Title	Experimental ecological study on spawning and development of four genera, four species of pufferfishes distributed in freshwater and brackish water regions
Author(s)	百田, 和幸
Citation	北海道大学. 博士(環境科学) 乙第7187号
Issue Date	2023-12-25
DOI	10.14943/doctoral.r7187
Doc URL	http://hdl.handle.net/2115/91176
Type	theses (doctoral)
File Information	Kazuyuki_Momota.pdf

Doctoral Thesis

Experimental ecological study on spawning and development of four genera, four species of pufferfishes distributed in freshwater and brackish water regions

(汽水・淡水域に生息するフグ科魚類4属4種の繁殖と成長に関する実験生態学的研究)

Division of Biosphere Science

Graduate School of Environmental Science

Hokkaido University

Kazuyuki Momota

CONTENTS

List of Figures

List of Tables

GENERAL INTRODUCTION

Tetraodontidae (pufferfishes), including 30 genera and 200 species, is distributed in shallow warm, tropical seas, and freshwaters of the world (Matsuura, 2015; Nelson et al. 2016). This family has some unique morphological features such as: a head that is large and blunt; jaws that are modified to form a beak of heavy, powerful teeth, with two above and two below; gill opening, as a simple slit in front of pectoral fins; and pelvic fin that is absent (Matsuura 2015).

Tetraodontidae is a family of Tetraodontiformes, one of the most derivative monophyletic groups in Teleostei (Yamanoue 2015; Matsuura 2015). Tetraodontiforms have long attracted the attention of ichthyologists and biologists because of their peculiar morphological characteristics (Matsuura 2015). Among tetraodontiforms, Tetraodontidae is the only family adapted to freshwater habitats (Yamanoue 2015). The boundary between freshwater and sea is one of the most difficult barriers to expand habitats for aquatic animals because there are many difficult gaps to overcome between these habitats (Hutchinson 1957). In this respect, this family is thought the most unique, interesting group in tetraodontiforms.

Tetraodontidae has expanded their habitat from coastal waters into other habitats like freshwaters, brackish waters, and deep seas in its evolutional history (Yamanoue et al. 2011). Their life cycles are various. Some species in this group are known to migrate between ocean and river in their life span, others are known to spend their entire existence in oceans or rivers without migration (Ebert 2001). They have also acquired diverse reproductive biology, such as large group spawning in the genus *Takifugu,* pair spawning in *Canthigaster rivulata* (see Matsuura 2017), and harem spawning in *Canthigaster valentini* (see Gladstone, 1987). In addition, *Takifugu niphobles* spawns scattered eggs (Uno 1955), *Canthigaster rivulata* spawns in territories without parental egg protection (Arai and Fujita 1988), and *Torquigener albomaculosus* makes a territory with a circular structure, spawns there and guards the eggs until hatching (Kawase et al. 2015).

When considering the diversity of Tetraodontidae, the adaptation to inland water must be one of its essential factors. In tetraodontid fishes, eight genera and 41 species (five *Auriglobus*, five *Carinotetraodon*, one *Chonerhinos*, three *Colomesus*, six *Dichotomyctere,* one *Leiodon*, 14 *Pao* and six *Tetraodon* species) are distributed in freshwater or brackish water (Ebert 2001). Their invasions into freshwater habitats were investigated using molecular phylogenetic analysis in recent years (Yamanoue et al. 2011; Igarashi et al. 2013; Santini et al. 2013a). Yamanoue et al. (2011) and Igarashi et al. (2013) reports included seven genera of freshwater and brackish water pufferfishes, which showed almost the same phylogenetic tree (Fig. gi. 1). Yamanoue et al. (2011) considered that six genera [*Leiodon, Pao, Tetraodon* (these three genera were treated as *Tetraodon*), *Auriglobus, Carinotetraodon, Colomesus*], distributed in freshwater, had invaded into freshwater at least three separate times in different continents and thus separated them into the following three groups: South American group (*Colomesus*), Central African group (*Tetraodon*) and Southeast Asian group (*Auriglobus, Carinotetraodon, Leiodon, Pao*). They positioned *Dichotomyctere* in a different clade from the clade of the other Asian freshwater pufferfishes. However, they thought *Dichotomyctere* (treated as *Tetraodon*) a marine genus and did not give any comments about this genus. Igarashi et al. (2013) considered that six genera, except for *Colomesus,* were separated into three slightly different groups by their invasion time into freshwater or brackish water as reported from Yamanoue et al. (2011). These groups were as followed: Asian freshwater group (*Auriglobus, Carinotetraodon, Leiodon, Pao*), Asian brackish water group (*Dichotomyctere*), and African freshwater group (*Tetraodon*). Yamanoue et al. (2011) and Igarashi et al. (2013), however, did not examine *Chonerhinos,* as the position of it was unrevealed. On the other hand, Santini et al. (2013a), analyzed all the eight genera of freshwater and brackish water pufferfishes (Fig. gi. 1), showing that the genus *Chonerhinos* was nested in the same clade with Asian freshwater group [*Leiodon*, *Pao* (these two being treated as *Tetraodon*), *Auriglobus*, *Carinotetraodon*] and Asian brackish water group (*Dichotomyctere*, treated as *Tetraodon*). Then Santini et al. (2013a) considered the invasion of the ancestral Asian freshwater and brackish water pufferfishes had been once. However, *Chonerhinos* is an euryhaline genus throughout its evolutionary life (Roberts 1982; Ahmad-Nasir et al. 2016, 2020). Moreover, the four Asian freshwater genera (*Auriglobus*, *Carinotetraodon*, *Leiodon*, *Pao*) were separated into some other subclades within the phylogenetic tree of Santini et al. (2013a). Thus, the question concerning the diversification and adaptation of the freshwater and brackish water pufferfishes had remained unsolved and would be answered following clarification of their phylogenetic relationship and accumulation of more detailed knowledge of their life history.

Each reproductive behavior in fish has a close relation with an evolutional background (Sokabe 2018; Sunobe 2018). Therefore, information on various reproductive behaviors of tetraodontid fishes may be helpful to reveal their evolutional background such as the invasion into freshwater of Tetraodontidae. However, the divergence of the reproductive behaviors of freshwater and brackish water pufferfishes have yet been surveyed insufficiently. There have been only 10 reports about captive breeding of freshwater and brackish water pufferfishes as follows: African *Tetraodon schoutedeni* was known to perform tandem swimming and spawning with one or some males while biting and clinging to female abdomens, scattering demersal eggs (Feigs 1955); In the Southeast Asian genera, which has a limited species and five genera except for the genus *Auriglobus*, there have been reports on their breeding in aquariums; Three species of *Carinotetraodon* scatter

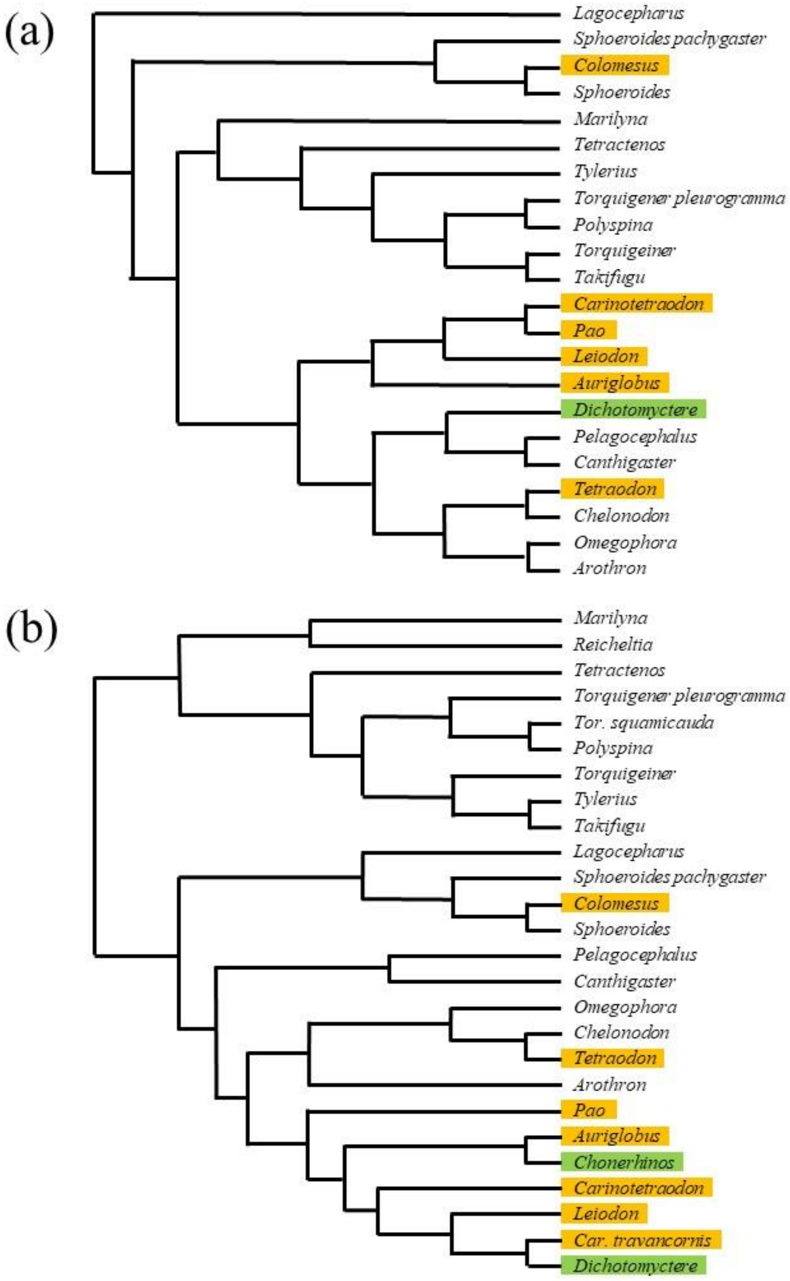

Fig. gi.1. Phylogenetic relationships among tetraodontid genera and their distribution. Cladogram (a) and (b) are based on Yamanoue et al. (2011) and Santini et al. (2013a), respectively. Distribution is based on Ebert (2001). Orange, green and colorless mean freshwater genera, brackish water genera, and marine genera, respectively.

small adhesive demersal eggs (Doi et al. 2015; 2022a); Six species of *Pao* and *Leiodon* lay relatively large eggs in a single layer batch with male protection of these eggs (Doi et al. 2015, 2022a, b); Two species of *Dichotomyctere* are known to scatter large numbers of small eggs (Watson et al. 2009; Doi et al. 2015); and *Chonerhinos naritus,* on the other hand, returns to the river for spawning and spawns oval eggs at lower salinity conditions (Ahmad-Nasir et al. 2016, 2020). To consider their divergence and adaptation, however, more detailed information including other species and another genus such as *Auriglobus* would be necessary.

Besides, tetraodontid fishes were also recognized as utility animals for genetic studies because of their small genome size (the smallest genomes among vertebrata, approximately 400 Mb, one-eighth of human genome) (Crnogorac-Jurcevic et al. 1997; Venkatesh et al. 2000); whole genomes of the following two species were published [*Takifugu rubripes* (Aparicio et al. 2002); *Dichotomyctere nigroviridis* (Jaillon et al. 2004)]. The improvement of their breeding techniques may facilitate the advancement of genetic studies. Tetraodontid fishes also have sustained interest from the commercial field. Many species are traded as ornamental fish (Ebert 2001; Livengood and Chapman 2007). However, most of them have been caught in the wild. If this continued, there might be a danger of extinction in the future. In the viewpoint of fishery, some Asian freshwater pufferfishes have nutritional value (Ahmad-Chowdhury et al. 2019) and have been eaten (Arakawa 2013; Zhu et al. 2020). However, most pufferfishes accumulate some toxins, and sometimes have caused food poisoning (Arakawa 2013; Barman et al. 2018). Utilizing them as food safely, we need more information of their toxin uptake abilities. In addition, artificial breeding of pufferfishes would be helpful for toxicological research because most of their toxins are ingested from their diet (Zhu et al. 2020; 2021). Thus, the improvement and establishment of their breeding techniques are very important not only for ecological and genetic studies but also for future conservation programs of tetraodontid fishes.

In this thesis, I aimed to improve and establish captive breeding techniques of freshwater and brackish water pufferfishes, while clarifying the reproductive manner and early morphological development of four genera and four accessible species, *Leiodon cutcutia*, *Auriglobus modestus*, *Dichotomyctere fluviatilis*, and *Tetraodon schoutedeni*, which are major commercial species. I also tried to consider the evolutional background of their invasion into freshwater environments from the viewpoint of their reproductive biology. In this study, hormone injection was done for three species (*L. cutcutia, A. modestus*, and *D. fluviatilis*) as it is difficult to induce spontaneous maturity in captivity.

In chapter 1, through the examination of captive breeding with hormone injection of *Leiodon cutcutia*, one of the Asian freshwater pufferfishes, details of its reproductive behavior and early morphological development were described, and its mating system was discussed.

In chapter 2, to clarify the reproductive manner of *Auriglobus*, one genera of Asian freshwater pufferfishes, and establish its breeding techniques, artificial insemination and breeding of *Auriglobus modestus* were attempted. Through phylogenetic analysis and artificial insemination of *A. modestus*, relationships between the *Auriglobus* species and *Ch. naritus* were reviewed, and the morphological features of eggs and larvae were described.

In chapter 3, captive breeding with hormone injection of *Dichotomyctere fluviatilis,* one of the Asian brackish water pufferfishes, was observed. The spawning manner and morphological development of larvae and juveniles were observed, and similarities of these characteristics to those of marine species were discussed.

In chapter 4, through the observation of captive breeding of *Tetraodon schoutedeni*, its reproductive behavior and early morphological development were investigated, and the biological significance of its tandem swimming and spawning was discussed.

In general, I conducted a comprehensive comparison of the morphological features of egg and larval stages of freshwater and brackish water pufferfishes and their reproductive features. From this information, the evolutional background, and adaptation of freshwater and brackish water pufferfishes was reconsidered.

CHAPTER 1: Spawning and development of ocellated pufferfish *Leiodon cutcutia* in captivity

Leiodon cutcutia is one of many freshwater species distributed in Asia, being a potential ornamental fish. This species has also attracted attention as a food because of their contents of ash and lipid (Ahmad-Chowdhury et al. 2019) and is eaten in Bangladish. However, it sometimes causes outbreaks of poisoning when eaten (Barman et al. 2018).

Leiodon is known to perform male egg protection (Doi et al. 2015), resembling the behavior of *Pao* species (Doi et al. 2015; 2022a, b). However, *Pao* is a sister group of *Carinotetraodon*, which is a genus that does not perform this protection (Doi et al. 2015), and a little far from *Leiodon* phylogenetically (Yamanoue et al. 2011; Igarashi et al. 2013), therefore, the origin of their egg protection is remained ambiguous.

In this chapter, through captive breeding with hormone injections, details of the reproductive behavior and early development of *Leiodon cutcutia* are described, and its mating system is discussed.

1.1. MATERIALS AND METHODS

1.1.1. Parental fish and hormone injection

Three wild individuals were purchased as parental fish, later found to be one female (36.5 mm SL, 6.0 g BW) and two males (30.5 mm SL, 4.0 g BW; and 28.0 mm SL, 4.0 g BW, respectively), caught in the Jorai River in eastern India from a Japanese fish trader (Rio Co. Ltd., Tokyo, Japan) on March 20, 2018. They were kept in a closed circulating–filtering aquarium (60 cm (L) × 45 cm (D) × 45 cm (H)) at the aquarium NIFREL (Osaka, Japan). The rearing water was replaced with 40 *l* of new water once a week. The water temperature was kept at 26.0 ˚C without any special pH control, with a lighting regime of 5710 Lux 12 h per day (7:00 AM to 7:00 PM) through a year. The aquaria were arranged with some stones and pipes without any bottom sand to simulate a cave. Parental fish were fed larvae of Chironomidae, pieces of fish, and *Macrobrachium* sp. once a day.

All fish were injected with human chorionic gonadotrophin (ASKA Pharmaceutical Co., Ltd.) (10 IU per g (body weight)) on the left side of their caudal peduncle once on April 25, 2018, these doses of hormone followed Takaku et al. (2007). No additional injection was performed.

1.1.2. Observation of spawning behaviors

The aquarium was observed from 8:30 AM to 5:00 PM every day, and digital photographs were taken of any egg mass found to count the number of spawned eggs. The behavior of pairs swimming around the male territory was recorded with a digital camera (Nikon COOLPIX AW130; NICON Co., Tokyo, Japan).

1.1.3. Observation of development

The development and growth of eggs were observed when the eggs spawned on July 14, 2018. The spawned eggs were initially transferred to a 2 *l* plastic tank, and the hatched and growing larvae were transferred to a 5 *l* plastic tank or a 30 *l* tank, depending on the number and size of larvae. Water temperatures were kept at 26 °C. All the water was changed twice a day. Larvae, juveniles, and young fish were fed live *Artemia* larvae (0–50 days after hatching) and crushed frozen chironomid larvae (From day 20 onward after hatching) twice a day.

Eggs, larvae, and juveniles were observed for morphological development and measured to 0.01 mm under a stereoscopic microscope. Young fish and adults were photographed digitally for measurements and observed with eyes directly following eugenol anesthetic for sketches. They were also photographed digitally to measure and sketch. Standard length in the present study includes notochord length in the larval stage and body length in the juvenile stage and thereafter.

The growth curve of *L. cutcutia* from its average size each day after hatching was estimated by the standard form from the model of Richards (1959) (Akamine 1988) by comparing the residual sum of the square (Akamine 2004), calculated by MS Excel (Microsoft Office 365).

1.2. RESULTS

1.2.1. Spawning

The first spawning was observed on April 27, two days after hormone treatment.

After hormone treatment, the posterior edge of the caudal fin became reddish in all parental fish, the body color became freshy green, and the lateral black spot became distinct in the female (Fig. 1.1-a); whereas in

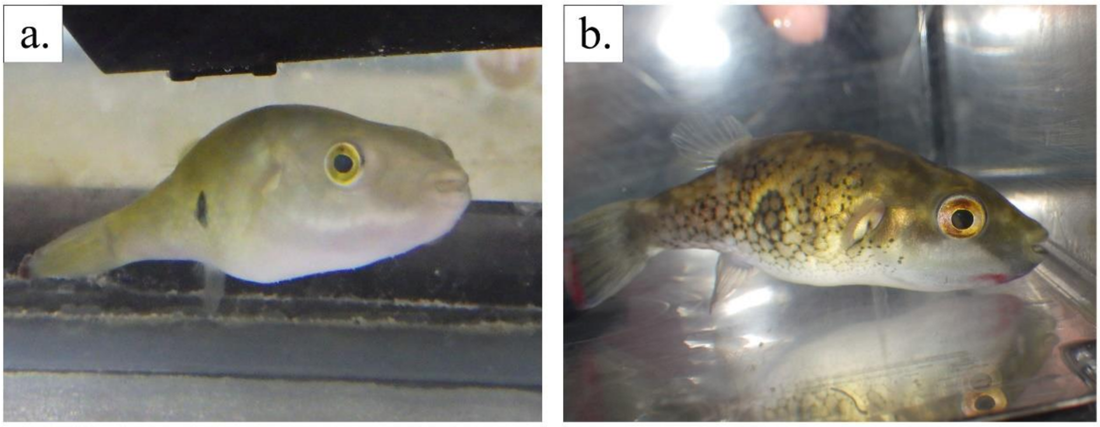

Fig. 1.1. Parent fish of *Leiodon cutcutia* in breeding season: a, female; b, male. The male chin turns red during breeding season.

the males, the net pattern appeared, the lateral black spot became indistinct, and the chin became reddish (Fig. 1.1-b).

Each male stayed in a limited area and attacked the other male or human hands approaching its area which he considered as his own territory, and the female visited both and spawned. The female spawned 47 times between April 27, 2018 and April 26, 2019, irrespective of season, laying 24–545 eggs [average 341.5 ± SD 105.002, median 353, $n = 42$ (egg laying could not be counted in five times)] at one laying. The interval between each spawning was about one week. Eggs were laid in a single-layered batch on the flat area of the glass floor of the aquarium, the cave floor of rocks, or the plastic pump cover.

1.2.2. Spawning behavioral sequence

A sequence of spawning behavior (shown in Fig. 1.2) continued for about 13 min (two observations; 10:12 June 15, 2018, and 12:25 June 26, 2018).

The male made a territory on a flat area (rock caves, top face of pump cover, and floor of aquaria) just before spawning and attracting the female (Fig. 1.2-a). The territorial male vibrated his body when the female approached there. The male stayed in a limited area and attacked another male or human hands approaching its area. However, male conflict was only rarely observed. When a territory was observed, the territorial male and female had already become a pair and expressed nuptial color.

Coupling (Fig. 1.2-b): They began swimming together around (shown with thin arrows) and sometimes vibrate their bodies (shown with wave lines) in the territory for about 5 - 6 min.

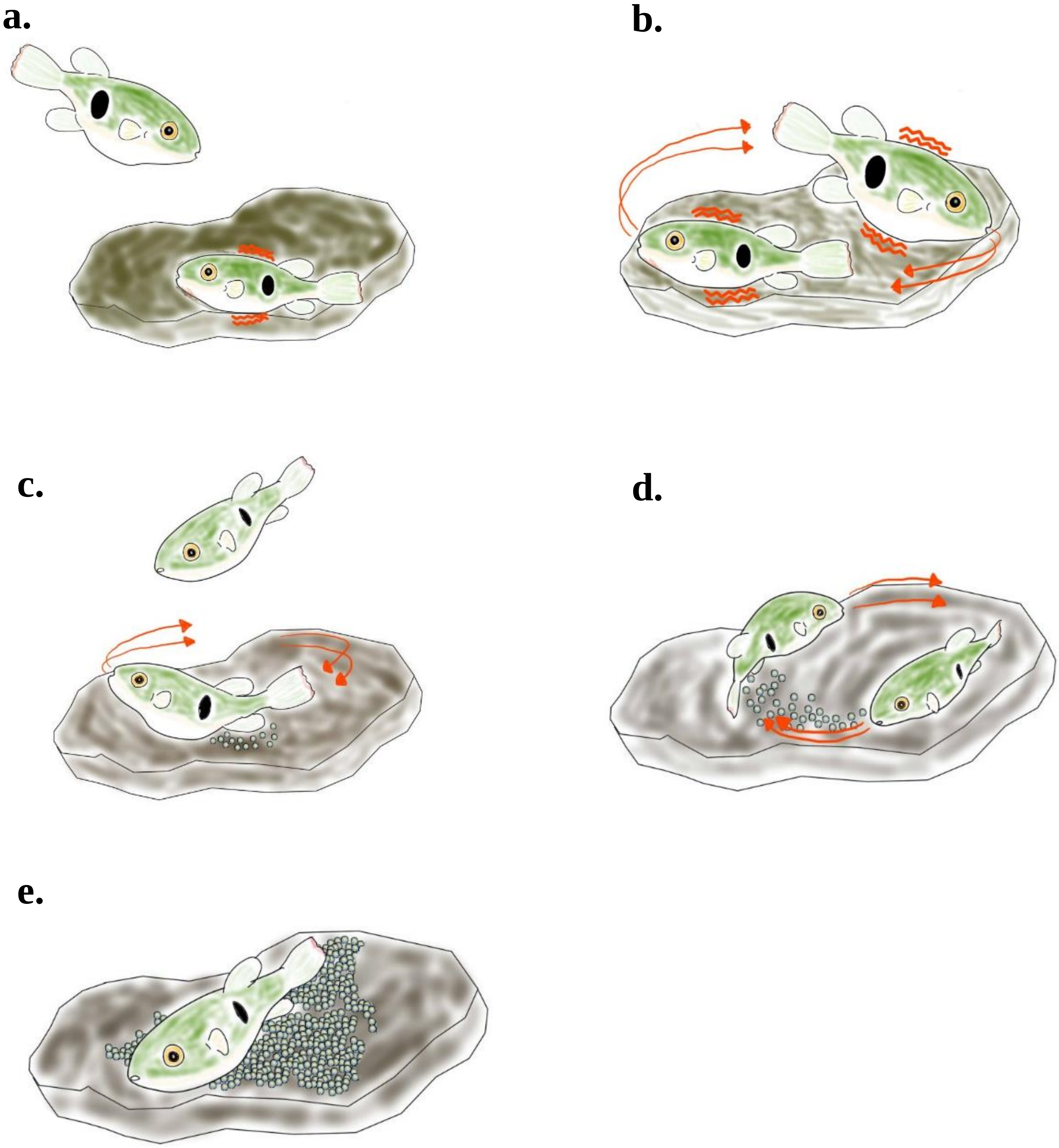

Fig. 1.2. A sequence of reproductive behaviors (shown with black arrows) of *Leiodon cutcutia*: a, male attracting female; b, female entering territory; c and d, spawning; e, male protecting eggs. Red arrows depict them swimming together; red wave lines show body vibrations.

Spawning (Fig. 1.2-c, d): The female would begin turning around, with the male being a little apart from the female. After a while, the female started laying eggs; the male returned, followed the female, and fertilized eggs with his sperm. Spawning finished about 3 min later since first egg was observed. After spawning, spawned eggs were sometimes afloat. They pressed spawned eggs on the spawning site with their bodies and anal fins to attach them. Parental fish both kept swimming on their eggs for about 5 - 7 min, after which the female would leave the territory.

Protecting (Fig. 1.2-e): The male remained at the spawning site and started protecting and fanning the eggs until they hatched. The guarding male kept swimming just above the egg layer, and sometimes would leave to run off or attack approaching individuals. Sometimes eggs were eaten by the protecting male or the spawned female.

The male reproductive behavior seemed to finish once when eggs were hatched. Spawning males was random, however the same male sometimes performed the spawning in succession. Another male sometimes alternately used the single territory.

1.2.3. Embryonic stages and Larval development

Fertilized egg (Fig. 1.3-a): The eggs were 1.42 mm (0.07 mm SD, *n* = 6) in diameter, translucent, spherical, demersal, and adhesive, with a mass of some small yellow oil globules.

Two days after spawning (Fig. 1.3-b): The embryo developed, and the optic vesicle appeared.

Four days after spawning (Fig. 1.3-c): The optic vesicle became black. Some melanophores appeared on the

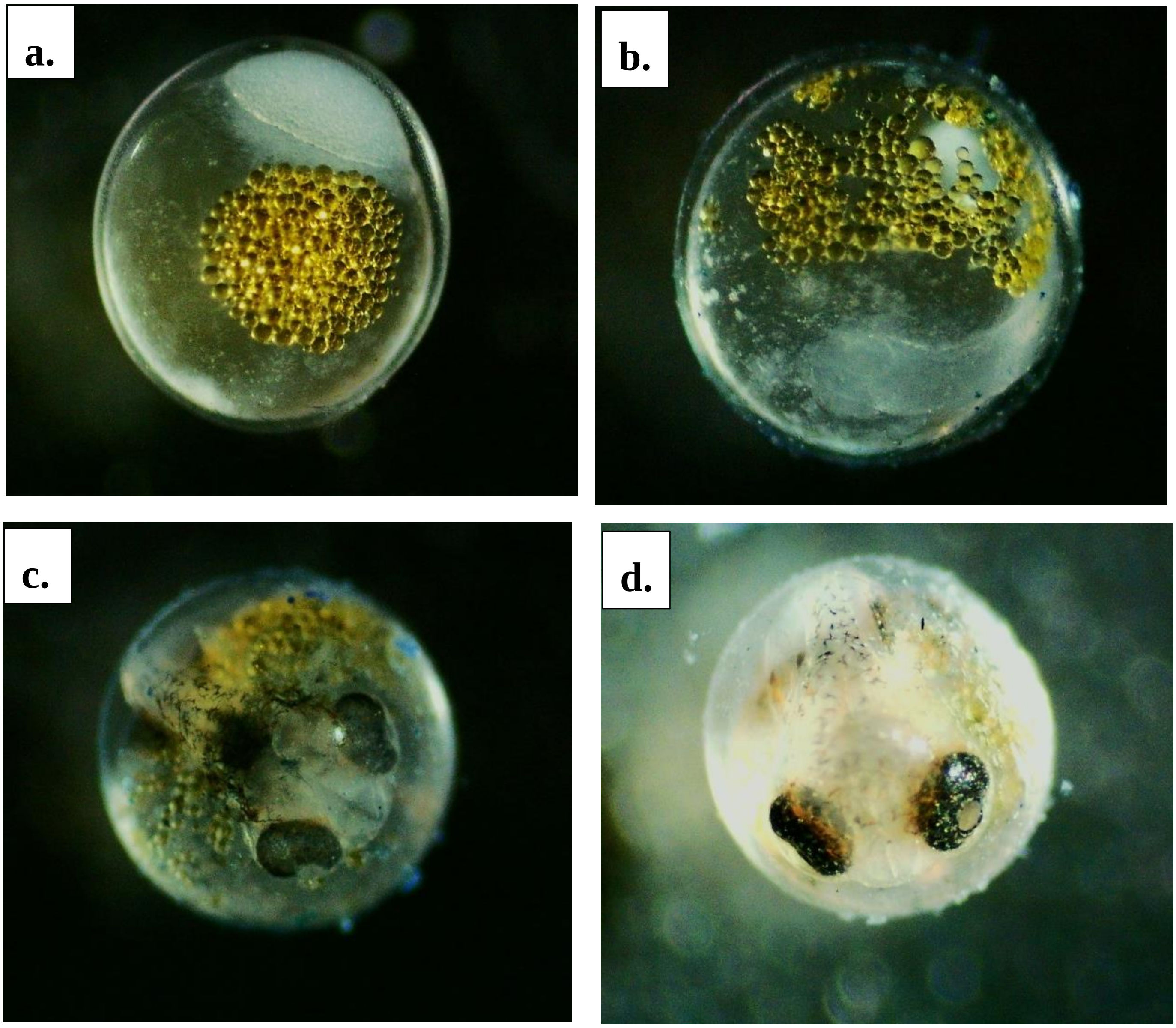

Fig. 1.3. Development of eggs of *Leiodon cutcutia*: a, fertilized egg; b, two days after fertilization; c, four days after fertilization; d, five days after fertilization.

dorsal area and caudal peduncle.

Five days after spawning (Fig. 1.3-d): One day before hatching. The larval notochord and optic vesicle developed completely.

Hatched larva [2.79 mm NL, Fig. 1.4-a. mean 2.71 mm NL, mean 2.87 mm TL (0.24 mm and 0.26 mm SD, respectively), $n = 3$]: The yolk sac larvae measured 2.39–2.96 mm NL, 2.53–3.15 mm TL ($n = 3$), and the mouth had already opened. Melanophores appeared across a wide range of the dorsal side from head to body trunk, surroundings the eyes, the lateral sides of the caudal peduncle, and both the dorsal and ventral parts of the gut. Xanthophores appeared on the head, chromophores comprising erythrophores and melanophores formed vertical lines from the edges of the eyes to the anus and a horizontal line formed on the lateral sides of the caudal peduncle.

One day after hatching (DAH) (mean 2.71 mm NL, mean 2.86 mm TL, $n = 3$): The yolk was digested. A brownish mottled pattern appeared around the eyes. Brownish horizontal bands appeared on the lateral sides of the caudal peduncle.

Four DAH (mean 3.26 mm NL, mean 3.52 mm TL, $n = 3$): Rudimental soft rays of the dorsal and anal fins formed. The body became greenish and chromatophores covered a wide range of the head to trunk.

Seven DAH (3.80 mm NL, 4.00 mm TL in the mean of three individuals): The caudal fin rays formed.

Nine DAH (3.79 mm NL, Fig. 1.4-b. mean 3.98 mm NL, mean 4.23 mm TL, $n = 3$): Notochord flexion was initiated. The body color corresponded with that observed seven days after hatching, being greenish with some brown spots.

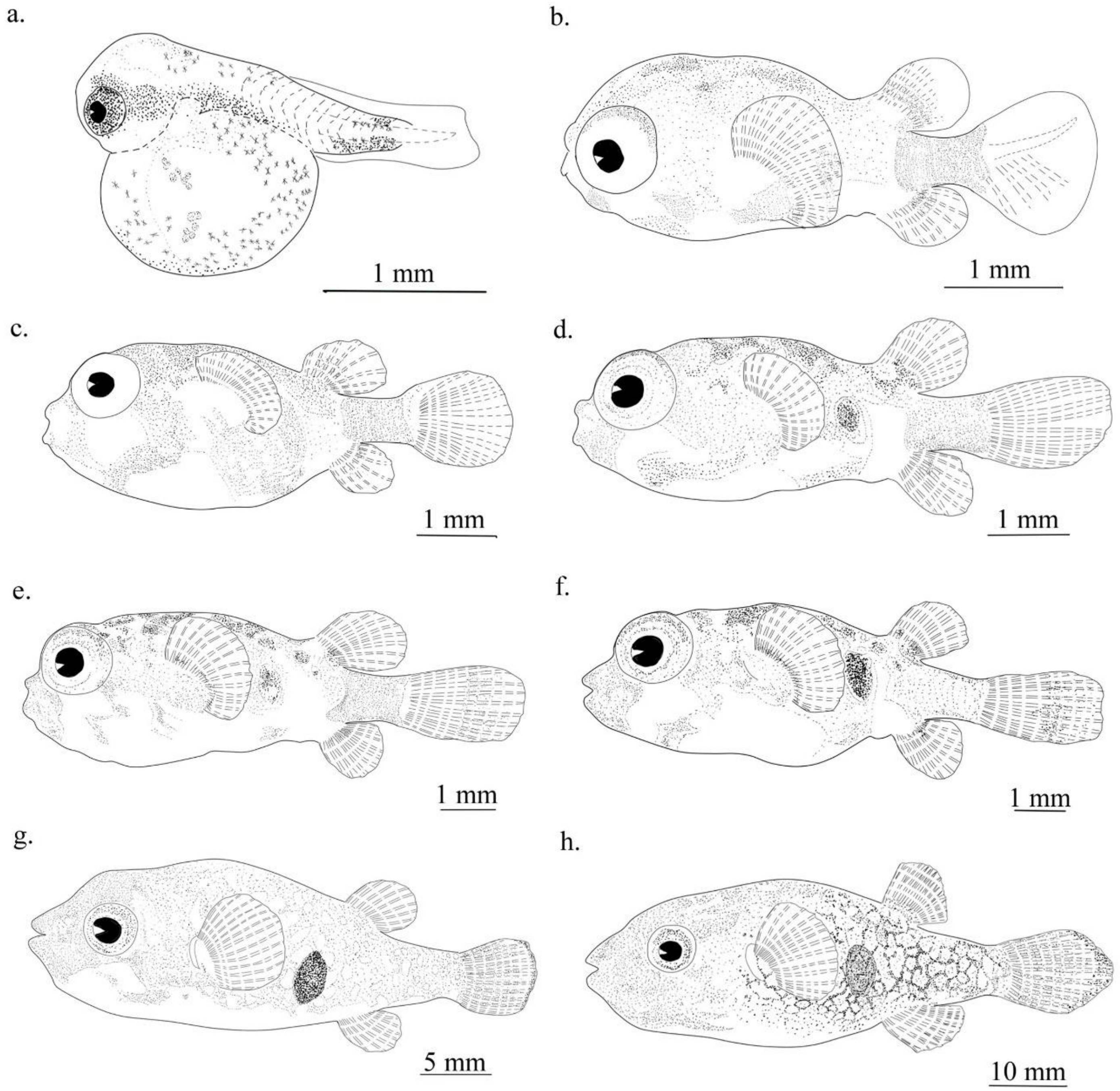

Fig. 1.4. Developmental series of *Leiodon cutcutia*: a, 2.79 mm newly hatched larva; b, 4.30 mm larva 10 day after hatching (DAH); c, 4.77 mm juvenile 13 DAH; d, 5.16 mm juvenile 20 DAH; e, 7.02 mm juvenile 30 DAH; f, 7.75 mm juvenile 39 DAH; g, 24.2 mm fish 180 DAH; h, 55.0 mm fish 360 DAH.

1.2.4. Juvenile development

13 DAH (4.77 mm SL, Fig. 1.4-c. mean 4.50 mm SL, mean 5.46 mm TL, *n* = 3): Notochord flexion was completed. A brownish horizontal band on the lateral sides became indistinct.

15 DAH (mean 4.52 mm SL, mean 5.60 mm TL, *n* = 3): An indistinct black spot on the lateral sides appeared in some individuals. The body color on the dorsal side was greenish with a brownish mottled pattern. A brownish horizontal band appeared on the caudal peduncle.

20 DAH (5.16 mm SL, Fig. 1.4-d. mean 5.19 mm SL, mean 6.62 mm TL, *n* = 3): The ventral part became whitish. The lateral black spot was indistinct.

30 DAH (7.02 mm SL, Fig. 1.4-e. mean 6.41 mm SL, mean 8.35 mm TL, *n* = 3): Some small brown blotches appeared on the dorsal region. The lateral black spot was indistinct.

39 DAH (7.75 mm SL, Fig. 1.4-f. mean 7.55 mm SL, mean 9.72 mm TL, *n* = 3): The characteristic lateral black spot became distinct. The brownish horizontal band on the caudal peduncle became indistinct.

50 DAH (mean 7.49 mm SL, mean 9.18 mm TL, *n* = 3): The dorsal part and lateral side were greenish with a clear brownish mottled pattern. The ventral part of the body was whitish. A black spot appeared on the lateral side. The body color corresponded with that of an adult fish.

1.2.5. Young and adult fish development

150 DAH (mean 26.8 mm SL, mean 30.68 mm TL, *n* = 3): The posterior margin of the caudal fin became

reddish.

180 DAH (24.2 mm SL, Fig. 1.4-g. mean 27.07 mm SL, mean 31.75 mm TL, *n* = 3): A mottled pattern of green and white appeared slightly on the dorsal and lateral sides of the body. One black spot existed in the middle of each lateral side.

270 DAH (mean 34.7 mm SL, mean 42.77 mm TL, *n* = 3): The chins of some males turned reddish.

360 DAH (55.0 mm SL, Fig. 1.4-h, male. mean 52.00 mm SL, mean 64.57 mm TL, *n* = 3): A mottled pattern clearly appeared on the side of the body. A black spot in the middle of the lateral side became indistinct.

486 DAH (about 60 mm SL in both sexes based on visual estimation from behavior patterns): The first spawning was observed, a male with a reddish chin guarding an egg batch in its territory; unfortunately, actual spawning behavior was not observed.

1.2.6. Growth

Growth in SL (NL and BL, *n* = 133) were summarized in Fig. 1.5. The growth model was approximated, containing both sexes as follows, the coefficient of determination being 0.9854, Lt is estimated length, and t is days after hatching:

$L_t = 67.60356 \times e^{(-e^{(-0.006654(t-161.6934))})}$.

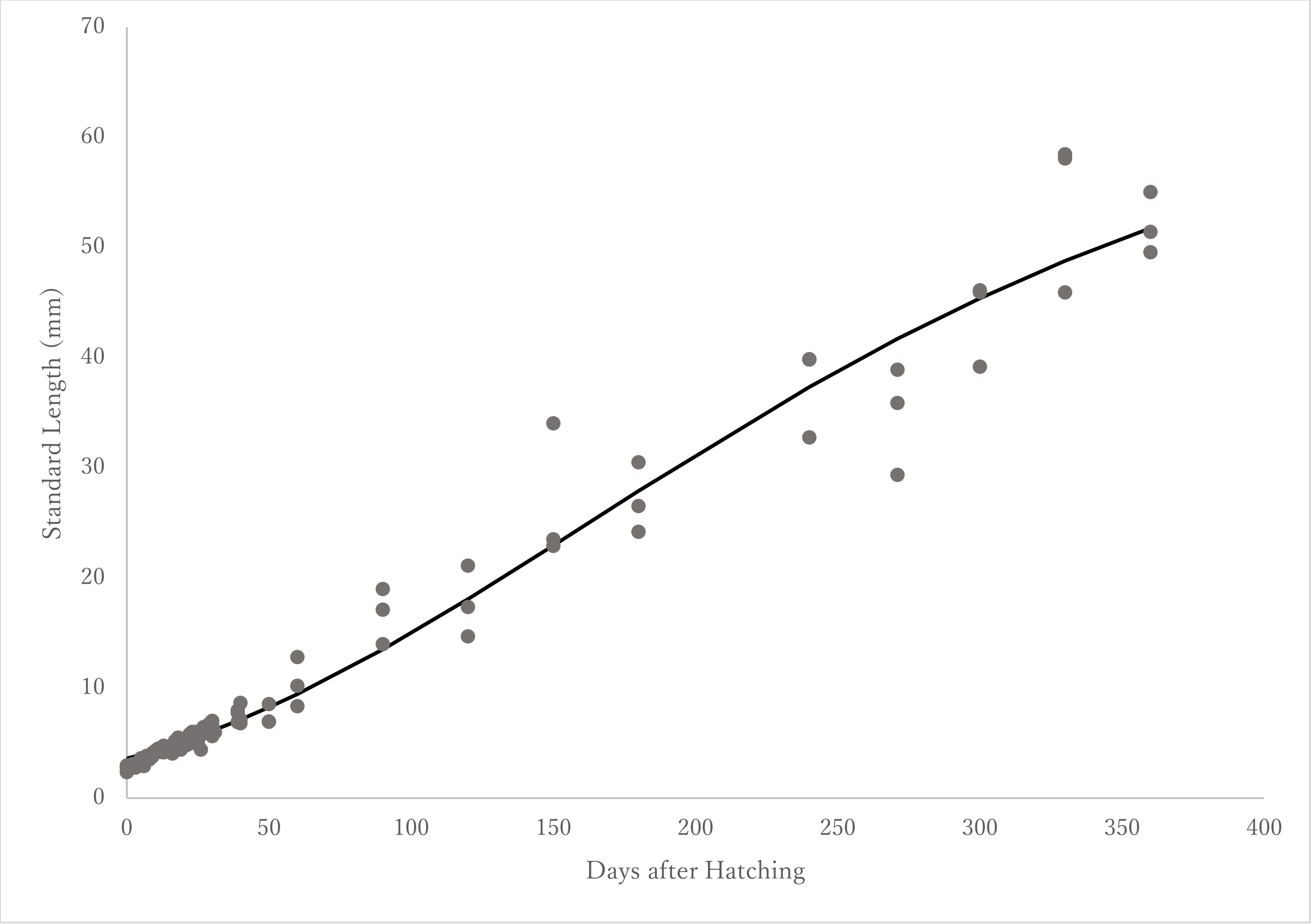

Fig. 1.5. Growth of *Leiodon cutcutia* spawned on July 14th, 2018. Growth curve approximated using the Richards (1959) model is shown in the figure. Grey markers indicate SL of each of the individuals.

1.3. DISCUSSION

In the present study, first spawning was observed at two days after hormone injection. Spawning was observed continuously throughout the year.

Artificial maturation with hormone injections was widely known in freshwater fishes. In most of them, spawning occurred within one or two days after hormone injection (Yamamoto et al. 1966; Suzuki and Yamaguchi 1975; Suguro 2001; Takaku et al. 2007; Takaku and Hosoya 2008). *Leiodon cutcutia* needs a relatively longer period to mature after hormone injection among freshwater fishes. In marine puffers, *Takifugu rubripes* ovulated over 90 hours after hormone injection, longer than other marine fishes (Miyaki et al. 1992). Therefore, pufferfishes tend to need relatively long periods for maturing after hormone injection.

Hormone injection was done only one time in this study. Hormone injections generally only have affects within a few days (cf. Yamamoto et al. 1966; Suzuki and Yamaguchi 1975; Miyaki et al. 1992; Suguro 2001; Takaku et al. 2007; Takaku and Hosoya 2008). Therefore, it is unlikely these injections continued to affect the subjects throughout the year. Accordingly, the year-round spawning in this observation was probably caused by the stable environmental conditions through a year in captivity (The water temperature: 26.0 ˚C, Lighting regime: 12 h per day), not by a temporary stimulation of the hormone injection.

In terms of parental fish size, the two males were slightly smaller than the female (30.5 mm and 28.0 mm SL in males, and 36.5 mm SL in female at the first spawning) in the present study. In contrast, Doi et al. (2015) reported the size of the spawning female (60 mm TL) as smaller than that of the male (70 mm TL). Karmakar and Biswas (2014) reported that the 50% maturity stage of wild females (60–75 mm) was larger than that of wild males (45–55 mm) (whether TL or SL is unknown); the size relationship being similar to that of this study. However, the spawning fish size of the present study was smaller than those reported by Karmakar and Biswas (2014) and Doi et al. (2015). The reared fish of this study matured and spawned by 486 days after hatching, at about 60 mm SL. From these results, it is possible that young and small fish were forced to mature by the hormone injection of this study.

Regarding body color patterns during spawning, the reddish caudal posterior edge, fresh green body color, and distinction of the lateral black spot in females or a single spot on the lateral body and net pattern appearing in males, were observed. These features were also reported by Doi et al. (2015). However, Karmakar and Biswas (2014) described the matured males as having two round black circles at the posterior end of the lateral body and yellow blotches on the dorsal body as sexual dimorphism. Features they mentioned were not observed in this study. It suggests the possibility of a local, subspecies, or species difference from the materials of Kalmaker and Biswas (2014). The spawning male chin was observed to become reddish (Fig. 1.1-b), which is not known in *Leiodon cutcutia* up to now. Therefore, it is a novel character of the matured male of the species.

Regarding the breeding season, spawning was observed throughout the year. Karmakar and Biswas (2014), in contrast, argued that spawning occurred during the rainy months, judging from matured wild individuals. In this present study, there were some artificial conditions including hormone injection, and stable environments in aquarium breeding. Therefore, with seasonal changes in nature, spawning season of this species can be during the rainy months, as Karmakar and Biswas (2014) argued.

In the present study, both males made their territories and a female visited both males and spawned. This suggested that the reproduction system of this species could possibly be classified as “male territory visiting

(MTV) polygamy (Kuwamura 1996; Kohda 2010), in which plural females visit a male territory one by one as seen in, for instance, Cyclopteridae (Abe and Sato 2009), Gobiidae (Kuwamura et al. 2020) and Hexagrammidae (Munehara et al. 2000). MTV polygamy is thought to have evolved under male competition for limited spawning grounds (Kuwamura 1987). However, in the present study, male conflict was rarely observed. Besides, each male sometimes alternately used a single territory. Territorial obsession of this species seemed weak. These results might be caused by breeding environments such as the number of males and spawning grounds. In the aquaria, there were only two males in this study and at least four spawning grounds, hence these were more than the number of males (two). A small number of males and an existence of many spawning grounds might reduce the male conflict. Further research like field observations is needed to clarify the details of male relationship and reproduction system. MTV polygamy has been known only in *Torquigener albomaculosus* (see Kawase et al. 2015) in Tetraodontidae. In Tetraodontiformes, on the other hand, some species have been known as MTV polygamy species in Balistoiodea, and most of them live in coastal water (Kawase 1998). The origin of Balistoidea was distributed in open ocean, spawned floating eggs in middle layer, and the MTV system in this group was thought to be developed with its adaptation to coastal waters (Kawase 1998). Therefore, the MTV system of *L. cutcutia* might be considered its adaptation to shallow waters, such as a large river. In freshwater pufferfishes, male egg protection is also observed in the genus *Pao*. However, in contrast to *Leiodon cutcutia*, the *Pao* species were reported as pair spawning (Doi et al. 2015; 2022a, b). Santini et al. (2013a) showed that the *Pao* and *Leiodon* clades branched off relatively earlier in Asian freshwater pufferfishes. Therefore, male egg protection of *Leiodon* and *Pao* might be similar with a little difference between each other, resulting from convergent evolution under similar environments. However, information of *Pao* was obtained from the limited observation in the aquaria and Doi et al. (2015;

2022a, b) did not reference the mating system. Future research for details of the reproductive behaviors of both genera might clarify their relationship of mating systems.

In conclusion, *Leiodon cutcutia* can breed in captivity under appropriate conditions with a suitable spawning bed. Under a stable environment, year-round breeding would also be possible. The hormone injections would possibly cause breeding by comparatively young and small pufferfish. Its reproductive behavior, male egg protection, was considered to have evolved from different origins from the genus *Pao* because of their differences in reproduction systems.

CHAPTER 2: Hormone injection-induced maturity and insemination of the bronze puffer *Auriglobus modestus*

Auriglobus modestus, distributed in Asian freshwater, is contained in the genus *Auriglobus* which is thought to be closely related to euryhaline genus *Chonerhinos*, from its morphological features (Tyler 1980; Roberts 1982) and phylogenetic analysis (Santini et al. 2013a). About its reproductive biology, *Chonerhinos naritus* is known to spawn oval eggs, which is a rare shape in fish eggs (Ahmad-Nasir et al. 2016; 2020). However, there is no such information on *Auriglobus* as well as its reproductive biology.

The species of *Auriglobus* are popular as ornamental fish, however they are very similar to each other, difficult to identify, and often misidentified.

In this chapter, DNA barcoding, morphological observation, and artificial insemination of *Auriglobus modestus* were attempted, its spawning manner, and egg and larval development were described. From these results, the spawning and phylogenetic relationship between *Auriglobus* and *Chonerhino*s was evaluated first and the differences in reproduction was discussed.

2.1. MATERIALS AND METHODS

2.1.1. Parental fish

Sixteen wild individuals collected from the Bangpakong River, Chachoeng Sao, Thailand, were purchased from a Japanese fish trader (Rio Co. Ltd.), June 10, 2019 and October 6, 2020. Three of these died before the reproductive experiments began in December 2019. The fish were separated into five closed circulating/filtering freshwater tanks, each accommodating two or three individuals, to avoid any conflict or biting behavior [2 tanks: 90 cm (L) × 36 cm (D) × 45 cm (H); 2 tanks: 60 cm (L) × 36 cm (D) × 45 cm (H); 1 tank: 70 cm (L) × 70 cm (D) × 60 cm (H)]. Water temperatures were maintained at 26 ℃, pH between 7.59-7.69, and the lighting regime of 1200 Lux 12 hours per day (7:00 AM - 7:00 PM). Half of the water was replaced once a week. Commercially prepared frozen chironomid larvae and dried meal worms (Natural Pet Foods Co., Ltd., Ibaraki, Japan) were provided to the fish satiation level once a day, with remaining food and excrement being removed daily.

2.1.2. DNA extraction, PCR, and DNA sequencing

Partial nucleotide sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene (652 bp) in the 16 parental fish were used for species identification by DNA barcoding. In addition, sequences of four additional pufferfish species [*Pao cochinchinensis* (purchased from Japanese fish trader, Rio Co. Ltd., the locality and date unknown) *Tetraodon miurus* (Rio Co. Ltd., the locality and date unknown), *Arothron firmamentum* (caught in Murotsu, Muroto city, Kochi Pref., on March 17, 2017), and *Sphoeroides pachygaster* (caught in Numazu city, Shizuoka Pref., on March 24, 2017)] were also determined in order to include them in the dataset for phylogenetic analysis and enhance the comparison among Tetraodontidae species (Yamanoue et al. 2006, 2009, 2011; Santini et al. 2013a; Yamada et al. 2021). In every individual, genomic DNA was extracted from the right pectoral fin using the QuickGene DNA tissue kit S (Kurabo Industries Ltd., Osaka, Japan). COI partial sequences were amplified by PCR with a primer set slightly modified from those designed by Ivanova et al. (2007) (COI-FishF2_t1: 5'-TCGACTAATCATAAAGATATCGGCAC-3, COI_FishR2_t1_Amod: 5'-ACCTCTGGGTGGCCAAAGAATCAAAA-3). The PCR amplification method involved initial denaturation at 95 ºC for 1 min, followed by 30 cycles of denaturation at 95 ºC for 30 sec, annealing at 52 ºC for 40 sec, and extension at 72 ºC for 1 min using Ex-Taq DNA Polymerase (Takara Bio). PCR products were purified using Agencourt AMPure XP (Beckman Coulter, Inc., California, USA) and sequenced on an automated DNA sequencer SeqStudioTM Genetic Analyzer (Thermo Fisher Scientific K. K., Tokyo, Japan) using amplification primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Tokyo, Japan). The nucleotide sequences obtained in the present study were submitted to the DNA Data Bank of Japan (Accession Nos. LC704694 - LC704713).

2.1.3. Species identification by BLAST search and phylogenetic analysis

To confirm the species of the parental individuals used in the present study, a BLASTN search was carried out against the NCBI non-redundant (nr) database, using COI partial sequences of every individual as a query.

The phylogenetic analysis compared sequences of the 16 parental *Auriglobus* specimens, four newly sequenced pufferfish species (*Pao conchinchinensis*, *Tetraodon miurus*, *Arothron firmamentum*, and *Sphoeroides pachygaster*), and published data for 19 additional pufferfish species (23 individuals) (Table 2.1). The ocean sunfish (*Mola mola*) was used as an outgroup. Nucleotide sequences were aligned by MAFFT v7.392 (Katoh and Standley 2013), and the best substitution model selected based on the Bayesian Information

Table 2.1. Species used for phylogenetic analysis.

Accession No. in NCBI	Species	Reference
LC704694-703	Amod01-Amod10	Present study
LC704704-709	Amod11-Amod16	Present study
LC704710	*Pao_cochinchinensis*	Present study
LC704711	*Tetraodon_miurus*	Present study
LC704712	*Arothron_firmamentum*	Present study
LC704713	*Sphoeroides_pachygaster*	Present study
LC586271	*Pao_suvattii*	Yamada et al. (2021)
NC_015348	*Auriglobus_modestus*	Yamanoue et al. (2011)
JQ681769	*Auriglobus_modestus*	Santini et al. (2013a)
AP011917	*Auriglobus_modestus*	Yamanoue et al. (2011)
JQ681770	*Auriglobus_nefastus*	Santini et al. (2013a)
JQ681848	*Chonerhinos_naritus*	Santini et al. (2013a)
KF027531	*Chonerhinos_naritus*	Santini et al. (2013b)
LC586270	*Pao_abei*	Yamada et al. (2021)
NC_015353	*Pelagocephalus_marki*	Yamanoue et al. (2011)
NC_015349	*Omegophora_armilla*	Yamanoue et al. (2011)
NC_010978	*Canthigaster_coronata*	Yamanoue et al. (2009)
NC_015365	*Leiodon_cutcutia*	Yamanoue et al. (2011)
NC_015369	*Chelonodontops_pleurospilus*	Yamanoue et al. (2011)
NC_015363	*Tetraodon_mbu*	Yamanoue et al. (2011)
JQ681839	*Pao_palembangensis*	Santini et al. (2013a)
NC_015366	*Pao_cochinchinensis*	Yamanoue et al. (2011)
JQ681838	*Dichotomyctere_nigroviridis*	Santini et al. (2013a)
NC_015339	*Polyspina_piosae*	Yamanoue et al. (2011)
NC_011636	*Torquigener_brevipennis*	Yamanoue et al. (2009)
AP006045	*Takifugu_rubripes*	Yamanoue et al. (2006)
NC_015352	*Carinotetraodon_salivator*	Yamanoue et al. (2011)
NC_011637	*Lagocephalus_wheeleri*	Yamanoue et al. (2009)
NC_015368	*Colomesus_asellus*	Yamanoue et al. (2011)
AP006238	*Mola_mola*	Yamanoue et al. (2004)

Criterion (BIC). A phylogenetic tree was then constructed by the maximum-likelihood (ML) method; the model selection and ML tree construction being carried out using the MEGA X program package (Kumar et al. 2018). The reliability of each tree node was assessed by the bootstrap method with 1,000 replicates.

2.1.4. Morphological identification

Parental fish were also identified by morphological features, based on the caudal peduncle height and the direction of skin spines below the pectoral fin, according to the identification key for five species of *Auriglobus* given by Roberts (1982). Total length, standard length, and caudal peduncle height were measured to 0.1 mm. Spines on the body surface of dead specimens were stained with Alizarin Red and observed under a stereoscopic microscope.

2.1.5. Hormone injection and artificial insemination

Mature females (79.6 - 91.9 mm SL, 25.4 - 35.3 g BW) were larger and had the edge of the genital pore more expanded than those in mature males (54.9 - 64.5 mm SL, 7.4 - 10.7 g BW) (Fig. 2.1). The nasal pores became black in both sexes. Human chorionic gonadotrophin (ASKA Pharmaceutical Co., Ltd.) was injected (15 IU per g) on the left side of the caudal peduncle of specimens on four occasions: January 4th, and February 1st, 13th, and 26th in 2021. Two to five days following such injection, artificial insemination (one female with one to four males) was conducted five times: January 7th, February 4th, 6th, 17th, and March 1st. The first four utilized a wet method (eggs and sperm mixed slowly in 5 ml freshwater, with a quantity of freshwater

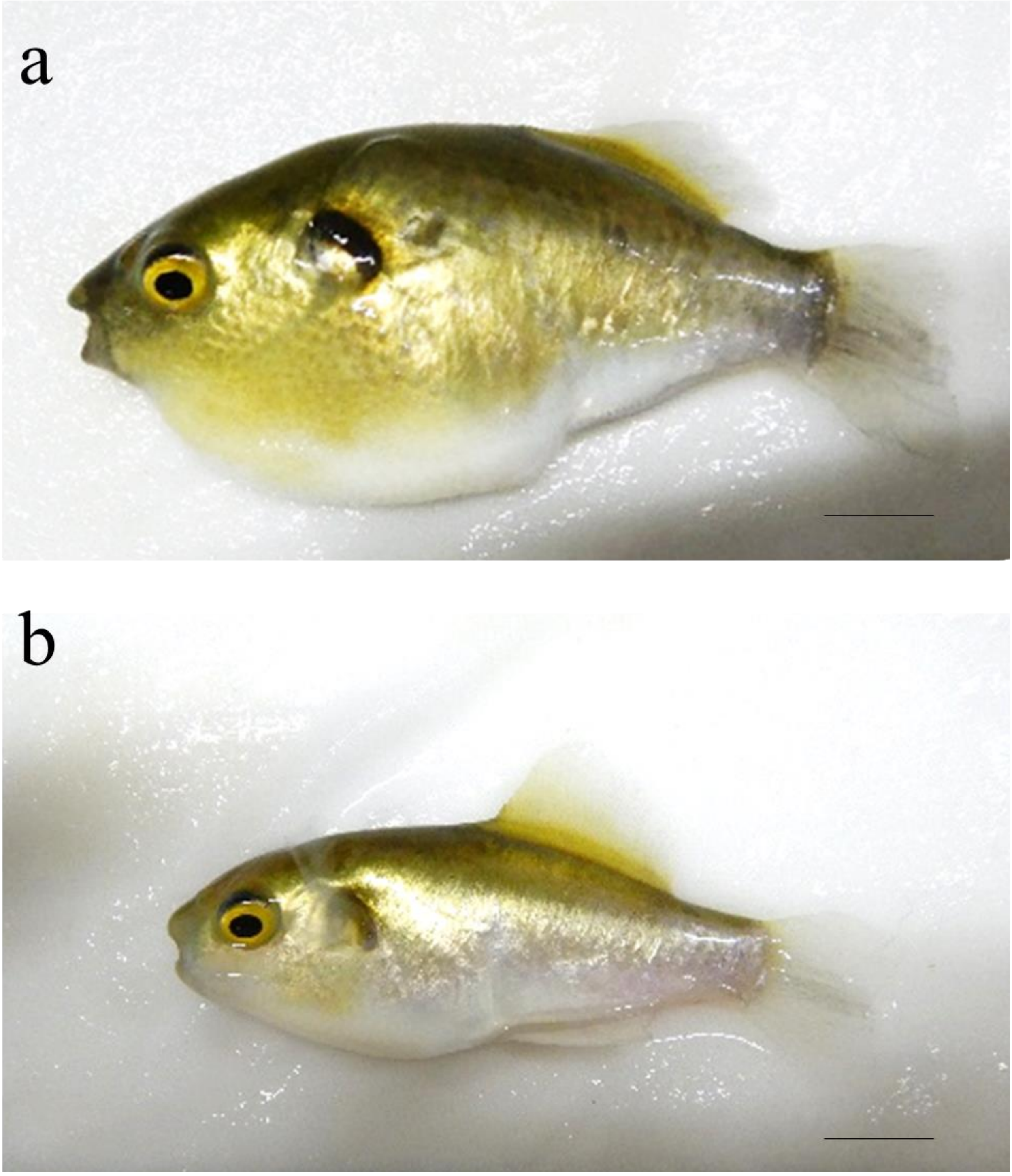

Fig. 2.1. Mature parental *Auriglobus modestus*: a, adult female; b, adult male. Each scale bar shows 1.0 cm.

added subsequently), and on the final occasion, an isotonic method [eggs and sperm mixed slowly in 5 ml L-sodium lactate ringer solution (TERUMO SOLULACT; TERUMO Co., Tokyo, Japan), with a quantity of freshwater subsequently added].

2.1.6. Incubation, rearing and observation of eggs and larvae

The fertilized eggs were transferred into a 1.5 *l* bottle or 5 *l* plastic tank containing freshwater with 1 ppm methylene blue. The water temperature was maintained at 26 ℃ throughout incubation and rearing. Half of the water was replaced three times a day. The hatched larvae were reared in a 5 *l* plastic tank containing 1‰ brackish water, since the relatively low hatching rate of eggs in freshwater (described below) suggested that freshwater was unsuitable for incubation and rearing. Half of the water was replaced twice a day. Larvae were fed with *Brachionus plicatilis* from two days after hatching.

Live eggs and larvae (unanesthetized) and specimens fixed in 1% formaldehyde solution were observed under a stereoscopic microscope equipped with a 3R Anyty WiFi Microscope ver.6.9.3 (Three R Co., Fukuoka, Japan) and photographed digitally. TL and NL of live fish were measured to 0.01 mm.

2.2. RESULTS

2.2.1. Identification of parental fish

DNA barcoding:

Mitochondrial COI sequences in each of the 16 parental fish were identical or nearly so (pairwise nucleotide sequence identity among individuals: 99 - 100 %). The BLASTN search against the NCBI nr database revealed that COI sequences of the parental fish corresponded very closely to those of *A. modestus*, *A. nefastus*, and *Ch. naritus*. The phylogenetic analysis indicated that the parental fish and these three species formed a monophyletic clade (Clade A) with 100 % bootstrap probability (Fig. 2.2). COI sequences (652 bps) within the Clade A differed by 1 - 4 bps. From these results, it was considered impossible to identify species of *Auriglobus* and *Chonerhinos* by DNA barcoding. Therefore, species identification of parental fish was done by morphological method.

Morphological identification:

Bodies were compressed and oval; height was 37.9 - 53.0 % SL in females and 38.1 - 45.8% SL in males. The body height became the largest in the middle part of the trunk. Eyes were positioned above, formed roundly. Mouth opened at the anterior edge of the body; upper jaws were little elongated than lower jaw. The posterior edge of the upper jaw did not reach to the eyes. A couple of nostrils opened with the flap. Opercular pores were small and opened in front of the base of the pectral fin. The lateral line was single, indistinct. Scales were

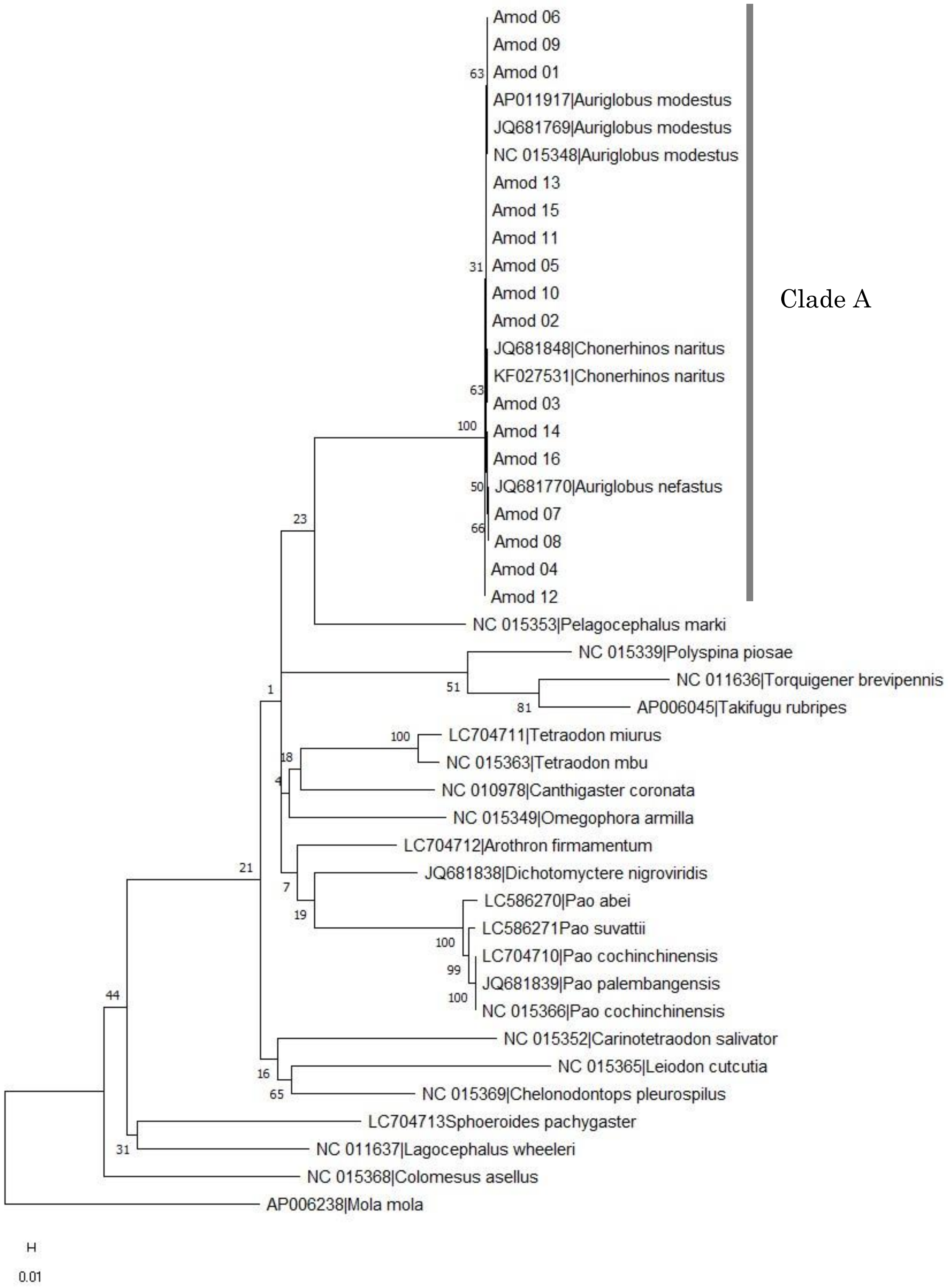

Fig. 2.2. Maximum likelihood tree of the COI partial sequences in the 16 parental fish (Amod_01-16) and 23 representative pufferfishes (four sequenced in present study and 23 obtained from NCBI database).

usually retracted; scales with spines on side of body anterior and ventral to pectral fin were close-set with these spines directed dorsally. In the living specimens, the caudal peduncle height was 14.4 - 17.1 % of the SL. Single dorsal fin and anal fin occurred posteriorlly; their outline formed roundish. The caudal fin was a little concaved. D: 22 - 25; A: 21 - 22; C: 9.

The color of living specimen: the upper part on side of the body was metallic yellowish green, and the lower part was pearlish. The dorsal and anal fin were opal, translucent. Caudal fin was opal and translucent with black on its central part.

Based on the morphological features identifying *Auriglobus* species (see Roberts 1982), such as the spine conditions below the pectoral fin and the caudal peduncle height, the present parental fish were corrected with *A. modestes* with the spines close-set and directed dorsally and the caudal peduncle height 13.5 - 14.9 % of SL (other *Auriglobus* species have the spines less close-set and directed dorsoposteriorly or posteriorly, and the caudal peduncle height 10.1 - 13.9 % of SL). Furthermore, the features such as: bodies were compressed, a paired nostril that opened with flap, shape of each fin (dorsal and anal: roundly; caudal: concave), lateral lines were indistinct, and anus opened anterior of anal fin; these agreed with the features of *A. modestus* shown in Bleeker (1850). Therefore, all parental fish were identified as *A. modestus*.

2.2.2. Artificial fertilization and hatching

The mean number of spawned eggs was 625.6 (range 272 - 928, SD 281.7, *n* = 5). No eggs were fertilized in the trials utilizing the wet method, whereas the isotonic method yielded 458 fertilized eggs (fertilized rate: 61.3 %). Three days later, eight larvae hatched successfully (hatching rate: 1.7 %).

2.2.3. Eggs and Larvae

Fertilized eggs: The oval eggs [mean long and short axes 2.05 mm (0.06 mm SD) and 1.68 mm (0.06 mm SD), respectively, *n* = 5] were demersal, adhesive, and transparent, with a mass of many small yellow oil globules (Fig. 2.3-a; three hours after fertilization). Two days after fertilization, the embryo had already appeared with transparent optic vesicles and a few melanophores dorsally on the gut (Figs. 2.3-b, 2.4-a). The eggs hatched three days after fertilization.

One DAH [Fig. 2.4-b, 4.58 mm TL; mean 4.85 mm TL, and mean 4.54 mm NL (0.35 mm and 0.34 mm SD, respectively), *n* = 3], the yolk sac was large, with many oil globules remaining. The mouth remained closed, and optic vesicles were translucent. Small pectoral fin rudiments were observed in a fixed specimen. Finfolds were apparent along the dorsal aspect from the central nuchal region to the anus. Melanophores were present on the nuchal region, lateral gut surface, and ventral edge of the tail. Myotomes numbered 11 + 12 =23.

Two DAH [Fig. 2.4-c, 5.10 mm TL; mean 5.06 mm TL, and mean 4.77 mm NL (0.03 mm and 0.04 mm SD, respectively), *n* = 3], the yolk sac had diminished, and the mouth opened, although feeding was not observed. The optic vesicles remained translucent. The dorsal and ventral finfolds had become slightly notched at the caudal peduncle. Melanophores were apparent on the snout, dorsal surface of the head, dorsal gut surface, and the edges of the dorsal and anal finfolds.

Five DAH (4.51mm TL), some oil globules remained. The optic vesicles were black and pectoral finfolds larger. Melanophores covered the lateral gut surface and the entire body, except the caudal peduncle. Feeding on *Brachionus* larvae was observed.

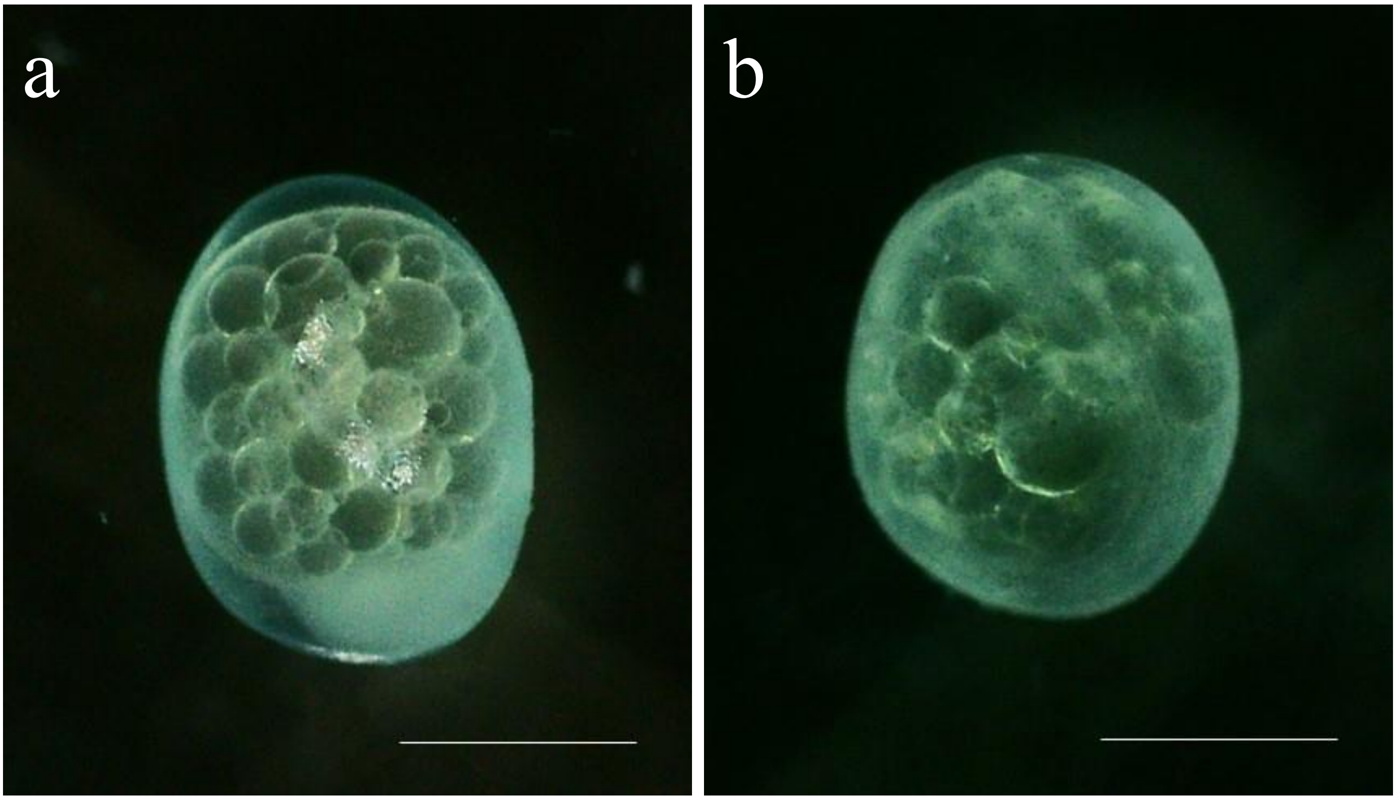

Fig. 2.3. Fertilized eggs: A, three hours after fertilization; B, two days after fertilization. Each scale bar shows 1.0 mm.

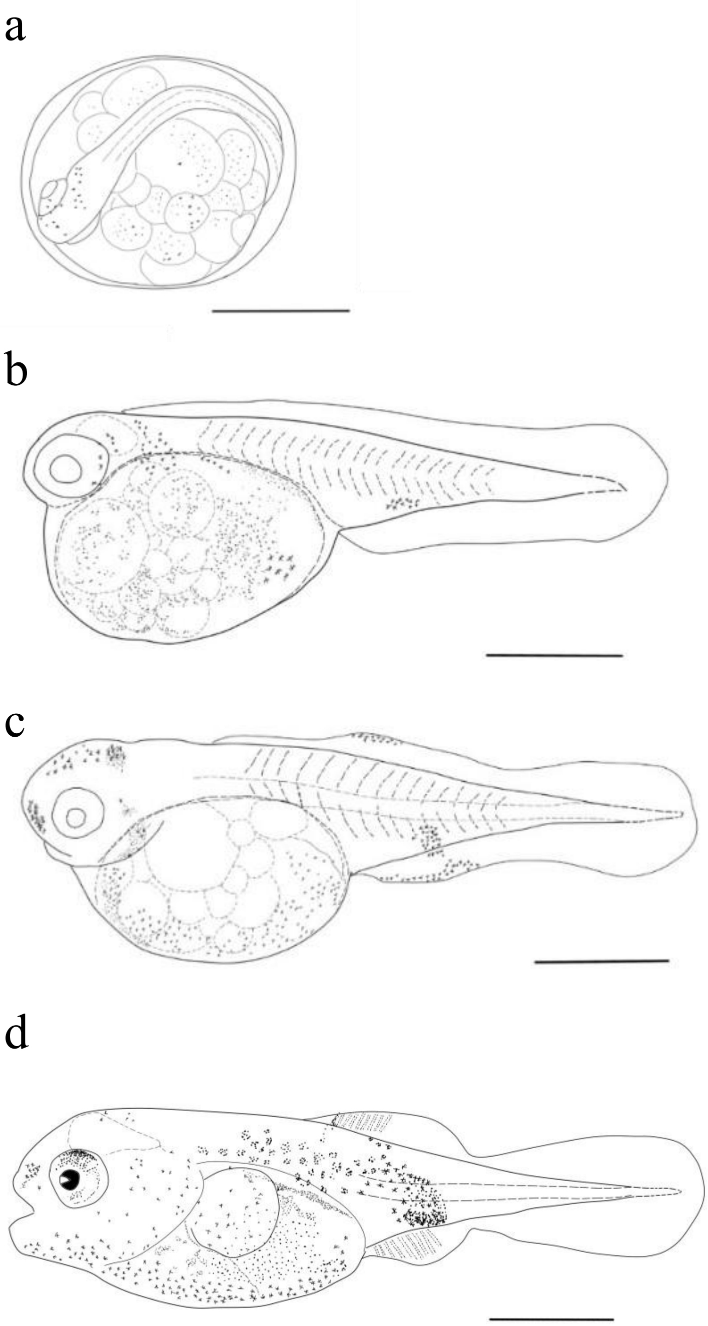

Fig. 2.4. Developmental series of *Auriglobus modestus*: a, fertilized egg two days after fertilization; b, 4.58 mm larva 1 DAH; c, 5.10 mm larva 2 DAH; d, 5.50 mm larva 7 DAH. Each scale bar shows 1.0 mm.

Seven DAH [Fig. 2.5-d, 5.50 mm TL; mean 5.47 mm TL and mean 5.22 mm NL (0.05 mm and 0.10 mm SD, respectively), *n* = 3], the body was spherical and the yolk sac completely absorbed. Rudimentary soft rays in the pectoral, dorsal and anal fins were apparent.

All larvae had died by the eight day after hatching; the reason unknown.

2.3. DISCUSSION

DNA barcoding in the present study showed a high similarity of COI sequences (652 bps) between three species: *A. modestus*, *A. nefastus*, and *Ch. Naritus*; with 1 - 4 bps differences. Individuals with 99 % homology of COI sequence are generally considered to be at the same species level in DNA barcoding (Ward et al. 2009). A close phylogenetic relationship of *Auriglobus* and *Chonerhinos* was also shown in some previous studies (Tyler 1980; Roberts 1982; Santini et al. 2013a).

Regarding reproductive biology, *Auriglobus modestus* and *Chonerhinos naritus* are similar in spawning oval eggs, resulting in relatively undeveloped hatched larvae with a large yolk sac, unopened mouth, unpigmented optic vesicles, and without membranous pectoral fins (present study, Ahmad-Nasir et al. 2016, 2020). These features are not shared by other fresh- and brackish water pufferfish genera, such as *Carinotetraodon* (see Doi et al. 2015), *Dichotomyctere* (see Doi et al. 2015; Watson et al. 2009; Zaucker et al. 2014), *Leiodon* (see Doi et al. 2015; Momota et al. 2022), *Pao* (see Doi et al. 2015), and *Tetraodon* (see Feigs, 1955); all of which have spawned spherical eggs and have more developed hatched larvae. The similarity apparently supports both in life-history aspects and/or genetic similarities between *A. modestus* and *Ch. naritus*.

In the present study, the hatching rate of artificial insemination of *Auriglobus modestus* was low under freshwater incubation. It was possibly caused by inappropriate rearing conditions for *A. modestus* eggs. The closely related species *Chonerhinos naritus* can be artificially fertilized and resultant larvae reared utilizing brackish water conditions (18 psu) (Ahmad-Nasir et al. 2016, 2020). Because *A. modestus* is also distributed in rivers and estuaries (Ebert 2001), it is likely to spawn and develop in such conditions, as *Ch. naritus*.

As one of their common features, both *Auriglobus* and *Chonerhinos* spawn oval eggs (present study; Ahmad-Nasir et al. 2016, 2020). Oval eggs have been known in groups such as bitterlings (Cyprinidae) (spawning into bivalve shells) and gobies (Gobioidea) and damselfishes (Pomacentridae) (spawning adhesive eggs thickly onto a substrate). They are relatively rare in open-water spawning fishes, except in species such as *Engraulis japonicus* and *Scarus* spp (Winn and Bardach 1960; Mito 1960, 1962; Hirai 2003). The functional significance of the oval shape of such eggs is unknown (Winn and Bardach 1960; Mito 1960, 1962; Hirai 2003). On the other hand, the oval eggs of *A. modestus* and *C. naritus* (present study; Ahmad-Nasir et al. 2016, 2020) are demersal and adhesive, and therefore the ecological significance of their eggs is possibly different from those of *E. japonicus* and *Scarus* spp. However, solving this question would require further investigation.

In this chapter, information on the morphological features of eggs and hatched larvae in *Auriglobus modestus* was shown. Future studies to establish an artificial breeding method for *A. modestus* should consider the salinity conditions best suitable to insemination, incubation, and rearing.

Phylogenetically close relationships between *Auriglobus* and *Chonerhinos* has been argued from a morphological perspective for a long time (Tyler 1980; Roberts 1982). In recent years, the same phylogenetic relationship was also shown by molecular analysis (Santini et al. 2013a). Moreover, the genus *Auriglobus* includes several species that are very difficult to identify (Roberts 1982).

Besides, the present study also showed close phylogenetic relationships of species in *Chonerhinos* and *Auriglobus*, suggesting a possible existence of some cryptic species in these groups. Future sophisticated genetic analyses will solve the mysteries of their relationships and classification.

CHAPTER 3: Reproduction and early development of the green pufferfish *Dichotomyctere fluviatilis* in captivity

Dichotomyctere fluviatilis, one of the euryhaline puffer distributed in Asia, is a potentially beneficial species for genetic study because its related species *D. nigroviridis* was published and its whole genomic information as a model animal (Jaillon et al. 2004). It has also attracted attention as an edible fish having nutritional value (Ahmad-Chowdhury et al. 2019).

In an evolutional viewpoint, Igarashi et al. (2013) treated this genus as an Asian brackish group and argued that it invaded into brackish- and freshwaters independently from the other genera. On the other hand, Santini et al. (2013a) showed that *Dichotomyctere* should be included in the same clade with other Asian genera sharing the same origin. Thus, there has been a controversy about their origin.

About the reproductive biology of *Dichotomyctere*, there has been information on two species, *D. nigroviridis* and *D. ocellatus* (see Watson et al. 2009; Yamada 2013; Zaucker et al. 2014; Doi et al. 2015). Both are known to scatter many relatively small, spherical adhesive demersal eggs.

In this chapter, through captive breeding with hormone injection, spawning manner and development of eggs, larvae, and juveniles of *Dichotomyctere fluviatilis* were described, and compared with marine and other freshwater puffers.

3.1. MATERIALS AND METHODS

3.1.1. Parental fish

Nine wild individuals (later determined to be five males and four females) caught in the Sunderbun Delta, eastern India, were purchased from a Japanese fish trader (Rio Co. Ltd.) on February 1, 2017. They were kept in a closed circulation–filtering aquarium (2200 *l*) at Osaka aquarium NIFREL. The water temperature was maintained at 27.0 ˚C without any special pH control (7.5 - 8.4), with a lighting regime of 100 Lux 12 hours per day (7:00 AM - 19:00 PM). The water conditions initially approximated to full-strength sea water (33 ‰) for about 20 days, before changing to brackish water [4 - 16 ‰ for about 11 months (from February 2017 to January 2018)], and subsequently reverting to sea water. Pieces of fish (mainly *Trachurus japonicus*) or invertebrates (mainly *Pandalus eous*) were fed to satiation level once a day (not fed on Sundays). The leftovers and excrement were removed daily.

3.1.2. Hormone injection

All fish were injected once with human chorionic gonadotrophin [10 IU per g (body weight, BW hereafter)] following Momota et al. (2022), into the left side muscle of caudal peduncle on May 12, 2019.

Just before injection, the standard lengths (SL) of parental fish were 123.6 – 148.9 mm (mean 132.7 mm, *n* = 5) in males and 142.6 - 183.6 mm (mean 157.1 mm, *n* = 4) in females. The BW of parental fish were 112.0 - 231.0 g (mean 151.6 g, *n* = 5) in males and 235.0 - 400.5 g (mean 319.8 g, *n* = 4) in females.

3.1.3. Number of eggs spawned

Because all the four females spawned and scattered lots of eggs on the aquarium floor at the same time, the number of spawned eggs by each female was roughly estimated from BW difference of female before (May 12th) and after spawning (May 16th), as follows:

> Estimated number of spawned eggs = BW decrease (g) of spawned female × average number of fertilized eggs per g (counted three times) collected from the aquarium bottom.

The counted eggs did not collect any sand or suspended dust on the surface. However, the number of spawned eggs was likely to have been underestimated because they were weighed after water absorption.

3.1.4. Observation of development

Development of the eggs (spawned on May 14, 2019) was observed after collection of eggs from the aquarium tank. Eggs, larvae, and juveniles were removed gently from the rearing tank (unanesthetized), photographed digitally under a stereoscopic microscope, sketched, and subsequently returned to the tank. Total length (TL) and SL (notochord length NL in larvae) were measured.

3.1.5. Rearing of eggs and offspring

Spawned eggs were transferred with a siphon to 303 *l* glass aquaria on May 17, 2019. The water temperature was kept at 27.0 ˚C, and salinity at 33‰, with moderate aeration. Half of the water was changed once a day.

Larvae, juveniles, and young fish were fed *Branchionus* species complex (0 - 34 days after hatching), *Artemia* larvae (24 - 60 days after hatching) and frozen *Artemia* larvae and pieces of fish (mainly *Trachurus japonicus*) or crustacean meat (mainly *Pandalus eous*) (from 42 days after hatching) twice a day.

3.1.6. Growth

Growth regression equations between TL, SL (y), and days after hatching (x) were calculated by simple regression analysis.

3.2. Results

3.2.1. Spawning

Spawned eggs were found scattered on the substrate in the afternoon of May 14, 2019, two days after hormone injection (observation of spawning behavior was missed). The demersal adhesive eggs were translucent, spherical [mean 0.73 mm (0.05 mm SD, n = 5) in diameter], and unguarded by parental fish. Judging from decreases in body weight, all four females were thought to have contributed to the spawning event. Based on the decreased female body weights, the number of spawned eggs per female was estimated to have been more than 70,000 (Table 3. 1).

Table 3.1. The female standard length (SL mm), body weight (BW g) before and after spawning, weight difference (g) and estimated number of spawned eggs.

SL	BW before spawning	BW after spawning	Weight difference	Estimated number of spawned eggs
183.6	400.5	358.0	42.5	150747.5
145.3	235.0	215.0	20.0	70940.0
156.8	314.0	271.0	43.0	152521.0
142.6	329.5	307.5	22.0	78034.0
				113060.6 (mean) ± 38660.2 (standard deviation)

3.2.2. Egg development

One day after spawning (Figs. 3.1-a, 3.2-a): Notochord apparent, with a mass of small translucent oil globules.

Three days after spawning (Figs. 3.1-b, 3.2-b): Melanophores and erythrophores were apparent on ventral surface of the abdomen, with a few melanophores associated with yolk sac; optic vesicle apparent.

Four or five days after spawning: Eggs hatched.

3.2.3. Larval development

Hatched larva (Figs. 3.1-c, 3.2-c): Yolk sac larvae measured mean 1.87 mm NL and mean 2.00 mm TL (0.19 mm SD and 0.17 mm SD, respectively, $n = 5$), with mouth already opened, and optic vesicle black; pectoral and dorsal finfold apparent on side of the body and from the central body region through notochord end to anus respectively; melanophores apparent on the ventral abdominal surface, and xanthophores on the dorsal gut surface; myotomes numbered 12 or 13 + 9 or 8 = 21.

Nine DAH (Fig. 3.2-d): Larvae measured mean 2.42 mm NL (SD; 0.26 mm, $n = 3$), mean 2.57 mm TL (SD; 0.31 mm, $n = 3$), with a spherical body; oil globules remaining dorsally on gut, although feeding initiated; melanophores widespread dorsally on head and body; notochord flexion not started; myotomes numbered 13 + 8 =21.

17 DAH (Fig. 3.2-e): Larvae measured mean 3.48 mm NL (0.53 mm SD, $n = 3$), mean 3.73 mm TL (0.61 mm SD, $n = 3$); finfolds greatly reduced, and rudimentary soft rays of pectoral, dorsal and anal fins apparent; Chromatophores widespread dorsally on head and body, and melanophores ventrally on body.

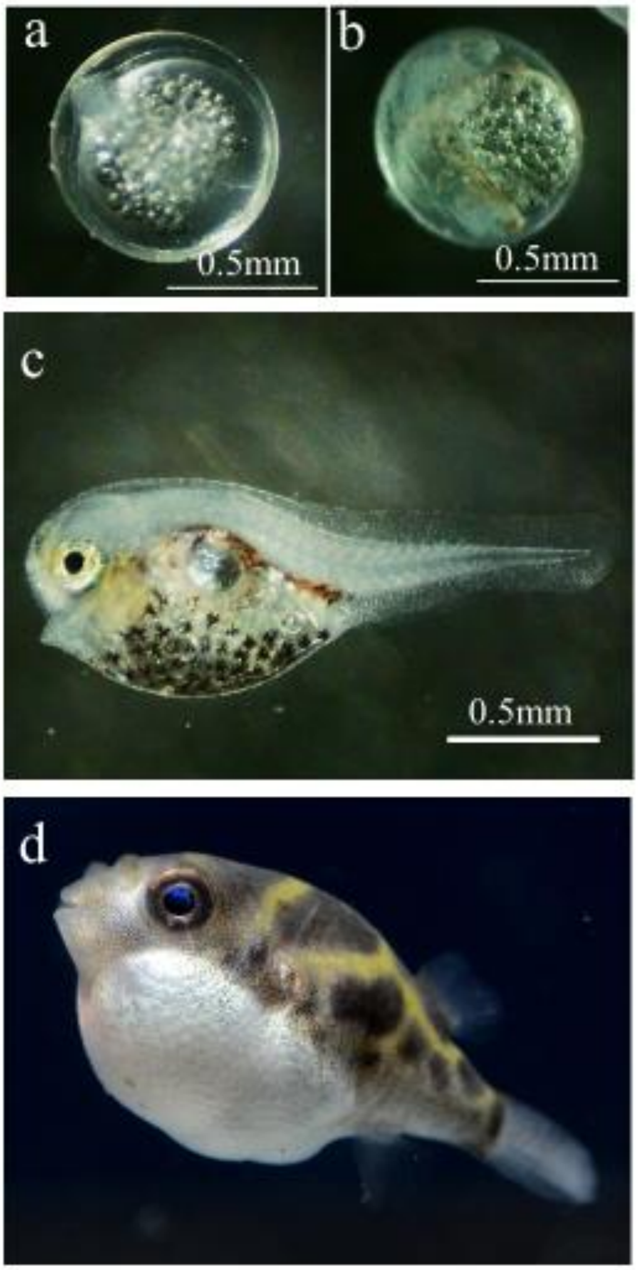

Fig. 3.1. Photos of eggs, larvae, and young fish of *Dichotomyctere fluviatilis*: a, egg one day after fertilization; b, egg three days after fertilization; c, newly hatched larva; d, young fish after three months (ca. 30 mm TL).

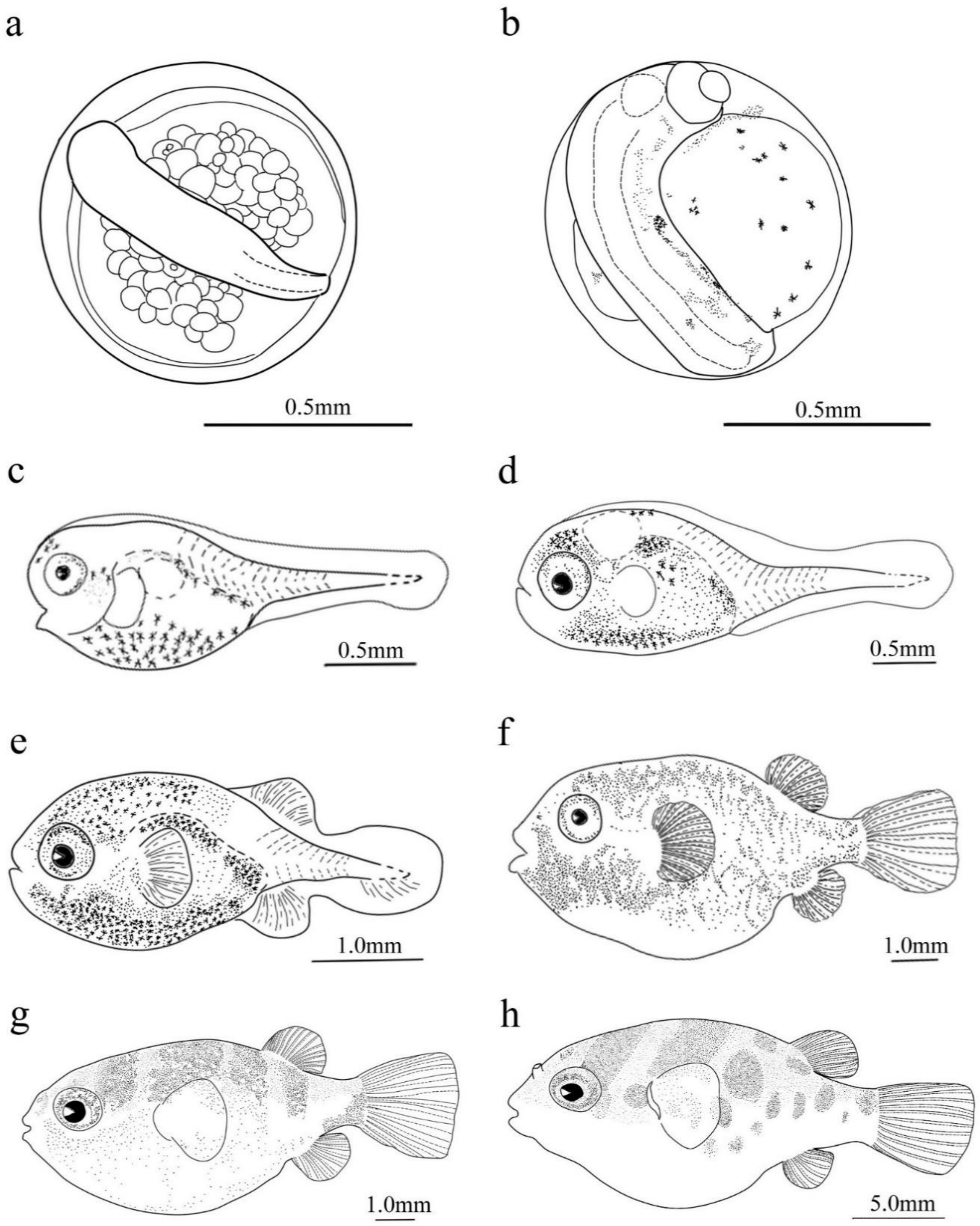

Fig. 3.2. Developmental series of eggs, larvae, and juveniles of *Dichotomyctere fluviatilis*: a, 0.76 mm egg, one day after fertilization; b, three days after fertilization; c, 1.87 mm newly hatched larva; d, 2.43 mm larva, 9 DAH; e, 3.48 mm larva, 17 DAH; f, 7.12 mm juvenile, 27 DAH; g, 8.97 mm juvenile, 35 DAH; h, 19.39 mm juvenile, 79 DAH.

3.2.4. Juvenile development

27 DAH (Fig. 3.2-f): Body length measured mean 7.12 mm SL (0.24 mm SD, n = 3), and mean 7.39 mm TL (0.73 mm SD, n = 3); notochord flexion completed, and fin ray numbers had attained adult complement (pectoral fin 22, dorsal fin 13, anal fin 11, and caudal fin 11); entire body brownish, covered with chromatophores.

35 DAH (Fig. 3.2-g): Body length measured mean 8.82 mm SL (0.19 mm SD, n = 3), and mean 11.57 mm TL (0.26 mm SD, n = 3); body greenish dorsally, with three or four blackish saddle bands, and pearly white ventrally, with many blackish dots.

80 DAH (Figs. 3.1-d, 3.2-h): Body length measured mean 18.65 mm SL (1.62 mm SD, n = 3), and mean 1.78 mm TL (1.62 mm SD, n = 3); body greenish dorsally, with two blackish saddle bands over eye and central body, and pearly white ventrally, as in adults; a few black blotches apparent posteriorly on body and tail.

3.2.5. Growth

TL and SL growth measurements (n = 60) are shown in Fig. 3.3 [Regression equation for TL, $y = 0.3077x + 0.779$ (determination coefficient 0.9751, black circles and solid line); regression equation for SL, $y = 0.2529x + 0.5479$ (determination coefficient 0.9758, gray triangles, and dotted line)].

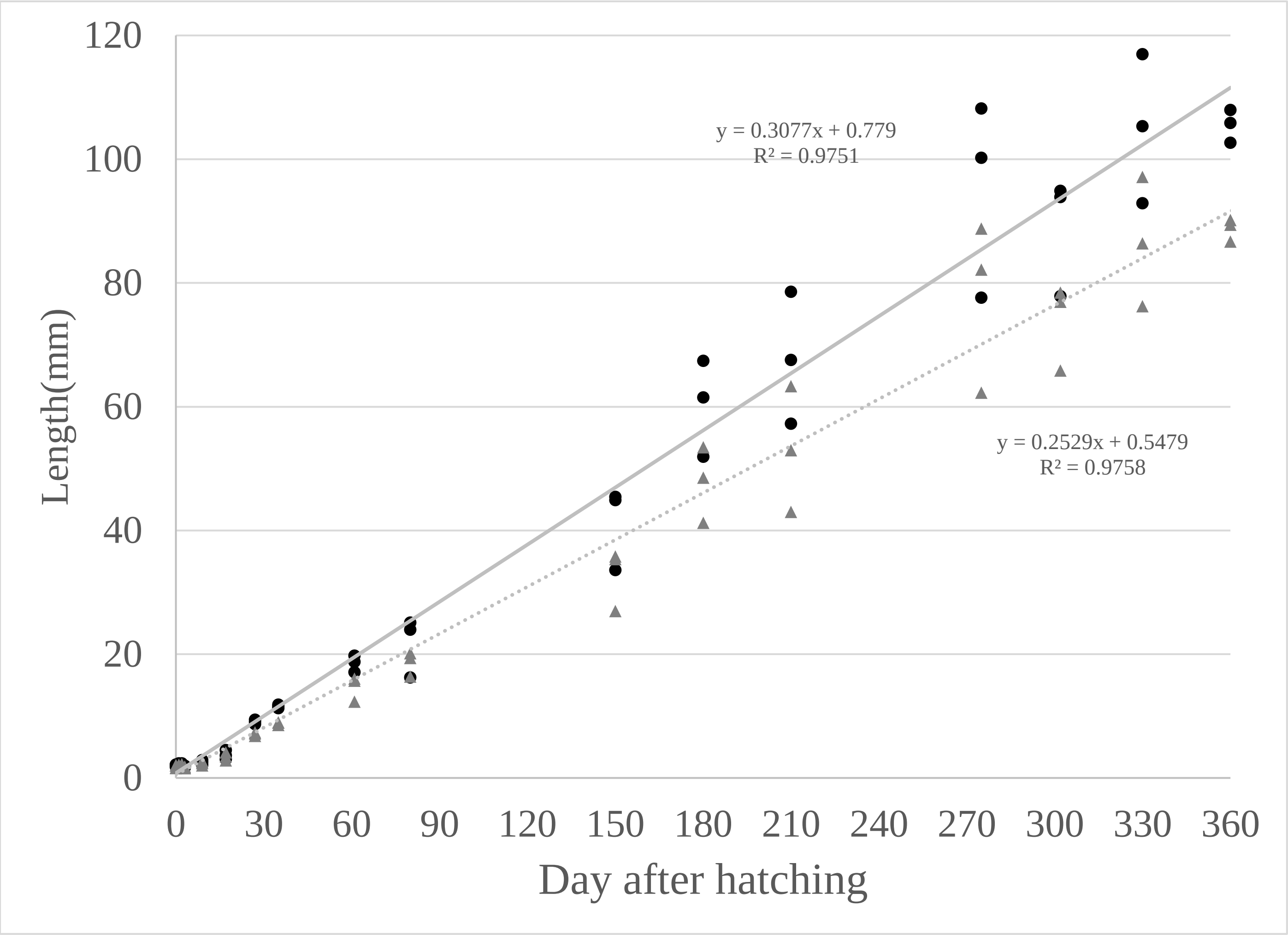

Fig. 3.3. Growth of *Dichotomyctere fluviatilis* spawned on May 14th, 2019 (black circles indicate TL of each individual; solid line indicates regression line of TL; gray triangles indicate NL or SL of each individual; dotted line indicates regression line of NL or SL)

3.3. DISCUSSION

Dichotomyctere fluviatilis spawned in full-strength sea water (33 ‰ salinity) two days after hormone injection, the first instance reported for the species. The demersal spherical, translucent, and adhesive eggs were scattered on the tank floor, without any parental protection. Some information on the reproductive biology of congeners, including *D. ocellatus* (treated as *Tetraodon biocellatus*) and *D. nigroviridis*, is available as shown in Table 3.2 (Watson et al. 2009; Yamada 2013; Zaucker et al. 2014; Doi et al. 2015). All the three species (*D. fluviatilis*, *D. ocellatus*, and *D. nigroviridis*) reported to spawn demersal adhesive eggs scattered on the bottom with no parental protection (Watson et al. 2009; Yamada 2013; Doi et al. 2015). Species of *Dichotomyctere* are likely to scatter eggs without egg protection. Compared with other freshwater puffer genera, the spawning manner of *Dichotomyctere* is similar to *Carinotetraodon* (Doi et al. 2015) with features such as no parental egg protection and spawning a large number of small eggs. However, numbers of eggs spawned by *Dichotomyctere* species are over 70,000 in *D. fluviatilis*, over 1000 in *D. ocellatus* (Doi et al. 2015), and 8000 -10000 in a female of *D. nigroviridis* (Watson et al. 2009; Yamada 2013). This is much more than other freshwater pufferfishes such as *Carinotetraodon* (<600). In this point, their reproduction resembled most marine puffers, known to spawn a large number of small eggs without egg protection [*Canthigaster rivulata*: 0.53 - 0.73 mm, 10000 - 17000; *Canthigaster rostrata*: 0.66 mm, 971; *Cantigaster valentini*: 0.68 - 0.72 mm, 15 - 876 (per a batch); *Takifugu niphobles*: 0.87 mm, 15000; *Takifugu pardalis*: 1.31 mm, 68000; *Takifugu poecilonotus*: 1.14 mm, 24000; *Takifugu snyderi*: 1.00 mm, 70000; *Takifugu stictonotus*: 1.09 mm, 50000 (Fujita 1962; Fujita and Shinohara 1986; Arai and Fujita 1988; Stroud et al. 1989; Sikkel 1990; Doi et al. 2014)].

Table 3.2. Spawning data for three species of *Dichotomyctere fluviatilis*, *D. ocellatus*, and *D. nigroviridis*.

	D. fluviatilis	*D. ocellatus* (as *Tetraodon biocellatus*)	*D. nigroviridis*		
	present study	Doi et al. (2015)	Watson et al. (2009)	Yamada(2013)	Zaucker et al.(2014)
Establishment date	1st February 2017	9th August 2002	unknown	unknown	unknown
Spawning date	14th May 2019	8th Decenver 2008	unknown	unknown	unknown
Aquaria volume (*l*)	2200	120	38	210	unknown
Hormone injection	10 IU/body weight (g) hCG*	No	3 IU/g hCG*	No	3 IU/g hCG*
Salinity (ppt)	33	9	33	>14	33
Water temperature (°C)	27	25-26	26	26-28	27.3
Male size (mm)	145.8 - 174.9 TL (*n* = 5) 123.6 - 148.9 SL	80 TL (*n* = 1)	unknown (*n* = 1)	unknown (*n* = 2)	unknown (*n* = 6)
Female size (mm)	170.0 - 216.9 TL (*n* = 4) 142.6 - 183.6 SL	75 TL (*n* = 1)	unknown (*n* = 1)	unknown (*n* = 2)	unknown (*n* = 6)
Egg diameter (mm)	0.73±0.05 (*n* = 5)	0.71±0.01 (*n* = 6)	0.39	0.8	unknown
Approximate number of eggs	>70000	>1000	8000	10000	unknown
Egg condition	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	unknown
Male protection	No	No	unknown	No	unknown
Spawning manner	scattered	scattered	scattered	scattered	unknown
Days until hatching	4-5	5	4	4	3
TL of hatched larvae (mm)	2.00±0.17 (*n* = 5)	1.69±0.04	2.00	2.00	1.29

*: human chorionic gonadotrophin

In salinity conditions for spawning Asian freshwater and brackish water pufferfishes, information of six genera is available. Spawning on brackish or full-strength sea water was known in *Dichotomyctere* and *Chonerhinos*. Spontaneous maturation and spawning without hormonal treatments have been observed only under brackish water conditions in *D. ocellatus* (9 ‰ salinity, Doi et al. 2015) and *D. nigroviridis* (>14 ‰ salinity, Yamada 2013). On the other hand, all instances in full-strength sea water (33 ‰ salinity) such as in *D. fluviatilis* (present study) and *D. nigroviridis* (Watson et al. 2009; Zaucker et al. 2014) required hormone injection. Moreover, the trials by Watson et al. (2009) and Zaucker et al. (2014), which failed to result in spontaneous spawning, and required artificial insemination instead, resulted in uncertain salinity requirements for reproduction. *Dichotomyctere* species were known as euryharine species, distributed a wide area of coastal, brackish, and river waters (Ebert 2001). Brackish water conditions may be appropriate not only for spontaneous maturation and spawning, but also for rearing larvae and juveniles. Therefore, future investigation of suitable salinity conditions should improve the breeding of *Dichotomyctere* puffers in captivity.

The growth rate of *D. fluviatilis* (present study: 60.27 mm mean TL 180 DAH; growth coefficient GC 0.3077) was about 2.5 times that of *D. ocellatus* (Doi et al. 2015: 22.56 mm mean TL 180 DAH; GC 0.116), reflecting their adult size difference; *D. fluviatilis* attaining twice the size of *D. ocellatus* (Ebert 2001). However, offspring obtained in the present study were reared in only full-strength sea water. As described above, this species is widely distributed in freshwater, brackish, and coastal waters (Ebert 2001). Not only its adults but also eggs, larvae, and juveniles might be able to survive in a variety of salinities. Hence, there remains room for the clarification of the relationship between salinity conditions and their growth.

Regarding to pigmentation patterns of hatched larvae, it was shown that chromatophores are distributed only on the gut, xianthophores on the dorsal side, and melanophores on the ventral side in *D. fluviatilis*, here. In *Dichotomyctere* species, morphologilcal information of larvae has been described in two species, *D. nigroviridis* and *D. ocellatus* (Zaucker et al. 2014; Doi et al. 2015). *D. nigroviridis* was described as: the body is covered with rich and colorful pigmentation except the tail (Zaucker et al. 2014). *D. fluviatilis*, in contrast, had a poor pigmentation pattern and limited ventral side and dorsal side of the gut. Therefore, in hatched larvae, this species was identified from *D. nigroviridis* by its pigmentation pattern. *D. ocellatus* described that melanophores covered the surface of the gut and bush-like erythrophores covered the head and body to the anus, rich pigmentation covered everywhere except the tail in hatched larvae (Doi et al. 2015). *D. fluviatilis*, in contrast, had only melanophores and xanthophores. The color of chromatophore in larvae was thought to become a taxonomic character between the two species. From this information, color and pigmentation patterns of chromatophores were considered to be useful characters for identified *Dichotomyctere* larvae. However, there is few information about three out of the six species in *Dichotomyctere*. Therefore, more information about the unknown three species is needed for discussion about identification in *Dichotomyctere* larvae.

The results of the present study should provide useful information for future breeding trials of *Dichotomyctere* species. The aquarium-bred offspring as the present study should also be available for future aquaculture studies such as the pufferfish toxicology as shown by Zhu et al. (2021).

Besides, *Dichotomyctere* is thought to be like marine pufferfish from an ecological viewpoint, as shown in this chapter. These results suggest the possibility that this genus was not well adapted to freshwater habitats yet. Therefore, *Dichotomyctere* might be a relatively primitive group among freshwater and brackish water pufferfishes.

CHAPTER 4: Tandem spawning and development in the African freshwater pufferfish *Tetraodon schoutedeni*

Tetraodon schoutedeni is a freshwater pufferfish, distributed in Africa. *Tetraodon* is the only freshwater genus distributed in Africa, and phylogenetically far from the other freshwater genera (Yamanoue et al. 2011; Igarashi et al. 2013; Santini et al. 2013a). About their reproductive behavior, Feigs (1955) reported that the female swims with males while biting her belly during spawning (tandem spawning). However, the details were not shown, and there is no morphological information about its larvae and juvenile.

In this chapter, through captive breeding of *Tetraodon schoutedeni*, details of their reproductive behavior and early development were described, and the biological significance of their tandem spawning was discussed.

4.1. MATERIALS AND METHODS

4.1.1 Parental fish and rearing

Eight individuals of *Tetraodon schoutedeni* (Fig. 4.1), approximately 60 mm total length (TL), were all wild caught in the Lua River, Ubangi River basin, Republic of Congo, were purchased from a Japanese fish trader (Rio Co. Ltd.) in October 2015, and maintained in a closed circulation/filtering cylindrical aquarium (170 *l* capacity) containing freshwater, at Osaka Aquarium NIFREL. One-third of the rearing water was replaced

Fig. 4.1. Tandem swimming of *Tetraodon schoutedeni*, the male biting and clinging to the female.

with new water once a week. Water was maintained at 24 – 25 ˚C and pH 6.4 – 7.7, with a daily light period from 07:00 – 19:00. The aquarium floor was covered with sand [4 mm ⌀ (MeritSand, Matsuda Co. Ltd.)] to 3 cm depth, together with sunken wood and the water plants *Aubias barteri, Hemionthus micronthemoides*, and *Microsorium pteropus.* Commercially prepared chironomid larvae and compound feed (NEOPROS) (Kyorin Food Industries, Hyogo, Japan) were provided to fish satiation level once a day, and uneaten food and excrement removed daily.

4.1.2 Observation

Spawning behavior of *T. schoutedeni* spawning behavior were recorded intermittently by video camera or mobile phone, and photographed by a digital camera (Table 4.1). Visual observations were also noted.

Spawned eggs scattered on the substrate were collected with a glass pipette, counted, and measured in a glass Petri dish. In one instance, when spawning was videoed, eggs just released into the water were counted from the video.

Eggs and larvae were observed and measured using a binocular microscope equipped with a measuring system, following anesthetization with FA100 (Bussan Animal Health Co., Ltd., Osaka, Japan). Standard length (SL, notochord length of early stage and body length of later stage larvae) and total length (TL) were measured.

Definition and terminology of developmental intervals followed Okiyama (2014): larval period, the stage before the complete development of countable characters; preflexion stage, the larval stage before notochord

Table 4.1. Summary of tandem swimming and spawning of *Tetraodon schoutedeni* observed in captivity from May 2016 to November 2017.

Spawning or tandem date	Tandem duration (minutes)			Time of spawning or finding eggs	Number of collectable eggs	Observation	Remarks
	before spawning[1]	between two spawnings	after spawning				
10th May 2016	unknown	—	unknown	17:00	20	eyes	only spawned eggs were found
20th May 2016	90	—	0	14:30	50	eyes	actual spawning failed to observe
7th July 2016	65	—	0	17:05	3	eyes movie recorded 2 times for 47 seconds in total	two males attatched for several minutes
27th July 2016	70	—	0	17:10	21	eyes	two males attatched for several minutes
9th November 2016	70	—	10	19:10	31	eyes movie recorded for 459 seconds	spawned eggs were counted from video movie
1st January 2017	70	—	30	17:10	20	eyes movie recorded 6 times for 54 seconds in total	two males attatched for several minutes
25th August 2017	10	—	—	—	0	eyes	not spawn
3rd September 2017	50	—	—	—	0	eyes	not spawn
4th November 2017	150	100	0	16:00 17:40	38	eyes movie recorded 3 times for 165 seconds in total	eggs were collected after the second spawning
9th November 2017	unknown	—	unknown	17:00	32	eyes	only spawned eggs were found

[1] From noticing of tandem swimming to spawning or finding eggs

flexion; flexion stage, the larval stage incorporating notochord flexion; postflexion stage, the larval stage following completion of notochord flexion; and juvenile period, the stage immediately following the larval stage, characterized by adult complements of countable characters (e.g., fin rays).

1.3 Rearing of eggs and larvae

The eggs were subjected to antibacterial treatment with 1 ppm methylene blue solution after collection. Subsequently, both collected eggs and hatched larvae were reared in 5 or 7 *l* plastic tanks set in a water bath (24–25 ˚C). From two days after hatching, fish larvae were fed initially with small type *Brachionus* (*B. plicatilis* spp. complex), with *B. calyciflorus* added from 18 days, and the latter only from 20 days. Moinidae sp. were added from 48 days, Moinidae sp. only from 50 days, and *Artemia* larvae added from 60 days, *Artemia* larvae and chironomid larvae were supplied from 70 days, and chironomid larvae only from 90 days. One-third of the tank water was changed, and leftovers and excrement removed daily.

4.2. RESULTS

4.2.1. Spawning

The sex of each parent fish was not apparent at the time of carry in October 2015, but became clear in the 2016 summer (three females and five males), the former being slightly larger and more swollen than the latter, as indicated by Preston John Aquatics (PJA) (2021). Although Kyoto Freshwater Pufferfish Institute (KFPI)

(2021) indicated that males had a shorter snout and darker cheek spot than females, such were not confirmed in present study.

Spawning occurred six times between May 10, 2016 and January 1, 2017, and twice on 4th and 9th November 2017 (Table 4.1). Tandem swimming with no actual spawning was also observed twice, on August 25, and September 3, 2017. Tandem swimming always started in the afternoon, with spawning occurring in late afternoon. The largest pair at first spawning comprised 55.0 mm SL (70.9 mm TL) female, and 51.5 mm SL (68.5 mm TL) male. Whether or not the same female and male were involved on other spawning occasions was uncertain due to the sizes of the other fish being similar in both sexes.

Four video recordings of tandem spawning (July 7, and November 9, 2016; January 1, and November 4, 2017). There were no signs of male pursuit or ambushing of females. Despite that the female sometimes avoided or swam away from nearby males before attachment, the female in tandem swimming pair delivered little warning to other surrounding individuals (Figs. 4. 1, 4. 2-a).

Successive spawning behavior, from pre-spawning tandem, spawning to post-spawning tandem, occurred on November 4, 2017 and was recorded by video (NIFREL 2021). A male was observed biting and clinging to the abdomen of a female, midway between the pectoral fins and anus. The female sometimes tried to shake off the male, but without inflating her body. Instances of tandem swimming followed by successful spawning which lasted for 65 – 150 minutes (Table 4. 1, Fig. 4. 2-b). At such times, the female continued swimming, searching for and pecking at food as normal, with the male clinging without any action like pushing (Fig. 4. 2-c). A second male bit and clung onto the female on three occasions, July 7 and 27, 2016, and January 1, 2017 (Fig. 4. 2-d), the initial male making no attempt to prevent or block the second male biting. The second

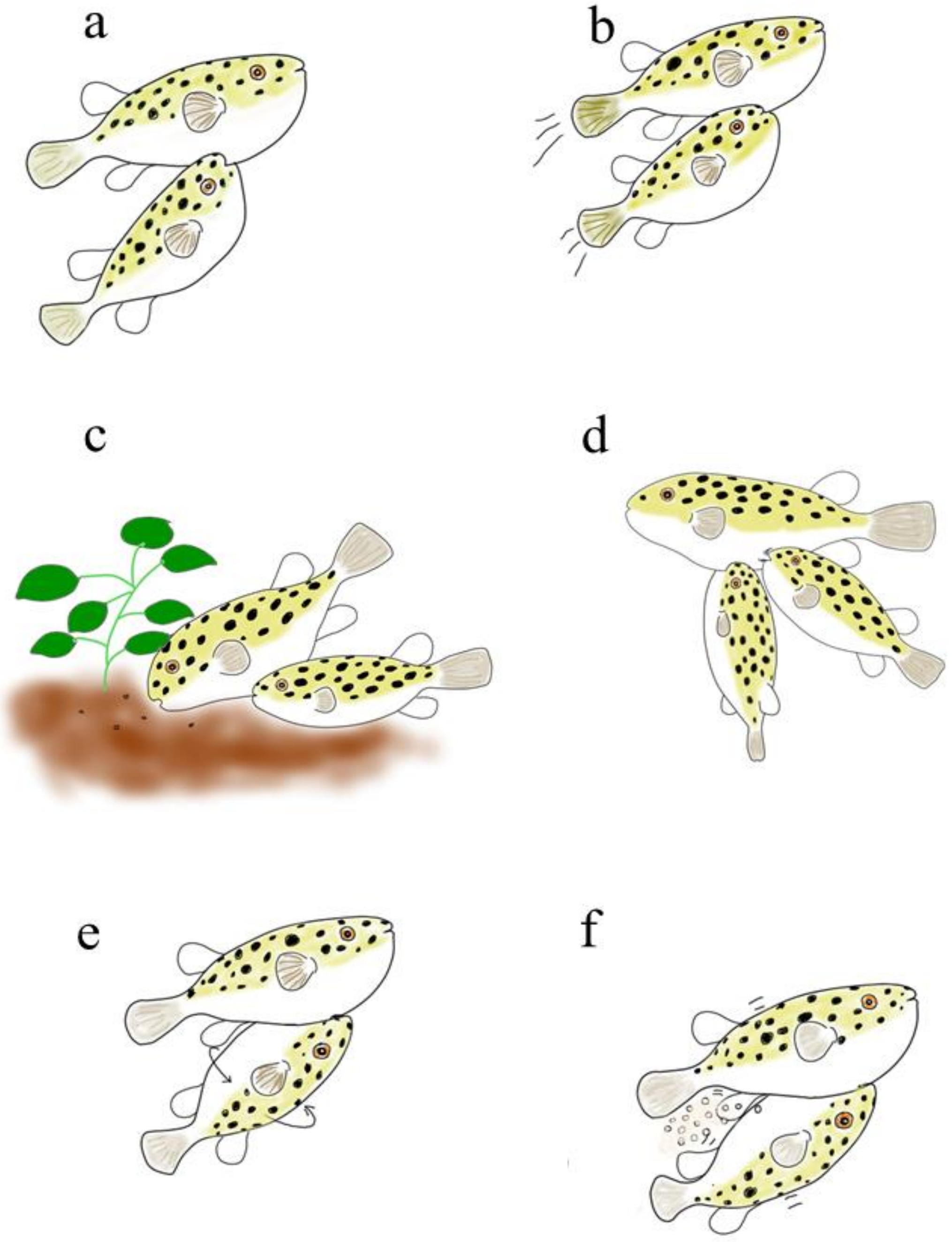

Fig. 4.2 Diagrammatic tandem spawning behavior of *Tetraodon schoutedeni*: a, a male biting and clinging to the female abdomen; b, tandem swimming; c, the female pecking at food while the male clings passively; d, two males biting and clinging to the abdomen of a female; e, reorientation of the male prior to spawning; f, spawning of the tandem pair.

male bit the female on a more lateral aspect (anteriorly or posteriorly) on the abdomen than the initial male, maintaining its position for several minutes before departing. Just prior to spawning, the male pushed the abdomen of the female repeatedly with its head. Both fish bodies then trembled, with the male upturning its body, thereby enabling its abdomen to oppose that of the female (Fig. 4. 2-e). Shortly thereafter, spawning took place, the female rising upwards swiftly (Fig. 4. 2-f). The weakly adhesive eggs were scattered on the sand, sunken wood, and leaves, being unguarded by either parent. Batch sizes ranged from 3 – 50 eggs, partially eaten by cohabitants. A video of spawning on November 7, 2016 revealed 31 eggs released.

Repeated spawning over an interval of 100 min of tandem swimming, occurred on November 4, 2017. This spawning series was interrupted by the brief departure of the male for a while. After the second spawning, the male quickly departed from the female. In other cases, the male also departed quickly from the female after spawning, although in two instances the male clung continuously to the female, for a further 10 min (November 9, 2016) and 30 min (January 1, 2017), but without subsequent spawning. A weak bite mark was seen on the abdomen of the female, but no apparent of a wound or bleeding.

4.2.2. Development

The following observations were based on eggs spawned on May 20, 2016 (to 10 days after hatching) and on July 27, 2016 (from 20 days after hatching).

Mean egg diameter was 0.91 mm (0.01 mm SD, *n* = 12), the eggs being spherical, transparent, demersal, and weakly adhesive (typically picking up incidental matter on the surface), with a mass of small oil globules (Fig.

4. 3-a, 0.90 mm diameter, two hours after spawning). The embryo was obvious after two days, and the eyes after five days. Hatching occurred seven days after spawning.

Larvae at hatching (Fig. 4. 3-b, 2.15 mm SL, preflexion stage) were mean 2.21 mm SL (0.08 mm SD, *n* = 5) and mean 2.34 mm TL (0.02 mm SD, *n* = 5), with the eyes well developed, mouth and anus opened slightly, and a pectoral finfold and continuous dorsal, caudal, and anal finfolds apparent. Melanophores and erythrophores occurred mainly dorsally on the head, under the brain and around the eyeballs, around the anus and under the mid-caudal region, as well as anteriorly on the yolk surface. Weak xanthophores were apparent dorsally on the head to the caudal region, and on the caudal finfold. A large yolk persisted at this stage. Larvae 1 DAH (Fig. 4. 3-c, 2.46 mm SL, preflexion stage) were mean 2.48 mm SL (0.03 mm SD, *n* = 5) and mean 2.62 mm TL (0.04 mm SD, *n* = 5), and 2 DAH (Fig. 4. 3-d, 2.59 mm SL, preflexion stage), mean 2.57 mm SL (0.07 mm SD, *n* = 5) and mean 2.72 mm TL (0.02 mm SD, *n* = 5). Chromatophores became scattered and denser. Although the yolk was fully absorbed, larvae remained on the bottom without feeding. After three days, the mouth and eyes moved, and larvae began to swim and actively feed. Prey of *Brachionus* spp. were observed in the gut on day 4.

Ten DAH, larvae had attained mean 2.94 mm SL (0.05 mm SD, *n* = 3) and mean 3.08 mm SL (0.05 mm SD, *n* = 3), and actively swam and fed (Fig. 4. 3-e, 2.97 mm SL, preflexion stage), although still lacking rudimentary rays of any fins. Melanophores had developed in bands between the eyes and on the dorsum, with xanthophores obvious on the head, body sides and caudal region. Erythrophores were present on top of the eyes, around the pectoral fins and on the body sides.

Notochord flexion had already started by day 20 in mean 4.95 mm SL (0.20 mm SD, *n* = 5) and mean 6.08

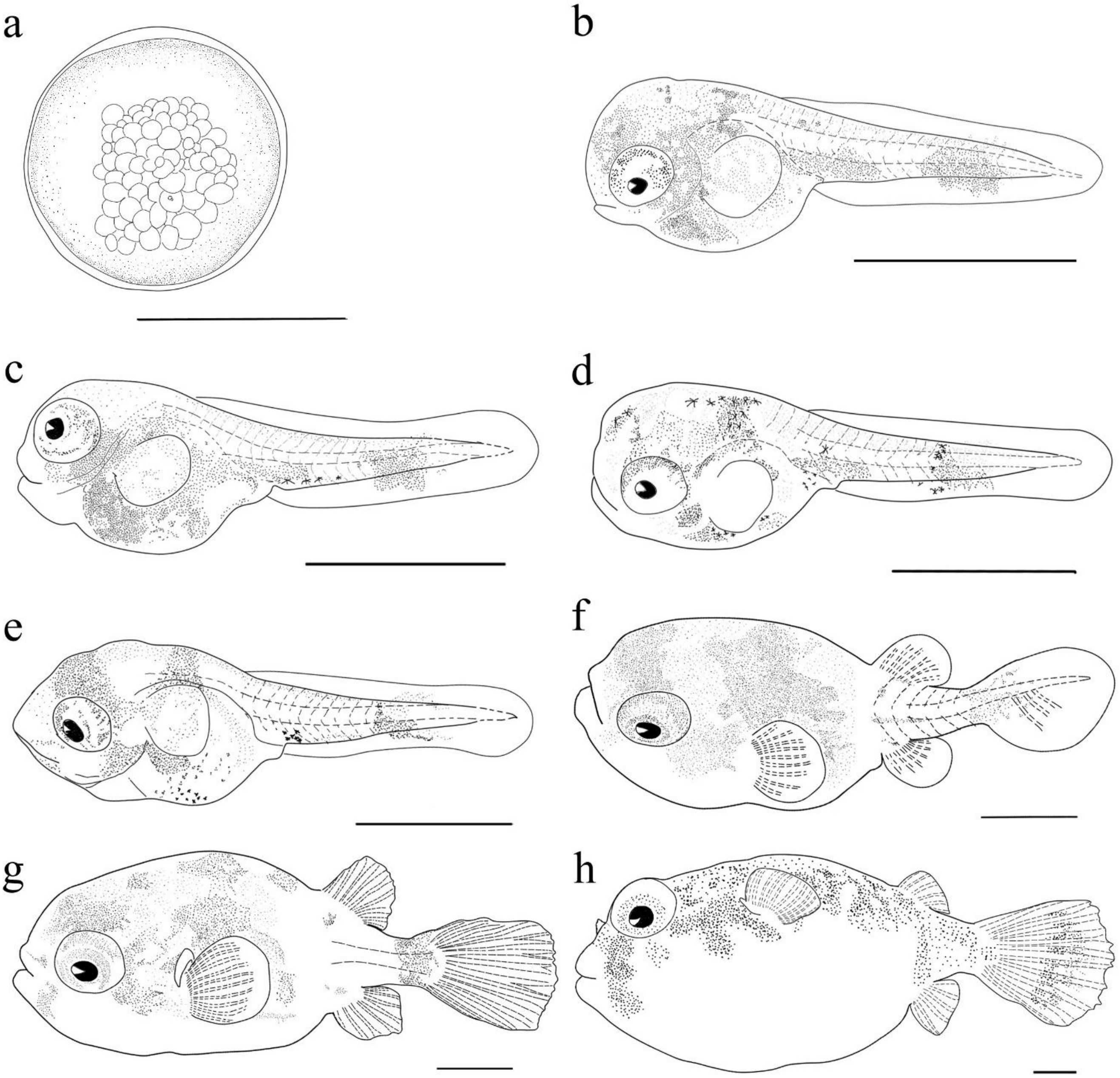

Fig. 4.3 Development of *Tetraodon schoutedeni*: a, egg, two hours after spawning, 0.90 mm diameter; b, just-hatched larvae, 2.15 mm standard length (SL); c, one DAH larva (preflexion stage), 2.46 mm SL; d, 2 DAH larva (preflexion stage), 2.59 mm SL; e, 10 DAH larva (preflexion stage), 2.97 mm SL; f, 20 DAH larva (flexion stage), 5.38 mm SL; g, 30 DAH juvenile, 5.51 mm SL; h, 50 DAH juvenile, 9.55 mm SL.

mm TL (0.18 mm SD, *n* = 5) larvae, with rudimentary fin rays obvious in all fins, and chromatophores spread over the back and sides of the body (Fig. 4. 3-f, 5.38 mm SL, flexion stage).

Notochord flexion and all fin rays were completed by day 30 in mean 5.55 mm SL (0.30 mm SD, *n* = 5) and mean 6.95 mm TL (0.30 mm SD, *n* = 5), being the onset of the juvenile stage (Fig. 4. 3-g, 5.51 mm SL, juvenile). Irregularly shaped xanthophores had developed on the pectoral and caudal fin bases, and erythrophores on the dorsal and anal fin bases, caudal region and caudal fin edge. Individuals had grown to 9.55 mm SL and 12.81 mm TL by day 50 (Fig. 4. 3-h, *n* = 1, juvenile), mean 9.13 mm SL (0.59 mm SD, *n* = 5) and mean 11.87 mm TL (0.97 mm SD, *n* = 5) by day 60, mean 23.30 mm SL (1.00 mm SD, *n* = 3) and mean 30.32 mm TL (1.22 mm SD, *n* = 3) by day 180, and mean 27.92 mm SL (2.36 mm SD, *n* = 3) and mean 35.84 mm TL (2.91 mm SD, *n* = 3) by day 360.

4.3. DISCUSSION

4.3.1. Breeding of Tetraodon schoutedeni in captivity

Feigs (1955) observed tandem swimming and spawning of *Tetraodon schoutedeni* on two occasions; on 16th February 1955, two males successively bit the abdomen of a female and formed a tandem trio for about 2 hours from 7:00 PM, but actual spawning was not seen; on March 14, 1955, a tandem trio formed at 7:30 PM, although the third male tried to participate, it departed soon. Spawning by the trio occurred at 8:40 PM, yielding about 80 eggs, followed by additional spawning at 1–2 min intervals. After the observer removed other cohabitants to protect the spawned eggs, the trio again started spawning repeatedly (seven times from 9:23 PM to 9:39 PM), resulting in 30–60 eggs each time (over 400 eggs in total). The trio separated after 20 min.

The present observations and those of all the videos recently published online (AQUAPICASSO 2022; GeoPhilgus Fishkeeping 2018; KFPI 2018a,b; Monaco Fish 2020; NIFREL 2021; PJA, 2018a,b,c,d;2021; TOEKIEWORLD–Marc 2018) were essentially similar to those of Feigs (1955), though a sequence of the male initially biting and clinging to the female abdomen was not observed in any case of both previous videos and present study.

In the present observation, there were no signs of male pursuit or ambushing of females before tandem swimming. In contrast, the female sometimes avoided or swam away from nearby males and seemed to evade approach of the male. Therefore, to succeed spawning, males might need to approach the female sneakily. Besides, despite that the female sometimes avoided or swam away from nearby males, tandem swimming pair with little warning and surrounding individuals (Figs. 4. 1, 4. 2-a). The males biting behaviors might stimulate females into spawning. Therefore, the behaviors might be the initial sign of its spawning without any indication. However, the present observation was intermittent, long-term observation such as recording by fixed pointed camera is necessary to clarify the detail of the male initially biting and clinging to the female abdomen.

After tandem swimming for a while, the tandem pair suddenly rose toward the near surface and spawned dozens of eggs in a single release (Feigs 1955; KFPI 2018b; PJA 2018c, 2018d; present study). Although PJA (2018c) and TOKIEWORLD-Marc (2018) suggested cave spawning, contrary to the above observations, its behavior could not be observed in their videos. PJA (2018a) and GeoPhilgus Fishkeeping (2018) both suggested male protection of spawned eggs, but also could not be observed.

The number of spawning times in a single overall spawning period varied considerably, once or twice at most yielding fewer than 100 eggs in total. In contrast, in previous information, the number of spawning times in a single overall spawning period were described as several times, more than the present study (Feigs 1955; PJA 2018d). Moreover, the total number of eggs were also larger, over 400 (Feigs 1955) or 300 eggs (PJA 2018d). A possible reason for such differences may be the nutritional status of the fish [fed with commercially prepared feed during the present study, and with freshwater snails, possibly natural food in the wild, during Feigs' (1955) and PJA's (2018a) observations]. Snail feeding of *T. schoutedeni* is also recommended by many aquarium hobbyists, such as GeoPhilgus Fishkeeping (2018), Sykes (2020) and PJA (2022), not only for nutrition but also for keeping teeth short and healthy. The male clung continuously after spawning during the present study, but further spawning did not occur, possibly due to the poorer conditioned female caused by unbalanced food condition of this study.

Some of the spawned eggs were devoured by cohabitants, as Feigs (1955) and PJA (2018d) noted, and possibly by the parental male as well. Whether or not such egg cannibalism occurs in the wild is also unknown.

The sizes of eggs (mean 0.91 mm diameter) and hatched larvae (mean 2.21 mm SL) of *T. schoutedeni* were similar to those of marine puffers, such as *Takifugu* spp. (Doi et al. 2014, 0.87–1.31 mm diameter and 2.17–2.90 mm SL, respectively). This reflects the similar sizes of natural food of both *T. schoutedeni* larvae and marine puffers, such as *Takifugu* spp. In any case, organisms such as small type *Brachionus* spp., as used for initial food in the present study, are important for rearing early larval *T. schoutedeni*. Elsewhere, KFPI (2022) and PJA (2022) bred larvae by providing rotifers and *Paramecium* with rotifers, respectively, as initial food.

In contrast, the hatching days of this species was seven days in the present study, relatively longer in freshwater pufferfishes without egg protection (Doi et al. 2015). This result might be caused by the rearing temperature. In the previous study, eggs hatched at the fifth day after spawning, and the author reared eggs at 77 - 80 °F (about 25 - 26 °C) (Feigs 1955). In the present study, rearing temperature was maintained at 24 - 25 °C, lower than previous studies of other freshwater pufferfishes (25 – 26 °C, Doi et al. 2015). Therefore, this result was caused by temperature.

4.3.2. Function of tandem spawning

The tandem spawning of *T. schoutedeni* (GeoPhilgus Fishkeeping 2018; KFPI 2018a,b; Monaco Fish 2020; NIFREL 2021; PJA, 2018a, b, c, d, 2021; TOEKIEWORLD–Marc 2018; present study) and *T. lineatus* (e. g. Fang 2020) was unknown in other tetraodontids. In the latter species, a pair performed tandem swimming for a while (Adrian 2016; Crazypufferfish 2022), abruptly rising to the near surface and releasing eggs (Fang 2020), a behavioral sequence similar to that observed in *T. schoutedeni*.

As function of biting and clinging (tandem) behavior of *Tetraodon*, two functions would be hypothesized.

① Preoviposition mate guard

② Ensuring successful fertilization

“Preoviposition (or precopulatory) mate guard” by males prevails among many animals, such as arthropods and anuran amphibians (e. g. Ridley 1983). For example, in many crab species, male precopulatory guarding of females persists from hours to days, with preoviposition guarding also occurring in possible multi-copulating species (e. g. Koga 2007). Tandem flying in many dragonflies is observed in detail, males undertaking pre- and post-copulation (namely, preoviposition) guarding by clasping the neck or head of the female with the tip of its abdomen (e. g. Corbet 1999; Watanabe 2015). In frogs, preoviposition guarding is common, wherein the male clasps the female with its front legs for hours to days (e. g. Light 1976; Ridley 1983). Such behavior functions as female monopolization in the male competitive system (Ridley 1983). In *T. schoutedeni,* on the other hand, such monopolization of females is less important than in the above examples, since the first clinging male did not exclude a second male. The clinging position on the female would be more significant for the male as discussed below. Therefore, tandem spawning in *T. schoutedeni* and *T. lineatus* would have exclusionary effect on monopolization by the first male.

Male lampreys use their sucking disc to attach to the head of a female, curling their tail around the genital pore of the latter, a body position ensuring successful fertilization of eggs (e. g. Hardisty and Potter 1971; Scott and Crossman 1973). Many male elasmobranchs bite the cheek or fin of the female to maintain a copulating posture (e. g. Uchida et al. 1990; Parsons et al. 2008). In the same manner, the male *T. schoutedeni* apparently maintains the best spawning posture by clinging to a specific position on the abdomen of the female. A second male, limited to a less appropriate position, may be restricted to less successful spawning.

An extreme example of tandem mating is that of deep sea Ceratoidei, wherein a male is attached permanently to a female as a sexual parasite (e. g. Pietsch 1976, 2005). Such male-female associations would be crucial for the timing of successful spawning in the isolating deep-sea environment. Obligatory sexual parasitism is known for six genera, facultative parasitism for three genera, and temporary nonparasitic attachment for five families, among Ceratoidei (11 families, 35 genera, and 160 species) (Pietsch 2005). The females of obligatory parasitic species do not develop ovaries without an attached male. Females of facultative parasitic species, however, can develop ovaries with or without a parasitic male. On the other hand, small free-living males (third category) attach temporarily to females for spawning (Pietsch 1976). In obligatory parasitism, the male apparently stimulates maturation of the female. If neither male nor female is ready to spawn in a facultative parasitic or non-parasitic species, the male remains attached until spawning occurs. Therefore, the longer the male remains attached, the greater the chance of becoming permanently fused as a parasite (Pietsch 1976, 2005). The attached male possibly stimulates not only maturation but also ovulation of the female. Being able to sense the latter accurately when attached would ensure successful fertilization. Therefore, tandem mating of deep sea Ceratoidei was thought to have a functional role not to guard the preoviposition mate or ensure successful fertilization, but to stimulate its female into maturation and ovulation. Tandem spawning in *T. schoutedeni* and *T. lineatus* are similar to that of the temporary nonparasitic attachment species of Ceratoidei, wherein the clinging male stimulates the pre-ovulatory female, waits for ovulation, and spawns at the appropriate time in the most appropriate position.

However, a behavioral difference between these species was apparent during tandem swimming. Whereas the female *T. schoutedeni* swims and drags the clinging male, the male *T. lineatus* often initially swims and pushes the female from the underside, shaking and rubbing its abdomen against that of the female. Additionally, whereas the male *T. schoutedeni* actively pushes the female and upturns its body immediately prior to spawning, probably sensing female ovulation, the male *T. lineatus* clings in the upturned posture, continually rubbing its abdomen against the female, seemingly stimulating female ovulation. In both instances, the proximity of the males to the females enables them to sense the readiness of the latter to spawn. At the moment of spawning, the cloacas of the male and female fish appeared to be closely adjacent, likely ensuring fertilization. Therefore, it would be important for the male to bite and cling at the best position on the abdomen of the female for gaining the optimal spawning posture. In a case of *T. lineatus*, a second male deprived the initial male from the best position, thereby monopolizing the female (Crazypufferfish 2022). In *T. schoutedeni*, on the other hand, the first clinging male did not exclude subsequent males (Feigs 1955; PJA 2018b; Monaco Fish 2020; present study). Whereas the clinging position on the female was significant for the initial male, the subsequent males seemingly accepted less appropriate positions (PJA 2018b; Monaco Fish 2020), which would result in less successful spawning. Therefore, tandem spawning in *T. schoutedeni* has a secondary, exclusionary effect of monopolization by the first male. The overall function of tandem spawning in *T. schoutedeni* and *T. lineatus* is therefore hypothesized as follows: semi-monopolization of the female, stimulation and sensing of ovulation, and maintenance of a spawning posture ensuring fertilization.

The only genus known as African freshwater pufferfish, *Tetraodon*, is considered phylogenetically far from other freshwater genera (Yamanoue et al. 2011; Igarashi et al. 2013; Santini et al. 2013a). The unique behaviors like biting, clinging, and tandem swimming of this species are shared with *T. lineatus*, considered to have separated relatively earlier among *Tetraodon* (see Igarashi et al. 2013). Therefore, these behaviors might be common in the genus *Tetraodon*.

GENERAL DISCUSSION

Spawning features of freshwater and brackish water pufferfish

Information on eggs, larvae, juveniles, spawning manner of eight genera, and 18 species of freshwater and brackish water pufferfishes is accessible (Araujo-Lima et al. 1994; Watson et al. 2009; Yamada 2013; Zaucker et al. 2014; Doi et al. 2015, 2022a, b; Ahmad-Nasir et al. 2016, 2020) (Table gd. 1). In marine pufferfishes, spawning manner tends to be resembled among intra-genetic species (Matsuura 2017). This tendency is also recognized in freshwater and brackish water pufferfishes (Table gd. 2).

In freshwater and brackish water pufferfishes, the reproductive biologies of eight genera were reported (Araujo-Lima et al. 1994; Watson et al. 2009; Yamada 2013; Zaucker et al. 2014; Doi et al. 2015, 2022a, b; present study) (Table gd. 2). Although the spawning manner of *Colomesus* is unknown, the other seven genera can be separated into two types: egg protection types (*Leiodon*, *Pao*) (Doi et al. 2015, 2022a, b) and scattering egg types (*Carinotetraodon*, *Dichotomyctere, Tetraodon, Auriglobus, Chonerhinos*) (Watson et al. 2009; Yamada 2013; Doi et al. 2015, 2022a). Observing the number of genera of each type, the egg protection type seems to be a minor feature among them. Also in marine pufferfishes, parental egg protection is quite rare, only known in *Torquigeiner* species (Kawase et al. 2015; Matsuura 2017).

Regarding the egg shape, only two genera, *Auriglobus* and *Chonerhinos* spawn oval eggs. Two genera are phylogenetically very close with each other as shown in Chapter 2. However, the egg diameter and size of hatched larvae were slightly different between the two (Table gd. 2). *Auriglobus* is larger than *Chonerhinos*

Table gd. 1. Spawning data for 18 brackish and freshwater pufferfish species

	Dichotomyctere fuluviatilis	*D. ocellatus*	*D. nigroviridis*		*Carinotetraodon irrubesco*	*Ca. lorteri*	*Ca. travancornis*	*Leiodon cutcutia*	*Pao cochinchinensis*
Hormone injection	Yes	-	Yes	-	-	-	-	Yes	-
Used hormone (amount)	human chorionic gonadotrophin (HCG) (10 IU/g)	-	HCG (3 IU/g)	-	-	-	-	HCG (10 IU/g)	-
Salinity	Sea water	brackish (6ppt)	brackish (6ppt)	brackish (>14ppt)	Fresh water	Fresh water	Fresh water	Fresh water	Fresh water
water temperature	25-27	25-27	26-27	26-28	25-26	25-26	25-26	25-26	25-26
Egg shape	spherical	spherical	spherical	spherical	spherical	spherical	spherical	spherical	spherical
Egg diameter	0.73±0.05mm	0.71±0.01mm	0.39mm	0.8mm	0.88±0.02mm	1.37±0.07mm	1.43±0.09mm	1.42mm	1.94±0.03mm
Approximate number of eggs	70000	1000	8000	10000	200	200	1-5	24-545	250
Egg condition	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal
Male protection	No	No	-	No	No	No	No	Yes	Yes
Spawning manner	Scatterd	Scatterd	Scatterd	Scatterd	Scatterd	Scatterd	Scatterd	Layered batch	Layered batch
Days until hatching	4-5	5	4	4	2-3	4	5	6	7
TL of hatched larvae	2.00±0.17mm	1.69±0.04mm	1.29-2.00mm	2.00mm	2.90±0.07mm	2.25±0.02mm	3.15±0.16mm	2.87±0.26mm	4.26±0.09mm
Refferance	present study	Doi et al. (2015)	Watson et al. (2009) Zaucker et al. (2014)	Yamada (2013)	Doi et al. (2015)	Doi et al. (2015)	Doi et al. (2015, 2022a)	Doi et al.(2015); present study	Doi et al.(2015)

	P. turgidus	*P. palembangensis*	*P. suvatti*	*P. abei*	*P. bayleyi*	*Auriglobus modestus*	*Chonerhinos naritus*	*Tetraodon schoutedeni*	*T. lineatus*	*Colomesus asellus*
Hormone injection	-	-	-	-	-	Yes	-	-	-	-
Used hormone (amount)	-	-	-	-	-	HCG (10 IU/g)	-	-	-	-
Salinity	Fresh water	Fresh water	Fresh water	Fresh water	Fresh water	Fresh water	Brackish water	Fresh water	-	Fresh water
water temperature	25-26	26	25-27	26	26	26	28	24-25	-	28.3
Egg shape	spherical	spherical	spherical	spherical	spherical	oval	oval	spherical	spherical	-
Egg diameter	2.13±0.05mm	2.25±0.01mm	2.41±0.41mm	2.26±0.04mm	2.74±0.09mm	2.05±0.06mm（L.A） 1.68±0.06mm（S.A.）	1.28±0.17mm	0.91mm	-	-
Approximate number of eggs	300	90-130	590	63	117	787	unknown	3-50	-	-
Egg condition	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	-
Male protection	Yes	Yes	Yes	Yes	Yes	No	No	No	No	-
Spawning manner	Layered batch	Layered batch	Layered batch	Layered batch	Layered batch	Scatterd	Scatterd	Scatterd	Scatterd	-
Days until hatching	8	10	10	8	10	3	3	7	-	-
TL of hatched larvae	4.41±0.13mm	4.58±0.10mm	4.87±0.18mm	4.50±0.20mm	5.27±0.05mm	4.85±0.35mm	3.42±0.23mm	2.34±0.02mm	-	-
Refferance	Doi et al.(2015)	Doi et al.(2015, 2022a)	Doi et al.(2022b)	Doi et al.(2022b)	Doi et al.(2022b)	present study	Ahmad-Nasir et al.(2016, 2020)	present study	Fang (2020)	Araujo-Lima (1994)

Table gd. 2 Spawning data for seven brackish and freshwater pufferfishes genera.

	Dichotomyctere	*Carinotetraodon*	*Leiodon*	*Pao*	*Auriglobus*	*Chonerhinos*	*Tetraodon*
Salinity	Sea water or brackish	Fresh water	Fresh water	Fresh water	Fresh water	Brackish water	Fresh water
water temperature	25-27	25-26	25-26	25-26	25-26	28	24-25
Egg shape	spherical	spherical	spherical	spherical	oval	oval	spherical
Egg diameter	0.39-0.73mm	0.88-1.43mm	1.42mm	1.94-2.74mm	2.05±0.06mm（L.A） 1.68±0.06mm（S.A.）	1.28±0.17mm	0.91mm
Approximate number of eggs	1000-70000	1-200	24-545	90-590	787	unknown	3-50
Egg condition	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal	adhesive demersal
Male protection	No	No	Yes	Yes	No	No	No
Spawning manner	Scatterd	Scatterd	Layered batch	Layered batch	Scatterd	Scatterd	Scatterd
Days until hatching	4-5	2-5	6	7-10	3	3	7
TL of hatched larvae	1.69-2.00mm	2.25-3.15mm	2.53-3.15mm	4.26-5.27mm	4.85±0.35mm	3.42±0.23mm	2.34±0.02mm
Refferance	Doi et al. (2015); Watson et al. (2009); present study	Doi et al. (2015, 2022a)	Doi et al.(2015); present study	Doi et al.(2015, 2022a,b)	present study	Ahmad-Nasir et al.(2016, 2020)	present study

in both sizes [*Auriglobus*: 1.68±0.06mm (short axis), 4.85±0.35mm TL; *Chonerhinos*: 1.28±0.17mm, 3.42±0.23mm TL] (Ahmad-Nasir et al. 2016, 2020). These differences are thought to be affected by the ecology of their habitats. To stay in the river, diameters of fish eggs are generally larger in freshwater species than marine species (Nishida 2001). *Auriglobus* spends almost of its life in freshwater in contrast to *Chonerhinos* which usually lives in coastal water and rises up to the river for spawning. These differences in lifecycles might cause the differences of sizes of eggs and larvae.

Except for these two genera, all species spawned spherical, adhesive, demersal eggs (Table gd. 2); the same in marine pufferfishes (Fujita and Shinohara 1986; Arai and Fujita 1988; Stroud et al. 1989; Sikkel 1990; Doi et al. 2014). The egg diameters were 0.53 - 1.4 mm in marine species (Fujita and Shinohara 1986; Arai and Fujita 1988; Stroud et al. 1989; Sikkel 1990; Doi et al. 2014) and 0.39 - 2.25 mm in freshwater and brackish water species (Table gd. 2); hence, they seem almost similar. As intraspecific variation in amphidromous fish like sculpins and species of *Rhinogobius*, eggs of landlocked types (living in freshwater areas for entire life) are known to become larger than the original amphidromous one (Nishida 2001). The reason for this tendency is generally thought that the larvae from larger eggs develop to larger sizes for staying in the river (Hutchinson 1957: Nishida 2001). This tendency, however, was not observed in pufferfish. It could be unnecessary to be a large larvae at hatching because pufferfishes invade into the continental large rivers without strong stream such as the Mekong River, Congo River, and Amazon River.

On the other hand, some genera (*Leiodon*, *Pao*, some of *Carinotetraodon*) spawn relatively large eggs (0.88 - 2.25 mm). These three genera were phylogenetically clustered into the same clade (Table gi. 1), being thought to share the same origin (Yamanoue et al. 2011; Igarashi et al. 2013; Santini et al. 2013a). Therefore, spawning larger eggs has possibly evolved uniquely in this clade. Therefore, this clade may have been more adapted to freshwater habitats than other genera.

Morphological features of eggs and larvae are generally thought to be controlled by reproductive strategy and behavior (Moser 1981; Senta et al. 2001; Minami 2019; Iida 2019). The egg protection genera (*Leiodon*, *Pao*) spawn larger eggs (diameter over than 1.42 mm) than the eggs of the scattering genera (*Carinotetraodon*, *Dichotomyctere*, *Tetraodon*) (less than 1.43 mm) (Watson et al. 2009; Yamada 2013; Doi et al. 2015, 2022a, b). When eggs become larger; the numbers must become smaller. Then the parental cost for one egg (e. g. parental egg protection) become higher (Smith and Fretwell 1974). Similarly, the size of hatched larvae of the egg protection genera are larger (2.38 - 5.27 mm) than the egg scattering genera (1.69 - 3.15 mm). Thus, the size of hatched larvae was thought to be related to the egg size (Fig. gd. 1). The egg scattering genus *Carinotetraodon*, however, yields middle sized eggs (0.88 - 1.43 mm) and larvae (2.25 - 3.15 mm) between the egg protection genera and the other egg scattering genera, rather being about the same size with the egg protection genus *Leiodon* (1.42 mm eggs and 2.53 - 3.15 mm larvae). These size differences might be related to their differences in their ecological as well as phylogenetical background as described above and in preceding chapters. The early-stage morphological features were also slightly different between *Pao* and *Leiodon*, which might reflect the similar difference in their phylogenetic or ecological (including mating system) background as shown in Chapter 1.

Regarding the morphological features of hatched larvae, *Auriglobus*, *Chonerhinos*, *Carinotetraodon*, and *Dichotomyctere ocellatus* are hatched with undeveloped optic vesicles (Chapter 2; Doi et al. 2015). On the

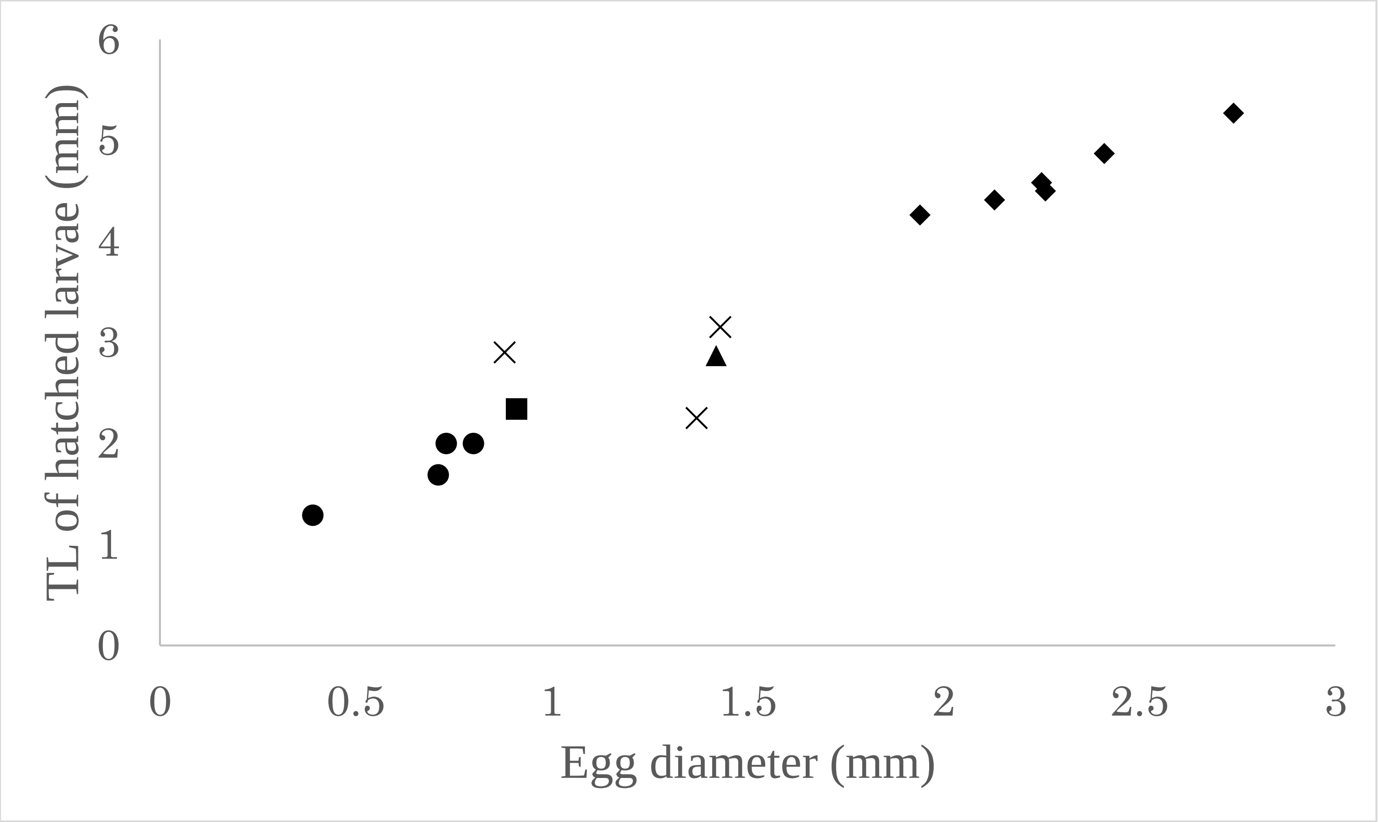

Fig. gd. 1 Relatiionship between egg diameter and TL of hatched larvae in each genus spawning spherical eggs (crosses indicate *Carinotetraodon*; circles indicate *Dichotomyctere*; triangle indicates *Leiodon*; diamonds indicate *Pao*; square indicates *Tetraodon*) other hand, *Leiodon*, *Pao*, *Tetraodon*, and other *Dichotomyctere* species are hatched with complete optic vesicles (Chapter 1, 3, 4; Zaucker et al. 2014; Doi et al. 2015; 2022a, b). The hatching days of each genus were three days in *Auriglobus* and *Chonerhinos* (Chapter 2; Ahmad-Nasir et al. 2020), two to five days in *Carinotetraodon* (Doi et al. 2015), four to five days in *Dichotomyctere* (Chapter 3; Watson et al. 2009; Yamada 2013; Doi et al. 2015), six days in *Leiodon* (Chapter 1), seven days in *Tetraodon* (Chapter 4), and seven to ten days in *Pao* (Doi et al. 2015; 2022a, b). Basically, the hatching days tend to be shorter in the genera (or species) with undeveloped optic vesicles than in those with developed optic vesicles. Besides, the hatching days in the egg protection genera tend to be longer than the genera without egg protection. It may show that the larger protected eggs produce more developed larvae, while taking longer hatching days. The larger developed larvae from larger protected eggs will make their survival rate higher in the river environment (Hutchinson 1957; Nishida 2001). The genus *Tetraodon* took relatively long days to hatch despite its no egg protection, probably caused by its rearing temperature, as shown in Chapter 4.

Keys to eggs and hatched larvae of Asian and African freshwater and brackish water pufferfish genera

Morphological keys to the eggs and hatched larvae of all genera of Asian and African freshwater and brackish water pufferfishes based on the information from the present and previous studies are arranged. The South American genus *Colomesus* is omitted because of deficiency of information.

1a. Eggs shape is oval … 2

1b. Eggs shape is spherical … 3

2a. Egg diameter is small (1.03 - 1.53 mm), TL of hatched larvae is small (3.42 mm)

…*Chonerhinos*

2b. Egg diameter is relatively large (1.68 mm in short axis), TL of hatched larvae is large (4.85 mm)

…*Auriglobus*

3a. Egg diameter is less than 0.8 mm … *Dichotomyctere*

3b. Egg diameter is over than 0.9 mm … 4

4a. TL of hatched larvae is less than 3.2 mm … 5

4b. TL of hatched larvae is over than 4.2 mm … *Pao*

5a. Egg diameter is large (1.42 mm), larvae are hatched with complete optic vesicle

… *Leiodon*

5b. Egg diameter is relatively small (0.88 - 1.43mm), larvae are hatched with incomplete optic vesicle.

… *Carinotetraodon*

5c. Egg diameter is small (0.91 mm), larvae are hatched with complete optic vesicle, Africa

… *Tetraodon*

As showing above, the shape of eggs, egg diameter, developmental status of hatched larvae (e. g. optic vesicle, mouth opening), and TL of hatched larvae are thought to be important characters to identify genera in Asian and African freshwater and brackish water pufferfishes. Besides, the pattern and kind of chromatophore might be identical in character for species as the case of *Dichotomyctere* (Chapter 3). However, the information collected in the present and previous studies has been only scarce, therefore the accumulation of comprehensive and detailed knowledge being necessary in the future.

Invasion to freshwater and brackish water of pufferfishes

Phylogenetic studies of Tetraodontidae (Yamanoue et al. 2011; Igarashi et al. 2013; Santini et al. 2013a) (Fig. gi. 1) uncovered the polyphyletic origin of freshwater and brackish water pufferfishes invading separately into the inland waters of Southeast Asia, Central Africa, and South America.

Southeast Asian freshwater pufferfishes include four genera (*Auriglobus*, *Carinotetraodon*, *Leiodon,* and *Pao*) (Matsuura 2017), being closely related phylogenetically with each other (Yamanoue et al. 2011; Igarashi et al. 2013). Among them, the latter three genera form a single clade, all spawning relatively larger eggs than the other genera as shown above. Especially the genera *Leiodon* and *Pao* perform a special reproductive behavior, the male protecting the egg batch. However, in their mating system, details of reproductive behaviors are possibly slightly different between the two genera as shown in Chapter 1. Moreover, *Pao* is genetically not a sister of *Leiodon* but of *Carinotetraodon* (Yamanoue et al. 2011; Igarashi et al. 2013). Therefore, male egg protection habit would have different origins between *Leiodon* and *Pao* as discussed above. On the other hand, *Auriglobus* has unique features among Southeast Asian freshwater genera in spawning oval eggs (Chapter 2) and having relatively compressed body (Tyler 1980; Roberts 1982). Santini et al. (2013a) showed the monophyly of *Auriglobus* and *Chonerhinos*, treating *Chonerhinos* as a freshwater genus. However, *Chonerhinos* is an euryhaline genus, distributed in coastal water (Ahmad-Nasir et al. 2016). Their close relationship is also supported by morphological similarities (Tyler 1980; Roberts 1982) and in their spawning of oval eggs (Chapter 2). *Auriglobus* would be invaded to the freshwater environment at different occasions from the other Asian freshwater puffers. It means there might have been at least two separate times for the pufferfish invasion to freshwater habitats in Southeast Asia.

The phylogenetic position of *Dichotomyctere* has not yet been determined among researchers. While Yamanoue et al. (2011) and Igarashi et al. (2013) positioned this genus in a different clade from the other Southeast Asian freshwater pufferfishes, Santini et al. (2013a) clustered it in the same clade. Regarding its reproduction, *Dichotomyctere* resembles marine pufferfishes rather than the other freshwater and brackish water individuals as shown in Chapter 3. Anyway, it might be possible that *Dichotomyctere* had invaded Southeast Asian brackish water separately from the other genera as argued by Igarashi et al. (2013).

The Central African *Tetraodon* and South American *Colomesus* are thought to invade each freshwater environment separately from Southeast Asian genera (Yamanoue et al. 2011; Igarashi et al. 2013; Santini et al. 2013a). While the information of reproductive behavior on *Colomesus* has been scarce, *Tetraodon* is known to have such an unique behavior as tandem spawning (Chapter 4). In recent years, a similar biting and clinging behavior by some males to a single female was observed in the marine puffer genus *Arothron* (Doi et al. 2022c), genetically and closely related to *Tetraodon* (Yamanoue et al. 2011; Igarashi et al. 2013; Santini et al. 2013a) (Table gi. 1). Therefore, the biting and clinging behavior might have originated earlier than its invasion into the freshwater environment.

From the considerations above, my opinion about the invasion of pufferfishes to the freshwater and brackish water are as follows:

①In the Southeast Asian freshwater group, there are at least two separate times for the invasion to the freshwater habitat, for *Auriglobus* and *Chonerhinos*, and for the other freshwater genera, *Carinotetraodon*, *Leiodon* and *Pao*.

②The genus *Dichotomyctere* has invaded the Southeast Asian brackish water separately.

③Freshwater and brackish water pufferfishes have invaded the inland waters of each continent separately, adapting their reproductive behaviors uniquely to each environment clade by clade.

Future study and possibility of study in Aquarium

Although some information on the reproductive biology and ecology of all eight freshwater and brackish water pufferfish genera have been accumulated through the present study and previous studies (Araujo-Lima et al. 1994; Watson et al. 2009; Yamada 2013; Zaucker et al. 2014; Doi et al. 2015, 2022a, b), the knowledge is still nothing more than limited. For example, only the distribution pattern of larvae in the Amazon River has been reported about the genus *Colomesus* (Araujo-Lima et al. 1994). Furthermore, knowledge of many species of the speciose genera, such as *Pao* and *Tetraodon* is lacking yet. In addition to the future field observation, aquarium-based observation and description like the present study must be helpful and necessary to clarify more of the adaptation of freshwater and brackish water pufferfishes.

Reproductive behaviors in fishes are rich in diversity, being closely related with environmental and evolutional background of each species (Sunobe 2018; Sokabe 2018). Pufferfishes, which were distributed and adapted in various environments all over the world, might become an optimal model group to study the relationship among the environmental factors, ecology, and reproductive behaviors in fishes.

In aquariums, the improvement and establishment of breeding techniques of animals are very important to sustain their exhibition without invasively influencing their nature. The Japanese Association of Zoos and Aquariums (JAZA) has been concerned about the current situations that the numbers of many wild animals have been reduced and some of them are endangered, and thus prepared the “Reproductive rewards (as Certification of reproduction for first time)”, and encouraged the improvement and establishment of breeding techniques (Uchida et al. 2014) in order to protect wild animals from destruction of nature, species extinction, and sustain species diversity (Kobayashi et al. 2019).

Aquarium would be an excellent place for the observation and investigation of the reproductive behaviors of fishes. Through such studies, aquariums would be able to contribute not only to the species diversity protection but also to public education by deepening the meaning of animal exhibition.

SUMMARY

CHAPTER 1. To clarify the origin of male egg protection of freshwater pufferfish, I induced artificial maturity in ocellated pufferfish *Leiodon cutcutia* from India through a hormone injection and observed its spawning behavior, morphological development, and growth. The male first marked his territory on a flat surface, and then invited the female by vibrating his body. The female entered the territory and laid eggs on the spawning bed, whereafter the male followed. After the departure of the female, the male protected the eggs till hatching. The eggs were spherical, translucent, demersal, and adhesive, with some small yellow oil globules, with a diameter of 1.42 ± 0.07 mm (n = 6). The hatched larvae, measuring 2.39–2.96 mm NL (n = 3), emerged with their mouths open. They reached juvenile stage 13 days after hatching (4.77 mm SL). From its reproductive behavior, its mating system was thought MTV polygamy. Therefore, its origin was thought to differ from the egg protection of *Pao,* which mating system was pair spawning.

CHAPTER 2. To clarify phylogenetic relationships between *Chonerhinos* and *Auriglobus*, DNA barcoding, hormone injection-induced maturity, and insemination of *Auriglobus modestus* from Thailand were undertaken; its reproductive manner and morphological development of eggs and early stage larvae observed. DNA barcoding comparisons of several pufferfish genera indicated a close genetic relationship between *Auriglobus* and *Chonerhinos*, being nested in the same clade with 99 % homology of COI sequence. Although no fertilized eggs resulted from artificial insemination utilizing a wet (freshwater) method, an isotonic (sodium lactate ringer solution) method produced 458 fertilized eggs out of 787 eggs (fertilization rate 61.7%) spawned

by one female, although only eight larvae (1.7%) eventually hatched. The eggs were oval [long axis 2.05 ± 0.06 mm, short axis 1.68 ± 0.06 mm (*n* = 5)], translucent, demersal, and adhesive, and contained several small yellow oil globules. Oval eggs are unusual among pufferfishes, although common in the genus *Chonerhinos*, an indication of the genetic closeness of the latter and *Auriglobus*, but also suggesting ecological similarity. The eggs hatched after two days, larvae one day after hatching [4.54 ± 0.34 mm NL (*n* = 9)] having a large yolk sac. However, the mouth remained unopened, the optic vesicle uncolored, and the pectoral fin membrane undeveloped, except for small knob-like rudiments. Seven days after hatching, larvae [5.22 ± 0.10 mm NL (*n* = 3)] had lost the yolk sac and acquired rudimentary soft rays in the pectoral, dorsal and anal fins. All larvae died after eight days. In this chapter, closely phylogenetic relationship between two genera was suggested from both genetic and reproductive biological perspective.

CHAPTER 3. To clarify the phylogenetic position of *Dichotomyctere* among fresh- and brackish water pufferfishes, artificial reproduction was induced in green pufferfish (*Dichotomyctere fluviatilis*) specimens from India, following hormone injection in full-strength sea water, and subsequent spawning behavior, morphological development, and growth observed. Parental fish scattered more than 70,000 demersal adhesive eggs (0.73±0.05mm in diameter) on the tank floor, without any subsequent egg protection. Hatching occurred four to five days after spawning. The larvae (1.87±0.19 mm in NL) were reared comparatively easily, being initially fed *Branchionus plicatilis* sp. complex, and grew to 88.75 mm mean standard length after 360 days. The reproductive manner of *Dichotomyctere* such as scattering large numbers of small eggs, resembled one of the marine puffers rather than fresh- and brackish water puffers. Therefore, this genus was thought to be a more ancestral genus than those of the other fresh- and brackish genera.

CHAPTER 4. To clarify the biological significance of tandem spawning of the African freshwater pufferfish *Tetraodon schoutedeni*, the reproduction of captive individuals was monitored. Eight spawning sessions (stable water temperature 24 – 25 °C; daily light period 7:00 AM – 7:00 PM) occurred between May 2016 and November 2017. After 65 – 150 min of tandem swimming (the male biting and clinging to the abdomen of the female), 3 – 50 spherical, weak, and adhesive eggs were spawned, being scattered onto the sandy substrate or water plants. The removal of cohabitants (potentially eating spawned eggs) and provision of small initial food items, such as small-type *Brachionus* spp., for larval fish are essential for successful breeding. A broad comparison with other online information of tandem spawning of *T. schoutedeni* as well as of *T. lineatus* led to the hypothesis of tandem spawning function as: semi-monopolization of females, stimulation and sensing of ovulation, and maintenance of a spawning posture. Besides, because tandem spawning was shared with *T. lineatus*, this behavior was thought to be shared in *Tetraodon* broadly.

GENERAL DISCUSSION. From the view of reproductive biology and early life history, the adaptation and the invasion into the inland water habitat of Tetraodontidae were discussed. Sizes of eggs and hatched larvae of fresh- and brackish water puffers were thought to be controlled by reproductive strategy and behavior. On the other hand, there are no differences in the sizes of eggs between marine puffers and this group. The general tendency of fish that produces eggs of the landlocked type is they become larger than those of the original amphidromous type and this was not observed in Tetraodontidae. It may be due to their invasion into the continental large rivers without strong streams. About the invasion into inland water of Tetraodontidae, Asian four freshwater genera were thought to invade at one time in previous studies. However, as shown in Chapter 2, *Auriglobus* was thought to have a different origin from other Asian freshwater genera because of their closely phylogenetic relationship with *Chonerhinos* shown in Chapter 2. Besides, the other three Asian genera, although *Pao* and *Leiodon* perform male egg protection, their behavior would have different origins between *Leiodon* and *Pao* as shown in Chapter 1. Therefore, this behavior was acquired two times in Asian freshwater pufferfishes. Opinions of the *Dichotomyctere* invasion were divided in previous studies. However, this genus has some ancestral reproductive features as shown in Chapter 3 and be thought to have different origins from other Asian fresh- and brackish water puffers. *Tetraodon*, the only African genus, has unique reproductive behavior such as tandem spawning as shown Chapter 4. Similar biting behavior was also observed in *Arothron*, a marine genus closely related to *Tetraodon*. Therefore, this unique behavior might have been acquired before its invasion. The hypothesis of the polyphyletic origin for each continent was strongly supported from the perspective of reproductive biology. In the present study, some novel views for the invasion into the inland water of this family were shown from the perspective of reproductive biology. However, previous information was limited in captivity. In future, more information such as field observation might be help to clarify further the evolutional background of fresh- and brackish water puffers.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Prof. Hiroyuki Munehara for his helpful advice and guidance.

I am grateful to Prof. Harumi Sakai (National Fisheries University), Prof. Hisashi Imamura (Hokkaido University Fisheries sciences) and Prof. Norishige Yotsukura (Hokkaido University Field science center for Northern Biosphere) for their fruitful advice and discussions.

I would like to thank Dr. Hiroyuki Doi for his cooperation in planning and accomplishment of each project. I would also like to thank Mr. Hiroshi Obata (Director of Osaka Aquarium NIFREL), Mr. Hiroyuki Murakami (Director of Osaka Aquarium Kaiyukan), Dr. Kiyonori Nishida, Mr. Hiroyuki Murai, and Mr. Nen Miwa for their kind support.

I thank Drs. Hiroshi Kawase (Natural History Museum and Institute, Chiba), Hiroko Aoki (Chiba University) and Satoshi Awata (Osaka Metropolitan University) for assisting with references. I also thank Dr. Yasuyuki Hashiguchi (Osaka Medical and Pharmaceutical University), Dr. Shigefumi Kanao (Lake Biwa Museum), and Mr. Koki Ikeya (Director of World Freshwater Aquarium Aquatotto Gifu) for their technical advice and helpful cooperation.

I would like to thank Dr. Graham Hardy and Editage [http://www.editage.com] for editing and reviewing this manuscript for English language.

Sincere thanks go to Messrs. Hideto Nakagawa, Shoki Murakami, Satoru Miyagawa, Shohei Nagao, Shunsuke Sawa, Mrs. Akari Nagao and all staff at Osaka Aquarium NIFREL.

Finally, I deeply appreciate Mrs. Kumiko Momota for her kind support and encouragement.

REFERENCES

1. Abe, T. and N. Sato (2009) Reproductive biology of lumpsucker fish *Lethotremus awae* in Shizugawa Bay, northern Honshu, Japan. *Jpn. J. Ichthyol.*, **56**, 159-163 (In Japanese).

2. Adrian, D. (2016) Fahaka’s mating behavior. https://www.youtube.com/watch?v=6Ob1lZ9zab4 (accessed 11 November 2022).

3. Ahmed Chowdhury, I., Md. R. Hasan, S. Parveen, S. K. Dey and Md. B. Billah (2019) Proximate composition of two puffer fish species, *Leiodon cutctia* and *Dichotomyctere fluviatilis* of Bangladesh. *Jahangirnagar Univ. J. Biol. Sci.*, **8**, 25-33.

4. Ahmad Nasir, A. S., S. Mohamad and M. Mohindin (2016) The first reported artificial propagation of yellow puffer, *Xenopterus naritus* (Richardson, 1848) from Sarawak, Northwestern Borneo. *Aquacul. Res.*, **2016**, 1-8.

5. Ahmad Nasir, A. S., S. Mohamad and M. Mohidin (2020) Early life history of yellow puffer, *Chonerhinos naritus* (Richardson, 1848) from Sarawak, Northwestern Borneo. *Aquacul. Res.*, **2020**, 1-10.

6. Akamine, T. (1988) Estimation of parameters for Richards model. *Bull. Jap. Sea Reg. Fish. Res. Lab.*, **38**, 187-200.

7. Akamine, T. (2004) Statistical test and model selection of fish growth formula. *Bull. Jpn. Soc. Fish. Oceanogr.*, **68**, 44-51 (In Japanese).

8. Aparicio, S., J. Chapman, E. Stupka, N. Putnam, J.-M. Chia, P. Dehal, A. Christoffels, S. Rash, S. Hoon, A. Smit, M. D. S. Gelpke, J. Roach, T. Oh, I. Y. Ho, M. Wong, C. Detter, F. Verhoff, P. Predki, A. Tay, S. Lucas, P. Richardson, S. F. Smith, M. S. Clark, Y. J. K. Edwards, N. Doggett, A. Zharkikh, S. V. Tavtigian, D. Pruss, M. Barnstead, C. Evans, H. Baden, J. Powell, G. Glusman, L. Rowen, L. Hood, Y. H. Tan, G. Elgar, T. Hawkins, B. Venkatesh, D. Rokhsar and S. Brenner (2002) Whole-Genome Shotgun Assembly and Analysis of Genome of *Fugu rubripes*. *Science*, **297**, 1301-1310.

9. AQUAPICASSO (2022) Mating *Tetraodon schoutedeni* / Paarung & Ablaichen. https://www.youtube.com/watch?v=HLx_UKtwRBg (accessed 11 November 2022).

10. Arai, H. and S. Fujita (1988) Spawning Behavior and Early Life History of the Sharpnose Puffer, *Canthigaster rivulata*, in the Aquarium. *Jpn. J. Ichthyol.*, **35**, 194-202. (In Japanese).

11. Arakawa, O. (2013) Distribution, accumulation mechanism, and physiological functions of pufferfish toxins. *Nippon Suisan Gakkaishi*, **79**, 311-314. (In Japanese).

12. Araujo-Lima, C. A. R. M., D. Savastano and L. C. Jordao (1994) Drift of *Colomesus asellus* (Teleostei: Tetraodontidae) larvae in the amazon river. *Rev. Hydrobiol. trop*., **27**, 33-38.

13. Barman, A. A., S. K. Mustafi, M. Hasan, N. C. Roy, M. M. Hossain, P. P. Barman, and M. G. Rasul (2018) Puffer fish poisoning: an assessment of consumer awareness and availability in Sylhet region, Bangladesh. *Int. J. Fish. Aquat. Stud.*, **6**, 110-116.

14. Bleeker, P. (1850) Bijdrage tot de kennis der ichthyologische fauna van Borneo, met beschrijving van 16 nieuwe soorten van zoetwatervisschen. *Natuurkundig Tijdschrift voor Nederlandsch Indië*, **1**, 1-16.

15. Corbet, P. S. (1999) *Dragonflies: behavior and ecology of Odonata.*, Cornell University Press, New York. 829 pp.

16. Crazypufferfish (2022) Fahaka puffer breeding. https://www.youtube.com/watch?v=GqQl4Cl2O44 (accessed 11 November 2022).

17. Crnogorac-Jurcevic, T., J. R. Brown, H. Lehrach and Schalkwyk, L. C. (1997) *Tetraodon fluviatilis*, a new puffer fish model for genome studies. *Genomics*, **41**, 177-184.

18. Doi, H., T. Sonoyama, C. Imai, H. Sakai and T. Ishibashi (2014) Early Development of Five Tiger Puffer Species of the Genus *Takifugu*. *J. Nat. Fish. Univ.*, **62**, 109-114. (In Japanese).

19. Doi, H., H. Sakai, Y. Yamanoue, T. Sonoyama and T. Ishibashi (2015) Spawning of eight southeast Asian brackish and freshwater puffers of genera *Tetraodon* and *Carinotetraodon* in captivity. *Fish. Sci.*, **81**, 291-299.

20. Doi, H, T. Akita and H. Sakai (2022a) Reproduction and development in captivity of the Southeast Asian freshwater pufferfish *Pao palembangensis*. *Aquacul. Sci.*, **70**, 197-199.

21. Doi, H, K. Momota, H. Obata and H. Sakai (2022b) Reproduction in captivity of three Southeast Asian freshwater pufferfish species (genus *Pao*). *Aquacul. Sci.*, **70**, 221-229.

22. Doi, H., K. Maei, T. Yoshitake, K. Momota and H. Sakai (2022c) Spawning-related biting behavior of *Arothron melagris* (Tetraodontiformes, Tetraodontidae). *Journal of National Fisheries University*, **71**, 21–25.

23. Ebert, K. (2001) *The Puffers of Fresh and Brackish Waters*. Aqualog, Rodgau

24. Fang, X. (2020) Fahaka puffer breed (*Tetraodon lineatus*). https://www.youtube.com/shorts/t0iTGWQt7mE (accessed 11 November 2022).

25. Feigs, G. (1955) Fresh-water puffers spawn. *The Aquarium*, **24**, 373-374.

26. Fujita, S. (1962) Studies on life history and aquaculture of Japanese puffer fishes. *Rep. Nagasaki Pref. Inst. Fish.*, **2**, 1-121.

27. Fujita, S and M Shinohara (1986) Development of Eggs, Larvae and Juveniles of the Puffer, *Takifugu chrysops*, Rears in the Laboratory. *Jpn. J. Ichthyol.*, **33**, 186-194. (In Japanese).

28. GeoPhilgus Fishkeeping (2018) *Schoutedeni* puffer care 2018 (spotted Congo puffer) | species special. https://www.youtube.com/watch?v=j2MkU0sZQhg (accessed 11 November 2022).

29. Gladstone, W. (1987) The courtship and spawning behaviors of *Canthigaster valentini* (Tetraodontidae). *Environmental Biology of Fishes*, **20**, 255-261.

30. Hardisty, M. W. and Potter, I. C. (Eds.) (1971) *The biology of lampreys, Vol I*. Academic Press, New York. 424pp.

31. Hirai, A. (2003) Topic about eggs of fish. Seizando, Tokyo.182pp. (In Japanese).

32. Hutchinson, G. E. (1957) A treatise on Limnology. Volume II Introduction to lake biology and the limnoplankton. Chapman and Hall, London, 1115 pp.

33. Igarashi, Y., H. Doi, Y. Yamanoue, S. Kinoshita, T. Ishibashi, H. Ushio, S. Asakawa, M. Nishida and S. Watanabe (2013) Molecular phylogenetic relationship of *Tetraodon* pufferfish based on mitochondrial DNA analysis. *Fish. Sci.* **79**, 243-250.

34. Iida, M. (2019) The Survival in early life history. In "The Encyclopedia of Ichthyology" (ed. By Nihongyoruigakkai), Maruzen press. Tokyo. pp. 210-211. (In Japanese).

35. Ivanova, N.V., T. S. Zemlak, R. H. Hanner and P. D. N. Hebert (2007) Universal primer cocktaiols for fish DNA barcoding. *Mol Ecol Notes*, **7**, 544–548.

36. Jaillon, O., J. M. Aury, F. Brunet, J. L. Petit, N. Stange-Thomann, E. Mauceli, L. Bouneau, C. Fischer, C. Ozouf-Costaz, A. Bernot, S. Nicaud, D. Jaffe, S. Fisher, G. Lutfalla, C. Dossat, B. Segurens, C. Dasilva, M. Salanoubat, M. Levy, N. Boudet, S. Castellano, V. Anthouard, C. Jubin, V. Castelli, M. Katinka, B. Vacherie, C. Biémont, Z. Skalli, L. Cattolico, J. Poulain, V. Berardinis, C. Cruaud, S. Duprat, P. Brottier, J. P. Coutanceau, J. Gouzy, G. Parra, G. Lardier, C. Chapple, K. J. McKernan, P. McEwan, S. Bosak, M. Kellis, J. N. Volff, R. Guigó, M. C. Zody, J. Mesirov, K. Lindblad-Toh, B. Birren, C. Nusbaum, D. Kahn, M. Robinson-Rechavi, V. Laudet, V. Schachter, F. Quétier, W. Saurin, C. Scarpelli, P. Wincker, E. S.

Lander, J. Weissenbach and H. R. Crollius (2004). Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature*, **431**, 946-957.

37. Karmakar, P. and S. P. Biswas (2014) Reproductive biology of *Tetraodon cutcutia* (Pisces: Tetraodontidae) from Meleng River in Jorhat District, Assam. *Int. J. Sci. Technol.*, **2**, 23-27.

38. Katoh, K and D. M. Standley (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.*, **30**, 772-780.

39. Kawase, H. (1998) Reproductive behavior and evolution of triggerfish (Balistidae) and filefish (Monacanthidae). *Jpn. J. Ichthyol.*, **45**, 1-19.

40. Kawase, H., Y. Okata, K. Ito and A. Ida (2015) Spawning behavior and parental egg care in a circular structure constructed by pufferfish, *Torquigener albomaculosus* (Pisces: Tetraodontidae). *Bull. Mar. Sci.*, **91**, 33-43.

41. Koga, T. (2007). Mating behaviors of brachyuran crabs: with special reference to reproductive strategy, sexual selection and predator-prey interactions. *Jpn. J. Ecol.*, **57**, 1-12. (In Japanese).

42. Kobayashi, S., T. Hoshino and K. Tokusawa (2019) *New theory of Museum*. Doseisha, Tokyo. 317pp. (In Japanese).

43. Kohda, M. (2010) Chapter 4 Social. In “The basis of fish ecology” (ed. by K. Tsukamoto), KoseishaKoseikaku. Tokyo. pp. 27-41. (In Japanese).

44. Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.*, **35**, 1547–1549.

45. Kuwamura, T. (1987) Evvolution of parental care in fishes with reference to determinants of the care-taker’s sex. *Jpn. J. Ecol.*, **37**, 133-148. (In Japanese).

46. Kuwamura, T. (1996) Introduction of Reproductive strategies in fishes. In “Reproductive strategies in fishes” (ed. by T. Kuwamura, Y. Nakajima). Kaiyusha. Tokyo. pp.1-41. (In Japanese).

47. Kuwamura, T., T. Sunobe, Y. Sakai, T. Kadota and K. Sawada (2020) Hermaphroditism in fishes: an annotated list of species, Phylogeny, and mating system. *Ichthyol. Res.* **67**, 341-360.

48. Kyoto Freshwater Pufferfish Institute (KFPI) (2018a) *Tetraodon schoutedeni* breeding behavior. https://www.youtube.com/watch?v=gRMCKfdafWs (accessed 11 November 2022).

49. KFPI (2018b) *Tetraodon schoutedeni* spawning eggs [spotted Congo puffer]. https://www.youtube.com/watch?v=Lv9MFBrU6NE (accessed 11 November 2022).

50. KFPI (2021) How to identify male and female *Tetraodon schoutedeni*. https://www.youtube.com/watch?v=MKnRUcayGkE (accessed 11 November 2022).

51. KFPI (2022) Q & A for freshwater puffers, No. 37. https://puffersisland.com/tegalog/tegalog.cgi?postid=37 (accessed 11 November 2022).

52. Light, L. E. (1976) Sexual selection in toad (Bufo americanus). *Canadian J. Zool.*, **54**, 1277–1284.

53. Livengood, E. J. and F. A. Chapman (2007) The ornamental fish trade: an introduction with perspectives for responsible aquarium fish ownership. *IFAS Extent. Univ. Florida*, FA124: https://edis.ifas.ufl.edu/publication/FA124., accessed on 28 Aug. 2022.

54. Matsuura K. (2015) Taxonomy and systematics of tetraodontiform fishes: a review focusing primarily on progress in the period from 1980 to 2014. *Ichthyol. Res.*, **62**, 72-113.

55. Matsuura, K. (2017) *Pufferfishes and their allies of Japan*. Tokai university press, Tokyo. 127pp. (In Japanese).

56. Minami, T. (2019) Metamorphosis. In "The Encyclopedia of Ichthyology" (ed. By Nihongyoruigakkai), Maruzen press. Tokyo. pp. 406-409. (In Japanese).

57. Mito, S. (1960) Pelagic fish eggs from Japanese waters-I Clupeina, Chanina, Stomiatina, Myctophida, Anguillida, Belonida and Syngnathida. *Fac. Agr. Kyushu Univ.*, **18**, 285-310.

58. Mito, S. (1962) Plagic fish eggs from japanese water-VI Labrina. *Fac. Agr. Kyushu Univ.*, **19**, 493-502.

59. Miyaki, K., K. Tachihara, R. Ebisu, Y. Tsukashima, Y. Matsumura, S. Fujita, G. Hayashida and O. Tabeta (1992) Induction of ovarian maturation of tiger puffer, *Takifugu rubripes* by gonadotropic hormone injection. *Suisanzoshoku*, **40**, 439-442. (In Japanese).

60. Momota, K., H. Doi, H. Obata and H. Sakai (2022) Spawning and development of ocellated pufferfish *Leiodon cutcutia* in captivity. *Acuacul Sci*, **70**, 9-15.

61. Monaco Fish (2020) Tetraodon schoutedeni spawning. https://www.youtube.com/watch?v=mQn9YefMV5E (accessed 11 November 2022).

62. Moser, H. G. (1981) Morphological and functional aspects of marine fish larvae, In “Marine Fish Larvae: Morphology, ecology, and relation to fisheries” (ed. by R. Lasker), pp. 89-131. Univ. Washington Press, Seattle.

63. Munehara, H., Z. Kanamoto and T. Miura. (2000) Spawning behavior and interspecific breeding in three Japanese greenlings (Hexagrammidae). *Ichtyol. Res.*, **47**, 287-292.

64. Nelson, J. S., T. C. Grande and M. V. Wilson (2016) *Fishes of the World fifth edition.* John Wiley and Sons, New Jersey.707pp.

65. NIFREL (2021) Spawning of *Tetraodon schoutedeni*. https://www.youtube.com/watch?v=Lfa7b2nQhqg (accessed 21 June 2022).

66. Nishida, M. (2001) The Process of large egg formation in *Rhinogobius*. In “Evolutionary Biology of Egg Size in Aquatic Animals” (ed. by A. Goto and K. Iguchi), Kaiyusha. Tokyo, pp.149-170. (In Japanese).

67. Okiyama, M. (Ed.) (2014) *An atlas of early stage fishes in Japan, Second edition*. Japan: Tokai university Press, Tokyo. 1639 pp. (In Japanese).

68. Parsons, G. R., E. R. Hoffmayer, J. Frank and W. Bet-Sayad (2008) A review of shark reproductive ecology: life history and evolutionary implications. In “Fish reproduction” (ed. by M. J. Rocha, A. Arukwe and B. G. Kapoor), CRC Press, Boca Ratan, pp. 435–469.

69. Preston John Aquatics (PJA) (2018a) Breeding and fry - spotted Congo puffer, *Tetraodon schoutedeni* "Shout-uh-denny". https://www.youtube.com/watch?v=72tWLCcNGr0 (accessed 11 November 2022).

70. PJA (2018b) Spawning trio!?! *Tetraodon schoutedeni* puffers. https://www.youtube.com/watch?v=odWANEH-ujc (accessed 11 November 2022).

71. PJA (2018c) Spotted Congo puffer - spawning (eggs) caught on video, *Tetraodon schoutedeni* "Shout-uh-denny". https://www.youtube.com/watch?v=lYFjtxE20sk (accessed 11 November 2022).

72. PJA (2018d) Breeding *Tetraodon schoutedni* - the spotted Congo puffer. https://www.youtube.com/watch?v=jEBfZf4rfFo (accessed 11 November 2022).

73. PJA (2021) Updated!!! Sexing spotted Congo pufferfish (*Tetraodon schoutedeni*). https://www.youtube.com/watch?v=KPI8KstaKhE (accessed 11 November 2022).

74. PJA (2022) Spotted Congo puffer knowledge, FAQ. https://www.spottedcongopuffer.com/faq (accessed 11 November 2022).

75. Pietsch, T. W. (1976) Dimorphism, parasitism and sex: reproductive strategies among deep-sea ceratioid anglerfishes. *Copeia*, **1976,** 781–793.

76. Pietsch, T. W. (2005) Dimorphism, parasitism, and sex revisited: modes of reproduction among deep-sea ceratioid anglerfishes (Teleostei: Lophiiformes). *Ichtyol. Res.*, **52**, 207-236

77. Richards, F. J. (1959) A flexible growth function for empirical use. *J. Exp. Bot.*, **10**, 290-300.

78. Ridley, M. (1983) *The explanation of organic diversity. The comparative method and adaptations for mating.* Clarendon Press, Oxford, 280pp.

79. Roberts, T. R. (1982) The southeast Asian freshwater pufferfish genus *Chonerhinos* (Tetraodontidae), with descriptions of new species. *Proc. Calif. Acad. Sci.*, **43**, 1-160.

80. Santini, F., M. T. Nguyen, L. Sorenson, T. B. Waltzek, J. W. Lynch Alfaro, J. M. Eastman and M. E. Alfaro (2013a) Do habitat shifts drive diversification in teleost fishes? An example from the pufferfishes (Tetraodontidae). *J. Evol. Biol.*, **26**, 1003-1018.

81. Santini, F., L. Sorenson, T. Marcroft, A. Dornburg and M. E. Alfaro (2013b) A multilocus molecular phylogeny of boxfishes (Aracanidae, Ostraciidae; Tetraodontiformes). *Mol. Phylogenet. Evol.*, **66**, 153-160.

82. Scott, W. B. and E. J. Crossman (1973) *Freshwater Fishes of Canada, Bulletin 184*. Fisheries Research Board of Canada, Ottawa, 966pp.

83. Senta, T., T. Minami and I. Kinoshita (2001) Natural history of Larvae. Hokkaido Univ. Press, Hokkaido, 303pp. (In Japanese).

84. Sikkel, P. C. (1990) Social organization and spawning in the Atlantic sharpnose puffer, *Canthigaster rostrata* (Tetraodontidae). *Env. Biol. Fish.*, **27**, 243-254.

85. Smith, C. C. and S. D. Fretwell (1974) The Optimal Balance between Size and Number of Offspring. *Am. Nat.*, **108**, 499-506.

86. Sokabe, A. (2018) *Mating system*. In "The Encyclopedia of Ichthyology" (ed. By Nihongyoruigakkai), Maruzen press. Tokyo. pp. 288-289. (In Japanese).

87. Stroud, G. J., B. Goldman and W. Gladstone (1989) Larval Development, growth and Age Determination in the Sharpnose Pufferfish *Canthigaster valentini* (Teleostei: Tetraodontidae). *Jpn. J. Ichthyol.*, **36**, 327-337.

88. Suguro, N. (2001) Trials on Raising *Pseudobagrus tokiensis* in a nursery – I. *Bull. Kanagawa Pref. Fish. Res. Inst.*, **6**, 97-107 (In Japanese).

89. Sunobe, T. (2018) *Reproductive behavior*. In "The Encyclopedia of Ichthyology" (ed. By Nihongyoruigakkai), Maruzen press. Tokyo. pp. 278-279. (In Japanese).

90. Suzuki, R. and M. Yamaguchi (1975) Hormone effect and water temperature on egg collection of loach *Misgurnus anguillicaudatus*. *Acuacult. Sci.*, **22**, 135-139. (In Japanese).

91. Sykes, M. (2020) *Tetraodon schoutedeni* pufferfish care sheet. https://www.pufferfishenthusiastsworldwide.com/post/tetraodon-schoutedeni (accessed 11 November 2022).

92. Takaku, H., I. Kim and K. Hosoya (2007) Artificial propagation of a small endangered cyprinid, Green chub, *Aphyocypris chinensis* Günther. *Mem. Fac. Agric. Kinki Uni.*, **40**, 63-70 (In Japanese).

93. Takaku, H. and K. Hosoya (2008) Artificial propagation of a small Small Endangered Cyprinid, Golden Venus Chub *Hemigrammocypris rasborella* Fowler. *Acuacul. Sci.*, **56**, 13-18 (In Japanese).

94. TOEKIE WORLD – Marc. (2018) At rivers end - update – mating of *Tetraodon schoutedeni*. https://www.youtube.com/watch?v=dfz52DwnX5M (accessed 11 November 2022).

95. Tyler, J. C. (1980) Osteology, phylogeny, and higher classification of fishes of order Plectognathi (Tetraodontiformes). *NOAA Tech. Rep. NMFS Circ.*, **434**, 1-422.

96. Uchida, S., M. Toda and Y. Kamei (1990) Reproduction of elasmobranchs in captivity. In "Elasmobranchs as living resources: advances in the biology, ecology, systematics and status of the fisheries., NOAA Technical Reports NMFS 90" (ed. by H. L. Pratt, S. H. Gruber and T. Taniuchi), NOAA, Silver Spring, pp. 211–237.

97. Uchida, S., K. Arai and K. Nishida (2014) *Japanese Aquarium* Tokyo Univ. Press, Tokyo. 215pp. (In Japanese).

98. Uno, Y. (1955) Spawning habit and early development of a puffer, *Fugu (Torafugu) niphobles* (Jordan et Snyder). Journal of Tokyo University of Fisheries, 41, 169–183.

99. Venkatesh, B., P. Gilligan and S. Brenner (2000) Fugu: a compact vertebrate reference genome. *FEBS letters*, **476**, 3-7.

100. Ward, R. D., R. Hanner and P. D. N. Hebert (2009) Review paper: The campaign to DNA barcode all fishes, FISH-BOL. *J. Fish Biol.*, **74**, 329-356.

101. Watanabe, M. (2015) *Ecology of Odonata.* University of Tokyo Press, Tokyo. 255pp. (In Japanese).

102. Watson, C. A., J. E. Hill, J. S. Graves, A. L. Wood and K. H. Kilgore (2009) Use of novel induced spawning technique for the first reported captive spawning of *Tetraodon nigroviridis*. *Marine Genomics*, **2**, 143-146.

103. Winn, H. E. and J. E. Bardach (1960) Some aspects of the comparative biology of parrot fishes at Bermuda. *Zoologica*, **45**, 29-34.

104. Yamada, A., A. Hamaguchi, H. Sakoda, M. Kakmu, H. Doi, S. Hasin and O. Arakawa (2021) Complete mitochondrial genomes of the Southeast Asian freshwater pufferfishes, *Pao abei* (Roberts, 1998) and *Pao suvattii* (Sontirat and Soonthornsatit, 1985) (Tetraodontiformes: Tetraodontidae) and an insight into the taxonomic status of *Pao* species. *Mitochondrial DNA Part B*, **6**, 1448-1450.

105. Yamada, M. (2013) Reproduction of *Dichotomyctere nigroviridis* in aquaria. *Fish MAGAZINE*, **553**, 108-110 (In Japanese).

106. Yamamoto, K., Y. Nagahama and F. Yamazaki (1966) A method to induce artificial spawning of goldfish all through the year. *Bul. Jpn. Soc. Sci. Fish.* **32**, 977-983 (In Japanese).

107. Yamanoue, Y. (2015) Diversity, phylogeny and taxonomy of tetraodontiform fishes. *TAXA, Proc. Jpn. Soc. System. Zool.* **39**, 1-16 (In Japanese).

108. Yamanoue, Y., M. Miya, K. Matsuura, M. Katoh, H. Sakai and M. Nishida (2004) Mitochondrial genomes and phylogeny of the ocean sunfishes (Tetraodontiformes: Molidae). *Ichthyol. Res.*, **51**, 269-273.

109. Yamanoue, Y., M. Miya, J. G. Inoue, K. Matsuura and M. Nishida (2006) The mitochondrial genome of spotted green pufferfish *Tetraodon nigroviridis* (Teleostei: Tetraodontiformes) and divergence time estimation among model organisms in fishes. *Genes. Genet. Syst.*, **81**, 29-39.

110. Yamanoue, Y., M. Miya, K. Matsuura, S. Miyazawa, N. Tsukamoto, H. Doi, H. Takahashi, K. Mabuchi, M. Nishida and H. Sakai (2009) Explosive speciation of *Takifugu*: another use of fugu as a model system for evolutionary biology. *Mol. Biol. Evol.*, **26**, 623-629.

111. Yamanoue, Y., M. Miya, H. Doi, K. Mabuchi, H. Sakai and M. Nishida (2011) Multiple invasions into freshwater by pufferfishes (Teleostei: Tetraodontidae): a mitogenomic perspective. *PLoS ONE*, **6**, E17410.

112. Zaucker, A., T. Bodur, H. R. Crolius, Y. Hadzhiev, J. Gehrig, F. Loosli, C. Watson and F. Müller (2014) Description of Embryonic Development of Spotted Green Pufferfish (*Tetraodon nigroviridis*). *Zebrafish*, **11**, 509-517.

113. Zhu, H., A. Yamada, Y. Goto, L. Horn, L. Ngy, M. Wada, H. Doi, J. S. Lee, T. Takatani and O. Arakawa (2020) Phylogeny and toxin profile of freshwater pufferfish (Genus *Pao*) collected from 2 different regions in Cambodia. *Toxins*, **12**, 689.

114. Zhu, H., T. Sakai, Y. Nagashima, H. Doi, T. Takatani and O. Arakawa (2021) Tetrodotoxin/Saxitoxins Selectivity of the euryhaline freshwater pufferfish *Dichotomyctere fluviatilis*. *Toxins*, **13**, 731. https://doi.org/10.3390/toxins 13100731., accessed on 28 Aug. 2022.