Spawning of Schizopygopsis microcephalus (Cyprinidae) in the unique high-altitude conditions of the Tanggula mountains on the Tibetan plateau

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Abstract

Schizothoracine fish spawning habitats in the Tibetan Plateau are poorly understood but critical for conservation amidst anthropogenic threats and climate change. In a 2019–2021 study, we located the spawning grounds of Schizopygopsis microcephalus Herzenstein, 1891, at an astounding 4800 m above sea level, the highest known fish spawning site. This occurred in mid-June, before seasonal flooding. We found approximately six circular egg burial nests per square meter, measuring 20–30 cm in diameter and 10–15 cm deep, nestled within gravel (4–5 cm max diameter, D50 of 2.2 cm). Egg fertilization rates reached 98%, with an 82% hatching rate. Intriguingly, eggs displayed delayed shell hardening during natural incubation, possibly to protect against intense high-elevation ultraviolet radiation. Further investigations are needed to understand the role of the pliable membrane. Survival of the species requires that this kind of rare habitat be conserved.
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Abstract

Schizothoracine fish spawning habitats in the Tibetan Plateau are poorly understood but critical for conservation amidst anthropogenic threats and climate change. In a 2019–2021 study, we located the spawning grounds of *Schizopygopsis microcephalus* Herzenstein, 1891, at 4806 m above sea level, the highest known fish spawning site. This occurred in mid-June, before seasonal flooding. We found approximately six circular egg burial nests per square meter, measuring 20 – 30 cm in diameter and 10 – 15 cm deep, nestled within gravel (4 – 5 cm max diameter, D$_{50}$ of 2.2 cm). Egg fertilization rates reached 98%, with an 82% hatching rate. Intriguingly, eggs displayed delayed shell hardening during natural incubation, possibly to protect against intense high-elevation ultraviolet radiation. Further investigations are needed to understand the role of the pliable membrane. Survival of the species requires that this kind of rare habitat be conserved.

KEYWORDS

*Schizopygopsis microcephalus*, spawning period, spawning ground location, egg burial, shell softening, grain size analysis, Tibetan plateau
INTRODUCTION

The Tibetan Plateau, commonly referred to as the Asian Water Tower and the Third Pole, serves as the headwaters for several significant rivers across the Asian continent (Gao et al., 2019). This vast expanse of land is of immense ecological value and is exceptionally vulnerable to climate change (Immerzeel et al., 2020). Warming will affect the hydrological regime of rivers, impacting the quality of fish spawning grounds (Miller et al., 2012; Dalke et al., 2018; Chase et al., 2020; Dalke et al., 2020). The Chinese government has implemented a conservation strategy for the Yangtze River, and the protection of fish habitats is explicitly mandated by the Yangtze River Protection Law of the People’s Republic of China and the Qinghai-Tibet Plateau Protection Law of the People’s Republic of China. Hence, locating Tibetan Plateau spawning grounds is fundamental to identifying and conserving habitat hydrology requirements and urgent for legal compliance.

Over 80% of schizothoracine fishes are endemic to China, including 70 species and nine subspecies belonging to 11 genera (Cao et al., 1981; Wu and Wu, 1992). Among them, *Schizopygopsis microcephalus* Herzenstein 1891 [synonym *Herzensteinia microcephalus*, Herzenstein 1891] is unique as the world’s highest-altitude cyprinid fish. This niche-conserved fish occurs only in the northern and southern regions of the Tanggula Mountains, particularly in the Yangtze River headwaters (Figure 1A), contributing to its scientific value in understanding how fish adapt to the extreme cold, low oxygen, and intense ultra-violet radiation (Wu and Wu, 1992). *Schizopygopsis microcephalus* provides a prime example of spawning adaptation to an extreme environment.

The location of schizothoracine fish spawning grounds is largely unknown. The natural
spawning grounds of only three species have been reported (Hu et al., 1975; Chen and Luo, 1997; Yan et al., 2017), and spawning occurred in non-flood periods (Hu et al., 1975; Yan et al., 2017). Schizothoracine fish spawn in rivers during spring and summer at times varying with elevation (Cao et al., 1981). Because of the harsh conditions of the area, reports of the spawning period have been based on gonad development stage and capture of larvae rather than direct observation.

Our objective for this study was to locate the spawning ground of *S. microcephalus* on the Tibetan plateau. We made three assumptions to assist our search: (i) spawning occurs during non-flood periods when river water is clear, (ii) spawning involved nest excavation, and (iii) eggs would be covered after being laid. If *S. microcephalus* exhibited the dual actions of pebble excavation and subsequent egg burial, the superficial substrate would be successively disturbed during the removal of substrate overlaying the pebbles/gravel, creating a conspicuous visual difference between the nest sites and the surrounding undisturbed substrate.

**MATERIALS AND METHODS**

**Study area**

The study area was centered on the 150 m long core overwintering grounds located at 4816 m asl in the upper reaches of the southern Yangtze River headwaters where 32,000 *S. microcephalus* overwinter (personal observation, unpublished). An adjacent 10 km stretch (Figure 1B) was investigated, as it was supposed that the fish would spawn near their overwintering grounds. An upstream 2.5 km segment was characterized by a shallow single
channel measuring 20–50 m in width. The downstream 7.5 km segment consisted of a braided system featuring multiple branched channels, varying in water depth and velocity, with river width ranging from 600–1000 m.

**Spawning period**

The spawning period was identified based on gonad development status of the adult *S. microcephalus* in the overwintering grounds. The gonad development cycle of *S. microcephalus* was determined in 2019 through 2020 in adult fish collected from the overwintering ground and during the spawning season in the adjacent 10 km. The spawning period was roughly determined in 2019 by monthly sampling. The precise spawning period was identified in 2020 by daily sampling. Intensive monitoring was conducted on April 6–10, 2019; May 12–17, 2019; June 27–30, 2019; August 6, 2019; November 4, 2019; and daily June 1–18, 2020. Gonad stage was determined based on secondary sex characteristics, compression to release eggs and milt, fish dissection, and egg diameter. Five to ten female fish were sampled each time. Stage IV females were dissected because of absence secondary sex characteristics and eggs could not be expressed. Ripe females were identified by pearl organs and compression. Spawning was determined by compression. The point when the fish ripened but had not spawned was defined as T0. An empty ovary indicated cessation of spawning (T1). The spawning period was designated as time from T0 to T1.

**Identification of the spawning area**

We attempted to determine the spawning area in June 2020. Using the parent fish method, we
identified areas in which mature fish cruised with males pursuing females. Egg production with compression and the presence of pearl organs confirmed that females were ripe and gravid. We also collected stray eggs flowing in the downstream current as well as newly hatched larvae. Eggs were collected in fixed nets, and larvae were captured in shallow waters with a hand-net at 500 m intervals. In the upstream reaches, the spawning area was confirmed by collection of eggs and larvae. In the parent fish cruising area, we placed a water temperature gauge (Onset HOBO /U20-01) to record water temperature every hour and measured water depth (using a ruler) and water flow rate (using a FlowTracker).

**Identification of nests**

In 2021, when daily monitoring showed that some female fish had spawned, it was determined that the spawning period had begun, and a search for nest in the river was immediately carried out along the target 10 km stretch for clusters of fertilized eggs or substrate of contrasting color. When eggs were found, they were compared for size and color to those we artificially propagated in 2020. Fertilization rate was conservatively estimated as live eggs/total eggs, and embryo development stage was determined immediately using a portable microscope (Bresser, Germany) based on standard fish egg developmental stages. Seven to eight eggs per nest were randomly collected for analysis of hatching rate. The 7–10 days after the time spawning was confirmed was identified as the hatching period. During this time, fertilized eggs were still to be found in the area, providing direct evidence of an active spawning ground.

Once the spawning ground was confirmed, we measured the water depth of the spawning
site using the ruler method and the flow velocity using a FlowTracker. Daily water temperature data was obtained from the nearest installed water temperature gauge. Daily maximum ultraviolet intensity data was sourced from the Agricultural Meteorological Big Data System V1.5.9 (http://wheata.cn/page/1-0.html). The substrate of the spawning ground was sampled for grain size analysis. The substrate was sieved at 0.9 mm, 1 mm, 2 mm, 4 mm, and 8 mm and grains >8mm were measured by Vernier caliper (Greener Ltd, 300mm). The grain grade curve was plotted by OriginPro 2019b, and weight D50 was determined by the screen reader function. The ratio of mean maximum grain diameter to mean total length of all spawned female fish was calculated.

**Incubation and taxonomic Identification**

Collected eggs were transported to the hatchery center in Zaduo County (4000 asl) of Yushu Prefecture for hatching and species identification. We incubated the wild fertilized eggs in an indoor basin-style setup with a diurnal temperature variation of 9.5°C to 20°C, similar to spawning ground. The incubation water was pumped from a well with a natural temperature of 9.5°C while the 20°C temperature was achieved through mixing with warmer water. Room temperature was maintained at 13°C. To simulate the natural conditions, a pump was used to ensure dissolved oxygen level of 6 mg/L, and the concentration of ammonia nitrogen concentration was below the detection threshold of 0.001 mg/L, light regime was 13L/11D, and 2/3 of the water was renewed each day. Dead eggs were removed until all eggs hatched.

**RESULTS**

**Spawning period**
From early November through late May 2019, the ovaries were at stage IV. In early June adult fish left the winter grounds and ripened. Secondary sex characteristics included differing body size and the presence of pearl organs in both sexes. Gravid females were significantly larger than ripe males, with a total length of $37 \pm 7$ cm ($n=14$, 21.4–45.2 cm) and body weight $396 \pm 237$ g, 1.5 times the length of males $24 \pm 6$ cm ($n=26$, 16.6–39.7 cm), and four times the male weight ($99 \pm 94$ g) (Figure 2A), although we observed some large males (30–38 cm). Pearl organs were more pronounced in males, particularly in the fins, head, skin on the posterior of the body, and the posterior three fin rays of the anal fin converted to hard spines. Most pearl organs of females were seen in the anal fin, with fewer in other fins. Gravid females exhibited a flow of eggs even without pressure, and slight pressure on the abdomen of ripe male fish resulted in discharge of milt, accompanied by open mouth and vigorous tail swaying. The spawning period from female ripening to releasing eggs extended to June 3–15 in 2020. By June 17–18, examination of gonads revealed that 30% had spawned (3/10). The spawning window lasted at least 12 days in the upstream river stretch.

Egg diameter at gonad phase IV was 2.20 ±0.20 mm on April 6. On May 13, eggs were still at phase IV, with a diameter of 2.37 ±0.15 mm. By early June, ovaries were mature and egg diameter was 2.40 ±0.18 mm. On June 29, the ovaries were mostly depleted eggs, diameter of remaining eggs was shrunk to 1.50 ±0.22 mm. On August 6, anal fins showed damage and ovaries were depleted of eggs, showing stage II–III, with egg diameter of 0.63 ±0.17 mm. On November 4, the female gonad was again at phase IV with egg diameter of 1.83 ±0.11 mm, increasing to 2.4 ±0.15 mm by June 4, 2020.
Spawning area

On the morning of 30 June 2019, no eggs or larvae were collected in the fixed net. We surveyed four sites in a 7.5 km segment downstream of the overwintering pond in the afternoon. A muddy 100 m zone ~3 m wide containing 400–500 empty nests, widely distributed as single or multiple pits, each with a diameter of ~30 cm, was located 2.2 km downstream of the overwintering site. A mixture of mud and white Kobresia humilis root was scattered throughout. A low-density (32 ind/m²) population of larvae (TL 1.69 ±0.11 cm) was observed. In addition, a separate 3 m × 1 m larva nursery was identified in a grassy area at the river center containing larvae estimated at a density of 1953 ind/m². Although S. microcephalus larvae were detected in the area of excavated pits, the ultimate evidence of fertilized eggs was not observed, hence, spawning was not confirmed.

From 1–10 June 2020, eight micro-habitats with mature fish congregating and milling about were discovered within the 10 km target area. The number of females ranged from a few to dozens, each pursued by 5–8 males. These courtship areas were characterized by water depths exceeding 0.3 m and flow velocities <0.37 m/s. The fish stayed within the area and returned immediately after an enforced absence.

Broader spawning area

An expanded search for spawning sites upstream and downstream of spawning grounds discovered in 2021 revealed no spawning activity on June 15, but by June 18, extremely clear and sunny weather, female fish were found to have spawned, and we commenced searching for the spawning sites.
No egg clusters were detected. Areas (80m × 4m) at 4806 m asl differing in substrate appearance were found ~500 m downstream of the empty nests discovered in 2020 (Figure 2B). Water was clear and at a depth of <5 cm with flow rate of 0.1 m/s. In the yellowish riverbed, numerous clean bluish-black gravel patches were observed (Figure 2C). We carefully excavated the bluish-black gravel substrate, and found evenly distributed golden-colored fertilized eggs at a depth of 3-5 cm (Figure 2D). Discovery of the fertilized egg proved that this was a real nest. The area contained averaged 6 ind/m² circular nests. The nests surface substrate consisted of a mixture of coarse and fine particles, with finer gravel predominating. The largest diameter was 4–5 cm and the smallest ~1 mm. In contrast, the surface substrate in the yellowish area consisted of uniform 4–5 cm gravel. The gaps in the gravel were exceptionally clean, with almost no sand content. The particle size distribution of the gravel is shown in Figure 2E, the diameter boundary of 50% cumulative weight percentage (D50) of the spawning substrate was 22 mm. The largest particle size observed was approximately 1/10 of the length of the sampled spawned female. After flooding in September, 2021, we observed uniform color and surface gravel size in this area (Figure 2F).

The spawning grounds were characterized by diurnal temperature range of 7 – 20 °C through June 17- 18. The highest ultraviolet intensity occurred at 2:00 PM (UTC+8), with a maximum intensity of 442,808.0 J/m² and 439,080.0 J/m² on the 17th and 18th day, respectively. The color and size (3.5 mm) of the fertilized eggs were similar to that of eggs obtained through artificial reproduction (Li et al., 2020). Shells were soft, even softer than the phase V unfertilized eggs. A light touch caused wave-like deformation of the shell, as might occur with the impact of gravel (Figure 3A), but the eggs did not rupture. Embryos were in the early
blastula stage.

**Incubation and species Identification**

Among 58 eggs observed in three nests, only one egg appeared to be unfertilized (white), resulting in a fertilization rate exceeding 98%. Twenty-two fertilized eggs were randomly collected from the three nests and transferred to the laboratory for incubation. Within 24 h, the shells became firm. After incubation for seven days, 18 larvae hatched almost simultaneously, with length ranging from 9 to 11 mm. The hatchlings lacked barbels (Figure 3B) and had small spots distributed along the body. Considering that only one schizothoracine fish, *S. microcephalus*, reproduces in our studied headwaters (unpublished observation) it was determined that the larvae were those of *S. microcephalus*. The hatching rate was 82%. The high fertilization rate and high hatching rate confirmed eggs of good quality.

**DISCUSSION**

The spawning grounds reported here in the Yangtze River headwaters is, at 4806 m, the highest-altitude fish spawning ground reported to date. The covering of eggs by the gravel substrate makes locating spawning grounds a challenge. Our extended observations took place over the course of three years living in extreme primitive conditions at high elevation.

Our investigation showed females to ripen in early June 2020–2021. *Schizopygopsis microcephalus* exhibits characteristics of egg covering and hatching in clean gravel substrate, similar to the spawning habits of salmonids (*Stuart, 1954; Groot, 1996*). These fish differ in spawning periods; the former spawns before the summer flood season, while salmonids can
spawn in a broader range of seasons (Groot, 1996). Fish spawning grounds require specific hydrological features to support the survival of fertilized eggs (Slotte and Fiksen, 2000; Bakun, 2006; Bakun, 2013; Ciannelli et al., 2015). For spawning and hatching periods prior to the flood season, little sand is present and gaps in gravel allow water flow-through to ensure sufficient oxygen for hatching. Covering serves as a protective mechanism, preventing the eggs from being washed away during flooding (Burril et al., 2010; Reiser et al., 2019), protecting them from predation (Gauthey et al., 2017), and shielding from the intense ultraviolet radiation of a high-altitude environment (Lawrence et al., 2020).

The blastula stage of the fertilized eggs when discovered indicated that spawning had occurred within the past 24 h, which calculated the spawning time as 17–18 June. Since <5 cm water depth is not sufficient for spawning behavior, we inferred that the water level had declined rapidly after spawning. Although the spawning date is known, due to significant daily water temperature variations, the water temperature requirements for spawning still need further research in the future.

The substrate particle size in salmonid spawning grounds is also limited to 1/10 of their body length (Kondolf and Wolman, 1993; Meredith, 2018), and a minimum flow rate is required to enhance fertilization and provide sufficient oxygen for hatching (Groot, 1996). The muddy spawning pits found in our survey may have resulted from fish fidelity to particular spawning grounds or to the ease of digging in mud as opposed to gravel. This may be a concern for the conservation of the population. If S. microcephalus exhibits fidelity, they may return to an original spawning site that has become unsuitable for nesting; for example, degraded by anthropogenic activity such as building or road construction.
Differences exist in spawning behavior among subfamilies of Schizothoracinae. *Gymnocypris przewalskii* exhibits digging behavior but does not cover the eggs (Hu et al., 1975). *Schizothorax wangchiachii*, does not dig, but sweeps eggs into gravel crevices (Yan et al., 2017). The spawning behavior of *S. microcephalus* combines digging and egg-covering, advantageous to hatching. As the altitude increases, the need for egg protection rises. It is likely that excavation and egg burial is a primary adaptation for preventing ultraviolet damage, as we observed that the completely exposed eggs of other schizothoracine species died at 4275 m asl.

Salmonid anal fins provide the function of detecting depth during nest digging but do not break (Groot, 1996). In our study, the anal fin of *S. microcephalus* exhibited severe degradation after spawning, but could regenerate. All observations indicate that *S. microcephalus* does not die after spawning. Its anal fin is longer relative to body length than that of salmonids, possibly related to the functional differences.

The time from the discovery of the spawning grounds to noting the softness of the egg shells was at least 30 min. In contrast, cultured fertilized eggs harden within five minutes (Li et al., 2020). The soft eggshell can withstand impact of gravel without damage during burial. This characteristic is seen in salmonids, in which the shell begins to harden after 1–2 h, completing the hardening process in 3–7 days (Zotin, 1958). We observed soft shells for more than 24 h. High water temperature and high dissolved solids can lead to eggshell softening (Cousins and Jensen, 1994; Brix et al., 2010; Pérez-Atehortúa et al., 2023). The mechanism causing the differences in eggshell hardening in natural and artificial breeding requires further research. High water temperature during fertilization under global warming may be a cause of
the soft shells of naturally fertilized eggs, because at the spawning site, due to shallow water
and low flow velocity, water temperature may be higher under intense solar radiation and we
installed a thermometer here later. The level of dissolved solids is less likely to be a factor, as
the water was clear and the spawning took place prior to flooding.

CONCLUSIONS

This study reports the first locating of spawning grounds of the world's highest-altitude
Cyprinidae fish species. In mid-June, the Schizopygopsis microcephalus fish reach sexual
maturity, spawning in the gravelly rivers of the Qinghai-Tibet Plateau. Burying of eggs for
incubation is a unique characteristic not previously reported among schizothoracine fish.
Fertilized eggs show the phenomenon of shell softening delayed hardening. The identification
of the precise spawning location and its hydrological characteristics provide the foundation
for habitat conservation as well as research into mechanisms of adaptation to extreme
conditions and potential impact of climate change. Further research is needed to explore the
reasons underlying eggshell softening and delayed hardening.

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**AUTHOR CONTRIBUTIONS**


D.H.A. performed grain size analysis, C.Y.L. collected data, K.F.Z. performed plotting work, H.L. analyzed output data. W.L. wrote the first draft of the manuscript, and all authors contributed substantially to revisions. All other authors approved the text in the manuscript.

**COMPETING INTERESTS**

The authors declare no competing interests.

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**Embedded Video**

Video 1: The discovery of spawning ground consisting of egg-burial nests