A Technique for Distinguishing Virgin and Mated Males of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae)[1]

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Abstract Studies on the mating history of male Lepidoptera are generally lacking because of difficulties in determining male mating status. In previous studies, presence/absence of pigmented fluids inside the male primary simplex have been used successfully for determining mating status. However, males of European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), have creamy white fluids in the primary simplex instead of pigmented fluids, so presence/absence of pigmented fluids inside the primary simplex cannot be used to assess mating status in this species. We developed a scoring system for the fluids inside the primary simplex that allowed us to classify *O. nubilalis* male mating status and the timing of copulation. The scoring system relies on differences in the fluids in the 1st, 2nd and 7th segments of the primary simplex. Males known to be virgins, mated <1d previously and mated >1d previously could be distinguished with a 6.3% error rate. The method was verified on 41 males in a blind study, with 100% accurate classification of the males.

Key Words mating history, *Ostrinia nubilalis*, internal reproductive system, high dose plus refuge strategy

There are many reports on mating history of Lepidoptera females in the field (Drummond 1984, Ehrlich and Ehrlich 1978, Hinton and Andow 2003). But, field research on mating history of Lepidoptera male is not common, probably because of a limitation of study methods. The first published methodology to determine male mating status was based on the color of the fluids inside the male primary simplex of internal reproductive system of fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) (Snow and Carlyse 1967). Newly-emerged virgin fall armyworm males had light-to-medium brown or dark fluid inside the primary simplex, and this colored pigment was incorporated into the spermatophore. Females that contained the pigment in the transferred spermatophore probably copulated with a virgin male. Mated males had unpigmented (regenerated) fluids in their primary simplex, and produced unpigmented spermatophores. Haines (1981) reported that newly-emerged (<24 h old) virgin male Egyptian cotton leafworm, *S. littoralis* (Boisduval), gradually accumulated pigmented fluids inside their primary simplex. The pigmented fluids disappeared after copulation and accumulated again 24 h after male copulation. Similar disappearance of pigmented fluids after male copulation also was reported in tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) (Henneberry and

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Clayton 1984), spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae) (Bergh and Seabrook 1986), and oblique-banded leafroller, *C. rosaceana* (Harris) (Lepidoptera: Tortricidae) (Evenden et al. 2003). The dynamics of pigmented fluids inside the male primary simplex before and after copulation has provided reliable information to distinguish male mating status in several species of Noctuidae and Tortricidae. Mated males of some of the species regenerated the pigmented fluids after copulation, but others did not.

Morphological and histological examination of the internal reproductive system of male silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae) showed that female bursa copularices became swollen with seminal fluid and secretions transferred from the male primary simplex (Osanai et al. 1987). Therefore, the disappearance of fluids inside the male primary simplex should be a reliable indicator of male mating history.

Our purpose is to develop and verify a technique for inferring male mating history of an important lepidopteran pest, European corn borer, *O. nubilalis*. Unlike the other successful, published cases that could rely on the pigmented fluids in the primary simplex, both virgin and mated *O. nubilalis* males have an unpigmented, creamy white fluid inside their primary simplex. Thus, it is not possible to infer mating history by simply examining the color of the fluid inside the primary simplex. Here we establish a scoring system for estimating the transparency levels of the creamy white fluids inside the primary simplex, and use this scoring system to distinguish male mating status and the timing of the most recent copulation.

Based on the technique, we can infer the mating history of *O. nubilalis* males caught in the field, which has important implications for evaluating the efficacy of the high dose plus refuge strategy (Y. Hu, unpubl. data). This strategy is being used to delay the rate of resistance evolution of *O. nubilalis* to *Bacillus thuringiensis* (Bt) maize (EPA 2001). As required, the Bt fields and refuge (nonBt) fields must be planted no more than a half mile (~800 m) away from each other. The strategy works best when there are a large number of virgin males emerging from refuge fields that move into Bt fields before their first mating attempt. In addition, this technique also could be used to evaluate the efficacy of mating interruption measures in the field.

For the purpose of estimating male age in field conditions, we also measured the testes size of *O. nubilalis* males. We might expect that the mated proportion of younger males would be lower than the mated proportion of older males. If a correlation were found between age and testes size, we might be able to infer male age in the field by measuring testes size. Mating status together with age might be much more informative than mating status alone.

Materials and Methods

Source of insects. Feral *O. nubilalis* larvae (n = 166) and pupae (n = 72) were collected from Rosemount, MN, during May 2006 by dissecting corn stalk litter. The collected larvae were treated with a phenylmercuric nitrate solution to sterilize the cuticle, thus reducing the inoculation potential of a fungus, *Beauveria bassiana* (Vuillemin) Balsamo. Larvae were fed with the overwintering larval diet (Andow and Stodola 2003) and incubated at 27°C with constant light. Larvae and pupae were maintained under a 16L:8D photoregime at 27:18°C and ~80% RH (Andow and Stodola 2003). All adults were kept inside a mating cage (55 × 25 × 27 cm). The adults used in these experiments were the 3rd generation removed from the field.

Insect dissection and measurements. All adults were killed by submerging in 70% ethanol for 5 - 15 min. Preliminary trials showed that this method did not cause any noticeable change in the primary simplex compared with live dissections in physiological saline. However, if adults were kept in 70% ethanol solution for overnight, the clear and soft walls of the 1st and 2nd segments of the primary simplex became cloudy, and the entire primary simplex became hardened.

Moth bodies were stored and dissected in physiological saline (Dulbecco's Phosphate-Buffered Saline, 1× with calcium and magnesium). All dissections were completed within 12 h after killing because: (1) there was no noticeable change in the primary simplex within 12 h; (2) the primary simplex began to harden after 24 h; and (3) after 24 h storage in saline at room temperature, the clear wall of the primary simplex became cloudy. The male was pinned with ventral side up on a wax pan. His abdomen was carefully opened with 2 fine forceps under a dissecting microscope. The internal reproductive system was exposed by carefully pulling out and removing attached fat bodies.

Chaudhury and Raun (1966) showed that the testes volume of *O. nubilalis* males was largest during their pupal stage and reduced thereafter. After eclosion, the volume of testes reduced continuously throughout their adult stage. Thus, we hypothesized that testes size varied with male age, and the diameter of the fused testes was measured using an ocular micrometer under a dissecting microscope at 40X. If the testes were not spherical (<5% of total individuals) both the longest and shortest dimensions were taken and the average was used. Furthermore, the testes size might be related to body size, so we also measured the length of left forewing. The wing length was measured from the conjunction between wing and thorax to the tip of the wing. The measurements were taken under the same dissecting microscope at 6.6X.

A qualitative scoring system was developed for estimating transparency levels of the creamy white fluid inside the 3 longest (1st, 2nd, 7th) segments of the primary simplex. There were 5 levels of transparency in this scoring system: level 0 is clear (we can see the texturing on the surface of the dissecting wax through the tube, although some fluid may be present) (A1, Fig. 1); level 1 is a little cloudy (remains translucent, but the texturing of the dissecting wax cannot be distinguished clearly (A2, Fig. 1); level 2 is cloudy and hard to see through (the fluids inside tube are not evenly distributed) (A3, Fig. 1); level 3 is creamy white and opaque (7th segment of virgin males contains this level of fluid) (A4, Fig. 1); level 4 is condensed, white, with occasional light blue granules (only located at the proximal end of the 7th segment) (A5, Fig. 1).

Experimental design. The pupae were sexed and aged (Gelman and Hayes 1982). Female and male pupae were maintained separately in different Petri dishes. Each Petri dish was covered by a single pair cage which allowed emerged females and males to expand their wings. Each day, the newly-emerged females and males were randomly assigned for mating treatments.

Virgin males were kept without females and provided with a ~0.5 cm^3 adult diet (Andow and Stodola 2003) and a ~2 cm^2 piece of water-saturated paper towel. Virgin males were killed 0 - 2 h, 12 - 14 h, 24 - 26 h, 36 - 38 h, 68 - 72 h, and 168 - 172 h after emergence. The 0 - 2 h age provided the status of internal reproductive system upon eclosion. The 12 - 14 h and 36 - 38 h ages provided the status during the first and second nights for the males that would eclose during the morning (Dalecky et al. 2006, Y. Hu, unpubl. data). The 24 - 26 h age provided the status during the first full night for the males that would eclose during the night. The 68 - 72 h and 168 - 172 h ages provided information about older virgin males up to 7 d old.

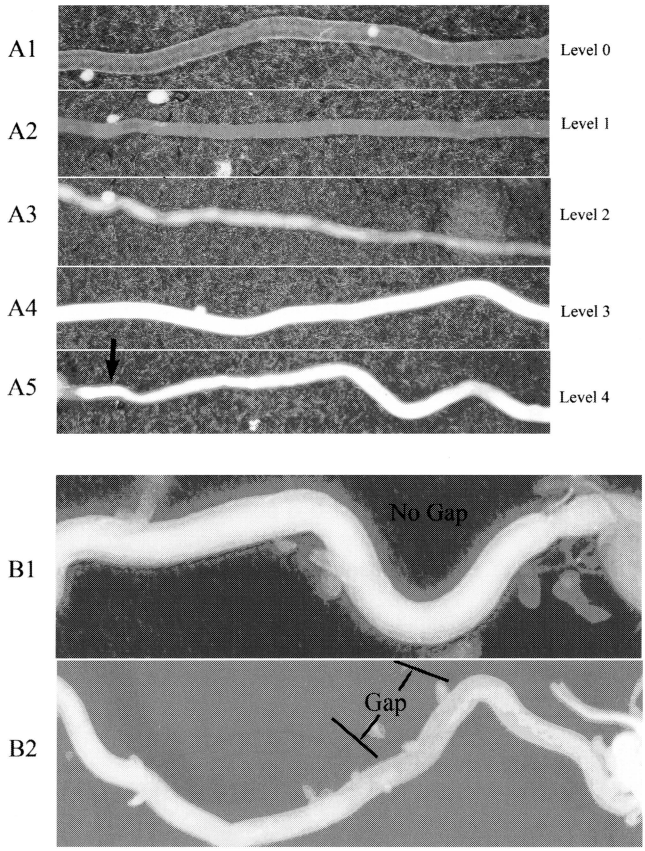

Fig. 1. Five levels of transparency (A1-A5) of creamy white fluids inside the primary simplex and no gap (B1) and a gap (B2) in the creamy white fluids between the anterior and posterior parts of 7th segment of primary simplex of *Ostrinia nubilalis* males.

Mated males were produced by combining newly-emerged virgin males with virgin females in a mating cage (55 × 25 × 27 cm). Over the next 7 h, the cage was checked every hour, and copulating pairs were carefully collected so that mating was not disturbed. Thus, all males had mated before they were 24 h old. Paired and collected couples were allowed to complete mating and provided 0.5 cm^3 adult diet and a water-saturated paper towel. Copulation termination time was recorded at hourly intervals, and the pairs were killed at 0 - 2 h, 6 - 8 h, 20 - 22 h, 30 - 32 h, 40 - 43 h, and 68 - 72 h after copulation terminated. The 0 - 2 h postcopulation time provided information on males just after mating. The other times corresponded to the same time intervals as for the virgin males, under the assumption that in the field mating would occur and terminate 4 - 6 h after eclosion. For each class of virgin and mated males, there were 20 or 21 replications.

Statistical analysis. Because our primary goal was to distinguish mating status in relation to the time after copulation, the PROC DISCRIM procedure was used (SAS Institute 2002). Discriminant analysis classifies our observations into groups according to the different variables (testes size, length of the parts of the 7th segment of primary simplex, transparency of each of the segments of the primary simplex and uniformity of the 7th segment). The nonparametric option was used for the discrimination analysis.

If the size of testes was related to age and independent of body length, we might estimate the age of field caught males by measuring the testes size. For determining the independence of body length and testes size, we calculated Pearson correlation coefficients (r) between left forewing length and testes size of virgin and mated males with different ages with PROC CORR. Thereafter, we analyzed the relation of testes size and age for virgin and mated males with PROC GLM. All analyses were conducted with SAS 9.1 (SAS Institute 2002).

Validation. To verify the accuracy of this technique, we tested the technique by classifying the mating status of males from another laboratory colony using a blind experiment. The colony was collected from corn fields at Lamberton, MN, during 1998. We established virgin, <1 d, and 1-3 d postcopulation males, with 12-14 individuals per group. They were randomly labeled by one person (S. McCrindle) and given to another person (Y. Hu) for dissection and classification using the results from

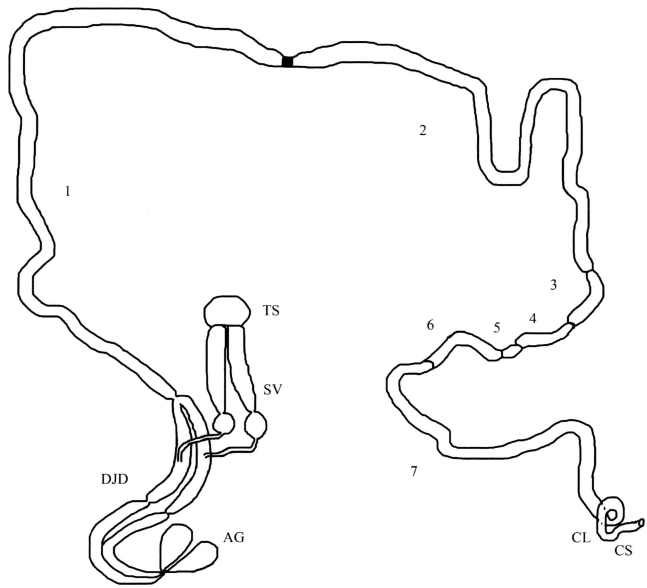

Fig. 2. Internal male reproductive system of *Ostrinia nubilalis*. TS, testes; SV, seminal vesicle; DJD, ductus ejaculatorius duplex; AG, accessory glands; PS, 1 - 7 segments of the ductus ejaculatorius primary simplex; CL, cuticle loop; CS, cuticle segment of the ductus ejaculatorius simplex.

the discriminant analysis. The scores were rectified to the randomized labels, and the number of correctly classified males was calculated for each group of males.

Results and Discussion

Internal male reproductive system. The internal reproductive system of *O. nubilalis* virgin males was similar to that of spruce budworm (Outram 1970, Fig. 2). In comparison with spruce budworm, the main differences were that *O. nubilalis* males had a shorter cuticle segment (the cuticle loops of both species were similar) of the ductus ejaculatorius simplex as well as a pair of much reduced accessory glands. The remaining structures were similar. Thus, our terminology follows Outram (1970). Like other studies on male moth mating status, our focus was also on the primary simplex. The primary simplex of *O. nubilalis* consisted of 7 segments (Fig. 2). The total length of the primary simplex was about 40 - 50 mm, nearly 5X the body length. The approximate lengths of the 1st through 7th segment were 15 - 20, 12 - 15, 4 - 5, 3 - 4, 1, 5 - 6, and 10 - 12 mm, respectively. The diameter of the primary simplex tube did not vary much, and the diameter was about 0.3 mm. The junctions of these segments can be identified by a narrowing of the primary simplex. Furthermore, some segments can be differentiated by the fluids inside. For example, the 7th segment can be easily distinguished from the 6th segment when it is filled with level 3 creamy white fluids, which were never found at level 3 inside the 6th segment.

Determination of mating status. Preliminary dissections ($n > 100$) of both wild-captured and laboratory-reared individuals showed noticeable variation in the creamy white fluid inside the 1st, 2nd, and 7th segments of the primary simplex in virgin males. These are the 3 longest segments of the primary simplex. Virgin males had level 3 fluids inside the 7th segment and level 0 fluids inside the 1st and 2nd segments at the time of emergence. Transparency levels of these 3 segments generally remained unchanged from emergence to 172 h (7 d) old virgin males. The 7th segment of some individuals can be further distinguished into two parts in older males, an anterior part and a posterior part, by a differentiation of the transparency levels. The anterior part remained filled with level 3 fluids and was about 90% of the length of the 7th segment. When there was differentiation, the posterior part was about 10% of the length of the 7th segment and contained level 4 fluids.

Just-mated males 0 - 2 h postcopulation ($n = 20$), had transparency level 0 (A1, Fig. 1) fluids inside their 7th segment. In contrast, virgin males had level 3 transparency fluids inside the 7th segment (A4, Fig. 1). This was the greatest change caused by copulation. In addition, the transparency level of the entire 1st and 2nd segments increased from level 0 in virgins to level 1 in just-mated males, which may indicate a process to regenerate fluids and refill the primary simplex immediately after copulation. Males 6 - 8 h postcopulation ($n = 20$) had level 1 fluids in all 3 of these longest segments (1st, 2nd, and 7th). Males 20 - 22 h postcopulation ($n = 21$) also had level 1 transparency in the entire 1st and 2nd segments (Table 1). However, at this time, the transparency level of the anterior part of the 7th segment had recovered back to level 3, but there was a large gap in the fluid inside this segment. Males <1d postcopulation were easily distinguished from virgin males by examining the transparency level of these 3 segments.

The pattern of regeneration of the fluids in the 7th segment was particularly important for distinguishing between 1 - 3 d postcopulation and virgin males. It appeared likely that the refilled fluids occurred in the anterior section of 7th segment, because

Table 1. Averaged scores of transparency level of 1st, 2nd, and two parts of 7th segments of virgin and mated males of *Ostrinia nubilalis* (means±sd)

Known mating status	n	Average transparency score			
		1st segment	2nd segment	Anterior part 7th segment	Posterior part 7th segment
Virgin, 0 < 7 d old					
0 - 2hr	21	0 ± 0	0 ± 0	3 ± 0.00	3.5 ± 0.51
12 - 14hr	21	0 ± 0	0 ± 0	3 ± 0.00	3.6 ± 0.50
24 - 26hr	21	0 ± 0	0 ± 0	3 ± 0.00	3.4 ± 0.50
36 - 38hr	21	0 ± 0	0 ± 0	3 ± 0.00	3.4 ± 0.50
78 - 82hr	21	0 ± 0	0 ± 0	3 ± 0.00	4.0 ± 0.00
168 - 172hr	21	0 ± 0	0 ± 0	3 ± 0.00	3.7 ± 0.48
Mated, 0 < 1d postmating					
0 - 2hr	20	1 ± 0	1 ± 0	0 ± 0.00	0.0 ± 0.00
6 - 8hr	20	1 ± 0	1 ± 0	1 ± 0.00	1.0 ± 0.00
20 - 22hr	21	1 ± 0	1 ± 0	3 ± 0.20	4.0 ± 0.20
Mated, 1 - 3d postmating					
30 - 32hr	21	0 ± 0	0 ± 0	3 ± 0.00	4.0 ± 0.00
40 - 43hr	21	0 ± 0	0 ± 0	3 ± 0.00	4.0 ± 0.00
68 - 72hr	21	0 ± 0	0 ± 0	3 ± 0.00	3.9 ± 0.30

the anterior section is closest to the probable source of the regenerating fluids. The residue of the initial fluids might have been pushed down to the posterior end of 7th segment, leaving a gap between the new and old fluids (B2, Fig. 1). The gap is a section inside 7th segment with higher transparency than on either side. Initially, the gap was large and closed gradually as the fluids were regenerated. But the regenerated fluids remained distinguishable from the original fluids by a gap of higher transparency or some other discontinuity in the fluids. At the same time, the transparency levels of the 1st and 2nd segments returned to level 0 (Table 1). Because the gap occurred in the mated males only, we can distinguish most mated males from virgin males by the presence of a gap in the fluids in the 7th segment (Fig. 3A).

Virgin males and males 1 - 3 d postcopulation could be nearly fully distinguished by (1) a gap between the anterior and posterior parts of 7th segment, and (2) the length of posterior part of the 7th segment. All males in these two classes that had a gap were 1 - 3 d postcopulation (Fig. 3A). But, some of 1-3 d postcopulation mated males (19/63) had no gap (B1, Fig. 1). Mated males without a gap could be distinguished from virgin males by measuring the length of posterior part of the 7th segment (Fig. 3B). Virgin males generally had a relatively shorter posterior length (≤2 mm) than that of mated males (>2 mm). By this, we could distinguish most mated males from virgin males. However, the length of the posterior part of the 7th segment in a few 1 - 3 d

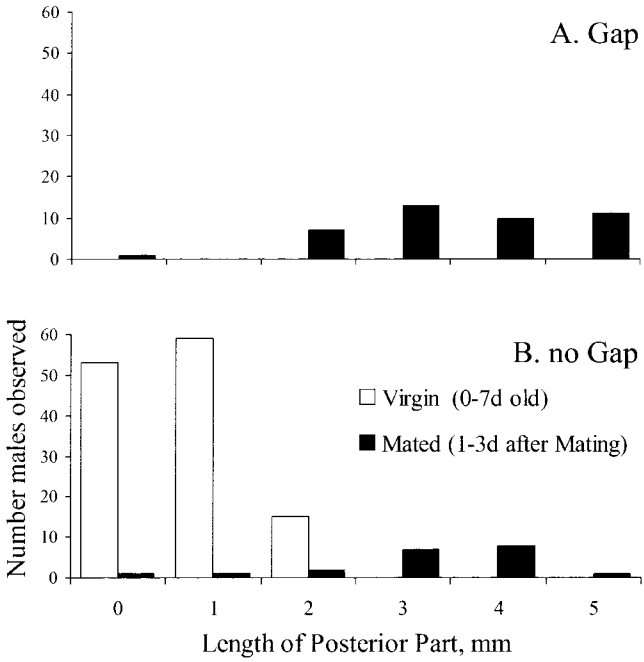

Fig. 3. Existence of a gap (upper panel) or no gap (lower panel) in creamy white fluids, and length of posterior part of 7th segment of primary simplex of *Ostrinia nubilalis* males.

postcopulation males was also <2 mm, resulting in a 6.3% misclassification of 1 - 3 d postcopulation males (n = 63) (Table 2). Although we did not observe males >3 d postcopulation, we expect that males >3 d postcopulation would have similar characters to males 3 d postcopulation because changes in the refilling process slowed down considerably after 1 d postcopulation.

In most cases, the transparency level in the 7th segment of mated male was not as uniform as in virgin males because of a mixture of remnants of the original fluids and regenerated fluids. An experienced investigator can classify the mating status by reading the uniformity of the 7th segment alone. However, uniformity is not a simple character, and becomes a reliable character for distinguishing the mating status only after considerable experience.

Unlike other successful studies which were based on the pigmented fluids inside the primary simplex (Bergh and Seabrook 1986, Evenden et al. 2003, Haines 1981), both virgin and mated *O. nubilalis* males contained only creamy white, unpigmented fluids. However, the dynamics of the creamy white fluids provided sufficient information to distinguish *O. nubilalis* male mating status and timing of copulation. Mating status of *O. nubilalis* males could be categorized into 3 classes: virgin males, <1 d postcopulation, and 1-3 d postcopulation. Males <1 d postcopulation were easily distinguished from other 2 classes. The level 1 transparency and 100% filled fluids in the 1st and 2nd segments were 100% reliable characters to conclude that the male was recently mated.

Table 2. Discriminant classification of male mating status of *Ostrinia nubilalis* males

Known mating status	Total number observed	Classified mating status		
		Virgin	Mated, <1d postmating	Mated, 1 - 3d post-mating
Virgin, 0 < 7d old	126	126	0	0
0 - 2hr	21	21	0	0
12 - 14hr	21	21	0	0
24 - 26hr	21	21	0	0
36 - 38hr	21	21	0	0
78 - 82hr	21	21	0	0
168 - 172hr	21	21	0	0
Mated, 0 < 1d postmating	61	0	61	0
0 - 2hr	20	0	20	0
6 - 8hr	20	0	20	0
20 - 22hr	21	0	21	0
Mated, 1 - 3d postmating	63	4 (6.3%)	0	59
30 - 32hr	21	0	0	21
40 - 43hr	21	2	0	19
68 - 72hr	21	2	0	19

In addition, we found that the testes size of *O. nubilalis* was not related to wing length within each age group. The correlation coefficients of testes size and wing length of virgin and mated *O. nubilalis* males varied (Table 3), which suggested the independence of testes size and wing length. The average diameter of the testes decreased as male age increased (Fig. 4). This result is consistent with those of Chaudhury and Raun (1966), who reported that the testes size of *O. nubilalis* was largest at the 3rd day of the pupal stage and decreased as age increased. Both virgin and mated males had similar slopes of decrease in testes size with age (Fig. 4). In addition, copulation had a significant effect about the testes size ($F = 371.64$; df = 1, 119; $P < 0.0001$). On average, a single mating event reduced the testes size by 0.0585 mm over all age groups. However, we cannot make a good prediction of male age by measuring the testes size because of the large variation within age and mating categories (Fig. 4).

Validation. Males used for validation of the method were identified blind to their actual mating status. All of the 14 virgin males, 13 < 1 d postcopulation males, and 12 1-3 d postcopulation males were correctly classified using the method developed above. This suggests that the method is capable of considerable accuracy, within the limits of the predicted 6.3% error rate for males 1 - 3 d postcopulation.

Summary of method. In Table 4, we list the steps for diagnosing the mating status of field-captured *O. nubilalis* males. Overall, it is critical to keep the moth body in fresh and soft condition to allow easy dissection and correct scoring. Sometimes, the

Table 3. Testes size (mm) and left wing length* (m m) of *Ostrinia nubilalis* (means ± sd)

Virgin males (n = 21)					Mated males (n = 20 or 21)				
Age Hrs	Left Wing	Testes diameter	Correlation Coefficients†	P	Age Hrs	Left wing	Testes Diameter	Correlation Coefficient†	P
0 - 2	11.96 ± 0.38	1.25 ± 0.11	0.3437	0.1272	24-26	11.90 ± 0.70	1.02 ± 0.09	0.3110	0.1820
12 - 14	11.70 ± 0.46	1.17 ± 0.12	0.3551	0.1142	30-32	12.05 ± 0.44	1.03 ± 0.09	0.0868	0.7083
24 - 26	11.84 ± 0.58	1.03 ± 0.07	–0.0583	0.8017	44-46	12.03 ± 0.48	1.00 ± 0.06	0.1331	0.5651
36 - 38	11.93 ± 0.55	1.04 ± 0.06	0.2249	0.3405	54-56	12.02 ± 0.39	0.99 ± 0.09	0.3789	0.0903
78 - 82	12.15 ± 0.45	0.90 ± 0.06	0.1818	0.4303	64-67	11.85 ± 0.64	0.93 ± 0.12	–0.1099	0.6354
168 - 172	11.67 ± 0.38	0.75 ± 0.05	0.2815	0.2165	93-96	11.99 ± 0.64	0.84 ± 0.09	0.7071	0.0003

* the wing length was measured from the junction of the costal and thorax to the tip of wing under a dissecting microscope.
† Pearson correlation coefficients were calculated for this analysis.

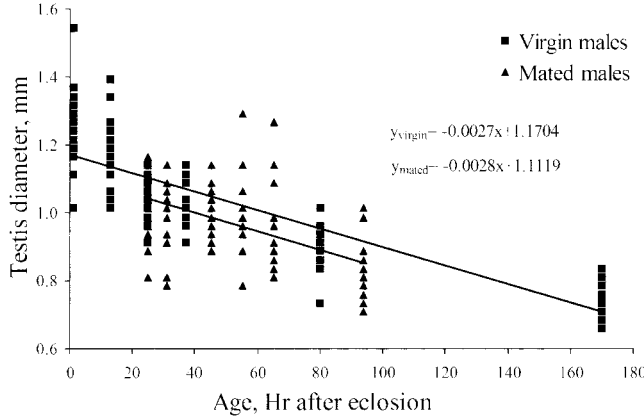

Fig. 4. Regression of testes size on age for virgin and mated *Ostrinia nubilalis* males.

Table 4. Steps of classification mating status of field caught *Ostrinia nubilalis* males

1. All captured males must be preserved fresh and dissection must be finished within 12h after they are killed.
2. First examine the transparency level of 7th segment. If it is clear or less than level this male can be classified as a < 1 d mated male. The classification can be confirmed by the presence of level 1 transparency in the 1st and 2nd segments. Otherwise, he is either a virgin or mated more than 1 d ago.
3. Continue examination of the 7th segment. If there is a gap between the posterior and anterior fluids in the segment, then the male is a mated male who mated >1 d ago.
4. If there is no gap in the white fluid in segment 7, then examine the length of the posterior part of the white fluid in segment 7. If the length >2 mm, the male has mated 1 - 3 d ago. If the length is ≤2 mm or there is no posterior part, then the male should be classified as a virgin.

moth will become dehydrated by noon in a pheromone trap during the summer flight, which makes scoring impossible. Thus, collection of *O. nubilalis* males during the early hours of the morning is essential. If the male cannot be dissected within 12 h of collection, moths may be stored in saline solution at 4°C. However, the shorter is the period between collection and dissection, the better is the accuracy and reliability of the scoring procedure.

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