Oral Delivery of dsRNA Targeting a Female-Biased Flight Muscle Actin Impairs Flight in the Malaria Vector, Anopheles Albimanus.

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Research

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Abstract

Background

Despite the progress to eliminate malaria in Central America, focalized transmission persists, and insecticide resistance is on the rise in the primary vector, *Anopheles albimanus*. Many of the new control methods being developed depend on the release of a large number of male mosquitoes that must be sorted prior to release. However, *An. albimanus* manual pupal-sex-sorting is not feasible, and therefore, we explored the use of RNA interference (RNAi) targeting genes with a sex-biased expression for female elimination. Here, we evaluated the effect of feeding larvae with dsRNA for a female-biased orthologue of the flight muscle actin gene.

Results

Two sex-biased actin forms were identified in *An. albimanus*. Gene expression analysis showed a >40-fold higher expression of the AALB015469 transcript in female pupae ($p = 0.0048$) and adults ($p = 0.0078$) when compared to males. Tissue-specific analysis also suggests this female-biased actin can be an orthologue of the flight muscle actin of *Aedes aegypti*. At the same time, the AALB015481 transcript showed a >40-fold higher expression in male pupae and adults when compared to females, with no detectable expression in flight muscle. The potential effects of oral-induced RNAi for the female-biased actin were evaluated. Larvae were fed a diet containing either dsRNA for the female-biased actin 3'-UTR alone or for the UTR with an adjoining portion of the C-terminal coding region. A significant number of flightless females resulted from feedings with 3' UTR alone (10.50 ± 5.92 %, $p < 0.05$) or with the coding region (6.00 ± 2.16 %, $p < 0.01$). Treatment with the 3' UTR alone resulted in a significant number of flightless males (8.25 ± 3.10 %, $p < 0.01$). Both diets produced significant mortality in both female and male adults ($p < 0.0001$).

Conclusions

Feeding of *An. albimanus* larvae with dsRNA targeting the female-biased flight muscle actin orthologue impairs flight in both sexes and affects the overall survival of female and male mosquitoes. Providing dsRNA in the larval diet shows promise as a method for screening other differentially expressed genes as potential targets for female elimination in mosquito breeding facilities.

Background

Malaria is a parasitic disease that affects millions of people worldwide. It is caused by *Plasmodium* parasites, and it is transmitted by the bite of female *Anopheles* mosquitoes. In the Mesoamerican region, northern South America and the Caribbean, *An. albimanus* is one of the main vectors of the disease (1–4). The incidence of malaria in this region decreased 20% between 2010 and 2014, but it has been increasing since then (5). Indoor residual spraying (IRS) and the use of insecticide-treated mosquito nets (ITN) have been effective in reducing the vector population (6), but new vector control strategies are

needed as insecticide resistance is widespread (7–12). New potential vector control strategies using genetically modified or sterile mosquitoes are being considered to overcome this problem. However, these strategies depend upon the release of large numbers of males, and therefore separating males from females before releasing the mosquitoes to the field is imperative (13, 14).

The methodologies for sex-sorting and ultimately sex-separation of mosquitoes vary greatly and depend on the sexual dimorphisms of each species (15). For many years, the efforts to develop sex-separation methods have been limited by the difficulty to scale up the technologies, and the complexity of building functional genetic systems (16). Previous research with transgenes in *Ae. aegypti* to obtain flightless females (17) has opened the consideration of such approaches as a genetic sexing tool in mosquitoes. In this strategy, one of the four *actin* genes found in *Ae. aegypti*, *actin-4*, was found to be essential for female flight. This and other studies in model organisms, such as *Drosophila melanogaster*, have shown that the identification of the specific functions of particular actin genes in each species can be complex, as they are highly conserved and can show more than 90% similarity between isoforms (18–23). *AeAct-4* and a similar orthologue have been reported as a female-biased gene in adults of *Ae. aegypti* and *Anopheles stephensi* (24, 25). Interruption of the promoter of this gene has been used to produce flightless females using the Release of Insects carrying a Dominant Lethal (RIDL) technology (16, 24–26).

The last decade has seen staggering progress in the creation of genetic tools for mosquito studies, with major breakthroughs in genetic analysis derived from the development of germline transformation and RNA interference (RNAi) (reviewed by Adolfi and Lycett (29)). RNAi could provide a viable and inexpensive technology (30–33) to knock down the expression of sex-biased genes (30, 34, 35) for sex-sorting and sex-separation in *Anopheles*. RNAi is part of the natural cellular defense systems that insects have against viruses (36). It is triggered by double-stranded RNA (dsRNA), and therefore its response is sequence-specific (37). When exogenous dsRNA is delivered to the cells, mRNA complementary to the dsRNA is cleaved and degraded, effectively silencing the gene expression of the target gene (38). The method has been used successfully to silence a wide variety of genes in insects, mostly for reverse genetic studies (reviewed by Huvenne & Smagghe in 2010 and updated by Jin et al. in 2020 (32, 33)).

Currently, several pest-control and vector-control strategies based on RNAi are being proposed. In most of these strategies, the delivery of the dsRNA is the crucial most challenging part of the method development, and several delivery methods have been proposed for mosquitoes. Encapsulation of the dsRNA in chitosan, agarose, yeast, and bacteria have been evaluated for oral delivery to mosquito larvae, all with various degrees of success (30, 35, 39–41). To our knowledge, to date there have been no reports of RNAi experiments in *An. albimanus*, and one of the main goals of this work, is to set grounds for more reverse genetic studies in this species. Additionally, in the recent annotation of the genome of *An. albimanus* (42), the muscle actins were not characterized. Thus, the second aim of this study was to search for a sex-biased actin in *An. albimanus* and to determine the viability of using RNAi to silence it to obtain flightless phenotypes, similar to *Ae. aegypti.*

Results

Primary identification of sex-biased actins

Due to conservation in their coding regions, actin genes can be distinguished by differences in their unique 3'-UTR sequences (25). Using 3'-UTR analysis, we found several actins expressed during the larval (Additional File 1), pupal, and adult stages (Fig. 1a). 3' Rapid amplification of cDNA ends (3'-RACE) resulted in a fragment of 600 bp that showed higher expression in females than males, here designated as female-biased actin. Conversely, a 500 bp fragment showed higher expression in males and designated as male-biased actin. The other putative actin genes (~ 700 and ~ 900 bp), although expressed in both male and female pupae and adults, were not studied further in this study. Both PCR products (500 bp and 600 bp) represent different 3'-UTR sequences preceded by a highly conserved coding region. Each sex-biased UTR was identical in pupae and adults. VectorBase nucleotide BLAST of the sequences of each product confirmed they are different *actin* genes located on different chromosomes. VectorBase BLAST showed 99.6% identity between the *An. albimanus* Sanarate strain female-biased actin and the *An. albimanus* Stecla strain AALB015489 gene transcript within the 3R chromosome (Fig. 1b, 1c). A BLAST search with other anophelines and aedines showed a coding region plus 3'-UTR conservation only in the new world anopheline, *An. darlingi* Coari strain in 86.5% (Additional File 2). A homologue search of AALB015489 transcript within VectorBase showed 98.94% similarity with the *Ae. aegypti* flight muscle actin (*actin-4*). BLAST analysis of the male-biased actin showed a 99.3% identity between the *An. albimanus* Sanarate strain and the *An. albimanus* Stecla strain AALB015481 gene transcript within the 2R chromosome (Fig. 1c). VectorBase homologue search of this transcript showed 100% similarity with several genes annotated as actins of several anopheline mosquitoes and 98.4% identity with *Ae. Aegypti actin-3* (Fig. 1b).

Spatiotemporal analysis of the sex-biased actins expression

Quantitative Real-Time PCR (qPCR) analysis showed that the female-biased actin (AALB015489) was expressed > 40-fold higher in female pupae ($p = 0.0048$) and female adults ($p = 0.0078$),compared to males of the same stages, and remained low in the larval stages (Fig. 2a).Expression was detected in flight muscle, and to a lesser extent, in ovaries, abdomen, and gut (Fig. 2b). qPCR showed that the male-biased actin (AALB015481) was expressed > 90-fold higher in male adults ($p = 0.0103$) than in female adults and remained low in the larval stages (Fig. 2a). In addition, expression was detected in the male abdomen, midgut and reproductive tissues, but not in the flight muscle (Additional File 3).

Feeding female-biased actin dsRNA affects flight and survival in both sexes

RNAi experiments were performed to determine if the female-biased actin was implicated in the flight of *An. albimanus*. We fed larvae with a dsRNA fragment that contains the 3'UTR sequence only (designated as Actin A) and another fragment that contains the carboxy-terminal coding region with its 3'UTR sequence (designated as Actin B). Some of the adults from both groups presented a flightless phenotype (Fig. 3, Additional File 4). A significant effect was observed on the flight in both sexes of adults emerging

from larvae fed with 3'UTR dsRNA (Actin A), with approximately 10% females (10.50 ± 5.92%, Student's t, $p < 0.05$) and 8% males (8.25 ± 3.10%, Student's t, $p < 0.01$) unable to fly one day after emergence. With Actin B (3'UTR plus the C-terminal coding region), the percentage of flightless mosquitoes (6.00 ± 2.16%, Student's t, $p < 0.01$) observed was significantly different from controls (fed the ANT dsRNA) only in females. Survival was affected considerably in both females and males fed with Actin A or Actin B (Log-rank test, $p < 0.0001$). To verify the association of the flightless phenotype with gene expression, transcript levels of the female-biased actin were measured in pupae. Our results showed a measurable decrease in the mRNA expression, but due to sample size and deviation, no statistically significant reduction was obtained (Additional File 5).

Discussion

Current insecticide-based control methods, such as IRS and ITNs, are threatened by increasing insecticide resistance in malaria vectors. The design of novel vector control strategies is a pressing need to alleviate or eliminate the burden of vector-transmitted diseases, particularly in developing countries where most vulnerable populations have limited access to medical services (43). Innovation is limited in regions where local vector species are not actively studied. As a vector with a geographical distribution limited to the Meso-American region, Caribbean and northern South America (2–4), research on *An. albimanus* has been limited compared to other malaria vectors such as *Anopheles gambiae*. The genome of *An. albimanus* was sequenced as part of the 16 Anopheles Genomes Project (42), and published only in 2015, more than ten years after the *An. gambiae* genome (44). Given the divergence of the neotropical anopheline species from their African counterparts, the importance of the study of this species' genome cannot be overstated, and more transcriptomic studies, such as the comparative transcriptomic study by Papa et al, 2017 (45), are needed.

Using the current version of the *An. albimanus* genome (Vectobase Gene Set AAlbS2.6), we found an orthologue of the *Ae. aegypti* female-biased flight muscle actin gene (*actin-4*) and determined that the *An. albimanus* form also had higher expression in female pupae and adults as compared to males. Given the high similarity between coding regions of different actin genes or actin isoforms within an organism, we confirmed the previous expression analysis of the *An. albimanus* sex-biased actin genes by the use of the highly divergent 3' UTR and qPCR to complement the RNA-seq available data (45), as short RNA-seq reads within coding region of homologous genes could be mapped to multiple locations (46).

Females and males have different morphological, behavioral and physiological characteristics, many of which can arise from sex-biased gene expression (45). We confirmed that this actin gene is involved in flight in both sexes after obtaining flightless phenotypes upon oral delivery of the actin 3' UTR dsRNA. However, feeding dsRNA for this actin gene accelerated mortality in males three weeks after emergence, suggesting that even the low expression levels that were detected in this sex, are essential for flight or other biological processes that we did not characterize. As the flightless phenotype only occurred in a limited sub-section of the treated groups, further analysis will be needed to identify the factors that made these mosquitoes more susceptible to the treatment or why the RNAi effect was more pronounced in

those individuals. The low frequency of the phenotype may also be related to the efficiency of delivery RNAi for this target gene. Given that the targeted actin gene in *An. albimanus* is expressed in the flight muscle; the dsRNA delivered in the midgut has to travel a significant distance to reach the target cells. Marked differences in the stability and production of short interfering RNAs after dsRNA oral delivery occur among insect species (50). Different tissues are also differentially susceptible to RNAi in various insects, as reviewed by Wynant et al, 2014 (51). Finally, the dose and the construct size can also produce different effects, sometimes with a saturation effect due to high doses (reviewed by Joga et al, 2016 (52)). Despite the high variability in RNAi efficiency across insect species and target genes, dsRNA produced in bacteria or yeast has a high potential as heat-killed mosquito larvicide, as recently reviewed by Wiltshire and Duman-Scheel (53).

We believe that, to obtain higher percentages of individuals with the phenotype, several parameters in the delivery system must be optimized (30, 54, 55). In our experiments, we designed enough negative controls for the delivery system (Additional File 6), to allow us to rule out an association of the phenotype with the method rather than with the specific RNAi for actin. When appropriate genes are available, positive controls will be useful to help optimize the feeding parameters, and further research to identify such genes in *An. albimanus* should be done. The partial and short-lived effect on both sexes may also be due to non-specific degradation in the gut, to variability in the doses ingested by individuals and/or possible compensation with other actin isoforms (56–59). Additionally, functional studies using RNAi injections could determine if higher concentrations of the dsRNA and crossing tissue barriers can effectively induce a longer lasting flightless phenotype.

Sex-biased genes can arise via gene duplication, in which the parental copy of the gene may remain unchanged, whereas the new copy may present sex-biased expression (47). A sex-biased expression can vary during different times of development and between tissues (48, 49). Given that the expression of the flight muscle actin begins during the larval stages, we fed larvae heat-inactivated bacterial lysates with isoform-specific dsRNA produced by *E. coli* HT115(DE3), starting at the L2 stage until pupation. We saw a flightless phenotype in both female and male adults; however, the phenotype was observed only in around 10% of individuals of either sex. We did not detect a significant effect on gene expression in pupae, possibly because of the low frequency of the flightless phenotype and a dilution from pooling individuals for therelative expression analysis.

Our results indicate that the female-biased actin gene is involved in flight in both sexes and silencing of this gene resulted in the reduced female and male survival. In *Drosophila*, mutations in the jumping muscle actin gene can be compensated by a low level expression of the flight muscle actin, although there is no evidence of actin compensation in the flight muscle (39). Mosquitoes may require sex-biased flight muscle actin due to different flight requirements for each sex. After a blood meal, females can double or triple their total weight and they must carry this additional weight until the blood meal is processed (60). This extra weight requires that the mosquitoes exert additional aerodynamic forces generated by beating wings to take off; these aerodynamic forces are generated by indirect flight power muscles (61). Furthermore, differences in wing beat frequencies exist between males and females during

the swarming and mating process that may require a different pattern of expression of actin in thoracic muscles (62).

Altogether, our results show for the first time that there is a female-biased, sexually-differentiated expression of the flight muscle orthologous actin gene in *An. albimanus*, and that it is possible to target this gene through oral mediated RNAi using its 3' UTR sequence, affecting flight in both sexes. We recently showed in *An. gambiae* that oral delivery of dsRNA for a female-specific doublesex gene could skew sex ratios towards males (35). Development of an efficient means of separating the sexes by targeting other sex-biased genes could provide new tools for female removal in genetic-based strategies to improve vector-control strategies in the region.

Conclusion

We isolated a female-biased flight muscle actin gene from pupae and adults of *An. albimanus* that is implicated in the flight and survival of both females and males. Future studies will characterize other sex-biased genes as potential targets for *An. albimanus* control strategies.

Methods

Mosquito rearing: Two strains of *An. albimanus* were used for this work: 1) *An. albimanus* Sanarate strain (Biosamples ID: SAMN10341946) (8), was used for gene characterization and quantification of basal gene expression. Rearing of this strain took place at the Universidad del Valle de Guatemala (UVG). Insectary conditions were 28 °C +/- 1 °C and relative humidity of 80% +/- 10%, with a photoperiod of 12:12 light-dark cycle. Colony larvae were fed daily a mixture of baby food Nestum® 5 cereals (Nestlé) and active yeast. Adults were fed ad libitum on a 10% sugar solution in sterile water and females were fed commercially available defibrinated sheep blood (Actividades Lucrativas, Guatemala, Guatemala) via a membrane feeder (Hemotek, United Kingdom) covered with Parafilm. 2) *An. albimanus* Santa Tecla (Stecla) strain (MRA-112 BioDefense Emerging Infections (BEI), Malaria Research and Reference Reagent Resource Center (MR4), Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA) was used for RNAi experiments. Rearing conditions at the CDC were 27 °C, relative humidity of 80%, and a photoperiod of 12:12 light-dark cycle with a 30-minute dawn and dusk period. Colony larvae were fed Damien's diet (63) according to the MR4 rearing protocol (64). Adults were fed ad libitum on a 10% sugar solution with 0.2% methylparaben dissolved in sterile water, and females were fed commercially available defibrinated rabbit blood (Hemostat, Dixon, CA, USA) via a Parafilm covered glass feeding bell (Lily, Atlanta, GA, USA).

RNA extraction: For 3' RACE and PCR evaluation of the sex-biased actins expression, we extracted RNA from pools of: 1) 15 one-day-old virgin females, 2) 15 one-day-old virgin males, 3) five female pupae, 4) five male pupae, 5) seven L4 larvae, and 6) 40 L3 larvae of *An. albimanus* strain Sanarate. The number of individuals per pool was selected based on previous normalizations of RNA obtained per wet body weight. The extractions were performed using the SV total RNA isolation system kit (Promega, WI, USA),

following the manufacturer's protocol. Briefly, mosquitoes were homogenized using a pellet pestle cordless motor and sterile pestles in 175 µl of lysis buffer and incubated in a cold rack for 20 min. 350 µl of RNA dilution buffer was added and mixed by inversion. This lysate was centrifuged at 13,000 rpm, 10 min at room temperature, the supernatant was removed and mixed with 200 µl 95% ethanol, transferred to spin column and centrifuged at 13,000 rpm for 1 min. The column was washed with 600 µl of RNA wash solution, and the sample was treated with DNAse incubation mix for 30 min, followed by the addition of 200 µl stop solution. The column was centrifuged at the same conditions as before and washed twice with RNA wash solution. RNA was eluted with 100 µl of RNase free water.

For the evaluation of female-biased actin expression in several adult tissues, two pools of five individuals per biological replica (n = 3) were dissected in ice-cold PBS. RNA extractions were performed using the RNeasy Micro kit (Qiagen, MD, USA) according to the manufacturer's protocol.

For cloning of the female-biased actin, RNA was extracted from a pool of eight adult female mosquitoes and five female pupae of *An. albimanus* Stecla strain using the RNeasy Mini Kit according to manufacturer´s protocol (Qiagen, MD, USA). Briefly, samples were homogenized using a pellet pestle cordless motor and sterile pestles in 350 µl of RLT buffer. After centrifugation, one volume of 70% ethanol was added to the lysate and transferred to the RNeasy Mini spin column. Two consecutive washes with 700 µl of RW1 buffer and one with 500 µl of RPE buffer were done before resuspending the RNA in 30 µl of RNase-free water. Quantification of RNA was done with a Nanodrop OneC (Thermo Fisher, MA, USA) and 1 µg of RNA was treated with 1.5 units of RQ1 DNase (Promega, WI, USA) for 1 h at 37 °C, followed by inactivation for 10 min at 65 °C.

3' Rapid amplification of cDNA ends (RACE): We performed 3' RACE with the kit 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen, MA, USA). One µg of total RNA was used to generate the cDNA by the manufacturer's instructions. Then 1 µl of this cDNA was used to amplify the 3' UTR region using 0.4 µM of actin primer FMActRace_1F (5'- CGATCAAGATCAAGATCATTGCC-3') and 0.4 µM of UAP primer provided by the manufacturer in a 25 µl reaction using the GoTaq Hot Start colorless Master Mix (Promega). We performed the amplification as follows: 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C-1 min and a final extension of 72 °C for 5 min. We gel pricked the 600 bp and 500 bp bands that showed differential expression in females and males and amplified using the same conditions described earlier, with the only modification that 20 cycles of amplification were used. Both fragments were ligated to the pGEM-T easy vector (Promega) and transformed it into *E. coli* XL-1 blue. Clones of each fragment were sequenced and the sequences used to design female-biased and male-biased actin specific primers within the 3' UTR.

Sex-biased actin expression: Quantitative PCR was done to assess the expression level of the sex-biased actins during the life cycle and in the different tissues of the adults of *An. albimanus* mosquitoes. RNA extraction was performed as previously described. The cDNA was generated from 1 µg of total RNA using the GoScript Reverse Transcription Kit with a mixture of Random primers plus oligo(dT) (Promega) as per manufacturer's instructions. The following primers were used: qPCRfemaleUTR_2F (5´-

GACATCCAATCACTACATCC-3´) and qPCRfemaleUTR_2R (5´-CAAAGGCAAACACAGCTAAC-3´) for the female-biased actin, and qPCR_Male3UTR_1F (5´-CCAACATCAACACCAACTTC-3´) and qPCR_Male3UTR_1R (5´-GTCGAAGAAGATTTGCACAG-3´) for the male-biased actin amplification. The RpS4 (AALB005332) and RpL49 (AALB008968) reference genes were amplified with the following primers: qPCR_RpS4_1F (5'-AGGTGATGGAGGTGCTGAAG-3´) with qPCR_RpS4_1R (5'-CGATGATGAACACGTTGGAG-3') and qPCR_RpL49_1F (5'-AGGGTGGAAGGTGATATCTG-3') with qPCR_RpL49_1R (5'- ACATGGTCGCCCTTAAATG-3'). To measure expression in each biological replicate of the different life-stages, qPCR was carried out with Power Up SYBR green master mix (Thermo Fisher) with 0.33 µM of each primer and 2.5 µl of cDNA diluted at 1:500 in a final volume of 10 µl. All samples were run in technical triplicates. Genes were amplified in the Lightcycler 96 (Roche, Switzerland) thermal cycler as follows: 50 °C for 60 s and 95 °C for 120 s, followed by 50 cycles of: 95 °C for 15 s and 60 °C for 60 s. Melting curves of 95 °C for 15 s, 60 °C for 60 s and 95 °C for 16 s were done after each run. Delta cycle threshold (ΔCt) method was performed using the average Cts of both Rps4 and Rps49 reference genes and performing the comparison of the relative expression to the L3 stage. Samples were analyzed using ANOVA test with Tukey's comparisons. To measure expression in each biological replicate of the different tissues, qPCR was prepared as before with the difference that the cDNA was diluted at 1:20. All samples were run in technical duplicate. PCR was run in a Quant Studio 6 (Applied Biosystems, CA, USA) thermal cycler as follow: 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curves of 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 sec were done for each run. Delta-delta Ct (ΔΔCt) analysis was performed using the average of Cts of both Ribosomal protein S7 (Rps7) and Actin housekeeping HK reference genes for analysis of transcript levels of pupa that receive actin A dsRNA and Rps7 only for pupae that were fed with Actin B and compared to pupae fed dsRNA of the *ant* gene as a control.

Female-biased actin cloning: Primers for two dsRNA were designed based on the sequenced regions. For 3´-UTR: AaACT3UTR_F: (5´-GCACAAATGATGGTGGCTAAAG-3´) and AaACT3UTR_R_T7: (5´-TAATACGACTCACTATAGGGCTCAAAGGCAAACACAGCTAAC-3´) (designated Actin A) and for coding region plus 3´-UTR: FMActRace_1F and AaACT3UTR_R_T7 (designated Actin B). The T7 promoter of the pGEM-T Easy vector and the T7 added in the reverse primers (underlined) were used to produce both strands of the dsRNA. Complementary DNA was produced using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) with a mixture of random primers and oligo(dT). Conventional PCRs were performed with 0.4 µM of each primer and the AccuStart™ II GelTrack™ PCR SuperMix (Quantabio, MA, USA), using a 1:5 dilution of cDNA from female and male pupae and adults of *An. albimanus* as a template to corroborate the expression and the following program: 94 °C for 3 min, 30 cycles of 94 °C for 45 sec, 61 °C for 30 sec, and 72 °C for 30 sec, followed by a last step at 72 °C for 5 min. The obtained fragments were cloned into the pGEM-T-easy vector and then transformed into *E. coli* HT115 (DE3) for production of dsRNA. Transformation of *E. coli* HT115 (DE3) was performed as described by Timmons et al, 2001 (37).

Preparation of dsRNA: Pricks of fresh bacteria colonies were used for inoculum of overnight cultures. The bacteria used were either *E. coli* HT115(DE3)-Actin (Actin A or B) and *E. coli* HT115(DE3)-Ant, a non-

related gene from *Arabidopsis thaliana* (GenBank: U41339.1), which is an APETALA2-like gene with pleiotropic roles in ovule development and floral organ growth, that we had previously cloned into *E. coli* HT115 (DE3) for control(65)). Colonies from Luria-Bertani (LB) agar plates with tetracycline (12.5 µg/ml) and ampicillin (100 µg/ml) were used as inoculum for each starting culture (10 ml). An overnight incubation in LB broth with tetracycline (12.5 µg/ml) and ampicillin (100 µg/ml) was done at 37 °C and 170 rpm. This culture was diluted 15:100 with 2xYT medium plus both antibiotics (same concentration as before) and incubated at 37 °C at 170 rpm until they reached OD_{600} = 0.4 (approximately 0.5 h). Once the culture reached OD_{600} = 0.4, the dsRNA production was induced with the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.4 mM final concentration, and a two-hour incubation at the same conditions (3.35×10^7 – 4.02×10^7 CFU/ml). This culture was centrifuged for 10 min at 4,000 rpm and 10 °C, and the cell pellet was washed twice in one volume of sodium phosphate buffer (PBS) (Thermo Fisher) and later re-suspended in half the initial volume in PBS. The *E. coli* cells were heat-killed by placing the tube with the resuspended pellet in boiling water for 15 min. The resuspended inactivated cells were aliquoted and stored at -70 °C.

dsRNA quantification

We used 3 aliquots of 250 µl of the inactivated cells to extract and quantify dsRNA. We centrifuged each tube of cells at 3,700 x g for 10 min at 4 °C, removed the supernatant, resuspended the pellet with 50 µl of 1% SDS in PBS and boiled them for 2 min in a water bath. After 10 min at room temperature, 64 µl of RNAse buffer (300 mM sodium acetate, 10 mM Tris-HCl pH 7.5 and 5 mM EDTA) and 2 µg of RNase A (Sigma Aldrich, MO, USA) were added and the mixture incubated at 37 °C for 5 min to degrade single stranded RNA. We extracted the dsRNA with 750 µl of Trizol LS Reagent (Invitrogen, MA, USA) according to manufacturer instructions. The RNA pellet was resuspended in 20 µl of DEPC water and 1 µl of the suspension was quantified on a Nanodrop one C (Thermo Fisher).

RNAi feeding experiments in larvae: Groups of 20 larvae (L2) of *An. albimanus* Stecla were set in individual 150 mm x 25 mm Petri dishes with 80 ml of water. Insectary conditions of temperature, humidity, and light were maintained as described in the mosquito rearing section. Feeding conditions were as following: an artificial bacterial diet (ABD), previously described by Taracena et al. 2019 (35), was added to the Petri dishes with the larvae, for a space of 4 h per day. Briefly, 200 µl of inactivated bacteria in PBS were complemented with 200 mg of a mixture of food (40% fish food (Goldfish, Tetra, Germany), 43% guar gum (Sigma-Aldrich) and 17% Active Yeast) and fed to each replica. After the 4 h, the dsRNA mixture was removed and we fed the larvae with conventional food (in this case, Baby 5 cereals Nestum® and active yeast). Negative controls for method validation were established in the following groups: 1) food without bacteria, 2) food with bacteria without plasmid for dsRNA production, 3) food with bacteria with a plasmid for the target dsRNA, but non induced, and 4) food with bacteria with non-related dsRNA. After the determination that no significant difference was observed amongst the negative controls (Additional File 6), the group of Ant-dsRNA was kept for reference in all experiments with actin genes. The larvae were fed daily with ABD until they reached pupation. Quantification of the dsRNA allowed to determine that the feedings were delivering an equivalent of approximately 5.75 µg of Ant-

dsRNA, 11.90 µg for Actin A, and 9.0 µg for Actin B at the beginning of each feeding. Degradation of the dsRNA in the medium or inside the larvae was not measured. For each biological replicate (N = 80) we had four internal replicates, each with 20 larvae. Observed phenotypes were recorded for all experiments. T-test was performed in comparison to the control group for the flightless phenotype. The Log-Rank Mantel-Cox test was performed for survival curves in comparison to the control group.

Expression analyses: Real-time expression analysis was done from 3 samples per group, each sample was composed of a pool of 3 male pupae or 3 female pupae. RNA extractions and cDNA synthesis were done as described in previous sections with the RNeasy Mini Kit (Qiagen) and High-Capacity cDNA Reverse transcription kit (Thermo Fischer). Actin housekeeping (HK) (AALB015483) and Ribosomal protein S7 (Rps7) (AALB010399) were used as reference genes and were amplified with the following primers: Actin F (5'-TACAACTCGATCATGAAGTGCGA-3´) and ActinR (5'-CCCGGGTACATGGTGGTACCGCCGGA-3´) and RpS7F (5'AGAACCAGCAGACCACCATC-3') and RpS7R (5'-ACAACCAGCAACGGTTATGT-3´). The q-RT PCR reaction was carried out with the Power Up SYBR green master mix (Thermo Fisher) with 0.33 µM of each primer and 5 µl of cDNA diluted at 1:500 in a final volume of 15 µl. All samples were run in technical triplicate. The genes were amplified as described previously for the tissue-specific qPCR. Delta-delta Ct (ΔΔCt) analysis was performed using the average of Cts of both Rps7 and Actin HK reference genes for analysis of transcript levels of pupae that received actin A dsRNA and Rps7 only for pupae that were fed with Actin B and compared to pupae fed dsRNA of the *ant* gene as a control. T-test was performed in comparison to the control group.

Bioinformatics analysis

A nucleotide BLAST search using the 500 bp or 600 bp fragment was performed using the VectorBase blast tool with default parameters (66), using the Aedes and Anopheles data sets. Clustal Muscle software was used to align the two actins with gene transcript accession numbers AALB015469-RA and AALB015489-RA (*An. albimanus* Stecla Gene set AalbS2.6 in VectorBase). The female-biased sequence was aligned with *A. darlingi* Coari strain (Gene set AdarC3.8 from Vector Base) genomic scaffold_1503 using Clustal muscle software. A homologue search was done for the AALB015469 and AALB014489 genes within the VectorBase gene tab.

Molecular phylogenetic analysis: Phylogenetic tree was built using the Maximum likelihood method based on the Tamura-Nei model (67). Analysis was done with cDNA sequences of the *An. albimanus* sex-biased actins, *An. albimanus* Stecla strain actins (Vector base: AALB015481 and AALB015469), *Aedes aegypti* flight muscle actin-4 (VectorBase: AAEL001951), muscle actin-1 (VectorBase: AAEL001928) and actin-3 (VectorBase: AAEL0094551); *Anopheles gambiae actin-4* homolog (VectorBase: AGAP011515) and *D. melanogaster* flight actin 88F (Ensembl: FBtr0083143). An initial tree was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Analyses were conducted using the Mega 7.0.18 software (68).

Data Analysis

One-way ANOVA with Tukey's multiple comparisons, Student's t-tests, and Log-Rank Mantel-Cox test were performed with GraphPad Prism Software version 8.02 (Graphpad Software, CA, USAS). All significant values have a p-value of less than 0.05.

Abbreviations

3'RACE: 3'Rapid amplification of cDNA ends

ABD: Artificial bacterial diet

ANOVA: Analysis of variance

ANT: Aintegumenta gene

bp: base pair

CDC: Centers for Disease Control and Prevention

cDNA: complementary DNA

Ct: Cycle treshold

dsRNA: double-stranded RNA

EDTA: Ethylenediaminetetraacetic acid

FA: female adult

FP: female pupae

HK: housekeeping

IPTG: Isopropyl b-thiogalactopyranoside

L3: third instar larva

L4: fourth instar larva

LB: Luria-Bertani broth

MA: male adult

MP: male pupae

MR4: Malaria Research ad Reference Reagent Resource Center

PBS: Sodium phosphate buffer

PCR: Polymerase Chain Reaction

qPCR: quantitative-Real Time PCR

RNAi: RNA interference

RNAse: Ribonuclease

rpm: revolutions per minute

SDS: Sodium dodecyl sulfate

SIT: sterile insect technique

Tris-HCl: Tris hydrochloride

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: The data that support the findings of this study are available on request from the corresponding author on reasonable request [PP].

Competing interests: The authors declare that there are no competing interests.

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Authors' contributions

CP, MT, PF, PP and ED developed the experiment design. CP, PF, MT, CH, KI, MM and AB performed biological assays and experiments. CP, MT and PP analyzed and interpreted experimental data. CP and MT wrote the manuscript. NP contributed with laboratory and insectary installations. PP, ED and NP made substantial contributions for data acquisition and data analysis and interpretation and revised the final manuscript. All authors read and approved the final manuscript.

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Figures

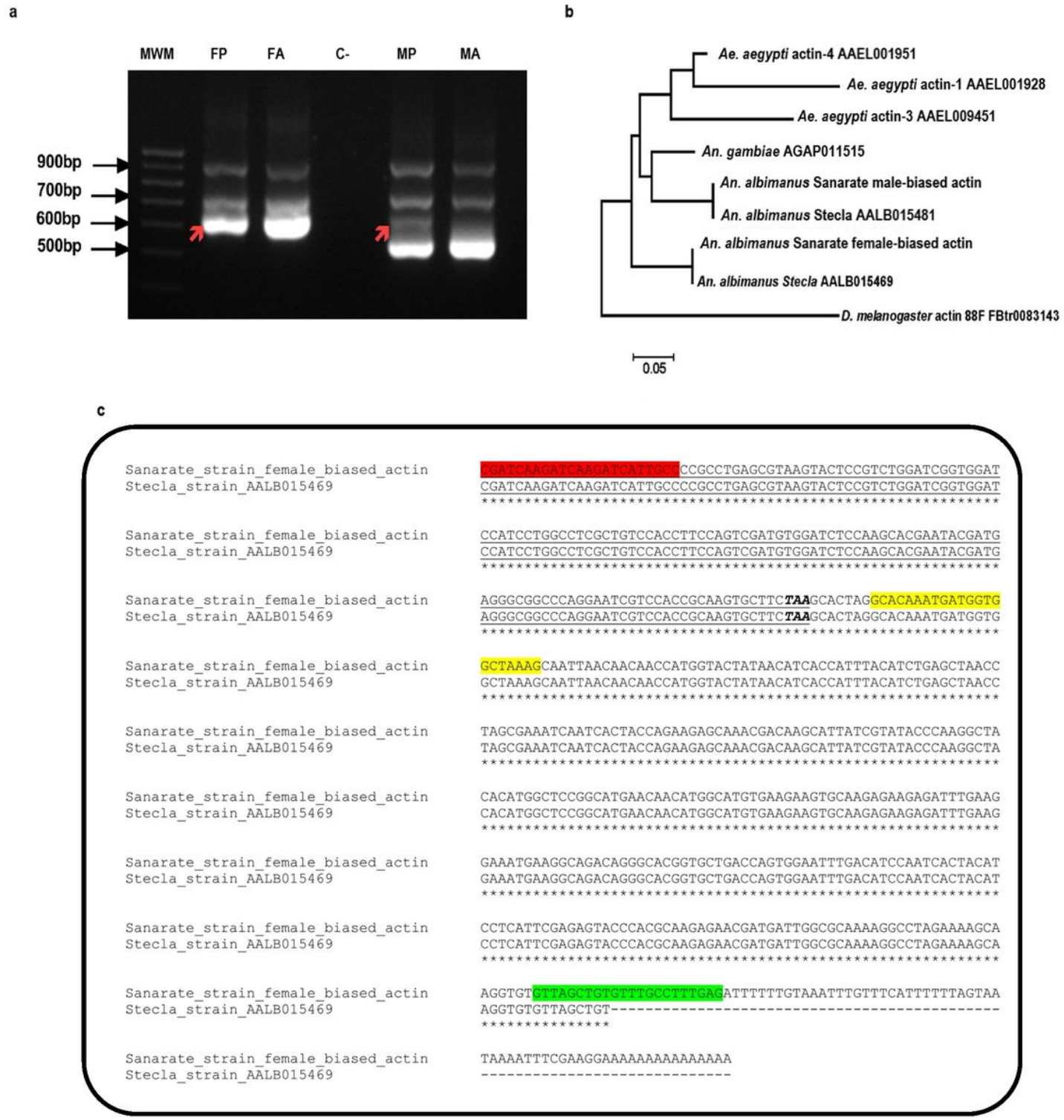
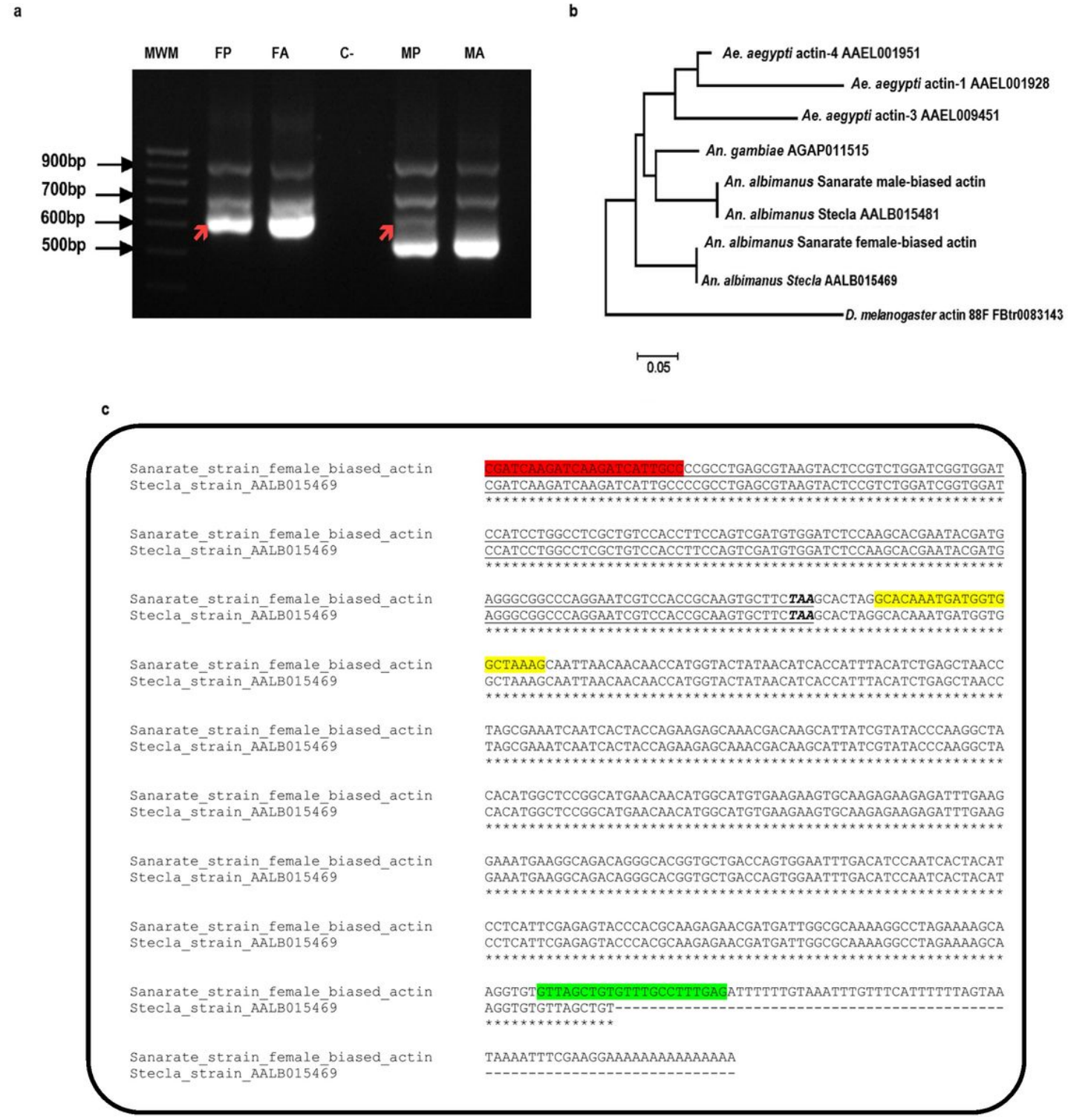


Figure 1

The An. albimanus Sanarate female-biased actin is a cytoplasmic actin orthologue mapping to chromosome three. a. The 3' UTR was amplified with conserved actin primers using 3'RACE. Female-biased actin fragments (red arrow). b. Maximun likehood tree of sex-biased actins and other mosquito species flight muscle and muscle actins c. Alignment between 600bp fragment and An. albimanus Stecla strain AALB015469 partial transcript sequence. Primers FMActRace_1F (red) and AaACT3UTR_F (yellow)

were used to produce the template sequence for dsRNA for the 3'-UTR alone (designated Actin A) and the forward primer (red) and reverse primer AaACT3UTR_R (green) to generate the template for dsRNA fragment to include a short C terminal coding region (designated Actin B). Coding region underlined, stop codon in italics and bold.



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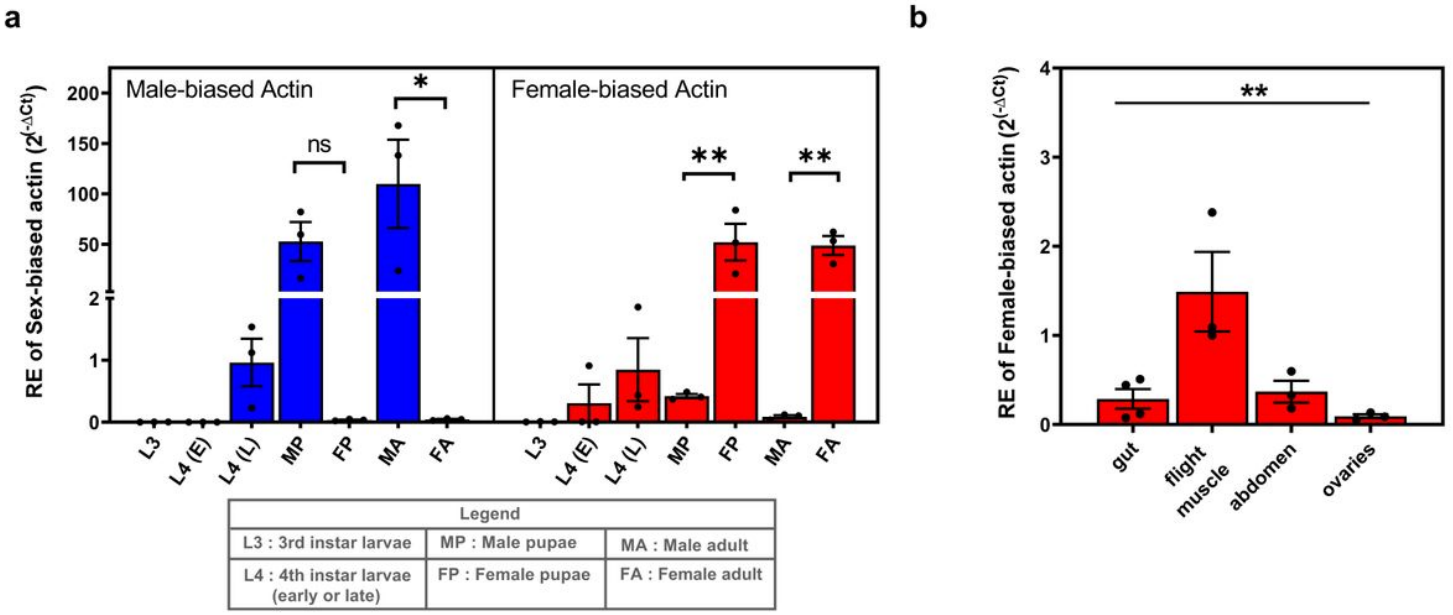
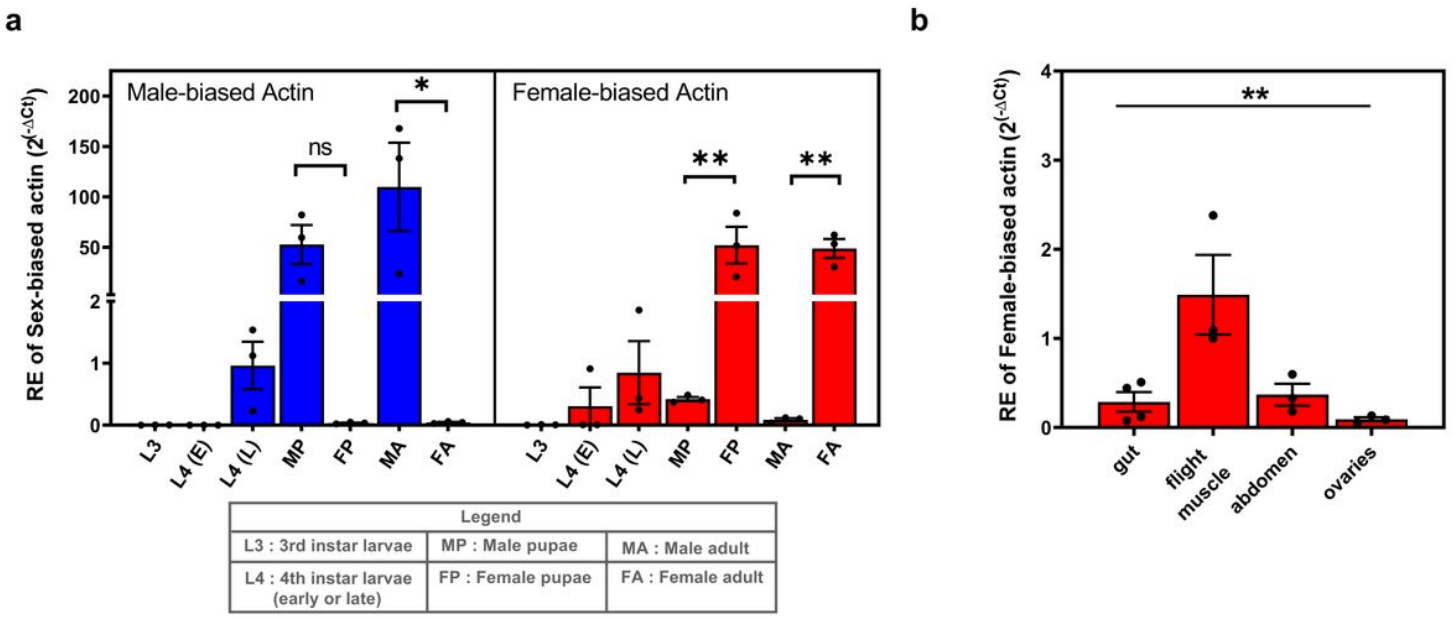


Figure 2

Sex-biased actin genes are differentially expressed across stages with female-biased actin expression in flight muscle. a. Relative expression across developmental stages and sexes compared to third instar larvae. b. Relative expression of female-biased actin in adult female gut, flight muscle, abdomen and ovaries. Delta Ct (ΔCt) analysis was done using Rps4 and RpL49 as reference genes for three biological replicas. Data are presented as a mean of relative expression and bars represent standard deviation. Data was analyzed using ANOVA test with Tukey´s comparisons * ($p < 0.05$); **($p < 0.01$); ns, not significant.



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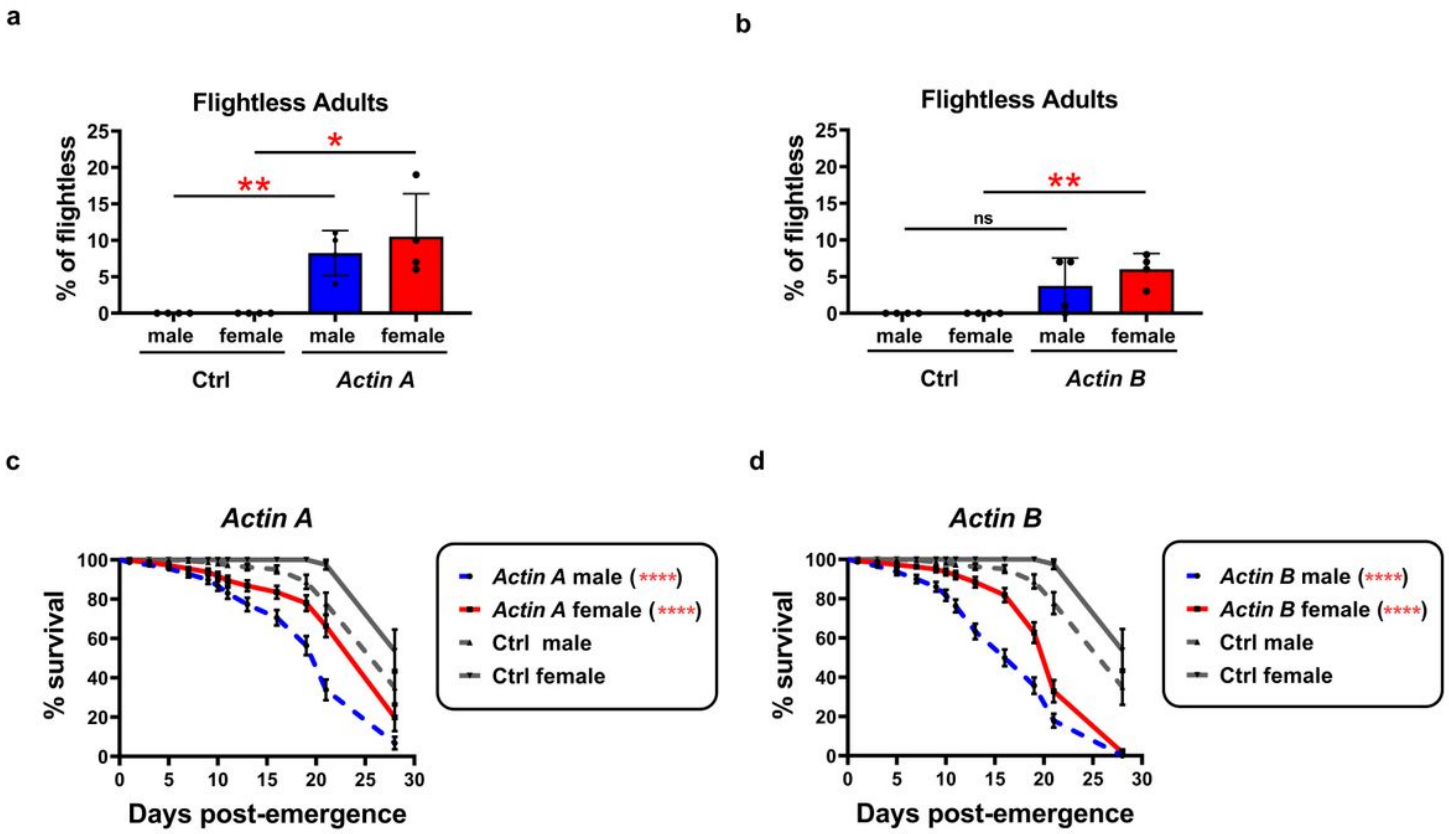
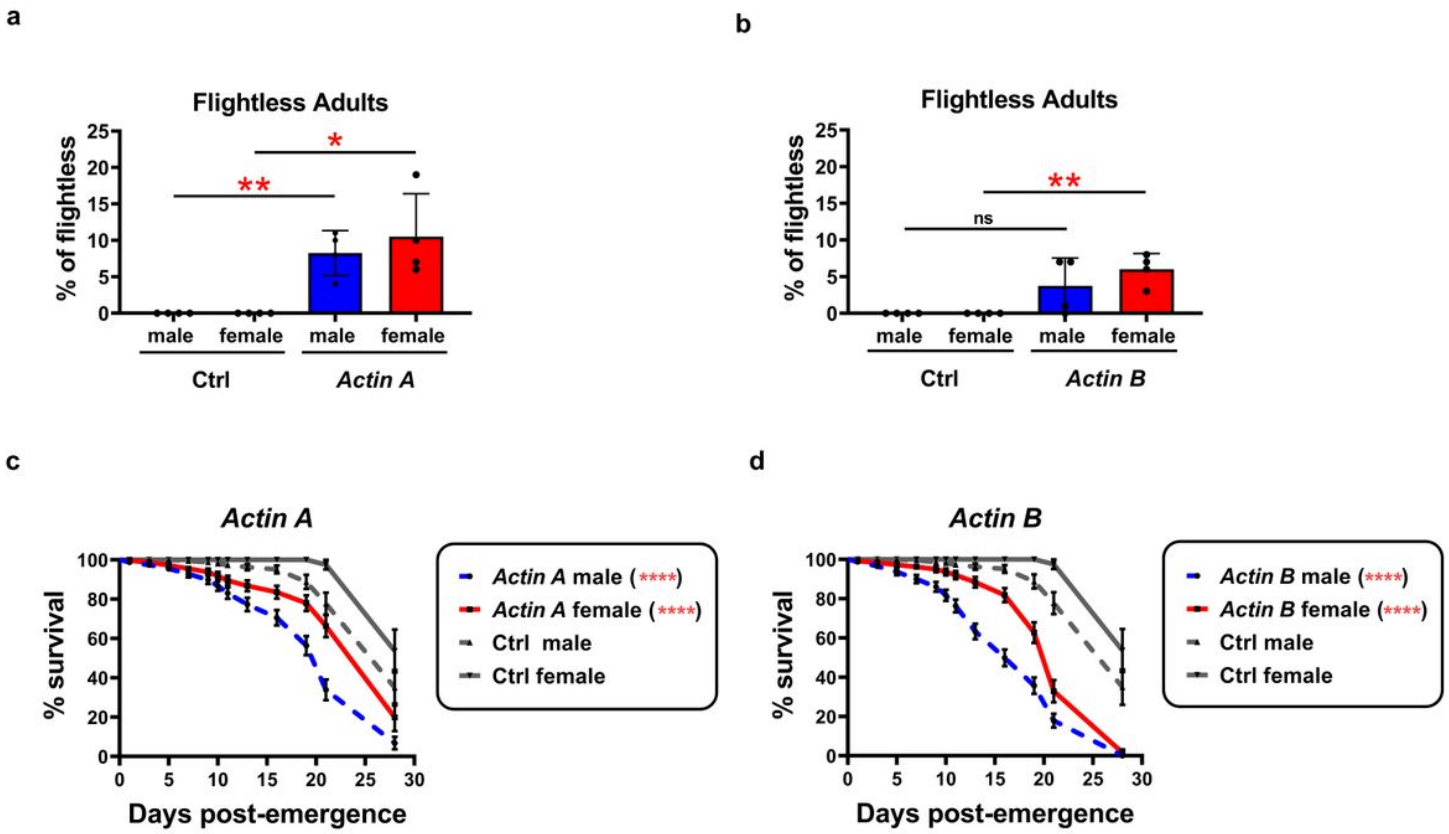


Figure 3

Flight and adult survival are affected after feeding larvae the female-biased actin 3' -UTR dsRNA. The proportion of flightless adult females and males one-day post-emergence after feeding on a) Actin A or b) Actin B. Adult survival over 30 days is presented for mosquitoes fed c) Actin A or d) Actin B. Ctrl, dsRNA targeting aintegumenta gene from A. thaliana as a control. The analysis includes four biological replicates (n=80 each), each one with 3-4 groups of 20 individuals. Data are presented as mean of relative expression and bars represent standard deviation. T-test was performed in comparison to Ctrl group for flightless phenotype *($p = 0.0121$) ** ($p = 0.00015$) for females and ** ($p = 0.0018$) for males; ns, non-significant. Log-Rank Mantel-Cox test was performed for survival curves in comparison to Ctrl group *** ($p < 0.0001$).



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Supplementary Files

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