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Research

Slow Larvae Mutant and Its Potential to Improve the Pupal Color-Based Genetic Sexing System in Mexican Fruit Fly, (Diptera: Tephritidae)

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Abstract

For many years, an area-wide fruit fly control campaign against the Mexican fruit fly, *Anastrepha ludens* (Loew) has been implemented in some regions of Mexico and Texas, using the sterile insect technique (SIT) as its principal component. To improve the efficiency of the SIT, a genetic sexing strain based on *black pupae* mutation (*bp*) was developed for *A. ludens*, namely, 'Tapachula-7' (Tap-7 genetic sexing strains [GSSs]). This strain was introduced into the AW-IPM program recently and allows male-only releases for SIT applications. Here, we report the genetic and biological characterization of a new mutation, *slow larvae* (*sl*), which was introduced to the original translocation of the Tap-7 GSS resulting in two new GSS (slow-7 and Tap/slow-7). In both GSSs, the translocated wild-type males emerge from brown pupae that develop faster than females. The females are homozygous for *sl* mutation in the slow-7 GSS and homozygous for *sl* and *bp* mutations in the Tap/slow-7 GSS, reaching larval maturity 2 d after most of the wild-type males, allowing the separation of most males during pupariation. The potential use of the slow-7 and Tap/slow-7 GSSs in mass rearing and large-scale population suppression programs is discussed.

Key words: Mexican fruit fly, mass rearing, sterile insect technique, mutation, genetic sexing strain

The Mexican fruit fly (Mexfly), *Anastrepha ludens* (Loew), is the most serious economic pest among fruit flies in the south of United States and Central America. Since 1992, an area-wide insect pest management (AW-IPM) campaign against this pest has been implemented in some regions of Mexico and Texas. The sterile insect technique (SIT) is the principal component of this population suppression campaign (Gutiérrez 2010). The SIT application consists of rearing, irradiation, marking, and release of a large number of insects into the field, which after mating will transfer sterile sperm to wild females and inhibit their reproduction, thus introducing sterility into the target wild population (Knipling 1966).

The Mexfly SIT program has been releasing both females and males into target areas during several years. However, it has been shown that the exclusive release of males by using genetic sexing strains (GSSs) is much more effective, e.g., avoids preferential mating among released flies, documented in bisexal strains (Robinson et al. 1986, McInnis et al. 1994, Rendon et al. 2000, 2004), and reduces postproduction handling costs to the control programs as the marking, irradiation, transport, and release activities are reduced by half. All GSSs used so far in SIT projects have been based on the same sex-specific pseudo-linkage principle (Y-autosome translocation), using different recessive markers. The combination of Y-autosome translocation and a selectable marker results in heterozygous males (wild-type phenotype) and homozygous females for the marker (mutant phenotype; Robinson et al. 1999). The markers used to construct the sexing mechanism and determine the sex separation strategy can be functional at any developmental stage. Thus, several GSSs have been developed using a variety of gene markers such as: wing mutation (*bent wing*) where the females are unable to fly and only males are active in the field (Meats et al. 2002), pupal color markers which allow the separation of male from female pupae through a sorting machine (Rössler 1979, Busch-Petersen and Kafu 1989, McCombs and Saul 1995, McInnis et al. 2004), as well as mutations affecting embryonic and larval development (Cladera 1995). However, the most successful GSS has been developed in the Mediterranean fruit fly *Ceratitis capitata* using two mutations: *white pupae* (*wp*; Rössler 1979) and *temperature-sensitive lethal* (*tsl*; Franz et al. 1994). In this GSS, males emerge from brown pupae and are resistant to elevated temperatures (34 to 35°C), whereas females emerge from white pupae

and are sensitive to high temperatures. The thermal sensitivity allows male-only production by killing all females through an embryonic heat shock treatment. Additionally, the *tsl* marker has been associated with slow larval development phenotype (Franz 2005), thus inducing an additional self-sexing mechanism in the GSS, since males develop 1 or 2 d faster than females (Cáceres 2002).

Using the same sex-specific pseudo-linkage principle (Y-autosome translocation) and as selectable marker the *black pupae* mutation (*bp*) located on chromosome 2, a GSS for Mexfly has been recently developed, the Tapachula-7 (Tap-7 GSS; Zepeda-Cisneros et al. 2014). In this GSS, males emerge from brown pupae (wild-type), whereas the females emerge from black pupae (mutant), thus allowing the separation of males from females at the pupal stage using a color-sorting machine. The Tap-7 GSS has been introduced into action programs in Mexico and United States for male-only releases. As is the case for Mediterranean fruit fly, the integrity of this genetic sexing system is maintained through a filter rearing system (FRS) which eliminates any recombinant insects (mutant males emerging from black pupae and wild-type females emerging from brown pupae; Fisher and Caceres 2000).

In this study, we report the genetics of *slow larvae* (*sl*), a new morphological mutant for *A. ludens*, which displays two traits: slow development during the larval stage and light pigmentation at the pupal and adult stage. The new mutation was integrated into the current black pupal color-based sexing system of the Mexfly, allowing self-sexing during the pupal stage. The potential use of the new GSS developed in the frame of this study in SIT programs is discussed.

Materials and Methods

Insect Strains and Rearing Conditions

All seven strains used in the present study are maintained at the Insect Pest Control Laboratory of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Seibersdorf, Vienna, Austria (Table 1). During the screening for new phenotypes in several established wild-type (WT) isolines, one of them yielded 16 light brown pupae. Emerged adults of these pupae (4 males and 12 females) showed light pigmentation and were interbred. All F_1 offspring displayed light pigmentation and delayed larval development time (*sl* mutation) and were used to establish the purebred colony used in this study. Using the original T:Y(2*bp*$^+$)/*bp* translocation from Tap-7 GSS, two new GSSs were constructed by backcrossing wild males to mutant females: Slow-7 GSS [T:Y(2*sl*$^+$)/*sl*] and Tap/slow-7 GSS [T:Y(2*bp*$^+$*sl*$^+$)/*bp sl*]. Flies were maintained in small cages (1,500 cm^3 of volume) with traditional food used under mass rearing condition (sugar:protein, ration 3:1) and water ad libitum at a density of 30 pairs per cage. Once the flies reached sexual maturity (10 d after emergence), one oviposition substrate (egg collector) was placed on top of the cage for egg collection. To collect fresh eggs only, the females had access to the egg collector only 5 h per day. In all experiments no especial conditions were used, the eggs were transferred to the larval carrot diet and the temperature used in all stages was 25°C (Tanaka et al. 1969).

Genetics of *sl* Mutant

Reciprocal crosses, in single mating pairs, were made between *sl* mutants and WT insects in order to determine the mode of inheritance of the delayed larval development phenotype. The F_1 progeny were interbred in groups of five pairs and the resulted F_2 phenotypes were observed and recorded. Crosses between the two mutant lines, *bp* and *sl*, were also carried out to assess their potential linkage. The F_1 progeny were interbred and the F_2 phenotypes were observed and recorded. To determine the genetic distance between the *bp* and *sl* loci in males and females, heterozygous insects in repulsion phase (*bp*$^+$ *sl*/*bp sl*$^+$) and in coupling phase (*bp sl*/*bp*$^+$ *sl*$^+$) were reciprocally backcrossed to double mutant insects (*bp sl*/*bp sl*).

Biological Characteristics

The biological characteristics of wild-type, single mutants (*slow larvae* and *black pupae*), double mutant (*slow larvae* and *black pupae*), and GSSs (Tap-7, Slow-7 GSS, and Tap/slow-7 GSS) were assessed in a comparative way. For determining the survival from egg to different development stages (larva, pupa, and adult) and the male ratio (number of males by females produced), 100 eggs from all the evaluated strains were placed on a net over a wet blotting black filter for 4 d at 26°C. The net with the eggs was then transferred to the larval diet in a Petri dish. After 3 d, the hatch rate was recorded and the Petri dishes were opened and placed inside a plastic container with sawdust. Pupae were collected from the sawdust and recorded by phenotype. Finally, the percentage of adults from each sex emerged from pupae was recorded. Twelve replicates of 100 eggs each were carried out per strain (n = 12 × 7 strains = 84 experimental units).

For estimating the time required for embryonic development, 100 eggs per replicate were placed on a net over a wet black filter paper in a small Petri dish (100 × 15 mm). The hatch rate was recorded daily until reaching maximum hatching. Fifteen replicates per strain were carried out. The larval development time was estimated as the number of days from when eggs were placed on the larval diet to the larval pupariation phase (Fraenkel and Bhaskaran 1973, Meza et al. 2005). For timing the larva stage, a sample of 0.1 ml of eggs of uniform age, close to hatching, was transferred to the larval diet, contained into a large Petri dish (150 × 15 mm). To avoid collecting immature larvae, 9-d-old larvae were recovered using a sieve and diluting the larval diet in water. The larvae collected were returned to another large Petri dish with fresh carrot diet and placed inside a plastic box with sawdust. The postfeeding larvae that came out of the fresh diet during the wandering larvae phase were collected daily from the sawdust as immobile larvae and prepupa (pupariation phase) and recorded (Denlinger and Zdárek 1994). Six replicates per strain were carried out.

Table 1. List of Mexfly, *A. ludens* strains used during the study

Name of the strain	Genotype	Reference
Wild-type	*bp*$^+$*sl*$^+$/*bp*$^+$*sl*$^+$	
Black pupa mutation	*bp*/*bp*	Zepeda et al. 2014
Slow larvae mutation	*sl*/*sl*	This study
Black –pupa–slow larvae double mutation	*bp sl*/*bp sl*	This study
Tapachula 7 genetic sexing strain (Tap-7 GSS)	T(Y;2*bp*$^+$)-7	Zepeda et al. 2014
Slow 7 genetic sexing strain (Slow-7 GSS)	T(Y;2*sl*$^+$)-7	This study
Tapachula/slow 7 genetic sexing strain (Tap/slow-7 GSS)	T(Y;2*bp*$^+$*sl*$^+$)-7	This study

The same procedure was used for the GSSs recording the brown pupae (males) and mutant pupae (females).

Data Analysis

The genetic crosses data were evaluated by contingency tables followed by Pearson chi-squared tests. The recombination distance was estimated as $r = R/n$ and the standard error was calculated using the formula $SE = \sqrt{r(1-r)/n}$ (Serra 1965), where R is the number of recombinants phenotype and n is the total number of individuals observed. Each biological characteristic was analyzed by one-way analysis of variance (ANOVA) using the 'strain' as predictor of survivorship across development stages (larva, pupa, and adult) and the male ratio (number of males by females produced). The Tukey's HSD test was used as a post hoc method to compare means between strains on significant factors. To normalize the data distribution and stabilize the variances, the data in percentages were transformed following *arcsin* $\sqrt{x+1}$ (Zar 2010). The genetic load of the pure mutations was estimated as the difference in average survival of the transformation from egg to adult between mutant and wild-type insects. Embryonic developmental time data were corrected based on maximum egg hatch using Abbott's formula (Abbott 1925) to avoid the effect of different fitness between them. The embryonic and larval developmental time were analyzed using a generalized linear model with Poisson distribution and a log-link function (Agresti 1996). A matched pairs analysis was performed to analyze the difference in larval developmental time between males and females for each GSS. All data were analyzed with Statistical Discovery JMP 11.0.0 software (SAS Institute).

Results

The Morphology and the Genetics of the *sl* Mutation

The typical yellowish-brown pigmentation in *A. ludens* wild-type insects (Fig. 1A) is significantly attenuated in the new *sl* mutation. The puparium of *sl* mutation has a golden-like color and the adults exhibit colorless dorsal fringes, which can be clearly distinguished from the wild-type phenotype by visual examination (Fig. 1B).

The mode of inheritance of *sl* in *A. ludens* was tested by appropriate genetic crosses between the mutant and the wild-type line. As shown in Table 2, all F_1 offspring of the reciprocal crosses between the mutant and the wild-type lines were wild-type. In F_2 offspring of reciprocal crosses, the proportion of the wild-type and the *sl* phenotypes was according to the segregation of a recessive autosomal gene (3WT:1*sl*), although significant deviation was observed in some replicates, explained by the low viability of *sl*, perhaps accentuated when both phenotypes compete each other as larvae (Table 2). It is worth noting that, as expected, *sl* individuals were having a golden-like puparium and exhibited colorless dorsal fringes at the adult stage which significantly facilitates the monitoring of the *sl* phenotype.

The potential genetic linkage between *sl* and *bp* in *A. ludens* was assessed by appropriate genetic crosses. All F_1 offspring of the reciprocal crosses between the two homozygous mutant lines *sl* and *bp* were 100% wild-type. In both reciprocal crosses, the proportion of the F_2 offspring phenotypes was significantly deviated from the expected (9WT:3*bp*:3*sl*:1*bp sl*), if the two genetic loci (*sl* and *bp*) were segregating independently. In addition, there was complete absence of double recessive homozygous insects (*bp sl*). Taken together, these data strongly suggest that the two genes, *sl* and *bp*, are linked, being located on the same chromosome. Due to crossing-over event during the F_1 interbred, some F_2 *sl* insects were heterozygous for *bp* and some F_2 *bp* insects were heterozygous for *sl*; thus, double mutant insects (*bp sl*) were obtained after the interbreeding of F_2 *sl* and F_2 *bp* insects. Interestingly, the phenotype of the *bp sl* insects showed an additive interaction between the two mutations resulting in a dark pupal color phenotype which is lighter than the typical of the *bp* mutant (Fig. 1C and D).

The genetic distance between the *bp* and *sl* genetic loci was determined by appropriate genetic crosses involving the F_1 wild-type progeny of the direct cross between the two homozygous mutant

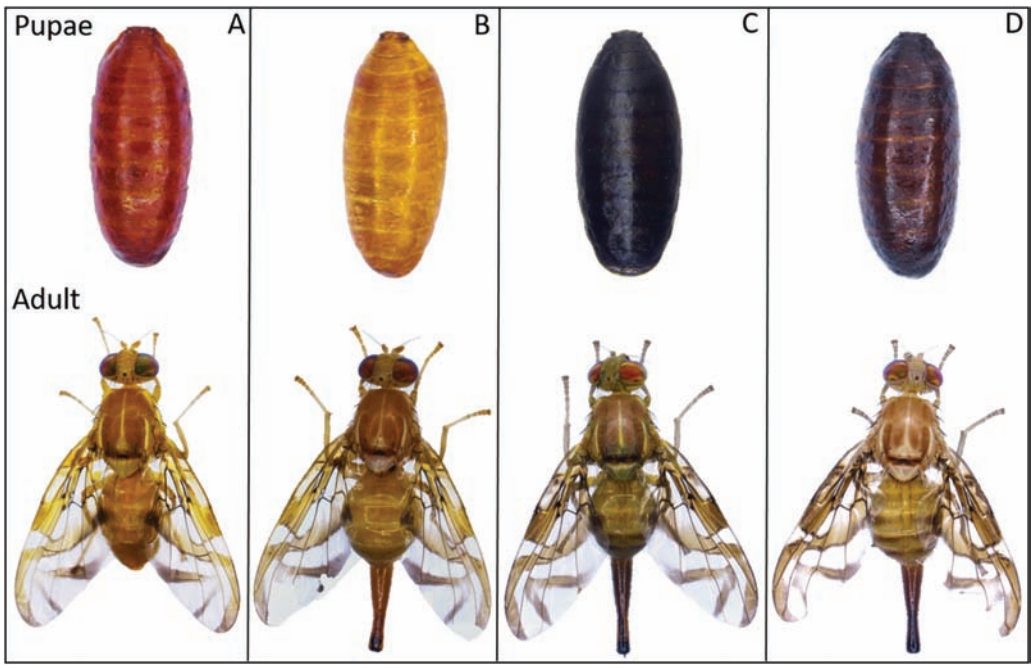

Fig. 1. Adult and pupal phenotypes of the selectable markers involved in the *A. ludens* GSS. (A) Wild-type male. (B) *Slow larvae* female mutant (*sl*) in slow-7 GSS. (C) *Black pupae* female mutant (*bp*) in Tap-7 GSS. (D) Double mutant female (*bp sl*) in Tap/slow-7 GSS.

lines. These F_1 wild-type insects, which are heterozygous for both genes in the repulsion phase, were reciprocally test-crossed to *bp sl* and again a significant deviation from the proportion expected (1WT:1*bp*:1*sl*:1*bp sl*) for independent segregation was observed. The same result was obtained when the recombinant wild-type (WT Tc) insects, in coupling phase, were test-crossed to *bp sl* insects (Table 3). In both experiments (repulsion and coupling), recombinant individuals were observed only in the female offspring while no recombination was observed in males. Based on the female recombinants, the estimated distance between the *bp* and *sl* genetic loci was estimated to be, in average, 0.43 cM.

Comparative Analysis of the Biological Characteristics

Significant differences were detected between the strains tested with respect to hatch rate ($F_{6,75}$ = 108.32, P < 0.001), egg to pupa survival ($F_{6,75}$ = 211.75, P < 0.001), and egg to adult survival ($F_{6,75}$ = 190.98, P < 0.001). Comparative analysis between the wild-type and the mutant lines regarding the survival showed that the genetic load was minimal for single mutant individuals (2.13% for *bp* and 2.17% for and *sl*), with no significant difference between the *bp* and *sl* mutations. However, in the double mutant line (*bp sl*), the genetic load increased up to 14.25%, significantly affecting the survival in the Tap/slow-7 GSS (Table 4).

Significant differences were also detected between the strains tested with respect to the sex ratio ($F_{6,75}$ =34.98, P < 0.001) and as shown in Table 4, it is clearly in favor of males in the slow-7 GSS and even more pronounced in the Tap/slow-7 GSS. So, the male production was almost similar in all GSS (Tap-7 = 20.33%, Slow-7 = 23.50% and Tap/slow-7 = 19.33%); however, the female production was more reduced in the GSS that integrated the *sl* marker (Tap-7 = 16.92%, Slow-7 = 12.92% and Tap/slow-7 = 9.33%).

As shown in Table 5, comparative analysis between the wild-type and the purebred mutant (*bp*, *sl* and *bp sl*) lines did not reveal any significant differences with respect to the embryonic developmental time. For all strains tested, the neonate larvae hatching started when the eggs were 4 d old, more than 80% of the eggs had hatched when they were 5 d, whereas the rest of them (up to 100%) when the eggs were 7 d old (Fig. 2). By contrast, significant differences were detected between the wild-type and the purebred mutant (*bp*, *sl* and *bp sl*) lines regarding the larval development time (Table 5). Wild-type and *bp* mutant insects started their pupariation phase at 10 d after the eggs were put on the larval diet (egg seeding) and it took 12 d in total for all individuals to achieve their pupariation phase. On the other hand, the single mutant line *sl* and double mutant line *bp sl* were delayed by almost 2 d to start the pupariation phase (12 d), and it took 15 d for all individuals to reach it (Fig. 2).

Regarding the genetic sexing strains, the pupariation started 10 d after egg seeding for all of them. No significant difference was detected in the pupariation rate between males and females of Tap-7 GSS (F = 1.55; P = 0.217); however, significant differences were detected for the slow-7 GSS and Tap/slow-7 GSS (F = 90.44; P < 0.001 and F = 47.41; P < 0.001, respectively).

Table 2. Mode of inheritance of *slow larvae* (*sl*) in *A. ludens*

Cross				F_2 phenotype					
				WT		*sl*			
Male	Female	Pair	F_1 (genotype)	male	female	male	female	TOTAL	X^2 (3:1)
WT	*sl*	1	WT (*sl*/*sl*$^+$)	414	428	119	121	1082	4.59
		2		328	342	92	103	865	2.78
		3		375	368	109	92	944	6.92
sl	WT	1	WT (*sl*$^+$/*sl*)	283	279	90	83	735	0.84
		2		368	372	108	92	940	6.95
		3		264	212	63	72	611	2.75

$X^2_{0.05,1}$ = 3.841.

Table 3. Linkage analysis of the *bp* and *sl* genetic loci of *A. ludens*

Crosses Phenotype (Genotype)								
Male	Female	Generation	WT	*bp*	*sl*	*bp sl*	TOTAL	X^2	Recombination r (SE)
sl (*bp*$^+$ *sl*/*bp*$^+$ *sl*)	*bp* (*bp sl*$^+$/*bp sl*$^+$)	F_2	603	261	170	0	1034	91.6[a]	–
bp (*bp sl*$^+$/*bp sl*$^+$)	*sl* (*bp*$^+$ *sl*/*bp*$^+$ *sl*)		362	156	99	0	617	55.7[a]	–
WT F_1 (*bp sl*$^+$/*bp*$^+$*sl*)	Double mutant (*bp sl*/*bp sl*)	Test-crossed	0	385	240	0	625	692.3[b]	0.00
Double mutant (*bp sl*/*bp sl*)	WT F_1 (*bp sl*$^+$/*bp*$^+$*sl*)		249	350	247	130	976	99.5[b]	0.39 (0.015)
WT Tc (*bp sl*/*bp*$^+$*sl*$^+$)	Double mutant (*bp sl*/*bp sl*)		658	0	0	429	1087	1183.5[b]	0.00
Double mutant (*bp sl*/*bp sl*)	WT Tc (*bp sl*/*bp*$^+$*sl*$^+$)		663	537	364	307	1871	170.0[b]	0.48 (0.011)

[a]9:3:3:1 hypothesis; [b]1:1:1:1 hypothesis; $X^2_{0.05,\,df=3}$ = 7.82.

Tc = Insect from test-cross.

Table 4. Survival from egg to different developmental stages and sex ratio (number of males by female produced) (Mean ± SE)

Strain	Larva neonate[a] (%)	Pupa (%)	Adult (%)	Males/females
WT	88.50 ± 0.66a	84.58 ± 0.65a	82.83 ± 0.60a	1.05 ± 0.03a
bp	86.30 ± 0.89a	84.40 ± 1.00a	80.70 ± 1.75a	0.98 ± 0.04a
sl	84.91 ± 1.11a	83.33 ± 0.97a	80.66 ± 1.02a	0.99 ± 0.02a
bp sl	78.50 ± 0.89b	70.50 ± 0.75b	68.58 ± 1.75b	1.03 ± 0.02a
Tap-7 GSS [T(Y;2*bp*$^+$)-7]	66.25 ± 1.2c	40.58 ± 1.74c	37.25 ± 1.66c	1.21 ± 0.05a
Slow-7 GSS [T(Y;2*sl*$^+$)-7]	65.75 ± 1.57c	39.66 ± 1.72c	36.41 ± 1.69c	1.86 ± 0.12b
Tap/slow-7 GSS [T(Y;2*bp*$^+$*sl*$^+$)-7]	56.16 ± 1.29d	33.41 ± 1.36d	28.66 ± 1.48d	2.13 ± 0.14c

[a]Egg hatch; for each column, lower case letters represent significant differences between strains ($P < 0.05$).

Table 5. Generalized linear model analysis of embryonic and larval developmental time (Poisson distribution, log-link)

Factor	Source	df	X^2	*P*
Egg hatch	Strain	3	0.001	0.99
	Egg hatching day	1	0.056	0.81
	Strain*Egg hatching day	3	0.001	0.99
Larval development	Strain	3	12.27	0.006
	day/ time	1	16.96	<0.001
	Strain*Pupariation day	3	11.74	0.008

In the Tap-7 GSS, the percentage of pupariation was daily 50:50 for males and females and it took 4 d for all individuals achieve the pupariation phase. However, this was not the case for the Slow-7 and Tap/slow-7 GSSs in which it was observed that only males achieve the pupariation phase at 10 d after egg seeding. At the 12 d, about 89.70% and 84.74% of the male achieve the pupariation phase and only 47.51% and 16.84% of the females for slow-7 GSS Tap/slow-7 GSS, respectively. In general, the pupariation process for the two new GSS required 1 d more (5 d) and in the last day, the percentage of female in pupariation in both slow-7 GSS and Tap/slow-7 GSS was higher than the Tap-7 GSS (Fig. 3).

Discussion

This work presents evidence that the *sl* mutation of the Mexfly, *A. ludens*, is due to a recessive and autosomal gene (*sl*) which is linked to the black pupae (*bp*) locus located on the mitotic chromosome 2 (Zepeda et al. 2014). The linked segregation of light pigmentation and slow larval development traits in the *sl* mutation suggests that both traits are either pleiotropic effects of a single gene or they are due to two genes which are extremely close to each other. The integration of the *sl* mutation into the current pupal color-based GSS used in mass rearing and SIT applications resulted in two new GSSs for *A. ludens*, which were named slow-7 and Tap/slow-7. Both GSSs produce wild-type males emerging from brown pupae, which develop faster than females. In the slow-7 GSS, the females are homozygous for the *sl* mutation and emerge from golden-color pupae, whereas in the Tap/slow-7 GSS, the females are homozygous for both selectable markers (*bp sl*) and emerge from light black pupae.

It is worth noting that the females of both GSS reach larval maturity significantly later (hence the name 'slow larvae') than most of the wild-type males, conferring self-sexing and allowing the separation of the majority of males from during late larval—early pupal stage. A similar pupariation process has been observed in two GSS for *C. capitata*: Vienna 8 and Cast 191. The Vienna 8 GSS carries a translocation (Y;5) marked with two mutations: *white pupae* (*wp*) and *temperature-sensitive lethal* (*tsl*). This GSS is also characterized by the presence of a slow larval development trait which was attributed to a pleiotropic effect of the *tsl* gene (Franz 2005). The Cast 191 GSS was also shown to carry the mutation *slow* (*sw*) located on chromosome 2, which affects the rate of embryonic and larval development and has pleiotropic effects on eye color and iridescence (Manso and Lifschitz 1992, Cladera 1995). In contrast with the *C. capitata sw* mutation, the *A. ludens sl* mutant does not show a delayed embryonic development, and it is similar to the effect induced by the *tsl* mutation in the Mediterranean fruit fly Vienna 8 GSS, where slow developmental rate has been reported only at the larval stage (Caceres 2002). However, this finding could be influenced by the observation period (daily), a reduction of this period (e.g., 8 h) and the use of different temperatures in the incubation could be addressed in the future to confirm whether *sl* mutation in *A. ludens* do not have any effect in the embryo stage. In the case of slow-7 GSS (brown pupae = male, golden pupae = female), the efficiency to distinguish male from female pupae was low, because brown and golden spectral colors are close resulting in some wild-type pupae being misclassified as *sl* pupae, because of displayed depigmentation (phenocopy). Nevertheless, to maintain the integrity of the sexing system, all wild-type phenocopies can be detected through the adult observation, because unlike *sl* adults, the phenocopies do not show depigmentation. Detecting phenocopy in adults stage is a strategy frequently used in the FRS for the mass production of Tap-7 GSS, as the black pupae mutation affects pigmentation at several stages, the observation of adult pigmentation conferring additional reliability to the pupal color-based sexing system (Zepeda et al. 2014). The puparium depigmentation phenomenon in wild-type insects can be explained as an effect of poor protein ingestion during the larval stage in some individuals, as dietary protein is associated with sclerotization and cuticular melanization (Lee et al. 2008, Andersen 2010), which can contribute to the presence of phenocopies in this slow-7 GSS.

In Tap/slow-7 GSS (brown pupae = male, clear dark pupae = female), the wild-type phenocopies were scarce and the two genders were easily sorted. Additionally, the self-separation during the pupation could be more efficient because the *bp sl* double mutants exhibited a longer delay than the *sl* mutants alone (e.g., in the GSS at 12 d of the larval development time, females *bp sl* showed a 16.81%

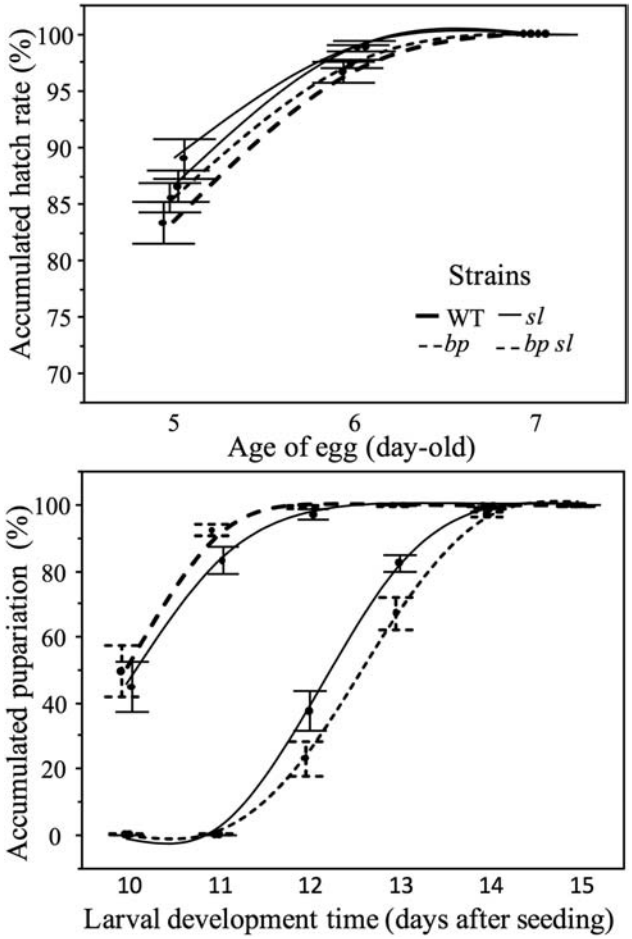

Fig. 2. Daily pattern of egg hatching and pupariation of wild-type (WT) as well as *black pupae (bp), slow larvae* (*sl*), and double homozygous mutant (*bp sl*) lines of *A. ludens*.

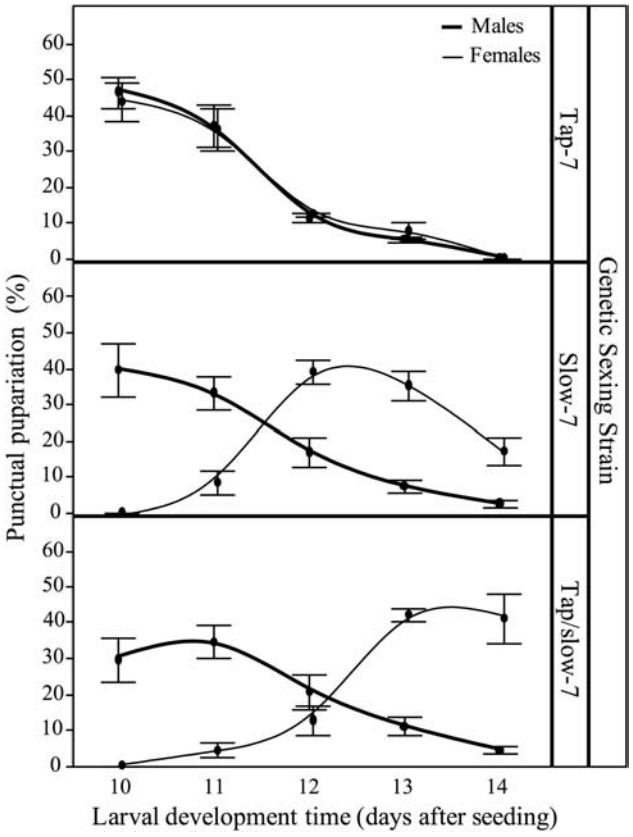

Fig. 3. Male and female daily pupariation patterns of *A. ludens* GSS.

of accumulated pupariation, whereas *sl* females already 47.51%). In both slow-7 and Tap/slow-7 GSSs, a small proportion of pupae recovered consists of a mixture of males and females, which in the case of the Tap/slow-7 GSS can be separated by the sorting machine currently used in the mass-rearing production of Tap-7 GSS in the Moscafrut SAGARPA-IICA facility (Flores et al. 2015). However, due to the long genetic distance between the alleles *bp* and *sl*, and if the translocation is not between them, the risk of recombination type 1b (recombination between the two genetic loci) could be high (Franz 2002). Nevertheless, this constrain can be minimized if the FRS is fed with pupae from the individuals that achieve their pupariation earlier, which will be wild-type males only, as well as pupae from individuals that achieve their pupariation later, which will be highly enriched in *sl* females in the case of slow-7 GSS and *bp sl* females in Tap/slow GSS. So, in the FRS the recombinant individuals that need to be removed for Slow-7 GSS are wild-type females and *sl* males, whereas for Tap/slow-7 are three phenotypes of females (wild-type, *bp*, and *sl*) and males (*sl*, *bp*, and *sl bp*). In addition, the induction of homozygous viable chromosomal inversions into the chromosome 2 and its introduction in the GSS could drastically reduce recombination rate and increase the genetic stability, ideally the inversion should cover the translocation break point and the *bp* and *sl* genetic loci (Franz 2005).

The large-scale production of a GSS which integrated the *sl* mutation could have some advantages. For example, in comparison with the Tap-7 GSS, the lower fitness observed in the GSS with *sl* marker is mainly due to the low production of female mutants, which significantly increases the rate of male production, thus decreasing the production cost of male for release, but will need to be evaluated the colony reproduction. Differing from *C. capitata* mass rearing, where the larva left the diet by themselves once they are matured and ready to enter in the pupariation phase, in *A. ludens* mass rearing, the whole larvae have to be separated from the diet using a mechanical sieve (Orozco-Davila et al. 2017). The competition for food among larvae is quite high under this rearing method. Therefore, under mass rearing conditions, the slow larval development exhibited by females in the GSS would favor males because they could fed in competition with youngers larva females, which demand less food and, interestingly, it has been reported that individuals with faster larval development are heavier and more sexually competitive (Meza et al. 2005). Further research should be conducted to evaluate the potential use to the SIT, e.g., survival in the field, mating success, dispersal ability, and to develop practical protocols for rearing the Tap/slow-7 GSS that can allow the separation of both genders by combining critical time-points during development with mechanical and optical sorting based on pupal color to ensure the delivery and release of only sterile males. Excess female larvae or black pupae females could be used for the production of larval or pupal parasitoids, respectively (López et al. 2006).

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