Differential Sperm Motility Mediates the Sex Ratio Drive Shaping Mouse Sex Chromosome Evolution

Highlights
- The sex ratio skew in the offspring of Yq-deleted male mice is abolished by IVF
- In Yqdel males, Y sperm are more severely morphologically distorted than X sperm
- Similarly, Y sperm in these males have relatively impaired motility
- This motility difference explains the sex ratio skew in offspring of these males

Authors
Claudia Cattoni Rathje, Emma Elizabeth Philippa Johnson, Deborah Drage, ..., Julie Cocquet, Benjamin Matthew Skinner, Peter James Ivor Ellis

Correspondence
p.j.i.ellis@kent.ac.uk

In Brief
Mouse sex chromosome evolution has been shaped by widespread gene amplification, due to genomic conflict over offspring sex ratio. Rathje et al. show that male mice with a partial Y chromosome deletion show impaired morphology and motility of Y- versus X-bearing sperm, revealing the physiological basis of the sex ratio drive underpinning the conflict.
Differential Sperm Motility Mediates the Sex Ratio Drive Shaping Mouse Sex Chromosome Evolution

Claudia Cattoni Rathje, Emma Elizabeth Philippa Johnson, Deborah Drage, Christina Patinioti, Giuseppe Silvestri, Nabeel Ahmed Affara, Côme Ialy-Radio, Julie Cocquet, Benjamin Matthew Skinner, and Peter James Ivor Ellis

1School of Biosciences, University of Kent, Canterbury CT2 7NJ, UK
2Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK
3University Biomedical Services, University of Cambridge, Cambridge CB2 2SP, UK
4Department of Development, Reproduction and Cancer, INSERM, U1016, Institut Cochin, Paris, France
5CNRS, UMR8104, Paris, France
6Sorbonne Paris Cité, Faculté de Médecine, Université Paris Descartes, Paris, France
7School of Life Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK
8These authors contributed equally
9Lead Contact
*Correspondence: p.j.i.ellis@kent.ac.uk
https://doi.org/10.1016/j.cub.2019.09.031

SUMMARY

The mouse sex chromosomes exhibit an extraordinary level of copy number amplification of post-meiotically expressed genes [1, 2], driven by an “arms race” (genomic conflict) between the X and Y chromosomes over the control of offspring sex ratio. The sex-linked ampliconic transcriptional regulators Slx and Sly [3–7] have opposing effects on global transcription levels of the sex chromosomes in haploid spermatids via regulation of postmeiotic sex chromatin (PMSC) [8–11] and opposing effects on offspring sex ratio. Partial deletions of the Y chromosome (Yq) that reduce Sly copy number lead to global overexpression of sex-linked genes in spermatids and either a distorted sex ratio in favor of females (smaller deletions) or sterility (larger deletions) [12–16]. Despite a large body of work studying the role of the sex chromosomes in regulating spermatogenesis (recent reviews [17–20]), most studies do not address differential fertility effects on X- and Y-bearing cells. Hence, in this study, we concentrate on identifying physiological differences between X- and Y-bearing sperm from Yq-deleted males that affect their relative fertilizing ability and consequently lead to sex ratio skewing. We show that X- and Y-bearing sperm in these males have differential motility and morphology but are equally able to penetrate the cumulus and fertilize the egg once at the site of fertilization. The altered motility is thus deduced to be the proximate cause of the skew. This represents the first demonstration of a specific difference in sperm function associated with sex ratio skewing.

RESULTS

In Vitro Fertilization (IVF) Abolishes Offspring Sex Ratio Skewing for MF1-XYRIIIqdel Males

Intracytoplasmic sperm injection (ICSI) was previously shown to abolish the skew, showing that Yq-deleted males produce equal numbers of X- and Y-bearing sperm that are functionally different from each other [21]. Consequently, we focused first on mechanisms through which X and Y sperm may differ in their ability to enter the oocyte. Based on existing data showing an interaction between sex chromosome complement and transmission ratio distortion mediated by Spam1 hyaluronidase deficiency in males heterozygous for Robertsonian (Rb) fusions involving chromosome 6 [22–25], our initial hypothesis was that the sex ratio skew in Yq-deleted males was related to differential penetration of the cumulus cell complex surrounding the oocyte and that removal of the cumulus would reduce or abolish the sex ratio skew.

To test this, we focused on an XYRII partial Yq deletion [13] that in our colonies leads to at least a 7.7 percentage point difference in sex ratio (Cambridge colony on outbred MF1 background, 47.2% females for control XYRII males and 54.9% females for XYRIIqdel males, p = 0.002; Kent colony on outbred MF1 background, 45.7% females for control XYRII males and 58.9% females for XYRIIqdel males, p < 0.001; Paris colony on inbred C57B16/N background, 50.1% females for control XYRII males, 61.4% females for XYRIIqdel males, 87.5% females for XYRII shSLY males, p = 0.01; all statistics two-tailed two/three-proportions Z-test). In our Cambridge colony, we introduced an X-linked GFP transgene [26] to allow genotyping of preimplantation embryos. Using sperm from the resulting X<sup>GFP</sup>Y<sup>RiIIqdel</sup> males, we generated matched sets of IVF offspring with and without hyaluronidase pre-treatment of the oocytes to remove cumulus cells (n = 6 males tested). Surprisingly, the female skew was abolished in both the cumulus-on and cumulus-off experimental groups, with both instead showing a slight male bias consistent with the strain background sex ratio (Figure 1; Table S1).
on the cumulus cell properties of daughters of B10.BR-Ydel IVF-derived XGFPYRIIIqdel embryos show a slight skew in favor of males. Error but this is abolished in IVF experiments. XYRIIIqdel animals and XGFPYRIII and bryos show a marked sex ratio skew in favor of females when mated naturally, there was again a significant skew toward females in the offspring of XGFPYRIIIqdel males (two-tailed binomial p = 0.0285 relative to null expectation of 50:50 ratio; p = 0.0162 relative to MF1-XGFPYRIII control males), there was no effect of the maternal background.

**Yq Deletion Affects Y-Bearing Sperm Morphology More Severely Than X-Bearing Sperm**

The female tract has been shown to discriminate between morphologically normal B10.BR sperm versus abnormal B10.BR-Yqdel sperm at the uterotubular junction [28]. If Y-bearing sperm are more severely morphologically distorted than X-bearing sperm in Yq-deleted males, this might impair their ability to pass this junction. This in turn would enrich for X-bearing sperm among the population reaching the site of fertilization in the oviduct. We therefore systematically tested for morphological differences between X- and Y-bearing sperm, using a novel image analysis tool for quantitative sperm morphometry [29, 30], and a repeat-imaging protocol (Figure S1) that allowed us to capture pre-fluorescence *in situ* hybridization (FISH) morphology data and correlate this with post-FISH identification of X- versus Y-bearing status.

We analyzed the effect of Yq deletion on both MF1 and C57Bl6 genetic backgrounds, using animals from all three of our colonies. This allowed us to also analyze shSLY males, which are maintained on a C57Bl6 background [3]. shSLY males have no loss of Y chromosomal DNA, allowing us to test whether any morphological differences between X- and Y-bearing sperm are a consequence of the reduction in DNA content in the Yq-deleted sperm. On both genetic backgrounds, our morphological analysis confirmed the known manifestations of Yq deletion, including increased acrosomal curvature and shortening of the apical hook (Figures 2A and 2B). Additionally, both Yq-deleted sperm and shSLY sperm showed a marked reduction in all linear dimension measures and in overall cross-sectional area relative to wild-type sperm, which has not been previously described (Figure 2C). When comparing X- and Y-bearing sperm, for the majority of parameters tested, whether size dependent (linear dimensions and area) or size independent (circularity and regularity), Y-bearing sperm in both mutant genotypes were significantly different from X-bearing sperm and deviated more from wild-type values. A focused analysis of acrosomal curvature and sperm head area (Figure S2) showed that, for both parameters, Y-bearing sperm were more severely affected than X-bearing sperm in the mutant genotypes.

**Cluster Analysis Reveals an X/Y Gradient of Morphological Abnormalities**

Following this morphometric analysis of sperm dimensions, we carried out dimensional reduction on size-independent angle profiles using t-SNE and used hierarchical clustering to identify different sperm head shape groups within each genetic background. On the MF1 strain background, this separated sperm shapes into two major clusters, the first containing predominantly, but not exclusively, XYRIII sperm and the second containing predominantly, but not exclusively, XYRIIIqdel sperm. These could
then be subdivided into grades of abnormality, i.e., clusters N1/ N2 and A1/A2 (Figures 3A and 3C; Table S2). On the C57Bl6 strain background, the picture was more complex. Once again, there was a cluster of normal-shaped sperm and a continuous spectrum of increasing shape abnormality (clusters A1/A2/A3) corresponding to increasing severity of the Yq gene deficiency phenotype of acrosomal curvature, hook shortening, and area reduction. However, the C57BL6 dataset additionally presented a second axis of shape abnormalities (clusters B1/B2) that encompassed shortening of the sperm nucleus, "flaring" of the base of the sperm head, and exaggeration of the dorsal angle. Because this second type of abnormality was also present at comparable levels in the wild-type (WT) controls, we interpret these as being a strain characteristic unrelated to the Yq gene deficiency phenotype. Finally, a third type of abnormality (cluster S), which encompassed narrowing of the base of the sperm head, almost total loss of acrosomal hook, and effacement of the tail attachment site, was almost exclusive to shSLY sperm (Figures 3B and 3D; Table S2).

On both genetic backgrounds, the wild-type sperm showed an even sex ratio across different shape categories, and the normal-shaped sperm from the mutant genotypes—i.e., cells in categories N1/N2 for MF1 background and N for C57Bl6 background—were preferentially X-bearing sperm; MF1 background: WT X% = 51.3 ± 1.1, Yqdel X% = 59.1 ± 4.6; C57BL6 background: WT X% = 50.2 ± 1.1, Yqdel X% = 58.0 ± 4.7, shSLY X% = 66.7 ± 27.2. Therefore, despite the confounding effects of unrelated background sperm morphology distortions, the cluster analysis overall supports the circularity and regularity measurements showing that, on both genetic backgrounds, Yq deletion and shSLY knockdown lead to more severe morphological defects in Y-bearing sperm. On the C57Bl6 background, we also analyzed sex chromosome localization within the nucleus using dynamic warping [30] (Figure S3). This showed no change.
in chromosome territory position, indicating that the morphological changes are likely driven by changes in cytoskeletal dynamics during spermiogenesis rather than differences in chromatin organization.

### Y-Bearing Sperm Have Lower Motility Than X-Bearing Sperm in XY<sup>RIII</sup>qdel Males

Because the sex ratio skew is abolished by IVF, the morphological differences alone cannot be the full explanation for the sex ratio skew—i.e., most or all shapes of sperm must be competent to fertilize the oocyte in an IVF context, consistent with prior findings via ICSI in BALB/c wild-type males [31] and in Sly knock-down males [6]. Rather, the morphological distortion must impair the sperm’s ability to transit in vivo from the site of deposition to the fertilization site.

Because sperm selection at the uterotubular junction is largely on the basis of progressive motility [32], we therefore directly tested whether there are motility differences between X- and Y-bearing sperm in XY<sup>RIII</sup>qdel males, using MF1 strain background animals from our Kent colony. We used a swim-up protocol to separate sperm into six fractions according to their relative motility (STAR Methods). MitoTracker staining confirmed the swim-up fractionation enriched for sperm with longer midpieces in both XY<sup>RIII</sup> and XY<sup>RIII</sup>qdel genotypes (Figure S4). This showed a significant enrichment for X-bearing sperm in the upper (more motile) fractions that was not seen in either the wild-type control or the killed sperm control (Figure 4), indicating that Y-bearing sperm from Yqdel males have impaired motility relative to X-bearing sperm.

### DISCUSSION

The question of whether there are any morphological or physiological differences between X- and Y-bearing sperm other than their DNA content has provoked controversy for decades [33–37]. Many potential differences have been proposed but none validated, and accumulating understanding of syncytial sperm development has cast doubt on whether such differences are possible even in principle [38–40]. Here, we show that, in Yq-deleted males with offspring sex ratio skewing, the skew is evoked specifically during natural mating/fertilization, is abolished by IVF, is not modified by the epididymal transit time of the sperm, and is not affected by the maternal genetic backgrounds tested. We conclude that the physiological mechanism of the skew cannot be related to any of cumulus penetration, zona pellucida or oolemma binding, sperm/egg fusion, or subsequent embryonic development but must relate to the transport of the sperm to the site of fertilization.

Consistent with this, we show that the morphological abnormalities induced by Yq deletion and/or shSLY knockdown are more severe in Y-bearing sperm than in X-bearing sperm on both genetic backgrounds tested and that Y-bearing sperm from Yqdel males have relatively worse motility than X-bearing sperm on an MF1 background. Because the degree of X sperm...
e.g., by ruling out genes involved in sperm/egg recognition and focusing on genes contributing to sperm motility. A further theoretical clue is given by the only two other examples of male-mediated transmission ratio distortion in mice. Both of these involve at least one “responder” gene whose products escape sharing across the cytoplasmic bridges between syncytially developing spermatids: *SpmT1* in the case of the Rb fusions [23–25] and *Smok1Gr* in the case of the *t* complex [45, 46]. An alternative is that there is no “responder” gene and that a gene on the X chromosome directly targets a Y-specific DNA element, as in the case of *HP1D2* in *Drosophila* [47]. Because *Six* and *Sly* act as a “thermostat” that can generate both male and female skewings depending on the balance of X- and Y-linked am- plicons [5], this is hard to reconcile with a simple X-borne, DNA-directed toxin system. We therefore predict that there is a “responder” gene on the X or Y chromosomes that escapes sharing between syncytial spermatids. Recent work indicates that X-linked Toll-like receptors *TLR7/8* appear to escape sharing; however, *TLR7/8* activation leads to impaired X-bearing sperm function and a reduced number of female offspring [48], opposite to that seen in our model system. Thus, the identity of the responder in Yqdel-mediated distortion remains as yet undetermined.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT AND MATERIALS AVAILABILITY**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Mice
- **METHOD DETAILS**
  - Superovulation and IVF
  - Embryo recovery
  - Timed mating
  - Sperm collection and fixation
  - Test tube swim-up motility fractionation
  - Image capturing and FISH of sperm
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Sperm morphological analysis
  - Sperm motility analysis
- **DATA AND CODE AVAILABILITY**

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.09.031.

**ACKNOWLEDGMENTS**

We thank the animal handling staff at the University of Kent, University of Cambridge, Cochin Institute, and Charles River Laboratories. B.M.S. was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) (BB/N000129/1), P.J.I.E. and C.C.R. were supported by H.E.F.C.E. (University of Kent) and by the BBSRC (BB/N000463/1). E.E.P.J. was supported by BBSRC training grant BB/L502443/1 and Genus PLC. J.C. and C.I.-R. were supported by INSERM. Their research was funded by ANR (Agence Nationale pour la Recherche) (ANR-12-JSV2-0005-01 and ANR-17-CE12-0004-01 to J.C.).
AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


## STAR★METHODS

### KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemicals, Peptides, and Recombinant Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMSG</td>
<td>NVS</td>
<td>Cat#859448</td>
</tr>
<tr>
<td>hCG</td>
<td>NVS</td>
<td>Cat#804745</td>
</tr>
<tr>
<td>HTF media</td>
<td>[43]</td>
<td>N/A</td>
</tr>
<tr>
<td>KSOM media</td>
<td>[44]</td>
<td>N/A</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>Sigma</td>
<td>Cat#H4272</td>
</tr>
<tr>
<td>Mouse chromosome X paint</td>
<td>Cytocell</td>
<td>Cat#AMP-0XG</td>
</tr>
<tr>
<td>Mouse chromosome Y paint</td>
<td>Cytocell</td>
<td>Cat#AMP-0YR</td>
</tr>
<tr>
<td>MitoTracker Red</td>
<td>Life Technologies</td>
<td>Cat#M7512</td>
</tr>
</tbody>
</table>

| **Experimental Models: Organisms/Strains** | | |
| Mouse: MF1-XY<sup>III</sup> | Colonies were sourced from Dr Paul Burgoyne, NIMR. | [13] |
| Mouse: MF1-X<sup>GFP</sup>Y<sup>III</sup> | Colonies were sourced from Dr Paul Burgoyne, NIMR. | [22] |
| Mouse: MF1-XY<sup>R III</sup>qdel | Colonies were sourced from Dr Paul Burgoyne, NIMR. | [13] |
| Mouse: MF1-X<sup>GFP</sup>Y<sup>III</sup>qdel | This study (F1 cross of above two lines) | N/A |
| Mouse: C57Bl6/N-XY<sup>R III</sup> | Colonies were sourced from Dr Paul Burgoyne, NIMR, and backcrossed onto C57Bl6/N background. | [13] |
| Mouse: C57Bl6/N-XY<sup>R III</sup>qdel | Colonies were sourced from Dr Paul Burgoyne, NIMR, and backcrossed onto C57Bl6/N background. | [13] |
| Mouse: C57Bl6/N-XY<sup>R III</sup>-shSLY | Colonies were sourced from Dr Paul Burgoyne, NIMR, and backcrossed onto C57Bl6/N background. | [3] |
| Mouse: MF1 (females used to maintain breeding colonies in Cambridge and Kent, and as oocyte donors for some IVF experiments) | Sourced from Charles River Laboratories (discontinued strain) | |
| Mouse: B6CBA/F1 (females used as oocyte donors for some IVF experiments) | Sourced from Charles River Laboratories | https://www.criver.com/products-services/find-model/B6CBAf1-mouse |
| Mouse: C57Bl6/6NRj (background strain for breeding colonies in France) | Sourced from Janvier Labs | https://www.janvier-labs.com/en/fiche produit/c57bl-6nrj_mouse/ |

| **Software and Algorithms** | | |
| Nuclear Morphology Analysis 1.15.1 | [25] | https://bitbucket.org/bmskinner/nuclear_morphology/wiki/Home |
| R 3.5.1 | [47] | https://www.r-project.org/ |
| Rtsne v0.15 | [49] | CRAN (https://cran.r-project.org/) |
| cluster v2.0.7-1 | [50] | CRAN (https://cran.r-project.org/) |
| betareg v3.1.1 | [51] | CRAN (https://cran.r-project.org/) |
| rcompanion v2.1.1 | [52] | CRAN (https://cran.r-project.org/) |

| **Other** | | |
| Nuclear morphological measurements and analysis scripts | This paper | https://github.com/bmskinner/Yqdel_physiology |
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Peter Ellis (P.J.I.Ellis@kent.ac.uk). The mouse strains used were originally obtained from colonies developed by Dr Paul Burgoyne (NIMR, Mill Hill, London) under an MTA that precludes further transfer - requests for these strains should be directed to the Francis Crick Institute, London as current owners of the strains.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

All animal procedures were in accordance with the United Kingdom Animal Scientific Procedures Act 1986 and were subject to local ethical review in UK and France (Comite d’Ethique pour l’Experimentation Animale, Universite Paris Descartes). Animals on MF1 strain background were bred on Home Office licenses 80/2451 and 70/8925, held by PE. These strains were originally sourced from colonies developed by Dr Paul Burgoyne (NIMR, Mill Hill, London), and were subsequently bred at Cambridge University Central Biomedical Services, or on contract by Charles River Laboratories (Manston, Kent, UK). Animals on C57Bl6/N background were bred at the Cochin Institute animal facility (license held by JC, registration number CEEA34.JC.114.12). These strains were originally sourced from colonies developed by Dr Paul Burgoyne (NIMR, Mill Hill, London) and were subsequently backcrossed to C57Bl6/N animals at the Cochin Institute animal facility (to reach > 95% of C57Bl6/N background). On both backgrounds, all males studied carry an RIII-derived Y chromosome. This provides the appropriate comparison for the 2/3 Yq deletion, which arose on a RIIl background. Unless specified otherwise, breeding animals were housed as pairs or trios, and progeny housed singly or in small groups (< 4 per cage), with ad libitum access to food and water throughout and a 12/12 hour light/dark cycle. Animals bred at Cambridge were housed in individually ventilated cages, animals bred at Kent or in France were housed in standard cages within a barrier facility. Animals used in this study were sacrificed via CO2 followed by cervical dislocation (motility experiments) or cervical dislocation only (morphology experiments) and tissues collected post mortem for analysis. Full details of the animals used in this study are given in Table S3. All animals were older than 10 weeks, after which the testes produce mature morphologically normal sperm [49].

METHOD DETAILS

Unless otherwise stated, room temperature (RT) was approximately 25 °C.

Superovulation and IVF

For the IVF work, and for in vivo experiments requiring superovulation, 5-6 week old females were induced to superovulate with injections of 5 IU PMSG (NVS Cat no. 859448) and 5 IU hCG (NVS Cat no.804745) in 100-150 μL of PBS, given 48 h apart. Donor males were sacrificed by cervical dislocation. One cauda epididymis was dissected and transferred to a 250 μL droplet of HTF media supplemented with 5% BSA, under embryo safe mineral oil (Sigma). The cauda was opened with a scalpel and the sperm allowed to swim out and capacitate for 1 hr at 37 °C, 5% CO2. Next, superovulated females (4 for each cumulus-on and cumulus-off experimental sample) were sacrificed. Cumulus/oocyte complexes were retrieved from the ampulla of the oviduct and transferred either into a 500 μL pre-equilibrated droplet of either HTF media or HTF media with hyaluronidase (300 μg/ml, Sigma H4272) and incubated for 3 minutes. The decumulated oocytes were rinsed briefly through a second HTF droplet and transferred to a final 500 μL droplet of HTF media without hyaluronidase. 3 μL of capacitated sperm were added to each fertilization droplet and incubated for 4 hr to allow fertilization. After fertilization, embryos were rinsed briefly through a fresh HTF droplet and transferred to culture wells containing 1ml KSOM media + 3mg/ml BSA for overnight incubation. The following morning, cleavage-stage embryos were transferred to KSOM media without BSA and cultured to blastocyst stage for GFP scoring.

Embryo recovery

For the 2-cell embryo recovery experiment, superovulated females were housed with males overnight (2 per male tested), and checked for plugs the following morning. The day of plug identification was recorded as day 1. Females were sacrificed on the day following plug identification. Two-cell embryos were recovered and cultured to the blastocyst stage to score GFP expression. Experiments were performed using both CBAB6/F1 egg donors (9 X_{GFP}^{RIII} males and X_{GFP}^{RIII}qdel males tested) and MF1 egg donors (4 X_{GFP}^{RIII} males and X_{GFP}^{RIII}qdel males tested).

For the series of experiments with CBAB6/F1 egg donors, each male was mated again to a second pair of superovulated CBAB6/F1 females 3 days after the first mating, and embryos collected and scored as above. The sperm for this second mating must have had only a short residence time in the epididymis, i.e., it is “ fresher” than the sperm from the original mating. For four X_{GFP}^{RIII} males and four X_{GFP}^{RIII}qdel this cycle was repeated again, with a third mating after a further 2-3 weeks (i.e., fertilization with aged sperm), and then finally a fourth mating 3 days later (i.e., fertilization with fresh sperm). This method of varying the mating interval to ensure that
eggs are fertilized either by fresh sperm (3 day interval since previous mating) or sperm that have undergone aging in the epididymis (virgin mating or > 14 day interval since previous mating) has been previously shown to affect the magnitude of transmission ratio skewing in Rb fusion carriers [22]. We found no effect of mating interval for either of the male genotypes, and the difference in sex ratio (defined as GFP+ve / total number of blastocysts) between CBAB6/F1 and MF1 oocyte donors was also not statistically significant for either of the male genotypes (p > 0.1, two-tailed two-proportions Z-test). The results were thus pooled across all experiments when comparing between X^{GFP}Y^{RIII} and X^{GFP}Y^{RIIIqdel} genotypes. 95%–97% of recovered 2-cell embryos progressed to blastocyst stage (327/329 genotypes (p > 0.1, two-tailed two-proportions Z-test). The results were thus pooled across all experiments when comparing number of blastocysts) between CBAB6/F1 and MF1 oocyte donors was also not statistically significant for either of the male genotypes (p > 0.1, two-tailed two-proportions Z-test).

Timed mating
For timed mating experiments looking at the effects of female background, estrus cycle progression was staged via visual inspection, females in estrus were housed with males overnight, and checked daily until plugged. Each male was mated successively at 1-week intervals to 4 different females: 2 from the XY^{RIII} colony and two from the XY^{RIIIqdel} colony. This allowed replicate data to be collected for both maternal backgrounds for each male tested. Once plugged, females were boxed out, sacrificed mid-gestation (~day 13.5 post coitus), and the number of green (XX) versus non-fluorescent (XY) embryos counted. The number of reabsorbing embryos was counted and found to be independent of both male and female genotypes (male: 20/254 XY^{RIII}, 17/302 XY^{RIIIqdel}, p = 0.38; female: 12/254 XY^{RIII} daughters, 25/302 XY^{RIIIqdel} daughters, p = 0.13, two-tailed two-proportions Z-tests).

Sperm collection and fixation
Mice were sacrificed by cervical dislocation and sperm collected from cauda epididymis and vas deferens as previously described [29]. The sperm samples were rinsed in 3 washes of 1xPBS and fixed in 3:1 methanol:acetic acid. Table S1 shows details of the sperm samples analyzed in the work reported here. Initial experiments used pooled samples, subsequent experiments analyzed individual males. No differences were observed in summary statistics from pooled versus individual samples (Wilcoxon rank sum tests, p > 0.05).

Test tube swim-up motility fractionation
2 caudae epididymides from freshly dissected males were transferred to 200 μL pre-equilibrated (37°C, 5% CO2) HTF droplets under embryo safe mineral oil (Sigma), opened with a scalpel and the sperm allowed to swim out and capacitate for 1 hr at 37°C, 5% CO2. Following capacitation, general motility was checked under a light microscope before carefully transferring 50 μL sperm suspension to the bottom of a test tube (Greiner Bio-One #120160) with 3 mL of pre-equilibrated (37°C, 5% CO2) HTF media. Sperm were allowed to swim up through the overlying column of media for 30 minutes, and then successive 0.5ml aliquots were carefully pipetted from the top of the liquid meniscus, yielding six successive fractions from different swim depths.

In one experiment (Figure 4, n = 9 XY^{RIII}, 10 XY^{RIIIqdel} males, MF1 background), these fractions were immediately spun down onto glass slides, processed for X/Y FISH, and counted blind (i.e., the counter was not aware of which slide contained which fraction or genotype). However, since the best swimming fractions had fewer sperm, and the Yq-del phenotype is readily visible, perfect blinding was not possible.

In another experiment (Figure S3, n = 5 XY^{RIII} and 5 XY^{RIIIqdel} males), the top and bottom fractions only were stained using 200 nM MitoTracker Red (M7512, Life Technologies) for 30 minutes at 37°C, 5% CO2, and then fixed as described above. Sperm were spun down and imaged at lower magnification (x60 objective) to allow visualization of the whole midpiece. 5 males were analyzed per genotype, and 55 images taken per fraction per male, each image containing 1-3 cells. Consistent image exposure times were used to allow accurate relative quantification of fluorescence intensity. Midpiece length and average staining intensity were measured using ImageJ [51].

Image capturing and FISH of sperm
In order to compare X and Y-bearing sperm we performed a capture / recapture analysis in which we first imaged sperm nuclei using DAPI fluorescence microscopy, then subjected them to FISH labeling and re-imaged them to determine their X/Y status. This was necessary because the chromatin swelling step required for FISH probe penetration distorts the detailed morphology of the sperm head (Figure S1).

Fixed sperm were dropped onto poly-L-lysine coated slides, air-dried, stained using Vectorshield Antifade mounting medium with DAPI (Vector Laboratories, Peterborough, UK), and covered with 22x50mm coverslips. Slides were imaged on an Olympus BX61 epifluorescence microscope equipped with cooled CCDs and appropriate filters, and a motorised stage (Prior Scientific, Cambridge, UK). Images were captured using SmartCapture 3 (Digital Scientific, Cambridge, UK) and exported in TIFF format for downstream analyses. XYZ coordinates were recorded for each image taken to allow re-identification of cells. Slides were washed in 2xSSC for 2x10 minutes to remove mounting medium, and dehydrated through an ethanol series (70%, 80%, 100%, 2mins at RT). FISH was performed as previously described [30] using X and Y chromosome paints (Cytocell, Cambridge, UK), and images were captured as described above. The saved slide positions were used to perform automated batch capture of the previously imaged nuclei. Similarities between chromosome positions were assessed using MS-SSIM* [52], as described in [30].
**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical parameters including the statistical tests used, values of \( n \), and statistical significance are reported in the Figure Legends and in this STAR Methods section. Results are expressed as mean ± standard error of the mean (S. E. M.) for directly measured parameters, and as mean ± standard error of proportion (S. E. P.) for proportional measures.

**Sperm morphological analysis**

Morphology analysis was performed using the ImageJ plugin ‘Nuclear Morphology Analysis’ [29], version 1.15.1, available at [https://bitbucket.org/bmskinner/nuclear_morphology/wiki/Home](https://bitbucket.org/bmskinner/nuclear_morphology/wiki/Home). In addition to the previously-described measurements, we developed a simple user interface to allow FISH images and pre-FISH images to be displayed side by side. This allowed simple manual sorting of pre-FISH sperm images into groups of X-bearing versus Y-bearing sperm to allow comparison. Data were exported for further processing in R 3.5.1 [54]. Summary statistics were calculated per sample and per strain for each chromosome, with errors propagated by quadrature. Two-dimensional Barnes-Hut tSNE [55] was run on angle profiles using Rtsne, version 0.15 [56] (perplexity = 100, max_iter = 1000) and a consistent seed. Parameters were chosen following confirmation of plot consistency across a range of values. Hierarchical clustering was performed by agglomerative nesting [57] using Ward’s method via the cluster package, version 2.0.7-1 [58]. Ward’s method was selected as giving the highest agglomerative coefficient. Dendrograms were cut, with cluster number determined following visual inspection, and cell cluster membership was imported back into Nuclear Morphology Analysis to allow consensus nucleus building. FISH images from males on the C57BL6 background were further used to assess nuclear organization via dynamic signal warping, as previously described [30]. Differences in measured strain parameters between X- and Y-bearing sperm were tested using non-parametric Wilcoxon rank sum tests. Significance was assessed as \( p < 0.01 \) following Bonferroni multiple testing correction.

**Sperm motility analysis**

For X/Y counting of swim-up slides, the cell-containing area of the slide was systematically scanned at high magnification (100x objective), and all observed cells scored for X/Y status. Counting was continued until at least 400 cells had been counted for each slide stained, or until all cells on the slide had been counted if fewer than 400 were present in any given fraction. The data were modeled using a beta regression using a logit link, with X proportion explained as an interaction of strain, live/dead state, and segment using the betareg R package, version 3.1.1 [59], and likelihood ratio testing was performed using the rcompanion package, version 2.1.1 [60]. For comparisons of MitoTracker staining of best-swimming \( (n = 495 \) WT, 401 Yq-del) and poorest-swimming fractions \( (n = 285 \) WT, 656 Yq-del), differences between fractions within each genotype were assessed using two-sample two-tailed Kolmogorov-Smirnov tests, with significance assessed as \( p < 0.01 \).

**DATA AND CODE AVAILABILITY**

Full data for breeding and embryo recovery experiments as well as the measured parameters for X and Y bearing nuclei, and the R code used to perform the analysis are available on Github ([https://github.com/bmskinner/Yqdel_physiology](https://github.com/bmskinner/Yqdel_physiology)).