INTRODUCTION

Organisms living in temperate regions face seasonal challenges, such as absence of food, harsh winter conditions, and the need to synchronize reproduction with suitable conditions for breeding. Insect adaptations to these challenges include migration, dormancy, and seasonal phenotypic variation; various combinations of these traits constitute a diapause syndrome (Andrewartha 1952; Tauber et al. 1986; Danks 1987; Leather et al. 1993). Diapause is a state of arrested development, characterized by low metabolic activity, reduced motor activity, and increased resistance to environmental extremes, and is usually hormonally controlled in insects (Nijhout 1994). Diapause can occur during any insect life stage; “reproductive diapause” refers to delayed reproductive development in the adult stage.

The course of development during diapause includes changes in the insect’s physiology and sensitivity to environmental stimuli against a backdrop of seasonal changes. After the onset of diapause, there are three steps: intensification, maintenance, and termination. These steps are referred to as “diapause development” (Tauber et al. 1986). Diapause development either follows a predetermined course not influenced by external cues, or terminates in response to external stimuli. Hodek (1983) refers to these pathways as “horotelic” (Greek hora = right time, telos = fulfillment) and “tachytelic” (Greek tachys = quick), respectively. The pathways may operate singly or together. The rate of diapause development is often driven by temperature, but other stimuli include photoperiod, food, moisture, parasitoid-host interactions, and mating (reviewed in Tauber et al. 1986; Danks 1987).

In reproductive diapause, once diapause development is complete, characteristics such as reduced metabolism and cold-hardiness disappear, and the insect is capable of reproducing. If prevailing environmental conditions are not suitable for reproduction, the insect may remain dormant, in a postdiapause “quiescence” (Tauber et al. 1986).

Many researchers have noted a lack of reproductive activity and undeveloped reproductive organs in overwintering monarchs, and referred to this as “reproductive diapause” (Urquhart and Urquhart 1976; Brower et al. 1977). Herman (1981) reported a period of minimal reproductive tract development in monarchs collected in Californian overwintering sites from September to December even after they were incubated under conditions that are normally favorable to reproductive development (16-h:8-h light-dark cycle; 25°C), clearly demonstrating the refractory period necessarily associated with diapause. Subsequent work comparing California and Mexico overwintering butterflies revealed qualitatively similar diapause patterns, with longer diapause in the eastern population (Herman et al. 1989). More recent work in Mexican overwintering sites followed male reproductive development and mating strategies (Van Hook 1993, 1996). Van Hook identified significant variation in the degree of male diapause and suggested that this leads to multiple mating strategies in the overwintering colonies. In Australia, monarch ovarian dormancy is more accu-
rately classified as “oligopause,” an intermediate condition that lacks the refractory period associated with diapause (James 1982).

Goehring and Oberhauser (2002) reported on environmental factors influencing the induction of monarch diapause. Here, we report studies on the effects of photoperiod, access to milkweed, and mating on diapause completion and postdiapause ovarian development in eastern-population females.

METHODS

Collection from the overwintering colony

We collected adult monarchs from the Arroyo Barranca Honda colony in the Sierra Chincua, located in the transvolcanic mountains of Central Mexico, during the 1996 and 1997 overwintering seasons. In both years, we collected during a time of increased activity (e.g., flying, drinking, nectaring, and mating), but before mass dispersal for spring migration. We collected 324 females and 72 males on 28 February 1996, and 300 females and 100 males on 5 March 1997. These dates roughly correspond to the average start of the mass mating period (Van Hook 1996).

We sampled butterflies from three hypothesized subpopulations—roosting, mating, and active—following Van Hook (1996). Roosting butterflies were collected from trees in the center of the colony. These butterflies were hanging immobile on tree trunks or branches 3.5 to 6 m from the ground. Mating butterflies were collected in copula, also in the colony center. Active butterflies were collected in flight in a clearing or near a stream downhill from the colony. Collections from the three populations were made at the same time. In 1996, equal numbers of adults were collected from each subpopulation. In 1997, to ensure male mating readiness, all males were collected in copula. In 1997, the nucleus of the colony moved downhill on the day prior to collection, possibly mixing subpopulations.

We marked butterflies at the time of collection and then placed them in glassine envelopes. Mating pairs were stored together in the same envelope and allowed to separate on their own. Butterflies were kept on ice in a cooler and transported to St. Paul, Minnesota, within 3 days of collection.

Dissections and general measurements

We measured butterfly mass, wing length, and wing condition (scale loss and tatter along the edges) immediately on arrival in Minnesota. We assessed scale loss on an ordinal scale from 1 to 5 (no loss to severe loss) and wing edge tatter on an ordinal scale from 0 to 4 (no wing tatter to all 4 wings tattered). Scale loss may approximate age, and wing tatter may indicate distance traveled, damage from mating struggles, or encounters with predators (Frey and Leong 1995; Van Hook 1996; Borland et al., this volume).

Females in reproductive diapause have small ovarioles with no ovarian development (Herman 1973). We examined ovaries for the presence of unyolked, yolked, and mature (chorionated) oocytes, and counted mature oocytes, if present (for details on dissection methods, see Oberhauser and Hampton 1995). Although we measured and report all of the stages in diapause development, we used the presence of mature oocytes as the indication that diapause was completed.

We determined mating status by examining the contents of the bursa copulatrix for spermatophores and spermatophore remains (Van Hook 1996). Thus we had two indicators of mating: mating activity at the time of collection (i.e., “mating” if collected in copula) and mating status found at dissection (i.e., “mated” if female had one or more spermatophores).

Experimental design

To establish a baseline on degree of ovarian development and mating status at the time of collection, we dissected a random sample of females from each activity category (active, mating, and roosting) (1996 \( n = 20 \) each category; 1997 \( n = 16 \) each). These butterflies were dissected on day 0 of the experiments. Remaining butterflies were used in environmental chamber experiments designed to test the hypothesis that exposure to certain environmental cues (host plant access, increasing daylength, and mating) will stimulate postdiapause ovarian development.

1996 Experiment: Host plant and daylength

In 1996, we tested the effects of photoperiod and access to milkweed on postdiapause reproductive development. Butterflies in the overwintering colony experience a daylength of 11 h and 45 min at the end
of February; this increases with time and northward movement. By early April, monarchs in the southern United States (approximately 30° north latitude) experience 12 h and 30 min of daylight, an increase of 45 min or a gain of over 1 min/day. To simulate these changes, we used programmable timers on standard shop fluorescent fixtures with 40-watt fluorescent tubes to control daily light-dark cycles, increasing the amount of daylength 2 min/day. To test the effect of access to milkweed on ovarian development, we used potted *Asclepias curassavica* in host plant treatments and potted *Cassia fasciculata* (partridge pea) in nonhost plant treatments.

We used a $2 \times 2$ factorial design, testing increasing daylength (12-h:12-h light-dark +2 min/day) versus constant daylength (12-h:12-h light-dark), and access to milkweed versus no access. We used two controlled-environment growth chambers ($4 \times 3 \times 2$ m), each subdivided with black opaque (157 μm) plastic sheeting to allow two light treatments in the same chamber ($2 \times 3 \times 2$ m each). Milkweed and nonmilkweed treatments were in separate chambers.

Sixty females (20 randomly selected from each activity category) and 12 males were put in each treatment, divided evenly into two net cages ($0.75$ m$^3$). We kept temperatures at 24 ± 1°C, which is suitable for ovarian development (Barker and Herman 1976; Malcolm et al. 1987). Adults were fed ad libitum from sponges soaking in honey-water (20% honey). Relative humidity was 59 ± 3.3% in the milkweed chamber and 60 ± 2.7% in the nonmilkweed chamber.

To assess ovarian development, we randomly selected one fourth of the females from each activity category on days 1, 3, 7, and 12 for dissection.

1997 Experiment: Host plant and mating

After observing an ad hoc effect of mating on ovarian development in 1996, we explicitly tested the effects of mating in 1997. We omitted the photoperiod treatment after observing no effect of this independent variable but included a host plant access variable. We conducted a $2 \times 2$ factorial experiment, with access to males versus no access to males, and potted *Asclepias physocarpa* for host plant treatments versus potted *Leucanthemum x superbum* (chrysanthemum) in nonhost plant treatments. Temperature and humidity were the same as in 1996, and the photoperiod was 12-h:12-h light-dark.

We used 63 females in each treatment (21 randomly selected from each activity category) and 40 males in mating treatments. To keep cage densities relatively even, we divided mating treatments into two cages. We recorded mating daily. Butterflies were fed as before, and temperature and humidity were similar to those in the previous experiment. On days 2, 4, 6, and 8, we dissected one fourth of the females from each activity category. We dissected these as before with the exception of more precise examination of the bursa copulatrix to count the number of spermatophores present.

**Statistical analyses**

We assessed postdiapause development by the presence or absence of mature oocytes (a yes/no, or binary, variable) and used stepwise analysis of deviance and logistic regression models to examine the relationship between oocyte presence and the independent variables of subpopulation, butterfly size and wing condition, mating status, and experimental treatment (Hardy and Field 1998; K. Chaloner, pers. comm.). Probabilities of 0.05 or less indicate statistical significance.

**RESULTS**

**Baseline condition**

Figure 23.1 shows the degree of ovarian development in females before the experimental treatments. In both years, most females had undeveloped ovaries at the time of collection: 70% in 1996 and 92% in 1997. Ovarian development differed between activity category as well as between years. Females collected *in copula* were more likely to contain oocytes than roosting or active females, and fewer females showed any degree of ovarian development in 1997 even though we collected them 5 days later that year.

In both years, many females were mated at the time of collection (figure 23.2): 54% in 1996 and 25% in 1997 (spermatophores received during the mating in progress for females collected *in copula* were not included in the data illustrated in figure 23.2). The proportion of females that had mated previously differed between activity categories, as did mating frequency. Butterflies collected *in copula* were more likely to have mated previously and more frequently in both years.
Figure 23.3 shows the degree of ovarian development for baseline females by mating status at the time of collection, with all activity categories combined. Given the small degree of ovarian development, there is not sufficient power to test for a relationship between mating status and ovarian development. However, there is an association between mating status and the presence of any oocytes. In 1997, mated females were significantly more likely to show some degree of ovarian development than nonmated females. This relationship was almost statistically significant in 1996 (figure 23.3).

We tested for relationships between wing condition and the degree of ovarian development; given the minimal ovarian development in 1997, we analyzed only 1996 baseline data. There is a suggestion that females with more scale loss had more developed ovaries, although this relationship was not statistically significant (correlation coefficient $r_s = 0.1612$, $t = 1.24$, $df = 58$, $p = 0.11$). There is a significant correlation between wing tatter and ovarian development (correlation coefficient $r_s = 0.3829$, $t = 3.16$, $df = 58$, $p = 0.001$). Wing tatter was not greater than expected in mated females (Kruskall-Wallis [KW] statistic $H = 1.99$, $df = 1$, $p = 0.158$) or greater with increased mating frequency (KW statistic $H = 4.85$, $df = 4$, $p = 0.435$).

Postdiapause reproductive development

In the following description, it is important to distinguish different mating categories. In 1996, all
Experimental females were kept in cages with males. However, they did not all mate during the experiment. In 1997, half of the experimental females had access to males (the mating treatments), but not all females in these treatments actually mated. In addition, since females were randomly assigned to treatments, some females in all treatments had mated before they were collected (their mating status).

Photoperiod and host plant access

Figure 23.4 illustrates the progression of ovarian development for 1996 females. Across all treatments, 59% (36/61) of the females had some degree of ovarian development after 1 day, compared with 30% (see figure 23.1) of the females from the baseline group. By days 7 and 12, over 77% and 100% had mature oocytes, respectively. Although our experimental design did not explicitly test for an effect of mating, we determined whether females had mated when we dissected them and included their mating status as a predictor in our model. Table 23.1 summarizes the analysis of postdiapause ovarian development, as measured by the presence of mature oocytes. In addition to the experimental variables of photoperiod and host plant access, we tested the effects of activity at the time of collection, mating status, mass, and wing length. The most parsimonious model for predicting mature oocyte presence includes days in treatment, mating status, and access to milkweed. The odds of full reproductive development in mated females, given access to milkweed, are 11 times the odds for unmated females. The odds of reproductive development in females with access to milkweed, given having mated, are 5 times the odds for females without access to milkweed. There were no significant effects of photoperiod, activity at collection, mass, or wing length.

Host plant access and mating

Figure 23.5 shows the progression of ovarian development in 1997. A greater proportion of females with access to milkweed and males developed mature oocytes than did those without access to milkweed or males. A large proportion of those in treatments without milkweed or males were still undeveloped 6 days into the experiment, and the majority contained only yolked oocytes by the end of the experiment. Other treatments reveal intermediate patterns of ovarian development.

Table 23.2 summarizes the analysis of postdiapause ovarian development in the 1997 experiment.
We tested the effects of access to milkweed and males, activity at the time of collection, mating status, mass, and wing length. The resulting model includes days in treatment, access to milkweed, mating status, and activity at the time of collection. Figure 23.6 shows the proportion of females with mature oocytes over time by milkweed treatment and accession.

Access to milkweed had the greatest effect on postdiapause ovarian development, followed by mating status and activity at the time of collection. Figure 23.4. Degree of ovarian development over time in each treatment of photoperiod and host plant experiment (1996).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>SE</th>
<th>p value</th>
<th>Log-odds</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-26,213.0</td>
<td>3,895.9</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days in treatment</td>
<td>0.7383</td>
<td>0.110</td>
<td>&lt;0.001</td>
<td>2.18</td>
<td>1.74–2.73</td>
</tr>
<tr>
<td>Access to host plant</td>
<td>2.919</td>
<td>0.459</td>
<td>&lt;0.001</td>
<td>19.60</td>
<td>7.79–49.35</td>
</tr>
<tr>
<td>Mated</td>
<td>1.826</td>
<td>0.399</td>
<td>&lt;0.001</td>
<td>6.42</td>
<td>2.88–14.3</td>
</tr>
<tr>
<td>Activity at time of collection</td>
<td>1.155</td>
<td>0.418</td>
<td>0.006</td>
<td>3.17</td>
<td>1.40–7.21</td>
</tr>
</tbody>
</table>

**Note:** Summary of binomial regression model for 1997 experiment. SE, standard error; CI, confidence interval.
mating status. Most unmated females without access to milkweed remained undeveloped.

These variables are not independent. Access to milkweed has an effect on the probability of mating within the experiment. In treatments with males, more females mated when milkweed was present than when it was not present: 55 (89%) of 62 and 43 (72%) of 60, respectively, \( \chi^2 = 5.6, p = 0.018 \). They also mated more frequently (table 23.3, KW statistic \( H = 20.66, p < 0.001 \)). Although some matings occurred prior to collection, the frequency of mating prior to collection was not different between milkweed and nonmilkweed treatments (25% and 23%, respectively, \( \chi^2 = 0.11, p = 0.741 \)).

Table 23.3. Mating frequency in milkweed versus nonmilkweed treatments

<table>
<thead>
<tr>
<th>No. of spermatophores</th>
<th>Milkweed treatments</th>
<th>Nonmilkweed treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>&gt;3</td>
<td>15</td>
<td>1</td>
</tr>
</tbody>
</table>

*Note: Only females in treatments with males are included.*
DISCUSSION

Diapause development is a progressive physiological process. While diapause induction is initiated in response to particular cues (e.g., Goehring and Oberhauser 2002), diapause development typically follows a predetermined course not necessarily terminated by specific cues (Tauber et al. 1986). The progression may be influenced by external factors that speed the completion of diapause, and response to these factors often varies as a function of diapause intensity. Given the wide geographic range and environmental conditions experienced by North American monarchs, diapause intensity is likely to be variable, making characterization of diapause development complex.

Monarchs used in this study were collected during the last third of the overwintering period, a time of increasing mating activity. Mating dynamics change dramatically during the overwintering period, from very little mating in early November to mass mating during the last 6 weeks in the Mexican colonies (Brower et al. 1977; Brower 1985; Calvert and Brower 1986; Van Hook 1996). The onset of mating is quite consistent from year to year; mass mating begins in mid-February and increases until monarchs disperse from the overwintering sites (Van Hook 1996). Although mating marks the end of diapause in males, it does not necessarily signify the same in females because they can be forced to mate. Females with undeveloped ovaries during this period either may be nearing completion of diapause or are in a postdiapause quiescence awaiting suitable conditions for reproduction.

Status of diapause development and mating at the time of collection

In both years, most females showed no ovarian development at the time of collection. Herman and coauthors (1989) reported similar findings for monarchs obtained in March 1983 and 1984 from two Mexican sites. However, we found that oogenesis had commenced in some females in late February and early March. Females with ovarian development were most likely to be collected while mating (see figure 23.1) or to have mated prior to collection (see figure 23.3), suggesting an association between ovarian development and mating. However, 15% of unmated females in 1996 showed ovarian development, indicating that mating is not required for postdiapause development.

The fact that more females in our samples had produced oocytes in 1996 than in 1997 suggests that females were further along in postdiapause development in 1996, even though they were collected earlier. Previous work has shown that the course of diapause is primarily influenced by temperature (Hodek 1983; Tauber et al. 1986; Danks 1987); unfortunately temperature data were not available for comparison.

Postdiapause ovarian development in experimental treatments

Ovarian development progressed rapidly under experimental conditions. While a significant proportion of females without access to milkweed or males remained undeveloped, most of those exposed to milkweed and allowed to mate developed mature oocytes within 3 days (see figures 23.4 to 23.6). This rapid development suggests that females were in a state of postdiapause quiescence (Tauber et al. 1986) awaiting stimuli to activate and accelerate postdia-
pause morphogenesis. Mating and access to milkweed appear to serve as such stimuli.

Given the importance of synchronizing reproduction with host plant availability, it is not surprising that milkweed is an external stimulus for postdiapause development in monarchs. Similarly, the presence of host plant Vigna unguiculata pods stimulate diapause termination in the seed beetle Bruchidius atrolineatus (Lenga et al. 1993; Tran et al. 1993; Glitho et al. 1996), as does host plant presence for the leek moth, Acrolepiopsis assectella (Abo-Ghalia and Thibout 1983).

Although mating has been shown to stimulate the rate of nondiapause oocyte production in monarchs, it is not required for oogenesis (Herman and Barker 1977; Oberhauser and Hampton 1995; for other insects, see review in Barth and Lester 1973). Few studies have assessed the effect of mating on postdiapause ovarian development (review in Danks 1987). In A. assectella, Abo-Ghalia and Thibout (1983) found that along with host plant presence, mating significantly increases the postdiapause mean number of oocytes, and that these factors have a synergistic effect. In our study, mating was associated with postdiapause oogenesis, both in the baseline analysis and in the experimental treatments. However, our results do not allow us to distinguish whether mating stimulates diapause completion or if it just results in faster postdiapause development.

Photoperiod

Although photoperiod has been shown to affect diapause completion, it does not usually affect postdiapause development (e.g., McNeil and Fields 1985; Tanaka and Sadoyama 1997). An increasing daylength, simulating that experienced by northward migrants, did not affect monarch postdiapause ovarian development in our 1996 experiment. Photoperiod may affect diapause termination, but our study did not address that question.

Variation in diapause development

Given the effect of mating on postdiapause ovarian development, it is instructive to consider male diapause development. While the course of insect diapause is influenced by environmental conditions experienced by both sexes, it does not always proceed identically in males and females. In general, it has been postulated that given the greater energy requirement to maintain eggs versus sperm and the smaller metabolic change required to enter diapause in males, male diapause is usually less intense and of shorter duration (Danks 1987). In monarchs, diapause appears to last longer in females than males (Herman 1981; Lessman and Herman 1983; Herman et al. 1989). Wiklund and colleagues (1992) proposed that the difference in the timing of diapause between sexes is an example of protandry, where males are expected to benefit by emerging or being ready to mate before females; Nylin and coworkers (1995) suggested this is the case in overwintering monarchs. Males may complete diapause earlier and begin mating at the overwintering sites to maximize their number of matings.

Diapause termination typically occurs over a considerable time span subject to individual and environmental variation, even in species with synchronous cohorts. Overwintering populations of monarchs are composed of individuals coming from a wide geographic area, subjected to a wide range of environmental conditions. The range of physiological ages and diapause intensities could lead to an even greater span of time for diapause termination. In addition, there are probably complicated relationships among postdiapause development and mating. The fact that females with access to milkweed mated more suggests that females may be more amenable to male mating attempts when they contain mature oocytes.

While the factors that trigger the development of diapause in monarchs remain to be determined, our results suggest that postdiapause reproductive development is strongly influenced by external factors such as host plant availability and mating, serving to synchronize reproduction with the seasonal availability of breeding habitat.

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