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RESEARCH ARTICLE

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Genetic tools for the study of the mangrove killifish, *Kryptolebias marmoratus*, an emerging vertebrate model for phenotypic plasticity

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Abstract

Kryptolebias marmoratus (Kmar), a teleost fish of the order Cyprinodontiformes, has a suite of unique phenotypes and behaviors not observed in other fishes. Many of these phenotypes are discrete and highly plastic—varying over time within an individual, and in some cases reversible. Kmar and its interfertile sister species, *K. hermaphroditus*, are the only known self-fertile vertebrates. This unusual sexual mode has the potential to provide unique insights into the regulation of vertebrate sexual development, and also lends itself to genetics. Kmar is easily adapted to the lab and requires little maintenance. However, its internal fertilization and small clutch size limits its experimental use. To support Kmar as a genetic model, we compared alternative husbandry techniques to maximize recovery of early cleavage-stage embryos. We find that frequent egg collection enhances yield, and that protease treatment promotes the greatest hatching success. We completed a forward mutagenesis screen and recovered several mutant lines that serve as important tools for genetics in this model. Several will serve as useful viable recessive markers for marking crosses. Importantly, the mutant *kissylips* lays embryos at twice the rate of wild-type. Combining frequent egg collection with the *kissylips* mutant background allows for a substantial enhancement of early embryo yield. These improvements were sufficient to allow experimental analysis of early development and the successful mono- and bi-allelic targeted knockout of an endogenous *tyrosinase* gene with CRISPR/Cas9 nucleases. Collectively, these tools will facilitate modern developmental genetics in this fascinating fish, leading to future insights into the regulation of plasticity.

KEYWORDS

hermaphroditism, killifish, mutagenesis, plasticity, self-fertility

1 | INTRODUCTION

Model organisms, or those species especially amenable to experimentation in a laboratory setting, have been critical in revealing fundaments aspects of developmental biology. Key models such as laboratory mice, the fly *Drosophila melanogaster*, the nematode *C. elegans*, and the zebrafish have permitted broad systematic analysis of genetic regulation of development and physiology. However, their characteristics that make them useful experimental genetic models do not capture some important types of regulation observed in nature (Bolker, 1995), and thus cannot be captured in phenotypic screens. One area that has been difficult to study in these workhorse experimental organisms is phenotypic plasticity and its regulation in development and evolution. Notable exceptions include work in nematodes. For example, in *Caenorhabditis elegans* regulation of dauer entry and exit by environmental factors has been extensively studied (Fielenbach & Antebi, 2008). Similarly, genetic screens in *Pristioncius pacificus* have revealed factors comprising a novel signaling network that regulates plasticity in formation of the pharynx, which supports alternative feeding behaviors (Bento et al., 2010; Bui et al., 2018; Sieriebriennikov et al., 2017).

New models that support the study of plasticity should be easily adaptable to the laboratory and provide unique morphological, physiological, or behavioral variants. They should be small, require little care, and have a short generation time. Additionally, ovipary permits easy imaging and manipulation of embryos, and access to the one-cell stage bottleneck critical for introduction of transgenes, gene products (e.g., RNA), reagents for genetic engineering. An experimental model should also have a genome of manageable size for analysis of variation and mutation.

The mangrove killifish, *Kryptolebias marmoratus* (henceforth "Kmar"; Figure 1a), has attracted considerable attention since the pioneering work of Robert W. Harrington, Jr. in the 1960s (e.g., Harrington & Kallman, 1968; Harrington & Rivas, 1958; Harrington, 1961; Orlando, 2012). Kmar harbors a broad array of unique developmental and physiological adaptations to the extreme environments in which it lives. Some of these traits expose extreme plasticity in their response to environmental change. For example, Kmar can be found out of the water for extended periods (Taylor et al., 2008) (Figure 1b). During these emergence times, Kmar epidermis remodels and establishes extensive intra-epithelial capillary networks on the anterior dorsal aspect of its skin (Heffell et al., 2018). Longer periods of emersion lead to reversible gill remodeling (Leblanc et al., 2010; Ong et al., 2007). These traits are reversed once fish reenter the aqueous environment (Turko et al., 2012).

Kmar early development also exhibits a developmental arrest, interpolated between the completion of embryonic development (Stage 32 of Mourabit et al., 2011) and hatching from the chorion (Figure 1c). This prehatching state lasts roughly 6 weeks (Sakakura & Noakes, 2000), is accompanied by gradual yolk consumption (Wells et al., 2015), and is not associated with substantially altered gene expression (Mesak et al., 2015). This state is distinct from the early embryonic diapause of annual killifish, which can occur either pre-gastrulation (Diapause I) or during somitogenesis (Diapause II). On a shift of environmental conditions these animals will hatch and progress with development and growth. Breaching of the chorion through natural or exogenous hatching enzyme, or by mechanical removal, leads to an abrupt awakening. While the trigger for rousing is not yet known, the accompanying rise in dissolved oxygen is a strong candidate. To avoid confusion with diapauses I and II, we will refer to prehatching arrest in *K. marmoratus* as "delayed hatching."

Most uniquely, Kmar also presents a suite of unusual reproductive adaptations. It and its sister taxon, *K. hermaphroditus* (with which it hybridizes in some locales; Berbel-Filho et al., 2022; Tatarenkov et al., 2018) are the only known self-fertilizing hermaphrodite vertebrates. Frequent selfing in Kmar has purged most recessive deleterious alleles, allowing completely homozygous strains to be collected from wild populations and maintained without inbreeding depression (Tatarenkov et al., 2007, 2010, 2012). Second, Kmar is also a sequential hermaphrodite, with some (but not all) hermaphrodite animals transitioning to become functional males with age (Harrington, 1967). Further, the frequency and timing of sex change is polymorphic between geographical isolates (Turner et al., 2006), impacting the operational sex ratio and the degree of outcrossing. In both the lab and in nature, Kmar is functionally androdioecious, in that males can fertilize the small proportion of unfertilized eggs

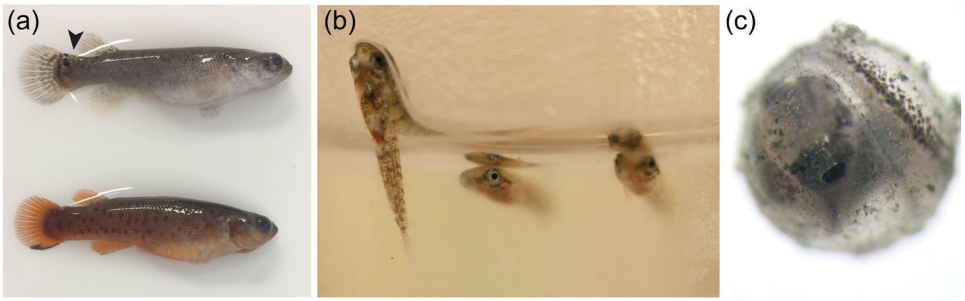

FIGURE 1 Kmar manifest plastic phenotypes not seen in current vertebrate experimental models of development. (a) Kmar reach adulthood as self-fertilizing hermaphrodites (top). Hermaphrodites have a prominent ocellus on tail (arrow head). Males (bottom) are distinguished by orange coloration and a ventral melanic tail bar. These fish are approximately 4 cm long. (b) Emersion behavior and skin respiration are often seen in laboratory culture. (c) Prehatching developmental arrest.

released by hermaphrodites (Lubinski et al., 1995; Mackiewicz et al., 2006a, 2006b, 2006c). This combination of selfing and outcrossing speeds production of novel homozygous genotypes while still allowing unification of alleles between different strains, much as is done in genetic experimentation in the nematode *C. elegans* and the model angiosperm *Arabidopsis thaliana*. These and other phenotypes in Kmar (e.g., King et al., 1989) offer an unusually tractable system in which to study plasticity and comparative genetics. This is further supported by an assembly of the approximately 650 MB euchromatic portions of its approximately 900 MB genome (Kanamori et al., 2016; Kelley et al., 2016). This is similar in size to medaka, and more compact than zebrafish (Howe et al., 2013; Kasahara et al., 2007).

Despite the appealing features of Kmar, it differs from medaka and zebrafish in two ways that hinder its use in developmental genetics. First, hermaphrodites rarely lay more than three eggs at a time, unlike the large clutches in zebrafish. Second, due to internal fertilization only a small fraction of the embryos that are laid are in the zygote or two-cell stage. These attributes hinder the systematic injection and production of morphant, transgenic, or genome-edited strains required to take full advantage of forward genetics. For example, morpholino knockdowns that characterized two mutations impacting Kmar tail development found in a forward screen (Sucar et al., 2016) were conducted with medaka because "it is difficult to obtain many one-cell-stage embryos for … injections" (Saud et al., 2021). Manual coaxing of egg laying, however, can be done for IVF (Nakamura et al., 2008). Further, without intervention, a large fraction of Kmar embryos never hatch after completion of embryonic development. To tap the potential of this species for research, it is essential to improve the yield of eggs collected and to increase hatching rates.

Here, we describe new genetic resources and approaches to improve Kmar egg yield and hatching success. The combination of these efforts has supported production of targeted gene knockouts in the species using CRISPR-Cas9 technology.

2 | METHODS

2.1 | Basic husbandry

Work with Kmar in both the Haag and Harris laboratories' was conducted using protocols approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Maryland, College Park and Boston Children's Hospital, respectively. Animals were housed in covered, noncirculating tanks, often in isolation from others. For the genetic screen (Harris Lab, Boston MA), fish were maintained in mouse cages with specialized insets having slots to let eggs fall to the bottom, away from fish. These cages were half filled with zebrafish culture water (sterile, RO with minor salts) amended with 10 parts per thousand (ppt) Instant Ocean salts. Live *Artemia* brine shrimp nauplii were provided ad libitum and allowed to coculture until needing to be replenished (1–2x a week).

For egg-laying, hatching, and CRISPR experiments (Haag Lab, MD), fish were held at 26°C–28°C with a 12 h light, 12 h dark cycle. Animals were housed singly in 1 L polypropylene food containers (Rubbermaid Take-along). Tank lids were tightly closed, with five 2 mm diameter holes drilled near the top to allow gas exchange and feeding via a Pasteur pipette. Each tank contained 750 mL of culture water made with house distilled water and Instant Ocean salts. Egg-laying and hatching experiments were performed with 25 ppt salts unless otherwise noted. Based on recent studies that indicate reduced salinity improved embryonic and larval survival (McCain et al., 2020), the Haag lab subsequently reduced all fish and embryo cultures to 12.5 ppt culture water. This reduced salinity water was used for injections and is the lab's current standard.

Adults and juveniles were fed six times a week with approximately 1 mL of a 25% suspension of live *Artemia* nauplii made with distilled water just before use. To encourage oviposition, adult hermaphrodites are provided roughly 150 mL of loosely packed polyester fiberfill, either draped in the water by trapping a small portion under the tank lid, or maintained as a floating mass. Eggs deposited on this substrate were gathered with gloved hands, and placed in a 3-inch diameter covered plastic condiment cup containing culture water and held at 26°C–28°C. Water was changed every 7–14 days.

2.2 | Forward genetic screen

Hon11 is a highly inbred strain originally collected in the Bay Islands of Honduras in 1996 (Tatarenkov et al., 2010), and has been maintained by pure selfing since. Adults were obtained from Brian Ring (Valdosta State Univ., Georgia USA). A total of 16 mature hermaphrodites were mutagenized with 3.0 µM N-ethyl-N-nitrosourea (ENU) once a week for 4 weeks. Once completed, eggs were collected in batch and collected only once eggs showed consistent viability. Eight hermaphrodites were used as the basis for the screen due to their laying consistent number of progeny (K1-K8). F1 progeny from a single P0 were raised in Petri dishes at 26°C–29°C until hatching, at which point they were transferred in groups of 5–6 to mouse cages on a shelf until maturity. Upon maturity, individual F1 were placed in a mouse cage individually and eggs were collected to screen for phenotypes. Each F1 was given a Roman numeral label to permit phenotype tracking to particular lineages. F2 were collected weekly and were grouped in Petri dishes.

We performed three phenotypic screens:

Hatching delay. After a week at room temperature viable eggs are at the start of prehatch diapause, and unmutagenized animals do not hatch spontaneously for at least 4 weeks. F2 fish from mutagenized lines were scored again after 2 weeks for any batch showing premature hatching at expected Mendelian ratios.

Larval patterning defects. F2 larvae were raised until hatching, and morphology was scored before moving to larger tanks.

Adult phenotypes. F2 progeny were raised until mature at which point outward morphology and reproductive phenotypes were scored. Reproductive phenotypes screened included sex ratio and sterility. A large proportion of F2 progeny were scored for regeneration potential of the caudal fin.

2.3 | Egg laying experiments

These experiments used both the Hon11-derived *kissylips* mutant line identified in the above screen and unmutagenized strains of the lines SOB8 and SOB10 (collectively "SOB"), which were collected in Florida by R.E. Earley and maintained for 2–3 generations by selfing before this study. Egg counts for weekly collections were established for each fish over 2 months. For twice daily treatments, each experiment began by clearing the fiberfill mop of all eggs that may have been present. Eggs were subsequently collected shortly after the lights coming on at 8 a.m. and again at 7 p.m. (i.e., just before the lights went off), repeated for 5–7 days. Fish that failed to lay any embryos were not included in analysis. One SOB10 fish in the "teabag experiment" (Figure 4b) produced 16 eggs in one 10 h period, an extreme outlier that was also excluded.

2.4 | Embryo hatching success

Embryos that received control conditions were reared using the conditions previously described, with a single water change approximately 2 weeks after oviposition. By this age, all viable embryos have completed development and are in a pre-hatch arrest state. For embryos in the "Reduced Salinity" treatment group, the water change was with half-strength (12.5) ppt salinity water. Embryos in the "Agitation" treatment group received fresh 25 ppt salinity water, but were placed on an orbital platform shaker set at 60 rpm. Two pronase treatments were initially used—Protocol 1: 0.02% pronase in 25 ppt water, held without a water change, and Protocol 2: 0.05% pronase in 25 ppt water for 2 days, followed by a change to 25 ppt water lacking Pronase. The higher concentration of pronase effectively stimulated hatching, but was accompanied by high rates of perinatal larval death. The data reported in Figure 5 were all from Protocol 1. We note that after these controlled experiments were completed, we found that more frequent changes with plain culture water (either 25 or 12.5 ppt) before pronase treatment elevated hatching rates beyond the approximately 50% reported in Figure 5. This appears to retard microbial and/or fungal contamination that is associated with fish that succumb during prehatch arrest.

2.5 | CRISPR-Cas9 mutagenesis

We modeled our targeting of the *Tyrosinase* gene on similar work in the cichlid *Astatotilapia burtoni* (Li et al., 2021).

2.5.1 | Design and synthesis of guide RNAs (gRNAs)

The Kmar *Tyrosinase* gene sequence from NCBI Genbank (Gene ID: 108244618, NCBI Reference Sequence: NC_051431.1). The IDT Custom Alt-R™ CRISPR-Cas9 guide RNA online tool (https://www.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE) was used to design gRNA targeting exon 4. Cleavage within this exon has successfully abolished melanocyte formation in *A. burtoni* (Li et al., 2021). We chose two gRNA targeting sites 51 bp apart (Figure 7a), to allow for double cut-mediated deletions while still preserving overall messenger RNA splicing. gRNA sequences were used as BLAST queries (Altschul et al., 1990) against the *K. marmoratus* genome to confirm absence of obvious off-target effects. Duplexed synthetic trans-activating CRISPR RNA (tracrRNA) and target-specific CRISPR RNA (crRNA) (both from IDT, Coralville, IA) were used instead of single guide RNA (sgRNA), as this improves editing rates in zebrafish (Hoshijima et al., 2019).

2.5.2 | Injection cocktail

crRNA and tracrRNA were first individually diluted as 100 µM stock solutions in manufacturer-provided duplex buffer (IDT; 30 mM Hepes pH 7.5, 100 mM Potassium acetate). To create crRNA:tracrRNA duplexes, equal volumes of crRNA and tracrRNA stock solutions were mixed and annealed in a thermocycler using this program: 95°C, 5 min; cool at 0.1°C/s to 25°C; 25°C, 5 min; cool to 4°C rapidly. The 50 µM crRNA:tracrRNA duplex stock was further diluted 1:1 with duplex buffer to produce a 25 µM working stock. Cas9 protein (IDT Alt-R S.p. Cas9 Nuclease V3, 1081058) was adjusted to 25 µM stock solution in 20 mM HEPES-NaOH (pH 7.5), 350 mM KCl, 20% glycerol. Injection mixtures combined the following: 5 µM Cas9 protein; 5 µM crRNA:tracrRNA duplex; 0.25% Texas Red conjugated dextran (final conc. 0.25%).

2.5.3 | Microinjection of embryos

On the day of the injection, we checked and collected eggs from individually house *kissylips* tanks twice: the first collection in the morning between 08:30 and 09:00 (shortly after lights on); the second one was between 12:00 and 12:30. To increase the genome-editing rate, only embryos between 1- and 16-cell stage (Figure 6a) were injected. We performed five rounds of injections in a total of 77 such early cleavage stage embryos.

Before microinjection, the gRNA:Cas9 ribonucleoprotein complex solution was incubated at 37°C for 5 min and then backfilled into 3 microinjection needles using an Eppendorf GELoader tip (Cat# 022351656; Eppendorf). After loading embryos into the embryo holder, which was covered with 12.5 ppt salinity water containing 0.0001% methylene blue, we injected them with 4 pulses of 1 nL, using 2.5 ms pulse duration at 22 psi. To further explore the editing efficiency in different conditions, 53 eggs were directly injected into

blastomere, and 24 eggs were injected into yolk. Once injections were complete, embryos were gently removed from trenches using a micro laboratory spatula (Fisher, blade size: 19 × 4.8 mm) and then transferred individually into 12-well plates with 2 mL of 12.5 ppt water containing 0.0001% of methylene blue. Embryo survival and pigmentation were monitored for approximately 28 days postinjection.

Microinjections were performed under a stereomicroscope (Nikon, SMZ745) equipped with a 3D micromanipulator (Narishige, M-152). Injection pulses were driven by a tank of compressed dry air (size 200, adapter CGA-590) fed into a Milli-Pulse Pressure Injector (Applied Scientific Instrumentation; MPPI-3). Embryos were immobilized for injection in grooves cast in a 2.0% agarose block prepared with 12.5 ppt salinity water. Grooves were formed by addition of several parallel Micropet Disposable Pipettes (ACCU-FILL90, 20 µL, #4618; Clay Adams) in a standard Petri plate. Removal of the micropipettes after gelling produces 1.5-mm-wide trenches (Figure 6b). Microinjection needles were made from glass capillary tubes (GC100F-10, 1.0 mm O.D; 0.58 mm I.D; Harvard Apparatus) pulled with a Sutter P-97 micropipette needle puller. Initially sealed needle tips were broken by holding taut a Kimwipe and gently tapping the tip straight into it. Needles with the ideal needle bore size of 10–12.5 µm outer diameter were identified using a microscope equipped with an ocular micrometer. This diameter of needle delivers approximately 1 nL of solution when a 2.5 ms air pulse at 22 psi.

2.5.4 | Indel mutation quantification

PCR and Sanger sequencing were used to assess genome-editing efficiency in each CRISPR-injected P0 fish. We designed primer pairs to amplify approximately 300 bp region surrounding the Exon 4 target sites. At 3 months of age, we collected about approximately 1 mm^2 of tailfin tissue from injected fish to identify those carrying the high rates of mutation. These fish with somatic mutations are preferred for breeding, as they are most likely to also carry mutations in the germline. Genomic DNA was extracted using a modified HotSHOT approach (Truett et al., 2000). The fin-clipped samples were first transferred into 0.2 mL PCR tubes, followed by adding 180 µL of NaOH (50 mM). The samples were then heated at 95°C for 15 min and cool to 4°C. Last, 20 µL of Tris buffer (1 M, pH 8.0) was added to each sample to neutralize pH.

PCR primers used for genotyping: 5′-TGTAAAACGACGGCC AGTtgctgtcgtttctgctcctgtg-3′ (capitalized portion of the primer sequence is an M13 universal tag, permitting subsequent reamplification with a fluorescent dye-conjugated M13 primer for fragment analysis). Reverse primer 5'-agcgagattgcaagcgtattgc-3'. A total of 35 cycles of amplification were performed with 94°C, 2 min; 94°C, 15 s, 60°C, 15 s, 72°C, 30 s; followed by a polishing step of 72°C, 7 min. A total of 2% agarose gels were run to confirm successful amplification. Most indels are <15 bp in size and cannot be resolved by this method, so amplicons were Sanger sequenced. The indel mutation rates were analyzed by using the web tool Synthego ICE (v3.0. https://ice.synthego.com/) and TIDE (Tracking of Indels by Decomposition; Brinkman et al., 2014).

To assess if the mutations were transmitted into the germline cells and passing to the offspring, we also collected >50 eggs from each surviving P0 injected fish, and screened for pigment loss in the F1 generation.

3 | RESULTS

3.1 | Chemical mutagenesis screen to identify markers for genetic analyses

To assess feasibility of forward genetic screens in Kmar as well as to establish tools to support genetic analyses, we initiated a chemical mutagenesis in the Hon11 wild-type background. Hon11 was previously identified as being hearty and a consistent egg producer in the lab (Sucar et al., 2016). We mutagenized 11 Hon11 hermaphrodites 4 times with 3.0 µM ENU, using protocol identical to that used for zebrafish (Rohner et al., 2011). Unlike a prior screen in Kmar (Sucar et al., 2016), we did not collect eggs in batch from selfing groups, but rather separated individual P0 founders and collected eggs daily, raising F1 of each founder to track potential clonal mutations in individual germlines. In this genetic screen design (Figure 2) we could more precisely associate expressivity of particular phenotypes as well as assess frequency of phenotypes within a particular founder lineages.

In sum >450 F1 founders were produced. Progeny of 301 founders were screened for mutations affecting entry or maintenance of prehatching arrest and for any abnormal larval or adult morphologies severe enough to be noticed upon quick inspection. Of these, 261 F1 progeny were also screened for regeneration and 196 for fertility phenotypes (Table 1). No F1 (presumably dominant) phenotypes were observed in the screen, nor were dominant mutations observed in the

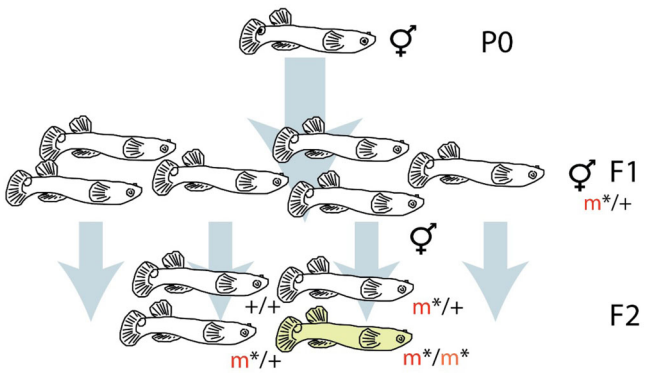

FIGURE 2 Design of mutagenesis screen in Kmar. Adult hermaphrodite parents (P0) were mutagenized and allowed to lay F1 self-progeny, which are often heterozygous for newly induced recessive mutations (m*/+). Selfing of these founders produces roughly 25% homozygous F2 progeny (m*/m*), which manifest a range of phenotypes. This path to novel homozygous mutants requires far fewer animals and one fewer generation than similar screens in obligately outcrossing species.

F2. We identified a large spectrum of mutants in the F2, many of which were subsequently reidentified in crosses of isolated homozygous founders, or in carriers in the case of larval lethal or adult sterile phenotypes (Table 1). Mutants were identified in most classes analyzed, however we did not detect any that affected tailfin regeneration. Although Kmar has distinct background pigmentation and has a prominent ocellus on the dorsoposterior caudal peduncle, only one pigmentation mutant was identified, resulting in a classic *albino* phenotype. Due to the number of mutants identified, re-identification efforts were performed only on a subset of these mutants having the most reproducible and useful phenotypes (see below).

Unlike zebrafish and medaka screens, in which mutagenized P0 and F1 are outcrossed, our Kmar screen selfed mutagenized founders. As a result, mutation burden is maintained in the population. Replicated phenotypes suggestive of a generalized response to mutational burden were mainly seen in mutants affecting early development. Such mutants often had short embryonic axes or lack of eyes. Some survived to be axially deformed or eyeless adults, respectively. These were not maintained.

3.2 | Increased tolerance for high-density rearing

Kmar live as solitary animals in confined spaces within mangrove forests (Taylor, 2012). In both the field (Huehner et al., 1985) and in captivity (Hsu & Wolf, 1999), adult hermaphrodites show high levels of agression when in close quarters with conspecifics. In our experience this usually leads to lower fecundity and often to death. As it relates to lab husbandry and genetic screens, such antagonistic behavior is a detriment to optimizing housing and increases space requirements for fishes. However, it has been reported that while rearing from hatching with conspecifics does not eliminate aggressiveness *per se*, it does reduce exploration and boldness, which may suffice to reduce mortality in group housing (Edenbrow & Croft, 2013). We, therefore, tested whether long-term bulk culture was feasible.

In the breeding strategy as part of our mutagenesis screen, individuals were housed in moderate densities that increased over time as the number of unique populations were refined in the F2 mutants and more individuals with comparable genotypes were raised. Addition of fiberfill and small vinyl tubing permitted local environments and barriers that helped increase rearing densities. Kmar raised in such manner became tolerant of communal living, being able to be kept up 10–12 per 500 mL without noticeable mortality or apparent conflict. As the lines were initially isogenic, the changes in these lines were presumably due to acclimation rather than selection of particular background or induced variants.

3.3 | Genetic lines maintained for use as genetic markers

Five healthy mutant lines with interesting phenotypes were retained for further characterization:

3.3.1 | Male hermaphrodite (mher-1, mher-2)

These two lines initially produced a high frequency of male F2 progeny, which were easily identified given their bright orange color (Figure 1a). This was initially suggestive of a segregating sex determination defect. Over time, however, excess male production waned, and microsatellite-verified mapping crosses between *mher* males and hermaphrodites of the SOB10 wild-type strain (following the methods of Mackiewicz et al., 2006) failed to produce F2 males (not shown). These lines were abandoned, but their existence indicates that sexual plasticity can be impacted by mutagenesis.

3.3.2 | Albino

Albino mutants lack pigment, a defect that can be observed very early in larval development (Figure 3a). Such mutants are of great utility in visualization of development and localization of gene expression by in situ hybridization or transgenesis. In addition, crosses between *albino* hermaphrodite founders and pigmented males allow early recognition of outcross progeny without more complex genotyping.

TABLE 1 Summary of forward screen phenotypes.

Affected phenotype	#Mutants identified/ #genomes	#Re-identified	Designation of retained mutant
Larval morphology	81/602	2	*Albino, stumpy*
Diapause	28/602	6	
Larval total	109/602; 0.18		
Adult morphology	91/602	1	*kissylips*
Regeneration	0/512		
Sex determination	2/602	2	*mher-1, mher-2*
Fertility	11/392	-	
Adult total	104/602; 0.17		

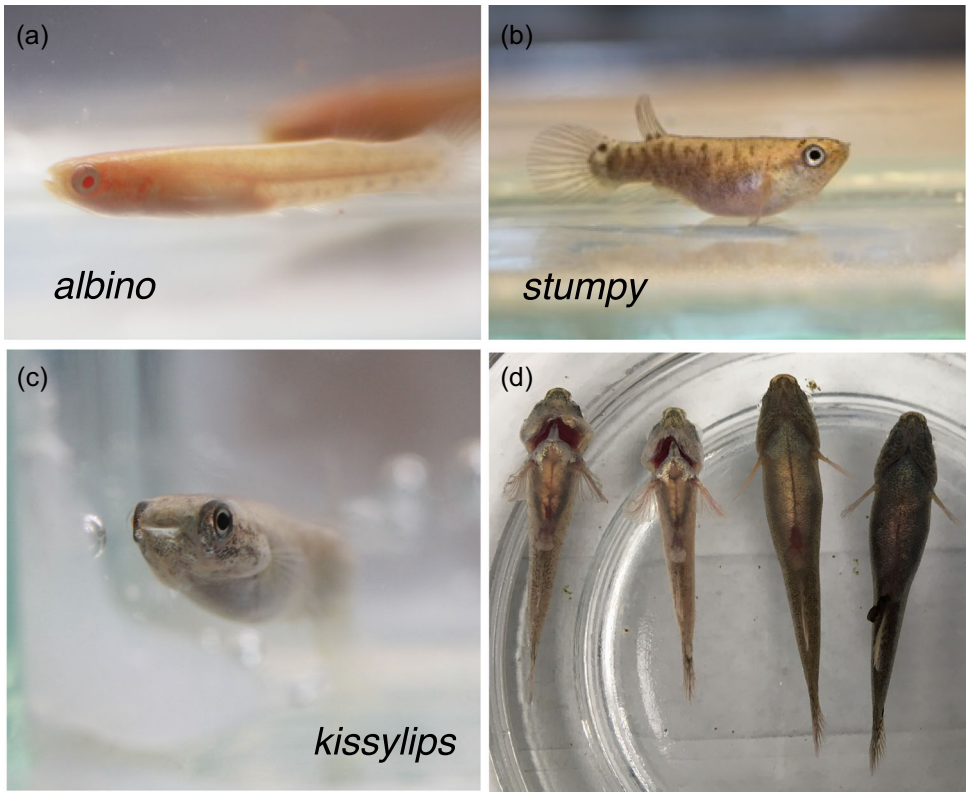

FIGURE 3 Forward genetic mutant lines bolstering Kmar experimental utility. (a) Adult *albino* mutant Kmar show lack of melanin differentiation both in body and retinal pigmentation. No other phenotypes are observable. (b) *stumpy* mutants are compressed along their rostral-caudal axis. (c) *kissylips* mutant adults have a irridiophore stripe along the distal jaws. (d) At 8 months of age, *kissylips* (two at left) are smaller than 5–6-month-old wild-type fish (two at right), and have constitutively flared opercula. *kissylips* is also hyperfecund (Figure 4).

3.3.3 | Stumpy

These mutants (Figure 3b) have a shortened body, but are otherwise normal hermaphrodites.

3.3.4 | Kissylips

Fish with this mutation have a general craniofacial shape change and may have postaxial skeletal phenotype, however not dysmorphic or pathological. *kissylips* gets its name from the novel pattering of a stripe of irridiophores across the distal jaw resembling the appearance of lipstick (Figure 3c,d). We fortuitously discovered that this mutant line is superfecund and the line was set aside for continued analysis (Figure 4).

3.4 | Husbandry factors impacting egg yield

Harrington (1963) reported that for his Floridian strains grown in a laboratory, hermaphrodite egg production is impacted by both season (peaking in Spring and summer) and time of the day (peaking mid-day). More recently, Lomax et al. (2017) confirmed seasonality in lab populations, and found that variation in egg output is not clearly linked to strain genotype. To understand the laying patterns of our various Kmar strains, we monitored the number of eggs collected from hermaphrodites of the inbred wild-type lines SOB8 and SOB10 (both from Florida), and also for the Hon11-derived *kissylips* mutant. After 2 months of weekly collections, we began collecting eggs from these same fish twice a day, once within 2 h of lights-on (8 a.m.) in the morning, and again within 2 h of lights off (8 p.m.). We did not find any qualitative difference in yield between collection times (not shown), indicating that these three strains do not have a strong preference for mid-day laying. However, we did observe that the twice daily collection roughly doubled the yield of eggs per day over the weekly collections in both wild-type and *kissy-lips* strains (Figure 4a). Yield from *kissylips* mutants were roughly double SOB strains in both collection regimes, such that frequent collection of from the mutants quadruples the egg yield compared to weekly collection of wild-type stocks.

The increased yield seen with more frequent collections might be due to the stimulation of oviposition. For example, the presence of an egg in the spawning mop may repress egg laying via a chemosensory mechanism. However, as is common in zebrafish, Harrington (1963) observed that hermaphrodites often eat their eggs soon after oviposition. Thus, the improved yield from frequent collections may simply indicate that Kmar eat a greater fraction of their eggs when left for long periods in their tanks. To distinguish between these hypotheses, we repeated the

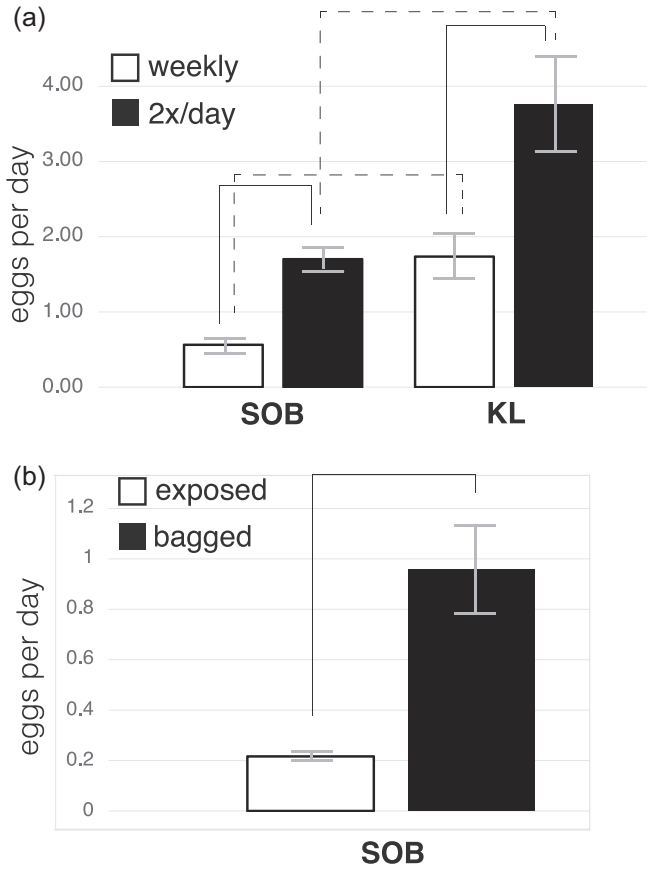

FIGURE 4 Factors influencing egg yield. (a) Frequency of egg removal. Eggs were collected from wild-type SOB8 and SOB10 strains (collectively "SOB", $N = 10$) and the HON11-derived mutant *kissylips* line ("KL," $N = 7$) weekly, and then twice per day. Solid lines indicate significant increases in egg yields within a group (paired sign test, $p < 0.01$). In both collection schemes, *kissylips* yield significantly more eggs than SOB8/10, as indicated by dashed lines (two-tailed *t*-test, $p < 0.01$). (b) Protection of eggs. Eggs from SOB8/10 hermaphrodites ($N = 14$) were either collected weekly as in (a) ("exposed"), or transferred up to twice per day into paper tea bags and left in the tanks to accumulate ("bagged"). Bagging of eggs significantly increased yield, as indicated by the solid line (two-tailed *t*-test, $p < 0.01$).

twice daily collections for wild-type fish, but instead of removing the eggs they were instead placed inside unbleached paper tea bags, and then returned the tank. We reasoned that this would allow fish to sense and possibly interact with eggs without the ability to eat them. With eggs accumulating in the tea bags, the enhanced yield in the twice-daily collections remained (Figure 4b).

3.5 | Factors influencing larval hatching

Fully developed (Stage 32) *K. marmoratus* embryos undergo pre-hatching arrest of several weeks, during which time they slowly consume yolk (Mourabit et al., 2011). However, in our lab colonies many otherwise viable embryos fail to hatch into larvae and eventually die, a phenomenon dubbed "stubborn eggs" (Turner et al., 2006). We tested two factors that had been suggested by colleagues as possible stimulators of hatching: abrupt introduction of lower-salinity culture water (from 25 to 10 ppt) and agitation of embryos using a rotary shaker. We also tested the fungal protease cocktail pronase, which has been reported to stimulate hatching (Kanamori et al., 2006). We found that agitation had no impact on hatching, but that reduction of salinity did increase hatching somewhat (Figure 5). However, pronase had a much larger effect. Since these controlled experiments were performed, pronase treatment has become our standard protocol to maximize hatching. It is common to obtain over 90% success if (a) embryos receive weekly water changes, (b) pronase treatment is initiated at 6–8 weeks after oviposition, and (c) juveniles are removed from treated water shortly after hatching.

3.6 | CRISPR/Cas9 gene disruption

A general strategy for injection of *K. marmoratus* embryos has been described (Mourabit & Kudoh, 2012; Mourabit et al., 2011). By combining a large colony of *kissylips* mutants with frequent egg collections, enough embryos in the 1–4-cell stages of development can be obtained to allow daily injections (Figure 6). We used this to achieve a targeted knockout.

We injected 77 eggs with CRISPR/Cas9 nucleases targeting the *tyrosinase* gene (Figure 7a); 53 directly into blastomeres, and 24 eggs into yolk. A total of 18 injected embryos survived to adulthood, but survival after blastomere injection was half of that of yolk injection (yolk injection: 9/24 survived; blastomere injection: 9/53 survived; Figure 7b). However, blastomere injection significantly increased the editing efficiency in embryos that did survive (*Mann–Whitney* test, $df = 1$, $p = 0.0023$;

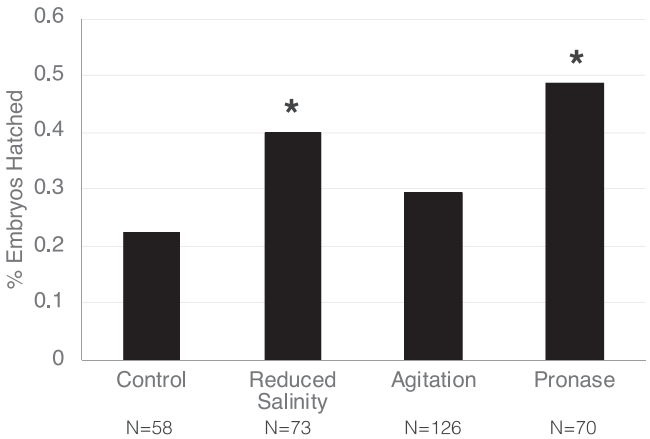

FIGURE 5 Impacts of various treatments on hatching of larvae. Compared to control embryos left in standard culture water without agitation, arrested embryos that were shifted to reduced salinity or to culture water amended with pronase hatched at a significantly higher rate (as indicted by asterisks, Chi-squared test, 1 *df*, $p < 0.001$). Agitation, in contrast, did not significantly impact hatching success.

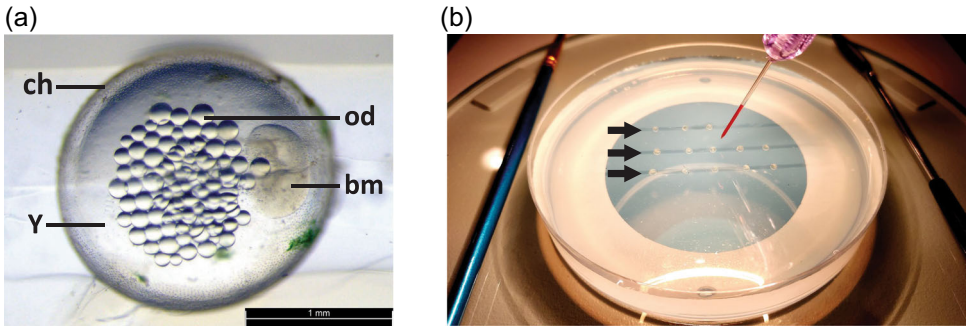

FIGURE 6 Injection of *K. marmoratus* embryos. (a) A representative two-cell Kmar embryo, showing the blastomeres (bm), chorion (ch), oil droplets (od), and yolk (y). (b) Injection configuration, on the stage of a stereomicroscope. Black arrows indicate grooves cast into an agarose plate, with embryos lined up in each. To the left is the small spatula used for inserting and retrieving embryos, and at upper right the needle and a dye-filled needle are visible.

Figure 7c). One P0 CRISPR-injected embryo grew to be a complete albino larva, while another had only sparse pigment (Figure 7d). This indicates CRISPR-Cas9 editing occurred to a variable extent in P0 injectees, with biallelic *tyrosinase* knockouts in some or all melanocytes. Sequencing analysis confirmed the existence of indel mutations at one of the two target sites (Figure 7e and Table 2). Synthego ICE analysis further revealed that these mutant animals carried multiple mutant alleles (Figure 7f). To identify pigmented carriers, pigmented progeny of P0 injectees were screened for pigment-loss phenotypes. Two produced multiple albino F1 offspring (Figure 7g and Table 2). The fraction of albinos, between 4% and 15%, is significantly less than the 25% expected for a homogenous heterozygote, indicating that these individuals have mosaic germ cells, with some wild-type and some bearing monoallelic mutations (Figure 7f).

4 | DISCUSSION

4.1 | Comparison of efficiency in genetic screens among small laboratory fish models

Despite the advent of targeted gene editing in a growing range of organisms (Reardon, 2019), unbiased forward genetic screens still remain a powerful tool for the genetic analysis of phenotype. The execution of large-scale forward genetic screens in the zebrafish, and complemented with similar designs in the medaka, proved the utility of such approaches in a vertebrate model, as had been previously shown in invertebrates such as Drosophila and *C. elegans*. One of the demands on doing such screens is the number of individuals needed to identify novel homozygous recessive mutants. In obligate outcrossing animals, one must carry analysis into the F3 progeny of isolated F2 families, only ¼ of which will have two mutation-carrying parents. A strength of *C. elegans* is the use of selfing within screen designs to reveal new recessives in F2 progeny of single F1 founders. Kmar's unique sexual strategy provides a similar approach (Figure 2).

We successfully screened mutagenized Kmar for recessive alleles affecting a varied array of larval and adult phenotypes. Since circulating water was not necessary, the work here was completed on only one tank rack, or shelf space. Additionally, we only used about 1/300th of the fish that would have been necessary in a comparable zebrafish screen. Unlike prior screens described in Kmar (Moore et al., 2012; Sucar et al., 2016), we kept individual lines isolated to assess for lineage-bias in alleles and potential mitotic clonal mutations arising. We isolated a wide array of mutant phenotypes. No apparent bias in mutation type with founder was seen. Overall mutagenesis efficiency was comparable to that seen in other screens (Table 3), with 36 recessive mutants observed in larvae and 81 in adults. These data confirm that chemical mutagenesis screens are a viable experimental approach in Kmar, further bolstered by the low numbers of individuals and associated resources required to carry out such screens.

A caveat of our screen design is the lack of outcrossing. This presumably leads to maintenance of multiple deleterious alleles in F2 lines, so that some observed phenotypes may be synthetic rather than monogenic. We believe that many of the early phenotypes that showed graded severity of axial defects, eye formation, and cephalic defects were members of this class of complex or compounded phenotypes, as they showed some lineage association with particular founders. In designing future screens, this can be addressed with use of a lower mutagen dose or outcrossing of the first mutagenized stocks, e.g. by mutagenizing only pigmented males, and crossing them to otherwise wild-type albino hermaphrodites. The mutants we maintained were clearly from individual F1 founders and presented with a clean Mendelian ratio in F2 progeny. Mutants were retained with a bias towards useful or informative phenotypes for use in genetic analysis more broadly; all others were not kept.

4.2 | Optimizing recovery of prehatch embryos

We further optimized a last hurdle for use of Kmar in genetic analysis. Once eggs are collected, Kmar larvae frequently fail to hatch from the chorion in the laboratory. We found that simple addition of pronase (Kanamori et al., 2006) was the most effective treatment and alleviated this experimental barrier (Figure 5). This suggests that

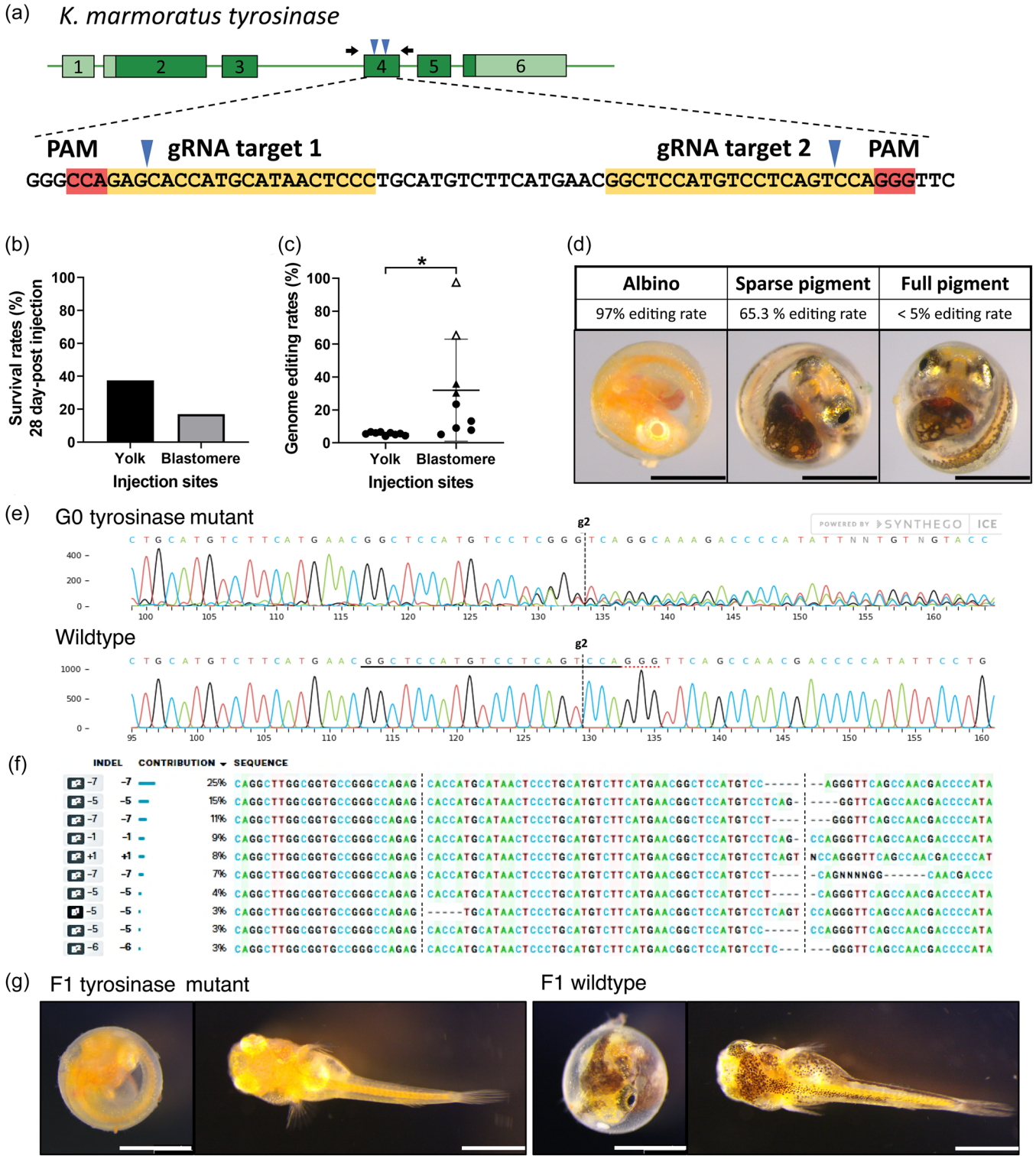

FIGURE 7 Targeted disruption of *tyrosinase* gene by CRISPR/Cas9 mutagenesis. (a) Structure of the *K. marmoratus tyrosinase* gene, with boxes representing exons and darker shading coding sequences. Blue carets depict the locations of target sequences for guide RNAs, with the sequence (target 2) or the sequence's complement (target 1) highlighted in yellow below. Black arrows indicate the primers used for PCR amplification of the target region. Survival rates (b) in embryos receiving CRISPR injection from blastomere ($N = 53$) or yolk ($N = 24$). (c) Genome editing rates in the 18 surviving fish, as measured by target amplicon sequencing. Open triangles represent the two embryos that had severe pigment-loss phenotype; black triangles represent the two individuals producing F1 albino progeny. (d) Three representative phenotypes and the corresponding gene editing rates in P0 CRISPR-injected embryos. (e) Sanger sequencing data of an exon 4 amplicon from a P0 albino that died shortly after hatching, aligned with the gene sequence around the gRNA target 2. Abrupt sequence heterogeneity beginning near the cut site suggests a mixture of mutations were induced in this sample. (f) Synthego ICE analysis showed all the possible insertions/deletions (indels) in this P0 albino mutant. (g) Sibling F1 embryos from a normally pigmented P0 progenitor, showing normal (right) and melanin-free mutants (left), both in prehatching diapause and after hatching. gRNA, guide RNA. PAM, protospacer-adjacent motif sites. Scale bar = 1.0 mm.

TABLE 2 Gene editing rates in P0 CRISPR-injected individuals and F1 mutant recovery from P0 crispant.

Embryo ID	Injection sites	Phenotype	P0 editing rates	F1 Albino/total	F1 Albino %	Note
8	Blastomere	Wildtype	**30.40%**	**3/78**	**3.85%**	~39% Mutant gametocytes
13	Blastomere	Wildtype	9.10%	0/60	0.00%	Mutant cells only in soma
16	Blastomere	Wildtype	23.40%	0/89	0.00%	Mutant cells only in soma
17	Blastomere	Wildtype	7.70%	*N/A*	*N/A*	Did not produce eggs
19	Yolk	Wildtype	6.60%	0/84	0.00%	Mutant cells only in soma
22	Yolk	Wildtype	5.60%	0/52	0.00%	Mutant cells only in soma
29	Blastomere	Wildtype	5.10%	0/91	0.00%	Mutant cells only in soma
32	Blastomere	Wildtype	**35.80%**	**17/129**	**13.18%**	~73% Mutant gametocytes
34	Yolk	Wildtype	5%	0/119	0.00%	Mutant cells only in soma
37	Yolk	Wildtype	6.10%	0/91	0.00%	Mutant cells only in soma
40	Yolk	Wildtype	6.60%	0/140	0.00%	Mutant cells only in soma
43	Yolk	Wildtype	4.30%	0/143	0.00%	Mutant cells only in soma
44	Blastomere	**Sparse pigment**	**65.30%**	*N/A*	*N/A*	Died as larva
47	Yolk	Wildtype	5.90%	0/110	0.00%	Mutant cells only in soma
52	Blastomere	**Albino**	**97.20%**	*N/A*	*N/A*	Died as larva
59	Yolk	Wildtype	4.10%	0/13	0.00%	Mutant cells only in soma
60	Yolk	Wildtype	5.40%	*N/A*	*N/A*	Died as larva
66	Blastomere	Wildtype	13.20%	0/32	0.00%	Mutant cells only in soma

Note: Bold font indicates cases of successful editing, i.e. the number of albino mutants obtained was not zero.

TABLE 3 Mutatagenesis in fish models.

Organism	phenotype	Mutants/genome screened (*n*)	Reference
Zebrafish	Larval	1.1 (3857)	Driever et al. (1996); Haffter et al., (1996)
Medaka	Larval	0.45 (300)	Loosli et al. (2000)
Zebrafish	Adult	0.18 (1000)	2004 ZFMODELS, *Harris unpublished*
Kmar	Larval	0.13 (568)	Moore et al. (2012); (Sucar et al. (2016)
Kmar	Larval	0.061 (36/586)[a]	This study
Kmar	Adult	0.156 (81/518)	This study

[a]This value is likely an underestimate, as only clear embryonic phenotypes were counted. More generalized embryonic inviability was not counted here, and could be a result of mutational load.

arrested larvae hatch when the chorion becomes permeable or otherwise breached, and that perhaps environmental microbes or other cues that stimulate production of hatching enzyme are often lacking in laboratory cultures. In general support of this, hatching of one embryo in a clutch, which would release hatching enzyme into a small volume of water, often triggers synchronous hatching in others (JAF and ESH, *unpublished observation*). In addition, viable embryos that have spent weeks in a diapause-like state become active immediately upon mechanical rupture of the chorion.

4.3 | Improved egg production supports genetic manipulation

We fortuitously discovered that the *kissylips* mutant has an unusually large egg output. As shown in Figure 4a, this is true whether eggs are collected frequently or infrequently. We observe that *kissylips* hermaphrodites are much more likely to deposit eggs in the bottom of their tank than wild-type fish, which strongly prefer to oviposit in the polyester fiber mop.

These can remain uneaten for weeks, often resulting in hatchlings sharing a tank with the mother. Because protection from maternal consumption greatly increases egg yield in wild-type SOB strains (Figure 4b), these observations suggest that *kissylips* hermaphrodites have both unusually frequent oviposition and are less likely to eat eggs once they are laid. Whether the inclination to consume kin is behavioral (Wells & Wright, 2017) or due to a mechanical inability related to their craniofacial abnormalities is unclear. In any case, the combination of the *kissylips* mutant with frequent removal of eggs overcomes a major barrier to Kmar experimentation, the inability to access suitable numbers of progeny for injections. This set the stage for generation of albino *tyrosinase* mutants with CRISPR/Cas9 nucleases, which are to our knowledge the first genome-edited *K. marmoratus*. From the frequency of albino F1, we can conclude that the founder was a genetic mosaic. This is consistent with our injections, which occurred after the first cleavage. Though early teleost fish blastomeres are often open to the yolk basally, diffusion of editing reagents between blastomeres is apparently not rapid in *K. mamoratus*.

4.4 | Plastic traits and generational variation

Many phenotypes obtained in our pilot mutagenesis screen were quite robust, remaining penetrant across generations. However, several lines showed considerable loss of expressivity despite being derived from isogenic, homozygous founders through selfing. This raises the possibility that acquired suppression of some phenotypes may be generated over generations. While we cannot exclude effects of subtle changes in husbandry, these instances harken to findings of epigenetic silencing of traits that can occur in a transgenerational manner (e.g., Bertozzi & Ferguson-Smith, 2020; Chey & Jose, 2022; Verdikt et al., 2023). Given the plasticity of Kmar development to environmental signals, there may be intrinsic mechanisms of trait suppression or modification not seen in traditional model species. Perturbations or challenges (e.g., pharmaceutical treatment or environmental shift) could open up this process to be studied using genetic approaches. Distinct *K. marmoratus* isolates also harbor substantial genotype-by-environment (GxE) interactions (reviewed by Earley et al., 2012). Thus Kmar supports complementation of both genetic study of plastic traits via mutagenesis screens with examination of natural variation in plasticity to understand the regulation of this malleability.

5 | SUMMARY

Kryptolebias marmoratus is a truly unique verterbate that invites investigation of dynamic phenotypic states not observed in other common laboratory species. Kmar demographics and variability in life history traits are well documented (Mackiewicz et al., 2006; Tatarenkov et al., 2007, 2009, 2012; Taylor et al., 2008), and plastic responses to environment are beginning to be analyzed (Rossi et al., 2020; Turko & Rossi, 2022). We provide advances in husbandry and genetic tools to enable the integrative exploration development and physiology in the context of a changing environment.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data and genetic stocks that support the findings of this study are available from the corresponding author upon request.

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PEER REVIEW

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