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The Effects on the Development and Survival of Monarch Caterpillars Marked with DayGlo ECO Aurora Pink Pigment Powder

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Abstract

In order to understand insect movement, it is necessary to track individuals. One of the most common marking methods is to dust the insect externally with fluorescent powder. However, it is important to know whether marking is detrimental to insect health. This study was conducted to determine whether marking with fluorescent powder is feasible and safe for use in movement studies of monarch butterfly (*Danaus plexippus*) larvae. Monarch larvae were exposed to DayGlo ECO Aurora Pink Pigment powder once during each instar to determine whether powder exposure affected their development and survival. One hundred 1st instar larvae were divided evenly into two treatment groups, control and dusted. Larvae in the dusted group were dusted with DayGlo ECO Aurora Pink Pigment powder during each instar. They were reared to adulthood on *Asclepius syriaca*; development and survival of each individual was recorded. Larval, pupal, and adult mass, pupal length and width, and adult wing length were recorded. Adult butterflies were observed to determine whether they mated and laid eggs successfully. The resulting eggs were monitored for hatching. Our results suggest that DayGlo ECO Aurora Pink Pigment powder did not affect development or survival of *Danaus plexippus* larvae. Twenty-three caterpillars in the dusted group and 21 in the control group survived through the end of the study. There were no significant differences in larval mass, pupal or adult measurements. Surviving adults mated successfully and laid viable eggs. This marking technique will be useful in future larval field studies.

Keywords: Insect Movement, Marking Methods, Monarch Butterfly

1. Introduction

In order to understand insect movement in natural habitats, it is necessary to track individuals. Various marking techniques, such as genetic, protein, paint, powders, and pollen have been used to assess insect behavior, population dynamics, movement, dispersal, feeding behavior, and ecological interactions[6,7,22]. Although externally marking insects is common, finding an effective marker to track the behavior of individuals can be challenging. The insect's size, life span, mobility, and the duration of the study must be considered when choosing a marking method[20]. The marker should not inhibit the insect's behavior, development, or survival and should be environmentally safe.

Marking techniques range from sophisticated, expensive methods to simple and inexpensive methods. Sophisticated, expensive methods include genetic, radio-isotope, elemental, and protein marking[6,7]. It is, however, easier and more convenient to use inexpensive, simple marking techniques, such as paints, pollen, powders, dyes, and inks to study behavior and movement. The use of ball point paint markers, to mark individual fire ants, did not affect their mortality and will be useful in accessing basic behavioral characteristics[22]. Pollen grains have been used to

identify and track the distribution of cross-pollination of honey-bees on almonds[4] and to track the long-distance movement of bollworm *Heliothis zea*, moths[8]. The honey-bees and moths were examined for pollen and the pollen was used to map their distribution[4,8].

One of the most common marking methods is dusting insects with fluorescent powder[2,7,9,13] because it is cost-effective and easy to use. The fluorescent color provides a good contrast to many insect's green habitat, allowing them to easily be observed. This method has been used extensively in mosquito research for studies determining population sizes, and examining host-seeking behavior, and distribution[18,19]. Fluorescent powder has been used to mark adult *Dendroctonus frontalis* Zimmermann and *Ips grandicollis*, bark beetles[2], *Trichogramma brassicae*, a parasitoid wasp[5], *Plutella xylostella*, diamondback moths[13], and *Diaphorina citri*, Asian citrus psyllids[14]. DayGlo fluorescent pigment powders are non-toxic and earth-friendly[14] making them popular for use in insect studies[12,21].

Studies on the use of fluorescent powders on different insect taxa found no significant differences between the survival of the dusted and control groups[5,12,14]. In particular, the results of a study on the use of DayGlo fluorescent powder on *Vanessa cardui*, painted lady butterfly, larvae suggested that there were no significant adverse effects when the powder was applied to a cohort of 101 second and third instars[21]. Similarly, DayGlo fluorescent powder externally applied once to adult *Diabrotica virgifera virgifera* LeConte, western corn rootworm beetles, had no effect on flight behavior or survival[15].

Over 150 years ago, Iowa's landscape was predominantly tallgrass prairie, with a large diversity of plants, animals, birds, butterflies, and other insects. However, this is not the case anymore as Iowa's landscape has been converted to mainly agricultural fields. 99.9% of Iowa's native prairies are completely gone. Due to this loss and recent agricultural practices, milkweed *(Asclepias)*, a common plant in Iowa's prairies and one that is crucial to the monarch's survival, has declined by over 50% in the last several years[17]. In turn, the monarch population has declined by over 84% and they could face quasi-extinction in the next 20 years[10]. Due to the monarch's imperiled conservation status, their health and well-being need to be taken into consideration before initiating a marking study.

This study was conducted to determine whether marking with fluorescent powder is feasible and safe for use in a larval movement study to determine the host finding abilities of *Danaus plexippus* larvae. The use of a colored powder is necessary to distinguish each larva from the green background of its host plants, *Asclepias species*[11]. Before using this powder in the field for larval movement studies, we wanted to be sure that the presence of this powder does not affect the survival or development of monarch larvae. Monarch larvae were exposed to DayGlo ECO Aurora Pink Pigment powder (DayGlo Color Corp., Cleveland, OH, USA) throughout their larval life stages to determine whether powder exposure affected their development and survival. We also assessed the effects of the powder on the adults' mating and the viability of laid eggs. We hypothesized that DayGlo ECO Aurora Pink Pigment powder would not affect development or survival of *D. plexippus* larvae, nor would it affect mating success or viability of eggs.

2. Materials and Methods

Danaus plexippus eggs were obtained from the 2014 *D. plexippus* colony, which is maintained by Iowa State University (ISU) and the United States Department of Agriculture (USDA) in Ames, Iowa. The colony was started by collecting *D. plexippus* eggs found on *Asclepias syriaca* along Iowa's roadways in Boone and Story counties. Eggs laid by colony females were collected, placed in a single, large Falcon petri dish, 100 x 15 mm (Corning Inc., Corning, NY, USA), lined with damp Whatman 90 mm filter circles (GE Healthcare, Chicago, IL, USA), and stored in a 27°C incubator (Percival Scientific, Perry, IA, USA) until hatching.

Common milkweed (*A. syriaca)* was harvested one time near Randall, IA in a recently mowed area and brought back to the laboratory. Milkweed leaves were placed in water and then soaked in a 10% bleach solution. Afterwards, the leaves were rinsed in three water baths for a total of 30 minutes. The leaves were then dried in a salad spinner (Chef-Master Salad Dryer, Blue Rhino, Winston-Salem, NC, USA) and allowed to air dry. Dry leaves were stored in the refrigerator and were used to feed the larvae throughout the study.

Agar plates for larval rearing were prepared using 60 x 15 mm Falcon petri dishes (Corning Inc., Corning, NY, USA). They were filled with Bacto Agar (Becton, Dickinson, and Co., Sparks, MD, USA), a solidifying agent that will provide a moist environment for the larvae. Once the agar had set, 15 mm holes were cut out of the center, removed, and disposed in order to reduce the amount of material in the petri dish.

Before a larva was added to a petri dish, a 2.54-cm^2 piece of *A. syriaca* leaf was placed in each agar plate. Fifty 1st instars were randomly chosen as the control group (n=50, one larva per plate) and were placed onto the milkweed leaf in their own dish. Fifty 1st instars were randomly chosen as the dusted group (n=50, one larva per plate). Each larva

was gently dusted with DayGlo Aurora Pink Pigment Powder (DayGlo Color Corp., Cleveland, OH, USA) with a small, soft brush and placed onto each milkweed leaf. The larvae were reared in an incubator at 27ºC with a 16-hour light/8-hour dark cycle. Individuals were assigned a number to track development and survival throughout the study.

The larvae were checked daily for both mortality and molting; the dates of each molt and any mortality were recorded for each individual. Milkweed leaves were replaced daily. After each molt, pink powder was reapplied to larvae in the dusted group. Fifth instars were weighed to the nearest 0.1 mg on an AND GR-202 Balance (A&D Company, Limited, Tosima-ku, Tokyo, Japan) and the larvae were transferred to large Falcon petri dishes, 100 x 15 mm (Corning Inc., Corning, NY, USA) lined with Whatman 90 mm filter circles (GE Healthcare, Chicago, IL, USA) with one whole milkweed leaf. Larvae were fed a new leaf daily and the debris in the petri dish was discarded. The 5^{th} instars were put back into the rearing incubator for 24 hours, then removed and reared on a table in the lab at room temperature (25-27ºC) with natural light.

When larvae began to pupate, leftover milkweed was removed from the petri dish. Twenty-four hours after pupation, the pupae were gently removed from the top of the cup. Pupal mass was recorded to the nearest 0.1 mg on an AND GR-202 Balance (A&D Company, Limited, Toshima-ku, Tokyo, Japan) and pupal length and width were measured to the nearest 0.1 mm using Neiko Digital Calipers (Neiko Tools, USA). Pupae were glued to the top of a plastic cup using a hot glue gun to ensure proper eclosion. The pupae were monitored for eclosion daily; eclosion dates were recorded.

After eclosion, adults were placed in the rearing incubator (Percival Scientific, Perry, IA, USA) at 16ºC for 24 hours to allow their wings to dry. They were checked for the presence of *Ophryocystis elektroscirrha* spores by observing abdomen scale samples under a compound microscope (Olympus BH-2, Olympus Corp., Shinjuku-ku, Tokyo, Japan). The butterfly was discarded if the parasite was present to avoid dispersal of the parasite to the colony. The butterflies were processed in a chamber at 16ºC and 60% humidity to reduce their activity during handling.

The individual number assigned to each larva was written on the bottom of each hindwing of both treatments using an ultra-fine black Sharpie marker (Sharpie, Oak Brook, IL, USA). Adult butterflies were not dusted with powder in this study. Butterflies were placed individually into a Glassine envelope (MeadWestvaco Corp., Richmond, VA, USA) to reduce movement during wing measurements and weighing. Both forewing and hindwing lengths were measured to the nearest 1/10 mm using Neiko Digital Calipers (Neiko Tools, USA), and adult mass was recorded to the nearest 1/10 mg on an AND GR-202 Balance (A&D Company, Limited, Toshima-ku, Tokyo, Japan). We recorded the sex of each butterfly and placed groups of adults into 29 cm^3 BugDorm-1 insect rearing cages (MegaView Science Co. LTD, Taichung, Taiwan), in their respective treatment groups to mate. The enclosures were moved into an incubating cupboard with a temperature range of 25-27ºC and 50-90% relative humidity, which are conducive to breeding[1]. Adults were fed artificial nectar *ad libitum* and observed daily to determine if females had mated.

Mated females were transferred to pop-up Backyard Safari Butterfly Habitats (Alex Brands, Fairfield, NJ, USA) by treatment group, in the incubating cupboard. Each enclosure contained one common milkweed plant on which they could oviposit. After 24 hours, the milkweed leaves with eggs were collected and placed in a large Falcon petri dish, 100 x 15 mm (Corning Inc., Corning, NY, USA) lined with a damp Whatman 90 mm filter circle (GE Healthcare, Chicago, IL, USA) by treatment group. The petri dishes were placed in a 27ºC incubator (Percival Scientific, Perry, IA, USA) and checked daily for hatching.

All data were analyzed in R v3.3.2/RStudio v1.0.136 (RStudio, Inc., Boston, MA, USA). A Welch Two Sample t-test was used to determine whether there were significant differences in mean larval mass, mean pupal mass, mean pupal width, mean pupal length, mean adult mass, mean forewing length, and mean hindwing length. A log rank test was used to determine whether there was a significant difference in survival, throughout life stages, between the two treatment groups. Percentages were compared with a one sample t-test using Statistics Calculator (StatPac, Inc, Northfield, MN, USA).

3. Results

Externally marking *Danaus plexippus* larvae with DayGlo Eco Aurora Pink Pigment powder had minimal effects on development and survival. Twenty-three caterpillars in the dusted group (46%) and 21 caterpillars in the control group (42%) survived through the end of the 32-day study. There was no significant difference in survival between the treatments (Log rank test; $\chi^2 = 0.1$, df = 1, p= 0.78; Figure 1).

Sixty percent of the dusted larvae pupated and 58% eclosed compared to 66% and 52% of control larvae. There were no significant differences in the pupation and eclosion rates between the treatment groups. The days to pupation

and pupal development time were nearly identical between the dusted (15.07 ± 0.28; 8.62 ± 0.12) and control (15.09 ± 0.27; 8.46 ± 0.10) groups (Table 1).

There were no significant differences between treatment and controls in mean larval mass (p= 0.46), mean pupal mass (p= 0.52), mean pupal length (p= 0.43), mean pupal width (p= 0.07), mean adult mass (p= 0.77), mean forewing length (p= 0.24), or mean hindwing length (p= 0.30) (Table 2).

Surviving adults successfully mated. Nine of the 23 surviving adults in the dusted group were female and of the 21 surviving adults in the control group there were also 9 females. The females in each treatment laid viable eggs and the eggs successfully hatched.

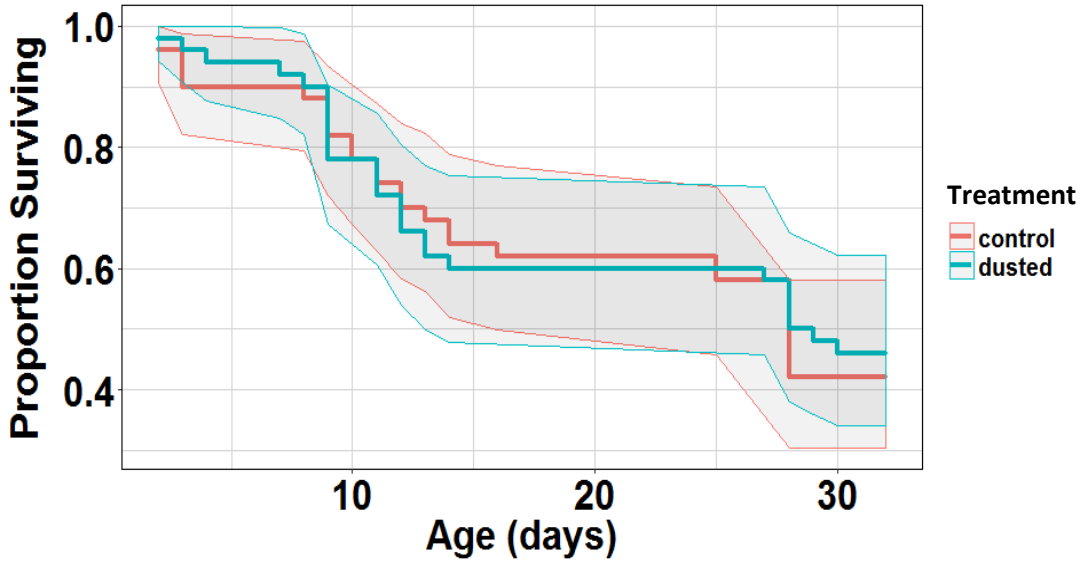

Figure 1. Kaplan-Meier survivorship curve of monarch larvae dusted with powder vs. control group. The shaded areas represent 95% confidence intervals (dusted = blue; control = pink). Starting n = 50 dusted and n = 50 control. (Log rank test; χ^2=0.1, df=1, p=0.78). There were no significant differences between the dusted and control groups.

Table 1. Mean (± SEM) days to pupation, pupal development time, pupation percentage, and eclosion percentage of *D. plexippus*. Sample sizes are given in brackets.

Treatment	**Days to Pupation**	**Days as Pupa**	**Pupation (percent)**	**Eclosion (percent)**
Control	15.09 ± 0.27 (33)	8.46 ± 0.10 (26)	66.0 (50)	52.0 (50)
Dusted	15.07 ± 0.28 (30)	8.62 ± 0.12 (29)	60.0 (50)	58.0 (50)
P-value	P = 0.95	P = 0.30		
Test Statistic	T = -0.06	T = 1.04	T = 0.54	T = 0.57
Degrees of Freedom	df = 60.80	df = 52.58	df = 99	df = 99

Table 2. Mean (± SEM) larval mass, pupal mass, pupal length, pupal width, adult mass, forewing length, and hindwing length of *D. plexippus.*

Treatment	Mean larval mass (g)	Mean pupal mass (g)	Mean pupal length (mm)	Mean pupal width (mm)	Mean adult mass (g)	Mean forewing length (mm)	Mean hindwing length (mm)
Control	361.53±9.61	1015.42±26.01	21.89±0.26	10.25±0.21	475.38±24.33	46.27±0.54	30.91±0.24
Dusted	351.25±9.81	992.17±24.22	21.62±0.22	9.80±0.29	465.69±21.20	45.41±0.48	30.54±0.25
P-value	P = 0.46	P = 0.52	P = 0.43	P = 0.07	P = 0.07	P = 0.24	P = 0.30
Test Statistic	T = -0.75	T = -0.65	T = -0.80	T = -1.88	T = -0.30	T = -1.19	T = -1.04
Degrees of Freedom	df = 83.97	df = 59.92	df = 59.43	df = 46.58	df = 49.11	df = 49.55	df = 50.29

4. Discussion

The fate of the eastern migrating monarch population is precarious, at best. It is important to have safe marking methods that do not affect their development or survival. Our results supported our hypothesis that DayGlo ECO Aurora Pink Pigment powder would not significantly affect development or survival of *Danaus plexippus* larvae. Twenty-three caterpillars in the dusted group (46%) and 21 in the control group (42%) survived through the end of the study (figure 1), which was slightly lower than ~50% reported elsewhere[16]. There was no difference in survival between the treatments, which correspond with the results of Cook and Hain[2], Mo et al.[13], and Nakata[14].

Like Warner and Bierzychudek[21], we documented no adverse effects on survival or development of monarch butterfly larvae when larvae were externally dusted with DayGlo fluorescent powder. DayGlo fluorescent powder also had no significant effects on survival when used on mountain pine beetles[12], parasitoid wasps[5], and western corn rootworm beetles[15].

There were no significant differences between treatment groups in any of the response variables. There was a minute difference (<0.02 days) until pupation, with 33 control larvae (66%) and 30 dusted larvae (60%) pupating after ~15 days. Larvae from both groups spent ~8.5 days as pupa with 26 eclosing from the control group (52%) and 29 eclosing from the dusted group (58%) (Table 1).

The control group had slightly larger mean larval mass, mean pupal mass, mean pupal length and width, mean adult mass, and mean forewing and hindwing lengths, but they were not significant. There was <1 mm difference between treatment groups in mean forewing and hindwing lengths (Table 2).

The surviving adults successfully mated and laid viable eggs which concurs with the Warner and Bierzychudek study[21]. Although this study was conducted in the laboratory under controlled conditions, we expect that this marking technique would also be safe to use in field experiments.

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