NOTE

Submerged spawning and larval dispersal of the mudskipper *Periophthalmus variabilis*

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ABSTRACT: Since the discovery of mudskipper aerial embryonic incubation in 1998, it has been unclear whether spawning and fertilization take place inside an air-filled or water-filled egg chamber, and whether the parent animals help the newly hatched larvae leave the hypoxic water inside the burrow. I can confirm submerged spawning, observed in video recordings of the egg chamber of a mudskipper *Periophthalmus variabilis* burrow. Initial indirect evidence was obtained by video recordings of water level increases at the entrances to the burrow caused by the male adding air into the egg chamber, displacing the water there. Furthermore, single video recordings show that the male can create a strong water current that causes water to flow out of the burrow, which may help the larvae leave the burrow.

KEY WORDS: Mudskipper · Reproduction · Air-adding behavior · Egg chamber · Larval transport · Parental care · Tail-undulation behavior

1. INTRODUCTION

Mudskippers are tropical and subtropical fishes naturally living in the shallow zones of the Indo-West-Pacific and eastern Atlantic. Ten genera including 43 species are currently recognized. These fishes commonly occur in soft-bottom habitats, especially mangrove forests and exposed mudflats. Species such as *Periophthalmodon septemradiatus* and *Periophthalmus weberi* also live further upriver in areas with lower salinity. They have developed morphological, physiological and behavioral adaptions to habitats which are unusual for fishes.

Until the discovery of air storage in mudskipper burrows (Ishimatsu et al. 1998), the development of mudskipper embryos in burrows had long been an enigma since the burrows are always filled with hypoxic water that does not permit embryonic development (Brillet 1976, Martin 2015, Mai et al. 2020).

More recently, data on the Japanese mudskipper *Periophthalmus modestus* indicated that egg-guarding males gulp mouthfuls of air and expel it into the egg chamber during low tide when the mudflat is exposed (Ishimatsu et al. 2007). As a result, the P_{O2} of the egg chamber increases steadily during low tide but declines during high tide. The discovery of mudskipper aerial embryonic incubation has raised new questions related to their reproductive strategy, such as whether spawning takes place prior to or after the egg chamber is filled with air.

The aerial spawning behavior of the rockhopper *Andamia tetradactyla* (Bleeker) (Shimizu et al. 2006) and low oxygen content prevailing during the immersion phase in mudskipper burrows suggest that both spawning and fertilization occur in air (Ishimatsu et al. 2007, 2009, Ishimatsu & Graham 2011, Toba & Ishimatsu 2014), although this had not yet been observed. The presence of air

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in spawn-free egg chambers of male *P. modestus* engaged in courtship (Ishimatsu et al. 2007), the finding of 3 egg chambers of *Periophthalmodon schlosseri* that contained air but no eggs (Ishimatsu et al. 2009) and data on the prolonged burrow residence of female *P. modestus* during the reproduction phase (Mai et al. 2020) suggest that the chambers are filled with air prior to spawning. If spawning and fertilization occur, contrary to expectations, in a submerged state, air-adding must start soon thereafter (Ishimatsu et al. 2009).

Furthermore, larval hatching is known to be induced by the male's removal of egg-chamber air upon completion of embryonic development, which increases the water level in the egg chamber and immerses the eggs in water. This process normally takes place during nocturnal high tide. The male transports egg-chamber air in its mouth and expels it into the burrow shaft (Ishimatsu et al. 2007). At that time, the burrow entrances are covered by water, and the larvae can exit the burrow. Etou et al. (2007) showed that the survival time of freshly hatched larvae of *P. modestus* declines rapidly in hypoxic water; at dissolved oxygen levels of 10% saturation, all larvae died within 1 h. It is therefore essential that larvae leave the burrow as quickly as possible. However, when larval hatching is artificially induced in the burrow after removing the male, only a few larvae find their way out of the burrow (Ishimatsu et al. 2007). This raises the question of how freshly hatched larvae find their way out of the burrow as quickly as possible under natural conditions (Ishimatsu et al. 2007, Ishimatsu & Graham 2011). As little information is available about the behavior of the male and female inside the burrow (Lee & Graham 2002), the objective of this study was to answer 2 key questions (Ishimatsu et al. 2007, Ishimatsu & Graham 2011, Martin 2015, Martin & Ishimatsu 2017). Do mudskippers spawn above or below water? Are parent animals involved in larval dispersal from the burrow?

2. MATERIALS AND METHODS

Periophthalmus variabilis was chosen for resolution of these questions and for breeding experiments (Mleczko & Rupp 2017, Rupp 2014, 2015a,b). For more than 2.5 yr, individuals were kept and filmed in an experimentation tank under semi-natural conditions (details in Text S1 in the Supplement at www.int-res.com/articles/suppl/b030p113_supp/).

3. RESULTS

3.1. Submerged spawning

3.1.1. Indirect evidence

The male *Periophthalmus variabilis* excavated a Y-shaped burrow by first digging downward and then changing direction and digging upward, making a second entrance. From the lowest point of this 'U', the male dug a vertical shaft with 1, rarely 2 and in one case 3 chambers at the end. Occasionally, the burrow had only one entrance and was J-shaped. Once the male had completed excavation of the burrow, the male and female appeared together, each in a separate entrance of the burrow, for several hours, but only during low tide. Usually, each mudskipper remained alone in one of the 2 openings (Fig. S2a). As the burrow was always built in the upper area of the mud slope, the burrow entrances were above water at that time. During all 42 observed breeding cycles, video recordings showed that both the male and female dove deeply into the burrow for only a short period per dive; in some cases, one individual followed the other before it resurfaced (Video S1). During the alternate diving phase of 3 exemplary cycles, the female stayed submerged for an average (±SD) of 79.1 ± 31.7 s, but not for more than 2.5 min dive^{-1}. The male stayed submerged for 39.7 ± 15.8 s, but not for more than 2 min dive^{-1}. However, the male dove much more frequently than the female per alternate diving phase (♂: 81 ± 9.6 times; ♀: 42 ± 7.5 times). At the end of this alternate diving phase (between 2.5 and 5.5 h after the initial dive), the female left the burrow and did not return. Occasionally, she remained in the burrow entrance for between 1 and 12 min, frequently without diving again.

In the final phase of the alternate diving or soon thereafter, the male began gulping mouthfuls of air and vigorously diving down into the burrow with a strong flick of the tail (Video S2). A simultaneous increase in the water level inside the shaft and at the burrow entrances was observed during 11 breeding cycles (Fig. S2b). During this air-adding phase, the male stayed submerged for 17.6 ± 25.5 s and up to 4 min dive^{-1}, when air-adding behavior had lasted long enough to allow a prolonged stay under water (Fig. S3a–c). The male continued air-addition only during low-tide phases until larval hatching. This behavior was observed a few times during high tide as well. After 8–9 d, the male removed the air during the evening or nocturnal high tide and once during

morning high tide. This behavior immerses the eggs and triggers the larval hatching process (Ishimatsu et al. 2007). The number of larvae varied between 10 to more than 500. On 12 occasions, no larvae hatched (Table S1, Fig. S4, Video S3); the cause is not known. During 3 breeding cycles, a few larvae hatched 1 night before mass hatching took place. During 1 cycle, early hatching of 1 larva was observed 2 nights before mass hatching.

The average volume of entire egg chambers was ca. 48 cm^3 based upon measurements of 16 plaster cast burrows. Only the upper section of the egg chambers could be filled with air (Fig. S5).

3.1.2. Direct evidence

When the burrow was built adjacent to the glass wall of the tank, individuals could be observed diving through the vertical shaft and entering the egg chamber. Due to the murky water, both individuals — swimming supinely near the dome-shaped ceiling — could be recorded only when they touched the glass plate (Video S4). Spawning underwater onto the upper part of the glass wall inside the egg chamber was clearly visible (Fig. 1, Video S5).

Video recording also showed that air gulping by the male is indeed associated with air addition to the upper part of the egg chamber. After the male transported and expelled air into the egg chamber, eggs could be seen on the glass wall near the rim of the egg-chamber ceiling due to the higher contrast between the yellowish-colored eggs and the dark, air-filled background (Fig. S6, Video S5). Eggs could not be seen up to that point because of the murky water. The total number of eggs laid could not be estimated because the eggs on the glass wall obscured the view of the egg-chamber ceiling.
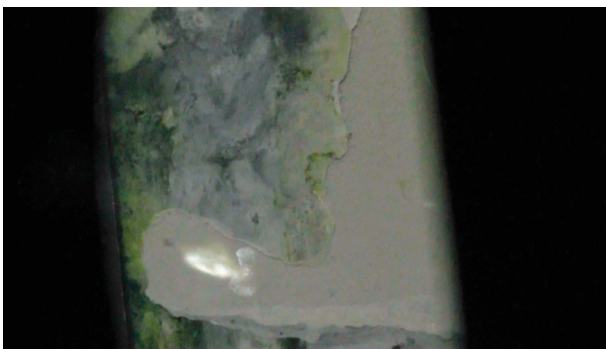

Fig. 1. Female mudskipper spawning onto the glass plate in the water-filled egg chamber using the pelvic fins to maintain contact with the glass plate

Within about 90 min, all eggs on the egg-chamber ceiling, the chamber wall and the glass wall were enclosed by air. Air-adding behavior was completed before high tide set in. Although an increase in the volume of air in the egg chamber was observed, no permanent increase of the water level in the vertical burrow shaft could be detected. Video images of larval development were also obtained (Fig. S7).

3.2. Larval dispersal

The question of whether parent animals help freshly hatched larvae leave the burrow is addressed by video footage of 7 hatching events (Table S2a). In 5 of these recordings, no obvious parental help was detected. The male began removing egg-chamber air a short time after the burrow entrances were flooded by the evening high tide before the light was switched off. Three of these hatching events were recorded completely (Fig. S8); large bubbles and extremely turbid water flowed out of the burrow (Video S6). The male stopped air removal several times and resurfaced before resuming this activity. Air-removal behavior lasted a total of 13.3 ± 2.3 min. The first larvae appeared on the water surface 8 min after the male began to remove egg-chamber air. After removing the air, the male still frequently dove into the burrow for short periods.

One further hatching event was filmed during nocturnal high tide while the light was switched on but strongly dimmed, and another one during the first morning high tide after a period of neap tides. In the first case, air-removal took 22.3 min, which was longer than in the evening hatchings mentioned above. Furthermore, it was preceded, interrupted and followed by tail-undulation behavior of the male inside the burrow (Fig. S9, Video S7). After the burrow entrances were inundated, the male dove down into one of the 2 burrow entrances every 3.1 ± 1.7 min and waved its tail vigorously back and forth for 34.1 ± 14.4 s per tail-undulation phase. While doing so, it held itself upside down in the burrow entrance area with its pelvic and pectoral fins. This action created a strong water current flowing out of the burrow (Fig. 2). After removing egg-chamber air, the male continued tail undulation every 3.4 ± 2.3 min for 48.8 ± 14.2 s per phase until the ebb tide began. Because of the strongly dimmed light, larvae exiting the burrow could not be detected; however, the next morning they were visible in the plankton net below the experimentation tank.

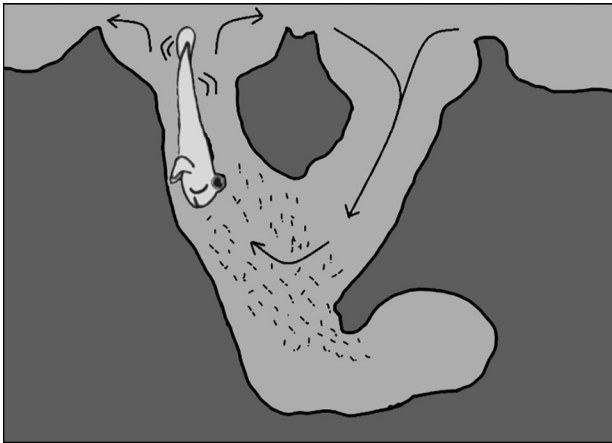In the second case, air-removal behavior lasting more than 1 h was also preceded, repeatedly inter-

Fig. 2. Mudskipper tail undulation causes water to flow out of the burrow, which could help larvae to exit the burrow

rupted and followed by the tail-undulation phases. Tail-undulation phases lasted 37.6 ± 20.7 s and were clustered around the air-removal behavior, i.e. they were not evenly distributed during the entire duration of the high tide (Fig. S10). This hatching event occurred during morning high tide after a prolonged period of artificial neap tides during which the burrow entrances had never been inundated. In this case, larvae exiting the burrow were observed.

Furthermore, 27 high tides with tail-undulation phases were recorded, but all without a hatching context (Table S2b). In addition to the observation after a neap tide period mentioned above, tail-undulation phases during 2 other breeding cycles after a previous series of neap tides were recorded. In 1 case, they were recorded during 2 daytime and 2 nocturnal high tides; in the other case, during 3 daytime and 1 nocturnal high tides. During the first daytime high tide after the neap tide period, they lasted 18.2 ± 6.3 s and were therefore shorter than during the first high tide after the neap tide period when larvae hatched. Furthermore, they were evenly distributed during the entire duration of the high tide (Fig. S11).

During cycles with normal tides, tail-undulation phases were also recorded. In addition to a single tail-undulation phase during 1 cycle, there were 2 complete breeding cycles in which 19 high tides with tail-undulation behavior were recorded: 16 daytime and 3 nocturnal high tides (Fig. S12). Only at the end of one of these 2 breeding cycles (cycle A) did the nocturnal hatching mentioned above take place (Fig. S12A). In the other one (cycle B), in which no hatching occurred, the burrow was used for a second time and the number and total time of tail-undulation

phases were always fewer than in cycle A, although they increased somewhat at the end of the cycle. In cycle A, the number and total time of tail-undulation phases were much greater during nocturnal hatching (number of tail-undulation phases: 46; total time: 29.3 min) than during the previous daytime high tides (number of tail-undulation phases: 12.5 ± 10.4; total time: 9.1 ± 7.4 min).

I was able to study tail-undulation behavior within these cycles more closely during daylight. When tail waving began, water flowing out of the burrow was very turbid but gradually became increasingly clearer. Water flowed out of the burrow entrance in which the male was sitting, while freshwater streamed into the other burrow entrance at the same time (Fig. 2, Video S8).

4. DISCUSSION

4.1. Submerged spawning

The 2 video recordings of the egg chamber clearly show that *Periophthalmus variabilis* is able to spawn successfully underwater. The additional observation of 11 water level increases within the entrances to the burrow caused by the male adding air into the egg chamber (thereby displacing the water inside the egg chamber) shows that *P. variabilis* seems to have frequently spawned while submerged. Also, an initial thin layer of air near the egg-chamber ceiling was not detected. Such a small amount of air would not have been sufficient for all eggs to be laid above the water since spawning was observed not only on the egg-chamber ceiling but also on the upper section of the egg-chamber walls. The periodically observed hatching of only a few larvae 1 night prior to mass hatching or of a single larva 2 nights prior to mass hatching supports this observation. *Periophthalmus modestus* eggs have a 5–6 d window for hatching competence (Ishimatsu et al. 2007); if this is also the case for *P. variabilis*, it can be assumed that this 'early hatching' was triggered by immersion of eggs attached to the egg-chamber walls directly above the water surface resulting from water movement caused by the air-adding male.

There are convincing arguments that *P. variabilis* not only frequently but even routinely spawned submerged, even though a water level increase was never observed in the recordings from inside the burrow and not always from the outside:

(1) the absence of an increase in water level may be related to the following factors:

• The large diameter of some burrow shafts and especially their entrances

• Excavation of mud from the burrow during the air-addition phase, which would lower the water level

• The porosity of the mud walls erected by the male around both burrow openings

• Water splashing over the rim of the burrow entrances caused by the air-adding behavior. The diving behavior during air addition with a strong flick of the tail was, in fact, very different from that of the alternate diving phase and led—depending on the height of the burrow rim—to an overflow (Video S2).

(2) Although hypoxic water and ammonia are noxious for embryogenesis (Mai et al. 2020), there was apparently adequate time for air addition after spawning, which can also be concluded from observations of *P. modestus*. Its eggs succumb within 2 d if placed in hypoxic water (Ishimatsu et al. 2007).

(3) The data on *P. variabilis* clearly show that the female remained submerged for less than 2.5 min dive^{-1} and the male for less than 2 min. Only after air-addition could the male remain longer. Nevertheless, the pair remained in the burrow for long periods, leaving it only to chase away intruders (for example) (Fig. S3a–c). Mai et al. (2020) observed a female *P. modestus* remain inside the burrow for an extended period without exiting onto the mudflat surface. Therefore, they assumed that the egg chamber is filled with air prior to spawning because hypoxic water would prevent metabolism during an extended submerged residence. But the data on the extended residence of female *P. modestus* inside the burrow cannot preclude the possibility that during the prolonged stay in the burrow the female had resurfaced from the hypoxic water and gulped air at the burrow entrance, as I observed in *P. variabilis*.

Due to the murky water in the burrow, it remains unclear when spawning began during the alternate diving phase. I hypothesize that the female spawned when the diving rate and diving duration of both individuals were particularly high.

The absence of fertilizing behavior after air addition that would have been observed in the air-filled egg chamber indicates that not only spawning but also fertilization took place underwater, possibly during the phase when both individuals dove one after the other supinely near the dome-shaped ceiling.

The burrows had a depth of approximately only 20 cm. It appears possible that *P. variabilis*, when digging deeper burrows under natural conditions, and other mudskipper species that excavate deeper

burrows would develop different breeding strategies. Due to the hypoxic water, the need for frequent resurfacing would cost a great deal of energy. Therefore, the results presented here cannot be transferred to the natural situation of *P. variabilis* and to other mudskipper species without further investigation. However, relatively heavy mudskippers, for instance *Periophthalmodon schlosseri*, build deep burrows up to 1.3 m depth (Ishimatsu et al. 1998), and it is difficult to imagine how spawning and fertilization could be accomplished on the ceiling of an air-filled burrow in a supine position by such heavy animals (Ishimatsu et al. 2009, 2018, Martin & Ishimatsu 2017, Mai et al. 2020).

4.2. Larval dispersal

Only 7 hatching events were recorded since they normally take place at night, making it difficult to understand the significance of the tail-undulation phases. In these 7 recordings, only 2 hatching events with tail-undulation behavior were documented. As the larvae are positively phototactic (Kobayashi et al. 1972), the ambient light present during the 5 video-recorded larval dispersals without the male's tail-undulation behavior may have aided orientation (at least in the upper part of the burrow) despite the turbid water. However, since the male always left the burrow during high tide and never displayed tail-undulation behavior during most other breeding cycles, I assume that tail-undulation behavior also did not occur during the nocturnal hatching events, which were not recorded.

But why were there isolated breeding cycles with tail-undulation behavior during larval hatching? Tail undulation by the male inside the burrow is well known but has not yet been described in the context of air removal during a hatching event. According to Brillet (1975), tail-undulation behavior could serve to flush the burrow. So, not surprisingly, it was recorded during 3 breeding cycles after a period of neap tides, when there had been no water exchange. In 1 of these cases, hatching occurred during the first high tide, and tail-undulation phases were intensified, clustered around and interrupting air-removal behavior. Although nocturnal hatching during the other 2 cycles was not recorded, tail undulation could also have been present there, as this behavior was also recorded during all daytime and 3 of the 5 evening high tides within these cycles. However, there were also cycles where no tail undulation occurred at high

tide after a neap tide period, and there were also tail-undulation phases during normal tides.

What is the significance of the tail-undulation phases that were more frequent and longer lasting in the context of hatching compared to this behavior independent of hatching? It is clear that if tail undulation occurs during hatching, it will have an effect on larval dispersal. It increases the oxygen content and clarity of the water inside the burrow, which might help the freshly hatched larvae find the burrow exits, at least in the U-part of the Y-shaped burrow. Furthermore, I hypothesize that the larvae could be washed passively out of the burrow by the water current caused by the male, or they could swim actively against or with the water current towards one of the burrow openings, depending on whether the mudskipper larvae are positively or negatively rheotactic (which is not known). Thus, tail-undulation behavior occurred not only prior to but also after and even during egg-chamber air removal and was actually intensified. Further studies are needed to understand why tail-undulation behavior does not occur during every breeding cycle and, when it does occur, under which conditions. This information is important since both types of hatching events (i.e. with and without tail-undulation behavior) have also been observed in *Periophthalmus gracilis* under semi-natural conditions (C. Schell unpubl. data).

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