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RELIABILITY OF ABDOMINAL PALPATION IN DETERMINING THE MATED STATUS OF OVERWINTERING MONARCH BUTTERFLY (NYMPHALIDAE: DANAINAE) FEMALES IN CALIFORNIA

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ABSTRACT. The abdominal palpation technique is commonly used to evaluate the mated status of monarch females overwintering in both Californian and Mexican winter sites. We found that this method was not reliable in determining the mated status of California overwintering monarch females that had not mated recently. The unreliability of this method was attributed to the reliance of detecting a swollen "pea" (bursa copulatrix) within the female's abdomen by palpation or "feel". The majority of the spermatophores recovered from overwintering females from November to January were too small to cause a significant swelling of the bursa copulatrix that could be accurately and consistently detected by tactile senses. We determined that bursa content weight containing single or multiple spermatophores 10 mg or greater could be detected by palpating the females' abdomen. The palpation method, thus allows quick and easy determination of recently mated females as occurs in February during the mass mating period just prior to the spring migration.

Additional key words: Monarch Butterfly mated status; abdominal palpation; spermatophore

Each fall, monarch butterflies (*Danaus plexippus* L.) migrate to coastal California to winter in groves with specific microclimatic conditions (Leong 1990, 1999; Leong *et al*. 1991, 2004) that will support their winter aggregations. The monarch butterflies begin to arrive in central California by late October and by mid- or late November, winter aggregations ranging from a few hundred to several thousand individuals can be observed on lower branches of wind sheltered inner grove trees (Leong *et al*. 1995, Leong 1999). Overwintering monarch butterflies continue to arrive until late December when their population levels generally reach peak abundance (Leong *et al*. 1995). The population remains at this level until mating activity begins in mid-January (Hill *et al*. 1976; Leong *et al*. 1995). The winter population begins to decline as the butterflies disperse from the winter site to recolonize their spring and summer ranges.

Upon arrival at central California winter sites, > 40% of overwintering butterflies were mated (based on spermatophore counts), and this percentage remained statistically unchanged (Leong *et al*. 1995) through most of the winter season (October to mid-January). This observation conforms with Hill *et al*.'s (1976) findings that mating behavior was infrequent or rare during the winter months and was mainly restricted to few days prior to the spring migration. Tuskes & Brower (1978) reported that mating occurred at a low level throughout the winter, especially during the cluster formation period. They determined the mated status of monarch females by palpating the abdomens with the thumb and forefinger for the presence or absence of a "pea-like" swelling within the abdomen. A female with this swelling was classified as a mated individual while those without were classified as non-mated or virgin females. The "pea" swelling was due to the presence of one or more spermatophores within the female's bursa copulatrix. Van Hook (1999) reported that abdominal palpation of monarchs in the eastern North American population had 95% accuracy for detecting fresh spermatophores and 84% for the detection of extremely deteriorated spermatophores.

This study evaluates the efficacy of the abdominal palpation method in determining the mated or non-mated status of monarch butterfly females overwintering at a central California winter site.

MATERIALS AND METHODS

Winter Site. The Morro Bay Golf Course winter site is located in San Luis Obispo County, California, ≈ 100 m southwest of the 5th putting green (35° 21' 02" latitude; 120° 50' 30" longitude) and the grove is ≈2 km from the ocean. The grove consists of Blue Gum trees (*Eucalyptus globules* Labill.) in the center, while the trees comprising its northern, southern and eastern borders consist of a mixture of old established Monterey pine (*Pinus radiata* Don) and new seedlings of Monterey cypress (*Cupressus macrocarpa* Gord.). Monterey cypress seedlings (now 2 years old) were planted to replace dead or dying Monetery pine trees affected with pine pitch canker. Beneath the Eucalyptus canopy, the understory consists of a mixture of ice plant (*Carpobrotus edulis* (L.) L. Bolus) and poison oak (*Toxicodendron diversilobum* (Torr. & Gray) Greene). The area beneath the grove had a north-south slope of 10°.

Butterfly samples. Butterflies were collected from clusters during the early morning hours (0700–0730 PST), twice monthly (7 and 29 November 1999, 13 and 23 December 1999, 3 and 17 January 2000, and 8 February 2000), using a special (7.2-m telescopic) long-handled net (BioQuip, Gardena, California). Forty females were randomly selected from each sample of netted butterflies and placed individually in numbered envelopes and stored in a cooler for transportation to the laboratory. Within 24 hrs, each butterfly was independently evaluated by the authors and placed into one of two categories using the abdominal palpation method: (1) mated, with a noticeable "pea" and (2) not mated, without a noticeable "pea". The palpation method assumes that the weight or mass of one or more spermatophore within the bursa copulatrix would cause a significant swelling that can be detected tactilely. The same three investigators independently evaluated the biweekly samples of butterflies throughout this study and the order of evaluating the biweekly samples was randomly assigned. After being evaluated, the butterflies were killed by freezing and held in the freezer until they were dissected and examined by the senior author for presence of spermatophores within their bursae copulatrices and spermatozoa within their sperm receptacles.

The bursa copulatrix of each butterfly was carefully dissected for spermatophores. If present, the number and the weight of each spermatophore were recorded to the nearest 0.01 mg using a Mettler-Toledo Balance (AG245). Importantly, the spermatheca (sperm receptacle) of each butterfly was examined for the presence of spermatozoa to confirm a successful mating, as the spermtophore or the neck of the spermatophore was not always recovered from the bursa copulatrix. A spermatheca with spermatozoa appeared opaque white, and the presence of spermatozoa was confirmed by examining the spermatheca contents with a compound microscope using 100X magnification. The spermatozoa appeared as bundles of fine hairs. Each spermatozoan had an oval head with a very long tail.

Statistical Analysis. The data were analyzed using the statistical program Biostat 1 (Pimentel & Smith 1990) for analysis of variance (ANOVA) and Chi-square.

RESULTS

Upon arrival in early November, 16 of 40 females (40%) captured from their winter aggregation were mated (Table 1) based on dissection of their spermatophores and/or the presence of spermatozoa within the sperm receptacle. The proportion of mated

TABLE 1. The average and range of spermatophore weights (mass) of butterflies collected from Morro Bay Golf course winter site during the 1999–2000 winter season. Based on the % mated female (expected) in the monthly samples and the average monthly palpation accuracy scores (observed) of the investigators to predict the mated status of the female, the Chi-square test was not significantly different from a 50-50 determination hypothesis through most of the winter season (November to January) X^2= 5.7; df=5; p>0.05. When the February sample was included in the analysis, the results show a significant deviation from a 50-50 determination; X^2=34.97, df = 6; p= 0.01.

Sample Date	N	Spermatophore weight (mean mg±SD)	Range (mg)	% Mated female	Average Palpation Accuracy scores
7 November	40	1.5±2.6	0 – 8.6	40	49
29 November	40	1.2±2.7	0 – 8.3	40	52
13 December	40	1.7±2.4	0 – 8.6	58	54
23 December	40	2.7±3.3	0 – 11.0	53	53
3 January	40	1.1 ±2.0	0.3 – 6.2	40	60
17 January	40	1.3±3.9	0.1 – 16.7	43	43
8 February	40	20.6±13.2	1.8 – 52.7	98	85

to non-mated females in the biweekly samples remained statistically unchanged (F=0.804, p=0.548, df=5, 234) until 8 February (F=67.33, p=0.001, df=6, 273), when mating activity had increased prior to the spring migration. Our data agree with earlier findings (Leong *et al*. 1995) concerning the seasonal variation of mated females within populations of overwintering butterflies in San Luis Obispo County, California.

The number of correct (hits) and incorrect (misses) assignments based on abdominal palpation of each evaluator was confirmed by dissecting the female. A female was classified as being mated if all or part of the spermatophore was recovered from the bursa copulatrix and/or the spermatheca contained spermatozoa. Notably, the bursae copulatrices of eight females (8 of 280) were void of spermatophores or the necks of spermatophores but were classified as mated because they had spermatozoa within their spermathecae. A female was classified as non-mated if the bursa copulatrix lacked a spermatophore and the spermatheca was void of spermatozoa.

The scores for abdominal palpation ranged from 43% to 60% in accuracy for females collected during most of the winter season (November to January), but increased to 85% in accuracy for females collected in February (Table 1). To test the significance of abdominal palpation accuracy, a Chi-square test was used to determine if our ability to differentiate a mated female from a non-mated female is better than chance (50-50 accuracy determination). The average accuracy scores (observed) of the three investigators were used to predict the actual mated status (expected) of the monthly samples of females, based on the dissection of the bursa copulatrix for the presence (mated) or absence (non-mated) of spermatophores or spermatozoa. The result of the Chi-square test of monthly samples from November to January was not significant from a 50-50 accuracy determination (X^2 = 5.7; df= 5; p> 0.05), suggesting that the abdominal palpation technique was not reliable in differentiating mated from non-mated females during most of the winter season. With the inclusion of the February scores, however, the results revealed a significant deviation from a 50-50 reliability hypothesis (X^2 = 34.97, df= 6, p=0.01) suggesting that recently mated females can be detected with a high degree of accuracy. Ninety-eight percent of the February females were mated and the abdominal palpation technique detected 85% of the recently mated females (Table 1). Those females that were incorrectly misclassified as "virgin" in the February sample had small or flattened spermatophores within their bursae copulatrices.

Since the abdominal palpation method relied on an investigator's ability to feel a "pea-shaped" bursa copulatrix, the data were sorted to eight weight categories along with their corresponding abdominal palpation accuracy scores (Table 2). A series of Chi-square tests were run, starting with the first two categories, progressively adding additional categories until heavier spermatophores caused a deviation from 50-50. The results revealed that the abdominal palpation method was unable to differentiate swellings of the bursa copulatrix containing the spermatophore weights of the first six categories (0.0 mg to 10.0 mg) from those of non-mated females (X^2= 6.79, df= 5, p>0.05). The inclusion of spermatophores weighing greater than 10 mg, however, resulted in better than a coin toss probability (X^2= 23.7, df= 6, p=0.05).

TABLE 2. The average spermatophore weights (mg ±SD) sorted into 8 categories, along with the corresponding sample size and the accuracy scores of the investigators. The "No spermatophores" category also includes highly degraded ones that could not be weighed. To test the 50-50 determination hypothesis, chi-square tests were run on the first two categories, then categories were progressively added until a significant X^2 value resulted. The spermatophore weight category that deviated from the 50-50 determination was >10≤20 mg, which suggests that a bursa copulatrix with spermatophores of this weight would cause a swelling that can be detected by abdominal palpation.

Spermatophore category (mg)	N	Average Spermatophore weights (mg±SD)	Accuracy %
No spermatophores	139	0±0.0	50.4
> 0 ≤2	36	1.2±0.6	37.0
>2 ≤4	36	2.8±0.4	60.2
>4 ≤6	16	4.9±0.6	56.2
>6 ≤8	8	6.9±0.7	70.8
>8 ≤10	8	8.4±0.2	70.8
>10 ≤20	22	15.3±0.1	83.3
>20	15	33.4±13.2	100.0

Spermatophores weighing an average of 15.3 ± 0.1 mg were detected with 83% accuracy, while spermatophores weighing an average of 33.4 ± 13.2 mg had a 100% detection rate (Table 2). With the exception of three females (two from 23 December and one from 17 January), spermatophores weighing >10 mg were all from the February samples.

The percentage of females that were multiply-mated (two or more spermatophores) during the months of November to January ranged from 9.5% to 31.3%. The swelling of the bursae copulatrices of these females could not be differentiated from virgin females because the cumulative weight of their spermatophores was less than 10 mg per multiply-mated female.

DISCUSSION

Based on the accuracy scores, the abdominal palpation method was subjective and only as reliable as a coin toss during most of the overwintering period (November through January). The subjective nature of the method may be attributed to its reliance on tactile senses to differentiate the enlargement of bursae copulatrices of mated females from non-mated females. The weights of spermatophores recovered from overwintering females from 7 November 1999 through 17 January 2000 (n=240) averaged from 1.1 mg to 2.7 mg (range 0 – 16.7 mg). At these weights, the presence of single or multiple spermatorphores within the bursa of mated females did not cause a discernable swelling that could be differentiated tactilely from a non-mated female. Chi-square tests showed that we were not able to accurately detect spermatophores less than 10 mg in mass (Table 2). Van Hook (1999) reported that her use of abdominal palpation was 85% accurate in detecting old spermatophores in 32 females collected in March 1997 from the Sierra Chinqua overwintering site in Michoacan, Mexico. The masses of the spermatophores were not reported. Van Hook (1999) also reported that spermatophores in butterflies in her study deteriorated at a slow rate as compared with rates reported by Oberhauser (1992) for summer breeders. We were able to detect spermatophores with masses between 10 and 20 mg with 83% accuracy (Table 2). The old spermatophores detected by Van Hook could have been within this size range since her March 1997 samples had not yet begun their active migration activities, which could cause a more rapid degradation of the spermatophores.

We were able to accurately detect the larger, fresh spermatophores in recently mated females. Ninety-eight percent of the 7 February 2000 sample were mated and had spermatophores averaging 20.6 mg (range 1.8 – 52.7 mg), 85% of which we accurately detected (Table 1). Spermatophores weighing > 20 mg (ave. 33.4±13.2) were detected 100% of the time (Table 2). Van Hook (1999) reported that abdominal palpation was 95% accurate in detecting fresh spermatophores for butterflies sampled between 15 January and 25 March 1985 from the Sierra Chinqua overwintering site in Michoacan, Mexico. Females surveyed were collected during the mating activity period prior to their spring migration. Like Van Hook, we misclassified five February females (12.5%) as being non-mated. These females contained "old" spermatophores from earlier matings. Monarch butterflies at California overwintering sites may transfer smaller spermatophores (Frey 1999) than butterflies in Mexico and this may account for the lower accuracy in our study.

Oberhauser (1992) estimated that the rate of spermatophore degradation within the bursa copulatrix was 3.3 mg/day. We believe that a spermatophore needs to be a certain critical weight before its presence within the bursa copulatrix can be consistently and accurately felt. Consequently, spermatophores weighing less than 10 mg would have a 50-50 chance of being detected using the abdominal palpation method. The mass of spermatophores recovered from females of the February samples ranged from 11.2 mg to 52.7mg. Using Oberhauser's estimated rate of degradation, the smaller spermatophore would not be reliably detectable after a day and the larger one after ≈ 15 days. Van Hook (1999) found that the rate of degradation was much slower and reported that the spermatophore remained in the freshly mated category for 10 to 11 days. Van Hook, however, hypothesized that the rate of spermatophore degradation would be faster under field conditions where the females are actively flying, feeding, ovipositing and/or migrating to their winter sites.

Since the proportion of mated to non-mated females collected and analyzed during this and an earlier study (Leong *et al*. 1995) remained statistically unchanged during most of the overwintering period, we believe that the majority of the spermatophore contents are broken down and absorbed into the female's system within 15 days of being deposited in the bursa copulatrix. What persists, at least for mated, overwintering females at California winter sites, is the chitinous neck and/or deflated sacs containing remnant amounts of the spermatophore materials. Of the 280 bursae copulatrices examined, 8 had no recoverable neck and/or sac of the spermatophore. The only evidence of mating in these females was the presence of spermatozoa in the their sperm receptacle. All females with "old" spermatophores had spermatozoa in their

sperm receptacles. While Van Hook (1999) did not look for spermatozoa, she noted that 31% of all females in her samples had mated long before the mass mating period in February, which is similar to our findings.

With the exception of three females, the weights of the spermatophores recovered from California overwintering females (November to January) fell below the minimal mass of 10 mg, and it is not surprising that our scores did not differ from 50-50 reliability. Significantly, the three females with spermatophores >10 mg likely were mated during the winter period prior to the mass spring mating period, and comprised just 1% (3 of 240) of the females collected and examined. This low level of mating supports the hypothesis by Hill *et al*. (1976) and observations by Tuskes & Brower (1978) that mating among butterflies in California is rare or infrequent during the winter months and does not significantly influence the proportion of mated to non-mated females within the overwintering population. Tuskes & Brower (1978) reported that 16% of females were mated upon arrival at the winter site but their determination was based on abdominal palpation and was not confirmed by examining the females for spermatophores or for spermatozoa. We believe that their estimate was lower than the actual numbers of mated females because of their inability to differentiate between the bursa swellings of mated females with spermatophores <10 mg from non-mated females. We found that 40% of the females were mated and our results agreed with an earlier study (Leong *et al*. 1995) where 51% of bursae copulatrices of the arriving females had spermatophores.

Van Hook (1999) determined levels of polyandry by counting the number of spermatophore stems extending out of the ostium of the bursa using a dissecting microscope (10–30X). We found examination of the bursa ostium for the presence of the spermatophore (thread) stem impractical and difficult under field conditions using a 10–20X hand lens because it required finding the spermatophore (thread) stem extending out of the bursa ostium in the dark recess of the female's genital orifice. In contrast to the findings of Van Hook (1999), in which the stem extending from the ostium bursa opening was undetected in only 6% of mated females, we were unable to find the stem in more than 20% of confirmed mated females. We therefore conclude that inspection for spermatophore stems without dissection in California overwintering monarchs may greatly underestimate the number of mated females surveyed.

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