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Tolerance to a Combination of Low Temperature and Sterilizing Irradiation in Male *Glossina palpalis gambiensis* (Diptera: Glossinidae): Simulated Transport and Release Conditions

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Abstract

Recently, aerial delivery of sterilized adult tsetse flies has been developed based on the release of chilled adult sterile males. The long-distance transport of irradiated male tsetse pupae for chilled adult release systems requires exposure of the mature pupae to irradiation and to low temperatures for both the pupae and adults. The effect of these treatments on mating of adult *Glossina palpalis gambiensis* (Vanderplank, Diptera: Glossinidae) males was investigated. Male *G. p. gambiensis* pupae were stored at 10°C for 5 d and irradiated with 110 Gy within the first 24 h of cold storage. In addition, to simulate a chilled adult release environment, 6-d-old adult males were stored at 5.1 ± 0.4°C for 6 or 30 h. Mating performance was compared to untreated controls in walk-in field cages. A significantly lower proportion of males that had been irradiated and stored at low temperature succeeded in securing a mating compared to untreated males. Premating time, copulation duration and spermathecal fill were similar. Insemination levels were slightly lower for adult males stored at low temperature for 30 h compared to 6 h or control. Although the mating behavior of the males was affected by the treatments given, the data presented confirm the suitability of using long-distance transport of chilled and irradiated male *G. p. gambiensis* pupae followed by releasing the emerged adult male flies using a chilled adult release system. However, the data indicate that the chilling duration of the adults should be minimized.

Key words: cold storage, sterile insect technique, chilled adult release system, tsetse

Most countries in sub-Saharan Africa have to endure the presence of tsetse flies and the diseases they transmit, i.e., human African trypanosomosis or sleeping sickness and African animal trypanosomosis or nagana. With the exception of a few cases, the sustainable management of these diseases, either through eradication or suppression of the vector or the treatment of livestock with trypanocidal drugs, has proven to be challenging and often not sustainable (Allsopp 2001). Tsetse flies are grouped in one genus *Glossina* (Wiedemann, Diptera: Glossinidae), are widespread in sub-Saharan Africa, and are considered one of the root causes of poverty and hunger (Vreysen 2006). In the past decades, concerted efforts have been made to control or eradicate tsetse fly populations from large tracts of land (Davies 1964, 1971; Jordan 1978, 1985; Dame et al. 1980; Oladunmade et al. 1990; Dyck et al. 1997; Kabayo and Feldmann 2000). Appreciation of good environmental management has led to development of control methods that have minimal deleterious effects on the livestock holder, the consumer and secondary, beneficial organisms (Thompson et al. 1991, Douthwaite 1992, Bauer et al. 1999, Allsopp and Phillemon-Motsu 2002). Preferably a combination of control tactics should be used sequentially or simultaneously for a sustained area-wide effect to ensure enhanced economic benefits (Green 1994, Allsopp 2001). The sterile insect technique (SIT) (Knipling 1959) was used as part of area-wide integrated pest management (AW-IPM) (Klassen 2005, Vreysen et al. 2007) campaign to eradicate a population of *Glossina austeni* (Newstead, Diptera: Glossinidae) on Unguja Island, Zanzibar. The release of sterile male flies was initiated after the fly population was suppressed using insecticide 'pour on' formulations on livestock and insecticide-impregnated blue cloth targets (Green 1994, Vreysen et al. 2000).

The sterile male flies were released from fixed-wing aircraft using biodegradable cardboard boxes through a chute in the aircraft's fuselage (Vreysen et al. 1999). The global positioning system (GPS) was used for accurate flight navigation allowing the release of the boxes along flight lines at pre-determined intervals to achieve the desired release density. Alternative options for aerial delivery of sterilized adult insects have been developed since then (Tan and Tan 2013), including the release of chilled sterile males that has been

used routinely for releases of sterile fruit flies (Dowell et al. 2005, Reynolds and Orchard 2011), screwworms and various Lepidoptera (Klassen 2005). Improved civil air navigation systems can now be used to control the operation and the delivery rate of chilled adult release systems that enhances dispersal accuracy and results in better sterile male coverage (Tween and Rendón 2007).

Tsetse flies have been sterilized at the pupal or adult stage for different field programs (Offori 1993). In most of these past programs, the production source of sterile tsetse flies for release was close to the target area but there have been proposals in the recent past to ship the flies from distant sources, even across national boundaries as was the case for the Libyan New World screwworm (*Cochliomyia hominivorax* (Coquerel, Diptera: Calliphoridae)) campaign (Cunningham et al. 1992, Vargas-Terán et al. 1994). The prospect of long-distance transport of sterile flies has led to consideration of processing steps that may include storage of pupae, adults, or both prior to release. Storage of pupae at low temperature is recognized as a means to delay emergence and allow several days production to be dealt with as a single batch (Curtis and Langley 1972, Williamson et al. 1983, Opiyo et al. 1999, Mutika et al. 2014). A full load of sterile flies can thus be delivered in a more cost-efficient way.

In 2005, the Government of Senegal initiated a project entitled 'Projet de lutte contre les glossines dans les Niayes' (Tsetse control project in the Niayes) with the aim of creating a zone free of *Glossina palpalis gambiensis*. A feasibility study indicated that the tsetse-infested area was limited to 1,000 km^2 (Bouyer et al. 2010), and based on the study data, an AW-IPM strategy (Klassen 2005) was proposed that included an SIT component to eradicate the *G. p. gambiensis* populations from the Niayes (Dicko et al. 2014). The size of the target area was deemed too small to justify constructing a mass-rearing facility in Senegal, so the strategy developed included the procurement of the sterile males from existing productions centers abroad.

Therefore, protocols for the shipment of irradiated male *G. p. gambiensis* pupae were required that would allow the production of pupae in different countries, i.e., Austria, Burkina Faso and Slovakia, and the effective use of the emerged adult sterile male flies in Senegal. In a previous study, storage at low temperatures and irradiation of the *G. p. gambiensis* pupae were found to be non-detrimental for various behavioral parameters in relation to mating in comparison to untreated male flies (Mutika et al. 2014). The protocol has been transferred to the tsetse project in Senegal, and the effects of the handling and shipments have been assessed under operational conditions during weekly pilot trials that lasted more than 1 yr (Pagabeleguem et al. 2015). The handling and transport protocol was found to be adequate for releases of adult flies from the ground and for aerial releases using boxed flies that were dropped from gyrocopters, i.e., mating competitiveness of the sterile males was similar to that observed in Burkina Faso (Bouyer et al. 2012).

The availability of a chilled release system (Mubarqui et al. 2014) would make the release of sterile *G. p. gambiensis* males in the Senegal project much more cost-efficient. However, that would entail exposing the sterile adult flies (after being chilled, irradiated, and transported as mature male pupae under low temperatures) again to low temperatures that they would not encounter in nature (Bursell 1960, Pilson and Pilson 1967, Challier 1982). This paper presents data on the mating performance of adult male *G. p. gambiensis* in walk-in field cages after exposing the pupae to sterilizing irradiation and the pupae and adults to low temperatures. The results of this research are likewise valid for other economically important species of tsetse flies where SIT can conceivably be a component of the pest management program.

Materials and Methods

All tsetse flies used in the experiments were *G. p. gambiensis* that were originally colonized at the Centre International de Recherche-Développement sur l'Élevage en zone Subhumide (CIRDES), Bobo Dioulasso, Burkina Faso and reared at the Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria since 2009 (Mutika et al. 2013). Adults were fed 3 times a week on defibrinated bovine blood that was collected from an abattoir, frozen and stored at minus 20°C and irradiated with 1,000 Gy in a commercial 220 PBq ^{60}Co wet storage panoramic shuffle irradiator. Aliquots of the blood were thawed and used as required. Fly-holding rooms had a 12:12 (L:D) h regimen, 24 ± 1°C, 75 ± 5 % RH and light intensity up to 400 lux (hereinafter referred to as standard colony conditions). Pupae were collected daily and incubated at 24.1 ± 0.3°C, 77.7 ± 2.4 % RH in an ES2000CDM reach-in chamber (Luwa Environmental Specialities, United States).

When nearly all females had emerged (35 d post-larviposition) the remaining pupae ('male') were stored at 9.2 ± 0.4°C and 78.7 ± 4.1% RH for 5 d to simulate the chilling during transport. In this article, the term pupae includes the pharate adult stage. A total of 2,649 male pupae in eight replicates with unequal sample sizes were observed to calculate emergence rate. Twenty-four hours after placement in 9.2 ± 0.4°C storage, the pupae were exposed to sterilizing radiation in a Gammacell 220 ^{60}Co gamma source (MDS Nordion, Canada). Pupae and adults were stored at low temperature in a Weiss environmental chamber (Weiss Umwelttechnik GmbH, Germany). For irradiation, the pupae were placed in a 4 cm^3 glass bottle with a screw top. A maximum of three glass bottles each holding up to 120 pupae were then placed on ice in a 130 cm^3 glass container that was in turn placed at the center of a 1L polycarbonate jar that fitted the irradiation chamber of the Gammacell 220 for irradiation with 110 Gy. The central dose rate was calibrated in 2010 using transfer alanine/EPR dosimetry against a secondary standard at Risø, Denmark, with 95% CI of ± 2.7%. Dose distribution mapping was performed using Gafchromic HD810 film (95% CI ± 4%). The transfer of pupae from the low-temperature environmental chamber to irradiation and back to the low-temperature environmental chamber was done in less than 10 min. The above protocol was developed to simulate the handling and transport conditions of the male *G. p. gambiensis* pupae from Austria, Burkina Faso, and Slovakia to Senegal.

After removal from the low-temperature environmental chamber, the pupae were placed in a petri dish that was covered with an emergence cage under standard colony conditions and all adults emerged within 48 h. The males were then apportioned into 200-mm diameter by 50-mm deep fly rearing cages with 50 flies in each cage. The adults were kept under standard colony conditions except that the experimental flies were last fed on the day before storage at low temperature. On the 6th day, post-emergence cohorts of adults were then placed in an environmental chamber maintained at 5.1 ± 0.4°C and 78.6 ± 5.8% RH for 6 or 30 h and thereafter returned to standard colony holding conditions overnight prior to the field cage test. The low-temperature storage was intended to simulate the expected minimum and maximum exposure of adult flies to chilling conditions when using a chilled adult release system for sterile male dispersal.

Field cage tests were carried out the day after removal from the low-temperature environmental chamber starting at 0900 and 1400 hours. The cylindrical netting cage, 2.9 m in diameter and 2.0 m high (Calkins and Webb 1983) was set up in an insect greenhouse with temperature and humidity controls under natural light that had to

penetrate a double-layered clear plastic roof. A potted *Citrus sinensis* (L., Sapindales: Rutaceae) that was pruned to enable the observer to have clear vision to all points on the shrub was placed at the center of the cage. The mean (±SD) temperature was 27.2 ± 3.1°C and relative humidity 42.2 ± 7.0% with the range during the 3 h observation period not exceeding 7°C and 12 percentage points respectively on any day. Light intensity was variable during the 3 h observation period ranging from 1,000 Lux to 4,520 Lux. Alternatively, the field cage was set up in the insectary building with temperature and humidity controls, and overhead constant fluorescent illumination from 8 × 58 W cool white tubes. The temperature was 24–25°C and relative humidity 60–65%. The walls and floor were covered with ceramic tiles. An Onset Hobo data logger was used to monitor temperature and humidity; light was monitored using a Testo 545 lux meter.

Prior to running the main experiment, a secondary experiment was carried out to assess the possibility of reducing the overall experimental period through running two tests per day, i.e., one in the morning and one in the afternoon. In the secondary experiment, the mating activity of untreated colony flies was assessed in the morning or afternoon, i.e., 40 virgin flies of each sex were used during each of 12 field cage tests in the insectary building. Six of the field cage tests were carried out in the mornings and six tests in the afternoons. Untreated colony males that were 8 d old were offered mating opportunities with 3-d-old females from the standard colony. Mating activity was observed in the field cage from 0900 to 1200 hours and from 1400 to 1700 hours. A fresh group of flies was used in each session.

In the main experiment carried out in the greenhouse, 24 field cage tests lasting 3 h each were carried out on 12 different days, 12 tests in the mornings and 12 in the afternoons. The males that were exposed to low temperature and irradiation competed for mating opportunities with 8-d-old males emerged from pupae that were incubated at 24 ± 1°C and 75 ± 5% RH. In the morning, 40 females were released at the center of the cage at 0850 hours and in the afternoon at 1350 hours. Males were released at 0900 hours and 1400 hours after the females had settled. The rearing cage (200 mm diameter, 50 mm high) was opened with the 26 mm diameter exit hole facing up to allow flies to leave, such that non-flyers, if there were any, remained in the fly-holding cage. The observer remained in the cage for the 3 h with the flies and occasionally some flies fed on the observer.

For each field cage test two groups of 40 males were used, giving a ratio of 2 males to 1 female at the start of the observations. One group was the control with males reared under standard colony conditions, the second group were males that emerged from pupae that were irradiated and stored at low temperature (9°C) followed by the storage of sexually mature adults at low temperature (5°C) for either 6 or 30 h at 6 d after emergence. Males in each group were marked with a small dot of a different color of acrylic paint on the thoracic tergum to differentiate between treatments. The paint was placed on the thorax using a wooden tooth-pick. The flies were restrained in a netting pouch placed on a flat surface during marking at room temperature. Females, reared under standard colony conditions, were 3 d old at the time of the test.

The premating time for each pair was taken as the period from the end of release of males to the time when a successful engagement of genitalia occurred. The time when each successful mating pair was seen was recorded and the pair immediately collected into a transparent acrylic tube with netting at both ends. The tubes were individually labeled and were observed to record the time when the copulating pairs separated. The total number of pairs collected each day that the test was run was expressed as a proportion of the total possible pairs to give the mating propensity. For each test, the number of males of each treatment as a proportion of the total number of males that mated gave the relative mating index (Mutika et al. 2001, FAO/IAEA/

USDA 2014). Mating duration was calculated as the difference between premating and the end of copulation in minutes. The females and males that did not mate were discarded at the end of each test. The males that mated were discarded after termination of copulation and the females kept in the normal colony conditions until dissection. Copulating pairs that had not separated at the end of the observation period were also kept in the normal colony conditions until the end of copulation. Dissection of females that mated was carried out in saline solution under a Carl Zeiss stereo microscope at 50× magnification to estimate the quantity of seminal fluid in the spermathecae (Nash 1955). The values for the two spermathecae were added.

An analysis of variance (ANOVA) on the number of mating pairs was carried out in a two-way classification. ANOVA was carried out on premating time and mating duration in a General Linear Model, followed by one-way analysis for significant factors (Snedecor and Cochran 1989) after normality test and confirmation of homoscedasticity using Levene's test. Spermathecal values were analyzed using the Kruskal–Wallis test (Sokal and Rohlf 1995). Statistical analyses were carried out using Minitab software (Minitab 13 2000). To calculate the overnight survival of the adult flies that were stored at low temperature, mortality was corrected to take into account control deaths (Abbott 1925).

Results

Emergence Rate, Sex Ratio, and Survival

Emergence rate of the chilled and irradiated pupae ranged from 83 to 91% with a mean (±SD) of 86 ± 3.5% in seven replicates compared to the control emergence rate of 94.9 ± 1.4% (not significantly different). The lowest calculated emergence rate of 76% was recorded in the eighth replicate when there was an accidental loss of emerged flies before counting; this result was excluded from the comparison. In the evaluation, all flies that had wing deformities (3.84 ± 1.2%), partly extricated from pupae (1.64 ± 1.2%) and pupae that were intact on the third day after removal from low temperature (10.29 ± 6.1%) were considered as unemerged. Females that emerged from the pupae presumed to be male constituted only 2.45 ± 3.3% of the total number of flies that emerged. The average (±SD) corrected overnight survival of adult flies stored at low temperature for 6 h was 94.2 ± 4.6% and for 30 h was 87.5 ± 5.2%.

Mating Activity in Insectary Building, Secondary Experiment

There were similar proportions of mating pairs in the morning and afternoon when untreated colony males were offered opportunities to mate in a field cage ($F = 0.44$; df = 1,5; $P = 0.54$). For the 298 males that mated, the mean premating time ($F = 0.36$; df = 1,296; $P = 0.55$), copulation duration ($F = 2.56$; df = 1,296; $P = 0.11$) and spermathecal fill were similar for the morning and afternoon (Fig. 1). There were some replicates where more than half of the mating pairs were formed within 10 min of release of the males.

Mating Activity in Field Cage, Chilled Flies

Significantly more untreated males were successful in mating than the chilled males when pupae were stored at low temperature and adults were stored at low temperature for either 6 or 30 h ($F = 5.08$; df = 2,22; $P = 0.015$). The relative mating indices for the untreated control were 0.58 ± 0.17 and for the chilled males 0.42 ± 0.17 when males were chilled for 6 h. When the males were chilled for 30 h the relative mating index (±SD) for the untreated control males was 0.62 ± 0.16 and for the chilled males 0.37 ± 0.16. A total of 383
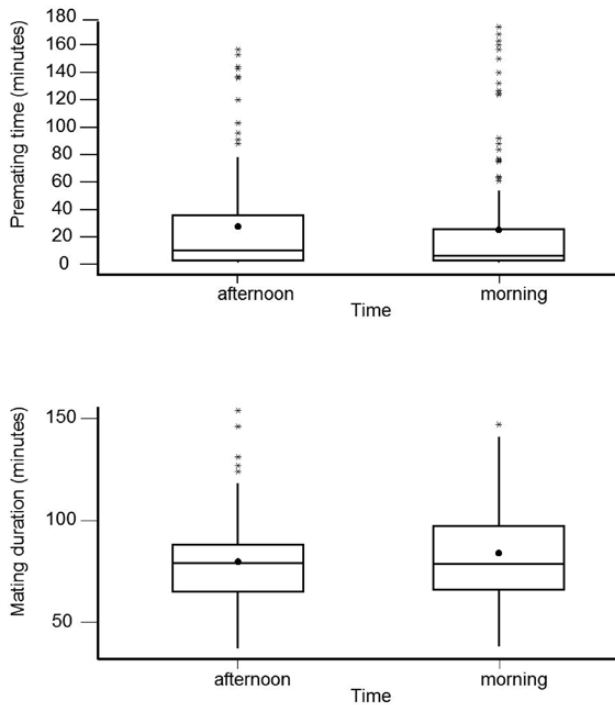
Fig. 1. Mating parameters for *Glossina palpalis gambiensis* when normal colony males mated with normal colony females—comparing observations in the morning against afternoon at 25°C, 63% RH and 500 Lux. The boxplots show outliers (*), mean (•), whiskers for highest and lowest values, ends of box are 1st and 3rd quartiles and the line across is the median.

Fig. 2. Mating parameters when male *Glossina palpalis gambiensis* were chilled (9.2°C) and irradiated as pupae then chilled for 6 or 30 h (5.1°C) as adults competed for mating opportunities with normal colony males. The boxplots show outliers (*), whiskers for highest and lowest values, ends of box are 1st and 3rd quartiles and the line across is the median.

untreated control males, 140 males chilled as adults for 6 h, and 109 chilled as adults for 30 h mated. The duration of copulation was significantly shorter ($F = 3.63$; $df = 1,629$ and $P = 0.03$) when males were chilled for 6 or 30 h compared to the control (Fig. 2). The premating time was similar, irrespective of treatment ($F = 1.70$; $df = 2,629$; $P = 0.18$), however a significantly longer premating time was recorded in the morning than in the afternoon ($F = 23.24$; $df = 1,628$ and $P = 0.00$). There was also a significantly shorter copulation duration in the afternoon ($F = 5.88$, $df = 1,628$; $P = 0.02$) than in the morning. The median spermathecal values for the females that mated with untreated males, males stored for 6 h and 30 h was 2. The insemination levels were similar for all treatments with an insignificant proportion that failed to transfer seminal contents for copulation in the morning or afternoon ($H = 0.57$; $df = 1$; $P = 0.45$).

Discussion

The use of aerial release systems for the SIT offers an opportunity to improve dispersal of sterile insects in comparison with ground releases, where insects are released from point sources and important energy resources are required for the flies to disperse to the areas where the virgin females are located. Aerial releases using biodegradable carton boxes that contain non-chilled adults have proven their efficacy for tsetse flies in the eradication program against *G. austeni* on the Island of Unguja, Zanzibar. Although dispersal of the sterile flies was acceptable using this system, and the sterile male flies were found to congregate in the same micro-habitat as the wild males (Vreysen et al. 2011), the boxes were expensive and increased overall project cost. The use of a chilled adult release system would reduce costs as it would entail only the initial investment in the release machine.

However, there are several important physiological issues that have to be considered in order to apply this system for tsetse flies.
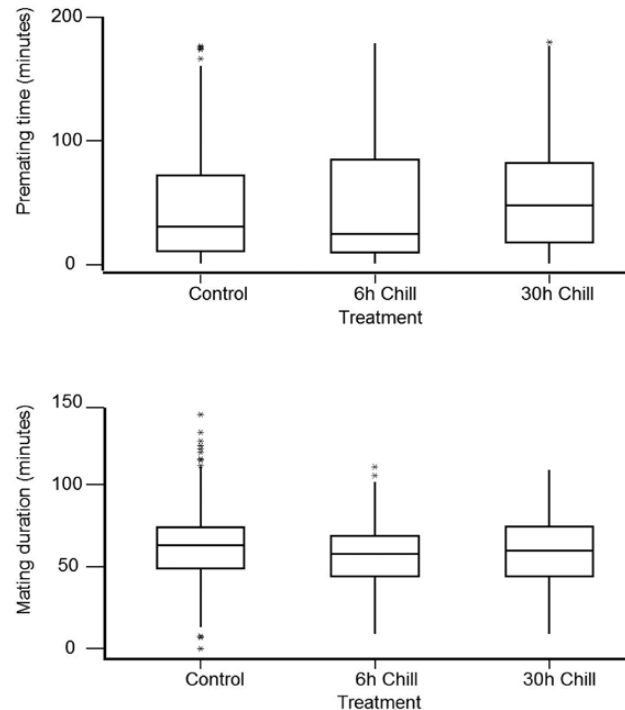
In nature, tsetse flies hardly experience temperatures below 10°C (Bursell 1960), yet in order to apply the chilled adult release system, the flies have to be chilled below 6°C to reduce mobility and allow regulated release with minimal mechanical damage. Recently fed flies with fully distended stomachs may rupture during passage in the release machine leading to losses. It is, therefore, necessary to arrange the feeding of the tsetse flies destined for release at a suitable time so that the period spent at low temperature does not negatively interfere with metabolic activities. The current work showed that the timing of feeding was appropriate since the level of male survival was over 80% even for the longer 30 h storage at low temperature. Survival of the sterile males to at least a successful mating with a wild female is one among many parameters of quality in which program managers would be interested. Mating behavior is critical for the SIT component of AW-IPM programs and the behavioral quality assessment encompasses several aspects, e.g., for the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann, Diptera: Tephritidae), the determination of the effects of low temperature includes direct assessment of flight ability (Andress et al. 2012). Laboratory studies on tsetse fly flight activity have been carried out on several species, and it has been determined that the total amount of time that is spent in flight per day by tsetse flies is less than 1 h. It is also accepted that in nature, a complex set of stimuli are involved leading to the initiation of flight (Adlington et al. 1996). The flight activity that is associated with mating in the field cage may conceivably constitute a smaller proportion of factors conducive to stimulate maximum fight activity. It would, therefore, be worthwhile to expand the current study and include an assessment of the impact of the double chilling (as pupae and adult) and irradiation (as pupae) on the flight ability of the resulting male flies.

The emergence rate of male pupae that were irradiated and stored at low temperature was around 83–91%, higher than the 64% that was recorded for *Glossina morsitans morsitans* (Westwood, Diptera:

Glossinidae) male pupae stored for 4 d at 10 ± 1°C prior to irradiation in a nitrogen atmosphere (Williamson et al. 1983). In comparison, the emergence rate of male *G. p. gambiensis* from the standard colony conditions from where the untreated males were collected was close to 94%. The overnight mortality figures for the flies that were stored at low temperature at both pupal and adult stage may point to the danger that lengthy exposure to low temperature may pose to the life-span of tsetse flies even though survivors retain some stamina to carry on reproductive activity. Despite the lower proportions that succeeded in copulating as compared to the untreated control males, there was successful transfer of sperm by males that were stored at low temperature at both pupal and adult stages, including irradiation. The overall quality of the stored and irradiated flies in terms of premating and duration of mating remained similar to untreated males (Fig. 2).

In insects such as tsetse flies, where the numbers available for release are limited primarily because of the low reproductive rate of the fly, a good dispersal system invariably makes a huge impact on the efficiency of the SIT. It is, however, important to take into account operational considerations such as the distance between the parallel release lines as these critically influenced the mating frequencies of sterile male New World screwworms: reduced swaths significantly increased the induced sterility in the native population (Krafsur et al. 1980). The prospect of storing some of the life stages of the tsetse fly with minimal damage may also be a bonus in the application of SIT in that program managers could schedule releases with some flexibility in the event of inclement weather or to allow several days of production to facilitate the release of a large number of flies in a single sortie (Williamson et al. 1983). The mating behavior is not fundamentally negatively impacted, but there are drawbacks in that a substantial number may be unable to participate in mating if stored at low temperature for lengthy periods. The reduction in 'operational' flies that are actually capable of mating may be compensated for by increasing the sterile to wild male ratio, which in practice is often already beyond the required sterile:wild male ratio of 10:1 (Vargas-Terán et al. 1994, Sow et al. 2012). The processing should be such that sterile male tsetse flies that are released as part of an AW-IPM program are able to successfully compete for mating opportunities against wild male tsetse flies (Opiyo et al. 1999, Mutika et al. 2002, Calkins and Parker 2005, Cáceres et al. 2007).

The data in this study have clearly indicated that the additional chilling of the sterile male flies as adults needs to be minimized in operational programs in order to limit any loss of biological quality. Although it is conceivable that a chilling period of 30 h is most likely never attained during an operational program, the differences obtained in several parameters between a chilling period of 6 and 30 h are indicative that the longer the adults are kept at the low temperatures, the lower their biological quality.

The differences in mating parameters when observations were carried out under near-constant environmental conditions inside the insectary building versus partially controlled conditions in the greenhouse confirm the importance of natural light and temperature in the modulation of tsetse behavior. The results in this paper reaffirm the possibility of improving the efficiency of the SIT through low-temperature storage as envisaged several decades back (Langley et al. 1974) and contributes to development of a protocol similar to the one for *C. capitata* (Cáceres et al. 2007). It is necessary to give attention to the importance of diurnal differences in behavior in order to derive full benefits from the application of the SIT (Curtis and Langley 1972).

Acknowledgments

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