Description of Embryonic Development of Spotted Green Pufferfish (*Tetraodon nigroviridis*)

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Abstract

Pufferfish species of the Tetraodontidae family carry the smallest genomes among vertebrates. Their compressed genomes are thought to be enriched for functional DNA compared to larger vertebrate genomes, and they are important models for comparative genomics. The significance of pufferfish as model organisms in comparative genomics is due to the availability of two sequenced genomes, that of spotted green pufferfish (*Tetraodon nigroviridis*) and fugu (*Takifugu rubripes*). However, there is only a very limited utilization of pufferfish as an experimental model organism, due to the lack of established husbandry and developmental genetics protocols. In this study, we provide the first description of the normal embryonic development of *Tetraodon nigroviridis*. Embryos were obtained by *in vitro* fertilization of eggs, and subsequent development was monitored by brightfield microscopy at constant temperature. Tetraodon development was divided into distinct stages based on diagnostic morphological features, which were adopted from published literature on normal development of other fish species like medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), and fugu. Tetraodon embryos show more similar morphologies to medaka than to zebrafish, reflecting its phylogenetic position. The early developmental stage series described in this study forms the foundation for the utilization of tetraodon as an experimental model organism for comparative developmental studies.

Introduction

SPOTTED GREEN PUFFERFISH (*Tetraodon nigroviridis*, hereafter tetraodon) is prevalent in the rivers, estuaries, mangroves, and seas of Southeast Asia, where it can attain a length of 17 cm. It is a popular aquarium fish, best reared in brackish water. In the 1960's, an extensive survey of nuclear DNA content in a range of teleost fish showed that species of the Tetraodontidae family, including spotted green pufferfish, possess the smallest genomes of all vertebrates known to date.¹ This prompted an initiative in the late nineties to sequence pufferfish genomes² (also see www.fugu-sg.org/) to aid in the annotation of human genes. Through genomic sequence comparisons, the tetraodon genome directly contributed to the annotation of protein-coding genes on 11 human chromosomes. Sequence similarity analysis of predicted coding se-

quences provided the first accurate prediction of the total number of coding genes in humans.³ Later, the analysis of the complete genome sequence of tetraodon made a compelling case supporting a whole genome duplication in an ancestral teleost species some 300 million years ago.⁴

Tetraodon, together with the marine pufferfish *Takifugu rubripes*, has contributed to genomics and vertebrate evolutionary biology (reviewed in Cusack and Roest Crollius⁵). Both species share a remarkably compact genome, only one eighth of the genome size of humans, and yet possess a similar set and number of genes to other teleosts and humans. This suggests that the difference in genome size was due to the loss of intergenic, functionally redundant junk DNA, as was originally proposed by Brenner.² In contrast to mammalian and other sequenced fish genomes, the compact 350 Mb tetraodon and 400 Mb fugu genomes are largely

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devoid of transposable elements. This property, together with the large evolutionary distance to mammals, contributes to their usefulness in genomic comparisons aimed at identifying sequences under purifying selection. In particular, pufferfish genomes have been successfully used to discover highly conserved noncoding elements (CNEs), which are useful guides in the identification of cis-regulatory elements regulating the expression of nearby or distantly located genes.^{6,7} The compact nature of pufferfish genomes could greatly facilitate the characterization of CNEs, by enabling the isolation of entire regulatory domains in a single genomic clone. For example the 741 kb intergenic region between the *ATP5G3* and *LNP* genes 5' of the human *HOXD* locus contains a cluster of 59 CNEs. The corresponding intergenic region 5' of the fugu *HoxDa* locus has a size of only 74 kb.⁸ This compact genomic size makes it possible to assay a complete cluster, for example, perform targeted deletions and mutations, within a single bacterial artificial chromosome (BAC) clone. Thus, long-distance interactions between coding gene and noncoding elements (both *cis*-regulatory elements and noncoding RNA) could be studied in tetraodon BAC transgenics. This necessitates the establishment of pufferfish as experimental models, to fully realize their potential for scientific research.

For fugu, a developmental stage series has been published, and there are sporadic publications with use of the fugu embryo.^{9,10} However, a standard and cost-effective laboratory breeding protocol is not available. In contrast, spotted green pufferfish can be bred in laboratory conditions, but developmental genetic profiling lags behind. As a result, both species have remained virtual models, mostly confined to genome sequence analyses.

In the lack of pufferfish embryos amenable to genomic manipulation, biological validation of comparative genomics findings has been carried out by testing pufferfish sequences in other model vertebrates, such as mouse and zebrafish.⁷ Such interspecies assays are invaluable tools for the annotation of function to genomic elements. However, they suffer from an inherent limitation. CNEs with transcriptional regulatory activity do not necessarily drive conserved expression patterns when tested in multiple species,¹¹ due to differences in the reading of the regulatory code by *trans*-acting machineries. This can be a problem considering the approximately 150 million years of divergent evolution between fugu and zebrafish. Thus, there is a need for coupling genome annotation with intraspecies experimental validation also in pufferfish, to understand the true and precise function of genomic elements.¹¹

Watson *et al.* demonstrated a simple and reproducible breeding protocol for tetraodon, which involves *in vitro* egg fertilization after ovarian lavage.¹² Tetraodon embryos are transparent similar to zebrafish or medaka embryos, their development takes around 80 h to hatching, and over 2000 eggs are produced by the female. The generation time of tetraodon is around 9 months (Watson *et al.*, unpublished data).

Based on the above described needs, and prompted by the availability of a successful breeding protocol, we have set out to develop spotted green pufferfish as a laboratory model for functional and comparative genomics projects. We have raised embryos under laboratory conditions and documented the normal development of tetraodon embryos. We, thus, provide essential morphology information for developmental

analyses. In addition, our findings give important basic information for developing reporter assays to test genomic elements or for molecular analysis of development by *in situ* approaches. Furthermore, we define developmental stages for future genome and transcriptome analysis. The developmental stages described in this study provide reference to the first developmental transcriptomics resources generated, including CAGE (Cap Analysis of Gene Expression) and RNA-seq datasets from several embryonic stages (¹³ and data not shown). CAGE data analysis led to a genome-wide compendium of tetraodon gene promoters and to the discovery of a novel core promoter motif also present in humans. These findings underscore the usefulness of tetraodon in vertebrate genomics.

Materials and Methods

Induced spawning of T. nigroviridis embryos and in vitro fertilization

A total of 12 adult tetraodon (6 males and 6 females) were reared and prepared for breeding at the University of Florida (Ruskin). Embryos for recording of the stage series were produced using the induced spawning technique developed by Watson *et al.*¹² In brief, female gonads were treated with Chorulon[®] (human chorionic gonadotropin; Merck Pharmaceuticals, Inc.) to stimulate ovulation. Chorulon was directly injected into the ovary of anaesthetized females through a catheter inserted into the oviduct. Mature eggs were expressed from anaesthetized females 36 h post Chorulon treatment and then fertilized with fresh sperm squeezed from anaesthetized males. The procedures applied in this article were carried out in agreement with the respective animal experimentation licenses (USA, Bham Home Office license no 40/3131).

Embryo culture

Thousands of embryos were obtained from two adult females by stripping and *in vitro* fertilization as described.¹² Batches of approximately 200 embryos were kept in Ø 9 cm Petri dishes filled with artificial seawater (33 ppt salinity). The seawater was replaced twice a day. For imaging, approximately 100 embryos were moved into an agarose-coated Ø 9 cm Petri dish with approximately 1 mm long, 1 mm wide, and 0.7 mm deep wells. Those wells were generated by a custom-made plastic mold similar to the one used by Kemp *et al.*¹⁴ The embryos for imaging in the well plates were replaced at least once a day. Embryos were cultured and imaged at 27.3°C. A second clutch of embryos from another breeding pair was used to document an independent stage series until the second day post fertilization, to control for reproducibility, and to fill gaps of missing stages.

Data collection and identification of developmental features

Embryos were imaged at varying intervals from fertilization until the third day post hatching (sixth day post fertilization). The embryos were imaged using a stereomicroscope SMZ800 with the digital camera DXM 1200 controlled by the ACT-1 2.70 software (Nikon). The images taken at different time points were of different individuals of the same clutch. Since the development of embryos of one clutch appeared

fairly synchronized, this strategy eliminated the risk of consistently imaging a nonrepresentative outlier. Stage defining characteristics of embryos were identified by comparison to the description of the normal development of medaka,¹⁵ zebrafish,¹⁶ and fugu.¹⁰

Data analysis

An eyepiece reticle was used for length (micrometer) measurements of features of live embryos. ImageJ¹⁷ software was used for area and length measurements of features from images. Standard deviations were calculated using Microsoft Excel.

Presentation of the stage series

To describe tetraodon embryonic development in a standardized way, the recorded tetraodon embryogenesis was divided into periods following the scheme used for zebrafish and fugu: zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching. Images of individual embryos were cropped and arranged into figures using the Adobe Photoshop CS. The stages are presented by stage number (stage name, developmental time) and a brief description of the appearance of the embryo under a dissecting microscope, with focus on stage defining morphological characteristics. We used a combination of numbers and specific terms to name individual stages. All given developmental times represent elapsed time post fertilization.

Results

Zygote (0 h 2 min)

Upon fertilization, cytoplasm streams toward the animal pole where it forms a blastodisc (bd). The micropyle (mp) in the chorion (ch) is visible above the blastodisc. Embryo yolk (yk) is transparent and contains an estimated 300–400 oil droplets (od) varying in size ($23.61\text{--}138.60\text{ }\mu\text{m}$ in diameter). They form an aggregate, but this aggregate does not have a fixed position until segmentation stages. The thin perivitelline space between the embryo and chorion is hardly discernible. Embryo diameter is $0.590 \pm 0.008\text{ mm}$ (SD, $n = 12$).

Cleavage

During the cleavage period of tetraodon embryonic development, a single cell (1st blastomere), formed at the animal pole by separation of cytoplasm from the yolk, is divided (cleaved) into an increasing number of smaller cells, decreasing in size with each division (Fig. 1).

Stage 1 (1 cell, 1 h 06 min)

Segregation of cytoplasm and yolk leads to formation of the first blastomere (bm) at the animal pole. This first blastomere is well separated from the yolk by a constriction (cs) running around the margin. This constriction also reveals the perivitelline space (ps).

Stage 2 (2 cell stage, 1 h 59 $\pm$ 4 min)

Meridional cleavage of the first blastomere generates two symmetric blastomeres. This and the following cleavage divisions appear incomplete, meroblastic. However, this observation was not verified by labeling cell membranes. The

FIG. 1. Brightfield images of zygote and cleavage stage embryos. Side views in the *top row* and top views (animal pole) in the *row below*. bd, blastodisc; bm, blastomere; bt, blastomere tier; ch, chorion; cs, constriction; la, long axis; mp, micropyle; od, oil droplet; ps, perivitelline space; sa, short axis; yk, yolk.

first cleavage generates a long and a short axis of the blastodisc (la, sa). This asymmetry in the blastodisc is present also at the following cleavage stages, but disappears at blastula stages. Subsequent cleavages occur at intervals of approximately 38 min. Blastomeres are rounded immediately after cleavage divisions and flatten before the next cleavage.

Stage 3 (4 cell stage, 2 h 32 $\pm$ 6 min)

The second cleavage is also meridional. It is directed perpendicular to the first cleavage plain and generates four cells of similar size, but somewhat variable in shape.

Stage 4 (8 cell stage, 3 h 06 min)

The meridional cleavages leading to the 8 cell stage generate two symmetrical rows of blastomeres.

Stage 5 (16 cell stage, 3 h 52 min)

The fourth set of cleavages produces a 4×4 array of cells. The regular arrangement of blastomeres into columns and rows is disappearing and the cleavage plains become hard to discern at this and the following stages.

Stage 6 (32 cell stage, 4 h 14 $\pm$ 6 min)

Thirty-two cells cover most of the animal pole of the embryo (see top view). The blastodisc is compacted and the blastomeres are irregularly shaped. The blastomeres may

form two tiers only in the central region of the blastodisc, similarly to medaka embryos.¹⁵

Stage 7 (64 cell stage, 4 h 47 min)

In the central region of the embryo, three irregular tiers of blastomeres (bt) can be discerned.

Blastula

The cleavage of blastomeres has resulted in a cap of small cells sitting on top of the yolk cell (Fig. 2). The yolk syncytial layer (ysl), nuclei in a ring of syncytial cytoplasm, forms below the blastodisc margin. At the end of this stage, the yolk bulges dome-like into the blastodisc. This stage marks the beginning of extensive cell movements during epiboly and gastrulation.

Stage 8 (128–256 cell stage, 5 h 34 min)

The cells are arranged in four irregular tiers (bt). No obvious sign of a blastocoele can be detected at this stage, or at other blastula stages.

Stage 9 (256–512 cell stage, 6 h 59 min)

Cleavage divisions have produced a cap of small cells sitting on top of the yolk cell.

Stage 10 (sphere, 9 h 49 ± 93 min)

The embryo developed into a sphere, with a flat border between blastodisc and yolk. Below the blastodisc margin the yolk syncytial layer (ysl) is visible in side views. The ysl nuclei (see insert) are presumably derived from marginal cells, which had collapsed into the yolk.

Stage 11 (dome, 11 h 27 ± 42 min)

A dome-shaped bulging of the yolk into the blastodisc indicates commencement of epiboly. The clearly visible ysl contains several tiers of nuclei.

Gastrula

The blastoderm moves over the yolk (epiboly) and starts becoming internalized at the margin (gastrulation) (Fig. 3).

FIG. 2. Brightfield images of blastula stage embryos. Side views in the *top row* and top views (animal pole) in the *row below*. *Insert* next to stage 10 embryo shows zoom in on the ysl nuclei. bt, blastomere tier; ysl, yolk syncytial layer.

FIG. 3. Brightfield images of gastrula stage embryos. Side views in the *top row* and top views (animal pole) in the *row below*. The *lower row* image for stage 14 is a vegetal pole view. The *middle row* image for stage 15 is focused on the anterior and the *lowest row* image is focused on the posterior of the embryo. The side view images for stages 13 and 14 are *right side* images, which have been flipped horizontally. The *arrowhead* points to a thickening of the blastoderm. bdm, blastoderm margin; ea, embryonic axis; kv, Kupffer's vesicle; ob, optic bud; sh, shield.

Convergence of cells on one side of the embryo generates a shield-shaped thickening, the first manifestation of the embryonic axis. This embryonic axis extends in anterior–posterior direction and neurulation generates an axial CNS (neural rod). Optic buds develop on the anterior end of the CNS.

Stage 12 (germ ring, 13 h 57 min)

Epiboly is well under way and the blastoderm appears as an inverted cup of mostly uniform thickness. The margin reaches approximately 40% of the distance between the animal and vegetal poles. Accumulation of cells at one position of the blastoderm margin is the first sign of formation of the embryonic shield (arrowhead). This stage is reminiscent of the germ ring stage in zebrafish, where internalization of cells at the blastoderm margin generates multiple distinct cell layers (germ layers). The presence of multiple separated layers manifests as a ring within the blastoderm in animal pole views and marks the beginning of gastrulation.

Stage 13 (shield, 13 h 43 min)

The blastoderm covers 50% of the yolk cell. The embryonic shield (sh), a thickening of the blastoderm at one position above the margin, is visible. The embryonic axis will develop from that structure. Shield formation breaks symmetry within the embryo. For the first time, all three major body axes can be distinguished morphologically: the dorsal–ventral, anterior–posterior, and left–right axes.

Stage 14 (80% epiboly, 15 h 17 ± 64 min)

The blastoderm margin (bdm) reached 80% of the distance between the animal and the vegetal pole. A streak forms on one side of the embryo, which projects from the position of

the embryonic shield to the animal pole. The streak corresponds to the embryonic axis (ea). Kupffer's vesicle is clearly formed at the posterior end of the shaping body axis (kv).

Stage 15 (neurula, 20h 17min)

Late neurula stage. At the anterior end of the neural axis, which appears as a solid rod, optic vesicle evagination is visible as lateral buds (ob).

Segmentation

During the segmentation period, reiterative structures, like distinct portions of mesoderm (somites) and the rhombomeres of the hindbrain, form along the anterior–posterior axis (Fig. 4).

Stage 16 (1 somite, 23h 28min)

The embryonic body encircles more than half of the yolk sphere. Head starts to form and eye vesicles (optic vesicle, ev) are developing. The first somite (s) appears in the middle

of the trunk. Kupffer's vesicle (kv) is observed at the posterior end of the embryo. Oil droplets coalesce on the ventral side of the embryo. This makes it difficult to follow the development of inner organs like liver, gut, and pancreas.

Stage 17 (3 somites, 1 day 2h 17min)

A groove (g) is discernible in the optic vesicles. It separates presumptive neural retina (lateral) and retinal pigment epithelium (medial). Kupffer's vesicle (kv) is well formed. Three somites (s) are discernible. The yolk assumes an ellipsoid shape.

Stage 18 (5 somites, 1 day 3h 35min)

Five somites (s) can be distinguished. Olfactory placode (op) can be seen in top views on the head. Otic vesicles (ov) appear.

Stage 19 (9 somites, 1 day 7h 32min)

Yolk is oval and the embryo encircles approximately 80% of the yolk circumference. Nine somites (s) have formed. The optic vesicle has transitioned into the optic cup (oc). The lens placode (lp) thickens to form the lens.

Stage 20 (brain subdivisions, 1 day 8h 30min)

Subdivisions of the brain are appearing: forebrain (fb), midbrain (mb), and hindbrain (hb). Neurocoele (nc) formation results in a visible channel along the CNS. The aggregate of oil droplets remains at its position ventral to the anterior trunk. However, it is more spread out over the surface of the yolk.

Stage 21 (brain ventricles, 1 day 9h 47min)

The embryonic body encircles most of the yolk. Somites gradually become more chevron shaped, indicating their transition into myomeres. Ventricles form in the brain (vt). Olfactory placodes (op) and otic vesicles (ov) are prominent. Hindbrain rhombomeres (rh) are observed in side views. The notochord (nt) is visible in the trunk and tail. The boundary between the midbrain and hindbrain can be discerned (mhb). The pericardium (pc) has formed.

Pharyngula

In the previous stages, the principle body plan of a vertebrate embryo was established. Tissues derived from all three germ layers were regionalized along the different body axes and organ primordia developed. Now the different organ systems, like the digestive tract, grow to gain functionality by the time the embryo hatches and becomes a free-feeding larva (Fig. 5).

Stage 22 (blood circulation, 1 day 15h 3min)

Heart beat (45 ± 3 beats per minute, $n=8$) generates blood circulation (bc). Slight tail movement can be observed. Red body pigmentation of erythrophores¹⁸ (ery) becomes visible. Optic tectum (opt) and midbrain–hindbrain boundary (mhb) can be clearly distinguished. Kupffer's vesicle (kv) remains visible, but is reduced in size.

Stage 23 (lens, 1 day 17h 18min)

Rounding of the lens suggests that lens (lns) formation is complete.

FIG. 4. Brightfield images of segmentation stage embryos. The top row contains left side images, with anterior to the left and dorsal to the top. The row below contains dorsal view images, focused on the anterior (head) of the embryo. The lowest row image for stage 16 is a dorsal view focused on the posterior (tail) of the embryo. The side view images for stages 16, 18, 20, and 21 are right side images, which have been flipped horizontally. ev, eye vesicle; fb, forebrain; g, groove; hb, hindbrain; kv, Kupffer's vesicle; lp, lens placode; mb, midbrain; mhb, mid–hindbrain boundary; nc, neurocoele; nt, notochord; oc, optic cup; op, olfactory placode; ov, otic vesicle; pc, pericardium; rh, rhombomeres; s, somite(s); vt, ventricle.

FIG. 5. Brightfield images of pharyngula stage embryos. The *top row* contains left side images of embryos, with anterior to the *left* and dorsal to the *top*. The *row below* contains dorsal view images focused on the anterior (head) of the embryo. The lowest row for stage 25 contains an additional dorsal view image, with better visibility of the erythrophores lining the side of the embryo. The side view images for stages 22, 27, and 28 are right side images, which have been flipped horizontally. bc, blood circulation; br, brain; cv, cranial vessels; dt, digestive tract; ery, erythrophores; kv, Kupffer's vesicle; lns, lens; mel, melanophores; mhb, mid-hindbrain boundary; opt, optic tectum; rpe, retinal pigment epithelium; tl, tail.

Stage 24 (free tail, 1 day 22 h 54 min)

Morphology of the embryo has changed. The head has a more roundish shape and the tail (tl) has lifted from the yolk. The posterior part of the presumptive digestive tract (dt) is visible, but no other internal organs can easily be identified.

Stage 25 (2 days 10 h 59 min)

Head appears much broader in top views. The body bends sideward over the yolk to fit into the chorion. A stripe of red erythrophores (ery) runs along both sides of the body.

Stage 26 (2 days 13 h 34 min)

Tail (tl) reaches in front of the head.

Stage 27 (eye pigmentation, 3 days 3 h)

Black eye pigmentation of the retinal pigment epithelium (rpe) appears. Cranial vessels (cv) are visible in top view.

Stage 28 (pre hatching, 3 days 5 h 50 min)

Melanophores (black, mel) become visible at various positions on the body. The brain (br) appears enlarged compared to earlier pharyngula stages and gives the head a bulky appearance. Eye movement starts.

Larval stages

On the third day post fertilization (dpf), the tetraodon embryo hatches from its chorion (Fig. 6). The larvae display rich and colorful body pigmentation. The tail is completely pigment free, and there is a sharp margin where the pigmentation ends in front of the tail. The 3 dpf (stage 29) larva still has plenty of yolk and oil droplets. The 6 dpf (stage 32) larva, however, seems to have used up all of its maternally supplied nutrients.

Stage 29 (hatching, 3 days 8 h 43 min)

The hatched larva is 1.29 mm in length and displays various types of chromophores distributed all over the body, with the exception of the tail. The tail is pigment free, and there is a sharp margin (pm) where the pigmentation ends. The larva has a large pear-shaped yolk sac (ys), with, unlike zebrafish larvae, no obvious yolk sac extension. Oil droplets (od) are observed atop the yolk.

Stage 30 (4 days larva)

The larva displays the characteristic roundish body shape of a pufferfish. A thin fin fold (ff) is running around the trunk and tail. The jaws (jw) can be seen in side views. Dorsal and ventral views reveal the pectoral fins (pf), the iris (is), and the swim-bladder (sb). The brain (br) appears very large in side views. Oil droplets (od) occupy the middle of the body and obscure the view on internal organs. The larvae display extensive body pigmentation, with melanophores (black, mel), erythrophores (red, ery) and xanthophores (yellow). There is a sharp margin (pm) of the pigmentation in front of the pigmentless tail.

Stage 31 (5 days larva)

A medial (ms) and a ventral (vs) stripe of black pigment runs along the side of the body of the 5 days larva. The otic vesicle (ov) is visible in side views and dorsal views. The yolk seems to be completely used up, but oil droplets (od) are still present around the gut region. Besides in the iris (is), iridiophores (iri) are also observed on the body. The pectoral fins (pf) appear more developed, with a smooth outline, as compared to stumpy at stage 30.

Stage 32 (6 days larva)

There are no obvious depots of yolk or oil droplets observed in the 6 days larvae. Their heads appear larger than at previous larval stages. The area of the eye at stage 32 is 1.2 times larger than the area of the eye of the stage 31 larva in side views. The ear is 1.45 times larger and the height of the head at the level of the optic tectum is 1.11 times higher than the one of the stage 31 larva.

FIG. 6. Brightfield images of larvae. The *top* row contains left side images of larvae, with anterior to the *left* and dorsal to the *top*. The row *below* contains dorsal view images of the larvae, with anterior to the *left*. The *lowest* row contains the corresponding ventral view images. The image for stage 29 shows a larva, which has just hatched from its chorion. br, brain; ery, erythrocytes; ff, fin fold; iri, iridiophores; is, iris; jaw, jaws; mel, melanophores; ms, medial stripe; od, oil droplets; ov, otic vesicle; pf, pectoral fin; pm, pigment margin; sb, swim bladder; vs, ventral stripe; ys, yolk sac.

We terminated the recoding of tetraodon development after stage 32, a stage just before the time when the larvae start to feed. It was the aim of this study to provide a description of tetraodon early development in a format comparable to stage series for zebrafish, medaka, and fugu. Therefore, we concluded the tetraodon stage series at a developmental stage similar to the last stage of the stage series for other fish species.

Discussion

In this study, we report to our knowledge the first *Tetraodon nigroviridis* stage series of embryonic development.

Tetraodon embryonic development followed recognizable periods similar to the ones in zebrafish, including zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. We also provided a brief description of the first 3 days of postembryonic development. Not surprisingly, embryonic and larval development of tetraodon is most similar to its closest relative used for comparisons, fugu. However, fugu embryos need more than 5 days until they start to hatch,¹⁰ which might simply reflect the lower temperature at which they were raised. Reflecting their phylogenetic relationship, tetraodon embryos appear more similar to medaka than zebrafish embryos. With medaka, they share the presence of oil droplets, the lack of a wide perivitelline space, the appearance of the eye rudiment before formation of somites, short or no yolk extension, an embryonic axis deeply embedded into the yolk, and a relatively large Kupffer's vesicle, which remains visible well into the late segmentation period.¹⁵

Kupffer's vesicles appear largest during mid-segmentation stages. We compared the relative size of Kupffer's vesicle between tetraodon, medaka, and zebrafish mid-segmentation stage reference embryos. The diameter of the Kupffer's vesicle of the 9 somite stage tetraodon embryo is 58% of the diameter of the trunk on the level of the most posterior somite in side views. It is 81% in case of the Kupffer's vesicle of the 9 somite stage medaka reference embryo¹⁵ and 38% in case of the Kupffer's vesicle of the 10 somite stage zebrafish reference embryo.¹⁶ The Kupffer's vesicle is a transient embryonic organ in teleosts, which directs biased left-right asymmetry (laterality) of internal organs, analogous to the node in mice.¹⁹ Signore *et al.* found evidence for a higher degree of canalization (robustness) of laterality regarding epithalamic and heart looping asymmetries in medaka compared to zebrafish.²⁰ Notably, medaka has a relatively larger Kupffer's vesicle than zebrafish, which is also more similar to the mouse node. The Kupffer's vesicle of medaka contains an epithelial layer of ciliated cells only in the dorsal roof of the organ, whereas ciliated cells are found also in the ventral floor of the organ in zebrafish. The architecture of the medaka organ more closely resembles the situation in the mouse node, which contains a planar layer of ciliated epithelium. Therefore, it has been proposed that differences in the morphology and architecture of laterality organs might account for differences in the robustness of laterality between species.²⁰ It would be interesting to see if the same is true for tetraodon.

Medaka embryos start to hatch much later (9 dpf) than zebrafish (2 dpf), and are also more developed by the time of hatching. In that respect, tetraodon embryos (3 dpf) are more similar to zebrafish. Also, tetraodon and zebrafish cleavage stage embryos seem to have a higher cytoplasm to yolk ratio than medaka embryos. The blastodisc covers 33% of the area of the embryo in side view images of the 2 cell stage tetraodon embryo, 28% of the area of the 2 cell stage zebrafish reference embryo,¹⁶ and only 8% of the area of the 2 cell stage medaka reference embryo.¹⁵ The values are 70%, 54%, and 16% in the corresponding top view images.

In this study, two independent stage series were recorded to monitor reproducibility of course and timing of embryonic development. As shown in Figure 7, the two recorded stage series were mostly in phase and the embryos looked highly similar.

FIG. 7. Comparison of the timing of progression through the stages between the two recorded stage series. The first embryos of both series having reached a specific stage are shown together with the respective developmental time. In the *bottom* of the figure the appearance of embryos at the time point when the second stage series was terminated is compared between the two stage series.

As demonstrated in Figure 8, the coverage of the documentation of tetraodon embryonic development is high for early periods of tetraodon embryonic development, but decreasing toward larval stages. This prompts the need for further, more detailed analysis of late embryonic and larval stages in the future.

During this study a number of issues have been raised that need addressing in the future, for the efficient use of tetraodon as an experimental model. We have attempted microinjections of fertilized eggs using borosilicate needles similar to those routinely used for zebrafish embryo injections. The tough chorion surrounding the tetraodon embryos appeared as an obstacle to experimental manipulation, and most embryos died during the procedure. Attempts of dechoriation by forceps or proteolytic enzyme were unsuccessful (data not shown), which suggests that a medaka protocol based on the use of hatching enzyme may be an alternative. We also found that visual observation of the development of inner organs is difficult in tetraodon embryos, due to the oil droplets, which form a dense aggregate. From the segmentation period on the droplets occupy the space where the inner organs develop, obscuring their visibility. Therefore, to follow the development of the inner organs in tetraodon would require labeling them with fluorescent protein encoding transgenes. Alternatively, staining methods like RNA *in situ* hybridization or antibody staining could be used to visualize the inner organs.

Most pufferfish, like tetraodon or fugu, are nonpoisonous when bred in captivity.²¹ This fact abrogates concerns about handling them during husbandry and experimentation. However, tetraodon is better suited to experimentation than its close relative fugu, for its small adult size (up to 17 cm compared to 70 cm) and its ability to survive in freshwater. In theory, it may be possible to keep tetraodon in the same facilities used for zebrafish and medaka. Aquarists claim that tetraodon does better in brackish water, or full-strength seawater (www.aquahobby.com/gallery/e_puffer1p.php, <http://animal-world.com/encyclo/fresh/Puffers/GreenSpottedPuffer.php>). However, tetraodon males and females reached sexual

FIG. 8. The bar chart depicts the developmental time points when pictures were taken during recording of the first (*dark gray*) and the second (*light gray*) stage series. The resolution is 1 min. On the *x*-axis the developmental time is given in minutes, hours, and days. *Above* the bar chart the time span of different periods of tetraodon development is indicated with *arrows*. The course of tetraodon development is displayed in images of embryos and larvae. *Dashed lines* indicate the time points when images were taken.

maturity in a wide range of salinities at the University of Florida (Watson, unpublished data).

In this study, we provide a starting point for the comprehensive description of tetraodon development. The developmental stage series described now, together with the first published experimental genomics data for tetraodon embryos,¹³ are the first steps toward the establishment of tetraodon as an experimental model for developmental biology.

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Disclosure Statement

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