



Effects of Irradiated Sterile Male and Mating Sequence on the Fertility of *Drosophila suzukii* (Diptera: Drosophilidae)

Yongzhuo Chen, Hui Pan, Jing Li, Deng Pan, Pengcheng Liu, and Haoyuan Hu^{1,*}

Collaborative Innovation Center of Recovery and Reconstruction of Degraded Ecosystem in Wanjiang Basin Co-founded by Anhui Province and Ministry of Education, School of Ecology and Environment, Anhui Normal University, Wuhu 241000, Anhui, P. R. China and ¹Corresponding author, e-mail: haoyuanhu@126.com

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Abstract

The sterile insect technique has been explored in the laboratory to control populations of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), a globally invasive pest. We studied the reproductive behavior of *D. suzukii* including mating frequency, time between matings, and mating duration among non-irradiated flies. Irradiation doses were tested at 0, 60, 90, 110, 120, 150, and 180 Gy to select the optimal dose for producing sterile males. In addition, we examined the effects of mating sequence on offspring production where females were presented with irradiated males first and then wild males, or the reverse. Female *D. suzukii* were found to mate twice on average through their lifespan, with 16.53 ± 12.05 d between matings. The first mating duration was 24.64 ± 1.52 min shorter than the second mating. A dose of 90 Gy was suitable where irradiated males lived as long as non-irradiated males, and few eggs hatched from matings. The mating sequence experiment revealed first-male parentage preference. Wild females that mated with a wild male and then irradiated male produced more offspring than females mated with an irradiated and then wild male. Overall, the influence of mating sequence should be taken into consideration when applying the sterile insect technique (SIT) to control *D. suzukii* populations.

Key words: sterile insect technique, invasive pest

The spotted wing *Drosophila*, *Drosophila suzukii* (Matsumura), was first reported in Japan (Kanzawa 1939) and was widely distributed as of 2008 in North America (Hauser 2011) and Europe (Calabria et al. 2012). It was also recorded in South America in 2013 (Deprá et al. 2014). The serrated ovipositor used by female *D. suzukii* to pierce the skin of ripening soft fruit (i.e., hosts) and lays eggs that develop into larvae, causing fruit to rot rapidly (Walsh et al. 2011). This pest causes very large economic losses to the fruit industry every year. Based on 2008 production values, a yield loss of 20% could lead to \$US 39.8 million in revenue losses for raspberries (Farnsworth et al. 2017), \$US 56.7 million for blueberries, \$US 156.6 million for cranberries, and \$US 174.8 million for cherries in California, Oregon, and Washington combined (Bolda et al. 2010). In Italy, *D. suzukii* causes great crop loss (approximately 30 ~ 40%), especially for blueberries, strawberries, and raspberries (Lee et al. 2011). To date, many methods have been employed to manage *D. suzukii* infestations. Insecticides are widely used to control this pest (Bruck et al. 2011, Van Timmeren and Isaacs 2013, Cuthbertson et al. 2014), but their efficiency is hampered by the development of resistance (Diepenbrock et al. 2016). Traps baited with apple cider vinegar, vinegar and wine, or yeast/sugar water mixtures are also used to catch *D. suzukii*; however, many nontarget insects are caught (Cha et al. 2015), as these traps are not highly specific to *D. suzukii*

(Burrack et al. 2015). Parasitoids, a native enemy of *D. suzukii*, can be used to control *D. suzukii* populations by attacking larvae or pupae (Schetelig et al. 2018, Rossi Stacconi et al. 2019). However, when considering protecting local ecosystems, native enemies are not always available and they may not be possible to introduce.

Irradiated sterile males have the ability of mating with fertile females, eventually leading to a decline in the number of progeny produced (Krafsur 1998, Hendrichs and Robinson 2009). The target population will finally collapse under the condition that with high frequencies of sterile matings and without strong density-dependent phenomena. The sterile insect technique (SIT) has been applied to protect against invasion by the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) from South and Central America to Mexico (Hendrichs et al. 1983), to eradicate the melon fly *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) in Okinawa, Japan (Kuba 1996), and to eradicate tsetse fly *Glossina austeni* (Newstead) (Diptera: Glossinidae) from Newstead in Zanzibar, Tanzania (Vreysen et al. 2000). The SIT can be also an alternative strategy to control *D. suzukii* populations.

According to the principles of the sterile insect technique, the ideal irradiation dose completely eliminate fertility but causes little damage to somatic cells; therefore, insects can perform all their

normal activities except reproduction (Dyck et al. 2006). Recent research showed that at irradiation doses of 170 or 220 Gy, the emergence, longevity, and flight ability of sterilized *D. suzukii* were not affected, but their fertility significantly decreased (Sassù et al. 2019). In addition, applying a 200 Gy dose to pupae before emergence can induce sterility in *D. suzukii* (Peter Krüger et al. 2018). An irradiation dose of 120 Gy applied to 4-d-old pupae did not affect male mating performance under either noncompetitive or competitive conditions (Lanouette et al. 2020). Polyandry is common in *Drosophila* species where one female mates with multiple males (Singh et al. 2002). Thus, the ability of sterile males to compete with wild males for mates can be affected by sterilization through debilitating effects on either sperm competition or the behavior of adults (Barclay 2005). Despite the large influence of the SIT, the effectiveness of mating between sterile males and wild females can be lost partially or entirely if the females also mate with wild males and preferentially use sperm from the latter for fertilization. Previous research has shown that female *D. suzukii* remate and that male sterility has no effect on mating or remating frequency (Krüger et al. 2019). Although sperm competition is almost inevitable if female *D. suzukii* mate with both wild and sterile males, the consequence of this competition on fecundity is still unknown.

In recent *D. suzukii* SIT research, the researchers are mainly focused on *D. suzukii* remating behavior (Krüger et al. 2019, Lanouette et al. 2020). However, remating frequency and remating sequence also need to be studied because the SIT efficiency would be decreased if wild females can remate with wild males to recover fertility. Therefore, we aim to determine how female fertility is influenced by remating with wild and sterile males.

Materials and Methods

Drosophila suzukii Stocks

Drosophila suzukii were collected from the Hui Wang blueberry orchard in Nanling, Anhui Province, China (Chen et al. 2021) and reared for approximately 50 generations. Rearing was done in three nylon cube cages (35 × 35 × 35 cm) with 25 ± 1°C, 14:10 photoperiod, and 60 ± 5% RH in incubator. The flies were fed daily and supplied with a water-rich solid food diet (hereafter, diet; Dalton et al. 2011).

Remating With Wild Males of Wild Female *D. suzukii*

Drosophila suzukii adults were individually reared for 2 d after emergence in 1.5-ml perforated centrifuge tubes with diet. Twenty virgin female and male pairs were transferred into a 10-ml cryotube containing diet by using CO₂ anesthesia, respectively. Female mating was observed in a climate chamber at 25 ± 1°C and 60 ± 5% RH with a 14:10 photoperiod. The observation time was from 9 a.m. to 12 a.m. and 1 p.m. to 8 p.m. every day. New adult males were provided daily to the female. In addition, when not observing mating, all cryotubes containing flies were kept in an opaque box to prevent *D. suzukii* from mating (Roy and Gleason 2019). For each female, the total mating times, the lengths of the first and second matings, and the intervals between the first and second mating were recorded. Twenty-two females were used to conduct this experiment. The diet was replaced every morning until females died.

Determining the Suitable Irradiation Dose

The pupae with both red eye and grey wing (4 d old) were collected from the medium and placed on a water-soaked paper (to keep them moist). The irradiation treatment was conducted at the Institute for

Application of Atomic Energy, Zhejiang Academy of Agricultural Sciences, by using a ¹³⁷Caesium source. The irradiation dose was measured by a Fricke gel dosimeter and was applied at 0, 60, 90, 110, 120, 150, and 180 Gy in our research. After irradiation treatments, all irradiated and non-irradiated pupae were placed individually into 1.5-ml centrifuge tubes until emergence. All irradiated females were discarded after eclosion. In our experiments, the longevity of irradiated males and the number of hatched eggs were recorded to assess irradiation quality. Twenty irradiated males used to record longevity were reared individually. For the experiment related to the hatched eggs, a 2-d-old irradiated male and a 2-d-old nonirradiated female were placed into *drosophila* tubes (not 1.5-ml centrifuge tubes) with diet using ten replicates. Pairs were transferred daily into new tubes to provide fresh diet until the two flies died. All replaced tubes were kept in an incubator at 25 ± 1°C and 60 ± 5% RH with a 14:10 photoperiod, and the number of larvae were counted after 3 d. By comparing two parameters, longevity and the number of hatched eggs, we selected the optimal irradiation dose for sterilizing males to test the effect of mating sequence on female fertility.

Mating Sequence on Female Reproduction

After applying the suitable irradiation dose, irradiated 4-d-old pupae were placed into 1.5-ml perforated centrifuge tubes until they emerged as adults. Two-day-old adults were used in this experiment, and the first male was replaced by the second male 2 d after the beginning of the experiment. To form a control (CON) group, a wild virgin female and male pair was immobilized using CO₂ anesthesia and transferred into a *drosophila* tube with diet. Then, the mated male was removed and a virgin wild male was placed into the tube after the mating with the previous wild male. In the irradiated-wild (IW) group, an irradiated male and wild female were transferred into a *drosophila* tube, and then the mated irradiated male was replaced by a virgin wild male; in the wild-irradiated (WI) group, a wild female and male pair was transferred into a *drosophila* tube, and then the mated wild male was replaced by a virgin irradiated male. Each group had 30 replicates. The matings were directly observed from 9 a.m. to 12 a.m. The *drosophila* tubes with diet were replaced every day. All replaced tubes were placed in an incubator for 3 d, at which point the number of larval offspring was counted.

Data Analysis

Prior to analysis, data were checked for normality prior to using parametric tests. The first and second mating times were tested by Student's *t* test. Male longevity was compared by irradiation doses in a one-way ANOVA. The number of hatched eggs in the irradiation dose or mating sequence experiment was analyzed by a generalized linear model (GLM) with dose or sequence as the effect using a quasi-Poisson error due to large residual deviance value on the degrees of freedom. All data were analyzed using R version 3.6.1.

Results

Remating With Wild Males of Wild Female *D. suzukii*

The average number of mating frequency by female *D. suzukii* was 2.68 ± 1.14, with a maximum of five and a minimum of two. Approximately 86.4% of the females mated at least twice throughout their lifetime; however, there was a long interval, 16.53 ± 12.05 d, between the first mating and second mating. In addition, the duration of the first mating 24.64 ± 1.52 min was significantly shorter than that of the second mating (33.74 ± 2.64 min, *t*₃₉ = -3.08, *P* < 0.001).

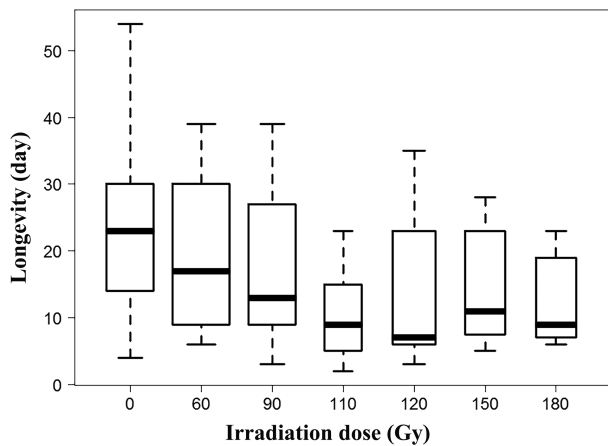


Fig. 1. The longevity of male *D. sukuzii* adults treated with different irradiation doses. Box: 25th and 75th percentiles; heavy line: median; whiskers: 1.5 times the interquartile range of the data.

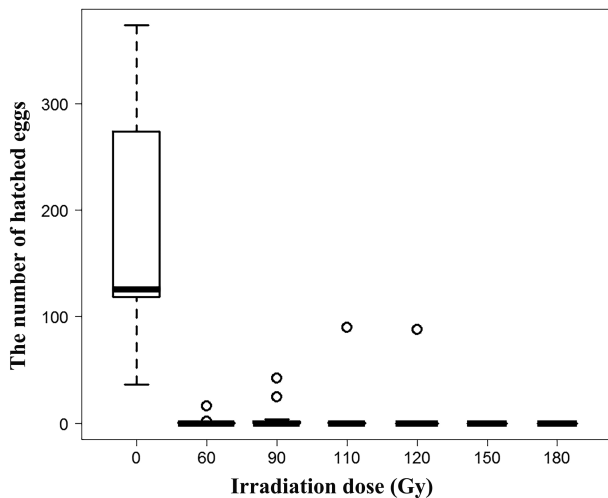


Fig. 2. The number of hatched *D. sukuzii* eggs at different irradiation doses. Box: 25th and 75th percentiles; heavy line: median; and whiskers: 1.5 times the interquartile range of the data; and data points: outliers.

The Suitable Irradiation Dose

The longevity of the males was not affected by irradiation doses of 60 or 90 Gy, but it decreased significantly with irradiation doses higher than 90 Gy ($F_{7,65} = 2.768$, $P = 0.014$; Fig. 1). Compared to the nonirradiated group, the number of hatched eggs was significantly impacted by any irradiation dose ($F_{7,65} = 24.957$, $P < 0.001$; Fig. 2). In addition, the egg hatch rate in the 90 Gy dose treatment group was 3.13%, which was lower than that in control group, 72.85%. Thus, in our research, 90 Gy was identified as a suitable irradiation dose for sterilizing male *D. sukuzii* based on the results of the longevity experiments and number of hatched eggs.

Effect of Mating Sequence on Female Fertility

The number of hatched eggs decreased significantly when the wild female mated first or second mated with irradiated male ($F_{2,85} = 8.66$, $P < 0.001$), and the first mating had a greater effect than the second mating on reducing the number of offspring produced, i.e., the wild-irradiated group (28.64 ± 7.31) had more offspring than the irradiated-wild group (14.03 ± 4.52 ; Fig. 3).

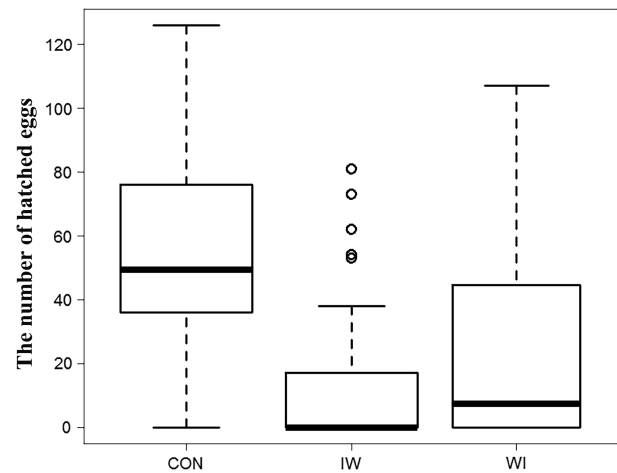


Fig. 3. The number of hatched *D. sukuzii* eggs of different mating sequences. CON = both the first and second mating of wild *D. sukuzii* females were mated with wild *D. sukuzii* males; IW = wild *D. sukuzii* females mated sequentially with irradiated *D. sukuzii* males and wild *D. sukuzii* males; and WI = wild *D. sukuzii* females mated sequentially with wild *D. sukuzii* males and irradiated *D. sukuzii* males. Box: 25th and 75th percentiles; heavy line: median; whiskers: 1.5 times the interquartile range of the data; and data points: outliers.

Discussion

There is little information on the effects of female remating on the fertility of *D. sukuzii*. However, the effects of irradiation on its reproductive behavior have been reported by Krüger et al. (2019). In our research, we studied the remating behavior and female fertility of *D. sukuzii*. We found that most wild female *D. sukuzii* can mate with wild males more than once in their lifetime and that there was a long interval between the first and second matings. In addition, the length of the second mating was significantly longer than the length of first mating time. Additionally, we determined the optimal irradiation dose for male *D. sukuzii* through longevity and hatched egg rate parameters. Compared to the other irradiation dose, 90 Gy had less significant influence on longevity while causing a decrease in the number of hatched eggs. In addition, we found that the order in which fertile females mated with sterile males first could reduce the number of offspring produced. Mating with sterile males first led to more apparent negative effects on the rate of hatched eggs than mating with sterile males second.

A low remating frequency was found in previous research, which differed from our results (Krüger et al. 2019, Lanouette et al. 2020). This can be explained by the long interval between the first and second matings of *D. sukuzii*; if the interval time is too short, the remating frequency can be reduced. A previous study showed that there was no significant difference between the length of the first mating and the second mating among *D. sukuzii* (Krüger et al. 2019). However, our results showed that the second mating was much longer than the first mating. This can be explained by the complicated mating system of *Drosophila*; even the same species from different regions can process different mating preference (Markow 1996). Compared to the length of first copulation, longer copulation times were observed for the second mating in two isofemale lines. However, the other three isofemale lines did not follow the same trend. Therefore, the variation of *D. sukuzii* copulation duration can be linked to the place in which the pest was collected. As for mating frequency, the mean number of matings among *Drosophila buzzatii* (Patterson & Wheeler) (Diptera: Drosophilidae) is approximately two (Markow 1996), which is consistent with the results of our research.

The optimal irradiation dose for sterilizing *D. suzukii* has been studied by other researchers with different results. The results of Lanouette et al. (2017) showed that there was no apparent morphological damage to male and female *D. suzukii* at dose less than 120 Gy and that the egg hatch rate declined from 82.6% in the control group to 4% in the treatment group. However, Krüger et al. (2018) suggested that the sterility of F₁ progeny could reach 99.5% by using a 200 Gy dose. In addition, in our experiment, with a 90 Gy dose, the egg hatch rate declined from 72.85% in the control group to 3.13% in the treatment group. This may have been due to the different irradiation environments in which low-oxygen tension, such as in nitrogen or hypoxia, can reduce radiation damage (O'Brien and Wolfe 2015). Because low-oxygen tension provides the same protection to both somatic tissue and sperm, the irradiation dose must be higher in nitrogen to achieve the same level of sterility as irradiation in air (Parker 2005). Similar to the variations in the mating system, we speculate that the irradiation environment as well as irradiation resistance might differ among *D. suzukii* lines such that the optimal irradiation dose is not a single value.

How the SIT affects the mating behavior of pests and how to confirm that sterile males have the ability to be competitive with wild males both need to be addressed. In addition, the influence of irradiation under a certain dose on mating behavior is limited. The effects of irradiation on mating behavior have been studied in Tephritidae: in *Anastrepha fraterculus* Wiedemann (Diptera: Tephritidae) treated with doses of 40, 70, and 100 Gy, male mating behavior had no significant change compared to that of the control group (Allinghi et al. 2007). As for *D. suzukii*, there was not a significant impact on the competitiveness of mating behavior of adult males treated with irradiation doses of 120 and 200 Gy (Krüger et al. 2019, Lanouette et al. 2020). In view of the abovementioned experimental results, we determined that wild female *D. suzukii* mating sequence affects fertility when mating with irradiated and wild males and showed that the first mating has the most influence on offspring production. To develop SIT for *D. suzukii*, it is necessary to: 1) achieve sex separation in the final pupal stage before irradiation and 2) monitor the effect of SIT releases. Only released sterile males mating with wild females can produce a sufficient sterilizing effect. If sterile females are also released, the SIT would be inefficient and uneconomical. However, it is difficult to separate sex relying on external morphology in the pupal stage and to achieve automation. This problem should be solved well before applying the pupal irradiation procedure. To assess the effect of the SIT, marking sterile male *D. suzukii* is important for field monitoring of sterile to wild ratios so as to follow program progress. In view of its successful application on mosquitoes, *D. suzukii* can be marked with stable nitrogen and carbon isotopes before release (Opiyo et al. 2016).

For sperm competition to occur, the sperm from a sterile male and a fertile male must coexist within the reproductive tract of the female. Generally, fertile males perform an average of 13% more fertilizations as second mates than do irradiated males (Simmons 2001). However, there are exceptions to this pattern. In *Bactrocera cucurbitae*, the irradiated males had more fertilizing capacity than did fertile males (Yamagishi et al. 1992), which is similar to our results showing that the IW group produced fewer offspring than the WI group.

Female insects can obtain a variety of benefits from copulating with several males, such as increased number of fertilized eggs compared to singly mated females or increased longevity compared to females that do not mate (Shuker and Simmons, 2014). Sperm competition would promote opposing adaptations in males that, on the one hand, allowed a male to pre-empt the sperm stored by females

from her previous mates, whereas on the other, ensure future rivals are unable to pre-empt a male's own sperm (Parker 1970). Sperm length is an important factor that can affect the value of P₂ (proportion of offspring sired by the second male in mating). Long sperms have many benefits in sperm competition: 1) longer sperm can block storage organs so that future sperm competition can be prevented (Ladle and Foster 1992, Briskie et al. 1997), and 2) longer sperms have better resistance to displacement once they reach sperm stores (Dybas and Dybas 1981, Sivinski 1984). We deduce that long sperm can be the reason for the first mating preference of offspring due to blocking storage organs. In addition, for *D. suzukii*, the detailed mechanism regarding the sperm length effect on multi-mating should be further studied.

There is another theory to explain the first-male parentage: in Lepidoptera, female sperm storage organs were filled by the ejaculate of the first male, leaving no room for sperm from the second male. Overall, future research should focus on the mechanism of *D. suzukii* sperm competition, which might improve our understanding on the efficiency of using the SIT to control *D. suzukii* populations.

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Authors' Contributions

Y.C.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing—original draft. H.P.: Conceptualization, Investigation, Methodology, Resources, Validation. J.L.: Conceptualization, Investigation, Methodology, Resources, Supervision, Validation. D.P.: Investigation, Methodology, Resources. P.L.: Conceptualization, Resources, Supervision. H.H.: Conceptualization, Funding acquisition, Supervision, Validation, Writing—review and editing.

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