Embryonic Development of the Self-fertilizing Mangrove Killifish *Kryptolebias marmoratus*

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The mangrove killifish, *Kryptolebias marmoratus*, is a self-fertilizing vertebrate offering vast potential as a model species in many biological disciplines. Previous studies have defined developmental stages but lacked visual representations of the various embryonic structures. We offer detailed photographic images of *K. marmoratus* development with revised descriptions. An improved dechorionation method was developed to provide high resolution photographs, in addition to a microinjection technique enabling cell marking in the yolk syncytial layer. Embryos were also treated with PTU (1-phenyl 2-thiourea), an inhibitor of melanogenesis, to provide optical transparency revealing internal structures in late stages of development. Chemical exposures (PTU and retinoic acid) demonstrated that *K. marmoratus* embryos were sensitive to chemicals, illustrating further their usefulness in developmental biology studies. Our data suggest that *K. marmoratus* embryos are easily used and manipulated, supporting the use of this hermaphroditic vertebrate as a strong comparative model system in embryology, evolution, genetics, environmental and medical biology. *Developmental Dynamics* 240:1694–1704, 2011. © 2011 Wiley-Liss, Inc.

**Key words:** *Kryptolebias marmoratus*; *Rivulus marmoratus*; hermaphrodite; embryogenesis; staging; dechorionation; PTU; retinoic acid; yolk syncytial layer

Accepted 20 April 2011

**INTRODUCTION**

The mangrove killifish *Kryptolebias marmoratus*, previously known as *Rivulus marmoratus* (Poey, 1880), has been the only known simultaneous hermaphroditic vertebrate able to self-fertilize (Harrington, 1961). However, Tatarenkov et al. (2009) recently demonstrated that a related nominal species, *Kryptolebias ocellatus*, is also capable of self-fertilization. Internal fertilization occurs in the gonadal lumen, containing both ovarian and testicular tissue, where spermatozoa are directly discharged (Sakakura et al., 2006). *K. marmoratus* are andro-dioecious, indicating that populations are composed of males and hermaphrodites (Tatarenkov et al., 2009). This reproductive mode provides a genetic architecture composed mainly of homozygous inbred strains, and a low percentage of highly heterozygous individuals induced by outcrossing, providing the basis for the formation of new clonal lines (Mackiewicz et al., 2006). Recently, 21 distinct clonal lines have been genetically verified (Tatarenkov et al., 2010). The Hon9 line, used in the present study, is amongst these distinct and established clonal lines. Adults grow up to 75 mm and maturity is achieved 3 to 4 months after hatching (Kanamori et al., 2006; Lee et al., 2008). Late in their life cycle (3–4 years), approximately 60% of the hermaphroditic individuals transform into secondary males (Lee et al., 2008). Harrington (1968) reported that true males could be induced directly by exposing embryos to low temperatures (below 20°C) during late stages of embryonic development (Harrington, 1967, 1968). Recently, Kanamori et al. (2006) demonstrated that males could be more efficiently induced by treating embryos to 17α-methyltestosterone. *K. marmoratus* is widely distributed in North, Central, and South
Developmental Dynamics

It is often necessary to remove the egg-dures in developmental biology, it is purposes and experimental proce-

Furthermore, for various imaging (PTU), an inhibitor of melanogenesis. embryons were generated by exposing optical clarity. Thus, transparent embryos become heavily pigmented reducing development, (1984). In later stages of embryonic various descriptions of development of K. mar-

marmoratus is found in marine and brackish water or hypersaline pools, and is capable of surviving rapid and extreme salinity changes (Lee et al., 2008). It is an extremely tolerant euryhaline and thermohaline species, enduring salinities ranging from freshwater to 114% (King et al., 1989) and temperatures of 7° to 38°C (Taylor et al., 1995).

Due to their unique ability to self-fertilize, mangrove killifish offer a vast potential as model fish species, with a recent review suggesting their use in toxicity studies (see Lee et al., 2008), and great interest being displayed for their physiology, ecology, and developmental biology. Asynchronies in embryonic development are known to occur even in eggs within a single clutch, and such delays can be intensified in K. marmoratus due to internal self-fertilization and oviposition at various stages. Embryonic development expressed as time postfertilization offers an approximate idea of the elapsed time period since fertilization of an ovum; however, due to the aforementioned variability, the need for defined stages depicting the formation of apparent morphological features becomes clear. Although the literature contains some descriptions of developmental stages for this species (Harrington, 1963, 1968; Koenig and Chasar, 1984), none has provided detailed visual representations of the various embryonic stages and structures.

The present study offers comprehensive photographic images of the embryonic development of K. marmoratus based on the stages previously described by Koenig and Chasar (1984). In later stages of embryonic development, K. marmoratus embryos become heavily pigmented reducing optical clarity. Thus, transparent embryos were generated by exposing embryos to 1-phenyl 2-thiourea (PTU), an inhibitor of melanogenesis. Furthermore, for various imaging purposes and experimental procedures in developmental biology, it is often necessary to remove the egg-shell (chorion) of embryos. Previous enzymatic procedures for dechorionation of mangrove killifish embryos required two days of treatment (Kanamori et al., 2006). Here, a new method was designed based on a protocol for the medaka, Oryzias latipes (Porazinski et al., 2010). Finally, K. marmoratus embryos were subjected to two chemical treatments (PTU and retinoic acid [RA]) to assess their usability for the study of signaling pathways during early embryonic development. The sensitivity of the embryos to water pollutants was also assessed by means of these chemical exposures, to confirm their use in environmental biology and toxicity studies. Such data will help establish K. marmoratus as a model vertebrate system for a variety of disciplines in biology.

RESULTS AND DISCUSSION

Normal Embryonic Development of Kryptolebias marmoratus

The following descriptions and photographs of embryonic development in K. marmoratus provide a series of stages illustrating the formation of discernable embryonic structures during development under a dissecting stereo microscope.

Stage 1: One-cell stage (Fig. 1A).

The fertilized egg possesses a hard chorion of rough texture, separated from the yolk (vitellus) by a thin periviteline space. The thin blastodisc gradually swells into a large blastomere. Large oil droplets are observable in the transparent yolk and appear to be mobile within the yolky cytoplasm, aggregating at the surface of the vegetal pole irrespective of the egg’s angle.

Stage 2: Two-cell stage (Fig. 1B).

The first cleavage gives rise to two rounded blastomeres of equal size. Cleavage of the blastomeres is mesoblastic, leaving the vegetal hemisphere undivided.

Stage 3: Four-cell stage (Fig. 1C).

The second cleavage furrow forms at a right angle to the first and produces four rounded blastomeres of similar size.

Stage 4: Eight-cell stage (Fig. 1D).

The four blastomeres divide vertically forming eight blastomeres.

Stage 5: 16-cell stage (Fig. 1E).

The blastomeres divide horizontally and are arranged in two tiers with cells on top being dissociated from the yolk.

Stage 6: 32-cell stage (Fig. 1F).

The fifth cleavage leads to 32 blastomeres, with more cells present in the upper tier. It becomes increasingly difficult from this stage onward to enumerate accurately the number of cells and distinguish between them. However, stages can still be determined by their morphological appearance.

Stage 7: Early blastula (Fig. 1G).

The dividing blastomeres are decreasing in size and the blastodisc has less rounded edges.

Stage 8: Mid-blastula (Fig. 1H).

The blastodisc adopts a distinct dome shape and flattens significantly. The marginal blastomeres have less defined edges and yolk syncytial layer (YSL) formation occurs at this stage (Fig. 3 transition between B and C).

Stage 9: Late blastula (Fig. 1I).

Coalescence of cytoplasm in the marginal tier of cells becomes more obvious and the blastodisc flattens further.

Stage 10: Gastrulation begins (Fig. 1J).

The blastoderm flattens greatly and begins to expand over the yolk by epiboly as gastrulation begins. The thin sheet of cells curving over the yolk is asymmetric as it is thicker on one side forming the dorsal lip.

Stage 11: Early gastrula (Fig. 1K).

The expanding blastoderm covers approximately 1/3 of the yolk.

Stage 12: Mid-gastrula (Fig. 1L).

Half of the yolk is now covered by a thin layer of blastoderm. The germ ring is well defined. The oil droplets,
although still largely mobile in the yolk cytoplasm, appear to aggregate under the embryonic shield.

Stage 13: Pre-late gastrula (Fig. 1M).

The blastoderm covers approximately 2/3 of the yolk and the embryonic shield grows in size.

Stage 14: Late gastrula (Fig. 1N).

The embryonic shield lengthens longitudinally and the blastoderm covers roughly 3/4 of the yolk sphere forming a large yolk plug. At this stage, it becomes increasingly clear that the mobility of oil droplets within the yolk cytoplasm is restricted as they aggregate around the embryonic shield.

Stage 15: 100% epiboly (Fig. 1O).

Gastrulation completes as the blastoderm covers the entire yolk. The embryo appears as a long bulge on the surface of the egg with oil droplets amassing under or near it. This stage can be confused with a recently fertilized egg at low magnification as the embryonic axis is fairly flat and difficult to see.

Fig. 1. Early embryonic development in *Kryptolebias marmoratus* showing stage transition from cleavage (A–F) to blastula (G–I) to gastrula (J–P). All images are lateral views of the embryos. From gastrula onward, the dorsal side was positioned on the right. Stage numbers are indicated at the bottom left, and time in hours postfertilization (hpf) at the bottom right of each picture. bd, blastodisc; bm, blastomeres; ch, chorion; dl, dorsal lip; em, embryo; od, oil droplet; ps, perivitelline space; y, yolk. Scale bar = 500 μm.
Stage 16: Head and tail regions recognizable (Fig. 1P).

The embryonic body becomes more distinct as it increases in size longitudinally and laterally. The head and tail regions are discernible later in this stage with the tail bud bulging out slightly compared with the smoother head region.

Stage 17: Optic vesicle and somite formation (Fig. 2A,A').

The optic vesicles form lateral to the forebrain and somitogenesis begins.

Stage 18: Otic vesicle formation (Fig. 2B,B').

The forebrain, midbrain, and hindbrain become visible as the center of the neural tube swells slightly. A ventricle is visible in the eyes (Fig. 2B' arrowhead) and the otic vesicles form lateral to the hindbrain. The pericardial cavity can be seen underneath the head region of the embryo.

Stage 19: Lens formation (Fig. 2C,C').

The compartmentalization of the brain increases and the midbrain enlarges. A furrow is visible from the midbrain to the hindbrain (Fig. 2C' arrowhead) and the midbrain–hindbrain boundary starts to form. The lens and the retina can now be distinguished within the eye. The otic vesicles are now prominent and more discernable, and the olfactory vesicles are visible. The heart is visible within the enlarged pericardial cavity but is not beating.

Stage 20: Heart beats, no circulation (Fig. 2D,D').

The midbrain–hindbrain boundary appears as a furrow between these two regions. The line running through the midbrain extends to the posterior forebrain, and a ventricle is visible in the hindbrain (Fig. 2D' arrowhead). The lens and the otic vesicles enlarge. The notochord becomes clearly distinguishable and the tip of the tail is partially detached from the yolk surface. The heart beats within the pericardial cavity but blood circulation is not established.

Stage 21: Body movements (Fig. 2E,E').

The hindbrain ventricle expands and the cerebellum is distinguishable. The midbrain ventricle is visible (Fig. 2E' arrowhead). The posterior end of the tail dissociates further from the yolk. Sporadic muscular contractions are observable in the tail region.

Stage 22: Circulation (Fig. 2F,F').

A ventricle is visible in the diencephalon (Fig. 2F' arrowhead). The midbrain expands laterally and folds, leaving the optic tectum discernable. This folding is accompanied with an expansion of the midbrain and hindbrain ventricles. Blood now circulates through the dorsal blood vessel and circulates around the yolk by means of one vessel.

Stage 23: Increased vitelline circulation (Fig. 2G,G').

Additional vitelline vessels are developing and oil droplets are redistributed around the entire yolk sphere. Two small blood vessels are observable from either side of the embryonic body, anterior to the first somite. These vessels increase in size in further embryonic stages, and branch into a network of vitelline vessels. The brain sections enlarge and the diencephalon, midbrain and hindbrain ventricles expand further (Fig. 2G').

Stage 24: Otolith formation (Fig. 2H,H').

Small otoliths are visible within the otic vesicles and melanocytes are seen on the head, trunk and yolk surface near the embryo. The telencephalon, diencephalon, optic tectum, and cerebellum are more easily observed. Tail mobility increases.

Stage 25a: Pectoral fin development (Fig. 2I,I').

The pectoral fins appear lateral to the first few somites and the otoliths increase in size.

Stage 25b: Erythrophore formation (Fig. 2J,J').

Erythrophores (orange pigments) are visible posterior to the otic vesicles. The optic tectum expands laterally and overlaps with the posterior side of the retina and part of the lens.

Stage 26: Liver formation (Fig. 2K,K').

The pectoral fins develop and protrude halfway toward the dorsal surface of the trunk. Melanophore pigmentation increases on the embryo and the yolk surface, and is especially dense on the yolk near the two blood vessels anterior to the first somite. Erythrophores are further scattered on the embryonic body, mainly posterior to the otic vesicles, on the pectoral fins and along the tail. The liver bud forms behind the left pectoral fin of the embryo; however, it is more readily observed in the next stage.

Stage 27a: Increased pigmentation and body movement (Fig. 2L,L').

Pigmentation increases greatly on the head, and the dorsal side of the trunk. The eyes are no longer transparent. The liver increases in size and tail movements are able to reach the head.

Stage 27b (Fig. 2M,M').

The embryo lengthens and the head increases in size. Blood circulation is seen in the brain. Pigmentation of the peritoneum is observed and a band of erythrophores is visible laterally to the notochord.

Stage 28: Caudal fin formation (Fig. 2N,N').

The eyes have greatly grown in size and the body cavity is enlarging. The caudal fin is developing and blood circulation is visible in the pectoral fins. Melanophores develop as a network on the surface of the yolk, covering vitelline vessels, particularly on the two large vessels anterior to the pectoral fins.

Stage 29: Air bladder and anal fin formation (Fig. 2O,O'; Fig. 4A).

The pectoral fins further develop protruding to the top of the trunk. The anal fin is developing and later in this stage the dorsal fin can be observed.
Fig. 2. Embryonic development in *Kryptolebias marmoratus* showing stages ranging from somitogenesis to hatching. A–R: Lateral views of the embryos. A’–N’ are head views and O’–R’ are dechorionated embryos for stages of the same letter. Stage numbers are indicated at the bottom left, and time in hours postfertilization (hpf) at the bottom right of each picture. af; anal fin; cer, cerebellum; cf, caudal fin; df, dorsal fin; di, diencephalon; ey, eye; fb, forebrain; fr, fin ray; gt, gut; hb, hindbrain; i, lens; lj, lower-jaw; mb, midbrain; mhb, midbrain–hindbrain boundary; no, notochord; ot, otolith; ov, otic vesicle; pf, pectoral fin; s, somite; sc, spinal cord; tec, optic tectum; tel, telencephalon; uj, upper-jaw. Arrowheads indicate: B’, eye ventricle; C’, brain furrow; D’, hindbrain ventricle; E’, midbrain ventricle; F’, diencephalon ventricle. Scale bars and associated photographs = 200 μm in A’–J’, 200 μm in K’–N’, 500 μm O’–R’, 500 μm A–R.
as a thin transparent membrane. Erythrophores are visible near the olfactory pits (Fig. 4A1), and a dense aggregate is seen around the otic vesicles (Fig. 4A2 arrowhead). The air bladder forms, although it is more readily observable in PTU-treated embryos at stage 30 (Fig. 4B3).

Stage 30: Jaw formation (Fig. 2P, P', Fig. 4B).

Erythrophore pigmentation has spread and can be seen in the midbrain and forebrain regions (Fig. 4B2). The midbrain has greatly enlarged and the eyes have further developed with the retina now surrounding over half of the lens (Fig. 4B2). Of interest, erythrophores are also visible surrounding the vertebrae in a segmented manner (Fig. 4B3 arrowhead). The liver, gut, and air bladder are enlarging in the body cavity (Fig. 4B3). Rays and pigments are
forming in the caudal fin. The jaws are developing and the anterior part of the head is raised off the yolk surface.

Stage 31: Pectoral fin movement (Figs. 2Q, Q', 4C, 5).

Unsynchronized movements are observed in the pectoral fins and blood circulates in the caudal fin. The green gallbladder is developing in the body cavity near the liver (Fig. 4C). The upper and lower jaws are more easily distinguished (Fig. 2Q'). Irregular heartbeats and movements occur at this stage in all embryos. The network of melanophores covering vitel- line blood vessels is easily observed when comparing control and PTU-treated embryos (Fig. 5F1 and F2).

Stage 32: Hatching (Fig. 2R, R', 4D).

Rays have developed in the dorsal and anal fins and pigmentation is observed on the dorsal fin. The

Fig. 3. Development of the yolk syncytial layer (YSL). Yolk syncytial nuclei (YSN) were labeled by injecting sytox green into the YSL. A1–H1, transmitted light images; A2–H2, fluorescent images. A: At stage 7, the prospective YSL cells have not formed a syncytium, and are still separated by cell membranes. B: At stage 8, the YSL starts to form and the cytoplasm of marginal cells is partially fused. C: Late in stage 8, a single row of YSN is formed and the border between the YSL and the blastoderm is straight. D: At stage 9, multiple layers of YSN are observed on the surface of the yolk. E–G: During gastrulation (stage 11, E; stage 12, F; stage 14, G) YSN are observed to gradually spread over the entire yolk sphere. H: At the end of epiboly, the YSL and YSN cover most of the yolk surface except for the posterior end of the embryonic axis (arrowheads) where YSN appear to aggregate. Arrows indicate location of the embryonic axis.

Fig. 4. Late embryonic development in PTU (1-phenyl 2-thiouracil)-treated Kryptolebias marmoratus embryos focusing on head and body cavity development. Eggs were exposed to 0.003% PTU starting before the onset of pigmentation (stages 19–21). ab, air bladder; fb, forebrain; fmb, forebrain-midbrain boundary; gb, gallbladder; gt, gut; hb, hindbrain; hv, hindbrain ventricle; l, lens; lv, liver; mb, mid- brain; mhb, midbrain–hindbrain boundary; op, olfactory pit; ot, otolith; pf, pectoral fin. Arrowheads indicate erythrophores.
pectoral fins move co-ordinately, and the mouth opens and closes with associated opercular movements. An embryonic diapause may occur at this stage, delaying hatching for 2 weeks or more (Koenig and Chasar, 1984; Taylor, 2000).

**Variety of Cleavage Patterns in Small Model Fish Species**

We found unique characteristics in the cleavage patterns of *K. marmoratus* embryos. Similarly to *Fundulus heteroclitus*, the first cleavage (Fig. 1B) gives rise to two high and round blastomeres (Armstrong and Child, 1965), whereas in medaka these blastomeres are flatter (Iwamatsu, 2004). Of interest, at the 16-cell stage in *K. marmoratus* (Fig. 1E), some blastomeres are already separated from the yolk (as cells are distributed in an upper and lower tier). In contrast, in other model species, such as medaka, *F. heteroclitus* and zebrafish (*Danio rerio*), separation of blastomeres from the yolk only occurs at the 32-cell stage. This difference may correlate to the high shape of the blastomeres in *K. marmoratus*, which leads to a smaller base connection to the yolk compared with the other species. These differences may arise from variations in cytoskeletal networking and membrane dynamics. The distinct shape of the blastomeres remains until the early blastula stage (Fig. 1G), after which the blastodisc becomes significantly flatter (Fig. 1H). Thus, it appears that even within killifish species (e.g., *Kryptolebias, Fundulus*, and *Oryzias*), cleavage patterns are quite variable. Such variation of cleavage patterns within the different killifish species may provide a useful model to study evolution of embryonic morphogenesis.

**Monitoring Development of the Yolk Syncytial Layer by Sytox Green Injection**

The yolk syncytial layer (YSL) is one of the first cell types that differentiates around the mid-blastula stage in many fish species (Trinkaus, 1993; Kimmel et al., 1995). Differentiation of the YSL occurs as the plasma membrane in the vegetal margin collapses, forming a gigantic mononuclear layer with multiple nuclei (syncytium). Here, YSL development was visualized by injection of a fluorescent dye (sytox green), which labels cell nuclei with a strong fluorescent signal and cytoplasm with a weaker signal. This enables monitoring of morphological changes in the cells during YSL formation, as well as tracing of the yolk syncytial nuclei (YSN), which dynamically move during gastrulation (D’Amico and Cooper, 2001).

*K. marmoratus* embryos were injected with sytox green at the early, mid-, and late blastula stages to determine the onset of YSL formation. During the early blastula stage (Fig. 1G), marginal cells marked with sytox green are still separated from neighboring cells by a membrane (Fig. 3A). At the mid-blastula stage (Fig. 1H), marginal cells fuse synchronously (Fig. 3B) and rapidly form a single multinucleate cell as the embryo progresses to stage 9 (Figs. 1I, 3C). It is noteworthy that the onset of YSL formation coincides with morphological changes in the blastodisc itself. At stage 7 (Figs. 1G, 3A), the blastodisc has a high and spherical shape, whereas during stage 8 and 9 (Fig. 1H, 3B,C) the blastodisc flattens significantly. This suggests that dynamic changes occur at these stages, in cytoskeletal and membrane structures, potentially correlating with formation of the YSL.

Throughout gastrulation, the YSN divide and proliferate, spreading along the yolk surface toward the vegetal pole (Fig. 3D–G), eventually covering roughly the entire yolk sphere, except for the posterior end of the embryonic axis (Fig. 3H arrowheads). During the early gastrula stage (stage 10), the dorsal side of the blastoderm becomes thicker forming the embryonic shield (dorsal lip, Fig. 1J), and the YSN accumulate in that region in higher density (Fig. 3E). The aggregation of YSN in this area enables visualization of the embryonic axis developing in the blastoderm (Fig. 3H1 and H2 arrows).

These data illustrate the dynamic changes in both cell shape and location during YSL formation and YSN migration, coinciding with morphological changes of the blastodisc during transition to the gastrula stage. The data also demonstrate that microinjection can be applied to *K. marmoratus* embryos, despite their thick chorion membrane.

**PTU Treatment Reveals Internal Structures Visible in Late Embryonic Stages of K. marmoratus**

*K. marmoratus* embryos were treated with PTU (0.003%) before the onset of pigmentation (between stages 19 and 21), and exposures were continued throughout to maintain the potency of PTU. No significant mortalities or developmental abnormalities were observed during treatment at this concentration. PTU was shown to inhibit the biosynthesis of melanin in *K. marmoratus* embryos at equal concentrations to those used in zebrafish (Westefield, 2000) suggesting a similar sensitivity to the chemical in these two species. *K. marmoratus* embryos were observed to lack melanocytes at all stages of development in which these should be present. However, erythrophore formation was not affected by PTU (Figs. 4, 5) even at high concentrations (0.75 mg/ml, toxic).

From stage 28 onward (Fig. 2O–R), it becomes increasingly difficult to observe different organs due to the dense pigmentation of the embryos. Differentiation and growth of the brain sections and their ventricles, as well as internal organs in the body cavity, were more readily observed in PTU-treated embryos (Fig. 4A–D). During this period (stage 29–32), the midbrain becomes notably larger in contrast with the forebrain (diencephalon), which does not show such growth (Fig. 4A2–D2).

Furthermore, assessment of the degree of transparency was conducted by comparing PTU-treated and non-treated embryos (Fig. 5A–H). It was observed that, despite erythrophores, the optical clarity of the embryos was enhanced. Thus, various tissues in older embryos including liver, gut, gallbladder, air bladder, nephric duct, heart, blood vessels, otic and optic vesicles, and the brain, are more easily observed using PTU-treated transparency.

**Retinoic Acid Suppresses Head Formation and Tail Elongation in a Dose Dependent Manner in K. marmoratus Embryos**

To effectively use *K. marmoratus* as a model animal in developmental
biology, it is important that specific signaling pathways may be modified by treating embryos with chemicals. As this species possesses a thick chorion, and the size of its embryos is relatively larger than popular model fish embryos (e.g., zebrafish and medaka), such methods may prove less effective. To assess the susceptibility of this species to chemicals, we exposed *K. marmoratus* embryos to RA from early cleavage (8 to 16 cells; stage 4 and 5) onward, without removing the chorion and examined resulting morphological changes on day 2 (stage 18–19) and 3 (stage 23–24) postfertilization (dpf).

In zebrafish embryos, addition of RA suppresses forebrain and midbrain development (Kudoh et al., 2002). In *K. marmoratus*, RA exposures at a concentration of $10^{-6}$ M lead to 100% mortality of the embryos after 72 hr. Concentrations of $10^{-7}$ M produced a headless and reduced tail phenotype (Fig. 6C,F, arrowheads indicate ablated head), whilst embryos exposed to $5 \times 10^{-8}$ M RA displayed a small head and tail morphology (Fig. 6B,E, arrowheads indicated reduced head). The dose sensitivity and phenotype (suppression of head and tail formation) are comparable to those of zebrafish embryos subjected to similar treatments (Holder and Hill, 1991; Kudoh et al., 2002, data not shown).
These data, in conjunction with previously described experiments using PTU (Figs. 4, 5), suggest that, despite their thick chorion and larger size, *K. marmoratus* embryos are responsive to chemical treatments, which proves to be highly useful for embryological research.

**CONCLUSION**

In this report, we demonstrate that *K. marmoratus* embryos can be easily manipulated and monitored during embryonic development. The visibility of various organs and structures in late stages of embryonic development was enhanced by dechorionation and exposure to PTU. In addition, we show that microinjection is applicable to *K. marmoratus* embryos; injection of sytox green in the yolk syncytial layer facilitated observation of cell movement during gastrulation. We further demonstrate that mangrove killfish eggs, despite their hard chorion, are amenable to chemical exposures (RA and PTU) at low concentrations, enabling the study of various developmental pathways by chemical treatment. PTU-induced optical transparency during development is promising for labeling and detecting late embryonic tissues by GFP and other fluorescent proteins.

*K. marmoratus* represents a very unique genetic model system due to its hermaphroditic nature, established clonal lines, and its androdioecious reproductive system which allows mixing between strains by outcrossing using primary or secondary gonochoristic males. Laboratory use of *K. marmoratus* has already been suggested in a variety of fields such as carcinogenicity testing (Koenig and Chasar, 1984) and aquatic toxicology studies (Lee et al., 2008). We further support that *K. marmoratus*, with its easily obtainable and handled embryos, can be used in a variety of embryological research fields in the same light as other species such as the zebrafish or medaka.

**DECHORIONATION**

The dechorionation procedure was designed based on a protocol for *O. latipes*, with some modifications, which uses both hatching enzyme (HE) and Pronase (Porazinski et al., 2010). HE extract was prepared by homogenizing, on ice, 20 to 30 *K. marmoratus* embryos at the hatching stage placed in 1.5-ml Eppendorfs with equal volume brackish water. The tube was then placed in 4°C at least overnight, and then centrifuged at 14 krpm for 10 min. The supernatant was collected and stored at −20°C; this stock solution was diluted twice by brackish water (14‰) to produce a working solution of HE for the aforementioned protocol. It is possible to further dilute the stock to decrease mortalities during the dechorionation procedure; however, this will also increase the time required for the chorion to be degraded.

**EXPERIMENTAL PROCEDURES**

**EXPERIMENTAL ANIMALS**

Hon9 clonal lineage was obtained from Valdosta State University, Valdosta, Georgia. This clonal line is homozygous at all loci tested by microsatellite analysis and is identical to all laboratories that culture it (Tatarenkov et al., 2010). Hermaphroditic individuals were kept individually in 1.5-L plastic aquaria in a controlled environment (25 ± 1°C; 14‰ salinity; 12 hr:12 hr light:dark photoperiod), and were fed daily ad libitum on Artemia. Brackish water was produced by mixing demineralized water and marine salts (Tropic Marin, Germany). Spawning mops were added in the tanks to provide a substrate for oviposition. The majority of eggs were found attached to these spawning mops. *K. marmoratus* hermaphrodites oviposit all year during day light hours, usually at midday; eggs can be laid at varying developmental stages due to intra-parental development (Harrington, 1963; Koenig and Chasar, 1984). It is difficult to assess the exact fertilization time of *K. marmoratus* embryos, as ovulated eggs are fertilized within the gonadal lumen (Sakakura et al., 2006). Thus, all timings expressed as hours post fertilization are back-calculated estimations based on previous literature (Koenig and Chasar, 1984; Grageda et al., 2005).

**EXPERIMENTAL PROTOCOLS**

**Imaging.**

For all controlled conditions, embryos were reared at 25 ± 1°C and 14‰ salinity, with daily water changes. Developmental timings of *K. marmoratus* were based on previous stages described by Koenig and Chasar (1984). For photographic purposes, rotation of the embryos maintained within the camera frame was made possible by placing eggs on 1% (using 14‰ brackish water) Agarose (Sigma, A9539) beds. These were produced by leaving Agarose to set on 1.2 mm diameter glass tubes laid in a Petri dish, moulding “channel” like depressions in the gel. Embryos were handled gently with forceps into these conduits, maintaining them in position without physical damage (note that the embryos could still rotate freely within the chorion). All photographs were taken using a Nikon Digital Sight DS-U2 camera mounted on a Nikon SMZ1500 microscope.
regularly and removed as soon as the chorion was partially degraded. Remnants of the eggshell were then removed with fine forceps and embryos were washed in brackish water. Dechorionated embryos were reared on 1% agarose gel (Sigma, A9539) beds and handled with a glass pipette of sufficient diameter to avoid damage.

**PTU.**

Similarly to procedures used in the zebrafish (Westerfield, 2000), K. marmoratus embryos were treated with a concentration of 0.003% PTU (Sigma, P7269) prior the onset of pigmentation (stage 19–21). Working solutions of PTU were prepared using brackish water (14%) and water changes were carried out daily to maintain the potency of PTU.

**Retinoic acid.**

Stock solutions for all trans retinoic acid (RA) were prepared as 10⁻³ M dissolved in demethyl sulfoxide (DMSO) and diluted in 14% brackish water to final concentrations. Embryos at early cleavage (8 to 32 cells) were treated with RA at three different concentrations (10⁻⁶ M, 10⁻⁷ M, 5 × 10⁻⁸ M). Mortality and phenotype were recorded; photographs were taken on day 2 and 3 postfertilization (dpf).

**Microinjection of sytox green in the yolk syncytial layer.**

Yolk syncytial nuclei appearance in K. marmoratus was monitored by injecting sytox green (Invitrogen, 0.5 mM) in the yolk syncytial layer (YSL) at three different stages of development (early, mid, and late blastula). Microinjections were carried out by placing embryos on 1% agarose bed with channels of 1.2 mm diameter (see above). Embryos were handled gently with forceps into these conduits, which maintained them in a fixed position. Eggs were lined up with the animal pole facing upward, allowing microinjection into the YSL with minimum damage. Microneedles (1 mm O.D., 0.58 mm I.D.) were pulled using a Narishige PC-10 puller (theater level set on 52 to 53, 186.8 g weight) mounted on a micromanipulator arm (Narishige MN-153). The tips of the needles were broken by touching the extremity with fine forceps or on the eggshell of the embryo; injections were carried out using a Pneumatic PicoPump PV820 (World Precision Instrument). Approximately 1–2 nl of the material was injected in the YSL region (interface between the blastoderm and yolk). Following injection, embryos were transferred back to 25 ± 1°C and left for 10–15 min. Fluorescence was then observed on a Nikon SMZ1500 microscope.

**ACKNOWLEDGMENTS**

We thank Michael Wetherell and Janice Shears for their help with maintenance of adult fish and Makoto Furutani-Seiki for his medaka dechorionation protocol. We also thank Brian Ring, Akira Kanamori, Robert Kelsh, Yoshitaka Sakakura, and Jae-Seong Lee for helpful comments on the data and manuscript and David Bechler for providing us with the fish to establish a population of Hon9 clonal line of Kryptolebias marmoratus at Exeter. Funding was provided to D.P.C. by the Nuffield Science Foundation. S.M. is a PhD student at the University of Exeter and his PhD studentship is provided by the Natural Environment Research Council in the United Kingdom.

**REFERENCES**


