Tetracycline-suppressible female lethality and sterility in the Mexican fruit fly, *Anastrepha ludens*

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Abstract

The sterile insect technique (SIT) involves the mass release of sterile males to suppress insect pest populations. SIT has been improved for larval pests by the development of strains for female-specific tetracycline-suppressible (Tet-off) embryonic lethal systems for male-only populations. Here we describe the extension of this approach to the Mexican fruit fly, *Anastrepha ludens*, using a Tet-off driver construct with the Tet-transactivator (tTA) under embryo-specific *Anastrepha suspensa serendipity α* (As-sry-α) promoter regulation. In the absence of tetracycline, tTA acts upon a Tet-response element linked to the pro-apoptotic cell death gene lethal effector, *head involvation defective* (hid), from *A. ludens* (*AlhidAla2*) that contains a sex-specific intron splicing cassette, resulting in female-specific expression of the lethal effector. Parental adults double-homozygous for the driver/effector vectors were expected to yield male-only progeny when reared on Tet-free diet, but a complete lack of oviposited eggs resulted for each of the three strains tested. Ovary dissection revealed nonvitellogenic oocytes in all strains that was reversible by feeding females tetracycline for 5 days after eclosion, resulting in male-only adults in one strain. Presumably the sry-α promoter exhibits prezygotic maternal expression as well as zygotic embryonic expression in *A. ludens*, resulting in a Tet-off sterility effect in addition to female-specific lethality.

Keywords: female-specific lethality, apoptosis, cell death, sterile insect technique, insect pest management.

Introduction

Tephritid fruit flies are critically important agricultural pest insect species owing to their invasiveness and large plant host range. One of them is the Mexican fruit fly (*mexfly*), *Anastrepha ludens*, which is indigenous to Mexico and much of Central America as far south as Costa Rica. Besides permanent presence in northeast Mexico and Texas (eradication on-going) there have been outbreaks not only in California, but also in Arizona and in Florida (eradication programmes in Florida in 1934, 1972 and 2003).

Control of endemic populations and prevention of their spread to non-infested areas is the primary goal of biologically based control strategies as part of integrated pest management programmes. The most effective of these strategies is the sterile insect technique (SIT; Knipling, 1955; Krafsur, 1998), which has been successful in suppressing the populations of several tephritid species. SIT is based on the idea that over-flooding a field population with sterile males (sterilized by irradiation or other means) over successive generations will lead to infertile matings and thus suppress, and thereby control, populations of the targeted pest species (Knipling, 1955). Thus, effective SIT requires the mass rearing and release of sterile pharate adult male insects that can competitively mate with females in the field. SIT is being used in various regions of Mexico as part of an integrated approach against *A. ludens*. It is also being used to eradicate *A. ludens* from the citrus production areas in southern Texas, and has been used to eliminate small outbreaks of *A. ludens* in California. Since 1991, the government of Mexico has been operating a country-wide fruit fly control programme aimed at suppressing and eradicating endemic fruit flies in order to establish and maintain low prevalence and fly free
areas. A retrospective economic assessment conducted for the period 1994–2008 indicated a positive return on investment for several Mexican states including Baja California, Guerrero, Nuevo Leon, Sinaloa, Sonora and Tamaulipas (Salcedo-Baca Diznarda et al., 2010). The benefit to cost ratio ranged from 4.2 to 25.7. Without the control programme, it was estimated that direct losses owing to yield reduction and restrictions in commercialization would have been US $397.7 million, apart from the substantial indirect losses including lost jobs and environmental impact (Enkerlin, 2005). The study also analysed the economic returns for two of the major production and export crops produced in these states, mango and citrus. During the study period, the net benefits accrued from production and exports were estimated to be US $960 million and US $1.06 billion, respectively. This clearly demonstrates that investments to control these insect pests paid out.

For many species including A. ludens, however, females cannot be eliminated or separated from males early in development, requiring their rearing, sterilization and/or field-release with their male siblings (Benedict & Robinson, 2003), which diminishes programme efficiency, increases costs and prevents its use for many additional pest and vector species.

Therefore, sexing strains have been created for several tephritid species using pupal colour mutations in females that can be distinguished from the wild type (WT) by automated sorters. Males, however, retain the WT phenotype by a translocation of the WT allele onto the Y-chromosome, allowing their separation from females during the pupal stage. The same strategy has been used in the medfly, Ceratitis capitata, but using a temperature-sensitive lethal (tsl) mutation to eliminate female embryos by elevated temperature. Both systems are highly preferable to female release, but separation of females during pupation still requires their rearing, and the tsl approach requires genetic markers and maps not available in most other species. An alternative to these ‘classical’ genetic approaches to sexing in tephritid species is molecular genetic manipulations that eliminate females during rearing. This has been achieved by creating transgenic female-specific conditional lethal strains, primarily by using the tetracycline-suppressible (Tet-off) regulatory system to control lethal effector expression. To develop male-only strains for sterilization and release, we previously tested a female-specific lethality system that acts early in embryogenesis in the Caribbean fruit fly, Anastrepha suspensa (Schetelig & Handler, 2012a,b). This used the Tet operon system from Escherichia coli (Gossen & Bujard, 1992) where the Tet transactivator (tTA) was controlled by the embryonically active A. suspensa serendipity z (As-sry-z) promoter, and the Tet response element (TRE; which is activated by tTA), which was linked to the phospho-mutated pro-apoptotic cell death gene, A. ludens head involution defective (hidAla2 (Al-hidAla2), for the lethal effector construct (Schetelig & Handler, 2012a). To limit Al-hidAla2 expression to female embryos, the C. capitata transformer first intron (Cctra; Pane et al., 2002), which is sex-specifically spliced revealing a stop codon only in males, was inserted 3’ to the ATG translational start site, to create the female-specific lethal effector, TRE-Cctra1-Al-hidAla2 (Schetelig & Handler, 2012b). This largely heterologous tTA promoter-driver and lethal effector combination was highly successful in double homozygotes, yielding complete female lethality in several caribfly strains when parental females were removed from Tet-diet. A similar strategy was proven successful in the Mediterranean fruit fly (medfly), C. capitata (Ogaugwu et al., 2013). Importantly, unlike the Tet-off female-specific release of insects with dominant lethality (tsRIDL) system where female lethality is limited to pupae (Fu et al., 2007), the transgenic embryonic sexing system (TESS) eliminates females prior to the larval stage, avoiding the high cost of rearing. Release of female-specific lethal transgenics has also been proposed as a population suppression strategy, in which female progeny of surviving males would die as pupae (Leftwich et al., 2014), although surviving male and female larval progeny would damage host plants at each new generation.

Given the effectiveness of TESS in other tephritids, especially in A. suspensa, it was logical to test the system in the more agriculturally important species, A. ludens, with the expectation that the constructs used in caribfly would be similarly effective in mexfly. Importantly, as the Al-hidAla2 lethal effector was highly effective in caribfly, its function in A. ludens was assumed, although heterologous function of the As-sry-z promoter was less certain. In this report we, indeed, show that the caribfly Tet-off embryonic sexing system is equally effective in mexfly, except for a significant difference. In addition to the expected lethality effect, sterility was also observed in parental females not fed with Tet-diet immediately after eclosion, by preventing formation of vitellogenic oocytes. This has implications for how this female-specific lethality system is implemented in mexfly, and also illustrates the more general necessity to fully understand orthologous and heterologous regulatory element function in other species before their use in genetic modification strategies.

Results

Generation of transgenic lines for female-specific lethality

To create a TESS for A. ludens, piggyBac transformation vectors that include the Tet-off cassettes for the embryonic tTA driver (pBacXLII(UBsDsRed.T3, As-sry-z-tTA); D-419) and female-specific lethal effector (pBacXLII(UBsnBlEgFP, TRE-Cctra1-Al-hidAla2); E-443), previously tested in A. suspensa (Schetelig & Handler, 2012a,b), were transformed
expression of hemizygous driver lines was quantified by relative quantitative real-time PCR (qPCR) on the assumption that the strongest lines would be the most promising for TESS development. Of seven driver lines tested, relatively high tTA expression was detected in lines D-419_2x, D-419_11 and D-419_17, in order of increasing expression (Fig. 1, Table S1).

The genomic insertion sites of the three driver lines as well as the single effector line, E-443-1, were then sequenced to elucidate potential insertion site effects on transgene expression or host fitness owing to insertional mutations. The vector insertion sites were sequenced by inverse PCR gene amplification of ligated MspI-digested DNA pools of each transgene. Successful amplicons were isolated from the strains D-419-7, D-419-17 and E-443-1 with integration sites identified by BLAST analysis (Altschul et al., 1997; Data S1). Interestingly, the transgene in the line D-419-11 integrated into the transformer intron and the E-443-1 transgene integrated in a mariner Mos1-like transposase gene, whereas there was no hit for the D-419-17 integration site.

Female-specific lethality of progeny double-hemizygous for the Tet-off transgenes

To evaluate sex-specific lethality in progeny double-hemizygous for both the driver and effector transgenes, the three double-homozygous lines, D-419-2x_E-443-1, D-419_11_E-443-1 and D-419-17_E-443-1, were outcrossed to A. ludens WT females. Progeny of line D-419-17_E-443-1, carrying single copies of the driver and effector constructs, resulted in 99% male progeny when parentals and progeny were reared on Tet-free diet (Fig. 2, Table S1). Based on the percentage of surviving larvae, it could be concluded that lethality occurred during embryogenesis and larval stages. By contrast, crosses of D-419-2x_E-443-1 and D-419-11_E-443-1 males to WT females had little to no effect on the sex ratio of their progeny. Possibly, the spatial expression of the driver transgene was insufficient because of the D-419-2x transgene being integrated on the X chromosome and the D-419-11 being located in an intron of the sex-specifically spliced transformer gene.

Female-specific lethality of progeny double-homozygous for the Tet-off transgenes

The practical application of a successful sexing system requires the separation or elimination of females from males at an early developmental stage to avoid the significant costs for their mass rearing. Previous studies in caribly and medfly indicated that for several driver/effector lines, sexing was more efficient in double-homozygous lines relative to double-heterozygous lines for the same transgenes, presumably because of a

Figure 1. Relative embryonic tetracycline-transactivator (tTA) expression of D-419 driver lines. Expression of tTA relative to the D-419-3 strain was recorded by quantitative real-time PCR. Data were normalized against Histone 3 expression levels.
dosage effect (Schetelig & Handler, 2012a,b; Ogaugwu et al., 2013). As use of a double-homozygous strain maintained on Tet-diet is also the simplest means of generating progeny for sexing (rather than mating a homozygous strain to WT), it was necessary to test the efficacy of female lethality for these progeny on Tet-free diet, which we expected to be more efficient relative to hemizygous progeny. Therefore, the double-homozygous driver/effector sexing lines D-419-2x_E-443-1, D-419-11_E-443-1 and D-419-17_E-443-1 were tested for female-specific lethality when parental females and their progeny were reared on Tet-free diet.

Parental adults from the homozygous lines were reared on Tet-free adult diet for egg collection, although none of the females from any of the three lines oviposited eggs. Upon dissection, only nonvitellogenic ovaries were observed in parental females (Fig. 3) whereas WT female control flies on Tet and Tet-free diet exhibited normal vitellogenic oocytes. To determine if this result was a Tet-dependent effect, newly emerged adults were fed on Tet-containing diet A100 (100 µg/ml Tet) for either 5 or 10 days. Normal ovarian development was observed in the parental females, and oviposition was initiated by 10 days after eclosion, similar to WT controls.

To compare larval hatching, total survival rate and sex-specific lethality in these progeny, the three lines were reared on Tet-free adult diet for egg collection, although none of the females from any of the three lines oviposited eggs. Upon dissection, only nonvitellogenic ovaries were observed in parental females (Fig. 3) whereas WT female control flies on Tet and Tet-free diet exhibited normal vitellogenic oocytes. To determine if this result was a Tet-dependent effect, newly emerged adults were fed on Tet-containing diet A100 (100 µg/ml Tet) for either 5 or 10 days. Normal ovarian development was observed in the parental females, and oviposition was initiated by 10 days after eclosion, similar to WT controls.

To compare larval hatching, total survival rate and sex-specific lethality in these progeny, the three lines were reared for 5 or 10 days after eclosion on A100 diet, and then transferred to restrictive (Tet-deficient diet) conditions. Under these conditions the line D-419-17_E-443-1 yielded 99.7% male progeny, with early lethality occurring in female embryos and larvae, when parents were reared on A100 for 5 days (Figs 4, 5, Table S1). Rearing the parents on A100 for 10 days after eclosion resulted in an average of 55, 14 and 6% females after 1–4, 5–8 and 9–12 days, respectively (Fig. 5), which we assume is the result of a tetracycline storage effect, acting to suppress lethality, similar to previous observations in A. suspensa (Schetelig & Handler, 2012b). The other two sexing strains D-419-2x_E-443-1 and D-419-11_E-443-1 exhibited larval hatching of 81 and 70% with an adult male to female ratio of 79:21 and 53:47, respectively, when parents were reared on A100 for 5 days after eclosion (Fig. 4). This demonstrates that varying genomic position effects on tTA driver expression resulted in significantly different efficiencies for female lethality, ranging from no lethal effect in the D-419-11_E-443-1 line to almost 100% female lethality in line 419-17_E-443-1. By decreasing the Tet concentration and duration of feeding, as tested for other tephritids, the ovarian development and Tet storage effect can possibly be further optimized.

Reversibility of the sexing mechanism
To test for reversibility of the sexing mechanism, adults from D-419-17_E-443-1 were reared on A100 for 5 and 10 days after eclosion. The adults were then placed on Tet-free diet for 20 days before putting them back on A100 diet. Eggs were collected during the complete time frame of fertility. In both cases, most female progeny died 5 days after parents had last been fed with A100. Three days
after the flies were put back on A100, the sex ratio normalized, with 47% male and 53% female progeny (200 eggs collected: 84 larvae hatched; 53 pupated; and 28 female and 25 male adults eclosed). This demonstrated the reversibility of the sterility and lethality mechanisms, and suggests that the initial observation of females having nonvitellogenic oocytes was a Tet-dependent effect that could be rescued by Tet feeding. This would also suggest that the lethal effect, and thus \textit{As-sry-\alpha} promoter function, is likely to be active in the \textit{A. ludens} nurse cells and/or ovarian follicular epithelium, in addition to early embryos. The observed low hatch rate was presumably because of reduced fertility of the flies, reaching the end of their life span.

\textbf{sry-\alpha} promoter activity

The possibility that the female sterility effect was a result of prezygotic activity of the \textit{sry-\alpha} promoter was tested by performing qPCR for \textit{Al-sry-\alpha} and \textit{tTA} transcripts in D-419-17_E-443-1 homozygous virgin male and female adults and embryos. Both \textit{tTA} and \textit{Al-sry-\alpha} were found to be expressed throughout adulthood in males and females, which is consistent with a prezygotic lethality effect in developing oocytes (Figs S2, S3, Table S2).

\textbf{Discussion}

Here we report the development of a tetracycline-suppressible female-specific embryonic lethal system for the Mexican fruit fly, \textit{A. ludens}, which has the potential for use as a highly effective sexing system for mass-reared mexflies used in SIT. This system utilizes the same \textit{tTA} embryonic driver and cell death lethal effector cassettes that were similarly effective in the caribfly, \textit{A. suspensa}, resulting in double-homozygous hybrid strains that eliminated survival of all females when reared on Tet-free diet. However, a significant difference between the two species is that in \textit{A. ludens} adult females, the Tet-off lethality system has a secondary tissue-specific effect on developing oocytes, not observed in \textit{A. suspensa}. The reversibility of the sterility effect, demonstrated by the resumption (or initiation) of oogenesis in Tet-fed females, supports the conclusion that this is a direct effect of the Tet-off lethality system. However, as all lines shared the same lethal transgene effector
cassette, the possibility exists that its transposition into the host genome created an insertional female-sterile mutation, but if so, the effector line by itself should have also exhibited the recessive effect. Additional effector and driver lines were generated independently (Data S2) and confirm the integration-independent female sterility of the system in *A. ludens*. The lack of a relationship between the sterility effect and an insertional mutation or position effect is also supported by a separate nonsex-specific lethality experiment (data not shown) where both the driver and the effector construct, without the *Cctra* intron, were inserted within a single transformation vector. In all *A. ludens* transmantant lines recovered, the Tet-dependent female sterility effect was also observed, demonstrating that the *Cctra* intron was not responsible for the sterility effect. In two of the dual-vector hybrid strains and the single-vector strain, the sterility effect was highly penetrant, whereas total female lethality occurred in only the D-419-17_E-E443-1 strain. This would suggest that either the driver or lethal effector component of the system is more highly active during oogenesis, or that either one or both are less affected by suppressive genomic position effects.

The most straightforward explanation for the oocyte-specific sterility is an unexpected sry-α promoter function in adults, which was proven by the detection of endogenous sry-α gene expression in virgin females and males up to 10 days post-eclosion. This maternal expression of sry-α had not been previously observed in other species and most likely resulted in the female sterility effect (not observed in males owing to female specific expression of *Cctra-AlhidAla2*, Figs S2, S3, Table S2). As sterility was reversible, a hid-induced cell lethal effect would have to be expressed in previtellogenic oocytes, but not in the germ cells or sterility would be irreversible and result in rudimentary ovaries lacking oocytes. The *A. suspensa sry*-α promoter was used in the tTA driver and tTA expression was also verified in both virgin males and females from *A. ludens* TESS.

In *Drosophila melanogaster* (Dm), the sry-α gene is specifically transcribed during embryonic cellularization for blastoderm formation (Schweisguth et al., 1990), making it the initial candidate promoter for Tet-off unisexual embryonic lethality in that species (Horn & Wimmer, 2003). Homologous promoters to *Dmsry*-α were subsequently found to be similarly effective for both uni-sex and female-specific embryonic lethality in *C. capitata* (Schetelig et al., 2009; Ogaugwu et al., 2013) and *A. suspensa* (Schetelig & Handler, 2012a,b). In all of these tests, whether using a homologous or heterologous promoter, only the embryonic lethal effect was observed in the absence of tetracycline, inferring a similar zygotic-specific sry-α-regulated expression in the three species. However, *Drosophila melanogaster* also has related sry β and δ genes that are expressed both maternally in nurse cells and during embryogenesis (Payre et al., 1989). In addition, although functionally distinct, sry-α and sry-β are transcribed both as di-cistronic and monocistronic transcripts in embryos, varying in different *Drosophila* species (Ibsonouda et al., 1998). Thus, despite similarities amongst different genera, sry transcriptional regulation can be complex within a genus, perhaps resulting in *As-sry*-tTA expression in nurse cells or the follicular epithelium, causing a termination in oocyte development.

Although the *A. ludens* sexing strain described can be highly effective for obtaining a male-only population, it requires feeding newly eclosed adult females on a Tet-diet for up to 5 days, and subsequently transferring them to a Tet-free diet. This would make rearing slightly more complex, but refreshing adult diet and water at that frequency is normal. A more important consideration is that released double-homozygous males, if not otherwise sterilized (eg by irradiation), would still have female progeny that die during early development. If female survivors did occur, they would be sterile, providing an internal safeguard against the release of fertile females. Release of these males could also have a significant suppressive effect on a field population after several generations of adult female lethality, as postulated for fsRIDL releases (Leftwich et al., 2014). An added benefit of TESS relative to fsRIDL would be the lower number of damaging female larvae expected to survive from each new generation.

On a more fundamental level, relatively little is known about the comparative relationships of gene expression regulatory systems, even for highly conserved genes in related organisms. Thus, the prezygotic activity of sry-α in *A. ludens* should not have been totally unexpected, and should serve as a reminder that genetic elements require functional evaluation prior to their applied use in heterologous systems, including closely related species.

**Experimental procedures**

**Insect rearing**

An inbred WT colony of *A. ludens* (adults) and all transgenic lines were maintained at 23 °C and reared under standard laboratory conditions (Handler & Harrell, 2001). All embryonic, larval and pupal stages were reared at 27 °C and 60% humidity on a 12 h light : 12 h dark cycle.

**Cloning**

The driver and effector transformation vectors pBXLI_{attP220_PubDsRed.T3_tTA-Assrya} (Assrya-tTA; #419) and pBXLI_{PU-bEGFP_TREhs43-Cctra-AlhidAla2_loxN-3xP3-FRT-AmCyan_lox2272loxP_attP235} (TRE-Cctra-AlhidAla2; #443) were generated as described previously (Schetelig & Handler, 2012a,b). © 2016 The Royal Entomological Society, 00, 00–00
Germline transformation

Germline transformation was performed by microinjection of the piggyBac effector vector construct (500 ng/µl) together with the phsp-pBac transposase helper plasmid (200 ng/µl) into WT embryos as described previously (Handler & Harrell, 1999; Handler & James, 2000). G1 offspring were selected by EGFP or DsRed epifluorescence using a Leica M165C microscope (Wetzlar, Germany) and a GFP2 (ex: 480/40; em: 510 LP) or TxRed filter set (ex: 560/40; em: 610 LP). Independent homozygous strains were established by single-pair inbreeding for successive generations with testing by segregation analysis of transformants outcrossed to WT flies.

qPCR

Total RNA was isolated from embryonic samples (0–24 h collection, including the cellularization stage in A. ludens) using TRIzol (Invitrogen, Carlsbad, CA, USA). An iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and 1 µg RNA was used for cDNA synthesis. qPCR was performed on ~100 ng cDNA using iQ SYBR Green Supermix (Bio-Rad) in a CFX96™ real-time PCR detector (Bio-Rad). PCR cycling conditions were: 95 °C for 3 min; 40 cycles of 95 °C for 15 s, 65 °C for 10 s, 72 °C for 10 s and 81 °C for 5 s with a plate read at the end of each cycle. All reactions were performed on three biological replicates. Gene-specific primers for tTA (qTATTa-f: GCTGCTTAATGAGTGCGGAAATCG; qTATTa-r: TTGGGCTCTTACACGTCTCAA TG) and Histone 3 (qHist3_F: CGAATTTCAGCAGACTGG; qHist3_R: CTCTGAAATCTGCCTCAACC) were designed using the Geneious software (Biomatters). Amplified products from randomly selected samples were analysed on a 2% agarose gel, subsequently cloned into the pCR4 vector (Invitrogen) and sequenced. Relative accumulation of His3 was calculated from the formula 2 – ΔΔCt (Livak & Schmittgen, 2001) where 2 is the reaction efficiency and ΔΔCt is the difference in His3 Ct values between the calibrator (D-419_3) and the other samples, subtracted from the difference in tTA Ct values between the calibrator and the other samples.

Developmental gene expression profiles

Freshly emerged and virgin males and females from the sexing strain D-419-17_E-443 reared on Tet100 diet were collected for 10 consecutive days (A1–A10). In addition, 0–24 h-old embryos were total RNA. Total RNA was isolated from all samples using ZR Tissue & Insect RNA MicroPrep™ (Zymo Research, Irvine, CA, USA). An iScriptTM cDNA synthesis kit (Bio-Rad) and 1 µg RNA was used for cDNA synthesis. qPCR was performed on ~100 ng cDNA using the Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a Step One Plus Real-Time PCR System (Applied Biosystems). PCR cycling conditions were: 95 °C for 20 s and 40 cycles of 95 °C for 3 s, 60 °C for 30 s. Melt curve analysis consisted of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s, with the ramp rate between 60 and 95 °C in +0.3 °C steps. The fluorescence signal was acquired at the annealing step of each cycle during amplification and throughout the final ramp between 60 and 95 °C.

All reactions were performed on three biological replicates. Gene-specific primers for iTA (qTAtTa-f: GTTGGCGTATTGGAAAGAT CAAG; qTAtTa-r: CAATTCAAGGCGCAATTAAAGAG); sry-α (qAl-sry-α-f: TCAATGGAGCACATGAGCAGCA; qAl-sry-α-r: CGTTCTCGTACATGCTATTGC) and His3 (qHist3_F: CGAATTTCAGCAGACTGG; qHist3_R: CTCTGAAATCTGCCTCAACC) were designed using the Geneious software (Biomatters). Relative accumulation of iTA and sry-α normalized against His3 and the respective statistics were calculated with the REST 2009 software package (Pfaffl et al., 2002; see Figs S2, S3 and Table S2). REST (relative expression software tool) is able to compare two groups, with up to 16 data points in a sample and 16 in a control group, for a reference and up to four target genes. The mathematical model used is based on the PCR efficiencies and the mean crossing-point deviation between the sample and control group. The expression ratio results of the transcripts are tested for significance by a randomization test.

Inverse PCR

Inverse PCR was performed to obtain a flanking region of the integrated transgenes. First, 1 µg genomic DNA from D-419_2, D-419_11, D-419_17 and E-443-1 was digested with MspI for 6 h. Digested DNA was precipitated, self-ligated in 100 µl at 16 °C for 24 h using T4 DNA Ligase (NEB, Ipswich, MA, USA) and precipitated again. The pellet was dissolved in 150 µl TE. For each inverse PCR, 5 µl circularized DNA solution and Platinum Taq polymerase (Life Technologies, Rockville, MD, USA) was used with the primer pair P830 (CTTTATCGAATTCC TGCAG C: AH144 (CCTCGATATACGAGCAGTAAACAC) and P778 (ACGACCAGTGAGTCAATTGAGC): AH122 (ATCGT GACATTACCGATTGACA), respectively. PCR conditions were: 1 min at 95 °C; six cycles of 20 s at 94 °C, 30 s at 65 °C (–2 °C each cycle), 2 min at 72 °C; 30 cycles of 20 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C; and 2 min at 72 °C. PCR products were cloned into the pCR4 vector (Invitrogen) and sequenced. The flanking sequences are shown in Data S1.

Female-lethality tests

Fluorescent-marked A. ludens strains carrying the PUb-EGFP or PUb-DsRed markers exhibit different ‘patterns’ and intensity in adult flies that depend on the genomic integration site of the transgene that can be differentiated by epifluorescence microscopy. Thus, marker expression allows multiple integration sites, and heterozygous and homozygous transgenes, to be distinguished even when the same fluorescent marker is used. Fluorescent-marker tissue specificity was therefore used to verify lines with single vector integrations after three consecutive backcrosses of transgenic males or females to WT adults that resulted in a transgenic/WT progeny ratio of 1:1. Homozygous transgenic effector lines were then generated by inbreeding and selection of the strongest green fluorescent adult flies. To test for female-specific lethality, homozygous driver lines D-419_2x, D-419_11 and D-419_17, all homozygous for the construct #419, were crossed to the homozygous effector line E-443-1 on non-Tet diet, with 510 eggs (double-hemizygous) per cross collected and their L1 larval hatch rate recorded.
To generate double-homozygous conditional sexing lines, the same crosses as described before were performed. Larval and adult diet contained 100 μg/ml Tet (L100 and A100, respectively). Hemizygous combinations were inbred with progeny visually screened by fluorescence intensity for homozygous individuals that were subsequently inbred to generate lines homozygous for the driver and effector transgene constructs (using Tet-containing larval and adult diet). To generate homozygous lines with multiple fluorescent markers having variable intensity, use of highly specific filter sets to distinguish individual markers was essential. For screening double-homozygous lines expressing DsRed and EGFP, this was achieved using the Texas Red (ex: 560/40; em: 610 LP) and yellow fluorescent protein (YFP) (ex: 500/20; em: 535/30) filter sets (Leica). Double-homozygous lines were then tested for their ability to produce male-only strains by rearing them on Tet-free diet. The number of eggs, L1 larvae, pupae, and adult males and females were recorded for each cross.

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References


**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Data S1. Flanking insertion site sequences for piggyBac germline transformation vector integrations.

Data S2. Additional driver and effector lines generated and evaluated for *A. ludens* sexing.

Figure S1. Fluorescence expression of DsRed in *Anastrepha ludens* driver lines.

Figure S2. Relative expression of tetracycline-transactivator (tTA) and serendipity α (sry-α) of the sexing strain D-419-17_E-443-1. tTA and sry-α transcript levels were analysed by quantitative real-time PCR on female and male adult cDNA of different stages and cDNA from embryos. A1–A10 are virgin adult samples 1–10 days after eclosion, respectively. Flies were fed with A100 tetracycline-containing diet (100 μg/ml).

**Figure S3.** Relative tetracycline-transactivator (tTA) expression of the sexing strain D-419-17_E-443-1, compared with the wild type. tTA transcript levels were analysed by quantitative real-time PCR on female and male adult cDNA of different stages and cDNA from embryos. Expression of tTA was detected in males, and females throughout adulthood and in embryos with strongest expression detected in late, adult stages (A9–A10) and embryos (0–24 h post-oviposition). A1–A10 are virgin adult samples 1–10 days after eclosion, respectively. Flies were fed with A100 tetracycline-containing diet (100 μg/ml).

**Table S1.** Statistical analysis. Differences in the mean values amongst the specific treatment groups were calculated by one-way analysis of variance using the Holm–Sidak method for pairwise multiple comparison procedures within SIGMAPLOT 12.

**Table S2.** Statistics on relative gene expression of tetracycline-transactivator (tTA) and serendipity (sry) in line D-419-17_E-443-1. Relative quantification of mRNA expression levels by qPCR of sample X compared with sample Y and normalized to the expression of the reference gene Histone 3. The relative quantification results were obtained and tested for significance using the REST 2009 software. Significantly different samples (up- or down-regulated; a value of 1 demonstrates no change) with a threshold of *P* < 0.05 are marked in green. A1–A10 describe adult virgin samples 1–10 days after eclosion, respectively, reared on A100 diet (100 μg/ml tetracycline).