PLOS GENETICS

REVIEW

Advances and challenges in genetic technologies to produce single-sex litters

Charlotte Douglas, James M. A. Turner*

Sex Chromosome Biology Laboratory, The Francis Crick Institute, London, United Kingdom

* james.turner@crick.ac.uk

Abstract

There is currently a requirement for single-sex litters for many applications, including agriculture, pest control, and reducing animal culling in line with the 3Rs principles: Reduction, Replacement, and Refinement. The advent of CRISPR/Cas9 genome editing presents a new opportunity with which to potentially generate all-female or all-male litters. We review some of the historical nongenetic strategies employed to generate single-sex litters and investigate how genetic and genome editing techniques are currently being used to produce all-male or all-female progeny. Lastly, we speculate on future technologies for generating single-sex litters and the possible associated challenges.

Citation: Douglas C, Turner JMA (2020) Advances and challenges in genetic technologies to produce single-sex litters. PLoS Genet 16(7): e1008898. https://doi.org/10.1371/journal.pgen.1008898

Editor: Jacob L. Mueller, University of Michigan Medical School, UNITED STATES

Published: July 23, 2020

Funding: Work in the Turner lab is supported by the European Research Council (CoG 647971) and the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001193), UK Medical Research Council (FC001193), and Wellcome Trust (FC001193). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Animal models remain indispensable experimental reagents for understanding fundamental biology and translational research. Despite this utility, there is an ongoing issue with the production of animals that are surplus to requirement. For example, in 2017, in Great Britain alone, over 1.8 million laboratory animals were culled without ever being used for a scientific procedure [1]. Globally, the Reduction, Replacement, and Refinement (3Rs) principles are common factors that encourage the reduction of unnecessary animal use [2]. For example, in the European Union, evidence of adhering to the 3Rs is a legal requirement, and in the USA, the goal of the Animal Welfare Act is to encourage alternative experimental strategy to minimise animal pain and distress. One factor contributing to excess production of animals is sex-specific research; for example, studies of reproductive biology or sex-specific cancers in which only one sex is required (Table 1). A genetic method of producing single-sex litters, in which the unrequired sex is nonviable in utero and therefore is never born, would remove the need for postnatal culling, in line with the 3Rs.

The requirements for all-female or all-male litters is not limited to laboratory models (Table 1). For example, it would also be extremely advantageous for agriculture, with the layer hen industry representing a prominent example. Approximately 6 to 7 billion male chicks are culled worldwide per year, generating a well-known and highly controversial ethical issue [3]. Conversely, in pest control, reducing or controlling the female mosquito population, the vector for the malaria parasite, found in over 100 countries including large parts of Africa and Asia, would be extremely advantageous, and similarly for the eradication of invasive pest species such as rodents in island countries such as New Zealand [4]. In these examples, a genetic

Table 1. A summary of major examples of different species and the current requirements for single-sex litters.

Species	Sex chromosomes	Sex required	Why are single sexes required
Mosquito	XX female XY male	Males	Females carry the malaria parasite
Mouse	XX female XY male	Males	Male-specific scientific research Laboratory-controlled sterility of all-male litters for population control
Mouse		Females	Female-specific scientific research
Chicken (layers)	ZW female ZZ male	Females	Egg-laying
Cows (dairy)	XX female XY male	Females*	Milk production for dairy products
Cows (meat)		Males	Greater mass for meat
Silkworm	ZW female ZZ male	Males	Greater quality of silk production
Fruit fly	XX female XY male	Males	Laboratory-controlled sterility of all-male litters for pest population control
Insects	Variable	Males	Laboratory-controlled sterility of all-male litters for pest population control

There is often a requirement for a single sex in food production because only one sex is able to produce an animal product: for example, eggs by female layer hens. However, there is also a requirement to produce all-male litters that can be sterilised in controlled laboratory or factory conditions prior to release in the wild. In this strategy, the overall population size can be reduced for pest-control measures, such as for insects and rodents. This strategy for pest control is called SIT. **Abbreviations:** SIT, Sterile Insect Technique.

*In some countries—for example, the USA—male offspring produced in the dairy cow industry are repurposed for the meat supply chain.

https://doi.org/10.1371/journal.pgen.1008898.t001

method of producing all-male litters in a controlled laboratory and factory environment for sterilisation, prior to release in the wild, would eliminate or reduce the population size. One alternative method of controlling malaria spread would be to repurpose engineered gene drives in order to produce single-sex progeny.

The production of all-female or all-male litters by genetic methods is feasible because in some species, males and females differ in their sex-chromosome complement (Table 1). Eutherian female mammals, such as mice and humans, are homogametic, producing only X-chromosome–carrying gametes. Eutherian male mammals are heterogametic, producing mature sperm that, with rare exceptions (for example, [5–7]), carry either the X or Y chromosome. Early studies on differences of sex determination (DSDs) showed that in eutherian mammals, sex determination is not regulated by the number of X chromosomes [8,9]. Instead, it is driven by the presence of the Y chromosome via a locus originally coined the Y-linked testis-determining factor (TDF; [10]). The TDF was later identified to be *SRY/Sry* (Sex-determining region Y), which is expressed in Sertoli cell precursors [11–17]. It is important to note that the *SRY/Sry* mode of sex determination is not the primary method of sex determination for all mammals. For example, the platypus, a prototherian mammal, does not have an *SRY* gene [18,19].

Conversely, in many bird species, including chickens, females are heterogametic and carry a single Z and a single W chromosome. Males are homogametic and carry 2 Z chromosomes. Avian sex determination is controlled by the dosage of a Z-linked gene called DMRT1 (Doublesex and mab-3–related transcription factor 1; [20]). Female birds carry 1 copy of DMRT1, whilst males have 2 copies. DMRT1 is an orthologue of *doublesex* that undergoes sex-specific alternative splicing to regulate sex determination in many insect species, including *Drosophila melanogaster*, reviewed in [21].

In this Review, we investigate some of the current requirements for single-sex litters in research and in other industries such as agriculture and pest control. We describe some of the historical methods for sexing-sorting and advantages and disadvantages associated with them.

We then discuss current methods performed to generate single-sex progeny, including by genome editing methods such as CRISPR/Cas9 [22–26]. Finally, we assess the challenges associated with generating single-sex litters and future perspectives of the technologies.

Previously developed nongenetic methods for producing single-sex litters

Historically, sex selection is performed by investigating sex-specific biological differences. For example, male and female chick embryo allantoic fluid contains differential levels of estrone sulfate [27]. However, such methods of determining sex by allantoic fluid extraction are invasive and generally give the most reliable results at day 9 of development, which is close to the onset of pain perception [3,27]. Furthermore, invasive procedures frequently result in reduced viability [28,29]. Recently, Galli and colleagues performed fluorescence and Raman spectroscopy to determine differential hormone levels in male and female chick embryonic blood at day 3.5 [30]. Although this method does not require fluid extraction, it requires a window to be made in the shell and therefore may still be considered invasive. Moreover, although accurate, this and other methods still result in culling of chick embryos during late stages of development. Therefore, the sex-selection field is now developing alternative approaches to produce single-sex litters by genetic methods, whereby the unrequired sex is eliminated at an earlier embryonic stage in utero without the need for mechanic or spectroscopic testing.

Physical separation of mammalian X- or Y-carrying sperm

Given the risk of embryonic nonviability and pain perception associated with invasive procedures for sexing embryos, one superior method to selecting offspring sex is by separation of X- or Y-carrying sperm. Prior selectivity of sex-specific gametes for in vitro fertilisation (IVF) or artificial insemination (AI) ensures that the offspring sex is predictable, which may be more economically viable and ethically justifiable than invasive sexing procedures.

Many techniques have been previously attempted to isolate X- and Y-sperm, including fluorescence in situ hybridisation (FISH) and swim separation [31]. The most successful method for selective separation of X- or Y-carrying sperm for IVF/AI is by flow cytometry [32]. Sperm nuclei are stained using Hoechst 33342 and sorted based on DNA content. Bull X-carrying sperm, for example, have approximately 3.8% greater DNA content compared to Y-sperm [33]. The main caveat of this flow cytometric approach is that the sperm exhibit reduced fertilisation ability [34–37]. Increasing the quantity of sperm used for AI does not appear to significantly rescue the conception rate [38], suggesting that the reduced fertility results from sorting and postsorting procedures and may possibly be due to residual Hoechst dye [35,36].

Flow cytometry is associated with large economic and time costs in sperm sorting and postsort procedures (reviewed in [37]), leading to the investigation of alternative methods. One such strategy is to separate X- and Y-carrying sperm by sex-chromosome–specific differential gene expression. However, this strategy has remained extremely challenging because during spermatogenesis, the X- and Y-sperm are connected via cytoplasmic bridges. The cytoplasmic bridge connections are essential to ensure that the haploid X-carrying sperm receive Y-carrying sperm products (and vice versa), as well as mRNAs [39,40] and organelles [41]. However, an intriguing recent study by Umehara and colleagues highlighted that cell-surface marker Toll-like receptor 7/8 (TLR7/8) was expressed on X-carrying, but not Y-carrying, sperm [42]. Ligand activation of the TLR7/8 receptor using Resiquimod or Imiquimod suppressed X-sperm motility, allowing for X- and Y-carrying sperm separation prior to IVF procedures. Following IVF with the X-carrying 'slow' sperm, the proportion of female offspring was 81%, whilst following IVF with Y-carrying 'fast' sperm, the proportion of male pups was 83% [42].

Umehara and colleagues performed the work using mouse as a model but speculated that the strategy was translatable to many agricultural species.

Another technique also utilising cell-surface markers for separating X- and Y-sperm, used in bulls, is 'WholeMom' [43]. In this approach, a monoclonal antibody selectively binds an epitope only present on the bull Y-chromosome–carrying sperm plasma membrane. Epitope binding results in agglutination of the Y-carrying sperm heads, whilst the X-carrying sperm are unaffected and fertilise oocytes [43].

In summary, current approaches rely on the differential DNA content or surface markers of X- and Y-carrying sperm in order to physically separate the sperm. These strategies rely on IVF of sex-sorted sperm to skew offspring sex ratios. Although these and other methods not described in detail in this Review (listed in Table 2) are occasionally feasible approaches to sex selection, many are expensive, time-consuming, and often inefficient or inaccurate. Genetic approaches to sex selection are therefore being developed as alternative approaches.

Current genome editing methods to generate single-sex litters

The development of genetic methods to produce single-sex litters relies on sex-specific genomic differences, such as a different sex-chromosome complement (Table 1). For example, in many insect species and eutherian males, the Y chromosome is inherited by sons, and the paternal X chromosome is inherited by the daughters. This sex-specific inheritance of the father's sex chromosomes can be exploited in order to control the inheritance of transgenes. In many insect species, the sex-specific alternative splicing of genes such as *doublesex* can also be harnessed to ensure sex-specific expression of transgenes.

Transgene-based sex-selection systems

Producing all-male litters would be advantageous for mosquito and insect population control. Broods of all-male litters could be generated, sterilised in controlled laboratory conditions, and then released into the wild to induce population collapse. This strategy is called the 'Sterile Insect Technique' (SIT; [79]). To select for males, insect species carrying sex-chromosome–specific or sex-specific fluorescent markers have been generated [80,81]. However, these strategies require manual sorting of insects.

A refinement of SIT is called 'Release of Insects carrying a Dominant Lethal' (RIDL; [82]). RIDL is a system of sex-specific transgene-induced lethality, thereby overcoming issues with manual sex sorting. Early successful transgenic methods for sex-specific lethality were carried out on a Lepidopteran species, *Bombyx mori* (Mulberry silkworm). Male silkworms are desirable over females because they produce higher-quality silk [83], but RIDL can also be used for generating male-only broods prior to sterilisation and release. Tan and colleagues cloned a tetracycline-repressible transactivator (tTAV) construct into an orthologous *doublesex* minigene from *Pectinophora gossypiella* (*Pgdsx*; pink bollworm) and inserted the transgene into the *B. mori* genome [84]. Endogenous *Pgdsx doublesex* undergoes sex-specific alternative splicing; therefore, in *B. mori*, tTAV expression was specific to females. The female-specific tTAV protein accumulation induced female-specific lethality, resulting in male-only cocoons surviving. Moreover, the female-specific lethality could be largely repressed by the addition of dietary tetracycline [84], allowing for control of the sex-specific lethality system. Interestingly, however, similar *doublesex*-regulated lethality constructs integrated into other pest insect genomes—for example, the olive fly and Mediterranean fruit fly—did not have the same lethality effect [85,86].

More recently, Kandul and colleagues generated an antibiotic-resistance–based sex-selection transgene system in *D. melanogaster* [87]. Two drug-resistance transgenes are expressed

Table 2. Nongenetic methods for sex selection.

Method	Advantages	Disadvantages	Species performed in	References
Hormone quantification	Accurate (in later developmental stages)	Invasive (affects hatching and viability) Can only be applied after day 9 (post onset of pain perception)	chicken	[27,44]
Egg shape	Noninvasive	Accuracy is variable		[45]
Egg odour	Noninvasive	Accuracy is variable		[46,47]
Raman and fluorescence spectroscopy (optical spectroscopy)	Accurate Near-infrared excitation prevents damage to cells	Invasive High background fluorescence signal		[30,48–50]
Hyperspectral imaging	Noninvasive	Limited to species with sex-specific feather colour Accuracy is variable		[51,52]
Fourier transform infrared spectroscopy	Performed on nonincubated eggs on the germinal disk	Invasive		[53]
'Hologic Invader'	Relatively quick molecular sexing method Accurate	Has only been established in laboratory conditions		[54]
Sperm separation				
(a) Flow cytometry	High purity	Requires detectable differences in DNA size between sex chromosomes Compromised fertilisation ability of sperm High cost and time	cow, rabbit, sheep, pig	[37,55,56]
(b) Swim separation by agglutination of X- or Y-specific epitopes	No mechanical damage to the sperm	X- and Y-carrying sperm are connected by cytoplasmic bridges, controversy as to whether there is sex-specific expression	cow, buffalo, mouse	[42,43,57]
(c) Immunological assays for male-specific H-Y antigens	No mechanical damage to the sperm	Controversy as to whether the H-Y antigen is uniquely on Y-sperm	cow, mouse	[58,59]; reviewed in [60]
(d) FISH	Accurate Generally used for flow-cytometry–sorted sperm purity check	Sperm heads have to be decondensed	cow, mouse, pig, dog	[61–65]
(e) Raman spectroscopy	Efficient Noninvasive		cow	[66]
(f) Labelling with nanoparticles	Efficient for labelling	Could be toxic for sperm	cow	Reviewed in [67]
Embryo sexing				
(a) Karyotyping	Accurate Inexpensive	Difficulty in producing high quality metaphase spreads Time-consuming Embryo biopsy may affect viability		[68]
(b) Metabolomic differences	Accurate in cow embryos	Limited by the amount of quantifiable enzyme Assay may be toxic to embryos	cow	[69]
(c) Analysis of sex chromatin	Inexpensive Simple method	May not be able to detect Barr body	rabbit	[70]
(d) FISH	Low risk of contamination from other cell types. Confirmation of embryonic cell type by visualisation of FISH Highly accurate	Requires highly Y-specific probe Embryo biopsy may affect viability Time-consuming	cow	[71]
(e) H-Y antigen	Noninvasive Fairly accurate	Assay may lower embryo viability	cow, sheep, pig, horse, goat, mouse	[72]
(f) ccffDNA	Fairly accurate Noninvasive	Requires a downstream PCR analysis for sex-specific polymorphisms	sheep	[73]

(*Continued*)

Table 2. (Continued)

Method	Advantages	Disadvantages	Species performed in	References
Pupal size	Could be effective in small laboratory settings	Requires manual sorting Highly error-prone Species variability	insects, including mosquito	[74,75], reviewed in [76]
Behavioural differences				[77,78]

Historically, there have been many methods to attempt to produce single-sex litters. We summarise some of the main methods utilised, with the major advantages and disadvantages for each method. **Abbreviations:** ccffDNA, circulating cell free foetal DNA; FISH, fluorescence in situ hybridisation

https://doi.org/10.1371/journal.pgen.1008898.t002

in opposite sexes by integration of each transgene into a sex-specific intron of the *transformer* or *doublesex* genes. Male and female flies have normal viability until the dietary addition of either puromycin or geneticin, which selects for males or females, respectively, producing progeny of 100% the required sex.

Although the use of fluorescence-transgene–based sexing systems were inefficient in insect species, in chickens, they are currently the most promising genetic approach for chick sexing prior to hatching. In Australia, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) is championing a chick-marker approach whereby a fluorescent marker is integrated onto the male-determining Z chromosome so that male and female chicks can be segregated prior to hatching [88].

Transgene-induced destruction of sex-specific sperm

A superior system of generating single-sex litters in the laboratory and agriculture would be to produce a single type of sex-chromosome–carrying gamete, i.e., only X- or only Y-carrying sperm, by selective destruction of the unrequired sperm. Evidence for the first genetic methods to skew offspring sex ratios by destruction of the X-carrying sperm was demonstrated in the *Anopheles* mosquito model [89]. *Anopheles* males are heterogametic, XY, whilst females are XX. An I-PpoI (*Physarum polycephalum* intron-encoded endonuclease) cassette was genetically engineered onto the Y chromosome. The I-PpoI endonuclease selectively targeted the X chromosome, resulting in endonuclease-driven shredding of the X [89,90]. The damage to the X-carrying gametes meant that only Y-carrying gametes were able to fertilise oocytes, resulting in a male-biased sex ratio skew [89–92]. Using CRISPR/Cas9, the strategy was refined to target X-linked repetitive ribosomal DNA sequence by single guide RNA (sgRNA)-guided Cas9 endonuclease activity (Fig 1A). Again, the X-shredding resulted in the loss of X-carrying gametes and a male-biased sex skew in offspring, ranging from 86.1% to 94.8% [93]. Most recently, Simoni and colleagues described a successful male-biased distorter system that harnesses a CRISPR-gene drive, inserted into the conserved *doublesex* intron 4–exon 5 boundary, driving I-PpoI to induce X-shredding [94].

Faluso and colleagues expanded on the Galizi and colleagues [90,93] studies by modelling X-shredding and a new strategy called X-meddling (Fig 1B) in *Drosophila* [95]. They produced germline-expressed Cas9 endonuclease lines and bred them with engineered sgRNA-encoding lines, targeting multiple repeat sequences on the X chromosome (for X-shredding) and putative haplo-insufficient genes on the X chromosome (for X-meddling). The authors noted that the majority of X-shredding sgRNAs, bar one, did not substantially affect the progeny sex ratio. Conversely, however, sgRNAs targeting the haplo-insufficiency genes *RpS6* (Ribosomal protein S6) and *RpS5a* (Ribosomal protein S5a) generated a male-biased offspring sex ratio skew from 93.8% to 56.6%, respectively. This study highlighted that although the X-shredding

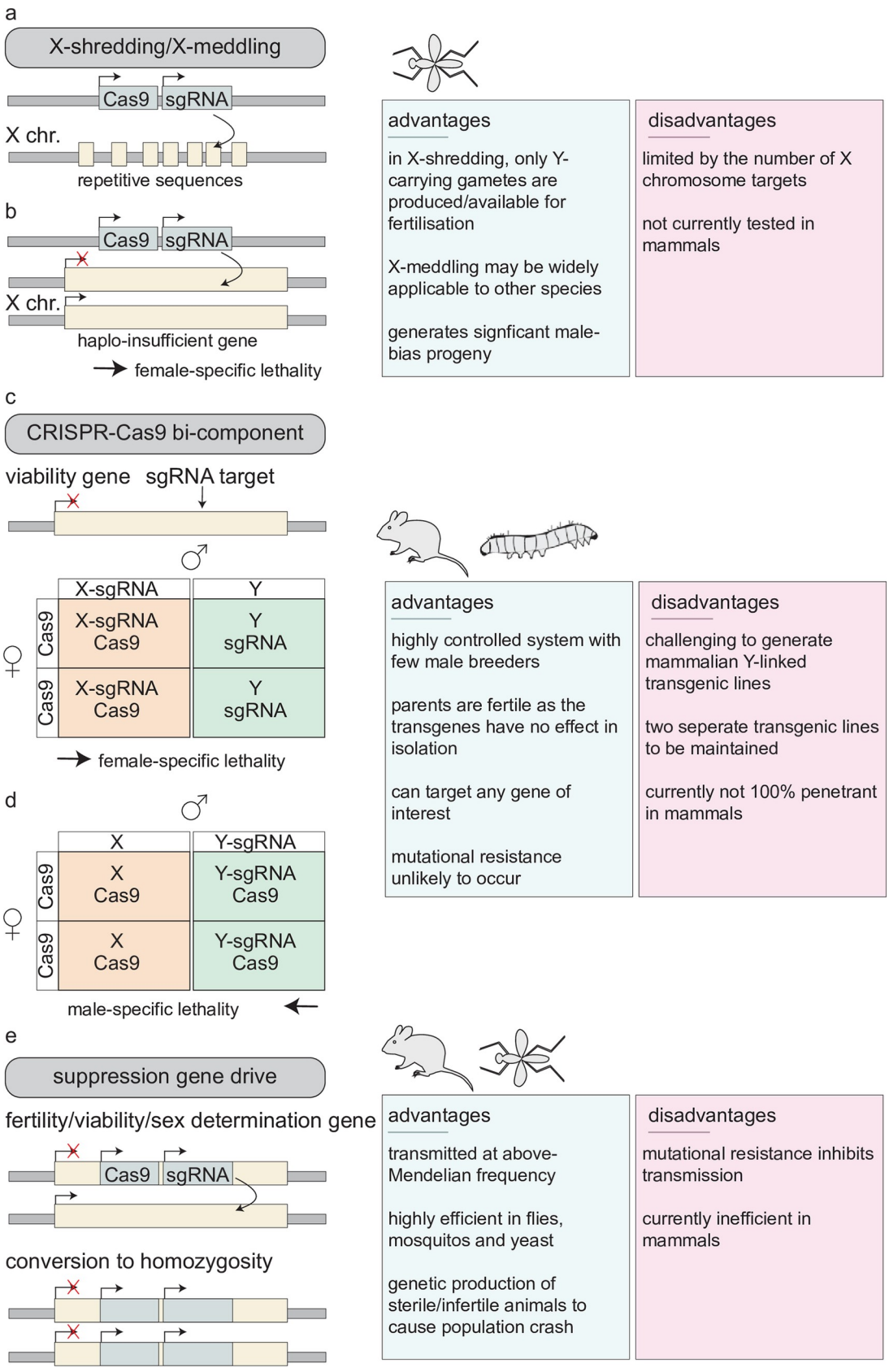
a
X-shredding/X-meddling
Cas9
sgRNA
X chr.
repetitive sequences
b
Cas9
sgRNA
X chr.
haplo-insufficient gene
female-specific lethality
advantages
in X-shredding, only Y-carrying gametes are produced/available for fertilisation
X-meddling may be widely applicable to other species
generates signficant male-bias progeny
disadvantages
limited by the number of X chromosome targets
not currently tested in mammals
c
CRISPR-Cas9 bi-component
viability gene
sgRNA target
♂
X-sgRNA
Y
Cas9
X-sgRNA Cas9
Y sgRNA
X-sgRNA Cas9
Y sgRNA
♀
female-specific lethality
advantages
highly controlled system with few male breeders
parents are fertile as the transgenes have no effect in isolation
can target any gene of interest
mutational resistance unlikely to occur
disadvantages
challenging to generate mammalian Y-linked transgenic lines
two seperate transgenic lines to be maintained
currently not 100% penetrant in mammals
d
♂
X
Y-sgRNA
Cas9
X Cas9
Y-sgRNA Cas9
X Cas9
Y-sgRNA Cas9
♀
male-specific lethality
e
suppression gene drive
fertility/viability/sex determination gene
Cas9
sgRNA
conversion to homozygosity
advantages
transmitted at above-Mendelian frequency
highly efficient in flies, mosquitos and yeast
genetic production of sterile/infertile animals to cause population crash
disadvantages
mutational resistance inhibits transmission
currently inefficient in mammals

Fig 1. Genetic methods of producing single-sex litters. (a,b) X-shredding and X-meddling techniques are engineered to utilise CRISPR/Cas9 components to target specific regions on the X chromosome. During spermatogenesis, the CRISPR/Cas9 components are expressed and induce mutations on the X-chromosome–linked targets. X-shredding involves the sgRNA targeting X-linked repeats, resulting in 'shattering' of the X chromosome. The sperm carrying the shattered X chromosome cannot produce viable offspring after fertilisation, resulting in all-male offspring. X-meddling involves targeting X-linked haploinsufficient genes. Therefore, when the knock-out allele containing sperm fertilises the oocyte, the female is nonviable, resulting in single-sex progeny. (c,d) CRISPR/Cas9 bicomponent systems have been generated in the mouse and silkworm. Offspring coinheritance of a Cas9 and sgRNA-transgenic allele targeting an essential viability gene results in mutation and loss of function of the target. Inheritance of a single transgene is predicted to have no effect. (e) Suppression gene drive has been generated in the mouse and mosquito models, amongst others. The CRISPR/Cas9 transgene is targeted to an essential male/female-specific fertility or viability gene in order to disrupt gene function. Expression of the CRISPR-Cas9 transgene converts the transgene from hemizygosity to homozygosity. Loss of function of the target gene renders the target female or male population sterile or nonviable. In order to generate single-sex litters, the CRISPR-Cas9 gene target is a sex determination gene such as *doublesex*, which theoretically would skew sex offspring sex ratios. chr., chromosome; sgRNA, single guide RNA.

https://doi.org/10.1371/journal.pgen.1008898.g001

strategy was applicable to other species outside mosquitos, X-meddling proved to be a more efficient method of producing sex-biased progeny and may be more greatly applicable to other species.

Bicomponent CRISPR/Cas9 systems

Another CRISPR/Cas9-based method for generating single-sex litters is a bicomponent system. A bicomponent CRISPR/Cas9 system refers to the genetic isolation of Cas9- and sgRNA-encoding transgenes. The isolated Cas9 and sgRNA transgenes are coinherited independently of each other from either parent. Integration of either the Cas9 or sgRNA transgene onto a sex chromosome of a heterogametic parent allows for sex-specific inheritance of the transgene. Monoinheritance of either the Cas9 or sgRNA transgene in isolation is predicted to have no mutational effect and therefore is advantageous because monoallelic stocks can be bred normally. Furthermore, it is known that constitutive expression of transgenic Cas9 is not detrimental to mice [96–98]. Conversely, the coinheritance of both transgenes, i.e., one from each parent, would generate loss-of-function mutations at the sgRNA target viability locus, thereby resulting in embryonic lethality of the unrequired sex (Fig 1C and 1D).

The first implementation of a CRISPR/Cas9 bicomponent system to breed all-male offspring was in *B. mori* [99]. Similarly to birds, female silkworms are the heterogametic sex (ZW) and males are the homogametic sex (ZZ). Zhang and colleagues generated a female-specific W-linked Cas9 transgenic line, and the transgene was therefore uniquely inherited by daughters [99]. Second transgenic lines were produced carrying an autosomal sgRNA targeting the essential gene *Bmtra2* (*B. mori* transformer 2). Coinheritance of the W-Cas9 and sgRNA transgenes in daughters resulted in *Bmtra2* CRISPR/Cas9-induced mutations, 50% of the progeny did not hatch, and the surviving progeny were 100% male. This study was the first to highlight that sex-specific coinheritance of CRISPR/Cas9 components targeting an essential gene produces single-sex litters. Developments in generating single-sex offspring by genetic methods has the potential to be extremely advantageous for the silkworm industry.

In the Zhang and colleagues silkworm study, the aim was to produce all-male progeny. However, laboratories or agricultural applications may require all-female litters; for example, female rodents to study female-specific biology or in producing layer hens (Table 1). In the Zhang and colleagues study, the Cas9 transgene was W-linked and therefore inherited uniquely by females, alongside the autosome-linked sgRNA, to induce *Bmtra2* mutations and female-specific lethality. The same principle is relevant to eutherian mammals; integration of a Cas9 transgene onto the Y chromosome would ensure unique inheritance by sons. Coinheritance of a Y-linked CRISPR/Cas9 component transgene and autosome-linked CRISPR-Cas9

component will induce mutations in the target gene uniquely in sons. If the target gene is an essential viability gene, the sons will be embryonic lethal.

However, the Y chromosome has been extremely challenging to genetically modify because of its highly heterochromatic and repetitive nature. The modern eutherian X and Y chromosomes diverged from a pair of ancestral autosomes [100] following the acquisition of the male-determining gene *SRY/Sry* approximately 148–166 million years ago [101]. Recombination was thereafter suppressed between the X and Y chromosomes, most likely through a series of Y-chromosome inversions [102]. The Y chromosome became recombinationally inert, accumulating deleterious mutations and losing most of its ancestral genes [103]. The clonal inheritance of the Y chromosome through the male lineage contributed to the sexual conflict between the X and Y, leading to further specialisation of Y-genes for male-function [18,103,104] and testis-specific expression [105]. The loss of Y-chromosome genes led to the male-specific Y chromosome being greatly reduced in size compared to the X chromosome. Similarly, in many bird species, the female-specific W chromosome is also greatly reduced compared to the Z chromosome. The comparative sizes of the X and Y or Z and W chromosomes are highly diverse amongst species.

Given the complexity associated with Y-gene targeting, the first study generating a targeted mammalian Y-gene reporter was not published until 2013, using transcription activator-like effector nucleases (TALENs; [106]). Previous attempts to mimic Y-linked transgene expression have utilised Y-gene promoter-driven transgenic lines; however, transgenes are instead randomly integrated into an autosome. For example, an enhanced green fluorescent protein (eGFP) reporter, driven by the *Sry* promoter, was randomly integrated into the genome by zygotic microinjection [107]. The main disadvantage of *Sry*-promoter–driven transgenic alleles, however, is that mouse *Sry* expression is tightly regulated in the gonad, occurring between embryonic day (E) 10.5 and E12.5 [14–16]. Therefore, Cas9 or sgRNA transgene expression would also be limited to the gonad within these developmental time points, and expression would be insufficient to drive sgRNA-guided mutations to produce single-sex litters.

Given that *Sry*-promoter–driven expression is restricted, a preferable choice would be a promoter driving a Y-gene with ubiquitous expression. There are multiple Y-linked genes ubiquitously expressed in the mouse, including *Uty* (Ubiquitously transcribed tetratricopeptide repeat containing, Y-linked) [108], *Eif2s3y* (Eukaryotic translation initiation factor 2 subunit 3, Y-linked) [109], *Ddx3y* (DEAD-Box helicase 3, Y-linked) [110], and *Kdm5d* (Lysine demethylase 5D) [111]. Of these, *Uty* is expressed during embryonic development and also in embryonic stem cells (ESCs) [112]. Furthermore, previous studies have successfully generated in-frame knock-in *Uty*-eGFP reporter ESC lines [106]. Moreover, Zhao and colleagues recently generated a Y-linked reporter mouse line wherein eGFP expression was driven by a constitutive promoter and shown to be expressed in preimplantation embryos [113]. The transgene was inserted into an intergenic region between *Uty* and *Ddx3y*, thereby opening up new possibilities of Y-chromosome knock-in targets.

In 2019, the first mammalian CRISPR/Cas9 bicomponent system was described, with the intention of generating all-female litters by CRISPR/Cas9-induced knock-out of essential genes in male embryos. Yosef and colleagues utilised the ubiquitous Y-linked *Uty* locus to integrate a constitutively expressed sgRNA transgene into the second intron [114], which would therefore be uniquely inherited by sons. Therefore, upon the male-specific coinheritance of the Y-linked sgRNA transgene targeting essential genes *Atp5b* (ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit), *Casp8* (Caspase 8), and *Cdc20* (Cell division cycle protein 20) and an autosomal constitutively expressing Cas9 transgene resulted in knock-out of the target loci and significant male-biased offspring sex ratio [114]. Whether *Uty* expression was affected in these sgRNA-transgenic males was not addressed.

Bicomponent systems are advantageous because mono-transgenic stocks can be maintained as separate lines and bred when necessary. The human control of using original stocks maintained independently ensures that genetic mutational resistance at the target loci is unlikely to occur. Furthermore, if mutational resistance did arise in any offspring, the individuals can be removed from the population without any negative impact on the breeding stocks. Refining the technologies further, it may be possible to generate transgenic laboratory models wherein the transgenes that induce the embryonic lethality effect are inherited by maternal deposition of mRNAs. In an analysis of embryos derived from Gt(ROSA)26Sor (*Rosa26*)-Cas9 hemizygous transgenic mothers, maternally loaded Cas9 was sufficient to induce mutations even in non-transgenic offspring [96].

Gene drive

Gene drive refers to the process of a selfish genetic element transmitting through a population at above-mendelian frequency [115–117]. Laboratory engineered gene drives can therefore be harnessed to spread genetic traits quickly through a population, and moreover, they can be adapted for producing single-sex progeny. Burt first postulated that endonuclease-driven gene drives could be used for pest-control management [91]; however, the advent of CRISPR/Cas9 has enhanced the potential for synthetic gene drives to be transmitted highly effectively in wild populations [118]. Engineered gene drives function by the insertion of an endonuclease transgene into a target locus. The transgene-encoded endonuclease then copies itself into the other wild-type allele, thereby converting from hemizygosity to homozygosity, and ensuring inheritance by all offspring [91]. 'Suppression drive' is a refined engineered gene-drive system, whereby the endonuclease-encoding transgene is inserted into an essential fertility or viability gene [91,118]. Upon active drive and transgene conversion to homozygosity, the individual becomes infertile or nonviable. In CRISPR/Cas9-engineered gene drives, a transgene encoding an sgRNA and Cas9 is integrated into a target viability or sex-specific fertility locus. Transgene expression converts the transgenic allele to homozygosity, thereby rendering individuals nonviable or infertile. These gene-drive methods could be highly efficient for reducing population size. Moreover, the engineered gene drive could be modified to target genes for sex determination. In this strategy, gene-drive–induced modifications of the target locus could produce single-sex progeny (Fig 1E).

In 2018, Kyrou and colleagues generated a gene-drive system in mosquitos, targeting the *doublesex* gene [119]. In this strategy, a gene-drive construct was engineered targeting the female-specific exon of *doublesex*, leaving the male *doublesex* splice variant unaffected, aiming to produce male-biased progeny. Heterozygous targeted females were unaffected, confirming that *doublesex* is functional with a single copy (haplo-sufficient). Interestingly, homozygous targeted females were not sex reversed but instead showed an intersex phenotype and were infertile [119]. This study highlights that currently gene drives cannot be used for generating sex-biased litters but instead could be used to cause a population collapse by sex-specific sterility.

To examine whether the synthetic suppressive drive systems could also be applied to mammals, Grunwald and colleagues performed the first proof-of-principle gene-drive system in mice [120]. To assess success, they utilised the *Tyrosinase* (*Tyr*) gene, which generates white-coated mice upon homozygous knock-out. An sgRNA transgene targeting *Tyr* and also encoding an mCherry reporter was inserted into the *Tyr* locus to produce a *Tyr*-heterozygous knock-out and hemizygous transgenic mouse line. When bred with Cas9-expressing mice, functional gene-drive systems would transmit to offspring at above-mendelian frequency. A successful gene drive should produce *Tyr* loss-of-function white mice that also express

mCherry. In this approach, gene-drive success varied from 0% to 72%. Often, the mice were white-coated but did not express mCherry, suggesting that transmission of the sgRNA and Cas9 transgenes produced mutations at *Tyr* but without copying the sgRNA/mCherry transgene [120]. The repair pathway after CRISPR/Cas9-induced mutations at *Tyr* was likely non-homologous end-joining (NHEJ), consistent with previous reports that NHEJ is the dominant mode of repair over homology-directed repair [121,122]. Therefore, although relatively efficient in mosquitos [123], mammalian synthetic gene drives require further optimisation.

Disadvantages of genetic methods

Genetic or genome editing systems have the potential to effectively generate single-sex litters; however, they currently have some disadvantages. The superior method of generating single-sex litters is by selective destruction of the nonrequired sperm. One method to selectively destroy X-carrying sperm is by X-shredding or X-meddling. Although this technique was shown to be highly efficient in mosquitos [90,93], it was variable in *Drosophila* [95]. An important consideration of harnessing X-shredding in other nonmosquito species is the availability of X-targets because this is likely to strongly influence the success rate. One alternative method is by targeting the X-shredding transgene to the Y chromosome for germline expression. The question of whether the Y chromosome could be used for *Drosophila* CRISPR/Cas9 transgene expression for X-meddling or X-shredding is still open; however, great strides have been made in determining possible transgene integration sites [124].

Although CRISPR/Cas9 bicomponent systems are advantageous in that the mono-transgenic stocks can be easily maintained independently, current bicomponent systems are not 100% efficient. In the Yosef and colleagues study [114], the sex skew was imperfect; i.e., some males were born despite mutations in the target housekeeping genes. Furthermore, these males were often born with severe developmental abnormalities, which raises further ethical questions in line with the 3Rs. Another interesting question regarding bicomponent systems is that of the number of offspring born. The bicomponent CRISPR/Cas9 system selectively induces nonviability in a target sex by CRISPR/Cas9-induced mutations in a target gene. Therefore, by estimates of mendelian frequency, approximately half of the offspring are embryonic lethal. Although the unrequired sex is not born, the number of pups of the required sex remains unchanged. Moreover, there is potentially a risk that the in utero embryonic lethality of the unrequired sex could also stimulate abortion of the required sex. As earlier described, a superior method to generating single-sex litters would be via selective destruction of the unrequired sex-chromosome–carrying sperm, such as by X-shredding. In this strategy, the surviving sperm are free to fertilise all available oocytes, and therefore, all of the offspring that are born are of the required sex.

One possible disadvantage of a released gene-drive genetic system is through mutational resistance arising at the sgRNA target site. If a nucleotide variant arises at the sgRNA target or the neighbouring protospacer-adjacent motif (PAM), then the CRISPR/Cas9 system becomes immediately dysfunctional. Mutational resistance to an embryonic-lethal gene drive could potentially spread efficiently through the population because of conferring a fitness advantage. Indeed, evidence for rapidly arising resistance alleles has been shown recently in flies [125] and mosquitos [126]. Current studies suggest that prevention of newly arising resistance mutations at gene-drive target loci would therefore require continuous intervention [91,127,128]. However, in laboratory or factory-maintained stocks, the issue of mutational resistance would not occur because these individuals could be simply removed from the population. One possible circumvention of the mutational resistance risk is to engineer many sgRNA transgenes targeting multiple loci. Therefore, even if mutational resistance occurs at one locus, the remainder are intact.

A second disadvantage associated with CRISPR/Cas9 gene drive is the risk that the above-mendelian frequency of inheritance spreads so rapidly through the population that the original wild-type allele is completely lost. The complete loss of the wild-type allele may cause apprehension against gene drive. Adaptations to gene-drive methods have been developed in order to prevent the uncontrolled spread of synthetic gene drives, called 'self-exhausting' gene drives, for example, the killer-rescue [129] or daisy-chain [130] models.

Summary and future outlook

Overall, there are many challenges still associated with producing transgenic mouse lines in order to produce single-sex litters. However, with careful consideration of which genes are targeted to induce embryonic lethality or sterility, bicomponent CRISPR/Cas9 methods could be widely employed to skew sex ratios. For example, targeting genes that are essential in postimplantation development may not result in a complete loss of the unrequired sex. Instead, an alternative approach may be to target essential housekeeping genes with roles in preimplantation development at embryonic genome activation. Embryonic lethality can then be induced very early, before the onset of organogenesis. Inducing embryonic lethality at preimplantation ensures firstly that embryos are nonviable prior to the onset of pain perception. Secondly, it would be interesting to determine whether embryo loss due to nonviability prior to implantation may allow extra viable embryos to implant, thereby compensating the litter size.

Even if all of the necessary optimisations are made and the technology for generating single-sex litters is consistent, there may be some apprehension regarding using genetically modified animal produce in agricultural industries. Utilising maternally loaded mRNAs such as the earlier described *Rosa26*-Cas9 may circumvent issues with genetically modified offspring for agricultural produce. Moreover, it may be that some consumers consider the use of transgenic animals in agriculture ethically preferable to the widespread culling of the unrequired sex. Indeed, there are some examples of genetically modified produce currently in the food industry, for example, a modified salmon species with increased growth rate [131], although it should be made clear that many regulatory limitations remain in place regarding the sale and consumption of genetically modified animal produce. It is important to continue the global conversation regarding the role of genetic modification in the agricultural industries. However, in the short term, it is more likely that the sex-selection strategies could be quickly and easily implemented for immediate reduction of postnatal animal culling in laboratory animals such as mice in line with the 3Rs.

Acknowledgments

The authors would like to thank Matthew Bentham and Daniel Snell for critical reading of the manuscript.

References

1. Home Office Statistics. Additional statistics on breeding and genotyping of animals for scientific procedures, Great Britain 2017 [Internet]. 2018 [cited 2020 Jun 26]. Home Office Statistical Bulletin 27;18. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/754408/breeding-genotyping-animals-scientific-procedures-2017-hosb2718.pdf
2. Russell WMS, Burch RL. The principles of humane experimental technique: London: Methuen & Co. Ltd.; 1959.
3. Krautwald-Junghanns ME, Cramer K, Fischer B, Forster A, Galli R, Kremer F, et al. Current approaches to avoid the culling of day-old male chicks in the layer industry, with special reference to spectroscopic methods. Poult Sci. 2018; 97(3):749–57. https://doi.org/10.3382/ps/pex389 PMID: 29294120

4. Dearden PK, Gemmell NJ, Mercier OR, Lester PJ, Scott MJ, Newcomb RD, et al. The potential for the use of gene drives for pest control in New Zealand: a perspective. Journal of the Royal Society of New Zealand. 2018; 48(4):225–44.
5. Just W, Rau W, Vogel W, Akhverdian M, Fredga K, Marshall Graves JA, et al. Absence of Sry in species of the vole Ellobius. Nature Genetics. 1995; 11(2):117–8. https://doi.org/10.1038/ng1095-117 PMID: 7550333
6. Soullier S, Hanni C, Catzeflis F, Berta P, Laudet V. Male sex determination in the spiny rat Tokudaia osimensis (Rodentia: Muridae) is not Sry dependent. Mammalian Genome. 1998; 9(7):590–2. https://doi.org/10.1007/s003359900823 PMID: 9657859
7. Sutou S, Mitsui Y, Tsuchiya K. Sex determination without the Y Chromosome in two Japanese rodents Tokudaia osimensis osimensis and Tokudaia osimensis spp. Mammalian Genome. 2001; 12(1):17–21. https://doi.org/10.1007/s003350010228 PMID: 11178738
8. Ford CE, Jones KW, Polani PE, De Almeida JC, Briggs JH. A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's syndrome). Lancet. 1959; 1(7075):711–3. https://doi.org/10.1016/s0140-6736(59)91893-8 PMID: 13642858
9. Jacobs PA, Strong JA. A case of human intersexuality having a possible XXY sex-determining mechanism. Nature. 1959; 183(4657):302–3. https://doi.org/10.1038/183302a0 PMID: 13632697
10. Eicher EM, Washburn LL, Whitney JB, Morrow KE. Mus poschiavinus Y chromosome in the C57BL/6J murine genome causes sex reversal. Science. 1982; 217(4559):535. https://doi.org/10.1126/science.7089579 PMID: 7089579
11. Berta P, Hawkins JR, Sinclair AH, Taylor A, Griffiths BL, Goodfellow PN, et al. Genetic evidence equating SRY and the testis-determining factor. Nature. 1990; 348(6300):448–50. https://doi.org/10.1038/348448A0 PMID: 2247149
12. Burgoyne PS, Buehr M, Koopman P, Rossant J, McLaren A. Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX—XY chimaeric mouse testes. Development. 1988; 102(2):443. PMID: 3166423
13. Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, et al. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature. 1990; 346(6281):245–50. https://doi.org/10.1038/346245a0 PMID: 2374589
14. Kashimada K, Koopman P. Sry: the master switch in mammalian sex determination. Development. 2010; 137(23):3921–30. https://doi.org/10.1242/dev.048983 PMID: 21062860
15. Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R. Male development of chromosomally female mice transgenic for Sry. Nature. 1991; 351(6322):117–21. https://doi.org/10.1038/351117a0 PMID: 2030730
16. Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature. 1990; 348(6300):450–2. https://doi.org/10.1038/348450a0 PMID: 2247150
17. Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, et al. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature. 1990; 346(6281):240–4. https://doi.org/10.1038/346240a0 PMID: 1695712
18. Cortez D, Marin R, Toledo-Flores D, Froidevaux L, Liechti A, Waters PD, et al. Origins and functional evolution of Y chromosomes across mammals. Nature. 2014; 508(7497):488–93. https://doi.org/10.1038/nature13151 PMID: 24759410
19. Veyrunes F, Waters PD, Miethke P, Rens W, McMillan D, Alsop AE, et al. Bird-like sex chromosomes of platypus imply recent origin of mammal sex chromosomes. Genome Res. 2008; 18(6):965–73. https://doi.org/10.1101/gr.7101908 PMID: 18463302
20. Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ, et al. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature. 2009; 461(7261):267–71. https://doi.org/10.1038/nature08298 PMID: 19710650
21. Capel B. Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nature Reviews Genetics. 2017; 18(11):675–89. https://doi.org/10.1038/nrg.2017.60 PMID: 28804140
22. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013; 339(6121):819–23. https://doi.org/10.1126/science.1231143 PMID: 23287718
23. Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. Elife. 2013; 2:e00471. https://doi.org/10.7554/eLife.00471 PMID: 23386978

24. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. Science. 2013; 339(6121):823–6. https://doi.org/10.1126/science.1232033 PMID: 23287722

25. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell. 2013; 153 (4):910–8. https://doi.org/10.1016/j.cell.2013.04.025 PMID: 23643243

26. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell. 2013; 154 (6):1370–9. https://doi.org/10.1016/j.cell.2013.08.022 PMID: 23992847

27. Weissmann A, Reitemeier S, Hahn A, Gottschalk J, Einspanier A. Sexing domestic chicken before hatch: a new method for in ovo gender identification. Theriogenology. 2013; 80(3):199–205. https://doi.org/10.1016/j.theriogenology.2013.04.014 PMID: 23726296

28. Rosenbruch M. [Early stages of the incubated chicken egg as a model in experimental biology and medicine]. ALTEX. 1994; 11(4):199–206. PMID: 11178387

29. Rosenbruch M. [The sensitivity of chicken embryos in incubated eggs]. ALTEX. 1997; 14(3):111–3. PMID: 11178496

30. Galli R, Preusse G, Schnabel C, Bartels T, Cramer K, Krautwald-Junghanns ME, et al. Sexing of chicken eggs by fluorescence and Raman spectroscopy through the shell membrane. PLoS ONE. 2018; 13(2):e0192554. https://doi.org/10.1371/journal.pone.0192554 PMID: 29474445

31. Eftekhaari TE, Nejatizadeh AA, Rajaei M, Soleimanian S, Fallahi S, Ghaffarzadegan R, et al. Ethical considerations in sex selection. J Educ Health Promot. 2015; 4:32. https://doi.org/10.4103/2277-9531.157184 PMID: 26097846

32. Pinkel D, Gledhill BL, Lake S, Stephenson D, Van Dilla MA. Sex preselection in mammals? Separation of sperm bearing Y and "O" chromosomes in the vole Microtus oregoni. Science. 1982; 218 (4575):904–6. https://doi.org/10.1126/science.6753153 PMID: 6753153

33. Johnson LA, Welch GR, Rens W. The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. J Anim Sci. 1999; 77 Suppl 2:213–20.

34. Dejarnette JM, Leach MA, Nebel RL, Marshall CE, McCleary CR, Moreno JF. Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: is comparable fertility of sex-sorted and conventional semen plausible? J Dairy Sci. 2011; 94(7):3477–83. https://doi.org/10.3168/jds.2011-4214 PMID: 21700034

35. Frijters AC, Mullaart E, Roelofs RM, van Hoorne RP, Moreno JF, Moreno O, et al. What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? Theriogenology. 2009; 71 (1):64–7. https://doi.org/10.1016/j.theriogenology.2008.09.025 PMID: 19004486

36. Garner DL. Hoechst 33342: the dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology. 2009; 71(1):11–21. https://doi.org/10.1016/j.theriogenology.2008.09.023 PMID: 18952273

37. Seidel GE Jr., Overview of sexing sperm. Theriogenology. 2007; 68(3):443–6. https://doi.org/10.1016/j.theriogenology.2007.04.005 PMID: 17512976

38. Maicas C, Holden SA, Drake E, Cromie AR, Lonergan P, Butler ST. Fertility of frozen sex-sorted sperm at 4×10^6 sperm per dose in lactating dairy cows in seasonal-calving pasture-based herds. Journal of Dairy Science. 2020; 103(1):929–39. Epub 2019 Oct 23. https://doi.org/10.3168/jds.2019-17131 PMID: 31668438

39. Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmiter RD. Genetically haploid spermatids are phenotypically diploid. Nature. 1989; 337(6205):373–6. https://doi.org/10.1038/337373a0 PMID: 2911388

40. Morales CR, Lefrancois S, Chennathukuzhi V, El-Alfy M, Wu X, Yang J, et al. A TB-RBP and Ter ATPase Complex Accompanies Specific mRNAs from Nuclei through the Nuclear Pores and into Intercellular Bridges in Mouse Male Germ Cells. Developmental Biology. 2002; 246(2):480–94. https://doi.org/10.1006/dbio.2002.0679 PMID: 12051831

41. Ventelä S, Toppari J, Parvinen M. Intercellular organelle traffic through cytoplasmic bridges in early spermatids of the rat: mechanisms of haploid gene product sharing. Mol Biol Cell. 2003; 14(7):2768–80. https://doi.org/10.1091/mbc.e02-10-0647 PMID: 12857863

42. Umehara T, Tsujita N, Shimada M. Activation of Toll-like receptor 7/8 encoded by the X chromosome alters sperm motility and provides a novel simple technology for sexing sperm. PLoS Biol. 2019; 17(8): e3000398. https://doi.org/10.1371/journal.pbio.3000398 PMID: 31408454

43. Chowdhury MMR, Lianguang X, Kong R, Park BY, Mesalam A, Joo MD, et al. In vitro production of sex preselected cattle embryos using a monoclonal antibody raised against bull sperm epitopes. Anim Reprod Sci. 2019; 205:156–64. https://doi.org/10.1016/j.anireprosci.2018.11.006 PMID: 30472064

44. Tran H, Ferrell W, Butt T. An estrogen sensor for poultry sex sorting. Journal of animal science. 2010; 88:1358–64. https://doi.org/10.2527/jas.2009-2212 PMID: 20081077

45. Yİlmaz-Dİkmen B, Dİkmen S. A morphometric method of sexing white layer eggs. Brazilian Journal of Poultry Science. 2013; 15(3):203–10.

46. Webster B, Hayes W, Pike TW. Avian Egg Odour Encodes Information on Embryo Sex, Fertility and Development. PLoS ONE. 2015; 10(1):e0116345. https://doi.org/10.1371/journal.pone.0116345 PMID: 25629413

47. Whittaker DJ, Soini HA, Gerlach NM, Posto AL, Novotny MV, Ketterson ED. Role of Testosterone in Stimulating Seasonal Changes in a Potential Avian Chemosignal. Journal of Chemical Ecology. 2011; 37(12):1349–57. https://doi.org/10.1007/s10886-011-0050-1 PMID: 22173888

48. Galli R, Preusse G, Uckermann O, Bartels T, Krautwald-Junghanns M-E, Koch E, et al. In Ovo Sexing of Domestic Chicken Eggs by Raman Spectroscopy. Analytical Chemistry. 2016; 88(17):8657–63. https://doi.org/10.1021/acs.analchem.6b01868 PMID: 27512829

49. Galli R, Preusse G, Uckermann O, Bartels T, Krautwald-Junghanns ME, Koch E, et al. In ovo sexing of chicken eggs by fluorescence spectroscopy. Anal Bioanal Chem. 2017; 409(5):1185–94. https://doi.org/10.1007/s00216-016-0116-6 PMID: 27966169

50. Harz M, Krause M, Bartels T, Cramer K, Rösch P, Popp J. Minimal Invasive Gender Determination of Birds by Means of UV-Resonance Raman Spectroscopy. Analytical Chemistry. 2008; 80(4):1080–6. https://doi.org/10.1021/ac702043q PMID: 18197696

51. Göhler D, Fischer B, Meissner S. In-ovo sexing of 14-day-old chicken embryos by pattern analysis in hyperspectral images (VIS/NIR spectra): A non-destructive method for layer lines with gender-specific down feather color. Poultry Science. 2017; 96(1):1–4. https://doi.org/10.3382/ps/pew282 PMID: 27591278

52. Pan L-q, Zhang W, Yu M, Sun Y, Gu X, Ma L, et al. Gender determination of early chicken hatching eggs embryos by hyperspectral imaging. 2016; 32:181–6.

53. Steiner G, Bartels T, Stelling A, Krautwald-Junghanns M-E, Fuhrmann H, Sablinskas V, et al. Gender determination of fertilized unincubated chicken eggs by infrared spectroscopic imaging. Analytical and Bioanalytical Chemistry. 2011; 400(9):2775–82. https://doi.org/10.1007/s00216-011-4941-3 PMID: 21479544

54. Clinton M, Nandi S, Zhao D, Olson S, Peterson P, Burdon T, et al. Real-Time Sexing of Chicken Embryos and Compatibility with in ovo Protocols. Sexual Development. 2016; 10(4):210–6. https://doi.org/10.1159/000448502 PMID: 27559746

55. Johnson LA, Flook JP, Hawk HW. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod. 1989; 41(2):199–203. https://doi.org/10.1095/biolreprod41.2.199 PMID: 2804212

56. Seidel GE Jr. Sexing mammalian sperm—Where do we go from here? J Reprod Dev. 2012; 58 (5):505–9. https://doi.org/10.1262/jrd.2012-077 PMID: 23124700

57. Husna AU, Azam A, Qadeer S, Awan MA, Nasreen S, Shahzad Q, et al. Sperm preparation through Sephadex™ filtration improves in vitro fertilization rate of buffalo oocytes. Reproduction in Domestic Animals. 2018; 53(2):377–84. https://doi.org/10.1111/rda.13117 PMID: 29239046

58. Ali JI, Eldridge FE, Koo GC, Schanbacher BD. Enrichment of Bovine X-and Y-Chromosome-Bearing Sperm with Monoclonal H-Y Antibody-Fluorescence-Activated Cell Sorter. Archives of Andrology. 1990; 24(3):235–45. https://doi.org/10.3109/01485019008987580 PMID: 2353847

59. Bennett D, Boyse EA. Sex Ratio in Progeny of Mice Inseminated with Sperm treated with H-Y Antiserum. Nature. 1973; 246(5431):308–9. https://doi.org/10.1038/246308a0 PMID: 4586316

60. Yadav SK, Gangwar DK, Singh J, Tikadar CK, Khanna VV, Saini S, et al. An immunological approach of sperm sexing and different methods for identification of X- and Y-chromosome bearing sperm. Vet World. 2017; 10(5):498–504. https://doi.org/10.14202/vetworld.2017.498-504 PMID: 28620252

61. Kawarasaki T, Sone M, Yoshida M, Bamba K. Rapid and simultaneous detection of chromosome Y- and 1-bearing porcine spermatozoa by fluorescence in situ hybridization. Molecular Reproduction and Development. 1996; 43(4):548–53. https://doi.org/10.1002/(SICI)1098-2795(199604)43:4<548::AID-MRD18>3.0.CO;2-V PMID: 9052947

62. Kobayashi J, Kohsaka T, Sasada H, Umezu M, Sato E. Fluorescence in situ hybridization with y chromosome-specific probe in decondensed bovine spermatozoa. Theriogenology. 1999; 52(6):1043–54. https://doi.org/10.1016/S0093-691X(99)00193-4 PMID: 10735111

63. Oi M, Yamada K, Hayakawa H, Suzuki H. Sexing of dog sperm by fluorescence in situ hybridization. The Journal of reproduction and development. 2013; 59(1):92–6. https://doi.org/10.1262/jrd.2012-098 PMID: 23059640

64. Rens W, Yang F, Welch G, Revell S, O'Brien PC, Solanky N, et al. An X-Y paint set and sperm FISH protocol that can be used for validation of cattle sperm separation procedures. Reproduction. 2001; 121(4):541–6. PMID: 11277872

65. Whyte JJ, Roberts RM, Rosenfeld CS. Fluorescent in situ hybridization for sex chromosome determination before and after fertilization in mice. Theriogenology. 2007; 67(5):1022–31. https://doi.org/10.1016/j.theriogenology.2006.11.014 PMID: 17215034

66. De Luca AC, Managó S, Ferrara MA, Rendina I, Sirleto L, Puglisi R, et al. Non-invasive sex assessment in bovine semen by Raman spectroscopy. Laser Physics Letters. 2014; 11(5):055604.

67. Falchi L, Khalil WA, Hassan M, Marei WFA. Perspectives of nanotechnology in male fertility and sperm function. International Journal of Veterinary Science and Medicine. 2018; 6(2):265–9. https://doi.org/10.1016/j.ijvsm.2018.09.001 PMID: 30564607

68. Hare WCD, Mitchell D, Betteridge KJ, Eaglesome MD, Randall GCB. Sexing two-week old bovine embryos by chromosomal analysis prior to surgical transfer: Preliminary methods and results. Theriogenology. 1976; 5(5):243–53.

69. Tiffin GJ, Rieger D, Betteridge KJ, Yadav BR, King WA. Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. J Reprod Fertil. 1991; 93 (1):125–32. https://doi.org/10.1530/jrf.0.0930125 PMID: 1920281

70. Edwards RG, Gardner RL. Sexing of Live Rabbit Blastocysts. Nature. 1967; 214(5088):576–7. https://doi.org/10.1038/214576a0 PMID: 6036172

71. Cotinot C, Kirszenbaum M, Leonard M, Gianquinto L, Vaiman M. Isolation of bovine Y-derived sequence: Potential use in embryo sexing. Genomics. 1991; 10(3):646–53. https://doi.org/10.1016/0888-7543(91)90447-m PMID: 1679747

72. Anderson GB. Identification of embryonic sex by detection of H-Y antigen. Theriogenology. 1987; 27 (1):81–97.

73. Asadpour R, Asadi MH, Jafari-Joozani R, Hamidian GH. Ovine fetal sex determination using circulating cell-free fetal DNA (ccffDNA) and cervical mucous secretions. Asian Pacific Journal of Reproduction. 2015; 4(1):65–9.

74. Focks DA. An Improved Separator for the Developmental Stages, Sexes, and Species of Mosquitoes (Diptera: Culicidae). Journal of Medical Entomology. 1980; 17(6):567–8. https://doi.org/10.1093/jmedent/17.6.567 PMID: 6111610

75. Zacarés M, Salvador-Herranz G, Almenar D, Tur C, Argilés R, Bourtzis K, et al. Exploring the potential of computer vision analysis of pupae size dimorphism for adaptive sex sorting systems of various vector mosquito species. Parasites & Vectors. 2018; 11(2):656.

76. Papathanos PA, Bossin HC, Benedict MQ, Catteruccia F, Malcolm CA, Alphey L, et al. Sex separation strategies: past experience and new approaches. Malaria Journal. 2009; 8(2):S5.

77. Lowe REL, Fowler JEF, Bailey DL, Dame DA, Savage KE. SEPARATION OF SEXES OF ADULT ANOPHELES ALBIMANUS BY FEEDING OF INSECTICIDE-LADEN BLOOD. Mosquito News. 1981; 41(4): 634–638.

78. Yamada H, Soliban SM, Vreysen MJ, Chadee DD, Gilles JRL. Eliminating female Anopheles arabiensis by spiking blood meals with toxicants as a sex separation method in the context of the sterile insect technique. Parasites & vectors. 2013; 6:197.

79. Knipling EF. Possibilities of Insect Control or Eradication Through the Use of Sexually Sterile Males1. Journal of Economic Entomology. 1955; 48(4):459–62.

80. Catteruccia F, Benton JP, Crisanti A. An Anopheles transgenic sexing strain for vector control. Nature Biotechnology. 2005; 23(11):1414–7. https://doi.org/10.1038/nbt1152 PMID: 16244659

81. Condon KC, Condon GC, Dafa'alla TH, Fu G, Phillips CE, Jin L, et al. Genetic sexing through the use of Y-linked transgenes. Insect Biochemistry and Molecular Biology. 2007; 37(11):1168–76. https://doi.org/10.1016/j.ibmb.2007.07.006 PMID: 17916503

82. Thomas DD, Donnelly CA, Wood RJ, Alphey LS. Insect Population Control Using a Dominant, Repressible, Lethal Genetic System. Science. 2000; 287(5462):2474. https://doi.org/10.1126/science.287.5462.2474 PMID: 10741964

83. Traut W, Sahara K, Marec F. Sex Chromosomes and Sex Determination in Lepidoptera. Sexual Development. 2007; 1(6):332–46. https://doi.org/10.1159/000111765 PMID: 18391545

84. Tan A, Fu G, Jin L, Guo Q, Li Z, Niu B, et al. Transgene-based, female-specific lethality system for genetic sexing of the silkworm, Bombyx mori. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(17):6766–70. https://doi.org/10.1073/pnas.1221700110 PMID: 23569267

85. Ant T, Koukidou M, Rempoulakis P, Gong H-F, Economopoulos A, Vontas J, et al. Control of the olive fruit fly using genetics-enhanced sterile insect technique. BMC Biology. 2012; 10(1):51.

86. Gong P, Epton MJ, Fu G, Scaife S, Hiscox A, Condon KC, et al. A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. Nature Biotechnology. 2005; 23(4):453–6. https://doi.org/10.1038/nbt1071 PMID: 15750586

87. Kandul NP, Liu J, Hsu AD, Hay BA, Akbari OS. A novel drug-inducible sex-separation technique for insects. bioRxiv 875716 [Preprint]. 2019 [cited 2020 Apr 20]. Available from: https://www.biorxiv.org/content/10.1101/2019.12.13.875716v1

88. Doran TJ, Morris KR, Wise TG, O'Neil TE, Cooper CA, Jenkins KA, et al. Sex selection in layer chickens. Animal Production Science. 2018; 58(3):476–80.

89. Windbichler N, Papathanos PA, Catteruccia F, Ranson H, Burt A, Crisanti A. Homing endonuclease mediated gene targeting in Anopheles gambiae cells and embryos. Nucleic Acids Res. 2007; 35 (17):5922–33. https://doi.org/10.1093/nar/gkm632 PMID: 17726053

90. Galizi R, Doyle LA, Menichelli M, Bernardini F, Deredec A, Burt A, et al. A synthetic sex ratio distortion system for the control of the human malaria mosquito. Nat Commun. 2014; 5:3977. https://doi.org/10.1038/ncomms4977 PMID: 24915045

91. Burt A. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. Proc Biol Sci. 2003; 270(1518):921–8. https://doi.org/10.1098/rspb.2002.2319 PMID: 12803906

92. Windbichler N, Papathanos PA, Crisanti A. Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in Anopheles gambiae. PLoS Genet. 2008; 4(12):e1000291. https://doi.org/10.1371/journal.pgen.1000291 PMID: 19057670

93. Galizi R, Hammond A, Kyrou K, Taxiarchi C, Bernardini F, O'Loughlin SM, et al. A CRISPR-Cas9 sex-ratio distortion system for genetic control. Sci Rep. 2016; 6:31139. https://doi.org/10.1038/srep31139 PMID: 27484623

94. Simoni A, Hammond AM, Beaghton AK, Galizi R, Taxiarchi C, Kyrou K, et al. A male-biased sex-distorter gene drive for the human malaria vector Anopheles gambiae. Nature Biotechnology. 2020. Epub 2020 May 11.

95. Fasulo B, Meccariello A, Morgan M, Borufka C, Papathanos PA, Windbichler N. A fly model establishes distinct mechanisms for synthetic CRISPR/Cas9 sex distorters. PLoS Genet. 2020; 16(3): e1008647. https://doi.org/10.1371/journal.pgen.1008647 PMID: 32168334

96. Cebrian-Serrano A, Zha S, Hanssen L, Biggs D, Preece C, Davies B. Maternal Supply of Cas9 to Zygotes Facilitates the Efficient Generation of Site-Specific Mutant Mouse Models. PLoS ONE. 2017; 12(1):e0169887. https://doi.org/10.1371/journal.pone.0169887 PMID: 28081254

97. Chu VT, Weber T, Wefers B, Wurst W, Sander S, Rajewsky K, et al. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotechnol. 2015; 33(5):543–8. https://doi.org/10.1038/nbt.3198 PMID: 25803306

98. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, et al. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell. 2014; 159(2):440–55. https://doi.org/10.1016/j.cell.2014.09.014 PMID: 25263330

99. Zhang Z, Niu B, Ji D, Li M, Li K, James AA, et al. Silkworm genetic sexing through W chromosome-linked, targeted gene integration. Proc Natl Acad Sci U S A. 2018; 115(35):8752–6. https://doi.org/10.1073/pnas.1810945115 PMID: 30104361

100. Ohno S. Sex chromosomes and sex-linked genes. (Monographs on endocrinology, Vol. 1.): Berlin, Heidelberg, New York: Springer Verlag.; 1967.

101. Bininda-Emonds OR, Cardillo M, Jones KE, MacPhee RD, Beck RM, Grenyer R, et al. The delayed rise of present-day mammals. Nature. 2007; 446(7135):507–12. https://doi.org/10.1038/nature05634 PMID: 17392779

102. Lahn BT, Page DC. Four evolutionary strata on the human X chromosome. Science. 1999; 286 (5441):964–7. https://doi.org/10.1126/science.286.5441.964 PMID: 10542153

103. Bachtrog D. Signs of genomic battles in mouse sex chromosomes. Cell. 2014; 159(4):716–8. https://doi.org/10.1016/j.cell.2014.10.036 PMID: 25417148

104. Bellott DW, Hughes JF, Skaletsky H, Brown LG, Pyntikova T, Cho TJ, et al. Mammalian Y chromosomes retain widely expressed dosage-sensitive regulators. Nature. 2014; 508(7497):494–9. https://doi.org/10.1038/nature13206 PMID: 24759411

105. Hughes JF, Page DC. The Biology and Evolution of Mammalian Y Chromosomes. Annual Review of Genetics. 2015; 49(1):507–27.

106. Wang H, Hu YC, Markoulaki S, Welstead GG, Cheng AW, Shivalila CS, et al. TALEN-mediated editing of the mouse Y chromosome. Nat Biotechnol. 2013; 31(6):530–2. https://doi.org/10.1038/nbt.2595 PMID: 23666012

107. Albrecht KH, Eicher EM. Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. Dev Biol. 2001; 240(1):92–107. https://doi.org/10.1006/dbio.2001.0438 PMID: 11784049

108. Greenfield A, Scott D, Pennisi D, Ehrmann I, Ellis P, Cooper L, et al. An H-YDb epitope is encoded by a novel mouse Y chromosome gene. Nat Genet. 1996; 14(4):474–8. https://doi.org/10.1038/ng1296-474 PMID: 8944031

109. Ehrmann IE, Ellis PS, Mazeyrat S, Duthie S, Brockdorff N, Mattei MG, et al. Characterization of genes encoding translation initiation factor eIF-2gamma in mouse and human: sex chromosome localization, escape from X-inactivation and evolution. Hum Mol Genet. 1998; 7(11):1725–37. https://doi.org/10.1093/hmg/7.11.1725 PMID: 9736774

110. Mazeyrat S, Saut N, Grigoriev V, Mahadevaiah SK, Ojarikre OA, Rattigan A, et al. A Y-encoded subunit of the translation initiation factor Eif2 is essential for mouse spermatogenesis. Nat Genet. 2001; 29(1):49–53. https://doi.org/10.1038/ng717 PMID: 11528390

111. Agulnik AI, Mitchell MJ, Lerner JL, Woods DR, Bishop CE. A mouse Y chromosome gene encoded by a region essential for spermatogenesis and expression of male-specific minor histocompatibility antigens. Hum Mol Genet. 1994; 3(6):873–8. https://doi.org/10.1093/hmg/3.6.873 PMID: 7524912

112. Shpargel KB, Sengoku T, Yokoyama S, Magnuson T. UTX and UTY demonstrate histone demethylase-independent function in mouse embryonic development. PLoS Genet. 2012; 8(9):e1002964. https://doi.org/10.1371/journal.pgen.1002964 PMID: 23028370

113. Zhao X, Wei W, Pan H, Nie J, Chen D, Zhang P, et al. Identification of the Sex of Pre-implantation Mouse Embryos Using a Marked Y Chromosome and CRISPR/Cas9. Scientific Reports. 2019; 9 (1):14315. https://doi.org/10.1038/s41598-019-50731-x PMID: 31586114

114. Yosef I, Edry-Botzer L, Globus R, Shlomovitz I, Munitz A, Gerlic M, et al. A genetic system for biasing the sex ratio in mice. EMBO Rep. 2019; 20(8): e48269. https://doi.org/10.15252/embr.201948269 PMID: 31267640

115. Curtis CF. Possible use of translocations to fix desirable genes in insect pest populations. Nature. 1968; 218(5139):368–9. https://doi.org/10.1038/218368a0 PMID: 5649682

116. Hamilton WD. Extraordinary Sex Ratios. Science. 1967; 156(3774):477. https://doi.org/10.1126/science.156.3774.477 PMID: 6021675

117. Serebrovsky A S. On the possibility of a new method for the control of insect pests. In: Sterile-Male Technique for Eradication or Control of Harmful Insects. Vienna: International Atomic Energy Agency; 1969. p. 123–237.

118. Esvelt KM, Smidler AL, Catteruccia F, Church GM. Concerning RNA-guided gene drives for the alteration of wild populations. Elife. 2014; 3: e03401. https://doi.org/10.7554/eLife.03401 PMID: 25035423

119. Kyrou K, Hammond AM, Galizi R, Kranjc N, Burt A, Beaghton AK, et al. A CRISPR–Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes. Nature Biotechnology. 2018; 36(11):1062–6. https://doi.org/10.1038/nbt.4245 PMID: 30247490

120. Grunwald HA, Gantz VM, Poplawski G, Xu XS, Bier E, Cooper KL. Super-Mendelian inheritance mediated by CRISPR-Cas9 in the female mouse germline. Nature. 2019; 566(7742):105–9. https://doi.org/10.1038/s41586-019-0875-2 PMID: 30675057

121. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem. 2010; 79:181–211. https://doi.org/10.1146/annurev.biochem.052308.093131 PMID: 20192759

122. Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, Ploegh HL. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol. 2015; 33(5):538–42. https://doi.org/10.1038/nbt.3190 PMID: 25798939

123. Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C, Katsanos D, et al. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. Nat Biotechnol. 2016; 34(1):78–83. https://doi.org/10.1038/nbt.3439 PMID: 26641531

124. Buchman A, Akbari OS. Site-specific transgenesis of the Drosophila melanogaster Y-chromosome using CRISPR/Cas9. Insect Molecular Biology. 2019; 28(1):65–73. https://doi.org/10.1111/imb.12528 PMID: 30079589

125. Champer J, Reeves R, Oh SY, Liu C, Liu J, Clark AG, et al. Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations. PLoS Genet. 2017; 13(7):e1006796. https://doi.org/10.1371/journal.pgen.1006796 PMID: 28727785

126. Hammond AM, Kyrou K, Bruttini M, North A, Galizi R, Karlsson X, et al. The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito. PLoS Genet. 2017; 13(10):e1007039. https://doi.org/10.1371/journal.pgen.1007039 PMID: 28976972

127. Noble C, Olejarz J, Esvelt KM, Church GM, Nowak MA. Evolutionary dynamics of CRISPR gene drives. Sci Adv. 2017; 3(4):e1601964. https://doi.org/10.1126/sciadv.1601964 PMID: 28435878

128. Unckless RL, Clark AG, Messer PW. Evolution of Resistance Against CRISPR/Cas9 Gene Drive. Genetics. 2017; 205(2):827–41. https://doi.org/10.1534/genetics.116.197285 PMID: 27941126

129. Gould F, Huang Y, Legros M, Lloyd AL. A killer-rescue system for self-limiting gene drive of anti-pathogen constructs. Proc Biol Sci. 2008; 275(1653):2823–9. https://doi.org/10.1098/rspb.2008.0846 PMID: 18765342

130. Noble C, Min J, Olejarz J, Buchthal J, Chavez A, Smidler AL, et al. Daisy-chain gene drives for the alteration of local populations. Proc Natl Acad Sci U S A. 2019; 116(17):8275–82. https://doi.org/10.1073/pnas.1716358116 PMID: 30940750

131. Waltz E. First genetically engineered salmon sold in Canada. Nature. 2017; 548(7666):148. https://doi.org/10.1038/nature.2017.22116 PMID: 28796219