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REVIEW

Bringing immersive science to undergraduate laboratory courses using CRISPR gene knockouts in frogs and butterflies

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ABSTRACT

The use of CRISPR/Cas9 for gene editing offers new opportunities for biology students to perform genuine research exploring the gene-to-phenotype relationship. It is important to introduce the next generation of scientists, health practitioners and other members of society to the technical and ethical aspects of gene editing. Here, we share our experience leading hands-on undergraduate laboratory classes, where students formulate hypotheses regarding the roles of candidate genes involved in development, perform loss-of-function experiments using programmable nucleases and analyze the phenotypic effects of mosaic mutant animals. This is enabled by the use of the amphibian *Xenopus laevis* and the butterfly *Vanessa cardui*, two organisms that reliably yield hundreds of large and freshly fertilized eggs in a scalable manner. Frogs and butterflies also present opportunities to teach key biological concepts about gene regulation and development. To complement these practical aspects, we describe learning activities aimed at equipping students with a broad understanding of genome editing techniques, their application in fundamental and translational research, and the bioethical challenges they raise. Overall, our work supports the introduction of CRISPR technology into undergraduate classrooms and, when coupled with classroom undergraduate research experiences, enables hypothesis-driven research by undergraduates.

KEY WORDS: *Vanessa cardui*, CRISPR, *Xenopus laevis*, Teaching

Introduction

Information is cheap, experience is expensive.

Attributed to Manu Prakash

The things we hear about in other biology courses about genome editing were actually performed in this class and we got to see real organisms with the result of the injections we did […]. This class revealed how important it is for us to contribute to the right sort of conversation in scientific innovations as we graduate from college.

Anonymous student feedback

Genome editing using CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology is sweeping through the field of biology with a transformative impact on basic research, biotechnology, medicine, agriculture and policy making. These rapid developments make it a priority to educate the next generation of bio-workers about the potential of genetic engineering. While most biology and pre-medical students leave college with a lecture-based understanding of CRISPR-related techniques, few will have had the opportunity to perform it in a laboratory. There are increasing numbers of classroom laboratory modules involving CRISPR experiments performed in bacteria (Pieczynski et al., 2019), yeast (Sehgal et al., 2018) and *Drosophila* (Adame et al., 2016), signaling increasing incorporation of this important technology into science education (Wolyniak et al., 2019). To enrich this repertoire of pedagogical approaches, we devised undergraduate laboratory courses involving gene editing in live animals. Student involvement begins with target design, includes injecting live eggs/embryos and performing phenotypic analysis. Our two undergraduate laboratory courses take advantage of the fast development and large eggs of the frog *Xenopus laevis* and of the butterfly *Vanessa cardui*. Here, we report the efficient generation, by course-sized cohorts of undergraduate students, of frog and butterfly CRISPR gene-knockout mutants (or 'crispants') generated by the injection of programmed CRISPR ribonucleoprotein (Burger et al., 2016). We describe practical aspects for their scalable implementation and share our experience integrating learning outcomes in developmental biology and bioethics.

Although our courses have mostly been targeting genes with already published phenotypes, our end goal is to run these classes as course-based undergraduate research experiences (CUREs). CURE courses are enhanced active-learning variants of teaching laboratories, where students are exposed not only to scientific methods as in a traditional laboratory course, but also participate in the authentic scientific process by producing data of potential interest to the scientific community (Auchincloss et al., 2014). CUREs expand the accessibility of research experiences to many more participants, while also fostering collaborative working skills, facilitating immersion into the process of scientific inquiry and discovery and increasing aspiration for scientific careers (Auchincloss et al., 2014). Based on our experience over several semesters, we discuss how CRISPR-based experiments can yield an ideal framework for innovative CUREs, although we note that the CRISPR-based experiments described here can also be readily implemented in regular non-CURE laboratory courses.

Choosing the right animals and equipment

Butterflies and frogs: availability, scalability, fecundity and injectability

There are many potential organisms where the delivery of CRISPR tools is scalable in the classroom. In particular, bacteria, yeast, mammalian cell cultures, plant protoplasts (Woo et al., 2015) and ascidian embryos (Stolfi et al., 2014) all benefit from relatively simple transformation methods that could be used for genome editing experiments in the laboratory classroom. However, our intention while designing these laboratory courses was to give students an opportunity to witness the complexity of animal development and to create gene knockouts that would produce an obvious external phenotype such as changes in pigmentation and/or

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patterning. We also wanted students to execute a high-precision task on a biological entity, such as the micro-injection of animal embryos. A class of 10–20 students brings the following challenges: (1) the reliable production of hundreds of eggs with adequate control of fertilization timing; (2) the installation of at least 5 affordable micro-injection stations and avoiding the use of expensive high-precision injection controllers; (3) large eggs that can be injected under standard dissection binoculars by untrained students; (4) relatively soft eggs that do not frequently clog or break the injection needle; and (5) a relatively fast development time to allow phenotyping within the timeframe of a standard laboratory course. Following a quick survey of the available laboratory organisms, we settled on *Xenopus laevis* for vertebrates and *Vanessa cardui* for invertebrates for their unique suite of traits, as summarized below:

Commercial availability, rearing and regulation

Stocks of both species can be purchased in the USA to scale up egg production prior to classroom use. *X. laevis* embryos, tadpoles and adults can be readily obtained through local *Xenopus* labs, the National *Xenopus* Resource or Nasco (a commercial supplier in the USA). As frogs are vertebrates, the appropriate local regulatory body approvals are needed (Institutional Animal Care and Use Committees in the USA). These regulatory committees should be consulted on whether approved protocols are needed for egg injections and how long larvae can be reared in laboratory classrooms. Many institutions allow the injection of embryos and euthanasia of larvae prior to hatching. Once tadpoles are free-swimming and eating, animal experimentation approvals are needed prior to classroom research activities. All *X. laevis* must be housed in appropriate facilities and must be euthanized prior to discarding as biohazardous waste. In particular, care must be taken to not let embryos or tadpoles escape through drains, as laboratory *Xenopus* escapees are ecological menaces (Measey et al., 2012).

'Painted Lady' *V. cardui* caterpillars can be easily obtained from a commercial supplier (e.g. '30 Live Caterpillars', Nature Gift Store). Obtaining egg-laying adults takes 2–3 weeks depending on temperature. Injected (G_0) *V. cardui* can be reared to the adult stage in 5 weeks using commercially available artificial diets (Fig. 1; Appendix 3). All G_0 individuals are subject to complete containment: individual rearing in plastic containers, closed rearing rooms with no sink or window escape route, no release, discarding as biohazardous waste and euthanasia by freezing upon adult emergence. Usually, no animal ethics protocols are required for classroom research involving butterflies, although instructors should consult with local regulations regarding the production of genetically modified organisms.

High fecundity with fertilization on demand

Scalability and fecundity are important considerations as each classroom with 10–20 untrained students needs hundreds of eggs. *X. laevis* females can routinely produce roughly 300 viable zygotes from *in vitro* fertilization within 1 h in a dedicated rearing facility (for instance, associated with a research lab) (Showell and Conlon, 2009). We recommend using several female *X. laevis* to produce eggs for a course as there is batch-to-batch variability in embryo quality. *V. cardui* can also produce hundreds of eggs on demand by the presentation of a host plant into the colony. While conveniently maintained at room temperature, a colony of 30 adults can produce 200–400 eggs within 3 h (Fig. 1). Butterfly embryos are fertilized at oviposition and start developing as a multi-nucleated cell (syncytium).

Tailoring micro-injections to the classroom

Butterflies and frogs are ideal for classrooms, as both have large eggs that tolerate relatively large injection volumes. This is critical in a classroom set-up, since injection needles do not need to be perfectly shaped and will resist multiple injections before breaking. These features allow students to inject in parallel without constant interventions from instructors. Importantly, this also allows the use of coarse and affordable micro-injection setups, instead of the high-precision micro-injectors that are classically needed for smaller organisms like nematodes. We recommend an air-pressure injection system that is affordable, easy to maintain, and allows back-loading by the students or co-instructors in less than 1 min (see Materials and Methods).

X. laevis eggs are spherical (1–1.3 mm diameter). *Xenopus* eggs can be injected at the one cell stage, which often produces mutant G_0 animals. Alternatively, one cell of the two cell-stage embryo can be injected to create within-animal controls where often only one side of the animal shows a phenotype.

V. cardui eggs are elongated (0.7–0.9 mm width and length) (Fig. 1). We recommend the injection of eggs within 3–7 h of egg laying (i.e. before cellularization) in order to generate mosaic G_0 'escapees' that survive embryogenesis. As only a fraction of the animal is affected, knock-outs are randomly obtained in non-essential tissues such as the wings and allow a local loss of function, bypassing the embryonic lethality that is expected with important developmental genes (Livraghi et al., 2017). Scalability is easily achieved by increasing colony size or the time of host plant presentation.

Other organisms deserve consideration for undergraduate injection sessions, such as killifish (Dodzian et al., 2018), beetles (Gilles et al., 2015), crickets (Watanabe et al., 2014) and fruit flies (Adame et al., 2016). However, we advocate by experience that the unique combination of accessibility, injectability and fecundity of butterflies and frogs make them optimal for the classroom. These protocols are likely to be adaptable to a number of amphibian and lepidopteran species. Moreover, amphibians and lepidopteran species are charismatic animals that easily capture the attention of undergraduates, and also have long and rich histories of addressing fundamental questions in biology by cross-cutting fields of evolution, development, behavior, ecology and genetics.

Course design and content

As undergraduate teaching laboratories are increasingly styled toward incorporating genuine research experiences, course design is more important than ever. Instructors should define and adhere to specific pedagogical goals while being flexible with student-driven hypotheses that contribute to new scientific discoveries. Moreover, instructors are obligated to discuss challenging topics such as bioethics, and develop course assessments that allow later refinement and improvement of outcomes. In this section, we describe the design, content and learning outcomes of our laboratory courses.

Course design

We have found that the 'backwards' course design is useful for planning an inquiry-based undergraduate laboratory, where learning goals are defined first and then curriculum is designed with specific learning outcomes to achieve those research and pedagogical milestones (Cooper et al., 2017). Our course learning goals are for students to: (1) apply critical thinking skills to a scientific question; (2) develop analytical skills to quantify and analyze phenotypic data; (3) communicate knowledge on a CRISPR-related topic

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Fig. 1. Overview of the *Vanessa cardui* CRISPR procedure for laboratory courses. See Appendix 3 for details. (A) Oviposition occurs on hollyhock or mallow for 1–4 h; fertilization occurs at egg laying. (B) An egg with micropyle side up (as during injections). (C) Transfer of eggs from a metal bowl to each inverted cup lid lined with double-sided sticky tape (white arrow). (D) Egg flipping and positioning is done by the students under a binocular stereomicroscope. (E) An example of simple and economic microinjection set-up. (F,F′) Egg microinjection to the micropyle within 7 h of egg laying. Injection volume (as measured in air) should be minimal, for instance, half the size of the micropyle structure (minimum and maximum droplet size indicated by filled red circles). (G,H) Hatchlings are fed artificial diet in small cups or plastic dishes and transferred at the third instar stage (G) to individual small plastic cups until pupation (H), after which they are frozen before characterization.

(journal-style article on results or a bioethics position piece, see below); and (4) gain practical skills for joining the scientific workforce. Once these learning goals were defined, we identified learning outcomes to achieve these goals, including technical, knowledge-based and analytical outcomes, and their associated assessments (Table 1).

Course logistics

The genome editing laboratory class is taught to 12–16 third and final year Biology undergraduates (junior and senior college years) each semester. The students meet for 2–3 h once a week for 14 weeks. This includes BLAST analysis and sgRNA design (one session), butterfly CRISPR injections (six sessions) and frog CRISPR injections (one session). The rest of the semester includes lectures on genome editing and bioethics, butterfly larval and pupal wing dissections, student short presentations and phenotypic analyses of the crispants generated in the lab.

The organismal biology laboratory course is taught to 10–20 second and third year undergraduates (sophomore and junior college years). Students meet for 4 h once per week for 10 weeks. This course is evenly divided into two research modules, one of which involves CRISPR manipulations (five sessions). Students are required to read background material prior to each session so that laboratory time can be dedicated primarily to laboratory

Table 1. Student learning outcomes and assessments

	Learning outcomes	Assessment
Technical	Record experimental protocols and results in a laboratory notebook	Laboratory notebooks are graded weekly for completeness of protocol notes and results
	Use a microinjector to deliver gene editing tools into animal embryos	Successful injection of animal embryos to create mutants
	Phenotype crispants	Phenotyping appropriate to the gene target is quantified by comparing crispants with controls
	Analyze genetic mutations	Analysis of mutation types (indels, deletions) using PCR and sequencing
Knowledge	Describe how Cas9 interacts with DNA to create mutations	Writing in laboratory notebooks and journal-style articles conveys understanding of Cas9 function
	Understand the gene pathway leading to a phenotype of interest	Reasonable prediction of phenotype associated with gene pathway
	Relate gene editing technology to a bio-ethical challenge in society	Presentation and in-class discussion on a topic relating to gene editing ethics
Analytical	Generate a hypothesis relating gene function to phenotype	Hypothesis about genotype–phenotype relationships with testable predictions is created
	Examine experimental results	Statistical analysis and visualization of data recorded in a laboratory notebook
	Communicate interpretation of results and updates on biological models	Write a journal-style article that includes a discussion of results within a broader context

experiments after roughly 30 min of discussion of assigned readings. For the module involving CRISPR, sessions include: (1) experimental design, selection of target genes and short guide RNA (sgRNA) design; (2) CRISPR injections and a discussion of bioethics; (3) phenotyping crispant morphology; (4) data analysis, data visualization and structuring of laboratory report; and (5) team presentations and finalizing a joint journal-style report.

Introduction to CRISPR and selection of gene targets

Lectures are appropriate to introduce the students to the functions and mechanisms of CRISPR interference in bacteria, and then to the repurposing of Cas9 and sgRNAs for generating targeted DNA double-strand breaks in the laboratory. For students to gain familiarity with manipulating sequence information and designing a pair of sgRNAs in its coding sequence, each student is assigned a gene with a well-known phenotype. Instead of directly working with a *V. cardui* or *X. laevis* nucleotide sequence, students begin with an orthologous protein sequence ID to fetch in NCBI GenBank (Table 2). The students quickly explore the GenBank entry, acquire the protein sequence in FASTA format, and following an introduction to BLAST analysis and sequence conservation, perform a TBLASTN against the *X. laevis* or *V. cardui* CDS database (coding sequences) at www.xenbase.org or www.butterflygenome.org, respectively. Students interpret the results to identify their most likely ortholog and acquire the full transcript sequence before identifying the coding frame in ORFfinder.

Table 2. Gene targets used for student assignment on designing sgRNAs

Protein name	Species	GenBank ID	Species for TBLASTN analysis
Abdominal-A	*Bombyx mori*	ACD10794	*V. cardui*
Distal-less	*Bicyclus anynana*	AAL69325	*V. cardui*
Membrane-associated transporter protein (SLC45A2/MATP/OCA4)	*Homo sapiens*	AIK67168	*X. laevis*
Optix	*Heliconius erato*	AEO13434.1	*V. cardui*
Tyrosinase (Tyr-a+Tyr-b)	*Homo sapiens*	NP_000363.1	*X. laevis*
WntA	*Heliconius erato*	AFC75683	*V. cardui*

After obtaining the full transcript sequence, students are asked to design a sgRNA target consisting of 20 nucleotides, with a GC-rich 3′ end and immediately preceding a 5′-NGG-3′ protospacer adjacent motif (PAM) sequence of the transcribed strand. Similarly, a second sgRNA is designed on the reverse strand, i.e. immediately 3′ of a 5′-CCN-3′ sequence on the coding frame, which clarifies that targeting is independent of transcript directions and reminds students about the bi-directionality of the DNA strand. Students are advised that since they have designed their target to a transcript, they risk an overlap with a splice junction. Students then confirm the lack of a splice junction using genome sequences of the same (*Xenopus*) or related (*Heliconius melpomene* butterfly) species. We also note that courses using species with sequenced genomes can design sgRNAs directly from the genome sequence. At the end of this session, the students have gained familiarity with simple sequence database functions, BLAST analysis and sgRNA design. Students are then provided with published sgRNA sequences for well-established targets for comparison. Students can also be encouraged to explore potential off-target effects of sgRNAs using common web interfaces (Bae et al., 2014; Stemmer et al., 2015). Subsequent lectures include more detailed information on generating null alleles using the non-homologous end-joining (NHEJ) repair pathway, achieving knock-in insertions and precise edits using homology-directed repair, and the *in vivo* delivery of genetic material to somatic cells for potential gene therapy, with emphasis on the promising use of viral vectors such as AAV9 (Mout et al., 2017).

Assessment of student learning outcomes and/or adapting this workflow to a CURE course can be achieved by having students choose their own gene of interest and designing sgRNAs. After verification of sequence by instructors, these student-designed sgRNAs can then be synthesized or purchased and used in subsequent experiments. For example, examining the function of genes related to pigmentation, patterning or behavior can be easily amenable to laboratory courses. Instructors developing new courses may consider beginning with targeting known gene targets prior to expanding into CURE approaches. For novel experimentation where students choose gene targets, we suggest directing students to curated genotype–phenotype lists, such as the Monarch Initiative database (Mungall et al., 2016), OMIA (Nicholas, 2003) or GepheBase (Courtier-Orgogozo et al., 2019 preprint). Note that in addition to

students choosing their own gene target, individualized phenotyping protocols will also need to be developed. Thus, we recommend having a single biological theme with established phenotyping protocols that all students can implement (for example, visual system related gene targets with easy behavioral phenotyping). Finally, we recommend that when students choose a novel gene candidate, they also target a gene with a known phenotype to serve as a positive control and keep student enthusiasm high even when their own gene of interest does not produce an easily observable phenotype.

Frog CRISPR experiments

An advantage of working with *X. laevis* is the large literature of gene-to-phenotype relationships and the availability of injection and rearing protocols optimized by the large community of researchers using *Xenopus* frogs as a model system in development and many other fields of research. There are many available protocols on obtaining eggs from females, *in vitro* fertilization, microinjection of *Xenopus* eggs, and how to successfully rear embryos to tadpoles for phenotyping (Karimi et al., 2017; Kay and Peng, 1992).

There have been several publications utilizing CRISPR methods in *X. laevis* that include validated sgRNAs for pigmentation pathways involving genes such as *tyrosinase* and *MATP* (DeLay et al., 2018; Wang et al., 2015). In our courses, targeting of the melanogenesis factor *MATP* (sgRNA sequence: 5′-GGUUACAU-AGGCUGCCUCCA-3′) provided a highly efficient CRISPR with a rapid and a spectacular effect on melanophore maturation (DeLay et al., 2018). Following injection of CRISPR/Cas9 and sgRNA at the one-cell and two-cell (single blastomere injection) stages by students, and proper embryo care by the instructor, between 11% and 50% of the injected zygotes survived to the 72 h tadpole stage depending on the egg batch, experimenter and injection set-up (e.g. needle size). Across two sessions (one per semester), 94% and 62% of embryos surviving the student injections showed pigmentation defects at 72 h (Fig. 2). Some student experimenters failed to obtain phenotypes, either because of rough manipulation or voluminous injections that decreased survival rate, or in rare cases because they may have not injected anything (clogging). Regardless, the high efficiency of the class experiment allows material sharing so every student can compare a batch of mosaic mutants and uninjected controls for phenotypic analysis.

For phenotyping tadpoles and determining efficiency, we encourage the use of two within-clutch controls: (1) uninjected controls to have a baseline survival rate and wild-type phenotype for each clutch, and (2) embryos injected with either sgRNAs and/or dye only to serve as an injection control to determine the survival rate of injected embryos and allow students to observe other phenotypes that may result from mishandling. For example, some amelanic mutants sometimes show unexpected morphological defects such as a missing eye or craniofacial defects compared with controls (Fig. 2A,A′), likely due to injection stress or off-target effects. The students are then encouraged to think about what would be additional control groups (e.g. injection of a mock mix with a random sgRNA). The first sections of the original publication (DeLay et al., 2018) are given as a mandatory pre-reading and is used to guide the students through a rapid evaluation of the penetrance and mosaicism of the amelanic phenotypes (note that the first two figures of DeLay et al., 2018 are ideal companion material for the students and we recommend their discussion in the class). First, each student scores each side of the tadpoles for levels of melanogenesis in the retinal pigment epithelium (‘WT’: completely pigmented eye; ‘mild’: <50% of the eye is amelanic; ‘severe’: >50% of the eye is amelanic). Consistent with DeLay et al. (2018), students generally find that embryos injected as 1-cell zygotes show the same category of phenotype between left and right, while the melanogenesis defects of 2-cell embryos (one-of-two cell injection) are almost always unilateral. Rarely, we find occurrences of mild phenotypes on presumably uninjected sides, most likely because those 1-of-2 cell embryos were injected before complete separation of the two blastomeres. Knockout of the *Xenopus tyrosinase* gene, another determinant of oculocutaneous albinism in humans (human synonym *OCA1*), produces similar phenotypic outcomes but requires the co-targeting of two gene homeologs because of the allo-tetraploidy of *X. laevis* (Wang et al., 2015). Finally, students are asked to discuss in their laboratory reports why mosaicism is observed.

Overall, the *MATP* sgRNA provides a highly repeatable and simple introduction to a practical CRISPR course in *X. laevis*, as well as a pedagogic platform with insights that can be discussed at shallow and deep levels, including the mosaicism of G_0 injected animals, melanin/pigmentation biology and basic principles of cell lineage restriction (fate maps, neural crest cell migration). Moreover, this experiment allows for discussion of the generation of animal models for studying human genetic conditions such as oculocutaneous albinism.

In addition to well-described *tyrosinase* and *MATP* knockouts, there are several other genes that could be targeted to produce easily observed phenotypes. For example, the pigmentation gene *hsp6* produces the *no privacy* mutation in the diploid species *Xenopus tropicalis* (Nakayama et al., 2017), resulting in transparent tadpoles that could be used for other laboratory physiology experiments. Other candidate genes could be involved in limb development (e.g. *nephronectin*; Abu-Daya et al., 2011), or target muscle-related pathways (e.g. muscle-specific myosin chaperones; Geach and Zimmerman, 2010), resulting in paralysis, which is an easy phenotype for students to score. Overall, when choosing genes to target with students, we recommend choosing pathways that could take advantage of easily scored phenotypes within the time frame of an undergraduate course.

An example of undergraduate CURE-like CRISPR result in butterflies

In an attempt to quickly obtain loss-of-function phenotypes in butterfly larvae, the Hox gene *abdominal-A* was targeted. When injecting at 1.5–3.5 h after egg laying (AEL), 20% of 121 injected eggs survived embryogenesis. Among the survivors, 5 individuals showed segmental fusion defects at the final larval stage and one individual gave rise to a similar fusion at the pupal stage (Fig. 3A-D). The expression of *abd-A* is localized to abdominal segments A2–A8 (Warren et al., 1994), consistent with the abdominal restriction of the observed phenotypes. These results replicate in *V. cardui* the outcome of *abd-A* KOs in other lepidopterans (Bi et al., 2016; Huang et al., 2016). We reasoned that the high mortality in this experiment was due to lethal effects of *abd-A* loss-of-function during embryogenesis. The Ubd-A epitope is shared by Ubx and abd-A and recognized by the FP6.87 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa). In wild-type embryos dissected at ∼80% of development, Ubd-A showed a strong signal in the nerve cord segmental ganglia from T3 to A8, and a small cell population in the posterior end of T2 (Fig. 3E). We infer from previous work in silkworms and butterflies that this signal is partitioned by Ubx from T2 to A2 and abd-A from A3 to the posterior end (Tong et al., 2017; Warren et al., 1994). In *abd-A* knockouts (injection at 30–90 min AEL), we observed a complete loss of the putative abd-A signal, with Ubx still detected in T2–A2 and acting as a positive control for the immunostaining (Fig. 3F). This mutant phenotype,

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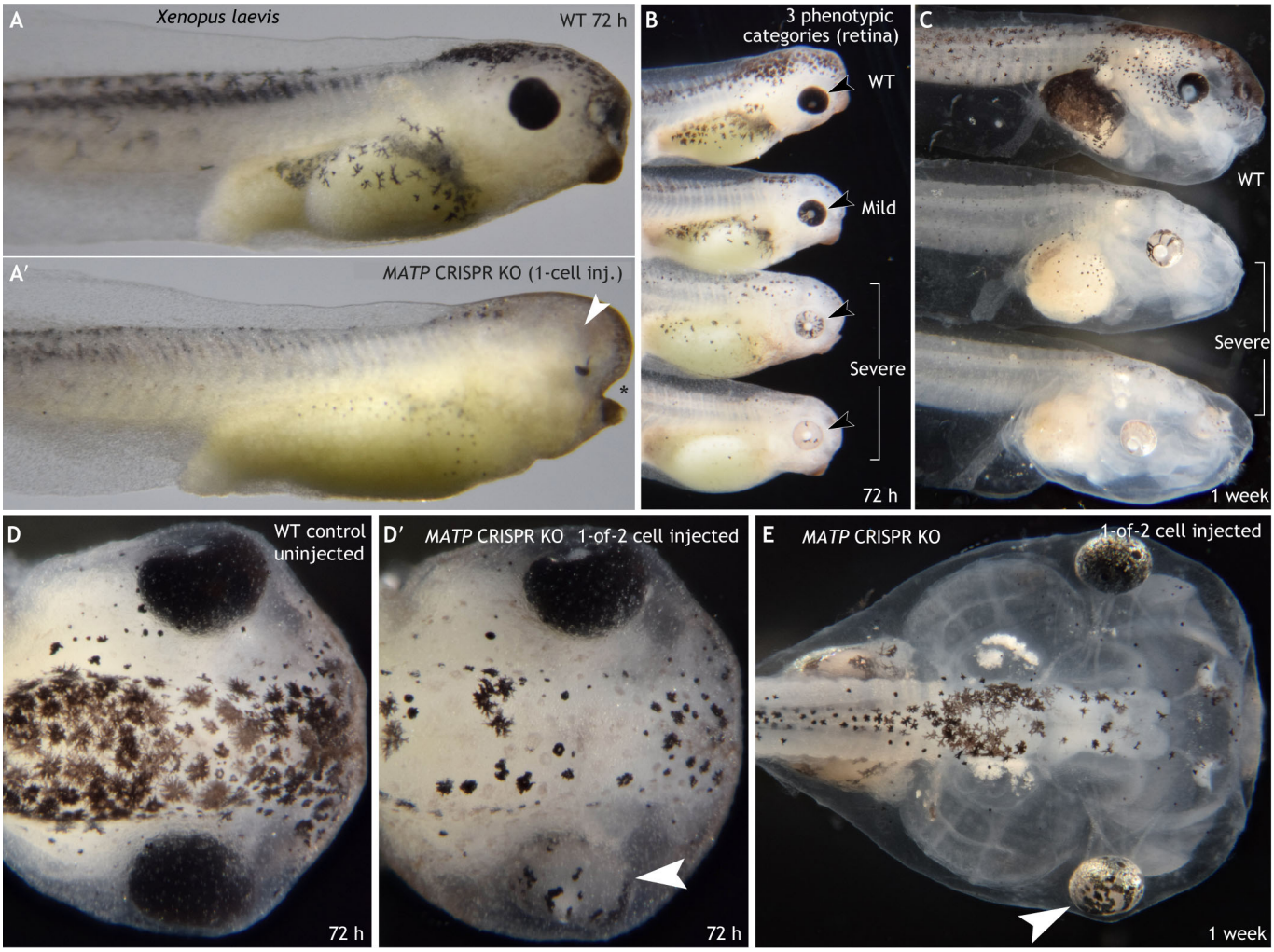

Fig. 2. *MATP* (syn. *slc45a2*) CRISPR bilateral and unilateral knockouts in *Xenopus laevis* embryos. See main text and a previous report (DeLay et al., 2018) for details; phenotypes shown here were obtained following classroom injections by the students. (A,A′) Example of a strong amelanic phenotype in *MATP* CRISPR knockouts (KO) following one-cell injection, shown here with non-specific phenotypes (arrowhead indicates missing optic cup; asterisk indicates oral/craniofacial abnormality). Such defects may be due to injection stress, but can be used to introduce the value of various control experiments. (B,C) Representative spectrum of mosaic melanic deficiencies obtained after one-cell injections. Students were asked to score phenotypes in the retinal pigmentary epithelia (arrowheads) and observed bilateral effects of comparable mosaicism between left and right within individuals. (D,E) One of 2-cell injections generate visible unilateral effects in the eye (arrowhead); dorsal phenotypes were typically mosaic compared with the wild type (WT) (D,D′).

accompanied with deformations of the abdomen, was observed in two-thirds (54/82) of the embryos that were mounted for microscopy. It is plausible that we approached 100% mutagenic efficiency, generating up to two-thirds of frameshift protein-null mutants, with the remaining third of injected individuals showing abd-A protein product due to in-frame indel mutations. Overall, this experiment suggests that injecting within 90 min of fertilization can produce biallelic knockouts at high efficiency and with limited mosaicism. For obtaining larval and wing phenotypes, it is thus desirable to increase mosaicism by delaying the injection time to >3 h (which is convenient for obtaining more eggs), or by injecting more-diluted CRISPR mixes, as previously suggested (Perry et al., 2016). These data also give an example of CURE-like research valuable to the community and immersing the students in a genuine discovery process.

Published targets for butterfly CRISPR

An increasing number of CRISPR results are being described in *V. cardui*, providing immediate opportunities to replicate published mutant phenotypes that illustrate key principles in genetics and developmental biology (Livraghi et al., 2017). The transcription factor *spineless*, as well as the melanin synthesis pathway genes *Ddc* and *yellow* reliably induce melanin-deficient wing clones, with *Ddc* also affecting larval pigmentation (Perry et al., 2016; Zhang and Reed, 2016; Zhang et al., 2017b). Mosaic KOs of the enzymes *ebony* and *black* yield the opposite effect with ectopic darker melanization (Zhang et al., 2017b). The selector gene *optix* both represses melanin and activates red-orange-yellow pigments. Butterflies with *optix* knocked out, have striking melanic expansions on their dorsal surfaces and loss of color tone on ventral surfaces due to the lack of ommochromes (Zhang et al., 2017a). In order to make orange color, the butterfly wing needs to locally activate the synthesis of orange pigments while also repressing melanin. This dual role – activation of orange and repression of melanin – is performed by *optix* in a variety of butterflies (Zhang et al., 2017a) and gives a good conceptual example of a switch-like regulatory effect by a single transcription factor.

The transcription factor and eyespot activator gene *Distal-less* (*Dll*) exemplifies how targeting different section of a gene can have different, even opposite effects. Indeed, targeting of *Dll* exon 2 triggers a gain-of-function in the wings and additional eyespots,

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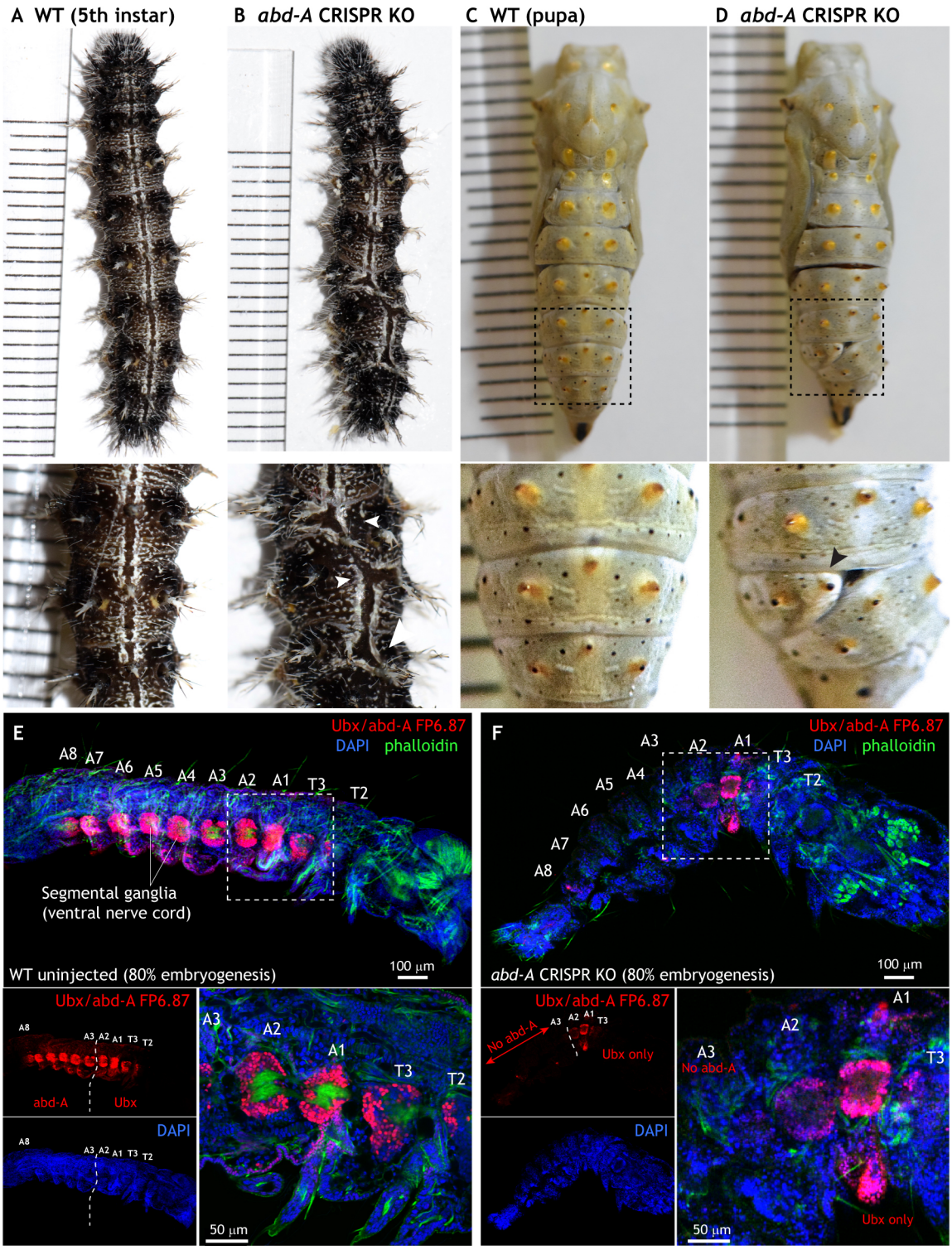

Fig. 3. *abdominal-A* CRISPR knockouts in *V. cardui* butterflies. All results shown are from experiments performed by undergraduate students, with the embryo section carried out during a follow-up summer internship. (A–D) CRISPR knockout (KO) of *abd-A* results in mosaic aberrant segmental fusion phenotypes as observed on dorsal views of fifth instar larvae (A,B, white arrowheads) and pupae (C,D). Boxed region shows dorsal view of segments A6 (top) to A8. Black arrowhead indicates aberrant segment 7, seemingly missing its right side. (E) Restricted expression of the UbdA (Ubx and abd-A antigen; red) in a wild-type embryo dissected at ∼80% of development. (F) CRISPR knockout of *abd-A* by injection within the first 90 min of development gives rise to aberrant embryos with a contracted abdomen, and with UbdA signals restricted to A2 to T2. A weak T2 signal was observed in other embryos but is not visible on this projection. Nuclei are stained blue with DAPI; phalloidin staining of actin is green.

while targeting exon 3 creates a 'null' loss-of-function and a loss of eyespots (Connahs et al., 2019; Zhang and Reed, 2016). Knockouts of the transcription factor *Spalt* also produce eyespot losses in *V. cardui* (Zhang and Reed, 2016), bringing the possibility of additional targets for a teaching module focused on eyespot development and patterning rather than pigment content.

Finally, our current favorite target in the classroom so far is the Wnt ligand gene *WntA*, a signaling gene involved in the determination of pattern shapes in butterflies, with multiple effects across the wing patterning surfaces of injected butterflies (Mazo-Vargas et al., 2017). We particularly recommend this gene as an experimented injector can routinely obtain 30–50% survival at time of hatching, even for early injection times such as 1–2 h AEL, and generates 10–30% of adult butterflies with mutant clones after injection by novices. Overall, our main challenge has been to successfully rear injected individuals on an artificial diet because of sporadic issues with moisture, diet quality and temperature, and we therefore provide here detailed and optimized instructions to facilitate implementation elsewhere. In summary, there are already a significant number of options available for instructors, including *V. cardui* sgRNA sequences for direct replication that should fit a variety of teaching plans. We expect this menu to grow quickly, especially if adventurous instructors dare to explore the knockouts of other genes and start building a community of butterfly 'CRISPR-ers'.

Key concepts for genome editing lecture series

The lectures introduce a number of distinctions essential for genetic literacy.

Human applications – germline versus somatic editing

At the beginning of the course, students are taught the differences between somatic editing and germline modifications. It is essential to contrast gene or stem cell therapies with edits that can be passed to the next generation. The website of the National Academy of Sciences compiles a variety of resources related to these topics that can help in crafting instructional material (National Academies of Sciences, Engineering and Medicine, 2017).

Medical versus enhancement

Biotechnologies can target conditions that can range from life-threatening to vanity-based, or be motivated by a complex mix of altruistic, utilitarian, profit-making and utopian ideals. We simply encourage instructors to make the students think about human applications of genetic techniques in terms of equity, accessibility, beneficence, consent and safety. A possible approach may be to first highlight a real-life example of gene therapies that have shown impressive rescues of orphan diseases in human infants or mouse models (Beyret et al., 2019; Mendell et al., 2017). The reversal of color-blindness, which used a similar viral delivery technique in adult squirrel monkeys, can be interesting for discussion in comparison (Mancuso et al., 2009).

Transgenesis versus replacement

There is heated debate on the international scene about what constitutes a genetically modified organism (GMO), and under which circumstances organisms tweaked by programmable nucleases will be allowed in our food supply. A possible distinction can be made on the nature of the edit that is made. For guidance, an advisory council commissioned by the European Union recommended the re-creation of an allelic variant that exists in the gene pool of the same species to be considered non-GMO, and also issues general views on population gene drives and edited micro-organisms (see European Academies Science Advisory Council website and Fears and Ter Meulen, 2017).

Teacher assessment and student reporting

After the practical sessions, students are evaluated on three components: participation, keeping an adequate laboratory notebook, and writing a journal-style article on their results from one of two modules. Laboratory notebooks are evaluated based on the student's rationale for experiments, including why they chose their gene of interest, as well as their detailed description of experimental procedures and interpretation of results. In CURE courses where students are doing science that is an original contribution to the field and thus the answer is unknown *a priori*, it is important that students not be graded on the success of the project, but rather on their interpretation of the results within the larger literature, thinking about potential next steps and revising existing biological models. The course also requires a significant writing component in the form of a journal-style article, including an abstract, introduction, methods, discussion and reference section. Students write their own report individually and are given detailed feedback from instructors. Then, students working on the same research theme combine their individual articles into a group-written article that serves as their final course project. In our experience, obtaining a jointly written manuscript prior to the end of the course makes the publication of results generated by students much more likely. With some editing of language and data visualizations, some articles may be suitable for publication with students as co-authors either as a stand-alone article or as a part of a larger project from the instructor's laboratory (e.g. Moskowitz et al., 2018).

Towards the end of the genome editing course, students are asked to find a topic related to genome editing (or genetics) and society, compile a bibliography of primary literature, present the topic in the classroom and as a final project, write an essay. Presentations are ∼5 min long, with 3–4 slides rich in visual content and a mandatory final slide with 2–3 questions directed at the audience for initiating a ∼5-min-long discussion among the students. A final course bioethics essay is 2500–3000 words long, excluding the bibliography (at least 8 relevant academic, peer-reviewed articles), structured in four sections, including an introduction and at least one of the subsequent sections addressing bioethical questions. A majority of students, perhaps due to the predominance of the pre-medical track in these cohorts, choose projects in relation to specific pathologies (Table 3). Other students choose to write more in-depth about bioethical themes (germline modifications, xeno-transplantation, personalized genetics), or to explore issues and potentials of genome editing in engineering domesticated species (e.g. agricultural genetics) or wild populations (e.g. gene drives).

Genome editing bioethics

With great power comes great responsibility.

Variably attributed to a decree from the French National Convention in 1793 and to Spider-man's Uncle Ben

While STEM undergraduate students are given knowledge about the tools of tomorrow – for instance, in data science, machine learning, genome editing – they may often leave college without having been trained to think about how those technologies can impact society. In the USA, only a handful of undergraduate STEM curricula integrate mandatory components related to 'Science and Technology Studies' or 'Science, Technology and Society' (both abbreviated STS), leaving compulsory ethical training to certain professional faculties such as medical schools. In our experience, biology students are often unequipped to participate in complex debates about the potentials and pitfalls of genome editing when graduating with a degree in biology. We advocate that empowering students with a technical understanding of genome editing comes with the obligation of

Table 3. Categories of the 65 'Genome Editing and Society' projects completed across five semesters

Category	No. of projects	Specific content
Cancer treatments	6	CAR-T immunotherapy, breast and ovarian cancers, HPV-related cancers, leukemia
Degenerative diseases	6	Duchenne's muscular dystrophy, Parkinson's disease, Huntington's disease, Alzheimer's disease, ALS, MS
Other clinical conditions (somatic approaches)	18	HIV, liver cirrhosis, hypertrophic cardiomyopathy, Rett Syndrome, cystic fibrosis, cocaine addiction, major depressive disorder, osteoporosis, spinal muscular atrophy, inflammatory bowel disease, hypercholesterolemia, retinal degeneration
Human germline editing	11	Designer babies, *in vitro* gametogenesis, pre-implantation diagnosis, 'Lulu and Nana' controversy, 'Adam Nash', mitochondrial replacement
Xeno-transplantation	6	Human–pig chimeric embryos, Type-1 diabetes cell therapy, transplant response
Disease vectors and population control	5	Mosquito gene drives, disease vectors, applications and concerns about gene drives, sterile insect techniques
Environmental engineering	5	Conservation genetics and interventionism, de-extinction (e.g. 'Mammophants'), symbiotic nitrogen fixation
Domestication	4	Food production, genetically modified crops, genome editing in the developing world, farm animals, designer pets
Personalized genetics	4	Gene-to-phenotype and ancestry services, gamete donor selection, genetic forensics

bioethics discussions (Baumann, 2016). Such discussions should encourage students to reflect on any moral responsibilities of gene editing tools as well as on the broad impacts of other forms of genetic knowledge (e.g. genetic diagnosis and personalized medicine). For example, genome editing is rapidly breaking into the public sphere with many negative examples (i.e. the first CRISPR-edited humans; Normile, 2018) fueling talk of 'designer babies' and 'playing God' (Janssens, 2016; Peters, 2017), which overshadows the promise of using genome editing to treat serious genetic diseases for which there are currently no cures (Lundberg and Novak, 2015). Such portrayals of genome editing and the language surrounding the technology in the media can lead to severe misconceptions (O'Keefe et al., 2015), sometimes steering towards fear-mongering or its opposite, technological utopianism. In an undergraduate laboratory course involving genome editing, instructors bear the responsibility to address this gap in student bioethics training and prime them to participate in public discussions on the topic. In our own courses, we encourage students to explore and discuss topics related to genome editing and society by incorporating a combination of formal lectures, student presentations, and a writing assignment stemming from an ethical dilemma involving gene editing. Our goals for students are to foster inquisitiveness, encourage consideration of various stakeholders' perspectives, incorporate relevant scientific literature, and evaluate the strengths and weaknesses of alternative solutions (Chowning et al., 2012).

Student and instructor reflections

Student perspectives

Presently, we evaluate the perception of our classes using university-level evaluations, which rely on voluntary and anonymous feedback from the students. Our students (51 out of 64 enrolled) scored three aspects of the class, and provided indicators of maximal satisfaction regarding the genome editing lab course (Table 4).

Challenges in developing CRISPR experiments in our laboratory classrooms

A technical challenge in implementing the targeting of novel gene targets is the need for easy phenotyping for untrained students. We began with pigmentation genes as these are very easy for students to visually score. Moving into other gene targets such as those influencing organogenesis or behavior creates problems when standardized scoring of non-obvious phenotypes is required. Behavior assays in particular are difficult to implement as behavior assays take more time than simple visual scoring of pigmentation and the differences in behavior between crispants and wild-type animals may be subtle. Instructors should carefully choose target gene pathways where phenotyping by untrained students can be done in a standardized manner and within the time frame of a laboratory course.

Finally, a major challenge in refining our courses is identifying and implementing appropriate assessments that often go well beyond the generic university questionnaires typically given at the end of a course. Many CURE assessment tools have been developed that test personal gains in confidence, cognitive skills or understanding of the scientific process (Shortlidge and Brownell, 2016). Instructors should ideally choose assessment tools that have been validated in a similar test population (e.g. high school versus university students) with some consideration of effort required (e.g. in class versus online polls or questionnaires). Once research and pedagogical goals have been determined and an appropriate assessment identified, instructors can design the laboratory course to align with research goals in a way where evidence of impact on student learning can be rigorously assessed.

Concluding remarks: towards CRISPR cures?

We present suggestions on designing and implementing undergraduate laboratory courses utilizing genome editing technologies in butterflies and frogs, which combine unique practical advantages such as the scalable yield of large eggs and fast development. Genome editing is possible in animal embryos within the opportunities and constraints presented by undergraduate lab courses with different formats (long

Table 4. Generic metrics from anonymous student evaluations of the genome editing laboratory course (SmartEvals polls)

Course feature assessed	Genome editing lab (mean score out of 5; *N*=51)	Institution average (mean±s.d.)
Integration of discussion and lab into the course structure	4.9	4.2 (±1.0)
Increased conceptual understanding and/or critical thinking	4.9	4.0 (±1.1)
Overall rating	5	4.3 (±1.0)

Anonymous student comments validated the potential of the teaching and laboratory approach, for instance (representative excerpts): 'it provided a very interesting and unique look into the realities of scientific research: trial and error, troubleshooting, revision of methods'; 'really opened my eyes to genome editing and modern day science'; 'it gave us a great lab experience, and really a more hands-on approach to science than most other classes have'.

14 week formats and short 5 week modules) and end-products (a bioethics essay and a journal-style lab report). Moreover, we give detailed suggestions on obtaining economical sources for reagents and equipment, as well as detailed procedures of butterfly genome manipulations to complement to the already large literature in amphibians. We believe that implementation of these courses is valuable for student technical and bioethical knowledge of CRISPR and allows for genuine research experiences.

Most importantly, undergraduate students were successful in performing CRISPR-based tests of gene function, from sgRNA design, to injection and phenotypic observation. Although most of our course experience has utilized already published gene targets, our basic structure lays out a clear path for designing CURE-based courses where students perform gene knock-outs for targets that have not been tested before, and actively participate in the creation of scientific knowledge (Wolyniak et al., 2019). In butterflies, we assign untested transcription factor and Wnt pathway genes that are expressed in the wing transcriptome, and look for morphological effects with a focus on wing patterning. In frogs, we suggest that CURE-based testing of new genetic targets can add to the well-established contribution of *X. laevis* in various fields of molecular genetics, including development, physiology and behavior. We encourage instructors to explore the potential of either organism to bring CRISPR to their laboratory classrooms while considering logistic constraints (e.g. feasibility of husbandry, *in vitro* fertilization of frog embryos, and regulations concerning the use of vertebrate animals in research). Moreover, instructors can tune experiments and approaches tailored to their interests (e.g. developmental versus physiological genetics, preference for vertebrate or invertebrate systems), and add new components based on desired learning outcomes (e.g. genotyping of induced mutations).

APPENDIX 1

Micro-injection set-up

For replication purposes, we provide a list of tested equipment appropriate for the classroom micro-injection of butterfly and frog eggs by undergraduate students. In our experience, the frogs and butterflies do not require needle balancing or the use of holding pressure between injections. In the class, we have also tried positive displacement systems such as oil-filled glass syringes (Watanabe et al., 2014) and the Drummond Nanoject II and III. While students were successful at injecting and obtaining phenotypes, these systems require the needle to be front-loaded with the CRISPR injection mix: a process that needs to be done by a trained co-instructor and takes several minutes. Instead, we recommend an air-pressure injection system as follows:

- Borosilicate capillaries with filament (#18100F-3; World Precision Instruments).
- Gravity needle puller (PC-10; Narishige International). We pull our needles 1–2 days in advance, gently break the tips by touching the side of a clean razor blade or with clean fine forceps, and store the needles on a strip of modeling clay in a square 120 mm Petri dish. Students can also pull needles extemporaneously, but we recommend a single instructor breaks the tips in large batches for consistency. A good needle should release a minimal amount of fluid at 20 psi, and may gradually open as more eggs are punctured.
- Three-axis MM33 right-handed manipulator (3-000-024-R; Drummond Scientific Company; can be flipped for left-handed operators) with a support base (3-000-025-SB; Drummond). While untested, a much cheaper single-axis coarse manipulator such as the M-10 (Narishige International) may add simplicity and perform just as well.
- Single pressure micro-injectors with footswitch (MINJ-1; Tritech Research Inc.), pulse-length control module (MINJ-2) and needle holder (MINJ-4). To fit into the micromanipulator, the needle holder can be made thicker with packing tape. We inject at 15–20 psi with the shortest pulse-length available (∼0.1 s).
- Laboratory air compressor (56 dBA MINJ-CMPR1; Tritech Research Inc.). Alternatively, the injectors can be connected to a laboratory compressed air faucet using an adapter such as the Tritech MINJ-38NPT14QC (standard USA dimensions).
- Polyurethane tubing 1/4” OD, 1/8” ID (TT-1-4OD; Tritech Research Inc.).
- 3-way and 4-way Tee air splitters (MINJ-3TQC and MINJ-4TQC; Tritech Research Inc.); extra brass compression fittings (MINJ-5; Tritech Research Inc.); extra compression fittings (MINJ-6; Tritech Research Inc.).
- Binocular stereomicroscopes with a variable magnification range of 1–2.5×, i.e. 10–25× with 10× eyepieces. Models with compact bases and built-in LED incident illumination are convenient. Ideally, one trinocular microscope station equipped with a camera broadcasting to a screen or projector for demonstration purposes.

APPENDIX 2

CRISPR reagents

This procedure describes the preparation of a batch of Cas9/sgRNA mix (10 µl at 250 and 125 ng µl^{-1}, respectively) for a single sgRNA per gene, tried and tested in both frogs and butterflies. We recommend the instructor prepares these aliquots in RNase-free conditions and uses careful pipetting for mixing liquids. Aliquots are made in order to avoid multiple freeze–thaw cycles of the reagents, and stored at −80°C in a freezer box with 196 PCR tube inserts (e.g. Argos FBZ-1196W).

- Order 3 nmol of 18–20 nt unmodified synthetic sgRNAs from a commercial provider (e.g. Synthego). Spin down pellet, label lid, store at −80°C.
- When using the stock for the first time, resuspend 3 nmoles (97 µg) dry sgRNA in 200 µl Nuclease-free Low Tris-EDTA Buffer (10 mmol l^{-1} Tris-HCl, 0.1 mmol l^{-1} EDTA, pH 8.0; e.g. Quality Biological #351-324-721). Synthetic sgRNAs are about 32,327 g mol^{-1}, resulting in a 485 ng µl^{-1} stock (i.e. ∼500 ng µl^{-1}). Dispatch into 40×5 µl aliquots and store at −80°C.
- Resuspend 65 µg Cas9-2xNLS (QB3 Macrolab, UC Berkeley or PNA Bio are recommended) in 45 µl RNAse-free H_2O and 10 µl 0.5% Phenol Red Solution, which reconstitutes as ∼65 µl of Cas9 at 2× concentration (∼1 µg µl^{-1}). Dispense into 13×5 µl aliquots and store at −80°C.
- Before class begins, thaw 5 µl Cas9 and 5 µl of sgRNA, mix gently by pipetting, leave for 10 min at room temperature to allow the formation of duplexes, and keep on ice during the injection session.

APPENDIX 3

Butterfly rearing and egg injections

This procedure makes recommendations specific to the rearing of *V. cardui* butterflies. Ambient air conditions such as temperature and relative humidity may affect the rearing parameters, and we recommend that instructors perfect their protocol on uninjected

stock before proceeding to class injections. In addition, while negative control injections lacking a sgRNA never produced post-embryonic phenotypes, we recommend the inclusion of such a control injection in the classroom for educational purposes.

Protocol for obtaining butterfly eggs

- Obtain *V. cardui* caterpillars from a commercial supplier (e.g. Nature Gift Store '30 Live Caterpillars'). It will usually take at least 14 days to obtain egg-laying adults at 25°C, or up to 22 days at 22°C.
- Artificial larval diet should be prepared following the manufacturer's instructions (Multiple species insect diet, Southland Products Inc.). Each liter of diet is supplemented with 5 ml canola oil, thoroughly mixed in a cooking pot with an electric handheld mixer while hot, and stored in a closed container at 4°C for up to 2 weeks.
- Upon receipt, in a clean environment and using gloves, add 2–3 g of diet to the bottom of small plastic cups (e.g. Dart Solo T125-0090), press diet to the bottom using a clean cup, gently transfer a third instar larva with a soft paintbrush, cover with a tight-fitting lid (e.g. Dart Solo PL100N) and pierce a small hole to allow excess moisture to evaporate. The cups are then placed on trays (Frontier Agricultural Sciences #F9698B) and stored at 25°C with 40–60% relative humidity, without perceivable air movement (Fig. 1H). If the diet dries up before the larvae pupate, change the diet and adjust humidity in the room.
- After 10 days, hang the lids in a 30×30×30 cm pop-up cage with the pupae hanging upside down, and enough room for emergence, with paper towels underneath to collect meconium upon emergence. Pupae that are not attached to a lid can be re-attached by their cremaster to a piece of microfiber cloth. Expose the cage to a 12 h:12 h light:dark cycle or to indirect daylight, and spray every 1–2 days with water.
- Adults should emerge within 10 days of pupation. Add a feeding station filled with energy drink (e.g. Gatorade) diluted in water at a 1:1 ratio. Male mating behavior is observed during the last hour of daylight, starting at least 48 h after emergence. After this, the adults should produce fertile eggs for the next 10 days. Continue to spray the cage with water every 1–2 days.
- 3–5 h before the class, place 5 hollyhock or mallow leaves in a water cup with a pierced lid (or in a long sunflower stem), and position in the cage next to an incandescent light bulb to stimulate activity around the plant with light and heat. Antennal and foreleg drumming by the females precedes egg laying. Monitor the time spent by the leaves in and out of the cage as they give the maximum and minimum age of the embryos, respectively. The next steps can be performed by the students.

Protocol for student injection of butterfly eggs

- Remove the eggs by hand (whilst wearing gloves) from the leaves and collect them over a large glass dish. Avoid moisture on the eggs. Transfer all the eggs to a small metallic cup.
- Add a flat strip of double-sided tape (e.g. 6137H-2PC-MP) to the inside of a small cup lid (e.g. Dart Solo PL100N). Alternatively, add the tape to the inside of the lid of a 60 mm tissue culture-treated dish (e.g. NSTF90019, Nest Scientific USA Inc.), and cover with the bottom of the dish at the end of the procedure.
- To decrease the stickiness of the tape, press firmly with gloved fingers, and then dab with tissue to cover with fibers. This will allow the hatchlings to walk away from the tape without getting stuck
- Then, drop 20–40 eggs directly on the tape (Fig. 1C). All the eggs that fall outside the tape should be repositioned on the tape using a thin soft brush. An anti-static gun can help to cancel uncontrolled electrostatic movement of the eggs.
- Under a stereomicroscope, use a blunt (not pulled) glass capillary to position the eggs with the micropyle (thin top) upwards, and press gently on top to adhere the eggs to the tape (Fig. 1D).
- Move to an injection station (Fig. 1E). Take an injection needle, back-loaded by depositing 0.6–0.8 µl of injection mix to the back of the needle using a filter tip and P10 pipette. The red color of the fluid allows you to monitor the tip filling under capillary action. The needle can then be mounted to the micro-injector.
- Use the micromanipulator to position the needle in the center of the field of view at minimum magnification. Pressing the foot pedal should release a small amount of fluid. At 15–20× magnification move the lid with one hand so the needle is at a 45 deg angle, slightly above the egg top. Focus the microscope so the egg top is sharp.
- At the beginning, use a pressure of 20 psi and keep the injection diameter minimal. Change the needle or reduce pressure down to 10 psi at anytime during the injection if expelled droplets become more than twice the size of the micropyle (Fig. 1F,F′).
- Holding the egg-covered lid firmly with one hand, use the coarse knob to puncture the upper side of the egg, not going too deeply past the egg chorion. Release a minimal volume of CRISPR fluid. Backflow of yolk will occasionally occur, but is often fixed by repeated bursts. Holding the egg dish firmly, retract the needle and inject outside the egg to expel yolk. If nothing comes out, the needle will usually unclog after puncturing the next egg. Try to achieve consistency by working steadily.
- Once all the eggs have been injected, count them, record name, date, age range of the embryos and injected reagent on the lid, snap lid onto a matching cup (e.g. Dart Solo T125-0090) and make two small holes with closed forceps to allow ventilation (or cover the 60 mm tissue culture dish). Place the cups or dishes in a closed plastic container with a wet paper towel to avoid desiccation over the next 24 h. Include a control batch of uninjected eggs in a similarly prepared dish.
- Open the plastic container after 24 h to prevent condensation.
- Eggs will develop at a temperature-dependent rate, hatching at ∼78 h at 25°C or ∼96 h at 22°C. In the morning of the expected hatching day, press small crumbs of fresh diet to side and lid of the cup or dish. Larvae should be found feeding on the diet within 24 h.
- Record hatching rate (L1 survivor larvae/injected eggs), which is typically 30–40% following the CRISPR injection of *WntA* sgRNA by an experienced operator. Low rates of survival may indicate lethal phenotypes, in which case you can inject later, or with more dilute mix in order to generate mosaic 'escapees'.

- Incubate at 24–25°C, monitoring daily for excess or insufficient moisture. A temperature of 25°C (Poston et al., 1977) provides an optimal trade-off between developmental time and survival in *V. cardui*, with completion of the life cycle in 28–32 days.
- Examine the successive stages for morphological phenotypes every 2 days. Add small amounts of fresh diet and dispatch larvae to individual cups once they have reached the third instar larval stage (Fig. 1G). Dispose of all dead individuals as biohazardous waste. About 20–22 days after injection, hang pupae in a dedicated cage, and spray every 1–2 days (pupation usually occurs 16–20 days after fertilization at 25°C, a sensitive time period where pre-pupae and soft, young pupae should not be disturbed). Record pupation rate (healthy pupae/L1 larvae) and take notes on possible aberrations.
- Allow wings to dry for 24 h after emergence, and freeze all G_0 adults at −20°C for >24 h. Pinning is not necessary for this class and phenotypic analysis can be done on wings that have been detached from the insect body and stored in #1 glassine envelopes.

APPENDIX 4

abd-A knockout and immunostaining of butterfly embryos

V. cardui eggs were injected at various times (see main text) with a mix of QB3/Macrolab Cas9 (250 ng µl^{-1}) and Synthego synthetic sgRNA targeting and *abd-A* coding exon (125 ng µl^{-1}, sequence: 5′-GGACUAGGGGCGGCUGCGC-3′). For embryonic analysis, eggs incubated for 59–62 h at 27°C were transferred to a test tube, permeabilized for 6 min in 5% commercial bleach diluted with 1× phosphate buffered saline (PBS), washed in PBS, fixed with rocking in 1.85% formaldehyde in PBS with 2 mmol l^{-1} EGTA, washed in PBS with 0.1% Triton X-100. Primary and secondary antibody immunostaining and embryo mounting was performed following a published procedure (Brakefield et al., 2009), using a 1:5 primary dilution of the mouse monoclonal FP6.87 anti-Ubx/abd-A antibody (Kelsh et al., 1994) obtained from the Developmental Studies Hybridoma Bank, detected by a 1:200 dilution of a goat anti-mouse AlexaFluor555 secondary antibody (Molecular Probes), and counterstained with DAPI and OregonGreen488-Phalloidin (Molecular Probes). Embryonic phenotypes were scored under an epifluorescence transmission microscope and imaged with an Olympus FV1000 confocal microscope.

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Competing interests

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