**The single-cell consequences of an X-linked meiotic driver in stalk-eyed flies**

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# ABSTRACT

Sex-linked meiotic drivers limit the inheritance of the alternate sex chromosome in the heterogametic sex, subsequently skewing the ratio of males to females in the offspring. They consequently have large impacts on genome evolution, adaptation, and the emergence and maintenance of sexually selected traits. Despite this, their molecular basis and consequences for gametogenesis and sex chromosome regulation more broadly are poorly understood. Here, we employ single-cell RNA sequencing to investigate a sex-linked meiotic driver in the Malaysian stalk-eyed fly, *Teleopsis dalmanni*. First, we produce a comprehensive single-cell atlas of the male *T. dalmanni* gonad and identify major testes cell types. We then characterise the regulatory dynamics of the X chromosome during spermatogenesis, providing evidence for a lack of complete meiotic sex chromosome inactivation and complex trajectory of dosage compensation. Second, by contrasting single-cell expression data between drive and standard testes, we provide insight into how the driver increases its transmission during spermatogenesis and affects the transcriptomic landscape of the testes. We show that the presence of a meiotic driver does not perturb fundamental patterns of X-linked regulation, with most expression differences enriched in the latter stages of spermatogenesis, and that it likely acts by interfering with proper sperm development, rather than directly killing gametes.

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# INTRODUCTION

Following Mendelian genetics, the expectation at meiosis is that maternal and paternal alleles segregate equally. However, meiosis is often a battleground for inheritance. Intragenomic conflicts emerge through selfish genetic elements forcing unequal segregation of alleles, skewing their chances of being represented in the mature germline [(Jaenike, 2001; Lindholm et al., 2016; Sandler & Novitski, 1957)](https://paperpile.com/c/UqA1L6/vvjj+THUr+tqZA). These selfish genes, known as meiotic drivers, are widespread across eukaryotic life [(Ardlie, 1998; Fishman & Willis, 2005; Sandler et al., 1959; Taylor et al., 1999; B. C. Turner & Perkins, 1979)](https://paperpile.com/c/UqA1L6/Yl7h+RH1Z+1WFS+QGie+2kYA), and have large consequences for the ecology and evolution of populations [(Hamilton, 1967; Lindholm et al., 2016; Wilkinson et al., 2014; Zhang et al., 2015)](https://paperpile.com/c/UqA1L6/vvjj+h7R1+matP+TjAU).

Sex chromosome meiotic drive is the most common form of meiotic drive [(Hurst & Pomiankowski, 1991)](https://paperpile.com/c/UqA1L6/4WFo). Here, X-linked drivers reduce the inheritance of the Y chromosome and by altering of offspring sex ratios from 1:1, are easily detectable. Consequently, they have profound effects on reproductive traits [(Herbette et al., 2021; Holman et al., 2015)](https://paperpile.com/c/UqA1L6/H3wW+y7Eb), genome evolution [(Blackmon et al., 2019; Eickbush et al., 2019; Reinhardt et al., 2023; Úbeda et al., 2015)](https://paperpile.com/c/UqA1L6/5JhV+1skKe+Sro7+TS6V), adaptation [(Dyer et al., 2007; Mackintosh et al., 2021)](https://paperpile.com/c/UqA1L6/kQuk+DqNG), sexual selection [(Cotton et al., 2014; Pinzone & Dyer, 2013; Wilkinson et al., 1998)](https://paperpile.com/c/UqA1L6/GCwg+7RQn+Ucc7), and population persistence [(Hamilton, 1967; Jaenike, 2001; Mackintosh et al., 2021)](https://paperpile.com/c/UqA1L6/kQuk+THUr+TjAU). Characterising the molecular mechanisms of meiotic drivers and their consequences is therefore key to understanding a range of biological processes.

However, despite meiotic drive having been identified almost a century ago [(Gershenson, 1928)](https://paperpile.com/c/UqA1L6/aJuY), the evolutionary origins and the general mechanisms by which drivers operate remain unclear. Further, at the molecular level, they have been well studied in only a small handful of species, such as *Drosophila* [(Courret et al., 2019)](https://paperpile.com/c/UqA1L6/6dhM), *Anopheles* [(M. Li et al., 2020; Simoni et al., 2020)](https://paperpile.com/c/UqA1L6/xYRx+pu4F), house mouse [(Cocquet et al., 2012; Kelemen et al., 2022; Silver, 1985)](https://paperpile.com/c/UqA1L6/SwcP+Ilz7+Y5ST), monkeyflower [(Fishman & Willis, 2005)](https://paperpile.com/c/UqA1L6/2kYA), yeast (ref), and *Neurospora* [(Vogan et al., 2022)](https://paperpile.com/c/UqA1L6/KuR8). Recent work has started to highlight both the diverse mechanisms that are utilised by drivers to disrupt meiosis, but also shared elements and time points that are targeted [(Courret et al., 2019; Kruger & Mueller, 2021; Zimmering et al., 1970)](https://paperpile.com/c/UqA1L6/6dhM+DapU+LrMK). As such, meiotic drivers have been broadly divided into two classes. Meiotic-acting drivers disrupt proper segregation at meiosis, such as the *Paris* driver in *Drosophila simulans* which causes improper segregation of the Y in anaphase II [(Cazemajor et al., 1997, 2000)](https://paperpile.com/c/UqA1L6/aavG+Rx3E). In contrast, post-meiotic drivers disrupt motility of sperm or poison them. For instance, the *Winters* driver in *D. simulans* leads to a defect in nuclear condensation of Y sperm [(Tao et al., 2007)](https://paperpile.com/c/UqA1L6/XlOJ). In the latter class, the histone-protamine transition, an essential checkpoint in spermatid elongation, has been repeatedly identified as a post-meiotic target [(Gingell & McLean, 2020; Hauschteck-Jungen & Hartl, 1982; Herbette et al., 2021; Kettaneh & Hartl, 1976; Vedanayagam et al., 2021)](https://paperpile.com/c/UqA1L6/RArV+ttCI+jJ6a+H3wW+gSyJ).

One of the reasons that meiotic drivers have been studied in so few species is their complex genomic architecture. They are frequently housed by inversions that guard against the subsequent breaking up of their complex molecular phenotypes [(Lyttle, 1993; Reinhardt et al., 2023; Sandler et al., 1959; Silver, 1993)](https://paperpile.com/c/UqA1L6/9Dph+1skKe+DOKP+Yl7h) and prevent the formation of suicidal haplotypes bearing both driver and their target gene [(Dyer et al., 2007)](https://paperpile.com/c/UqA1L6/DqNG). The resulting high level of linkage disequilibrium between the driver and neutral variation across these inversions limits the use of traditional genetic mapping approaches [(Dyer et al., 2007)](https://paperpile.com/c/UqA1L6/DqNG). In addition, the processes they disrupt, such as spermatogenesis, are complex and operate alongside unique regulatory mechanisms acting on the sex chromosomes, including dosage compensation and meiotic sex chromosome inactivation (MSCI), that are often poorly characterised in non-model organisms. In particular, X chromosome expression dynamics in the gonad are somewhat in dispute across many organisms, with a lack of consensus on the status of both regulatory processes (ref). Transcriptomics therefore provides an important avenue for understanding the molecular underpinnings and consequences of meiotic drivers. Specifically, by providing fine-scale data on regulatory variation at single-cell resolution as well as data on the cell types present in a tissue with limited *a priori* knowledge, nascent single-cell expression approaches afford us a high-dimensional perspective of the impacts of meiotic drivers on gametogenesis, gonadal tissue structure and sex-linked expression.

Here, we combine single-cell RNA-sequencing (scRNA-seq) approaches with a classic sex-ratio distorter in *Teleopsis dalmanni*, the Malaysian stalk-eyed fly, to test how meiotic drivers increase their transmission during spermatogenesis and affect the transcriptomic landscape of the testes. *T. dalmanni* harbours an X-linked meiotic driver in both wild and captive populations [(Presgraves et al., 1997; Wilkinson et al., 1998)](https://paperpile.com/c/UqA1L6/3Hym+7RQn) where drive males produce in excess of 90% female offspring [(Presgraves et al., 1997)](https://paperpile.com/c/UqA1L6/3Hym). The driver X chromosome is thought to have originated around 500 Kya (ref) and has multiple impacts on individual fitness [(S. Bates et al., 2023; Bradshaw et al., 2022; Cotton et al., 2014; Finnegan et al., 2019; Meade et al., 2019)](https://paperpile.com/c/UqA1L6/GCwg+yVv8+RJea+0woXS+FkoMy). Most notably, it reduces eye-stalk length in drive males, a sexual ornament used by females to choose males, and so makes them less attractive to females [(Cotton et al., 2014; Wilkinson et al., 1998)](https://paperpile.com/c/UqA1L6/7RQn+GCwg). Interestingly, drive males do not suffer reduced fertility, even though half of their sperm are destroyed, due to compensatory evolution to match the ejaculate size of standard males [(S. Bates et al., 2023; Meade et al., 2019)](https://paperpile.com/c/UqA1L6/FkoMy+RJea). This is most likely achieved via increased testes size in sexually mature flies [(Bradshaw et al., 2022)](https://paperpile.com/c/UqA1L6/0woXS), however, exactly how this is achieved is not clear. Recent work has shown that the driver X harbours a number of inversions relative to the standard X across its entire length, and bulk expression analyses have revealed significant differential expression between driver and standard male testes [(Reinhardt et al., 2014, 2023)](https://paperpile.com/c/UqA1L6/1skKe+2jbD). However, nothing is known about the molecular mechanism of the driver and its consequences for spermatogenesis and gene regulation in the testes more broadly.

We first produce a comprehensive single-cell atlas of the *T. dalmanni* testes and identify seven major cell types, including somatic supporting cells, germline stem cells (GSC) and spermatogonia, primary and secondary spermatocytes, and spermatids. We then characterise the regulation of the X chromosome throughout spermatogenesis and provide evidence for a lack of complete meiotic sex chromosome inactivation and complex pattern of dosage compensation across germ cells. By contrasting single-cell expression data between drive and standard males and utilising the time-series nature of scRNA-seq, we provide insight into how the meiotic drive biases its transmission to the next generation and identify several candidate genes that show diverged expression patterns across spermatogenesis.

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# RESULTS AND DISCUSSION

We generated eight scRNA-seq datasets from the testes of four standard *T. dalmanni* males and four males carrying the X-linked meiotic driver, referred to as ST and SR respectively. Following quality control and filtering, we recovered 12,546 cells, in which 12,452 genes were expressed, with 4,548 cells from standard individuals and 7,998 cells from drive individuals (Table S1).

## Single-cell atlas of the Teleopsis dalmanni testes

Following the clustering of cells via expression patterns, we used orthologs of cell-type-specific markers for *Drosophila melanogaster* testes [(H. Li et al., 2022; Mahadevaraju et al., 2021; Witt et al., 2019)](https://paperpile.com/c/UqA1L6/pXpt+JVCS8+LeMK) (Figures 1b & S1c**,** Table S2) to identify seven distinct cell types, four of these comprising different stages of spermatogenesis (Figure 1a, Supplementary Results). We identified somatic muscle and two groups of cyst cells, the latter of which supports germline development. We also identified a cluster corresponding to the germline stem cells (GSC) and the spermatogonia they produce. Finally, we were able to distinguish the primary and secondary spermatocytes, which enter meiosis to produce haploid spermatids.

We then used several additional approaches to validate these cell types. First, we used the number of expressed genes to confirm the stages of the germline across spermatogenesis. Previous studies in insect testes have shown that the total number of genes expressed varies significantly across spermatogenesis. Transcriptional activity in the germline peaks before the onset of meiosis, in primary spermatocytes, following which transcription dramatically reduces in spermatids [(Barreau et al., 2008; Page et al., 2023; Raz et al., 2023; Wei et al., 2024; Witt et al., 2019)](https://paperpile.com/c/UqA1L6/hrAa+pXpt+quSM+Zk0x+Mf5E). Consistent with this, we find a clear decrease in the number of expressed autosomal genes over spermatogenesis (Figures 2a, 2b & S1b), supporting our separation of spermatocytes into primary and secondary spermatocytes. Our trajectory analysis, where cells are assigned pseudotimes across a trajectory, further supports this pattern of expression change over developmental time (Figure 2c). Second, we used eukaryotic classifiers of the mitotic cycle stage to corroborate our classification of primary and secondary spermatocytes (Figure S1a, Table S3, Supplementary Results). Notably, we were unable to use ploidy to distinguish pre- from post-meiotic cell types as proposed by a recent study [(Robben et al., 2024)](https://paperpile.com/c/UqA1L6/6uXf). We hypothesise several reasons for this and present data urging caution when undertaking this approach with scRNA-seq data (Supplementary Results). Finally, we generated a comprehensive list of markers which are robustly differentially expressed between these cell types for future studies (Table S4).

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## Figure 1: Single-cell atlas of the Teleopsis dalmanni testes

***(a)*** *Uniform Manifold Approximation and Projection (UMAP) of identified cell types from the Teleopsis dalmanni testes.* ***(b)*** *Dot plot of relative expression of orthologs of key Drosophila melanogaster cell-type-specific testes markers. Size of dots indicates the relative number of cells expressing the marker in a cluster and colour indicates the level of expression (blue lowest and red highest).*

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## Figure 2. Genome wide expression patterns across T. dalmanni spermatogenesis

***(a)*** *Number of autosomal genes expressed across spermatogenesis per cell (gene classified as expressed if counts > 1). Data shown for standard (ST) males. Colours indicate different cell types as shown in panel (b).* ***(b)*** *Boxplot of cell type abundances across pseudotime.* ***(c)*** *UMAP of germline cells, coloured by pseudotime. Plotted line is the principal curve fitted through the centre of the data by Slingshot.*

## Lack of meiotic sex chromosome inactivation in Teleopsis dalmanni

Next, we characterised patterns of expression across *T. dalmanni* testes cell types, with a particular focus on the X chromosome. Due to their unique inheritance pattern and characteristics, X chromosomes frequently exhibit sex- and cell-type specific gene regulation compared to the rest of the genome [(Bachtrog et al., 2014; Charlesworth, 1996; Mank, 2013; Rice, 1984; J. M. A. Turner, 2007)](https://paperpile.com/c/UqA1L6/darR+E0DN+h0YX+xiPR+4rLM+38IN+aPqh+fbh8).

Firstly, using our scRNA-seq data, we examined expression of the X chromosome to test for meiotic sex chromosome inactivation across the four stages of *T. dalmanni* spermatogenesis that we identified. Meiotic sex chromosome inactivation acts in many species, inhibiting expression of the X chromosome during the meiotic stages of spermatogenesis [(J. M. A. Turner 2015)](https://paperpile.com/c/UqA1L6/oyo2+aPqh) and a number of theories have been suggested to explain its evolution [(J. M. A. Turner, 2007)](https://paperpile.com/c/UqA1L6/aPqh). However, despite its suggested evolutionary importance, the status of meiotic sex chromosome inactivation in insects has remained controversial, with considerable variation reported across studies and species [(Djordjevic, Tran Van, Toubiana, Labedan, et al., 2024; Hense et al., 2007; Landeen et al., 2016; Mahadevaraju et al., 2021; Meiklejohn et al., 2011; Mikhaylova & Nurminsky, 2011; Vibranovski, 2014)](https://paperpile.com/c/UqA1L6/GZJQ+LAiA+JVCS8+hNr4+JALW+yhtL+zxeO+0h7J). In part, this uncertainty arises from the methodological challenges of manually dissecting specific cell populations from testes of multiple individuals. Recent scRNA-seq data in a handful of model species has circumvented these challenges and provided new insights into the status of X inactivation [(Mahadevaraju et al., 2021; Page et al., 2023; Wei et al., 2024)](https://paperpile.com/c/UqA1L6/Zk0x+TrjZ+JVCS8+quSM). However, it remains unclear how conserved this regulatory process is across insects more generally and why it might evolve in some species but not others.

Our scRNA-seq data reveals substantial X-linked expression across all stages of spermatogenesis in *T. dalmanni* and no evidence for a shutdown of the X (Figures 3a & 3b). Indeed, we find that the proportion of expressed genes on the X chromosome, relative to the autosomes, is actually equivalent to or even greater in meiotic and post-meiotic germline cells than in somatic cell types (Figure S3). This lack of meiotic sex chromosome inactivation is consistent with recent scRNA-seq data in other diptera including *Drosophila melanogaster* (Witt et al., 2021, but see Mahadevaraju et al., 2021)and *Drosophila miranda* [(Wei et al., 2024)](https://paperpile.com/c/UqA1L6/Zk0x).

Interestingly, recent observations of meiotic sex chromosome inactivation in insects have been limited to species exhibiting chiasmatic male meiosis, including *Anopheles gambiae* [(Benedict et al., 2003; Page et al., 2023)](https://paperpile.com/c/UqA1L6/quSM+hZdP), *Tribolium castaneum* [(Johnson, 1966; Robben et al., 2024)](https://paperpile.com/c/UqA1L6/6uXf+f0nv), *Timema poppense* [(Djordjevic, Tran Van, Toubiana, Labédan, et al., 2024; Parker et al., 2022)](https://paperpile.com/c/UqA1L6/8MkP+x5Uk) and *Artemia franciscana* (ref). The Brachycera suborder of diptera, of which *T. dalmanni* and *Drosophil*a are a part, exhibit achiasmatic meiosis, and therefore no recombination in males [(Gethmann, 1988)](https://paperpile.com/c/UqA1L6/xvCv). Importantly, [the](https://paperpile.com/c/UqA1L6/xvCv) *Teleopsis* X chromosome is not homologous to the *Drosophila* X but instead independently derived from Muller element B (ref). This apparent difference in the status of meiotic sex chromosome inactivation between achiasmatic and chiasmatic species lends further support to a prominent theory that inactivation occurs to protect against harmful recombination between heteromorphic sex chromosomes [(McKee & Handel, 1993)](https://paperpile.com/c/UqA1L6/zvat).

## Figure 3. Expression of the X chromosome across T. dalmanni spermatogenesis

***(a)*** *Relative number of X-linked genes expressed across spermatogenesis. Data shown for standard (ST) males. For each cell, the number of expressed X-linked genes divided by the number of expressed autosomal genes is shown (gene classified as expressed if counts > 1). Colours indicate different cell types as shown in panel (b).* ***(b)*** *Boxplot of cell type abundances across pseudotime. The GSC and spermatogonia are mitotic germline cell types whereas spermatocytes are meiotically active.* ***(c)*** *Box plots of X-linked gene expression compared to median autosomal expression across cell types. Data shown for standard (ST) males Line at 0 represents even expression of autosomal and X-linked genes and at -1 represents 50% X-linked expression. A two-sided Wilcox test was used to determine if ​​log2(X:A) values for each cell type deviate from 0. p < 0.00001 = \*\*\*, p < 0.001 = \*\*, p < 0.05 = \*.*

***Status of dosage compensation varies across testes cell types***

We then examined patterns of dosage compensation across testes cell types. Dosage compensation is predicted to evolve on the X chromosome when the X and Y diverge in sequence [(Ohno, 1966)](https://paperpile.com/c/UqA1L6/QfLj). This is thought to equalise the expression of sex chromosomes and autosomes in both sexes and mitigate the costs of hemizygous X expression in the heterogametic sex. However, the degree of compensation varies substantially across species and tissues, particularly between gonadal and somatic tissue [(Djordjevic, Tran Van, Toubiana, Labédan, et al., 2024; Julien et al., 2012; Mank, 2013)](https://paperpile.com/c/UqA1L6/8MkP+sFqc+38IN). For instance, previous work in the stalk-eyed fly has suggested equal expression between the X and autosomes in somatic tissue but incomplete compensation in the testes [(R. H. Baker et al., 2016; Wilkinson et al., 2013)](https://paperpile.com/c/UqA1L6/QYht+pypF). This pattern is found across many other species [(Djordjevic, Tran Van, Toubiana, Labedan, et al., 2024; Julien et al., 2012)](https://paperpile.com/c/UqA1L6/hNr4+sFqc) but the exact reasons still remain unclear [(Gu & Walters, 2017)](https://paperpile.com/c/UqA1L6/VF9X). It has been suggested that differences in the magnitude of sexual conflict over optimal expression levels could be responsible [(Mullon et al., 2015)](https://paperpile.com/c/UqA1L6/0NZQ). This debate is partly driven by the fact that, with a handful of recent exceptions [(Page et al., 2023; Robben et al., 2024; Wei et al., 2024; Witt et al., 2021)](https://paperpile.com/c/UqA1L6/6uXf+TrjZ+Zk0x+quSM), studies of dosage compensation measure expression in aggregate across entire tissues or body regions, masking variability in the degree of compensation across cell types and potentially leading to inaccurate conclusions about the presence or absence of compensation. This is particularly consequential for the testes which are composed of both somatic and germline cell types.

Our scRNA-seq dataset reveals a complex pattern of dosage compensation in the *T. dalmanni* testes across spermatogenesis (Figure 3c). We show that somatic cell types exhibit equal expression of the autosomes and the X chromosome in males, consistent with complete compensation. Interestingly, the early stages of spermatogenesis appear to lack dosage compensation, with expression of the X close to half that of the autosomes, whilst equal expression is restored during the later stages (Figure 3c). Notably, X:A expression ever drops below half and therefore cannot be attributed to meiotic sex chromosome inactivation. When this pattern is broken down by chromosome, we show that the expression of the two autosomes is constant across testes cell types, but the X exhibits a clear reduction in expression in the GSC/spermatogonia and primary spermatocytes (Figure S4a). This is very different to that recently observed using single-cell data in adult *D. miranda* [(Wei et al., 2024)](https://paperpile.com/c/UqA1L6/Zk0x) and *D. melanogaster* (), where there is a progressive shutdown of dosage compensation through spermatogenesis, and in *Anopheles* [(Page et al., 2023)](https://paperpile.com/c/UqA1L6/quSM), where dosage compensation is absent in germ cells.

Previous work has suggested that the *T. dalmanni* X chromosome is highly enriched for testes-specific genes, with almost twice as many as expected based on its size. This appears to be the result of the migration of testes-specific genes from the autosomes, the evolution of testes-specific expression for ancestral X-linked genes, and the emergence of new genes on the X with expression limited to the testes [(R. H. Baker et al., 2016)](https://paperpile.com/c/UqA1L6/QYht). This pattern of masculinisation is not found on the *Drosophila* X [(Sturgill et al., 2007; Vibranovski et al., 2009)](https://paperpile.com/c/UqA1L6/LRH6+IaKo) and so could explain the relative increase in X-linked expression we observe later in *T. dalmanni* spermatogenesis, where male-benefit genes are disproportionately expressed in mature sperm. It is also possible that the upregulation of the X we observe here is actually a false signal driven by Y-linked genes that share sequence similarity to the X and are expressed later in spermatogenesis. However, this is unlikely as the *T. dalmanni* sex chromosomes are highly diverged [(R. H. Baker & Wilkinson, 2010)](https://paperpile.com/c/UqA1L6/4BS0), with only one Y-linked coding gene being identified to date (ORF-126) [(R. H. Baker & Wilkinson, 2010)](https://paperpile.com/c/UqA1L6/4BS0) which was not expressed in our dataset. Furthermore, the increase in expression appears not to be limited to a handful of highly expressed genes on the X but looks to be evenly distributed across the entire chromosome (Figure S4b), hinting at a chromosome-wide mechanism of X upregulation.

Interestingly, whilst we found differences in patterns of dosage compensation in the germline between *Drosophila* and *T. dalmanni*, orthologs of male-specific lethal (MSL) genes, key components of the dosage compensation complex (DCC) that operates in the soma of *Drosophila* [(Lucchesi & Kuroda, 2015)](https://paperpile.com/c/UqA1L6/xiPR), show similar expression between the two species [(Wei et al., 2024; Witt et al., 2021)](https://paperpile.com/c/UqA1L6/Zk0x+TrjZ). As in *Drosophila*, MSL genes are generally lowly expressed in the germline and actually exhibit a gradual reduction in expression across stalk-eyed fly spermatogenesis (Figure S5). MSL recognition sites have yet to be identified in *Teleopsis*, and so we are unable to test directly whether differential gene activity correlates with physical proximity to chromatin entry sites. However, there is currently mixed evidence for the role of this complex in regulating dosage compensation more broadly in the insect germline [(Conrad & Akhtar, 2012; Robben et al., 2024; Wei et al., 2024; Witt et al., 2021)](https://paperpile.com/c/UqA1L6/Zk0x+TrjZ+6uXf+4UBY), particularly as components of the complex do not localise to the nucleus () or the X in the male germline in *D. melanogaster* [(Rastelli & Kuroda, 1998)](https://paperpile.com/c/UqA1L6/P44S). Given the lack of concordance we find between patterns of MSL and X expression, our results further support a non-canonical mechanism of dosage compensation in insect testes.

## Impacts of meiotic drive on the cellular landscape of the testes

The mechanisms by which drivers bias their transmission to gametes have only been studied in a handful of species [(Courret et al., 2019)](https://paperpile.com/c/UqA1L6/6dhM), however, they appear to operate through two main approaches, either by killing gametes directly or halting their maturation. We compared the cellular composition of the testes across spermatogenesis to test which of these mechanisms is operating in *T. dalmanni*. In this species*,* sperm are formed in bundles, composed of 128 germ cells housed in two cyst cells [(Presgraves et al., 1997)](https://paperpile.com/c/UqA1L6/3Hym). If the driver acts by killing Y bearing sperm directly, we expect to see a relative depletion in the number of germ cells found in later stages of spermatogenesis in drive males. This is because if Y bearing sperm were killed or not formed during meiosis, we would expect bundles to contain less than 128 germ cells, and thus a relatively smaller number of germ cells relative to cyst cells in drive compared to standard males. Alternatively, if the driver prevents Y bearing sperm from fully maturing, we expect to see no difference or even a relative increase in cell numbers towards the end of spermatogenesis. This is because immobilised or improperly elongated Y (or O if improper segregation at meiosis) sperm may be unable to migrate to the seminal vesicle and so temporarily accumulate in the testes.

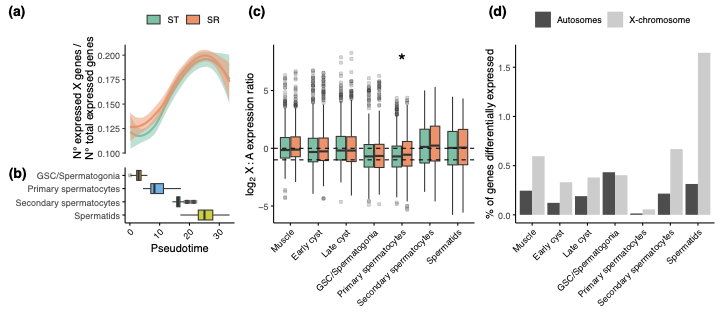
Notably, we see no significant effect of meiotic drive on cell type abundance when comparing the relative size of the cyst to the germline (Table S1, p = 0.48). This lack of a clear difference in the relative number of cells progressing through spermatogenesis between standard and drive males suggests there is no sudden sperm cull, but instead that the driver causes incomplete spermatid maturation. Therefore, once Y bearing sperm are immobilised, they may simply build up in the distal end of the testes before being eliminated by standard cellular programs. Indeed, we do observe a non-significant enrichment for post-meiotic germ cells in drive individuals (Table S1, p = 0.09). Together, our findings are consistent with previous cytological work in *T. dalmanni* where sperm of drive males reach the latter stages of spermatogenesis, but, just before individualization, sperm heads either deteriorate before leaving the bundle or appear overextended [(Presgraves et al., 1997)](https://paperpile.com/c/UqA1L6/3Hym). Together, this pattern is analogous to the *Segregation Distorter* (SD) male meiotic drive system in *D. melanogaster*, where the driver operates post-meiotically to prevent sperm maturation [(Herbette et al., 2021)](https://paperpile.com/c/UqA1L6/H3wW).

## Impacts of meiotic drive on the transcriptional landscape of the testes and sex chromosome regulation

We expect standard and drive males to exhibit differential gene regulation due to both direct and indirect consequences of meiotic drive. Meiotic drivers are frequently housed by inversions [(Dyer et al., 2007; Pieper & Dyer, 2016; Stalker, 1961)](https://paperpile.com/c/UqA1L6/euNS+BPgL+DqNG) and the *T. dalmanni* driver is no exception. Although it’s location on the X chromosome is unknown, the driver X is characterised by a series of large inversions across its entire length [(Johns et al., 2005)](https://paperpile.com/c/UqA1L6/LTsu). A consequence of these inversions is reduced recombination for both the standard and driver X chromosomes, leading to high sequence divergence between X types and low diversity within the drive X [(Reinhardt et al., 2023)](https://paperpile.com/c/UqA1L6/1skKe). Furthermore, inversions may directly disrupt cis-regulation by physically shuffling promoters and enhancers within a chromosome [(Kleinjan & Coutinho, 2009)](https://paperpile.com/c/UqA1L6/Xh3P). Therefore, using our scRNA-seq data, we tested the extent to which the meiotic driver perturbs fundamental patterns of sex chromosome regulation and gene expression more broadly across spermatogenesis (Figure 4).

First, as in standard males, we find no evidence for meiotic sex chromosome inactivation (Figures 4a, 4b & S6) and similar patterns of dosage compensation on the driver X (Figure 4c). The only exception is a marginal increase in relative X-wide expression in primary spermatocytes in drive relative to standard males (p < 0.05). Together, these patterns suggest that there is no widespread dysregulation of the X chromosome across spermatogenesis as a consequence of meiotic drive.

Strikingly, we observe only a limited number of genes that are differentially expressed between standard and driver males across either the autosomes or X chromosome in each cell type (Figure S7, Tables S6 & S8). This contrasts previous bulk RNA-seq studies in *T. dalmanni* [(Reinhardt et al., 2014, 2023)](https://paperpile.com/c/UqA1L6/1skKe+2jbD) that suggest a significant portion of the genome has diverged in expression (~600 genes) in response to the meiotic driver. Notably, only one of the genes, *mcm10*, that Reinhardt et al. [(Reinhardt et al., 2023)](https://paperpile.com/c/UqA1L6/1skKe) identified was also differentially expressed in our dataset. This discrepancy is most likely an outcome of measuring differential expression from bulk approaches, which represent an average of expression across entire populations of distinct cell types and can lead to false inferences of regulatory variation [(Montgomery & Mank, 2016; Price et al., 2022a, 2022b)](https://paperpile.com/c/UqA1L6/EDFZ+Cja4+kZee).



## Figure 4: Conservation of X-linked regulation in the germline of drive males

***(a)*** *Loess curves fit to the relative number of X-linked genes expressed across spermatogenesis for standard (ST) and drive (SR) male cell types separately. For each cell, the number of detected X-linked genes divided by the number of expressed autosomal genes (gene classified as expressed if counts > 1) is shown. Filled area is the standard deviation.* ***(b)*** *Boxplot of cell type abundances across pseudotime.* ***(c)*** *Boxplots of log2(X:A) ratios across cell types in standard (ST) and drive (SR) males. Line at 0 represents even expression of autosomal and X-linked genes and at -1 represents 50% X-linked expression and complete lack of dosage compensation. A two-sided Wilcox test was used to determine if values for each cell type varied between ST and SR individuals. p < 0.05 = \** ***(d)*** *Proportions of expressed autosomal (dark grey) or X-linked (light grey) genes in each cell type that were differentially expressed between ST and SR individuals.*

The differentially expressed genes we do observe are disproportionately located on the X chromosome across most cell types (Figure 4d, Tables S6, S7 & S8). Additionally, these genes are evenly distributed across the entire X and not localised to a specific region (Figure S8). Whilst we cannot distinguish whether X-linked enrichment is a result of indirect or direct effects of the driver, interestingly, this pattern is most pronounced in the spermatids (p = 0.006), with most differentially expressed genes being X-linked (42/50). This is consistent with the earlier finding that the driver likely acts through improper maturation of spermatids [(Presgraves et al., 1997)](https://paperpile.com/c/UqA1L6/3Hym).

Similarly, we also observe few genes with significantly differential trajectories between standard and drive spermatogenesis. Trajectory analyses allow us to test whether genes are differentially regulated across developmental time, rather than at distinct, self-assigned timepoints that include cells spanning developmental states. Briefly, we assigned pseudotime points to each germ cell and fitted a Generalised Additive Model (GAM) for standard and drive cells independently for expression of each gene. To ensure high confidence in the identified trajectories, genes with no association between pseudotime and expression were removed. Of the 5047 genes maintained for this analysis, only 260 had significantly differential trajectories between standard and drive spermatogenesis (Table S9). These genes are enriched for gene ontology terms including cilia, axoneme, and cell projection assembly (Table S10), suggesting that the driver may interfere with proper sperm development by affecting sperm motility.

Using our analyses of differential expression and trajectory, we sought to identify candidates for functional aspects of the driver. Of those we found, many are associated with sperm motility and function (Figure S9). Notably, *Grip75* is required for tethering of microtubules, and *Drosophila* mutants of this gene are sterile with defects in meiosis and sperm motility [(Vogt et al., 2006)](https://paperpile.com/c/UqA1L6/X0sz). Consistent with this phenotype, *Grip75* is X-linked and expressed at significantly lower levels in *T. dalmanni* drive individuals, particularly in the later stages of spermatogenesis. Similarly, we identify differential expression of *Tsr, Drosophila* mutants of which are unable to perform proper cytokinesis at meiosis I and II [(Gunsalus et al., 1995)](https://paperpile.com/c/UqA1L6/eU7K)*.* This gene is X-linked in *T. dalmanni* and has significantly lower expression in spermatocytes of drive individuals and exhibits a different trajectory between standard and drive spermatogenesis. Finally, *Ced-12* is required for apoptotic cell clearance [(Van Goethem et al., 2012)](https://paperpile.com/c/UqA1L6/DfGs) with *Drosophila* mutants showing significantly increased spermatogonia volume [(Zohar-Fux et al., 2022)](https://paperpile.com/c/UqA1L6/67f7). *Ced-12* is X-linked in *T. dalmanni* and downregulated in the later stages of spermatogenesis in drive individuals. With strong selection pressure for increased germline size acting in drive males [(Bradshaw et al., 2022)](https://paperpile.com/c/UqA1L6/0woXS), this shift in regulation of germline growth is a clear mechanism to mitigate the loss of Y-bearing sperm.

# CONCLUSION

In conclusion, we provide a comprehensive profile of the cellular and transcriptional landscape of the testes of the stalk-eyed fly. Specifically, we show limited evidence for meiotic sex chromosome inactivation and unique patterns of dosage compensation across spermatogenesis, relative to both other dipterans and insects in general. We show that the X-linked meiotic driver in this species likely acts by interfering with proper sperm development, rather than directly killing gametes, and suggest that it does so via affecting motility of sperm cells.

# METHODS

## Reference genome and mitochondrial genome assembly

A reference genome for *Teleopsis dalmanni* is publicly available (**DRYAD**) and consists of two autosomes and an X chromosome. However, it lacks a mitochondrial sequence. We therefore assembled a mitochondrial genome using publicly available PacBio Hifi reads generated from pooled *T. dalmanni* larvae (**DRYAD**) and MitoHifi v3.01 [(Uliano-Silva et al., 2023)](https://paperpile.com/c/UqA1L6/T9Pd). Specifically, we used raw Hifi reads as input, the rust fly (*Loxocera sinicia*) mitochondrial genome as a reference, and MitoFinder to annotate the genome, to produce a circularised assembly 20,708bp in length containing 37 genes. This *T. dalmanni* mitochondrial reference is available at <https://doi.org/10.5061/dryad.brv15dvk3>. The reference genome also lacks a Y chromosome, but it is highly diverged from the X and likely contains only a handful of genes [(R. H. Baker & Wilkinson, 2010)](https://paperpile.com/c/UqA1L6/4BS0).

## Sample collection

Flies were reared at University College London from a wild-caught population originating from the Gombak Valley, Malaysia. All flies and larvae were incubated and reared at 25°C and fed on a diet of sweetcorn. To ensure known genotypes of samples, a homozygous drive (SR) population is maintained through a series of crosses as previously described [(Presgraves et al., 1997)](https://paperpile.com/c/UqA1L6/3Hym). Eight adult males, four standard (ST) and four with drive (SR), were sacrificed before the dissection of both testes in iced phosphate-buffered saline (PBS). These adults were all virgins, reproductively mature and reared from egg lays collected on the same day.

## Tissue collection, dissociation and single-cell sequencing

Testes pairs were individually dissociated by incubation in a collagenase-TrypLE lysis solution (10mg/ml collagenase in 10X TrypLE) at 37.5°C for one hour with three sets of mechanical dissociation by triturations of wide then narrow bore Pasteur pipettes. Digestion was inhibited by the addition of iced Schneider’s Serum. The solution was then gently triturated with a narrow-bore Pasteur pipette before filtering through a 35μm filter pre-rinsed with Schneider’s Serum. The sample was then spun in a swing bucket centrifuge for 5 minutes at 1000xg and 4°C. The supernatant was removed and the pellet resuspended in 50μl of iced PBS with gentle pipetting of a wide-bore pipette. To count cells, 10μl of the suspension was combined with 10μl of trypan blue and placed onto a humidified haemocytometer plate before counting in triplicate.

10X Genomics Chromium transcriptome libraries were generated at the NERC Environmental Omics Facility (NEOF) Liverpool before sequencing with Illumina NovaSeq using S2 chemistry, aiming for recovery of ~10,000 cells per sample and ~20,000 reads per cell. Raw scRNA-seq data for eight males is available at https://doi.org/10.5061/dryad.zkh1893kb.

## Single-cell RNA-seq data processing

Sequencing data for each sample was processed using Cell Ranger v7.2.0 [(Zheng et al., 2017)](https://paperpile.com/c/UqA1L6/OmsA). First, a custom reference genome was built with the *T. dalmanni* reference genome using mkref. Using cellrangers count function, fastq reads were then aligned against the custom index and counted, creating gene-by-cell count matrices. Data filtering and downstream analyses were performed using Seurat v5.0.3 [(Hao et al., 2024)](https://paperpile.com/c/UqA1L6/Xc7Y) in R v4.3.2 [(R Core Team, 2021)](https://paperpile.com/c/UqA1L6/Xk4A).Cells in each sample were filtered for a minimum of 200 features expressed and less than 20% mitochondrial expression. Count data for each sample was also filtered by only keeping genes with expression in at least three cells. We used DoubletFinder v2.0 [(McGinnis et al., 2019)](https://paperpile.com/c/UqA1L6/kMWD)in R [(R Core Team, 2021)](https://paperpile.com/c/UqA1L6/Xk4A) with default parameters to identify and remove doublets. The filtered dataset consisted of 12,546 cells across the eight samples, expressing 12,452 genes, with 7,998 cells from drive individuals and 4,548 cells from standard individuals (Table S1). Seurat objects from all eight samples were then integrated post-filtering using the ‘SCTransform’ function [(Hafemeister & Satija, 2019)](https://paperpile.com/c/UqA1L6/9MmH).

## Cell-type identification

After running a PCA on the integrated Seurat object, we used the ElbowPlot function to identify how many PCs were necessary to describe a significant amount of variation. Subsequently, a nearest neighbour graph was created using FindNeighbors, and clusters at varying resolutions identified with FindClusters. From this, an appropriate resolution for the number of clusters was determined using the clustree package v0.5.1 [(Zappia & Oshlack, 2018)](https://paperpile.com/c/UqA1L6/ER83), giving a final cluster number of 16.

Using a series of cell-type-specific markers for *Drosophila melanogaster* testes (Table S2) [(H. Li et al., 2022; Mahadevaraju et al., 2021; Witt et al., 2019)](https://paperpile.com/c/UqA1L6/pXpt+JVCS8+LeMK), clusters were assigned into biological groupings. Orthology between *T. dalmanni* and *D. melanogaster* reference genome (dm6) was established using OrthoFinder v2.5.5 with default parameters [(Emms & Kelly, 2019)](https://paperpile.com/c/UqA1L6/kRYm) giving a total of 9,883 reciprocal orthologs. Distinguishing cell populations in non-model organisms relies primarily on databases of marker genes from model species, which are often distantly related. Our comparison between *T. dalmanni* and *D. melanogaster* (a divergence time of ~150 MY) is within the range of species pairs previously employed to identify cell types using orthologous marker genes in recent single-cell RNA-seq studies [(Darolti & Mank, 2023; Robben et al., 2024; Segami et al., 2022)](https://paperpile.com/c/UqA1L6/VKKD+flyx+6uXf).

To further validate cell types, we used information on the number of features expressed and classifiers of the mitotic cycle stage (Table S3). Finally, to remove unwanted or unknown clusters, we cleaned the data by removing clusters that (a) had no clear biological classification, (b) were predominantly represented by a single sample (a cluster must have at least two samples from a treatment representing >12.5% of the cells, weighted by total cell number for each sample) and (c), had doublet-like expression profiles (high numbers of features and expression of markers from divergent cell types). After identifying cell types, new markers were identified on the basis of differential expression using FindAllMarkers from the Seurat package.

***Dosage compensation analysis***

For each sample and cell type, expression values were aggregated using a pseudobulk approach with scuttle v1.14.0 [(McCarthy et al., 2017)](https://paperpile.com/c/UqA1L6/k0oq). Specifically, the expression counts of all cells belonging to a cell type were summed for each gene across the genome. Using this approach instead of the expression of each cell reflects that the sample is the biological replicate and not the cell itself (Lun and Marioni 2017). Genes for each cell type were then filtered in two ways. First, genes were kept if they were expressed in >= 5% of cells (> 1 count). Second, genes had to have a pseudo-bulk log2(CPM) (counts per million) > 2 in more than half of standard or drive males. Dosage was measured as the ratio of expression of X-linked genes to the median autosomal expression. In each cell type, a non-parametric two-sided Wilcoxon test was used to test for deviations of log2(X:A) from 0 in standard males, with μ set to 0, and differences in log2(X:A) between standard and drive males.

***Differential cell type abundance analysis***

To test for differences in cell type abundance between standard and drive males, a series of binomial models were fit comparing cell counts of germline to cyst, early cyst to late cyst, and pre-meiotic germline to post-meiotic germline. All models were run using glmer from lme4 [(D. Bates et al., 2015)](https://paperpile.com/c/UqA1L6/paEs) in R [(R Core Team, 2021)](https://paperpile.com/c/UqA1L6/Xk4A) with sample as a random effect (count~treatment + (1|sample)).

***Differential gene expression analysis***

A quasi-likelihood (QL) approach from EdgeR v4.0.16 [(Robinson et al., 2010)](https://paperpile.com/c/UqA1L6/vPNv) was used to identify differentially expressed genes between standard and drive males in each cell type (|log2(fold-change)| >  1 and FDR < 0.05). Enrichment of the number of differentially expressed genes across chromosomes and cell types was modelled with a generalised linear model of family ‘binomial’ where genes were classified as biased or unbiased and regressed against cell type and chromosome. Nested models were then compared using chi-squared in anova.glm from the R ‘stats’ package [(R Core Team, 2021)](https://paperpile.com/c/UqA1L6/Xk4A).

## Trajectory Analysis

To identify genes with differential trajectories between standard (ST) and drive (SR) males across spermatogenesis, data was subset to include only germline cells (GSC/spermatogonia, primary and secondary spermatocytes, and spermatids). The Seurat object was then converted into a SingleCellExperiment class for downstream analysis using tradeSeq v1.18.0 [(Van den Berge et al., 2020)](https://paperpile.com/c/UqA1L6/RRYi) and slingshot v2.12.0 [(Street et al., 2018)](https://paperpile.com/c/UqA1L6/HYYZ). First, pseudotimes were assigned to each cell within the germline. Then a negative binomial generalized additive model (NB-GAM) with 10 knots was fit to each gene for ST and SR individuals separately. Genes were kept if they were expressed in at least 10% of either ST or SR cells with 2 or more counts [(Van den Berge et al., 2020)](https://paperpile.com/c/UqA1L6/RRYi), and if their expression was significantly associated with pseudotime in either ST or SR cells (*p* < 0.05 & log2 fold-change > 1). GAM smoothers were then compared between ST and SR cells to identify genes with significantly different trajectories using the conditionTest function. Genes were classed as significant if false discovery rate *p* < .05 & log2 fold-change > 2. Additionally, the remaining genes were then only kept if they were expressed in at least 10% of either ST or SR cells with 2 or more counts [(Van den Berge et al., 2020)](https://paperpile.com/c/UqA1L6/RRYi). The ST and SR NB-GAMs are then compared to find differential trajectories.

***Gene Ontology enrichment analysis***

A gene ontology term enrichment of the genes with significant differential trajectories was performed using the clusterProfiler package v4.12.2 [(Yu et al., 2012)](https://paperpile.com/c/UqA1L6/IjjH). The background gene set used was the genes that were previously identified as having a significant association with pseudotime. The org.Dm.eg.db v3.19.1 [(Carlson, 2019)](https://paperpile.com/c/UqA1L6/LvWy) package for *D. melanogaster* was used as a reference database.

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# SUPPLEMENTARY MATERIAL

## SUPPLEMENTARY RESULTS

## Expression of marker genes across cell types in Teleopsis dalmanni testes

First, we used a series of known *Drosophila melanogaster* cell-type-specific testes markers [(H. Li et al., 2022; Mahadevaraju et al., 2021; Witt et al., 2019)](https://paperpile.com/c/UqA1L6/pXpt+JVCS8+LeMK) to identify *T. dalmanni* cell types (Figures 1 & S1c, Table S2). We used the germ-cell-specific marker *vasa* to separate the germline from somatic tissues [(Ohlstein & McKearin, 1997)](https://paperpile.com/c/UqA1L6/ONoN). Somatic tissues were split using *Mhc* to identify muscle cells [(Hess et al., 2007)](https://paperpile.com/c/UqA1L6/Q0GM), *eya* expression to identify cyst cells associated with post-mitotic germline cells [(Zoller & Schulz, 2012)](https://paperpile.com/c/UqA1L6/JHqr), and *Impl2*, *fng*, *tj* and *Nrt* to identify cyst cells associated with the mitotic germline [(Terry et al., 2006)](https://paperpile.com/c/UqA1L6/jMo0). To then separate the germline stem cells (GSC) and spermatogonia from meiotic phase cells, *bb8* was used, a key gene in mitochondrial derivative development, which is expressed from spermatocytes onwards [(Vedelek et al., 2016)](https://paperpile.com/c/UqA1L6/9iUU). The lack of *twe* expression was used to split spermatocytes from spermatids, with *twe* expression peaking in spermatocytes and not detected in spermatids [(Courtot et al., 1992)](https://paperpile.com/c/UqA1L6/Kxkv). The presence of *cup* genes could also validate spermatids as whilst the post-meiotic germline of *Drosophila* is transcriptomically nearly inactive, *cup* and *comet* genes are expressed [(Barreau et al., 2008)](https://paperpile.com/c/UqA1L6/Mf5E). To then separate primary from secondary spermatocytes, we used expression of *CycB*, a core G2/M cell cycle component, whose expression peaks at the meiosis I transition between primary and secondary spermatocytes, and *Fest*, a regulator of *CycB*, whose expression begins in primary spermatocytes and extends into late-stage germ cells [(C. C. Baker et al., 2015)](https://paperpile.com/c/UqA1L6/y16b). These key markers, used to define cell types, were highly expressed in our dataset and shown in Figure 1c. However, there is a wider set of markers in the literature that we used to corroborate our findings, shown in Figure S1c and Table S2, but some of these genes were lowly expressed in our dataset and so less reliable in defining cell types.

We then used a large set of eukaryotic mitotic cycle stage classifiers, from the Harvard Chan School: Bioinformatics Core (<https://github.com/hbc/tinyatlas/blob/master/cell_cycle/Drosophila_melanogaster.csv>), (Figure S1a, Table S3) to refine the stages of spermatogenesis with expression of G2M markers enriched in the spermatogonia through to primary spermatocytes [(Robben et al., 2024)](https://paperpile.com/c/UqA1L6/6uXf).

### Using ploidy to distinguish cell types across spermatogenesis

A recent study proposed using SNP-based haploid/diploid phasing to distinguish pre- from post-meiotic cell types [(Robben et al., 2024)](https://paperpile.com/c/UqA1L6/6uXf). We expect the GSC/spermatogonia and primary spermatocytes to be diploid and secondary spermatocytes and spermatids to be haploid. Therefore, we followed the approach of Robben et al (2024) to call SNPs across our cells in standard (ST) males. Briefly, using the cellranger generated BAM files for each sample, duplicate reads were marked and removed using GATK’s MarkDuplicates, reads with Ns in their cigar string were split with SplitNCigarReads and finally, SNPs were called using HaplotypeCaller. Variants were then filtered for a minGQ of 20, minDP of 4 and minQ of 30. Using this set of SNPs, we identified single-cell level variants using scAlleleCount (<https://github.com/barkasn/scAlleleCount>). We then calculated the level of heterozygosity for each cell as an estimate of ploidy, classifying cells as haploid if heterozygous at < 95% of SNPs.

As we observed a decrease in transcriptional activity over spermatogenesis, we first checked for any confounding relationship between coverage depth and heterozygosity level that might bias our ability to robustly distinguish haploid and diploid cell types. We focused on diploid cell types (somatic tissue, cyst, GSC/spermatogonia and primary spermatocytes) for this test. We found a negative and significant relationship (*p* < 0.0001, ρ = -0.65) where cells with less coverage exhibited lower heterozygosity (Figures S2a & S2b). This is likely due to reduced power to confidently identify the minor allele when coverage is low. Therefore, to mitigate this bias we significantly increased our filtering for SNP calling to require at <= 2 reads for homozygotes to be called, and 4 for heterozygotes (2 reads mapping to both ref and alt) and a cell requiring variant information at >= 10 SNPs. However, even with this strict filtering, whilst the significant relationship between coverage and heterozygosity was weaker (*p* < 0.0001, ρ = -0.25), we still failed to identify differences in ploidy across cell types where expected (i.e., between somatic cells and spermatids) (Figures S2c & S2d). We hypothesise several reasons for our inability to detect ploidy differences between cell types, discussed below.

First, levels of heterozygosity in our population might be generally low as a result of inbreeding. This would lead to the incorrect assignment of diploid cells as haploid. We investigated this using DNA-sequencing data from five standard males collected from the same population (Supplementary Methods). However, levels of heterozygosity are high and there is no evidence of inbreeding (Table S5).

Second, the stringent filtering we used to mitigate our reduced power to identify the minor allele when coverage is low means we had very few cells and sites remaining from which to assign ploidy. Specifically, after filtering, there were 2,359 cells remaining from the 4,469 genotyped cells with relaxed filtering. This will significantly reduce our power to robustly identify haploid and diploid cells.

Finally, across much of eukaryotic life, from *Drosophila* to humans, the post-meiotic germline becomes close to transcriptionally inert [(Erickson, 1990)](https://paperpile.com/c/UqA1L6/3AAG) after a huge peak in activity in the primary spermatocytes [(Witt et al., 2019; Xia et al., 2020)](https://paperpile.com/c/UqA1L6/s4WVR+pXpt). Transcripts that are transcribed in primary spermatocytes can remain dormant in the cytoplasm of the cell to be transcribed later in spermatogenesis [(Barreau et al., 2008; Raz et al., 2023)](https://paperpile.com/c/UqA1L6/Mf5E+hrAa). Furthermore, in many species (e.g., rats [(Ventelä et al., 2003)](https://paperpile.com/c/UqA1L6/gkRT) and *Drosophila* [(Greenbaum et al., 2011)](https://paperpile.com/c/UqA1L6/1sWr) the spermatids within a bundle are joined by large cytoplasmic bridges that enable the movement of mRNA [(Braun et al., 1989)](https://paperpile.com/c/UqA1L6/efzk), proteins [(Kaufman et al., 2020)](https://paperpile.com/c/UqA1L6/PlGf) and structures such as large as organelles [(Ventelä et al., 2003)](https://paperpile.com/c/UqA1L6/gkRT). This means the cytoplasmic content does not just reflect the transcriptomic activity of one particular cell but also, to an extent, that of those neighbouring it [(Braun et al., 1989; Greenbaum et al., 2011; Ventelä et al., 2003)](https://paperpile.com/c/UqA1L6/1sWr+efzk+gkRT). Thus, if SNP calling for ploidy determination is reliant on RNA-seq approaches, reads sequenced from these cells were transcribed not just from themselves but also from earlier diploid cell states and from their neighbours. This could therefore give the appearance of diploidy even if the underlying genomic ploidy is haploid.

In summary, whilst assigning ploidy offers a valuable and alternate route to defining cell types in principle, particularly in non-model organisms where marker genes are either not reliable or absent, we urge caution when interpreting these results, and if necessary propose the use of joint sequencing [(Vandereyken et al., 2023)](https://paperpile.com/c/UqA1L6/VNeR) or single-nucleus approaches instead.

## SUPPLEMENTARY METHODS

***Alignment, SNP calling and heterozygosity calculations***

DNA for nine *T. dalmanni* individuals was extracted using standard approaches. Illumina DNA-seq libraries were generated at the NERC Environmental Omics Facility (NEOF) Liverpool before sequencing with Illumina NovaSeq using S4 chemistry, aiming for a coverage of 30X.

FASTQ files were quality trimmed and aligned to the indexed *T. dalmanni* reference genome **(DRYAD)** using the BWA-MEM algorithm implemented in BWA v0.7.17 [(H. Li & Durbin, 2009)](https://paperpile.com/c/UqA1L6/gouL). BAM files were sorted and indexed using Samtools v1.11 [(Danecek et al., 2011)](https://paperpile.com/c/UqA1L6/9CtX). Read group information was added to the BAM files using Picard tools v2.27.5 (http://broadinstitute.github.io/picard) and PCR duplicates removed using Picard tools MarkDuplicates. Next, variant calling was performed using GATK HaplotypeCaller v4.3.0 [(van der Auwera & O’Connor, 2020)](https://paperpile.com/c/UqA1L6/CBAw) to generate GVCF files and remove reads with a mapping quality < 20 and base quality score < 20. GVCFs were merged into a single GVCF using GATK CombineGVCFs and genotypes called using GATK GenotypeGVCFs. Variant filtering was performed using BCFtools v1.11 [(Danecek et al., 2021)](https://paperpile.com/c/UqA1L6/iKV9). Genotypes of sites were set to missing (.) if they met one of the following parameters: Depth (DP) < 5 or > 98, genotype quality (GQ) < 50, or SNP quality (QUAL) <= 50. Additionally, only biallelic SNPs were kept. Then, BCFtools view was used to extract only the autosomes (Chr1; Chr2) and the extracted vcf file was indexed using Tabix (htslib) v1.13 [(Bonfield et al., 2021)](https://paperpile.com/c/UqA1L6/0H2k). Levels of heterozygosity and the inbreeding coefficient (F) per individual were calculated using VCFtools (-het) v0.1.17 [(Danecek et al., 2011)](https://paperpile.com/c/UqA1L6/9CtX).

## SUPPLEMENTARY TABLES

[https://docs.google.com/spreadsheets/d/1mhxI3Y3DTd2TuhsC98rfOgc8GkgXti8Q1v1CD3kxz48/edit#gid=0](https://docs.google.com/spreadsheets/d/1mhxI3Y3DTd2TuhsC98rfOgc8GkgXti8Q1v1CD3kxz48/edit" \l "gid=0)

## SUPPLEMENTARY FIGURES

<https://docs.google.com/document/d/1Cg7fKGho5o4yLeMeTk7nM1QfGMx4Dkj4vlBBMTVi3WE/edit>

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**AUTHOR CONTRIBUTIONS**

AEW and PDP designed the research. SLB, SB and AP reared the flies and provided expertise with dissections. PDP collected and processed the data that was sequenced by SP and NEOF Liverpool. PDP, SP, VJL analysed the data with assistance from AEW, ID, SB and AP. AEW and PDP wrote the manuscript with input from all authors.

**DATA AND CODE AVAILABILITY STATEMENT**

*T. dalmanni* mitochondrial reference is available at https://doi.org/10.5061/dryad.brv15dvk3.

Raw scRNA-seq data for 8 males is available at https://doi.org/10.5061/dryad.zkh1893kb.

Code is available at https://github.com/petedprice/scStalkie\_Drive

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