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Embryonic development of the grass pufferfish (*Takifugu niphobles*): From egg to larvae

V. Gallego [a, b], M. Yoshida [b], D. Kurokawa [b], J.F. Asturiano [a], G.J. Fraser [c, *]

[a] Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain
[b] Misaki Marine Biological Station, Graduate School of Science, University of Tokyo, Miura, Kanagawa, 238-0225, Japan
[c] Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN, United Kingdom

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ABSTRACT

Tetraodontidae (pufferfish) family members carry the smallest genomes among vertebrates, and these pocket-sized genomes have directly contributed to our understanding of the structure and evolution of higher animals. The grass pufferfish (*Takifugu niphobles*) could be considered a potential new model organism for comparative genomics and development due to the potential access to embryos, and availability of sequence data for two similar genomes: that of spotted green pufferfish (*Tetraodon nigroviridis*) and Fugu (*Takifugu rubripes*). In this study, we provide the first description of the normal embryonic development of *T. niphobles*, by drawing comparisons with the closely related species cited above. Embryos were obtained by *in vitro* fertilization of eggs, and subsequent development was monitored at a constant temperature consistent with natural conditions. *T. niphobles* development was divided into seven periods of embryogenesis: the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods; and stages subdividing these periods are defined based on morphological characteristics. The developmental stage series described in this study aims to provide the utilization of *T. niphobles* as an experimental model organism for comparative developmental studies.

1. Introduction

The grass pufferfish (*T. niphobles*) is a teleost fish with a wide distribution in the Northwest Pacific Ocean. This species is one of around twenty four pufferfish species in the genus Takifugu, and there are some reasons that justify its study: i) it is placed on the IUCN Red List due to the reduced knowledge about the stage of its current populations, making it a possible endangered species [1]; ii) the closely related species (like Takifugu rubripes) is widely-kept by scientists as a model organism for genomics [2,3]; and *iii*) some species of this genus are considered a popular food in Japan.

The genome of the congeneric species *T. rubripes* (Fugu) has been sequenced and assembled recently, the second vertebrate genome to be sequenced and the shortest known genome of any vertebrate species [4]. In this respect, the pocket-sized genome of Fugu should help to resolve contentious estimates of human gene number, where the genome of Fugu has directly contributed to the annotation of protein-coding genes on 11 human chromosomes and has also helped unearth nearly 1000 new human genes [5,6]. In this regard, closely related species such as *T. niphobles* could be similarly applied in this purpose due to its small and similar genome. One advantage of *T. niphobles* over the other pufferfish species currently used for genomic studies is the potential for the study of essential steps in development: staging series based on morphological traits will provide in-depth knowledge of the developmental processes governing teleost fish [7,8].

Staging by morphological criteria is an useful tool for generating developmental comparisons between different species and, in this sense, to determine the underlying mechanisms of evolutionary changes among them [9]. For Fugu (*T. rubripes*), a developmental stage series has been published [10], but a standard and cost-effective laboratory breeding protocol is not available. In contrast, *T. niphobles*, with a high fertility rate during a wide spawning period (offering the availability of thousands of eggs [11]), can be kept and maturated in laboratory conditions [12]. As a result, both species have remained virtual models, mostly confined to genome sequence analyses. In this study, we have set out to promote

* Corresponding author. Department of Animal and Plant Sciences, Alfred Denny Building, University of Sheffield, Western Bank, Sheffield S10 2TN, United Kingdom.
E-mail address: g.fraser@sheffield.ac.uk (G.J. Fraser).

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T. niphobles as a laboratory model for functional and comparative genomic and developmental projects. We report the embryonic development of *T. niphobles*, raised under laboratory conditions, describing the series of embryonic stages and provide fundamental data to facilitate its use for future developmental studies.

2. Materials and methods

2.1. Fish handling

Takifugu niphobles shows a singular spawning behavior at Arai Beach near Misaki Marine Biological Station (MMBS, Japan). Large schools of fish (200–1000 [13]) arrive to the beach around the new or full moon at spring tide during the spawning season, which occurs between May and July. Spawning takes place repeatedly from 2 h before the sunset and includes a beach-spawning behavior, where the fish are routinely found out of the water on the beach until the next wave. During this time, males and females of *T. niphobles* were caught and moved to the MMBS seawater facilities. Fish were kept in running seawater tanks at 18 °C and the trial was carried out under the approval of the animal guidelines of the University of Tokyo on Animal Care.

2.2. Gamete collection and in vitro fertilization process

Genital area was cleaned with freshwater and thoroughly dried to avoid the contamination of the samples with faeces, urine or seawater, and gentle abdomen pressure was applied to obtain the gametes both in males and in females. Eggs from two females were divided into batches of approximately 100 eggs and placed into 60 × 15 mm Petri dishes (x4) using a micropipette with the tip cut off to prevent compression of the eggs. An aliquot of sperm from only one male (10^5 sperm/egg ratio) was put on to the batches of eggs and 5 ml of seawater was then added in order to activate the sperm and achieve fertilization success as described in Gallego et al. (2013) [14].

2.3. Embryo culture

The fertilized eggs were transferred into clean Petri dishes and were then incubated in darkness at a controlled temperature of 20 °C (each Petri dish with approximately 50 eggs). Embryos were observed every 30 min using a Leica M165FC microscope to check the embryonic staging of pufferfish and detailed descriptions of each development stage were performed (see Table 1). Images were taken with a camera (MicroPublisher 5.0; QImaging, Surrey, Canada). Dead eggs were removed during daily inspections, and seawater was exchanged once a day.

2.4. Presentation of the stage series

To describe *T. niphobles* embryonic development in a standardized way, embryogenesis was divided into periods following the scheme used for other model organisms as zebrafish (*Danio rerio* [7]) and medaka (*Oryzias latipes* [8]): zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching. Images of individual embryos were cropped and arranged into figures using the Adobe Photoshop CS3 (Adobe Systems).

2.5. In situ hybridisation

T. niphobles MyoD2 and Myogenin cDNA fragments were isolated by RT-PCR with 96 hpf total RNA. Primer sets were designed using *F. rubripes* genome information available from Ensemble database [3]. Primers used and lengths of amplified products are: MyoD2, 370bp with sp (5′-AGAAGGCCACCAGCACCTCCATCAC-3′) and ap (5′-. CAGCGGTGGGTAGAAGCTCTGGTCT-3′); Myogenin, 394 bp with sp (5′- CCTACGACCAAGGCACCTAC-3′) and ap (5′-TCAGTGTCCTGCTGGTTGAG-3′).

Whole mount *in situ* hybridisation was perfomed using digoxigenin-11-UTP labeled antisense RNA probes as described [15].

2.5.1. T. niphobles MyoD2 partial cDNA seq

AGAAGGCCACCAGCACCTCCATCACCACGTCCCCAGTGCAGAGGAG
GAGCTGGAGGAGGAGACGGTGGTGGAAGAGCACGTGAGAGCACCGG
GGGGCCTCCACCAGGCCGGCCGATGCCTGCTCTGGGCCTGCAAAGCCT
GTAAAAGGAAGACGACCCACGCGGACCGGCGGAAGGCGGCGACCATG
CGGGAGCGGCGACGACTGAGCAAAGTCAACGACGCCTTTGAGACGCT
AAAGCGCTGCACCGCCTCCAACCCCAACCAGAGGCTCGCCAAGGTGGA
GATCCTGCGCAACGCCATCAGCTACATCGAGTCCCTGCAGGCCCTGCTG
AGGACTTCGGGTCAAGACCAGAGCTTCTACCCACCGCTG.

2.5.2. T. niphobles myogenin partial cDNA seq

CCTACGACCAAGGCACCTACCAGGATAGGAACACCATGATGGGCTT
GTGTGGGAGTCTGTCCGGAGGTGTGGATGTTGGAGTGACAGGGACAG
AGGACAAAGCCTCTCCATCCAGCCTGTCACCTCACTCTGAGCCACACTG
CCCGGGCCAGTGCCTTCCCTGGGCCTGCAAGTTATGCAAGAGGAAGAC
GGTCACCATGGACCGCCGGAGAGCGGCCACGCTGAGAGAGAAGAGGC
GCCTGAAGAAGGTGAACGAGGCCTTCGACGCTTTGAAGAGGAGCACG
TTGATGAACCCAAACCAGAGGCTGCCCAAGGTGGAGATCCTCAGGAGC
GCCATCCAGTACATCGAAAAGCTACAGGCCCTGGTGTCCTCCCTCAACC
AGCAGGACACTGA.

3. Results

3.1. Zygote period

The zygote period started from *in vitro* fertilization until the onset of cleavage period, when the embryonic polar cell mass transitioned from the 1-cell stage to the 2-cell stage (Fig. 1A–B). Zygote period spanned 0–1.7 hpf for reaching the cleavage.

Table 1
Timing-stages of embryonic development of grass pufferfish (*T. niphobles*).

Period	Stage	Time (h)
Zygote period	None	0.0
	1 cell	0.9
Cleavage period	2 cell	1.7
	4 cell	2.4
	8 cell	2.7
	16 cell	3.7
	32 cell	4.1
	64 cell	4.6
Blastula period	128 cell	5.1
	256 cell	5.8
	512 cell	6.5
	1024 cell	7.3
	Epiboly 20%	12.7
Gastrula period	Epiboly 40%	14.1
	Epiboly 90%	15.7
	Tail bud 1	23.7
	Tail bud 2	25.6
Segmentation period	3-somite	32.9
	14-somite	48.9
	21-somite	65.9
Pharyngula period	Prim-5	77.1
	Prim-24	108
	Fin stage	147
Hatching period	First eclosion	191
	Last eclosion	214

3.2. Cleavage period

During the cleavage period of *Takifugu niphobles* embryonic development, a single cell (1st blastomere), formed at the animal pole by separation of cytoplasm from the yolk, was divided (cleaved) into an increasing number of smaller cells, decreasing in size with each division (Fig. 1C–H). This period took approximately 2.9 h.

3.3. Blastula period

During the early blastula period from the 128-cell stage to dome stage (Fig. 1I–M), the number of cells and the shape of the cell mound were used as criteria for staging. *T. niphobles* embryos began this phase of development at 5.1 hpf when 100% of the embryos were consistently dividing into the blastula dome. This period included the stages up to 20% epiboly, where the embryo forms

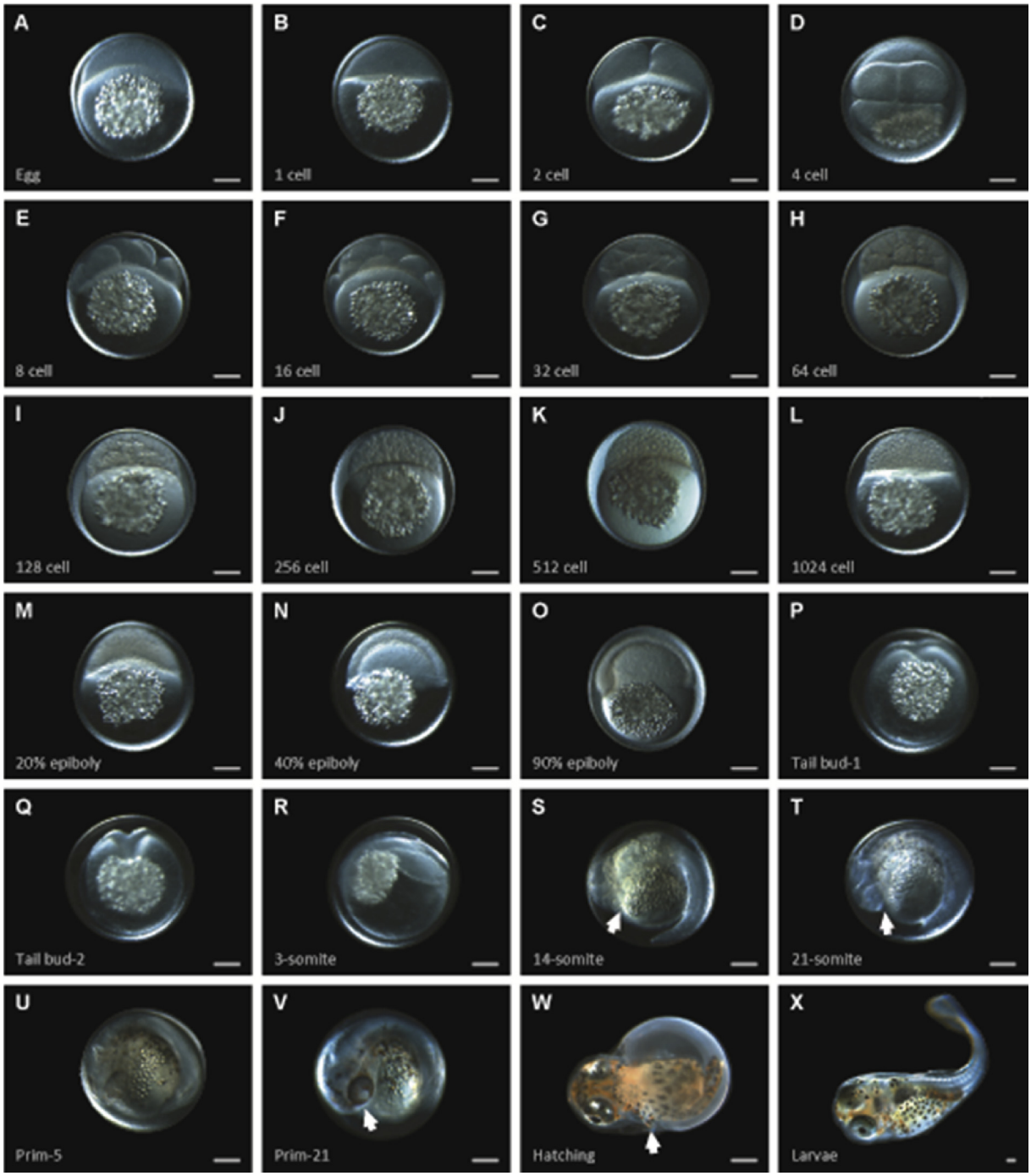

Fig. 1. Developmental process of grass pufferfish (*T. niphobles*) embryo from zygote to hatching. A) External appearance of egg. B) 1-cell stage. C) 2-cell stage. D) 4-cell stage. E) 8-cell stage. F) 16-cell stage. G) 32-cell stage. H) 64-cell stage. I) 128-cell stage. J) 256-cell stage. K) 512-cell stage. L) 1024-cell stage. M) 20% epiboly stage. N) 40% epiboly stage. O) 90% epiboly stage. P) Tail bud-1 stage. Q) Tail bud-2 stage. R) 3-somite stage. S) 14-somite stage. T) 21-somite stage. U) Prim-5 stage. V) Prim-21 stage. W) Hatching. X) Larvae. Scale bar = 200 μm. White arrow in S, T, V indicate the position of the mouth opening. White arrow in W demarcates the pectoral fin, during emergence from the chorion. Orientation of images show anterior to the left and posterior to the right, dorsal is toward the top and ventral is toward the bottom of the images.

multiple sheets of cells through to gastrulation. During this period, the yolk pushed into the embryonic cells (animal pole; Fig. M) as the embryo develops.

3.4. Gastrula period

During the gastrula period, the extent to which the blastoderm covers the yolk cell and the form of blastoderm were used as criteria for staging. The gastrula period from 40% epiboly to the tail bud stage proceeded during 14.1–25.5 hpf (Fig. 1N–Q). We marked the beginning of the gastrula period when the majority of the embryos in a given brood reached 40% epiboly (Fig. 1N). During the stages from 50 to 70% epiboly the germ ring started to appear and soon after the embryonic shield developed as a thickening at the germ ring poles (shield stage, Fig. 1O). Towards the end of epiboly (90%) the margin of the blastoderm (germ ring) progressed around the yolk cell (between 15.8 and 21.5 hpf) and the dorsal indentation occurred to mark the start of the tail bud period (Fig. 1P–Q).

3.5. Segmentation period

Segmentation refers to the division of territories and the emergence of somitogenesis – this began soon after tail bud stage at approximately 32.9 hpf and when the embryo developed 3-somites the first indication of optic placode formation begins (Fig. 1R). The number and division of somites is a universal indicator of embryonic staging (Fig. 2), and in order to fully appreciate the staging during somitogenesis, we conducted *in situ* hybridisation experiments to determine the precise number of somites during development (6 and 8 somites; Fig. 2, *myoD* and *myogenin*, respectively). Without any indication of gene expression the formation of somites is relatively unclear in *T. niphobles*. *myoD* and *myogenin* (*myog*) are two Muscle Regulatory Factors (MRFs); these genes encode related myogenic basic helix-loop-helix (bHLH) transcription factors involved in myogenesis [16] and are associated with establishing myogenic potential and delineating the process of somitogenesis [16,17]. During segmentation the tail began its extension and separation from the yolk membrane (Fig. 1S). Within the latter stages of the segmentation period (approximately the 18 to 21-somite stage) the first signs of pigmentation emerged with black melanophores appear ahead (Fig. 1T) of the orange xanthophores that spread concurrently in ventral regions of the embryo near to and covering the ventral boundary between the embryo and the yolk.

3.6. Pharyngula period

Eye pigmentation emerged at the start of the pharyngula period,

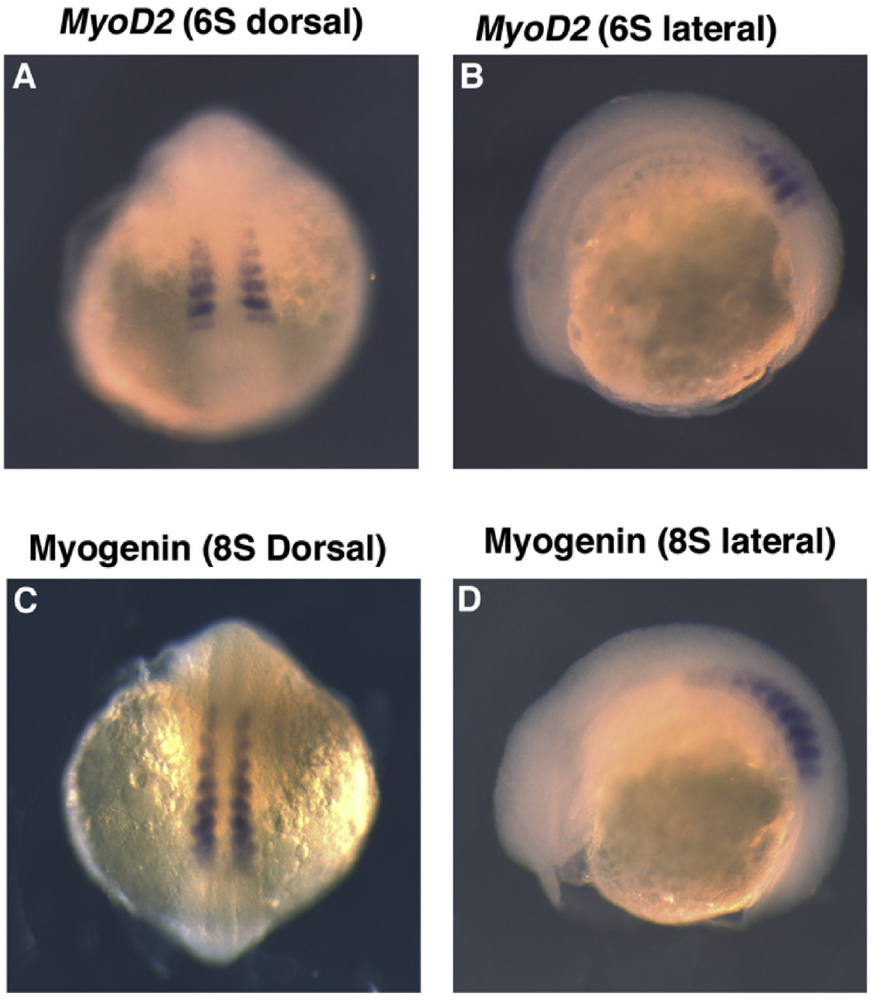

Fig. 2. In situ Hybridisation of *MyoD2* and *Myogenin* (*myog*) during somitogenesis in *T. niphobles*. **A**, **B**, *MyoD2* expression in developing somites at the 6-somite stage embryo (A) dorsal view and (B) lateral view. **C**, **D**, *Myogenin* expression demarcating the early bilateral somite blocks in the 8-somite stage *T. niphobles* embryo; dorsal view (**C**) and lateral view (**D**).

with a weakly darkened retinal pigment equivalent to the Prim (primordial) −10 to Prim-21 stages of development (in zebrafish). The pigmentation of the embryo by both the xanthophores and melanophores spread during these pharyngula stages and covered the dorsal regions of the exposed yolk, the ventral trunk of the embryo and began migration to anterior and dorsal regions of the head image (Fig. 1U). During these migratory periods of the pigment cells, the retinal pigment became darker (Fig. 1V).

3.7. Hatching period

The hatching period of *T. niphobles* was variable within a batch of embryos, where this process can take from 24 h (in this trial) to several days [18]. Typical landmarks of this period of development were the formation of the jaw cartilages, which defines the period by which the mouth develops. At these stages prior to hatching the pigmentation in the retina began to transition from the dark/black to reflective iridophore pigmentation. The mouth was clearly visible and began to protrude beyond the limit of the eyes. Interestingly, the pigment of the ventral trunk and the anterior head region became dominated by xanthophores, giving the embryo a distinctive orange colouration (Fig. 1W). This colour pattern then appeared to permeate throughout the body and on hatching the emerging *T. niphobles* hatchlings were orange dotted with large dark melanocytes. The emerging *T. niphobles* fry were free-swimming (Fig. 1X) and although still retaining a considerable yolk for several days after hatching, they began to feed on zooplankton (rotifer) between 3 and 5 days after emergence from the chorion.

4. Discussion

In this study, we report the developmental stages of *T. niphobles* based on morphological characteristics. This information is anticipated to allow the use of pufferfish as a model for developmental studies [19], uncovering the morphological diversification of this group of highly derived teleost fishes. Regarding the different embryonic stages during the egg development, cell division cycle from the 2-cell to the 1k-cell stage lasted approximately 12 h in *T. niphobles*. These intervals were quite similar to the other closely related species, the green spotted pufferfish (*T. nigroviridis,* [18]) and another model teleost species, as the medaka (Killifish; *O. latipes;* [20]). However, the cleavage and blastula period of Fugu (*T. rubripes*) embryogenesis was a little longer (about 16 h) compared to *T. niphobles*; and approximately a threefold shorter in zebrafish (*D. rerio*), probably due to the fast embryo development in the model species par excellence.

The early embryonic development of *T. niphobles* to the start of the segmentation phase of development was approximately 11 h faster than *T. rubripes* [10] and 7 h shorter than *T. nigroviridis* [18]. This shows that even among closely related species inhabiting a similar environment with equivalent standard temperatures for development, there is a great degree of developmental heterochrony and potential diversification. *T. niphobles* embryos at this stage are vastly more heavily pigmented than the closely related *T. rubripes* embryos [10] suggesting the diversity in development even during these later stages of embryogenesis. In contrast to *T. niphobles*, *T. rubripes* appears almost clear of pigmentation in areas other than the trunk (i.e. the head and majority of the body), although pigmentation only appears in the head region of *T. rubripes* at the protruding mouth stages of development (188 h [10]). In comparison pigmentation in *T. nigroviridis* appears early, at 3 days and 5 h (77 h [18]) and this obviously reflects the speed of development towards an earlier hatching period in *Tetraodon*. It is clear that pigmentation becomes more pronounced in the stages closest to the hatching period in all species. Pectoral fins have developed towards the end of this protruding mouth stage of development (equivalent to the mid-high 'pec' stages observed by Uji et al. (2011) [10]).

Hatching time of *Takifugu niphobles* was relatively similar to it closely related species Fugu (*T. rubripes*): pufferfish embryos needed approximately 8 days until they start to hatch while fugu embryos needed 6 days [10]. In contrast, another species of pufferfish genus *Tetraodon* (*T. nigroviridis*) needed only a little more than 3 days for hatching [18]. In this respect, embryo development period is widely variable in marine fish [21]: from a few hours in some carangid fishes to several days (even weeks) in some species of gadids, and this variation is directly related to the combined effects of body size, temperature and life-history attributes [22].

In the case of pufferfish, long incubation times (about 8 days) are due to its peculiar reproductive strategy, where pufferfish larvae must hatch within a narrow window during the next high tides from the egg fertilization [11,23]. In this regard, this slow developmental rate could enable analysis of gene expression patterns in greater detail, as occur in other species like the model medaka fish (*O. latipes*) [20]. At the hatching period, the time between the first and the last hatching larvae in *Takifugu niphobles* showed a 24 h interval, while in other species for example Fugu (*T. rubripes*) or zebrafish (*D. rerio*) this period seems to be two-fold longer, about 48 h. In this respect, the hatching synchrony in *T. niphobles* could be due to intertidal reproductive behavior, where every larvae should hatch in close succession at the right time (high tide) in order to reach the seawater [24].

On the other hand, regarding *Takifugu* as a model organism for future research, it is important to keep in mind that *Takifugu niphobles* is better suited to experimentation than its close relative Fugu, for its small adult size (up to 15 cm compared to 80 cm) and its ability to survive in different salinity waters: seawater, brackish and freshwater. However, one important caveat to the emergence of *T. niphobles* as a comparative lab-based model for developmental biology is the fact that the fishes cannot breed easily in captivity without hormone stimulation. The study of the embryonic stages in captivity is therefore more accessible with proximity to beach breeding adults.

A great deal of research has been conducted on *T. niphobles* in several fields as sperm physiology and quality, gamete storage, ecotoxicology, tooth evolution and neuroscience [25–30]; and this study aims to provide a starting point for the comprehensive description of *T. niphobles* development with the aim to enhance all these research areas. The developmental stage series described in this study is one of the essential steps toward the establishment of *T. niphobles* as an experimental model for developmental biology.

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