Title: **Development and validation of real-time PCR method to estimate stored sperm in the spermathecae of *Ceratitis capitata* (Diptera: Tephritidae)**

Short title: *Medfly sperm quantification by RT-qPCR*

To be submitted to: *Journal of Economic Entomology*

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Abstract

The development of polymerase chain reaction (PCR) markers to identify the Y chromosome of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) has permitted the detection of sperm transferred to females during mating. However, a molecular technique to quantify the sperm transferred has not yet become available. The current method to quantify the amount of sperm has been the direct counting of sperm heads. Thus, the purpose of this research was to develope and validate an accurate molecular method of diagnosis based on the application of an absolute quantitative real-time PCR, which allows the assessment of the quantity of sperm stored in the spermathecae. For this, Y-specific sequences were used to re-design and test distinct sperm markers. From the amplification product of samples detected as strong positives in conventional PCR, a cloning process of the target sequence was carried out to build the required standard curve. A series of known dilutions of this standard material was prepared for the absolute quantification process. A Roche Lightcycler ® 480 Real-Time PCR System and SYBR™Green fluorescent dye were used to quantify the sperm contained in the spermathecae of 4-day-old mated females and virgins. Wild type and Vienna-8 strain sterile males were used to quantify the sperm transferred at four mating durations (10, 30, 60 and 90 minutes) under laboratory conditions. To validate the reported quantitative method, our results were compared by counting sperm heads under a fluorescent microscope using the same experimental design. In addition, DNA samples were also evaluated and compared by conventional PCR.

Key words: Mediterranean fruit fly, tephritidae, sperm transfer, quantification, molecular technique

1. Introduction

Tephritid fruit flies are among the most damaging pests of agricultural crops worldwide and have an important economic impact in many cultivated fruits (White and Elson-Harris 1992, Enkerlin 2005, Aluja and Mangan 2008). In the Mediterranean area, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) is considered a major pest in many citrus growing countries. Producers and agricultural authorities are focused on controlling this pest to reduce potential fruit production losses and to minimize effects of restrictive quarantine regulations in international trade. In Spain, to maintain medfly populations below economic thresholds, integrated pest management strategies are well established; area-wide programs such as mass-trapping and the sterile insect technique (SIT) are being implemented (Eduardo Primo Millo, R. Argilés Herrero 2003, Navarro-Llopis et al. 2008, Sabater-Muñoz et al. 2012, Pla et al. 2018).

The SIT consists of the mass-production, sterilization, and release of mass-reared individuals into infested areas to reduce matings between wild individuals. The germ cell genetic damage of sterilized males brought about by irradiation causes chromosomal imbalance in the developing zygote through the breakage-fusion-bridge cycle (Muller 1954, Lachance and Riemann 1964, Curtis and Hill 1971, Robinson 2005) subsequently reproduction fails. Despite this chromosome fragmentation at the cytological level, normal sperm function is required in terms of motility and fertilizing ability (LaChance 1967). In fact, the efficacy of the SIT depends greatly on the sperm transfer of the released sterile males that should locate, attract, court, copulate and effectively inseminate wild female (Pérez-Staples et al. 2013).

To date sperm transfer in medflies has been estimated by techniques such as egg hatchability measurement or direct observations under microscope (Farias et al. 1972, Seo et al. 1990, Yuval et al. 1996, Marchini et al. 2001, Twig and Yuval 2005), nevertheless an effective

method to assess and quantify this mating success in *C. capitata* it has not been used until now. The development of biotechnological tools and the recent sequencing of the whole medfly genome (Papanicolaou et al. 2016, United States National Library of Medicine 2017), offer the possibility to establish new, more accurate methods suited for high throughput analysis. A first step towards this goal was a protocol able to determine the presence of Y chromosome and identify the sterile sperm (Vienna type) in the Mediterranean fruit fly female spermathecae (San Andres et al. 2007) based on known Y-chromosome sequences (Willhoeft and Franz 1996, Zhou et al. 2000) and a Vienna-8 strain mitochondrial polymorphism (Gasparich et al. 1995, Spanos et al. 2000). This diagnostic method consists of spermathecae dissection, DNA extraction, and two polymerase chain reactions (PCR). The first PCR was carried out to detect the presence of sperm and the second, along with a restriction fragment length polymorphism analysis (PCR-RFLP), to identify Vienna type sperm. The original method was evaluated at a later stage by checking the universality of the markers and testing new DNA preservation and sperm extraction methods (Juan-Blasco, Urbaneja, et al. 2013).

Despite these biotechnological advancements, until the study presented here it has not been possible to quantify sperm transfer by molecular tools. We adapted and validated a molecular method based on the application of an absolute quantitative Real-Time PCR to estimate the amount of sperm stored in the spermathecae after mating with wild type and Vienna-8 males. To do so, we established an experimental procedure that involves the re-design of sperm markers and the establishment of a standard curve for the absolute quantification real-time PCR. The validation of the sperm abundance was quantified with this new technique by comparison to microscope sperm counts and conventional PCR.

2. Material and methods

2.1 Insect colonies

Wild flies used in the establishment of this method come from a laboratory colony maintained in the Instituto Valenciano de Investigaciones Agrarias (IVIA) facilities (Valencia, Spain) since 2014. The colony was reinforced with regular additions of flies emerged from pupae collected on naturally infested fruits on field. The maintenance and diet of the fly populations followed the methodology described by Jacas et al. (2008). The conditions of the environmental chamber were 25±2ºC, 60-70% relative humidity with a photoperiod of 14:10 h (light: dark). The adults were fed with a mixture of sugar and hydrolyzed yeast extract in a 4:1 (w:w) proportion and water *ad libitum*.

The sterile males used in the experiment were obtained from the mass-rearing Vienna-8 genetic sexing strain (GSS) colony at the biofactory of Caudete de las Fuentes (Valencia, Spain). The rearing process was initiated in this biofactory managed by the TRAGSA Company with the current standard production of eggs, larvae, and pupae (Caceres, 2002). Vienna-8 males were irradiated as pupae by an electron accelerator with a dose from about 90 to 110 Gy. After irradiation they were transferred under hypoxia to Generalitat Valenciana emergence facility located in the facilities of IVIA at Moncada (Valencia, Spain) where the emergence and sexual maturation of the adult males took place. The production parameters and auxiliary treatments (ginger aromatherapy and cold treatment) were the same as those performed on the sterile males produced for the field-release program (Juan-Blasco et al. 2013).

2.2 Mating experiments

All mating experiments were performed with four-day old virgin males and females, an age in which they are considered to be sexually mature (Papadopoulos et al. 1998). To ensure virginity, wild type flies were segregated by sex within the first 24 hours of adult emergence and maintained in methacrylate-ventilated cages (20 X 20 X 20 cm) until experimental

120 testing. All of them, including sterile males, were fed a protein diet (4:1 w: w, sugar: yeast) to
121 simulate field conditions (availability of protein source).

122 The mating arena consisted of methacrylate cages (30 x 30 x 50 cm) with mesh openings on
123 two sides. Under laboratory conditions (23-25ºC, 60-70% relative humidity), virgin males
124 (wild-type or Vienna-8 depending on cross) were placed in the mating arena alone for ten
125 minutes to settle, thereafter virgin females were offered to the males in 1:1 sex ratio. During
126 two hours after the first couple was isolated all the mating pairs were carefully collected in
127 plastic test tubes (12 mm diameter x 75 mm tall; approximate volume 5ml) and the copulation
128 start time was recorded. Matings were interrupted after four established periods: 10, 30, 90
129 and 120 minutes.

130 Mated females considered for DNA extraction were preserved at -20ºC and females used for
131 microscope observations were dissected the same day the experiments were conducted; in
132 both cases they were frozen or dissected one hour after the separation moment. This time
133 interval was fixed because most sperm have reached the spermathecal receptacles one hour
134 after the end of mating with only a few sperm still present in the spermathecal ducts (mostly
135 in the apical part close to the spermathecae) (Marchini et al. 2001). A pool of females mated
136 with wild males for 120 minutes were used to test the Real-time PCR markers and to obtain
137 the standard curve for the absolute quantification. Mating experiments were repeated several
138 times until 30 females for each experimental combination were obtained. Virgin females from
139 each test day were set aside and preserved at -20ºC to be used as negative controls.

140 2.3 Dissection of female spermathecae and DNA extraction

141 The reproductive tract of female medfly contains two types of sperm storage organs, the
142 spermathecae used for long-term storage, and the fertilization chamber as the staging point for
143 fertilizing sperm (Marchini et al. 2001, Twig and Yuval 2005). Nevertheless, we focused the

144 analysis on the sperm lodged in the spermathecae as the amount of sperm stored in the
145 fertilization chamber can represent less than the 20 % of the total amount of sperm stored
146 (fertilization chamber and spermathecae) (Twig and Yuval 2005, Bertin et al. 2010).

147 Each pair of spermathecae were isolated from the female abdomen using microdissection
148 tweezers under a stereomicroscope. Because sperm storage asymmetry in the female's two
149 spermathecae has been reported (Yuval et al. 1996, Taylor and Yuval 1999), we processed the
150 two spermatechae together as a single sample. The spermathecal ducts were dissected in the
151 apical tract and the maternal tissue surrounding the spermatheca were removed to reduce non-
152 target female DNA which can interfere in the DNA extraction process and PCR of the sperm.
153 The intact spermathecae were transferred with autoclaved micropins into a 1.5ml Eppendorf
154 tube containing 100 µl of the TNES buffer used in the DNA extraction. After being crushed
155 gently with a micropestle, the DNA extraction protocol was performed according to the
156 'salting-out' method (Sunnucks and Hales 1996) with the following modifications. The
157 composition of the extraction buffer (TNES) contained 50mM Tris-HCl pH 7.5, 400mM
158 NaCl, 20mM EDTA pH 8.0, 0.5% SDS. DNA was precipitated with 250 µl of isopropanol
159 and washed with 200 µl of 70% ethanol. DNA samples were finally dissolved in 15 µl LTE
160 (50mM Tris-HCl pH 7.5, 0.1mM EDTA pH 8.0), spun, and frozen (-20ºC) until PCR
161 amplification.

162 2.4 Real-time PCR marker design

163 Sperm detection markers were obtained to detect the presence of sperm in medfly female
164 spermathecae (San Andres et al. 2007), however, because shorter amplicons are optimal for
165 most real-time PCRs (from 50-200 bp), Y-specific sequences were used to redesigned new
166 sperm markers using GenBank database, sequence alignment program Clustal Omega, and
167 primer analysis software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Two markers were

tested at four different temperatures (55, 56, 59 and 60ºC) using 1 µl of DNA extract of spermathecae (from virgin and mated females) (Tab.1). The archived DNA from male and female abdomen was used as the template in a mixture containing 0.2 mM dNTPs (Thermo Scientific), 1x Taq buffer, 1.75 mM $MgCl_2$, 0.75u DNA polymerase (Biotools) and 0.25 µM of each primer in a final volume of 20 µl. Conventional PCR was performed in a thermal cycler (Applied Biosystems™ Veriti™96-Well) and the amplification conditions were one denaturation step at 94ºC for 4 min, 35 cycles at 92ºC for 30 s, the tested temperatures for 30 s and 72ºC for 30 s, followed by a final extension at 72ºC for 10 min. PCR products were separated by electrophoresis in 1.5% agarose D-1 low EEO gel in 1x TAE buffer, stained with GelRed™ Nucleic Acid Gel Stain, and visualized under ultraviolet light.

2.5 Sperm quantification

The concentration of the sperm cells contained in the spermathecae was estimated by an absolute quantification analysis on a Real-Time PCR system where the sample values were obtained by comparing them to a standard curve generated from known amounts of the target Y-sequence.

2.5.1 Standard curve

The DNA material required to obtain the standard curve was taken from the amplification of the spermathecae DNA extract samples previously preserved for that purpose. The target sequence was multiplied by conventional PCR using the selected markers and the annealing temperature. PCR conditions were the same as described in the previous section. A commercial kit (Invisorb ® DNA Cleanup (250) Stratec Molecular) was used to purify the PCR products.

A cloning process of the purified products was performed according to a conventional method (Hanahan 1983). The DNA target sequence (insert) was ligated into a pGEM®-T vector (Easy

Vector Systems, Promega) to produce pGEM-TCcYsp and introduced into *E. coli* JM109. Plasmid DNA from the selected transformed bacteria selected by ampicillin resistance, were extracted using the Invisorb® Spin Plasmid Mini Two kit (Stratec Molecular). The concentration of the isolated and purified pDNA was measured with a NanoDrop 2000c spectrophotometer (Thermo Scientific). To confirm the presence of the target insert, a sample of the purified pDNA was digested with the restriction enzyme EcoRI at 37ºC for one hour and analyzed by electrophoresis in a 2% agarose gel. The quantification of the pGEM-TCcYsp in picomoles was performed considering the average weight of a one pair base (660 Da) and the number of base pairs of vector and insert (N_b). The following mathematical formula was applied: pmol of dsDNA = (µg of dsDNA× 10^6) / (660 ×N_b). Avogadro constant was used to estimate the number of pGEM-TCcYsp (6.023 × 10^{23} molecules/ mol). Number of copies was calculated per microliter, which was the volume used as template in each quantitative Real Time PCR was estimated. A ten-fold series of dilutions of the selected clones with correct insert size were prepared and stored at −20ºC until use. Dilutions from 10 to 10^6 copies were employed to generate the standard curve.

2.5.2 Absolute Real-Time PCR

The quantification analysis was carried out in a real-time thermal cycler (LightCycler® 480 Real-Time PCR Roche System) using the fluorescent dye SYBR Green. The master mixture contained 5 µl of Maxima SYBR Green I /ROX qPCR Master mix (2x), 0.5 µM of each primer and 1 µl of DNA template in a final volume reaction of 10 µl. Standard materials and non-template control were added to the 96-well plate in duplicate at each running. Real Time PCR protocol consisted of one step of 95ºC for 10 min, 40 cycles of amplification (95ºC for 15 s, 59ºC for 30 s and 72ºC for 30 s) followed by a final cycle of 95ºC for 5 s and 60ºC for 1 min. Data acquisition, standard curve fittings and analysis were performed with the LightCycler® 480 software that translated, for each sample, the threshold cycle value into the

starting amount of copies of the target Y-sequence, which was the sperm abundance in the spermathecae.

2.6 Validation of the method

The Real Time qPCR method for quantification analysis of sperm stored in the spermathecae was validated through conventional PCR and sperm microscope counts.

2.6.1 Semi-quantitative conventional PCR

A semi-quantitative conventional PCR was one of methods used to test the validity of the Real Time qPCR results. Taking the standard materials as reference, a pool of DNA samples were analyzed by conventional PCR using the designed markers and the same amplification conditions as described in section 2.4 except for the volume of the DNA template was 2 µl. PCR products were separated by electrophoresis on the same 1.5% agarose gel plate and visualized under ultraviolet light. Abundance values for each sample were assigned by comparing them with the amplicon density obtained for the standard materials.

2.7.2 Sperm microscope counts

The second validation method employed direct counts with a microscope; the only method used in previous sperm transfer studies. The same experimental design used in section 2.2 was carried out to obtain mated females of each male type and four copula duration groups (10, 30, 60 and 90 minutes). One hour after the separation time, females were numbed in a freezer and dissected under a stereomicroscope. Each pair of isolated spermathecae was individually placed onto a microscope slide in a drop of 0.5% Triton X-100 solution (5-10 µl) (Abraham et al. 2016) and crushed using a ball head pin. Once the spermathecae was broken into micro-fragments and the sperm content released, the sample was stirred and gently spread on the slide-grids; immediately after drying out, the content was fixed and preserved in a freezer (-

20ºC) until being stained. For the microscope counting, sperm was stained in 5 µl of 4µg/ml DAPI (4',6-diamidino-2-phenylindole) solution (Bertin et al. 2010) that was spread on the sample using a pin, and fixed before drying out with a glass coverslip. After 10 min of incubation in darkness to avoid light fluorescent signal loss, the sperm heads were counted from half of the area of the column grids under ultraviolet light microscope (40x) and the total number calculated using a multiplying factor.

2.8. Statistical analysis

All statistical analyses were performed with *STATGRAPHICS® Centurion XVI* statistical package. Where needed, the data were transformed log(x+1) to normalize it before analysis. The quantification data from Real-time PCR assay were subjected to analysis of variance (One- Way or Multifactorial ANOVA) to test the sperm transfer with respect to copula duration and male type (wild and Vienna-8 sterile males). A correlation analysis was used to compare the values of the sperm estimated from the real-time qPCR and semiquantitative PCR. The average of the total sperm counted by microscope was obtained with the standard error for each copula duration group. Finally, a comparison of regression lines were considered to compare the relationship between sperm transfer and copula duration obtained from real-time qPCR and microscope counting. Results were reported using significance criteria at 0 and 0.05 levels.

3. Results

3.1 Development of the quantitative method

The selected Y-specific markers employed were CcYsp-dir (5'-CGA AGC CAG ACA TAC ACG AGG AG-3') obtained by (San Andres et al. 2007) and the designed marker CcYsp-R2 (3'-TCGTTTGGAAAAGTGTGGTTC-5') at the optimum melting temperature of 59ºC. This melting temperature was determined experimentally and became key due to the lack of

mismatches with the female DNA. It was tested with DNA from the abdomen of females and males (wild type and Vienna 8 strain), and from spermathecae of mated females (Fig. 1). The provided markers generated a 112 pb DNA amplicon; their sequences are available in the GenBank database with the accession number MN062263.

The mating experiment was replicated thirty times; more than 300 matings were obtained. An average of 32 females per male type and mating duration were collected. Overall, 280 samples of dissected females and extracted DNA were evaluated. DNA concentration obtained from spermathecae was found to be 5-20 ng/µl whereas DNA extracted from the abdomen totaled 600-700ng/µl. The DNA sequences obtained from the cloning process at 10^{11}copies/µl were diluted serially to establish the required standard concentrations. The cycle threshold (Ct) values observed ranged from 26.6 to 12.9 which corresponded to a concentration between 10 to 10^6 copies of the cloned sequence. The suitability of the generated standard curve was evaluated by the means of estimated slopes (-3.0 ±0.06) and amplification efficiencies of the standards (2.2 ± 0.03) from the replicated calibration curves. The linearity of the concentration range was also assessed ($r^2 = 0.73$) (Fig. 2).

The minimum sperm detection was established based on the analysis of Ct values and concentrations obtained for non-template controls, samples from virgin females, and standard materials. Samples with Ct values above or at 26 were negative in Y-sequence detection; positive samples showed values ranging from 16.8 to 25.8. The detection limit concentration was close to 80 copies/µl.

The presence of sperm in the tested spermathecae was determined as positive in 78% of all samples analyzed (mated females with wild and sterile males). The number of samples with a positive PCR reaction varied significantly between male type ($F_{1,261} = 17.08$; $P < 0.001$) and mating duration ($F_{3,261} = 8.11$; $P < 0.001$), being higher for wild males and when the mating

duration lasted more than sixty minutes. The interaction between these two factors was not significant ($F_{3,261} = 0.14$; $P = 0.94$) (Tab. 2).

The values obtained in the real time quantification showed a positive significant correlation between the copula duration and the sperm stored in the spermathecae after females were mated with wild ($F_{3,164} = 25.16$; $P < 0.001$) or Vienna-8 sterile males ($F_{3,97} = 4.24$; $P = 0.01$) (Filled circles in Fig.4). The longer the copula duration (for the four evaluated durations), the greater the amount of sperm transferred and allocated in the spermathecae.

3.2 Validation of the real-time qPCR method

The results of the real-time qPCR were compared to the values obtained by semiquantitative conventional PCR. The sperm samples from wild-mated females were used in both methods and the semiquantitative PCR values were represented as levels of intensity bands observed in the agarose gel electrophoresis. The correlation analysis showed a strong relationship between the values determined from each method ($P < 0.001$; r = 0.81), which indicates comparable sperm abundance. Only five of the 93 samples which tested as positive in real-time qPCR showed a negative result by conventional PCR; data that could be attributed to a higher sensitivity of the real-time qPCR in sperm detection and quantification (Fig. 3).

The microscope sperm head counting method produced satisfactory results as an additional validation for the real-time qPCR analysis. Under the UV light microscope the amounts of sperm allocated in the spermathecae of 259 females were quantified. Numbers of sperm per female varied significantly between male type ($F_{1, 261} = 58.06$; $P < 0.001$) and mating duration ($F_{3, 261} = 34.04$; $P< 0.001$) (Tab. 3). The interaction between both factors was significant ($F_{3, 261} = 21.06$; $P < 0.001$); which can probably be attributed to a greater transfer of sperm with increasing copula duration observed in wild-mated females as opposed to that in the V8-mated females ($F_{3,261} = 21.06$; $P < 0.001$).

312 Comparing the regressions of sperm abundance and mating duration obtained in both
313 methods, no significant differences between the slopes were found. As was confirmed for
314 wild-mated ($F_{1, 287}$ = 162.67; P= 0.23) and V8 sterile-mated females ($F_{1,230}$ = 54.96; P = 0.66)
315 (Fig. 4).

316

317 **4. Discussion**

318 The complexity of the medfly mating system has generated numerous and relevant studies
319 that widely examine the factors involved in this reproductive system. However, the influence
320 of these factors, in some cases, still remains unclear. With the qPCR method reported here, a
321 potential and valuable tool is provided to enhance this understanding. We were able to detect
322 and quantify the amount of sperm transferred during mating in the female spermathecae. The
323 method was successfully applied to sperm of wild and Vienna-8 GSS fly strains.

324 The quantification of the sperm was possible at low concentrations of the target nucleic acid
325 (i.e. the sperm stored in spermathecae of females mated for ten minutes). This sensitivity had
326 not been reported in previous medfly molecular detection studies. The DNA of sperm had
327 only been evaluated in females after copula completion (between forty-five minutes and three
328 hours) which contained a greater stored quantity (San Andres et al. 2007, Juan-Blasco,
329 Sabater-Muñoz, et al. 2013, Juan-Blasco, Urbaneja, et al. 2013, Juan-Blasco et al. 2014). This
330 result (presence of sperm in spermathecae in 10-minute-matings) is in agreement with the
331 microscope observations reported in other studies where sperm was found after 4 to 15
332 minute, interrupted, copula (Farias et al. 1972, Seo et al. 1990). The real-time qPCR method
333 also quantified the increase in amount of sperm transferred with the increase in copula
334 duration for the four tested mating durations (10, 30, 60 and 90 minutes); results that are again
335 consistent with those reported by Farias et al. (1972) and Seo et al. (1990). Other studies have

not clearly suggested this increasing tendency of sperm stored during copula duration although in these cases the sperm transfer was evaluated in natural uninterrupted matings (Taylor and Yuval 1999, Taylor et al. 2000, Costa et al. 2012). Otherwise, microscope results conducted in this study to validate the proposed method confirmed the relationship between copula duration and the quantity of sperm transferred to the female for the tested periods.

The question of whether the chromosomal breaks induced by irradiation could affect the regions where the markers were designed was also addressed in the validation procedure by the microscope counting. As our results showed, values of relative sperm abundance obtained by qPCR method were in conjunction with the sperm heads counted, both in normal wild sperm and in irradiated V8 sperm.

All spermathecae were extracted from dry females preserved at -20ºC for up to three months. Longer conservation times were observed to increase the difficulties in obtaining spermatheca without ruptures and consequent sperm loss. Special care should be taken in this DNA extraction process, especially in the removal and isolation of the spermathecae and their further handling. The spermathecal capsules are sclerotized (Dallai et al. 1993) and need to be broken gently to release their content and obtain a homogenized extract in each individual tube. This sample processing is unavoidable and time-consuming, but alternative extraction protocols (Juan-Blasco, Urbaneja, et al. 2013) and modifications (data not showed) were examined without reliable results. According to our records, time required for spermathecae dissection and DNA extraction was estimated at ten minutes per sample.

The real-time qPCR method herein described has been designed as a specific and valuable method to assess the sperm transfer and storage in the female spermathecae. It offers us an accurate estimation and certain advantages from microscope diagnosis, such as the possibility of researching with frozen preserved flies and a lack of difficulties associated with sperm

bundle counting. Several studies have focused on evaluating the influence of different factors (age, diet, size) on the sperm transfer and storage (Churchill-Stanland et al. 1986, Orozco and Lopez 1993, Blay and Yuval 1997, 1999, Taylor and Yuval 1999, Taylor et al. 2001, Kaspi et al. 2000, Taylor et al. 2000, Papanastasiou et al. 2011, Costa et al. 2012, de Aquino and Joachim-Bravo 2013); nevertheless, the variability in outcomes is striking. Within this context, the molecular method herein presented can contribute to the clarification of some knowledge gaps in this multifactorial approach.

In SIT programs, the method represents a useful tool in the diagnosis of quality parameters for the mass-rearing medfly especially when laboratory studies on dietary or chemical manipulation of the adult environment are to be implemented in mass production. Another question, not previously regarded, is the ability of re-mating along with the sperm transfer of the released sterile males which undoubtedly has an impact on the efficiency of SIT in the field.

In summary, our results showed the molecular method herein reported allows a reliable quantitative estimation of the sperm transferred and stored by females in their spermathecae. This research will simplify the study of aspects related to the reproductive biology of this tephritid fly, especially those related to sperm quantification and hence those involved in the male competitiveness assessments within Sterile Insect Technique (SIT) programs.

5. Acknowledgements

The research leading to these results was funded by the Conselleria d'Agricultura, Pesca i Alimentació de la Generalitat Valenciana. We thank SIT emergence facility (GVA-IVIA) personnel for technical assistance and supply of material along with Miquel Alonso and Megan Hassane for technical support in the IVIA laboratory. MP-H was the recipient of a

384 research fellowship from the INIA Spain (Subprogram DOC-INIA-CCAA) and MC the
385 recipient of a grant EU-FSE (Fondo Social Europeo).

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Table 1 PCR primers tested to assess Y chromosome presence by real-time quantitative PCR. The nucleotide sequences of the PCR primers (5'-3') and the amplicon size of the PCR product are included.

Primer pair name	Forward primer sequence	Reverse primer sequence	Band size (pb)
CcYsp-Dir/CcYsp-R2	CGA AGC CAG ACA TAC ACG AGG AG	TCG TTT GGA AAA GTG TGG TTC	112
CcYsp-FF/ CcYsp-RR	AGT GAT GCA CCA CAC ACA CA	ATT GAT TCC TGG ACG AGT GG	96

534

535 **Table 2** Percentage of positive sperm identification (mean ± SE) in spermathecae of *Ceratitis*
536 *capitata* females (wild- or V8-mated) at four mating durations analyzed by the absolute real-
537 time qPCR analysis. Differences between superscript letters denote significant differences
538 between male type (uppercase) and mating duration (lowercase) (multifactorial analysis of
539 variance, $P < 0.001$).

540

Copula duration (min)	**Positive samples in Wild-mated females**	**Positive samples in V8-mated females**
10	66.7 ± 8.0 Aa	44.4 ± 12.1Ba
30	79.1 ± 6.3 Aa	60.9 ± 10.4 Bab
60	95.5 ± 3.2 Ab	70.4 ± 9.0 Bab
90	97.2 ± 2.4 Ab	80.0 ± 7.4 Bb

541

542 **Table 3** Number of sperm (mean ± SE) counted under microscope in spermathecae of
543 *Ceratitis capitata* **f**emale**s** (wild- or V8-mated) at four mating durations. Each value was
544 calculated from at least 30 females in each male type and mating duration category.
545 Differences between superscript letters denote significant differences between male type
546 (uppercase) and mating duration (lowercase) (multifactorial analysis of variance, $P < 0.001$).
547

Copula duration (min)	**Number of sperm from Wild-mated females**	**Number of sperm from V8-mated females**
10	15.2 ± 3.4 Aa	5.4 ± 2.2 Ba
30	41.8 ± 7.1 Aa	14.6 ± 2.6 Bab
60	142.8 ± 32.4 Ab	32.1 ± 4.6 Bbc
90	349.7 ± 49.2 Ac	48.2 ± 8.9 Bc

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Figure captions

Figure 1. Specificity of selected markers which discriminate the presence of the Y-chromosome. The amplification success in DNA extraction from mated female spermathecae (lane 3) and male abdomen (10^{-2} diluted) to wild-type (lane 5) and Vienna-8 (lane 6) where a yield band of 112pb are shown. Negative amplification corresponds to a negative control (lane 1), virgin female spermathecae (lane 2) and female abdomen (10^{-2} diluted) (lane 4). PCR products were run on electrophoresis in 2% agarose gel and stained with GelRed^{TM}. The weight marker included is a 100-pb ladder (Thermo Scientific).

Figure 2. Absolute sperm quantification standard curve set up in a real time qPCR test with paired samples of the six known dilutions (10 to 10^6 target gen copies per microliter).

Figure 3. Correlation between real-time qPCR values (displayed on y-axis) and semiquantitative conventional PCR (on x-axis). Analysis performed on 93 samples from wild-mated females with three copula durations (10, 30, and 90 minutes). The picture with the six reference levels of the amplicon density corresponds to known concentrations of standard materials.

Figure 4. Comparison of the regression lines estimated by the qPCR method and microscope counting in females mated with wild males (A) and Vienna 8 sterile males (B). Each point represents the average of at least 30 females in each method and mating duration category. Vertical bars indicate standard errors.

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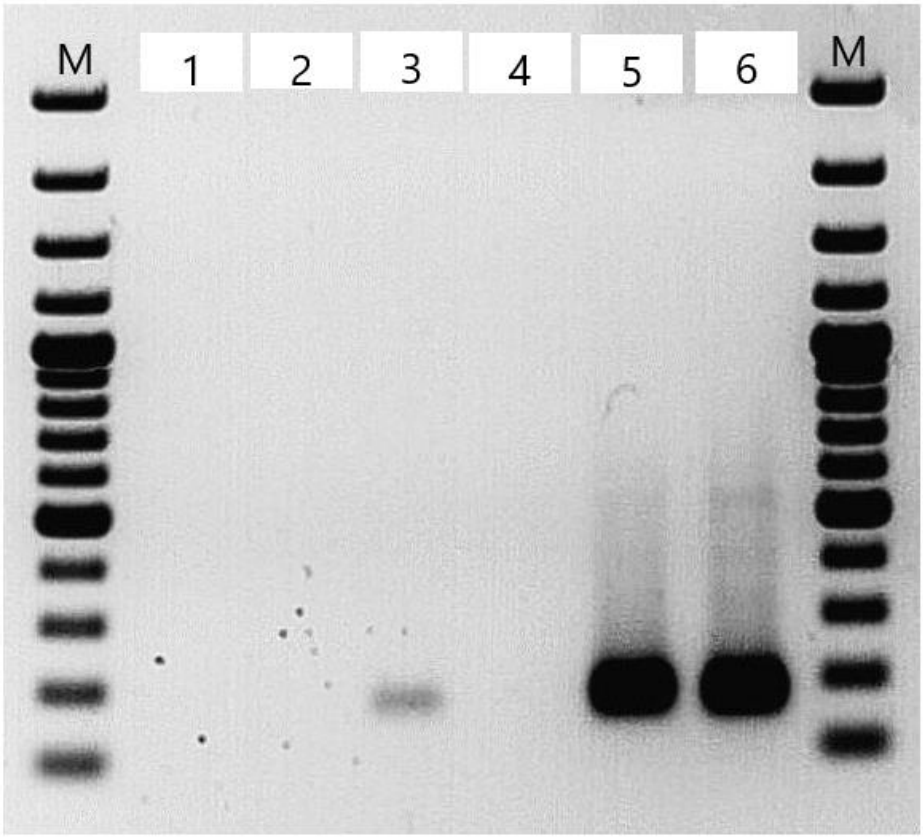

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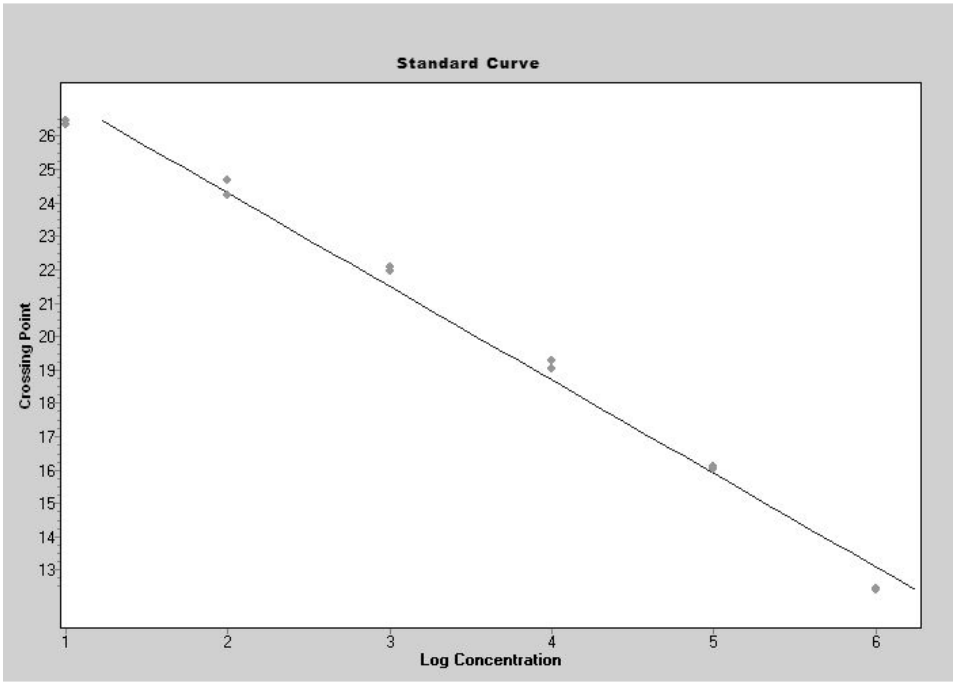

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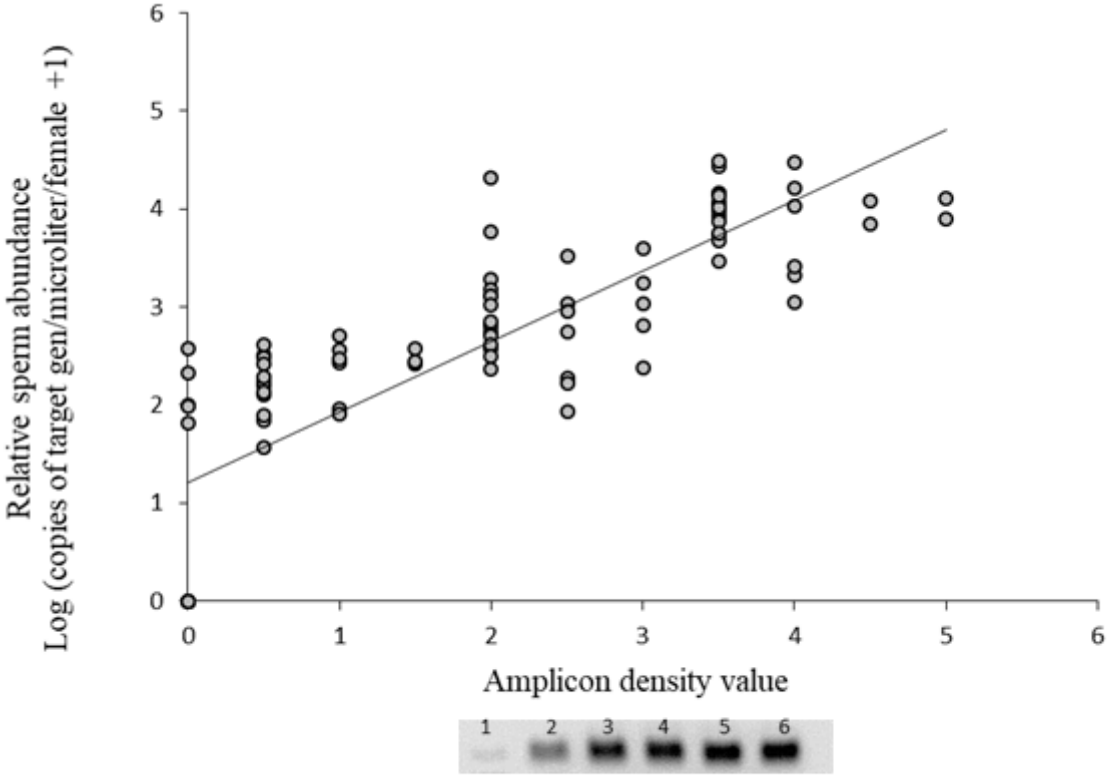

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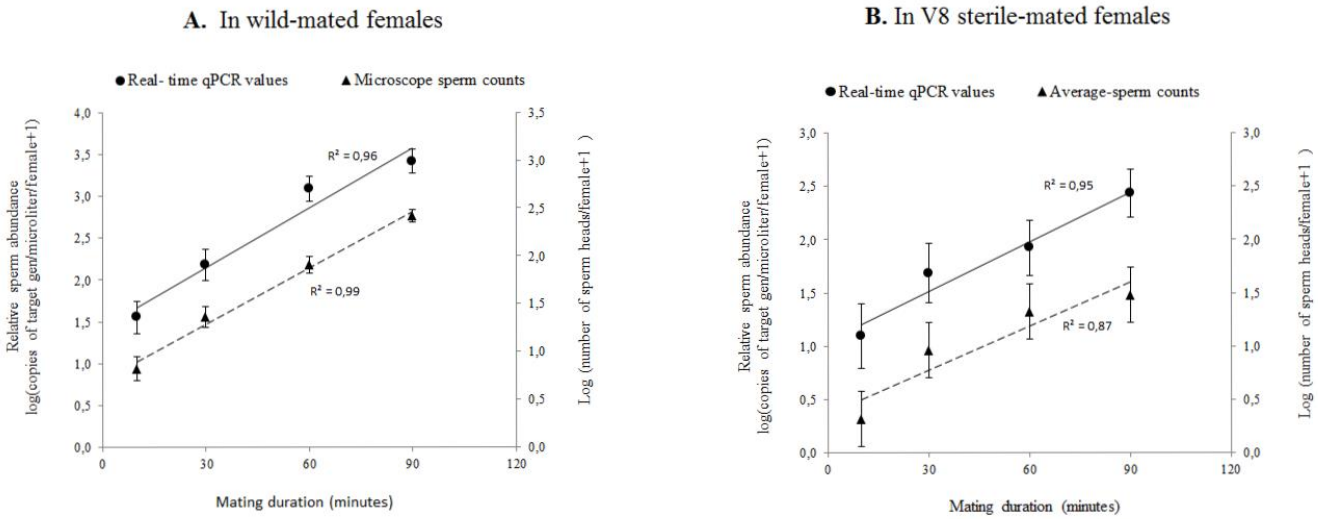

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