A Re-examination of Reproductive Arrest in the Monarch Butterfly, *Danaus plexippus.*

BY

Victoria M. Pocius

Submitted to the graduate degree program in the Department of Ecology and Evolutionary Biology and the Graduate Faculty of the University of Kansas
In partial fulfillment of the requirements for the degree of Master of Arts

_____

Chairperson: Dr. Orley Taylor

_____

Dr. Jennifer Gleason

_____

Dr. Justin Blumenstiel

Date Defended: August 20, 2014

The Thesis committee for Victoria M. Pocius certifies that this is the approved version of the following thesis:

A Re-examination of Reproductive Arrest in the Monarch Butterfly, *Danaus plexippus.*

_____

Chairperson: Dr. Orley Taylor

Date Accepted: 8/20/2014

Abstract

Migratory and overwintering monarch butterflies, *Danaus plexippus*, are observed in a non-reproductive state classified as either reproductive diapause or oligopause. The stimuli that lead to this reproductive condition have been characterized as changes in photoperiod, declining host plant quality, and temperature (Goehring and Oberhauser 2002), and in another study simply as temperature (James 1982). This study was conducted to examine cool temperature as the stimulus for the induction of reproductive arrest and to correctly classify reproductive arrest as either reproductive diapause or oligopause.

Reproductive arrest was studied using monarchs reared in the laboratory. Butterflies were allowed to fly, bask, and nectar freely within screened cages. Cages were kept in temperature controlled growth chambers. Oocyte presence and ovarian development score were used to determine reproductive status. The mean number of mature oocytes was dependent on temperature. Females exposed to a mean temperature of 15°C failed to develop mature oocytes during the course of the experiment. All females held in the moderate temperature treatment (17°C), did not develop any mature oocytes, but many immature oocytes were observed in 50% of the butterflies. Females held in the warm temperature treatment (25°C) developed mature oocytes. Females exposed to the cool temperature treatment three days post eclosion showed similar patterns. Temperature is the main factor controlling reproductive arrest in *D. plexippus.*

A second experiment was conducted to classify reproductive arrest. Reproductive arrest was induced and maintained by exposing females to a constant

12°C temperature regime for either 4 or 8 days. These cold pretreated females did not retain ovarian dormancy after two days of exposure to 28°C temperatures. Pretreated females showed reproductive development similar to control females that were never exposed to 12°C. No observable refractory period was seen in cold-exposed females. Due to the lack of such a refractory period, reproductive arrest in the female monarch is better classified as oligopause, not reproductive diapause.

Acknowledgments

The completion of this work would not have been possible without the experience, guidance, and time of several individuals. First, I would like to thank my graduate advisor, Chip Taylor, for teaching me how to develop and execute a research project independently. I would also like to thank Jennifer Gleason and Justin Blumenstiel for taking time to serve on my committee and providing insightful edits and helpful input over the last two years as I finished each requirement for my masters degree.

I thank Lincoln Brower for being a surrogate advisor and for taking the time to edit and read multiple drafts of this document; his support from Virginia as I completed this degree was invaluable. I appreciate his advice on continuing both my research and career in science.

I thank Ann Ryan and the students at Monarch Watch for their help rearing larvae and pupae for these experiments. Their help was vital when laboratory conditions were not ideal to rear butterflies. Special thanks to Taylor Patterson for his help taking infrared photographs of active monarchs and for analyzing the images in FLIR.

I would like to thank Kaila Colyott, Paula Roy, Desiree Harpel, Lucas Hemmer, Alex Erwin, Sally Chang, and many other graduate students for providing companionship as I encountered problems during the course of both experiments and for enduring hundreds of monarch butterfly facts over the past two years.

I would also like to thank my family for their encouragement to pursue a career that I love and supporting my decision to be an advocate for the monarch butterfly and other integral pollinators.

Completion of this degree was supported by funds from the University of Kansas Department of Ecology and Evolutionary Biology. My research was partially funded through a S.C.I.O.N. grant and additional funds were provided by Monarch Watch. Special thanks to the Ida Hyde Scholarship committee for funding to travel to an insect hormone conference, and to the Entomology Endowment Fund for financial support during my final semester.

Table of Contents

Chapter One

Cool temperatures induce reproductive arrest in the female monarch butterfly, *Danaus plexippus*.

Introduction

Insects that reside in arctic, temperate, and subtropical climates face seasonal challenges that require specific adaptations (Danks 1987, 2004). Extreme seasonal temperatures, lack of water and/or nutrients can limit activity and reproduction (Andrewartha 1952, Danks 2004). Gradual changes in temperature, photoperiod, and nutrient availability or quality can signal oncoming extremes. Sensitivity to these changes in insects, followed by changes in behavior and physiology dictated by selection, has produced a broad array of adaptations including migration, aestivation, and diapause, which can involve any of the life stages in holometabolous insects. Monarch butterflies, the subject of my thesis, combine migration with adult reproductive arrest to survive the winter. My study focuses on the nature of ovarian dormancy in monarchs; this work examines temperature as the environmental stimulus that induces reproductive arrest referred to as both reproductive diapause (Herman 1981, Goehring and Oberhauser 2002), and oligopause by other authors (Herman 1981, James 1982, Goehring and Oberhauser 2002, 2004).

Any combination of migration, dormancy and seasonal polyphenism has been linked to a diapause syndrome, which is a state of arrested development often associated with lowered metabolic activity, reduced motor activity, and increased resistance to environmental extremes (Andrewartha 1952, Tauber and Tauber

1986, Danks 1987, Leather 1993, Goehring and Oberhauser 2002). Arrested development can be induced by external stimuli (Tauber and Tauber 1986, Kostal 2006) and is controlled neurohormonally in insects (Tauber and Tauber 1986, Nijhout 1994); external stimuli almost always precede adverse reproductive conditions and include abiotic components, such as photoperiod, humidity, and temperature (Tauber and Tauber 1986, Kostal 2006).

The monarch butterfly, *Danaus plexippus,* is a tropical butterfly whose distribution has expanded into temperate areas following the range expansion of its host plants, *Asclepias ssp.* (Brower 1985, Masters et al. 1988). Monarchs are not adapted to survive the winter, unlike temperate insects, and undertake an annual long distance migration each autumn to escape the temperature extremes of their North American range (Urquhart and Urquhart 1978). Migrants exhibit many unique characteristics: they fly directionally, exhibit redder coloration, accumulate increased lipid stores, enter reproductive arrest, and display increased longevity (Cenedella 1971, Brown and Chippendale 1974, Urquhart and Urquhart 1978, Herman 1981, James 1983, 1993, Brower 1995, Goehring and Oberhauser 2002, Davis and Garland 2004, Goehring and Oberhauser 2004, Brower et al. 2006, Zhan et al. 2011, Brower 2014). Migrants complete the autumn migration, and overwinter in a state of reproductive arrest, with little to no gonadal development, and delay reproduction until the following spring (Herman 1973, Brower et al. 1977, Herman et al. 1989). Autumn migrants exhibit reproductive arrest because of the cues they received from the environment including decreasing photoperiod, low overnight temperatures, and decreasing host plant quality as larvae, pupae and

freshly eclosed adults (Barker 1976, Herman 1981, James 1982, 1983, 1993, Goehring and Oberhauser 2002).

Hormone levels also play a role in reproductive arrest. Juvenile hormone (JH) is released by the *corpora allata*, and spurs reproductive development when it binds to gonadal tissue receptors (Lessman 1980, Herman et al. 1981, Kort and Granger 1981, Denlinger 2002, Bellés et al. 2005). Three days post eclosion, JH titer increases rapidly in female monarchs; potential inhibition of this rapid increase has not been examined as a contributor to reproductive arrest (Lessman 1980). The timing of JH release is important because high JH levels lead to gonadal development, ending reproductive arrest. Once a butterfly has begun development, it cannot return to a previous, undeveloped state (Herman 1973, Lessman 1980, Herman 1981).

Photoperiod and temperature influence reproductive development in monarch butterflies. Cool temperature (10°C) and a short photoperiod (9h) have been associated with both low JH titer, and little ovarian development (Barker 1973, 1976, Herman 1981, Goehring and Oberhauser 2002) indicating reproductive arrest. Reproductive arrest in Australian populations is associated with exposure to cool temperatures (<18°C) after adult eclosion (James 1982, 1983, 1993). The environmental stimulus causing reproductive arrest in the eastern migratory population of North America is attributed to multiple factors including decreasing photoperiod, cool overnight temperatures, and decreasing host plant quality (Goehring and Oberhauser 2002, 2004). Given that there are reasons to doubt that photoperiod is a factor since monarchs appear to enter reproductive arrest over a

broad range of latitudes on different dates with decreasing differences in photoperiod length and rates of change in day length per day and due to the complexities of assessing hostplant quality, I chose to determine if temperature alone, as in the studies conducted by James, governed the reproductive status of newly emerged monarchs.

The following studies were conducted to determine if reproductive arrest could be induced and maintained by exposing female monarchs to cool temperatures with a constant, photoperiod in order to attribute reproductive arrest to temperature alone. Another series of experiments examined if exposure to cool temperatures three days post eclosion in female monarchs affects reproductive arrest. Ovarian development score was recorded in response to each temperature regime to monitor reproductive development; if butterflies are in reproductive arrest, no ovarian development should be observed. Reproductive arrest was predicted to occur under cool temperature conditions because low JH titers had been associated with temperatures at or below 15°C (Herman 1981, James 1982).

Methods

Field Collection

Migrant monarch butterflies were collected on October 4, 2013 at the Baker wetlands (Lawrence, KS). Butterflies were netted while in flight or nectaring. Four males and four females that were negative for the parasite *Ophyryocystis elektroscirrha* (OE) were used to establish a breeding colony. Temperatures in the breeding cage ranged from 28˚C during the day to 22˚C overnight. Butterflies were observed mating after six days in the cage. The first eggs were seen three days later.

Milkweed plants were watered daily; plants were changed every three days. Nectar dishes were refilled daily and changed every ten days.

Larval Care

First instar larvae (3-6 generations from collection) were isolated from milkweed plants with paintbrushes dipped in a 15% bleach solution. Larvae were placed on a thin layer of artificial diet with a constant air temperature of 26 ± 1.2°C. First instars were isolated and transferred to new diet every four days until pupation. Pupae were placed in screen-lined cups after they had dried for two days. Upon eclosion, butterflies were sexed and tested for OE using standard procedures (Davis 2005).

Experiment 1: Temperature Control Trials

Each OE negative female was weighed on the day of eclosion after drying for 8-12 hours. Forewing and hind wing lengths were measured with a ruler to the nearest millimeter. Each individual was tagged with a unique number on the individual's left wings and a cohort color code on the right discal cell. A cohort of five or six butterflies was placed in a 32 in h x 24 in w x32 in d treatment cage with a clear plastic top, one fluorescent light bank, and four screened sides. Each treatment cage was assigned to one of three treatments: cold (15°C, 12 hours light; 10°C, 12 hours dark), moderate (17°C, 12 hours light; 17°C, 12 hours dark), or warm (25-28°C, 12 hours light; 25-28°C, 12 hours dark). Temperature was recorded each hour with a Thermocron sensor. Pans of distilled water were placed in the bottom of

each cage to raise the ambient humidity to 20%. Three trials were conducted for each treatment (N=3 butterflies per time point per treatment).

Every ten days up to 60 days, one female from a treatment group was weighed, frozen, and dissected to determine ovarian development and total mature oocyte count. In an initial trial at 15˚C, females were dissected on day five, but no mature oocytes were observed. All dead females were weighed and dissected upon discovery. Ovarian development was scored on a scale from 0-3. An ovarian score of zero indicated no development. A score of one indicated yolk deposition, while a score of two indicated multiple immature oocytes. A score of three indicated chorionated oocytes. Lifespan, mature oocyte counts, and ovarian score were also recorded for each individual that died naturally. Sacrificed individuals were excluded from the lifespan analysis (N=5 per treatment).

Experiment 2: Delayed Addition Temperature Trials

OE negative females were measured, and marked as described above. Females were held in ambient laboratory conditions (26 ± 1.2°C, 12 hours) for three days to allow the *corpora allata* to release JH (Lessman 1980). Afterwards, butterflies were added to the cool (15°C 12 hours light; 10°C 12 hours dark), moderate (17°C, 12 hours light; 17°C 12 hours dark), or warm (22-28°C, 12 hours light; 22-28°C 12 hours dark) treatment groups in cohorts of five or six individuals. Females were dissected every ten days as described above. Lifespan, mature oocyte count and ovarian development were recorded.

Results

Experiment 1: Temperature Control Trials

Even though butterflies died during the time course of the experiment, the mean age of all individuals, including those sacrificed and those that died naturally, did not differ significantly among any of the treatments (ANOVA, F=0.16 df=2; Figure 1). Thus, in subsequent analyses, values were averaged over all ages. Overall, the mean number of mature oocytes was dependent upon temperature (Figures 2,3; ANOVA, P=0.01, F=4.6, df=2). There was a significant difference between females from both the cold and moderate temperature treatments and females from the warm temperature treatment (ANOVA, P=0.01, F=4.87, df=2; Figure 3). There was no difference between females from the cold and moderate treatments, which had different temperatures (0 oocytes). The observed number of mature oocytes increased with age in females from the warm temperature treatment although mature oocyte count was not statistically different among age groups (Figure 2). Only females held in the warm temperature treatment developed mature oocytes (Figure 3).

Ovarian score was dependent on temperature (Figure 4). Females from the cool temperature treatment exhibited a lower ovarian score than females from the warm temperature treatment; ovarian scores were significantly different when females from the warm and cool temperature treatments were compared (t-test, P=0.03; Figure 4). Females from the warm temperature treatment exhibited the highest ovarian score (Figures 3,4). There was a significant difference in the number of females that developed in the cool vs. moderate temperature treatment

groups over 60 days (Fisher's exact test, P=0.009). Ovarian score increased with age in females from the moderate temperature treatment although ovarian scores were not statistically significant (t-test, P=0.18; Figure 5). Even though oocytes did not reach maturity, females exposed to 17°C developed more immature oocytes compared to females from the cool temperature treatment, though this was not a statistically significant difference (Figure 5; t-test, P=0.36).

Experiment 2: Delayed Addition Temperature Trials

Over the course of this experiment, the mean age of all individuals did not differ significantly among any of the treatments (ANOVA, F=0.16, df=2; Figure 1). Thus, in subsequent analyses, values were averaged over all ages as in the temperature trials above. In this experiment the mean number of mature oocytes was dependent on temperature (ANOVA, p=0.001, F=22.8, df=2;Figure 3). The number of mature oocytes differed between females from the delayed cool temperature treatment and females from the delayed warm temperature treatment (t-test, P=0.003) as well as females from the delayed moderate temperature treatment and females from the delayed warm temperature treatment (t-test, P=0.003). The number of mature oocytes did not differ between females from the delayed cool temperature treatment and females from the delayed moderate temperature treatment (0 oocytes, Figure 3). There was no significant difference in the number of females that developed mature oocytes or the number of mature oocytes in the warm treatment group vs. the delayed warm treatment group over 60 days (Fisher's exact test, P=0.15; t-test, p=0.19; Figure 3).

Ovarian score was dependent on temperature (Figure 4). Mean ovarian score was significantly different when all delayed temperature treatments were compared to each other (ANOVA, *P*=<0.001; Figure 4). Females from the delayed cool temperature treatment were significantly different from females in both the delayed moderate (t-test, P<0.001; Figure 4) and delayed warm temperature treatments (t-test, P<0.001; Figure 4). Females from the moderate delayed temperature treatment also differed from females in the warm delayed temperature treatment (t-test, P<0.001). Ovarian scores, even if oocytes remained immature, were higher in females from the cool delayed temperature treatment than females immediately exposed to the cool temperature treatment (t-test, *P*<0.001; Figure 4).

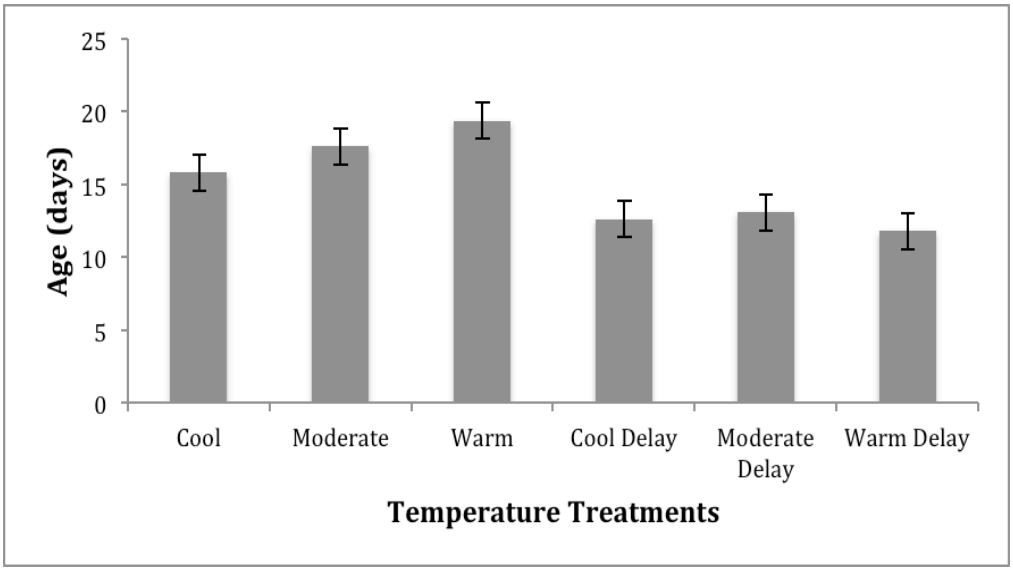

Figure 1. Mean age of female monarchs by treatment. N=16 for all groups; all individuals for the entire experiment are included. Error bars depict standard error. Although mean age varied slightly, none of the treatments are significantly different from each other (Non Dealyed: ANOVA, F=0.064, df=2; Delayed: ANOVA, F=0.16, df=2)

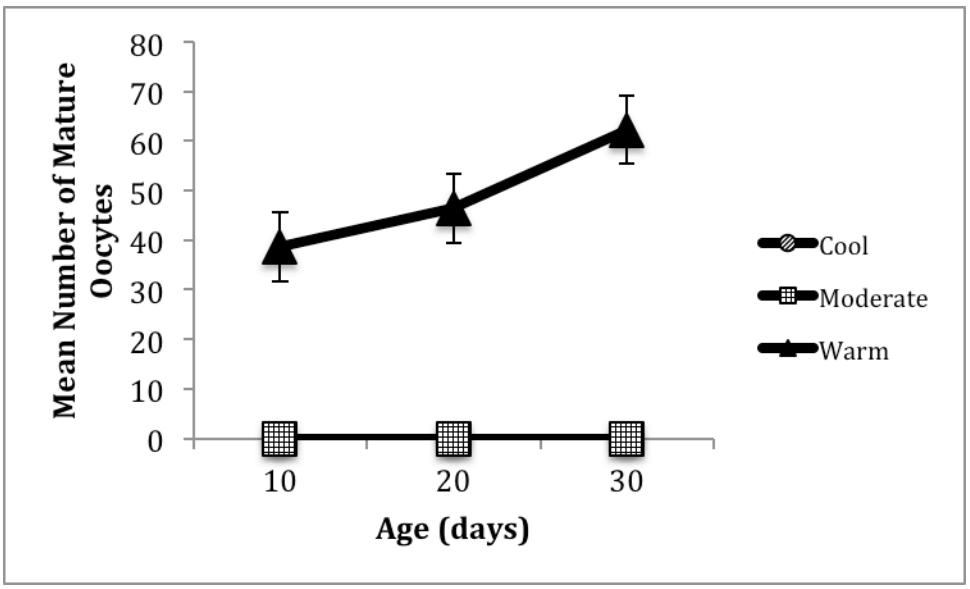

Figure 2. Mean number of mature oocytes counted in females from day 10 to day 30 in different temperature regimes. Error bars depict standard error. As temperature increased, the number of mature oocytes observed increased. Mean mature oocyte count is significantly different among the three treatments (ANOVA,F=4.9, df=2, P=0.01). When groups were separated, mature oocyte count was significantly different between femlaes in the cool and warm temperature treatments(t-test, p=0.05; n=16) as well as between femlaes in the moderate and warm treatments (t-test, p=0.05; n=16).

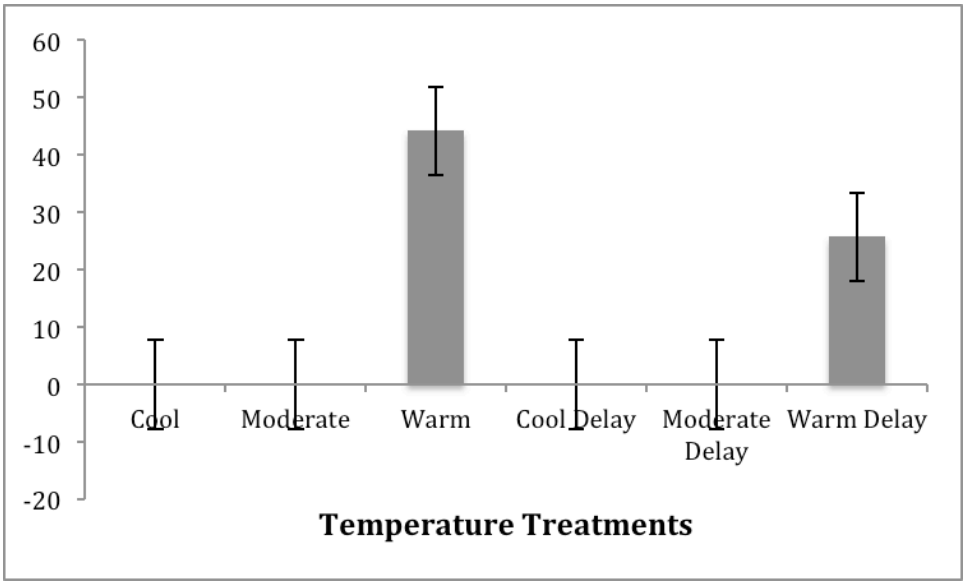

Figure 3: Mean number of mature oocytes by temperature treatment. Both delayed and non-delayed trials are included. Error bars depict standard error. N=16 for all treatment groups. Mature oocytes developed only in the warmest treatment. All other groups differ from the non-delayed warm group (ANOVA P=0.01, F=4.9 df=2).

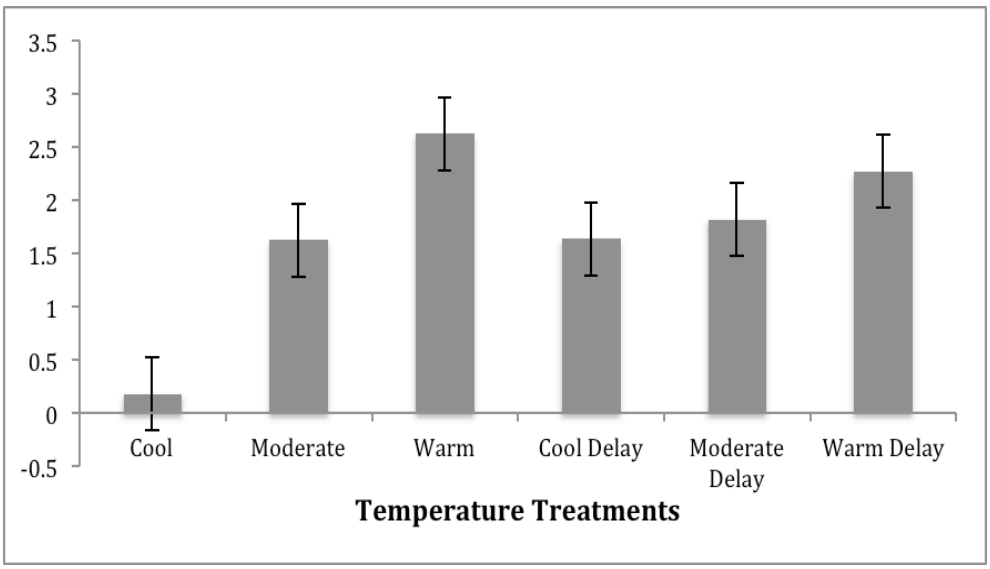

Figure 4. Mean female ovarian score by temperature regime. Both delayed and non-delayed trials are included. Females from the cool treatment were significantly different from females in the warm treatment in non-delayed butterflies (t-test, P=0.03). The delayed cool treatment was significantly different from both the delayed moderate (t-test, P<0.001) and warm treatments (t-test, P<0.001). The moderate delayed treatment also differed from the warm delayed treatment (t-test, P<0.001). Females from the cool treatment groups were significantly different from each other (t-test, P<0.001). All other comparisons among delayed and non-delayed butterflies were not significantly different. See Materials and Methods for details on scoring. N=16 for all groups. Error bars depict standard error.

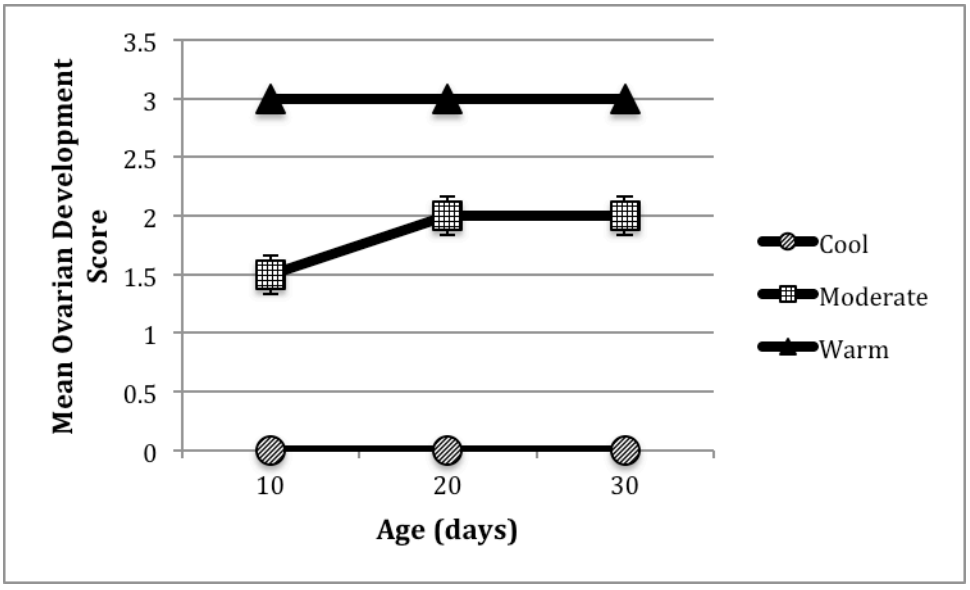

Figure 5. Mean ovarian development score observed in females from day 0 to day 30. Error bars depict standard error. As temperature increased, ovarian score also increased. When treatments were compared by age, females from the cool treamtent were significantly different from females from the moderatre treatment at 20 days (t-test, P=0.04). No other comparisons were significantly different.

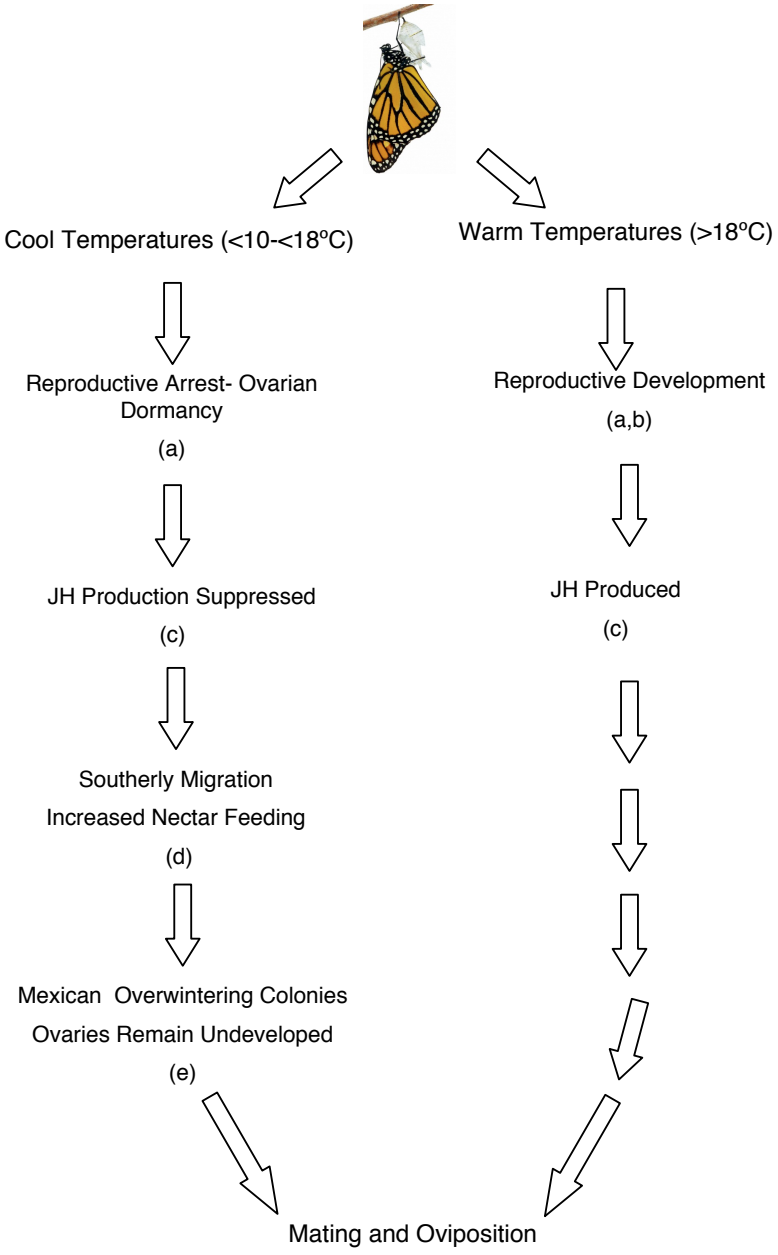

Figure 6. Proposed reproductive Path of a freshly eclosed adult female when exposed to cool temperatures vs. warm temperatures. (a) This thesis, (b) James 1983, (c) Lessman 1980, (d) Brower 2011, (e) Herman, et al 1989.

Discussion

Post eclosion temperatures had a major influence on the reproductive development of female monarch butterflies (Figure 6). Females exposed to temperatures under 18°C immediately after eclosion failed to develop mature oocytes (Figures 1, 3, 5). Conversely, females exposed to temperatures over 18°C developed mature oocytes within 10 days (Figure 2). At temperatures between 10 and 18°C, reproductive development was suppressed and the female entered reproductive arrest. The reproductive path of a freshly eclosed female is determined by the temperature to which she is immediately exposed.

In my experiments, monarchs failed to develop mature oocytes at temperatures under 15°C. Monarchs held at constant temperatures below 18°C developed many immature oocytes that failed to mature throughout the duration of the experiment. In contrast, monarchs held at 28°C developed mature oocytes in ten days. These results are consistent with the findings of James (1982, 1993) in which monarchs held at an average temperature of 12°C induced and maintained ovarian dormancy, as indicated by the absence of oocytes. Thus, monarch butterflies exposed immediately to temperatures below 18°C enter ovarian dormancy, regardless of population because the results from both North American and Australian females follow the same pattern. These populations are genetically similar (Lyons et al. 2012) and these results indicate that there is physiological similarity as well.

In contrast, others (Herman 1971, Herman 1973, Barker 1976, Herman 1981, 1985, 1993) found that females held at 25°C developed mature oocytes in only five

days and females held above 15°C developed mature oocytes within 10 days. However, these butterflies were stored in individual glassine envelopes and thus were not able to bask, fly, or eat regularly as they would during the migration when reproductive arrest is observed (Brown and Chippendale 1974, Brower 1985, Brower 1995). A basking butterfly experiences differences in temperature: the thoracic temperature is elevated to warm the flight muscles and enable flight (Kingsolver 1983). Because stored butterflies were not able to bask or move, they may have experienced different metabolic rates than butterflies able to bask and fly in both cool and warm temperatures. As a result, juvenile hormone production could have been affected and may have shown artificial differences when our results were compared. Thus, the results presented here were from a more naturally realistic treatment and may more appropriately approximate what is found in nature.

Butterflies from the cool treatment held for three days at room temperature (25 ±1.5°C) before to cold exposure (15°C days; 10°C nights) exhibited statistically different increased ovarian development from females in the non-delayed cool treatment. This suggests that in the three days post eclosion, reproductive development is initiated but subsequent development is arrested by transfer to cool temperatures (Lessman 1980). This is consistent with the early adult production of JH initiating ovarian development. For individuals with initiated ovarian development, experiencing subsequent cool temperatures unfavorable for development does not cause full reproductive dormancy because immature oocytes

are present. Instead, these individuals will experience slowed maturation of both ovaries and oocytes at temperatures between 18-20°C (James 1982, 1983, 1993).

In individuals that begin reproductive development prior to cold exposure, it is possible that at cool temperatures, juvenile hormone is not produced due to a slowed metabolic rate. This hormone also may not be able to reach its target tissues in the abdomen due to the absence or lowered production of juvenile hormone binding protein as speculated by Kort and Granger (Kort and Granger 1981). More molecular work needs to be done to understand how JH titer relates to metabolic rate and the role of juvenile hormone binding protein in adult insects.

Cool temperatures influence reproductive arrest in female monarch butterflies. This experiment did not address the impacts of temperature on hormone levels or effectiveness. Future experiments need to examine the mechanisms by which juvenile hormone is produced and released in adult monarch butterflies, the molecular pathways by which JH titer is managed, and the changes in juvenile hormone titer as monarchs in reproductive arrest begin ovarian development and produce mature, chorionated oocytes.

Distinguishing reproductive arrest (no reproductive development) from slowed reproductive development due to cool temperatures and associating this state with the timing of JH release is important for understanding reproductive arrest. Elucidating the induction stimulus of reproductive arrest is crucial for understanding the annual migration and for planning future conservation efforts. Differences between the North American and Australian populations need to be recorded because climatic and environmental conditions differ geographically.

After this state of reproductive arrest is properly characterized, research on this phenomenon can be expanded to include genetic characteristics of this state as well as genomic comparisons among monarchs and other danaids.

References

Andrewartha, H. 1952. Diapause in relation to the ecology of insects. Biological Reviews **27**:50-107.

Bale, J. S., and S. A. Hayward. 2010. Insect overwintering in a changing climate. Journal of Experimental Biology **213**:980-994.

Barker, J. F., and W.S. Herman. 1973. On the neuroendocrine control of ovarian development in the monarch butterfly. The journal of experimental zoology **183**:1-10.

Barker, J. F., and W.S. Herman. 1976. Effect of photoperiod and teperature on the reproduction of the monarch butterfly, *Danaus plexippus*. Journal of Insect Physiology **22**:1565-1568.

Bellés, X., D. Martín, and M.-D. Piulachs. 2005. The mevalonate pathway and the synthesis of juvenile hormone in insects. Annu. Rev. Entomol. **50**:181-199.

Brower, L. 1985. New perspectives on the migration biology of the monarch butterfly, *Danaus plexippus L.* Contributions in Marine Science. **68**.

Brower, L. P. 1995. Understanding and misunderstanding the migration of the monarch butterfly(Nymphalidae) in North America: 1857-1995. Journal of the Lepidopterists Society **49**:304-385.

Brower, L. P., W. Calvert, L. Hedrick, and J. Christian. 1977. Biological observations on an overwintering colony of monarch butterflies (*Danaus plexippus*, Danaidae) in Mexico. Journal of the Lepidopterists' Society. volume, pages

Brower, L. P., L. S. Fink, and P. Walford. 2006. Fueling the fall migration of the monarch butterfly. Integrative Comparative Biology**46**:1123-1142.

Brower, L. P., L.S. Fink, R.J. Kiphart, V. Pocius, R. Zubieta, and M.I. Ramírez 2014. The effect of the 2010-2011 drought on the lipid content of monarch butterflies (*Danaus plexippus* L., Danainae) migrating through Texas to their overwintering sites in Mexico. *In* K. S. Oberhauser, K.R. Nail, and S.M. Altizer, editors. Monarchs in a Changing World: Biology and Conservation of an Iconic Insect. Cornell University Press, Ithaca, NY.

Brown, J., and G. Chippendale. 1974. Migration of the monarch butterfly, *Danaus plexippus*: Energy sources. Journal of Insect Physiology **20**:1117-1130.

Cenedella, R. J. 1971. The lipids of the female monarch butterfly, *Danaus plexippus*, during fall migration. Insect biochemistry **1**:244-247.

Danks, H. V. 1987. Insect dormancy: an ecological perspective. Biological Survey of Canada (Terrestrial Artropods).

Danks, H. V. 2004. Seasonal adaptations in arctic insects. Integr Comp Biol **44**:85-94.

Davis, A. K. 2005. Project MonarchHealth. http://www.monarchparasites.org/

Davis, A. K., and M. S. Garland. 2004. Stopover ecology of monarchs in coastal Virginia: using ornithological techniques to study monarch migration. *In* K.S. Oberhauser and M.J. Solensky, editors. The Monarch Butterfly: Biology and Conservation. Ithaca: Cornell University Press, Ithaca, NY. p:89-96.

Denlinger, D. L. 2002. Regulation of diapause. Annual Review of Entomology **47**:93-122.

Goehring, L., and K. S. Oberhauser. 2002. Effects of photoperiod, temperature, and host plant age on induction of reproductive diapause and development time in *Danaus plexippus*. Ecological Entomology **27**:674-685.
Goehring, L., and K. S. Oberhauser. 2004. Environmental factors influencing postdiapause reproductive development in monarch butterflies. Monarch Butterfly Ecology and Behavior. Cornell University Press, Ithaca, NY:187-198.
Herman, W., C. Lessman, and G. Johnson. 1981. Correlation of juvenile hormone titer changes with reproductive tract development in the posteclosion monarch butterfly. Journal of Experimental Zoology **218**:387-395.
Herman, W. S. 1973. The Endocrine Basis of Reproductive Inactivity in Monarch Butterflies Overwintering in Central California. Journal of Insect Physiology **19**:1883-1887.
Herman, W. S. 1981. Studies on the Adult Reproductive Diapause of the Monarch Butterfly, *Danaus plexippus*. Biological Bulletin **160**:89-106.
Herman, W. S. 1985. Hormonally mediated events in adult monarch butterflies Migration Mechanisms and Adaptive Significance:800-815
Herman, W. S. 1993. Endocrinology of the monarch butterfly. Natural History Museum Los Angeles County Science Series **38**:143-146.
Herman, W. S., Lincoln P. Brower, and William H. Calvert. 1989. Reproductive tract development in monarch butterflies overwintering in California and Mexico. Journal of the Lepidopterists' Society **43**:50-58.
Herman, W. S. a. J. F. B. 1971. Reproductive endocrinology of Danaus plexippus. American zoologist **11**:645.
James, D. 1982. Ovarian dormancy in *Danaus plexippus (L.)*(Lepidoptera: Nymphalidae)—oligopause not diapause. Australian Journal of Entomology **21**:31-35.
James, D. 1983. Induction of Reproductive Dormancy in Australian Monarch Butterflies *Danaus plexippus (L.)*. Australian journal of zoology **31**:491-498.
James, D. G. 1993. Migration biology of the monarch butterfly in Australia. *In* K.S. Oberhauser and M.J. Solensky, editors. The Monarch Butterfly: Biology and Conservation. Ithaca: Cornell University Press, Ithaca, NY:189-200.
Kingsolver, J.G. 1985. Butterfly thermoregulation:organismic. Journal of Research on the Lepidoptera **24**:1-20.
Kort, C. d., and N. Granger. 1981. Regulation of the juvenile hormone titer. Annual Review of Entomology **26**:1-28.
Kostal, V. 2006. Eco-physiological phases of insect diapause. Journal of Insect Physiology **52**:113-127.
Leather, S. 1993. Diapause and overwintering. The Ecology of Insect Overwintering **3**:19-60.
Lessman, C. A. 1980. The Biology of Hemolymph Juvenile Hormone in the Adult Monarch (*Danaus P. plexippus*: Lepidoptera). University of Minnesota, Minneapolis, Mn.
Lyons, J. I., A. A. Pierce, S. M. Barribeau, E. D. Sternberg, A. J. Mongue, and J. C. De Roode. 2012. Lack of genetic differentiation between monarch butterflies with divergent migration destinations. Molecular Ecology **21**:3433-3444.

Masters, A. R., S. B. Malcolm, and L. P. Brower. 1988. Monarch butterfly (*Danaus plexippus*) thermoregulatory behavior and adaptations for overwintering in Mexico. Ecology:458-467.

Miskin, W. 1871. Occurrence of Danais archippus in Queensland. Entomologists Monthly Magazine **8**:17.

Nijhout, F. 1994. Diapause. Insects Hormones. Princeton University Press, Princeton, NJ.

Tauber, M. J., and C. A. Tauber. 1986. Seasonal Adaptations of Insects. Oxford University Press.

Urquhart, F. A., and N. R. Urquhart. 1978. Autmnal Migrtion Routes of Eastern Population of Monarch Butterfly (*Danaus P. Plexippus L*: Danaidae: Lepidoptera) in North America to Overwintering Site in Neovolcanic Plateau of MexicoCanadian Journal of Zoology-Revue Canadienne De Zoologie **56**:1759-1764.

Zhan, S., C. Merlin, J. L. Boore, and S. M. Reppert. 2011. The monarch butterfly genome yields insights into long-distance migration. Cell **147**:1171-1185.

Chapter 2

Is Reproductive Arrest in the Monarch Butterfly, *Danaus plexippus*, a True Diapause?

Introduction

As a group, insects have various strategies to cope with unsuitable conditions for growth and reproduction including migration, dormancy and seasonal polyphenism (Danks 1987, Leather 1993, Danks 2004, Bale and Hayward 2010). Many of these traits have been linked to a diapause syndrome, which is a state of arrested development often associated with lowered metabolic activity, reduced motor activity, and increased resistance to environmental extremes (Andrewartha 1952, Tauber and Tauber 1986, Danks 1987, Leather 1993, Goehring and Oberhauser 2002). Insect reproductive dormancy can be classified as several different phenomena: quiescence, oligopause, diapause, and reproductive diapause. There are slight physiological differences between each of these states.

Quiescence refers to slowed development as an immediate response to unfavorable conditions; environmental conditions directly limit development. Development resumes immediately when favorable conditions return; unfavorable conditions can include limited water and nutrient availability as well as hot or cold temperature extremes. Quiescence can occur during any life stage, including reproductive development as an adult, and the insect does not prepare for this dormancy by storing fats or altering physiological processes (Gullan and Cranston 2009).

Oligopause refers to dormancy that begins and ends gradually. It is a state of rest found in species inhabiting areas of moderate winter, with a fixed period of dormancy in response to a cyclic and rather long climatic change. An environmental factor of equal intensity both begins and ends this dormancy. For example, the loss of one hour in light exposure can begin dormancy and regaining that hour of daylight can end it. Limited physiological changes, such as increased fat storage, are associated with oligopause, but these changes are not as drastic as those associated with diapause. Oligopausing insects cannot withstand the same environmental extremes as true diapausers (Mansingh 1971).

Diapause is defined as arrested reproductive development combined with adaptive physiological changes such as increased fat storage or migration. Reproductive development begins only after specific physiological stimuli are experienced, not necessarily upon the return of suitable conditions. In many insects, decreasing photoperiod induces diapause, but the stimulus varies among species. In most overwintering insects, development recommences after two weeks at room temperature (Mansingh 1971). Diapause can take place during any life stage. Diapause involves more complex preparation for the dormant period than oligopause including building up nutritional stores and a lowering of metabolic rate (Mansingh 1971).

Reproductive diapause is the suspension of reproduction in mature insects (Gullan and Cranston 2009). Reproduction commences post migration or when conditions for successful oviposition and immature stage development return. Reproductive diapause is associated with a refractory period, defined as the

suspension of DNA and RNA synthesis and mitotic activity in all tissues except for reproductive tissues and hemocytes. During this refractory phase, the reproductive system is in preparation for the future reactivation of the endocrine system (Mansingh 1971).

Monarch butterflies exhibit reproductive arrest, a total suspension of reproductive development, during the annual fall migration and overwintering periods in North America (August-February) and resume reproduction before the spring movement northward (Herman 1981, Herman et al. 1989, Brower 1995, Van Hook 1996, Goehring and Oberhauser 2002). Reproductive arrest is associated with a substantial increase in lipid stores and low juvenile hormone titer (Herman 1973, James 1982, Herman 1985, Herman et al. 1989, Brower et al. 2006, Brower 2014). This observed reproductive arrest has been described as both diapause in the Western North American population (Herman 1981) and reproductive diapause in the Eastern North American population.

Reproductive dormancy in North American monarchs has been classified as reproductive diapause (Herman 1981, Herman et al. 1989, Goehring and Oberhauser 2002). Although the induction of ovarian dormancy seems to involve similar temperatures (15°C) to the Australian population (Barker 1976), a refractory period is associated with North American migrants (Herman 1981). This refractory period was defined in monarch butterflies as a significant reduction in the response of reproductive tissues to juvenile hormone despite reproductive temperatures (Herman 1981). Migrating monarchs collected in Minnesota during the autumn migration produced fewer mature oocytes when exposed to 25°C than

monarchs reared at 25°C in the laboratory. This decrease in mature oocyte production may be indicative of a refractory period denoting reproductive diapause.

The reproductive dormancy observed in Australian monarchs has been classified as oligopause(James 1993). Freshly eclosed monarchs exposed to cool temperatures (<18°C) displayed reproductive arrest as well as intensive nectar feeding. Reproductively dormant females removed from overwintering clusters and exposed to 28°C for six days displayed rapid ovarian maturation; the number of mature oocytes was not significantly different from newly eclosed females exposed to the same conditions (James 1982). Because no refractory period was observed in the cold exposed females, this phenomenon has been classified as oligopause.

The physiology of the North American population needs to be reexamined using the same temperature scheme as the Australian studies to determine if the reproductive arrest observed during the migration is a true diapause or oligopause and if any mechanistic differences actually exist between these two populations.

The following study was conducted using lab-reared descendants of North American migrants following the methods described in the Australian work(James 1982) to determine if a refractory period exists in the North American population. I aim to classify the reproductive arrest observed each autumn as diapause or oligopause. If reproductive arrest in *D. plexippus* were an example of a true diapause, a refractory period would exist in females exposed to cold temperatures. Observable ovarian inactivity would be maintained for some time after induction, regardless of environmental conditions (Mansingh 1971, James 1982, 1993).

Methods

Larval Care

Eggs were collected from a laboratory colony in April 2014, descendants of monarchs collected in late September 2014 from the Baker Wetlands (Lawrence, KS). First instar larvae were reared outdoors on tropical milkweed. Upon pupation, monarchs were moved into the laboratory. Additional pupae were ordered from Flutterby Gardens of Manatee, Inc (Bradenton, FL). Butterflies were OE tested, sexed and assigned to a treatment group upon eclosion.

Effect of Cold Pretreatment

To assess the effects of cold pretreatment on subsequent ovarian development at summer temperatures, two treatment regimes were devised: one in which females were pretreated with cold temperatures before shifting to summer temperatures and one (control group) in which females did not have the pretreatment. All ovarian development was assessed at summer temperatures. Female monarchs 8-12 hours post eclosion were assigned to pretreatment (N=22) or control (N=22) groups. All individuals were maintained in a 12 hour photoperiod. The control females were immediately placed at constant 28°C ± 1.5°C. Pretreatment females were placed at 12°C± 1.5°C for either 4 (n=12) or 8 (n=10) days before shifting to 28°C ±1.5°C. Once placed at 28˚C, two females from each treatment were dissected every two days up to 20 days and scored for mature oocyte count and ovarian development. Cohort differentiation, individual butterfly numbers, mature oocyte count procedures and ovarian development scoring were done as in Chapter 1.

Results

Ovarian development was not dependent on cold exposure. Neither 4- nor 8-day pretreated females had ovarian development at 4 days (ovarian score=0, N= 12 and N=10, respectively). Thus, in subsequent analysis their results have been combined. When cold pretreated females were compared to control females, mature oocyte production was not significantly different between the two treatment groups groups (t-test, P=0.53; Figure 1;Table 1). In both groups, ovaries began to develop by day 2, with mature oocytes appearing by day 4 (Table 1; Figure 1).

The mean number of mature oocytes increased from eclosion to day 6 in both the control and pretreatment groups (Figure 1). Total oocyte production in each group when time points were pooled was not different for each treatment (t-test, P-0.53). Thus, pretreatment females failed to retain ovarian dormancy imposed by cool temperatures when subsequently exposed to summer conditions (28°C).

Table 1. Ovarian development for pretreatment and control groups. N=2 for each timepoint and treatment.

Days at 28˚C	Ovarian Development Score	
	Pretreatment	Control
0	0±0.0*	0±0.0
2	2±0.0	2±0.0
4	3±0.0	3±0.0
6	3±0.0	3±0.0
8	3±0.0	3±0.0
10	3±0.0	3±0.0
12	3±0.0	3±0.0
14	3±0.0	3±0.0
16	3±0.0	3±0.0
18	3±0.0	3±0.0
20	3±0.0	3±0.0

*Mean and standard error

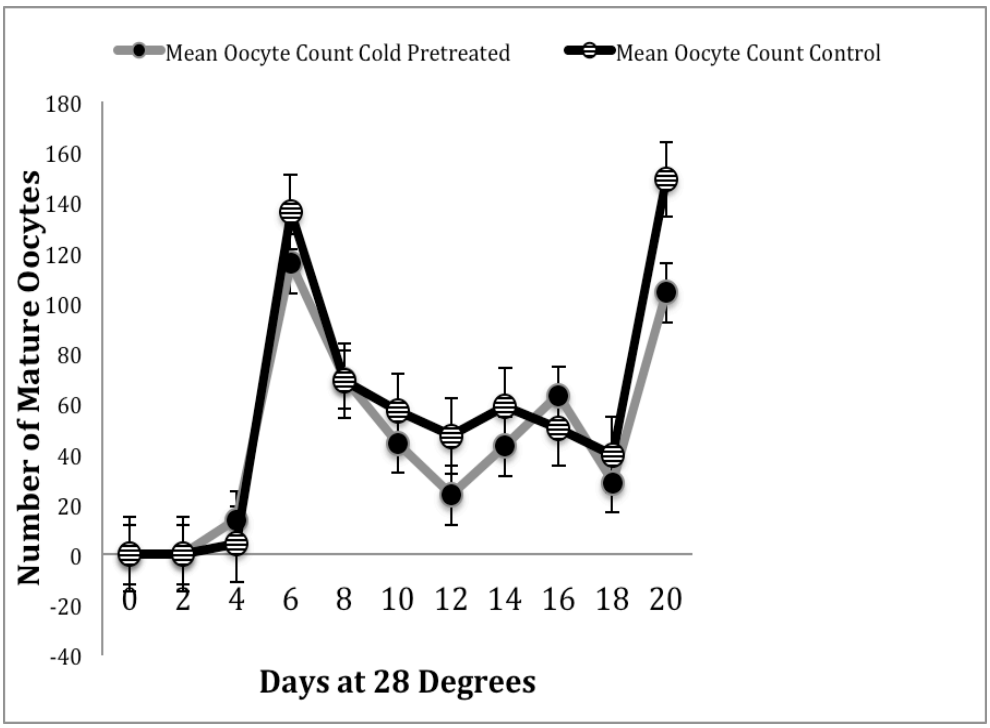

Figure 1. Mean mature oocyte count in females from day 0 to day 20. Oocytes appear after the same number of days in both cold exposed and non cold exposed females. Mature oocyte count was not significantly different between treatments for any two day time period.

Discussion

These data contradict the idea that monarch ovarian arrest should be classified as diapause. Ovarian development progressed at 28°C without delay even after either 4 or 8 days of exposure to 12°C. The first mature oocytes were observed after 4 days at 28°C regardless of cold exposure. Both ovarian score and mature oocyte count were not significantly different overall.

This work supports the work of James (James 1982, 1993) in which he found no refractory period in cold-exposed laboratory reared females when they were moved to summer conditions. Similarly in this experiment, no reproductive refractory period was observed in cold exposed females. Although confusion still surrounds the definition of diapause, one constant characteristic of this state is the occurrence of a refractory phase during which reproductive maturation is repressed

even during normally favorable conditions (Herman, 1981; Mansingh, 1971). The results described above clearly demonstrate the absence of such a refractory period.

In contrast, Herman (1981, 1985, 1993) defined monarch reproductive arrest as a true diapause because he observed a difference in the growth measured via ovarian weight and mature oocyte counts in wild caught migrants exposed to summer conditions vs. a non-migrant control group. In these studies, monarchs were collected and reared throughout the year from various North American populations and each butterfly was stored in an individual glassine envelope, which prevented natural flight, basking, and eating behavior. This experiment needs to be repeated with field-captured migrants in more natural conditions in order to make a direct comparison to the diapause studies of Herman (Herman 1981, 1985, 1993) and to facilitate comparisons between the work of Herman (1981, 1985,1993) and James (1982,1993). Migrants may experience other environmental cues as larvae or pupae that encourage reproductive arrest as a freshly eclosed adult, thus wild caught migrants must be compared to other wild caught migrants instead of laboratory-reared butterflies to ensure that all treatment groups experience the same environmental cues during both the larval and pupal stages.

This experiment may explain how monarchs migrating north from the Mexican overwintering colonies break reproductive arrest and are able to lay eggs soon after their departure from the overwintering colonies. When temperatures favorable for breeding return, a mass-mating event takes place at the Mexican overwintering sites before adults begin the migration north. Some females are not reproductively developed when this mating takes place (Van Hook 1996). Females

flying north have a limited time window to lay their eggs on new milkweeds sprouting in Texas and along the Gulf coast; these individuals need to develop mature oocytes quickly in order to exploit the rich milkweed resources of southern Texas in the early spring. My work may explain how females can easily break oligopause and develop mature oocytes quickly.

References

Andrewartha, H. 1952. Diapause in relation to the ecology of insects. Biological Reviews **27**:50-107.

Bale, J. S., and S. A. Hayward. 2010. Insect overwintering in a changing climate. Journal of Experimental Biology **213**:980-994.

Barker, J. F., and W.S. Herman. 1973. On the neuroendocrine control of ovarian development in the monarch butterfly The journal of experimental zoology **183**:1-10.

Barker, J. F., and W.S. Herman. 1976. Effect of photoperiod and teperature on the reproduction of the monarch butterfly, Danaus plexippus. Journal of Insect Physiology **22**:1565-1568.

Bellés, X., D. Martín, and M.-D. Piulachs. 2005. The mevalonate pathway and the synthesis of juvenile hormone in insects. Annual review of entomology **50**:181-199.

Brower, L. 1985. New perspectives on the migration biology of the monarch butterfly, *Danaus plexippus L.* Contributions in Marine Science **68**.

Brower, L. P. 1995. Understanding and misunderstanding the migration of the monarch butterfly (Nymphalidae) in North America: 1857-1995. Journal of the Lepidopterists Society **49**:304-385.

Brower, L. P., W. Calvert, L. Hedrick, and J. Christian. 1977. Biological observations on an overwintering colony of monarch butterflies (*Danaus plexippus, Danaidae*) in Mexico. Journal of the Lepidopterists' Society.

Brower, L. P., L. S. Fink, and P. Walford. 2006. Fueling the fall migration of the monarch butterfly. Integr Comp Biol **46**:1123-1142.

Brower, L. P., L.S. Fink, R.J. Kiphart, V. Pocius, R. Zubieta, and M.I. Ramírez 2014. The effect of the 2010-2011 drought on the lipid content of monarch butterflies (*Danaus plexippus L., Danainae*) migrating through Texas to their overwintering sites in Mexico.*in* K. S. Oberhauser, K.R. Nail, and S.M. Altizer, editor. Monarchs in a Changing World: Biology and Conservation of an Iconic Insect. Cornell University Press, Ithaca, NY.

Brown, J., and G. Chippendale. 1974. Migration of the monarch butterfly,*Danaus plexippus*: Energy sources. Journal of Insect Physiology **20**:1117-1130.

Cenedella, R. J. 1971. The lipids of the female monarch butterfly, *Danaus plexippus*, during fall migration. Insect biochemistry **1**:244-247.

Danks, H. V. 1987. Insect dormancy: An Ecological Perspective. Biological Survey of Canada (Terrestrial Artropods).

Danks, H. V. 2004. Seasonal adaptations in arctic insects. Integr Comp Biol **44**:85-94.

Davis, A. K., and M. S. Garland. 2004. Stopover Ecology of Monarchs in Coastal Virginia: Using Ornithological Techniques to Study Monarch Migration. Pages 89-96 *in* K. S. Oberhauser and M. J. Solensky, editors. The Monarch Butterfly: Biology and Conservation. . Cornell University Press, Ithaca.

Denlinger, D. L. 2002. Regulation of diapause. Annual review of entomology **47**:93-122.

Goehring, L., and K. S. Oberhauser. 2002. Effects of photoperiod, temperature, and host plant age on induction of reproductive diapause and development time in *Danaus plexippus*. Ecological Entomology **27**:674-685.
Goehring, L., and K. S. Oberhauser. 2004. Environmental Factors Influencing Postdiapause Reproductive Development in Monarch Butterflies. Pages 187-198 *in* K. S. Oberhauser and M. J. Solensky, editors. The Monarch Butterfly: Ecology and Conservation. Cornell University Press, Ithaca.
Gullan, P. J., and P. S. Cranston. 2009. The Insects: an Outline of Entomology. John Wiley & Sons.
Herman, W., C. Lessman, and G. Johnson. 1981. Correlation of juvenile hormone titer changes with reproductive tract development in the posteclosion monarch butterfly. Journal of Experimental Zoology **218**:387-395.
Herman, W. S. 1973. The endocrine basis of reproductive inactivity in monarch butterflies overwintering in central California. Journal of Insect Physiology **19**:1883-1887.
Herman, W. S. 1981. Studies on the adult reproductive diapause of the monarch butterfly, *Danaus plexippus*. Biological Bulletin **160**:89-106.
Herman, W. S. 1985. Hormonally mediated events in adult monarch butterflies Migration Mechanisms and Adaptive Significance:800-815
Herman, W. S. 1993. Endocrinology of the monarch butterfly. Natural History Museum Los Angeles County Science Series **38**:143-146.
Herman, W. S., L. P. Brower, and W. H. Calvert. 1989. Reproductive tract development in monarch butterflies overwintering in California and Mexico. Journal of the Lepidopterists' Society **43**:50-58.
Herman, W. S. a. J. F. B. 1971. Reproductive endocrinology of *Danaus plexippus*. American zoologist **11**:645.
James, D. 1982. Ovarian dormancy in *Danaus plexippus (L.)*(Lepidoptera: Nymphalidae)—oligopause not diapause. Australian Journal of Entomology **21**:31-35.
James, D. 1983. Induction of reproductive dormancy in Australian monarch butterflies *Danaus plexippus (L.)*. Australian journal of zoology **31**:491-498.
James, D. 1993. Migration biology of the monarch butterfly in Australia. Pages 189-200 *in* K. S. Oberhauser and M. J. Solensky, editors. Biology and conservation of the monarch butterfly. Cornell University Press Ithaca.
Kingsolver, J. G. 1983. Ecological significance of flight activity in Colias butterflies: implications for reproductive strategy and population structure. Ecology:546-551.
Kort, C. d., and N. Granger. 1981. Regulation of the juvenile hormone titer. Annual review of entomology **26**:1-28.
Kostal, V. 2006. Eco-physiological phases of insect diapause. Journal of Insect Physiology **52**:113-127.
Leather, S. 1993. Diapause and overwintering. The Ecology of Insect Overwintering **3**:19-60.
Lessman, C. A. 1980. The Biology of Hemolymph Juvenile Hormone in the Adult Monarch (*Danaus P. plexippus*: Lepidoptera). University of Minnesota, Minneapolis.

Lyons, J. I., A. A. Pierce, S. M. Barribeau, E. D. Sternberg, A. J. Mongue, and J. C. De Roode. 2012. Lack of genetic differentiation between monarch butterflies with divergent migration destinations. Mol Ecol **21**:3433-3444.

Mansingh, A. 1971. Physiological classification of dormancies in insects. The Canadian Entomologist **103**:983-1009.

Masters, A. R., S. B. Malcolm, and L. P. Brower. 1988. Monarch butterfly (*Danaus plexippus*) thermoregulatory behavior and adaptations for overwintering in Mexico. Ecology:458-467.

Nijhout, F. 1994. Diapause.*in* F. Nijhout, editor. Insect Hormones. Princeton University Press, Princeton, NJ.

Tauber, M. J., and C. A. Tauber. 1986. Seasonal Adaptations of Insects. Oxford University Press.

Urquhart, F. A., and N. R. Urquhart. 1978. Autmnal Migrtion Routes of Eastern Population of Monarch Butterfly (*Danaus P. Plexippus L*: Danaidae: Lepidoptera) in North America to Overwintering Site in Neovolcanic Plateau of Mexico. Canadian Journal of Zoology **56**:1759-1764.

Van Hook, T. S. 1996. Monarch butterfly mating ecology at a Mexican overwintering site: proximate causes of nonrandom mating. Ph.D. University of Florida, Ann Arbor.

Zhan, S., C. Merlin, J. L. Boore, and S. M. Reppert. 2011. The monarch butterfly genome yields insights into long-distance migration. Cell **147**:1171-1185.