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Article

Competitive Sperm-marked Beetles for Monitoring Approaches in Genetic Biocontrol and Studies in Reproductive Biology

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Abstract: Sperm marking represents a valuable monitoring tool for genetic pest control strategies such as the Sterile Insect Technique, but also provides a key tool for reproductive biology studies. Sperm-marked lines can be generated by introducing transgenes that mediate the expression of fluorescent proteins during spermatogenesis. Homozygous lines established by transgenesis approaches are going through a genetic bottleneck that can lead to reduced fitness. Transgenic SIT approaches have mostly focused on Dipteran and Lepidopteran pests so far. With this study, we provide sperm-marked lines for the Coleopteran pest model organism, the red flour beetle *Tribolium castaneum*, based on the *β2-tubulin* promoter/enhancer driving red (DsRed) or green (EGFP) fluorescence. The obtained lines are reasonably competitive and were thus used for studies on reproductive biology confriming the phenomenon of 'last male sperm precedence' and that the spermathecae are deployed for long term sperm storage enabling the use of sperm from first matings even after secondary matings for a long period of time. The homozygosity and competiveness of the lines will enable future studies to analyze the controlled process of sperm movement into the long time storage organ as part of a post-mating cryptic female choice mechanism of this extremely promiscuous species.

Keywords: Insect Biotechnology; molecular entomology; pest management; Sterile Insect Technique; sperm storage; transgenesis; *Tribolium castaneum*

1. Introduction

The Sterile Insect Technique (SIT) is a birth management practice geared towards efficient control of insect pest populations, which represents a safe species-specific and environmentally friendly management strategy (Dyck et al., 2021). SIT has been used successfully to eradicate insect pests such as the Tsetse fly in Zanzibar, the New World Screwworm in North and Middle America as well as in North Africa (Klassen et al., 2021) and is used to manage several Tephritid fruit fly pests around the world (Enkerlin, 2021). SIT represents a classical genetic pest control method, which reduces a pest population by mass rearing and releasing of reproductively sterile males that compete with wild males for wild females (Knipling, 1955).

Besides the mass rearing, sterilization and releasing of the pest species, monitoring is of major importance for this cost-intensive method. To calculate the ratio of released to

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wild insects, data from field traps are used (Enkerlin et al., 1996). At present, mass-reared pupae are dusted with fluorescent dyes that are expensive, dangerous for human health, and error prone (Hagler and Jackson, 2001). Fluorescently marked sperm using transgenesis approaches could serve as an excellent alternative. Such transgenic marking systems have been described for the mosquitoes *Anopheles stephensi* (Catteruccia et al., 2005) and *Aedes aegypti* (Smith et al., 2007) as well as the Tephritid fruit flies *Ceratitis capitata* (Scolari et al., 2008), *Anastrapha suspensa* (Zimowska et al., 2009) and the cherry vinegar fly *Drosophila suzukii* (Ahmed et al., 2019). All these systems are based on the endogenous spermatogenesis-specific β2-tubulin (β2t) promoter driving testes-specific expression of fluorescent markers, which enables an inheritable sex-specific marking. Such systems allow for detection of the sperm even in mated females (Scolari et al., 2008) extending the abilities to also monitor the receptiveness of the wild females regarding the released sterile males, and thus their effectiveness in mating and sperm transfer. Moreover, such transgenic lines also improve studies on reproductive biology related to the mating behavior of polyandrous species, such as sperm storage, sperm precedence, sperm use, and sperm competition (Scolari et al., 2014).

There is a historical bias of using SIT towards mostly Diptera and Lepidoptera, which is probably both based on the large number of pest species in these orders and the biological basis for the implementation of SIT (Lance and McInnis, 2021). Despite the fact that also the order Coleoptera includes a high number of pest species, they have only been addressed to a lesser extent, probably because SIT seems impractical for species that are highly destructive at the adult stage (Knipling, 1955). However, there are also quite a number of severe coleopteran pests, especially weevils, that do not cause much damage as adults and SIT can be considered (Klassen et al, 2021). Here, we present a proof of principle for transgenic sperm marking in the coleopteran model organism for development and pest biology, the red flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Brown et al., 2009). We show that such transgenic lines are reasonably competitive and can be used for monitoring as well as for reproductive biology studies in this extremely promiscuous species showing cryptic female choice (Fedina and Lewis, 2015).

2. Results

2.1. Generation of different Tribolium castaneum *sperm marking lines*

To establish a sperm marking system that drives florescent proteins sperm-specifically in the testis of *T. castaneum*, we identified the β2t gene (TC009035, GENBANK accession number XP_969993) in its genome (Siebert et al., 2008). The β2t gene codes for a 452 amino acid protein encoded by a 1347 bp Open Reading Frame that is bordered by a 121 bp 5'UTR and a 258 bp 3'UTR and contained on two exons that are interrupted by a single 46 bp intron (Figure 1a). To determine the regulatory elements that are needed for effective and efficient gene expression in the T. castaneum testes, we tried to identify the conserved sperm-specific regulatory elements: β2t Upstream Element 1 (β2UE1), β2UE2, the 7bp Initiator sequence, and the downstream element β2tDE1, which have been described as important for TATA-less promoters in *D. melanogaster* (Michiels et al., 1989) and *D. suzuki* (Ahmed et al., 2019). However, we could not identify these elements in the *T. castaneum* genome. Therefore, we used the 1 Kb Promoter/Enhancer (P/E) region upstream of the β2t gene to drive the red fluorescing protein DsRed (β2t-DsRed-SV40; Figure 1b) or the green fluorescent protein EGFP (β2t-EGFP-SV40; Figure 1c). The constructions were then inserted into *piggyBac* transformation vectors (Horn and Wimmer, 2001; Horn et al., 2002) carrying the respective opposite fluorescent eye transformation marker driven by 3xP3 (Berghammer et al. 1999).

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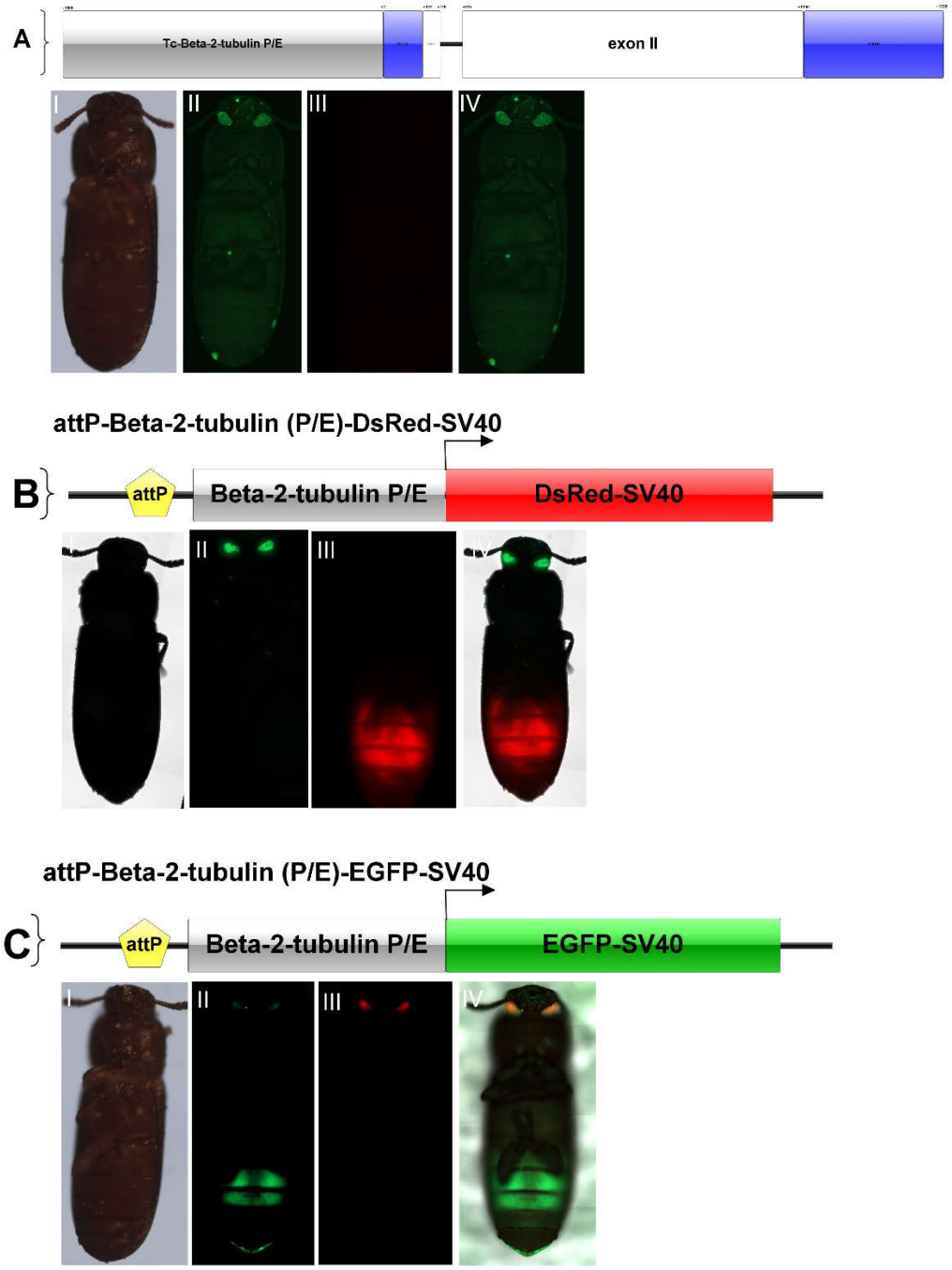

Figure 1. β2t gene and derived sperm marking constructs. (a) Schematic depiction of the endogenous β2t gene (TC009035) showing the coding region in white, intron as black line and 5′UTR and 3′UTR in blue. (a^{I}-a^{IV}) non-transgenic male photographed under white light, EGFP filter, DsRed filter, and compilation of the channels. (b) Construct for red fluorescent sperm. (b^{I}-b^{IV}) β2t-DsRed-SV40 carrying male photographed under white light, EGFP filter, DsRed filter, and compilation of the channels. (c) Construct for green fluorescent sperm. (c^{I}-c^{IV}) β2t-EGFP-SV40 carrying male photographed under white light, EGFP filter, DsRed filter, and compilation of the channels. The opposite fluorescence in the eyes is due to the respective transformation markers 3xP3-EGFP and 3xP3-DsRed.

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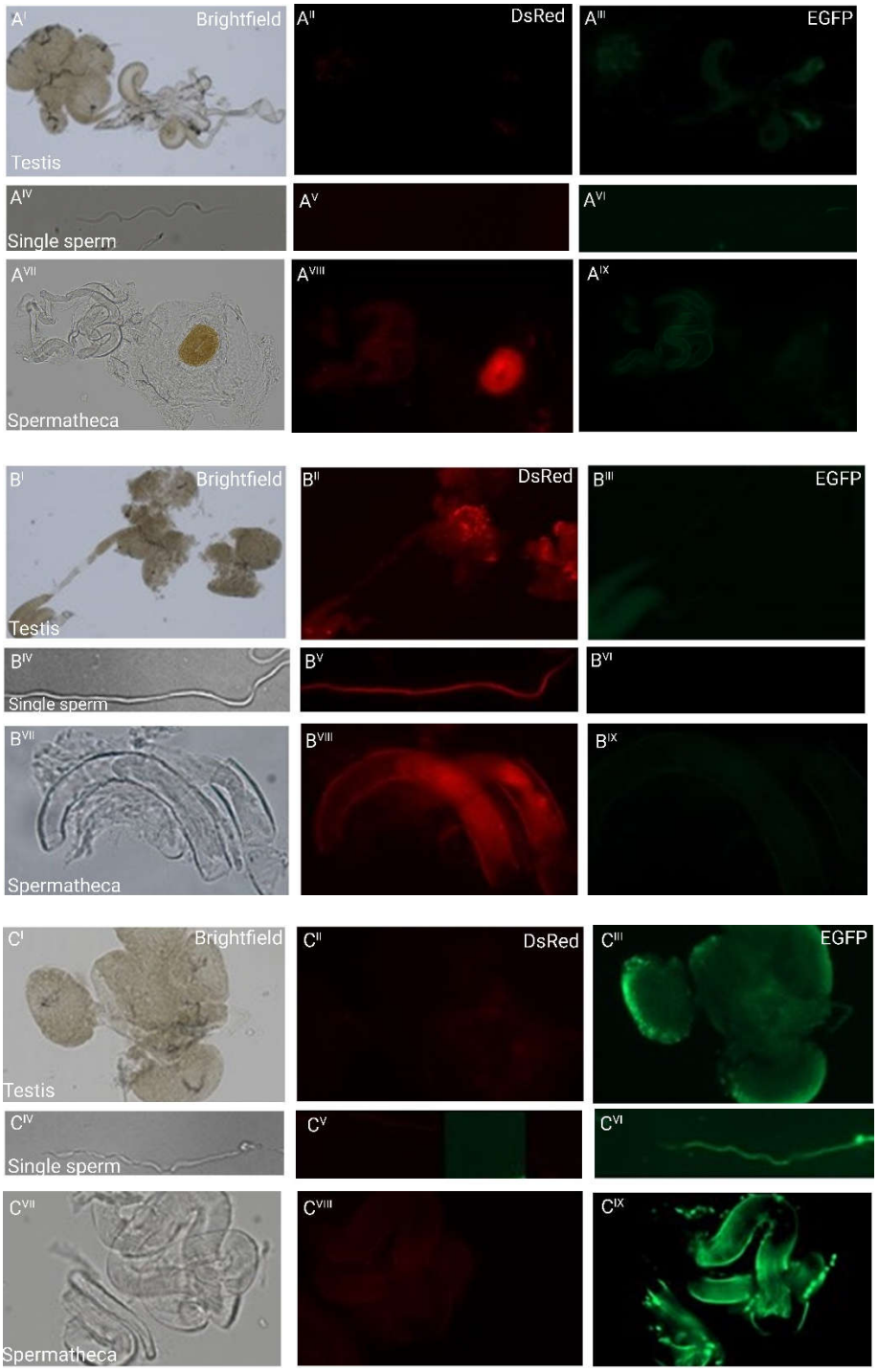

Figure 2. Fluorescent sperm detection in dissected testes (I-III), as individual sperm (IV-VI), and spermathecae (VII-IX) of non-transgenic females inseminated by transgenic males. (a) Weak green auto-fluorescence in the testis of non-transgenic males and red auto-fluorescence of the chitinous o-ring structure below the spermathecal of a non-transgenic female inseminated by a non-transgenic male. No other fluorescence pattern are observed. (b) The testes and single sperm from a transgenic male carrying pBac_a{3xP3_EGFP_SV40 _attP_β2t_DsRed_SV40} and the spermathecal tubules of a non-transgenic female mated to such a male show strong red fluorescence only. (c) The testes and single sperm from a transgenic male carrying pBac_a{3xP3_DsRed_SV40 _attP_β2t_EGFP_SV40} and the spermathecal tubules of a non-transgenic female mated to such a male show strong green fluorescence only.

To generate transgenic lines expressing the fluorescent proteins in their testes, we performed *piggyBac*-based germline transformation (Berghammer et al. 1999). For β2t-

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DsRed-SV40, we obtained three injected G_0 giving rise to transgenic progeny (transformation rate 5.5%; Supplementary Table S1). Two of the lines that could be homozygoused (F37_F1 and F37_M2) were kept and used for subsequent experiments. For β2t-EGFP-SV40, we obtained four injected G_0 giving rise to transgenic progeny (transformation rate 4,9%; Supplementary Table S1). Two independent lines that could be homozygoused (M1_F1 and F1_M1) were kept and used for subsequent experiments. The transgenic male and female individuals could be identified by their eye fluorescence while the testes fluorescence was only detected in males (Figure 1). To identify the genomic integration sites of these lines, inverse PCR was performed, which confirmed the independent integration of M1_F1 and F1_M1 (Supplementary Table S2).

To examine whether the β2t promoter-driven fluorescence would allow us to follow individual sperm, we analyzed dissected testes, individual sperm, and spermathecae of non-transgenic females inseminated by transgenic males (Figure 2). The sperm-specific fluorescence indicates that the isolated 1 kb region contains all regulatory elements responsible for sex- and tissue-specific expression confirming another recent study, in which about 700 bp of upstream sequence were used to drive EGFP (Khan et al., 2021). Single sperm derived from transgenic males showed the respective red or green fluorescence along the sperm tail and dissected spermathecae from non-transgenic females mated to transgenic males carried transferred red or green fluorescent sperm, respectively (Figure 2). This indicates that the transgenetically marked sperm does not in principle interfere with mating and paternity success supporting the idea to use such a marker for monitoring purposes in SIT programs also in coleopteran pests and to potentially use such lines for studies in reproductive biology.

To determine the sperm utilization and competition of sequentially twice mated females, we performed a laboratory assay, in which non-transgenic *vermillion*^{white} (*v*^{w}) *Tribolium* females were sequentially mated with transgenic males with first DsRed (M1_F1 and F1_M1) and second EGFP (F37_F1, F37_M2) marked sperm or *vice versa*. The sired progeny was followed up over twenty days after the remating (Supplementary Figure S1). The results (Supplementary Figure S2) confirm the notion that the females start utilizing the sperm of the last mated male most predominantly (Fedina and Lewis, 2004). However, over time there seems to be a balance of the use of the sperm of the first and second male, with sperm of the first male still being used after twenty days. Since the EGFP lines (F37_F1, F37_M2) seemed to perform weaker than the DsRed lines (M1_F1 and F1_M1) in this experiments especially when using them as second mates, we performed an outcrossing and line re-establishing procedure to overcome the potential problems of genetic bottlenecks in establishing transgenic lines.

2.2. Competitiveness of sperm marking lines

After outcrossing and re-establishing the homozygous sperm marking lines, we performed competition assays against non-transgenic males to evaluate the competitiveness of the transgenic males. For this purpose, we performed simultaneous competition in a relaxed, a normal, and a high competition as described in Supplementary Figure S3. The results show that all transgenic males are outperformed by the non-transgenic males (Supplementary Figure S4). However, all transgenic line sire between a fifth and almost half of the progeny, depending on the respective line (Figure 3), which indicates that the lines are reasonably competitive to be used in studies of reproductive biology. To evaluate, how the different transgenic lines compete with each other, we performed simultaneous competition experiments as outlined in Supplementary Figure S5. While right after setting the beetles together, the EGFP sperm marked beetles (F37_F1 and F37_M2) seem to perform slightly better, after removal of the males, both sperm sources are used more evenly (Supplementary Figure S6).

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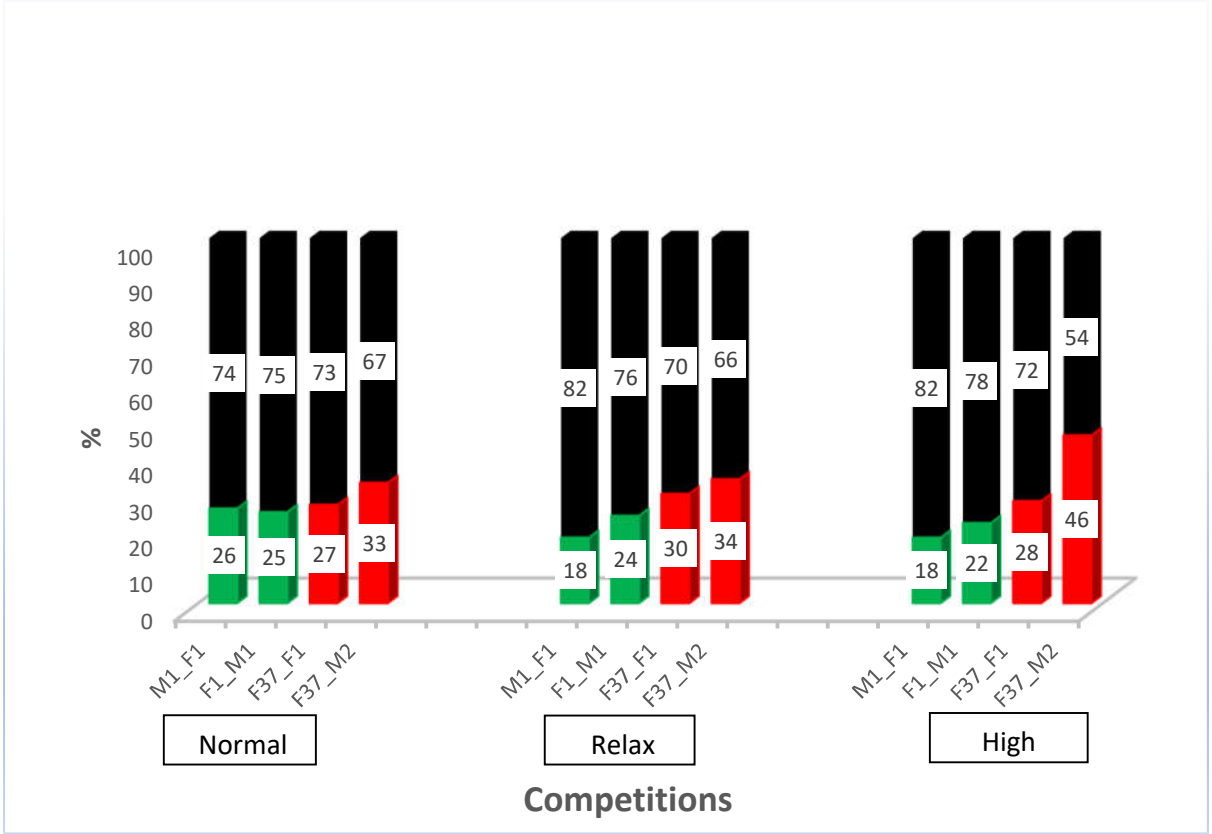

Figure 3. Simultaneous competition of transgenic males compared to non-transgenic males. Percentage of offspring sired by non-transgenic (black) and transgenic males (green or red, respectively) in a relaxed (5 transgenic males x 5 non-transgenic males x 10 non-transgenic females), a normal (5 transgenic males x 5 non-transgenic males x 5 non-transgenic females), and a high competition (10 transgenic males x 10 non-transgenic males x 5 non-transgenic females).

2.3. Sperm use in consecutive matings

With the outcrossed and re-established homozygous lines, we repeated the sperm utilization and competition in consecutive twice mated and followed the sired offspring over twenty days after the remating (Supplementary Figure S1). The results (Figure 4) again confirm that the female's last mate generally sires a majority of the immediate progeny (Fedina and Lewis, 2004). However, over time the sperm use is more balanced with sperm of the first male still being used after twenty days.

To determine the long term storage of sperm within the female reproductive tract after the first and the second mating, we dissected and cryosected the female reproductive tissue and evaluated the fluorescent sperm presence (Figure 5). After the first mating, the sperm seems to occupy both the spermathecal tubules as well as the bursa copulatrix, which actually provides the fertilization set (Droge-Young et al., 2016). After the second mating, the first sperm is still kept in the spermatheca, while the second sperm is still occupying the bursa copulatrix, which explains, why the second sperm is used more frequently for siring progeny at this time (Figure 4). Ten days after the second mating, there doesn't seem to be any longer a clear localization of the first or second sperm but both types of sperm are still present and more homogenously distributed, which is also reflected by the more equal use of both types of sperm to sire progeny at this time (Figure 5). First received sperm is still clearly visible in the female reproductive tract (Figure 5A^{XII}) as is secondly received sperm (Figure 5B^{XII}).

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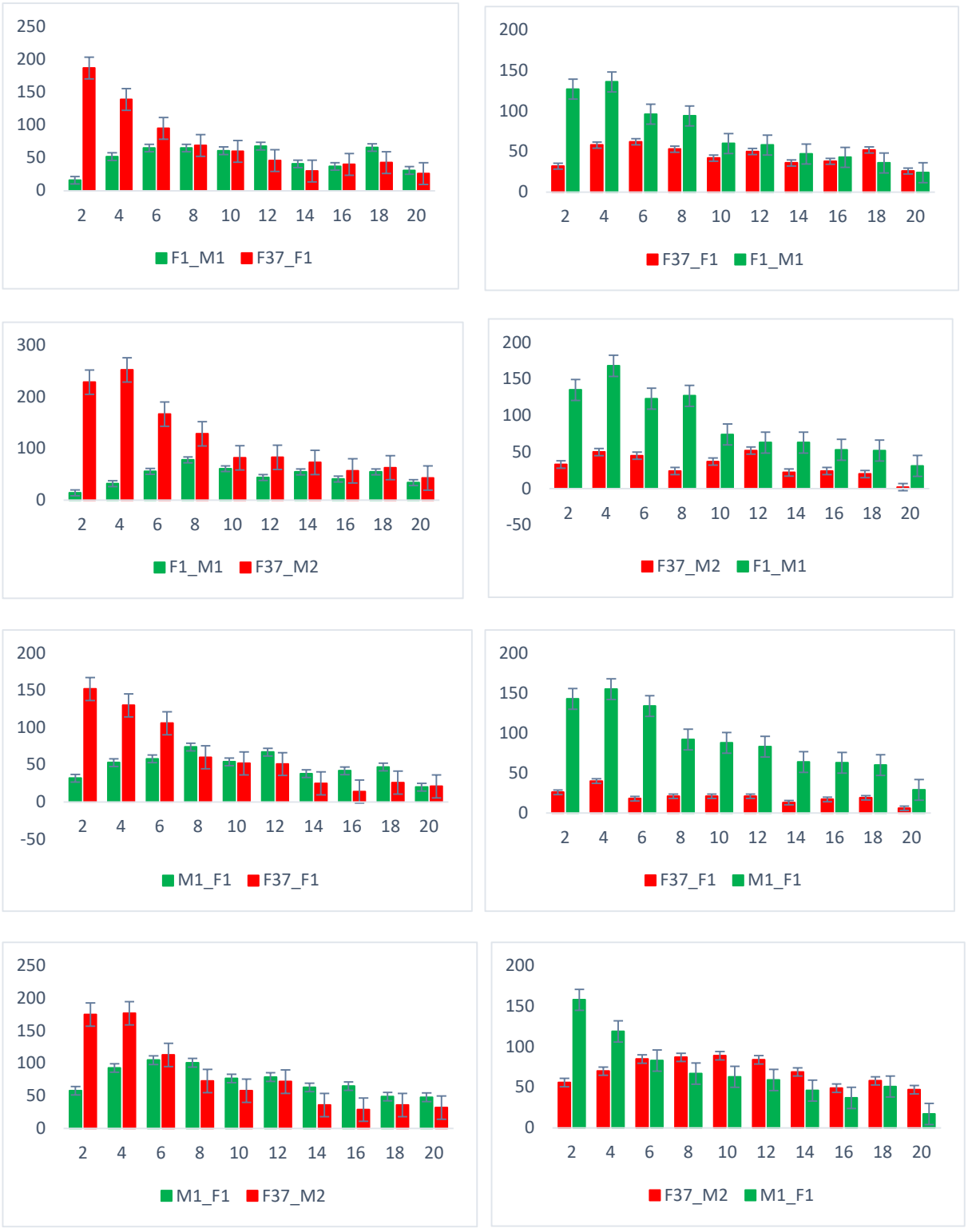

Figure 4. Sperm utilization and competition of sequentially twice mated females. Matings were performed as indicted in Supplementary Figure S1 with the order of the respective lines as indicated below of each chart.

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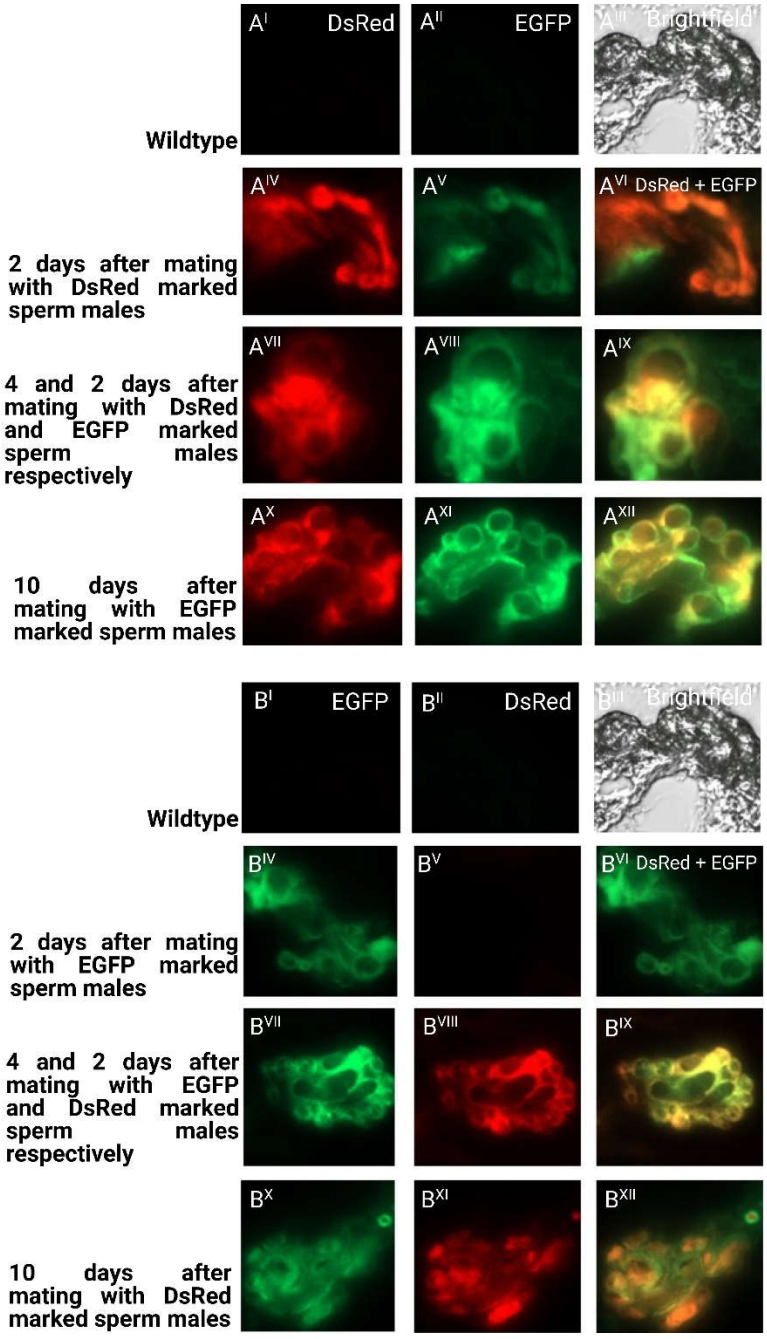

Figure 5. Cryosections of female reproductive tissue with fluorescent sperm after sucessive matings. (I-III) Non-transgenic female mated to non-transgenic males as negative control reagarding autoflourescence. (IV-VI) Two days after mating with first male before mating with second male. (VII-IX) Two days after second mating. (X-XII) Ten days after second mating. Pictures were taken for detection of EGFP (I, IV, VII, X), for detection of DsRed (II, V, VIII, XI), and overlay of channels (VI, IX, XII). (a) Mating first with males providing DsRed marked sperm and consecutively with males providing EGFP marked sperm. (b) Mating first with males providing EGFP marked sperm and consecutively with males providing DsRed marked sperm.

3. Discussion and conclusion

The successful establishment of differently marked lines in the red flour beetle *T. castaneum* shows that the β2t enhancer/promoter can also be used in Coleoptera for sperm marking purposes. This confirms on the one hand recent findings by Khan et al. (2021) but on the other hand also indicates that the promoter/enhancer alone suffices and that the 5′UTR is not needed for such constructs. Droge-Young et al (2016) also used green and red fluorescently marked transgenic *T. castaneum* lines but employed the *TcProtamine-1*

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gene to generate complete fusion proteins for marking. In that study, however, the only red fluorescent line could not be made homozygous hampering the progeny analysis in their reproductive biology studies to some degree. In addition, we could show that males of at least one EGFP sperm marked line (F37_M2) were siring almost 50% of the progeny compared to non-transgenic males in a highly competitive situation (Figure 3), which indicates that such sperm marked lines could be used for monitoring of genetic pest control strategies such as SIT also in Coleoptera.

Our results regarding the reproductive biology of *T. castaneum* confirm the notion, that the spermathecae are employed for long term storage of sperm to be used for months (Bloch Qazi et al., 1996), while sperm in the bursa copulatrix is used for fertilization (Droge-Young et al., 2016). *T. castaneum* females can actively discard sperm after copulation (Fedina and Lewis, 2007) and also actively transfer sperm from the site of deposition, the bursa copulatrix, to the long term storage organ, the spermathecae, (Bloch Qazi et al., 1998; Edvardsson and Arnqvist, 2000). Our results confirm and clearly visualize (Figures 4 and 5) that both first as well as secondly provided sperm are stored in the spermathecal tubules and used for siring progeny for a long period of time (Droge-Young et al., 2016). The first occupation of the long term storage organ by the fist sperm can also explain, why in multiple matings this first sperm is kept more predominantly then successive sperm (Lewis et al., 2005). However, right after a mating, mostly the newly provided sperm, which is still in the bursa copulatorix (Figure 5), is used for siring progeny (Figure 4) leading to the phenomenon of 'last male sperm precedence' (Fedina and Lewis, 2004). In the very promiscuous species *T. castaneum*, there are several well documented mechanism of cryptic female choice at pre-mating (allowing intromission), mating (spermatophore transfer and positioning) and post-mating (spermatophore ejection; re-mating) stages (reviewed in Fedina and Lewis, 2015). Our established lines could be used to further analyze the compelling process of sperm movement into the long time storage organ and how this is controlled by the female as part of a post-mating cryptic female choice mechanism.

4. Materials and Methods

4.1. Beetle culture

In all experiments unless otherwise stated, the *vermillion*^{white} (v^{w}) strain of the red flour beetle, *T. castaneum*, which includes the germ line transformation and the use as non-trangenic control. Beetle stocks were fed on full grain flour supplied with 5% yeast powder in a 4.5 L square plastic boxes (15cm x 15cm x 20cm) with a ventilation mesh fixed on the lid and were kept at 32°C with constant light (Schmitt-Engel et al., 2015).

4.2. Isolation of the β2t promoter region and cloning of the transfomation vectors

To establish a sperm marking system in *T. castaneum*, 1 Kb upstream region of the *β2t* gene (TC009035; GENBANK accession number XP_969993; Siebert et al., 2008) was amplified from genomic DNA of the wild type San Bernardino strain (SB-gDNA). SB-gDNA was extracted using the NucleoSpin® DNA insect kit (Macherey-Nagel GmbH α Co. KG, Düren, Germany). Polymerase Chain Reaction (PCR) was performed using primer pair MID#4/MID#7 (Supplementary Table S3) in a 50µl reaction consisting of water 28µl, gDNA 2µl, 5x Phusion buffer 10µl, Phusion polymerase 1µl, dNTPs 5µl, forward primer 2.5µl and reverse primer 2.5µl in a program of 2 min at 98°C, 30 sec at 98°C, 30 sec at 68°C, 2 min at 72°C 35 cycles, 10 min at 72°C. The amplified fragment was run on a 1% agarose gel and a distinct single band was excised and purified using the NucleoSpin®Gel and PCR Clean-up Kit (Macherey-Nagel GmbH α Co. KG, Düren, Germany).

The transformation vectors were constructed in a two step cloning procedure (Horn and Wimmer 2001). First, the 1Kb *β2t* enhancer/promoter fragment was cloned into the shuttle vector pSL_af_tTA-SV40, by cutting both the fragment and the vector with *NcoI*/*ClaI* and ligating their ends therby dispalcing tTA-SV40 from the pSL plasmid to generate pSL_af_β2t. Then, 5 µl each of *attP* forward and reverse oligos (Supplementary Table S3) were mixed with 90µl water and placed in a heat block set at 95°C for 5 min,

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after which the heat block was switch off and ramped to room temperature to anneal the oligos. pSL_af_β2t was cut using *EcoRI*/*NcoI*, and subsequently the *attP* annealed oligos were ligated upstream of the β2t to generate pSL_af_*attP*_β2t. The pSL_af_*attP*_β2t was opened by *ClaI*/*HindIII* and a 1 Kb DsRed-SV40 or EGFP-SV40 fragment, respectively derived from pSL_DsRed-SV40af (Horn et al., 2002) by cutting with the same enzymes or amplification from pSL_EGFP -SV40af (Horn and Wimmer 2001) with primers MID#103/MID#104 (Supplementary Table S3) and recutting of the isolated fragment, was ligated to obtain pSL_af {*attP*-β2t- DsRed-SV40} and pSL_af {*attP*-β2t-EGFP-SV40}, respectively.

In a second step, the fragments *attP*_β2t_DsRed_SV40 and *attP*_β2t_EGFP_SV40 were excised out of the pSL_af_*attP*-β2t- DsRed-SV40 and pSL_af_*attP*-β2t-EGFP-SV40 shuttle vector via *AscI* digestion and inserted independently into *AscI*-cut pBac_{3xP3_EGFP_SV40af} (Horn and Wimmer 2001) or pBac_ {3xP3_DsRed_SV40af} (Horn et al., 2002) transformation vectors carrying fluorescent transformation eye markers, respectively, to generate the transformation vectors pBac_a {3xP3_EGFP_SV40 _*attP*_β2t_DsRed_SV40} (Fig 1b) or pBac_a {3xP3_DsRed_SV40_*attP*-β2t_EGFP_SV40} (Fig 1c.), respectively.

4.3. Germ line transformation and strain establishment

Germline transformation was conducted as described (Berghammer et al., 1999; Eckermann et al. 2018). Beetles of the v^w strain were placed on white flour for 1 hour after which the embryos were sieved out and kept for an additional hour at room temperature. The embryos were washed twice with 1% Klorix solution and rinsed with clean tap water. Embryonic injection was done using a FemtoJet® Microinjector (Eppendorf, Hamburg, Germany). Needles for the microinjection were made from 10mm×1mm borosilicate capillaries by pulling them with a P-2000 micropipette puller (Sutter Instrument, Novato USA) with the following settings: Heat=350, Fil=4, Vel=50, Del=225, PUL=150. The pulled needles were opened and sharpened using a Bachofer Laboratoriumsgeräte beveller (Reutlingen, Germany). For both constructs, pBac_a{3xP3_EGFP_SV40 _*attP*_β2t_DsRed_SV40} and pBac_a {3xP3_DsRed_SV40_*attP*-β2t_EGFP_SV40}, 1000 (0-2hrs old) embryos each were injected using a concentrations of 500 ng/µl transformation vector along with 300 ng/µl helper plasmid *mhypBase* (Eckermann et al. 2018) in injection buffer (5 mM KCl, 0.1 mM KH2PO4, 0.1 mM NaH2PO4 pH 6.8). After injection, the embryos were transferred onto an apple agar plate and sealed in a plastic box, which was then kept in an incubator at 32°C for 2 days. After 2 days, the lid of the box containing the injected embryos was removed. Hatched larvae were picked with a hair thin brush individually and placed on full wheat flour until pupation. After pupation, the pupae (G_0) were sexed and individually crossed to 3 non-injected individuals of the opposite sex. F_1 individuals were screened for EGFP fluorescence (Figure $1B^{II}$) or DsRed fluorescence (Figure $1C^{III}$) in the eyes driven by 3xP3 promoter in both males and females using a LEICA M205 FA epifluorescence stereomicroscope with the filters EGFP-LP (excitation: ET480/40, emission: ET510 LP) or RFP (excitation: ET546/10x, emission: ET605/70m), respectively. The survival of the injected embryos and the transformation rate is documented in Supplementary Table S1 for each construct.

To obtain homozygous lines, single transgenic F_1 individuals were outcrossing with v^w beetles of the opposite sex and the heterozygous progeny obtained was pooled for each individual F_1, which subsequently resulted in a mixture of non-transgenic, heterozygous, and homozygous individuals. Homozygous individuals were identified based on the fluorescent intensity in the eyes and crossed to each other. Of their progeny, 10 virgin individuals (5 males and 5 females) were randomly picked and crossed to virgin v^w beetles of the opposite sex. The crosses were allowed to stand for 2 weeks to assure mating, after which the individuals are removed, separated and kept in separate vials. Their offsprings were allowed to grow to become adults and screened for respective fluorescence. If all the

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offspring found in a vial was transgenic, the founder transgenic parent was considered homozygous, kept and crossed to establish the respective line.

Genomic DNA sequences flanking the *pBac* insertion 5′ and 3′ were determine by inverse PCR. DNA was extracted from the sperm marked individuals using the Nucleo-Spin® DNA Insect kit (Macherey-Nagel GmbH α Co. KG, Düren, Germany). About 1 µg DNA of each transgenic line was digested with *Sau3AI* for 5′ arms and *Msp*I for 3′ arms for 4 hours and kept at 37°C in a 25 µl reaction, respectively. The Sau3AI reactions for 5′ arms were inactivated at 65°C for 20 minutes. The resulting cut DNA fragments were self-ligated in a reaction volume of 400µl (water 333µl, reaction 25µl, Buffer 40µl and T4 DNA ligase 2µl) and kept at 16°C overnight. PCRs were conducted on the self-ligated genomic DNA from several lines for both 5′ and 3′ arms using different PCR protocols. For the 5′ arm the first PCR (3 min at 98°C, 30 sec at 98°C, 30 sec at 68°C, 1 min at 72°C 35 cycles, 10 min at 72°C) was performed using primers iPCR5′F1- 5/ iPCR5′R1-6 (Supplementary Table S3). An aliquot of this first reaction served as template for the second nested PCR (3 min at 98°C, 30 sec at 98°C, 1:30 sec at 72°C, 35 cycles, 10 min at 72°C) carried out with primers iPCR5′F2-7/ iPCR5′R2-8 (Supplementary Table S3). For the 3′ arm just one PCR reaction was conducted using primers MFS227/MFS228 (Supplementary Table S3). The products of the PCRs were run on 1% agarose gel and distinct bands were excised and purified using the NucleoSpin®Gel and PCR Clean-up Kit (Macherey-Nagel GmbH α Co. KG, Düren, Germany). The purified fragment were cloned into Pjet 1.2 vector (Schmitt-Engel et al., 2015) and sequenced using primers Pjet 1.2 forward and reverse primers (Supplementary Table S3). Sequences obtained were used to search against the *T. castaneum* genome Tcas 5.2 using BLAST search to ascertain the localization of the *piggyBac* insertion as shown in Supplementary Table S2.

To outcross and re-establish the trangenic lines for counteracting the bottleneck effect of transgenesis, homozygous individuals of lines M1_F1 and F1_M1 carrying p*Bac* {a_3xP3_EGFP-SV40_attP-β2t_DsRed_SV40**}** as well as F37_F1 and F37_M2 carrying p*Bac* {a_3xP3-DsRed-SV40_attP-β2t_EGFP_SV40} were singly outcrossed to v^w beetles of the opposite sex and placed on a 50g full grain flour with 12 replications each. The set ups were left to stand for a period of 10 days to mate and lay embryos, then the adults were removed and discarded while the embryos laid were left to develop into heterozygous adults. From the 12 replications, 3 males from 6 replications each and 3 females out of the other 6 replications each were crossed to each other to set up 6 replications, which were again kept for 10 days to mate and lay eggs. The adults were subsequently taken out, and the embryos left to develop into adults comprising of non-transgenic, heterozygous, and homozygous individuals. The homozygous individuals were screened based on the high intensity of the fluorescent in the eyes. Subsequently, three homozygous males and females each were screened at pupal stages from the 6 vials, to give 3 sets of males or females individuals, which were then crossed to give a total of 3 crosses at the end. They were left to mate and lay eggs, and after 10 days the adults were removed and kept singly. The offspring were left until they were adults and screened, and when all the sired offspring was highly fluorescent indicating homozygosity, the lines were considered homozygous again and re-established.

4.4. Competition assays

To determine the sperm competition and utilization by sequentially twice mated females, we designed a laboratory as depicted in Supplementary Figure S1. Female v^w beetles were first mated with a males conatining either red fluorescent sperm **(**p*Bac* a_3xP3_EGFP-SV40{attP-ß2t_DsRed_SV40**)}** or green fluorescent sperm (p*Bac* a_3xP3-DsRed-SV40 {attP-ß2t_EGFP_SV40}) for 2 days. Then the males were replaced by new males with differently marked sperm and kept for additional 2 days before also the second males were removed and the females placed on a new flour. The females were then moved to new flour after every 2 days for a period of 20 days. Progenies of the assay were

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screened for eye transformation marker to ascertain the sperm competitiveness of the individuals to sire, and the numbers obtained were counted and recorded. Each set up was replicated 10 times.

To assess the direct mating competitiveness of outcrossed and re-established homozygous transgenically marked sperm lines, we competed them against non-transgenic v^w males as described in Supplementary Figure S3. The completion for non-transgenic females was at a normal level 1x1x1 (5 transgenic males: 5 non-transgenic males: 5 non-transgenic females), at a relaxed level 1x1x2 (5 transgenic males: 5 non-transgenic males: 10 non-transgenic females), and a high level (10 transgenic males: 10 non-transgenic males: 5 non-transgenic females). All experiments were replicated 3 times and left to stand for 4 days before the males were removed and the female transferred to new flour after every 2 days over a period of 20 days.

To assess the direct mating competitiveness between the different sperm marked lines, we used males from strains M1_F1 or F1_M1 (p*Bac* {a_3xP3_EGFP-SV40_attP-ß2t_DsRed_SV40}) and F37_F1 or F37_M2 (p*Bac* {a_3xP3-DsRed-SV40_attP-ß2t_EGFP_SV40}) and mated them to non-transgenic v^w females. The set up was made using 5 transgenic males per strain crossed with 10 females (Supplementary Figure S5). The experiment was allowed to stand initially for a period of 4 days, after which the adults were sieved, and the females were transferred to a new flour and kept for further 21 days while the males were discarded. After 21 days the adult females were then removed and discarded, and the offsprings of both sired after 4 days or 21 days were screened by epifluorescence using the eye marker to discriminate between the strains. The experiment was replicated 3 times.

4.5. Microscopy

Testis, spermathecal, and single sperms imaging (Figure 2) was done using Zeiss Imager Z2 equipped with 2 cameras, Axiocam 305 colour for EGFP-LP (excitation: ET480/40, emission: ET510 LP), DsRed (excitation: 533-558, emission: 570-640) or DAPI (excitation: 335-383, emission: 420-470) for cell nuclei staining observation and Axiocam 506 mono for brightfield. Dissection of testis or spermatheca was done by placing adults on ice for 10 minutes. Male or female individual to be dissected is later picked up and placed in a pool of insect saline solution, and by slightly squeezing the thorax of the individual with a pair of forcep the genitalia is exposed, and using another pair of forcep the exposed genitalia is pulled out and moved into ice cold 1xPBS before fixation. The testis or spermatheca were fixed as described by http://coleoguy.blogspot.com/2011/10/dissecting-tribolium-genitalia.html with some modification whereby the tissues were made to stand for 30 minutes using DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) for nuclei staining. Afterwards, they were mounted in 70% glycerol for image capturing. To obtain the single sperms, the dissected testis was put in 1.5µl plastic tubes and grinded several times using a pestle, and vortexed in between the grinding to facilitate single sperms going into solution. Afterwards, the dissected spermatheca and/or testis were placed on a slide singly and further separated carefully before mounting them for microscopy. While the suspended sperms in a solution were pipetted on a slide and covered with a slide before mounting on a microscope.

Spermatheca of single or twice mated females with EGFP and/or DsRed transgenically sperm marked male individuals (Figure 5) were dissected and embedded in OCT embedding matrix (Carl Roth GmbH + Co. KG, Schoemperlen straße 3-5, 76185 Karlsruhe) according to manufacturer instruction and kept in a -80°C freezer. The spermatheca was cryosected in a cryostat (Leica CM1950, Leica Biosystems Nussloch GmbH Heidelberger Str. 17 - 19 D-69226 Nussloch Germany) machine set at 8mm.

Supplementary Materials: Supplementary Figures S1-S6: S1: Experimental protocol for Sperm utilization and competition of sequentially twice mated females, S2: Sperm utilization and competition of sequentially twice mated females, S3: Experimental protocol for simultaneous competition, S4. Simultaneous competition of transgenic males compared to non-transgenic males, S5: Experimental

protocol for simultaneous competition between different transgenic males, S6: Simultaneous competition of different transgenic males, and Supplementary Tables S1-S3: S1: Transformation rate, S2: Integration sites, S3: List of primers.

Author Contributions: M.D.I., S.D., and E.A.W. conceived and initiated the project. M.D.I., B.A., and S.D. performed the experiments. M.D.I., S.D., and E.A.W. analyzed the data and M.D.I. created the figures. M.D.I. wrote the first draft and E.A.W. finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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