Assessing the Mating Status of Male Obliquebanded Leafrollers *Choristoneura rosaceana* (Lepidoptera: Tortricidae) by Dissection of Male and Female Moths

M. L. EVENDEN,[1] L. E. DELURY,[2] G.J.R. JUDD,[2] AND J. H. BORDEN

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada

Ann. Entomol. Soc. Am. 96(3): 217–224 (2003)

ABSTRACT We tested several methods for assessing the mating status of male obliquebanded leafrollers, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae). In four experiments, we established that the technique developed to determine mating status of male spruce budworms, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae), can be used for *C. rosaceana* populations in western and eastern North America. Section seven of the primary simplex of the *ductus ejaculatorius* appeared opaque, with either a white or yellow coloration in virgin *C. rosaceana* males, but was clear for up to 6 days after copulation in mated males. This technique was used successfully to determine the mating status of males captured in pheromone-baited traps during the first flight of *C. rosaceana* in two consecutive seasons. In three laboratory experiments, we determined that the presence or absence of sclerotized bars on the surface of spermatophores dissected from female *C. rosaceana* is a reliable indicator of the mating status of the females' partner. Spermatophores produced by virgin males had sclerotized barring, whereas those from once-mated males did not. This technique was used to demonstrate that the mating status of males mated to tethered, virgin females did not differ between pheromone-treated and nontreated control plots.

KEY WORDS *Chroristoneura rosaceana*, Tortricidae, mating status, *ductus ejaculatorius*, spermatophore

DETERMINATION OF MATING status is particularly important in assessing the effectiveness of pest management tactics that target insect mating systems, such as pheromone-based mating disruption and the sterile-insect technique (SIT) (Boake et al. 1996). The SIT has been developed and implemented against the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) in British Columbia apple orchards (Dyck et al. 1993) and may be applicable for other tortricid species (Butt 1991). Mating disruption has been developed and is available commercially for several tortricid species on a variety of cropping systems (Cardé and Minks 1995). For the obliquebanded leafroller, *Choristoneura rosaceana* (Harris), a pest of apples across North America (Chapman et al. 1968), mating disruption has been investigated, but not with consistent success (Reissig et al. 1978, Roelofs and Novak 1981, Deland et al. 1994, Agnello et al. 1996, Lawson et al. 1996, Knight et al. 1998, Evenden et al. 1999a, 1999b, 1999c). Easy and reliable techniques to establish mating status of males could assist researchers in determining the mechanisms of control by mating disruption (Evenden et al. 1999a) and in evaluating pheromone-based management of this species.

In the Tortricidae, the mating status of female moths can generally be determined by the presence or absence of a spermatophore in the *bursa copulatrix* (Horak and Brown 1991, but see George and Howard 1968, Outram 1971). Information about the mating status of the male is more difficult to assess, but the size of the spermatophore delivered to the female declines with multiple matings in some tortricids (Outram 1971, Carroll 1994, Delisle and Bouchard 1995, Foster and Ayers 1996, Torres-Vila et al. 1997). However, spermatophore size may also depend on male size, age at mating, time between matings, and the larval host plant (Carroll 1994, Delisle and Bouchard 1995, Delisle and Hardy 1997, Torres-Vila et al. 1997). Thus, determination of male mating status by spermatophore size based on mass, volume, or diameter measurement is not only time consuming but potentially misleading.

Despite the widespread use of pheromones for monitoring lepidopteran pest populations (Jutsum and Gordon 1989), indices of mating status of males captured in pheromone traps have been developed only for the tobacco budworm, *Helicoverpa virescens* (F.) (Lepidoptera: Noctuidae) (Henneberry and Clayton 1984), and the spruce budworm, *Choristo-*

[1] Department of Biology, West Chester University, West Chester, PA 19383 (e-mail: mevenden@wcupa.edu).

[2] Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, British Columbia V0H 1Z0, Canada.

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neura fumiferana (Clemens) (Lepidoptera: Tortricidae) (Bergh and Seabrook 1986b). In the spruce budworm, section seven of the primary simplex of the *ductus ejaculatorius* contains a yellow substance in virgin males and is clear in recently mated males (Bergh and Seabrook 1986a). This characteristic was used to determine that traps baited with virgin spruce budworm females or synthetic pheromone captured the greatest proportions of virgin males at the beginning and end of the flight period (Bergh and Seabrook 1986b).

We tested two techniques for determining the mating status of *C. rosaceana* males from both western and eastern North America: examination of the *ductus ejaculatorius* in males and the external morphology of spermatophores deposited in mated females.

Materials and Methods

Insects. The western *C. rosaceana* used in laboratory experiments came from a colony started from larvae collected from organic orchards in the Similkameen Valley of British Columbia in 1995. The eastern moths came from an established laboratory colony at the Pennsylvania State University, Tree Fruit Research and Extension Center, Biglerville, PA, which was initiated from larvae collected in conventional orchards in central Pennsylvania in 2001. Both colonies were maintained at a 16:8 (L:D) h and 24°C. Larvae were fed a pinto bean–based diet (Shorey and Hale 1965), and adults were provided with a 10% sugar solution. Experiments on western *C. rosaceana* were conducted from 1995 to 1997 and on eastern moths from 2001 to 2002.

Determination of Male Mating Status. Several experiments were conducted to determine if the technique developed by Bergh and Seabrook (1986a) could be adapted for *C. rosaceana.* Experiment 1 was conducted using western *C. rosaceana.* Male pupae were placed alone or with a female pupa in 150-ml cups, provided with a water source, and held under the same conditions as the colony. After eclosion, adult females were dissected to identify pairs that had copulated. Sixty mated and 80 virgin males 24- to 168-h-old and 6- to 144-h-old, respectively, were dissected. The male reproductive tract was removed by securing the moth at the thorax and gently pulling on the claspers at the tip of the abdomen. The color and transparency of the seventh segment of the primary simplex of the *ductus ejaculatorius* was recorded. This segment is easily identified by the loop in the cuticular simplex directly to the posterior, as described for *C. fumiferana* (Outram 1970). In experiment 2, we examined whether the technique could be extended to the eastern *C. rosaceana* population. Male and female pupae were maintained in shell vials, and newly eclosed male adults were placed alone or with a female in 150-ml cups. Twenty-one mated and 23 virgin males 24- to 48-h-old and 24- to 96-h-old, respectively, were dissected, as in experiment 1.

In experiments 3 and 4, we examined the effect of time since mating on the appearance of segment seven of the primary simplex of mated western (experiment 3) and eastern (experiment 4) *C. rosacena* males, respectively. Male and females were paired in cups as in experiments 1 and 2, but were held at 24°C under a reversed photoregimen of 8:16 (D:L) h. Cups containing pairs were observed 3 h into the scotophase and recorded as *in copulo* if they were mating. Females were removed at the onset of photophase and males were held for 3–4 or 5–6 d (experiment 3) or 3 d (experiment 4) before dissection as in experiment 1.

Mating Status of Western *C. rosaceana* in Pheromone-Baited Monitoring Traps. In experiments 5 and 6, we determined the mating status of western *C. rosaceana* males captured throughout the first flight in 1996 and 1997 in two pheromone-baited traps hung in an experimental apple orchard at the Agriculture and Agri-Food Canada Research Centre in the Okanagan Valley at Summerland, British Columbia (49°11′N, 119°46′W). Traps were modified wing-traps (Phero Tech, Delta, British Columbia, Canada) that were constructed using opposing wing-trap tops held 5 cm apart with pieces of drinking straw and with Stickem Special (Phero Tech) thinly applied to the inside surface of the trap bottom. Traps were hung from trees ≈1.5 m above ground by a wire hanger. Lures were suspended inside tops of traps using a straight pin and consisted of red rubber septa (West Company, Linville, PA) loaded with 3 mg of a 100:2:1.5:1 blend of Z11-tetradecenyl acetate (Z11–14:OAc), *E*11-tetradecenyl acetate (*E*11–14:OAc), Z11-tetradecenol (Z11–14:OH), and Z11-tetradecenal (Z11–14:Ald) (Vakenti et al. 1988, Thomson et al. 1991) in 200 μl of HPLC-grade hexane. Lures were replaced every 4 wk. Traps were checked daily, and trap bottoms with captured males were removed and replaced. Twenty (1996) or 10 (1997) males, or lesser numbers if captures were low, were dissected immediately after removal of the trap bottom, or trap bottoms were wrapped in plastic and stored at 4°C for 1–2 d before dissection. Degree day (DD) accumulations (°C) calculated for the Summerland orchard (Evenden and Judd 1999) were regressed against the percent of mated males captured in traps (SAS Institute 1996).

Assessment of Mating Status of Females' Partners. In two laboratory experiments, we tested the hypothesis that spermatophores transferred to western *C. rosaceana* females from virgin western males are larger than spermatophores transferred from mated males, as demonstrated for eastern *C. rosaceana* (Delisle and Bouchard 1995). In experiment 7, males were obtained from the laboratory colony, whereas in experiment 8, they were collected as larvae on apple hosts.

In experiment 7, male and female pupae were placed in 43 pairs in 150-ml cups, provided with water, and maintained under the same conditions as the colony. Adults remained together for 1–2 nights after eclosion before females were dissected. Mated males were then placed with another virgin female, and the procedure was repeated. The mating history of each male was followed, and measurements of the first spermatophore from 14 males that provided two spermatophores were excluded from analyses to ensure

independent samples. At the time of mating, virgin and mated males providing spermatophores were 6- to 72-h-old and 24- to 96-h-old, respectively, and females were 6- to 120-h-old. The second mating occurred 24–120 h after the first. Bursa copulatrices were excised from females and stored in 70% ethanol before further dissection to remove the spermatophore. Spermatophores had a spherical corpus and long collum that extended through the *ductus bursum*. Spermatophore diameters were measured perpendicular and parallel to the collum under a dissecting microscope using an ocular micrometer. The latter measurements were the consistently larger of the two and were used in analyses.

For experiment 8, larvae were collected from 6 to 20 May 1997, in an organically-managed apple orchard in the Similkameen Valley, British Columbia (49°11′N, 119°46′W), held in 58-ml cups under the same conditions as the laboratory colony, and fed apple leaves until pupation. Forty-four pairs of field-collected male and female pupae were placed in 150-ml cups, and the protocol used in experiment 7 was repeated, excluding measurements of the first spermatophore from 13 males that provided two spermatophores. At the time of mating, virgin and mated males were 6- to 120-h-old and 24- to 144-h-old, respectively, and females were 6- to 96-h-old. The time between matings ranged from 24 to 144 h. Spermatophores were measured directly after dissection of the females.

Diameter measurements of spermatophores from virgin and mated males in experiments 7 and 8 were compared using a two-sample *t*-test, $\alpha = 0.05$ (Zar 1984). Before analysis, data were checked for normality and homoscedasticity (SAS Institute 1996).

In experiment 9, we tested the hypothesis that the presence of sclerotized bars on the spermatophores (possibly the cornuti from the end of the male aedeagus that had been dislodged during mating) was a good indicator of the mating status of the female moth's partner. Ninety-two virgin females, 0- to 48-h-old, were placed with virgin males 48- to 72-h-old in 150-ml cups, provided with a constant water source, and held under the same conditions as the insect colony. Females were dissected the following morning, and the presence or absence of sclerotized bars on the surface of spermatophores was noted. Mated males were placed with a second 0- to 48-h-old virgin female during the next scotophase. These females were dissected the following morning, and their spermatophores were examined for the presence of sclerotized bars.

Mating Status of Western *C. rosaceana* Males in Pheromone-Treated Plots. In experiment 10, we tested the hypothesis that the mating status of males that mated with tethered females would differ in pheromone-treated and nontreated plots. *Bursa copulatrices* were excised from tethered females and used to assess the effectiveness of mating disruption against western *C. rosaceana* in three small plot field experiments. Plot design, pheromone treatments, and experimental handling of tethered females are described elsewhere (Evenden et al. 1999a). Spermatophores were stored in 70% ethanol until spermatophores were observed, as in experiment 9. The ratio of mated:virgin males was determined at the time of collection by dissecting males captured in pheromone-baited traps in the area but outside the experimental plots as in experiment 4. The ratio of mated:virgin males was also determined for males captured in monitoring traps baited with a 3-mg lure placed in clean air control plots or with a 10-mg lure placed in pheromone-treated plots. The hypothesis that mating status of males was independent of atmospheric treatment (pheromone versus clean air) was evaluated by a χ^2 test ($\alpha = 0.05$) using pooled data from three field experiments.

Results

Determination of Male Mating Status. Dissections of males in experiments 1–4 revealed that segment seven of the primary simplex of the *ductus ejaculatorius* of western *C. rosaceana* was opaque in 93.7% of the 80 virgin males dissected and clear in 93.3% of 60 mated males dissected (Fig. 1; Table 1). In eastern *C. rosaceana*, this segment appeared opaque in 86.9% of 23 virgin males and clear in 85.7% of the 21 mated males (Table 1). The color of the segment varied and appeared either yellow or white in virgin males from both populations.

There was no coloration of segment seven of the primary simplex in 7 of 10 mated western *C. rosaceana* males 3–4 d after mating, whereas 3 showed some coloration, but the segment was not opaque. Similarly, five of seven mated eastern *C. rosaceana* males showed no coloration in segment seven of the primary simplex 3 d after mating, and two showed some coloration, but the segment was not opaque. All of the eight western males dissected 5–6 d after mating showed no coloration.

Mating Status of Western *C. rosaceana* in Pheromone-Baited Monitoring Traps. The percentage of mated males in pheromone-baited traps in experiments 5 and 6 increased linearly over the duration of the first flight in both 1996 and 1997 (Fig. 2).

Assessment of Mating Status of Females' Partners. Spermatophores produced by either laboratory (experiment 7) or wild (experiment 8) mated western males were significantly smaller than spermatophores produced by virgin males (Table 2). Spermatophores produced by all except one virgin male in experiments 7 and 8 exhibited sclerotized barring (Fig. 3), but none of the mated males produced spermatophores with barring (Table 2). All of the spermatophores produced by laboratory-reared virgin males in experiment 9 had sclerotized bars, but only 1 of the 39 spermatophores produced by mated males exhibited sclerotized bars (Table 2).

Mating Status of Western *C. rosaceana* Males in Pheromone-Treated Plots. In pooled data from three field experiments, there was no significant difference in the percentages of mated males that provided spermatophores to tethered virgin females in control plots (49.5% of 93 males) and in pheromone-treated plots (57.5% of 153 males; $\chi^2 = 1.514$; $P > 0.10$). Similarly,

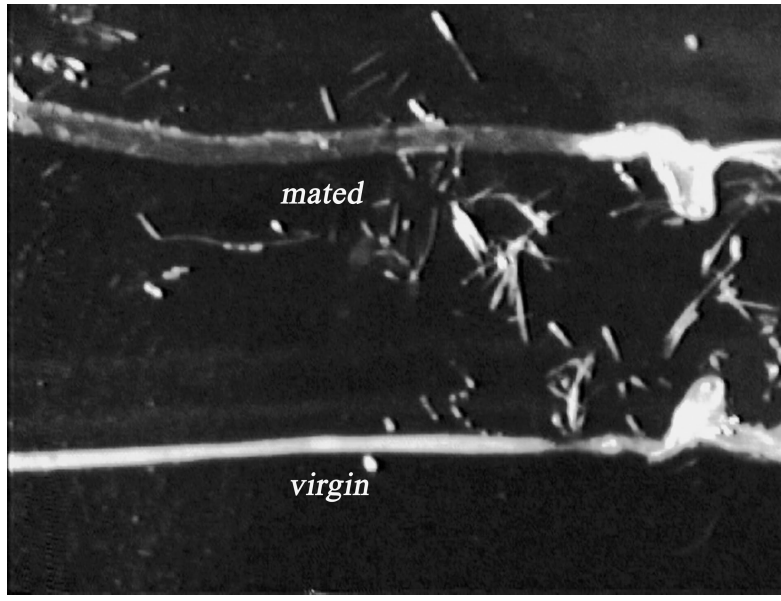

Fig. 1. Appearance of section seven of the primary simplex of the *ductus ejaculatorius* in mated and virgin *Choristoneura rosaceana* males.

the percentage of mated males trapped outside of the experimental area (46.7% of 90 males) was not significantly different from the 57.5% mated males that provided spermatophores to tethered virgin females in pheromone-treated plots ($\chi^2 = 2.639$; $P > 0.10$). Finally, there was no significant difference in the percentages of mated males trapped in control plots (46.3% of 82 males) and those trapped in pheromone-treated plots (42.9% of 268 males; $\chi^2 = 0.313$, $P > 0.50$).

Discussion

Our results demonstrate that the technique to assess mating of male *C. fumiferana* (Bergh and Seabrook 1986a) can be adapted for *C. rosaceana* (Table 1). However, unlike *C. fumiferana*, the coloration of segment seven of the primary simplex of the *ductus ejaculatorius* of *C. rosaceana* appeared either yellow or white in virgin males from both populations. It is generally assumed that the primary simplex of male Lepidoptera contains the spermatophore precursors (Callahan 1958, Outram 1970). The secretory fluid in the primary simplex of the *ductus ejaculatorius* varies in color among species in the Noctuidae (Callahan and Cascio 1963), and intraspecific variation in coloration occurs with moth age in *H. virescens* (Henneberry and Clayton 1984). However, the yellow coloration of the fluid did not vary with age in virgin *C. fumiferana* males (Bergh and Seabrook 1986a) nor did the ≈50:50 ratio of white to yellow coloration vary with age in virgin *C. rosaceana* (M.L.E., unpublished data). Despite the variation in coloration, section seven of the primary simplex of the *ductus ejaculatorius* of *C. rosaceana* was generally opaque in virgin males and clearly distinguishable from the clear section seven of mated males (Fig. 1) and the grainy appearance of a few mated males (Table 1). Lack of coloration in segment seven of the primary simplex persisted in the small number of *C. rosaceana* males dissected 3–6 d after mating, as found in *H. virescens* (Henneberry and Clayton 1984). In contrast, section seven of *C. fumiferana* became opaque as early as 24 h after copulation and was indistinguishable from that of similarly aged virgins 72 h after copulation (Bergh and Seabrook 1986a).

As expected for a protrandrous species (Onstad et al. 1985, Delisle and Bouchard 1995, Evenden and Judd

Table 1. Assortment of virgin and mated *C. rosaceana* males in relation to the condition of segment seven of the primary simplex of the *ductus ejaculatorius*

No dissected	Colony and experiment	Mating status	Percent of *C. rosaceana* sampled					
			Segment seven opaque		Segment seven clear		Segment seven grainy	
			White	Yellow	Colorless	Yellow	White	Yellow
80	western (experiment 1)	Virgin	52.5	41.2	1.3	0	1.3	3.7
60		Mated	1.7	0	93.3	0	5.0	0
23	eastern (experiment 2)	Virgin	30.4	56.5	0	0	0	13.1
21		Mated	0	0	85.7	0	0	14.3

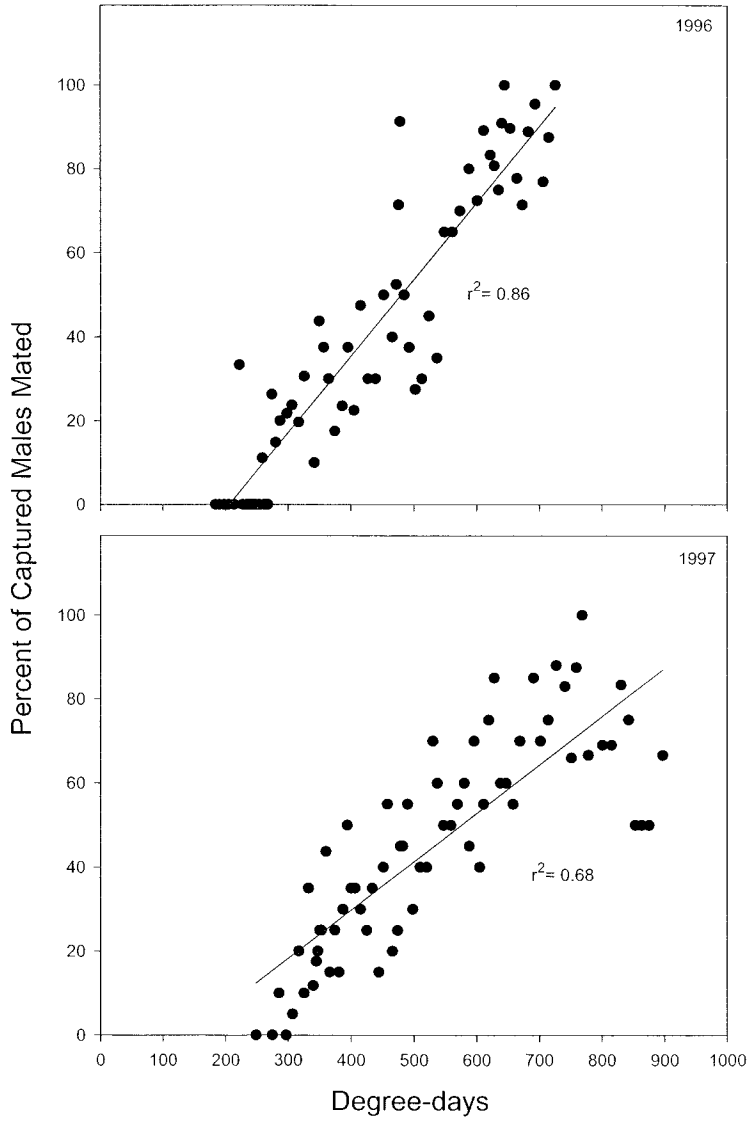

Fig. 2. Relationships between percent mated males captured in monitoring traps in experiment 6 in 1996 and experiment 7 in 1997 during the first-generation *Choristoneura rosaceana* flight and $DD_{10°C}$ air temperature after 1 January (Evenden and Judd 1999). Regression equations are y = 0.18x − 37.59 (1996) and y = 0.12x − 16.26 (1997).

Table 2. Size and description of spermatophores produced by previously mated and virgin western male *C. rosaceana*

Experiment no.	*N*	Male mating status	Male source	Mean ± SE[a] spermatophore diameter (mm)	Spermatophore diameter (mm) range	Spermatophores with sclerotized bars	
						N	%
7	29	Virgin	Laboratory	1.61 ± 0.04a	1.4–2.0	19	94.7
	14	Mated	Laboratory	1.08 ± 0.04b	0.8–1.4	11	0
8	22	Virgin	Wild	1.78 ± 0.06a	1.5–2.2	31	100
	13	Mated	Wild	1.20 ± 0.05b	0.7–1.6	13	0
9	72	Virgin	Laboratory	Not measured	Not measured	72	100
	39	Mated	Laboratory	Not measured	Not measured	39	2.6

[a] Within each experiment, means followed by different letters are significantly different, $P < 0.05$, two-sample *t*-test.

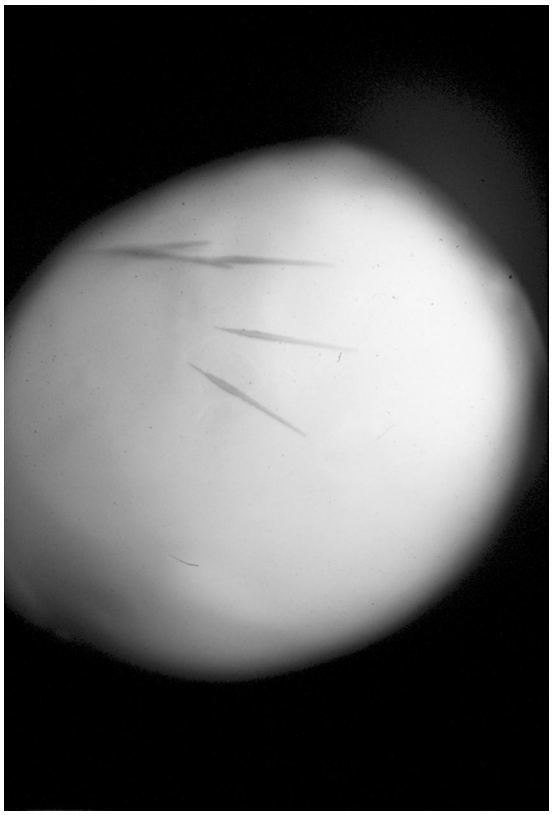

Fig. 3. Surface of spermatophore with visible sclerotized barring produced by virgin *Choristoneura rosaceana* males.

1999), the proportion of mated western *C. rosaceana* males captured in pheromone-baited traps increased linearly throughout the first flight in both 1996 and 1997 (Fig. 2). Bergh and Seabrook (1986b) observed a similar increase in mated male *C. fumiferana* until mid-flight, but more virgin males were captured at the end of the flight period. This may be because male *C. fumiferana* regenerate the fluid in section seven of the primary simplex or that virgin males are more responsive to sex pheromone than mated males (Bergh and Seabrook 1986a).

This technique will help to assess the effectiveness of mating disruption against *C. rosaceana* by determining if mated males are present in pheromone-treated orchards or the surrounding areas. However, the technique may be of limited use in predicting developmental events throughout the flight period. When 50% of trapped males were mated, 100% of both sexes had eclosed in the same orchard (Evenden and Judd 1999). This could indicate that males prefer to mate at an older age. Accordingly, Delisle (1995) demonstrated that mating success of male *C. rosaceana* increased with age over 3 d and then decreased, but female mating success decreased linearly with age, suggesting that when 100% female eclosion had occurred >50% of males should be mated.

In agreement with the results of Delisle and Bouchard (1995) for eastern *C. rosaceana*, spermatophores transferred to females from once-mated males were significantly smaller than those transferred by virgin males (Table 2). Both diets used in experiment 7 (artificial diet) and experiment 8 (apple foliage) are considered to be of high quality for *C. rosaceana* (Carrière 1992), and the ranges of spermatophore size produced by males on both diets overlapped (Table 2). Thus, the reduction in spermatophore size observed on a poor quality host (hazlenut) by Delisle and Bouchard (1995) would not have been expected.

In experiment 9, all spermatophores transferred by virgin males and only one transferred by mated males had visible sclerotized barring on the surface (Table 2). Many male *Choristoneura* species, including *C. rosaceana*, have terminal spines called cornuti on the aedeagus (Dang 1992). In the codling moth, cornuti interlock with a sclerotized plate in the *bursa copulatrix* of the female during mating, presumably augmenting the male claspers' hold on the female (Ferro and Akre 1975). In some tortricids, corunuti can remain in the bursa copulatrix after copulation (Horak and Brown 1991). In the case of *C. rosaceana*, it seems that they not only remain but become imbedded in the first spermatophore.

Delisle and Bouchard (1995) demonstrated that female *C. rosaceana* mated most readily with virgin males, from whom they received larger spermatophores than from mated males, imparting an hypothesized adaptive advantage in female mate choice. If female *C. rosaceana* do exhibit mate choice behavior, they may alter this behavior in a competitive situation when many females are calling or when the atmosphere is treated with synthetic pheromone. However, using the sclerotized barring technique to determine male mating status, we found no evidence that atmospheric treatment with pheromone influenced selection by tethered females for either virgin or mated males. Because there is only a 25% reduction in reproductive output by *C. rosaceana* females inseminated by mated males (Delisle and Bouchard 1995), it may take several generations of selection pressure to influence female mate choice for virgin males in the presence of high pheromone levels.

The mating status of male *C. rosaceana* can be reliably determined using the simple dissection techniques presented here. Researchers and pest managers will benefit from the adoption of these techniques to evaluate mating behavior and pheromone-based integrated pest management programs for this species.

Acknowledgments

We thank M.G.T. Gardiner for technical assistance and Drs. L. Hull and G. Krawcyzk for providing the eastern *C. rosaceana* colony. Kelly McVeigh of West Chester University helped to produce Figs. 1 and 3. Financial support was provided by the Science Council of British Columbia, British Columbia Fruit Growers' Association, Okanagan Valley Tree Fruit Authority, Similkameen Okanagan Organic Producers' Association, Natural Sciences and Engineering Research Council of Canada, Phero Tech, and a College of Arts and Sciences Development Award, West Chester University.

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Received for publication 11 October 2002; accepted 22 January 2003.