

Detailed Methods

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1 Experimental design

The objective of this study was to determine how *O. elektroscirrha* infection and plant diet impacts the gene expression of monarchs throughout their development. Infection status (infected and uninfected) and plant diet (*A. incarnata* and *A. curassavica*) were factorially manipulated, resulting in four treatment groups consisting of forty individuals each. The developmental stages sampled in this study were third instar, fifth instar, early pupa (1 day after pupation), late pupa (6-8 days after pupation), and adult (the day of eclosion). Eight caterpillars were sampled at each developmental stage, and from these eight, five were sent for transcriptome sequencing. The overall experimental design is depicted in Figure 1.

2 Milkweed cultivation

A. incarnata and *A. curassavica* seeds were purchased from Joyful Butterfly (Blackstock, SC, USA). To break cold dormancy, seeds were placed in sand-filled bags and kept in a refrigerator at 4°C for two months prior to sowing. Approximately two months before the start of the experiment, seeds were sown into Lambert LM-GPS germination soil and placed in a temperature controlled greenhouse room held between 25°C and 29.4°C. *A. incarnata* germination rates tend to be relatively low, so seed trays were topped with vermiculite to aid in moisture retention. Seedlings were fertilized with approximately X PPM of Jack's LX 15-5-15 with 4% Ca and 2% Mg fertilizer three times a week until the majority of plants grew two sets of true leaves. All plants were then re-potted into Pro-mix BK25 soil, moved to a new temperature controlled room held between 25.6°C and 29.4°C, and fertilized three times a week as described above. Approximately one week before the start of the experiment, plants were moved into the same greenhouse room that caterpillars were reared in (described below).

3 Monarch rearing

Monarch butterflies were caught and labeled near St. Marks, Florida, U.S.A. (30°09'33N, 84°12'26W) between October 21st and October 23rd, 2022. Clear tape was placed on the abdomen of each butterfly and examined under a stereomicroscope to check for *O. elektroscirrha* spores. Prior to mating season, wild-caught monarch butterflies were stored in glassine envelopes, placed in a refrigerator (14°C) to induce a state of diapause, and occasionally fed approximately 10-20% honey water. Between March 6th and March 15th, 2023, wild-caught monarchs were placed in mesh cages for mating. Each cage was set up in a climate controlled growth chamber (25°C, 16-hour/8-hour day/night cycle) and contained three to six male and three to six female butterflies (Figure 2). All cages were provided with a petri dish containing a sponge soaked in approximately 10-20% honey water for butterfly feeding. Mating cages were checked every 14 hours, and copulated butterflies were transferred to their own separate cage. After a copulated pair had detached the next day, the male was removed from the cage and the female was given a potted *A. curassavica* plant for oviposition, as well as honey water as described above (Figure 3). After a given female was done laying eggs, the plant was taken out of the growth chamber and placed in a temperature controlled greenhouse room held between 23.3°C and 27.8°C for the eggs to hatch.

F1 caterpillars were then reared on *A. curassavica* in the same greenhouse room previously described. After pupation, the silk attached to the end of the pupal cremasters was used to hot glue the pupae to the lid of clear solo cups, which were then taken from the greenhouse to the lab (22°C) for eclosion. A piece of paper towel was placed in the bottom of cups to help absorb liquids produced during the eclosion process. After eclosion, butterflies were placed in glassine envelopes and stored in a refrigerator as previously described.



Figure 2. Top: Monarch butterflies in mating cages. Image does not depict the actual cages used in this experiment. Bottom: Monarch butterfly feeding on honey water from sponge in petri dish. Images do not show actual butterflies used in this experiment.

Between April 23rd and May 1st, 2023, F1 butterflies not infected with *O. elektroscirrha* and from different lineages were mated as previously described in the F0 generation. F1 females were given either *A. curassavica* or *A. incarnata* for oviposition, and caterpillars were collectively placed on their treatment plant species upon hatching. Care was taken to make sure caterpillars that had taken bites of the plant they were oviposited on to were placed on the same milkweed species. Likewise, only caterpillars that had not taken any bites of the plant they were oviposited on were placed on the other milkweed species (Figure 1). To reach the sample size needed for this experiment, we used F2 caterpillars from two different lineages that did not share F0 or F1 ancestors. Treatments of parasite infection, plant species, and development stage were randomly distributed to caterpillars from both lineages to minimize confounding due to genetic background.

Figure 3. Top: Female monarch butterflies in cages with *A. curassavica* for oviposition. Bottom: Close-up of female monarch butterfly ovipositing on *A. curassavica*. Images do not show actual butterflies used in this experiment.

4 *O. elektroscirrha* inoculation

Once caterpillars reached the third instar, parasite treatments were administered. [Details about OE spore acquisition]. *O. elektroscirrha* inoculation was performed by placing caterpillars into petri dishes containing a milkweed leaf disc corresponding to their milkweed treatment group. Prior to placing milkweed leaf discs into the petri dishes, qualitative filter paper was placed into petri dishes and wetted with deionized water to prevent leaf discs from drying out before the caterpillars could finish eating them. Milkweed leaf discs (approximately 6.35 mm in diameter) were obtained by using a standard hole puncher to take sections of the leaf that included side veins but not the midrib, as third instar caterpillars often do not eat leaf midribs. The hole puncher was cleaned using 75% ethanol between plants to minimize contamination of leaf discs with the other plant species' chemicals. For infected treatments, 100 *O.*

elektroscirrha spores were counted using a stereomicroscope and placed on the veins of the milkweed leaf discs using a modified glass stir rod. Leaf discs fed to uninfected caterpillars were punched at the same time but did not have parasite spores placed onto them.

After all petri dishes and leaf discs were prepared, second instar caterpillars were taken from the plant they were feeding on and placed in a petri dish containing a leaf disc corresponding to their treatment (Figure 4). After caterpillars finished eating their whole leaf discs, they were placed on new plants corresponding to their treatment plant species. Transparent plastic tubes with mesh nets on top were placed around all plants to confine the caterpillars. All tubes were placed on the same shelf in the greenhouse room to minimize possible micro-climate variation, and tube positions were shuffled every three days (Figure 5).



Figure 4. Third instar caterpillar in petri dish with milkweed leaf disc.

5 Sampling across developmental stages

To minimize changes in transcription due to cold stress, all caterpillars, pupae, and adults were snap frozen in liquid nitrogen before storage in -80°C. Third instar caterpillars were frozen the day after parasite inoculation. Caterpillars were pulled from their feeding plant and quickly placed into a sterile 2mL microcentrifuge tube that was then dipped in liquid nitrogen. Fifth instar caterpillars were frozen in the same way, but were placed in sterile 5mL centrifuge tubes. Caterpillars that ate all of the leaves off of the plant they were originally placed on were placed on another plant of the same species.



Figure 5. The arrangement of plant tubes on the greenhouse shelf that they were placed on for the duration of the experiment.

One day after pupation, early pupa samples were placed in 5mL centrifuge tubes and frozen in liquid nitrogen as described above. Three days after pupation, pupae assigned to late pupa and adult stages were removed from their plant and taped to the lids of clear solo cups using silk attached to the cremaster (Figure 6). This allows for better assessment of the early signs of *O. elektroscirrha* proliferation, as indicated by pupal darkening (Figure 7). In some cases, not enough silk detached with the pupa, and tape was applied directly to the cremaster. Solo cups were then placed on the bottom rack of the same shelf that the caterpillars were reared on, and shade was provided by placing plastic trays above and to the southeast facing side of the shelf to prevent pupae from burning. A piece of paper towel was placed in the bottom of the cups containing adult samples to absorb fluids produced during the pupation process. *O. elektroscirrha*-infected late pupa samples were frozen in liquid nitrogen six to eight days after pupation, depending on when evidence of *O. elektroscirrha*

troscirrha proliferation was presented. To make the distribution of time spent as a pupa even across treatment groups, an uninfected sample that fed on the same plant species and had been a pupa for the same amount of time was also frozen at the same time as each infected sample (Figure 8). Adult samples were frozen on the same day that they eclosed. Here, adults were removed from their solo cup and quickly placed in glassine envelopes, which were then quickly frozen in liquid nitrogen.



Figure 6. Monarch pupae taped to the lids of clear solo cups.



Figure 7. Top: Monarch pupa showing early signs of *O. elektroscirrha* infection (pupal score=1). Bottom: An uninfected pupa (left) next to an *O. elektroscirrha*-infected pupa (right, pupal score=3)

After flash freezing in liquid nitrogen, samples were stored in a styrofoam cooler full of dry ice until all freezing for that day was completed. This process took approximately one hour or less on any given day, so no sample was on dry ice for more than an

hour before being transferred to the -80°C freezer. All freezing took place in the same greenhouse room that the caterpillars were reared in, and no caterpillar left said room throughout the duration of the experiment.

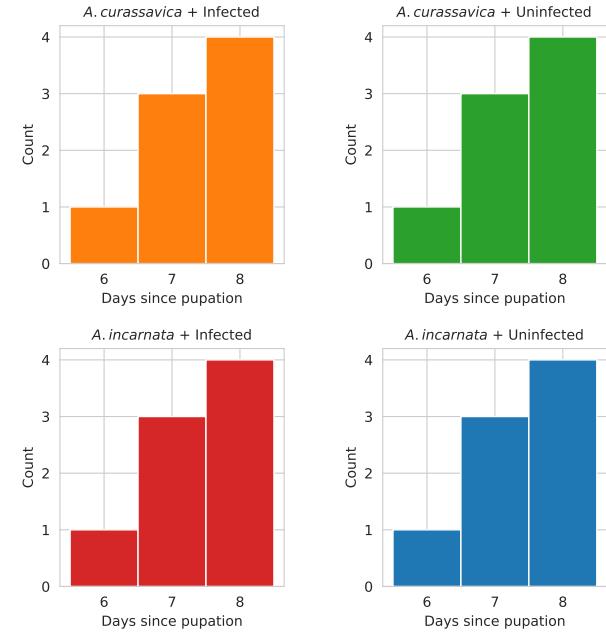


Figure 8. The distribution of days after pupation that late pupae samples were frozen.

6 RNA Extraction and Sequencing

6.1 Sample Preparation

Prior to RNA extractions, whole caterpillars or butterflies were homogenized by using a porcelain mortar and pestle to grind the tissues into a powder. Mortars and pestles were cleaned using 70% ethanol, and subsequently autoclaved at 121°C while wrapped in aluminum foil. Each sample for a given round of homogenization was removed from the -80°C freezer and placed on dry ice. Samples were individually placed in a sterile mortar and liquid nitrogen was immediately added to prevent samples from thawing. While submerged in liquid nitrogen, samples were ground using a sterile pestle, and liquid nitrogen was continuously added to ensure samples did not thaw throughout the homogenization process. After the sample was completely homogenized, homogenate quickly was collected using a disposable sterile polypropylene spatula (sterilized as described for the mortars and pestles) and placed in either a

2mL microcentrifuge tube (for 3rd instars) or a 5mL microcentrifuge tube (for all other samples). The microcentrifuge tube was then stored on dry ice while the remaining samples were completed. After all samples were homogenized, they were placed back in the -80°C freezer. Twenty samples were homogenized per round, and each round took approximately two to three hours.