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ADAPTIVE SIGNIFICANCE OF PREVIOUSLY MATED MONARCH BUTTERFLY FEMALES (*DANAUS PLEXIPPUS* (LINNEAUS)) OVERWINTERING AT A CALIFORNIA WINTER SITE

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ABSTRACT. In the fall, migrating monarch butterflies are in reproductive diapause when they arrive at winter sites in California. Approximately 1/3 of the overwintering females contain sperm of summer males within their spermatheca. When these females were subjected to conditions that terminated diapause, they were able to produce offspring with near equal fecundity throughout the overwintering period. The mated females also benefited by the added male nutrients to produce eggs when their body energy reserves were low. Spring matings of virgin females are necessary to obtain spermatozoa to fertilize their eggs and possibly to receive male nutrients to supplement their energy reserves. Several possible survival advantages of early mating are: (1) previously mated females are better able to withstand the rapid ovarian development towards the end of the overwintering season than virgin females; (2) females need not mate again to insure the fecundity of her eggs; and (3) mating with previous summer males broadens the genetic plasticity of the species.

Additional key words: Diapause, fecundity, multiple mating, spermatheca, sperm

In North America, the monarch butterflies, *Danaus plexippus* (Linneaus), have evolved an adaptive strategy for utilization of larval host plants, the milkweeds (*Asclepias* spp), that grow abundantly over much of the United States and as far north as lower regions of Canada during spring and summer months. In the fall, before the milkweeds die back to their rhizomes for the winter, monarch butterflies east of the Rocky Mountains begin their long distance migration to winter sites located in the high mountains of Mexico while monarchs west of this divide, migrate to selected forested areas along the California coastline (Leong et al. 2004). During the winter, monarchs are found in groves that offer protection against environmental extremes (Leong 1990) and are physiologically different from the summer generation in that they are long lived (5–6 months versus 4–6 weeks) and in reproductive diapause (Herman 1985; Herman et. al 1989; Herman & Tatar 2001).

In California, approximately 30% of the females captured from winter aggregations were mated (contained spermatophores) and this percentage remained statistically unchanged through most of the overwintering months until just prior to the spring migration (Leong et. al. 1995, 2008). Since overwintering butterflies are in reproductive diapause upon emergence (Herman & Peng 1976; Herman & Tatar 2001), the mated overwintering females had to have mated with the previous summer's non-diapausing males prior to the migration to winter sites. Mating between two physiologically different generations can occur because of the temporal overlap of diapausing females with non-diapausing males and the mating behavior of this species. Unlike many danaine species, the monarch male employs comparatively simple mating behavior where male pheromone plays a minor role and

in which "force mating" is common (Boppré 1993). Males locate females visually and capture them in flight (Urquhart 1960; Pliske 1975; Hill et al. 1976; Frey et al. 1998) or while sunning on foliage (Leong 1995). Mating between diapausing females and non-diapausing males is not unique to the monarch butterfly. The autumn morph females of the common grass yellow butterfly, *Eurema hecabe* (Linnaeus) often mated with summer non-diapausing males (Kato 1986). A similar mated condition may exist among monarch females migrating to the Mexican overwintering sites because "old" spermatophores were recovered within the bursa copulatrix of females in spring (Van Hook 1999).

The purpose of this study was to investigate the adaptive significance of previously mated overwintering females by comparing the fecundity, fertility and longevity of these mated females with virgin females under laboratory conditions.

METHODS AND MATERIALS

Winter site. The monarch butterflies were collected during the 2009–2010 overwintering season from the Pismo North Beach winter grove, located in the southeastern section of Pismo State Park, Pismo Beach, San Luis Obispo County, California (35°07'46" latitude; 120°37'53" longitude). The site consists mainly of blue gum trees, *Eucalyptus globulus* Labillardiere, few Monterey cypress, *Cupressus marocarpa* Gordon, and willow, *Salix* sp. on the northern edge and Monterey pines, *Pinus radiata* D. Don, on the southeastern corner. The grove (180 × 120 m) supports little to no understory beneath the eucalyptus canopy and slopes downward northwest toward a small creek.

Collection and laboratory rearing. Twenty female monarch butterflies were randomly collected from their

winter aggregation during the early morning hours (0700–0730 h PST) every 21 d starting on 6 November 2009 until 1 February 2010. The butterflies were placed in a large paper bag and stored in a cooler for transportation to the laboratory. The laboratory is located in the Biological Sciences Department, California Polytechnic State University, San Luis Obispo, California.

In the laboratory, the butterflies were initially placed in a cool room (10°C) to lower their body temperature so that they could be more easily handled. Each butterfly was tagged by writing a sequence number on the right hind wing with a marking pen and placed in a 18.8 liter ice cream container, one female per container, with the top open and covered with a plastic house screen (36 cm^2). A door "flap," 10 cm square, was cut on the bottom side of the cage. The door "flap" was secured to the side of the cage with tape.

Diapause was terminated by keeping the butterflies under laboratory conditions (temperature 20.6°C ± 3.2 SE; RH = 56.5 ± 6.2 SE) with continuous light. Lights were standard ceiling fluorescent lights approximately 2 meters above the containers. A honey-water mixture in 200 ml plastic tissue culture dishes with 1 mm perforations on top was provided and changed every 3 d. These conditions have been used previously to successfully rear several generations of monarch butterflies from eggs to adults. The butterflies were misted daily to provide water for them to imbibe. A bouquet of milkweed, *Calotropis gigantean* (L.), leaves was placed in each cage and changed every 2 d. Number of eggs laid and the emerging neonates were recorded daily for each female for a 21-d period.

Upon death or when killed at the termination of the 21 d period, the female's abdomen was removed, immersed in insect Yeager's ringer solution, and examined under a dissecting microscope (60–100X).
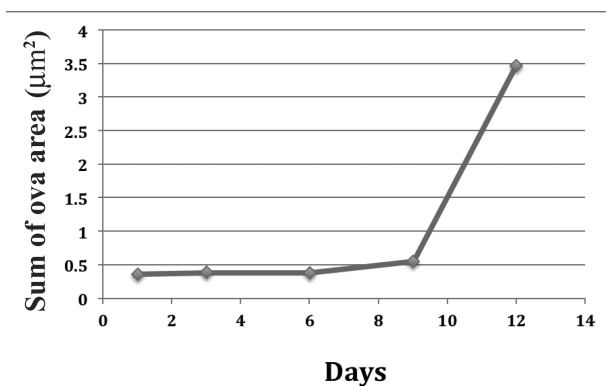

FIG. 1. The sum of the average area of the three largest ova measured with a compound microscope (100X) over a 12-d period after terminating diapause.

Each female was dissected and observed for the presence of spermatophores and sperm within the spermatheca. A female was considered to be virgin if it lacked spermatophores within the bursa copulatrix, and if sperm was not found within the spermatheca. Females were considered to be mated if spermatophores or sperm were detected.

To determine the rate of ovarian development under laboratory and 24 h light conditions an additional 15 females were collected on 27 November 2009. After holding intervals of 1, 3, 6, 9, and 12 d, three females were randomly selected per interval, killed and their left ovary dissected, placed on a glass slide and examined with a compound microscope (100X). The length and width of the three largest ova within one of the four ovarian strands were measured to the nearest 0.01-micrometer and the data presented as the sum of the product of these 2 measurements.

Statistical analyses. Data were analyzed using the statistical program of Biostat 1 (Pimentel & Smith 1990) for analysis of variance (ANOVA). Data that did not satisfy the assumption of ANOVA were subjected to nonparametric tests (chi-square and randomization test).

RESULTS AND DISCUSSION

The sample size for each collection date varied, due to errors in the number of females collected and to butterflies escaping from cages. The resulting sample sizes for 6 and 27 November and 18 December were 18, and for 8 January and 1 February were 19.

The proportion of non-mated to mated overwintering females collected from winter aggregations from November to January was 3:1 and remained statistically unchanged through most of the winter season (Table 1. X^2 = 4.02; df = 3; p=0.259). This relationship agreed with earlier investigations of California winter populations where mating among overwintering butterflies is infrequent (Hill et al 1976; Tuskes & Brower 1978; Leong et al. 1995, 2008). Mating among overwintering monarch butterflies in California is generally limited to the last few weeks prior to their spring migration. The inclusion of the 1 February (last) sample with the November to January data resulted in a deviation from the 3:1 proportional relationship between the non-mated to mated females (Table 1. X^2 = 17.10; df = 4; p> 0.01). All of the females in the February sample were mated which agreed with the previously reported truncated mating activity period prior to their spring dispersal (Frey et al.1998; Leong et al. 1995).

Under laboratory conditions where diapause was terminated, the longevity of virgin females collected

TABLE 1. Proportion of non-mated to mated individuals collected from winter aggregations from November 2009 to February 2010.

Collection date	Total/mated females	% mated
6 Nov	18/5a	28
27 Nov	18/10a	56
18 Dec	18/6a	33
8 Jan	19/11a	61
1 Feb	19/19b	100

Proportions within a column with different letters deviated significantly from a 3:1 ratio ($X^2 > 0.01$).

TABLE 2. Average longevity (days) of mated and virgin females fed honey water mixture, sprayed daily with water mist, and held under laboratory conditions (20.6 °C ± 3.2 SE, RH 56.5 ± 6.2 and 24 h light).

Collected	Mated	days ± SE	Virgin	days ± SE
6 Nov	5	14.8a ± 3.56	13	13.3a ± 0.8
27 Nov	10	14.9a ± 1.6	8	12.9a ± 2.5
18 Dec	6	12.3a ± 3.5	12	13.0a ± 2.2
8 Jan	11	17.5a ± 1.8	8	5.2b ± 1.5
1 Feb	19	11.1a ± 1.7		

Means within a column followed by a different letter were significantly different ($P > 0.01$).

TABLE 3. Number of virgin females collected from winter aggregations laying eggs under laboratory conditions.

Collected	N	No. laying eggs	Eggs laid (mean ± SE)
6 Nov	13	4	5.5 ± 1.3
27 Nov	8	6	17.5 ± 6.6
18 Dec	12	2	1.5 ± 0.5
8 Jan	8	0	0

TABLE 4. Number of eggs laid, hatches and % fecundity of mated overwintering females under laboratory conditions.

Collection date	No. females laying eggs	No. Eggs laid (mean ± SE)	No. Hatches (mean ± SE)	% Fecundity
6 Nov	4	139.0 ± 49.34	122.7 ± 55.26	88.3
27 Nov	5	71.2 ± 14.31	69.6 ± 12.21	90.4
18 Dec	3	102.3 ± 17.90	69.0 ± 13.58	67.6
8 Jan	10	117.1 ± 29.10	75.8 ± 14.58	64.8
1 Feb	10	142.2 ± 49.56	114.2 ± 39.12	80.3

from November to December was similar, ≈13 d, but was significantly shorter, 5.2 d ± 1.4 SE (F = 4.59; df 3, 37; p=0.008; Table 2) for those collected in January. In contrast, longevity among mated females showed no such decline (Table 2). Reduced longevity in January among the virgin females may be attributed to the demands of ovarian development when body energy reserve is low (Wells et al. 1990). The egg development of overwintering females under laboratory conditions showed little change for the first 6 days but began to increase geometrically starting by the 9th day, and by the 12th day the ovaries were filled with fully developed eggs with chorion (Fig. 1). Death occurred at an average of 5.2 days that corresponded well to the transitional period of rapid ovarian development. The marked decline in the survival in January among the virgin females suggests that under laboratory conditions favoring ovarian development, the amount of fat (energy) reserve remaining in these females (Wells et al. 1990) was not enough to accommodate both the full ovum development and survival.

Under field conditions, virgin females may be able to survive with low fat reserve in January because temperatures that induce ovarian development are not constant. The females can conserve their body energy and delay ovarian development by returning to the winter grove where temperatures are cool, thereby lowering their metabolic rate. In the spring or just prior to their migration, the available evidence suggests that they must mate not only to fertilize their eggs but also to gain added nutritional energy from the males (Boggs & Gilbert 1979; Boggs 1981; Marshall 1982; Shapiro 1982; Oberhauser 1989). Previous mated overwintering females, on the other hand, have the nutritional advantage to survive the winter and perhaps to compete better for mates than virgin females in the spring.

Virgin females collected from their winter aggregations in November and December were able to lay infertile eggs (1 to 45 eggs/female; Table 3) under laboratory conditions. None of the virgin females collected in January produced eggs. Seven of 8 females in the January sample died about 5 days after being collected from the winter grove and placed under laboratory conditions that would break diapause. Their deaths seem to correspond well to the interval prior to rapid maturation of eggs within the ovaries (Fig. 1), suggesting that their premature deaths were attributed to physiological demands of ovarian development when their body energy reserves were low. The sole survivor did not lay eggs, possibly due to the lack of sufficient body energy reserves.

Since one-third of the overwintering females were mated when they arrived at the California winter site

and winter matings were infrequent, the fertility (reproductive ability, i.e. eggs laid) and fecundity (number of offspring) of these females collected from November to January were the product of earlier mating with summer males. The average number of eggs laid (F = 0.89; df = 3, 18; p = 0.53), hatches (F=1.33; df = 3,18; p=0.29) and % fecundity (Rant test F = 1.01; p = 0.40) during this period were not significantly different (Table 4), indicating that overwintering females were able to store viable spermatozoa for more than four months and produced fertilized eggs with equal fecundity. This long-term storage of viable sperm in monarch butterflies has not been previously reported. The physiological aspect of storage and the maintenance of the viable sperm within the female's spermatheca should be investigated to determine if seminal fluids from the male (Shapiro 1982; Tram & Wolfner 1999; Gillot 2003; Baer & Schmid-Hempel 2005; Poiani 2006; den Boer et al 2008), spermathecal gland secretions (Filosi & Perotti 1975; Pitnick et al. 1999; den Boer et al. 2008), or a combination of the seminal fluid and spermathecal fluids (Baer et al. 2009) are involved in keeping the sperm viable.

The February sample of females deviated significantly from the 3:1 proportion of non-mated to mated females (Table 1. X^2 = 17.09; df = 4; p=0.002) because all were mated. The average number of eggs laid (F = 0.44; df = 4, 27; p=0.78), hatches (F = 0.72; df = 4, 27; p=0.59), and fecundity (Randomization test; F = 1.48; p = 0.26) did not, however, differ significantly from earlier samples of mated females. Although differences were not detected, the reduced fecundity of approximately 65% of the December and January samples (Table 4) suggests that the viability of the stored spermatozoa in fall-mated females was diminishing.

Mating occurred earlier this season due to mild January temperatures. The February sample may reflect an early mating behavior of California monarch butterflies. Prior to the monarch's spring migration, the monarchs at California winter sites undergo a short intense mating activity period where males capture females in flight or while they sun on foliage. Multiple mated females are common. Leong et al. (1995) reported that 80% of the females collected during the latter phase of this mating period had 2+ spermtophores within their bursa copulatrix. Although all of the females of the February sample were mated (Table 1), only 1 of 19 was multiple mated. Assuming mating is random in a winter population consisting of 3 virgin to 1 previously mated females, the expected number of multiple mated females in the February should have been 6 out of 19. The low frequency of multiple mated females in the February sample suggests that virgin females were

mated before the previously (fall) mated females. Another possibility is that the previously mated females had already left the winter grove.

We believe that the 1 February sample was taken during the initial phase of the mating activity period. Another sample on 8 February was unsuccessful because few butterflies remained and were clustered at heights too high to be captured by our 25 ft extension net.

This study revealed several possible survival advantages to mating in late summer. Firstly, the nutrients associated with the male's spermatophore improve the female's winter survival and possibly contribute towards maintenance of viable spermatozoa. Mated females are better able to withstand the physiological stresses of rapid egg maturation toward the end of the overwintering season than the virgin females, when their body lipid levels are at the lowest (Wells et al. 1990). A similar circumstance may exist for the females overwintering in Mexico since Van Hook (1999) reported finding many "old" spermatophores in overwintering females. This added survival advantage may also play a significant role for females overwintering in Mexico because they undergo a longer period of reproductive diapause (Herman et al. 1989).

Secondly, previously mated females need not mate again to secure fecundity and possibly begin spring migration earlier to be the first to exploit re-emerging milkweeds. Kato (1986) reported that the mated overwintering pierid butterfly, *Eurema hecabe*, began laying eggs in the spring without remating. By comparison, virgin females require spring mating to fertilize their eggs.

Thirdly, earlier mating connects the genes of late summer and the overwintering generations and thereby broadens genetic plasticity of the species, although numbers of offspring resulting from spermatozoa of the late summer males has not been determined. How many of the offspring will be sired by spermatozoa of the late summer males is yet to be determined.

By mating with females before they arrive at the overwintering site, males from the summer generation avoid competition with other males for females in the spring and the subsequent competition among spermatophores of different males within the bursa copulatrix for alignment with the ductus seminalis, the narrow pathway the spermatozoa must travel to the spermatheca (Drummond 1984; Solensky & Oberhauser 2009a, b). Once the spermatozoa gain entry into the spermatheca, however, mixing (Solensky & Oberhauser 2009a, b) and competition among the spermatozoa of different mating may occur before successfully uniting with the ova.

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