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A drug-inducible sex-separation technique for insects

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Here, we describe a drug-inducible genetic system for insect sex-separation that demonstrates proof-of-principle for positive sex selection in D. melanogaster. The system exploits the toxicity of commonly used broad-spectrum antibiotics geneticin and puromycin to kill the non-rescued sex. Sex-specific rescue is achieved by inserting sex-specific introns into the coding sequences of antibiotic-resistance genes. When raised on geneticin-supplemented food, the sex-sorter line establishes 100% positive selection for female progeny, while the food supplemented with puromycin positively selects 100% male progeny. Since the described system exploits conserved sex-specific splicing mechanisms and reagents, it has the potential to be adaptable to other insect species of medical and agricultural importance.
Insects play an important role in genetic research, as they have short life cycles and are simple to work with and contain. Their experimental tractability and similarity of biological pathways to those in humans make them great model systems for basic research. The *Drosophila melanogaster* fruit fly was introduced by Thomas Morgan for the study of heredity in the early 20th century. Since then, a plethora of genetic tools and assays accumulated and shared by the fly community transformed *Drosophila* into one of the most widely used genetic model systems. The high level of gene conservation between flies and humans, in conjunction with a single gene ortholog in flies versus multiple paralogs in humans and mice, placed the *Drosophila* model at the forefront of studies of conserved gene functions, including research into human development and diseases. In addition to serving as a relatively simple translational model organism, *Drosophila* provides a model system for diverse insect pest and disease-vector species and frequently acts as a proof-of-concept system for innovative technologies.

The identification and separation of male and female insects are necessary in any genetic study. In *Drosophila* and many other insect species, special care must be taken to separate female flies before they mate, as females will store the sperm from the first mating in the spermatheca. However, current techniques for the sex sorting of insects, which often require sorting them by hand, are labor intensive, time consuming, and error prone, making this approach unsuitable for large-scale insect production, and that can be adopted to different insect species are required. Here we describe a positive, drug-inducible GSS system for insects and demonstrate its proof-of-principle in *Drosophila melanogaster*. Two genes conferring resistance to specific drugs are expressed in opposite sexes by incorporating sex-specific introns disrupting the coding sequences of drug-resistance genes. In the absence of sex selection, the transgenic strain harboring a sex-sorting gene cassette is maintained on normal food. When insects of a particular sex are desired, the transgenic strain is raised on food supplemented with the corresponding drug. Members of the sex selected against will not be resistant to the selecting drug, resulting in the emergence of adults of the desired sex. The described GSS system will still be susceptible to genetic recombination, chromosomal rearrangements and other loss-of-function mutations; however, these types of mutations would be selected against by positive drug selection making this system more robust than previously described GSS approaches.

**Results**

**Antibiotics inhibit *Drosophila* development.** To engineer a drug-inducible sex-selection system in *D. melanogaster*, we used two common antibiotic-resistance genes, *puromycin N-acetyltransferase* (PuroR) and *aminoglycoside phosphotransferase* (NeoR). These genes were previously demonstrated to confer resistance in eukaryotic cells, *D. melanogaster* S2 cells, and *D. melanogaster* larvae to the corresponding water soluble antibiotics, puromycin and geneticin (Fig. 1a), respectively. To determine the toxic doses for these drugs in *D. melanogaster*, we first raised wildtype (wt) fly larvae on food supplemented with increasing concentrations of either puromycin or geneticin (0, 0.2, 0.4 mg/ml). From this experiment, we determined that a concentration of 0.2 mg/ml of either drug was toxic, though it permitted the survival of some *D. melanogaster* larvae to adulthood (2.2 ± 1.5% for puromycin; and 14.0 ± 6.5% for geneticin, Supplementary Data 1), while concentrations of 0.4 mg/ml and above completely inhibited development, with almost no larvae able to mature past the first instar stage, and 100% of larvae perishing before adulthood on the supplemented food (for each treatment, expected n > 500 found 0; replicates [N] = 5; P < 0.001; two-sample Student’s t test with equal variance: Fig. 1b, Supplementary Data 1).

**PuroR or NeoR rescues the induced lethality.** After determining the toxic doses, we then tested if we could rescue this toxicity by the transgenic expression of antibiotic-resistance genes *PuroR* or *NeoR* integrated into the *D. melanogaster* genome. We engineered a piggyBac (PB) transposable element that encoded a constitutive baculovirus promoter *Hr5IE1* that drove expression of dsRed as a selectable marker (*Hr5IE1-dsRED*). To provide a consistent and ample supply of an antibiotic-resistant protein, we used a

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strong ubiquitous baculovirus promoter Opie2 to express the PuroR or NeoR gene (Opie2-PuroR or Opie2-NeoR) and inserted the gene cassette in an opposite orientation relative to the marker (Hr5IE1-dsRED) to avoid any transcriptional read-through effects. We generated several transgenic lines harboring a copy of either Opie2-PuroR or Opie2-NeoR and permitted them to lay eggs on fly food supplemented with either puromycin or geneticin, respectively. When non-balanced transgenic fly lines, which contained both transgenic and wt flies, were raised on food supplemented with either puromycin (0.4 mg/ml) or geneticin

Fig. 1 Development of sex-sorter cassette in Drosophila. a Chemical structures of puromycin and geneticin (G418). b Supplementing fly food with puromycin or geneticin to a final concentration of 0.4 mg/ml completely arrests development of wildtype (wt) D. melanogaster. Both drugs are also toxic to wt larvae at 0.2 mg/ml, but a few adult flies do emerge. c Fly survival in two independent transgenic lines harboring one copy of either Opie2-PuroR or Opie2-NeoR mixed with wt flies on food supplemented with 0.4 mg/mL of puromycin or geneticin. The PuroR and NeoR genes expressed under the Opie2 promoter rescued transgenic flies harboring one copy of a transgene on the corresponding drug, while all wt flies perished. Bar plots show the average ± one standard deviation (s.d.) over five biological replicates. Statistical significance was estimated using a t-test with equal variance. (***P < 0.001). d Schematic of genetic constructs engineered and tested in the study. The expression of antibiotic-resistance genes (PuroR and NeoR) throughout Drosophila development confers resistance to puromycin and geneticin, respectively, supplemented on fly food. To ensure that functional antibiotic-resistance proteins will be produced only in one or the other sex, sex-specific introns from two sex-determination genes (tra and dsx) were inserted into coding sequences of PuroR and NeoR. The entire sequences of female-specific tra and male-specific dsx introns (highlighted in pink) are spliced out in the corresponding sex, but some sequences carrying a stop codon (TGA) are retained in the opposite sex (Supplementary Fig. 1). The transgenic flies harboring one copy of a genetic construct were identified by the strong ubiquitous expression of dsRed (highlighted in purple). e Survival of females and/or males carrying the respective constructs when supplemented with the indicated antibiotic. Source data available in Supplementary Data 1-3.
together, these results strongly indicate that by inserting either the \textit{traF} or \textit{dsX} sex-specific introns into the coding sequence of \textit{PuroR} or \textit{NeoR} the production of functional antibiotic-resistance genes can be sex limited and mediate drug-inducible sex selection.

A sex-sorter cassette enables selection of both sexes. To achieve positive drug selection of either sex from a single construct (herein termed a sex-sorter cassette), we next tested whether we could combine the two separate sex-selection systems (\textit{Opie2-NeoR} + \textit{Opie2-PuroR} \textit{dsX}M, Fig. 1d). We engineered a sex-sorter plasmid, generated three independent transgenic lines harboring the cassette, and tested them by raising heterozygous flies on food supplemented with either puromycin or genetin at 0.4 and 1.0 mg/ml. For two of these tested lines, only female flies emerged on food supplemented with 1.0 mg/ml of genetin (\( n = 335 \), \( N = 6 \), \( P < 0.001 \), two-sample Student’s \( t \) test with equal variance), and only males were recovered from vials containing 1.0 mg/ml of puromycin (\( n = 210 \), \( N = 6 \), \( P < 0.001 \); two-sample Student’s \( t \) test with equal variance; Supplementary Data 1). The lower drug concentration was not sufficient to enforce the emergence of 100% single-sex progeny for each of these tested transgenic lines harboring only a single copy of the sex-sorter cassette (Fig. 2c). These results strongly indicate that by raising flies harboring one copy of the sex-sorter cassette on the food supplemented with either drug at 1.0 mg/ml, we can dominantly control which sex survives to adulthood.

We next explored an opportunity to lower the drug concentration and still enforce 100% efficient sex sorting by doubling the copy number of the sex-sorting cassette. The homozygous sex-sorter line established from the flies carrying the \textit{Opie2-NeoR} \textit{dsX} \textit{M} + \textit{Opie2-PuroR} \textit{dsX} \textit{M} cassette integrated on the third chromosome (line \#3 on Fig. 2c) did not produce any obvious fitness defect and was therefore used for further analysis. We raised the homozygous flies on food supplemented with either puromycin or genetin, titrating concentrations down from 1.2 mg/ml, and we quantified the percentages of each sex in the emerging flies. We discovered that the addition of puromycin at the final concentrations of 1.2, 1.0, 0.8, 0.6, and 0.4 mg/ml resulted in 100% male progeny. Even 0.2 mg/ml of puromycin caused a significant increase in the male to female ratio, from 43.0 ± 1.3% to 62.7 ± 2.15% (\( N = 3 \), \( P < 0.001 \); two-sample Student’s \( t \) test with equal variance; Fig. 3a). Inversely, supplementing the food with genetin to the final concentrations of 1.2, 1.0, 0.8, 0.6, 0.4, and 0.2 mg/ml resulted in 100% female progeny. For genetin, even 0.1 mg/ml led to a significant increase in female progeny, from 56.1 ± 1.1% to 78.0 ± 4.6% (\( N = 3 \), \( P < 0.001 \); two-sample Student’s \( t \) test with equal variance; Fig. 3b; Supplementary Data 2).

We investigated why the selection was not effective at lower drug concentrations, and came up with two possible reasons. First, the concentration may be too low to enforce effective selection, as wt flies can also survive at these lower concentrations. We previously found that wt flies cannot be raised on 0.4 mg/ml of either puromycin or genetin—fly development arrests at the 1st instar larval stage. To test whether wt flies can survive on concentrations lower than <0.4 mg/ml, we raised wt flies on 0.200, 0.100, 0.050, and 0.025 mg/ml of puromycin or genetin. Notably, while the development of flies raised on food supplemented with each antibiotic was delayed, some flies repeatedly emerged on concentrations ≤ 0.2 mg/ml for each drug, indicating that these concentrations are indeed too low for complete selection. The second reason the selection might not be effective at lower drug concentrations is that some antibiotics may degrade over time, becoming ineffective in selecting against the opposite sex. In our experiments, we found that puromycin was indeed unstable over time, as we observed that after collecting
exclusively male flies from the vials with 0.4 mg/ml of puromycin for eight straight days at +25 °C, a few female flies would emerge starting at day 9 (Supplementary Data 3). However, for geneticin at the same concentration (0.4 mg/ml), only female flies emerged from the vials supplemented with the antibiotic. This indicates that both wt fly survival and antibiotic degradation contribute to the lack of effective sex selection at concentrations lower than 0.4 mg/ml.

The sex-sorter cassette is not costly to Drosophila fitness. As the fertility of flies is very important for genetic experiments, we tested whether the antibiotic-mediated selection would affect the fertility of the recovered flies. To do so, we repeatedly tested the fertility of males and females carrying two copies of the sex-sorter cassette raised on food supplemented with either puromycin (male selection) or geneticin (female selection) at 0.4 mg/ml.

To estimate the fitness costs to the carriers of two copies of the sex-sorter cassette, we compared the fitness of homozygous sex-sorter flies to that of wt flies. We found that the survival rates,

Fig. 2 Positive selection of a specific sex. Sex-specific drug resistance is achieved by inserting the female-specific traF or male-specific dsxM intron into the coding sequences of PuroR and NeoR. a The efficiency of drug-induced sex sorting was assessed for a few independent transgenic lines of the same genetic construct. Transgenic flies harboring one copy of the antibiotic-resistance genes were raised on drug-supplemented food. When sex sorting was not 100% efficient, the higher drug concentration of 1.0 mg/ml was used. b Expression of Opie2-PuroRdsxM or Opie2-PuroRtraF transgene rescues only transgenic males or females (red fluorescence) raised on the food supplemented with puromycin, while all wildtype (wt) flies (no red fluorescence) and the transgenic flies of the selected-out sex die during early development. c Both antibiotic-resistance genes expressed in the two sexes were combined into one sex-sorter cassette, Opie2-NeoRtraF + Opie2-PuroRdsxM. Three independent transgenic lines harboring one copy of the sex-sorter cassette were tested on food supplemented with either puromycin or geneticin at 0.4 and 1.0 mg/ml. We found two transgenic lines that can produce 100% males or 100% females when raised on food supplemented with 1.0 mg/ml of geneticin or puromycin, respectively. Bar plots show the average ± one standard deviation (s.d.) over at least three biological replicates. Statistical significance was estimated using a t test with equal variance. (ns *P ≥ 0.05, *P < 0.05, **P < 0.01, and ***P < 0.001). Source data available in Supplementary Data 1–3.
calculated as a percentage of embryos surviving to adults, for both lines raised on non-supplemented food, were not statistically different from wt flies: 91.2 ± 2.8% of wt embryos survived to adulthood versus 90.4 ± 8.1% of transgenic sex-sorter embryos (N = 5, P = 0.85; two-sample Student’s t test with equal variance, Fig. 3c). Moreover, no flies survived to adulthood when supplementing fly food with both puromycin and geneticin at either 0.4 or 1.2 mg/ml, which was expected since neither wt nor flies harboring two sex-sorter cassettes are resistant to both drugs simultaneously (Fig. 3c). To further measure fitness, we compared the survival rates of each sex, normalized as a percentage of male or female embryos surviving to adults, compared between wt flies raised on food without any antibiotics and the homozygous sex-sorter flies raised on food supplemented with either puromycin or geneticin. The percentage of wt males that emerged on non-supplemented food was similar to that of sex-sorter males that emerged from the food supplemented with puromycin at 91.2 ± 1.9% versus 90.6 ± 3.2% (N = 5, P = 0.93, two-sample Student’s t test with equal variance) and 91.2 ± 4.3% (N = 5, P = 0.77, two-sample Student’s t test with equal variance; Fig. 3d). Similarly, the percentage of hatched wt females was not significantly different from sex-sorter females that emerged on the food supplemented with geneticin at concentrations of either 0.4 or 1.2 mg/ml: 91.4 ± 1.7% vs 91.2 ± 2.8% (N = 5, P = 0.93, two-sample Student’s t test with equal variance) and 90.7 ± 4.5% (N = 5, P = 0.77, two-sample Student’s t test with equal variance; Fig. 3d; Supplementary Data 3).

Discussion

We describe the proof-of-concept of a GSS approach in Drosophila. Its design is based on the sex-specific expression of two antibiotic-resistance genes (NeoR or PuroR), which is accomplished by incorporating introns that splice in a sex-specific manner, traf and dsxM. Females or males are positively selected by rescuing their development on food supplemented with either geneticin or puromycin, respectively. The described drug-inducible GSS approach has several advantages over other traditional genetic and mechanical methods for insect sex sorting: genetic stability, positive selection, potential portability across different insect species, low maintenance requirements (i.e. does not need to be supplemented during maintenance), low fitness costs, and potential for adaptability for high-throughput sex sorting.

While this system is still susceptible to mutations such as chromosomal translocations, recombination, and loss-of-function mutations, these types of mutations would likely be selected against when exposed to the antibiotics, as opposed to other GSS which could be broken by these kinds of mutations. For example, traditionally, the construction of genetic sexing lines was based on linking a selectable marker gene, such as insecticide resistance, to a Y-autosome or X-autosome via induced chromosomal translocations. Such engineered lines are not stable, and chromosomal rearrangements will break the genetic linkage between a selective marker and a sex chromosome. In fact, it was found that when large...
numbers of insects were produced from an engineered GSS line, rare chromosomal rearrangements persisted or were selected for and would contaminate the original line, which made it unusable for sex sorting. Given that our GSS mechanistically relies on sex-specific alternative splicing—an splicing in a wrong sex may "break" function of the sex-sorter cassette.

The sex-sorter gene cassette was designed to be widely transferable through the use of the PB transposable element, which has been shown to be portable across many insect species. Pur-ferable through the use of the PB transposable element, which has a cassette.

Moreover, any breakage event that causes a loss-of-function mutation in the NeoR or PuroR antibiotic-resistance genes will be selected out during drug exposure, since the flies harboring loss-of-function mutations will not survive on food supplemented with the corresponding antibiotic assuring that surviving individuals harbor a functional sex-sorter gene cassette. Only gain-of-function mutations, which are very rare, conferring the dominant splicing in a wrong sex may "break" function of the sex-sorter cassette.

The sex-sorter gene cassette was designed to be widely transferable through the use of the PB transposable element, which has been shown to be portable across many insect species. Pur-ferable through the use of the PB transposable element, which has a cassette.

The sex-sorting gene cassette does not directly affect the fitness of its carriers. Unlike Tet-Off systems with conditional lethal transgenes, no antibiotic is required for survival and maintenance of the sex-sorter strain, meaning continuous drug feeding is not necessary during mass rearing. The antibiotics, puromycin or geneticin, are supplied only to enforce the sex selection by rescuing the selected sex (positive selection) and "killing" the opposite sex. We demonstrate that drug selection occurs early on, at the first instar stage, and thereafter the surviving sex can be maintained on a regular food. This transient exposure of antibiotics could further reduce any potential fitness costs and could also reduce costs associated with drug selection, as smaller quantities could be used at only the early instar stages—a factor that may play a significant role in large-scale projects.

The sex-sorting gene cassette does not directly affect the fitness of its carriers. Unlike GSS methods that use negative selection, the sex-sorter cassette does not include a toxin or suicide gene that could leak and affect the organismal fitness. However, the location of transgene integrations in the Drosophila genome can affect fitness of transgenic flies, and we therefore assessed multiple integration sites for each genetic construct (Fig. 2c). The homozygous sex-sorter transgenic line generated in the study harbors two copies of the sex-sorter cassette (i.e. homozygous) and has the same egg-to-adult survival rate for each sex as compare with wt flies, even when the transgenic flies were raised on food supplemented with antibiotics to enforce sex selection (Fig. 3d).

We also confirmed that males sex sorted on the highest antibiotic concentration were able to court and mate with wt females. The fitness of sex-sorted males is of great importance, since many insect control methods, such as sterile insect technique, release of insects carrying a dominant lethal, and Wolbachia-mediated incompatible insect technique rely on male releases. Taken together, our synthetic genetic circuit that relies on the positive, instead of negative, selection can ameliorate some of the effects of a negative selection on organismal fitness.

The positive drug-inducible GSS system presented here complies with the seven key requirements for efficient sex separation technology proposed to Papathanos et al., referred as the "7 Ses". (1) Small: the sex selected against dies early in development and does not compete for resources with the selected sex. (2) Simple: a required sex is positively selected by simply raising the transgenic insects on food supplemented with a drug. (3) Switchable: the sex-sorter transgenic flies can be maintained on a regular food. (4) Stable: the constitutive expression of functional antibiotic-resistance genes in a sex-specific manner guarantees survival of a specific sex on drug-supplemented food, and any loss-of-function mutations in the antibiotic-resistance gene are selected against. (5) Stringent: our data demonstrate that the sex sorting is enforced at 100%. (6) Sexy: the sex sorting happens genetically during insect development and does not required insect handling. It is mediated by positive selection, and its genetic circuit does not include any toxin or suicide genes, which results in minimal to no observable reduction to fitness. (7) Sellable: we designed this system to utilize mechanisms (e.g. mechanistically rely on sex-specific alternative splicing which is conserved in many insects) and components (piggybac, a transposon shown to function in many insects), and baculovirus promoters to drive expression of marker and resistance genes, antibiotics (e.g. puromycin and geneticin), and antibiotic resistance genes (e.g. PuroR and NeoR) that should be portable to many insects in the future.

Finally, it has not escaped our attention that the technology proposed herein, with its use of antibiotic resistance, could pose concerns due to the increasing antibiotic resistance worldwide. This feature needs to be taken into consideration if this technology is used to generate insects for pest control purposes. Notwithstanding, to mitigate these concerns we have included safeguards in our system to prevent function in prokaryotes. For example, the insertion of an intron into the coding sequence of an antibiotic-resistance gene will block its translation in prokaryotes since the introns will not be spliced. The antibiotic-resistance genes harboring introns in their coding sequences will not confer selective advantage and will not spread through the horizontal gene transfer to and between prokaryotes. Moreover, an antibiotic-resistance gene will have a real advantage only to the extent that the antibiotic concentrations are so high that they cause the death or slow the growth of other species in the environment, which would already suggest a problem of a different magnitude—that the antibiotic concentrations in the environment are literally sterilizing the environment, which seems unlikely. We suggest that the insertion of introns into transgenes to break their coding sequences could be both a useful strategy to improve their expression levels in Drosophila, but also provide a promising strategy to safeguard against potential spread in prokaryotes via any possible horizontal gene transfer, especially when transgenes are intended for field releases.

Methods
Antibiotics and antibiotic-resistance genes. Puromycin is a water soluble aminonucleoside antibiotic produced by the bacterium Streptomyces alboniger; it inhibits protein synthesis by disrupting peptide transfer on ribosomes, causing
premature chain termination during translation, and is thus a potent translational inhibitor in both prokaryotic and eukaryotic cells.\(^4\) Geneticin (G418) is a water soluble aminoglycoside antibiotic produced by the bacteria \(Neomycoris rhodanearia\); it interferes with 80S ribosomes and protein synthesis, and is therefore commonly used as a selective agent for eukaryotic cells.\(^{\text{www.thermoﬁsher.com}}\). Puromycin (Puro)\(^{\text{p}}\) from Streptomyces alboniger encodes puromycin \(N\)-acetyltransferase, and its expression in bacteria and mammalian cells confers resistance to puromycin (Neo)\(^{\text{n}}\) and has become a transposon of choice for genetic engineering of a wide variety of species, particularly insects.\(^7\) We used Gibson assembly to engineer the genetic constructs. The protein sequences of Puromycin (Puro)\(^{\text{p}}\) and Neomycin (Neo)\(^{\text{n}}\) were back translated, codon optimized for Drosophila in Gene Designer 2.0 (https://www.genes20.com/resources/genedesigner), and synthesized as gene blocks by Integrated DNA Technologies\(^8\). Both genes were expressed constitutively under Opie2 regulatory sequences that originated from the baculovirus \(Oryzias pseudotutugata\) multicapsid nuclear polyhedrosis virus\(^2\) and were amplified from the JC35276 plasmid\(^9\). The transformation marker dsRed, a red fluorescent protein, was amplified from the KC738375 plasmid\(^10\) and was ubiquitously expressed hormogonial \(UAS\)-mediated regulatory sequence amplified from the KC991096 plasmid\(^7\). The SV40 3UTR sequence from pAc5-1.5-V5-HisB (Invitrogen)\(^{11}\) terminatd the transcription of the transgenes. To confer the sex-specific translation of Puromycin and Neomycin, the \(D.\) melanogaster \(UAS\) female-specific intronic (traF) located between tra exon 1 and 2 (3L: 16591093-16590756, 248 bases, FlyBase.org) or the \(D.\) melanogaster \(UAS\) male-specific intronic (dazx exon 9) located between dazx exon 8 and 9 (3R: 7930688-7937567, 5080 bases) was inserted inside coding sequences of antibiotic-resistance genes. The plasmids generated in the study and their complete sequences (Fig. 1c) were deposited at Addgene.org (#131613–131618).

### Fly transgenesis

The generated PB plasmids were injected into \(w^{118}\) flies at Rainbow Transgenic Flies, Inc. (http://www.rainbowgene.com). Recovered transgenic lines were balanced on the 2nd and 3rd chromosomes using single chromosome balancer lines \(w^{118}, CyO,\text{smma}^{12}\) for II, and \(w^{115}, \text{TMsx}^{13}/\text{Tm6B}, \text{TB}\) for III) or a double-chromosome balancer line \(w^{115}, \text{CyO}/\text{Sp}, \text{Df(TM6C, Sb)}^{14}\). Multiple independent lines on the 2nd and/or 3rd chromosome were recovered for each plasmid and were tested on food supplemented with puromycin or genetin. We used heterozygous transgenic lines harboring one copy of a transgene to assess the antibiotic resistance and sex-sorting efficiency. One transgene line harboring the complete sex-sorter cassette integrated on the 3rd chromosome supported 100% sex sorting for both sexes, was homozygous fertile, and demonstrated especially good fitness. This line with two copies of the sex-cassette cassette was used for further analysis and was also deposited at the Bloomberg Drosophila Stock Center (BDSC #79015).

### Genetics and sex selection

Flies were maintained on cornmeal, molasses, and yeast medium (Old Bloomberg Molasses Recipe) at 25 °C with a 12:12H12L light/dark cycle. To assess drug resistance and/or sex selection, we used Instant Drosophila Food (Formula 4–24) from the Carolina Biological Supply Company. Per fly vial (FlyStamens.com), 1.1 g of dry food was mixed with 5 ml of distilled water supplemented with puromycin (Sigma #P8833) or geneticin (G418, Sigma #A1720) in varying concentrations from 0 to 1.2 mg/ml. To assess the drug resistance and/or sex sorting of the transgenic flies, a mixture of wt and transgenic flies harboring one copy of a transgene were allowed to breed on the supplemented food. Once the third instar larvae began to appear, the parents were removed. After hatching, the adult offspring, their transgenic markers, and their sex were recorded. Two or three independent transgenic lines integrated on the 2nd or 3rd chromosome were analyzed on food supplemented with puromycin and/or geneticin at 0.4 and 1.0 mg/ml. Since puromycin is known to be unstable over time in water solutions, we counted emerging flies and noted their sex for only 7 days after the first fly emerged. Three or five replicates were performed for each concentration.

### Fitness estimation

To compare fly fitness on different food regimens, we calculated the percentage of embryos that survived to adulthood on the Instant Drosophila Food (Formula 4–24). Large numbers of Drosophila embryos were staged and collected on grape juice agar plates that were fitted into embryo collection cages (Genesee Scientific, FS59-100) following the stated protocol. In short, 50–80 flies were transferred into embryo collection cages and laid many eggs on grape juice plates fitted on the bottom of a cage. The grape juice plates were replaced, and the embryos that were laid on the plates overnight were collected in the morning. Embryos of both wt and transgenic flies harboring two copies of the sex-sorter cassette were collected. Batches of seventy five embryos were placed on the insect food supplemented with 0.0, 0.4, and 1.2 mg/ml of puromycin or geneticin, and the number and sex of the emerging adult flies were recorded for each condition. For the sex-sorter line raised on foods with different antibiotic concentrations, the embryo-to-adult survival rate of either males or females on drug-supplemented foods was compared with that of the corresponding sex for wt embryos developed on the food without any antibiotics. In other words, the survival percentages for each sex were compared and presented as normalized percentages (Fig. 3d). The embryo-to-adult survival rate was estimated from five biological replicates. To assess the mating competence of the sex-sorted males, the males raised on the food supplemented with puromycin at the concentration of 1.2 mg/ml were placed into vials with virgin wt females and their progeny was scored for dsRed.

### Drug selection stage

To determine the larval stage at which sex selection occurs, we fed first instar wt larvae with a yeast paste with or without the drug at 0.4 mg/ml and observed the larval stage to which they survived. \(D.\) melanogaster embryos were staged and collected on grape juice agar plates for a comparison of the fitness. Then, the embryos were transferred on agar plates whose surfaces were spread with a yeast paste supplemented with drugs. The plates were incubated at 25 °C, and the endpoint of embryo development was observed and recorded.

### Statistical analysis

Statistical analysis was performed in JMP 8.0.2 by SAS Institute Inc. The percentages of a specific sex or embryo-to-adult survival were compared with the corresponding values estimated for the wt flies (Fig. 3a–c). \(P\) values were calculated for a two-sample Student’s t test with equal variance.

### Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

### Data availability

All data underlying Figs. 1, 2, and 3 are represented fully within Supplementary Data 1–3. The plasmids constructed in the study (Fig. 1c) were deposited at Addgene.org (#131613–131618). The homozygous sex-sorter lines was deposited at Bloomington Drosophila Stock Center (#79015). The remaining Drosophila lines will be made available upon request. Any other relevant data are available from the authors upon reasonable request.

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Author contributions
O.S.A conceived the idea, engineered plasmids, and carried out preliminary experiments. O.S.A and N.P.K designed experiments. N.P.K, A.D.H., and J.L. performed all molecular and genetic experiments. All authors analyzed the data, contributed to the writing of the manuscript, and approved the final manuscript.

Competing interests
O.S.A and B.A.H filed the US patent application (#20150237838) describing this technology. O.S.A has an equity interest in Agragene, Inc. and serves on the company’s Scientific Advisory Board. All the other authors have no competing interests.

Additional information
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