

Efficient CRISPR-Cas9 editing of major evolutionary loci in sticklebacks

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ABSTRACT

Background: Stickleback fish are widely used to study the genetic and ecological basis of phenotypic evolution. Although several major loci have now been identified that contribute to evolutionary differences between wild populations, further study of the phenotypes associated with particular genes and mutations has been limited by the difficulty of generating targeted mutations at precise locations in the stickleback genome.

Approach and aims: We compared different methods of expressing single-guide RNAs (sgRNAs) and Cas9 activity in fertilized stickleback eggs. We used an easily scored pigmentation gene (*SLC24A5*) to screen for molecular lesions, phenotypic effects, and possible germline transmission of newly induced alleles. We then used the optimized CRISPR methods to target two major evolutionary loci in sticklebacks, *KITLG* and *EDA*. We hypothesized that coding region mutations in the *KITLG* gene would alter body pigmentation and possibly sex determination, and that mutations in the *EDA* gene would disrupt the formation of most armour plates, fin rays, spines, teeth, and gill rakers.

Results: Targeted deletions were successfully induced at each target locus by co-injecting one-cell stage stickleback embryos with either *Cas9* mRNA or Cas9 protein, together with sgRNAs designed to protein-coding exons. Founder animals were typically mosaic for multiple mutations, which they transmitted through the germline at overall rates of 21% to 100%. We found that the copy of *KITLG* on the X chromosome (*KITLGX*) has diverged from the *KITLG* on the Y chromosome (*KITLGY*). Predicted loss-of-function mutations in the *KITLGX* gene dramatically altered pigmentation in both external skin and internal organs, but the same was not true for *KITLGY* mutations. Predicted loss-of-function mutations in either the *KITLGX* or *KITLGY* genes did not lead to sex reversal or prevent fertility. Homozygous loss-of-function mutations in the *EDA* gene led to complete loss of armour plates, a severe reduction or loss of most soft rays in the dorsal, anal, and caudal fins, and severe reductions in the numbers of teeth and gill rakers. In contrast, long dorsal and pelvic spines remained intact in *EDA* mutant animals, suggesting that common co-segregation of plate loss and spine reduction in wild populations is unlikely to be due to pleiotropic effects of *EDA* mutations.

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Conclusion: CRISPR-Cas9 approaches can be used to induce germline mutations in key evolutionary loci in sticklebacks. Targeted coding region mutations confirm an important role for *KITLG* and *EDA* in skin pigmentation and armour plate reduction, respectively. They also provide new information about the functions of these genes in other body structures.

Keywords: genome editing, stickleback, CRISPR-Cas9, *SLC24A5*, *KITLG*, *EDA*.

INTRODUCTION

Sticklebacks have become a powerful system for combining molecular and ecological studies of vertebrate evolution (Gibson, 2005). More than a century of detailed research has identified dramatic morphological and physiological differences that have evolved repeatedly among abundant marine, lake, and stream populations (Bell and Foster, 1994). More recently, the development of genetic mapping approaches has made it possible to identify some of the major chromosome regions that underlie classical skeletal, pigmentation, physiological, and behavioural differences (Kingsley and Peichel, 2007; Peichel and Marques, 2017). Unfortunately, the candidate regions from genetic mapping studies typically contain many different genes (Miller *et al.*, 2014). It can thus be challenging to determine which particular genes in a quantitative trait locus (QTL) interval are responsible for a phenotype of interest. In addition, multiple different phenotypes have often been mapped to the same or overlapping chromosome regions (Miller *et al.*, 2014; Peichel and Marques, 2017). It is usually not clear whether co-mapping of phenotypes is due to pleiotropic effects of single genes or to separate effects of multiple linked genes in a given genetic interval.

Transgenic methods have previously been used to study the regulatory or phenotypic effects of single genes in sticklebacks. However, conventional microinjection of foreign DNA sequences typically results in random integration, generating substantial variability between individuals and lines (Hosemann *et al.*, 2004; Colosimo *et al.*, 2005; Chan *et al.*, 2010; Mills *et al.*, 2014; Erickson *et al.*, 2015, 2016a; O’Brown *et al.*, 2015; Indjeian *et al.*, 2016; Howes *et al.*, 2017; Cleves *et al.*, 2018; Thompson *et al.*, 2018).

Targeted editing of endogenous loci in sticklebacks would provide a powerful additional tool for examining the function of engineered DNA sequence changes within a normal chromosomal context. Multiple methods have been developed in other organisms for targeting DNA nucleases to particular DNA sequences in the genome. For example, TALEN nucleases can be engineered to recognize and cut particular DNA sites (Miller *et al.*, 2011; Dahlem *et al.*, 2012), and have previously been used successfully to generate targeted germline deletions in sticklebacks (Erickson *et al.*, 2015, 2016a, 2018; Cleves *et al.*, 2018). However, substantial effort is required to generate TALEN nucleases with the desired specificity.

Recently, CRISPR-Cas9 has emerged as a simple and efficient system for genome editing. To create targeted mutations, two components are required: (1) the Cas9 protein, which cuts DNA, and (2) a single-guide RNA (sgRNA), which targets Cas9 protein to a specific DNA region. The complex formed by Cas9 and sgRNA generates double-stranded breaks at the site specified by the sgRNA (Jinek *et al.*, 2012). Repair machinery in cells will usually attempt to repair introduced breaks. However, this process is imperfect and mutations are often generated as a result. Induced mutations can range from single base pair substitutions to deletions of hundreds of base pairs. It is simple to design sgRNAs to target specified locations in the genome, and CRISPR-Cas9 targeting has been successfully implemented in many different model systems ranging from yeast (DiCarlo *et al.*, 2013) to cell lines (Cong *et al.*, 2013;

Jinek *et al.*, 2013; Mali *et al.*, 2013) to mice (Yang *et al.*, 2013). Promising initial CRISPR studies have also been reported in sticklebacks (Hart and Miller, 2017; Xie *et al.*, 2019). Here, we test additional ways of introducing Cas9 activity into stickleback embryos and use optimized protocols to achieve targeting and germline transmission of engineered mutations in three different stickleback genes of evolutionary interest: *SLC24A5*, *KITLG*, and *EDA*.

SLC24A5

To optimize and test whether we could efficiently induce stickleback mutations using CRISPR-Cas9, we initially targeted the *SLC24A5* gene. *SLC24A5*, also known as the *golden* locus in zebrafish, is required for normal pigmentation in zebrafish, and has also played an important evolutionary role in lightening skin pigmentation in humans at northern latitudes (Lamason *et al.*, 2005). *Golden* mutant phenotypes also provide an easy way to quickly and visually test the efficacy of different CRISPR-Cas9 conditions because loss of *SLC24A5* results in loss of eye and body pigmentation early in development at 5 days post fertilization (Swarup, 1958). These phenotypes are typically recessive, requiring biallelic loss-of-function in order to see a phenotype in a given cell, so CRISPR-Cas9 must highly efficiently introduce loss-of-function mutations to produce a visible phenotype (Jao *et al.*, 2013).

KITLG

KITLG encodes a ligand to the c-KIT receptor, also known as Steel (*Sl*) and mast cell growth factor (*MGF*) in mice (Copeland *et al.*, 1990; Williams *et al.*, 1990). The *KITLGX* locus has previously been linked to repeated evolution of pigmentation changes in both sticklebacks and humans (Miller *et al.*, 2007; Guenther *et al.*, 2014) and is known to be important for gametogenesis and the survival, proliferation, and differentiation of the primordial germ cells (PGC) in mice (Silvers, 1979; Brannan *et al.*, 1992; Bedell *et al.*, 1995; Gu *et al.*, 2009, 2011). Given its location on LG19 (Miller *et al.*, 2007), which is known to be the master sex-determining chromosome in stickleback (Peichel *et al.*, 2004), we hypothesized that the *KITLG* genes on the diverging sex chromosomes (*KITLGX* and *KITLGY*) might also play a role in sex determination. Teleost fish use a variety of different sex-determining mechanisms. Many characterized systems, such as those in medaka or mice, involve a master sex-determining gene, but no such gene has yet been found in sticklebacks (Gubbay *et al.*, 1990; Koopman *et al.*, 1991; Matsuda *et al.*, 2002). However, previous studies have found that an increase in the number of primordial germ cells may drive the unspecified gonad to an ovarian fate (Lewis *et al.*, 2008). Given their location in the genome and known roles in PGC migration and proliferation, *KITLGX* and *KITLGY* are promising candidates for genes that could determine gonad sex determination by altering the number of PGCs that arrive or proliferate in the unspecified gonad. In addition, generating loss-of-function mutations in the *KITLGX* gene can provide a critical genetic test of its previously postulated role in ecotypic colour differences in sticklebacks (Miller *et al.*, 2007).

EDA

EDA has been linked to the evolution of several traits in stickleback, including repeated armour plate reduction, changes in lateral line neuromast patterns, and changes in schooling behaviour (Colosimo *et al.*, 2005; Mills *et al.*, 2014; Greenwood *et al.*, 2016). Quantitative trait locus

(QTL) mapping has also identified peaks near *EDA* for a number of additional skeletal traits, including pelvic reduction; the length of dorsal spines one, two, and three; tooth number; and gill raker number (Shapiro *et al.*, 2004, 2009; Rogers *et al.*, 2012; Miller *et al.*, 2014; Erickson *et al.*, 2016b; Howes *et al.*, 2017). Loss-of-function *EDA* mutants in other species, such as zebrafish, lack all of their fin rays, including their pelvic fins. They are also missing scales, pharyngeal teeth, and gill rakers (Harris *et al.*, 2008). Given the mapping results and phenotypes in other species, we wanted to determine the range of skeletal phenotypes caused by introducing mutations into the stickleback *EDA* gene.

By conducting the current experiments with three different genes (*SLC24A5*, *KITLG*, and *EDA*), we examine whether CRISPR-Cas9 targeting provides a general approach for probing the functions of interesting evolutionary loci in sticklebacks.

MATERIALS AND METHODS

mRNA synthesis of *Cas9*

nls-zCas9-nls mRNA was transcribed from PT3TS-nCas9n from the Chen lab, obtained via Addgene (46757) (Jao *et al.*, 2013). The plasmid was linearized using *XbaI* (New England BioLabs, R0145S). The linear plasmid was purified (QIAquick PCR Purification kit, Qiagen), and 1 μ g was used for an *in vitro* transcription reaction (T3 mMessage mMachine, Life Technologies, AM1348M). The reaction was incubated at 37°C in a thermocycler for 1 hour, and the mRNA was purified by phenol-chloroform and isopropanol precipitation as detailed in the mMessage mMachine manual. A nanodrop spectrophotometer was used to check the concentration and purity of the *Cas9* mRNA. One microlitre of the product was run on a 1% agarose gel to check the integrity of the transcript.

Cas9 protein

Cas9-nls protein was purchased from the QB3 MacroLab Facility at the University of California, Berkeley.

Characterization of X- and Y-linked *Kitlga* genes

The stickleback ortholog of the zebrafish *kitlga* gene has been previously reported (Miller *et al.*, 2007). This gene maps to Linkage Group 19 (LG19), the evolving sex chromosome pair in sticklebacks (Peichel *et al.*, 2004). To avoid possible confusion between the divergent loci on the evolving female and male sex chromosomes, we here refer to the X-linked locus as *KITLGX* and the Y-linked locus as *KITLGY*. Expressed sequence tags (ESTs) derived from mixed adult organs of the Conner Creek (CC), Washington, freshwater population show partial homology to *KITLGX*, but also have regions of unique sequence (see Genbank accession numbers DN681761.1 and DN681762.1, produced from the ends of cDNA clone CGX20-B06). We completely sequenced the corresponding CGX20-B06 clone, which revealed a divergent *KITLG* predicted open reading frame. Predicted *KITLGX* and *KITLGY* protein sequence alignment was made with ClustalW, and alignment displayed using the output from the BOXSHADE server (https://embnet.vital-it.ch/software/BOX_form.html). We mapped this gene to the Y chromosome in a marine $\times$ freshwater F2

CROSS (Shapiro *et al.*, 2004; Miller *et al.*, 2007) using primers TTCTCCTTCGGCCTACTCCT and TGTGCGAAAGCCTATTTCAG, which amplify a ~200 base pair (bp) fragment off the Y chromosome and named this gene *KITLGY*. We screened all BAC filters from the stickleback CHORI 213 and 215 BAC libraries (Kingsley *et al.*, 2004), using two pairs of overgoes for each gene, designed to distinguish these two genes. Overgo screening was performed as described at www.chori.org/bacpac/overgohyb.htm. The *KITLGX* overgoes (all overgo and primers sequences are 5' to 3') were CAACGACGGACCTCCCCTAGTTAA and CGTTTTAGAGCCATCATTAAGTAG, and GAACCTGAAACCCTGTCTGGGGTG and GAAGGCTTTGCTCCAACACCCAG. The *KITLGY* overgoes were TGACACTGTCTCTGCAACCACCAA and GAGTCTGGCCATCGCCTTGGTGGT, and GAAACGAGAGCCCTGTCTGCGGTG and GAAAGCTTTGCTCCACCACCGCAG. *KITLGX* positives were screened by PCR with CTGTCGGCCTGATCAGTTTC and TGCAGTGTATCCTCCATGAATC, and TGCTGCTTTGAATAACTCACAT and ATTTAGGTCTGGACGGCATT. From this screening, two 213 and two 215 *KITLGX* BACs were found to overlap the *KITLGX* locus (213-42J11, 213-167N05, 215-250I22, and 215-264A13). These pairs of 213 and 215 BACs span ~360 kb and ~300 kb respectively in the reference assembly (Jones *et al.*, 2012), centred on the *KITLGX* gene.

KITLGY positives were screened by PCR with CACCACTCAGGCTTTGGCTG and CAGCTGCTAGAGGATTTGCT, and TTCCTGATTCACAGGTGACG and GTGGTCCACACTTTGCCTCT (designed to the SP6 and T7 ends of BAC 213-21C23, accession numbers CL641946.1 and CL641947.1, respectively), since 213-21C23 was found to be a *KITLGY* positive here. From this screening, one 213 and one 215 BAC was found to contain portions of the *KITLGY* gene (213-21C23 and 215-203C15). The 21C23 BAC lacks the 3' end of the *KITLGY* gene, while the 215-203C15 BAC spans the entire gene. These BACs were fully sequenced by the Stanford Human Genome Center as part of a larger sex chromosome sequencing project to be reported elsewhere.

We confirmed that *KITLGX* and *KITLGY* are both expressed in stickleback tissues by BLASTN (Altschul *et al.*, 1990) of the exon sequences of both against large RNA-sequencing datasets generated from testes and liver of the Bear Paw Lake (BEPa), Alaska, freshwater population [NCBI Sequence Read Archive (SRA): SRX146597 and SRX146599]. Recovered sequence reads were assigned to either *KITLGX* or *KITLGY* when they covered at least three different sequence positions that differ between *KITLGX* and *KITLGY*, and did not differ from both of the *KITLGX* and *KITLGY* reference sequences at more than one position. This analysis showed 131 and 22 reads that could be assigned to *KITLGX* and *KITLGY* respectively in the testes dataset; and 23 and 47 reads that could be assigned to *KITLGX* and *KITLGY* respectively in the liver dataset. Both datasets contained reads that supported the *KITLGY* exon-exon junctions at the skipped sixth exon (9 reads in testes, 6 reads in liver) and novel ninth exon (3 reads in testes, 2 reads in liver).

Design and synthesis of single-guide RNAs

Single-guide RNAs (sgRNAs) were designed using CHOPCHOP (<http://chopchop.cbu.uib.no/index.php>) based on the *Gasterosteus aculeatus* genome assembly (BROADS1) (Montague *et al.*, 2014; Labun *et al.*, 2016). To target *SLC24A5*, the target sequence 5'-GAAGCCGTCGGGGAAGTCTGGAGG-3' was chosen for three reasons. First, the target sequence starts with a G allowing for better transcription using T7 RNA polymerase

Table 1. sgRNA target sequences for *KITLG*

sgRNA1 designed in reference	GAAGTACATTCCGAGGGAAGAGG
<i>KITLGX</i> in MATA	GAAGTACATTCCAGGGAAGAGG
<i>KITLGY</i> in MATA	ACATTACGTTCTGAGGGAAAGG
sgRNA2 designed in reference	AGGGAAGAGGTAAGACATGGTGG
<i>KITLGX</i> in MATA	AGGGAAGAGGTAAGACATGGTGG
<i>KITLGY</i> in MATA	AGGGAAGAGGTAAGATGTGGTGG
sgRNA3 designed in reference	AGTGCCACTACAGGGAAGAGAGG
<i>KITLGX</i> in MATA	AGTGCCACTACAGGGAAGAGAGG
<i>KITLGY</i> in MATA	AGTGCCACTACAGGCAAGAGAGG
sgRNA4 designed in reference	GGTGTGTGGGACAGGGAGGAGG
<i>KITLGX</i> in MATA	GGTCTGTGTGGGACAGGGAGGAGG
<i>KITLGY</i> in MATA	GGTGTGTGGGACAGGGAGGAGG

than other possible bases (Chamberlin and Ring, 1973). Second, the closest possible off-target sequence, identified by a BLASTN search (Altschul *et al.*, 1990) of the *G. aculeatus* genome assembly (BROADS1), differed by eight bases and lacked the required NGG PAM site (shown in bold above), therefore minimizing potential off-target effects. Third, this sequence was highly ranked in CHOPCHOP's computational predictions of efficiency, with a score of 0.70. To target *KITLG*, four sgRNAs were used in combination because it is located on the stickleback sex chromosome (LG19), and there are coding differences (see Table 1) not only between the X- and Y-linked copies of the gene, but also between the reference genome (Bear Paw Lake, AK) and the population used for injection (Matadero Creek, CA).

The sgRNAs were made with a two oligo-PCR method that uses one gene-specific primer and one scaffold primer as described by Shah *et al.* (2015). The gene-specific guide contained a T7 RNA polymerase promoter sequence, the 20 bp targeting sequence in the gene of interest (N20; the target site excluding the NGG PAM sequence), and 20 bp of homology to the scaffold oligo. The sequence was 5'-AATTAATACGACTCACTATA(N20)GTTTTAGAGCTAGAAATAGC-3'. The scaffold oligo sequence was 5'-GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATTTCTAGCTCTAAAC-3'. The scaffold oligo was HPLC purified (synthesized by IDT), but the gene-specific oligo was not.

To make the sgRNA template for transcription, 2× Phusion Master Mix (New England BioLabs, M0531L) was used for a 20 μ L reaction with 5 μ L of 10 μ M gene-specific oligo and 5 μ L of 10 μ M scaffold oligo. The PCR cycle was 95°C for 30 seconds; 40 cycles of 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds; and 72°C for 5 minutes. The PCR product was run on a 2% agarose gel, and the correct size band (121 bp) was cut out and purified (QIAquick Gel Extraction kit) or purified if there was no double band (QIAquick PCR Purification kit) and eluted in 20 μ L of water. For transcription, 7 μ L of this gel-purified guide template was used in a T7 *in vitro* transcription (MEGAscript T7, Life Technologies, AM1334M). The transcription was incubated at 37°C in a thermocycler for 16 hours and cleaned up using LiCl as detailed in the MEGAscript manual. sgRNAs were resuspended in nuclease-free water from the kit and stored at a concentration of 1 μ g/ μ L at -80°C.

***SLC24A5* injection and assessment of efficiency**

Injections with sgRNAs targeting *SLC24A5* were done with both Cas9 protein and *Cas9* mRNA. For the *SLC24A5* *Cas9* mRNA injections, the mix contained 300 ng/ μ L of *Cas9* mRNA and 300 ng/ μ L of the sgRNA. For the *SLC24A5* Cas9 protein injections, the mix contained 1 μ g/ μ L of Cas9 protein and 300 ng/ μ L of the sgRNA. A final concentration of 0.05% Phenol red was used, and the volume was adjusted with 10 mM Tris pH 7.5. Two clutches of fertilized stickleback eggs were pooled and split into approximately three equal portions. One-third was injected with *Cas9* mRNA, another third was injected with Cas9 protein, and the remaining third was kept as non-injected controls. At 5 days post fertilization, a total of 26 controls, 38 mRNA-injected embryos, and 31 protein-injected embryos were screened for pigmentation defects. To assess the efficiency of the mutagenesis, five embryos were sacrificed (two injected with *Cas9* mRNA, two injected with Cas9 protein, and one control). To extract DNA, embryos were placed in lysis buffer (10 mM Tris pH 8, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) with Proteinase K (333 μ g/mL) at 55°C overnight, and then phenol chloroform extracted. The target *SLC24A5* genomic region was amplified with the forward primer 5'-AAACCCATTCCACCAGAGTG-3' and the reverse primer 5'-TACCACAGCATTCCAGACCA-3'. The PCR product was cloned into pCR Blunt-II TOPO using the Zero Blunt TOPO PCR Cloning Kit. DNA was isolated from approximately 30 colonies from each individual (except for 12 for the control individual) by miniprep and sequenced to determine the lesion.

To measure germline transmission of targeted mutations, mosaic founder fish were grown to sexual maturity (~7 months). A total of 34 *Cas9* mRNA-injected fish and 22 Cas9 protein-injected fish successfully hatched and were raised to adulthood. Sperm was isolated from the males and used to fertilize eggs of wild-type females from the same stickleback population (Matadero Creek, CA). Because no obvious pigmentation defect was seen in adult F0 animals, males were chosen for breeding based only on reproductive condition. Offspring from these crosses were sacrificed and screened for transmitted DNA lesions by amplifying the *SLC24A5* target region as above and directly sequencing the PCR product.

***KITLGX* and *KITLGY* injection and assessment of efficiency**

For the *KITLGX* and *KITLGY* injections, the mix contained 300 ng/ μ L *Cas9* mRNA and a total 125 ng/ μ L for the four sgRNAs combined (each sgRNA was at a concentration of 31 ng/ μ L). One clutch of eggs was injected, and eleven of the fish were raised to sexual maturity. To determine the transmission of the mutations and identify associated phenotypes, F0 fish were crossed either to each other (to create potential homozygotes) or to wild-type fish (to create potential heterozygotes). *KITLGX* was amplified using forward 5'-GCACTTTGCATGTTAGCCCC-3' and reverse 5'-ACAAACAAGCTCTCCCCGAG-3' primers. *KITLGY* was amplified using 5'-AGAAGACCCAGCGGAAAAGG-3' and 5'-TGAGGAACATCAGTGCACCC-3' primers. These products contained all four sgRNA sites. Because environment and age affect pigmentation, age-matched fish of each genotypic class were raised together for 8 months before comparing their pigmentation patterns.

EDA mutants

One clutch from fully-plated anadromous fish (Little Campbell River, British Columbia) was injected with Cas9 protein and an sgRNA (N20: 5'-CGCTCTTCACAGGGAAAAAG-3') targeting the fourth exon of *EDA* using the same procedures described above for *SLC24A5*. Fish were raised to sexual maturity and then F0 founders were crossed with each other. Fish skeletons were stained with Alizarin Red as described elsewhere (Peichel *et al.*, 2001; Miller *et al.*, 2007), except that fish were first fixed in 70% ethanol. Branchial skeletons were dissected and flat-mounted as described in Ellis and Miller (2016), with an additional 10 day clearing step in 1% potassium hydroxide after dissection. To examine DNA lesions, a 1394 bp region was amplified using 5'-ACACACGCAAACACACATGG-3' and 5'-GAAGCCCCACCTTCTCTCAC-3' primers. The resulting product was cloned into pCR Blunt-II TOPO and sequenced. Differences in lesions and linked SNPs were used to verify that both alleles were detected.

RESULTS

Generation of *SLC24A5* mutants

To optimize the efficiency of CRISPR-Cas9 mutations, we first tested two ways to introduce Cas9 activity and sgRNAs into stickleback embryos. Cas9 can be introduced either as a premade protein or as mRNA that will be translated by the machinery of the cell. The advantage of using Cas9 protein is that it can begin cutting almost immediately when injected into the cell, whereas if *Cas9* mRNA is injected, it must first be translated, and the injected message may also compete with endogenous transcripts for translation. While in theory Cas9 protein should be more efficient, previous studies have not always found increased efficiency using Cas9 protein (Gagnon *et al.*, 2014). We chose to test both by introducing either *Cas9* mRNA or Cas9 protein with a single sgRNA into single cell stage stickleback embryos by microinjection, using the *SLC24A5* locus as a test because of its easy-to-score phenotypes.

Sticklebacks generated by injecting fertilized eggs with *SLC24A5* CRISPR-Cas9 showed reduced melanization in the eye and body of the fish at 5 days post fertilization (Fig. 1). These defects were no longer visible at 7 days post fertilization. No obvious pigmentation defects persisted into adulthood, but more subtle differences may have been missed due to natural variation in stickleback pigmentation. Severely reduced melanin pigmentation was only seen when fish were injected with Cas9 protein, but the amount of pigment that remained varied widely among both protein- and mRNA-injected embryos (Fig. 1). To study the types of molecular lesions introduced by *SLC24A5* CRISPR targeting, we amplified and cloned a genomic region around the targeting site and then sequenced multiple sub-clones from two founder (F0) individuals injected with *Cas9* mRNA, and two F0 individuals injected with Cas9 protein. All fish were mosaic for multiple lesions, which ranged in size from single bp deletions to a 273-bp deletion around the sgRNA target (Fig. 2).

To determine the efficiency of male germline transmission, three male F0 fish injected with *Cas9* mRNA and three injected with Cas9 protein were crossed to wild-type fish from the same population. Sequencing of ~30 offspring from each cross demonstrated that the transmission rate was between 33% and 41% for fish injected with *Cas9* mRNA (Table 2).

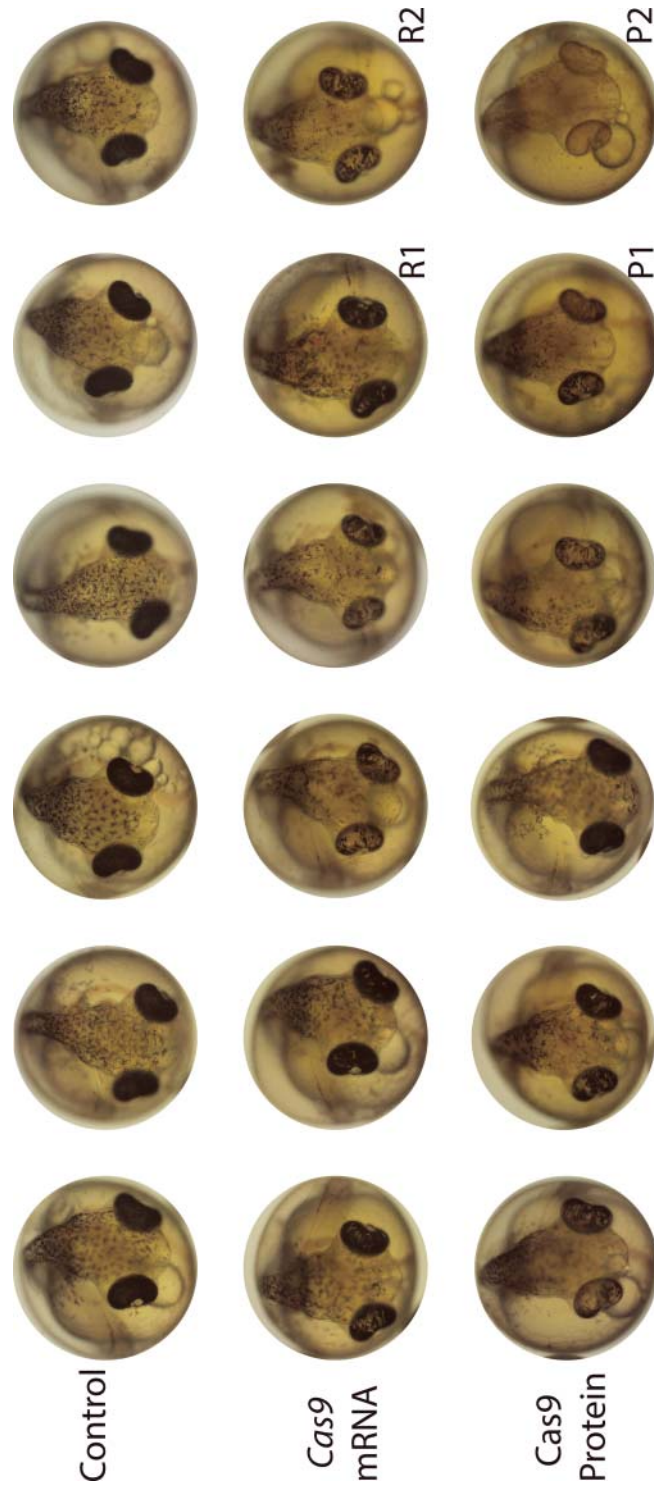


Fig. 1. Pigmentation phenotypes in *SLC24A5* targeted F0 mosaic stickleback embryos. Six representative embryos are shown for each condition: non-injected controls, fish injected with *Cas9* mRNA and sgRNA, and fish injected with *Cas9* protein and sgRNA. Images were chosen to represent the range of phenotypes seen at 5 days post fertilization. DNA lesions in R1 and R2 fish (injected with *Cas9* mRNA) and P1 and P2 fish (injected with *Cas9* protein) were sequenced and are shown in Fig. 2.

F1

sgRNA SLC24A5 exon 2

R1

WT	TCTCGGCCAGTCTCCGAGTTC	CCGACGGCTTCTTCACGGTG	Size(bp)	Count	
R1	TCTCGGCCAGTCTCCTCC--AGTTC	CCCGACGGCTTCTTCACGGTG	1 del	1	
	TCTCGGCCAGTCTCCTC--AGTTC	CCCGACGGCTTCTTCACGGTG	2 del	1	
	TCTCGGCCAGTCTCTCCG--TTCC	CCGACGGCTTCTTCACGGTG	2 del	1	
	TCTCGGCCAGTCTCCATCC--TTCC	CCGACGGCTTCTTCACGGTG	2 del	1	
	TCTCGGCCAGTCTCCTCCGAGTTC	CCCGACGGCTTCTTCACGGTG	5 ins	1	
	TCTCGGCCAGTCTCTCCGA-----	CGGCTTCTTCACGGTG	9 del	3	
	TCTCGGCCAGTCTC-----	CCGACGGCTTCTTCACGGTG	9 del	1	
	TCTCGGCCAG-----	ACGGCTTCTTCACGGTG	16 del	1	
	TCTC-----	TTCCCGACGGCTTCTTCACGGTG	16 del	1	
	-----	CCGACGGCTTCTTCACGGTG	185 del	1	
	-----		190 del	1	
		Total	13 mutant	15 WT	
R2	TCTCGGCCAGTCTCTCCG--TTCC	CCGACGGCTTCTTCACGGTG	2 del	2	
	TCTCGGCCAGTCTCC--CCAGTTC	CCCGACGGCTTCTTCACGGTG	2 del	12	
	TCTCGGC-----	AGTTC	CCCGACGGCTTCTTCACGGTG	11 del	1
	TCTCGGCCAGT-----	TTCCCGACGGCTTCTTCACGGTG	9 del	1	
	TCTCGGCCAGTCTCTCCGA-----	CGGCTTCTTCACGGTG	9 del	4	
	TCTCGGCCAGTCTCTCCG-GTGCAGGAGG-----	TG	11 del	1	
	TCTCGGCCAGTCTCTCCG-GT-----	G	23 del	1	
	-----	TTCCCGACGGCTTCTTCACGGTG	54 del	1	
	-----	TTCCCGACGGCTTCTTCACGGTG	64 del	1	
	-----	CACGGTG	218 del	1	
	-----	CGGCTTCTTCACGGTG	273 del	1	
		Total	26 mutant	2 WT	
P1	TCTCGGCCAGTCTCTCAGTCTTCC	CCGACGGCTTCTTCACGGTG	1 ins	1	
	TCTCGGCCAGTCTCTCC-----	CCGACGGCTTCTTCACGGTG	7 del	1	
	TCTCGGCCAGTCTCTC-----	TTCCCGACGGCTTCTTCACGGTG	6 del	1	
	TCTCGGCCAGTCTCTCCGA-----	CGGCTTCTTCACGGTG	9 del	2	
	GTTCGACCCAGT-----	TTCCCGACGGCTTCTTCACGGTG	8 del	4	
	TCTCGGCCAGTCTCTCCGA-----	CGGCTTCTTCACGGTG	26 ins + 9 bp del	1	
	TCTCGGCCAGTCTCC-----	CCGACGGCTTCTTCACGGTG	9 del	1	
	TC-----	CCGACGGCTTCTTCACGGTG	21 del	4	
	TCTCGGCC-----	CCGACGGCTTCTTCACGGTG	15 del	1	
	TCTCGGCCAGTCTCTCCG-----	GGTG	22 del	4	
	TCTCGGCCAGTCTCTC-----	TTACGGTG	19 del	1	
	TC-----	CCGACGGCTTCTTCACGGTG	21 del	1	
	TCTCGG-----	TG	36 del	1	
	-----	TCGTGAGTGTTC	CCCGACGGCTTCTTCACGGTG	38 del	1
	TCTCGGCCAGTCTCTCCG-----		45 del	1	
	-----	GGCTTCTTCACGGTG	48 del	2	
	-----	CCGACGGCTTCTTCACGGTG	197 del	1	
		Total	28 mutant	0 WT	
P2	TCTCGGCCAGTCTCTCCGT--TTCC	CCGACGGCTTCTTCACGGTG	1 del	4	
	TCTCGGCCAGTCTCTCCG--TTCC	CCGACGGCTTCTTCACGGTG	2 del	4	
	TCTCGGCCAG-----	ACGGCTTCTTCACGGTG	16 del	2	
	TCTCGGCCAGTCTCTCC-----	CCGACGGCTTCTTCACGGTG	14 ins+ 7 del	5	
	TC-----	GGTG	38 del	6	
	-----	TTCCCGACGGCTTCTTCACGGTG	52 del	10	
		Total	31 mutant	0 WT	

Fig. 2. Diversity of molecular lesions induced around *SLC24A5* targeting site. A region of the *SLC24A5* locus was amplified and sub-cloned from two mosaic F0 embryos injected with *Cas9* mRNA (R1 and R2) and two F0 embryos injected with *Cas9* protein (P1 and P2). Red indicates deletions, blue indicates substitutions, and pale green indicates insertions. If base substitutions were seen along with insertions or deletions (indels), the size is indicated in red and denotes only the size of the indel.

Table 2. Transmission efficiency from *SLC24A5* F0 mosaic fish

Cas9 type	F0 mosaic individual	% of F1 offspring with mutations
mRNA	Male #1	39% (11/28)
	Male #2	41% (12/29)
	Male #3	33% (10/30)
Protein	Male #4	26% (07/27)
	Male #5	100% (27/27)
	Male #6	21% (06/28)

The transmission rate for fish injected with Cas9 protein was more variable, between 21% and 100% (Table 2). All of these efficiencies are probably underestimates because the assay would not detect lesions that also remove one or both of the primer binding sites for PCR amplification.

Once we had successfully targeted the *SLC24A5* gene, we chose to target two major evolutionary loci in sticklebacks: *KITLGX* on Linkage Group 19 (LG19) (the sex chromosome) and *EDA* on LG4.

Effects of *KITLGX* and *KITLGY* on pigmentation

CRISPR targeted F0 *KITLGX* and *KITLGY* fish injected with *Cas9* mRNA and four sgRNAs had melanized pigmentation defects that were visible starting at 5 days post fertilization. These defects persisted into adulthood and were visible in both males and females (Fig. 3). Viable eggs and sperm were obtained from female and male F0 fish, respectively. Amplification, cloning, and sequencing of induced mutations (Table 3) revealed a range of lesions around all the sgRNA sites in *KITLGX* (sgRNA 1 and 2 could not be distinguished because they are so close). In contrast, *KITLGY* only had mutations at the site of sgRNA 4, probably due to many sequence differences between *KITLGX* and *KITLGY* (Table 1).

KITLGX and *KITLGY* contain numerous sequence differences and produce different transcripts. *KITLGY* skips the sixth exon and has a long divergent C-terminus (Fig. 4). To determine if *KITLGX* and *KITLGY* have different roles in pigmentation, F0 males and females were crossed to wild type and each other to generate all possible mutant genotypic classes following germline transmission. The survival rate among putative homozygous fish was low. Only 30% ($n = 27/88$) of the fish from two crosses that survived until 15 days post fertilization lived to 2 months.

Males with mutations in *KITLGX* (total, $N = 4$; *KITLGX* allele 1, $n = 3$; *KITLGX* allele 2, $n = 1$), *KITLGY* (total, $N = 5$; *KITLGY* allele 1, $n = 3$; *KITLGY* allele 2, $n = 1$; *KITLGY* allele 3, $n = 3$), or both *KITLGX* and *KITLGY* (*KITLGX* allele 2 and *KITLGY* allele 1, $n = 1$) all produced viable sperm (Fig. 5A–D; Table 3). The males only carrying frameshift mutations in *KITLGY* did not show dramatic pigmentation differences in the skin or testes compared with wild type, though sometimes appeared lighter in ventral regions (Fig. 5B). The males only carrying frameshift mutations in *KITLGX* lost much of their normal dark external melanophore pigmentation and had testes devoid of obvious melanized pigmentation (Fig. 5C). While the testes were consistently white, the extent of remaining external body pigmentation was somewhat variable even among fish of the same age, genotype, and

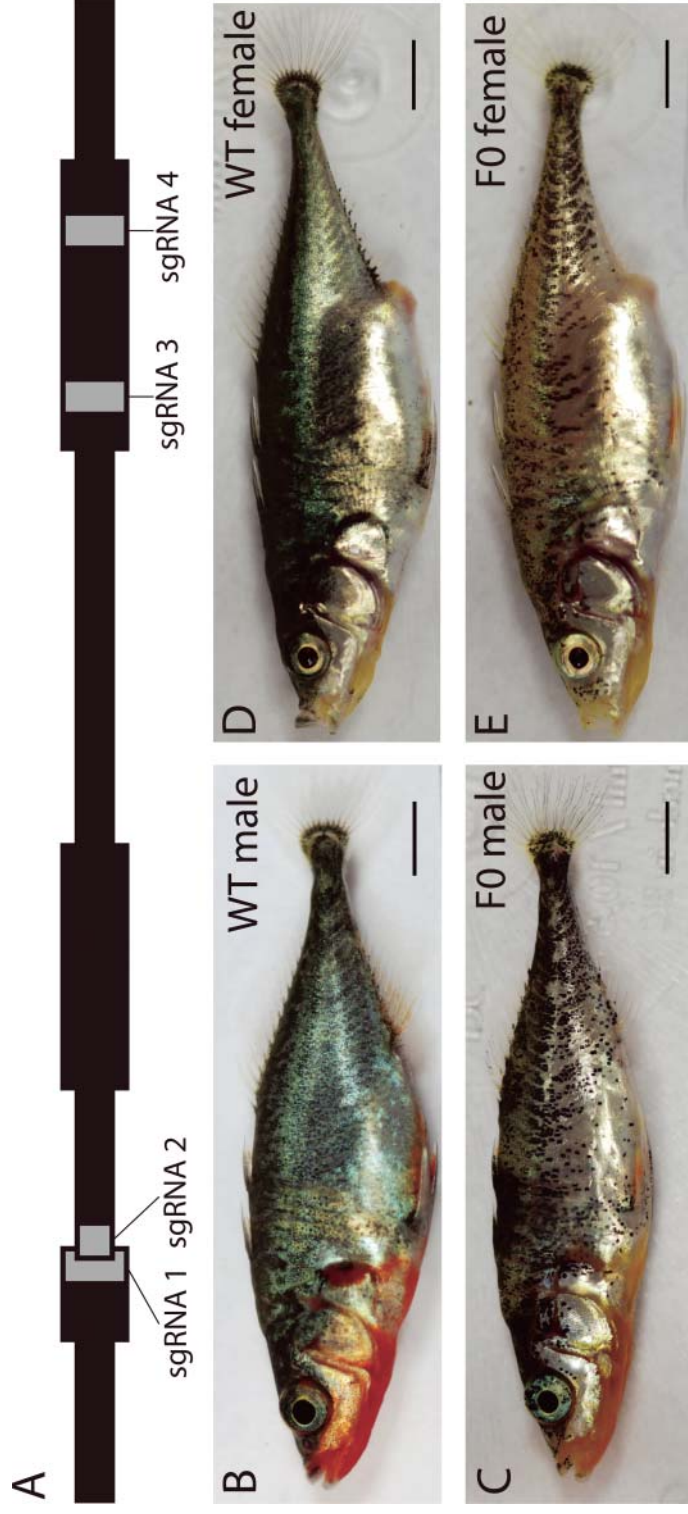


Fig. 3. External pigmentation phenotypes in F0 mosaic sticklebacks following *KITLGX* and *KITLGY* targeting. (A) Schematic showing exon 3–5 of *KITLGX* and *KITLGY* and the location of the four sgRNA sites that were targeted. (B) Wild-type male; (C) F0 mosaic *KITLGX* and *KITLGY* CRISPR male; (D) wild-type female; (E) F0 mosaic *KITLGX* and *KITLGY* CRISPR female. Scale bar = 5 mm.

<i>KITLGX</i> -LC	1	MKSKIWIRVCVHILLFITLGVHSSKFDVNPVTDDISRLSILRQNI PKDYKIPVNYIPRE
<i>KITLGX</i> -FTC	1	MKSKIWIRVCVHILLFITLGVHSSKFDVNPVTDDISRLSILRQNI PKDYKIPVNYIPRE
<i>KITLGY</i> -CC	1	MKKPKIWIRVCVHILLFITLGVHSSKFDVNPVTDDISRLSILRQNI PE DYKIPVHYVIRE
<i>KITLGX</i> -LC	61	EGGMCWVKLVNFYLEESLKGLAHKFGNISSNRKDISIFIQMFQELRLNMGLLEAIMNDFQ
<i>KITLGX</i> -FTC	61	EGGMCWVKLVNFYLEESLKGLAHKFGNISSNRKDISIFIQMFQELRLNMGLLEAIMNDFQ
<i>KITLGY</i> -CC	61	KGGMCWVKLVNFYLEESLKGLAHKFGNISSNRKDISIFIQMFQELRLNMGSLELIMYDFE
<i>KITLGX</i> -LC	121	CHYREERWQTARYFDFVKDFLIAAQNKEDSDYCDPPPCPTTPYAVTTADYLNATSEPGPP
<i>KITLGX</i> -FTC	121	CHYREERWQTARYFDFVKDFLIAAQNREDSYCDPPPCPTTPYAVTTADYLNATSEPGPP
<i>KITLGY</i> -CC	121	CHYREERWQTARYFDFVKDFLRAAQNKEDSDYCDPPPCPTTPYAVTTADYLNDS-----
<i>KITLGX</i> -LC	181	KCADCKPKPETLSGVLEQSLLSLLFIPLVALIFLLVWKVRSRNEENLQQSPGEGGLFPG
<i>KITLGX</i> -FTC	181	KCADCKPKPETLSGVLEQSLLSLLFIPLVALIFLLVWKVRSRNEENLQQSPGEGGLFPG
<i>KITLGY</i> -CC	175	-----ETRALSAVVEQSFLSLLFIPLVALIFLLVWKVRCRNEEDLEQSHGEGGLFPG
<i>KITLGX</i> -LC	241	AEATAPPLDTEISEKNMLNVIEIE-----
<i>KITLGX</i> -FTC	241	AEAAAPPLDTEISEKNMLNVIEIE-----
<i>KITLGY</i> -CC	228	AKATATPLDTEISEKKTFKDNERRNACAQTMRPDDTSRERPATPWQHDVLGRGGRDATP
<i>KITLGX</i> -LC		-----
<i>KITLGX</i> -FTC		-----
<i>KITLGY</i> -CC	288	DTVSATTKAMARLQLGDPGSNPDPDPLDSTRQHS GPKXPVPLRQLRRKKRTDLEQGDQRA
<i>KITLGX</i> -LC		-----
<i>KITLGX</i> -FTC		-----
<i>KITLGY</i> -CC	348	DRRATRPPSSRPSTCDGPRLSGRSAPTLP R

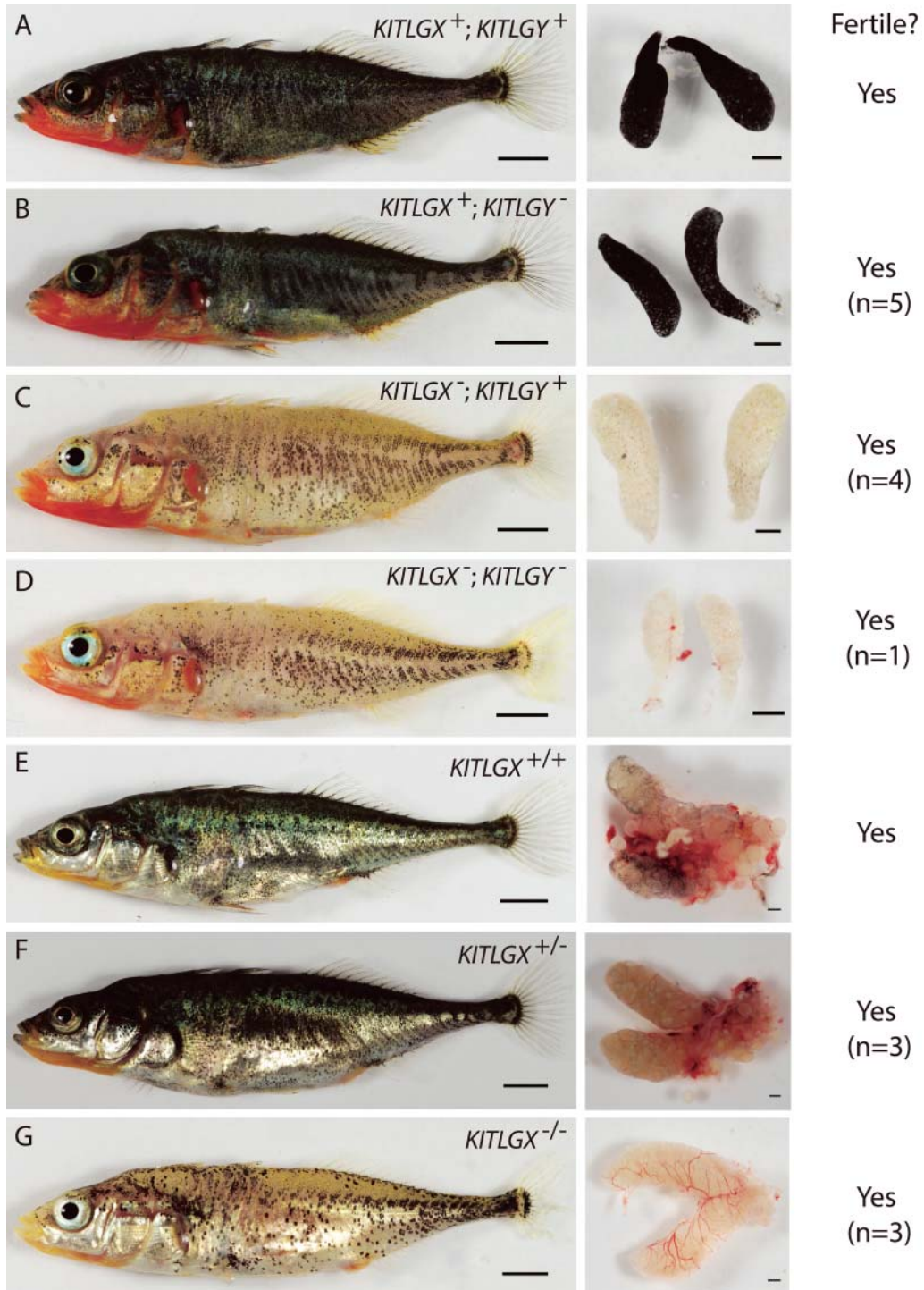
Fig. 4. *KITLGX* and *KITLGY* predicted amino acid sequence divergence. LC = Little Campbell anadromous marine (see Miller *et al.*, 2007), FTC = Fish Trap Creek freshwater (see Miller *et al.*, 2007), CC = Conner Creek freshwater (cDNA clone, see Methods).

tank. Nevertheless, *KITLGX* mutant genotypically male fish could be clearly distinguished from wild type and carriers of mutant *KITLGY* alleles. Only one fish was recovered with frameshift mutations in both *KITLGX* and *KITLGY* (Fig. 5D), and appeared similar to its genotypically male siblings that only had *KITLGX* mutations (Fig. 5C).

Both the females heterozygous for frameshift mutations (total, $N = 3$; *KITLGX* allele 3, $n = 1$; *KITLGX* allele 4, $n = 1$; *KITLGX* allele 5, $n = 1$) and the females homozygous for frameshift mutations (total, $N = 3$; *KITLGX* alleles 4 and 3, $n = 1$; *KITLGX* alleles 6 and 7, $n = 1$; *KITLGX* alleles 2 and 8, $n = 1$) produced viable eggs (Fig. 5E–G; Table 3). While the heterozygous female mutants did not show obvious external pigmentation defects, the homozygous female *KITLGX* mutants lacked most of the melanized pigment on the dorsal and ventral sides of the fish (Fig. 5F, G). The heterozygous females also showed less pigmentation of the dissected ovaries compared with the wild-type controls, and the homozygous females lacked all ovarian melanin pigmentation (Fig. 5F, G). *KITLG* is linked to pigmentation in the gills (Miller *et al.*, 2007); however, the Matadero Creek population, in which the mutants were made, already had little pigmentation in the gills, so the effect of *KITLG* loss on this trait could not be determined.

EDA mutants

We targeted the *EDA* protein coding region with an sgRNA, and subsequently intercrossed founder fish to transmit lesions through the germline. One F0 × F0 intercross produced 24 F1 siblings that were successfully raised to 7 months of age and >30 mm in length. Of



these surviving progeny, one was homozygous wild-type (Fig. 6A), and two were homozygous for mutations predicted to reduce or eliminate *EDA* function (Fig. 6B, C). The fish shown in Fig. 6B inherited two alleles with the same 2-bp deletion resulting in an early stop codon in the fifth of eight exons. One allele had an additional point mutation eight bases upstream that falls in the intron and probably does not affect the transcript. The fish shown in Fig. 6C had the same 2-bp deletion allele as well as a second allele with a 75-bp deletion that removed the splice acceptor of the fourth exon and the first 60 bp of the exon.

External skeletal armour

While the wild-type control sibling had a full assemblage of fins, spines, and armour plates, the homozygous mutants had no armour plates and severely deformed or absent caudal, anal, dorsal, and pectoral fins (Fig. 6B, C). The first and second dorsal spines were long and prominent, but the third dorsal spine and anal spines were absent in the mutants even though the underlying pterygiophores were present. The homozygous mutants also had a full assemblage of pelvic spines and processes, but the pelvic ray did not form.

Gill rakers and teeth

EDA homozygous mutants displayed reductions in gill raker and pharyngeal tooth number in the branchial skeleton. A 2-bp/2-bp deletion homozygote displayed severe reductions in posterior facing gill rakers, while anterior facing gill rakers appeared normal (Fig. 7A, B). A 2-bp/75-bp deletion mutant displayed mild reductions in gill raker number, again specifically affecting posterior facing gill rakers (Fig. 7A, C). Relative to wild type, both mutants displayed severely reduced dorsal and ventral pharyngeal dentitions, with smaller tooth fields but also reduced tooth number within each tooth field (Fig. 7A–C).

DISCUSSION

Our studies show that CRISPR-Cas9 is a powerful tool to induce mutations at defined loci in sticklebacks and to study the biology of major evolutionary loci.

CRISPR-Cas9

Both *Cas9* mRNA and Cas9 protein could be used to efficiently create deletions and insertions in pre-chosen regions of the stickleback genome. In targeting *SLC24A5*, severe

Fig. 5. Altered pigmentation but normal sex determination in F1 fish carrying different induced *KITLGX* and *KITLGY* alleles. (A) Wild-type male and testes. (B) Body and testes from male carrying frameshift *KITLGY* allele 1 (see Table 3). (C) Body and testes from male fish with frameshift *KITLGX* allele 1. (D) Body and testes from male carrying both frameshift *KITLGX* allele 2 and *KITLGY* allele 1. (E) Wild-type female and ovaries. (F) Body and ovaries from female carrying frameshift *KITLGX* allele 1. (G) Body and ovaries from female fish carrying both frameshift *KITLGX* alleles 1 and 8. Scale bar = 5 mm (left column) and 1 mm (right column).

Table 3. Transmitted frameshift alleles found in F1 *KITLGX* and *KITLGY* mutants

sgRNA 1 and 2 site		
<i>KITLGX</i> WT allele	TTCCTGTGAACATACATTCCAAGGGAAG AGGTAAGACATGGTGGCATCCG	
<i>KITLGX</i> allele 1	TTCCTGTG----- GCATCCG	34-bp Δ
<i>KITLGX</i> allele 2	TTCCTGTGAACATACATTCCA A ----- TGGTGGCATCCG	16-bp Δ
<i>KITLGX</i> allele 3	TTCCTGTGAACATACATTCCAAGGGAAG AGGTAAGACATGGTGGCATCCG	WT
<i>KITLGX</i> allele 4	TTCCTGTGAACATACATTCCAAGGGAAG AGGTAAGACA -----	262-bp Δ **
<i>KITLGX</i> allele 5	TTCCTGTGAACATACATTCCAAGGGAAGAG CCAGAAT TGGTGGCATCCG ^T	Splice*
<i>KITLGX</i> allele 6	TTCCTGTGAACATACATTCCAAGGGAAG AGGTAAGACATGGTGGCATCCG	WT
<i>KITLGX</i> allele 7	-----AAG AGGTAAGACATGGTGGCATCCG	24-bp Δ
<i>KITLGX</i> allele 8	TTCCTGTGAACATACATTCC----AAG AGGTAAGACATGGTGGCATCCG	5-bp Δ
<i>KITLGY</i> WT allele TCCTGTACATTACGTTCTTGAGGGAAA AGGTAAGATGTGGTGGCATCCG		
<i>KITLGY</i> allele 1	TCCTGTACATTACGTTCTTGAGGGAAA AGGTAAGATGTGGTGGCATCCG	WT
<i>KITLGY</i> allele 2	TCCTGTACATTACGTTCTTGAGGGAAA AGGTAAGATGTGGTGGCATCCG	WT
<i>KITLGY</i> allele 3	TCCTGTACATTACGTTCTTGAGGGAAA AGGTAAGATGTGGTGGCATCCG	WT
sgRNA 3 site		
<i>KITLGX</i> WT allele	ACTTTCA G TGCCACTACAGGGAAGAG AGGTGGCAG	
<i>KITLGX</i> allele 1	ACTTTCA G TGCCACTACAGGGAAGAG-- GTGGCAG	2-bp Δ
<i>KITLGX</i> allele 2	ACTTTCA G TGCCACTACAGGGAAGAG-- GTGGCAG	2-bp Δ
<i>KITLGX</i> allele 3	ACTTTCA G TGCCACTACAG----- AGAGGTGGCAG	5-bp Δ
<i>KITLGX</i> allele 4	ACTTTCA G TGCCACTACAGGGA----- GGTGGCAG	5-bp Δ
<i>KITLGX</i> allele 5	ACTTTCA G TGCCACTACAGGGAAGAG AGGTGGCAG	WT
<i>KITLGX</i> allele 6	----- AGGTGGCAG	53-bp Δ*
<i>KITLGX</i> allele 7	ACTTTCA G TGCCACTACAGGGAAGAG SCCCTG --	68-bp Δ
<i>KITLGX</i> allele 8	A----- GAGGTGGCAG	24-bp Δ
<i>KITLGY</i> WT allele	ACTTTGA G TGCCACTACAGGCAAGAG AGGTGGCAG	
<i>KITLGY</i> allele 1	ACTTTGA G TGCCACTACAGGCAAGAG AGGTGGCAG	WT
<i>KITLGY</i> allele 2	ACTTTGA G TGCCACTACAGGCAAGAG AGGTGGCAG	WT
<i>KITLGY</i> allele 3	ACTTTGA G TGCCACTACAGGCAAGAG AGGTGGCAG	WT
sgRNA 4 site		
<i>KITLGX</i> WT allele	TTACTGCGACC CCTCCTCCCTGTCCCACAAGACCAT	
<i>KITLGX</i> allele 1	TTACTGCGACC CCTCCT ----GTCCCACAAGACCAT	4-bp Δ
<i>KITLGX</i> allele 2	TTACTGCGACC CCTCCT ----GTCCCACAAGACCAT	4-bp Δ
<i>KITLGX</i> allele 3	TTACTGCGACC CCTCCTCCCTGTCCCACAAGACCAT	WT
<i>KITLGX</i> allele 4	-----GTCCCACAAGACCAT	24-bp Δ
<i>KITLGX</i> allele 5	TTACTGCGACC CCTCCTCCCTGTCCCACAAGACCAT	WT
<i>KITLGX</i> allele 6	TTACTGCGACC CCTCCTCCCTGTCCCACAAGACCAT	WT
<i>KITLGX</i> allele 7	TS CC TG CTCTACAGAGC CCCTGTCCCACAAGACCAT	No Δ
<i>KITLGX</i> allele 8	TTACTGCGACC CCTCCTCCCTGTCCCACAAGACCAT	WT
<i>KITLGY</i> WT allele	TGACTGCGACC CCTCCTCCCTGTCCCACAACACCAT	
<i>KITLGY</i> allele 1	TGACTGCGACC CCT ----CTGTCCCACAACACCAT	5-bp Δ
<i>KITLGY</i> allele 2	-----TCCCACAACACCAT	26-bp Δ
<i>KITLGY</i> allele 3	TGACTGC-----GTCCCACAACACCAT	13-bp Δ

loss of pigmentation was only seen when fish were injected with Cas9 protein, but both mRNA and protein injections resulted in a range of phenotypes. This result is consistent with other studies showing a general trend of increased efficiency using injections with Cas9 protein, but in some cases there is no significant difference between protein and mRNA (Gagnon *et al.*, 2014).

Even though targeting is highly efficient, injected founders are clearly mosaic for a variety of different alleles. Pigmentation traits (e.g. in *SLC24A5* and *KITLG* mutants) could easily be scored in F0 founder animals because even somatic patches of mutant cells produce visible phenotypes. In contrast, *EDA* founders had to be bred to homozygosity to reveal easily visible phenotypes. Germline transmission efficiencies were high, and founders typically transmitted more than one type of allele to various progeny in the next generation.

The sgRNAs used to target *KITLG* highlight that the sgRNA does not need to be an exact sequence match to cut the genome. As has been seen in other studies, mismatches that occur in the sgRNA target sequence furthest from the PAM site are tolerated and may not even reduce the cutting efficiency, whereas those close to the PAM significantly reduce the cutting efficiency (Hsu *et al.*, 2013; Hwang *et al.*, 2013). These are important considerations in sticklebacks due to numerous SNPs between populations. The possibility of polymorphisms should always be considered when designing sgRNAs based on the reference genome and doing experiments in other populations. At the same time, population polymorphisms might be used as an advantage to generate allele-specific CRISPR, targeting only the allele of one population but not another.

KITLG

Frameshift mutations in *KITLGX* and *KITLGY* demonstrate that the two different sex-chromosome versions of *KITLG* play different roles in the pigmentation of sticklebacks. Frameshift mutations in *KITLGY* in males did not result in dramatic external or testes pigmentation defects compared with the wild type, whereas frameshift mutations in *KITLGX* resulted in a loss of both external and testes pigment. In females, one copy of *KITLGX* appears sufficient for the wild-type external pigmentation of the fish, but less pigment is seen in the ovaries, suggesting that one copy is not fully sufficient for a wild-type phenotype. *KITLGX*'s role in pigmentation in sticklebacks was originally identified based on X-linked gill and ventral skin pigmentation differences in crosses between marine and freshwater fish (Miller *et al.*, 2007), but the likely severe loss-of-function mutations in *KITLGX* clearly affect both dorsal and ventral pigmentation patterning.

Although the *KITLGX* mutants confirm that this gene plays an important role in the pigmentation of sticklebacks, the sterility phenotype seen in mice was not seen in sticklebacks. While we cannot rule out that *KITLGX* and *KITLGY* mutant stickleback have reduced fertility (reduced sperm or egg production), we did not see the total sterility seen in some mouse mutants.

Footnote to Table 3 (opposite): Identified linked mutations near different sgRNA target sites are shown for the various alleles recovered from simultaneous injections with the four different sgRNAs shown in Fig. 3A. Underlining indicates the sgRNA sequence, **bold** type denotes the PAM sequence, and *italic* type is used to indicate intron sequences of *KITLGX* and *KITLGY*. – indicates the deletion of a base. * indicates the deletion or mutation of splice donor or acceptor sequence. ** indicates the deletion of the fourth exon. Blue bases are substitutions.

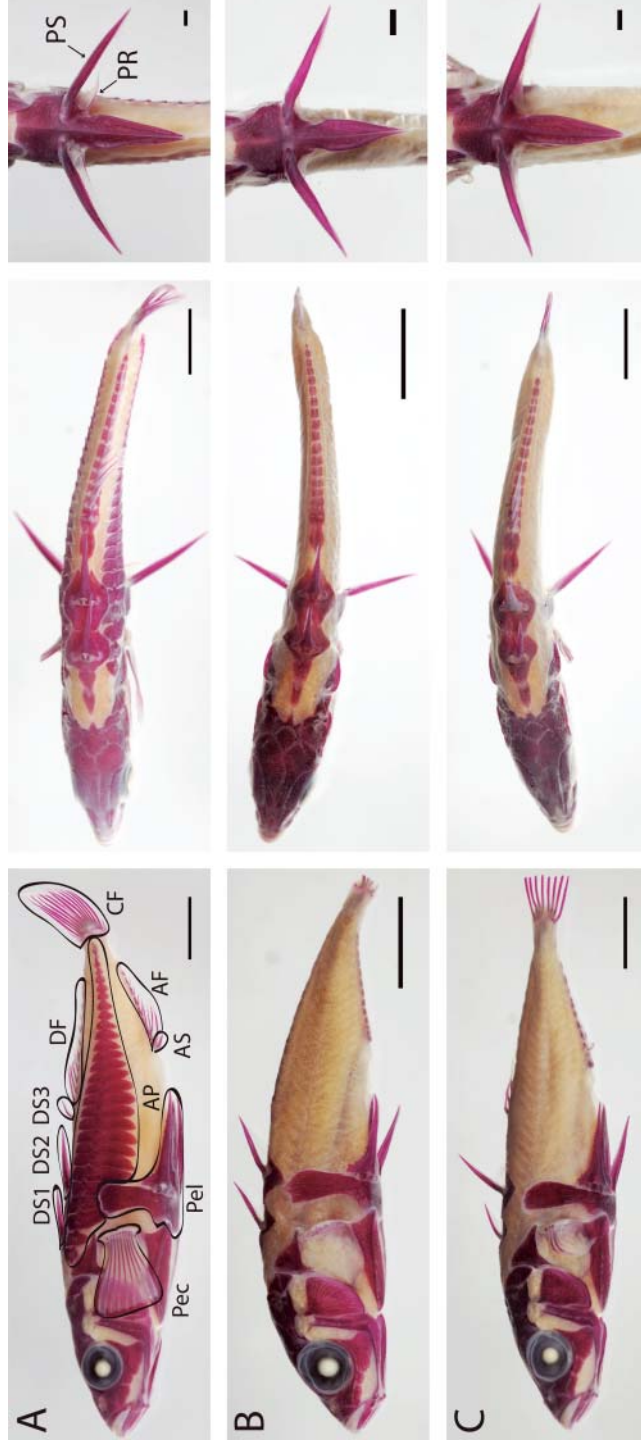


Fig. 6. Skeletal phenotypes in sticklebacks homozygous for induced *EDA* CRISPR mutations. (A) Lateral, dorsal, and ventral views of a wild-type sibling, stained with Alizarin Red to reveal calcified bones. (B) Fish carrying two mutant *EDA* alleles: allele 1 is a 2-bp deletion; allele 2 is a 2-bp deletion with an additional substitution in an intron. (C) Second fish carrying two mutant *EDA* alleles: allele 1 is the same 2-bp deletion as in (B); allele 2 is a 75-bp deletion that removes the last 60 bp of exon 4 and the first 15 bp of the following intron. The wild-type control fish shown in row A had long pectoral, dorsal, anal, and caudal fins, but the right pectoral fin was removed for DNA preparation. The two *EDA* mutant fish in rows B and C developed with no armour plates and largely missing pectoral, dorsal, and anal fins. Portions of the reduced caudal fins were removed for DNA preparation prior to photography. DS1 = dorsal spine 1; DS2 = dorsal spine 2; DS3 = dorsal spine 3; DF = dorsal fin; CF = caudal fin; AF = anal fin; AS = armour plates; AP = pectoral fin; Pec = pelvis; Pel = pelvic spine; PR = pelvic ray. Scale bar = 5 mm (left and centre columns) and 1 mm (right column).



Fig. 7. Branchial skeleton phenotypes in sticklebacks homozygous for induced *EDA* CRISPR mutations. (A, left to right) Flat-mounted branchial skeleton, zoomed view of gill rakers, ventral pharyngeal tooth plate, and large dorsal pharyngeal tooth plate in wild-type sibling. In the two left-most columns, anterior is the top; in the two right-most columns, anterior is to the left. Wild-type fish have both anterior facing gill rakers (odd rows) and posterior facing gill rakers (even rows). (B) Fish homozygous for 2-bp deletion display severe reductions in posterior facing gill rakers (white asterisks), as well as reduced tooth plate size and tooth number. (C) Fish trans-heterozygous for a 2-bp and a 75-bp deletion display more moderate reductions in posterior facing gill rakers (e.g. missing medial row 2 gill rakers labelled with white asterisk), as well as reduced tooth plate size and tooth numbers. Scale bars: left two columns = 1 mm, right two columns = 0.5 mm.

The fertility of the stickleback *KITLGX* and *KITLGY* mutants is consistent with what has been seen in zebrafish. Mutants have been made for both *kitlg* and its receptor *c-kit* in zebrafish. The ligand mutant, *kitlga* or *sparse-like*, and the receptor mutant, *kita* or *sparse*, have a reduced melanophore number (Kelsh *et al.*, 1996; Parichy *et al.*, 1999; Dooley *et al.*, 2013). In contrast to the mouse mutants, haematopoiesis and primordial germ cell development appeared normal, even though *kita* is expressed in the cells that give rise to the haematopoietic precursors. Although zebrafish *kitlga* mutants do not have documented reductions in viability, we do see a reduction in the viability of the fish homozygous for stickleback *KITLG* mutations, indicating that the stickleback gene likely plays important roles in addition to its contribution to pigmentation.

Although *KITLGX* and *KITLGY* map to the sex chromosomes in sticklebacks, our experiments indicate *KITLG* is unlikely to be the sex-determining locus. Genotypically female *KITLGX* heterozygous and homozygous mutants produced viable eggs and did not develop testes or produce sperm. Additionally, it does not appear that *KITLGY* is required for the production of sperm, as genotypic males with mutations in *KITLGY* still produced functional sperm. It is possible *KITLGY* has become a pseudogene on the young evolving stickleback Y chromosome. However, the gene is expressed and spliced based on a cDNA clone and RNA sequences from testes and liver, and these sequences predict a large encoded protein with intact KIT binding domains (Fig. 4). We only recovered a single viable stickleback that was simultaneously carrying induced mutant alleles at both *KITLGX* and *KITLGY*, suggesting that fish that inherit mutations in both genes may have reduced viability (Fig. 5). We thus think it is likely that *KITLGY* has some functions in development that are separate from pigmentation or sex determination, and this possibility can be tested in the future by additional phenotypic studies of fish carrying *KITLGY* mutations.

EDA

Many freshwater stickleback populations have prominent armour reduction, including common reduction in armour plates, shortening or loss of dorsal spines, and rarer loss of the pelvic hind fin. All of these phenotypes have been linked to the *EDA* region of LG4 in some genetic crosses (Shapiro *et al.*, 2004, 2009; Rogers *et al.*, 2012; Miller *et al.*, 2014; Erickson *et al.*, 2016b; Howes *et al.*, 2017), raising the possibility that armour, spine, and pelvic reduction may be due to pleiotropic effects of the *EDA* gene in one or more populations. Our homozygous *EDA* mutants confirm that *EDA* is critical for armour plate formation, as no armour plates formed in the homozygous mutants. However, the homozygous *EDA* fish do not support an obvious requirement for *EDA* in formation of the most prominent dorsal spines in sticklebacks, or in formation of the pelvis and pelvic spine, which remain robust in mutant fish even when armour plates and most fins were lost. It is possible that other types of mutations in the *EDA* gene might generate a different range of phenotypes, including dorsal spine and pelvic defects. However, it is striking that the spines and pelvis appear relatively unaffected in fish with drastic reductions in other skeletal structures, suggesting that the dorsal and pelvic spines of sticklebacks are less sensitive to changes in *EDA* activity than other armour structures. The only part of the pelvis that was clearly affected was the pelvic ray. Threespine stickleback generally have one pelvic ray on each side (Bell and Foster, 1994). The loss of the pelvic ray is consistent with the fact that most of the other soft fin rays are lost in the stickleback *EDA* mutants, and that the pelvic rays are lost in zebrafish *EDA* mutants (Harris *et al.*, 2008).

The severe reductions in pharyngeal tooth number support the hypothesis that natural variant *EDA* alleles modify tooth number, as previously suggested by a large-effect tooth number QTL detected in a large marine $\times$ freshwater F2 cross that mapped to LG4 and had a peak marker at *EDA* (Miller *et al.*, 2014). Freshwater alleles of this tooth number QTL reduced tooth number in this cross, a direction opposite to the overall pattern of increased tooth numbers observed in the grandparental freshwater fish (Cleves *et al.*, 2014; Miller *et al.*, 2014). However, the tooth reductions seen in the current experiments match the predicted direction for *EDA* alleles in freshwater populations being partial loss-of-function (Colosimo *et al.*, 2005; O'Brown *et al.*, 2015). In contrast, the posteriorly biased gill raker phenotypes do not resemble the phenotypic effects of the large-effect gill raker number QTL previously mapped to LG4, which have strong effects on anterior facing gill rakers as well (Glazer *et al.*, 2014; Miller *et al.*, 2014). However, this *EDA* mutant phenotype strongly resembles the gill raker phenotype described in *EDAR* hypomorphic mutant zebrafish (Harris *et al.*, 2008), suggesting posterior gill rakers are most sensitive to reductions in *EDA/EDAR* function in teleosts. These posterior-biased reductions in gill rakers seen in *EDA* mutants also resemble the natural phenotypes seen in some fish species (e.g. herrings) (Gibson, 1988).

The two homozygous mutant fish recovered in this study had somewhat different phenotypes, possibly related to the nature of the different mutant alleles and their predicted effects on *EDA* transcript and/or protein. The *EDA* ligand is synthesized as a transmembrane protein with a transmembrane, collagen, and tumour necrosis factor (TNF) domain. In the 2-bp deletion allele, a premature stop codon occurs before the TNF domain. Premature stop codons often lead to nonsense-mediated decay of the corresponding transcript (He and Jacobson, 2015), which could reduce transcript levels and *EDA* activity. In addition, the TNF domain is crucial for receptor binding, therefore any protein encoded by the mutant transcript would be unlikely to bind the receptor. In addition, the 75-bp deletion allele removes 60 bp at the start of the fourth exon and 15 bp in the intron and presumably prevents the fourth exon from being normally spliced. However, the mutation does not produce an obligate frameshift mutation that would disrupt the TNF domain. Instead, the mutation alters the collagen domain, which is thought to be required for the oligomerization of the ligand. Cell culture experiments suggest that similar mutations may decrease the activity of *EDA*, but not eliminate it entirely (Schneider *et al.*, 2001; Swee *et al.*, 2009).

The diversity of alleles generated by CRISPR targeting can be seen as both an advantage and disadvantage of current methods. The diversity clearly requires careful screening of fish to identify the types of mutations present. These mutations can range from single base pair substitutions, insertions and deletions on the order of tens or hundreds of bases (such as those detected in this study), to deletions larger than 1000 bases and rearrangements that have been reported in other studies (Kosicki *et al.*, 2018). However, having multiple induced alleles at a locus may also be helpful for revealing the range of phenotypic effects at a locus, or for recovering viable fish carrying mutations in powerful developmental control genes. The complexity of alleles present in founder fish becomes greatly simplified upon germline transmission of individual alleles, allowing the investigator to then choose which mutations to further propagate and study. In the future, it may be possible to further enrich for particular types of edited genome changes during targeting, by including oligo donor nucleotides or other DNA sequences containing homology arms to direct the repair process surrounding Cas9-induced lesions (Irion *et al.*, 2014; Albadri *et al.*, 2017; Zhang *et al.*, 2018). We have also recently used injections with multiple sgRNAs to recapitulate larger non-coding deletions upstream of the *PITX1* locus, which mimic

the type of regulatory alleles and phenotypes that repeatedly occur in wild stickleback populations (Xie *et al.*, 2019).

CONCLUSION

Our studies show that CRISPR-Cas9 can be used to efficiently generate targeted mutations to study the roles of key evolutionary loci in sticklebacks. We show that both Cas9 protein and *Cas9* mRNA can be used to efficiently induce mutations, although Cas9 protein may be slightly more efficient. *KITLG* mutants have pigmentation defects that differ depending on whether the X- or Y-linked copy is mutated, but they are fertile. Mutations in *EDA* cause a host of phenotypic effects, including the loss of armour plates and soft fin rays and severe reductions in tooth and gill raker number, while leaving the large dorsal spines and pelvis intact. The ability to edit defined locations in the genome will add a powerful new tool to the stickleback model system, making it possible to dissect the roles of individual genes and regulatory elements, rather than large linked chromosome regions, in a range of phenotypes.

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