A Simultaneous Genetic Screen for Zygotic and Sterile Mutants in a Hermaphroditic Vertebrate

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ABSTRACT

The mangrove killifish *(Kryptolebias marmoratus)* is unique among vertebrates due to its self-fertilizing mode of reproduction involving an ovotestis, analogous to the nematode *Caenorhabditis elegans.* It develops externally, is easy to maintain, and reaches sexual maturity in about 100 days, making it a desirable vertebrate model organism for developmental genetics. As a continuation of an initial pilot screen (Moore, Sucar, Newsome, Ard, Bernhardt, Bland, & Ring, 2012) in which 73 out of 284 F1 fish were found to display zygotic defects in their F2 offspring (25%), a three generation genetic screen was performed using N-ethyl-N-nitrosourea as a mutagen in order to a) confirm zygotic mutant allele heritability and b) simultaneously score for homozygous recessive mutant sterile F2 fish. One to ten F2 fish per family were set up and their progeny screened. From a total of 307 F2 fish screened, 10 were found to be 1^0 males and 16 were found to be sterile. From the remaining 281 fish, 92 showed a wild type phenotype and 189 were carriers of zygotic recessive alleles. These carriers produced 25% progeny exhibiting several zygotic phenotypes previously described in zebrafish (Mullins et al., 1994) as well as in a *K. marmoratus* pilot screen. Also, new phenotypes such as *golden yolk, no trunk,* and *short tail*, were discovered. In order to confirm inheritance of sterility, fish were grown from the F3 generation from siblings of sterile F2 mutants. From 284 F3 fish belonging to 10 previously identified sterile families, 12 were 1^0 males. From the remaining 272 fish, 69 were wild type, 83 were sterile and 120 were classified as */+ (either wild type or carriers) because their genotypes were undefined. This screen provides proof of principle that *K. marmoratus* is a powerful vertebrate model for developmental genetics and can be used to identify mutations affecting fertility.

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Chapter I

INTRODUCTION

Mangrove Killifish

Kryptolebias marmoratus, hereafter referred to as *K. marmoratus* is the scientific name used to designate the common mangrove killifish. This is a species of fish in the *Rivulidae* family, belonging to the order *Cyprinodontiformes*, that lives along the east coast of North, Central, and South America, extending from Florida to Brazil. Greater than 90% of these fish exist as hermaphrodites in nature and the laboratory (Mackiewicz, Tatarenkov, Taylor, Turner, & Avise, 2006b). They can reach 75 mm in length and have a unique pigmented dorsal ocellus on the tail (Figure 1). Primary males, which occur at low frequency, are easily discernible due to their bright orange coloration and lack of ocelli (Figure 1). They inhabit shallow intertidal waters in mangrove swamps. The nature of those environments is highly variable; they can dry up entirely during months in which the temperature is high or be suddenly inundated by fresh water when rain fall is heavy. This explains why they need to be able to adapt to different (ephemeral) abiotic factors such as salinity, temperature, oxygen, and ammonia levels. Emersion is one extreme adaptation in *K. marmoratus*. They can spend up to sixty-six consecutive days out of water, typically inside damp fallen logs or crab burrows, breathing air through the skin (Taylor, 2012).

The first descriptions of *K. marmoratus* reared in the laboratory were made by Robert W. Harrington Jr. and were unique because *K. marmoratus* was demonstrated to

be the only known synchronous, self-fertilizing vertebrate (Harrington, 1961). Harrington's observations documented *K. marmoratus'* basic life cycle, seasonal and diurnal fluctuations in fecundity (Harrington 1963; 1975), as well as temperature induction of functional "primary males" containing single testes in the laboratory by rearing embryos at 18°C degrees through several phenocritical periods of development (Harrington, 1968).

The percentage of primary males in natural populations can vary from < 1 to 25% (Mackiewicz et al., 2006a). Even though most habitats contain a very low percentage of males, a study focused on a small population at Twin Cays, Belize, showed that the relative abundance of primary males was much higher than at other sites (20% compared to 1%). However, the majority of this fish species both in natural populations and in the laboratory are hermaphrodites that have an ovotestis; a mixed gonad containing both functional ovarian and testicular tissue. Cole and Noakes (1997) carefully reevaluated gonad development by histological examination at defined time periods of development and found that juvenile fish primarily contain ovaries (between 60 and 100 days post hatching) and transition to the majority ovotestis configuration after 100 days.

Hermaphroditic reproduction can be sequential when animals change sex at some time during their lifespan, or simultaneous when an animal possess ovarian and testicular gonadal tissue at the same time (Michiels, 1998). Simultaneous hermaphrodites can have a separate testis and ovary or a combination in one organ known as ovotestis, as found in *K. marmoratus*. Hermaphroditism is common among invertebrates, such as, the model organism, *C. elegans*. Among vertebrates, hermaphroditism is not as common. Even though in some cases hermaphroditism can result in reproductive anomalies that cause

infertility, many fishes are asynchronous hermaphrodites. *K. marmoratus* is a synchronous hermaphroditic vertebrate in which self-fertilization is the natural mode of reproduction resulting in production of viable progeny. *K. marmoratus* exploits a mixed-mating strategy termed androdiecy. It mainly reproduces by self-fertilization but occasionally mates with males by sexual reproduction. Androdiecy confers evolutionary advantages directly related to the benefits of having two types of mating possibilities, autofertilization and sexual reproduction. To explain why such a system would be evolutionary stable, Mackiewicz, Tatarenkov, Taylor, Turner, and Avise (2006c) propose that it represents a 'best-of-both-worlds'option. An organism gains the reproductive-assurance of self-fertilization but also retains the ability to outcross when the option is available, and with it the ability to combine one's own genes with other favorable genotypes (Mackiewicz et al., 2006c). The ecology of this fish helps to further clarify why such a mode of reproduction constitutes a great benefit. As previously mentioned, natural populations consist mostly of self-fertilizing hermaphrodites but also include males occuring at varying frequencies in different populations (Tatarenkov, Ring, Elder, Bechler, & Avise, 2010). *K. marmoratus* inhabits estuarine and shallow intertidal locations within red mangroves and shows several adaptations to semi-terrestrial life, often utilizing crab burrows and driftwood boreholes as refugia during periods of low tide. Since its preferred habitats are red mangrove swamps, which can dry up entirely or, in heavy rain, be suddenly inundated by freshwater. *K. marmoratus* lives in a highly variable environment. These challenging conditions may act as selective pressures on *K. marmoratus* populations. Few other fish are capable of living in such severe conditions, however, these killifish have evolved a suite of extreme physiological and

behavioral adaptations. The extremities of this environment also influence the possibility of connecting different populations, which vary according to the conditions. This means that access to complementary mates (in a normal sexual breeding system) could therefore be sufficiently uncertain as to generate selective pressure on the evolution of self-fertilization. If local adaptation to specific environmental harshness is important, self-fertilization would be encouraged (Molloy & Gage, 2006).

Evolutionary theory predicts that one adaptive benefit of self-fertilization is the intact propagation and preservation of highly fit and environment-tested multilocus genotypes (Tatarenkov, Earley, Taylor, & Avise, 2012). But at the same time, it also predicts that if the environment is variable, outcrossing should be of special adaptive significance by promoting recombinational variation upon which natural selection might act. Either possibility applies to *K. marmoratus* depending on the environmental circumstance (Mackiewicz et al., 2006b). This reproductive tactic is adaptively significant especially in low-density colonizing species that occupy heterogeneous, ephemeral, or marginal habitats. Mackiewicz et al. (2006b) suggest that the occasional production of functional males inject genetic variation into the lineage preventing deleterious mutation accumulation and also allow a regaining of 'hybrid vigor' within a narrow lineage specific to the local environment.

Their mode of reproduction, together with the fact that they require low maintenance and are easily reared as a colony or as individual specimens, make *K. marmoratus* a desirable organism to use for research. However, possessing all these desirable features, it has not been widely utilized as a model organism.

Zygotic Mutants

Genetic screens have been performed to search for zygotic mutations in different organisms. Several zygotic defects have been described in the established zebrafish (*Danio rerio*) model (Haffter et al., 1996).

Zygotic mutations occur in genes that are expressed in the embryo after fertilization and are required for normal embryogenesis. As such, zygotic mutations produce phenotypic defects that arise in the embryo and usually lead to lethality dependent on the embryo genotype.

N-ethyl-N-nitrosourea (ENU; $C_3H_7N_3O_2$) is a highly potent mutagen used for inducing zygotic mutations (Mullins, Hammerschmidt, Haffter, & Nusslein-Volhard, 1994). ENU acts by transferring its ethyl group to nucleobases (usually thymine) in nucleic acids, mainly targeting are the spermatogonial stem cells, from which mature sperm are derived. This potent DNA alkylating agent induces point mutations in the germ-line that are heritable in the offspring. The mutational specificity of ENU was determined in *Drosophila melanogaster* using the vermilion locus as a target gene (Pastink, Vreeken, Nivard, Searles, & Vogel, 1989). In the fruit fly, transition mutations were the most prominent sequence change. Most frequent transitions were GC to AT (61%) and AT to GC (18%) substitutions. The spectrum of ENU mutations also included a significant fraction of transversions (21%) (Pastink et al., 1989).

ENU mutagenesis was investigated in zebrafish during the 1990s. Solnica-Krezel, Schier and Driever (1994) studied the efficiency with which ENU, as well as ethyl methanesulfonate (EMS), induced mutations at different stages of spermatogenesis in zebrafish (*Danio rerio*). Their results revealed that both EMS and ENU induced

mutations at high rates in post-meiotic germ cells, but that ENU was found to be more effective (Solnica-Krezel et al., 1994). Results revealed that embryonic lethal mutations induced with ENU were transmitted to offspring and that they could be recovered in an F2 screen (Solnica-Krezel et al., 1994). ENU is therefore a mutagen that is desirable for mutagenesis studies with fish, since high rates of mutations are achievable that allow for rapid identification of large numbers of mutant genes.

Mullins et al. (1994) developed a method to perform large-scale saturation screens for mutations affecting embryogenesis in the zebrafish. This screen was performed using zebrafish as an alternative model to higher vertebrates, such as mice, that develop *in utero*. To this end, zebrafish males were exposed to various mutagens leading to the induction of point mutations in premeiotic germ cells at a rate of one to three mutations per locus per 1000 mutagenized haploid genomes. They also performed a pilot screen in which mutagenized fish were inbred for two generations and scored for embryonic mutants. They were able to isolate 100 recessive mutations with phenotypes visible in the homozygous embryos (Mullins et al., 1994). This study demonstrates that it is possible to perform saturation mutagenesis screens in zebrafish, similar to those done in other vertebrates utilizing ENU.

Several other corroborative mutagenesis studies have been performed using ENU as a mutagen in zebrafish. Driever et al. (1996) induced mutations in spermatogonial cells by exposing male zebrafish to ENU. Mutations were transmitted to the F1 generation, and F2 families were raised. F3 embryos from sibling crosses within the F2 families were screened for developmental abnormalities. They identified mutants with phenotypes involved in specific aspects of embryogenesis. These mutations were maintained for

further characterization and were classified into categories according to their phenotypes (Driever et al., 1996). These genetic screens were fundamental because they demonstrated that mutations affecting a variety of developmental processes can be efficiently recovered from zebrafish; a vertebrate model that develops externally with a clear egg chorion. This constitutes a great advantage over other vertebrate models in which such mutations are not as easy to detect given the fact that they develop *in uterus*.

In a large-scale screen, Haffter et al. (1996) isolated mutants displaying a specific visible phenotype in embryos or early larvae of the zebrafish. Males were mutagenized with ENU and F2 families of single pair matings between siblings F1 fish, heterozygous for a mutagenized genome, were raised. Eggs were obtained from several crosses between F2 siblings and 3,857 mutagenized genomes were scored for zygotic defects (Haffter et al., 1996). They identified genes involved in early development, notochord, brain, spinal cord, somites, muscles, heart, circulation, blood, skin, fin, eye, otic vesicle, jaw and branchial arches, pigment pattern, pigment formation, gut, liver, motility, and touch response.

Driever et al. (1996) extended their pilot screens and determined the mutagenesis rate as 1 locus per 651 mutagenized genomes. Mutations were transmitted to the F1 generation, and 2,205 F2 families were raised. F3 embryos from sibling crosses within the F2 families were screened for developmental abnormalities. A total of 2,337 mutagenized genomes were analyzed, and 2,383 mutations resulting in abnormal embryonic and early larval phenotypes were identified. One of the important results of this study was that the phenotypes of 695 mutants indicated involvement of the identified loci in specific aspects of embryogenesis. They demonstrated that mutations affecting a

variety of developmental processes of interest can be efficiently recovered from zebrafish (Driever et al., 1996).

No large-scale screens have been performed to date using *K. marmoratus*. This organism has the advantage of potentially making the identification of mutations easier, because of genomic isogeny and the ease of self-reproduction. Recently, 21 clonal-isogenic laboratory stocks were established within the scientific community through microsatellite and mitochondrial DNA analysis (Tatarenkov et al., 2010). This further refined the stocks used in the first ENU pilot mutagenesis screen in *K. marmoratus* (Moore, Sucar, Newsome, Ard, Bernhardt, Bland, & Ring, 2012).

This thesis aims to demonstrate that the mangrove killifish is a useful vertebrate model for forward genetic screens. We established proof of principle that ENU can be used as a mutagen in the identification and confirmation of both zygotic and sterile mutations recovered in a F3 generation screen of *K. marmoratus*.

Sterile Mutants

The identification of sterile mutants is of particular importance due to the application of such results in reproductive biology of vertebrates. *Kmar* is useful as a vertebrate model for sterility screens because it develops externally with a clear egg chorion and egg/embryo features such as viability can be easily observed.

Sterile mutants found in zebrafish or medaka are defined by a characteristic type of oviposition: they lay non-viable eggs, infertile eggs or they do not lay eggs at all. Non-viability of embryos produced by a sterile parent is potentially the result of either a paternal effect in which sperm are defective or a maternal effect mutation. Maternal effect mutations are mutations in maternally-encoded gene products that are present in

the egg cytoplasm (Abrams & Mullins, 2009). Maternal factors control development before the activation of the embryonic or zygotic genome and are required for survival during early development. Infertile eggs are often the result of a paternal effect mutation that prevents fertilization (Bauer & Goetz, 2001). Lastly, absence of egg production can be caused by a defect in the ovotestis of the fish. Several scenarios can give rise to this phenotype, from anatomical problems preventing developing embryos to be laid, to internal gonadal defects that cause eggs or embryos to be arrested in a certain stage of development and subsequently deteriorate (Bauer & Goetz, 2001).

Sterile fish producing non-viable eggs with a maternal effect mutation were described in the zebrafish (Dosch, Wagner, Mintzer, Runke, Wiemelt, & Mullins, 2004; Wagner, Dosch, Mintzer, Wiemelt, & Mullins, 2004). These studies identified maternal effect mutants affecting development before and after the mid-blastula zygotic transition. In their screen, they were able to identify 68 maternal-effect mutants (Dosch et al., 2004; Wagner et al., 2004).

The two other sterile categories were also described in zebrafish by Bauer and Goetz (2001). They conducted a mutagenesis screen on zebrafish using ENU as a mutagen and an F2 crossing scheme to obtain homozygous mutants in the F3 generation. Histological samples of abdomens of the progeny were collected and analyzed for alteration in gonadal structure and gametogenesis and were compared with gonads of wild-type sibling fish *en masse*. A total of 125 mutagenized genomes in 81 families were screened and 11 mutations were observed that produced visible phenotypes in only one sex per family (Bauer & Goetz, 2001). They found both testes mutants and ovarian

mutations. Lastly, sterile fish that do not lay embryos were described in medaka by Morinaga et al. (2004).

One of the purposes of this thesis is to perform a genetic screen that allows the identification of not only maternal effect mutants but non-egg layers and paternal effect mutants that are all defined here as sterile.

Genetic Screening and Other Experimental Models

Reverse genetic techniques are also useful but they require knowledge of the gene that is being studied (Moore et al., 2012). On the other hand, forward genetic screens do not require pre-knowledge and are important because they enable the determination of gene function through phenotypic screening of desired mutants applicable to understanding of developmental pathways (Moore et al., 2012).

Unlike *K. marmoratus,* zebrafish is a dimorphic species, which makes genetic screens more laborious because of the time and effort devoted to husbandry and crossing, as well as the need for an extra generation for the identification of homozygous mutants (Pelegri & Mullins, 2004). The fact that *K. marmoratus* self fertilizes constitutes a benefit that makes this organism a unique vertebrate model for developmental genetic research. It reaches sexual maturity at approximately 100 days and is usually maintained in small groups in habitats having refugia, as the hermaphrodites are very aggressive. Early stages of embryonic development are visible through the translucent chorion similar to the one in zebrafish (Figure 2). The embryos are most often fertilized internally on a diurnal schedule (e.g., early morning oviposition) and nearly laid eggs can range in developmental stages significantly (Harrington, 1963). A typical well-fed fish, in the

range of 20-40 mm in length, at its peak reproductive capacity can produce 15-25 eggs per day (Moore et al., 2012).

Another advantage of *K. marmoratus* is related to its unique reproductive attributes. Since it self-fertilizes, the cycle of self-crossing can be maintained to clonal homozygosity. This does not happen with dimorphic species, like zebrafish, which need to be pair mated female X male, each generation. In this sense, *K. marmoratus* requires not only less laboratory space but also one less generation to detect homozygous recessive mutants (Dosch et al., 2004). Given the fact that males need to be paired up with females and that zebrafish researchers usually work with extended F3 families, it is only possible to detect a homozygous recessive mutant in the F3 generation in zebrafish. On the other hand, the utilization of a self-fertilizing organism like *K. marmoratus*, allows the identification of homozygous recessive mutants belonging to the F2 generation (refer to Discussion for details). Thus, *K. marmoratus* constitutes a desirable but underutilized vertebrate model organism for developmental genetics.

Purpose and Significance

K. marmoratus is being established as an alternative vertebrate model organism and it will potentially be further exploited by the scientific community in a near future. Studies on gonadal defects that make use of this fish could constitute a starting point to expand our knowledge on abnormalities causing gonadal malfunction in humans. This study can provide some insight on genetic screens in *K. marmoratus* and this will ultimately serve researches in other fields. This thesis provides novel data on developmental genetics in *K. marmoratus*, with a main objetive to discover novel genes involved in normal reproduction among vertebrates. This knowledge has the potential to

be relevant to human reproductive health and ultimately help in treating human infertility. Based on the data presented in this thesis, it is anticipated that *K. marmoratus* will become a more useful tool for genetic studies of multiple fields of comparative biology in the near future.

Chapter II

MATERIALS AND METHODS

Every experiment described in this thesis was approved by the Valdosta State University (VSU) Institutional Animal Care and Use Committee (Animal Use Protocol 00045-2012, Appendix A) under the National Institutes of Health's Office of Laboratory Animal Welfare Assurance Number A4578-01 (Moore et al., 2012).

Fish Husbandry

K. marmoratus used for this study were maintained in the VSU Aquatic Laboratory in a controlled environment at 27-30ºC on a 14 h light:10 h dark photoperiod. Larvae and adult fish were housed in either 1-L plastic food containers or 2-L breeder tanks (Fisher Scientific, Pittsburgh, PA; LBTANK, Aquatic Ecosystems Inc., Apopka, FL) in 17-18 ppt Instant Ocean (Instant Ocean Inc., Earth City, MO) [17 g/L prepared using water treated by reverse osmosis (RO water)] with biweekly water changes to avoid algae accumulation. Air holes were added to 1-L lids and all holes were covered with 10 cm x 10 cm Black UV Stabilized 1/8" mesh with a glue stick to prevent escape of fish (Industrial Netting, Minneapolis, MN). Embryos were collected from the 2-L breeder tanks by first removing the adult hermaphroditic parents using the basket inserts followed by retrieval with 3-mL disposable polyethylene pipettes. Embryos were observed with an Olympus SZX16 stereomicroscope (Olympus, Center Valley, PA). They were kept in sterile 100 X 15 mm polyestyrene petri dishes (Fisherbrand, Pittsburgh, PA) containing brackish water until hatching and then transferred to ~ 200 mL of brackishwater in plastic

food containers (Fisher Scientific, Pittsburgh, PA). Young hatchlings were reared in 250-mL containers for a month (to about 20 mm in standard length). Individuals were fed 1 mL of brine shrimp daily, with frequent water changes and subsequently transferred to either 1-L plastic containers for further growth and storage, or breeder tanks for embryo collection. Fish in 2-L breeder tanks were fed 3 mL of brine shrimp solution and fish in 1-L plastic containers were fed 2 mL, both every other day. Young hatchlings in 250-mL plastic containers were fed 1 mL daily. Fish were fed freshly hatched *Artemia nauplii* (Artemac; Biomarine, Inc, Hawthorne, CA) harvested from a commercial grade hatchery funnel daily (BS6; Aquatic Ecosystems Inc., Apopka, FL). Shrimp were hatched in a 26-32 ppt solution of brackish water that was prepared dissolving 160.0 g of Instant Ocean in 5 L of RO water plus 10 g of cysts. Salinity was confirmed using a hydrometer and the hatchery funnel was provided with constant aeriation. Cysts were given 24 h at constant aeriation to hatch. In order to prepare them for feeding, the air hose was removed to stop aeriation and the shrimp were allowed to settle for 10 min. Once they accumulated at the bottom, they were drained through a sieve made using 150 μm mesh to remove cyst shells (Aquatic Ecosystems Inc., Apopka, FL). Afterwards, brine shrimp were rinsed with zebrafish water (60 mg/1L Instant Ocean), and resuspended in 1.25 L of zebrafish water (~300 shrimp nauplii/mL). Unhatched cysts were left to settle to the bottom and discarded. This process was repeated twice. If an excess of unhatched cysts was still visible, they were removed with a 3-mL disposable plastic pipette.

Genetic Screen

The genetic screen described here is a continuation from a pilot screen where 34 fish from a parental generation were mutated using ENU (Sigma, St. Louis, MO) (Moore

et al., 2012). Briefly, from 284 fish screened in the first generation (F1), 73 were identified that produced zygotic mutants in their F2 progeny (Figure 3). Those 73 F1 fish were re-screened and 71 of them reproduced zygotic mutations in the F2 progeny in predicted Mendelian ratios.

This thesis focuses on a simultaneous screen to: a) confirm that the above zygotic mutants are heritable into the next generation (F3), b) simultaneously identify sterile F2 fish, and c) further confirm sterility in the F3 generation.

Confirmation of Zygotic Mutants

Mutations producing zygotic defects were originally identified as embryonic lethals. Therefore, surviving F2 fish are composed of a 2 (m/+): 1 (+/+) ratio (Figure 4). In other words, it is predicted that two carriers of the zygotic mutation per every wild-type fish will be found. These remaining fish (33% of them wild-type and 67% of them heterozygous carriers) were grown to adulthood and screened to confirm zygotic mutants from the previous generation. For each F2 family, approximately 10 F2 fish were raised to maturity to ensure that both m/+ and +/+ genotypes are represented and confirmatory of the zygotic mutant pattern of inheritance.

Since zygotic mutations manifest as obvious phenotypes in juveniles, they were relatively easy to identify under the dissecting microscope and compared to the previous observations. They were observed as defects in head morphology, eyes and/or tail structure, patterning, pigmentation, etc. Most of these mutations have been described in other fish species, like medaka or zebrafish, and those studies were used as a basis for comparison as previously described (Moore et al., 2012).

A minimum of 20 F3 embryos were collected from each F2 fish once or twice a week in petri plates to form an F3 clutch and both the collection date and total number of embryos was recorded. All non-viable embryos were removed and the remaining viable embryos were observed two days later. Viable versus non-viable embryos were also recorded. Embryos were observed for zygotic mutations and these were recorded as type of mutations and number of embryos displaying it. After scoring a minimum of 20 embryos, the zygotic mutation was confirmed if 25% of the F3 progeny showed the same phenotype observed for the F2 zygotic mutant of the same family and the observed was compared to the expected using a chi-square goodness-of-fit test.

Simultaneous Identification of Sterile Fish

F2 fish were simultaneously screened for sterility. A fish homozygous for a sterile mutation is predicted to fall into three different phenotypic classes. The first phenotypic class includes fish that produced an opaque 100% infertile clutch of eggs which lack the perivitelline membrane space indicative of fertilization (Class I). The second phenotypic class includes fish that produced a clutch of 100% nonviable eggs, which were fertilized due to the presence of the perivitelline membrane space, but were unable to develop beyond embryonic stages as compared to control siblings (Class II). The third and final predicted phenotypic class includes non egg-layer fish (Class III). After reaching a minimum of 20 F3 embryos collected, an F2 adult fish was considered sterile if a) 90% or more of the progeny were non-viable or non-fertilized or b) no progeny were observed for a period greater than two months after reaching sexual maturity (~7 months old).

Confirmation of Sterile Fish

In the case that an F2 fish was found sterile, it and its siblings were kept for further analysis. Since the mutation causing sterility makes it impossible to raise progeny from the sterile fish, their siblings were analyzed to confirm sterility into the next generation. In order to confirm sterile mutants, 2-8 F2 fish (siblings of a sterile F2) were allowed to self-cross to produce an F3 family (Figure 5). These F3 fish were raised to maturity and screened for sterility. Each of these was raised in a separate breeder tank and embryos were collected following the same procedure described before. Embryos were collected three times a week and classified as viable or non-viable. Upon initial collection, all non-viable embryos were discarded, while the remaining viable embryos were observed two days later and scored as viable or non-viable. If characterization was difficult, embryos were observed under a dissecting microscope to assess viability.

Once a fish was categorized as sterile (Class I or II), additional embryos were subsequently collected to a total of n = 100 to further confirm the result and assess penetrance. If a fish was found that did not meet the sterility criteria during this period, it was categorized as +/+ or m/+ depending on its siblings' phenotypes.

Photography of Embryos and Fish

The chorion of *K. marmoratus* is rather difficult to dissect away from developing embryos. Different protocols were tried (Mourabit, Edenbrow, Croft, & Kudoh, 2011) but all of the images in this study are of embryos with intact chorions. Digital images were recorded using an Olympus DP72 camera mounted to an Olympus SZX16 stereomicroscope (Olympus, Center Valley, PA).

Statistics

Sigma Plot (Systat Software, Inc., San Jose, CA) was used to run a Mendelian chi-square analysis for the Confirmation of Zygotic Mutants, Simultaneous Identification of Sterile Fish and, Confirmation of Sterile Fish.

Chapter III

RESULTS

From an initial pilot forward genetic screen in *K. marmoratus*, 34 fish from the parental generation (P) were mutagenized using 2.8-3.8 mM ENU (Moore et al., 2012). Briefly, P fish were exposed to ENU three times a week for one hour and allowed to self-cross to produce 7,350 embryos in the F1 generation. Approximately 18% hatched (n = 1,334) and 284 were screened to score for recessive zygotic mutant defects. As a result of this pilot screen, 73 F1 fish consistently produced 25% progeny showing zygotic defects in a recessive pattern as expected (m/m; Figure 3). Zygotic mutants were classified into eight main phenotypic categories: No tail, Curved tail, Eye/skull defects, Jaw/mouth defects, Dwarf, Fluid pouch, Unresolved, and Early embryonic lethal (see Moore et al., 2012 for classification details). Subsequently, these 73 F1 fish producing zygotic mutants were re-screened to further confirm the initial observations. From >2,000 F2 embryos produced by 73 self-crossing F1 fish, 71 F1 fish (97%) reproduced the previous recessive zygotic mutant phenotype in their F2 offspring, as previously identified in the pilot screen (Moore et al., 2012).

A continuing genetic screen into the next generation is described in order to: a) confirm zygotic mutant allele heritability and b) simultaneously score for homozygous recessive mutant sterile F2 fish (s/s). Furthermore, the sterile mutants were confirmed into the F3 generation to prove mutations were heritable.

From a total of 307 F2 fish screened, 10 were found to be 1^0 males and 16 were

found to be sterile (Table 1). From the remaining 281 F2 fish, 92 were wild-type and 189 were carriers of zygotic recessive alleles (m/+).

As part of this simultaneous screen, an average of 6.5 fish were screened per family. The range of F2 fish screened was from 2 to 13 (Table 1). Primary males were found ranging from 0 in some families up to a maximum of 2 in some others and totaled 3.6% of the total number of fish screened. This is consistent with natural observations of males (either primary or secondary) found in nature (Laughlin, Lubinski, Park, Taylor, & Turner, 1995; Tatarenkov et al., 2012).

Sixteen sterile mutant hermaphrodites showing maternal effects (non-viable embryos, NV) or ovotestis defects leading to sterility (s/s) were identified. On average 1.33 sterile fish were observed per family.

Confirmation of Zygotic Mutants

From the remaining 281 F2 fish, 92 F2 were wild-type (33%) and 189 carried the zygotic recessive allele (67%; Figure 4). Fish were identified as carriers of a zygotic mutation because they produced 25% of F3 progeny showing a zygotic defect that was found in the previous generation. As part of the zygotic confirmation, the zygotic mutant categories were expanded because some new phenotypes were consistently observed (Figure 6). Most of the zygotic phenotypes discovered in the pilot screen were confirmed. Examples of these phenotypes are Curly tail, Jaw/mouth defects, Dwarf or Eye defects. Interestingly, new categories were consistently found, such as Short tail, No trunk-no tail or Golden yolk. Finally, embryos classified as “Unresolved” were found, which were not further characterized but had consistent Mendelian patterns of inheritance.

The most obvious zygotic defects were those affecting morphology of the tail.

Curly, shortened, reduced, and absent tail fin fish were found. Curly tail mutants were found in which the tail was either curled-down or curled-up with various degrees of penetrance as compared to wild-type siblings (Figure 7).

One of the families screened, R228, consistently gave a Short tail phenotype (Figure 8). For example, F3 embryos from two different members descended within this family gave the same characteristic short tail morphology. The image on the left and the one in the center show two short tail F3 embryos from the same F2 fish in each case. The mutant shown on the right is an F3 embryo with the characteristic no tail/short tail that just hatched. They usually do not survive past this stage and more generally die before hatching. This phenotype was also found in zebrafish resulting from a mutation in a gene called *no tail* (*ntl*; Amacher, Draper & Summers, 2002). This is an ortholog of the *Brachyury* gene, a member of the T-box family of transcription factors, originally discovered in mice (Dobrovolskaïa-Zavadskaïa, 1927). Mutant alleles of the zebrafish *Brachyury* ortholog (to mice), *ntl*, showed graded effects on the development of posterior structures (Papaioannou & Silver 1998). A homozygous *Brachyury* mutation in mice is lethal at around embryonic day 10. In contrast, mutants of this gene in zebrafish thus far identified display different effects than in the mouse. Unlike the mouse, zebrafish heterozygous for mutations in the *Brachyury* orthologue of *ntl* do not exhibit any obvious phenotypic defects indicative of recessive mode of inheritance. Therefore, zebrafish have to be homozygous for *ntl* mutations to show a phenotype that closely resembles the mouse mutants (Showell, Binder, and Conlon, 2004). Zebrafish *ntl* mutant embryos fail to form a differentiated notochord, lack posterior structures, and have abnormally shaped anterior somites. In zebrafish, the *no tail* gene is transiently expressed in cells of the

endoderm and the mesoderm with a more persistent expression in the prospective notochord (Schulte-Merker, Eeden, Halpern, Kimmel, & Nüsslein-Volhard, 1994). In our putative *ntl K. marmoratus* mutant, we hypothezise that a mutation was induced in the *ntl* orthologue causing this shortened tail phenotype. Further molecular characterization of the *ntl* mutant alleles is required to confirm this hypothesis.

R109 was a family that consistenly showed a characteristic No trunk-no tail phenotype (Figure 9). It was hypothezised that this is caused by an ENU-induced mutation in the fetal growth factor receptor 1a gene (*fgf1a)*, that is required for proper mesoderm formation (Griffin, Patient, & Holder, 1995). During the blastula and gastrula stages, vegetal cells (presumptive endoderm), release signals to marginal zone cells resulting in the induction and patterning of the mesoderm. One of these signals, *fgf1a*, achieves this through the regulation of T-box transcription factors. FGF could be of importance to our study as a regulator of T-box genes. Griffin et al. (1995) analyzed the roles of *fgf1a* activity and *Brachyury* during gastrulation in zebrafish embryos. They compared the consequences of inhibiting the FGF-receptor signaling with the phenotype of the zebrafish *ntl* mutant and found that expression of *ntl* is regulated by *fgf1a*. Inhibition of FGF receptor-signaling leads to complete loss of the trunk and tail. However, the zebrafish *ntl* mutant lacks the tail and notochord but has an otherwise normal trunk. This demonstrates that trunk development is dependent upon an unidentified gene, or set of genes, referred to as no trunk (*ntk*) which is regulated by FGF (Griffin et al., 1995). The *K. marmoratus* mutants fit into this category (FGF function inhibited), since they showed no trunk. For example, F3 embryos from several F2 parents of the R109 family produced the No trunk phenotype characterized by the absence of tail

and trunk (Figure 9). It is important to mention that this phenotype, which is especially easy to visualize in the third picture of the upper panel (R109-6), was previously described as “Headfish” in medaka by Yokoi et al. (2007). The morphology of the mutant medaka embryo is quite similar to findings presented in this research and basically consists of a fully developed head devoid of trunk and tail.

Another new category referred to as *golden yolk,* consistently seen in members of the R058-2 family throughout several developmental stages, was identified during the simultaneous screen (Figure 10). It was predicted to be a maternal effect as the characteristic golden oil droplets are also visible in unfertilized embryos (Figure 11).

Phenotypes were found which confirmed our observations in the pilot screen. For example, F3 embryos with Jaw/mouth defects were observed in the R176 and R210 families (Figure 12). Eye/skull mutants were confirmed in several families (Figure 13). Embryos from R234-4 had a characteristic larger eye, while embryos from R182-2 s were missing an eye. Eyes were absent in members of the R201 family, which was also characteristic in members of the R152 family, where some of the embryos with no eyes actually survived to the larval stage. F3 embryos from the R240 and R201 families had forward eyes and skull defects, and members of the R201 family had a flat head.

Lastly, mutants were found that were categorized as “other mutants” (Figure 14). Embryos from the R159 family had an unresolved phenotype where no tail or fins are visible and basic structures such as eyes or mouth are not identifiable because the embryo looks somewhat like a mass of cells. In the center panel, an embryo from the R240 family looks normal but contains some kind of appendix in one of the sides that we cannot classify as any normal structure. The panel to the right shows an image from the family

R149 in which the embryo looks thinner than normal, the eyes are not fully developed and some pigmentation pattern defects are observable. Finally, the mouth/jaw does not look completely developed.

Zygotic confirmation screening revealed fish from several families that showed a characteristic hyperpigmentation; they were defined as hypermelanistic (Figure 15). It is not believed that these are true zygotic defects passed by Mendelian inheritance. There is a chance that they could be related to other type of mutation, such as eye defects. Regardless, these fish develop normally and grow to adulthood without any further complication. In two of these families, R096 and R152, the fish have no eyes and posses a typical V-shaped jaw. Their body structure is somewhat twisted. These fish have eye defects that follow Mendelian inheritance patterns. Alternatively, hyperpigmentation may result as an adaptive response to their environment. Since they are not able to see, this poses an extra selective force. They could potentially change their coloration to a darker one as a way of protection. Even though this is just a speculation, *K. marmoratus* exhibits phenotypic plasticity in response to environmental conditions (Earley, Hanninen, Fuller, Garcia, & Lee, 2012). In both of these cases, viable progeny could not be collected to determine if the phenotype is also visible in the next generation. In the R096 family, R096-9 lays 100% NV embryos. In the family R152, R152-10 does not lay embryos.

In another family, R058, embryos show the hypermelanistic phenotype but have normal eyes. In such a case, this could truly be a pigment defect that is passed by Mendelian inheritance. Interestingly, this family is the same one that produces the *golden yolk* phenotype that may be a carotene defect. Within this family, progeny from R058-7 were raised and hyperpigmentation observed in embryos belonging to the F4 generation.

Finally, several two-headed fish were found. These are visible very early in development and even though they are often lethal during pre-hatching stages, sometimes the embryos survive through larval stages, as seen in the last panel (Figure 16).

Among the F2 fish screened, wild-types (+/+) were found (ranging from 0 to 6 in some families) and carriers (m/+) were observed more often as expected (ranging from 0 to 9 per family). On average, two wild-type fish per family and four carriers of the zygotic recessive allele per family were found (Table 1). These observed genotypes of the F2 fish were as expected ($p < 0.05$).

Simultaneous Identification of Sterile Fish

A total of 9,095 F3 embryos were scored during the simultaneous screen. These embryos belonged to either wild-type F2 fish (+/+), carriers of the zygotic recessive allele (m/+), or sterile mutant hermaphrodites (s/s). Sterile F2 fish observed during the simultaneous screened had either maternal effects or ovotestis defects (see Materials and Methods regarding predicted sterile phenotypic classes). Fish with ovotestis defects typically do not lay embryos because they are arrested in early stages of development and remain in the gonad or never completely develop. In some cases, fish with these defects, laid a very low number of embryos that most likely die. This means that from the total number of F3 embryos scored that belong to s/s fish, the majority correspond to F2 parents showing a maternal effect. On average, 21 F3 embryos from each F2 sterile fish were collected. The total number of embryos collected from all F2 steriles was 334, with a range from 0-78 per fish (Table 2).

An average of 30 F3 embryos from each F2 fish, ranging from 0 to 164 embryos per fish were collected. Since carriers were found in greater numbers than wild-types, as

was expected, the number of F3 embryos laid by carriers is much higher than the total number of F3 embryos from wild-type fish. Carriers of the zygotic recessive allele laid 6,131 F3 embryos, while wild-type F2 fish laid 2,630 F3 embryos. On average, 29 embryos per wild-type F2 fish and 32 per carrier were scored. For wild-type F2s, embryos collected ranged from 3 to 164 and for carriers the number of embryos collected ranged from 4 to 89 per fish.

Finally, a new category identified during the simultaneous screen, previously mentioned as *golden yolk,* constitutes a maternal effect viable phenotype. This phenotype was consistently observed in members of the R058-2 family throughout several developmental stages from blastula to adult fish (Figure 10). The golden oil droplets are very easily seen during early stages (i.e., blastula or gastrula) and they become less apparent as the fish develops. They are still observable at the hatching stage followed by characteristic orange fins in the larvae and adult fish. These characteristic oil droplets are also visible in unfertilized embryos (Figure 11), revealing a maternal defect that could be pigment related possibly from a carotene defect passed through the oogenesis into the zygotic stages of development.

Goodness-of-Fit Analysis of Data

Data was also analyzed by a Mendelian chi-square test (Table 3). The null hypothesis states that observed values are significantly close to the expected ones ($p = 0.05$). Since there are two possible outcomes, either +/+ or m/+, degrees of freedom (df) = 1. From a total of 281 F2 fish scored, 94 (1/3) were expected to be wild-type and 187 (2/3) were expected to be carriers of the zygotic recessive allele. The observed numbers were very close to the X^2 expected ones, with 92 wild-types and 189 carriers

found. Using a p-value of 0.05, df = 1, with a critical value of 3.84, the null hypothesis failed to be rejected since the X^2 = 0.0510. Thus, the zygotic confirmation data closely matched X^2 expectations.

Confirmation of Sterile Fish

In order to confirm sterile mutants, fish from the F3 generation of siblings of sterile mutants were grown. A total of 284 F3 fish belonging to 10 previously identified sterile families were screened (Table 4). This coresponds to 46 F2 siblings that were raised to sexual maturity to produce the 284 F3 fish screened. An average of 4.6 F2 siblings were set up per family, ranging from 1 to 8 F2 fish per family.

From the 284 F3 fish screened, 12 were found to be primary males, with a range of 0 to 2 males per family; an average of 0.26 males per family (Table 4). From the remaining 272 F3 fish, 69 were wild-type (+/+), 83 were sterile (s/s), and 120 were either wild-type or carriers, referred to as */+ because their genotypes are as yet undefined. In order to determine their genotype, their progeny would need to be raised to maturity and scored for continued transmission of mutant alleles. Therefore, it should be possible to determine if they are either wild-type (+/+) or if carriers of mutant sterile alleles (s/+) in the future. For the confirmaton into the F3, this was not necessary and this is why they are only classified as */+ *en mass* in this study.

Sterile F3 fish ranged from 1 to 10 per family and in an average of ~ 2 F3 sterile fish (1.8) per family (Table 4). Wild-type F3 fish ranged from 0 to 9 depending on the family (avearge of 4.6 fish). An average of 4.14 fish with a */+ genotype were found per family, ranging from 0 to 8.

F4 embryos collected during the sterile confirmation screen were analyzed for viability (Table 5). A total of 8,439 F4 embryos were scored, with an average of 31 per F3 parent. The range per fish varied from 0 to 119. From the total of 8,439 F4 embryos, 3,922 were viable and 4,516 were non-viable. The number of non-viable embryos was observed to be higher than the number of viable ones, which is expected from a sterile confirmation screen. An average of 14 viable embryos were collected per F3 fish, ranging from 0 to 42 and an average of 17 non-viable embryos were scored per fish, ranging from 0 up to 112.

Data was analyzed using a chi-square test (Table 6). From the total number of 46 F2 siblings screened, 15 (1/3) were expected to show a wild-type phenotype (+/+) and 31 (2/3) were expected to be carriers of the recessive sterile allele. The observed number of wild-types was 14 and the observed number of carriers was 32. The resulting X^2 value was 0.1739, which means that the null hypothesis was not rejected using a p-value = 0.05.

Data was analyzed by observing what happened within each family (Table 7). Table 7 shows these results for the 10 families that made it through the confirmation screen. For the R176 and R247 families, the sterile mutation was lost since no sterile F3 fish were found during the confirmation screen probably due to low numbers of F3 fish raised to sexual maturity. For five of the families the percent of F3 sterile fish among siblings descended from F2 s/+ parents closely matched the expected 25%. For example, within the R015 line, exactly 25% F3 fish were sterile. For the R010 and R019 families the percent of F3 sterile fish was 30 and 27.66, respectively. For the R182 and R194 families the percent of F3 sterile fish was 17.9% and 16.7%, respectively. For 3 of the

families, we observed a higher than expected number of sterile fish. For the R002 and R103 families, 55.6% and 53.6% respectively of the F3 fish among siblings descending from F2 s/+ parents were sterile. Finally, 71.4% sterile progeny were found in the R152 family which was much higher than the expected percentage (25%) for simple recessive mutation. In some cases, all F3 fish from an F2 parent are sterile. These 3 sterile mutant alleles are likely the result of partial dominant maternal effects.

Data corresponding to the F3 generation from the sterile confirmation screen was also analyzed using a chi-square test (Table 8). Only data belonging to five of the recessive lines was included. The R176 and R247 lines were excluded because, according to the results, they lost the sterile alleles. The R002, R103 and R152 families were also excluded from the chi-square test because they unexpectedly resulted in a very high percentage of steriles. More specifically, in two of these lines, R103 and R152, all F3 fish from an F2 parent were sterile.

The test focused on F3 fish from families R010, R015, R019, R182, and R194 that descend from and F2 s/+ parent. If these families follow Mendelian inheritance, it is expected that 75% of the F3 progeny will result s/+, +/+ or +/s. Since these genotypes cannot be distinguished if the next generation is not raised, they are symbolized as */+. Following the same logic, the rest of the F3s, which correspond to 25% of the progeny, were expected to be recessive sterile mutants. From a total of 111 F3 fish descending from F2 s/+ parents that were analyzed, 83 were expected to be */+ and 28 were expected to be s/s (Table 8). Eighty-four */+ and 27 s/s fish were obtained. Using a p-value of 0.05, null hypothesis cannot be rejected given that $X^2 = 0.0270$ is below the critical value 3.84.

Few of these sterile families showed either a maternal effect or an ovotestis defect, whereas most of them showed a combination of both (see Table 8). Maternal effect sterile mutants are characterized by laying 100% non-viable progeny. Embryos are fertilized, as evidenced by a perivitelline space, but progress to non-viable during early development (Figure 17). For example, F3 embryos belonging to the R152 family developed into non-viable embryos by day 2. The yolk retracts and shrinks, the mass of cells degrades, and the embryo finally turns into a degraded chorion characteristic of death.

Fish from a category that termed embryo holders were also identified (Figure 18). These fish are suspected to posses an anatomical defect causing them to hold embryos. In this case, embryos are actually formed and fertilized, but the anatomical defect prevents the fish from laying their embryos. For example, the R171 family displays the embryo holding phenotype. In these mutants, the abdominal region enlarges as embryos accumulate (Figure 18A) and fish have severe difficulties laying embryos causing cloacal damage (Figure 18B). Figure 18C shows in more detail the large number of embryos that the fish is trying to hold and Figure 18D shows an enlarged view of an embryo in an advanced stage of development.

Pedigree Analysis

As a summary of results from the sterile confirmation, a pedigree of the R010 sterile mutant family is presented that serves as an example to better visualize inheritance of a mutant allele s within a family (Figure 19). A fish from a parental generation P was mutated utilizing ENU. After it was allowed to self-fertilize, heterozygous F1 fish carrying the sterile allele s were produced. When this heterozygous F1 fish (s/+) self-

fertilized, it was expected that they produce 25% homozygous recessive sterile mutants (s/s) in the F2 generation. In the case of family R010, 5 F2 fish were screened. By analyzing the F3 progeny and backward genotyping, one of them (20%) was classified as sterile because it either yielded no progeny or 100% non-viable progeny. The four remaining F2 fish analyzed were also characterized after screening the F3 progeny. In this family, two of them (40%) were wild-type for the sterile mutation s (+/+) and the two remaining progeny (40%) were carriers of the sterile allele s (s/+ or +/s). Wild-type F2 fish were characterized as such because all of their F3 progeny are wild-type for the sterile allele. On the other hand, carriers are identified as such because 25% of their F3 progeny are sterile homozygous recessive mutants.

Chapter IV

DISCUSSION

In the present study, it was demonstrated that ENU is a useful mutagen for forward genetic screens in *K. marmoratus*. Zygotic mutants in the F2 generation were identified that were confirmed into the F3 generation. This study, which is a continuation of the Moore *et al.* (2012) pilot screen, serves as proof of principle that *K. marmoratus* is a model organism that can be used in forward genetic screens in the future. Different common phenotypic classes of zygotic mutants were identified and confirmed, some of which have been previously described in zebrafish (*Danio rerio*) and/or medaka (*Oryzias latipes*) screens. For example, embryonic lethality, tail defects, or head structure defects have all been described in zebrafish (Mullins et al., 1994; Solnica-Krezel et al., 1994). A family was identified (R228) that consistently gave a Short tail phenotype (see Figure 8); a zygotic defect that has been previously identified in zebrafish (Amacher et al., 2002). The gene responsible for this phenotype in zebrafish is called no tail (*ntl*) and it is hypothesized that a mutant allele of an *ntl* orthologue in *K. marmoratus* is causing this shortened tail phenotype. Another interesting zygotic category that was consistently found during the screen is the No trunk phenotype (see Figure 9) which is potentially produced by a mutation in the *fgf1a* signaling factor. Studies in zebrafish have analyzed the role of the *fgf1a* signaling factor, which participates in mesoderm formation, during gastrulation (Griffin et al., 1995). Griffin et al. (1995) reported that expression of *ntl* is regulated by *fgf1a*. Inhibition of *fgf1a* receptor-signaling leads to complete loss of the

trunk and tail, however, the *ntl* mutant lacks the tail and notochord but has an otherwise normal trunk. This demonstrates that trunk development is dependent upon an unidentified gene, or set of genes, referred to as No trunk which is regulated by *fgf1a* signaling pathway (Griffin et al., 1995), which has also been reported (described as "Headfish") in medaka (Yokoi et al., 2007). Headfish mutants found in this study are analogous to the medaka headfish mutant since they show a characteristic absence of tail and trunk.

A future goal fo this research is to isolate DNA from embryos with the Short tail and No tail-no trunk phenotype to further characterize these mutations. The aim of this work is to sequence the DNA and search for point mutations using available data for the homologous genes in zebrafish and medaka.

Mutants from the golden yolk category that were discovered are currently being raised to build a homozygous recessive stock. This is the only maternal/zygotic defect that was not lethal and proved to be true-breeding during screening. At this writing, members of the F2, F3, and F4 generation all expressing this unique phenotype are being raised in the VSU Aquatic Laboratory (Figure 10). A future direction of interest is to analyze this mutation in detail to confirm its maternal effect expression and to determine if it is the result of a defect in a carotene synthesis biochemical pathway. This novel mutation is of special importance because it could result in useful markers for outcrossing and genetic mapping in *K. marmoratus* due to its zygotic phenotype in later stages of development.

It is anticipated that *K. marmoratus* will become a model organism for various genetic screens because of its evident advantages as a vertebrate model of development.

Compared to dimorphic species (male and female members), such as zebrafish, it requires one less generation (Dosch et al., 2004) during mutagenesis when utilizing the hermaphroditic parent. It is easy to maintain and grow in the laboratory and does not need as much husbandry effort as does zebrafish which requires growth in stagnant brackish water and feeding with a single source of brine shrimp from hatching to adulthood. As a result husbandry space is reduced in comparison to similar screens performed in other established models like zebrafish or medaka that require a higher number of F2 fish and extensive labor performing crosses between males and females.

Even though self-fertilization reduces by one the number of generations needed for genetic screens and simplifies animal husbandry, there is a possibility for deleterious mutations to accumulate through self-crossing which increases lethality via inbreeding. However, true breeding stocks such as golden yolk were selected, thereby overcoming the deleterious mutation load.

The approach used in the present study has the benefit of allowing the identification of zygotic mutants in the F2 generation and the confirmation of zygotic recessive mutant alleles and simultaneous identification of steriles into the F3 generation. When the F2 generation is grown to adulthood and their F3 progeny screened, it is possible to simultaneously confirm zygotic mutants and detect sterile F2s. It is not necessary to look at F4 embryos to identify steriles, only to confirm them. Analogous studies on zebrafish where natural crosses are performed following ENU treatment, for example, require screening into the F4 generation among extended cousin families with 1/16 fish among intracrosses predicted to find sterile mutants (Dosch et al., 2004; Wagner et al., 2004). Also, in order to avoid interbreeding, and since a mapping scheme is

involved in these type of zebrafish studies, F1 heterozygous fish cannot be crossed to their heterozygous siblings (which would allow for collection of homozygous steriles in the F2 generation). F1 fish are crossed with fish from a different strain that carry a mutation but are wild-type for the mutation of interest. This introduces one more generation for the identification of steriles. In an effort to reduce time and space, zebrafish researchers work with what they call extended F3 families, which consists of an artificial F3 generation resulting from several individual crosses that are pooled together in the same tank. The *K. marmoratus* screen has the advantages of reduced space and the identification of steriles, since the chance of finding a *K. marmoratus* sterile is 25% and only 6.25% in zebrafish.

A great advantage in using *K. marmoratus* is the small number of fish necessary and hence number of genomes to be screened required for identifying homozygous sterile mutants in comparison to zebrafish. This is not necessarily related to the type of screen performed but it is inherently derived from the utilization of the only known self-fertilizing fish. In order to obtain 90% maternal effect mutants, 35 individual F3 intracrosses are required in zebrafish sterile screens. When *K. marmoratus* are utilized, only 8 F2 fish need to be set up to obtain an equal probability of 90%. Not only is the number of fish needed much smaller but they also belong to a previous generation and do not need to be crossed. Therefore, multiple screens (zygotic and sterile) can be performed in *K. marmoratus*.

Results from the sterility screen allowed the identification of fish possessing either a maternal effect or ovotestis defects. Paternal effect mutants, analogous to those reported in zebrafish with reproductive defects affecting spermatogenesis (Bauer &

Goetz, 2001), were not found and this constitutes a potential avenue for future investigations.

Newsome (2011) identified three F2 sterile mutants (R002F, R010A, and R019C) out of 35 fish in a preliminary pilot screen. Upon further analysis of these lines, two produced 100% nonviable offspring (R002F & R010) and one was a non-egg layer mutant that did not produce any embryos after reaching sexual maturity (R019C). Histological examination of the ovotestis of the R019C fish were indistinguishable from tissue observed in wild-type fish. However, inflammatory cells surrounding oocytes were also observed, which are indicative of an ovarian anomaly (Newsome, 2011). To establish that this inflammation phenotype is indeed a heritable mutant allele, the non-egg layer phenotype was confirmed across F3 fish descended from the R019 family. Therefore, these confirmatory fish need only be subjected to histological examination to further confirm the phenotype from our confirmed genotype. Among the 21 non-egg laying mutants identified and confirmed in the extensive screen, gonadal defects were predicted to be seen at the histological level. This is of special importance for non-egg layer mutant fish because it is of interest to determine what happens inside the mixed gonad leading to sterility. In this way the specific ovotestis mutant phenotype, such as developmental arrest of oogenesis, or major anatomical defects leading to fish that are unable to ovideposit (hold) fully developed embryos, might be identified. In summary, we expect visible defects to be observed in the ovotestis of non-egg layer sterile fish by histology in future work.

Other future directions stemming from this project include the identification of genes responsible for sterility in *K. marmoratus.* The identification of these genes could

potentially contribute to a better understanding of sex determination anomalies in humans. In short, there is much research left to further characterize the sterile families identified in this genetic screening and to identify the stages in which the non-viable mutant embryos die within each family to explore the causes of lethality at the cellular level.

It would also be important to know more about sterile families showing both maternal effects and ovotestis defects because these have not been discovered simultaneously in other fish models. Other fish models, such as zebrafish, do not possess a mixed gonad characteistic of *K. marmoratus*. Maternal effects and ovotestis defects could potentially result from the same mutant allele that shows penetrance effects. Further research is needed to address this hypothesis.

K. marmoratus is a useful model organism and will potentially be utilized by more researchers in the future given the simplicity and advantages for genetic screenings. Studies on gonadal defects in *K. marmoratus* could constitute a starting point to expand the knowledge on abnormalities causing gonadal malfunctioning in humans. If single genes are discovered, or genetic pathways better understood, this type of work could eventually be used to advance human reproductive health, for example, in treating human sterility.

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APPENDIX A

Valdosta State University Institutional Animal Care and Use Committee Approval

Appendix A. Valdosta State University Institutional Animal Care and Use Committee (IACUC) Animal Use Proposal approval for the work presented in this Thesis.

VALDOSTA STATE UNIVERSITY
Building for Our Next Century

June 12, 2012

Dr. Brian Ring
Department of Biology
Valdosta State University

Dear Dr. Ring:

Your Animal Use Protocol, "*Krptolebias marmoratus* (mangrove killifish) research" (AUP-00045-2012) has been approved by the Institutional Animal Care and Use Committee (IACUC). This approval is for the period of June 12, 2012, through June 11, 2015. Each year, an annual review and report must be submitted to the IACUC to keep your protocol active. You will be contacted by the Office of Sponsored Programs and Research Administration approximately two months before the annual review and report is due.

Please remember that you must obtain IACUC approval before amending or altering the scope or procedures of the protocol. You are also required to report to the Attending Veterinarian, the IACUC Chair, and the IACUC Administrator any unanticipated problems with the animals which become apparent during the course, or as a result, of the research or teaching activity.

If you have any questions, please contact the IACUC at iacuc@valdosta.edu.

Sincerely,

Barbara Gray

Barbara Gray
IACUC Administrator

cc: Dr. Karla Hull, Institutional Official
Dr. Theresa Grove, IACUC Chair
Dr. Teresa Doscher, Attending Veterinarian
Dr. Robert Gannon, Biology Department Head

Office of Sponsored Programs & Research Administration
Location Regional Center for Continuing Education • 903 N. Patterson St. • **Address** 1500 N. Patterson St. • Valdosta, GA 31698–0429
Phone 229.259.5045 • **Fax** 229.245.3853 • **Web** www.valdosta.edu/ospra/

A Regional University of the University System of Georgia & an Equal Opportunity Institution

APPENDIX B

Figures 1-19

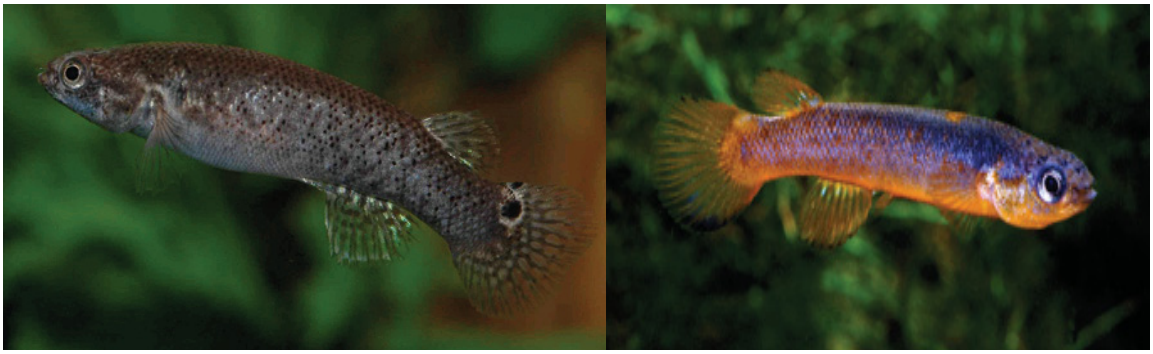

Figure 1. *K. marmoratus.* Left: hermaphrodite. Right: primary male.

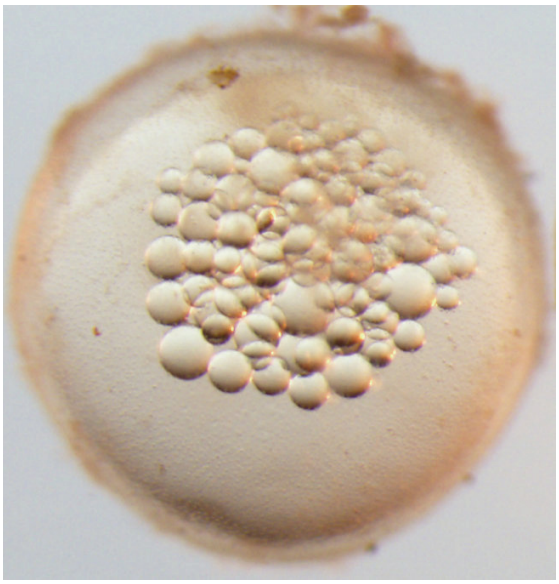

Figure 2. Recently fertilized *K. marmoratus* egg. Small translucent balls are oil droplets of yolk.

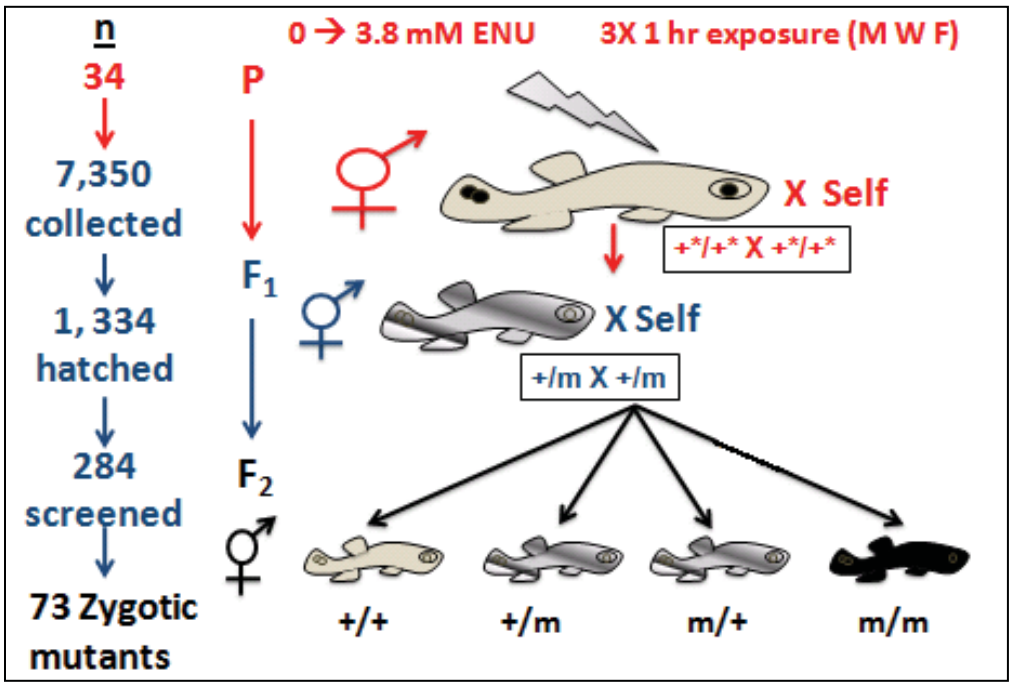

Figure 3. Pilot screen scheme.

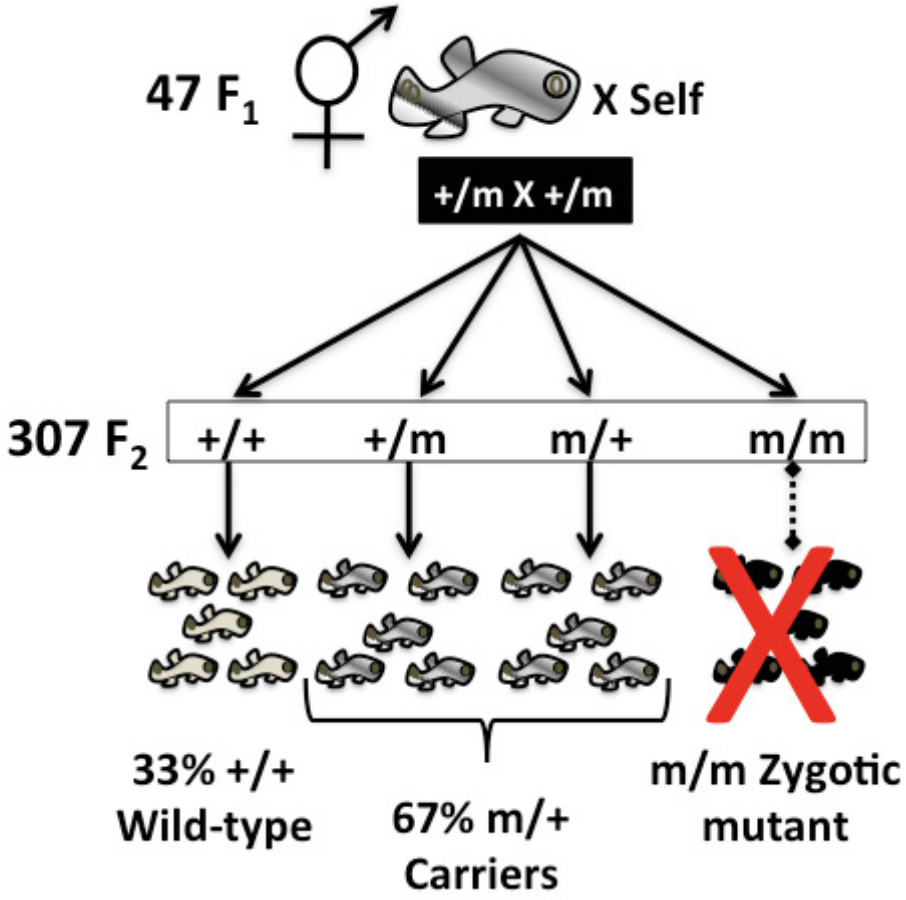

Figure 4. Confirmation of zygotic mutants.

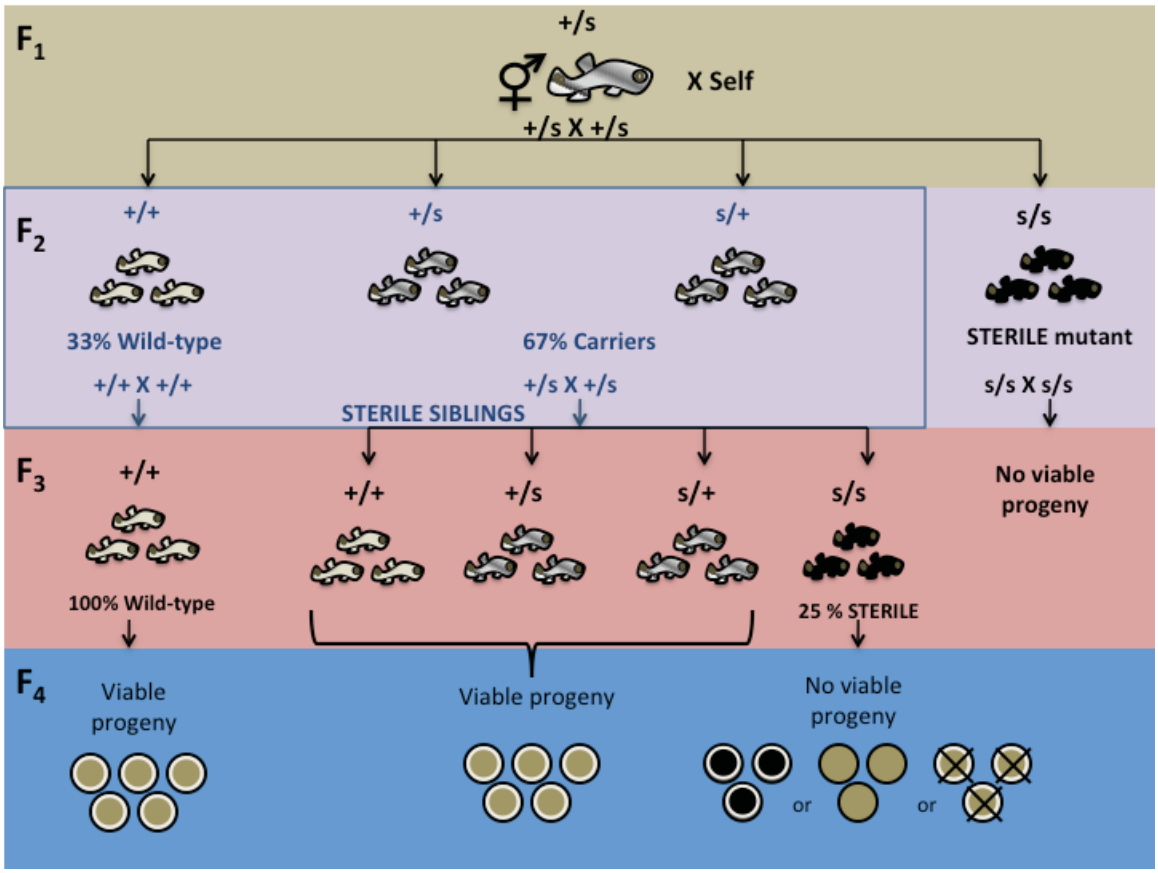

Figure 5. Sterile identification and confirmation squeme.

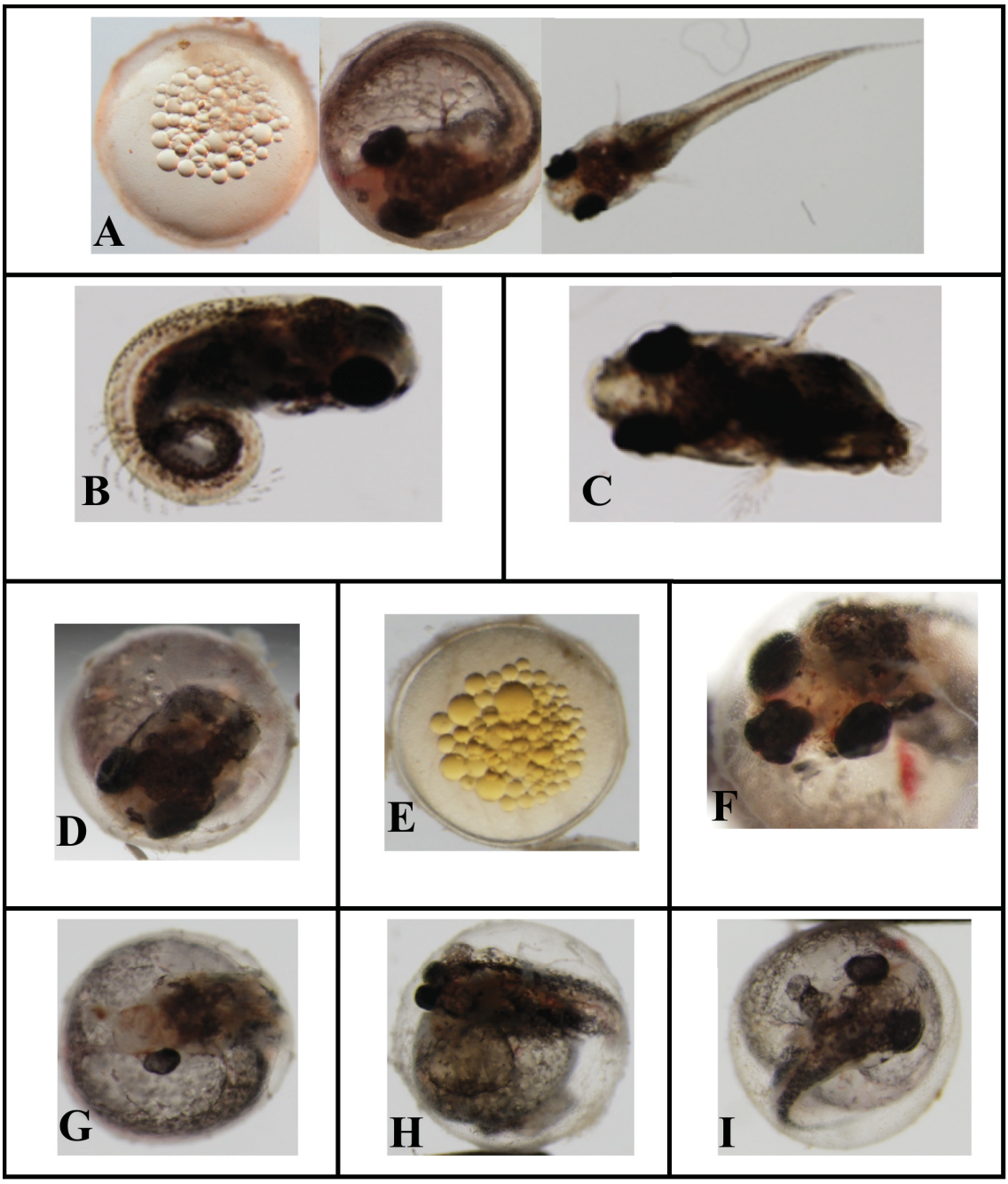

Figure 6. Zygotic phenotypes found in confirmation screen.

A. Wild-type *K. marmoratus* early stage embryo (left), 14 dpf embryo (center) and larvae (right).

B. Curly tail	C. Short tail	D. No trunk	E. Golden yolk
F. Jaw/mouth defects	G. Eye defects	H. Dwarf	I. Unresolved

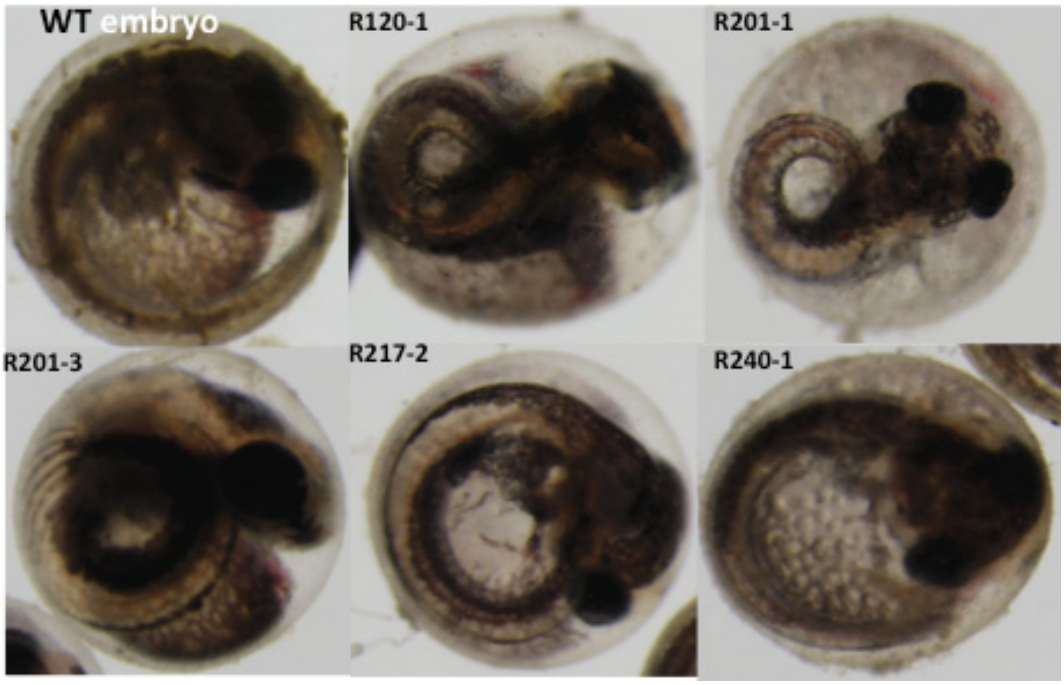

Figure 7. Curly tail mutants. Curled-up (top panel), curled-down (bottom panel).

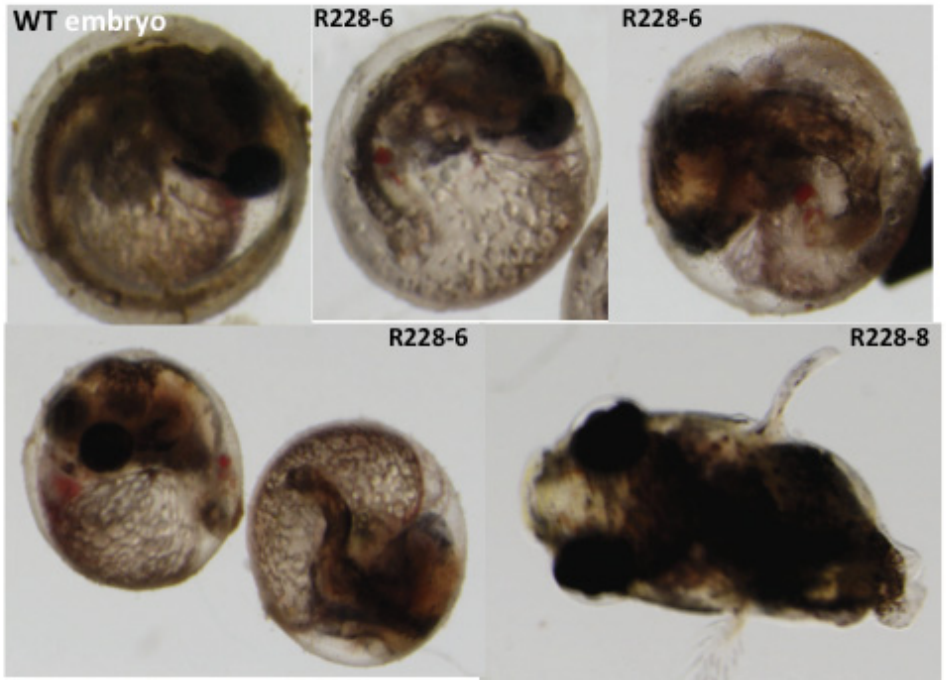

Figure 8. Short tail F3s from the R228 family.

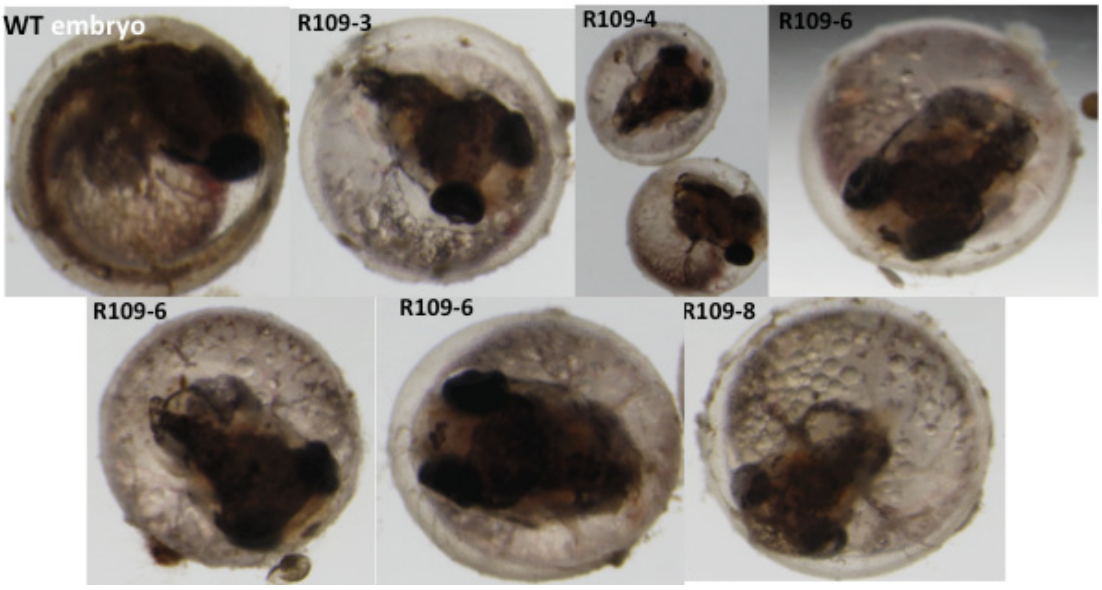

Figure 9. No trunk F3 embryos from the R109 family.

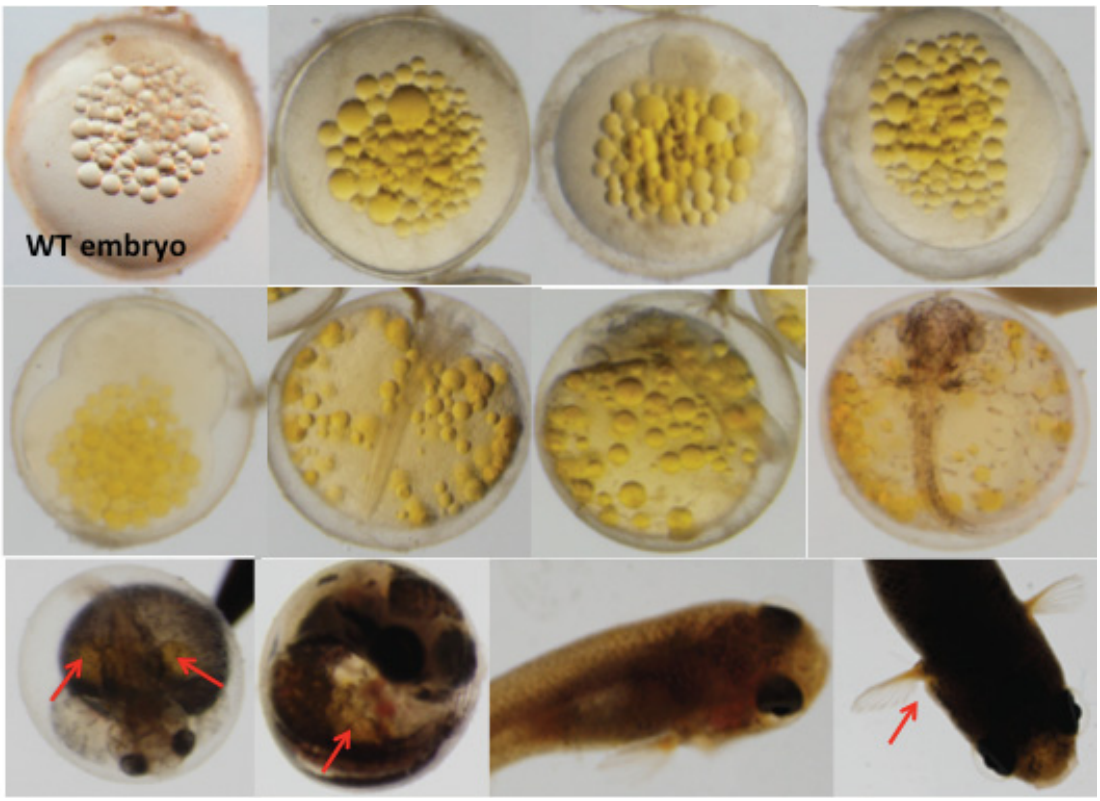

Figure 10. Golden yolk mutants from the R058-2 family. Developmental stages from blastula to adult fish.

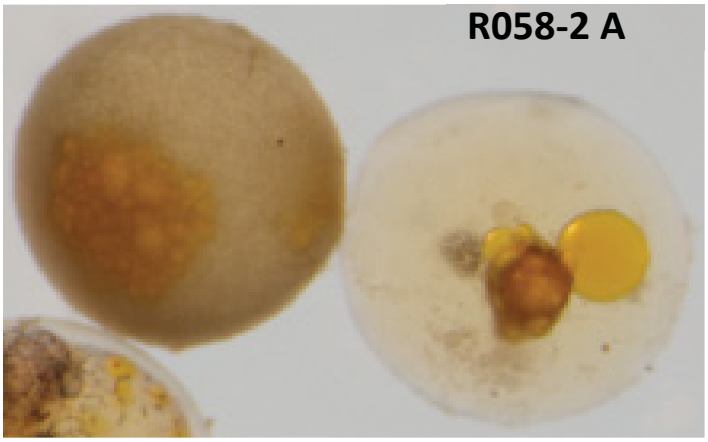

Figure 11. Non-fertilized golden yolk embryos.

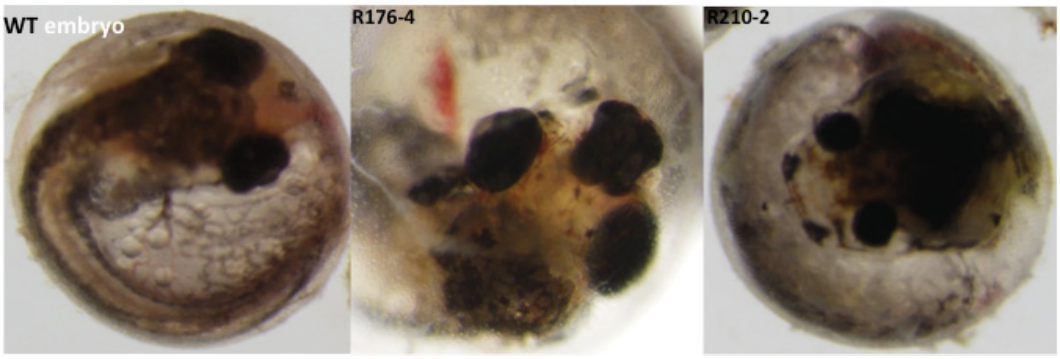

Figure 12. Jaw/mouth defects.

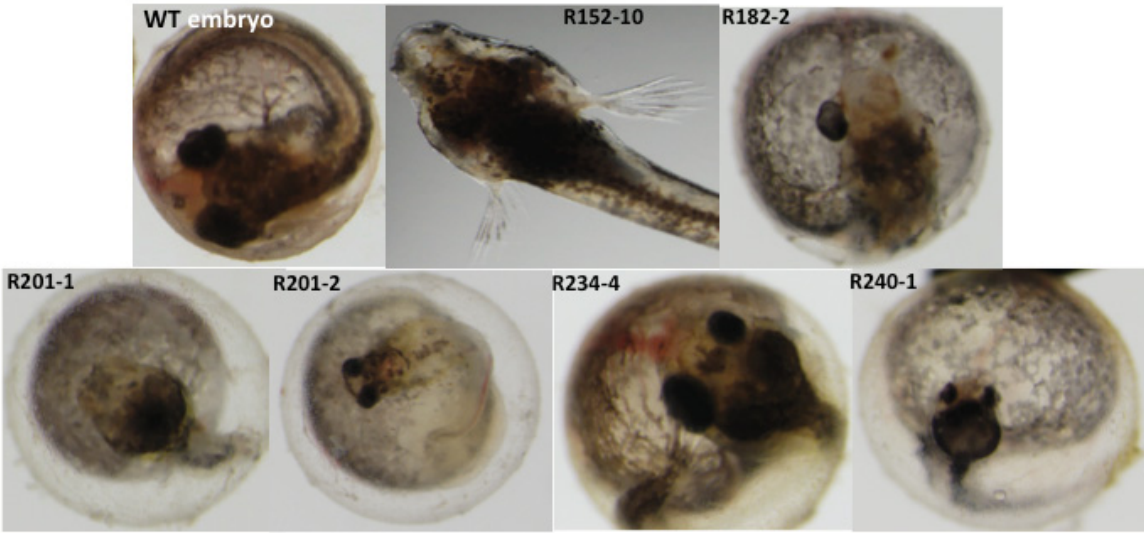

Figure 13. Eye/skull mutants.

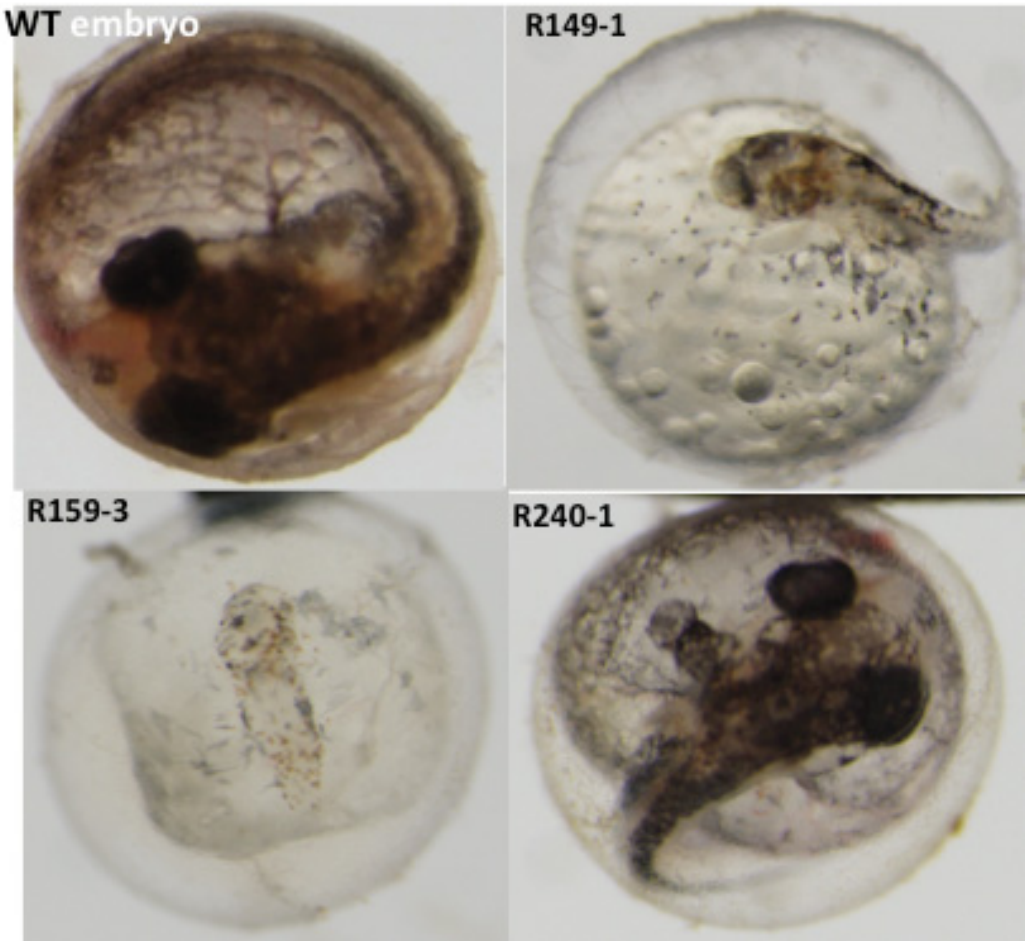

Figure 14. Unresolved.

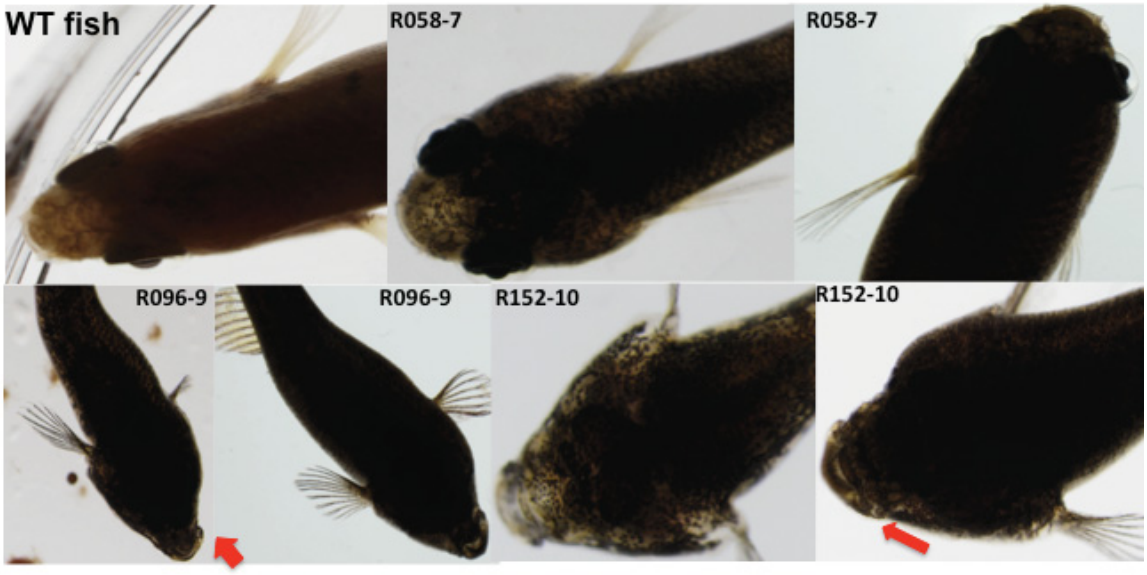

Figure 15. Hypermelanistic fish from the R058, R069 and R152 families.

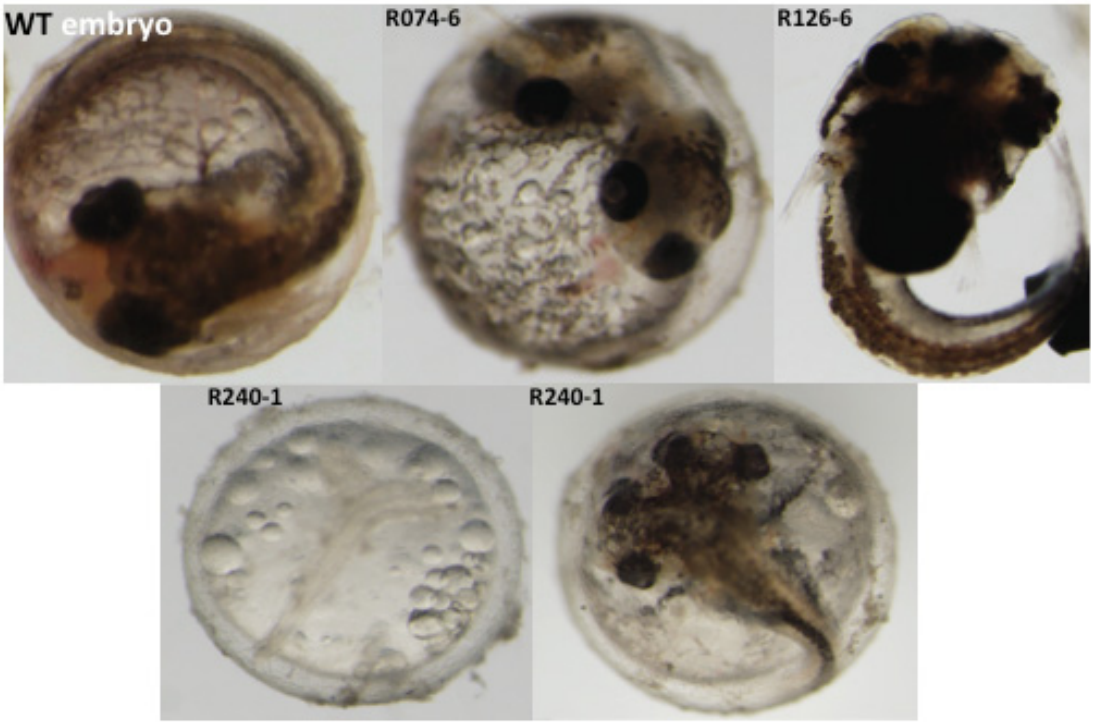

Figure 16. Two-headed fish.

Figure 17. Sterile Non-Viable Maternal Effect Mutant R152-1 embryo. Normal fertilized embryo (left), progression from viable to non-viable of an embryo from the R152-1 family (right).

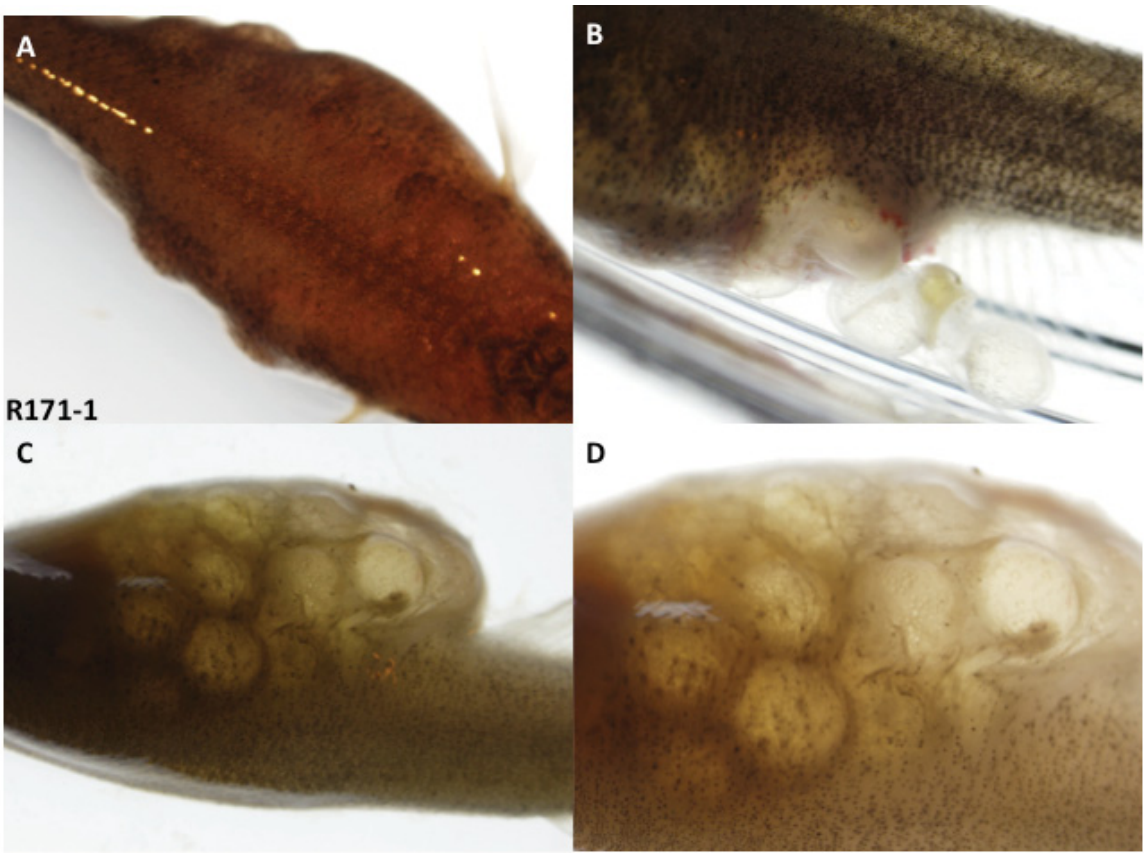

Figure 18. Sterile embryo holders F2 fish from R171 family.

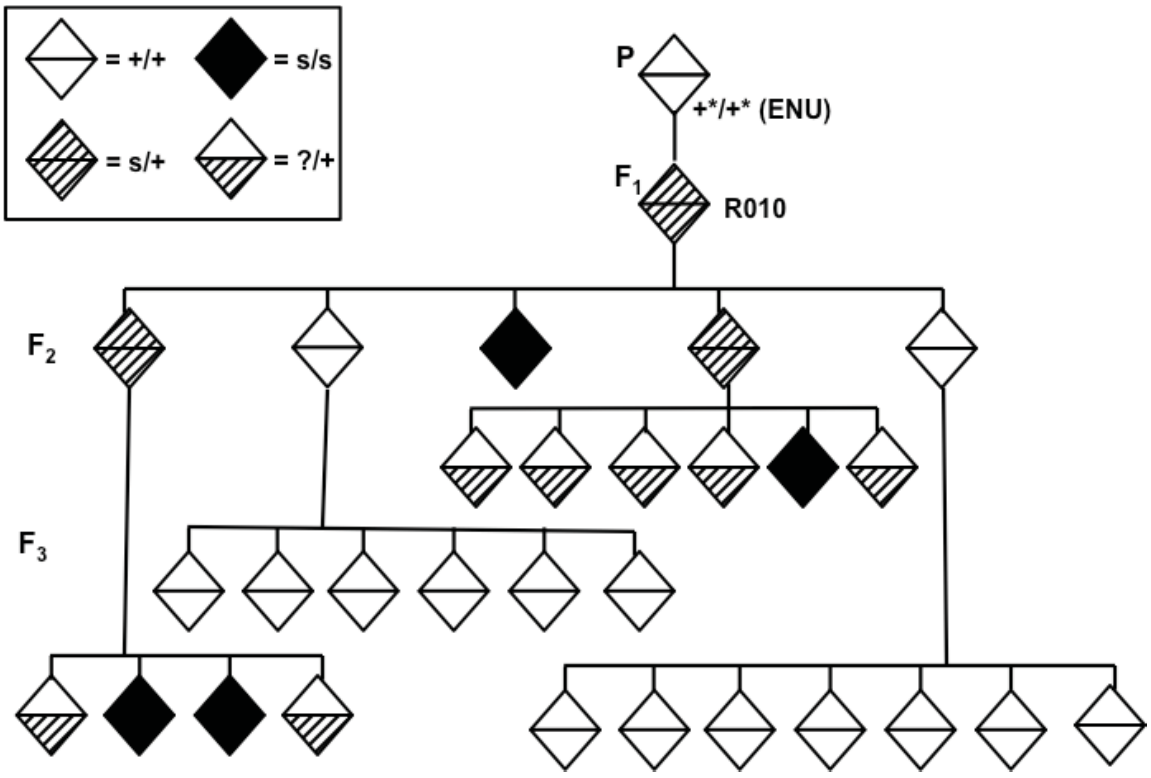

Figure 19. Pedigree of the R010 sterile mutant family.

APPENDIX C

Tables 1-9

TABLES

Table 1. Summary of Simultaneous Zygotic & Sterile F2 screen in *K. marmoratus*

F2 fish	**(+/+)**	**(m/+)[b]**	**(s/s)[c]**	**1^0 Males**	**N[a]**	**Genomes screened**
Sum	92	189	16	10	307	38-76
Mean	2	4	1.33	0.21	6.5	--
Range	0-6	0-9	1-3	0-2	2-13	--

[a] N = total F2 fish scored
[b] Confirmed carriers of zygotic recessive alleles (m).
[c] Sterile mutant hermaphrodites: maternal effects or ovotestis defects (s/s).
Updated from Moore et al. 2012.

Table 2. Summary of F3 embryos scored in the Simultaneous Screen.

F3 embryos from	(+/+)	(m/+)[b]	(s/s)[c]	N[a]
Sum	2630	6131	334	9095
Mean	29	32	21	30
Range	3-164	4-89	0-78	0-164

[a] N = total F3 embryos scored
[b] Confirmed carriers of zygotic recessive alleles (m).
[c] Sterile mutant hermaphrodites: maternal effects or ovotestis defects (s/s).

Table 3. Zygotic Mutants chi-square test summary table.

F2 fish	**(+/+)**	**(m/+)**	**TOTAL**
Observed	92	189	281
Expected	94	187	281
(Obs-Exp)^2/Exp	0.0297	0.0214	**0.0510**

Our null hypothesis states that observed values are significantly close to the expected ones. Expected values are 94 for wild-type F2 fish and 187 for F2 carriers of the zygotic mutant allele. $p < 0.05$, critical value 3.84, df = 1

Table 4. Confirmation of Sterile Mutants

	F2 families	F2 siblings	F3 +/+	F3 */+	F3 s/s	1^0 MALES	N[a]
Sum	10	46	69	120	83	12	284
Mean	--	4.6	4.6	4.14	1.8	0.26	6
Range	--	1-8	0-9	0-8	1-10	0-2	1-11

[a] N = total F3 scored

Table 5. Summary of F4 embryos scored in the Confirmation of Sterile Mutants.

F4 embryos	Viable	Non-viable	N[a]
Sum	3922	4516	8439
Mean	14	17	31
Range	0-42	0-112	0-119

[a] N = total F4 embryos scored

Table 6. Sterile siblings chi-square test summary table.

F2 fish	**(+/+)**	**(s/+)**	**TOTAL**
Observed	14	32	46
Expected	15	31	46
$(Obs-Exp)^2/Exp$	0.1159	0.0580	**0.1739**

Our null hypothesis states that observed values are significantly close to the expected ones. Expected values are 15 for wild-type F2 fish and 31 for F2 carriers of the sterile mutant allele. $p < 0.05$, critical value 3.84, df = 1

Table 7. Summary of Sterile Mutants across F2 families.

Sterile Line	F2 +/+	F2 s/+	F2 scored	F3 Scored	F3 (+/+)	F3 (*/+)	F3 s/s	%[a]
R002	2	3	5	21	3	8	10	55.56
R010	2	2	4	23	13	7	3	30.00
R015	1	3	4	21	1	15	5	25.00
R019	1	7	8	56	9	34	13	27.66
R103	1	6	7	44	5	18	21	53.58
R152	0	6	6	35	0	10	25	71.43
R176	2	0	2	4	4	0	0	--
R182	1	4	5	34	6	23	5	17.86
R194	3	1	4	30	24	5	1	16.67
R247	1	0	1	4	4	0	0	--

[a] Expressed as percent of F3 sterile fish among siblings descended from F2 s/+ parents.

Table 8. Chi-square test of 5 recessive lines in the F3 generation.

F3 fish	**(*/+)**	**(s/s)**	**TOTAL**
Observed	84	27	111
Expected	83	28	111
$(ObsExp)^2/Exp$	0.0068	0.0203	**0.0270**

Our null hypothesis states that observed values are significantly close to the expected ones. Expected values are 83 for */+ F3 fish and 28 for F3 sterile fish.
$p < 0.05$, critical value 3.84, df = 1

Table 9. Summary of *K. marmoratus* recessive mutants.

Family Stock	Category	Phenotype
R001	Zygotic not confirmed	Wild-type zygotic
R002	Sterile/ Zygotic	Maternal effect/ Dwarf
R007	Zygotic	Died early
R010	Sterile/ Zygotic not confirmed	Maternal effect & Non-egg layer/ Wild-type zygotic
R013	Zygotic	Dwarf
R015	Sterile/ Zygotic	Maternal effect & Non-egg layer/ Dwarf and curly
R019	Sterile	Maternal effect & Non-egg layer
R058	Zygotic	Golden Yolk, dwarf, eye defects
R062	Zygotic	Dwarf
R063	Zygotic	Dwarf, dwarf with skull defects
R064	Zygotic	Died early
R066	Zygotic	Died early
R074	Zygotic	Eye defects
R075	Zygotic	Eye defects, skull defects
R076	Zygotic	Curly tail
R077	Zygotic	Short tail, short tail with jaw defects
R084	Zygotic	Dwarf and jaw defects
R094	Zygotic	Curly with skull defects
R096	Zygotic	Dwarf
R097	Zygotic	Curly tail
R103	Sterile/ Zygotic	Maternal effect & Non-egg layer/ Died early, curly with eye and skull defects
R107	Zygotic	Died early
R109	Zygotic	No trunk-no tail
R113	Zygotic	Dwarf, eye and skull defects
R120	Sterile/ Zygotic	Non-egg layer/ Curly, died early
R126	Zygotic	Curly, died early
R129	Zygotic	Curly tail
R130	Zygotic	Dwarf, died early
R134	Zygotic	Eye defects, died early
R137	Zygotic	Died early, curly tail
R149	Zygotic	Curly, Eye and/or skull defects
R152	Sterile/ Zygotic	Died early, dwarf and curly, thin-forward eyes and pigmentation defects

R159	Zygotic	Died early, short tail
R171	Sterile/ Zygotic	Holding embryos/ pigmentation defects
R176	Sterile/ Zygotic	Maternal effect / Dwarf
R182	Sterile/ Zygotic	Maternal effect & Non-egg layer/ Dwarf and curly with eye defects
R194	Sterile/ Zygotic	Holding embryos/ Died early, curly, thin-forward eyes and pigmentation defects
R201	Zygotic	Curly, thin-forward eyes and pigmentation defects
R210	Zygotic	Dwarf, eye defects
R217	Zygotic	Curly
R228	Zygotic	No trunk- no tail
R234	Zygotic	Curly, skull/eye defects
R240	Zygotic	Died early, curly tail, thin-forward eyes and pigmentation defects
R247	Sterile/ Zygotic	Maternal effect/ Curly tail
R248	Zygotic	Died early
R249	Zygotic	Died early, curly tail
R257	Zygotic	Died early, curly tail