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REPRODUCTION AND DEVELOPMENT IN CAPTIVITY OF THE SOUTHEAST ASIAN FRESHWATER PUFFERFISH *PAO BAILEYI* (TETRAODONTIFORMES, TETRAODONTIDAE)

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ABSTRACT

The freshwater pufferfish *Pao baileyi* was experimentally bred in order to improve reproduction techniques in captivity. One pair was reared in a freshwater aquarium with a half tube of PVC pipe set on a flat stone as a spawning bed, at a water temperature (25–27 °C) and lighting (L12:D12). The pair spawned spontaneously two days after hormone injection for both sexes. An egg batch containing 233 eggs laid in a single layer on the floor of the spawning bed was protected by the male. Ten to 11 days after spawning, 171 individuals (5.05 ± 0.08 SD mm, $n = 6$) in body length (BL), 5.36 ± 0.10 mm in total length (TL) hatched (hatching rate 73.4 %). One-day old larvae were initially fed with *Artemia* nauplius larvae. To reduce mutual biting, larvae and juveniles were fed three times a day to satiation level for 44 days after hatching. Water plants and sunken driftwood were additionally set in the aquarium to provide hiding places. Juveniles of similar size and total weight were accommodated in each of six rearing tanks, also to reduce biting. From 181 days after hatching, growing young fish (10 individuals) were isolated in separate tanks. After 540 days, the fish had reached adult size of 93.3 mm in mean BL and 112.8 mm TL ($n = 9$). The Gomperz growth formulae were as follows: BL: $Lt = 91.363 \bullet \exp(-\exp(-0.01283(t-77.5439)))$; TL: $Lt = 111.2789 \bullet \exp(-\exp(-0.01243(t-80.2623)))$. Improvement to reduce mutual biting behavior is crucial in reproducing pufferfish of the genus *Pao* successfully.

Keywords: *Pao baileyi*, freshwater pufferfish, artificial reproduction, aquarium fish, prohibition of biting

INTRODUCTION

There are currently 14 known species of freshwater pufferfishes in the genus *Pao* from Southeast Asia (MATSUURA, 2015), and wild individuals of many of these species are commercially traded as aquarium fish (EBERT, 2001; SUBAMIA *ET AL.*, 2008). Therefore, development of aquaculture techniques applicable to freshwater pufferfishes is essential, not only for trade purposes but also for conservation of natural biodiversity (NG & TAN, 1997; LIVENGOOD & CHAPMAN, 2007; NUGRAHA *ET AL.*, 2011; DOI *ET AL.*, 2022a, b; MOMOTA *ET AL.*, 2022a, b). *Pao baileyi* (Sontirat, 1985), distributed in the Mekong River basin of Laos, Thailand, and Cambodia, is unique in that the head and body are usually sparsely or densely covered with epidermal outgrowths, or cirri (SONTIRAT, 1989, as *Tetraodon baileyi*; KOTTELAT, 2001, as *Monotrete baileyi*). It is also often traded as an aquarium fish (EBERT, 2001; So

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et al., 2018; Taki et al., 2021). This species inhabits rocky rapids of the Mekong mainstream and its larger tributaries, but current ecological knowledge of this species is limited (Roberts, 1998). It has been assessed as Endangered in Thailand due to habitat loss (Vidthayanon, 2005). Therefore, the development of aquaculture techniques for this species would be particularly important. Doi et al. (2022b) reported captive reproduction of three species of *Pao* species, *P. abei*, *P. baileyi* and *P. suvattii*. However, *P. baileyi* was not successfully reared beyond 43 days after hatching, mainly due to death caused by mutual biting behavior. In the present study, we improved the rearing methods of Doi et al. (2022b) in reducing biting among juveniles and were able to raise the larvae to adult size successfully.

MATERIALS AND METHODS

Parental Fishes

Two wild fish of *P. baileyi* (sex unknown at the time, later identified as one female and one male from their spawning behavior) from Thailand, were purchased from a Japanese fish dealer on June 28, 2020, and were reared in a freshwater closed-circulation filtration system tank (water capacity 95 liters) at the Osaka Aquarium NIFREL. At the beginning of rearing, the two individuals were 72.1 and 70.9 mm in body length (BL), 88.6 and 84.2 mm in total length (TL), and 20.2 and 16.7 g in body weight (BW), respectively. One-quarter of the rearing water was changed weekly, filter sand was cleaned monthly, and the rearing system was maintained at 25–27 °C, L12:D12 lighting cycle, with no specific pH control (pH approximately 7.0 to 7.6). Two sets of a half PVC pipe (110 mm wide, 110 mm long, and 40 mm high) set on two flat stones were prepared as shelters and spawning beds (Fig. 1). Parental fish were fed with pieces of river prawn (mainly *Macrobrachium* spp.), Tetra krill-E (Spectrum Brands Japan Co., Ltd., Tokyo), and commercially prepared Hikari Catfish (Kyorin Food Industries, Himeji) once a day to satiation level. Leftovers and feces were removed daily.

Hormone Injection

Parent fish were reared for 1 year and 9 months, and on March 24, 2022, human chorionic gonadotropin (10 IU per g, BW) was injected once into the left lateral muscle of the caudal peduncle of both parents, according to Momota et al. (2022a, b). Parental fish sizes at that time were 97.2 mm BL, 113.9 mm TL, and 51.3 g BW for the male and 103.4 mm BL, 123.9 mm TL, and 81.1 g BW for female (Fig. 2). After injection, we did not attempt artificial insemination, but waited for spontaneous spawning in the tank.

Developmental Observations

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

Figure 1. A shelter and a spawning bed of *Pao baileyi* in the present study: a half PVC pipe (110 mm wide, 110 mm long, and 40 mm high) set on a stone with flat surfaces (100 mm wide, 150 mm long, and 20 mm high). Photograph by Hiroyuki Doi.

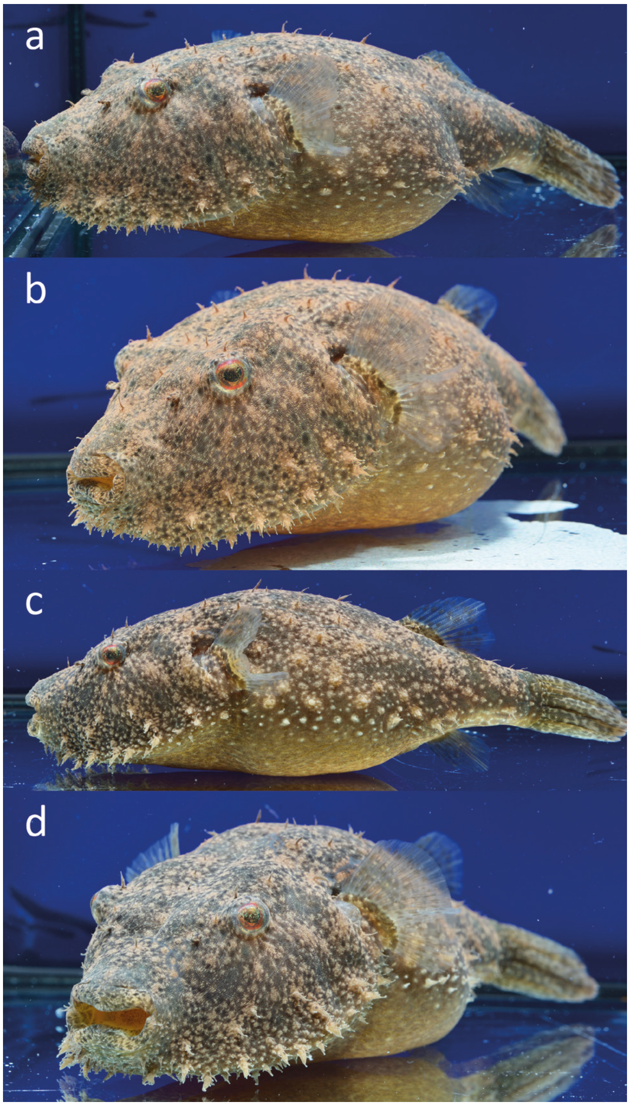

Figure 2. Parental fishes of *Pao baileyi*: male, 97.2 mm BL, 113.9 mm TL, in lateral (a) and diagonally front view (b); and female, 103.4 mm BL, 123.9 mm TL, in lateral (c) and diagonally front view (d). Photographs by Hiroyuki Doi.

Designation of developmental intervals followed Okiyama (2014): larval period, the stage before the 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). Measurement and counting methods followed Okiyama (2014).

Rearing of Eggs and Offspring

Eggs spilled from the egg batch in the spawning bed were collected with a pipette and reared in three plastic tanks (2 L, with 20–60 eggs for each tank) set up in a water bath (26 °C) and development was observed. One-half of the aerated tank water was changed and dead eggs and excrement were removed daily. All eggs protected by the male were removed before hatching and reared in three separate plastic tanks (2 L, with 20–60 eggs for each tank). The number of eggs laid was calculated by summing up the spilled ones and the other protected ones by the male.

The hatched larvae were reared in six plastic tanks (5 L), set up in a water bath (26 °C). One-third of the aerated water in the tanks was changed and the remaining food and excreta were removed daily.

Artemia nauplius larvae were initially fed three times a day from one day after hatching, and from 16 to 29 days after hatching, *Artemia* nauplius larvae and chironomid larvae were fed three times a day. From 30 days after hatching, *Artemia* nauplius larvae, commercially prepared feeds (VIBRA BITES, Hikari CARNIVORE, Hikari MINICAT, Kyorin Food Industries, Himeji) were fed three times a day. The larvae and juveniles were fed to satiation level in order to prevent them from mutual biting caused by hunger. Two pieces of water thyme *Hydrilla verticillata* (about 20 cm) from 15 days and one piece of driftwood (about 10 cm in length) from 22 days from hatching were additionally placed in all the tanks for hiding places which might help the fish avoiding biting of others.

From 45 days after hatching, the fish were fed twice to satiation level, the remaining food and excrement removed daily, and a quarter of the water was changed daily. Similar sized juveniles of similar total weight were accommodated in each six rearing tanks (5 L) also for reducing biting.

From 181 days after hatching, the grown young fish was kept alone in each tank (24 × 11.5 × 13 cm, 3.6 L, water capacity).

The pufferfish were fed once a day, the remaining food and excrement were removed, and one-fifth of the water was changed daily.

Growth

The Gomperz growth formulae of *P. baileyi* were estimated following the standard form of Richards' (1959) model by comparing the residual sum of squares (Akamine, 1988, 2004), calculated using MS Excel (Microsoft Office 365).

RESULTS

Spawning Behavior and Spawning

After the hormone injection to the pair, a series of spawning behaviors occurred, as follows:

1. Male and female usually stayed separately in their own pipes.
2. Two weeks before spawning, the male was observed approaching the female's pipe. The male remained at the entrance of the female's pipe. The male body color turned paler and speckled. The female's body color remained brown. When the male inflated his abdomen and shook his body, the female also inflated frequently.
3. The male entered the female's pipe, and the male and female began to go in and out of the pipe. While the female was outside, the male was observed to turn around inside the pipe, stirring up water and cleaning the bottom. The female occasionally swam up and down the tank for several minutes.
4. Thereafter, the male and female were always in the pipe. They chased each other, spinning around slowly.
5. Unfortunately, actual spawning itself was not observed. A single-layered egg batch was found on the bottom of the spawning bed protected by the male two days after hormone injection (March 26, 2022), the female being outside the pipe. Some eggs were observed to spill out from the spawning bed, and 106 eggs were collected.
6. The male protected the egg batch, fanning them with his pectoral fins. The male attacked and injured the female when she unintentionally swam in front of him. Therefore, a partition was placed in the tank to separate the female from the male.

Just before hatching, the spawning bed was isolated from the male and 127 eggs laid were collected. Therefore, at least 233 eggs were spawned (106 + 127), the number being an underestimate of the real number of spawned eggs, since the dead eggs may have been removed by the male during egg protection.

Egg Development

One day after spawning (Fig. 3a), eggs were 2.85 ± 0.11 (SD) mm in diameter (n = 10), spherical, demersal, and adhesive, with a mass of small oil globules. Two days after spawning (Fig. 3b), embryo body was formed. Four days after spawning (Fig. 3c), head and tail were formed, and melanophore appeared on the yolk. Nine days after spawning (Fig. 3d), embryo moved, eyes already developed, blood flew over the yolk, eyes were formed. Melanophores developed on the head, body, and upper side of the yolk, xanthophores in the caudal region, and erythrophores in the lower side of the yolk. Hatching occurred 10–11 days after spawning. Hatching rate was 73.4 % (171/233).

Larval, Juvenile and Young Fish Development

Larvae up to the first 90 days from hatching are illustrated in Figures 4a–h. The hatched larvae (Fig. 4a), were 5.1 ± 0.1 (SD) mm BL and 5.4 ± 0.1 (SD) mm TL (n = 6). They remained motionless on the tank bottom, with opened mouth and anus, fully developed eyes, pectoral fins, and many small oil globules in the yolk. The rudiments of the dorsal and anal fins were formed. Notochord flexion had started by two days. The yolk was almost absorbed by five

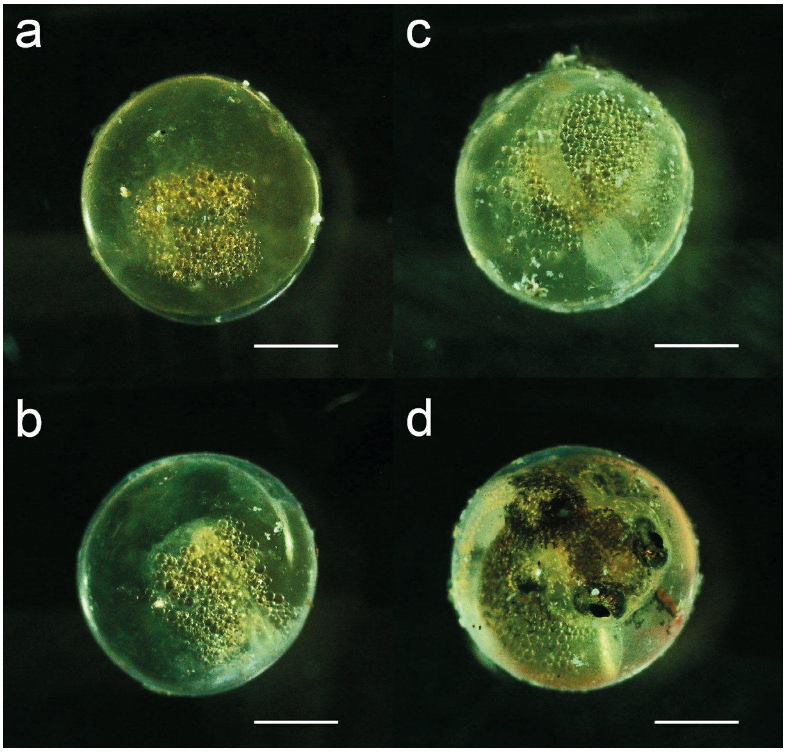

Figure 3. Development of eggs of *Pao baileyi*: a, 1 day after spawning; b, 2 days after spawning; 4 days after spawning; and, d, 9 days after spawning. Scale bars indicate 1 mm. Photographs by Hiroyuki Doi.

days. By 90 days (Fig. 4h), the larvae had reached 59.7 mm (TL), and 39.5 ± 8.8 mm (BL), and by 540 days they were 112.8 ± 6.7 mm TL (n = 9) and 93.3 ± 5.9 mm (BL).

At five days after hatching, the body was generally bright yellow. Twelve days after hatching (Fig. 4d), juveniles had 11 dorsal fin rays, 8 anal fin rays, 18 pectoral fin rays, and 7 caudal fin rays, reaching the adult complements. At 15 days, small white spots were scattered on the head, back, and abdomen. A white cross band appeared between eyes. At 30 days, juveniles were brown in basic color with scattered small white and dark brown spots and white abdomen. Rudimental epidermal cirri appeared from small white spots on the head and back. Forty days after hatching (Fig. 4g), juveniles had small white and dark brown spots on the body. Elongated epidermal cirri were present on the head, upper eyes, and back, and also appeared around the mouth and on the lower part of the body. The abdomen was pale brown. By 180 days after hatching body shape, color, and epidermal cirri were similar to those of adults.

At hatching, melanophores were present from the front of the eye to the parietal and body, the body melanophores forming a saddle pattern from the back to the abdomen. Xanthophores were distributed from the body to the tail. Erythrophores were present in the abdomen. At two days, melanophores were still present on the head, body, and yolk, xanthophores on the bases of dorsal and anal fins and on the tail. Five days after hatching (Fig. 4c), xanthophores were distributed on the body, bases of dorsal and anal fins, and tail, but erythrophores were obscure.

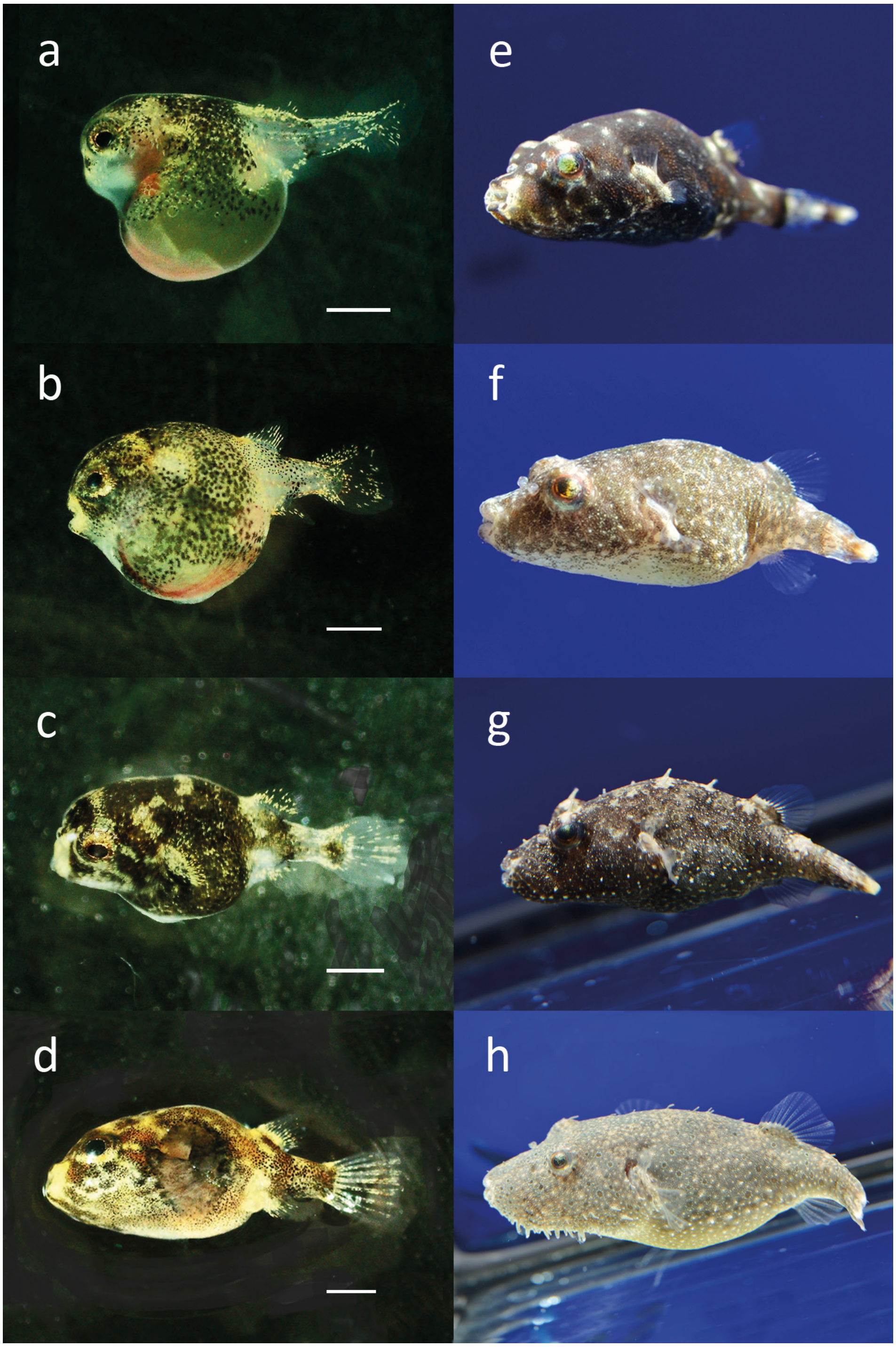

Figure 4. Development of larvae and juveniles of *Pao baileyi*: a, 5.3 mm TL newly hatched larva; b, 6.0 mm TL larva 2 days after hatching; c, 6.2 mm TL larva 5 days after hatching; d, 7.6 TL juvenile 12 days after hatching; e, 9.7 mm TL juvenile 15 days after hatching; f, 19.7 mm TL juvenile 30 days after hatching; g, 24.4 mm TL juvenile 40 days after hatching; and, h, 59.7 mm TL juvenile 90 days after hatching. Scale bars indicate 1 mm. Photographs by Hiroyuki Doi.

We observed changes in the behavior of larvae as they developed. Very young larvae swam near the bottom, gathering in the corners of the aquarium or around air stones. They did not exhibit any special behavior, keeping about one individual distance from each other. After five days, juveniles no longer gathered at the corners or around the air stones, but became solitary, responding, approaching and feeding food. At 22 days, strong individuals occupied the center of the tank in the hollow of sunken driftwood or among water plants, biting others, and monopolizing food, while the others were in the corners of the tank. At 30 days juveniles often fought for chironomid larvae, larger juveniles chasing and biting smaller juveniles except when feeding.

Rearing Density and Survival Rate

During the first 14 days after hatching the pufferfish were kept at an average density of about 5.4 individuals/L, and the survival rate was 76% (130 individuals survived). From 15 to 22 days, they were maintained at about 4.0 individuals/L, and the survival rate was 87% (113 individuals). The density declined thereafter, and the total survival rate from hatching to 180 days was 15%. From 181 to 540 days after hatching, individuals were maintained separately in 3.6 L tanks, and the survival rate during this period was 90% (9 individuals remaining). Hence, survival increased slightly with increasing age.

Growth

Approximated Gomperz growth formulae for BL and TL ($n = 328$, including both sexes) are summarized below, the coefficients of determination being 0.990 (BL) and 0.992 (TL). *Lt* is estimated length, and t is days after hatching (Fig. 5):

BL : $Lt = 91.363 \bullet \exp(-\exp(-0.01283(t - 77.5439)))$

TL : $Lt = 111.2789 \bullet \exp(-\exp(-0.012431(t - 80.26232)))$

DISCUSSION

Doi *et al.* (2022b) reported a case of spontaneous spawning of *P. baileyi* in a tank without any hormone injections. In the present study, hormone injections to both parents caused similar spontaneous spawning effectively two days after injections. However, juveniles raised by Doi *et al.* (2022b) often bit one another, and consequently died from bacterial infection. In the present study, biting behavior was also observed from 23 days after hatching. In order to reduce biting, we fed the pufferfish three times a day to satiation level, divided the tanks into several sections, and increased the number of hiding places by introducing water plants and sunken driftwood. From 35 days after hatching, larger juveniles responded quickly to food and began to chase and bite smaller juveniles. Therefore, similar-sized juveniles of similar total weight were accommodated in each of six rearing tanks, which further reducing biting behavior. Beyond 180 days after hatching, the pufferfish were separated into individual rearing tanks.

At 420 days after hatching, many individuals had grown to about the same size as the parental fish in the present study, as was observed by Doi *et al.* (2022b). Hence, this species is able to reach breeding size in approximately one year.

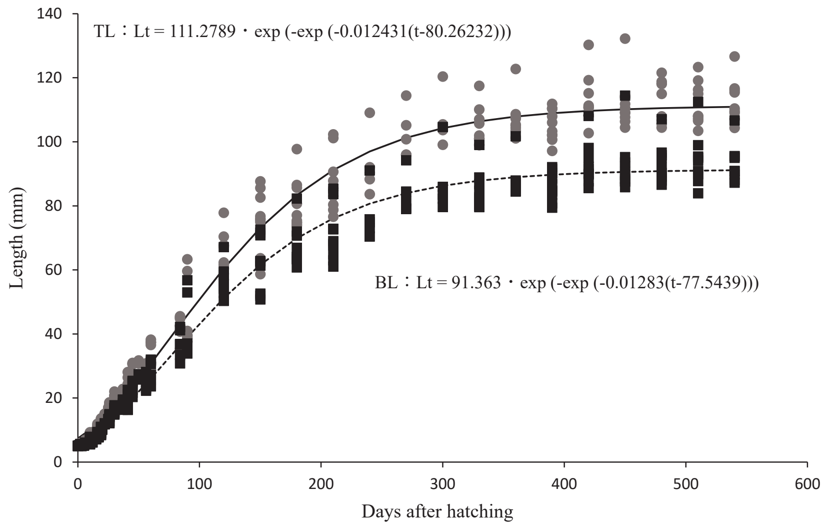

Figure 5. Growth of *Pao baileyi* spawned on March 26th, 2022. Gomperz growth formulae were approximated using Richards' (1959) model and shown in the figure. Black squares and a black dotted line indicate those of BL. Grey circles and a solid black line indicate those of TL.

Our results show that this species can be bred in captivity by providing appropriate hormone treatment to adult-sized individuals to promote spontaneous spawning, and by taking appropriate measures to reduce biting behavior during the juvenile stage to increase survival rate. These techniques may be useful for future conservation programs of *Pao* species. In the future, additional techniques to reduce biting behavior such as reducing lighting in the tanks during rearing, which was found to be highly effective in suppressing the biting behavior during seedling production of the tiger puffer *Takifugu rubripes* (Hatanaka, 1997; Furukawa *et al.*, 2016), may be worth examining.

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