Population Differences and Genetic Basis of Parasitic Resistance and Melanization in Monarch Butterflies

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Abstract

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Monarch butterfly *(Danaus plexippus)* larvae feed exclusively on the leaves of milkweed plants. They can consume secondary defense chemicals produced by milkweed called cardenolides. These chemicals are toxic to many other animals but have been linked to increased resistance to *Ophryocystis elektroscirrha* - an obligate, neogregarine protozoan parasite that infects monarch and queen butterflies with no other known hosts – in adult monarch butterflies. That being said, it is not yet known whether it is the cardenolides themselves that provide the resistance. *Asclepias curassavica*, a plant species in the milkweed family, is native to Puerto Rico and has a relatively higher cardenolide concentration than *Asclepia incarnata*, native to eastern North America. We crossed Eastern North American monarchs with Puerto Rican monarchs to generate a Hybrid population. We expected both monarch populations to be more effective at dealing with the protozoan parasites *O. elektroscirrha* when reared on *A. curassavica* as the consumption of milkweeds that produce more cardenolides has been linked to greater parasitic resistance in monarchs. We also expected differences in parasitic resistance between monarchs of different populations as previous work has shown that non-migratory monarchs are more resistant to infection when compared to migratory monarchs. As for the resistance of the hybrid monarchs, we projected that they would have intermediate levels of resistance between parental genotypes. We predicted this because it is likely that resistance to parasites in monarch butterflies is governed by multiple genes and, as a result, when hybridizing Eastern North American and Puerto Rican monarchs, which have different resistance profiles, their offspring are expected to inherit a blend

of alleles from each parent resulting in an intermediate resistance phenotype. To test this, monarchs captured from Puerto Rico and North Florida were crossed in a typical F2 mating pattern to produce pure Eastern monarch families, pure Puerto Rican families and Hybrid monarch families. We then raised half of all Puerto Rican monarchs on A. incarnata and the other half on A. curassavica, half of all Eastern monarchs on A. incarnata and the other half on A. curassavica and half of all Hybrid monarchs on A. incarnata and the other half on A. curassavica. After accessing the severity of infection of each adult monarch, we found that Puerto Rican monarchs had a greater resistance to the protozoan parasite than Eastern monarchs and that Hybrid monarchs showed intermediate parasitic resistance, suggesting that parasitic resistance is a polygenic trait. Additionally, monarchs reared on *A. curassavica* presented greater resistance regardless of which population they belong to. Following these findings, we used pictures of fifth instar monarch larvae to assess larval melanization as the latter has been found to be a conserved immune response in other arthropods. Studies have found that Puerto Rican monarch larvae are darker than Eastern North American larvae, and these findings were reflected in our results. Interestingly, hybrid monarch larvae mean corporal blackness was significantly greater than that of Eastern monarchs, but we found no significant difference when compared to the Puerto Ricans. As these results do not mirror the latter parasitic resistance observations, melanization does not seem to be correlated to resistance.

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This work was made possible through the collective efforts of these individuals, and their contributions are sincerely acknowledged.

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Introduction

From a single common ancestor, milkweeds have diversified to over a hundred species all over North America. Monarch butterflies, one of their few consumers, lay their eggs on these milkweed plants and as they hatch, the caterpillars devour the leaves of the plant, their only food source during this stage of their development. Monarchs and milkweed have coevolved over millions of years, the milkweed evolving to develop more defenses against the monarch and the monarch evolving to find new ways to bypass these defenses: a genetic “arms race” (Agrawal, 2017; Cornell & Hawkins, 2003).

In this project, we studied monarch butterflies from Eastern North America and from Puerto Rico on two different milkweed species, *A. incarnata* and *A. curassavica,* native to Eastern North America and Puerto Rico respectively. Relative to the former, *A. curassavica* produces more secondary defense chemicals (toxins called cardenolides) which have been associated with increased parasitic resistance of the larvae that were reared on it (Gowler et al., 2015). In nature, monarch butterflies are exposed to a wide range of selective forces. One of these selective forces is parasitic infection (Altizer & Oberhauser, 1999). Eastern monarch butterflies embark on an annual migration to overwintering sites in Central Mexico at the start of the winter and migrate back in the beginning of the spring. As part of this migration, they are able to leave areas of high parasite concentration and go towards areas where the parasite is not as present in a process known as “migratory escape” (Kendzel et al., 2023). Additionally, because migration demands considerable energy and effort, infected individuals often perish during the journey, thereby diminishing the likelihood of future parasite transmission. This phenomenon is referred to as "migratory culling" (Kendzel et al., 2023). While Puerto Rican monarchs belong to the same species as Eastern monarchs, they exhibit a different behavior pattern regarding migration. Instead of undertaking the long migratory journeys like their Eastern counterparts, Puerto Rican monarchs remain on the island throughout the year (Altizer & Davis, 2010). Consequently, they do not benefit from migratory escape or culling mechanisms to reduce the presence of infected individuals within their population. This leads to a higher prevalence of OE in Puerto Rican monarchs (S. Altizer & De Roode, 2015). The absence of migratory escape or culling mechanisms may have led to the selection of alternative forms of parasite resistance in Puerto Rican monarchs. In this project, we hypothesize that Puerto Rican monarchs may have adapted a genetic form of resistance as seen in other systems such as in the *Biomphalaria glabrata–S. mansoni* snail–parasite system (Carton et al., 2005).

To test this hypothesis, monarch butterflies captured from Eastern North America (North Florida) and Puerto Rico were crossed in a typical F2 mating design to produce pure Eastern monarch families, pure Puerto Rican monarch families and Hybrid monarch families - the role of the Hybrids being to determine whether the trait for parasitic resistance follows a monogenetic or polygenetic model of expression. The eggs were then transferred to either *A. incarnata* or *A. curassavica*. Two days after hatching, we infected the larvae with the protozoan parasite *O. elektroscirrha*. We then let the monarchs live out their life cycle, assigning pupal scores to assess their level of parasitic infection and analyzing pictures of caterpillars in fifth instar stage to evaluate melanin production which has been found to correlate positively with parasitic resistance in insects (Smith et al., 2021). Furthermore, if the results point to a genetic basis for parasitic resistance in monarch butterflies, we planned to explore what other genes could be involved in shaping parasite resistance.

One of the traits we plan to explore is melanin production. Melanin, a pigment synthesized by specialized cells known as melanocytes, is ubiquitous across all biological kingdoms (Freitas et al., 2021). Besides its well-documented role as a shield against harmful UV radiation, melanin contributes to bodily temperature regulation and offers protection against the detrimental effects of free radicals (Sugumaran, 2002). Notably, research has also identified melanization as a conserved immune response in arthropods (Bilandžija et al., 2017). Consequently, we hypothesize that melanin may influence monarch butterfly immunity, given prior observations that Puerto Rican monarch larvae and adults exhibit darker pigmentation compared to their Eastern North American counterparts. Pictures taken of fifth instar caterpillars and the corresponding adult wings from all three experimental groups were analyzed to investigate any pleiotropic correlations between corporal colorations (black and yellow) and parasitic resistance.

Methods

Matings

Four males and two females were placed in secured cages with a sponge soaked in honey water for consumption. The cages were checked every day for matings and when a mating was recorded, the mating pair was moved to a separate cage until the male and female detached. One day after detachment, females were moved to a new cage with *A. incarnata* to deposit their eggs onto. Offspring from a particular pair constituted distinct lineages. Multiple lineages of monarchs originating from the same population were employed to account for the innate genetic variability inherent within that population. All matings were set up with regular day-night cycles and with a controlled temperature and humidity. Hatching caterpillars were split onto *A. incarnata* or *A. curassavica* plants to ensure monarchs consumed either plant species as soon as they were born.

Inoculations

We used a paper-hole puncher to cut out small leaf discs from either *A. curassavica* or *A. incarnata* leaves. A single *O. elektroscirrha* spore was then placed on each leaf disk. The leaf disk with the single *O. elektroscirrha* spore was placed into a sterile petri dish along with a damp filter paper to prevent the leaf disc from drying out. Next, a single second instar caterpillar was placed into each petri dish and checked daily until it consumed the entire leaf disk – it would typically do so within 48 hours. Caterpillars that did not finish their leaf disk were excluded.

Handling larvae and butterflies

Tubing

Once they finished eating their leaf disk, the larvae were moved using forceps to a new, individual tube containing a new milkweed plant of the same species – *A. curassavica* or *A. incarnata* – as the larvae were placed on. Larvae remained in their individual tubes until pupation.

Feeding

When leaves were no longer visible on a milkweed plant, the latter was either replaced with a new plant of the same species or leaves from the same species were added into the tube.

Taking photos of fifth instar phase

The final stage of a caterpillar's development before it pupates is called the fifth instar phase. During this stage of development, which typically lasts 3 to 5 days, the caterpillar is at its largest size, appears plum and is often found looking for a place to pupate. Prior to this transformation, each caterpillar was carefully transferred onto a white filter-lined petri dish, with individual images captured from above using a Nikon DSLR camera. Representative pictures of Puerto Rican, Eastern and Hybrid fifth instar caterpillars raised on *A. curassavica* are shown in Figure 1.

Moving pupa to plastic cup

Once the caterpillars form pupae – also known as chrysalises- they were moved from the tube to a plastic cup which was appropriately labeled. To do so, one third of a paper towel was placed at the bottom of the cup to absorb moisture from the butterfly's wing when it ecloses and the chrysalis was glued to the top of the cup using a hot glue gun. A lot of care was placed as to not put any glue on the chrysalis itself but instead on the leaf stem it was attached to.

Moving butterflies into envelopes

After butterflies eclosed, they were moved to appropriately labeled envelopes and placed into incubator chambers kept at a temperature of around 14 °C

Assessing infection burden

We assigned pupal scores based on the proportion of discoloration of the pupa on day prior to adult eclosion. Proportion of discoloration has been shown to positively correlate with parasite load, where monarchs with high pupal scores also have high spore loads as adults. A single pupa can be scored on a scale from 1 to 5 with 5 being the most extreme discoloration.

Death checks

After fully grown monarchs were placed into envelopes then in the incubator chambers (kept at a temperature of around 14 °C), their status as living/dead was checked daily at around the same time of day over the course of weeks until all monarchs had died. To do so monarchs were taken out of the incubator and placed at room temperature for around 10 minutes then were checked for any signs of life such as movement in legs, wings, or abdomen. If no movement was observed, monarchs would be marked as dead and placed back in the incubator. Lifespan was then calculated as the difference in days between a single monarch's eclosion date and death date.

Color Analysis

Pictures of monarch butterfly larvae in their fifth instar stage of development were cropped as to only include the caterpillar body. Subsequently, the background was adjusted to blue (RGB: 0,0,255) so as to not conflict with the black color measurements of the caterpillar. Following this background adjustment, pixels representing the white, yellow and black bands of the caterpillars respectively were grouped to the three mean colors - k-means clustering. Following that, the euclidean distances between each picture was minimized so that all white pixels were recolored to RGB (255,255,255), all yellow pixels were recolored to (255,255,0) and all black pixels to RGB (0,0,0). Finally, the amount of yellow, white and black pixels comprising a caterpillar body were quantified as a percentage of the total area of the caterpillar body they covered. Due to poor lighting conditions, certain caterpillar pictures had yellow and white bands that were either badly separated or inseparable. These pictures were removed from the quantification process.

Band Patterning Analysis

Pictures of monarch butterfly larvae in their fifth instar stage of development were modified by the k-means clustering and put into Image J after which midlines of their abdomen were hand-drawn. Midlines were drawn through abdominal segments 1 through 8 as these regions were the most conserved and unaffected by the different body positions of the caterpillar was laying at the time the picture was taken. Gray value data was generated by Image J for each pixel encountered by the midline.

Statistical Analysis

In R, an analysis of variance (ANOVA) was employed to evaluate the variation in the proportion infected, infection burden, larval mean corporal blackness, and larval mean corporal yellow coloration across populations. Additionally, linear models in R were utilized to examine the correlation between infection burden and mean corporal blackness per population, as well as the correlation between larval body mean black coloration and adult wing black coloration. Results were considered statistically significant if the associated p-value was less than 0.05. A principle component analysis of caterpillar morphology was also done to visually represent the morphological diversity within and between Eastern North American, Puerto Rican and Hybrid monarch populations.

Results

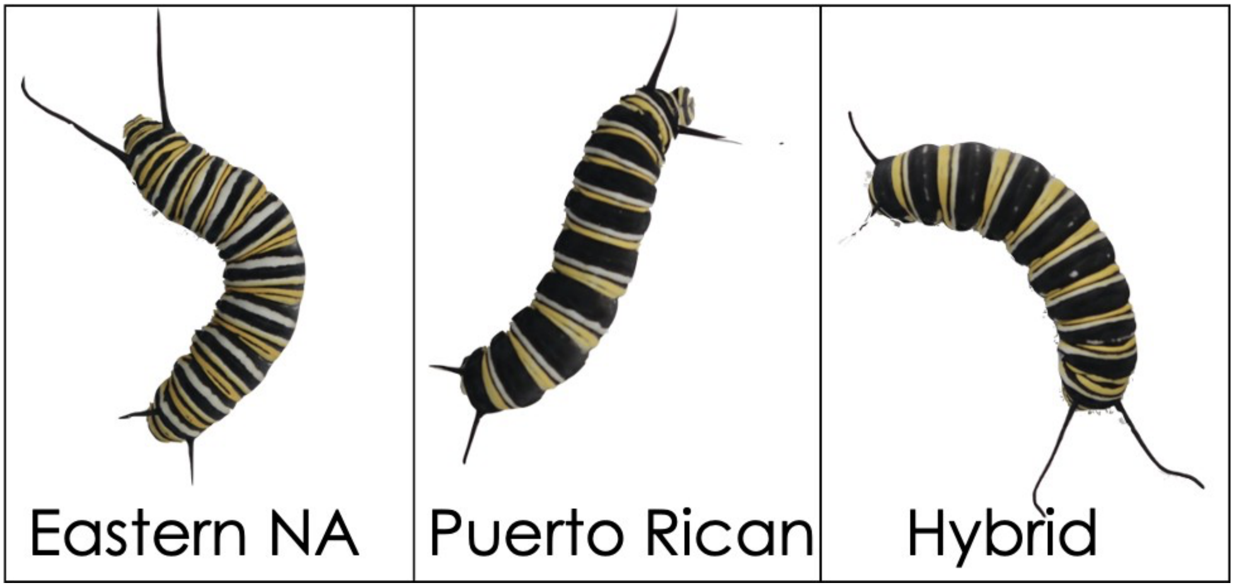


Fig. 1. Eastern, Puerto Rican and Hybrid monarch larvae in the fifth instar stage of development. Left, Eastern North American monarch larva; middle, Puerto Rican monarch larva; right, Hybrid monarch larva

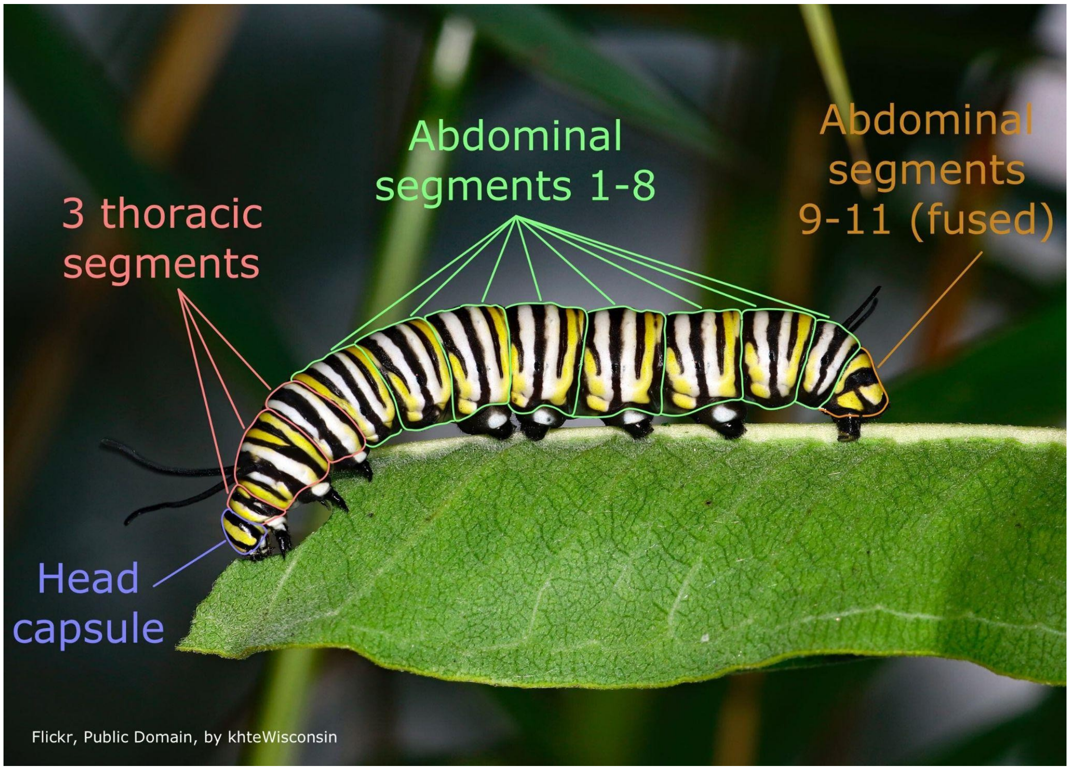


Fig. 2. Caterpillar body plan. The illustration includes the head capsule, the three thoracic segments and eleven abdomen typical of insects (nine through eleven being fused)

Parasitic Resistance

As presented in figure 3, a significant difference between the proportion infected of Eastern monarch butterflies and Hybrid monarch butterflies was found ($p < 0.001$) with Eastern monarchs being a more infected experimental group. Furthermore, a significant difference between the proportion infected of Eastern monarchs and Puerto Rican was found as well ($p < 0.05$), again with Eastern monarchs being the most infected experimental group. Puerto Rican monarchs were the least infected experimental group overall. Interestingly, these findings show that Hybrid monarch butterflies present an intermediate proportion infected, being significantly greater than that of the Eastern North American groups while being significantly lower than the Puerto Rican group.

We noticed the same trend for the severity with which each treatment group was infected as assessed by pupal score, presented in figure 4. A significant difference between pupal scores of Eastern monarch butterflies and Hybrid monarch butterflies was found ($p < 0.01$) with Eastern monarchs being more infected, and a significant difference between pupal scores of Eastern monarchs and Puerto Rican was found as well ($p < 0.001$), again with Eastern monarchs being the most infected. Puerto Rican monarchs were the least infected experimental group. Again, these findings show that Hybrid monarch butterflies present an intermediate severity of infection, with Hybrid pupal scores being significantly greater than that of the Eastern North American groups while being significantly lower than the Puerto Rican group.

Additionally, figures 2 and 3 show that monarchs reared on *A. curassavica* tended to have greater resistance to parasitic infection regardless of whether they were Eastern, Puerto Rican or Hybrid monarchs ($p=0.0694$). These findings would support previous findings that consuming milkweed with greater cardenolide concentration correlates positively with greater parasitic resistance since *A. curassavica* produces more of these secondary defense chemicals than *A. incarnata.*

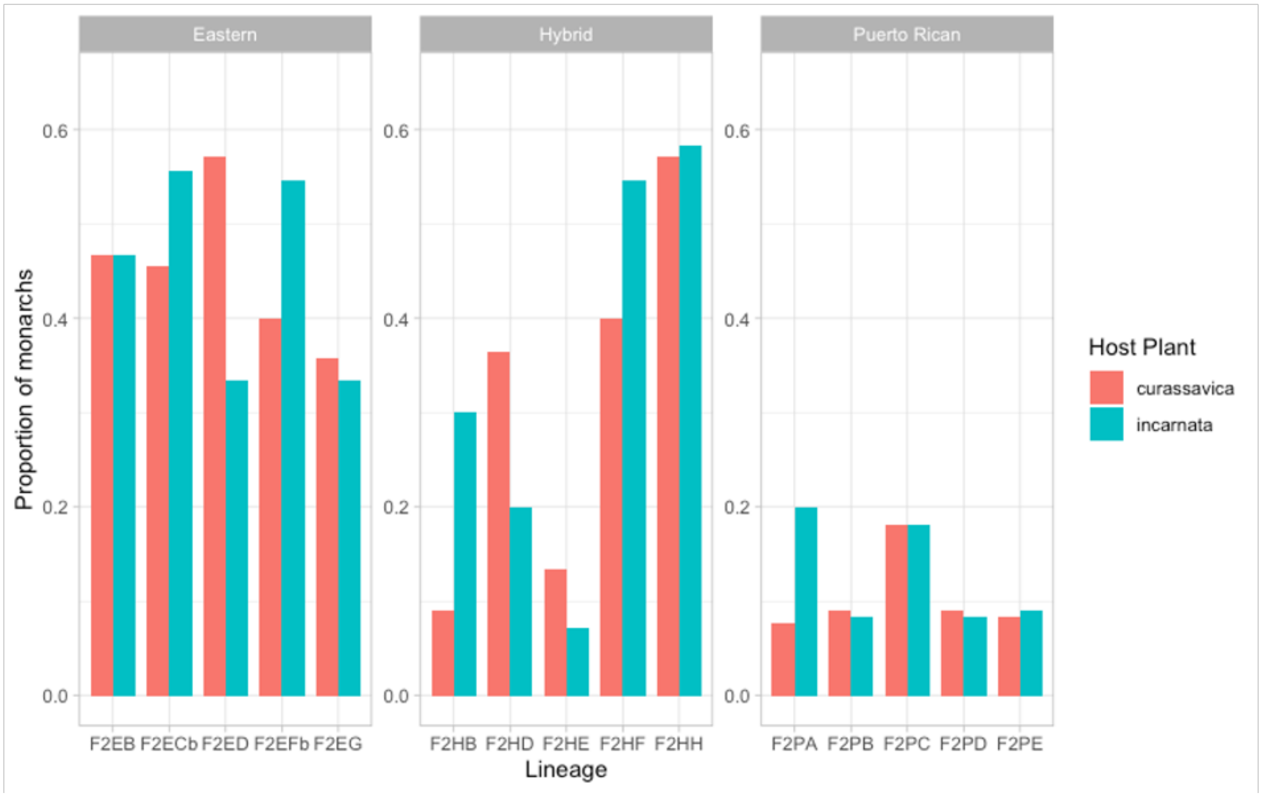


Fig. 3. Hybrid monarch butterflies have intermediate infection rate by the protozoan parasite O. *elektroscirrha* and Puerto Rican monarchs have lower infection rate than Eastern North American monarchs. Infection rates were determined as the ratio of infected monarchs to the total number of monarchs in the lineage.

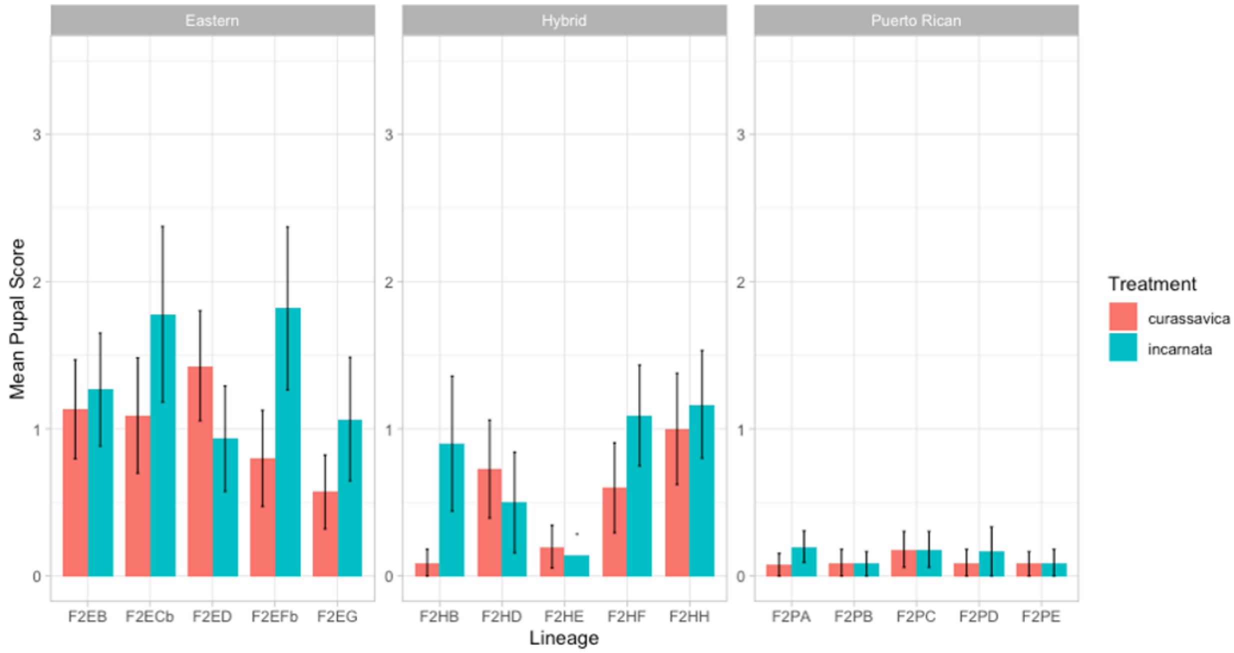


Fig. 4. Hybrid monarch butterflies have intermediate infection burden and Puerto Rican monarchs have lower pupal scores than Eastern North American monarchs. Pupal scores are a measure of parasite growth and were assigned based on the proportion of discoloration of the pupa on a scale from 1 to 5 with 5 being the most extreme discoloration and therefore greatest infection. Error bars represent standard error of the mean (SEM).

Larval Melanization

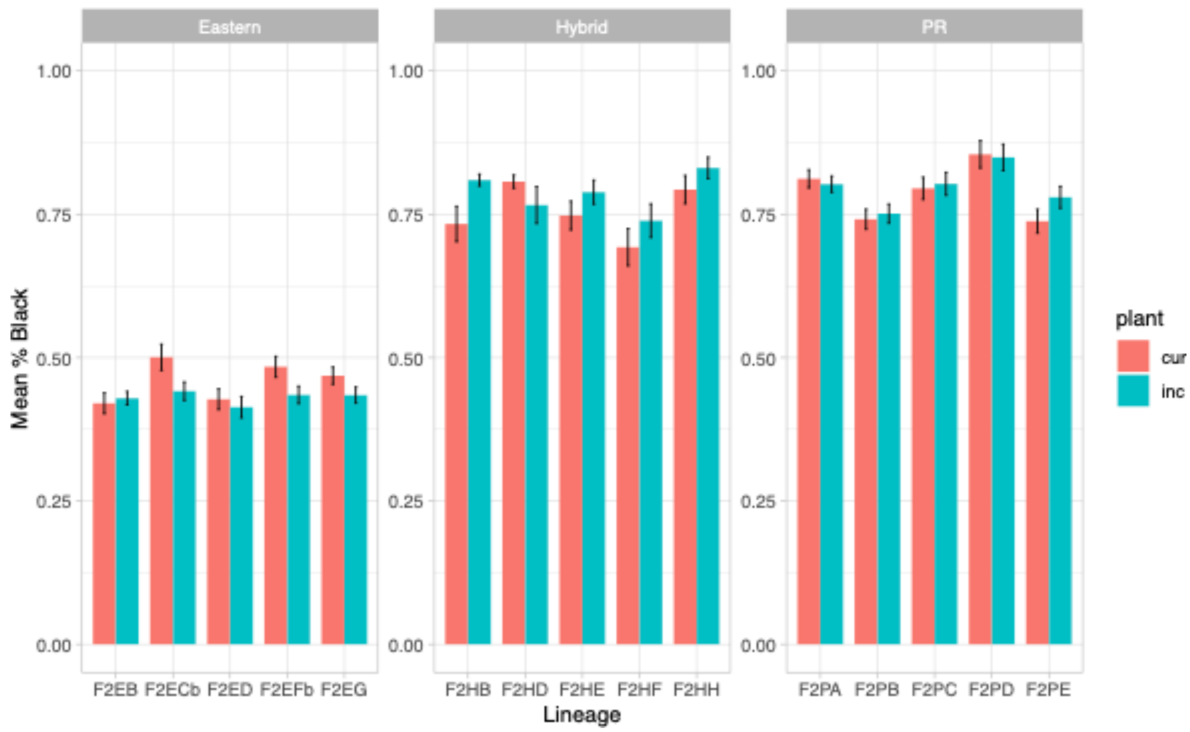


Fig. 5. Hybrid monarch butterflies exhibit phenotypic similarity to PR monarchs rather than displaying intermediate mean corporal blackness between ENA and PR monarchs. Error bars depict standard error of the mean (SEM).

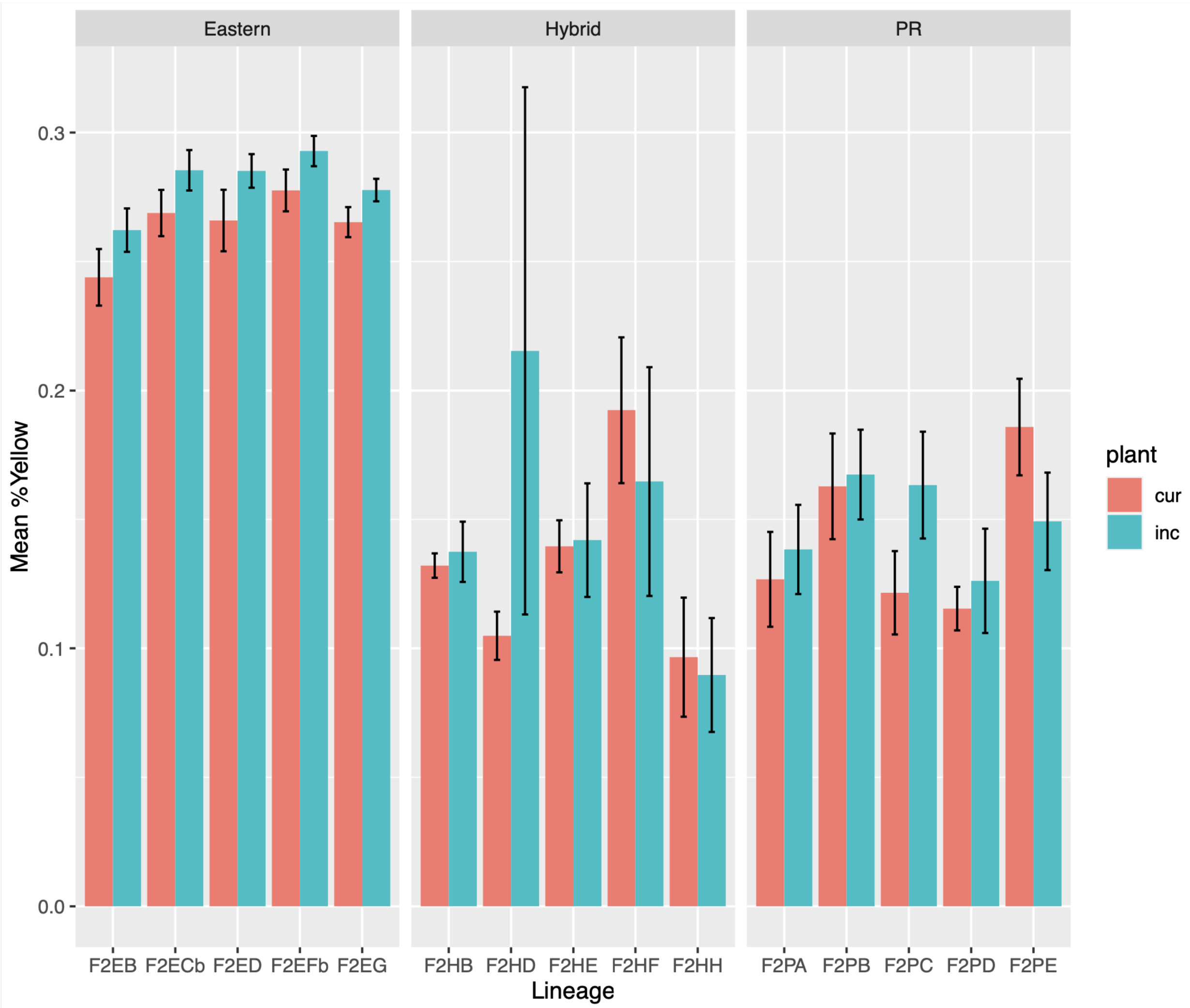


Fig. 6. Hybrid monarch butterflies again exhibit phenotypic similarity to PR monarchs rather than displaying intermediate mean corporal yellowness between ENA and PR monarchs. Error bars depict standard error of the mean (SEM).

Figures 5 and 6 show that larval melanization does not follow the same trend as parasitic resistance. In figure 5, we see that mean corporal blackness was significantly lower in Eastern monarch larvae compared to their Puerto Rican counterparts, but Hybrid monarchs presented with a phenotype similar to that of the Puerto Rican, instead of an intermediate level. Hybrid monarch larvae had significantly more black coloration than Eastern monarch larvae, yet exhibited a similar amount to that observed in Puerto Rican larvae

Mean corporal yellowness, presented in figure 6, follows a similar pattern. Eastern monarch larvae have significantly higher mean corporal yellowness compared to their Puerto Rican and Hybrid counterparts. Hybrid monarch larvae mean corporal yellowness is again similar to the Puerto Rican phenotype and does not follow the intermediate pattern seen for parasitic resistance. Furthermore, no significant difference in mean corporal blackness or mean corporal yellowness was found between lineages raised on *A.curassavica* compared to those raised on *A. incarnata*.

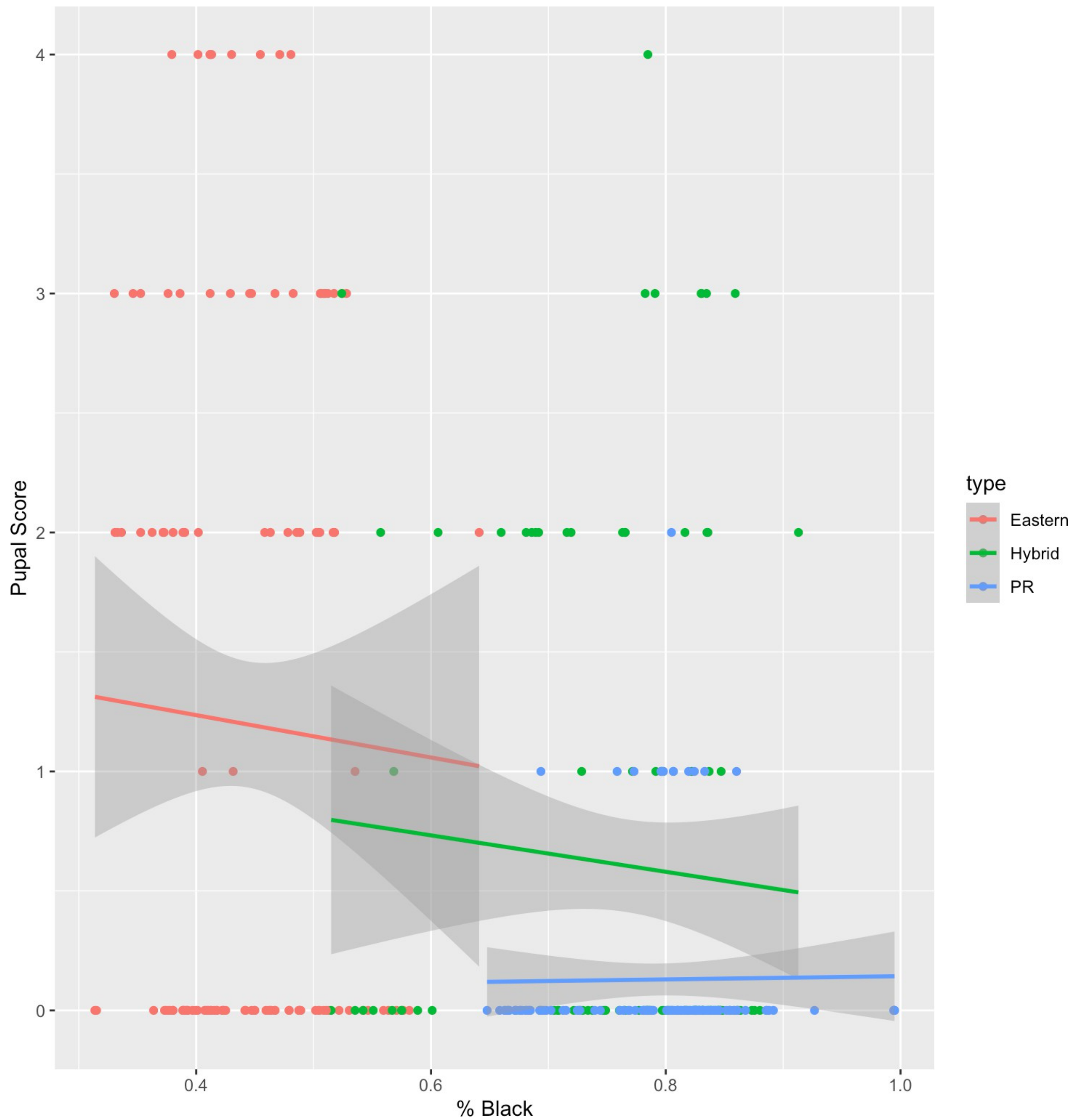


Fig. 7. Decreased infection burden is correlated with higher monarch butterfly mean corporal blackness between Eastern North American, Hybrid and Puerto Rican populations yet shows no significant impact on infection burden within individual populations.

Figure 7 illustrates a correlation between larval melanization, represented by the mean corporal blackness of monarch caterpillars, and infection burden, quantified by pupal discoloration (pupal score), at the population level ($p = 0.017$). This correlation, however, is not observed at the individual level within populations. That is to say that mean corporal blackness of Eastern North American, Hybrid, and Puerto Rican caterpillar populations serves as a predictor of infection severity for each population, but variations in blackness amongst individuals of a population do not correlate with the infection load endured by each individual. Consistent with figures 2 and 3, Eastern North American monarchs were the most heavily infected and least black population whereas Puerto Rican the least heavily infected and most black group. Hybrid monarchs had an intermediate infection burden.

Larval Band Patterning

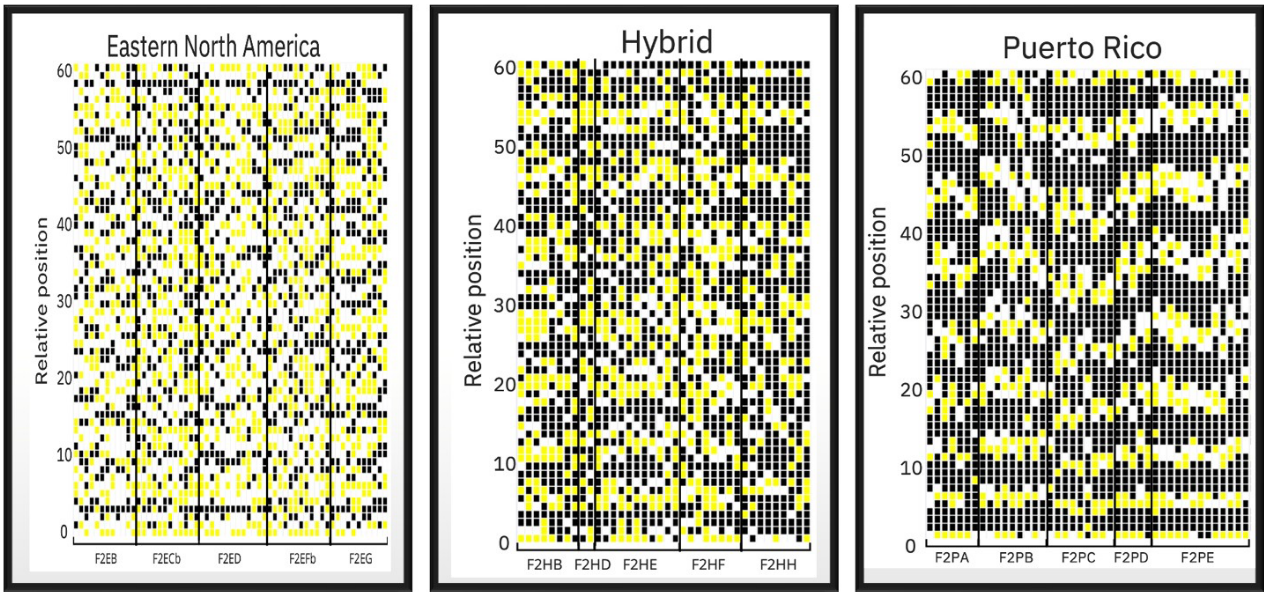


Fig. 8. Larval monarch butterfly abdominal segment coloration patterns. Eastern North America (left), Hybrid (middle), Puerto Rican (right). Block 0 represents the initial set of pixels located on the caterpillar's first abdominal segment, positioned closest to its head. Conversely, block 60 corresponds to the last set of pixels situated on the caterpillar's eighth abdominal segment, nearest to its posterior end.

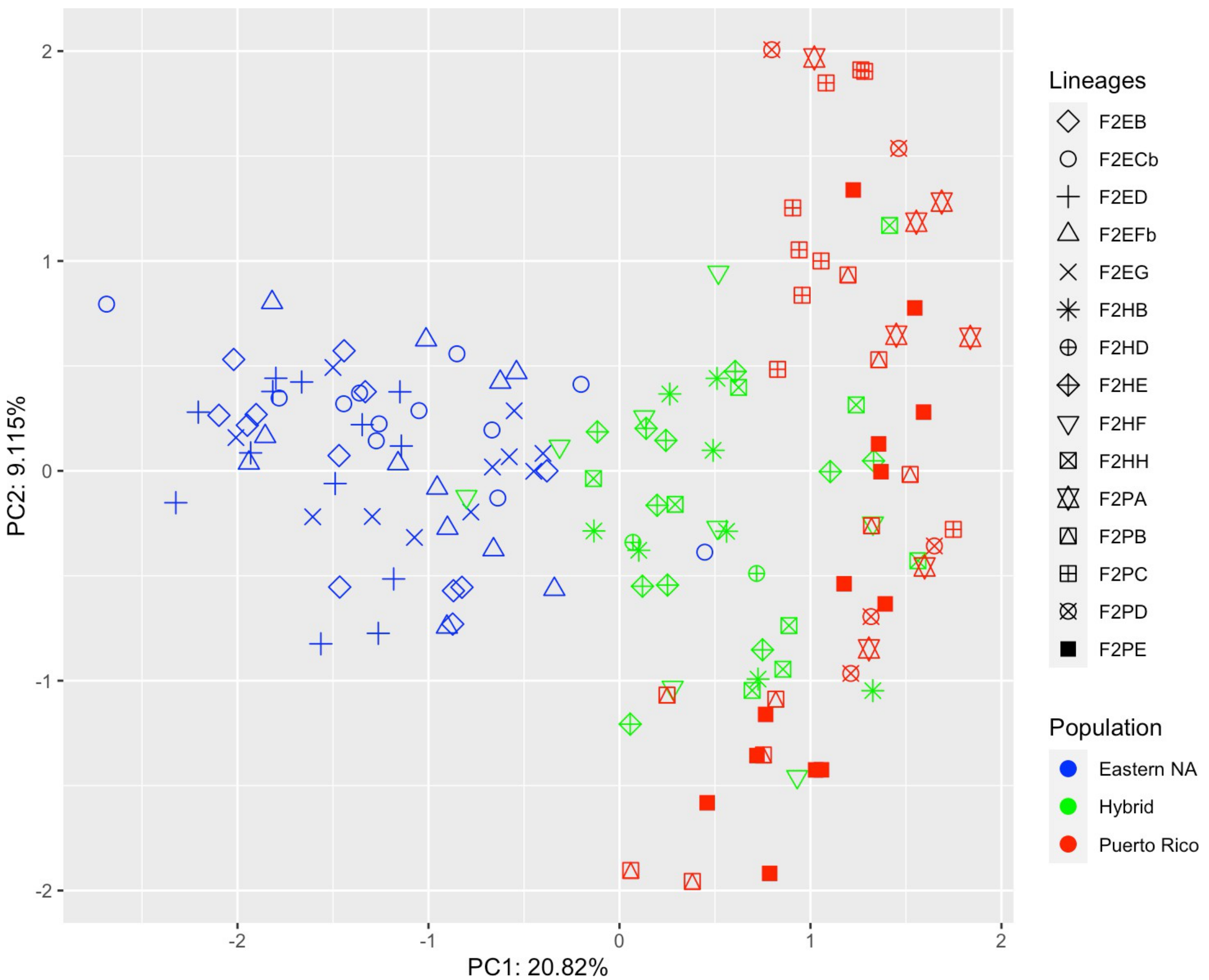


Fig. 9. Principal component analysis (PCA) of fifth instar monarch butterfly larvae body coloration and patterning. The two axes of the PCA presented here (PCA 1 and PCA 2) account for 20.83% and 9.115% of the variance respectively. Larval morphologies group into 3 populations: Eastern North American, Hybrids and Puerto Rican. Different shapes denote different lineages.

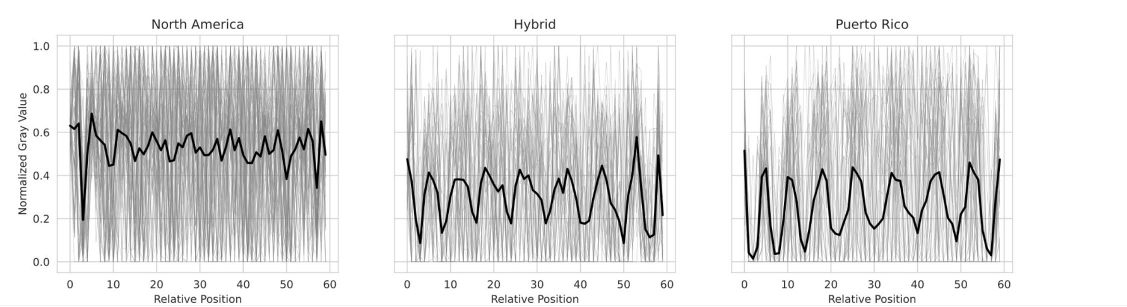


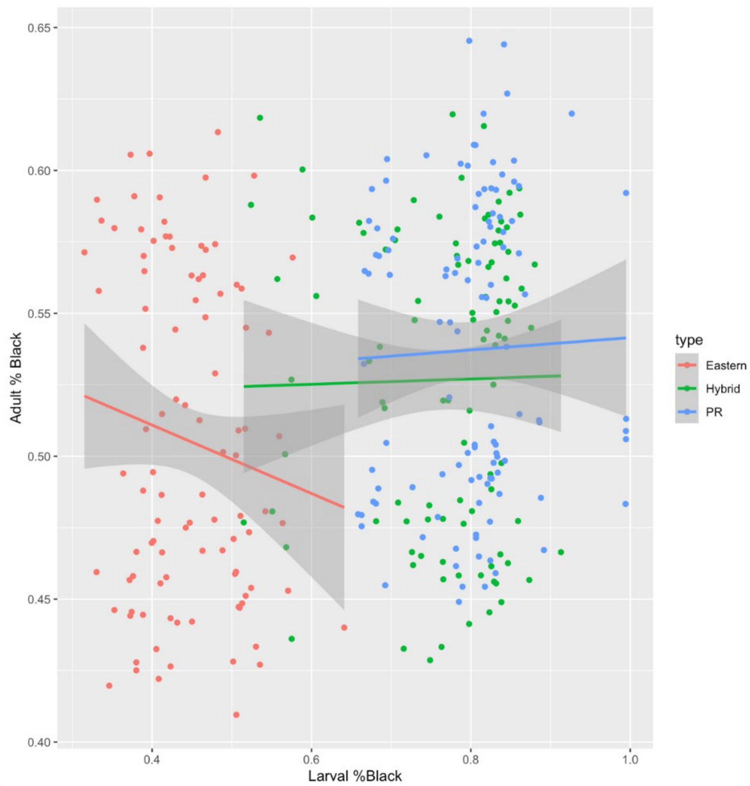
Fig. 10. Abdominal segments of Eastern North American monarch butterfly larvae exhibit higher normalized gray values compared to those of Hybrid and Puerto Rican monarch butterfly larvae. The peaks in gray values correspond to distinct color bands: the highest peaks represent white bands, mid-level peaks represent yellow, and troughs correspond to black bands

Figures 8, 9 and 10 all describe comparisons in larval morphology, including coloration and band patterns. Figure 8 presents a direct visual comparison of monarch larvae abdominal band patterns and sizes across populations and lineages. This illustration further corroborates the observations made in Figure 10, indicating that both Hybrid and Puerto Rican monarch larvae exhibit broader black bands in their abdomens compared to those of the Eastern North American population.

In Figure 9, we present the results of a Principal Component Analysis (PCA) conducted on the body coloration and patterning of fifth instar monarch butterfly larvae. The two principal components (PCA 1 and PCA 2) account for 20.83% and 9.115% of the variance, respectively. The PCA plot reveals distinct clustering of larval morphologies into three populations: Eastern North American, Hybrids, and Puerto Rican.

Finally, these data are supported by figure 10 which illustrates a comparative analysis of the abdominal segments of monarch butterfly larvae from our three treatment groups. Distinct color bands are discernible within the gray value peaks, with the highest peaks corresponding to white bands, mid-level peaks representing yellow bands, and troughs aligning with black bands. Through this assessment of normalized gray values, it is evident that Eastern North American larvae exhibit significantly higher values compared to their Hybrid and Puerto Rican counterparts, indicating a generally lighter abdomen. It is also clear that Eastern monarch larvae abdomens have narrower troughs compared to Hybrid and Puerto Rican larvae, indicating shorter black bands.

Larval vs Adult Mean Black Coloration



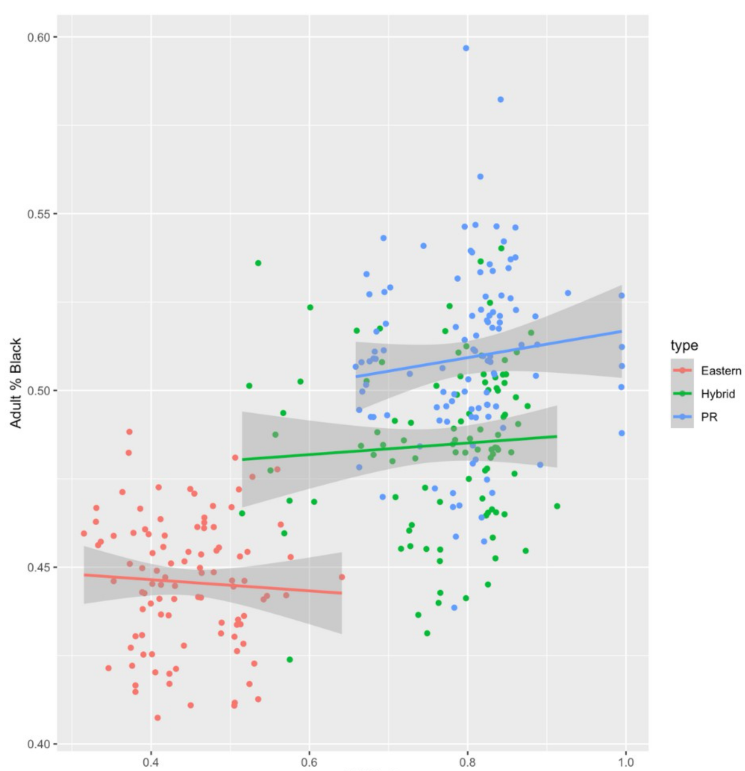


Fig. 11. The black coloration of the dorsal (above) and ventral (below) sides of adult monarch butterfly wings does not correlate with the black coloration of monarch larvae bodies across Hybrid, Eastern North American, and Puerto Rican monarch butterfly populations. Coloration was quantified by calculating the percentage of black surface area relative to the total surface area of the body or wing

The results depicted in figure 11 provide a quantitative analysis of coloration, measured as the percentage of black surface area relative to the total surface area of both monarch larval bodies and corresponding adult butterfly wings. These figures collectively reveal a lack of correlation between the mean corporal blackness present in the bodies of larval monarch butterflies and the mean black coloration displayed on both the dorsal and ventral sides of adult monarch butterflies wings, both within and across Eastern North American (dorsal side p-value = 0.2588, ventral side p-value = 0.6399), Puerto Rican (dorsal side p-value = 0.3126, ventral side p-value = 0.2964) and Hybrid (dorsal side p-value = 0.3637, ventral side p-value = 0.4067) populations.

Discussion

As indicated by our results for proportion of total population infected and severity of infection, Puerto Rican monarchs show greater resistance to parasitic infection than monarchs from Eastern North America. Furthermore, monarchs raised on *A. curassavica* tended to show greater resistance to infection when compared to those raised on *A. incarnata*, supporting previous work that monarch butterflies exhibit increased resistance when consuming milkweed with elevated cardenolide concentrations during their larval stage. These results are in line with previous work that has found that monarchs suffered less parasite growth and disease when reared on *A. curassavica* than on *A. syriaca*, the former being the more cardenolide-rich milkweed (Tan et al., 2018). While these findings were anticipated, what proved particularly intriguing was the observation that Hybrid monarchs did not adopt the phenotype of either of their parental populations. They were more resistant to parasitic infection than Eastern North American monarchs but less than Puerto Rican monarchs. This intermediate level of resistance suggests that parasitic resistance is a polygenic trait in monarch butterflies, similar to the polygenic expression of fenvalerate resistance in *Spodoptera exigua,* another member of the Lepidoptera order of winged insects (Brewer & Trumble, 1991). Fenvalerate is a synthetic pyrethroid insecticide commonly used in agriculture to control insect pests. It acts by affecting the nervous system of insects, causing paralysis and eventual death.

After discovering that parasitic resistance in monarch butterflies follows a polygenic mode of inheritance, we became interested in the potential involvement of additional genes in shaping this resistance trait We decided to look at melanization as it is a conserved immune response in arthropods (Bilandžija et al., 2017; Smith et al., 2021). We used pictures of monarch larvae in their fifth instar stage of development to determine the percent area of their abdomen that was black. As expected, we found that Puerto Rican monarch larvae were significantly darker than their Eastern North American counterparts. What proved intriguing, however, is that Hybrid monarch larvae, rather than displaying an intermediate phenotype as observed for parasitic resistance, exhibited a phenotype resembling that of Puerto Rican larvae. Similarly, we measured the percent area of the caterpillar abdomens which were yellow and found that although Eastern North American and Puerto Rican monarch larvae differed significantly from each other, with Eastern larvae being more yellow, Hybrids presented a phenotype that was similar to that of the Puerto Rican larvae. These results suggest that the genetic pathways determining monarch larvae melanization do not correlate with those involved in parasitic resistance in adults.

Further investigation of the melanization trait in monarch butterflies found that there is no correlation between adult and larval mean black coloration suggesting a decoupling of the melanization trait. This is in line with other work centered around the adaptive decoupling hypothesis which suggests that disrupting developmental links between different life stages allows for independent evolution when faced with varying selection pressures (Herrig et al., 2021). Future work investigating trait decoupling in the monarch butterfly - specifically the decoupling of the melanization trait as well as a potential decoupling of the immune response trait between larvae and adults- might prove important.

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