Parental RNAi Silencing of the *transformer-2* Gene in a Species of the *Anastrepha fraterculus* Complex of Cryptic Species (Diptera, Tephritidae)

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Abstract

In genera *Anastrepha, Bactrocera* and *Ceratitis* of the tephritid fruit flies the auto-regulatory function of gene *transformer* is assumed to be activated by maternal derived mRNA or the proteins of the gene *transformer* ($tra-2^{mat}$) and *transformer-2* ($tra-2^{mat}$). However, this maternal effect was not yet been demonstrated. The objective of the present study was to test the effect of absence of $tra-2^{mat}$ in the eggs on the sex determination of *A*. sp.1 affinis *fraterculus*. This was achieved by silencing gene *tra-2* in the parental females via the pRNA interference. The data showed that *tra-2* was transiently silenced in the female for three weeks period. The progenies sex ratio produced by these females during the silencing of *tra-2* depart from 1:1 in favor of males. The excess of males was due to the transformation of a fraction of genotypical female XX embryos into XX males, the so-called pseudomales, Individual F1 males from the offspring of treated females crossed to females from the stock, revealed that majority of them showed regular mating behavior and were fertile. However, no offspring was produced in the crosses by a fraction of males that have produced sperms, showed regular mating behavior but did not transfer sperms to the females. The data allow the conclusion that the absence of $tra-2^{mat}$ in the eggs had impaired the self-regulation of the embryonic gene *tra* resulting in the transformation of XX embryos into pseudomales and also that these pseudomales are sterile. This effect may be useful improve more sustainable technologies for fruit fly control such as SIT.

Keywords: sex determining, pRNAi, *Anastrepha*, *transformer-2*, pseudomales

1. Introduction

Some fruit fly species of the Tephritidae family are considered one of the most devasting insect pests of agriculture The species of economic and quarantine importance (ca.78 species) damage the horticulture industry worldwide, reaching losses of billions of US dollars every year (Rendón & Enkerlin, 2021). The most important genera include *Anastrepha* (Schiner) indigenous to the Americas, *Bactrocera* (Macquart) in Asia and Oceania, *Dacus* (Fabricius) in Africa, *Rhagoletis* (Loew) in more temperate areas of Europe, North and South America, and the *Zeugodacus* (Hendel) in Asia (White & Elson-Harris, 1992; Rendón & Enkerlin, 2021).

The genus *Anastrepha* is endemic to the American tropics and subtropics, and many of its pest species have a wide distribution within this range (Norrbom & Foote, 1989). The South American fruit fly, *Anastrepha fraterculus* (Wiedemann) is a complex of cryptic species that has a wide geographic distribution almost the same as the genus and uses many host plants for larval development (Zucchi & Moraes, 2008; Norrbom et al., 2013). This species involves a complex of cryptic species, the so-called *Anastrepha fraterculus* complex of cryptic species that is being currently characterized (Selivon et al., 2004, 2005; Hernández-Ortiz et al., 2012, 2015; Hendrichs et al., 2015; Vaníčková et al., 2015; Prezotto et al., 2019). Due to the economic importance of the *Anastrepha* species programmes for their control, SIT for example, have been established in several countries along the continent (Rendón & Enkerlin, 2021). However, the effectiveness of such programs can in some cases be increased with the establishment of so-called sexing system to eliminate females allowing male-only releases. Therefore, new strategies to improve the massive production of males in the biofabrics are ever since being pursued, one of which is based on the knowledge of the genetic/molecular mechanism of sex determination of

the targeted species in search of gene alteration that cause sex ratio modification in favor of males (Häcker & Schetelig, 2021).

Sex determination in insects is achieved by a variety of mechanism but are commonly based on a hierarchic cascade of genes, the product of one gene controlling the sex-specific splicing of the pre-mRNA of the gene downstream in the hierarchy. It has been demonstrated that sex determination in frugivorous Tephritidae flies is controlled by the activity of a set of three key genes, *transformer* (*tra*), *transformer-2* (*tra-2*) and *doublesex* (*dsx*). Differentially from *Drosophila*, in which gene *Sexlethal* (*Sxl*) is on the top of gene cascade of sex determination, in tephritid flies and other dipteran insects it does not have this function (Belote et al., 1989, Saccone et al., 1998, Saccone et al., 2002; Sánchez, 2008; Hediger et al., 2010; Verhulst et al., 2010; Gempe & Beye, 2011; Bopp et al., 2014; Bachtrog et al., 2014). In tephritids, gene *tra* is expressed in both sexes but produces functional Tra protein only in females by a self-regulatory mechanism that is imposed by the maternal *tra* (*tra^{mat}*) and *tra-2* (*tra-2^{mat}*) RNA or their proteins deposited in the eggs (Pane et al., 2002; Ruiz et al., 2007a, 2007b; Sarno et al., 2010; Luo et al., 2017) in conjunction with the embryonic *transformer-2* gene (Salvemini et al., 2009). The functional Tra protein plus the constitutive embryonic Tra-2 protein control the female-specific splicing of the pre-mRNA of gene *dsx*, producing a female Dsx^F protein. This protein acts controlling the expression of many cytodifferentiation genes, which results in the expression of female phenotype (Figure 1, left). The Y chromosome of the tephritid *Ceratitis capitata* carry a linked M factor that determines the male sex by repressing directly or indirectly the function of embryonic *tra* gene (Willhofet & Franz, 1996; Pane et al., 2002). The molecular nature of the M factor of *C. capitata* and of four species of *Bactrocera*, was identified and the gene named as *Maleness-on-the-Y* (*MoY*) (Meccariello et al., 2019). This factor is being hypothesized to exists also in other genera of frugivorous tephritid flies (Sánchez, 2008). Thus, in males no functional Tra protein is produced and the splicing of *dsx* displays its default form, giving rise to production of male specific protein Dsx^M which leads to development of male phenotype (Figure 1, right). The tephritid sex determination mechanism has been found in species of *Bactrocera* (Shearman & Frommer, 1998; Lagos et al., 2005; Chen et al., 2008; Permpoon et al., 2011; Liu & Wan, 2015; Liu et al., 2015; Morrow et al., 2014; Laohakieat et al., 2016; Luo et al., 2017), in *Ceratitis capitata* (Pane et al. 2007; Saccone et al., 2002, 2011; Salvemini et al., 2009) and in 12 species of *Anastrepha* (Ruiz et al., 2007a, 2007b; Sarno et al., 2010; Schetelig et al., 2012).
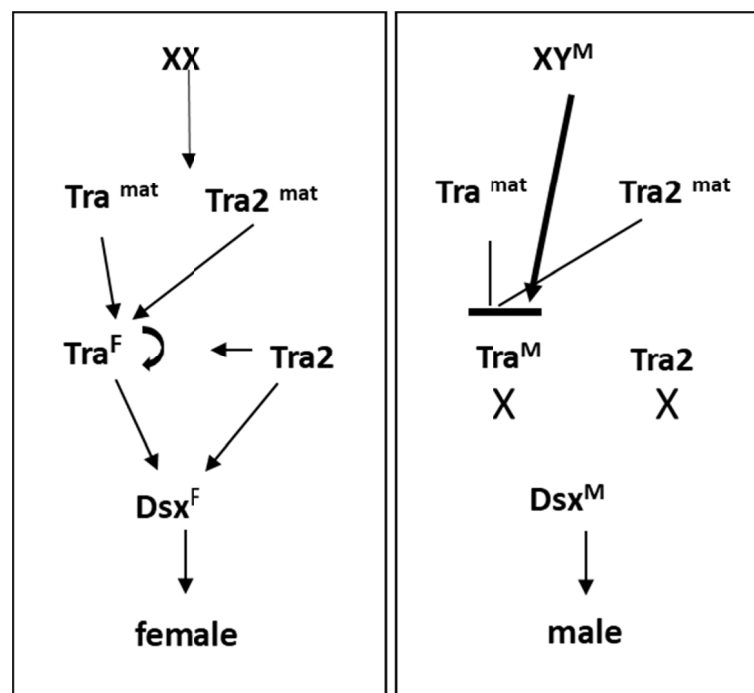

Figure 1. Sex determination cascade in Tephritidae flies. Tra^{mat} and Tra-2^{mat} are maternal Tra and Tra-2 products. Dsx^F and Dsx^M stand for Dsx proteins. Y^M is the putative M factor supposed to exists on the Y chromosome of *Anastrepha* species. Schemes redrawn based on Sánchez (2008)

Experimentally, the role of the three genes in sex determination of tepritid species has been elucidated by the embryonic RNAi methodology. Injection of *tra*dsRNA, of *tra2*dsRNA and/or *dsx*dsRNA, in the eggs of *Ceratitis* (Pane et al., 2002, 2007; Salvemini et al., 2009), *Bactrocera* (Chen et al., 2008; Liu et al., 2015; Peng et al., 2015), *Anastrepha* (Sarno et al., 2010; Schetelig et al., 2012) as well as of *MoY*dsRNA in *C. capitata* and species of *Bactrocera* (Meccariello et al., 2019), results in the male-specific splicing of pre-mRNA of *tra* and of *dsx* in genotypic female embryos. This leads to transformation of a fraction of females into XX male individuals, the pseudomales. Moreover, functional role of the three genes, *tra*, *tra-2* and *dsx* of *Anastrepha*, was also demonstrated by introduction of cDNA of the *Anastrepha* genes in intersexual *Drosophila melanogaster* and the development of sexually dimorphic structures were monitored. It was shown that genes *tra* (Ruiz & Sanchez, 2010), *tra-2* (Sarno et al., 2011) and *dsx* (Alvarez et al., 2010) of *Anastrepha* promoted the transformation *Drosophila* XY male flies into females, *i.e.*, the *Anastrepha* genes have changed the male splicing of the *Drosophila* pre-mRNAs into the female mode of splicing corroborating the role of the genes in *Anastrepha* sex determination. Similarly, it was demonstrated that the *dsx* gene of *Ceratitis capitata* induces masculinization of transgenic XX *Drosophila* flies (Saccone et al., 2008).

In the embryonic RNAi experiments with tephritid flies it was also detected that the transformation of females into XX pseudomales was usually not complete, and that fertility of pseudomales was also affected. In the XX pseudomales asymmetric and/or disformed testes and gynandromorphs were detected in *Ceratitis capitata* (Pane et al., 2002; Salvemini et al., 2009), *Bactrocera dorsalis* (Liu et al., 2015), and in *Anastrepha* sp.1 *aff. fraterculus* (Sarno et al., 2010). Although the XX pseudomales of *Anastrepha suspensa* had motile sperms, they were shown to be sterile (Schetelig et al., 2012). On the contrary, the XX pseudomales of *Ceratitis capitata* (Salvemini et al. 2009, Meccariello et al 2019) and of *Bactrocera dorsalis* (Liu et al., 2015) were fully fertile.

Most of sex determination experimental studies in tephritid fruit flies have been performed by injection of dsRNA into eggs which suppresses the function of the involved genes in the embryos. However, although sex determination also involves maternal components of genes *tra* and *tra-2* in eggs (Pane et al., 2002; Ruiz et al., 2007a, 2007b; Sarno et al., 2010; Luo et al., 2017), no experimental demonstration of their function has been made. The present report describes the results of silencing the *tra-2* expression in adult females demonstrating that the *tra-2^{mat}* is essential for the sex determination hierarchy in *Anastrepha* sp.1 aff. *fraterculus*.

2. Method

2.1 Biological Material

The present study was carried out with *Anastrepha* sp.1 *aff. fraterculus*, a species of the *Anastrepha fraterculus* complex of cryptic species (Selivon et al., 2005; Hernández-Ortiz et al., 2012, 2015; Prezotto et al. 2019). The flies derived from laboratory colonies initiated with specimens collected in guavas (*Psidium guajava*) from Poços de Caldas, MG, Brazil (21° 47'S, 46°33'W), which were maintained accordingly to standard procedures (Selivon et al. 2005).

2.2 dsRNA Preparation and Injection

For generating *Aftra2*dsRNA, *tra-2* cDNA fragments were produced using Superscript® III First-Strand Synthesis for RT-PCR kit from total RNA (5 μg per reaction), following manufacturer instructions using tra-2F primer. Afterwards, those fragments were amplified by PCR reaction using the following primers: tra-2F: ATGAGTCCACGTAC, tra-2R: CACGTCGCTTATCGTACGGA. The PCR was performed under the following conditions: 94 ºC for 1 min, followed by 35 cycles at 94 ºC for 20 sec, 55 ºC for 20 sec and 72 ºC for 30 sec, followed by a final extension step of 72 ºC for 10 minutes. PCR products were purified using GeneJET PCR Purification Kit (Thermo) and then sequenced using ABI Prism Big Dye Terminator Cycles Sequencing Ready Reaction kit, both following manufacturer instructions. Obtained sequences were manually inspected in BioEdit Sequencies Alignment Editor (v.7.0.9.0). Purified fragments were used in a second PCR using tra-2F and tra-2R primers containing T7 promoter sequences, T7tra-2F: ATGAGTCCACGTACTAATACGACTCACTATAGGGAGA and T7tra-2R: CACGTCGCTTATCGTACGGATAATACGACTCACTATAGGGAGA. The PCR cycling was as following: 94 ºC for 1 min, followed by 35 cycles at 94 ºC for 20 sec, 65 ºC for 30 sec and 72 ºC for 30 sec, followed by a final extension step of 72 ºC for 10 minutes. Product samples were analyzed by electrophoresis in 1% agarose gels in 1x TAE buffer with 1x GelRedTM Nucleic Acid Gel Stain, 1x using TAE as running buffer for 30 minutes at constant 60V and 60mA, as recommend by Sambrook et al. (1989). Electrophoresis gels were analyzed under UV transilluminator, recorded (Viber-Laumat) and transferred to computer for analysis. After confirming the presence of the fragments, DNA was measured by NanoDropTM 1000 Sepectrophotometer (Thermo Scientific), and this product was used for dsRNA synthesis.

About 1μg of the product was used to produce *Aftra2*dsRNA by *in vitro* reaction with T7 polymerase for 16 hours using Megasript kit (Ambion). dsRNA products were purified by precipitating in lithium chloride (LiCl), following manufacturer instructions, and resuspended in injection buffer (5 mM KCl, 0.1 mM NaH_2PO_4, pH 6.8) to a final concentration of 2 μg/μl, measured with NanoDropTM 1000 Spectrophotometer (Thermo Scientific). Samples were used immediately or stored at -80 ºC.

For injection, solution of *Af1tra2*dsRNA at the concentrations of 1.8 μg/μL, 1.2 μg/μL and 0.6 μg/μL were prepared in buffer containing 5 mM KCL, 0.1 mM, NaH_2-PO_4, pH 6.8 (Sarno et al., 2010). Groups of 10 adult females under cold anesthesia were injected with dsRNA with a fine tip glass needle (Eppendorf) at the ventro-lateral pleura between abdominal segments 3 and 4. Three volumes of dsRNA solution (1 μL, 0.7 μL and 0.5 μL) from each of the three concentrations were tested as well as control injections of plain buffer. The injected females were transferred to cages provided with water and food *ad libitum* and inspected for the next 24 h. The female survival was higher (70 to 80%) at the dsRNA concentration of 1.2 μg/μL in a volume of 0.7 μL, similarly to the survival of females injected with plain buffer. These parameters for the dsRNA injection were employed in all the experiments. Number of flies, as well as experiment details and statistical methods of analysis will be explained in the Results for the sake of clarity.

2.3 Experimental Design

The study comprises three sets of experiments: Firstly, tests for the demonstration that the *Af1tra2*dsRNA induces the transient silencing of gene *tra-2* in the adult females; secondly, analysis of sex ratio of progenies produced by the treated females during the period of *tra-2* silencing; third, analysis of the morphology, of karyotypes and fitness parameters of the F1 males and morphology of F1 females from these progenies.

2.3.1 Maternal tra-2 Silencing

Efficiency of the *Af1tra2*dsRNA in promoting *tra-2* gene silencing was tested by RT-PCR. For that, 18 females were injected with *Af1tra2*dsRNA and an equal number with plain buffer (BC). The injected females were separated in groups of 3 for RNA extraction in days 0, 3, 6, 9, 12 and 15 after injection (0 corresponding to the day of injection). RNA was extracted from total adult flies and employed to produce cDNA using SuperScript III[®] (Thermo Scientific) following the fabricant instruction with *tra-2* specific primers (F: 5' AGAGTTGGAATGAGTCCACGTAC 3'; R: 5' CACGTCGCTTATCGTACGGA 3). Primers were constructed using NCBI Primer-Blast tool, from *tra-2* cDNA of *Anastrepha obliqua* (GenBank FN658607). For the RT-PCR reaction 10% of cDNA was employed and the cycles were as follows: 1 minute at 94 ºC, followed by 32 amplification cycles at 15 seconds at 94 ºC, 15 seconds at 57 ºC and 30 seconds at 72 ºC, and finally and final extension step of 10 minutes at 94 ºC. Then, the reactions were analyzed by running gel electrophoresis (agar 1.5% in TAE buffer) stained with GelRed[®]. Gel runs were analyzed under UV transilluminator (Viber-Loumat), photographed and images were edited by Photoshop 6.0 (StatSoft, Inc[®]).

2.3.2 Analysis of Sex Ratio

Four replicate experiments (A, B, C, D) for testing the effect of dsRNA, and two controls, one with plain buffer (BC) and the other without any treatment (UC) were made. In each experiment, pupae of *A*. sp.1 aff. *fraterculus* from colony were transferred to cages until emergence of adults. Soon as they emerged males and females were separated in individual cages and provided with water and food *ad libitum*. For analysis of sex ratio, 50 pairs of mature virgin flies (15-17 days) were transferred to standard insect rearing cubic cages (20 cm edge), and guava fruits were provided for oviposition and larvae development. After a week, guavas were transferred to flasks, containing vermiculite. After 15-20 days, pupae were collected and maintained under standard condition until emergence of adults. Analysis of imagoes from each progeny revealed the sex ratio produced by females before of dsRNA and buffer (BC) treatments. At the end of this one-week pre-injection period, females of each replica were injected with *Af1tra2*dsRNA, others were injected with buffer (BC) and a non-injected untreated group of females (UC) was secured. To each group guavas were furnished for oviposition during a week to obtain progenies and this procedure was repeated for the next four weeks. The sex ratio of emerged adults produced at each experimental and control group during each of the five weeks the experiment lasted was registered. Additionally, groups of F1 males from the offspring of the experimental replicas were secured along the experiments for karyotype analysis of their as well as for their mating behavior, fertility, morphology of reproductive structures, production and transfer of sperms. Morphology of F1 females was also analyzed.

2.3.3 Karyotype Analysis

For this analysis, 40 F1 males derived from progenies of dsRNA injected females of each experimental replica and 40 from the control group (BC) were karyotyped as described by Selivon and Perondini (1967). Briefly, the

testicles of recent emerged males (1 to 2 days after emergence) were dissected, transferred to a 0.2% solution of sodium citrate for 10 min, immersed in a drop of 2% acetic orcein, and squashed between slide and coverslip. Analysis of the preparations was made under a BX60 Olympus microscope under regular illumination or phase contrast. Digital images were captured by a DC100 Leica camera coupled to the microscope and edited by Photoshop 6.0 (StatSoft, Inc.[®]).

2.3.4 Morphology and Reproductive Performance of Males

For these tests, 20 F1 males at the 1st week and 20 at the 3rd week from each experimental replica and from the control group (BC) were individually transferred to cages together with 2-3 virgin females from the stock. These cages were provided with water, food, and guavas as oviposition site for two weeks. The guavas from each replica were retrieved and maintained until pupae recovering. After emergence, adults were employed for in cross fertility tests. During this period, 5 males from replica A, 5 from replica B and 6 from replica C, died for unknown causes. Hence, the analysis of F1 male characteristics were based on 35 males from each experimental replica A and B, and 34 from replica C.

The males from each cross were observed daily for two weeks and their behavior related to courtship and mating were registered: everted anal pouch membranes (indicative of pheromone emission), wing fanning and copulas or copula attempts (Gomes-Cendra et al., 2011). After this period, the guavas were retrieved, maintained until adult emergence, and the presence of progenies derived from these fruits indicated fertility of the crosses.

After these two weeks, the F1 males were retrieved, anesthetized under cold and examined in a stereomicroscope for the external morphology of their terminalia. After, they were dissected for analysis of their internal reproductive organs. The testicles were removed and squashed between slide and coverslip in a drop of insect Ringer and examined for the presence of motile sperm. The external terminalia of the females involved in these crosses were also examined. After female dissection, their spermathecae were removed, squashed in drop of insect Ringer under a slide and coverslip, and examined for the presence of sperms. Hence, it was ascertained whether each one of the males was fertile or not and this information correlated with their morphological and other biological parameters analyzed. These analyses were made in a BX60 Olympus microscope under phase contrast. Digital images were taken by a DCC Leica camera coupled to the microscope and edited by Photoshop 6.0 software (Statsoft Inc.[®]).

3. Results

3.1 Maternal tra-2 Silencing

In females injected with buffer (BC control) amplification of a fragment of about 300 bp was detected along three weeks after injection with no visible differences in the size of the *tra-2* fragment compared to the fragment amplified from non-treated control females (UC). The fragments were sequenced and was confirmed that they correspond to *tra-2* sequences (Sarno et al., 2010) (data not shown). In *Af1tra2*dsRNA injected females, amplification of a fragment of 300 bp was observed at the day of injection (day 0). However, on the 3rd day after injection a faint band was still obtained, followed by a period in which no amplification of *tra-2* was detected, but on day 15 a faint fragment was detected again, indicating that expression of *tra-2* was being resumed (Figure 2).
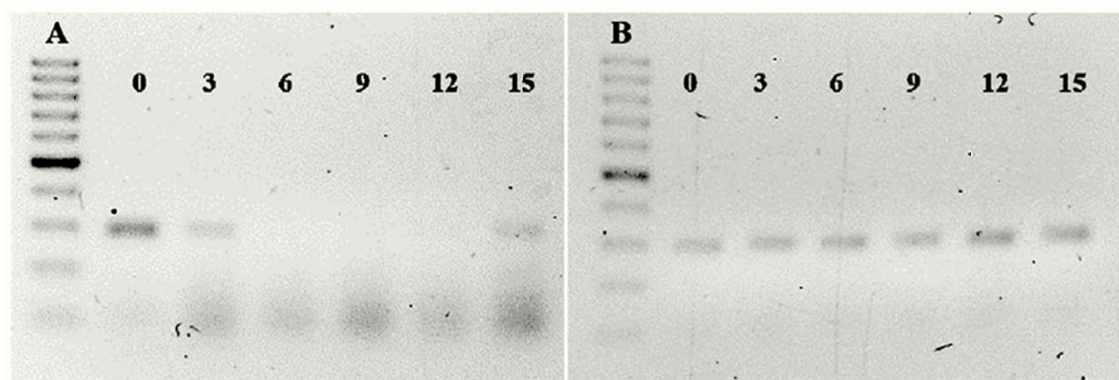
Figure 2. Silencing of gene *tra-2* in adult females. (A) Expression of *tra-2* in females injected with *Af1tra2*dsRNA and (B) in females injected with plain buffer. The numbers indicate the day of injection (day 0) and thereafter. A fragment of *tra-2* with 300 bp is seen in the controls (B) during the 15 days; on the treated females (A) a fragment of 300 bp is seen at day 0, a faint band was found in day 3, and no fragment was amplified from day 6 to day 12, but a faint band appears again at day 15. Black and white colors inverted in the gel photo for clarity. The left columns in both figures are the molecular scale 1000 bp

3.2 Progeny Analyses

3.2.1 Sex Ratio

The effects of parental RNAi were analyzed in a significant number of offspring produced by treated females of the four replicates (A, B, C, D) and two control groups (BC and UC) (Table 1). Firstly, analyses of the progenies produced by females previously to the dsRNA treatments (day 0) showed the expected 1:1 sex ratio (Table 1 and Figure 3). On the contrary, the sex ratio of total offspring produced during the following 4 weeks by females injected with *Af1tra2*dsRNA has deviated significantly from 1:1 in favor of males (Table 1). The proportion of males that were about 50% before treatment, gradually increased during the first three weeks and returned to about 1:1 in weeks 4 and 5 (Figure 3A). This pattern was observed for the four replicas of the experiment and although small differences between replicas have been observed the Kruskal-Wallis test indicate no statistical differences between the distributions (H (3, n = 24) = 2.04, p = 0.557). The progenies sex ratio produced by females from both control groups, BC and UC, fluctuate during the 5 weeks but did not differed significantly from the 1:1 ratio (Table 1 and Figure 3A). The highest deviation occurred during the 3rd week attaining 69.3% of males in replica D. The overall average increase in male frequencies above the expected 50% in weeks 1, 2, 3 and 4 was of 13.95% (Figure 3B). This figure also makes evident that the deviation in the sex ratio occurred in the progenies during the period that the *tra-2* gene was silenced in the injected parental females and progressively returned to about 1:1 after the resuming of *tra-2* activity.

Table 1. Sex ratio among adult flies of total progenies before and after injection of RNAi, and chi-square tests

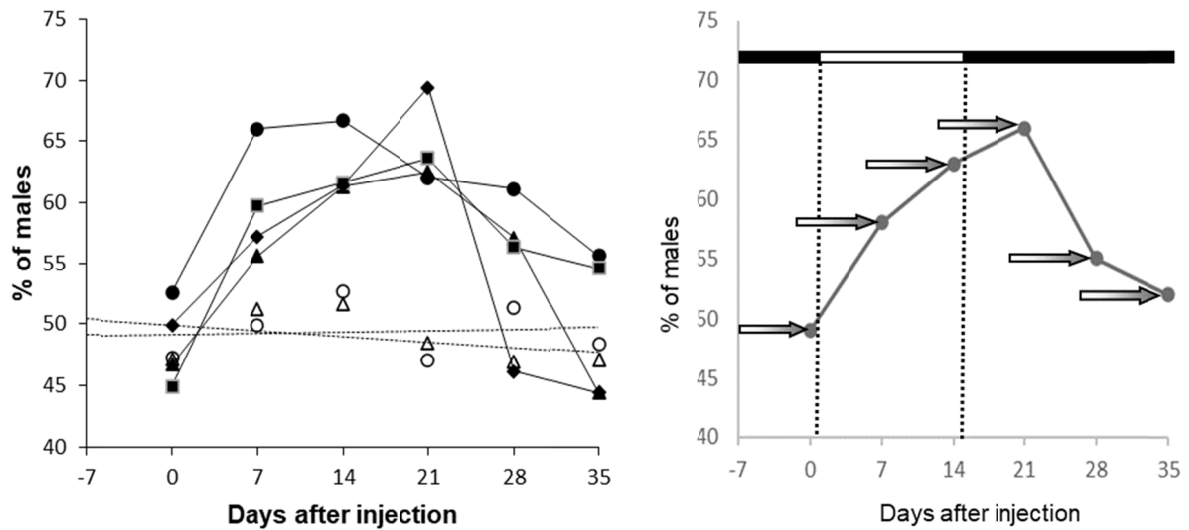| Treatment		Before injection				After injection			
	Groups	N	% Males	χ^2	P	N	% Males	χ^2	p
*Aftra2*dsRNA	A	169	52.6	0.379	0.538	213	63.8	15.793	<0.001*
	B	47	46.8	0.085	0.771	275	58.9	8.378	0.003*
	C	40	45.0	0.225	0.635	311	61.1	14.868	<0.001*
	D	30	50.0	0.033	0.856	311	59.2	10.084	0.001*
Buffer control	BC	169	47.3	0.378	0.538	305	50.2	0.004	0.949
Untreated	UC	36	47.2	0.028	0.867	405	50.4	0.010	0.920

Figure 3. Sex ratio of adults in progenies of injected females with *Af1tra2*dsRNA. (A) Injection was made on day 0. The filled points indicate the sex ratio of the four replicate experiments and the open points the sex ratio of the two control crosses. (B) The bar at the top indicates the period of activity (black) and silencing (clear) of *tra-2* in the parental females. The points show the mean sex ratio of the four experimental replicas, and the days were egg deposited by the females were collected and maintained until emergence of adults. The arrows indicate the approximated time when the collected eggs start their formation in the ovaries, *e.g.*, eggs deposited on day 7 started development 7-15 days before. As shown by the two vertical dashed lines, the eggs in samples with deviation of sex ratio in favor of males (days 7, 14, 21) had developed entirely or large part of time during the period that *tra-2* were inactive in the parental females

3.2.2 Karyotype Analysis

Analysis of 40 males per experimental and control groups showed that a proportion of the males produced by dsRNA injected females were pseudomales bearing a XX sex chromosome pair while the majority were regular males exhibiting the XY chromosomes constitution (Table 3 and Figure 4). This is clearly detected since *A.* sp.1 aff. *fraterculus* has a small Y small heterochromatic chromosome (Selivon et al 2005; Goday et al 2006). The mean frequency of pseudomales (12.8%) is not significantly different from the mean increase in male frequency of 13.95% (above 50%) as observed in the sex ratio analysis above described (χ^2_{Yates} = 0.091, p = 0.77). The data indicate that the sex ratio deviation is due to the transformation of a fraction of genotypic XX embryo into pseudomales and not to differential mortality of females as attested by the rate of adult emergence in progenies of the treated females (Table 2).

Table 2. Rate of adult emergence in control and in crosses of dsRNA injected females and chi-square tests

Samples	No. of pupae	Emergence		Contingence p-values
		N	%	
A	302	213	70.5	0.801
B	362	275	75.9	0.772
C	423	311	73.5	0.997
Control	417	305	73.1	X

Table 3. Number of males with XY or XX sex chromosomes in the experimental and control groups

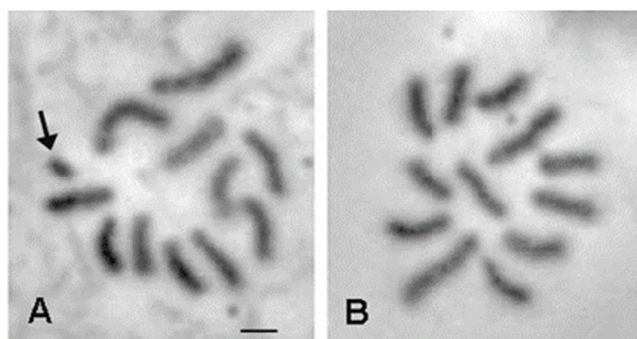| Groups	XY	XX (%)
Control	34	0
A	32	5 (13.5)
B	28	3 (9.7)
C	27	3 (10.0)
D	14	4 (13.3)
Total (A-D)	92	15 (12.8)

Figure 4. Mitotic divisions in male testicles. (A) Metaphase of a regular male with the small Y chromosome (arrow); (B) metaphase of a F1 male from the progeny of a *Af1tra-2*dsRNA treated *A*. sp.1 female with 12 chromosomes without the Y chromosome, thus being a pseudomale with a XX sex chromosome constitution. Scale bar, A, B = 10 μm

3.2.3 Morphology and Reproductive Performance of Males

For these analyses F1 males from the progenies of *Af1tra2*dsRNA treated females were individually crossed to females from the stock and guavas were furnished for oviposition and the flies observed for two weeks. After this, the guavas were retrieved and maintained until adult emergence for determination of each cross fertility. Similar crosses were made with F1 males in the progenies of buffer injected females (control group).

Analysis of adult emerged from the retrieved guavas showed that there were no significant differences in emergence rate between replicas (contingence table, $\chi^2_{Yates} = 0.31$, p = 0.856), hence the data were pooled. Among the 104 tested F1 males, 72 (69.2%) were fertile and the produced progenies were composed by male and female flies. However, for the other 32 (30.8%) F1 males no progenies were recovered. The number of fertile and unfertile males, however, are significantly different from the control crosses since 32 (94.1%) were fertile and only 2 (5.9%) were infertile (contingence table, $\chi^2_{Yates} = 8.547$, p = 0.035*).

The F1 males, after the 2 weeks period of observation, was retrieved from each cross and individually examined, no abnormalities being found in their external terminalia. It was observed that the males from each replica and from the control group showed normal courtship behavior, like everted anal pouch membranes, wing fanning and that all of them have attempted or have indeed copulated. Then, the males had their testicles dissected out and motile sperms were found in all of them. However, in 4.8% of them asymmetrical testicles were found, one was apparently normal, but the other was larger and spherical (Figure 5). These alterations were not found in the males of the control group.

Dissection of females from the 72 fertile crosses showed that sperms were clearly found in their spermathecae but among the females from the 32 infertile crosses two classes were found: In 24 (75.0%) of them sperms were not found in the spermathecae but in 8 (23.5%) sperms were indeed transferred. The morphological analysis of the F1 females from the progenies of *tra2*dsRNA treated females have normal internal structures but one female had the ovipositor shorter than normal and without the terminal opening (Figure 5).
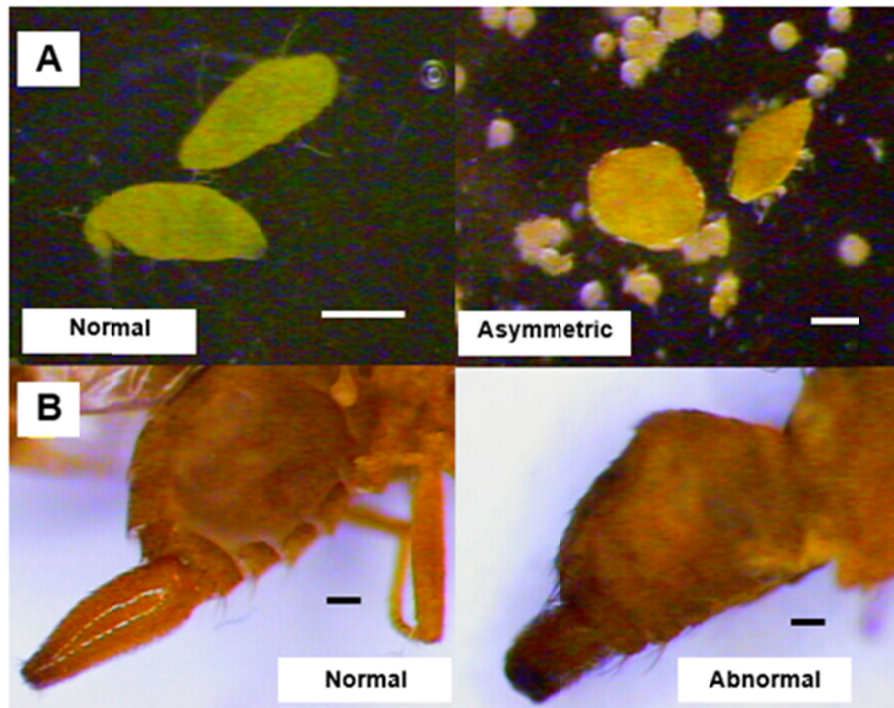
Figure 5. Morphological aspects of testicles and of the female external genitalia. (A) Regular and asymmetric testicles and in (B) regular and abnormal ovipositor found within the progenies of *Af1tra2*dsRNA injected females. Scale bars, A, B = 300 µm; C, D = 250 µm

4. Discussion

Silencing of gene *tra-2* by dsRNA injection in the adult females were achieved in the present study. The data suggested that inactivation of the gene might have occurred soon after injection. In *B. dorsalis* it was shown that inhibition of *dsx* gene by parental dsRNA treatment started 1 hour after injection (Chen et al., 2008). However, in the present analysis a faint amplified fragment was still visible on day 3 after treatment. It could be remnants of stocked *tra-2* RNA that were slowly being employed and depleted or due to silencing in distinct asynchronous females. On day 15, a faint fragment appears again indicating that *tra-2* activity was being resumed not abruptly but in increasing fraction of oocytes and/or due to asynchronous development of distinct females. Similar gradual recovery of gene activity after pRNAi silencing were described for *dsx* activity in *Bactrocera dorsalis* (Chen et al., 2008) and in *tra-2* activity in the Asian citrus psyllid *Diaphorina citri* (Yu & Killiny, 2017).

Since the amplification of *tra-2* was made from the parental females "in toto", including ovaries, the data indicate that during the three weeks of silencing, the treated females produced oocytes depleted of the $tra-2^{mat}$ RNA or its protein. Hence, if the auto-regulatory function of gene *tra* in the oocytes was really blocked by the absence of $tra-2^{mat}$ it would be expected that during the period of *tra-2* inactivity the females will produce offspring with altered sex ratio in favor of males and with the formation of XX pseudomales, as shown by embryonic silencing by dsRNA of the gene *tra-2* in this species (Sarno et al., 2010). Indeed, females under the *Af1tra2*dsRNA treatment produced progenies with altered sex ratio in favor of males. The frequency of male gradually increases in the weeks following the dsRNA treatment until the 3[dr] week and decrease toward 1:1 ratio thereafter. It is relevant to note that the frequency of males never reached 100% or closely, what would be expected if every oocyte produced during the inhibition of the gene *tra-2* did not had the $tra-2^{mat}$ factor. In species of frugivorous tephritid maturation of oocytes may take a variable timing interval to develop even among females of the same species as showed, for example, in *Anastrepha fraterculus* (Cardoso et al., 2006); Bartolucci et al., 2006), *A. serpentina* (Martinez et al., 1995), *A. suspensa* (Kendra et al., 2006) and *Bactrocera dorsalis* (Chou et al., 2012). Since the present analyses were made with pooled females the results might be explained by asynchronous development of the ovarioles or even the ovaries of a given female or between distinct females which could limit the number of oocytes depleted of $tra-2^{mat}$. However, the alteration in the sex ratio attained its maximum value (69.3% of males) at day 21 when the activity of gene *tra-2* in the parental female had already

been resumed. This apparent inconsistence may be explained, considering that the eggs collected at day 21 had initiated their development 7-10 days before, thus during the period that *tra-2* was still silenced in the parental female, as indicated in Figure 3B. Moreover, the fact that absorption of substances by the developing oocyte occurs only during the initial stages of their development (vitellogenesis stage), or in other words, before the stages the egg covers, vitelline and chorion membranes, are deposited in the oocyte (Telfer, 2009), may also be an explanation for the observed results.

In species of tephritid fruit flies, as in other insect species (Sánchez, 2008; Verhlust et al., 2010; Bopp et al., 2014), the increase in male frequency following injection of *tra*dsRNA and/or *tra2*dsRNA in embryos cause the default expression of zygotic *tra* leading to the development of a fraction of genotypic XX embryos to development as males, the so called XX pseudomales (Salvemini et al., 2009; Sarno et al., 2010; Schetelig et al., 2012; Liu et al., 2015). It shall made clear that in the present study the XX pseudomales were identified by chromosome analysis in a sample of F1 males derived from the sex ratio analysis. However, the karyotypes of another sample of 104 sister F1 males could not be determined because they were employed in the crosses to evaluate their reproductive status. Thus, one may assume that among these 104 F1 males a fraction must have had the XX chromosome pair while the majority were regular males bearing XY sex chromosomes. The analysis of these 104 F1 males showed they exhibited the typical mating behavior of the species herein analyzed (Selivon et al., 2005; Roriz et al., 2017). Hence, if pseudomales of *A*. sp.1 were present, they would show similar biological characteristics as the pseudomales of *A. suspensa* that although displaying regular mating behavior and producing motile spermatozoids, are sterile (Schetelig et al., 2012). Another aspect suggestive that the pseudomales herein produced were sterile is the fact that single sex progenies composed only by females were not found among the 72 fertile crosses of the present analysis. These only female offsprings would be produced by XX pseudomales if they were fertile as was observed in *C. capitata* (Savemini et al., 2009) and *B. dorsalis* (Liu et al., 2015). Hence, as pointed out by Schetelig et al. (2012), it seems that in *C. capitata* and *B. dorsalis* male fertility is not dependent of the Y chromosome while in *A. suspensa* the Y chromosome may carry factor(s) related to fertility. The results herein obtained for *A*. sp.1 aff. *fraterculus,* seems to be in line with these inferences.

Sterile crosses in which sperms were not transferred may be related to failure to complete the copula or to alteration in the female receptivity to mate, as shown in *A*. sp.1 from Argentina (Abrahams et al., 2011).

Since every male from the offspring of treated females, including the existent XX ones, was able copulate it was not expected morphological alterations in their terminalia, a fact that indeed was observed, as well as the presence of mobile sperms. Moreover, in the present study some of these males had altered testicles, usually hypertrophied ones and these characteristics were similar to those observed for males that develop from eggs of *A. suspensa* in which gene *tra-2* had been silenced by injection of *tra2*dsRNA (Schetelig et al., 2012). Another morphological alteration in the progenies of treated females is a rare case of a female that had a deformed ovipositor. This morphological malformation seems to be similar to that described in *B. dorsalis* treated by parental *dsx*dsRNA in which a fraction of the F1 females showed shorter ovipositor (Chen et al., 2008).

Hence, in the present study it is assumed that in the absence of *tra-2*^{mat} in XX embryos the auto-regulatory expression of the zygotic *tra* were not activated and truncated/default Tra protein must have been produced leading XX embryos to follow male development. Clearly, regular XY embryos are not affected by the treatment given origin to regular male flies. It must be noted that the sex ratio deviation in favor of males was not caused by differential mortality of females as attested by the rate of adult emergence in progenies of the treated females (Table 2) but was due to the transformation of a fraction of genotypic XX embryos into pseudomales. This effect may be useful to generate improvements in the mass rearing of flies, since sexing of the insects is a significant bottleneck for the application of the SIT to insect pest. The production of pseudomales may be enhanced by association of dsRNA of the genes *tra* and *tra-2* as reported for *A. suspensa* (Schetelig et al., 2012), and the dsRNAs for adult females could be delivered by other methods more suitable for the large-scale application in the biofabrics (Katoch et al., 2013; Zhang et al., 2013; Yu et al., 2013; Pomerantz & Hoy, 2015; Song et al., 2017). Improvements in modern and more sustainable technologies, as SIT, integrated with other control methods may lead to a more environmentally friendly strategies for insect pest control (Liedo et al., 2021).

5. Conclusion

The present report describes the results of the transient silencing of gene *transformer-2* by parental dsRNA interference methodology in *A*. sp.1 aff. *fraterculus*. It is concluded that the *tra-2*^{mat} in the egg is a necessary factor for the auto-regulatory activity of embryonic gene *transformer*, the key gene for the sex determination

hierarchy in *Anastrepha*. This effect may be useful to improve more sustainable technologies for fruit fly control such as SIT.

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