic ratio and MS/MS spectrum against the LipidBlast libraries embedded in MS-DIAL. To improve the quantification accuracy, the measured MS signal intensities were corrected using serial diluted QC samples following the reported workflow[125].

Quantification and statistical analysis

All microscopy images were quantified using Fiji software[122]. For lipid droplet counts, a single optical slice through the middle of the testis containing the hub was used with the exception of FLP-FRT experiment where all lipid droplets within a GFP-negative cyst were counted (**Figure 2I**). All statistical analyses were done using R (obtained from *https://cran.r-project.org*). With exception of data concerning spatial distribution, and lipidomic data, Shapiro-Wilk test (via *shapiro.test* in base R) was used to assess normality of distribution prior to testing for significance. Kruskal-Wallis rank sum test (from the R package *coin*) and Dunn's test (from the R package *dunn.test*) were used in place of Welch two-sample t-test and Tukey's multiple comparison test when the assumption of normality was not met. For testing differences in variance between two populations, F-test (via *var.test* in base R) was used. For testing differences in spatial distribution, two-sample Kolmogorov-Smirnov test (via *ks.test* in base R) was used. All *p*-values are indicated in figures; extremely small *p*-values are listed as $p<2.2 \times 10^{-16}$.

Resource table

REAGENT or RESOURCE	SOURCE	RESOURCE #
Antibodies		
Anti-Vasa (1:200)	Gift from Dr. R. Lehman, MIT	
Anti-Eya (1:50)	Developmental Studies Hybridoma Bank (DSHB)	eya10H6
Anti-zfh1 (1:1000)	Gift from Dr. J. Skeath, WUSTL	
Anti-boule (1:1000)	Gift from Dr. S. Wasserman, UCSD	
Anti-phospho-histone H3 (1:1000)	Millipore Sigma	05-1354
Experimental models: *Drosophila melanogaster*		
w^{1118}	Bloomington *Drosophila* stock center	3605
CantonS	Bloomington *Drosophila* stock center	64349
OregonR	Bloomington *Drosophila* stock center	25211
bmm^{1}	Gift from Dr. R. Kühnlein	
bmm^{rev}	Gift from Dr. R. Kühnlein	
mdy[Qx25], cn[1], bw[1]/CyO, l(2)DTS513[1]	Bloomington *Drosophila* stock center	5095
y[1],w[67c23];P{lacW}Cse1[k03802],mdy[k03902]/CyO	Bloomington *Drosophila* stock center	10536
w[1118];P{GD1749}v6367 (UAS-mdy-RNAi)	Vienna *Drosophila* resource center	6367

REAGENT or RESOURCE	SOURCE	RESOURCE #
nos-GAL4::VP16	Bloomington *Drosophila* stock center	7303
Tj-GAL4	Gift from Dr. D. Godt, University of Toronto	
c587-GAL4	Bloomington *Drosophila* stock center	67747
Bam-GFP	[40]	
bmm-GFP	Gift from Dr. K. Kamei[57]	
GFP-LD	Gift from Dr. M. Welte[36]	
P{neoFRT}82B, bmm[1]	This study	
P{neoFRT}82B, bmm[rev]	This study	
bam-GFP, bmm[1]	This study	
bam-GFP, bmm[rev]	This study	
Software and algorithms		
Fiji	https://imagej.net/software/fiji/	
R	https://cran.r-project.org	

Supplemental table and files

Supplemental table 1 – Table showing identified lipid species from untargeted lipidomic analysis.

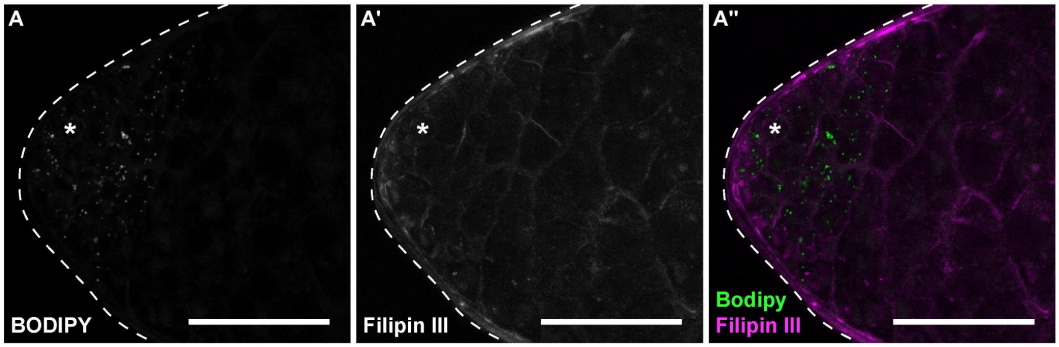
Supplementary file 1 – Raw data and statistical outputs from **Figure 1**.

Supplementary file 2 – Raw data and statistical outputs from **Figure 2**, Supplemental figure 2, and Supplemental figure 3.

Supplementary file 3 – Raw data and statistical outputs from **Figure 3** and Supplemental figure 4.

Supplementary file 4 – Raw data and statistical outputs from **Figure 4** and Supplemental figure 5.

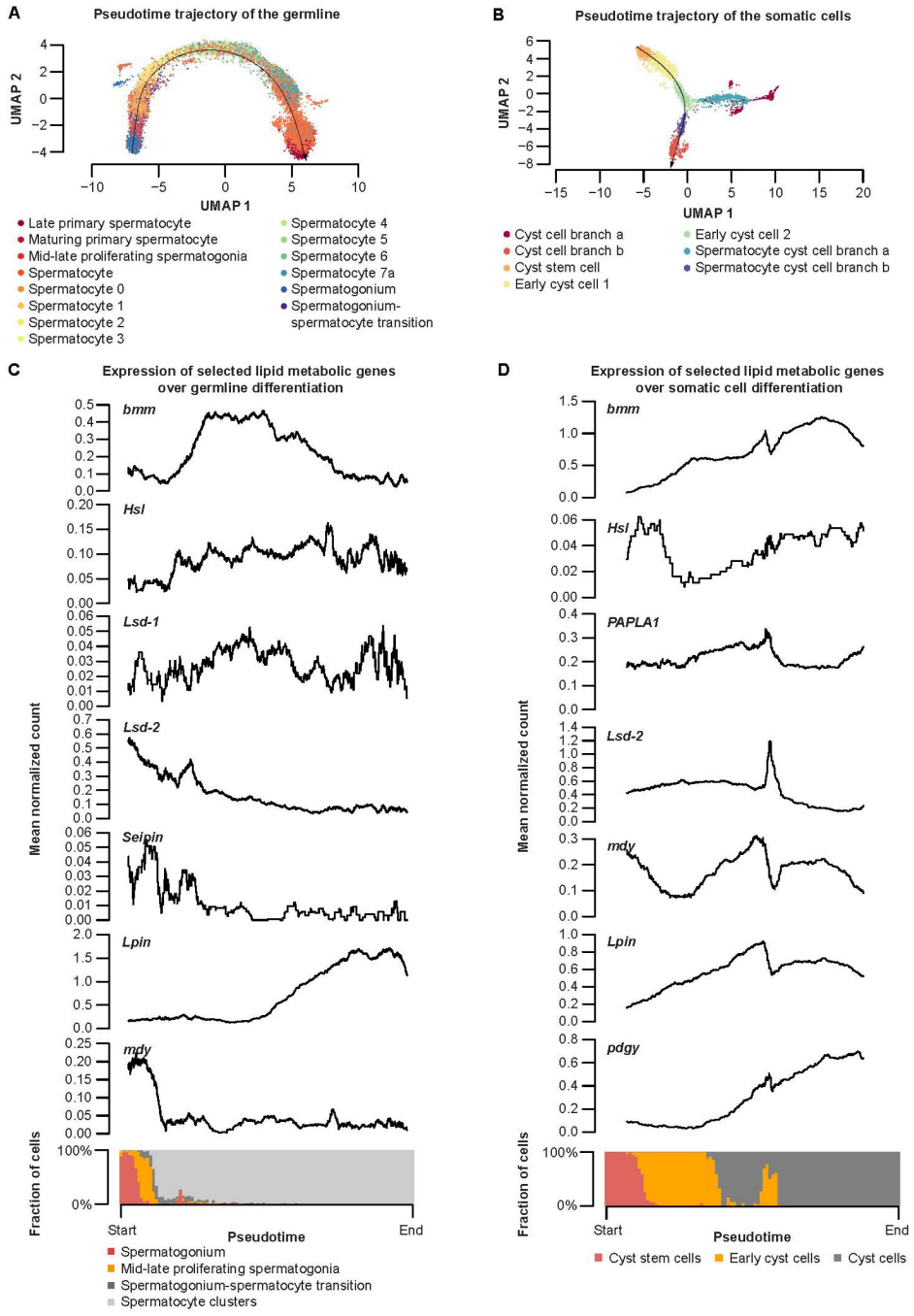
Supplemental figure legends



Supplemental Figure 1 related to Figure 1

Cholesterol is absent from testis lipid droplets.

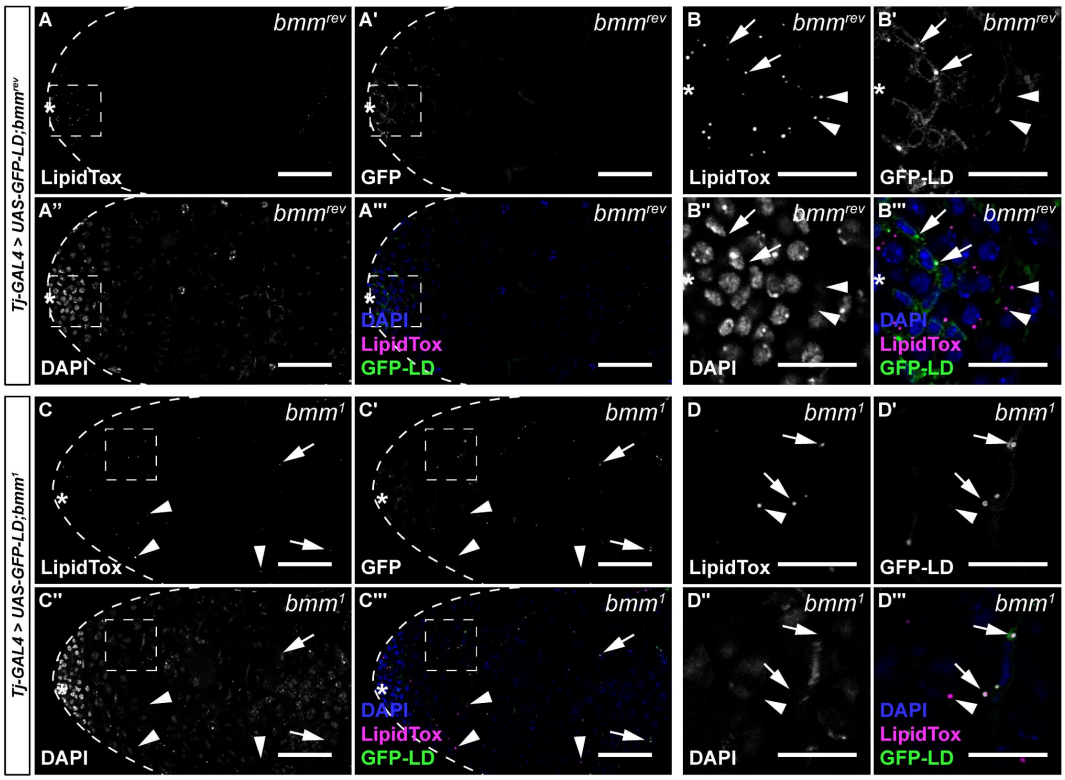
(A) Testes stained with BODIPY (A) to detect neutral lipids and Filipin III (A') to detect free cholesterol. Scale bars=50 μm.



Supplemental Figure 2 related to Figure 2

Expression of *brummer* and selected lipid metabolic genes during spermatogenesis in germline and somatic lineages.

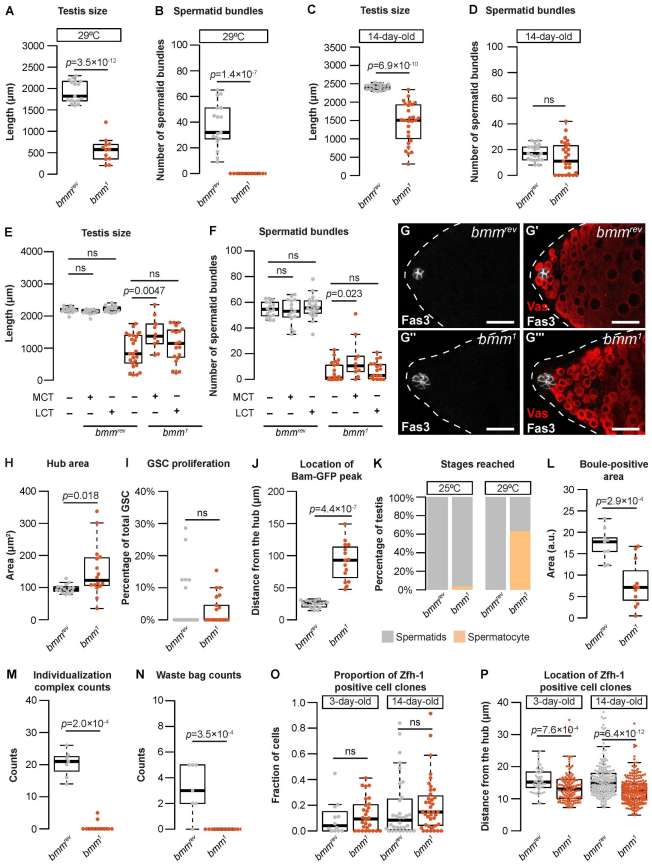
(A) Pseudotime trajectory of germline (black line) based on single-cell RNA sequencing data [78]. Individual cells are labeled according to the annotation within the data set. (B) Pseudotime trajectory of the somatic cells (black line) based on publicly available single-cell RNA sequencing data [78]. Individual cells are labeled according to the annotation within the data set. Only one trajectory (branch b) is marked and used for panel D. (C) Rolling average of normalized transcript counts in the germline along the trajectory shown in panel A are plotted as a black line on the upper panel. Composition of cell types mapped on to the trajectory at each time point is shown at the bottom of panel B. (D) Rolling average of normalized transcript counts in somatic cells plotted as a black line along the trajectory shown in C (upper panel). Composition of cell types mapped on to the trajectory at each time point is shown at the bottom of panel D.



Supplemental Figure 3 related to Figure 2

brummer **regulates lipid droplets in both germline and somatic cells of the testis.**

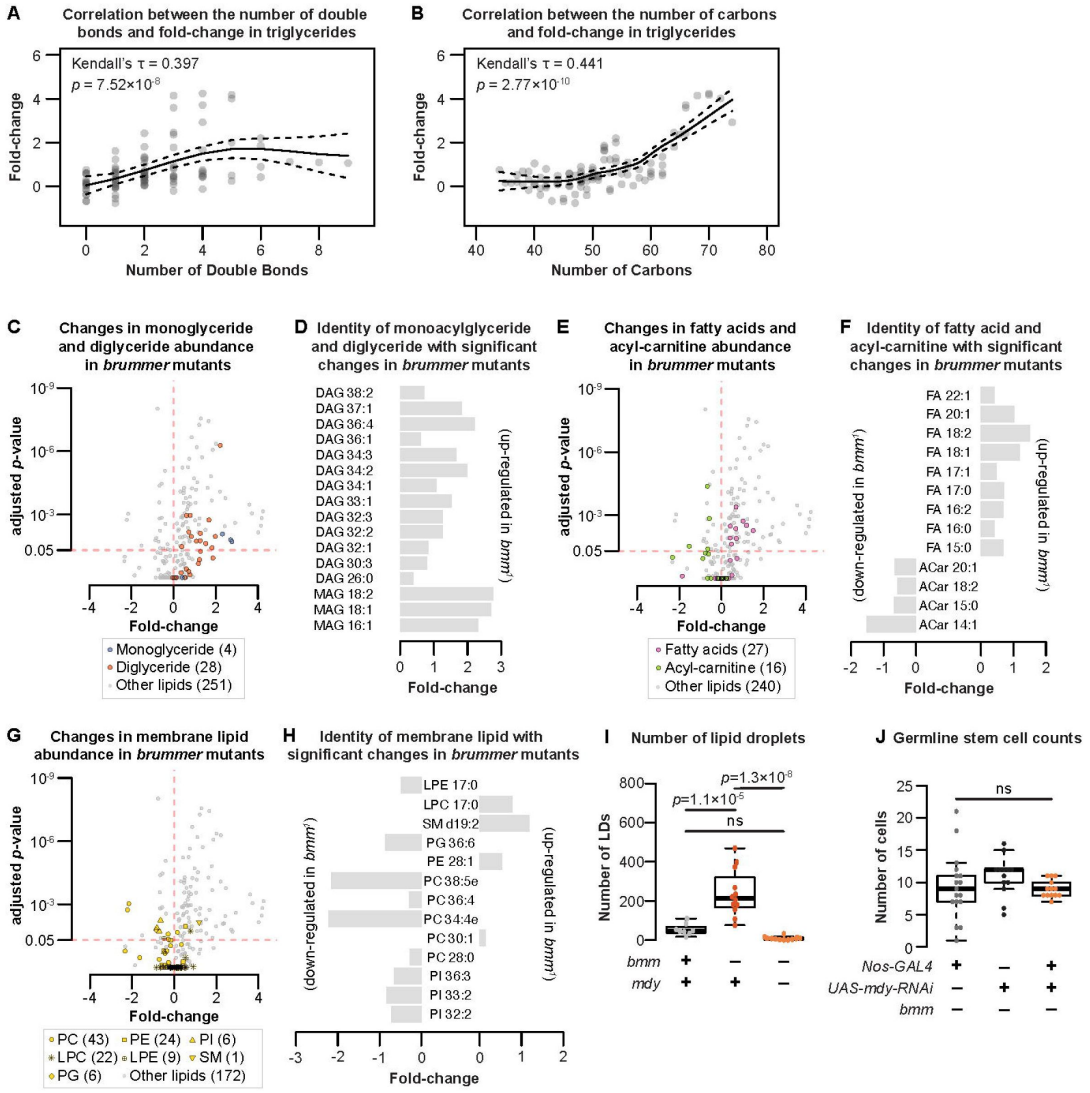
(A-D) Representative images of *bmm*^{rev}(A and B) and *bmm*^{1} (C and D) testes with somatic over-expression of *GFP-LD* (*Tj-GAL4>UAS-GFP-LD*). Panel F and H contain magnified images of the area indicated by the boxes in panel A and C, respectively. In *bmm*^{rev} testes, LD were restricted to a region near the apical tip (A) of the testis in both somatic (B–B''' arrows) and germline cells (B–B''' arrowheads). In *bmm*^{1}testes, LD were present in both somatic (C–D arrows) and germline cells (C–D arrowheads), near the apical tip of the testis in a region corresponding to early-stage germ cells and in the region corresponding to spermatocytes. (A,C) Scale bars=50 μm; (B,D) scale bars=20 μm.



Supplemental Figure 4 related to Figure 4

Additional characterization of testis development and spermatogenesis in animals lacking *bmm*.

(A) Testis size was smaller in *bmm*^{1} mutant animals compared with *bmm*^{rev} controls at <24 hr post-eclosion when raised at 29°C (A; Welch two-sample t-test). (B) The number of spermatid bundles was significantly lower in *bmm*^{1}mutant animals compared with *bmm*^{rev} controls at <24 hr post-eclosion when raised at 29°C (Kruskal-Wallis rank sum test). (C) Testis size was significantly smaller in *bmm*^{1}mutant males compared with *bmm*^{rev} control males at 14-days post-eclosion (Welch two-sample t-test). (D) While the median number of spermatid bundles was not significantly different between *bmm*^{1} mutant males and *bmm*^{rev} control males at 14 days post-eclosion (Welch two-sample t-test), 8/27 *bmm*^{1} testis had no spermatid bundles, a phenotype absent in age-matched *bmm*^{rev} males (0/22) (p=0.0163, Pearson's Chi-squared test), suggesting a subtle defect is present. (E) Food supplemented with 4% medium chain triglyceride (MCT), but not long chain triglyceride (LCT), significantly increased testis length in *bmm*^{1} animals but had no effect on this phenotype in *bmm*^{rev}control animals (one-way ANOVA with Tukey multiple comparison test). (F) Food supplemented with 4% medium chain triglyceride significantly increased the number of spermatid bundles in *bmm*^{1}testes but had no effect on this phenotype in *bmm*^{rev} control animals (one-way ANOVA with Tukey multiple comparison test). (G) Representative images of *bmm*^{rev} (G–G') or *bmm*^{1} (G''– G''') testes stained for Fas3 (G and G'') and Vas (G' and G'''). Scale bars=25 µm. (H) Quantification of hub area in *bmm*^{rev}or *bmm*^{1} testes showed a significantly larger hub size in *bmm*^{1}testes (Welch two-sample t-test). (I) The number of germline stem cells (GSC) undergoing mitosis (phospho-histone H3^{+} GSC/total GSC) was not significantly different between *bmm*^{1} and *bmm*^{rev}testes (Kruskal-Wallis rank sum test). (J) The distance between the hub and the first Bam-GFP positive cyst (**Figure 3H**) was significantly higher in *bmm*^{1} testes than in *bmm*^{rev}testes (Welch two-sample t-test). (K) All *bmm*^{rev} testes and most *bmm*^{1} testes contained spermatids when raised at 25°C; however, the most advanced stage of spermatogenesis observed in the majority of *bmm*^{1} testes isolated from animals reared at 29°C was the spermatocyte stage. (L) Testes isolated from *bmm*^{1} animals showed a significantly smaller Boule-positive area than control testes (Welch two-sample t-test). (M) Testes isolated from *bmm*^{1} animals contain fewer individualization complexes than *bmm*^{rev} control testes (Kruskal-Wallis rank sum test). (N) Fewer waste bags were present in testes isolated from *bmm*^{1}animals compared with *bmm*^{rev} control testes (Kruskal-Wallis rank sum test). (O) Proportion of Zfh-1 positive clones that were homozygous for either *bmm*^{1} or *bmm*^{rev} at 3 and 14 days post-clone induction (Kruskal-Wallis rank sum test). (P) The location of Zfh-1 positive clones homozygous for either *bmm*^{1} or *bmm*^{rev} at 3 and 14 days post-clone induction measured as the distance from the center of the hub. Clones homozygous for *bmm*^{1} locate significantly closer to the hub than the wildtype clones (Kruskal-Wallis rank sum test).



Supplemental Figure 5 related to Figure 5

Lipidomic analysis of animals lacking *bmm*.

(A) Higher fold-changes of triglycerides in bmm^{1} animals were associated with less saturation in the acyl-groups (Kendall's rank correlation test). (B) Higher fold-changes of triglycerides in bmm^{1} animals were associated with higher number of carbons in the acyl-groups (Kendall's rank correlation test). Each dot represents a single triglyceride species for panel B and C. (C) Volcano plot of identified lipids; monoglycerides shown in blue and diglycerides shown in orange. Many monoglycerides and diglycerides show increase in fold-change in bmm^{1} males. (D) The number of carbon and the degree of saturation of monoglycerides (MAG) and diglycerides (DAG) with significant changes in abundance between bmm^{1} and bmm^{rev} males. (E) Volcano plot of identified lipids; fatty acids shown in magenta and acyl-carnitine shown in green. Many fatty acids show an increase in fold-change while many acyl-carnitines show a decrease in fold-change in bmm^{1} males. (F) The number of carbon and the degree of saturation of fatty acids (FA) and acyl-carnitines (ACar) with significant changes in abundance between bmm^{1} and bmm^{rev} males. (G) Volcano plot of identified lipids; membrane lipids shown in yellow. (H) The number of carbon and the degree of saturation of membrane lipids with significant changes in abundance between bmm^{1} and bmm^{rev} males. For panel G and H, PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; SM: sphingomyelin; PG: phosphatidylglycerol. (I) Loss of *mdy* function rescued the elevated number of LD in bmm^{1} testes to control levels (one-way ANOVA with Tukey multiple comparison test). (J) Germline-specific loss of *mdy* in bmm^{1} animals did not reduce GSC numbers, but the variance in GSC numbers was significantly rescued (*nos-GAL4*>+; bmm^{1} vs *nos-GAL4>mdy RNAi*; bmm^{1}: $p=4.5 \times 10^{-5}$; +>*mdy RNAi*; bmm^{1} vs *nos-GAL4>mdy RNAi*; bmm^{1}: $p=0.0082$ by F-test).

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Reviewer #1 (Public Review):

In this study, the authors investigate the role of triglycerides in spermatogenesis. This work is based on their previous study (PMID: 31961851) on triglyceride sex differences in which they showed that somatic testicular cells play a role in whole body triglyceride homeostasis. In the current study, they show that lipid droplets (LDs) are significantly higher in the stem and progenitor cell (pre-meiotic) zone of the adult testis than in the meiotic spermatocyte stages. The distribution of LDs anti-correlates with the expression of the triglyceride lipase Brummer (Bmm), which has higher expression in spermatocytes than early germline stages. Analysis of a bmm mutant (bmm[1]) - a P-element insertion that is likely a hypomorphic - and its revertant (bmm[rev]) as a control shows that bmm acts autonomously in the germline to regulate LDs. In particular, the number of LDs is significantly higher in spermatocytes from bmm[1] mutants than from bmm[rev] controls. Testes from males with global loss of bmm (bmm[1]) are shorter than controls and have fewer differentiated spermatids. The zone of bam expression, typically close to the niche/hub in WT, is now many cell diameters away from the hub in bmm[1] mutants. There is an increase in the number of GSCs in bmm[1] homozygotes, but this phenotype is probably due to the enlarged hub. However, clonal analyses of GSCs lacking bmm indicate that a greater percentage of the GSC pool is composed of bmm[1]-mutant clones than of bmm[rev]-clones. This suggests that loss of bmm could impart a competitive advantage to GSCs, but this is not explored in greater detail. Despite the increase in number of GSCs that are bmm[1]-mutant clones, there is a significant reduction in the number of bmm[1]-mutant spermatocyte and post-meiotic clones. This suggests that fewer bmm[1]-mutant germ cells differentiate than controls. To gain insights into triglyceride homeostasis in the absence of bmm, they perform mass spec-based lipidomic profiling. Analyses of these data support their model that triglycerides are the class of lipid most affected by loss of bmm, supporting their model that excess triglycerides are the cause of spermatogenetic defects in bmm[1]. Consistent with their model, a double mutant of bmm[1] and a diacylglycerol O-acyltransferase 1 called midway (mdy) reverts the bmm-mutant germline phenotypes.

There are numerous strengths of this paper. First, the authors report rigorous measurements and statistical analyses throughout the study. Second, the authors utilize robust genetic analyses with loss-of-function mutants and lineage-specific knockdown. Third, they demonstrate the appropriate use of controls and markers. Fourth, they show rigorous lipidomic profiling. Lastly, their conclusions are appropriate for the results. In other words, they don't over-state the results. Overall, the rigorously quantified results support the major

aim that appropriate regulation of triglycerides are needed in a germline cell-autonomous manner for spermatogenesis.

This paper should have a positive impact on the field. First and foremost, there is limited knowledge about the role of lipid metabolism in spermatogenesis. The lipidomic data will be useful to researchers in the field who study various lipid species. Going forward, it will be very interesting to determine what triglycerides regulate in germline biology. In other words, what functions/pathways/processes in germ cells are negatively impacted by elevated triglycerides. And as the authors point out in the discussion, it will be important to determine what regulates bmm expression such that bmm is higher in later stages of germline differentiation.

https://doi.org/10.7554/eLife.87523.2.sa1

Reviewer #2 (Public Review):

Summary:

Here, the authors show that neutral lipids play a role in spermatogenesis. Neutral lipids are components of lipid droplets, which are known to maintain lipid homeostasis, and to be involved in non-gonadal differentiation, survival, and energy. Lipid droplets are present in the testis in mice and Drosophila, but not much is known about the role of lipid droplets during spermatogenesis. The authors show that lipid droplets are present in early differentiating germ cells, and absent in spermatocytes. They further show a cell autonomous role for the lipase brummer in regulating lipid droplets and, in turn, spermatogenesis in the Drosophila testis. The data presented show that a relationship between lipid metabolism and spermatogenesis is congruous in mammals and flies, supporting Drosophila spermatogenesis as an effective model to uncover the role lipid droplets play in the testis.

Strengths and weaknesses:

The authors do a commendably thorough characterization of where lipid droplets are detected in normal testes: located in young somatic cells, and early differentiating germ cells. They use multiple control backgrounds in their analysis, including w[1118], Canton S, and Oregon R, which adds rigor to their interpretations. The authors employ markers that identify which lipid droplets are in somatic cells, and which are in germ cells. The authors use these markers to present measured distances of somatic and germ cell-derived lipid droplets from the hub. Because they can also measure the distance of somatic and germ cells with age-specific markers from the hub, these results allow the authors to correlate position of lipid droplets with the age of cells in which they are present. This analysis is clearly shown and well quantified.

The quantification of lipid droplet distance from the hub is applied well in comparing brummer mutant testes to wild type controls. The authors measure the number of lipid droplets of specific diameters, and the spatial distribution of lipid droplets as a function of distance from the hub. These measurements quantitatively support their findings that lipid droplets are present in an expanded population of cells further from the hub in brummer mutants. The authors further quantify lipid droplets in germline clones of specified ages; the quantitative analysis here is displayed clearly and supports a cell autonomous role for brummer in regulating lipid droplets in spermatocytes.

Data examining testis size and number of spermatids in brummer mutants clearly indicates the importance of regulating lipid droplets to spermatogenesis. The authors show beautiful images supported by rigorous quantification supporting their findings that brummer mutants have both smaller testes with fewer spermatids at both 29 and 25C. There is also

significant data supporting defects in testis size, but not spermatid number, in 14-day-old brummer mutant animals compared to controls. Their analysis clearly shows an expanded region beyond the testis apex that includes younger germ cells, supporting a role for lipid droplets influencing germ cell differentiation during spermatogenesis.

The authors present a series of data exploring a cell autonomous role for brummer in the germline, including clonal analysis and tissue specific manipulations. The clonal data indicating increased lipid droplets in spermatocyte clones, and a higher proportion of brummer mutant GSCs at the hub are convincing and supported by quantitation. The authors also show a tissue specific rescue of the brummer testis size phenotype by knocking down mdy specifically in germ cells, which is also supported by statistically significant quantitation. The authors present data examining the number of spermatocyte and post-meiotic clones 14 days after clonal induction. Their finding is significant with a p-value of 0.0496, which they acknowledge is less robust than their other data reported in this study, and could be a result of a low sample size. They indicate that future studies might validate these results with additional samples.

The authors do a beautiful job of validating where they detect brummer-GFP by presenting their own pseudotime analysis of publicly available single cell RNA sequencing data. Their data is presented very clearly, and supports expression of brummer in older somatic and germline cells of the age when lipid droplets are normally not detected. The authors also present a thorough lipidomic analysis of animals lacking brummer to identify triglycerides as an important lipid droplet component regulating spermatogenesis.

Impact:

The authors present data supporting the broad significance of their findings across phyla. This data represents a key strength of this manuscript. The authors show that loss of a conserved triglyceride lipase impacts testis development and spermatogenesis, and that these impacts can be rescued by supplementing diet with medium-chain triglycerides. The authors point out that these findings represent a biological similarity between Drosophila and mice, supporting the relevance of the Drosophila testis as a model for understanding the role of lipid droplets in spermatogenesis. The connection buttresses the relevance of these findings and this model to a broad scientific community.

https://doi.org/10.7554/eLife.87523.2.sa0

Author Response

The following is the authors' response to the original reviews.

Response to reviews

We would like to extend our thanks to the reviewers who took the time to carefully read our paper and provide thoughtful insights and suggestions on how to strengthen our conclusions. All reviewers agreed that our study presented strong data supporting a role for triglyceride lipase brummer (bmm) in regulating testis lipid droplets and spermatogenesis in Drosophila, and that our findings advance our understanding of lipid biology during sperm development. Reviewers made several helpful suggestions on how to strengthen our manuscript even further. Below, we outline how we revised our manuscript in response to reviewer comments to ensure we clearly communicate our data and conclusions with readers, and properly contextualize our findings.

> ***REVIEWER 1***
>
> *In this study, the authors investigate the role of triglycerides in spermatogenesis. This work is based on their previous study (PMID: 31961851) on triglyceride sex differences in which they showed that somatic testicular cells play a role in whole body triglyceride homeostasis. In the current study, they show that lipid droplets (LDs) are significantly higher in the stem and progenitor cell (pre-meiotic) zone of the adult testis than in the meiotic spermatocyte stages. The distribution of LDs anti-correlates with the expression of the triglyceride lipase Brummer (Bmm), which has higher expression in spermatocytes than early germline stages. Analysis of a bmm mutant (bmm[1]) - a P-element insertion that is likely a hypomorphic - and its revertant (bmm[rev]) as a control shows that bmm acts autonomously in the germline to regulate LDs. In particular, the number of LDs is significantly higher in spermatocytes from bmm[1] mutants than from bmm[rev] controls. Testes from males with global loss of bmm (bmm[1]) are shorter than controls and have fewer differentiated spermatids. The zone of bam expression, typically close to the niche/hub in WT, is now many cell diameters away from the hub in bmm[1] mutants. There is an increase in the number of GSCs in bmm[1] homozygotes, but this phenotype is probably due to the enlarged hub. However, clonal analyses of GSCs lacking bmm indicate that a greater percentage of the GSC pool is composed of bmm[1]-mutant clones than of bmm[rev]-clones. This suggests that loss of bmm could impart a competitive advantage to GSCs, but this is not explored in greater detail. Despite the increase in number of GSCs that are bmm[1]-mutant clones, there is a significant reduction in the number of bmm[1]-mutant spermatocyte and post-meiotic clones. This suggests that fewer bmm[1]-mutant germ cells differentiate than controls. To gain insights into triglyceride homeostasis in the absence of bmm, they perform mass spec-based lipidomic profiling. Analyses of these data support their model that triglycerides are the class of lipid most affected by loss of bmm, supporting their model that excess triglycerides are the cause of spermatogenetic defects in bmm[1]. Consistent with their model, a double mutant of bmm[1] and a diacylglycerol Oacyltransferase 1 called midway (mdy) reverts the bmm-mutant germline phenotypes.*
>
> *There are numerous strengths of this paper. First, the authors report rigorous measurements and statistical analyses throughout the study. Second, the authors ulize robust genetic analyses with loss-of-function mutants and lineage-specific knockdown. Third, they demonstrate the appropriate use of controls and markers. Fourth, they show rigorous lipidomic profiling. Lastly, their conclusions are appropriate for the results. In other words, they don't overstate the results.*

We thank the Reviewer for their positive assessment of our paper.

> *There are a few weaknesses. Although the results support the germline autonomous role of bmm in spermatogenesis, one potential caveat that the mdy rescue was global, i.e., in both somatic and germline lineages. The authors did not recover somatic bmm clones, suggesting that bmm may be required for somatic stem self-renewal and/or niche residency. While this is beyond the scope of this paper, it is possible that somatic bmm does impact germline differentiation in a global bmm mutant.*

In the revised manuscript, we made several changes to address these points.

1. We now clearly state when we used global versus germline-only loss of mdy to rescue bmm mutant phenotypes in the testis.

“Notably, at least some of the effects of global loss of mdy on bmm1 males can be attributed to the germline:

RNAi-mediated knockdown of mdy in the germline of bmm1 males partially rescued the defects in testis size (Figure 4I; Kruskal-Wallis rank sum test with Dunn's multiple comparison test) and GSC variance (Figure S5J; p=4.5 x 10-5 and 8.2 x 10-3 by F-test from the GAL4- and UAS-only crosses, respectively)."

"Importantly, testes isolated from males with global loss of both bmm and mdy (mdyQX25/k03902;bmm1) had fewer LD than testes dissected from bmm1 males (Figures 5D, S5I; one-way ANOVA with Tukey multiple comparison test)."

1. We also discuss the possibility that somatic bmm may play a role in germline differentiation in a global bmm mutant, and present phenotypic data on somatic bmm1 clones.

"We also reveal a potential non-cell-autonomous role for somatic bmm. While there was no difference in the ratio of Zd-1-positive cells between homozygous clones and heterozygous clones in animals carrying the bmm1 or bmmrev alleles at 14 days post clone induction (Figure S4O; Kruskal-Wallis rank sum test), the distance from the hub to the Zd-1 positive clones reside was significantly decreased in bmm1 homozygous clones (Figure S4P; Kruskal-Wallis rank sum test). Together, these data indicate bmm may play a cell-autonomous role in germline cells, and potentially a non-cell-autonomous role in somatic cells, to regulate spermatogenesis."

1. Finally, we clarify that we were unable to assess somatic LD. Specifically, this was a technical issue as the dye we use to visualize testis LD is incompatible with staining protocols to identify somatic cells. As a result, we were unable to count LD in somatic clones with confidence.

"While we were unable to assess LD in bmm1 somatic clones, our data when taken together reveals a previously unrecognized cell-autonomous role for bmm as a regulator of testis LD in germline cells."

> *Regarding data presentation, I have a minor point about Fig. 3L: why aren't all data shown as box plots (only Day 14 bmm[rev] does).*

In our revised manuscript Figure 4L does present a boxplot across all genotypes and times; the appearance of 'no boxes' is simply due to the large number of datapoints with a value of zero, which compress the box near the X-axis.

> *Finally, the authors provide a detailed pseudotime analysis of snRNA-seq of the testis in Fig. S2A-D, but this analysis is not sufficiently discussed in the text.*

In the revised manuscript we added text to describe our pseudotime analysis of single-cell RNA seq data in more detail.

"Using pseudotime analysis, we arranged the germline (Figure S2A) and the somatic cells (Figure S2B) based on their annotated developmental trajectory. The expression pattern of bmm in the germline matched our observation with bmm-GFP reporter (Figure S2C). While levels of the bmm-GFP reporter were lower in somatic cells, single-cell RNA sequencing data identified bmm expression in the somatic lineage that was higher in cells at later stages of development (Figure S2D). Additional neutral lipid- and lipid droplet-associated genes such as lipid storage droplet-2, Seipin, Lipin, and midway also showed differential regulation during differentiation (Figure S2C, S2D). Combined with our data on the location of testis LD, these data suggest that bmm upregulation in both somatic and germline cells during differentiation corresponds to the downregulation of testis LD. Supporting this, germline GFP levels were

negatively correlated with testis LD in bmm-GFP flies (Figure 2A, 2C), suggesting regions with higher bmm expression had fewer LD."

> *Overall, the many strengths of this paper outweigh the relatively minor weaknesses. The rigorously quantified results support the major aim that appropriate regulation of triglycerides are needed in a germline cell-autonomous manner for spermatogenesis.*
>
> *This paper should have a positive impact on the field. First and foremost, there is limited knowledge about the role of lipid metabolism in spermatogenesis. The lipidomic data will be useful to researchers in the field who study various lipid species. Going forward, it will be very interesting to determine what triglycerides regulate in germline biology. In other words, what functions/pathways/processes in germ cells are negatively impacted by elevated triglycerides. And as the authors point out in the discussion, it will be important to determine what regulates bmm expression such that bmm is higher in later stages of germline differentiation.*

We agree with the reviewer about the many interesting future directions for this project. We added a model figure in the revised manuscript to visualize our findings and highlight remaining questions about how bmm and triglycerides support normal spermatogenesis in Drosophila (Fig. 6).

> ***REVIEWER 2***
>
> *Summary:*
>
> *Here, the authors show that neutral lipids play a role in spermatogenesis. Neutral lipids are components of lipid droplets, which are known to maintain lipid homeostasis, and to be involved in non-gonadal differentiation, survival, and energy. Lipid droplets are present in the testis in mice and Drosophila, but not much is known about the role of lipid droplets during spermatogenesis. The authors show that lipid droplets are present in early differentiating germ cells, and absent in spermatocytes. They further show a cell autonomous role for the lipase brummer in regulating lipid droplets and, in turn, spermatogenesis in the Drosophila testis. The data presented show that a relationship between lipid metabolism and spermatogenesis is congruous in mammals and flies, supporting Drosophila spermatogenesis as an effective model to uncover the role lipid droplets play in the testis.*

We thank the Reviewer for their positive assessment of our paper.

> *Strengths and weaknesses:*
>
> *The authors do a commendably thorough characterization of where lipid droplets are detected in normal testes: located in young somatic cells, and early differentiating germ cells. They use multiple control backgrounds in their analysis, including w[1118], Canton S, and Oregon R, which adds rigor to their interpretations. The authors employ markers that identify which lipid droplets are in somatic cells, and which are in germ cells. The authors use these markers to present measured distances of somatic and germ cell-derived lipid droplets from the hub. Because they can also measure the distance of somatic and germ cells with age-specific markers from the hub, these results allow the authors to correlate position of lipid droplets with the age of cells in which they are present. This analysis is clearly shown and well quantified.*
>
> *The quantification of lipid droplet distance from the hub is applied well in comparing brummer mutant testes to wild type controls. The authors measure the number of lipid droplets of specific diafteters, and the spatial distribution of lipid droplets as a function of distance from the hub. These measurements quantitatively support their findings that*

> *lipid droplets are present in an expanded population of cells further from the hub in brummer mutants. The authors further quantify lipid droplets in germline clones of specified ages; the quantitative analysis here is displayed clearly, and supports a cell autonomous role for brummer in regulating lipid droplets in spermatocytes.*
>
> *Data examining testis size and number of spermatids in brummer mutants clearly indicates the importance of regulating lipid droplets to spermatogenesis. The authors show beautiful images supported by rigorous quantification supporting their findings that brummer mutants have both smaller testes with fewer spermatids at both 29 and 25C. There is also significant data supporting defects in testis size for 14-day-old brummer mutant animals compared to controls. The comparison of number of spermatids at this age is not significant, which does not detract from the story but does not support sperm development defects specifically caused by brummer loss at 14 days. Their analysis clearly shows an expanded region beyond the testis apex that includes younger germ cells, supporting a role for lipid droplets influencing germ cell differentiation during spermatogenesis.*

We thank the reviewer for pointing out this inaccuracy in our manuscript. In the revised manuscript we chose more precise language to describe defects in 14-day-old bmm mutants:

“Defects in testis size were also observed at 14-day post eclosion; suggesting testis size defects persist later into the life course (Figure S4C; Welch two-sample t-test). In contrast, the number of spermatid bundles per testis was not significantly different between bmm1 and bmmrev males at this age (Figure S4D; Welch two-sample ttest), potentially due to a large decrease in the number of spermatid bundles in 14-day-old bmmrev males (Figure 4C, S4D).”

> *The authors present a series of data exploring a cell autonomous role for brummer in the germline, including clonal analysis and tissue specific manipulations. The clonal data indicating increased lipid droplets in spermatocyte clones, and a higher proportion of brummer mutant GSCs at the hub are convincing and supported by quantitation. The authors also show a tissue specific rescue of the brummer testis size phenotype by knocking down mdy specifically in germ cells, which is also supported by statistically significant quantitation. The authors present data examining the number of spermatocyte and post-meiotic clones 14 days aeer clonal induction. While data they present is significant with a 95% confidence interval and a p value of 0.0496, its significance is not as robust as other values reported in the study, and it is unclear how much information can be gained from that specific result.*

We thank the reviewer for raising this point. In the revised manuscript we displayed the p-value clearly in the text and on the figure to ensure our statistical output is clear for readers to evaluate our conclusions regarding bmm mutant clones 14 days after clone induction. We also state that the finding should be reproduced by others given that the statistical significance of this result was not as strong as our other data.

“Because we observed significantly fewer bmm1 spermatocyte and spermatid clones at 14 days after clone induction (Figure 4K,4L; p = 0.0496, Kruskal-Wallis rank sum test), these effects on germline development may represent a cell-autonomous role in regulating spermatogenesis for bmm in this cell type. Given that the statistical significance of this finding was not as strong as for our other data, future studies should repeat this experiment with more samples.”

> *The authors do a beautiful job of validating where they detect brummer-GFP by presenting their own pseudotime analysis of publicly available single cell RNA sequencing data. Their data is presented very clearly, and supports expression of brummer in older somatic and germline cells of the age when lipid droplets are normally not detected. The*

> *authors also present a thorough lipidomic analysis of animals lacking brummer to identify triglycerides as an important lipid droplet component regulating spermatogenesis.*
>
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>
> *The authors present data supporting the broad significance of their findings across phyla. This data represents a key strength of this manuscript. The authors show that loss of a conserved triglyceride lipase impacts testis development and spermatogenesis, and that these impacts can be rescued by supplementing diet with medium chain triglycerides. The authors point out that these findings represent a biological similarity between Drosophila and mice, supporting the relevance of the Drosophila testis as a model for understanding the role of lipid droplets in spermatogenesis. The connection buttresses the relevance of these findings and this model to a broad scientific community.*

We thank the Reviewer very much for their positive assessment of our paper!

> ***REVIEWER 3***
>
> *In this manuscript, Chao et al seek to understand the role of brummer, a triglyceride lipase, in the Drosophila testis. They show that Brummer regulates lipid droplet degradation during differentiation of germ and somatic cells, and that this process is essential for normal development to progress. These findings are interesting and novel, and contribute to a growing realisation that lipid biology is important for differentiation.*

We thank the Reviewer for their positive comments about our manuscript.

> *Major comments:*
>
> 1. *The data in Figs 1 and 2, while helpful in setting the scene, do not add much to what was previously shown by the same group, namely that lipid droplets are present in both early germ cells and early somatic cells in the testis, and that Bmm regulates their degradation (PMID: 31961851). Measuring the distance of lipid droplets from the hub, while helpful in quantifying what is apparent, that only stem and early differentiated stages have lipid droplets, is not as informative as the way data are presented later (Fig. 2I), where droplets in specific stages are measured. Much of this could be condensed without much overall loss to the manuscript.*

We thank the reviewer for this comment. In our revised manuscript we edited the first part of the paper while still preserving the detailed characterization that builds upon our previous paper.

> 1. *It would be important to show images of the clones from which the data in Fig. 2I are generated. The main argument is that Bmm regulates lipid droplets in a cell autonomous manner; these data are the strongest argument in support of this and should be emphasised at the expense of full animal mutants (which could be moved to supplementary data).*

We thank the reviewer for this comment. In the revised manuscript we added a figure showing lipid droplets in control and bmm mutant spermatocyte clones in Fig. 3A, 3B with a quantification of this data in Figure 3C.

> *Similarly, the title of Fig. S2 ("brummer regulates lipid droplets in a cell autonomous manner") should be changed as the figure has no experiments with cell (or cell-type)-specific knockdowns/mutants. This figure does show changes in lipid droplets in both lineages in bmm mutants, so an appropriate title could be "brummer regulates lipid droplets in both germ and soma".*

We thank the reviewer for this comment, we adjusted the Figure 2 legend title in the revised manuscript to “brummer regulates lipid droplets in both germline and somatic cells of the testis”.

> 1. *Interestingly, the clonal data show that bmm is dispensable in germ cells until spermatocyte stages, as no increase in lipid droplet number is seen until then. This should be more clearly stated, as it indicates that the important function of Bmm is to degrade lipid droplets at the transition from spermatogonial to spermatocyte stages. This is consistent with the phenotypes observed in which late stage germ cells are reduced or missing. However, the effect on niche retention of the mutant GSCs at the expense of neighbouring wildtype GSCs is hard to explain. Are lipid droplets in mutant GSCs larger than in control? Is there any discernible effect of bmm mutation on lipids in GSCs? Additionally, bam expression is delayed, suggesting that bmm may have roles on cell fate in earlier stages than its roles that can be detected on lipid droplets.*

We thank the reviewer for this comment. We included more text in the revised manuscript to clarify the key role bmm plays in regulating lipid droplets at the spermatogonia-spermatocyte transition.

“Because we observed no significant effect of cell-autonomous bmm loss on LD at any other stage of germline development (Figure 3C), this suggests bmm function is not required to regulate LD at early stages of germ cell development. Instead, our data suggests bmm plays a role in regulating LD at the spermatogonia-spermatocyte transition.”

We also added more detail to our description of how bmm affects lipid droplets in cells at the earliest stages of germline development.

“Given that we detected no effect of cell-autonomous bmm loss on the number of GSC LD (Fig. 3C), more work will be needed to understand how bmm regulates GSC at a stage prior to its effects on LD number.”

> 1. *The bmm loss-of-function phenotype could be better described. Some of the data is glossed over with little description in the text (see for example the reference to Fig. 3A-C). For instance, in the discussion, the text states "loss of bmm delays germline differentiation leading to an accumulation of early-stage germ cells" (p13, l.25960). However, this accumulation has not been clearly shown, or at least described in the manuscript. Most of the data show a reduction (or almost complete absence) of differentiated cell types. This could indeed be due to delayed differentiation, or alternatively to a block in differentiation or to death of the differentiated cells. The clonal data presented show a decrease in the number of cells recovered, but do not allow inferences as to the timing of differentiation, making it hard to distinguish between the various possibilities for the lack of differentiated spermatids. Apart from data showing that GSCs are more likely to remain at the niche, no further data are shown to support the fact that mutant germ cells accumulate in early stages. While additional experiments could help resolve some of these issues, much of this could also be resolved by tempering the conclusions drawn in the text.*

We thank the reviewer for these comments. In the revised manuscript we temper our conclusions regarding bmm's precise role in spermatogenesis by discussing different mechanisms (e.g. differentiation or death) that could lead to the phenotypes we observe.

"This regulation is important for sperm development, as our data indicates that loss of bmm causes a decrease in the number of differentiated cell types. This reduction in differentiated cell types may be attributed to a delay in differentiation, a block in differentiation, or to a loss of differentiated cells through cell death. Future studies will therefore be essential to resolve why bmm loss causes a reduction in differentiated cell types."

> 1. *In the discussion (p.14, l-273 onwards), the authors suggest that products of triglyceride breakdown are important for spermatogenesis. However, an alternative interpretation of the results presented here (especially those using the midway mutant) could be that triglycerides impede normal differentiation directly. Indeed, preventing the cells' ability to produce triglycerides in the first place can rescue many of the defects observed. A better discussion of these results with a model for the function of triglycerides and their by-products would be a great improvement to this manuscript.*

We thank the reviewer for this comment. To ensure our data is clearly communicated with readers, we added a model to the paper suggesting how triglyceride and its by-products influence spermatogenesis (Fig. 6) and text to clarify that triglyceride could potentially impeded differentiation.

"It will also be important to determine whether it is the loss of metabolites produced by bmm's enzymatic action, or an increase in triglycerides, that leads to the reduction in differentiated cell types during spermatogenesis. Together, these experiments will provide critical insight into how triglyceride stored within testis LD contributes to overall cellular lipid metabolism during spermatogenesis."

Together, these changes will strengthen our overall finding that bmm-mediated regulation of testis triglyceride is important for normal sperm development. Because our findings in flies align with and extend data from rodent models, the developmental mechanisms we uncovered about how triglyceride lipase bmm regulates testis lipid droplets and sperm development will likely operate in other species.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *I have a minor concern about methodology: how were spermatocytes identified? I ask because data in Figure 3 indicate that there is a significant delay in germline differentiation in the bmm[1] mutant, with relatively smaller germ cells throughout the apical half of the testis. Typical large spermatocyte-like cells are not clearly obvious to me in Fig. 3.*

We thank the Reviewer for suggesting we add more clarity to how we identified spermatocytes. We state in the revised manuscript how we identify spermatocytes:

"Cells in the testis region occupied by primary spermatocytes were identified by their large cell size and decondensed chromosome staining occupying three nuclear domains [120]."

Also, we note that while it is difficult to see where the bmm1 testis have spermatocytes in Fig. 4E, this is due to the large number of early-stage cells in this close-up image. The spermatocytes can be more easily seen in Fig. 4I and 4I' when the whole testis is included in the image.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *• Lines 197-198 mention "Boule-positive area," "individualization complexes," and "waste bags." It would be helpful to the reader to explain what these measurements are to help contextualize the data shown related to these statements.*

We thank the Reviewer for this comment. We added the following text to the revised manuscript:

“Because Boule-positive area, individualization complexes, and waste bags are all markers for later stages in sperm development, these data indicate the loss of bmm causes a reduction in differentiated cell types.”

> *• Line 162 states a defect in sperm development observed in 14-day-old bmm[1] males, but the data presented in Figure S3D does not show a significant difference. The words "sperm development" should be removed from this sentence.*

We thank the Reviewer for pointing out this inaccurate statement. We fixed the statement as follows in the revised manuscript:

“Defects in testis size were also observed at 14-day post eclosion; suggesting testis size defects persist later into the life course (Figure S4C; Welch two-sample t-test). In contrast, the number of spermatid bundles per testis was not significantly different between bmm1 and bmmrev males at this age (Figure S4D; Welch two-sample ttest), potentially due to a large decrease in the number of spermatid bundles in 14-day-old bmmrev males (Figure 4C, S4D).”

> *• Line 294 has a typo: "regulating" should likely be "regulated"*

We thank the Reviewer for pointing out this mistake, which we corrected.

> *• Line 456 should include the length of time for heat shock*

We thank the Reviewer for pointing out this omission. We now include these details:

“Adult males were collected at 3-5 days post-eclosion and heat-shocked three times at 37°C for 30 min followed by a 10 min rest period at room temperature between heat shocks.”

> *• Methods section beginning on Line 442 might include an explanation of how hub area was quantified.*

We thank the Reviewer for this suggestion. We now include the following information:

“Hub size was measured by quantifying FasIII-positive area of the testis.”

> *• Figure 1 legend could benefit from adding a statement on how spermatocytes (arrowheads) were identified*

We thank the Reviewer for this suggestion, we now refer the reader to the more detailed description in the methods section.

> *• Figure 2A should present the merged panel in A' first. The legend states that Panel A shows Lipid Droplets, but LipidTox is not shown until A'.*

We thank the Reviewer for this suggestion, we now clarify that the text refers to panels A-A"".

> *• Figure 2I would benefit from a key, to emphasize that these are individual cell clones, highlighting the idea of cell autonomous effects of bmm in the spermatocytes. Showing example images of spermatocyte clones with increased lipid droplets could also emphasize this result. The legend for this panel should note the statistical test done to confirm significance in the SC result.*

We agree with the Reviewer and have added images of the LD in bmm1 spermatocyte clones in Figure 3B, and the quantification in Figure 3C. We explicitly state the significance of this result and the statistical test in Figure 3 legend.

> *• In Figure 3, the cell autonomous data clearly indicates that there are higher proportions of bmm mutant GSCs occupying the hub compared to control GSCs. It could be worth stating whether this observation indicates an increased ability of bmm mutant GSCs to compete for occupying space at the hub.*

We thank the Reviewer for pointing out this potential implication of our data, which we acknowledge in the revised version of our manuscript:

“Future studies will also need to confirm whether bmm1 mutant GSCs show an increased ability to occupy space at the hub.”

> *• In Figure 4, I suggest changing the title of Panel B to "Proportion of significant species in each lipid class" for clarity.*

We made this change in the Figure 5 legend (Figure 5 is the corresponding figure in the revised manuscript).

> *• It could be valuable to quantify the number of spermatids in the germline specific mdy knockdown, which would lend additional support to a cell autonomous requirement for bmm in spermatogenesis*

We added a sentence to the revised manuscript recognizing that this is an interesting experiment for studies on the role of germline triglyceride in promoting spermatogenesis.

“While future studies will need to test whether germline-specific loss of mdy also rescues spermatid number defects in bmm1 males, our data suggest bmm-mediated regulation of testis triglyceride plays a previously unrecognized role in regulating sperm development.”

> ***Reviewer #3 (Recommendations For The Authors):***
>
> 1. *bmm-GFP does not show expression in somatic cells yet previous work by the same group has shown a requirement for bmm in the testis soma using C587-Gal4.*

We thank the Reviewer for raising this issue. While the reporter shows low GFP expression in the somatic cells, the single-cell RNA sequencing data we analyze suggests bmm is expressed in these cells. We address this issue in the revised manuscript as follows:

“While levels of the bmm-GFP reporter were lower in somatic cells, single-cell RNA sequencing data identified bmm expression in the somatic lineage that was higher in cells at later stages of development (Figure S2D).”

> 1. *p.11 l.200-202 "Because we recovered fewer bmm1 spermatocyte and spermatid clones 14 days after clone induction (Figure 3K,3L; Kruskal-Wallis rank sum test), this effect on germline development represents a cell-autonomous role for bmm." This sentence should be rephrased as the phenotype could be a combination of autonomous roles within the germline and non-autonomous roles in supporting cyst cells.*

“We also reveal a potential non cell-autonomous role for somatic bmm. While there was no difference in the ratio of Zd-1-positive cells between homozygous clones and heterozygous clones in animals carrying the bmm1 or bmmrev alleles at 14 days post clone induction (Figure S4O; Kruskal-Wallis rank sum test), the distance from the hub to the Zd-1 positive clones reside was significantly decreased in bmm1 homozygous clones (Figure S4P; Kruskal-Wallis rank sum test). Together, these data indicate bmm may play a cell-autonomous role in germline cells, and potentially a non-cell-autonomous role in somatic cells, to regulate spermatogenesis.”

> 1. *The labelling in Fig. 3 is confusing - presumably the graph in 3C refers to spermatid bundles [this comment applies to other figures showing spermatid bundle numbers], not individual spermatids, while the graph in 3G refers to the proportion of the total GSC pool that is contained within the clone. The data in Fig. 3C are not described in the main text.*

We adjusted the confusing labelling to ‘spermatid bundles’ from ‘number of spermatids’, as suggested. We also changed the title of panel Fig. 3G (now 4G) as suggested and men5oned Fig. 3C (now Fig. 4C) in the text.

> 1. *On p.9, comments are speculative or seek to draw comparisons with the broader literature and would seem to belong more to the discussion (eg "our data suggests flies are a good model to study how bmm/ATGL influences sperm development" - also there is a typo, it should be "suggest").*

We thank the Reviewer for raising concern about our speculative statement; we changed the text as follows in the revised manuscript:

“This identifies similarities between flies and mice in fertility-related phenotypes associated with whole-body loss of bmm/ATGL.”

> 1. *The length of the heat shocks used for clone induction should be specified in the methods (rather than just the period in between heat shocks).*

We now include more information on clone induction:

“Adult males were collected at 3-5 days post-eclosion and heat-shocked three times at 37°C for 30 min followed by a 10 min rest period at room temperature between heat shocks. Amer heat-shock, the flies were incubated at room temperature until dissection.”

> 1. *p.8 l.132 "bmm-GFP accurately reproduces changes to bmm mRNA levels". This sentence should be rephrased.*

We thank the Reviewer for this comment and rephrased the sentence:

“We first examined bmm expression in the testis by isolating this organ from flies carrying a bmm promoter driven GFP transgene (bmm-GFP) that recapitulates many aspects of bmm

mRNA regulation [77].”

> *1. p.9 l.172 "we used germline-specific marker" should read "we used an antibody against the germline-specific marker".*

We corrected this inaccurate statement in our revised manuscript.

> *1. p.10 several lines, "GSC" should be "GSCs".*

We corrected this inaccurate use of GSC in our revised manuscript.

> *1. p.13 l.247 should read "variance in GSC numbers".*

Thank you, this error was fixed.