Methods to Separate Lobesia botrana (Lepidoptera: Tortricidae) Males from Females for the Implementation of Sterile Insect-Inherited Sterility Technique Control Tactics

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Methods to separate *Lobesia botrana* (Lepidoptera: Tortricidae) males from females for the implementation of sterile insect-inherited sterility technique control tactics

*Hadass Steinitz, Adi Sadeh, Martin Tremmel and Ally R. Harari**

Abstract

The sterile insect technique (SIT) requires the release of a large number of irradiated moths in the infested crop area targeted for suppression or eradication. Irradiated males must compete strongly with wild males for mating with conspecific wild females. Irradiated female moths are fully sterile and therefore when released do not pose a potential risk to the crop. However the close proximity of the released males and females may result in assortative mating among the irradiated moths, thereby undermining the competitive ability of the irradiated males. Furthermore, released females have partially depleted the reproductive potential of the colony, and will not contribute to further increases in the size of the colony in a mass-rearing facility. We tested 4 methods to separate European grapevine moth, *Lobesia botrana* (Dennis & Schiffermüller) (Lepidoptera: Tortricidae) males from females based on differences between males and females with respect to: (i) the number of abdominal segments of the pupae, (ii) the colors of wandering larvae, (iii) speed of maturation of larvae (protandry), and (iv) the lengths of the pupae. The sexing of 500 moths by the number of abdominal segments in the pupae was accomplished without any errors, but it was time consuming, tedious and required skill and experience. However, it should be possible to develop an automated apparatus to sort pupae by gender. Sexing by color differences did not provide clean separation because of large overlaps between the sexes in the green, blue and red spectra. Although male larvae entered the wandering phase significantly earlier than female larvae, in a large portion of the population the time of departure, some males and females departed simultaneously so that clean separation was possible for only about ¼ of the males or about ¼ of the females. A similar situation prevailed for emergence of adult males and females from pupae. On average, male pupae were significantly shorter than female pupae, and pupal length was found to be the most practical method for separating *L. botrana* males from females. For example, if all pupae < 5.4 mm were irradiated and released, they would include about 86% of all males and about 22% of all females—all of which would be the smallest and least fecund ones. This would allow about 78% of the females—including the largest and most fecund ones—to be retained for mass-rearing. At this time separation of the sexes based on pupal length is the most practical method.

Key words: sterile moths; European grapevine moth; pupae separation; sexual dimorphism; larval color; protandry; pupa size

Resumen

La técnica del insecto estéril (TIE) requiere la liberación de un gran número de polillas irradiadas en el área de enfoque del cultivo infestado para la supresión o erradicación. Los machos irradiados deben competir fuertemente con los machos silvestres para aparearse con hembras salvajes de la misma especie. Las polillas hembras irradiadas son totalmente estériles y por lo tanto, cuando se liberan no representan un riesgo potencial para el cultivo. Sin embargo, la proximidad de los machos y las hembras liberados puede resultar en el apareamiento selectivo entre las polillas irradiadas, lo que socava la capacidad competitiva de los machos irradiados. Además, la liberación de hembras agota parcialmente el potencial reproductivo de la colonia, y las hembras liberadas no puede contribuir al aumento en el tamaño de la colonia en el futuro en las instalaciones de cria en masa. Hemos probado 4 métodos para separar los machos de las hembras de la polilla europea de la vid, *Lobesia botrana* (Dennis y Schiffermüller) (Lepidoptera: Tortricidae), en base a las diferencias entre machos y hembras con respecto a: (i) el número de segmentos abdominales de las pupas, (ii) el color de las larvas errantes, (iii) el tiempo de maduración de las larvas (protandria) y (iv) la longitud de las pupas. Se realizó la determinación del sexo de 500 polillas por el número de segmentos abdominales de las pupas con ningún error, pero llevaba mucho tiempo, fue tedioso y requieron habilidad y experiencia. Sin embargo, debería ser posible automatizar este método para separar las pupas por sexo. La determinación del sexo por diferencias de color no proveyó una separación limpia debido a el gran traslapo entre los sexos en cuanto del espectro verde, azul y rojo. Aunque las larvas machos entró en la fase errante significativamente antes que las larvas hembras en una gran parte de la población, algunos machos y hembras partieron simultáneamente, de modo que la separación clara fue posible para sólo alrededor de una ¼ parte de los machos y aproximadamente una ¼ parte de las hembras. Una situación muy similar con respecto a la emergencia de los adultos machos y hembras de las pupas. Por lo general, las pupas macho fueron significativamente más cortas que las pupas hembra, y se encontró que la longitud pupal fue el método más práctico para la separación de los machos de las hembras de *L. botrana*. Por ejemplo, si todas las pupas <5,4 mm fueron irradiadas y liberadas, que incluirían alrededor del 86% de todos los machos y el 22% de todas las hembras — todas las cuales serían las más pequeñas y menos fecundas. Esto permitiría que aproximadamente el 78% de las hembras — incluyendo las más grandes y más fecundas — serán retenidas para la cría en masa. Actualmente, la longitud de pupa es el método más práctico para separar los sexos.

Palabras Clave: polillas estériles; polilla europea de la vid; separación de pupas por sexo; dimorfismo sexual; color de las larvas; protandria; longitud de pupa.

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The sterile insect technique (SIT), i.e., the release of sterile or partial sterile (inherited sterility-IS) male insect pests into infected crop fields or orchards, is an environmental friendly control method which has gained significant importance in the last decades (Bloem et al. 2005; Dyck et al. 2005). Moth larvae are among the most devastating insect pests of agricultural crops worldwide (Bloem et al. 2005). Experimental SIT trials combined with field pilot trials against a few selected lepidopteran pests showed promising results in managing these pest populations (e.g., the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) (North & Holt 1969); the corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Carpenter & Gross 1993); the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae) (Maestro 1993) that facilitated the establishment and implementation of operational field programs for the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) (Carpenter et al. 2005), the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Henneberry et al. 2007) and the Australian painted apple moth, *Teia anartoides* Walker (Lepidoptera: Lymantriidae) (Suckling et al. 2007). Using this technique, sterile males are released in the infested area to outnumber the wild male population. In the case of the sterile insect technique (SIT), the fully sterile males—because of their numerical advantage—outcompete the wild males and matings of sterile males with wild females result in nonviable embryos, but in the case of partial sterility—also known as inherited sterility (IS)—matings of partially sterile males with wild females result in greatly male-biased sterile offspring (Carpenter et al. 2005).

Lepidopteran females, in general, are more sensitive to radiation than their conspecific males. Thus, the radiation dose can be lowered in most species to a level that causes complete female sterility, but only partial sterility in males that retain greater biological quality and competitiveness (North 1975; LaChance 1985). Partial sterility in males allows the release of smaller numbers of irradiated males in the target area (Knipling 1970). At the same time, the number of their F_1 offspring developing in the target area increases with the intrinsic increase rate of the species, contributing substantially to the presence and spread of sterile males in the field (Carpenter 1993). Typically both male and female moths are irradiated and released in the target area (Grefenstette et al. 2009; Tabashnik et al. 2010; Vreysen et al. 2010).

The production of high quality insects that will successfully compete with wild males is a prerequisite for the successful implementation of the SIT/IS (Calkins & Parker 2005). Irradiation and release of laboratory-reared females in programs that have a SIT component have 2 negative effects: (1) a reduced growth of the colony in the mass-rearing facility due to repeated releases of a large numbers of females, and (2) a high probability of mating between the released irradiated males with released irradiated females. The probability of matings of released irradiated males and wild females is eroded in codling moth programs involving the SIT because their capacity to disperse is genetically based (Keil et al. 2001) and varies substantially among individuals, and this could result in a lower effectiveness of the released irradiated males. The sedentary genotype has an inferior ability to fly, but is more fecund and survives longer than the dispersing genotype (Rankin & Burchsted 1992). Laboratory mass-rearing selects for sedentary genotypes (Gu et al. 2006) and the sedentary attributes increase the possibility that irradiated sedentary females will mate with laboratory-released males, rather than both mating with wild individuals.

Various approaches, especially for Diptera, have been used to eliminate female insects prior to the release of irradiated adults. One approach relies on linking an insecticide resistance gene to the Y chromosome, thereby eliminating females that are sensitive to insecticides (Seawright et al. 1978). Another approach uses a female-linked temperature sensitive lethal gene that makes the females sensitive to a higher temperature (Hendrichs et al. 1995; Franz et al. 1996; Robinson et al. 1999). However, a certain portion of females produced in a mass-rearing facility must be retained for subsequent production, and the partial sterility and overall reduced viability of these genetic sexing strains (Franz et al. 1994; Kerremans & Franz 1995) makes mass-rearing more challenging. Genetic sexing strains have so far not been developed for Lepidoptera, with the exception of balanced lethal strains for the silkworm, *Bombyx mori* (L.) (Lepidoptera; Bombycidae), and the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), following the scheme of Strunnikov (1975). Balanced lethal strains are, however, not easily employed in mass-rearing programs because they require the maintenance of 2 colonies. For this reason, balanced lethal strains have never been used in an operational program (Marec et al. 2005). Separating male from female moths may be accomplished by taking advantage of naturally existing sexual dimorphism in physiological attributes such as color and size and/or by taking advantage of differences in behavioral patterns of male and female moths.

Polymorphic coloration of the last instar and pupa is widespread in moths and butterflies (Rivnay & Meisner 1966; Hazel 1995). In several polymorphic species dimorphism is affected by environmental cues (reviewed in Hazel 1995). The observed polymorphism is density-dependent in some larvae of moth species, which have a lighter color when reared individually and darker when reared under crowded conditions (Ikemoto 1983). Altstein et al. (1994) suggested the involvement of a pheromone biosynthesis activating neuropeptide (PBAN) in color polymorphism in the African cotton leafworm, *Spodoptera litoralis* Boisduval (Lepidoptera: Noctuidae), with a high concentration in the hemolymph leading to lighter color of the late instar larvae. PBAN is known to regulate sex pheromone biosynthesis in females, and thus color polymorphism regulated through PBAN may also indicate the larva's gender.

In many moths and butterflies, as well as in other insects, males mature sexually before females, which is a type of protandry (Wiklund & Fagerström 1977; Harari et al. 2000). In monandrous species in which a female mates only once in her life time, the benefit for early-matured males is obvious, as the first male to arrive and successfully mate with a virgin female sires all of her offspring. Protandry is under sexual selection when the generations are discrete in a way that in each generation the emergence of males precedes that of the females (Wiklund & Fagerström 1977; Singer 1982; Muralimohan & Srinivasa 2008). By contrast, in polyandrous moth species in which a female may mate multiple times, the last male to mate with a female usually fertilizes most of her eggs (Drummond 1984). Nevertheless, a male that emerges earlier than others may be the first to encounter a calling or receptive female, regardless of her mating status, and this is especially consequential when the frequency of remating by females is relatively low. This is the case in most polyandrous species, because the number of matings also depends on population density and female age (Zonneveld 1992). A protandrous male optimizes his chances to encounter females by mating early in the flight period (Zonneveld 1992). Protandry is common in many of the moth pest species that are potential targets for control programs with a SIT/IS component.

Sexual size dimorphism is a well-known phenomenon in moths and is often accepted as a result of different selection pressures acting on males and females. Typically, female size is positively correlated with fecundity (Honek 1993; Harari et al. 1999; Davidowitz 2008), whereas body size of males is negatively correlated with the searching duration for females due to a lower energy intake. Moreover, body size in moths is positively correlated with development time. As a result of

selection for protandry of males, early matured males develop faster and have a smaller body size than conspecific females (Abrams et al. 1996; Blanckenhorn 2005; Morbey 2013). Male body size, in this regard, is considered an adaptive trait, resulting from tradeoffs between the consequences of reaching a large size and having a copious energy supply versus emerging early with a lesser energy supply but in better time to encounter receptive females. It follows that male body size is not primarily a result of environmental effects, such as food availability and seasonality (Abrams et al. 1996).

The aim of this study was to select a method that facilitates the separation of *L. botrana* males from females in a way that males can be irradiated and released in the target area, while females can be retained in the mass-rearing facility for the further increase of the colony in order to better meet the needs of the field program.

Materials and Methods

GENERAL PROCEDURES FOR REARING *LOBESIA BOTRANA*

Lobesia botrana was reared under laboratory conditions of 25 °C and a photoperiod of 14:10 h L:D, with a cycling time from egg to adult of ~25 d. Adult males and females were kept in mating screen cages (30 × 30 × 30 cm) that contained cotton wicks saturated with 10% sucrose as food. Mated females oviposited their eggs on a sheet of Parafilm wax paper hanging on the cage wall. Egg sheets were cut into small squares (~50 eggs each) and placed in a Petri dish (8.5 cm diam) that contained a premixed diet (WARD'S 38-0600, Rochester, New York). Petri dishes with last (5th) instar larvae were placed in a large plastic box (35 × 30 × 15 cm) covered with a glass plate. Pleated laboratory paper was placed on the bottom of the cage as a pupation substrate for the larvae. Pupae were collected from the paper sheets, and placed in screen cages where they could emerge and mate. In the spring of each year, the laboratory rearing was enriched with a few hundreds of wild collected larvae to minimize inbreeding effects and adaption to laboratory conditions.

GENERAL METHODS

Lobesia botrana in the last larval stage (5th instar) typically leave their food in search of pupating places (hereafter "wandering stage"). The 5th instar can be recognized by the change in its cuticular color from light brown to purple, green or various combinations of these 2 colors. In this research we separated the larvae as soon as they entered the wandering stage by placing them individually in small Petri dishes (5.5 cm diam). The larvae were then given time to pupate and the following information was recorded: the date of onset of the wandering stage (also referred to later as "d 1"), the d when the larva became a pupa (loss of legs) and the d when the adult emerged. We measured the pupa's size and recorded its sex using a stereoscope (see external morphology for details). The efficacy of 4 methods to separate *L. botrana* males from females before and during the pupal stage, based on external morphology was tested. Thus gender discrimination was explored based on: (1) the number of abdominal segments in the pupae, (2) the various colors of 5th instar larvae, (3) the time of onset of wandering (protandry) of larvae in a cohort (same egg sheet and collection time), and (4) the overall lengths of the pupae.

SEXING BY NUMBER OF ABDOMINAL SEGMENTS IN THE PUPA

The number of abdominal segments (from the tip of the abdomen to the segment adjacent to the wing edge) (Bathon et al. 1991) was counted for 500 pupae. Pupae having 3 segments were scored as females and pupae having 4 segments behind the wing were scored as males (Fig. 1). The success of the method was verified in the adult stage.

SEXING BY 5TH INSTAR LARVAL COLORATION

Fifth instar larvae change their color from light brown in the 4th instar to a colorful mix of purple and green. In order to analyze the association between sex and color of the 5th instar larvae, we collected larvae on the first d of the "wandering stage" and placed each of them in a small petri dish (5.5 cm diam). We photographed the larvae on the d of collection using a Dino-eye microscope eyepiece camera and analytical software (Dino capture 2.0 software version 1.3.8). Each larva was allowed to pupate in the petri dish and its sex was determined by counting the abdominal segments of the pupa. Magnification and light conditions remained constant for all pictures. Using Adobe Photoshop the background to the larva in the photograph was cut away following the outline of the larva. We used a Matlab procedure (MathWorks, Version 5.3.1 Inc., Natick, Massachusetts) to analyze the red-green-blue (RGB) values (an integer number between 0–255). Mean RGB values were compared between male and female larvae using multivariate analysis of variance (MANOVA).

SEXING BY PROTANDRY

Protandry (i.e., more rapid maturation of males than females) was calculated using the following recorded data: the time that 5th instar larva left the food and began wandering and searching for a pupa-

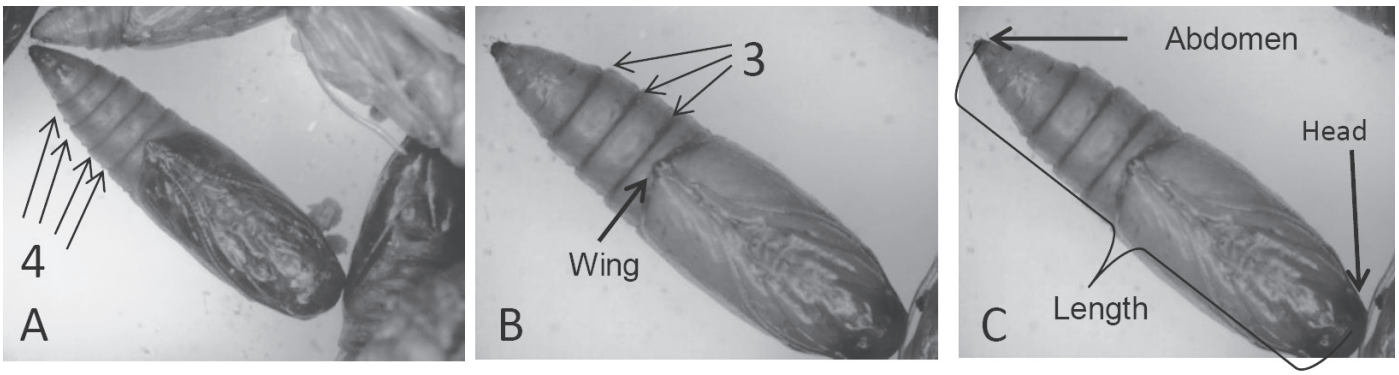

Fig. 1. *Lobesia botrana* pupae indicating the number of abdominal segments in (A) male (4 segments), and (B) female pupae (3 segments) as measured from the abdomen tip to the wing's point, and (C) distance between the head and the abdomen tip as a measurement of pupal length.

tion site (also referenced "d 1"), the onset of pupation (defined as the beginning of the metamorphosis), and time of adult emergence. Pupae were sexed by the number of abdominal segments, as described above. Time of "wandering stage", duration of the pupal stage (time that lapsed from the beginning of the "wandering stage" to adult emergence) were compared between males and females by *t*-test.

SEXING BY LENGTHS OF PUPAE

Using a digital caliper, the length of 7 d-old pupae (1 d before emerging to adult) was measured from the head to the tip of the abdomen (Fig. 1C). The difference between the lengths of male and female pupae was analyzed using a *t*-test.

Results

GENDER DISCRIMINATION BY NUMBER OF ABDOMINAL SEGMENTS OF PUPAE

Sexing moths by the number of abdominal segments in the pupae revealed no errors (n = 500 pupae). All pupae that had 3 segments under the wing tip were found to be males in the adult stage. Moreover all pupae that had 4 segments were scored as females and all of them developed into adult females. This method, although error free, was time consuming, tedious and required skill and experience.

GENDER DISCRIMINATION BY COLORATION OF 5TH INSTAR LARVAE

Photographs were taken of 267 fifth instar larvae (123 males and 144 females) (Fig. 2). Analyzing the mean green, blue and red values of all larvae revealed a high level of interaction among the colors (0.82–0.92 between the 3 colors). Comparisons of RGB means between males and females demonstrated a significant difference between the sexes (Wilks' lambda F = 0.8935; df = 3,263; P < 0.0001) (Fig. 3A). Although males and females varied significantly in their mean RGB values, the frequencies of larvae with each RGB value were similar for males and females. This resulted in large overlaps between the sexes in the green (72.4%), blue (78.1%) and red (79.1%) spectra. Therefore, practical discrimination of the sexes by their RGB values was not feasible (Fig. 3B).

GENDER DISCRIMINATION BASED ON PROTANDRY

The wandering of larvae from the food in the Petri dish in a search of a place to pupate began 13 d after the eggs had been placed in the petri dish and continued for an additional 11 d (Fig. 4). Male larvae left the food plates significantly earlier than female larvae (Mean ± SE), i.e., males: 3.7 ± 0.17 d; n = 149; females: 4.5 ± 0.13 d; n = 177 (t-test, t = 3.65; df = 324; P < 0.001).

The majority of wandering larvae on the first and second d were males. These males represented 34.7% of all males in the cohort, whereas wandering female larvae during the first and second d represented 5.6% of all females in the cohort (Fig. 4). On the third d similar numbers of male and female larvae were observed leaving the food. By this time 57.9% of all males but only 26.6% of all females had already left the food. From the 4th d onward, the percentage of both males and females wandering in search of pupal sites declined but the percentage of female larvae exceeded that of males on each d.

Mean duration of the pupal period was significantly longer for males (mean ± SE), i.e., males: 8.1 ± 0.07 d, n = 129; females: 7.8 ± 0.07 d (n = 15); (t-test; t = 4.78; df = 77; P < 0.001). Nevertheless, in each cohort males were the first to emerge after leaving the food plates (Mean ± SE), i.e., males: 4.9 ± 0.19 d; n = 129; females: 6.1 ± 0.18 d; n = 150 (t-test; t = 4.78; df = 77; P < 0.001).

Sex ratio of adults that emerged during the first 2 d was highly biased towards males (0.88) (Fig. 5). Taking the data of the first 3 d, the adult sex ratio was less male biased (0.68) and on the 4th d the sex ratio of emerged adults was 0.5. Thereafter, the majority of emerging adults, on each d, were females (Fig. 5).

GENDER DISCRIMINATION BASED ON PUPAL LENGTH

On average, male pupae were significantly shorter than female pupae (mean ± SE), i.e., males: 5.04 ± 0.03 mm; n = 149; females: 5.59 ± 0.04 mm; n = 177 (t-test; t = 10.9; df = 324; P < 0.001). Pupae shorter than 4.8 mm were males only, but these constituted only 14% of all males in the population (Fig. 6). Only 3.3% of the females were included among pupae shorter than 5 mm, but 37.2% of all of the males were included among pupae shorter than 5 mm; thus in the cohort of pupae up to 5 mm long, the proportion of males was 0.91. As the size of pupae increased progressively greater proportions of females were found in the population. Pupae with lengths up to 5.2 mm constituted of 66.7% of all of the males in the population, and 11.3% of the female population; thus in the cohort up to 5.2 mm long, the proportion of males was 0.83. However, most adults emerging from pupae with the length of 5.6 mm were females (Fig. 6); thus in the cohort up to 5.6 mm long the proportion of all of the males was 0.98, and the proportion of all of the females was 0.42. The male:female sex ratio of all pupae reaching the length of 5.6 was 0.66. All pupae of 6 mm or longer were females and they constituted 23.3% of all females in the population.

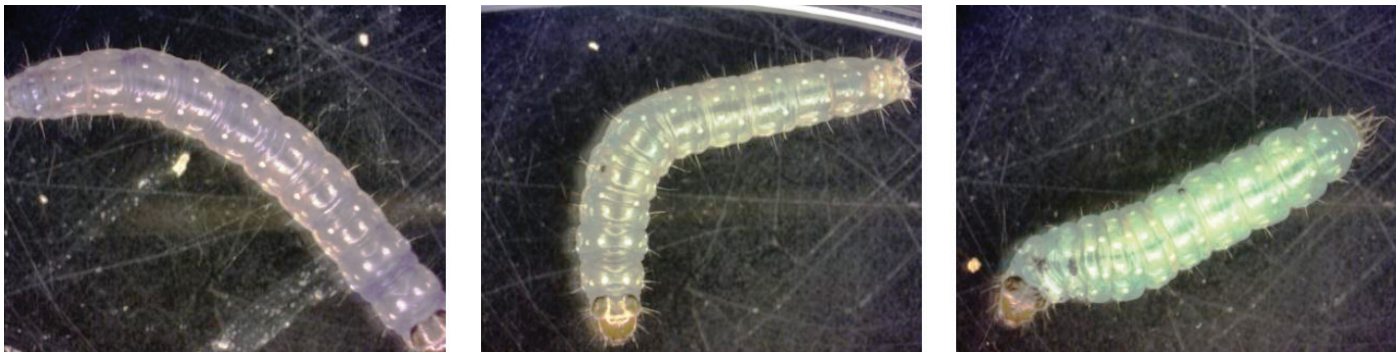

Fig. 2. Fifth instar larvae of *Lobesia botrana* indicating various shades of blue and green.

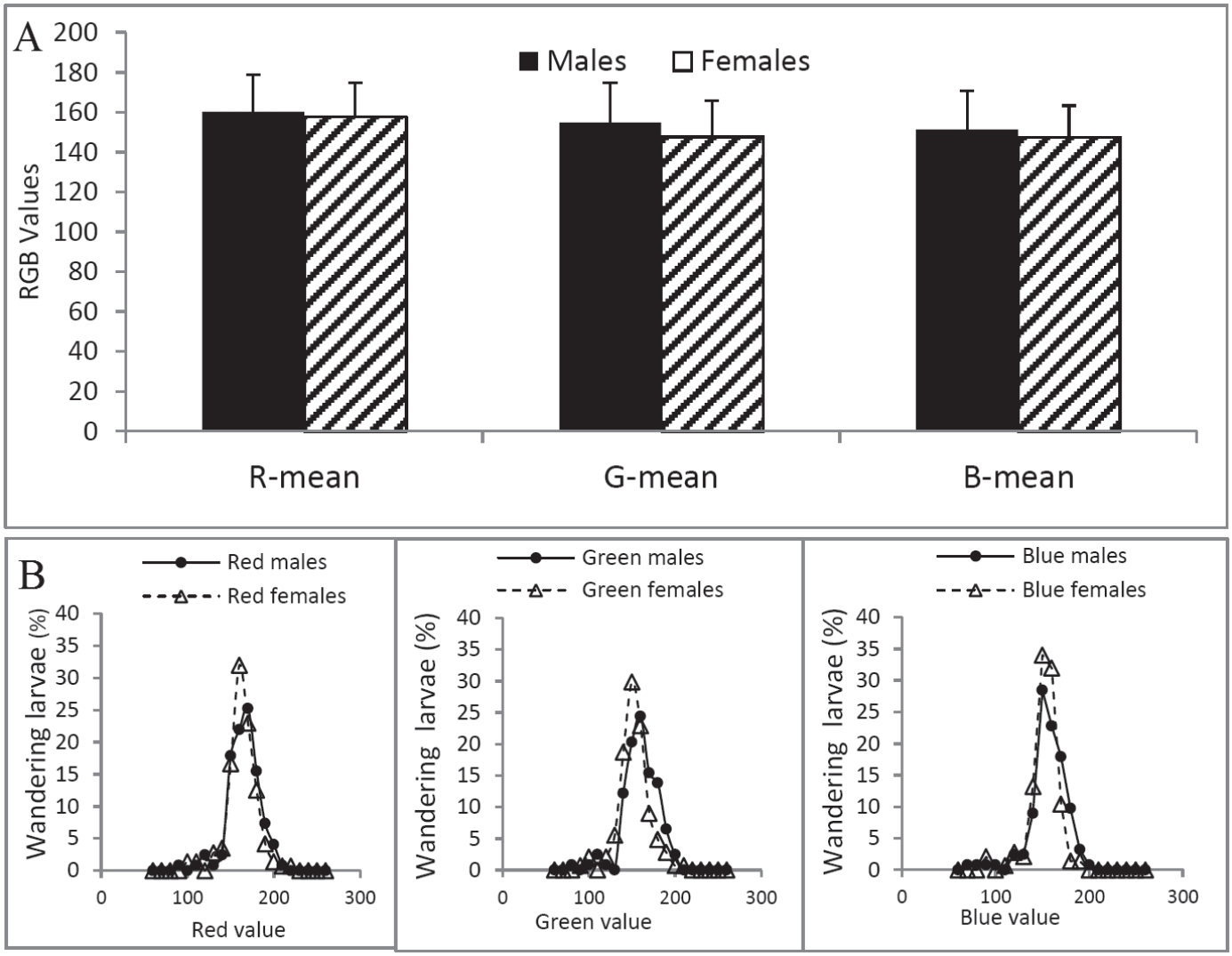

Fig. 3. R, G and B color values of male and female *Lobesia botrana* larvae during the wandering phase. A. Average R, G and B color values of wandering male and female larvae, and B. Percentages of wandering red, green and blue larvae in relation to the R, G and B values as a proportion of total male and female larvae. * $P = 0.001$, Tukey $\alpha = 0.05$.

Discussion

Insect pest control programs that include an SIT/IS component, involve mass-release of sterile males; hence the requirement to mass-rear the target pest species, which is labor intensive and costly. Improving rearing efficiency in these programs would facilitate increases in the number of ha of habitat covered by this environmentally friendly tool for a range of key pest species. One approach to save time and costs is to develop a routine, rapid and inexpensive way to separate male from female moths. The release of males only, or mostly males, may reduce program costs by: (1) retaining more females in the rearing units, thus increasing mass-production, and (2) increasing the efficacy of the SIT-IS component by minimizing the probability of matings between released irradiated males and wild females. Currently, in most operational SIT programs against lepidopteran pests both irradiated male and female adults are released (Bloem et al. 2001), although in a few cases males have been separated from females during the pupal stage (Suckling et al. 2002, 2007).

Four methods to separate *L. botrana* males from females were explored in this study, and these were based on (i) the number of abdominal segments of the pupae, (ii) the colors of wandering larvae, (iii) protandry, and (iv) the lengths of the pupae. All 4 of these explored methods would allow the irradiation of pupae on the last d of development when the pupae are fully sclerotized and easy to handle.

Counting the number of abdominal segments—4 in males and 3 in females—was the most accurate method to distinguish between the sexual genders with practically no errors. However, this method is time consuming, and counting the abdominal segments of millions of pupae per d is neither feasible nor sustainable. However, the development of an automated apparatus to sort pupae by gender should be possible.

The mean blue and green color values of wandering male larvae were significantly different from those of females, but because of the extensive overlapping of colors between males and females, the coloration method was not a reliable way for separating the sexes.

Typically, male larvae left the food source to pupate before the female larvae did so. Collecting paper sheets within the first 2 d of the onset of wandering by larvae yielded mostly males but these constituted less than 25% of all males present among the pupae. The percentage of males present on the paper sheets at later times decreased as more females left the food in a search of a substrate suitable for pupation. Starting on the 4th d after the onset of wandering, the fraction

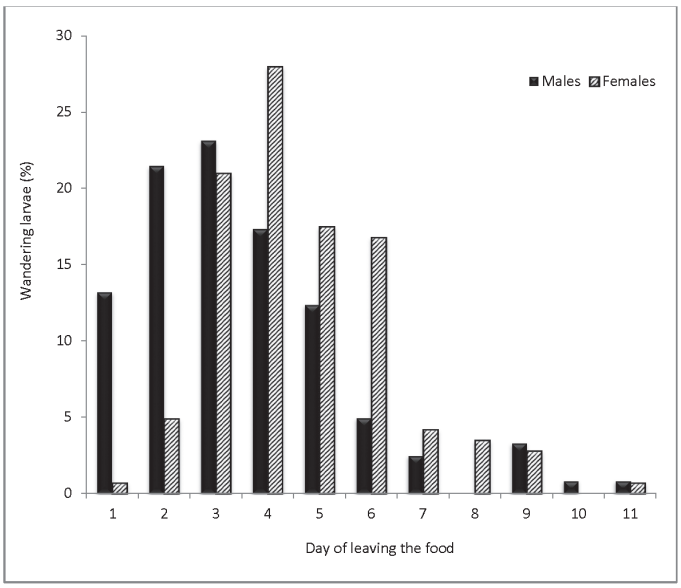

Fig. 4. The percentages of wandering *Lobesia botrana* larvae that departed from their food on each consecutive day of the wandering phase and that subsequently developed into either adult males or adult females.

of females among the pupae exceeded the fraction that were males. Thus collecting paper sheets within a few d of the onset of larval wandering resulted in a highly male-biased sex ratio. However the number of males for irradiation and release could be increased by including wandering larvae at a later d, but this would also increase the number of released females. Partial separation of males and females based on the time they leave the food source would be easily accomplished in mass-rearing facilities. However, this approach would not result in a very efficient use of the females for increasing the level of production of the mass-rearing facility Irradiation of pupae in sheets collected at the onset of wandering will yield mostly males but only a small portion of all mass-reared males.

Adult males emerged significantly earlier than females (i.e., protandry), thus collecting emerging individuals on the first d of emergence guarantees a strong male-biased sex ratio. However, here again, collecting all individuals that emerged in the first 2 d would result in a strong male biased sex ratio (0.88) but would contain only 26% of all males in the cohort. At the fourth d 74% of all males in the population would have emerged but the sex ratio of all emerged adults is expected to declined and reach 0.5. This sex ratio may hinder the efficacy of SIT/IS as some of the released males may mate with their co-released females.

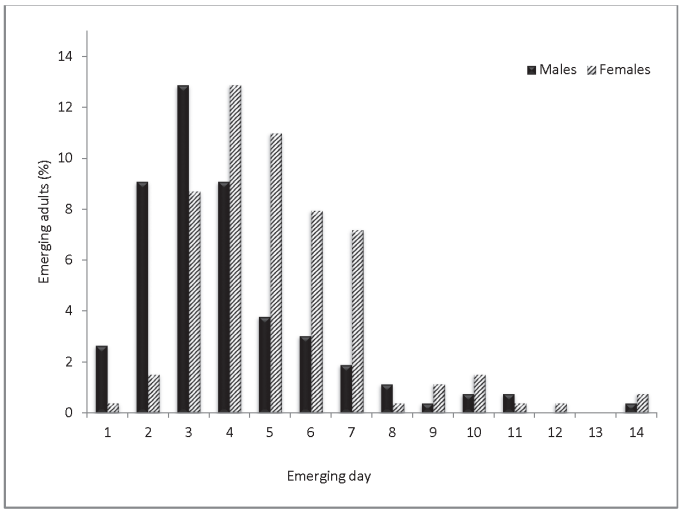

Fig. 5. The percentages of male and female *Lobesia botrana* adults that emerged from the pupal stage on each consecutive d after the onset of adult emergence.

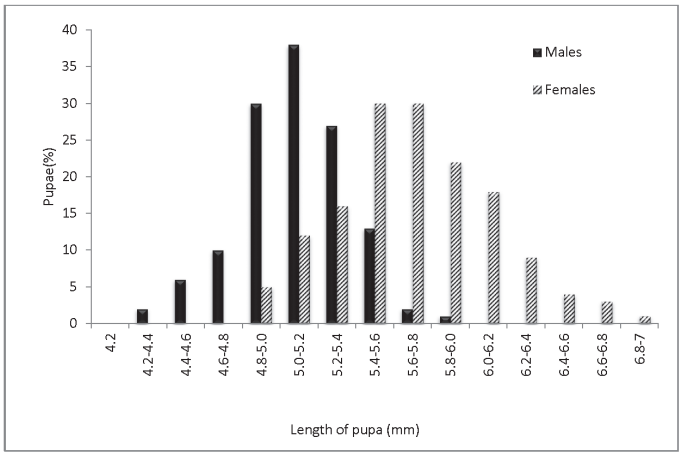

Fig. 6. Distribution of *Lobesia botrana* male and female pupae based on the overall lengths of the pupae.

Male pupae were significantly shorter than female pupae. All pupae that were equal in length or shorter than 4.8 mm yielded only male moths. Limiting the size of the collected pupae to 5.4 mm enabled the collection of 86% of all males of the population, a male-biased sex ratio of 0.77 and only 22% of the females in the population. A further increase in length of collected pupae would enlarge the proportion of released males, but it would also increase the number of females released. However, if for rearing purposes only females are needed, then the collection of larvae that are larger than 6.2 mm should result in the complete absence of male moths.

Pupal length was found to be the most practical method for separating *L. botrana* male from females, and a method based on this approach would result in the release of mostly males, and would enable the majority of females—including all of the largest and most fecund ones—to be retained in the rearing facility. By collecting pupae that are equal in length or shorter than a given length would allow the sex ratio of the released adults to be controlled, and thus minimize the probability of mating of released irradiated males with released irradiated females. Moreover, this procedure would result in retaining the larger, more fecund females to establish the next generation. This procedure, however, would entail an artificial selection process that may lead to the selection for larger sized males, because the largest males would remain in the colony and contribute more to the colony gene pool than smaller males that were mostly destined for the releases. This may also affect the body size of the females, albeit—depending on the nature of the genes that determine body length—to a lesser extent, as only a small portion of the smaller females would be excluded from further reproduction. In both sexes, selection pressure may bias the mass-rearing for larger individuals. Large body size is associated with greater fecundity in females (Honek 1993; Harari et al. 1999; Davidowitz 2008) and greater stamina and vigor in males (Serrano-Meneses et al. 2008). Here, an opposing direction of selection acting on males may come to act: the reproductive benefit of protandrous males, which selects for small body size (Gotthard 2004; Stillwell et al. 2010). The results of this dichotomy in selection pressures may differ among species (Stillwell et al. 2010) in relation to their mating systems (e.g. monandrous or polyandrous) and should be tested for each species independently.

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