Engineering mice for female-biased progeny without impacting genetic integrity and litter size

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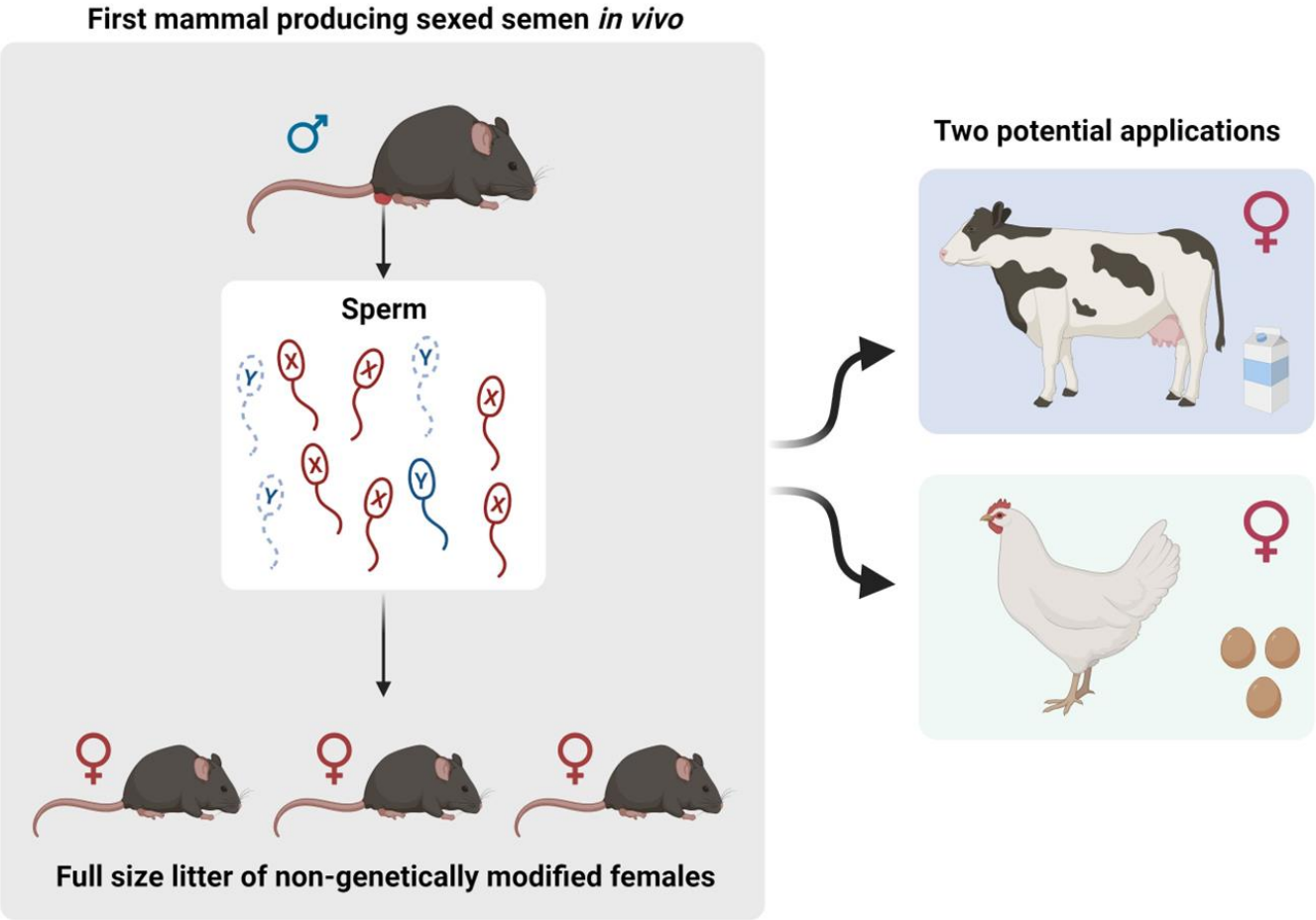


Graphical Abstract. A proof of concept for the first mammal producing sexed semen may revolutionize the way for breeding animals such as cows for the dairy industry and chickens for the eggs industry.

Abstract

Generating mammalian gametes with a skewed sex ratio has thus far eluded empirical confirmation. The utilization of such genetically engineered organisms would offer the potential to curtail the necessity for culling animals of undesirable sex, mitigate resource wastage, and alleviate superfluous labor burdens. In this study, we introduce a transgenic male mouse lineage, which consistently yields predominantly female progeny (comprising up to 91% of the total offspring). This accomplishment was made possible by integrating a controllable genetic cassette onto the Y chromosome. The cassette encodes dCas9 and RNA guides that selectively silence a spermatid maturation gene. After the separation of X and Y gametes during meiosis, gametes containing an X chromosome develop normally, while those harboring the engineered Y chromosome, subjected to dCas9 silencing of the spermatid maturation gene, do not mature properly. Indeed, some spermatozoa from the transgenic mice exhibit a unique morphology, associated with the absence of the maturation gene. Notably, the resultant female offspring do not inherit the genetically engineered Y chromosome and are thus not genetically modified. Importantly, the litter size of the transgenic mice remains unchanged compared to the wild type. These findings represent a groundbreaking demonstration of genetic engineering's potential to yield sex-biased litters of full size without compromising genetic integrity, marking a pioneering advancement in this field of study.

Keywords: Genetically unmodified organisms /sexed semen/ sex-ratio /mammals /Y-chromosome

Introduction

Generating animals of a preferred sex holds substantial advantages for various sectors dependent on animal husbandry. This extends to economically significant farm species, encompassing dairy cattle, egg-laying poultry, swine, wool-producing sheep, laboratory animals, and even companion animals. The practice of sex-biased animal production serves the dual purpose of alleviating the plight of unwanted sex animals while concurrently conferring economic benefits upon breeders through reduced labor and resource expenditure. To illustrate, consider the instance of mice, rabbits, and rats, which are essential in sex-specific research endeavors, particularly studies pertaining to the reproductive organs. The development of laboratory animals capable of producing sex-biased offspring serves to curtail the birth of undesirable sex animals, thereby minimizing both expenses and the associated ethical concerns. The successful engineering of laboratory mice for such purposes may also hold promise for implementation in larger farm animal populations, potentially ushering in transformative changes in animal breeding practices [1].

Farmers can significantly mitigate both economic expenses and the complexities associated with rearing unwanted animals by adopting a strategy that predominantly yields animals of the preferred sex. In agricultural sectors such as dairy farming, where females are the sole choice for milk production, male offspring are often confronted with a grim fate, being frequently euthanized at a tender age, sometimes as early as 3 to 4 days post-birth, prior to weaning. This practice stems from the financial burden they impose upon the farm [2]. Some male animals are subjected to distressing conditions in transportation to slaughterhouses, during which their physiological resilience and resistance to pathogens are significantly compromised. The adoption of a system that exclusively produces animals of the desired sex holds immense potential to substantially diminish the suffering and mortality of animals belonging to the undesired sex.

In the broader context, females typically represent the bottleneck in population expansion, given that their numerical abundance sets the upper limit on potential pregnancies, while a limited number of males suffice for mating with the entire female population. Consequently, to optimize the yield of a given product, such as wool, or for any other intended purpose, favoring females in the

population would facilitate the overall herd expansion. Moreover, it is worth noting that males often exhibit heightened aggressiveness towards each other, which can lead to injuries and compromise the overall welfare of the herd [3]. Hence, in cases where males are not the preferred choice for specific reasons (e.g., male calves in the meat industry due to their superior meat production mass [1]), skewing the sex ratio in favor of females would result in a huge economic benefit of swift expansion of the animal population with the added benefit of reduced aggressiveness within the herd.

The only method currently employed to influence the sex distribution of domesticated cattle livestock relies on the use of sexed semen, a product obtained through flow cytometry-based sorting of desired gametes [4]. This sorting process leverages the slight difference in weight between gametes carrying the X versus the Y chromosome. Sorted sexed semen undergoes approximately 20 procedures, yielding a potential sex bias of up to 90% while losing more than 50% of the total semen. However, this approach constitutes a relatively minor portion of the artificial insemination market [5]. This is primarily due to its economic costs, the associated labor-intensive nature, reduced fertility rates, and variable effectiveness in achieving the desired sex ratio. Additionally, this method is only applied in bovine breeding and is not prevalent in swine breeding due to the challenges posed by the high volume and count of pig sperm [6].

Hormonal-based approaches have been successfully implemented in various animal species to generate litters consisting entirely of one sex. These treatments involve the administration of hormones, which can feminize males or masculinize females, leading to the production of offspring of a single sex. Such methodologies have been effectively demonstrated in crustaceans [7] and fish [8-11]. However, these hormonal treatments are not practical or feasible for application in laboratory or farm animals.

Genetic-based methodologies for inducing sex bias in offspring have been successfully implemented in various insects, including mosquitoes and drosophila flies, through the manipulation of specific genes [12-20]. In mammals, our research group achieved a pioneering milestone by introducing a genetic system comprising two distinct mouse lines, the mating of which predominantly yielded female offspring [21]. The fundamental principles elucidated in our work were subsequently refined and successfully replicated by the Turner group, achieving complete sex bias for both males and females [22].

In our published study, zygote development within the uterus occurred predominantly when fertilized with an x gamete because the y gamete was lethal. Specifically, male sperm carried RNA guides on their Y chromosome, which, in conjunction with the CRISPR-Cas9 protein encoded by the female's autosomal chromosomes, led to the demise of developing male zygotes. This intricate genetic system necessitated the use of two distinct mouse lines: one carrying the Cas9 gene in females and the other encoding the RNA guides in males. Notably, the litter size resulting from such mating was reduced by approximately half due to the selective abortion of male progeny. Furthermore, it is essential to recognize that the female mice involved in this process were genetically modified organisms (GMOs), as they harbored the Cas9 gene within their genome.

Securing approvals for GMOs intended for human consumption is a stringent and intricate process. GMOs destined for consumption must conform to a set of stringent criteria outlined in the United States Food and Drug Administration (FDA) Act to ensure their safety and effectiveness. These criteria encompass several key aspects: safety for human consumption; safety for the modified organism; demonstration of enhanced traits, and minimal environmental impact. It is worth noting that the FDA has granted approvals for certain GMOs, including salmons that exhibit accelerated growth compared to their non-GMO counterparts [23], as well as genetically engineered cattle and pigs [24,25]. However, it is important to acknowledge that these approvals may not necessarily be universally accepted as safe, either by the public or by regulatory agencies outside the purview of the FDA. Consequently, the availability of non-GMO animals remains highly advantageous, particularly from a commercialization perspective.

The development of an efficient genetic system capable of yielding non-GMO progeny with regular litter sizes represents a monumental leap forward in the field. This system leverages natural reproduction processes, obviating the need for cumbersome technological interventions, hormone treatments, or other costly and intricate methods. Instead, it seamlessly delivers the desired outcome, which is of paramount importance. Of particular significance is the fact that normal litter sizes effectively double the production of animals of the desired sex. This doubling of output translates into substantial profitability, especially within sectors such as laying hens, the dairy and the swine industry, where efficiency and yield are pivotal. The anticipation for such a system in the industry has been long-standing, underlining its transformative potential.

In this study, we present a pioneering proof-of-concept, marking the first instance of achieving normal-sized litters with a sex bias in mammals. This accomplishment entails the production of non-GMO female offspring from genetically modified males. Crucially, the litter size of the resulting progeny remains within the typical range. Moreover, our system stands out for its simplicity in both production and maintenance when compared to previous genetic systems. Notably, genetic modification is exclusively applied to the male parent, leaving the female parent unaltered, thus enabling the maintenance of a single transgenic lineage. This streamlined approach underscores the practicality and feasibility of implementing this innovative methodology.

Results

To generate males whose sperm exclusively contains X-gametes, we engineered a cassette on the Y chromosome producing a repressor of a gene essential for gamete development. Our rationale was that after the separation of the X gametes from the Y gametes during meiosis, the Y gametes would fail to mature due to the repression of this maturation gene, while the X gametes, lacking the repressor post-meiosis, would undergo normal development. Consequently, these males would predominantly produce X gametes, leading to the generation of mostly female offspring (Figure 1a). The cassette itself was designed to be repressed through a tetracycline/doxycycline genetic switch to allow occasional production of Y gametes for maintaining the male lineage.

To genetically engineer these mice, we constructed a plasmid encoding dCas9, a modified version of Cas9 capable of gene silencing without DNA cleavage [26]. To enhance its repression activity, we fused dCas9 with the KRAB domain. The cassette featured five RNA guides that directed dCas9 to silence the *Spermatid maturation 1* (*Spem1*) gene, an essential component of spermatid maturation in mice [27]. Furthermore, a tetracycline-trans activator enabled dCas9-KRAB (hereafter referred to as dCas9) expression only in the absence of tetracycline [28] (Figure 1b). To facilitate integration into the Y chromosome, the entire cassette was flanked by sequences homologous to an intron in the *Uty* gene encoded on the Y chromosome [29], an intron previously targeted by us for insertion of RNA guides [21].

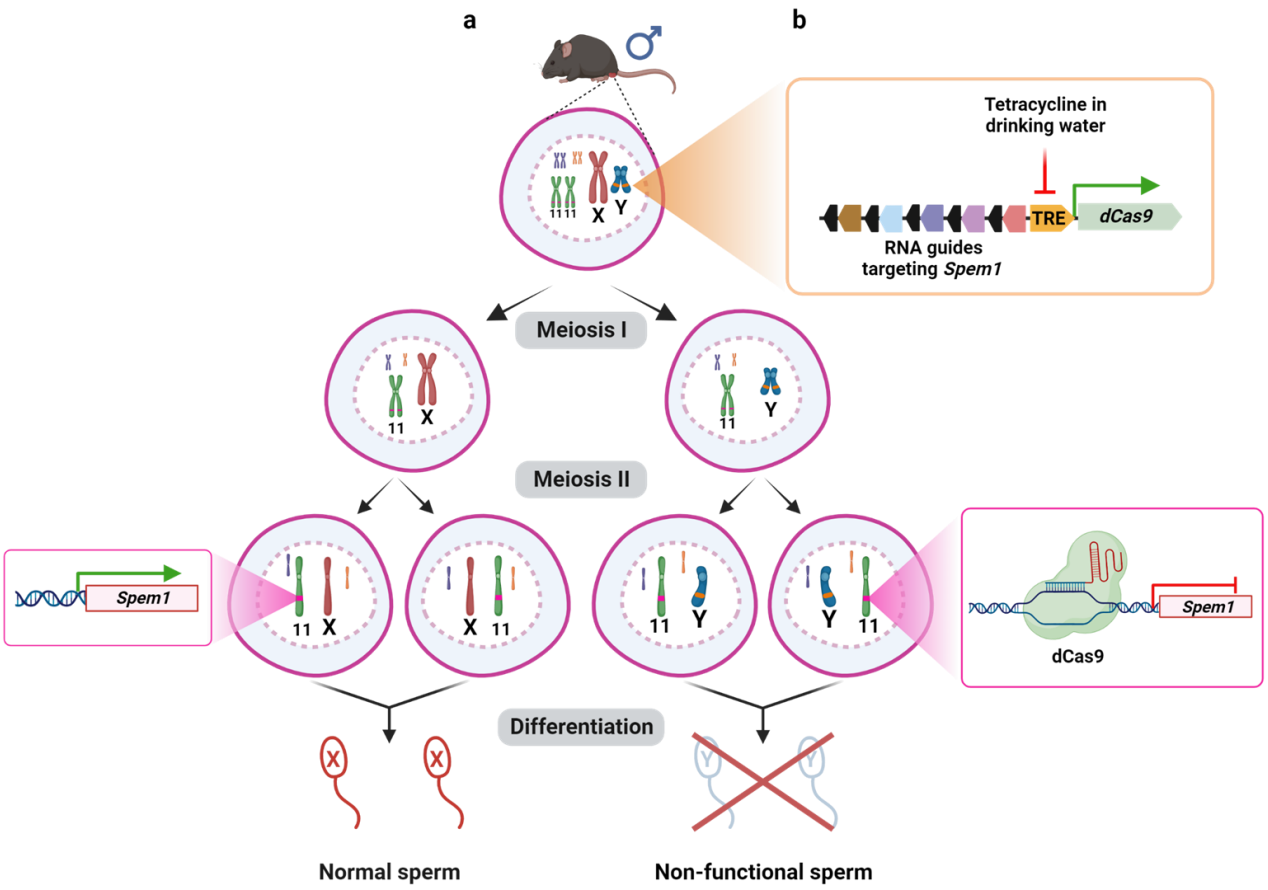


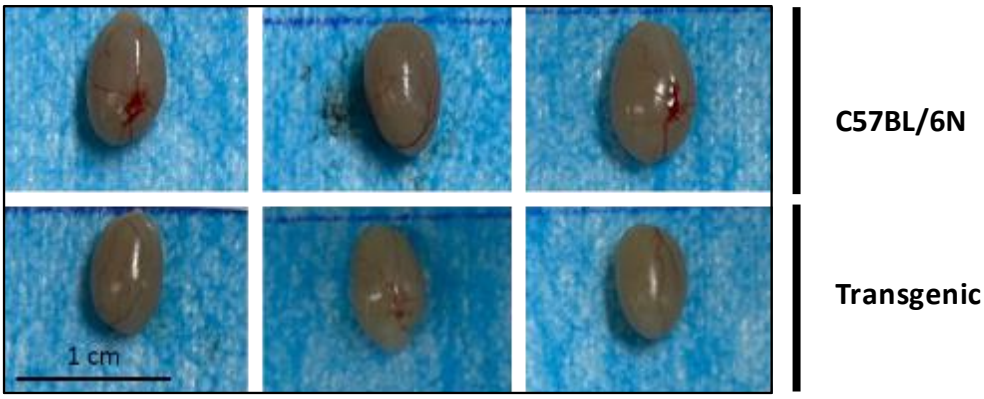
Figure 1. Sexing the semen *in vivo* in a male mouse. (**a**) A male mouse's Y chromosome is genetically engineered to contain a cassette repressing a gene essential for gamete development. After meiosis, the Y-gametes encode and express the repressing gene whereas the X gametes lack them. Consequently, the Y gametes do not mature whereas the x-gametes mature normally. (**b**) A schematic representation of the cassette inserted to the Y chromosome. dCas9 is expressed from a promoter encoding a tetracycline responsive element (TRE), repressed if tetracycline/doxycycline are present in the drinking water. The cassette also encodes 5 different RNA guides that target the *Spem1* gene encoded on chromosome 11. The representation is not adjusted to the actual scale. Additional elements of the cassette are described in Materials and Methods.

We introduced this cassette into mouse embryonic stem cells (mESC) and achieved integration into the Y chromosome through homologous recombination. Subsequently, we used these modified mESCs to generate transgenic mice using a proprietary technology, ensuring minimal to no chimerism in

the resulting mice [30]. These mice were treated with tetracycline in the drinking water and produced male mice, all carrying the transgene on their Y chromosome, as validated by PCRs (Figure S1).

Since the effect of the transgene insertion is expected in the sperm, we focused on comparing sperm from 3 transgenic and 3 wild type mice. The testes of the transgenic mice were similar to the control group in size and appearance, after normalizing to the overall weight differences between the mice (Figure 2a). Importantly, we observed ~5% of spermatozoa with distinct bending of the head toward the tail in the sperm of the transgenic mice, and none (0%) in that of the C57BL/6N mice (Figure 2b). Bent heads (Figure 2c) were observed as a hallmark morphological signature in the lack of *Spem1* [27]. These findings corroborate well with the hypothesis that the observed distorted gametes contain the Y gametes that cannot develop normally due to *Spem1* silencing. They also suggest that the penetration of dCas9 silencing is incomplete, or that some silenced gametes are cleared, as the expected percentage is 50% if complete silencing is achieved.

a



b

	C57BL/6N mice			Average	Transgenic mice			Average ± SD
Mouse number	**1**	**2**	**3**		**17**	**35**	**57**	
Not fully bent	100	100	100	100	96.2	94.5	94.3	95.0 ± 1
Fully bent	0	0	0	0	3.8	5.5	5.7	5.0 ± 1

c

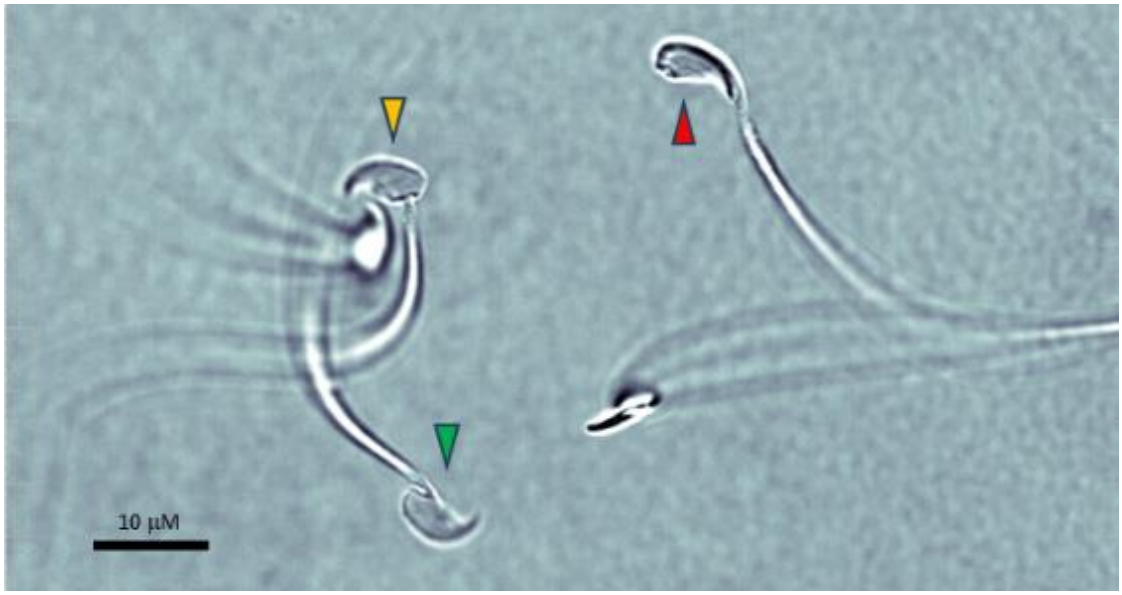


Figure 2. Testes and sperm comparison between the transgenic and C57BL/6N mice (**a**) Pictures of the left testes of 3 transgenic mice compared to 3 C57BL/6N mice. (**b**) Summary of the percentage of bent spermatozoa detected in the sperm of 3 transgenic and 3 C57BL/6N mice. (**c**) Image, taken by a benchtop confocal microscope (Andor BC43) showing spermatozoa with fully bent heads (red arrowhead), normal heads (green arrowhead), and a partially bent head (orange arrowhead) from a sample of a transgenic mouse sperm. The normal and partially bent heads were counted as "not fully bent".

Based on the morphological defects of the transgenic mice compared to the control mice, we hypothesized that at least some of their Y gametes are selectively defective. If indeed this is the case, then their progeny should show bias toward females. Furthermore, based on the construction of the cassette, we assumed that tetracycline or doxycycline would repress the dCas9 and consequently more Y gametes would be produced.

To evaluate our hypotheses, we formed four groups, each comprising 6 mice, and mated each of them with wild type C57BL/6N females. The first group did not receive doxycycline in their drinking water while the second and third groups received doxycycline at low and high concentrations (0.5 mg/ml, and 2 mg/ml, respectively). The doxycycline treatment is expected to repress dCas9, resulting in an unbiased ratio of females to males in these mice. The fourth group was a control group of C57BL/6N mice of a similar age, which did not receive doxycycline. The sex of the progeny was determined by physical examination and PCR tests were carried out on all the newborn mice. All the male progeny of the transgenic mice were confirmed positive by 3 PCRs carried out on their chromosomes and all females were found negative in these PCRs (Figure S1). Remarkably, the group of transgenic mice that did not receive doxycycline produced ~83% females and ~17% males (Figure 3a). The group treated with the low doxycycline concentration generated 70% females and 30% males. The group exposed to the high doxycycline concentration generated ~60% females and ~40% males. The control wild type group produced ~43% males and ~57% females. These results, with high statistical significance, indicate that the transgenic mice exhibit a sex ratio bias favoring females. Furthermore, they illustrate that the pronounced bias is significantly diminished with high doxycycline treatment, as evidenced by the absence of a statistical difference between this group and the control group.

The statistical analysis, employing One-Way ANOVA test and depicted in Figure 3b, reveals that the average litter sizes across all four groups are comparable to those of the wild type control group. These findings indicate that the transgenic mice exhibit normal litter sizes, irrespective of doxycycline treatment.

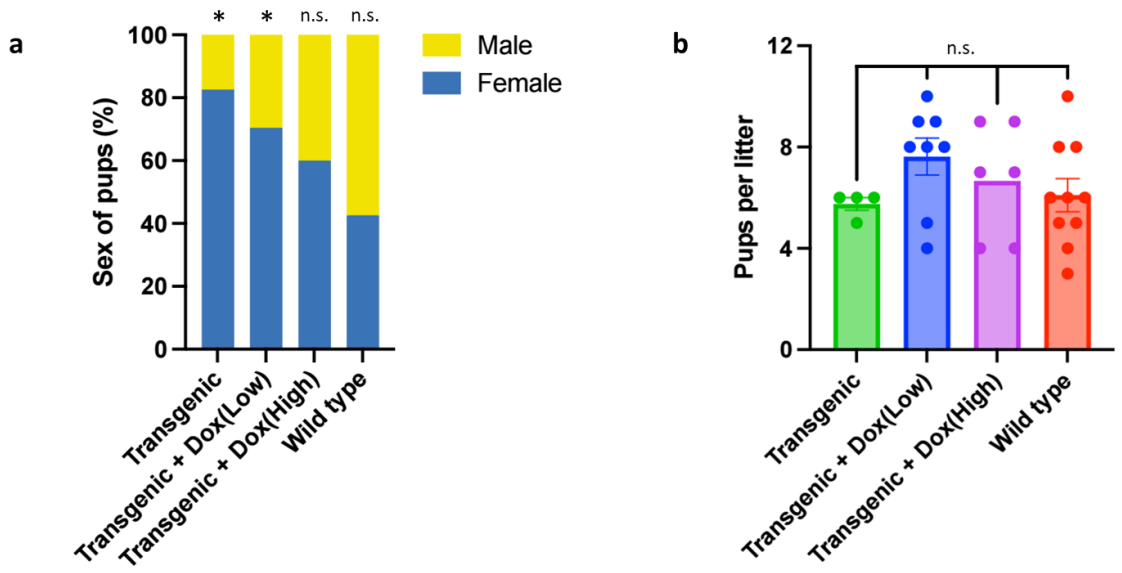


Figure 3. (**a**) The sex distribution of the total pups from the crosses C57BL/6N females with the transgenic males (n=23), transgenic males treated with 0.5 mg/ml doxycycline [Dox(low)] (n=40), 2mg/ml doxycycline [Dox(High)] (n=40), and the C57BL/6N males (n=61). Significance was determined using a two-tailed binomial test, assuming equal male to female ratio. (**b**) Litter sizes of the crosses of the transgenic vs. the wild type mice. Dots represent individual litter size and bars represent average litter size ± SE. Significance was determined using a One-Way ANOVA parametric test with Tukey correction for multiple comparison. Results recorded from two births of F0 generation of the transgenic mice were added to this group due to its low mating. Litters occurring within three days in the same cage were calculated as half or approximately half of the litter (if even or odd, respectively). *, $P < 0.005$; n.s., not significant.

An issue that we encountered is that the transgenic mice without the doxycycline treatment mated less efficiently than the doxycycline-treated mice and the wild type mice. This was quantified by the births observed for each group. Within two months of breeding, the females mated with the wild type, the low, and the high doxycycline treated transgenic mice gave 10, 8, and 6 total births, respectively, while the females mated with the transgenic mice (no doxycycline) gave only 2 births. This issue may be related to the generally smaller size of the transgenic mice. At 8 weeks of age, the transgenic mice exhibited a low weight, measuring 18 ± 2.2 g (n=17), which is at the lower scale for C57BL/6N mice (Table S1). Nevertheless, it should be noted that as we cannot generate littermate controls (i.e., males from the same parents, which are not transgenic), it is impossible to conclude if this

is significant and related to the genetic manipulation. Other than weight differences, the transgenic mice developed normally without any physiological or morphological unnatural appearance, as previously reported for *Spem1* knockout mice (Figure S2) [27].

We aimed to enhance the statistical significance of the results and verify that the observed decrease in births is linked to reduced mating behavior, rather than sperm infertility. We wished also to investigate the viability of extracted sperm for fertilization, akin to the application of sexed semen in cattle. Subsequently, sperm was extracted from the epididymis of four transgenic mice and employed in *in vitro* fertilization (IVF) procedures with four different wild type female counterparts. Notably, these IVFs resulted in the successful development of four pregnancies out of four IVF procedures, yielding a total of 35 offspring (litter size = 8.75 ± 4.86 pups). Subsequent analysis of the offspring's sex composition revealed that a remarkable 91% of the progeny (32 out of 35 pups) were females. These results effectively demonstrate that the sperm is functional, and further extends the potential applications of transgenic animals to include the production of sexed semen.

Altogether, these results demonstrate, for the first time, a genetic engineering of a mammal male resulting in the production of sexed semen *in vivo*. The sexed semen produces a non-GMO biased sex progeny. Importantly, a full litter size is observed, doubling the production of the desired sex.

Discussion

We present a groundbreaking achievement: the creation of a genetically engineered mammal with *in vivo* sexed semen, effectively eliminating most of the Y gametes. This demonstration represents a significant advancement compared to the current state-of-the-art method of *ex vivo* sexed semen for producing sex-biased litters. Sexed semen technology has been widely employed for generating offspring of a preferred sex in various species, including cattle and humans. In most species, the success rate of sex bias using sexed semen technology reaches up to 90%, with a typically lower fertility compared to unsexed semen [31,32]. In our approach, we achieve a sex bias of up to 91% with minimal ongoing effort after the initial engineering of the mammal. This genetically modified animal can be propagated without the need for additional labor or complex equipment. Importantly, this technology does not affect the litter size, likely because the sexed sperm can fertilize all the available eggs. The genetic manipulation on the male's Y chromosome does not pass to the females, maintaining the non-GMO status of the offspring. These qualities align this technology with the positive aspects of sorted sexed semen while avoiding its disadvantages.

Engineering mice to eliminate all the Y gametes (i.e., 0%) in sperm could prove even more advantageous. The partial elimination of Y gametes in the current mouse might stem from incomplete activity of dCas9, which results in incomplete silencing of the *Spem1* gene. This proposition gains support from the observation that only a minority, not constituting 50% of sperm, displayed a morphology indicative of complete *Spem1* inactivation [27]. Nevertheless, one would expect a higher percentage of morphology-defective cells considering the percentages of females obtained in the natural mating and in the IVFs. The low incidence, merely 5% of sperm, exhibiting this phenotype may arise from the incomplete suppression of *Spem1*, leading to other subtle morphological alterations challenging to discern microscopically. Despite being undetected, these subtle alterations likely make the gametes non-functional, elucidating the incongruity between distorted gametes observed and the resulting male offspring. Alternatively, certain Y gametes, featuring silenced *Spem1*, undergo apoptosis or are cleared through other mechanisms, and are thus not detected under the microscope. This could also explain the apparent contradiction between the observed morphology and the resulting sex percentage of the offspring. In addressing this incomplete silencing, consideration could be given to

employing a more robust promoter to enhance dCas9 expression levels. In addition, the incorporation of a stabilizing domain, such as UbM [33], to the dCas9 protein could bolster its stability and functionality. These suggested modifications hold the potential to significantly enhance the efficiency of generating single-sex litters, addressing the current limitations, and advancing the precision of this genetic engineering approach.

Transitioning to the production of single-sex males can be achieved straightforwardly by transferring the genetic construct to the X chromosome in the male parent. A similar approach, involving the construction of the same cassette on the corresponding sex chromosome to yield single-sex litters of the opposite sex, has been previously demonstrated by Turner and colleagues [22]. This method offers a versatile means of manipulating offspring sex ratios to suit specific breeding objectives.

The proof-of-principle described here holds promise for application in other mammalian species, including cows within the dairy industry. A similar genetic cassette can be designed and constructed on the Y chromosome of a bull, necessitating only minor modifications. Specifically, the *Spem1* gene can be targeted for silencing, given its high degree of conservation across mammals, with homologs identified in rat, dog, cow, chimp, and human [27]. Once established, the sperm from the genetically engineered bull may be utilized to fill insemination straws for fertilizing cows. This adaptability underscores the versatility and potential widespread applicability of the approach across various mammalian species.

The underlying principles of this approach can also be extended to animals in which the female represents the heterogametic sex, as exemplified by chickens. In chickens, the female carries the Z and W sex chromosomes, while the male possesses two Z chromosomes [34]. To implement this strategy, the Z chromosome within the maternal line of chickens can be genetically engineered to incorporate a cassette designed to selectively eliminate the Z-chromosome-bearing eggs within the ovaries (Figure 4).

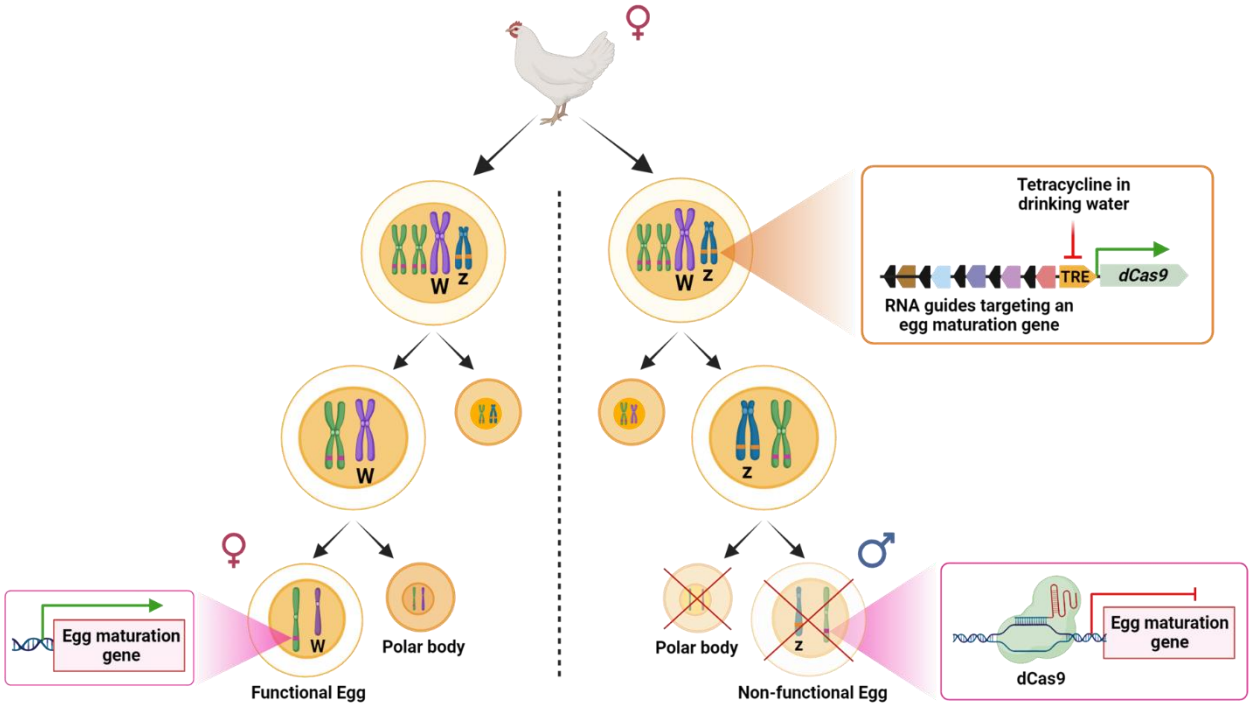


Figure 4. A schematic illustration of hypothetical birds producing only females using the proof of concept described in this manuscript. The female is the heterogametic organism in birds, and consequently, the Z chromosome should be engineered to produce female-biased sexed eggs. A gene associated with egg maturation should be targeted using the dCas9 and appropriate guides.

This genetic modification would result in the exclusive production of female progeny, potentially sparing billions of male chicks from annual culling [35]. Moreover, this approach offers resource-saving advantages, as it would necessitate less labor for segregating male and female chicks and would require less incubation space. Importantly, since the male elimination process occurs within the ovaries, as opposed to alternative methods like genetic or spectroscopic techniques for eliminating fertilized eggs [35], there would be no loss of fertilized eggs. Consequently, the implementation of this approach would effectively double the production of egg-laying females by replacing male chicks with females. Notably, the transfer of this cassette from mammals to birds may present unique challenges, given the distinct mechanisms and genes involved. However, the potential financial and ethical benefits that could be realized are substantial, underscoring the transformative potential of such an endeavor.

Several crucial features are required from a silenced gene, such as *Spem1*, to effectively eliminate sex-specific gametes: 1. Testis-specific expression: The gene should be exclusively required only for gamete development and expressed solely within the testis. Silencing a gene whose expression is required in other tissues could lead to undesired potential developmental defects in the animal. *Spem1* and numerous other testis-specific genes fulfill this criterion [36-38]. Notably, knockout studies of *Spem1* have demonstrated that mice develop normally in its absence, except in their sperm maturation [27]. 2. Essentiality for gamete formation: The gene must play a pivotal role in gamete formation and be indispensable for the process. In the case of *Spem1*, its absence leads to the production of deformed sperm with characteristic structural and motility abnormalities, ultimately resulting in male infertility [27]. 3. Expression timing during meiosis: The stage of meiosis during which the gene is silenced is also important. Silencing of the maturation gene should occur after the separation of gametes to ensure that only the targeted sex gamete is affected. Spermatogenesis involves several distinct stages, such as mitoses and two meiotic divisions [39-41]. The earliest point at which gamete selection, based on specific silencing, can be effectively implemented is after the first meiotic division, where the X and Y chromosomes segregate. However, even at this stage, the presence of cytoplasmic bridges, which exist early in meiosis and persist into the spermatid stage, can potentially allow for organelles and protein transfer between gametes [42,43]. Consequently, *Spem1*, chosen for its exclusive expression in the late stages of spermatid formation, aims to reduce the potential transfer of dCas9 into X gametes through these cytoplasmic bridges [27]. These stringent criteria ensure that the silencing of the gene selectively and effectively targets the desired gametes, minimizing off-target effects and preserving the overall integrity of the organism's development.

Considering the presence of cytoplasmic bridges and the potential for proteins' transfer, there has been speculation regarding whether the separation of X and Y gametes is sufficient to enable the selective elimination of one gamete while preserving the other. Some published literature supports the notion that X and Y gametes may indeed differ in their content, despite the existence of these bridges (e.g., [44-47]). Conversely, others have expressed doubts, suggesting that the cytoplasmic content of the gametes is balanced by the cytoplasmic bridges (e.g., [39,43]). Our system provides empirical evidence that, at the very least, certain elements—specifically, dCas9 and the RNA guides—may be differentially distributed between gametes. This observation may be attributed to the presence of a nuclear

localization signal (NLS) attached to the dCas9 protein, which localizes it within the nucleus of the Y gametes where it is encoded, while it remains absent from the X gametes. Thus, despite the presence of cytoplasmic bridges, it is plausible that proteins carrying an NLS are differentially distributed within the gametes. This finding contributes a valuable insight into the mechanisms governing the cytoplasmic distribution within the gametes, and ways to use them in the intricate processes involved in sex-specific gamete selection.

The principles and considerations applied in the design and production of the mouse line should serve as a valuable framework for transferring this technology to bulls and other animals. The potential to generate non-GMO, sex-biased litters with normal litter sizes holds the promise of revolutionizing animal breeding practices across various facilities engaged in large-scale animal production. In certain scenarios, this innovation could lead to a doubling of the desired yield, eliminate the ethically challenging practice of culling animals of undesired sex, and significantly conserve resources, energy, and labor. This groundbreaking approach has the potential to usher in a new era of efficiency and ethics within the field of animal breeding, offering a sustainable and humane solution to enhance productivity while minimizing waste and resource consumption.

Materials and Methods

Targeting vector for transgene insertion

The targeting vector VB201210-1271-bpw was synthesized by VectorBuilder (USA). It includes the following elements: Uty homology arms (a 4.6-kb 5' arm and 2.5-kb 3' arm) for insertion into intron 2 of Uty; cassette of 5 sgRNA against *Spem1*; Kruppel-associated box (KRAB) domain fused to dead Cas9 with a nuclear localization signal (KRAB-dCas9-NLS); Self-deleting Neomycine (SDN) cassette; tetracycline-controlled transactivator (tTA); a negative selection marker - diphtheria toxin A (DTA) cassette downstream of the 3' homology arm.

Housing and husbandry

All mice were housed and bred under specific pathogen-free conditions maintained in the Cyagen Transgenic Animal Center. Animals were housed in exhaust ventilated cage systems or individually ventilated cage systems. Air in the facility was controlled by fully enclosed ventilation systems. Temperature was kept at 24±2^{0}C. Humidity was kept at 40-70%. Light was automatically set from 6AM to 6PM. Pre-sterilized diet was manufactured according to the standard published by the Chinese government (No. GB 14924.3-2010, Laboratory Animals: Nutrients for Formula Feeds). The quality of diet was monitored via periodic testing by state-approved third-party test providers. Water was filtered from the city's public water system, placed in bottles with sipper tubes and autoclaved before use. The bedding was placed in cages and autoclaved before use. Water was added with either 0.5 mg/ml or 2mg/ml Doxycycline hyclate (TCI, catalog number D4116) where indicated and replaced weekly. Mating was carried out by placing one male with two female mice in a cage with nesting materials. The weight of the males was monitored weekly. Copulatory plugs, pregnancies, and births were monitored every three days.

Transgenic mice

Transgenic mice were produced by Cyagen Biosciences Inc., Suzhou, China. The linearized targeting vector VB201210-1271-bpw was introduced into Cyagen's proprietary TurboKnockout embryonic stem (ES) cells from C57BL/6N by electroporation. Clones that underwent homologous recombination were isolated using positive (neomycin) and negative (DTA) selection. 96 G418 resistant clones were picked

and amplified in 96-well plates. A duplicate of this 96-well plate served for DNA isolation and subsequent PCR screening for homologous recombination. The PCR screening identified 41 potential targeted clones, from which 5 were expanded and further validated to contain the insert by Southern blot and Sanger sequencing. Targeted ES clones were injected into albino B6N embryos and transplanted into CD-1 pseudo pregnant mice to generate F0 mice with 100% chimerism. F0 and F1 males were genotyped as described below for validating transgene insertion and germline transmission.

Sperm extraction and analysis

Sperm extracted from the epididymis was washed twice with PBS, resuspended in 200 μl of PBS. Ten μl of the suspension, or dilutions thereof, were placed on slides and smeared with an additional slide, as described [48]. The samples were air-dried, immersed in 75% ethanol and-air dried again. The preparations were viewed under the microscope and at least 100 spermatozoa were counted. The percentage of spermatozoa with heads that were bent backward (only those with an unequivocal morphology) was calculated from the total spermatozoa.

Genotyping and sex determination

Genomic DNA templates for PCR reactions were extracted from mice tails. The transgene and sex of the F0 and F1 pups were determined by 3 PCRs of the Y chromosome using oligonucleotides described in Table S2 and LongAmp DNA polymerase (New England Biolabs, USA). Thermocycling conditions were as follows: 94 °C (3 min), 34 X cycles of 95 °C (30 s), 60 °C (30 s), 65 °C (50 s/kb) followed by 72 °C (3 min) and 4 °C (hold). Sex was further confirmed at day 7 and at weaning by observing the genitals.

***In vitro* fertilization**

Sperm was collected from 4 different F0 mice and stored in liquid nitrogen. Oocytes were obtained from female mice 15-17 hours following chorionic gonadotropin injection. The fertilization dish containing the cumulus-oocytes complexes (COCs) was incubated on CARD medium for 30-60 min. before insemination. 20 µL of the sperm was thawed, diluted in FERTIUP sperm dish and incubated for 30 min. 10-20 µL of sperm was then added to the COCs and incubated for 3 h. The oocytes were then washed 3 times in a fresh mHTF medium. 6 H after insemination, oocytes that had only one pronucleus were removed. After overnight, the 2-cell stage embryos were then transferred to female mice.

Acknowledgements

UQ is supported by the European Research Council – Horizon 2020 research and innovation program, grant no. 818878. UQ received funding from the Israeli Ministry of Health in the framework of the ERANET-JPI-AMR (grant no. 15370). Cody Genetics Ltd. sponsored the IVF experiments. AM received funding from the US-Israel Bi-national Science Foundation (grant no. 2015163) and the Israel Science Foundation (grant no. 542/20). MG received funding from the US-Israel Bi-national Science Foundation (grant no. 2017176) and the Israel Science Foundation (grant no. 2174/22). We thank Nitzan Gonen and Anat Eldar-Boock for critical comments and suggestions.

Author Contributions

Conceptualization, U.Q.; Methodology, I.Y., H.B-J. R.S., A.M., M.G., and U.Q.; Investigation, I.Y., J.C., H.B-J. R.S., A.M., M.G., and U.Q.; Writing – Original Draft, U.Q.; Writing – Review & Editing, I.Y., T.M., R.S., A.M., M.G., and U.Q.; Funding Acquisition, A.M., M.G., and U.Q.

Competing interests

I.Y., M.G., and U.Q. have submitted a patent request on a related technology in January 2019.

I.Y., A.M., M.G, and U.Q. have submitted a patent request on the described technology in August 2023.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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