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CRISPR in butterflies: An undergraduate lab experience to inactivate wing patterning genes during development

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Abstract

CRISPR is a technique increasingly used in the laboratory for both fundamental and applied research. We designed and implemented a lab experience for undergraduates to carry out CRISPR technology in the lab, and knockout the wing patterning genes *optix* and *WntA* in *Vanessa cardui* butterflies. Students obtained spectacular phenotypic mutants of butterfly wings color and patterns, awakening curiosity about how genomes encode morphology. In addition, students successfully used molecular techniques to genotype and screen wild-type caterpillar larvae and butterflies for CRISPR edits in genes. Student feedback suggests that they experienced a meaningful process of scientific inquiry by carrying out the whole CRISPR workflow process, from the design and delivery of CRISPR components through microinjection of butterfly eggs, the rearing of live animals through their complete life cycle, and molecular and phenotypic analyses of the resulting mutants. We discuss our experience using CRISP genome editing experiments in butterflies to expose students to hands-on research experiences probing gene-to-phenotype relationships in a charismatic and live organism.

KEYWORDS

butterflies, CRISPR technology, gene-editing, *optix*

1 | INTRODUCTION

CRISPR was discovered as an adaptive immune system in bacteria against viruses, and has been adapted for gene targeting and editing in the lab with transformative impact across the life sciences.[1–4] CRISPR technologies are reshaping the landscape of possibilities in biological research due to their efficiency across a wide range of organisms, and in humans, are enabling promising therapeutic options for the treatment of genetic conditions such as sickle cell disease and childhood blindness.[5,6] Recently, CRISPR-based technology has been modified as gene-detection technology, with the FDA approving it as a COVID-19 diagnostic "rapid" assay to detect SARS-CoV-2 genetic material, with results produced in an hour.[7–9] CRISPR technology has arrived at mainstream media, being showcased in the 2018 movie Rampage and in the documentaries Unnatural Selection and Human Nature. As CRISPR technology continues to advance, we need to develop effective educational practices and experiences framed around CRISPR technology. This practical training at the forefront of contemporary advances may not only train our next generation of scientists and innovators, but also educate STEM students as informed citizens about the complex implications of programmable genomic engineering.

The molecular and technical aspects of CRISPR-based studies provide a framework in which CRISPR

technology can be used to apply biological principles such as the central dogma of molecular biology/flow of genetic information, genotype to phenotype relationships and the organizational structure of proteins/cells/tissue/organisms.[10,11] CRISPR technology has been recently used in undergraduate labs in small upper-level biology courses with established model organisms such as *Saccharomyces cerevisiae* yeasts,[12–14] *Drosophila melanogaster* fruit flies,[15] *Arabidopsis thaliana* thale cress,[16] *Xenopus laevis* frogs,[17] and *Danio rerio* zebrafish.[18] Here we aimed to develop an undergraduate lab experience where students immerse in a comprehensive process of CRISPR targeting by attempting to knockout a gene of interest in a multicellular organism. We envisioned students starting with the gene analysis of the target gene, delivery of CRISPR components into the target embryo of organisms, screening of CRISPR-edited organisms using molecular biology, and then conducting phenotypic analysis to visualize the effect of CRISPR gene knockouts (Figure 1).

We chose *Vanessa cardui* butterflies, commonly known as Painted Ladies, as our organism due to key advantages for both instructors and students (Table 1). Butterflies provide a unique opportunity to engage students with gene-to-phenotype relationships during development in an invertebrate organism. Additionally, students can gain expertise that span gene sequence analyses, experimental design, molecular biology techniques and phenotypic analysis in a living organism. Recent studies demonstrate that molecular tools can be conducted in butterflies and these are ideally suited to an undergraduate setting.[19–23] We capitalized on the idea that students would feel more invested in the experiment if they were responsible for the CRISPR delivery and rearing of the animals, rather than the instructor carrying out these steps out for the students as previously reported in *Drosophila* and zebrafish.[15,18] Students can easily rear Painted Ladies in the lab from egg to caterpillar to butterfly life stages in 3 weeks, which is a critical factor when using live organisms in a lab course setting with time constraints. Furthermore, rearing conditions are simple and economical in comparison to aquatic genetic model organisms like zebrafish and *Xenopus*. Artificial diet in plastic cups are used for caterpillars and butterfly cages for butterflies. Butterflies mate easily in butterfly cages and lay their eggs on mallow leaves, enabling students to collect hundreds of eggs in several hours from a small batch of butterflies for CRISPR delivery, which is extremely beneficial for the instructor in terms of having sufficient number of embryos for students to work with within a class period. The relatively large size of butterfly eggs (0.8–0.9 mm) is a key advantage for delivery of CRISPR components by allowing students to use a simple, affordable microinjection system using coarse air pressure and compressed Nitrogen gas or air. CRISPR delivery into embryos of commonly used genetic model organisms like *C. elegans*, is more complex, where

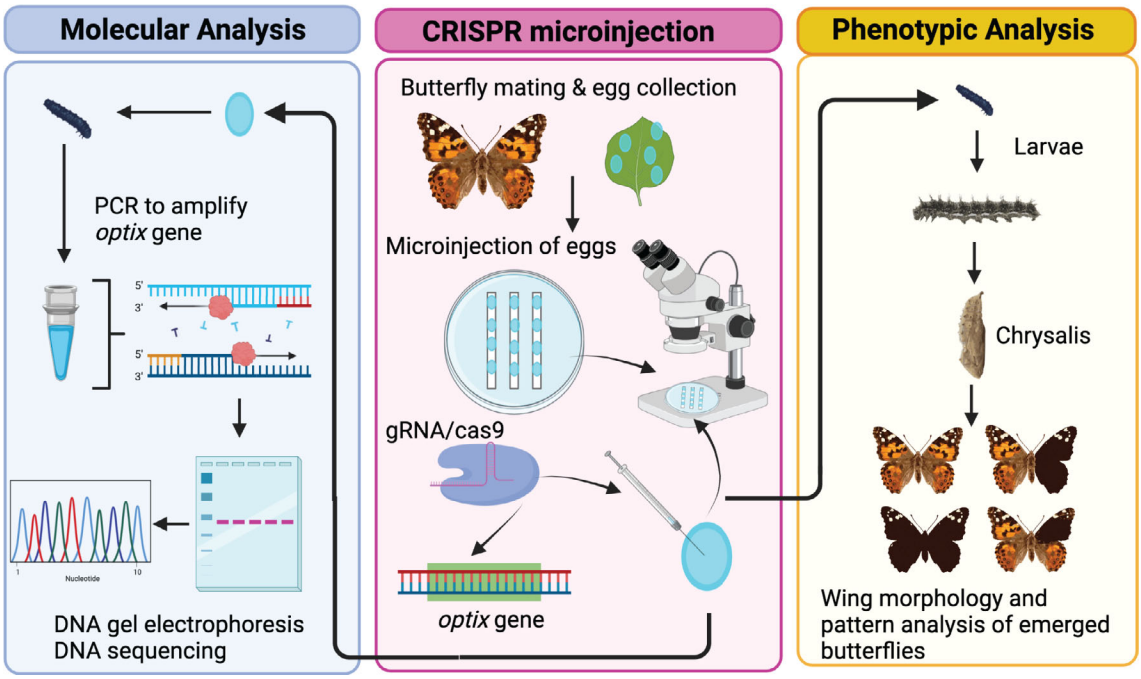

FIGURE 1 Schematic depicting the workflow of student lab activities. Student workflow begins with molecular analysis of wild-type *optix* gene (left column), and continues with microinjection of CRISPR components into butterfly eggs (middle column), and culminates in molecular (left column) and phenotypic analyses (right column) to characterize CRISPR modifications

TABLE 1 Advantages of using *Vanessa cardui* butterflies in undergraduate CRISPR experiments

Key Features	Details of advantages
Genomic database	The transcriptome is available online, allowing students to find DNA sequences of genes
Caterpillars and butterfly husbandry	*Vanessa cardui* caterpillars are bought from Carolina, Amazon or a local butterfly farm in Florida. Caterpillars are easily reared in plastic cups with artificial diet. Butterflies are kept in big mesh cages.
Mating and egg laying	Students can easily obtain newly hatched eggs from butterflies reared in cages in a greenhouse or indoors using an artificial diet that is easily prepared. *V. cardui* mate easily in cages and produce hundreds of eggs over the course of 7–10 days.
Egg size and ease of microinjection	The newly hatched eggs are about 1 mm big, thus visible to the naked eye. Egg size and ease of microinjection allow students to microinject the butterfly eggs with a micromanipulator and stereomicroscopes at 5× magnification. Stereomicroscopes are typically found in teaching labs.
Rearing of injected animals	Larvae from injected eggs are easy to rear in plastic cups with artificial food, with little maintenance. Larvae phenotypes can be identified quickly as lifecycle is fast (2–3 weeks). Butterflies emerge 3 weeks from egg, allowing phenotypes like butterfly wing color/patterning/eyespot or larvae body patterning or developmental changes to be observed within the timeframe of a semester-long class.
Phenotypic analysis	Documentation and analysis of the above phenotypes requires no specialized equipment, only cameras, which most students have access to via cell phones.
Molecular analysis	Direct PCR amplification of genes from larvae and butterfly tissue can be done and is straightforward, without the need to first extract genomic DNA using a separate genomic purification kit before molecular analysis of PCR and dNA sequencing.
Biosafety Animal Care & Safety	Currently, one cannot produce stable passaging lines of edited *V. cardui* as offspring are not produced, therefore any edited animals pose no risk to the environment. As invertebrates, butterfly work does not require student Institutional Animal Care and Use Committee (IACUC) training.

embryos are 50 μm in size and thus requires microinjection under high magnification microscope into the gonads of gravid adults where the embryos are located. Additionally, CRISPR delivery for *C. elegans* is expensive in comparison for undergraduate lab course setting, in terms of the microinjection system and inverted microscope required. Moreover, molecular analyses like Polymerase Chain Reaction (PCR) of genes from caterpillars and butterfly tissue can be done easily, without the need to do a separate genomic DNA purification, thus enabling students to characterize their CRISPR molecular changes within two to three class periods.

Here we first used CRISPR technology for the targeted gene knockout of the gene *optix*.[20] This gene is encoded as a single exon in butterflies, simplifying gene analysis because the absence of introns makes guide RNA (gRNA) design and annotation easier for students (Figure 2A, B). We used gRNA-Cas9 enzyme complexes targeting two sites within the *optix* gene to induce error-prone DNA repair and deletion mutations that lead to loss-of-function of the gene (Figure 2A, C). The butterfly eggs are syncytial embryos for about 7 h after egg laying, and CRISPR microinjection within this timeframe produces mosaic animals that show a mixture of wild-type and CRISPR-mutant tissues.[24] We set out to determine if undergraduate students could produce butterfly CRISPR mutants, and characterize phenotypic mutants through molecular analysis of caterpillars and adult muscle tissues using Polymerase Chain Reaction amplification and Sanger DNA sequencing (Figure 2).

2 | MATERIALS & METHOD

2.1 | Genotyping

- Frozen post-1 day egg hatched larvae (wild-type and CRISPR-injected) was subjected to Polymerase Chain Reaction (PCR) using the Thermofisher's Phire PCR protocol with final concentration of 0.3 μM forward and reverse primers, 1× master mix containing DNA Phire Polymerase, dNTPs and buffer, and a whole hatchling per reaction. We have also successfully used the manufacturer's "Dilution" protocol in lysing the whole hatchling in DNA Phire Dilution Buffer and DNA release as described below for butterfly tissue, and using 1 μl of the supernatant as template DNA for PCR. PCR primers for *optix* gene were: 5′-CTACTCGATCCTCGAGCGACAC-3′, 5′-TCGTCCACGTTGATCTCCGAGT-3′ from.[20] PCR rimers for *WntA* gene were: 5′-CCGTGAATCTGCATTCGTCCAT-3′ and 5′-GCGTGTATTGGTCTCAATTTCCTG-3′ from.[21]
- To genotype adult butterfly tissues, tissue samples were lysed by placing small amount of thorax muscle

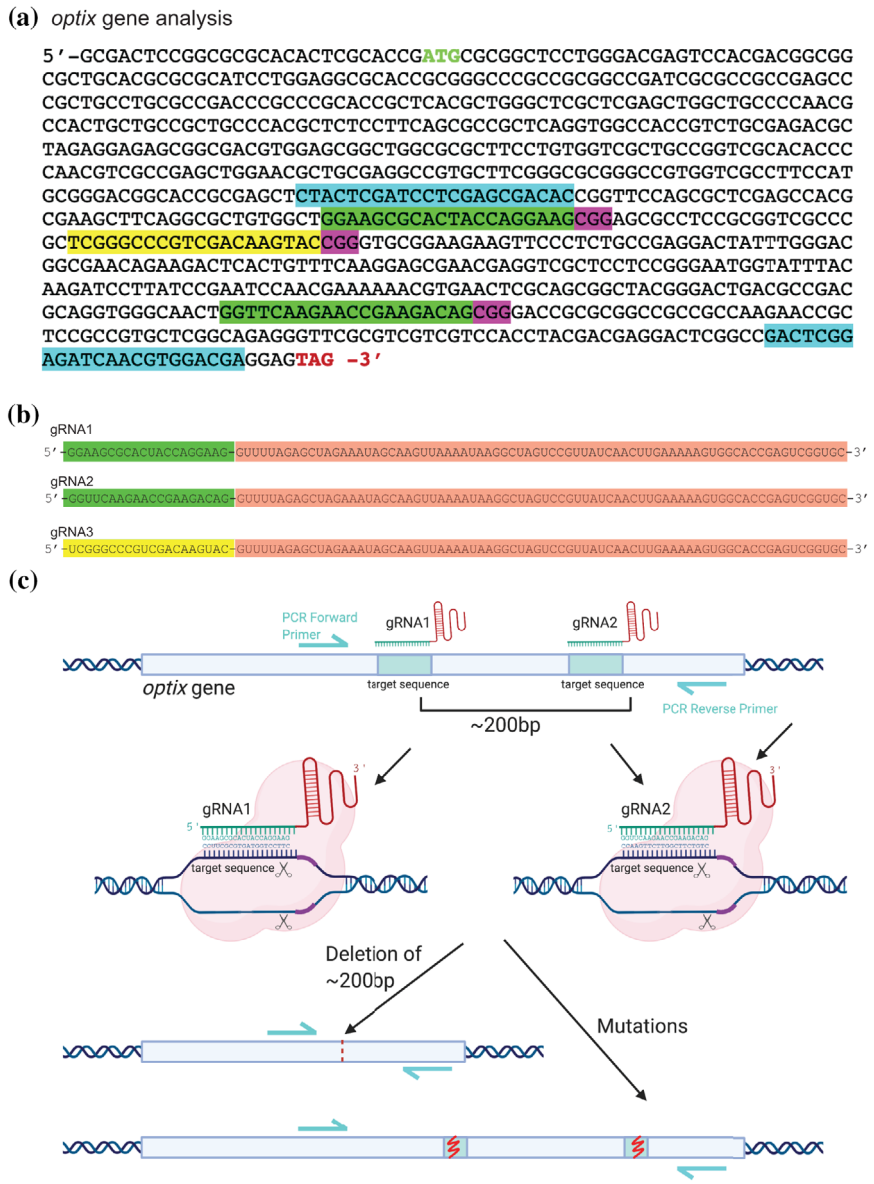

FIGURE 2 CRISPR targeting of *optix* gene. (a) *Vanessa cardui* optix gene sequence with gRNA/cas9 target sites highlighted in green and yellow, PAM sequence highlighted in purple, and primer sequences for amplifying DNA highlighted in blue. The start (ATG) and stop (TAG) codon are indicated in green and red. (b) gRNA sequences for sgRNA 1–3, where first 20 nucleotides are complementary to target DNA sequence, and last 80 nucleotides are the 80mer gRNA scaffold sequence for *Streptococcus pyogenes* cas9. The gRNA scaffold sequence from Synthego is propriety information and not available, thus here the generic scaffold sequence is used, highlighted in orange. gRNA3 (yellow) is student-designed. (c) Schematic of *optix* gene with primers used for PCR, gRNA/cas9-target sequences and corresponding gRNA 1 and 2, the gRNA-cas9 complex and the desired molecular outcomes

tissue (1–3 mm) in 40 µl of DNA Phire Dilution Buffer mixed with 1 µl of DNA release, incubating at room temperature for 5 min, then 98°C for 2 min. Tubes were centrifuged for a minute, the supernatant was removed and 1 µl was used as the template DNA source in PCR reaction as described above. PCR reaction was conducted in a thermocycler at: 1 cycle of 98°C for 5 min, 30 cycles of 98°C for 5 s, 55°C for 5 s, 72°C for 20 s (for *optix*) or 72°C for 50 s (for *WntA*), 1 cycle of 72°C for 1 min. PCR products were run on a 1.5% w/v agarose gel, with an expect DNA band size of 444 bp for wild-type *optix* and 2138 bp for wild-type *WntA*.

- PCR products were purified for DNA sequencing by either Exo-SAP-IT PCR Product Cleanup (The or gel extraction of DNA bands from gel using Zymo DNA gel extraction Kit.
- DNA sequencing of PCR amplicons was conducted by Eurofins with the PCR forward primer.

The protocol for caterpillar/butterfly husbandry, CRISPR reagents and microinjections were adapted from.[17,20] The modified protocol is briefly described below.

2.2 | Animal husbandry and diet

- Painted Lady Caterpillars were purchased from Carolina Biological Sciences (Catalog #144070) at least 2 weeks before the start of the lab experience. We recommend instructors rear one full life cycle of painted ladies and attempt butterfly egg collection prior to the class to get a sense of developmental timing at the

instructor's institution, as temperature can affect life cycle. The kit comes with sufficient artificial diet for the number of individuals that is included, usually around 60–70 caterpillars at the second or third larval instar. Caterpillars usually take about 1–2 weeks to form a chrysalis. After pupation, pupae were placed at the bottom of butterfly cages on top of paper towels or can be hung on strips of microfiber cloth, and take about 1 week to hatch. Butterfly cages were kept in the greenhouse with natural daylight/night cycle, and the butterfly cages were sprayed with water every 1–2 days. Butterflies were fed with 50% Gatorade in water mix using a feeding cup (cotton ball in cup, provided by kit, or can be made in lab).

- Artificial diet was prepared in advance for subsequent experiments (Frontier Scientific, Painted Lady Diet # F9698B) following manufacturer's protocol and hand-held immersion blender, except dried leaves are not included. While hot, the diet was poured into 5.5 oz plastic cups with lids (WebstaurantStore, #: 127P550C, #: 127PL400) and stored in a 4°C fridge until use. A square of Kimwipe or paper towel was laid in between lid and cup and 3 holes were punched into the lid for airflow.
- Alternatively, we recommend our previous single-cup rearing method.[17] Briefly, a batch of the Southland Inc. "Multiple Species Diet" is prepared per the manufacturer's recommendation (930 ml of water, 162 g of diet powder), supplemented with 5 ml of canola oil, and rapidly dispensed into about 180 individual cups (1.25 oz Dart Solo T125-0090) placed on 30-well trays (Frontier Agricultural Sciences, #9040) using a confectionery funnel (Food Grade Stainless Steel confectionery funnel, nozzle size 5.56 mm). Diet cups are left open on the bench to cool down and evaporate for 15 min, closed with the lids (Dart Solo PL100N), and kept at 6°C for up to 2 weeks until needed. Lids with injected eggs on double-sided tape can be directly transferred to a cup until hatching, as neonates will successfully reach the diet at the bottom and start feeding. Second or third instar larvae are then transferred to individual cup until pupation. Small cups always require pinholes in the lid to allow breathing and evaporation of excess humidity (e.g., two holes made with a pair of fine forceps). We routinely stack multiple trays of 30-cups, and students can be involved in the dispatching of larvae or the isolation of hardened pupae.
- CRISPR-injected caterpillars in cups and butterfly cages were kept in an indoor laboratory room with temperature set to 26°C with indoor heaters, and humidity of 40%. The laboratory light was turned on manually from 9 am–5 pm, Monday to Friday, in the first cohort. In the second cohort, the lights were set on timers and automatically turned on every day from 8 am–5 pm.

2.3 | Egg laying and preparation

- Mallow plants for egg laying were purchased from Carolina (Cat No 144042), and kept in the greenhouse and watered every day. Three days after hatching from chrysalis, two cups of water with three mallow leaves in each cup were placed in the butterfly cage 3 h before the desired microinjection time.
- It is optional but recommended to surface decontaminate the eggs for 2 min in 5% Benzalkonium Chloride, rinse the eggs in distilled water, and then air dry them with a gentle air flow. This procedure adds preparation time and can be skipped if the parental generation was directly obtained from the commercial supplier. We recommend it to limit the spread of viral disease if more than one generation is bred in the lab.
- Students prepared their own plate of eggs for microinjection. Using gloved hands, eggs were gently brushed off the leaves with fingers into glass finger bowls. Double-sided tape was made less sticky on one side by pressing a Kimwipe against the tape. Thin strips of the tape were cut with a blade and placed on the lid of a 10 cm plastic petri dish with the Kimwiped-sided tape facing up. Using a damp thin paintbrush, place eggs on the tape. Using a stereomicroscope, eggs were adjusted so that the thin top faces upward.

2.4 | CRISPR reagents and delivery

- CRISPR reagents were prepared in advance following protocol established by Martin et al.[17] gRNAs for *optix* were purchased from Synthego as CRISPRevolution sgRNA EZ Kit (3 nmol): gRNA1: GGAAGCGCACUACCAGGAAGG, gRNA 2: GGUUCAAGAACCGAAGACAG from.[20] (Figure 2B). A student-designed gRNA for *optix* was also tested: gRNA 3: UCGGGCCCGUCGACAAGUAC. gRNAs for *WntA* are as follows: student-designed gRNA1: UCUUUAAGUACAAGAUAUGA that targets sequence in Exon 6, and gRNA2: GGCAGCAUUGGCCCAUGCGG targeting a sequence in Exon 4 from.[21]
- 500 ng/μl stocks of gRNA were prepared and kept at −80°C. 1 μg/μl stocks of Cas9-2xNLS (QB3 Macrolab, UC Berkeley) with Phenol Red Solution (0.07%, final concentration) were prepared. Before microinjection, the microinjection mixture was prepared by mixing

5 μl Cas9 and 2.5 μl of each gRNA in a RNAase-free tube with pipetting. The Cas9/gRNA complexes were allowed to form by incubating at room temperature for 10 min, and then put on ice.

- In the first cohort, two microinjection systems were set up with Zeiss stereomicroscope (Stemi 305 with Stand K EDU) and side LED lights (Navlinge, Ikea) and Tritech Research microINJECTOR™ System with footswitch, pulse-control module, and dual-pressure. Compressed nitrogen gas was used to supply air pressure for microinjection. Single pressure can be used, but we found having a balancing pressure (dialed down to almost 0 psi, but providing a slight balance pressure) helps novice students during injection. In the second cohort, four more microinjection stations were set up using compressed air as the source of air pressure (California Air Tools 2010A Ultra Quiet and Oil-Free 1.0 HP 2.0-Gallon Aluminum Tank Air Compressor, Amazon). To position glass needle at ~45° angle to inject the eggs we used the 3-axis manipulator (Drummond Scientific Company) for two injection systems connected to compressed Nitrogen gas, and tested the 1-axis course manipulator from Narishige for the additional four injection systems connected to air compressor (UM-1C manipulator and GJ-1 magnetic stand). The 1-axis course manipulator is more economical and simpler for students to use, although the 3-axis manipulator feels more sturdy. Borosilicate glass capillary needles were pulled with PC-10 Gravity Needle Puller (Step 2 only, No.2 heater set at 58) the day before and stored on clay strip in 10 cm plates.
- The trinocular stereoscope was connected to a Moticam camera, which allows students to connect to the camera via their smartphone using the MotiConnect camera during demonstration of microinjection.
- 1 μl of Cas9/gRNA mix was back-loaded onto the needle. The tip of pulled needle was broken by lightly touching the tip on the plate or razor blade. The loaded needle was positioned in micromanipulator and connected to the microinjection system. Novice students break needles very easily, so lower volumes of Cas9/gRNA mix can be loaded into the needle.
- Microinjection of eggs was demonstrated to students, followed by students trying to microinject on their own. Students performed microinjection over 3 lab periods.
- Each microinjected plate was placed in a Tupperware with a damp papertowel/kimwipe. Students monitored their plate up to 5 days post microinjection for hatchlings. Using paintbrush, hatchlings were transferred to PCR tubes to freeze at -20°C for genotyping or transferred to plastic cups with artificial diet (5 hatchlings per cup) for phenotype analysis.
- Caterpillars in cups were monitored by students over next 3 weeks and moved to new food cups every ~3–5 days.

2.5 | Project ownership survey

The POS is a 16-item survey instrument measuring two subscales Cognitive Ownership and Emotional Ownership.[25] Students from the CRISPR lab (15 students) and a comparison lab Microbiology (16 students) completed the survey during the last week of the semester. The Cognitive Ownership subscale has 10 questions asking students to what extent they agree they had intellectual ownership of or responsibility of their lab work with a five-point response scale that ranges from strongly agree to strongly disagree. The Emotional Ownership subcscale has six questions measuring the strength of students' emotion towards their lab work with a five-point response scale that ranges from very strongly to very slightly. The POS is found in the Supplemental Material. The possible range of scores is 10–50 for Cognitive Ownership, and 6–30 for Emotional Ownership. Students' response scores were summed for each subscale, and the mean was calculated for each class. Two-sample t tests were conducted to compare the CRISPR lab and Microbiology lab mean scores on the Cognitive Ownership subscale and the Emotional Ownership Subscale.

3 | RESULTS AND DISCUSSION

3.1 | Overall design

We implemented CRISPR technology to deactivate the master wing regulator gene called *optix*[20] in *V. cardui* butterflies in an undergraduate lab classroom setting over an eight-week span. Specifically, students conducted both molecular and phenotypic experiments to characterize the CRISPR-d butterfly mutants generated in lab. In the first iteration of this lab experience, 17 students were enrolled in a 300-level Genetics course with a lab that met once a week for 3 hours in Fall 2019 semester. In the second iteration of this lab, 16 students were enrolled in a 300-level Molecular Biology and Biotechnology course with a lab that met twice a week for 3 hours in Spring 2022 semester. The overall goal was to immerse the students in the full process of gene analysis, modeling of CRISPR targeting, delivery of CRISPR components into embryos, animal husbandry, genotyping of CRISPR edits, and phenotypic characterization from CRISPR knockouts. Figure 1 contains the laboratory workflow and Table 2 contains weekly student activities and learning

TABLE 2 Timeline for students and student learning outcomes

Class	Student lab activity	Student learning outcomes: Students will:	Tools needed
1	Analyze *optix* gene sequence	• be guided to design appropriate PCR primers to amplify and sequence gene of interest • use SnapGene software to annotate gene, design primers	PCR primers Snapgene software/computer
1–2	PCR amplification of *optix* from wild-type caterpillar	• use PCR to amplify their region of interest from their gene using larvae • run DNA agarose gel and send PCR amplicons for DNA sequencings	PCR Primers PCR kit
3	*optix* sequence analysis of PCR product with reference gene from database	• senalign sequencing data with the reported gene sequence	Snapgene/computer
3	Analyze target sequence for cas9 and guideRNA. Design gRNA.	• be guided to determine target sequence and PAM sequence for cas9 • use SnapGene software to annotate these features • use CRISPR paper model to model gRNA/cas9 activity on target gene	Snapgene software/computer Paper model
1–4	Rearing of butterflies (2–4 weeks)	• maintain caterpillar/butterfly husbandry: Caterpillars will be ordered from Caroli, and students will maintain them until butterfly stage during first few weeks. Students will feed butterflies	Larvae artificial diet, plastic cups, butterfly food, mesh cages
2–4	Mating of butterflies to collect eggs (1–3 weeks, depending on success of microinjection)	• set up egg collection and collect newly hatched eggs from leaves	Mallow plants for egg collection (butterflies lay eggs on leaves)
2–4	Microinjection of gRNA/cas9 (3 microinjection attempts over 3 classes)	• microinject eggs with gRNAs/cas9 complex	Microinjection set up (stereoscope, needles, micromanipulator, cas9/gRNA complex, microinjector, gas), RNase free tips/tubes
3–6	Screening experimental larvae and control larvae by genotyping and phenotypic analysis of CRISPR-d animals (3–5 classes)	• Injected eggs will be maintained until hatching of larvae, some hatched larvae will be used for PCR/genotyping and DNA sequencing, others will be used for phenotypic analysis	PCR kit, Snapgene/computer
7	Work on Poster	• present their results in poster form	
8	In class lab exam	• be evaluated on student learning outcomes in an in-class exam.	

outcomes. The wet-lab experiments were combined with computer visualization and manipulation of DNA sequences using the SnapGene software.

3.2 | Sequence analysis of the *optix* gene and design of CRISPR targets

Students first conducted molecular analysis of the *optix* gene from wild-type *V. cardui* newly hatched caterpillars (1 day post hatch from egg). Using SnapGene, a computer software for analyzing nucleotide sequences, students were guided to visualize the *optix* gene, translate the sequence, and annotate the primers for PCR to determine the expected PCR product size (Figure S1). We conducted PCR amplification by utilizing the Phire DNA Polymerase from Thermofisher, which enables "direct tissue" PCR amplification in a tube with whole caterpillars, without the need for a separate step of genomic extraction from caterpillars. To visualize the success of PCR

amplification, PCR products were run out on a DNA 1.5% (w/v) agarose gel in Week 2. After the correct sized DNA band was visualized, students utilized Thermofisher's Exo-SAP-IT enzyme mix to prepare the PCR product for DNA sequencing, which is a quick (~30 min) method that rids the PCR sample of dNTPs and primers, saving time in undergraduate lab by not having to use traditional column purification method. The PCR products were sent for DNA sequencing, and students analyzed the DNA sequencing results by aligning the sequenced PCR product to the reference sequence in Snapgene. The reference sequence was obtained from the *V. cardui* transcriptome. Students were guided to two CRISPR target sequences and the adjacent PAM sequence in the *optix* gene and annotated these as features in the gene. The desired region to be deleted through CRISPR was also annotated. Paper modeling of the guide RNA targeting the complementary target sequence was conducted to support conceptual understanding and visualization of CRISPR process.

3.3 | Delivery of Cas9/guide RNA duplexes through microinjection of butterfly eggs

Butterfly eggs (~800 μm in length) are an ideal size for undergraduates to work with. They are big enough to be seen by the naked eye and are easily handled with paintbrushes. Hundreds of eggs can be easily collected by placing mallow leaves in a butterfly cage of 20 butterflies 3 hours prior to class. The butterflies lay on the leaves, and students can collect the leaves for their experiment and prepare plates with rows of eggs stuck to double-sided tape. To deliver single-guide RNA (sgRNA) and CRISPR into the eggs, we set up a microinjection system with a stereomicroscope that has up to 5× magnification. Microinjection of eggs is a process that requires practice and hand-eye coordination of driving the needle into the egg and injection using a foot pedal while visualizing the eggs under ~2–3× magnification. Thus, we built into the schedule for students to conduct three rounds of microinjections over three class times. Each student prepared and injected one plate of ~50 eggs so that each pair had about 100 eggs microinjected. We observed that students did improve in their ability to deliver non-lethal injections over the three rounds. However, students varied in their skills and abilities to successfully inject. One student who became proficient quickly said that it was very easy to pick up because she plays video games daily. Other students struggled with the hand-eye coordination and dexterity needed to consistently inject without crushing the eggs. After hatching of the eggs, students collected and froze some hatchlings for genotyping of caterpillars, and the remaining larvae were moved into cups with caterpillar food for phenotypic analyses.

3.4 | Genotyping analysis of CRISPR-injected larvae

Frozen CRISPR-treated larvae were subjected to PCR amplification as previously described, with wild-type larvae for comparison. One team observed two caterpillars with a DNA band at 200 bp in their agarose gel, suggesting CRISPR-mediated deletion (Figure 3a). The DNA band was gel extracted, purified, and sent for DNA sequencing. Students aligned the sequencing results to the reference gene on Snapgene, and observed a 201 bp and 208 bp deletion between the CRISPR target sequences, suggesting CRISPR delivery and modification was successful.

3.5 | Phenotypic analysis of butterflies

Students maintained caterpillars over a 2–3 week period, checking on them every other day, and moved them to new food cups every 3–5 days. After chrysalis formation, students moved the chrysalis to butterfly cages. After hatching of butterflies, students observed any changes in butterfly wing color and patterning. In the first cohort, all student teams obtained at least one phenotypic mutant, with some groups obtaining as many as four mutants. In the second cohort, 5 of the 9 groups obtained at least 1 phenotypic mutant, with one team obtaining 6 mutants. Compared to wild-type butterflies, mutants displayed striking color changes (Figure 4a), consisting of a conversion of orange scales to black scales in the upper side of mutant wings, and a lack of yellow and orange pigments on the under sides. These phenotypes replicate the published *optix* knock-outs, and corroborate the role of this gene in regulating color pigmentation by repressing black melanin pigments and promoting red-orange-yellow pigmentation in *V. cardui*.[20] Butterflies were frozen in cups for subsequent molecular analysis and wing dissection. Of note, CRISPR knock-outs of *optix* have been described in additional species of butterflies and can provide an interesting entry for students into the scientific literature as well as template for comparative insights.[20,26]

3.6 | Genotyping analysis of butterfly tissue

The CRISPR delivery to the eggs generates mosaic mutants with clonal tissue populations of CRISPR

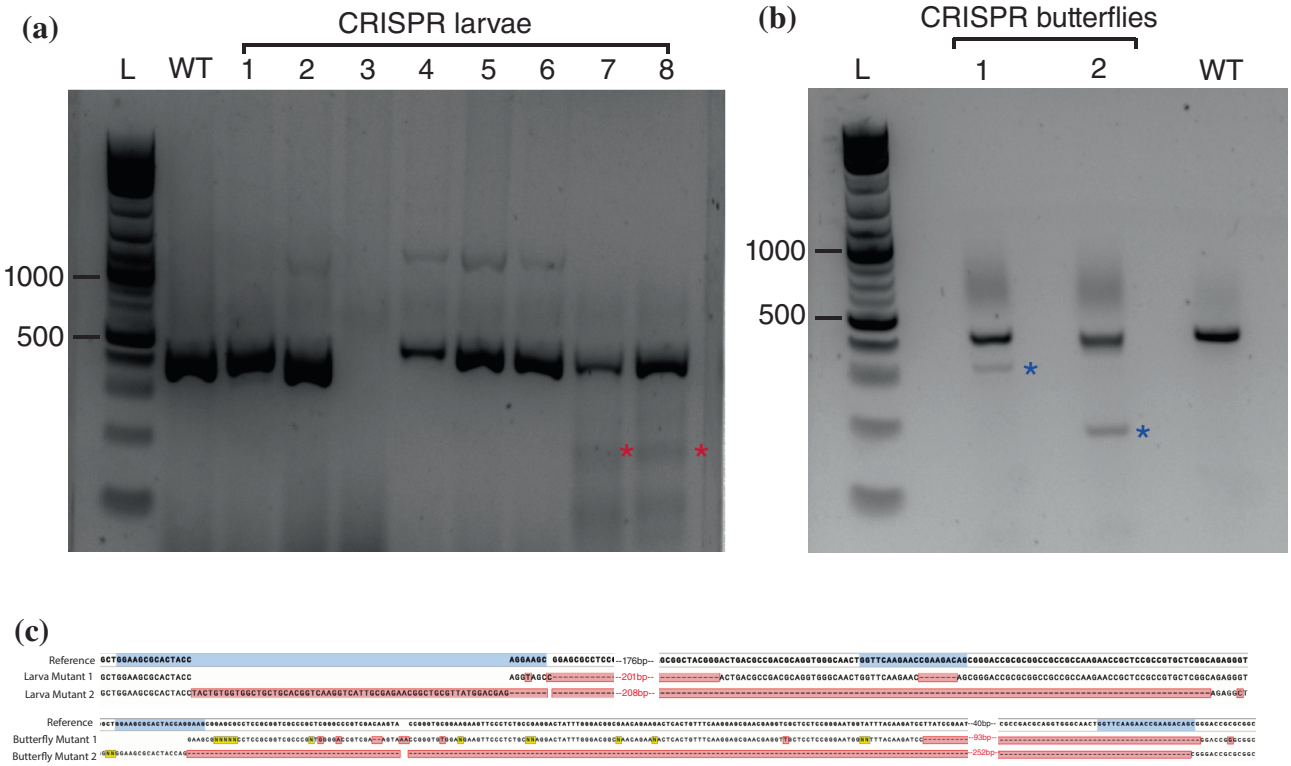

FIGURE 3 Genotyping of *optix* gene of larvae and butterflies from CRISPR-microinjected eggs. DNA gel electrophoresis of *optix* PCR product from wild type (WT) and CRISPR mutant caterpillar hatchlings 1-8 (a) and butterflies 1-2 (b). Asterisks (*) indicate deletion in larvae (red *, ~200 bp deletion) and butterflies (blue*, ~100 bp and 200 bp deletion). L indicated ladder in base pairs. (c) Sequencing alignment of *optix* gene from CRISPR larvae and butterfly mutants to reference gene. Nucleotides highlighted in blue represent guide RNA target sequences. The number of deleted nucleotides is indicated in red in mutant alleles

knockouts within the organisms. Therefore, if the wings display a mutant phenotype, we cannot directly assay the wing tissue as the wing is made up of chitin-based scales. So if the wings demonstrate an *optix* CRISPR knockout phenotype, the body tissue may or may not genotypically show CRISPR-mediated modification. Here we aimed to test the various phenotypic mutants obtained by students to determine if we could detect CRISPR modification in the thorax muscle tissue, as done previously.[21,23,27] After the students dissected the wings out, a small amount of thorax tissue was dissected out and used for genotyping by PCR amplification, DNA gel electrophoresis and DNA sequencing. Each team assayed 2–5 phenotypic mutants. Two teams observed a CRISPR-mediated deletion in their DNA gel (Figure 3b). DNA sequencing of these DNA bands revealed a 92 bp and 252 bp deletion in between the two CRISPR target sequences (Figure 3c). Students also obtained base substitutions at the CRISPR target sites, and one team observed a 6-nucleotide insertion of nucleotides at one CRISPR target site. Students utilized Snapgene to determine how the nucleotide changes affected the protein sequence and length.

3.7 | Evaluation of student learning outcomes

Prior to the lab experience, students from the first cohort were surveyed and asked if they had heard of CRISPR technology before. 13/17 students replied yes. However, when asked to explain their knowledge of CRISPR technology, 11/17 students responded with “I don't know” or provided no answer. Two students said it was a gene editing system, while two students knew of some molecular components and wrote that it involved a Cas protein and cutting of DNA or RNA. Clearly, although many students had heard of CRISPR technology, they were not familiar with how it works.

At the end of the semester, students from the first cohort worked in pairs to create a scientific poster of their findings. Examples of student posters are shown in Figure S2. Students were also evaluated with question-based assessment in a class lab exam at the end of the lab experience for both cohorts. The percentage of students achieving student learning outcomes is shown in Table 3. We evaluated whether students could effectively explain and communicate the experimental strategy of CRISPR

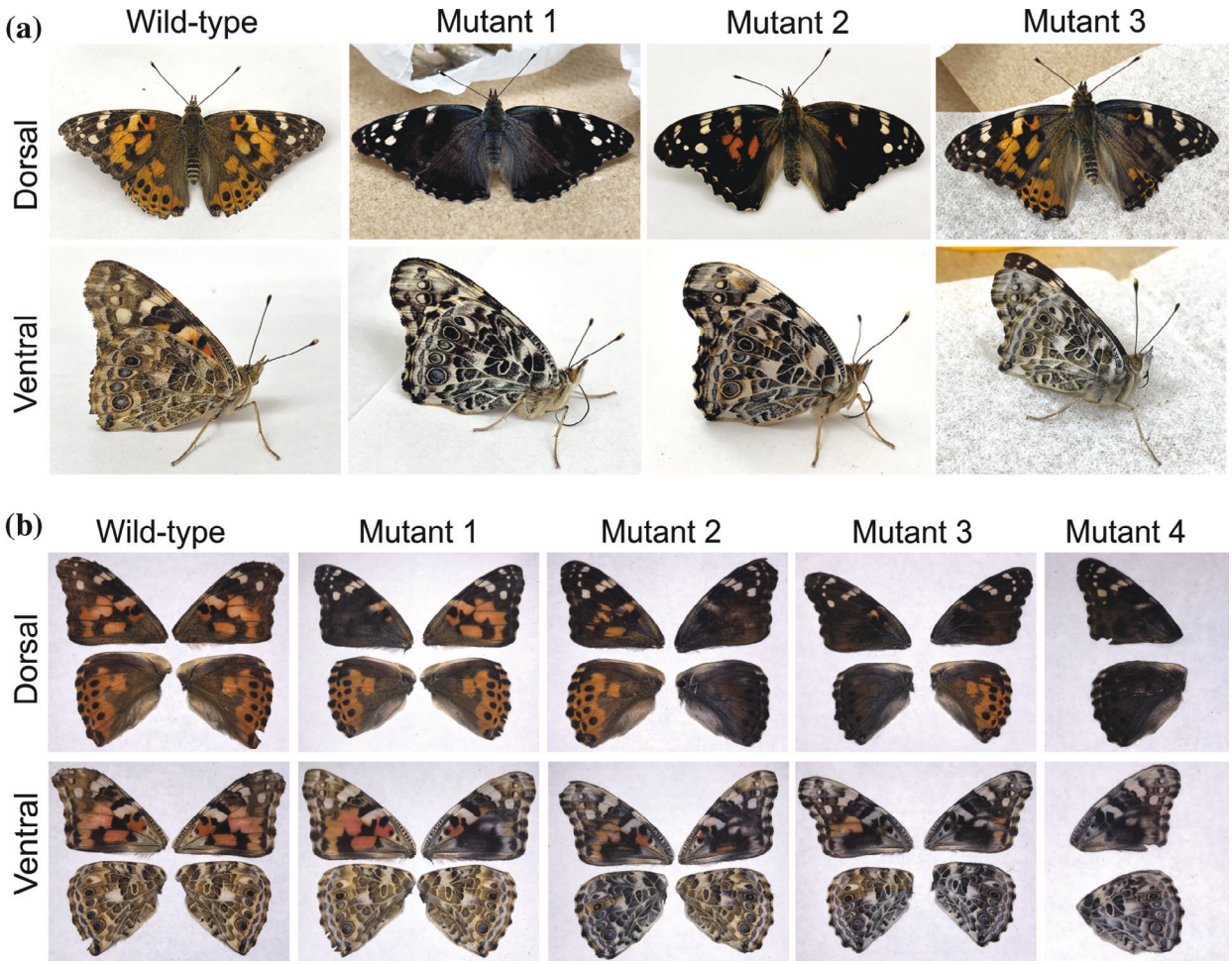

FIGURE 4 Student generated *optix* CRISPR knockout butterfly mutants. (a) Examples of dorsal and ventral wings of wild-type and mosaic CRISPR knockout mutants after hatching from pupa. (b) Mosaic CRISPR knockout mutants produced with student-designed gRNA3 paired with gRNA1

TABLE 3 Student learning outcomes and means of assessment

	Student learning objective	Means of assessment	Percentage of students achieving SLO
1	Explain the experimental strategy to deliver CRISPR technology and assay for CRISPR effects through molecular and phenotypic analysis	(A) Poster, Cohort 1 (B) Final in-lab exam, Cohort 1 (C) Final class exam, Cohort 2	(A) 100% $N = 8$[a] (B) 70% $N = 17$[b] (C) 75% $N = 16$[b]
2	Analyze and communicate the effect of CRISPR on *optix* gene through molecular biology experimentation	(A) Poster, Cohort 1 (B) Final in-lab exam, Cohort 1 (C) Final class exam, Cohort 2	(A) 75% $N = 8$[a] (B) 47% $N = 17$[b] (C) 43.75% $N = 16$[b]
3	Analyze the effect of *optix* CRISPR on butterfly wings and explain how results demonstrate the function of *optix* during development	(A) Poster, Cohort 1 (B) Final in-lab exam, Cohort 1 (C) Final class exam, Cohort 2	(A) 62.5% $N = 8$[a] (B) 58.8% $N = 17$[b] (C) 68.75% $N = 16$[b]

[a]Seven groups of two students, one group of three students.
[b]Individual students.

delivery and assaying for molecular and phenotypic effects in two different modes, in a lab poster and in a lab exam. In lab poster, 100% of lab groups achieved the outcome through written and schematic form in the background and methods section of the poster. When students were prompted to explain the strategy in written

and schematic form in a lab exam, 70% of the students achieved acceptable level of competence in the first cohort. The 30% of students at the developing level did explain how Cas9/gRNA was used to target *optix* gene through microinjection of butterfly eggs, but did not answer the part of the question explaining how both molecular and phenotypic analysis were both used to determine CRISPR effects (17.64%, $n = 3$) or described only molecular or phenotypic analysis and not both types of assays (11.76%, $n = 2$). To address this issue of students not fully answering the question, the question was broken down into two parts and scaffolded for the second cohort. For the second cohort, 75% of students achieved acceptable level of competence, with 25% at the developing level as they described only phenotypic analysis and either omitted the molecular analysis or described only part of the molecular analysis.

We evaluated the ability of students to analyze and communicate the effect of CRISPR on the *optix* gene (SLO2) and butterfly wings (SLO3). We observed that there was a difference in percentage of students achieving SLO2 (CRISPR effect on *optix* gene) between the poster (75%) and the in-lab exam (47%). Students worked in their groups to complete the lab poster, and a poster guideline and rubric were given to students, which may have helped a higher percentage of students achieving acceptable levels in the posters. For the in-lab exam, the students at the "developing" level could articulate that CRISPR modified the *optix* gene by causing mutations, but did not expand upon how the molecular techniques showed whether CRISPR modification was successful. One reason for this could be how students interpreted the exam question and did not answer the prompt in as much detail to achieve "acceptable/mastery" levels for assessment. In the second iteration of this lab, in addition to asking students to explain in written form how molecular analysis demonstrated the success of CRISPR activity on the target gene, they were also instructed to draw a schematic to describe their results and asked explicitly what they observed and concluded from their DNA gel and DNA sequencing results. The percentage of students who achieved acceptable and mastery levels was 43.75%, which was similar to the first cohort (47%). The remaining students were at developing levels, where they either (1) described and drew results, but did not fully explain the interpretation of the data, or (2) stated conclusions but did not provide a sufficient or accurate explanation of how results allowed them to make these conclusions. Interestingly though, 25% of the second cohort achieved mastery level with detailed and accurate explanations and schematics, whereas no individuals achieved mastery levels in the first cohort. In future iterations, questions will be modified such that actual images of data will be provided and students will be prompted in a series of scaffolded questions to interpret the data and then explain what the data suggests.

We observed that the level of students achieving SLO3 (explain CRISPR effect on butterfly wings) was similar between posters (62.5%) and the in lab exam (58.8% for first cohort, 68.75% for second cohort). Students assessed at the "developing" level primarily because students failed to articulate or expand upon the role of the *optix* gene or gene product in the context of its biological pathway that would result in the observed *optix* loss-of-function phenotype. Common responses receiving a "developing" assessment level simply stated that CRISPR produced the mutant phenotype they observed, which showed that "*optix* plays a role in regulating butterfly color", and did not address the specific function of *optix* of promoting orange pigmentation or repressing black pigmentation during normal development. This lack of specificity in student answers, suggests that students may have difficulty understanding and communicating, both verbally and in written form, how genes behave in biological context as a part of molecular pathways. This is not particularly surprising given previous data demonstrating student's weak conceptual understanding of stepwise fundamental processes such as transcription or the relationships between genes and chromosomes.[28,29] Our data further highlight the necessity for instructors to be explicit during instruction about basic concepts in developmental biology and how loss-of-function phenotypes allow us to interpret normal function of gene/protein. For instance, *optix* encodes a transcription factor that likely orchestrates the deployment of pigment synthesis pathways, and is specifically thought to activate ommochrome (yellow-red-orange) pigment synthesis, while repressing the synthesis of dark melanins. A gene knockout would result in increased dark pigmentation and loss of orange pigments. Based on this model, it is interesting to use *optix* as an example of a regulatory gene that coordinates the expression of other genes, resulting in complex morphology. Based on findings from the first cohort, in the second cohort we intentionally had students work in groups to discuss potential mechanisms that would lead to the loss-of-function phenotype observed in butterflies, and then came together as a class to discuss and draw out models. We observed that there was a 10% increase in students achieving acceptable levels between first and second cohort.

3.8 | Student feedback of CRISPR lab experience

At the end of the lab, we asked students "What aspect of our CRISPR experiment was most fun and exciting and why". Anonymous feedback included:

- "*Being able to phenotypically see the mutants because it made me feel like I was a real scientist and not just in an undergrad lab*."
- "*We were able to produce mutants, see the phenotypical difference between the wild type and the mutant, as well as their sequences aligned on SnapGene*."
- "*I loved being able to see the outcome of the gene editing on a living creature. By taking care of the butterflies I was more invested in the whole experiment*."

Overall, students' positive responses spoke to three main aspects of the lab: being able to phenotypically visualize the mutants after injection of eggs themselves, caring for caterpillars and butterflies themselves, and using molecular techniques to visualize nucleotide changes that they created in lab. Thus, in the second cohort we measured the students' ownership of the lab using the Project Ownership Survey (POS), a 16-item survey instrument developed to measure what extent students agree they have intellectual ownership and responsibility for their work (cognitive ownership) and the strength of students' emotion towards their lab work (emotional ownership).[25] The POS is found in Data S1. We compared POS results to that of a comparison lab class, Microbiology, where students were focused on an inquiry-based project characterizing oral bacteria from students own mouth using biochemical assays and genotyping of bacteria. We found that the students in the CRISPR lab developed higher cognitive ownership compared to the students from the microbiology lab (Figure S3A). Similarly, CRISPR students also reported significantly higher emotional ownership compared to students in the Microbiology lab (Figure S3B). There are many variables that could contribute to this difference, including, but not limited to students' perceived novelty in the technology, their level of engagement and collaboration with peers and instructor during the experience, and their enthusiasm for the discovery and relevance of the topic. One student stated in feedback, "*I feel a lot of purpose to be engaging in groundbreaking science*," while another student commented, "*CRISPR is at the intersection of research and reality*."

Interestingly, when asked "What aspect of the lab experience was most challenging and why?", students did not point to just one aspect, but commented on various aspects: the microinjection of butterfly eggs, setting up and understanding PCR, interpreting DNA gel electrophoresis data, linking the concepts from each lab period together. One student even thoughtfully reflected, "*I think the most challenging part of the lab was to be able to obtain sufficient number of mutants for PCR reactions and DNA sequencing. To achieve that, multiple injections and experiments are needed. The time aspect of the experiments was also another thing* (*normally an hour for each PCR and gel electrophoresis run*)." Indeed, a challenging aspect of this experience was the multi-week continuous nature of the experiment from injection to both molecular and phenotypic screening of butterflies. We wanted to expose students to a lab experience that modeled discovery-based research involving the process of iteration and in-depth genotype to phenotype analysis. From feedback, it was evident that some students found the more open-ended nature difficult, particularly when they had other classes and other demands. We attempted to scaffold the students' work by having them conduct PCR, gel electrophoresis and sequence analysis multiple times, first wild-type larvae, then CRISPR-d larvae and finally their CRISPR-d butterflies. However, one student from the first cohort noted that it was hard to link each week's lab activities together. One way to address this is to provide or build a roadmap with students or build in more scaffolds or metacognitive activities to ensure students are reflecting on their weekly lab activities. Another way to improve on this is to run this lab experience in a class that meets twice a week instead of once. This was addressed in the second cohort, where the class met twice a week for the full semester. In addition, in the second cohort, more emphasis was placed into maintaining a lab notebook, forcing students to intentionally document their steps, set experimental goals, write observations, and analyze their data. Student feedback in the second cohort mentioned that they found great value in maintaining their notebook and it became an important resource for providing additional clarity about their workflow and promoted reflection of their work as they progressed through the lab.

3.9 | Extensions and future directions

This lab experience can be modified by having students target different regions of the *optix* gene. One student outside of the class attempted to do this by designing a different gRNA that targeted a different region of *optix*, and the student obtained striking phenotypic mutants (Figure 4b). There are other published examples of color patterning genes that can be targeted in the class using *Vanessa* butterflies.[21,27,30,31] Concurrently with the class, our undergraduate teaching assistant (UTA) tested CRISPR targeting of *WntA* using a gRNA that was designed by the UTA. *WntA* has previously been shown to be a key patterning gene in various butterfly species.[21,32,33] Molecular analysis of *WntA* is more complex for undergraduates with multiple exons and introns. The UTA's molecular and phenotypic analysis demonstrates that CRISPR of *WntA* with a novel student-designed gRNA is successful at the undergraduate level (Figure 5).

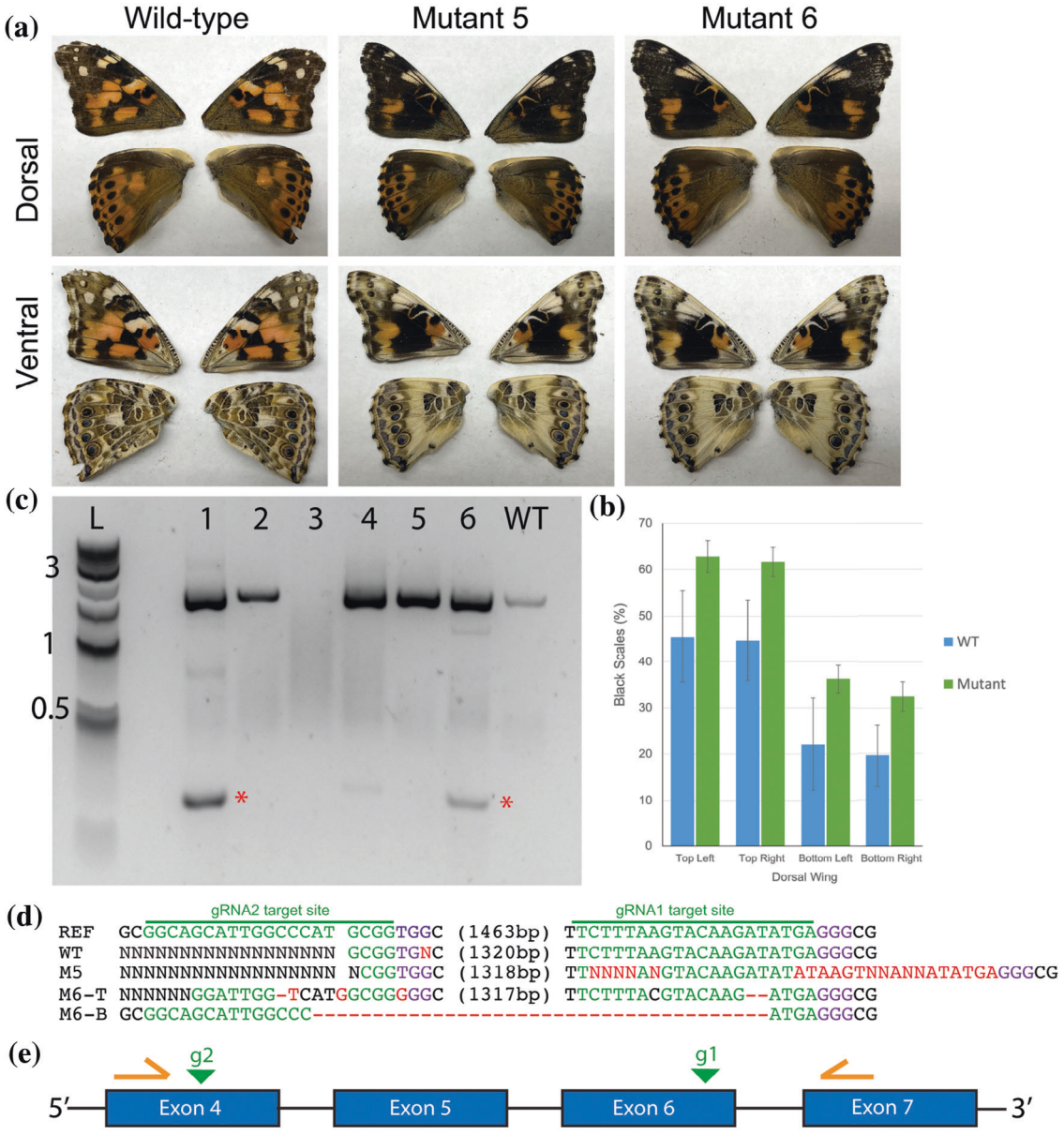

FIGURE 5 Phenotypic and molecular analysis of *WntA* CRISPR mutant butterflies. Comparison of (a) wild-type (WT) and two CRISPR mutants show increased black melanin pigments and drastic changes in wing patterning. (b) Quantification of the percentage of black pigmentation in each dorsal wing. Error bars represent standard deviation. $N = 5$ for wild-type and $N = 4$ for mutants. One-tail, two sample *t*-tests between wild-type and mutant for each wing type have $p < 0.05$. (c) PCR amplicons for *WntA* of WT and CRISPR butterfly mutants. Red asterisks (*) indicates desired 1.4 kb deletion between two gRNA sites. (d) DNA sequencing of *WntA* PCR amplicons from muscle tissue of WT, mutant 5 (M5) and mutant 6 (M6-T = top DNA band, M6-B = bottom DNA band) show mutants acquired CRISPR mutations (in red), including insertion in M5, single nucleotide changes and deletions in M6. Deletion of nucleotides are indicated with red dashes (−). gRNA target sites are shown in green and PAM sequences are shown in purple. (e) Schematic of exons with gRNA target sites for gRNA 1 and 2, labeled with green triangles, where gRNA 1 is student-designed. Orange arrows indicates PCR primers

A laboratory skill that students often work on in lab is the quantification of a dependent variable, graphing and statistical analysis. We were able to quantify the amount of black pigmentation using a stereoscope with camera and imaging capabilities. When comparing the different dorsal wings in wild-type versus CRISPR mutant wings, we observed a significant difference in the percentage of black pigmentation (Figure 5b).

Overall, we conclude that Painted Ladies are a research organism that is both interesting and accessible for undergraduate students to characterize at the phenotypic and molecular level. We hope that our lab

experience and findings can be utilized by others to bring meaningful lab experiences framed around CRISPR technology into undergraduate curricula. In the future, we hope to expand the CRISPR target genes within Painted Ladies to study basic principles in developmental biology and genetics.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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