Zygote

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Embryonic development of the world's smallest puffer fish, *Carinotetraodon travancoricus* – a threatened freshwater fish of the Western Ghats Biodiversity Hotspot

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Research Article

Cite this article: Chandana BL *et al*. (2024). Embryonic development of the world's smallest puffer fish, *Carinotetraodon travancoricus* – a threatened freshwater fish of the Western Ghats Biodiversity Hotspot. *Zygote*. page 1 of 8. doi: 10.1017/S0967199424000273

Received: 15 October 2023
Revised: 12 June 2024
Accepted: 17 June 2024

Keywords:
Endemic fish; ex situ conservation; fish biology reproductive biology; India

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Abstract

The Malabar dwarf puffer, *Carinotetraodon travancoricus* is the smallest known pufferfish (family Tetraodontidae) and one of the smallest freshwater fishes of the Indian subcontinent. Due to their miniature size, wacky behaviour and appearance, they are much preferred in the international aquarium fish trade, although little is known regarding their breeding activity in captivity and their embryonic development. The purpose of this study was to fill these knowledge gaps. Wild-caught Malabar dwarf puffers were acclimatised to conditions, and pairs were introduced to breeding tanks. Adult fishes were fed with live and frozen diets including Artemia nauplii, moina and bloodworm. During spawning seasons, adult fish displayed elaborate courtship behaviour around sunset. *Carinotetraodon travancoricus* is a batch spawner releasing 1 to 5 eggs per diem. The eggs were spherical, and non-sticky, with a diameter of 1.48 ± 0.1 mm, and hatching took place after 108 to 116 h post-incubation. The newly hatched larvae were 3.5 ± 0.2 mm in length, and weighed 2.9 ± 0.4 mg. The early larvae have substantial yolk and oil globules as an energy reserve. Histological studies on mature females suggested the batch spawning nature of the species and low fecundity. Given its unique reproductive behaviour and characters, in situ protected habitats are required to ensure their continued survival in the wild, apart from encouraging captive breeding to augment the demand in the international aquarium fish trade.

Introduction

Fishes of the family Tetraodontidae, popularly known as puffer fishes, or simply 'puffers' comprise the largest family within the teleostean order Tetraodontiformes. They occur in shallow waters of the tropical and temperate seas, as well as in brackish water and freshwater habitats, from where 27 genera and 193 species of tetraodontids are currently known (Fricke *et al*., 2024). Of these, 30 species occur exclusively in freshwater habitats (Stump *et al*., 2018).

The Malabar dwarf puffer *Carinotetraodon travancoricus* (Hora & Nair, 1941), occurring in the lowland rivers of south-western peninsular India is one of the smallest known pufferfish species (maximum size of 35 mm; Froese & Pauly 2022). They are endemic to the Western Ghats Biodiversity Hotspot (Dahanukar *et al.* 2004) and recorded from thirteen rivers in the State of Kerala and also from the Mavincar (Devi *et al*., 2000) and Aghanashini Rivers (Bhat, 2004) in the adjoining state of Karnataka. Malabar dwarf puffer, though primarily a freshwater fish, have often been reported from low-saline estuarine regions as well (Devi *et al*., 1996). The species is currently assessed as Vulnerable on the IUCN Red List of Threatened Species as a result of extensive habitat alterations and exploitation for the aquarium fish trade (Dahanukar, 2011).

Carinotetraodon travancoricus dominates the wild-caught aquarium fish exports from India, but its fishery and trade have been subdued by the popularity of the Redline Torpedo Barbs, *Sahyadria denisonii* and its sister species, *Sahyadria chalakkudiensis* (Raghavan *et al*., 2013). In recent times, the advent of captive breeding and exports of torpedo barbs from the Southeast Asian countries resulted in poor demand for its wild counterparts from the Western Ghats, which resulted in the resurgence of the popularity of dwarf puffers. Complete dependency on wild populations to meet the increasing demand for freshwater aquarium fishes, often causes overexploitation and therefore developing viable captive breeding techniques is important in augmenting the supply, but at the same time making sure that pressure on wild populations is minimal.

Limited studies are available on the taxonomy, distribution, biology and biocontrol capabilities (Inasu, 1996; Joshi, 2004; Anupama *et al*., 2019) of *C. travancoricus*, although the spawning and a brief description on the embryo and larval developments have been reported

(Doi *et al*., 2014). However, it is not an elaborative account of the embryogenesis, hatching and rearing of larvae. The staging of embryogenesis is one of the cardinal approaches to developmental studies, and such information has not yet been documented for the dwarf puffers. We aim to bridge this knowledge- gap by presenting baseline information on the captive breeding and embryonic development of *C. travancoricus*. In addition, the members of the family Tetraodontidae are known to have the smallest vertebrate genome (Hinegardner, 1968), and this makes captive breeding and embryonic studies essential to understand the genetic imprints with broader applications.

Materials and methods

Broodstock development

Adult individuals of *Carinotetraodon travancoricus* originating from Chalakudy River, Kerala, India were procured from an aquarium fish supplier. Fish were procured and transported to the Ornamental Fish Hatchery of the Kerala University of Fisheries and Ocean Studies, Kochi, India, and conditioned in 400 L planted, circular, cement cisterns. Adult fish with an average size of 2–2.5 cm and 0.4–0.5 g was transferred to breeding tanks of 2'x1'x1' size. Tanks were provided with pebbled substratum and aquatic plants to facilitate spawning. The brooders were fed with bloodworms and moina twice a day, in the morning and evening. The water parameters such as temperature and pH were monitored daily, while ammonia, alkalinity, hardness and nitrite were determined once a week. About half of the water in the broodstock tank was replenished weekly to compensate for the loss due to evaporation and routine waste removal. The experiment was conducted during the months of July to April, that is, for 10 months.

The structure and development of male and female gonads were determined through histology. The tissues were fixed in Bouin's fixative and dehydrated by passing through a series of alcohol concentrations. The clearing was done with xylene and dehydration by absolute methyl benzoate and benzene. The dehydrated and cleared tissues were subsequently embedded in paraffin wax for block preparation, resultant sections were mounted with DPX, and staining was carried out using hematoxylin and eosin.

Egg collection and incubation

After acclimation to the breeding tank, pairs exhibited courtship behaviour and spawned without external hormonal induction. As the eggs were deposited between pebbles, they were carefully collected using a Pasteur pipette, or by siphoning the tank bottom. The collected eggs were carefully rinsed to clear any attached particles and transferred to 2000 ml glass containers. Eggs collected from each tank were incubated separately for proper data collection.

Embryonic development

The embryonic development was observed and recorded using a stereo zoom microscope (Stemi 508, ZEISS) attached to a digital camera (AXIOCAM 105). Observations were made at room temperature with duration for each division and for documenting the changes that occurred during development. The description of the staging of *C. travancoricus* embryonic development followed the methods used previously for tiger puffer (Uji *et al.,* 2011), green-spotted puffer (Zaucker *et al.,* 2014), grass puffer (Gallego *et al.,* 2017), and also for laboratory models such as zebrafish (Kimmel *et al.,* 1995) and medaka (Iwamatsu, 2004). These were primarily based on their morphological characteristics, and stages were divided into zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching.

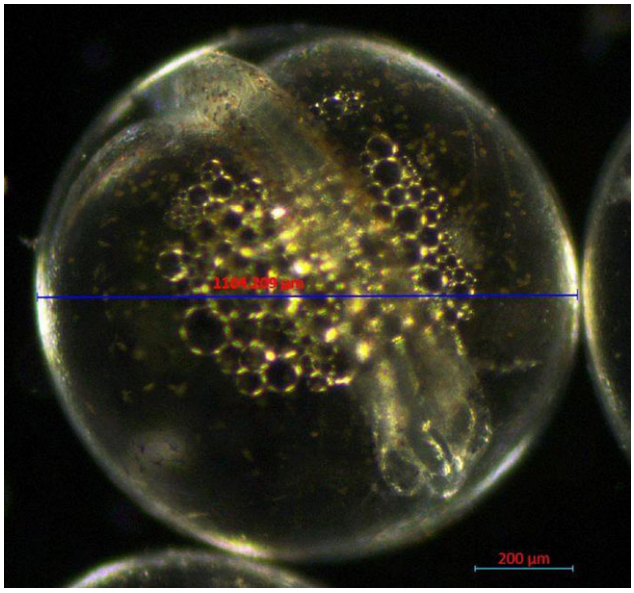

Figure 1. Developing egg of *Carinotetraodon travancoricus*.

Response variables/formulae used

Hatchability

The hatchability of eggs (expressed in percentage) was estimated as the ratio of the number of hatched eggs to the total number of eggs incubated.

$$\text{Hatchability (\%)} = \frac{\text{No. of eggs hatched}}{\text{Total no. of eggs}} \times 100$$

Condition factor

Fulton's condition factor (K) was calculated according to the following equation of (Htun-Han, 1978)

$$K = \frac{\text{Weight of fish (g)}}{(\text{Length of fish; cm})^3} \times 100$$

Results

Broodstock development

Distinct sexual dimorphism was observed in *C. travancoricus* with the mature male having (1) a prominent mid-ventral keel, with a solid black stripe and a yellowish-orange periphery, extending to most of the ventral side; (2) prominent round wrinkles around the eyes; (3) dorsally and ventrally curved body resulting in an oval shape and 4) caudal fin with numerous conspicuous black marks. The keel was observed to be erect, the fins spread out, and the overall body colouration darker during courtship. In contrast, the female had a whitish belly with a pale yellow periphery. In mature females, bulging of the belly was visible from both sides, and distinct black blotches were observed on the body and caudal fins. Both male and female brooders were more colourful and spread their fins during courtship.

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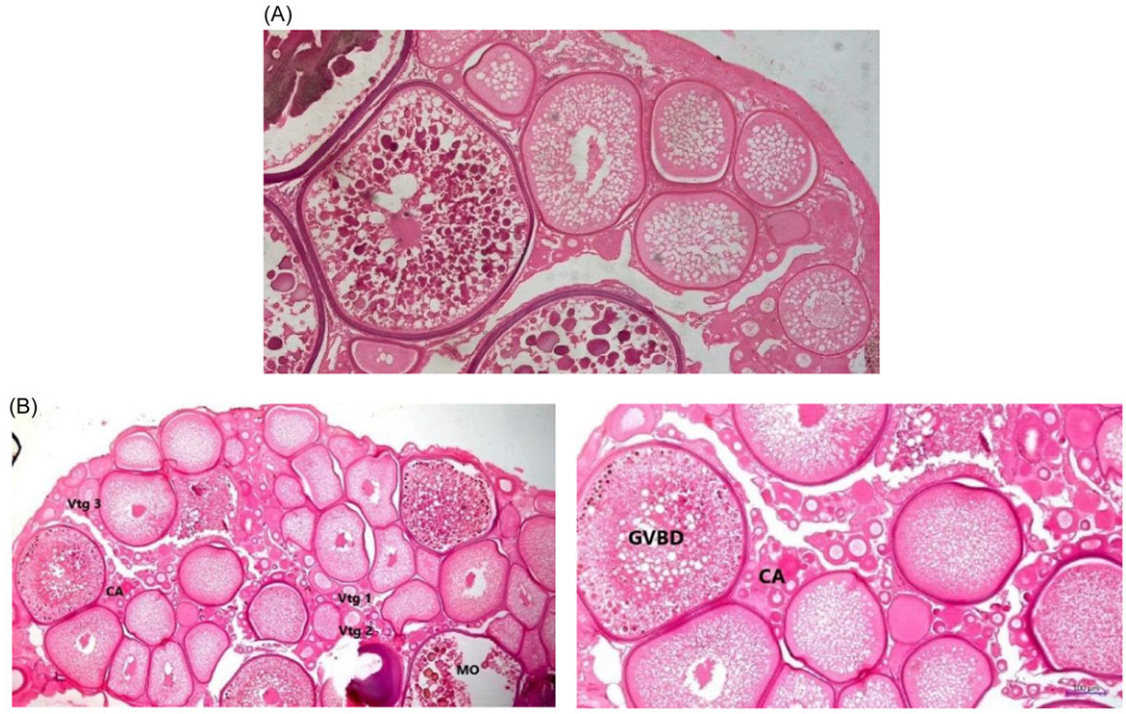

Figure 2. (A) Cross section of ovary in adult *Carinotetraodon travancoricus*; (B) Transverse section of the ovary showing CA – corticular alveolar oocytes; Vtg1 – primary vitellogenic oocyte; Vtg2 – secondary vitellogenic oocyte; Vtg3 – tertiary vitellogenic oocyte; GVBD – germinal vesicle breakdown and Mo – mature oocyte.

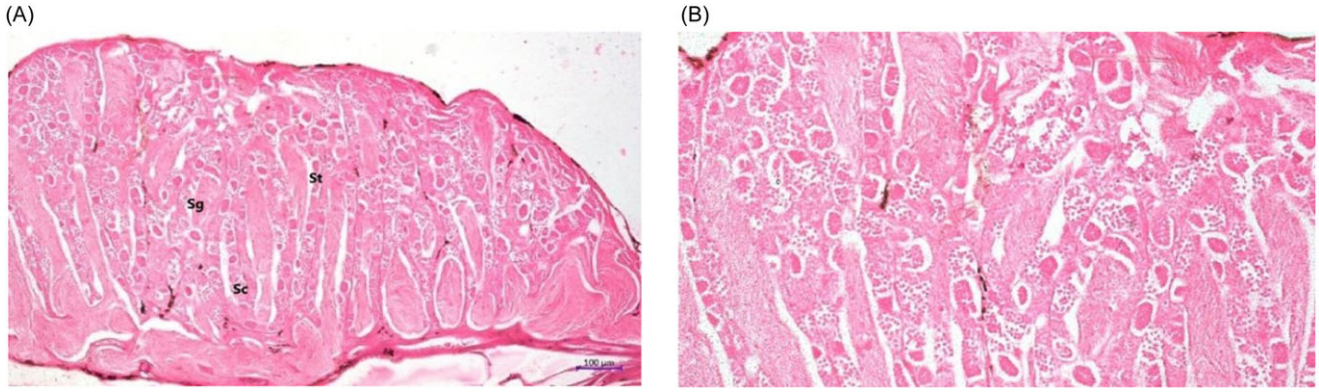

Figure 3. (A) Cross section of testis in adult *Carinotetraodon travancoricus* showing Sg –spermatogonia; Sc – spermatocyte and St – spermatid; (B) Transverse section of testis.

Carinotetraodon travancoricus exhibited intense courtship behaviour during the late evening which culminated in the release of eggs. The male was often observed to passively guard the spawning area. Females released 1 to 5 eggs per spawning at an interval of one to 3 days. The demersal eggs (diameter of 1.48 ± 0.1 mm (Figure 1)) were deposited between the pebbles or confined spaces in the tank. They were transparent, and non-sticky with pale yellow colouration, and characterised by the presence of multiple oil globules. The mean condition factor (*K*) of the brooders reared in captivity was 2.5 ± 0.1. A total of 161 spawning events were noted from twelve breeding tanks in 10 months.

The gonad histology revealed the presence of ova at different stages of maturity ranging from primary oocytes to fully mature ovum (Figure 2). This ova distribution pattern indicated the batch spawning nature of the dwarf pufferfish. The presence of mature ova near the peripheral region of the ovary resulted in external bumps in mature females, and the occurrence of 1 to 5 matured oocytes in the gonad conformed to the eggs released per spawning. The presence of many mature and maturing ova in the gonads indicated the peak seasonal spawning nature of the species. The developing ova which were irregular acquired a spherical shape during the later stages of maturity.

Histological observation of the male gonad revealed the presence of spermatozoa indicating its maturity, and the spermatocytes and spermatogonia were also visible (Figure 3). The gonadosomatic index (GSI) of the captive brooders had a mean value of 8.1 ± 1.8, and the mean condition factor (*K*) was 2.5 ± 0.13. The water parameters observed during the study period were temperature of 26.2 to 30.1°C, pH ranged from 7.4 to 8.1 and the photoperiod was maintained close to 12 h/day.

Embryonic development

The most significant embryonic developmental stages are presented in Table 1 and Figure 4. The eggs were collected from

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Table 1. Embryonic developmental stages of *Carinotetraodon travancoricus* in captivity

Period	Stages	Time (h)
Zygote stage	None	00:05 h
	Single-cell stage	01:07 h
	2-cell stage	01:23 h
	4-cell stage	01:59 h
Cleavage	8-cell stage	02:38 h
	16-cell stage	03:08 h‘
	32-cell stage	03:36 h
	64-cell stage	04:01 h
	128-cell stage	04:30 h
Blastula	256-cell stage	05:02 h
	512-cell stage	05:40 h
	20 % epiboly	12:46 h
Gastrula	40 % epiboly	14:40 h
	50 % epiboly	16:19 h
	80 % epiboly	19:00 h
	Tail bud stage 1	20:29 h
Segmentation	Tail bud stage 2	22:15 h
	Eye vesicle stage	24:10 h
Pharyngula	Twitching stage	40:46 h
	Eye pigmentation	60:21 h
Hatching	First eclosion	108:00 h
	Last eclosion	116:00 h

the breeding tanks immediately after fertilisation, or the following morning and subsequently incubated in glass beakers with mild aeration. As the development progressed, the egg colour changed from pale yellowish to reddish-brown. The incubation period varied from 108 to 116 h depending on the water temperature, which varied from 26.8°C to 29.8°C during the experimental period

Zygote stage: The zygote stage started soon after fertilisation and lasted for more than an hour. The cytoplasm of the egg streamed towards the animal pole of the egg to form the blastodisc. The yolk was transparent with multiple oil globules, and the cluster of oil globules kept changing its position until the onset of segmentation. The thin perivitelline space increased and became clearer with the formation of the blastodisc.

Single-cell stage: The cytoplasm after separation from the animal pole formed the blastomere, also called the single-cell stage. This stage lasted for an average of 16 min before the first division started. This single cell cleaved continuously to form numerous cells which kept decreasing in size.

Cleavage: First division started from 01:23 h post-fertilisation (hpf). The cleavage was meroblastic, and blastomere cleavage was meridional to form the 2-cell stage, and these cells were again divided in the same manner to form 4 cells. Meridional cleavage occurred for the first three divisions until the formation of 8 cells. From the fourth cleavage onwards, the plane of division became indistinguishable, and the subsequent cleavages occurred at approximate 30 min intervals.

Blastula: The morula stage of the embryo is followed by the blastula stage, which is represented by the 128-cell stage lasting until the dome stage. This occurred from 04:30 hpf, and continued up to 20% epiboly, and for a duration of 8.16 h. The continuous divisions during this stage resulted in a cap-like formation of the cells on top of the yolk. During the onset of epiboly, the individual smaller cells formed through the cleavage became indiscernible. The yolk was observed to push itself into the embryonic cells at the animal pole, and the yolk syncytial layer appeared below the blastodisc margin.

Gastrula: The different gastrula stages proceeded during the period from 40% epiboly to the tailbud stage. The blastoderm margin moved over the yolk towards the vegetal pole and gastrulation began when it covered > 40% of the distance. Up to 50% epiboly was achieved at 16.19 hpf, and the blastoderm was observed to have a uniform thickness. Afterwards, a thickening was noticed at the animal pole above the margin where the embryonic axis was formed. By the end of epiboly, the margin almost completely covered the yolk cell, and the small notch-like formation at the thickened region of the blastoderm indicated the onset of the tailbud stage. The tailbud stage was observed at 22:15 hpf, and the gastrula stage lasted for around 10 h.

Segmentation: The principal body plan of the embryo was formed at this stage, which followed the tailbud stage. Development of the head region started, and at the late stage of neurulation, the eye vesicles which were present as optical buds started developing at the fore-end of the neural axis. Segmentation was observed after 24 hpf. Repetitious structures called somites started to form along the anterior–posterior axis. Morphological analysis of somitogenesis was relatively unclear in *C. travancoricus* due to the presence of the aggregate of oil globules. Olfactory placodes and optic vesicles appeared during the initial stages of somitogenesis. During this stage, the embryo encircled around half of the yolk cell. The shape of the yolk remained circular with the embryo around it. Brain development was also observed. Notochord became visible along the trunk and tail regions, the caudal region became distinct, and eye vesicles became prominent by the end of the second day of incubation. Initial signs of pigmentation could also be observed on the embryo and yolk.

Pharyngula This stage was marked by the completely formed head region, and visible heartbeat along with circulation. A slight movement of eyeballs was observed indicating the formation of the lens. Pigmentation started to get prominent with the body becoming darker, and coloured dots were observed all over the yolk. Black coloured eye pigmentation was observed during the third day of incubation. Pectoral fin buds became visible, and the tail region detached from the yolk. To fit into the chorion, the embryo bended sideways over the yolk. After 72 hpf, the embryo was characterised by discontinuous bright pigmentation in the head and trunk region. The eye movement started on the fourth day of incubation. The brain became enlarged in comparison to earlier stages, and the head appeared bulky. The embryo was observed to encircle the yolk almost completely with circulation all over its body and yolk sac.

Egg hatching and larvae

The hatching was not synchronous, and it varied even within a batch, which indicates elongated courtship and spawning nature. Before hatching, the embryo showed frequent movements within the egg sac. The body and eye pigmentation were brighter, giving the egg a dark reddish morphological appearance. The process of hatching took around 35 min from initial breaking, to completely

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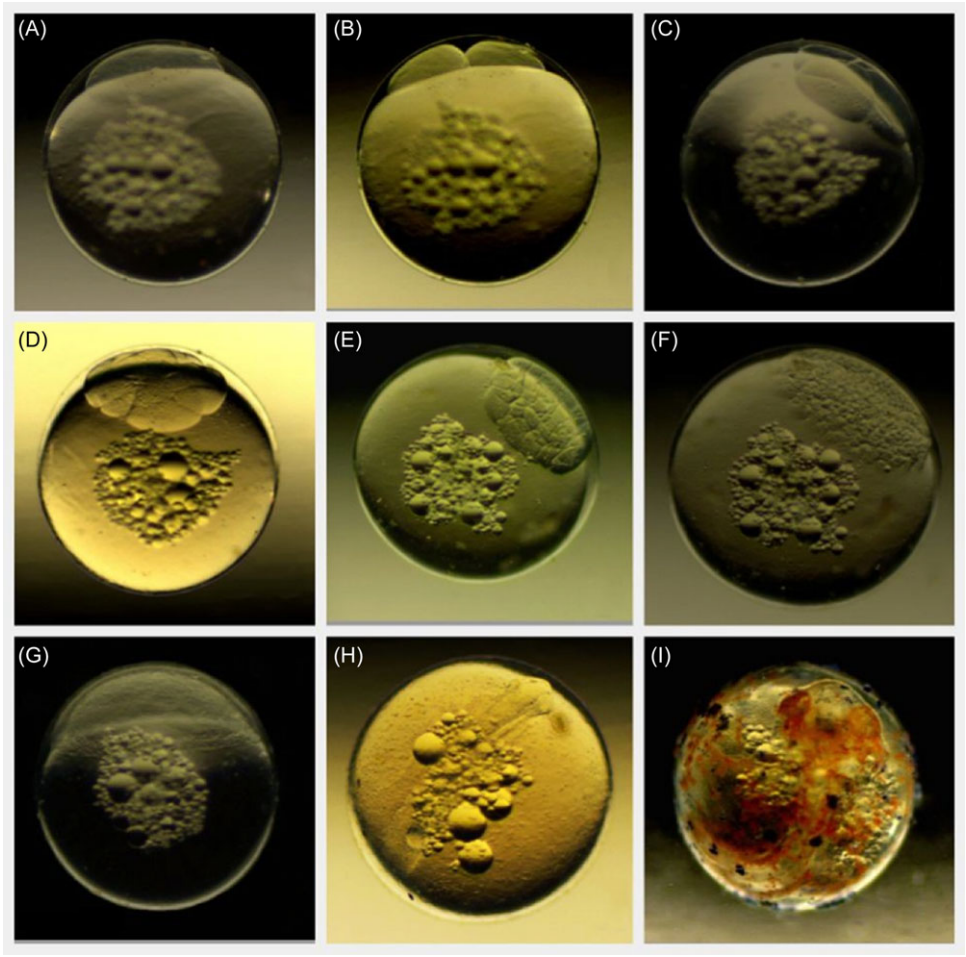

Figure 4. Stages of embryonic development of *Carinotetraodon travancoricus*. (A) single-cell stage. (B) 2- cell stage. (C) 4-cell stage. (D) 16-cell stage. (E) 64-cell stage, (F) blastula stage (check whether it is morula stage). (G) 20% epiboly (early blastula). (H) eye vesicle stage. (I) before hatching.

emerging out of the egg sac (Figure 5). The newly emerged hatchlings were highly pigmented with a transparent tail region. The larvae had a considerable amount of yolk and oil globules; however, they seem to have a partially opened mouth at hatching (Figure 6). The newly hatched larvae had a total length of 3.5 ± 0.2 mm and a wet weight of 2.9 ± 0.4 mg. The larvae start free swimming and start feeding 4 to 5 days after hatching.

Incomplete hatching of eggs was also observed, wherein the larvae were unable to detach completely from the thick outer covering. Many larvae perished during this process and could be saved by manually detaching from the capsule. The hatchability of dwarf puffer eggs was low and found to be about 52%.

Discussion

Developing captive breeding techniques is the key to maintaining the sustainability of an aquaculture production system. Despite the huge demand for *C. travancoricus* in the aquarium pet trade, research approaches on captive breeding and early development have been limited. Though sexual dimorphism in *Carinotetraodon travancoricus* was reported from wild collected specimens (Inasu, 1993; Britz and Kottelatt, 1999), direct visual observation and examinations were done on male and female brooders for the first time, in our study. The peculiar colour patterns exhibited by males on reaching maturity was permanent in nature. Similar distinct sexual dimorphism was also observed with freshwater pufferfish *Tetraodon cutcutia* on maturity, wherein females developed a dark reddish colour in eyes and caudal fin margin (Karmakar and Biswas, 2014).

The embryonic development stages of *Carinotetraodon travancoricus* were similar to that in other members of the family Tetraodontidae including fugu *Takifugu rubripes* (Uji *et al.*, 2011), spotted green pufferfish *Tetraodon nigroviridis* (Zaucker *et al.*, 2014) and grass pufferfish *Takifugu niphobles* (Gallego *et al.*, 2017). As reported in the cases of spotted green pufferfish (Zaucker *et al.*, 2014), the eggs of the dwarf puffer have oil globules, lack wide perivitelline space and the emergence of the eye rudiment before somitogenesis. The number of eggs released per spawning was less ranging from 1 to 5, much lower than that of *Carinotetraodon irrubesco*, and *Carinotetraodon lorteti*, wherein it was about 200 (Doi *et al.*, 2014). Eggs of *C. travancoricus* were non-adhesive unlike *Canthigaster valentini* (Gladstone & Westoby, 1988) and *Xenopterus naritus* (Ahmad Nasir *et al.*, 2017) which had adhesive eggs. The incubation period of *C. travancoricus* was found to be four and a half days, and these prolonged hatching periods in pufferfishes account for their particular reproductive strategy (Gallego *et al.*, 2017).

Newly fertilised eggs of *C. travancoricus* had a mean diameter of 1.48 mm which was in agreement with the observation of Doi *et al.* (2014). The time taken to achieve the single-cell stage was about an hour in dwarf puffers, similar to *T. nigroviridis* and *T. niphobles*, but lesser than that in the case of fugu *T. rubripes* (= 4 h) (Zaucker *et al.*,2014; Gallego *et al.*, 2017; Uji *et al.*, 2011). The time for blastulation in *C. travancoricus* (8.16 h) was similar to grass and

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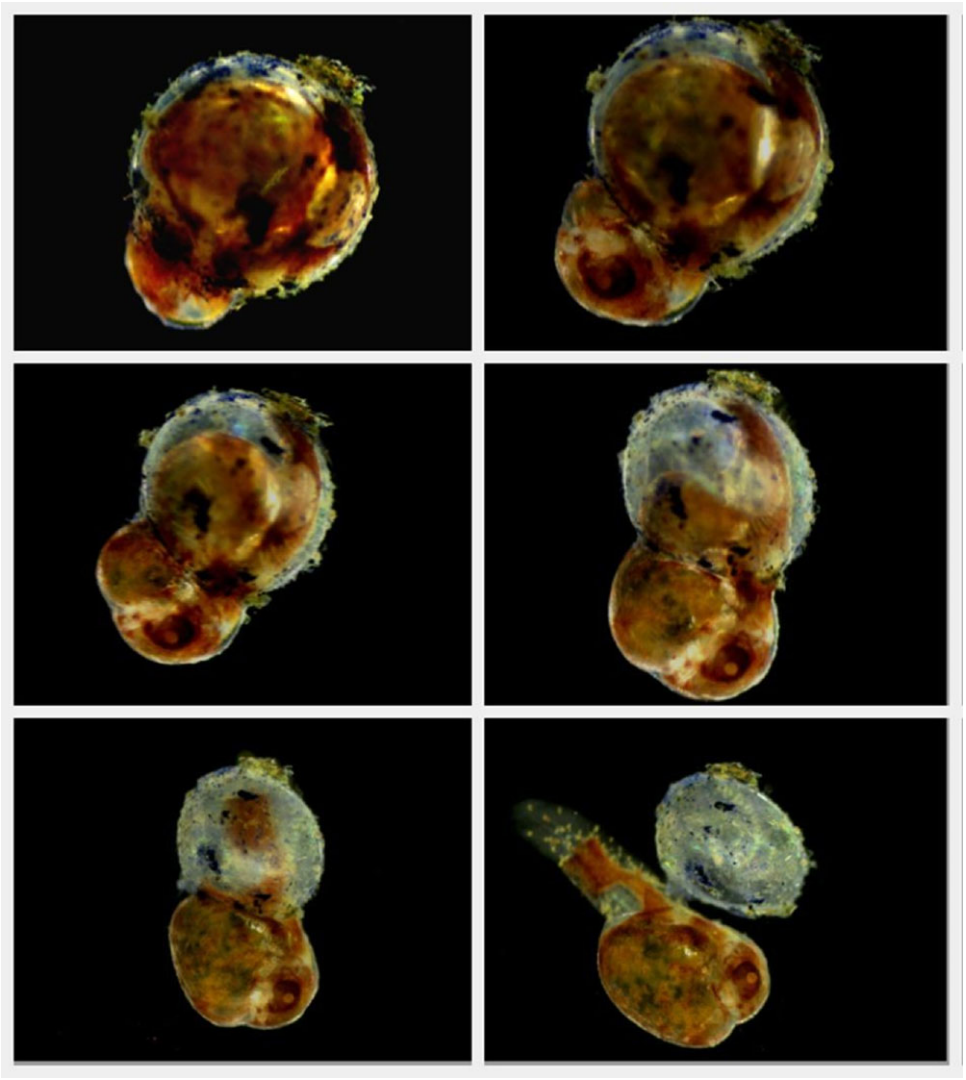

Figure 5. The struggle of *Carinotetraodon travancoricus* larvae to emerge from the embryo with blunt head and massive yolk sac.

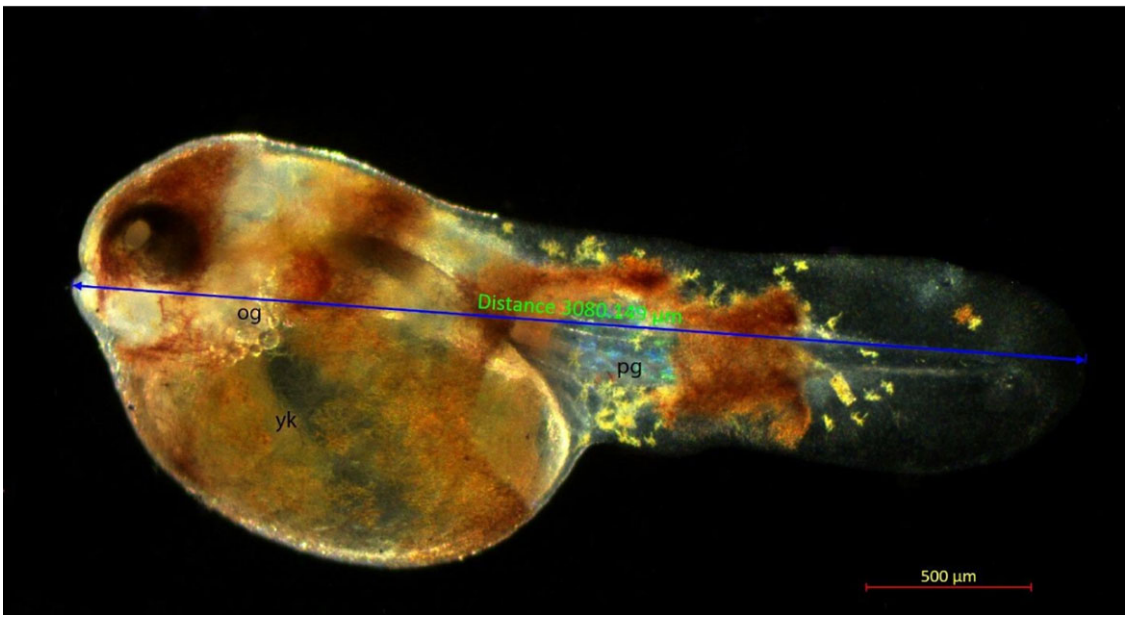

Figure 6. Newly hatched larvae of *Carinotetraodon travancoricus*. (og – oil globule; yk – yolk sac, pg - pigmentation).

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tiger puffers, but much lesser than that observed in fugu (*T. rubripes*) which was around 12 h (Uji *et al.,* 2011). The gastrula stage lasted for 9 h in dwarf puffers as in the case of *T. niphobles* (Gallego *et al.,* 2017), but lower than that observed in *T. rubripes* – 15 h (Uji *et al.,* 2011) and *T. nigroviridis* – 9 h (Zaucker *et al.,*2014) respectively. The early embryonic development of *C. travancoricus* up to the pharyngula stage was similar to that of *T. nigroviridis* whereas, it was 37 h quicker than *T. niphobles* (Gallego *et al.,* 2017) and 40 h quicker than *T. rubripes*. *Carinotetraodon travancoricus* embryos are heavily pigmented at this stage similar to that of *T. nigroviridis,* while *T. rubripes* appeared almost clear of pigmentation in areas other than the trunk. The dense aggregate of oil droplets obstructed the visual observation of the internal organ development. Suitable labelling or staining methods are therefore required in the case of dwarf puffer for a complete description of the organogenesis.

Hatching occurred in *C. travancoricus* at 108–116 h; a period much slower than that of T. *nigroviridis* which took 80 h 43 min (Zaucker *et al.,* 2014), despite the similarity in incubation temperature. However, this duration was quicker when compared to the 191 –214 hpf of *T. niphobles* (Gallego *et al.,* 2017) and 145–192 hpf of *T. rubripes* (Uji *et al.,* 2011). The egg incubation period varied between the same batch of eggs, with a gap of 8 h between emergences of larvae in *C. travancoricus*; in Fugu (*T. rubripes*) this period was about 48 h (Uji *et al.,* 2011), and in grass puffer, this interval was reported to be 24 h (Gallego *et al.,* 2017). The incubation period for other freshwater pufferfishes reported by Doi *et al*., (2014) such as *C. irrubesco* (2-3 days) and *C. lorteti* (4 days) was found to be shorter than that of *C. travancoricus,* while for species like *T. biocellatus* (5 days), *T. cochinchinensis* (7 days), *T. cutcutia* (6 days), *T. palembangensis* (10 days) and *T. turgidus* (8 days), it was longer. In the present study, the hatchability obtained was 52%, which was lower compared to those observed in induced-bred *Takifugu obscurus* (90%) during induced breeding (Yang and Chen, 2005). This could be due to inadequate water parameters, temperature fluctuation and lack of water movement in our study set-up. Further studies are warranted to determine whether modification of captivity conditions might improve breeding of this species.

Conclusion

The spawning and embryonic development of *Carinotetraodon travancoricus* based on morphological characters and the various stages during development were similar to closely related species. *Carinotetraodon travancoricus* is difficult to breed in captivity during the spawning season and the hatchability is low. There is hence a need for maintaining a larger brooder population to achieve bulk production under captivity and further refine conditions for captivity breeding. Additional in situ conservation strategies, such as the development of protected zones in the lowland areas of rivers during peak breeding season, and a seasonal ban on collection will ensure the long-term survival of this threatened species in the wild.

Data availability. The datasets generated during the current study are available from the corresponding author on reasonable request.

Acknowledgements. This research was supported by the KUFOS Aided Research Project (KARP), Kerala University of Fisheries and Ocean Studies, Kochi, India. The authors are thankful to the Directorate of Research and Dean, Faculty of Fisheries for the support.

Author contributions. Binu Varghese: investigation, conceptualisation, methodology. Rajeev Raghavan: methodology, manuscript editing. BL Chandana and Ashly Sanal: investigation, data collection, drafting, literature review. All authors contributed to the article and approved the submitted version.

Funding. Partial financial support was received from KARP, KUFOS. Grant no. DoR/4751/2019.

Competing interests. The authors have no conflict of interest to disclose.

Ethical standards. The authors confirm that the ethical policies of the journal, have been adhered to, and the research undertaken complies with the current animal welfare laws in India.

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