Reproduction and development of the Amazon puffer *Colomesus asellus* in captivity

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Abstract : To induce breeding of the freshwater pufferfish *Colomesus asellus*, artificial maturation by hormone injection (hCG, 10 IU per gram) and insemination were performed, and subsequent development observed. The rearing environment for the parental fish was freshwater, maintained at 26.0 ± 1.5 ℃ under L12:D12 lighting cycle. Two hormone treatments were applied, one month apart, to two females and four males, ovulation being confirmed 47 hours after the second treatment. Three hundred and sixty-three and 548 eggs with a mean diameter of 1.11 mm (n = 20) were collected from the two females, 334 (92.0 %) and 395 (72.1 %) being fertilized, respectively. The larvae and juveniles were fed with S-type *Brachionus* sp. fortified initially with freshwater chlorella, followed by *Artemia* larvae and Chironomid sp. larvae. The pufferfish were reared in a salinity of 7 ‰ for 24 days after hatching, the water then being gradually changed to freshwater over the following 10 days. Two individuals survived for 225 days after hatching, growing to average standard and total lengths of 29.24 mm and 38.72 mm, respectively.
The Gonperz growth formulae were as follows :
SL : $Lt = 29.4075 \times exp\ (-exp\ (-0.02301(t-39.1213)))$
TL : $Lt = 38.51177 \times exp\ (-exp\ (-0.023253(t-41.25841)))$
Further investigations of rearing conditions, such as salinity, are required to improve the breeding techniques for this species.

Key words : *Colomesus asellus*; freshwater pufferfish; hormone injection; artificial reproduction; aquarium fish

Introduction

The Amazon puffer *Colomesus asellus* (Müller & Troschel) is widely distributed in fresh and brackish water areas in northern South America, including the Amazon, Orinoco, and Essequibo River basins[1-4]. The species is often traded as aquarium specimens[4-6], resulting in the population declining in the Amazon basin[7].

The invasion of pufferfishes into freshwater environments is thought to have first occurred in Southeast Asia, followed by Africa, and most recently in South America[8-10]. As a representative species from South America, reproduction of *C. asellus* may provide an insight into the evolution of freshwater invasion by pufferfishes.

Current ecological knowledge of *C. asellus* is limited. Queiroz et al.[3] reported that it spawned in flooded rivers during the rainy season, and Araujo-Lima et al.[11] documented that it scattered numerous small eggs on the substrate, with hatchlings drifting downstream with the current. A few reports exist on condition factors[12, 13], food habits[14], and population differentiation within the Amazon River basin[15], the only report on captive breeding being a note by Shinkawa[16], who recorded the spawning of unfertilized eggs in freshwater.

The development of breeding techniques applicable to commercially traded freshwater pufferfishes is essential for conservation of their natural biodiversity[17-19]. In this study, induced maturation by hormone injection, artificial insemination, and rearing and development of *C. asellus* in captivity were documented. Some inferences on the

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relationship between reproduction and evolution are included. Further study of the larval rearing environment, particularly salinity conditions, should improve the breeding techniques used for this species.

Materials and Methods

Parental fish

Fifteen wild *C. asellus* from Colombia [detailed collection locality and sex unknown; mean standard length (SL) 41.16 ± standard deviation (SD) 3.57 mm, mean total length (TL) 49.50 ± 3.75 mm, and mean body weight (BW) 2.74 ± 0.35 g], purchased from a Japanese fish dealer on February 18, 2021, were reared in a closed filtration freshwater aquarium (120 × 60 × 40 cm, 480 L). The rearing system was maintained at 26.0 ± 1.5 ℃ , pH at 7.20 ± 0.99, with a lighting cycle of L12:D12 (light period 7:00-19:00; water surface brightness 106-147 lux]. The fish were fed with chopped river prawns (mainly *Macrobrachium* sp.), Chironomidae sp. larvae, mealworms (Tenebrionidae sp.), Tetrakrill-E (Spectrum Brands Japan Co., Ltd.), and Hikari Catfish processed fish food (Kyorin Food Industries Co., Ltd.) to satiation level. The tank water was changed once a week, filter sand cleaned once a month, and uneaten food and feces removed daily.

Hormone injection and artificial insemination

After the parental fish had been reared for approximately 2 years, human chorionic gonadotropin (hCG, Asuka Pharmaceutical Co., Ltd.) was injected (10 IU per gram) on the left side of the caudal peduncle on February 21 and March 20, 2023, the fish being 53.52 ± 5.66 mm SL, 64.65 ± 6.28 mm TL, and 8.84 ± 2.61 g BW. Abdominal swelling in the females was slightly greater

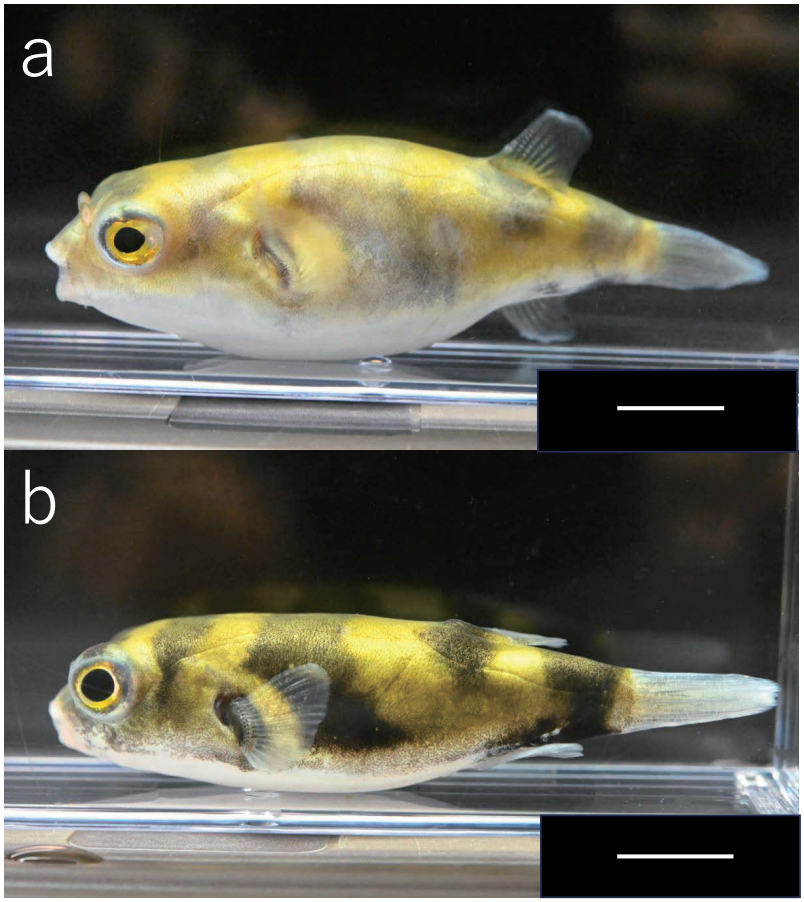

Fig. 1. Parental Amazon puffer, *Colomesus asellus*; a, 67.74 mm TL female; b, 62.50 mm TL male. Scale bars indicate 10 mm.

than in the males, there being no other obvious morphological differences between the sexes. Sex of the remaining five individuals was unknown.

Artificial insemination was conducted on March 22 utilizing the dry method as described below, between two females (A, B) and four males (A to D), from which sufficient quantities of eggs and semen had been obtained. Female A was mated with male A (Figs. 1a, 1b, respectively) and female B with males B-D. After wiping water from around the anus, the abdomen was gently squeezed to collect gametes. Semen was added to the eggs in a petri dish, followed by gentle mixing in freshwater (5 ml).

Developmental observation

Eggs, larvae and juveniles were collected from the rearing tanks with a pipette or a small hand net, observed without anesthesia under a binocular stereomicroscope [OLYMPUS SZ61 (equipped with measuring software Anyty Microscope with 3R-WDKMCO2, 3R System, Olympus Co., Tokyo)], measured to the nearest 0.01 mm, and photographed with a digital camera. Standard length (SL, notochord length NL in early larval stage) and TL were measured.

Designation of developmental intervals followed Okiyama[20]: larval period, the stage before complete development of countable characters; preflexion stage, the larval stage before notochord 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).

Rearing of eggs and offspring

Fertilized eggs and hatched larvae were reared in four aerated plastic tanks (5 L) set in a water bath (26 ℃). Two of the tanks contained freshwater and the other two saline water (7 ‰), considering this species spawns in the rainy flooding season[3] and the hatched larvae swiftly drift downstream[11]. One-half of the water was changed for fertilized eggs and one-third for larvae daily. Dead eggs were removed daily.

From 24 to 33 days after hatching, salinity of the rearing water was gradually reduced to freshwater level. From 60 days after hatching, the juveniles were maintained in a glass tank (30 × 21 × 20 cm, 12.6 L), about one-tenth of the rearing water being changed daily.

Four days after hatching, the larvae were initially fed with S-type rotifers *Brachionus* sp. fortified with freshwater chlorella at a density of 20 ind./ml, supplemented with artificial plankton (Fujiks Co., Ltd.) from 7 days. From 24 days, larvae were fed S-type rotifers, artificial plankton, and *Artemia* nauplii; from 30 days, *Artemia* nauplii only; from 87 days, *Artemia* nauplii and Chironomid sp. larvae; from 180 days, Chironomid sp. larvae only; from 190 days, Chironomid sp. larvae and commercially prepared Otohime B2 fish food (Marubeni Nisshin Feed. Co., Ltd.). Uneaten food and excreta were removed daily.

Growth

The Gonperz growth formulae of *C. asellus* were estimated following the standard form of Richards'[21] model by comparing the residual sum of squares[22, 23], calculated using MS Excel (Microsoft Office 365).

Results

Ovulation and spermiation

Thirty hours after the first injection, semen could be stripped from two males, but no female had ovulated. Forty seven hours after the second injection, ovulation occurred in three females (55.28 ± 10.45 mm SL, 69.09 ± 11.72 mm TL, 10.10 ± 3.93 g BW) and semen was observed in seven males (51.46 ± 4.27 mm SL, 63.82 ± 4.09 mm TL, 7.66 ± 1.27 g BW).

Artificial insemination and hatching

Eggs spawned numbered 363 (female A) and 548 (female B), 334 (92.0 %) and 395 (72.1 %) being fertilized, respectively. Three days later, a total of 64 larvae hatched from two batches (10.4 % of fertilized eggs).

Egg development

Fertilized eggs were 1.11 ± 0.04 mm in diameter (*n* = 20), spherical, separate, and adhesive, with small oil globule masses (Fig. 2a). Four days after fertilization, the embryonic body had formed, with melanophores and xanthophores apparent on the yolk (Fig. 2b).

Larval, juvenile and young fish development

Hatched larvae (Fig. 3a, 2.63 mm NL, 2.75 mm TL) were 2.68 ± 0.16 mm NL, 2.82 ± 0.10 mm TL (*n* = 5), with large yolks containing many oil globules. The mouth remained closed, and the optic vesicles were translucent. Melanophores were present anterior to the eyes, on the yolk, and around the anus, and xanthophores on the head, yolk, and around the anus.

Two days after hatching, preflexion larvae (Fig. 3b, 2.85 mm SL, 3.02 mm TL), were 2.83 ± 0.09 mm SL, 2.98 ± 0.08 mm TL (*n* = 10), with the mouth and anus opened, black eyes, and yolk still attached. Melanophores were present behind the eyes and on the yolk, and xanthophores behind the eyes, on the yolk, and around the anus.

Four days after hatching, preflexion larvae (Fig. 3c, 3.12 mm SL, 3.30 mm TL), were 3.14 ± 0.04 mm SL, 3.31 ± 0.05 mm TL (*n* = 8), with the yolk almost absorbed. Melanophores were present on the body, but xanthophores were not observed. Erythrophores were present in a punctate pattern from the body to the anus. No larvae had survived in the two freshwater tanks, whereas 12 larvae were alive (18.8 % survival rate) in the

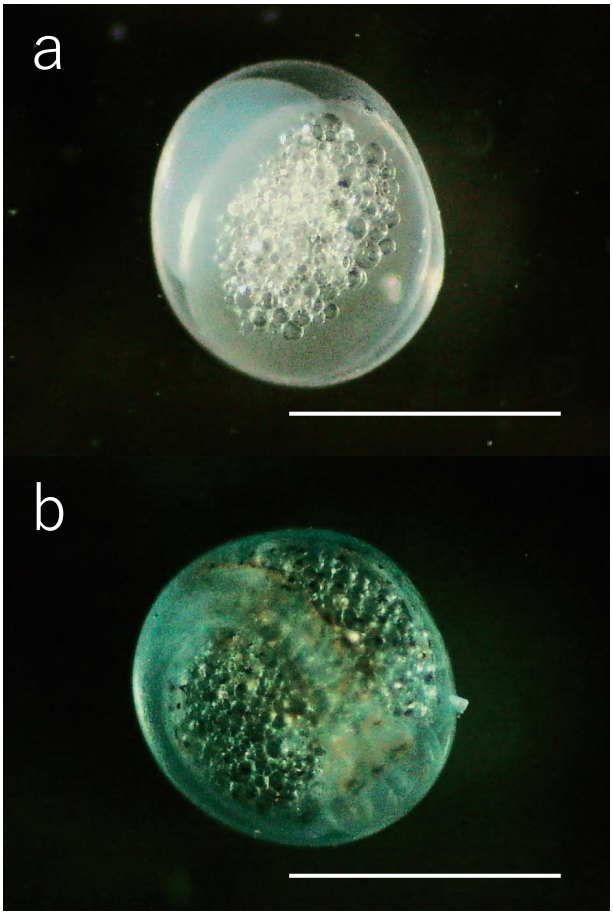

Fig. 2. Development of *Colomesus asellus eggs*: a, 1 hour after fertilization; b, 4 days after fertilization. Scale bars indicate 1 mm.

two tanks containing 7 ‰ saline water.

Five days after hatching, some individuals began to swim up from the bottom. Eleven days after hatching, five individuals had survived. After 16 days, the larvae exhibited bottom-pecking behavior, swimming mainly in the middle and bottom layers, although occasionally moving up to the surface. Twenty-two days after hatching, three individuals had survived.

Twenty-four days after hatching, the entire body of the remaining flexion larvae (Fig. 3d, 5.45 mm SL, 6.74 mm TL), were 6.51 ± 1.09 mm SL, 8.43 ± 1.58 mm TL (n = 3) was yellow, melanophores being present on the head, body, back, and caudal peduncle. From this point, the tank water salinity was progressively lowered from 7 ‰ to fresh water over the following 10 days. Twenty-six days after hatching, two individuals had survived.

Thirty days after hatching, juveniles were mean 7.92 mm SL and 10.36 mm TL (n = 2). After forty-five days,

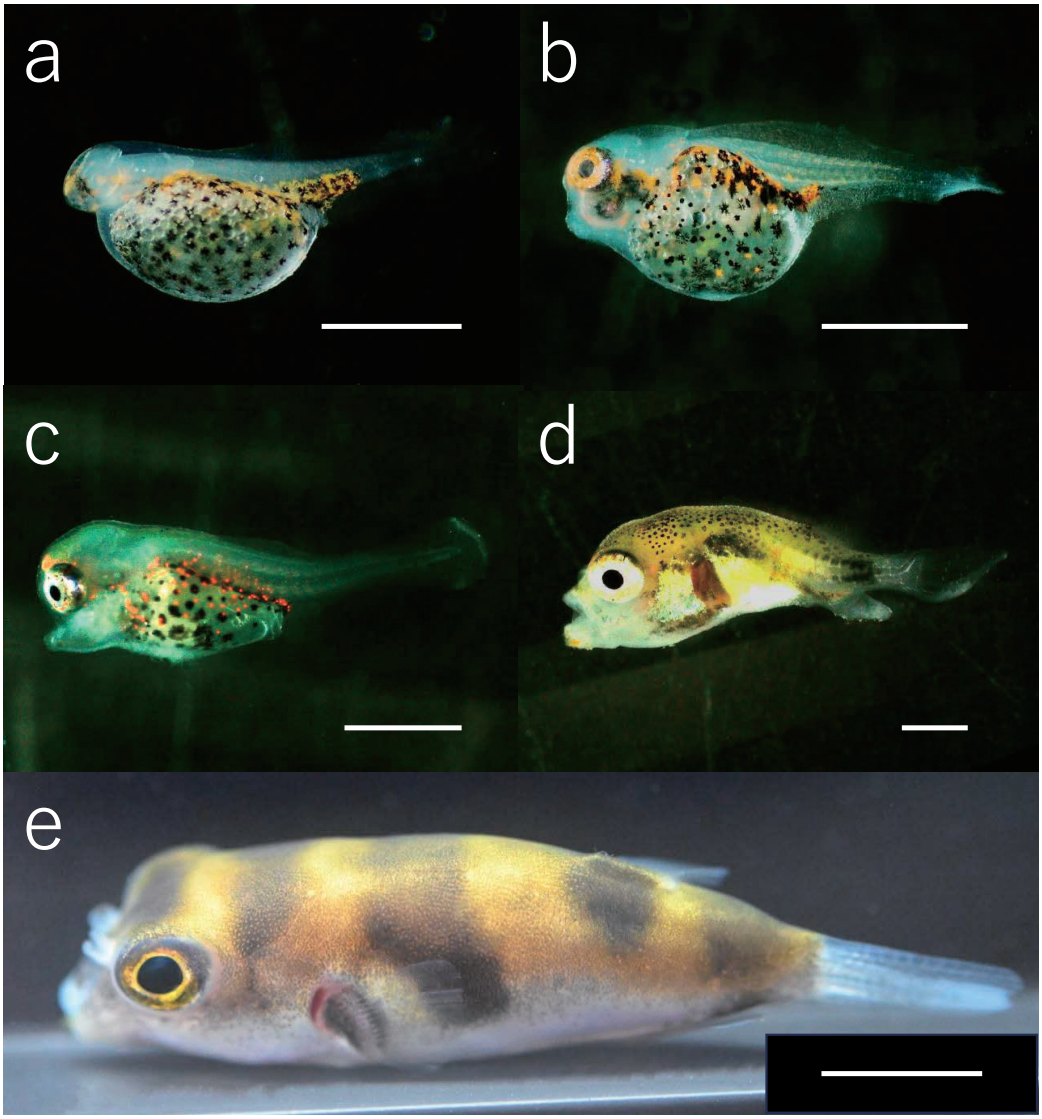

Fig. 3. Development of larval and juvenile *Colomesus asellus*: a, 2.75 mm TL newly hatched larva; b, 3.02 mm TL larva 2 days after hatching; c, 3.30 mm TL larva 5 days after hatching; d, 6.74 mm TL juvenile 12 days after hatching; e, 38.38 mm TL young fish 210 days after hatching. Scale bars indicate 1 mm for a to d, 10 mm for e.

juveniles (mean 13.36 mm SL and 17.84 mm TL; n = 2) swam easily throughout the tank, sometimes jumping above the water surface.

Juvenile sizes after sixty days after hatching were mean SL 16.98 mm and TL 21.45 mm (n = 2); after 90 days, mean SL 21.69 mm and TL 27.58 mm (n = 2); after 120 days, mean SL 25.26 mm and TL 32.20 mm (n = 2); after 150 days, mean SL 26.06 mm and TL 33.45 mm (n = 2); after 180 days, mean SL 28.35 mm and TL 37.55 mm (n = 2); after 210 days, mean SL 29.00 mm and TL 37.96 mm (n = 2) (Fig. 3e); and after 225 days, mean SL 29.24 mm and TL 38.72 mm (n = 2).

Growth

Approximated Gonperz growth formulae for SL (or NL) and TL (n = 54, including both sexes) are summarized below, the coefficients of determination being 0.99289067 (SL) and 0.994933415 (TL), where Lt is estimated length, and t is days after hatching (Fig. 4):

SL : $Lt = 29.4075 \times exp\ (-exp\ (-0.02301(t-39.1213)))$

TL : $Lt = 38.51177 \times exp\ (-exp\ (-0.023258(t-41.25841)))$

Discussion

Colomesus asellus was successfully bred in freshwater, following induced maturation by hormone injection and artificial insemination. Bartolette et al.[14] reported that adult Amazon puffers from central Brazil ranged from 26 to 45 mm SL, although Kullader[1] had reported the maximum size of the species as 128 mm SL. In the present study, the hormone-treated individuals were clearly sufficiently mature for breeding, having mean

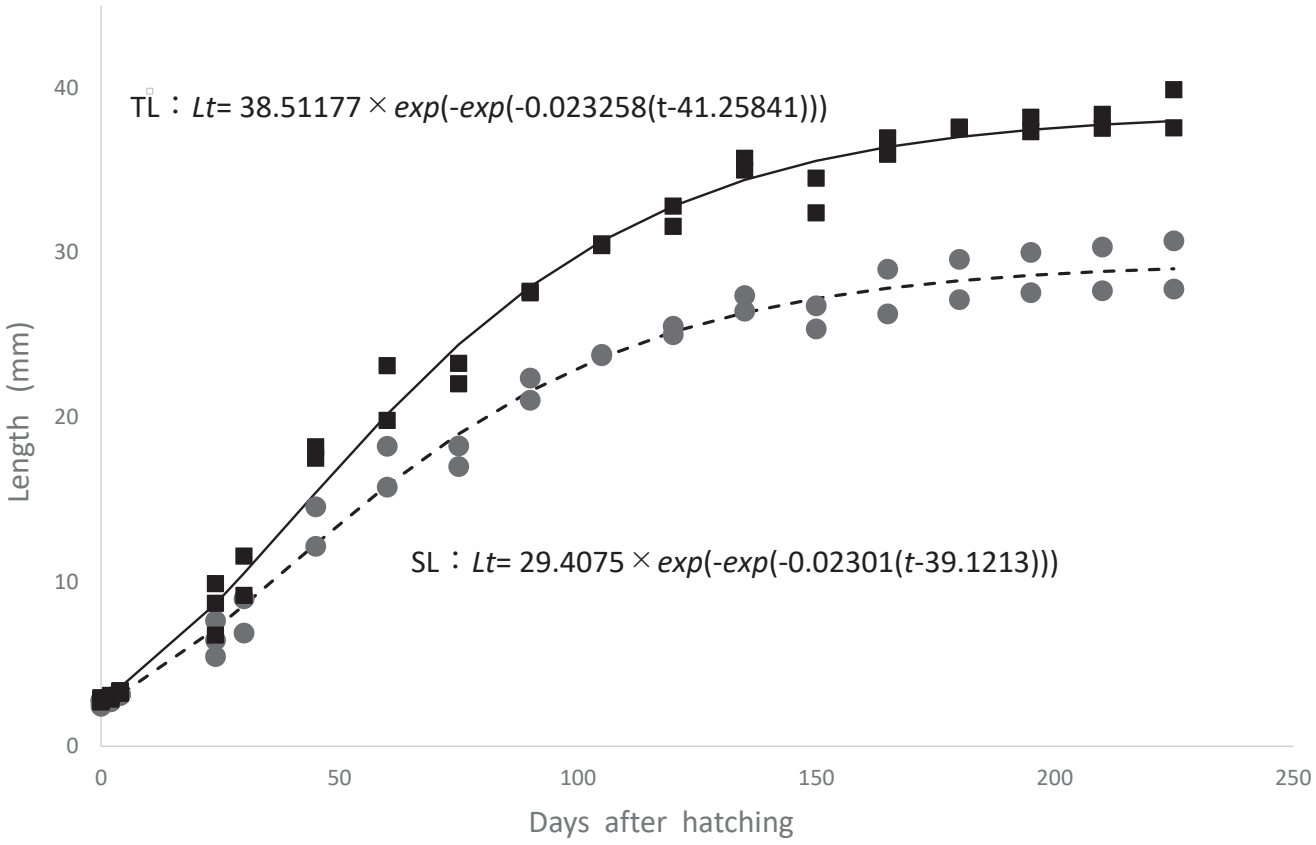

Fig. 4. Growth of *Colomesus asellus*, Gonperz growth formulae being shown on the figure. Gray circles and dotted line indicate SL; black squares and solid line, TL.

sizes of 51.46 mm SL (males) and 55.28 mm SL (females).

Some hatched larvae were able to develop successfully in 7 ‰ salinity (survival rate 4.7 %, 24 days after hatching), being fed S-type rotifers *Brachionus* sp. fortified with freshwater chlorella as the initial diet. On the other hand, all hatched larvae in the freshwater tanks died within 4 days.

Colomesus asellus is known to spawn in freshwater during the rainy season, with hatchlings drifting downstream. Its developmental series has been suggested as similar to that of marine pufferfishes[3, 11]. In fact, the egg diameter of *C. asellus* (mean 1.11 mm), which is considerably smaller than other egg-protecting freshwater pufferfish, such as *Leiodon cutcutia* (mean 1.42 mm)[18] and *Pao* spp. (1.92-2.74 mm)[17, 24, 25], is instead comparable to those of marine pufferfish, such as *Takifugu* spp. (0.87-1.31 mm)[26].

Hatched larvae of *C. asellus* had weakly pigmented optic vesicles and large yolks, similar to those of the euryhaline pufferfish *Dichotomyctere fluviatilis* (mean egg diameter 0.73 mm, spawning and developing in 33 ‰ salinity)[27], *D. ocellatus* (mean egg diameter 0.71 mm, spawning and developing in 9 ‰ salinity)[24], *D. nigroviridis* (mean egg diameter 0.39-0.80 mm, spawning and developing in 14-33 ‰ salinity)[28, 29], and anadromous *Takifugu obscurus* (mean egg diameter 1.48 mm, parents migrating upstream to spawn in freshwater, hatched larvae drifting downstream and develop in up to 8 ‰ salinity)[30-32]. Therefore, larvae and juveniles of *C. asellus* may possibly grow in brackish water, like other euryhaline pufferfishes. This is consistent with the species belonging to the most recent group to invade freshwater[8-10], suggesting a life history still dependent upon marine or brackish water.

Nevertheless, *C. asellus* is generally considered as a freshwater species, distributed primarily in upper river reaches, although also inhabiting brackish waters[1-4].

On the other hand, genetic population differentiation has been recognized in the Amazon River, probably according to watercolor difference among tributaries and vicariant biogeographic history[16]. It is possible that the parental fish in the present study may have originated from a relatively downstream population.

To improve the reproduction and growth of *C. asellus* in captivity, confirmation of the origin of the parental fish is necessary, with breeding and rearing salinity adjusted appropriately. Ultimately, improved breeding and reproduction techniques for this species will be important for captive maintenance in aquariums, as well as helping in conservation of the species and supporting biodiversity.

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