Video Article

Shifting Zebrafish Lethal Skeletal Mutant Penetrance by Progeny Testing

Elliott P. Brooks[1], James T. Nichols[1]

[1]Department of Craniofacial Biology, University of Colorado Anschutz Medical Campus

Correspondence to: James T. Nichols at james.nichols@ucdenver.edu

URL: https://www.jove.com/video/56200
DOI: doi:10.3791/56200

Keywords: Developmental Biology, Issue 127, Zebrafish, craniofacial skeleton, selective breeding, progeny testing, family selection, Kompetitive Allele Specific PCR, husbandry, cartilage, penetrance gene, mutant phenotype

Date Published: 9/1/2017

Citation: Brooks, E.P., Nichols, J.T. Shifting Zebrafish Lethal Skeletal Mutant Penetrance by Progeny Testing. *J. Vis. Exp.* (127), e56200, doi:10.3791/56200 (2017).

Abstract

Zebrafish mutant phenotypes are often incompletely penetrant, only manifesting in some mutants. Interesting phenotypes that inconsistently appear can be difficult to study, and can lead to confounding results. The protocol described here is a straightforward breeding paradigm to increase and decrease penetrance in lethal zebrafish skeletal mutants. Because lethal mutants cannot be selectively bred directly, the classic selective breeding strategy of progeny testing is employed. This method also includes protocols for Kompetitive Allele Specific PCR (KASP) genotyping zebrafish and staining larval zebrafish cartilage and bone. Applying the husbandry strategy described here can increase the penetrance of an interesting skeletal phenotype enabling more reproducible results in downstream applications. In addition, decreasing the mutant penetrance through this selective breeding strategy can reveal the developmental processes that most crucially require the function of the mutated gene. While the skeleton is specifically considered here, we propose that this methodology will be useful for all zebrafish mutant lines.

Video Link

The video component of this article can be found at https://www.jove.com/video/56200/

Introduction

The zebrafish is a powerful model system for understanding skeletal development. With mutant zebrafish strains, biologists can decipher gene function during skeletogenesis. However, zebrafish skeletal mutant phenotypes can present with variable penetrance[1,2,3,4] which can hinder developmental and genetic analyses. The purpose of this method is threefold. First, generating zebrafish mutant lines which consistently produce severe phenotypes enables downstream developmental studies like time-lapse recording[5] and transplantation[6]. These sorts of studies can be crippled by attempting to study phenotypes that only manifest inconsistently. Second, inbreeding zebrafish strains can decrease genetic background variation, thus promoting experimental consistency and reproducibility. For example, performing all *in situ* hybridization analyses on one selectively inbred strain can reduce confounding variability and strengthen conclusions. Third, generating severe and mild strains will reveal the entire phenotypic series that can result from a particular mutation.

At first glance, selective breeding of lethal mutants seems impossible. How can one breed for penetrance when the animals that are scored for selection are dead? Fortunately, methods for selective breeding by family selection, specifically progeny testing, have demonstrated effectiveness in livestock breeding programs for many years[7,8]. These programs are mainly used for selective breeding for traits that are only present in one sex, like milk production in cows or egg production in hens. The males of these species cannot be scored directly, but their progeny are scored and a value is then assigned to the parents. Borrowing from this strategy, the protocol presented here involves scoring the fixed and stained mutant offspring from a pair of zebrafish that are heterozygous for a mutant gene of interest. The penetrance of a phenotype in the homozygous lethal mutant offspring is assigned to the parents when deciding which individuals will produce the next generation in the line. We find that this method is an effective means of shifting penetrance in zebrafish lethal skeletal mutants[1].

Similar to other studies, this selective breeding protocol takes under consideration criteria like clutch size, survival of offspring, normal development of embryos, and sex ratio[9]. However, these factors are all considered in the context of a mutant background with the objective of shifting the mutant penetrance. Therefore, this protocol extends previous selective breeding paradigms by offering a method to strengthen developmental mutant analyses as well as increase background homogeneity.

This protocol requires extensive genotyping, so it is important to develop a reliable, rapid genotyping protocol in advance. There are many genotyping protocols available[10,11], however we find the KASP genotyping[12,13,14] is faster, more cost efficient, and more reliable than methods based on restriction enzyme cleavage of amplified sequences[10]. Therefore, we include a KASP protocol in this work. Additionally, we focus on skeletal mutant phenotypes in this protocol and include a procedure for Alcian Blue/Alizarin Red staining modified from previous protocols[15].

The method described here is a straightforward strategy for shifting lethal mutant penetrance upward or downward. While this protocol focuses on skeletal mutant phenotypes, we believe it will be a useful strategy for husbandry of all mutant zebrafish lines. In fact, the utility of this breeding

Copyright © 2017 Journal of Visualized Experiments

strategy likely extends beyond zebrafish. We predict that this protocol can be modified to shift penetrance in a broad range of organisms. Shifting lethal penetrance by progeny testing can help push forward the progress of any developmental geneticist.

Protocol

All experiments described in this protocol were completed in accordance and compliance with the University of Colorado and the University of Oregon Institutional Animal Care and Use Committees (IACUC).

1. Preparing the Unselected Starting Stock

1. Identify heterozygous carriers of the mutant allele of interest by fin clip[11] and genotyping a stock of full-sibling animals by a method of choice, such as KASP[12,13,14]. This protocol was performed with the zebrafish *mef2ca*^{b1086} strain.
2. **KASP genotyping**
   NOTE: Allele specific primers for KASP are designed by the company that provides the reagents (see **Table of Materials**). To obtain the appropriate 6-carboxyl-X-rhodamine (ROX) dye concentration for the specific real-time PCR machine visit the company website.
   1. Place the zebrafish tail tissue in 50 µL of lysis buffer (10 mM tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.3% non-ionic surfactant, 0.3% Octylphenyl-polyethylene glycol). Add 10 µL of 10 mg/mL Proteinase-K per well of a 96-well PCR plate and digest in a thermal cycler at 55 °C for 2 - 5 h followed by a 20 min 94 °C inactivation step. Refrigerate the lysis product after digestion.
      NOTE: Lysis products can be successfully used for several months after preparation. When tissue was lysed using 50 mM NaOH, the KASP reactions were unsuccessful. These findings suggest that the NaOH method of genomic DNA preparation is not compatible with KASP.
   2. Quantify the genomic DNA using a spectrophotometer. Dilute the genomic DNA template using molecular biology grade water so that each reaction contains approximately 20-30 ng.
      NOTE: Quantify 4 or 5 samples, pool them, then prepare dilutions for all samples based on the average. When using this protocol for adult tail tissue, a 1:100 dilution is often sufficient. The precise quantification of DNA concentration is not required.
   3. Add 0.14 µL of the KASP primer mix and 5 µL of the KASP master mix per sample on ice into a 1.5 mL microcentrifuge tube and mix well before briefly centrifuging to collect contents at the bottom of the tube.
      NOTE: KASP master mix is heat and light sensitive, keep stock on ice and avoid direct light.
   4. Pipet 5 µL of primer/master mix solution into the wells of a 96-well optical PCR plate suitable for the real-time PCR machine being used.
   5. Pipet 5 µL of diluted (approximately 5 - 7 ng/µL) DNA template samples into each well and aspirate with a pipette to mix.
   6. Add 5 µL of nuclease-free water to 3 wells to serve as no-template controls (NTC) and add 5 µL of diluted confirmed homozygous wild type, heterozygous and homozygous mutant DNA template for positive controls.
   7. Place an optically clear film seal over the plate ensuring that the film is fully sealed on each well.
   8. Briefly centrifuge the plate at 600 x g to collect the contents at the bottom and to remove bubbles from the mix.
      NOTE: Keep the plate on ice and shield from light until ready to proceed.
   9. Use the cycling program shown in **Table 1** to perform the main KASP reaction.
   10. View the resulting scatter plot produced by the analysis computer. A successful reaction will show 4 distinct groupings of points on the plot (**Figure 3**): three sample groupings corresponding to genotype, and one grouping of the NTCs near the origin. The positive controls should segregate with the appropriate sample grouping.
   11. If the computer program does not recognize the plot groupings as corresponding to specific genotypes, additional cycling sets may be required (**Table 2**). Repeat the additional program until the computer recognizes tight groupings by genotype.
   12. Export the genotyping results to a spreadsheet file for easier use and analysis.
3. House the identified heterozygous animals together on the main water system.

2. The First Round of Progeny Scoring

1. **Pairwise cross sibling heterozygotes**
   1. After the animals recover from the fin clip, set up pairwise intercrosses. Use dividers to ensure embryos are synchronous[16].
   2. Collect the embryos and keep the parental pairs isolated in breeding cages.
   3. Monitor isolated adults so that aggressive fish can be separated or provided with cover (shredded fish nets work nicely). Fish should be fed and their water changed following IACUC guidelines while in static water.
2. **Embryo rearing**
   1. Stage and raise the zebrafish embryos to larvae under standard conditions[17].
   2. Collect phenotypically wild-type animals using a glass pipette on day 5 or 6 when the swim bladder is inflated in 75% of the clutch. Place phenotypic wild types in the nursery recording of which parents yielded them.
      Note: With mutant alleles that are recessive lethal, which is true of many skeletal mutants, homozygous mutants fail to form swim bladders[18,19]. Therefore, phenotypic wild types can be easily discerned from their mutant siblings based on inflated swim bladders.
3. **Alcian Blue/Alizarin Red cartilage and bone staining**
   1. Anesthetize larvae that do not inflate their swim bladders using 0.17 g/L Tricaine-S in embryo media. Collect animals into 1.5 mL microcentrifuge tubes with a wide-bore glass Pasteur pipet. Add no more than 100 larvae per tube.
   2. Remove embryo water from each tube and fix animals in 1 mL of 2% paraformaldehyde in 1x PBS per tube.
      CAUTION: Aqueous PFA is combustible and will cause serious skin and eye irritation. Wear appropriate personal protective equipment such as gloves and eye protection, and wash hands thoroughly after use.

3. Rock for 1 h; longer fixation impairs bone staining. Check the tubes regularly during this and all subsequent rocking steps to ensure no larvae have become stuck to the side or clumped in the bottom of the tube.
4. Remove the fixative from tubes using a glass pipette, add 1 mL of 50% ethanol, and rock for 10 min.
5. Prepare fresh staining solution by adding 20 µL of 0.5% Alizarin Red per 1 mL of Alcian premix (0.04% Alcian Blue/10 mM $MgCl_2$/80% ethanol). Remove 50% ethanol, add 1 mL of staining solution to each tube, and rock overnight. Larvae can remain in the stain for up to 5 days.
6. Remove the stain solution from the tubes and add 1 mL of 80% ethanol/10 mM $MgCl_2$ solution to each tube. Rock the tubes for at least 1 h; rinsing overnight can produce a clearer Alcian Blue stain.
7. Remove the 80% ethanol/10 mM $MgCl_2$ solution from the tubes and add 1 mL of 50% ethanol; rock for 5 min.
8. Remove the 50% ethanol solution from the tubes and add 1 mL of 25% ethanol and rock for 5 min.
9. Remove the 50% ethanol solution from the tubes and add 1 mL freshly prepared 3% H_2O_2/0.5% KOH bleaching solution. Leave the caps open and let the tubes stand in in a micro-tube rack for approximately 10 min or until a faint brown coloration can be seen in the upper part of the solution.
   NOTE: A layer of bubbles form on top of the solution. Careful timing is required at this step, as over bleaching will result in a poor double stain.
10. Remove the bleaching solution and add 1 mL of 25% glycerol/0.1% KOH; rock for 10 min to 1 h.
11. Remove 25% glycerol/0.1% KOH solution from the tubes, add 1 mL of 50% glycerol/0.1% KOH solution to each tube, rock for 10 min to overnight.
12. Remove 50% glycerol/0.1% KOH solution from the tubes and again add 50% glycerol/0.1% KOH solution to each tube. Rocking overnight will help remove air bubbles that can accumulate inside larvae. Store stained skeletal preparations at 4 °C when not being used.

4. **Phenotype scoring**
   1. Score stained mutant offspring for the penetrance of the selected phenotype. In Nichols *et al.*[1] mutants were scored for any occurrence of ectopic bone.
      Note: Because the zebrafish parents are in static water and the Alizarin Red stain can fade with time, it is important to score skeletons within a few days of completing the skeletal preparation.
   2. Penetrance is the proportion of a genotype that has a phenotype. Calculate the percent penetrance by the following formula:
      % penetrance = (mutants with phenotype)/(total number of mutants) × 100

3. Family Selection

1. Choose two high penetrance and two low penetrance families for the next generation. In addition to penetrance, it is important to consider fecundity and vigor when choosing which families to propagate; see **Discussion** for more details on this.
2. Give each parental pair a sub stock identifier: family .01, .02, *etc.*
3. House parental pairs on the main system with several mixed sex 'companion' fish. Companion fish can be any zebrafish line with permanent, obvious distinguishing features, such as altered pigmentation or fin structure.
   NOTE: While many researchers successfully keep only breeding pairs in a tank, favorable results are seen by housing pairs with several companion fish. This optional step allows breeding pairs of animals to be kept in larger schools and easily retrieved so they can be repeatedly crossed as needed. Parental pairs can be crossed repeatedly to generate large full-sibling families; wait at least one week before repeat crossing the same parental pair.
4. Cull the larval families from step 2.2.2. that were not selected for the next generation.
5. Label the tanks containing the wild-type sibling larvae from selected families with the penetrance from their mutant siblings and raise to adulthood as normal.
6. The next generation will have at least four full-sibling families, two for the upward line and two for the downward line.
7. When larvae reach sexual maturity, repeat the protocol starting at step one with the new generation.

Representative Results

This protocol is a long-term husbandry technique useful for understanding zebrafish skeletal mutants (**Figure 1**). Selective breeding by progeny testing should yield a shift in overall penetrance both downward and upward in a few generations (**Figure 2**). In our previous work, two rounds of selective breeding drove the average penetrance downward from 17% to 3%[1]. Similarly, in our upward line, we shifted the average penetrance upward from 63% to 93% in three generations. If after four rounds of selective breeding the penetrance does not respond downward or upward, it is possible that the phenotype that is being scored is not sensitive to selective breeding by progeny testing.

In a successful KASP procedure, tight groupings of samples corresponding to genotype should be recognized by the computer program, and the NTCs should be located near the origin of the plot after sufficient cycles have been performed (**Figure 3**). If the KASP scatter plot data is not grouped tightly or contamination in one NTC is detected, the program will be unable to determine genotypes. If most samples are recognized by genotype grouping, but there are still some that are ambiguous, omitting undetermined samples from the data set may allow the program to recognize genotyping clusters. Undetermined sample calls and ambiguous genotype grouping may result from the following: contaminated reaction mixtures in which NTCs will not group near the origin, over-diluted template DNA causing samples to locate near the origin, under-diluted template DNA, or insufficient number of reaction cycles. Prepare fresh NTCs if contamination is suspected.

Copyright © 2017 Journal of Visualized Experiments

A successful Alcian Blue/Alizarin Red stain will have vibrantly stained bone and cartilage elements (**Figure 4A**) that are not transparent or faint (**Figure 4B**). The boundaries of the cartilage elements should appear crisp, and individual chondrocytes will be discernable. The Alizarin Red bone stain is usually the more finicky of the two stains. The fan-shaped opercle (op) bone and stick shaped branchiostegal rays (br) are good indicators of a successful 6 days post fertilization (dpf) bone stain (**Figure 4A**, op, br). The bleaching step should not fade the stains in the bone and cartilage while fully clearing the other tissues of color. The eyes will have a brown hue even after successful staining and clearing. Alcian Blue that is too concentrated will not clear from other tissues making analysis impossible (**Figure 4C**). Alcian Blue from vendors other than the one described in the material's table can also produce poor cartilage stains.

Copyright © 2017 Journal of Visualized Experiments

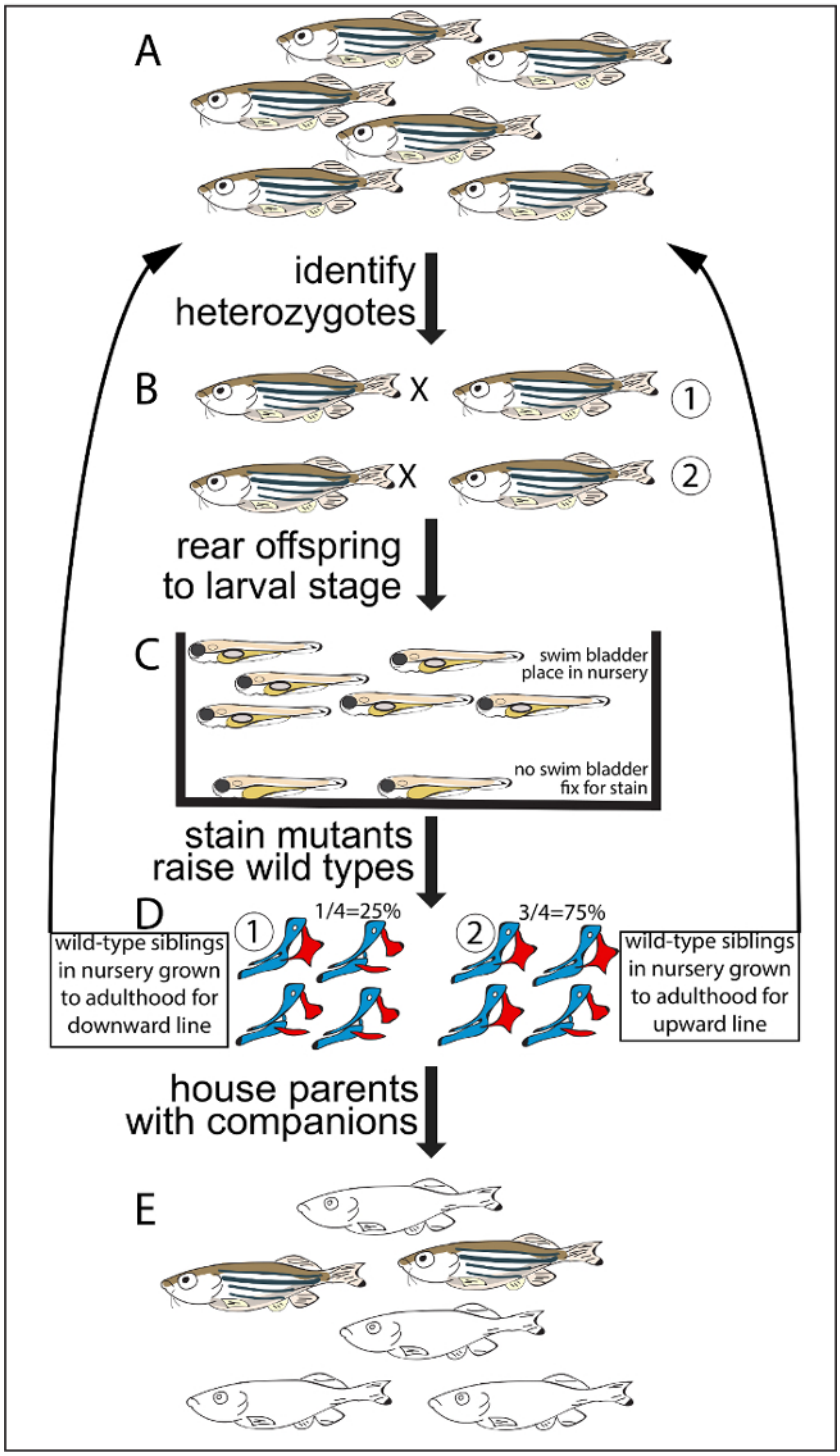

Figure 1. Schematic Overview of Selective Breeding for Skeletal Penetrance by Progeny Testing. (**A**) Genotype a full-sibling family from a mutant line to identify heterozygous carriers. **(B)** Pair-wise cross identified heterozygous siblings, and keep parents isolated until offspring can be scored. **(C)** Place phenotypically wild-type larvae with inflated swim bladders in the nursery to be raised to adulthood, and fix mutant larvae without inflated swim bladders for cartilage and bone staining. **(D)** Score each clutch of Alcian Blue/Alizarin Red stained mutant animals for penetrance. Select which families will be bred upward and downward and raise the phenotypic wild types from each family to adulthood. **(E)** House parental pairs with companions so that they can be repeatedly crossed to generate large full-sibling families. Please click here to view a larger version of this figure.

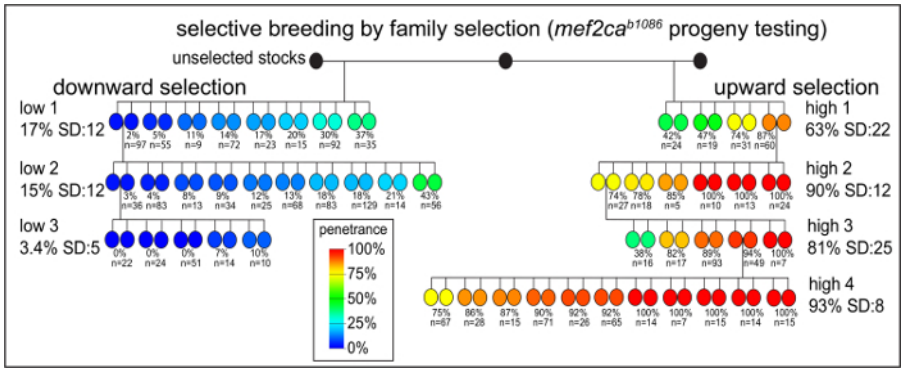

Figure 2. Example of Shifting Skeletal Mutant Penetrance by Progeny Testing. Selective breeding as described in this manuscript was applied to the zebrafish *mef2ca*^{b1086} mutant. In this experiment, we selected for high or low penetrance of ectopic bone between the opercle and the branchiostegal ray in lethal homozygous mutants. Penetrance of ectopic bone in mutant offspring was assigned to the parental pair who was color-coded by penetrance as shown. This figure was modified from[1]. Please click here to view a larger version of this figure.

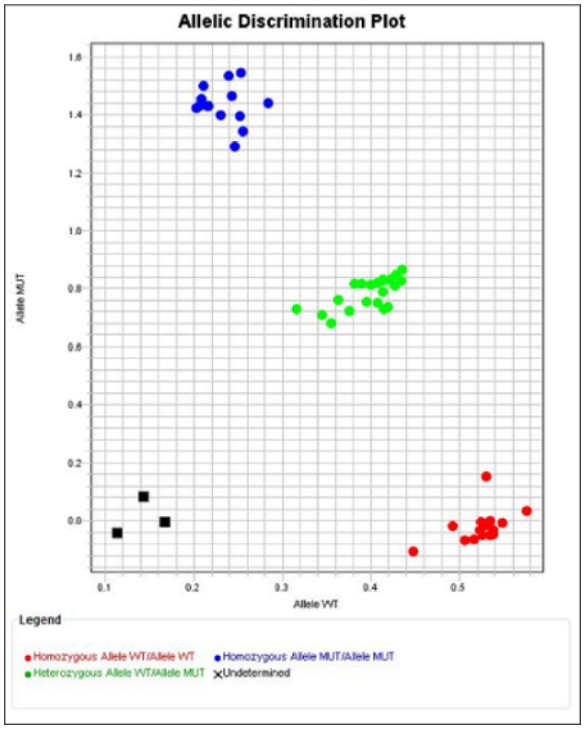

Figure 3. Screen Shot from Software used for KASP Genotyping and Results Analyses.
In this successful KASP reaction, tight sample groupings were formed. Blue points are homozygous mutant, green points are heterozygous, red points are homozygous wild-type, and black squares are the NTCs. Groupings were designated by the computer program. The NTCs are located near the origin of the plot signifying little to no reaction mixture contamination. Undetermined samples that are not yet assigned a genotype would be indicated with an x. There are no undetermined samples present in this successful completed run. Please click here to view a larger version of this figure.

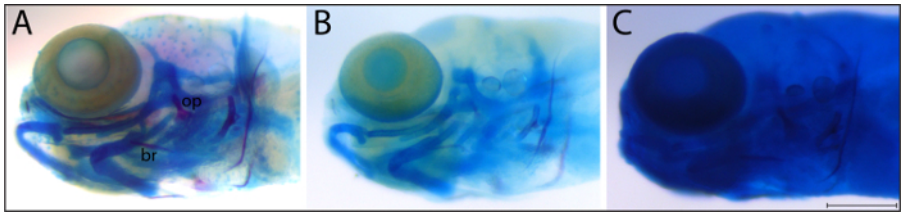

Figure 4. Good, Bad and Ugly Alcian Blue/Alizarin Red Skeletal Preparations.
(A) An example of a fresh, nicely stained and cleared cartilage and bone stain is shown. Note the distinct cartilage elements and easily visualized red opercle (op) and branchiostegal ray (br) bones. **(B)** A weak cartilage stain with faded Alizarin Red stain is shown. This sample sat at 4 °C for many months resulting in faded cartilage as well as opercle and branchiostegal ray bones that are difficult to distinguish. **(C)** An over-stained larva in which too much Alcian Blue was used. The scale bar is 200 µm. Please click here to view a larger version of this figure.

Discussion

Selective Breeding Unveils Subtleties of Gene Function

Shifting mutant phenotypes to be either more or less severe by selective breeding is a straightforward way to gain new insights into gene function. When compared with standard methods of unselected breeding, the protocol presented here can yield a much more complete understanding of mutant phenotypes. Specifically, by generating strains that are severe, the full breadth of mutant phenotypes may be revealed, including some that were unobservable with unselected stocks. Thus, new developmental roles for the gene under study can be discovered. Conversely, generating mild strains can reveal the most crucial roles for the gene of interest. Take, for example, only a single phenotype persists in mild mutants. Here it is likely that the developmental process that remains disrupted in the mild strain most critically requires the function of the gene. Hence, the full phenotypic series of a given mutation can be more fully understood through selective breeding.

Avoiding Inbreeding Depression

The offspring of related individuals demonstrate reduced survival and fecundity in many animal and plant systems, including zebrafish[20]. Known as inbreeding depression, most models posit that increased homozygosity at loci with either recessive deleterious alleles or alleles that are beneficial as heterozygotes underlie this phenomenon[21]. Studies with wild-caught zebrafish reveal a low frequency of deleterious alleles in natural populations[22]. Moreover, laboratory strains like AB were further cleared of recessive lethal mutations[23]. Thus, it seems that careful husbandry could allow indefinite inbreeding of selected zebrafish lines. Indeed, a recent report revealed that zebrafish can be maintained by full-sibling inbreeding for at least 16 generations[9].

This protocol emphasizes a few simple but critical steps to ensure that inbred zebrafish lines continue to produce enough offspring to be useful for developmental studies. The most crucial aspect of this selective breeding strategy is the selection process itself. First, developing animals must be carefully monitored so that families with developmental defects or abnormalities, unlinked to the mutation of interest, can be stringently selected against. Second, clutch size should be considered, as selective breeding requires large families. In addition to selecting pairs that yield large clutches, large families can be generated through repeat crosses of the same parental pair. Keeping parental pairs in tanks with many other companion fish, which are easily discerned from the breeding pair, such as pigmentation or fin mutants, allows for long-term housing of breeding pairs in large conspecific groups while allowing them to still be easily identified and retrieved. This practice promotes a healthy social environment where the parental pair are free to shoal with other zebrafish[24]. Yet the researcher can easily retrieve the parental pair so that they can be crossed repeatedly, enabling the generation of very large full-sibling families.

Choosing a Selectable Phenotype

This protocol involves a great deal of phenotype scoring. Thus, one limitation is that the selection needs to be applied to a phenotype that is easy to visualize and score rapidly. It is also important to decide at what stage animals will be fixed and stained for scoring. For bone phenotypes, it is advantageous to wait until 6 dpf because the bones are more developed[25] and will be more easily scored. For cartilage patterning mutant phenotypes, 4 dpf is often suitable for phenotype scoring[26,27]. In deciding when to fix animals, it is important to consider if the gene of interest has pleiotropic roles leading to developmental mutant phenotypes in other tissues, which can result in confounding secondary defects. For example, with skeletal mutants that also display heart edema it is important to fix at 4 dpf before secondary defects like a general delay in chondrogenesis manifest[26].

For simplicity, we chose a binary scoring system focusing on penetrance for our selective breeding. However, mutant phenotypes can also have variable expressivity. In future studies, it will be interesting to test if, like penetrance, expressivity is sensitive to selective breeding. That is, can the frequency of specific ectopic bone shapes be altered through selective breeding?

This protocol describes the classic selective breeding strategy of progeny selection and its application to zebrafish developmental genetic studies. The straightforward husbandry practices described here are possible in any laboratory, even in small facilities. Through selective breeding, one of the greatest strengths of the zebrafish system, tractable genetics, can be harnessed to learn about gene function in newly developed mutants as well as mutants that have been propagated for decades.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We would like to thank Chuck Kimmel for guidance, John Dowd for help in developing this breeding strategy, Macie Walker for her work in perfecting the skeletal stain, and Charline Walker and Bonnie Ullmann for helpful zebrafish advice. This work was supported by K99/R00 DE024190 to JTN.

References

1. Nichols, J. T., *et al.* Ligament versus bone cell identity in the zebrafish hyoid skeleton is regulated by mef2ca. *Development.* **143** (23), 4430-4440 (2016).
2. Sheehan-Rooney, K., Swartz, M. E., Zhao, F., Liu, D., Eberhart, J. K. Ahsa1 and Hsp90 activity confers more severe craniofacial phenotypes in a zebrafish model of hypoparathyroidism, sensorineural deafness and renal dysplasia (HDR). *Dis Model Mech.* **6** (5), 1285-1291 (2013).

3. Cox, S. G., *et al.* An essential role of variant histone H3.3 for ectomesenchyme potential of the cranial neural crest. *PLoS Genet.* **8** (9), e1002938 (2012).
4. DeLaurier, A., *et al.* Role of mef2ca in developmental buffering of the zebrafish larval hyoid dermal skeleton. *Dev Biol.* **385** (2), 189-199 (2014).
5. McGurk, P. D., Ben Lovely, C., Eberhart, J. K. Analyzing Craniofacial Morphogenesis in Zebrafish Using 4D Confocal Microscopy. *J Vis Exp.* (83), e51190 (2014).
6. Kemp, H. A., Carmany-Rampey, A., Moens, C. Generating chimeric zebrafish embryos by transplantation. *J Vis Exp.* (29), e1394-e1394 (2009).
7. Lush, J. L. Progeny test and individual performance as indicators of an animal's breeding value. *J Dairy Science.* **18** (1), 1-19 (1935).
8. Lerner, I. M. *Population Genetics and Animal Improvement.* Cambridge University Press. (1950).
9. Shinya, M., Sakai, N. Generation of Highly Homogeneous Strains of Zebrafish Through Full Sib-Pair Mating. *G3.* **1** (5), 377-386 (2011).
10. Neff, M. M., Neff, J. D., Chory, J., Pepper, A. E. dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in Arabidopsis thaliana genetics. *Plant J.* **14** (3), 387-392 (1998).
11. Xing, L. Y., Quist, T. S., Stevenson, T. J., Dahlem, T. J., Bonkowsky, J. L. Rapid and Efficient Zebrafish Genotyping Using PCR with High-resolution Melt Analysis. *Jove-Journal of Visualized Experiments.* (84), e51138 e51138 10.3791/51138, (2014).
12. He, C., Holme, J., Anthony, J. SNP genotyping: the KASP assay. *Methods Mol Biol.* **1145** 75-86 (2014).
13. Semagn, K., Babu, R., Hearne, S., Olsen, M. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. *Molecular Breeding.* **33** (1), 1-14 (2014).
14. Yuan, J., Wen, Z., Gu, C., Wang, D. Introduction of high throughput and cost effective SNP genotyping platforms in soybean. *Plant Genet Genomics Biotech.* **2** (1), 90-94 (2014).
15. Walker, M. B., Kimmel, C. B. A two-color acid-free cartilage and bone stain for zebrafish larvae. *Biotech Histochem.* **82** (1), 23-28 (2007).
16. Nasiadka, A., Clark, M. D. Zebrafish breeding in the laboratory environment. *ILAR J.* **53** (2), 161-168 (2012).
17. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev dyn.* **203** (3), 253-310 (1995).
18. Schilling, T. F., *et al.* Jaw and branchial arch mutants in zebrafish I: branchial arches. *Development.* **123** 329-344 (1996).
19. McCune, A. R., Carlson, R. L. Twenty ways to lose your bladder: common natural mutants in zebrafish and widespread convergence of swim bladder loss among teleost fishes. *Evol Dev.* **6** (4), 246-259 (2004).
20. Mrakovčič, M., Haley, L. E. Inbreeding depression in the Zebra fish Brachydanio rerio (Hamilton Buchanan). *J Fish Biol.* **15** (3), 323-327 (1979).
21. Charlesworth, D., Willis, J. H. The genetics of inbreeding depression. *Nat Rev Genet.* **10** (11), 783-796 (2009).
22. McCune, A. R., *et al.* A low genomic number of recessive lethals in natural populations of bluefin killifish and zebrafish. *Science.* **296** (5577), 2398-2401 (2002).
23. Streisinger, G., Walker, C., Dower, N., Knauber, D., Singer, F. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature.* **291** (5813), 293-296 (1981).
24. Dreosti, E., Lopes, G., Kampff, A. R., Wilson, S. W. Development of social behavior in young zebrafish. *Front Neural Circuits.* **9** 39 (2015).
25. Eames, B. F., *et al.* FishFace: interactive atlas of zebrafish craniofacial development at cellular resolution. *BMC Dev Bio.* **13** (1), 23 23 10.1186/1471-213x-13-23, (2013).
26. Nichols, J. T., Pan, L., Moens, C. B., Kimmel, C. B. *barx1* represses joints and promotes cartilage in the craniofacial skeleton. *Development.* **140** (13), 2765-2775 (2013).
27. Sasaki, M. M., Nichols, J. T., Kimmel, C. B. edn1 and hand2 Interact in early regulation of pharyngeal arch outgrowth during zebrafish development. *PLoS One.* **8** (6), e67522 (2013).

Copyright © 2017 Journal of Visualized Experiments