The Effect of In Vivo Deactivation of *Optix* Gene on Wing Pigmentation in Painted Lady Butterfly *Vanessa cardui*

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April 2020

Introduction

Color in nature is incredibly diverse, and an array of different plant and animal species have evolved over time to produce unique colors and patterns. These colors and patterns produced by organisms are often used as a medium to communicate with other organisms, whether it be for mate attraction, as warning signals to avert predators, or to evade detection (Zhang et al., 2017). Thus, external appearance and pigmentation have been strongly manipulated by natural and sexual selection, leading to the vast assortment of pigmentation patterns seen within and between many different species (Kronforst & Papa, 2015). Animal pigmentation has been widely researched in evolutionary biology, and much is known about the ecological processes that shape color patterns. However, the underlying molecular and genetic processes that induce color patterns are not as well known. One specific clade of animals that has recently emerged as a model system for this research are the *Rhopalocera*, or butterflies. Recent experimentation with butterflies has allowed for the linking of genetic and evolutionary processes that lead to morphological variations in the natural population (McMillan et al., 2002).

Butterflies are well known for their distinct pigmentation patterns, and the evolution of their wing patterns serves as a model for how morphological diversity develops (Mazo-Vargas et al., 2017). There are approximately 20,000 different species of butterflies, many of which can be distinguished solely based on their wing patterns (Nijhout, 1991). Butterfly wing patterns are essentially thin mosaics composed of numerous overlapping colored scales, which are organized into rows within a network of wing veins. Each scale is a modified sensory bristle that contains one color pigment and has a characteristic morphology. The size, shape, and position of clusters of similar scales are what produce the vast array of pigmentations and patterns seen in butterfly wings (McMillan et al., 2002). Butterflies are an excellent experimental system for understanding the molecular basis of patterning and pigmentation, partly due to the fact that

some species of butterflies are relatively easy laboratory models. Butterflies can be reared in large quantities, their wings produce enough pigment for chemical analysis, and cultivating them is fairly simple and affordable (Zhang et al., 2017). Furthermore, recent studies have shown that genome editing tools can now be used in butterflies to study gene function (Zhang & Reed, 2017).

To better understand butterfly wing diversity, the origin and variation of pigmentation must also be understood. Many different genes have been implicated in wing pattern and color development in butterflies. However, only a small subset of those genes play a causative role in wing pattern adaptation in nature: *WntA*, *optix*, *cortex*, and *doublesex* (Zhang et al., 2017). Of those four genes, *WntA* and *optix* are known as the major pre-patterning genes (Livraghi et al., 2017). *WntA* is a gene that encodes a signaling molecule of the Wnt family, and it has a large number of shape-tuning alleles involved in wing mimicry. Protein expression studies of the *WntA* gene performed in the past suggested *WntA* plays an important role in the induction of conserved pattern elements like the Central Symmetry System (CSS), which is a stripe commonly seen in the median region of many different butterfly species (Livraghi et al., 2017). Put more simply, *WntA* seems to control the formation of wing patterns associated with specific symmetry systems. *Optix*, on the other hand, is usually associated with the formation of red and orange ommochrome wing color patterns and encodes a homeobox transcription factor that is expressed later in wing development. For the butterfly family Nymphalidae, *optix* acts as an activator of wing color, but in other species it has been discovered that it can also control blue iridescence. These genes are particularly interesting and important because they behave as complex trait regulators, in which different alleles are associated with different spatial expression domains. This means that these genes control highly varied, complex pigmentation patterns, not just the presence or absence of a particular feature (Zhang et al., 2017). Furthermore, *WntA*, *optix*,

cortex, and *doublesex* are significant because they have been characterized as "adaptive hotspot" genes, or genes that repeatedly drive adaptive variation within and between different species (Papa et al., 2008).

Over time, strong interest has been shown in the research of these genes. However, researchers did not have the tools required to adequately study the genes' functions until the recent development of the clustered regularly interspaced short palindromic repeat/CRISPR-associated (CRISPR/Cas9) system (Livraghi et. al., 2017). The CRISPR/Cas9 system revolutionized the study of gene function by allowing for the modification of a gene in a predetermined way. CRISPR/Cas9 functions as an RNA-directed endonuclease complex that produces targeted double-stranded breaks (DSBs) in DNA. Depending how the cell repairs the break, a mutation can be introduced into the genome, or gene expression can be knocked out completely. Analogous to the search function on computers, Cas9 can be led to specific locations by specialized functional RNA molecules called single-guide RNAs (sgRNAs). The sgRNAs are composed of CRISPR RNA (crRNA), which encodes a sequence complementary to the desired target sequence, and transactivating CRISPR RNA (tracrRNA), which links crRNA to Cas9. The last component required for Cas9 to recognize the desired target sequence is a protospacer adjacent motif (PAM) sequence, immediately downstream of the target sequence. The PAM sequence is 5'-NGG-3' where N is any base and is the location at which Cas9 will create the DSB in DNA. These DSBs have two outcomes: an error ridden nonhomologous end-joining (NHEJ), or a more accurate homology directed repair (Barretto et. al, 2013). Homology directed repair requires either a repair donor or long stretches of sequence homology to repair DNA, which are not usually readily available to cells. If provided with the rest of the CRISPR/Cas9 system to the cell, however, the repair donor will be inserted into the gene. Cells more frequently utilize NHEJ repair mechanisms, which only requires few complementary bases for

the re-ligation of DNA (Sampson et al, 2014). The re-ligation usually results in a DNA sequence that is erroneous or encodes truncated proteins, which scientists use in lab to produce a gene knockout. For years, several attempts were made to manipulate gene function in butterflies, but the systems prior to the CRISPR/Cas9 were costly and very time-consuming to engineer, which limited use to a much smaller scale than today.

Through the usage of CRISPR/Cas9 systems in butterflies, considerable headway has been made in understanding wing patterning genes. Loss of *WntA* and *optix* function studies using CRISPR/Cas9 have shown that severe knockouts of these genes lead to mutant phenotypes. In the Painted Lady Butterfly *Vanessa cardui* (*V. cardui*), *optix* knockouts generated abnormal pigment formation, with complete loss of ommochrome pigments and replacement by melanin pigments (Zhang et al., 2017). In contrast, *WntA* knockouts in *V. cardui* produced elimination of the CSS, distal movement of the parafocal elements, reduction of dorsal forewing eyespots, and generated color composition defects in the ventral forewing eyespots (Mazo-Vargas et. al., 2017). In this study, I used CRISPR/Cas9 to deactivate the *optix* gene in the Painted Lady butterfly *Vanessa cardui*, targeting my sgRNA-guided Cas9 to a different area of the *optix* that had not been directly targeted before. I sequenced larva that were injected with my sgRNA and Cas9 to determine if a gene edit had been made, and reared the rest to adulthood to observe wing pigmentation and patterning defects. I hypothesized that deactivating the *optix* gene in *V. cardui* would lead to genotypic mutations within the *optix* gene and complete replacement of red/orange ommochrome pigments by melanin pigments in the wings.

Methods

Butterfly Rearing

Vanessa cardui caterpillars were ordered from Shady Oak Farm and maintained in a 16:8 hour light/dark cycle at 27^{o}C. Caterpillars were placed in a medium plastic cup that contained

one cup of synthetic food. The synthetic food is a Lepidoptera diet from Frontier Agricultural Sciences. Four caterpillars were placed in each cup, and caterpillars were transferred to a new cup once a week. Cups were covered with a Kimwipe and perforated plastic lids, to allow for ventilation while preventing escape of caterpillars. Three days after chrysalises formed, the Kimwipes to which they were attached were transferred into a 15”x15”x24”H mesh cage. Mesh cages were kept in the greenhouse of Sage Hall of Stetson University. Matured butterflies were fed a mixture containing one part Gatorade and one part water, which was replaced once a week; mixtures were placed in a cup with an opening in the lid, and a cotton ball sticking out of the opening. Adult butterflies used a Mallow plant for oviposition. One Mallow plant was placed in each mesh cage, and butterflies were allowed to oviposition for 3-5 hours. Mallow plants were then removed from the mesh cage and taken to the laboratory, where eggs were collected and separated into control and experimental groups. Experimental group of eggs was injected the same day eggs were collected. Adult wild-type (WT) butterflies were recorded as a phenotypic control using a camera.

Polymerase Chain Reaction

SnapGene was used to analyze a reference sequence of the *optix* gene in *V. cardui*, and forward (5’- CTACTCGATCCTCGAGCGACAC-3’) and reverse (5’-TCGTCCACGTTGATCTCCGAGT-3’) primers were created to isolate and amplify a portion of the gene. Primers were ordered from Integrated DNA Technologies. A polymerase chain reaction (PCR) was conducted using WT larva to test the primers and amplify the *optix* gene. The primers, larval extract containing genomic DNA, Taq Polymerase, PCR buffer and water were combined in a PCR tube and reaction was carried out in PCR machine. The PCR reaction included one cycle of heating for 2 min in 98^{o}C, 30 cycles of 30 seconds each of heating and cooling between 55^{o}C-66^{o}C, and a last cycle of heating for one minute at 72^{o}C. This amplified

the *optix* region of the larval DNA. The PCR product was analyzed via gel electrophoresis to determine the size of the PCR products. The gel was made using 150mL of tris-acetate-EDTA (TAE) buffer and 3g of agarose. The agarose gel was cut when DNA bands of the correct size, approximately 450 base pairs, were seen and sent for sequencing at Eurofins.

Designing the sgRNA

The DNA sequencing results were used to choose two target sequences within the optix gene using SnapGene, a DNA sequence analysis software. The target two sequences were immediately upstream of a PAM sequence (5'-NGG-3') and consisted of 20 nucleotides. Target sequence one was 5'-TCGGGCCCGTCGACAAGTAC-3', and target sequence two was 5'-GGTTCAAGAACCGAAGACAG-3'. Once the sequences were selected, the sgRNAs and Cas9 were ordered from Synthego.

Microinjection and Larvae Analysis

Butterfly eggs were collected 3-5 hours after being deposited on the Mallow plant. Some were used as a control and were not injected. The rest of the eggs were a part of the experimental group. Double sided tape was cut into strips, placed on a Kimwipe to soften the adhesive, and set up in columns in a petri dish. Eggs were lined up in the tape columns, using a paint brush to transfer them from the mallow plant to the petri dish. They were injected using a Microinjector with a mixture of sgRNAs and Cas9 proteins using borosilicate needles. Eggs were injected the same day they were collected to ensure the experimental conditions were introduced to the egg in an early stage of development. After injection, eggs were counted and petri dishes were placed in a closed Tupperware with a soaked cotton ball for moisture, to improve healing of the eggs after injection. Once the eggs hatched, the caterpillars were counted and transferred to a cup containing the synthetic Lepidoptera food, and were reared as previously described above. The percent of hatched eggs was recorded for each injection treatment. A portion of the hatched

larvae were collected so the larval DNA could be amplified via PCR and sequenced. The PCR was performed the same way as described above. Once the sequencing results became available, the sequence of CRISPR/Cas9 modified larvae and placebo injected larvae were compared to the sequence previously obtained of wild-type larvae. The sequences were compared and analyzed using SnapGene.

Mature Butterfly Analysis

Control and experimental larvae were reared in plastic cups until chrysalises were formed, then transferred to mesh cages. The number of butterflies that emerged was recorded. Twenty-four hours after emerging, the butterflies were caught and placed into separate plastic cups, and subsequently sacrificed by being placed in a -20^{o}C freezer. For analysis, butterflies were removed from freezer and allowed to thaw for 2 minutes, then forewings and hindwings were pulled by dislocation using forceps. Wings were arranged in a white background, any phenotypic changes in wing patterning and pigmentation were observed and analyzed in comparison to wild-type adult *V. cardui*. Butterfly wings displaying phenotypic differences from wild-type were recorded using a camera. The adult mutant butterfly thorax tissue was used for genotypic analysis via PCR and gel electrophoresis. The ratio of phenotypically mutant butterflies to wild-type butterflies was demonstrated graphically to quantify success of mutation in experimental group, by comparing the number of phenotypically WT and mutant butterflies to the total number of hatched butterflies.

Results

A)

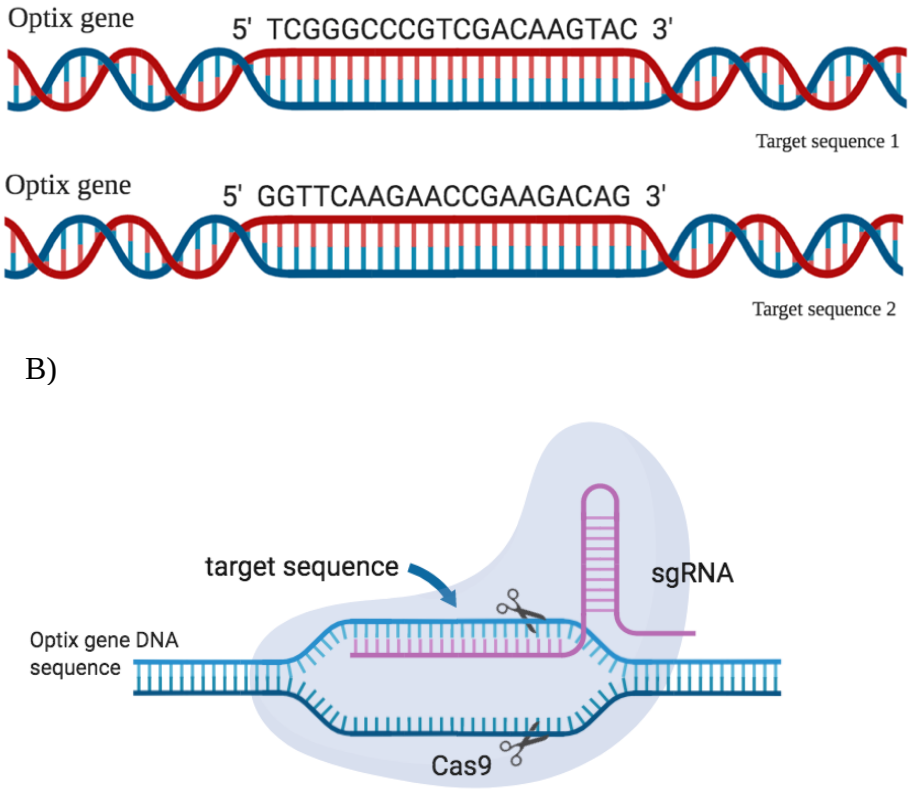

Figure 1. Schematic of sgRNA target sites and CRISPR/Cas9 system. A) Schematic of *optix* gene showing both designed sgRNA DNA target sites. B) General schematic of CRISPR/Cas9 complex showing position of the sgRNA and cas9 protein in relation to the target sequence.

A)

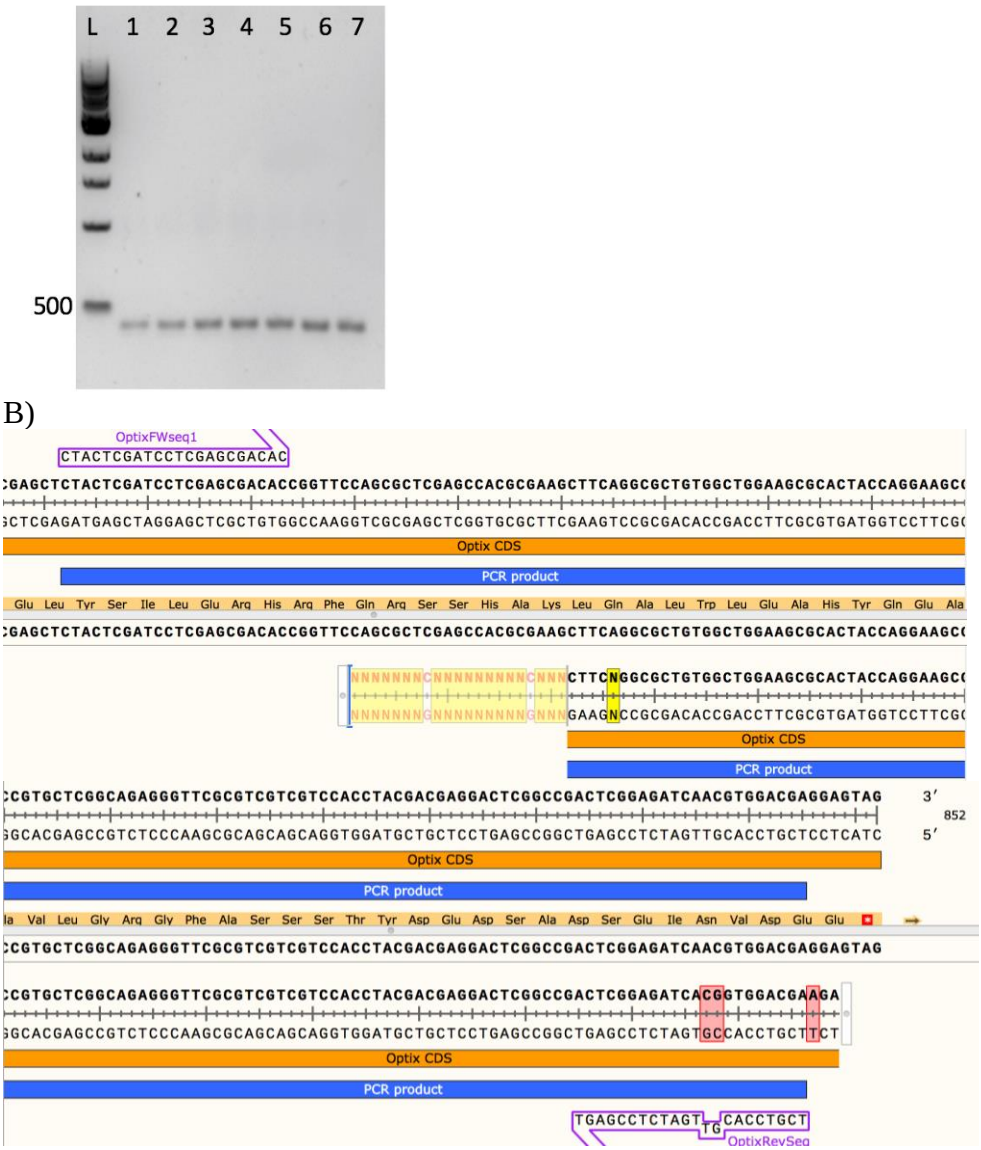

Figure 2. Comparison of reference *optix* gene from SnapGene with WT larva gene PCR product. A) PCRs of the *optix* gene in wild-type (control) larva, using temperature gradient of 58^{o}C -66^{o}C; 1kb ladder was used. Bands of expected size (~450 base pairs) are shown, and represent PCR product seen in B. B) Sequencing alignment of PCR product to reference gene from SnapGene. Top DNA strand represents the reference gene, bottom strand represents PCR product from WT larva. Forward and reverse primers used in the PCR are shown where they align with the DNA. Yellow nucleotides signify unknown nucleotide, and red nucleotides signify nucleotide differences between the two sequences being compared. Showing only beginning and end of alignment, middle portion showed no mutations.

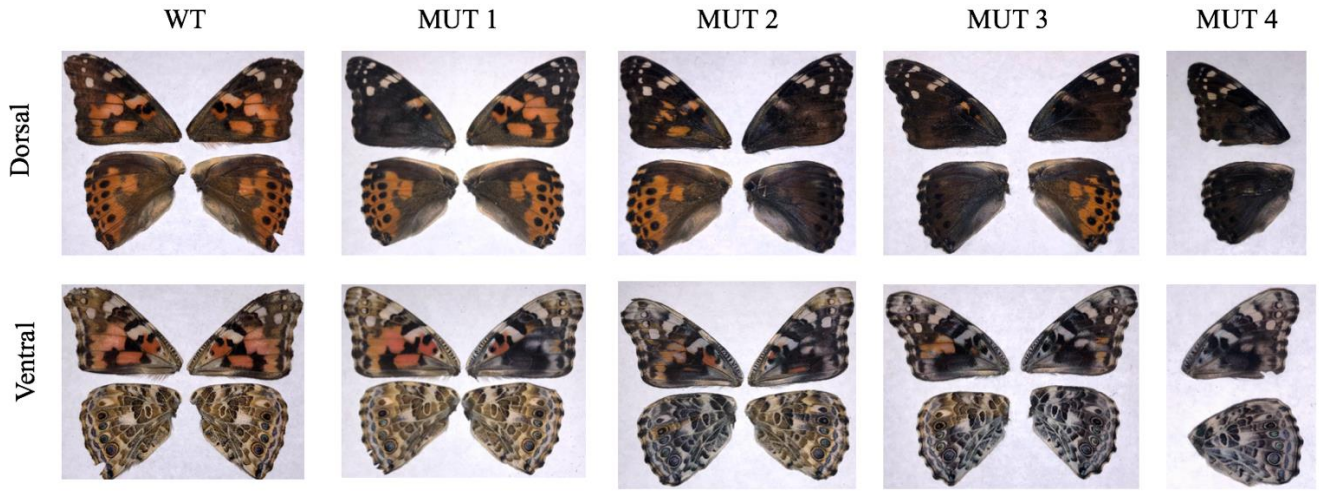

Figure 3. Phenotypic differences in wild-type and mutant butterfly wings. Dorsal and ventral views of the wings are shown. The WT butterfly has distinctive orange ommochrome pigments in both ventral and dorsal forewings, and dorsal hindwings. Ventral hindwings of WT butterfly contain network like combination of beige and brown mosaics and eye spots. Mutant butterflies display varying degrees of *optix* knockout. Mutant 1 only displayed replacement of orange pigments by melanin pigments in its left forewing. Mutant 4 exhibited complete replacement of orange pigments by melanin pigments (only left side is shown because right wings were damaged).

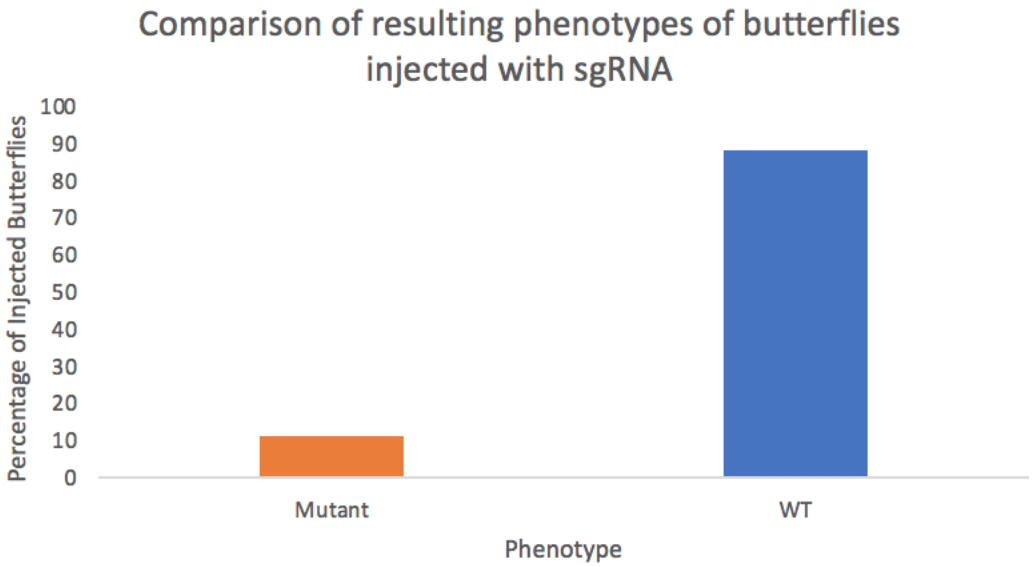

Figure 4. Percentage of emerged butterflies that displayed a mutant phenotype. All butterflies in the experimental group were microinjected with sgRNA and Cas9, however only 11% of butterflies were successfully effected, resulting a mutant phenotype. A total of 114 butterflies emerged in the experimental group, out of which 101 appeared phenotypically wild-type and 13 exhibited a mutant phenotype.

Discussion

The goal of this study was to test the function of the *optix* gene in the painted lady butterfly *Vanessa cardui* by creating a gene knockout using the CRISPR/Cas9 system. The *optix* gene is usually associated with the formation of red and orange ommochrome wing color patterns and encodes a homeobox transcription factor that is expressed later in wing development. *Optix* acts as an activator of wing color, and previous studies have confirmed that *optix* plays a fundamental role in the development and regulation of pigmentation in *V. cardui* (Livraghi, 2017). Deactivation of the *optix* gene, therefore, was expected to generate significant pigmentary and structural color mutations, with increased distribution of melanin pigments in areas containing ommochrome pigments in WT butterflies. My hypothesis was supported by my results, since *optix* deactivation did, indeed, lead to the formation of melanin pigments instead of ommochrome pigments. As depicted in Figure 3, the mutant phenotypes were observed as expected, arranged by increasing prevalence of melanin pigments in each mutant. Interestingly, however, only one of the mutants appears to have exhibited a complete gene knockout- mutant 4 in Figure 3. This mutant manifested a complete loss of orange pigments in dorsal and ventral forewings and in dorsal hindwings. In the ventral hindwings, the mosaic elements that appear beige and brown in WT butterflies were replaced by black and blue hues. Brown geometric spots seen in WT appeared black in this mutant, and the background color of the wing was grey subtle with hints of blue, instead of the typical beige.

The other observed mutants were all mosaic mutants, which means the mutation was only present in a fraction of the adult cells. And the mosaic mutants displayed varying degrees of phenotypic change. Mutant 1 in Figure 3 presents a phenotypic mutation only in one of its forewings, while mutants 3 and 4 exhibited phenotypic changes in 3 out of 4 wings. A possible explanation for the varying degrees of mutations observed involves the timing of the injections.

Zhang and Reed (2017) found that injecting eggs 1 hour after oviposition generated more and larger mutations compared to later injections. I performed injections between 2-5 hours after oviposition, which might account for not only why many of the emerged mutants exhibited smaller mutations, but also why such a large proportion of butterflies in the experimental group were WT, a pattern shown in Figure 4. Future studies should be done to fully understand the effect of injection timing on the phenotype of resulting mutants. This would provide information that not only would be beneficial for increasing rate of success of injections, but that could also add to the knowledge of at which stage in development a certain gene is upregulated. *Optix* encodes for a protein that is expressed very early in egg development, therefore it would make sense for earlier injections to be more successful.

Genotypic analysis of WT larva and mutant larva were performed, however, gel electrophoresis only showed bands of the correct size for WT larva, which can be seen in Figure 2.A. The same primers and PCR conditions were used in the PCR reactions of both WT and mutant larva, suggesting that the primers and PCR conditions were not the issue. The underlying reason in this case might be due to the fact that most mutants observed in this study were mosaic mutants, meaning their cells contained two genotypes, WT and mutant. Studies have found that genotypic analysis of mosaic mutants may yield unexpected results, since separating mutant DNA from WT DNA in an organism that contains both is not an easy task (Livraghi, 2017). Future studies should seek to compare the genotypes of phenotypically different mosaic mutants. In this study, the same sgRNA and target sequences produced mutants of significantly different from each other, such as Mutants 1 and 2 in Figure 3. It would be interesting to see where in the *optix* gene their mutations were, and if there was a correlation between area of DNA effected and part of wing containing mutant phenotype.

The development of the CRISPR/cas9 system has provided an effective way of manipulating DNA that has revolutionized genetic research. Although still technically a recent technology, CRISPR/Cas9 has already been extensively used and found to be effective in the research of butterfly wing pattern and pigmentation. This study differs from previous *optix* deactivation studies because of the use of new target sites within the *optix* coding sequence. Research on genes that generate patterns and pigments in organisms are essential to understanding the incredible biodiversity exhibited in nature. Butterflies are the ideal experimental systems for this type of research because they are relatively low-maintenance subjects, have short life cycles, and studies have proved that CRISPR/Cas9 can efficiently manipulate butterfly genes in a predetermined way. Butterfly research has the potential to serve as the link between evolutionary and developmental biology processes. Butterfly wing patterns and pigments owe their characteristics to the molecular processes that take place during development as much as they do to natural and sexual selection. Therefore, understanding patterning and pigmentation yields a greater scientific understanding of how evolutionary and developmental processes work together.

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