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Reproduction of marble-mouth frogfish *Lophiocharon lithinostomus* (Lophiiformes, Antennariidae) and the evolution of parental care among frogfishes

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Abstract

Here, we observed the reproductive behavior of marble-mouth frogfish (*Lophiocharon lithinostomus*) and the morphology of newly hatched juveniles under captive conditions. Adult males showed pursuit behavior towards females approximately seven days before spawning; spawning and ejaculation took place almost simultaneously. An adult female cared for a fertilized egg mass adhered to their right of the left side of the flank. The position of the adhered eggs on the flank was left-biased (3 on the right and 25 on the left). The females exhibited a proactive fanning behavior towards the egg masses using their dorsal fins; the fanning frequency increased over time after spawning. Meanwhile, the males did not display any form of parental care behavior. The eggs had hook-like structures that might enable the eggs to attach to the flank of the parent. Hatching occurred between 22 and 28 days after spawning. Newly hatched juveniles already had a full set of fin rays; their morphology was similar to that of adult fish. Moreover, we reviewed the evolution of parental care behaviors and egg types among frogfish family.

Keywords

Antennariidae, aquarium experiment, early ontogeny, egg care, parental care

Introduction

Antennariidae, the frogfish, belongs to the order Lophiiformes and consists of two subfamilies: Antennariinae and Histiophryninae, with 14 genera (Pietsch and Arnold 2020). Frogfishes are found in all tropical and subtropical oceans and seas except for the Mediterranean (Jordan and Richardson 1908; Pietsch 2004; Pietsch and Arnold 2020), and they are predatory fishes (Arnold et al. 2014). Almost all frogfishes resemble certain environmental objects, such as sponges, rocks, and coral (Arnold et al. 2014). Cryptic colorations can help frogfishes avoid predation and misidentification by prey (Arnold et al. 2014). Although the fishes in the family Antennariidae have diverse egg types and reproductive behaviors (Pietsch and Grobecker 1980; Pietsch and Grobecker 1987; Kuiter 1993; Liem 1998; Pietsch et al. 2009; Arnold et al. 2014; Arnold and Pietsch 2018; Pietsch and Arnold 2020; Bray and Gomon 2020), there are few detailed studies on their reproduction. According to the few reports in the literature (Mito 1960; Fries 1973; Pietsch and Grobecker 1987), the fishes in the family Antennariidae are divided into two types of species that lay an aggregated pelagic egg (e.g., *Antennarius* spp. and *Histrio* spp.) and demersal eggs (e.g., *Histiophryne* spp. and *Lophiocharon* spp.) (Friese 1974; Molter 1983; Fujita and Uchida 1985; Arnold et al. 2014).

Some species of Antennariidae demonstrate egg care behavior. For instance, *Lophiocharon* spp. cares for a demersal egg mass adhered to the flank of the parents (Pietsch and Grobecker, 1980). Nevertheless, the sex of the parent engaging in egg care remains unclear. For example, Pietsch and Grobecker (1980) reported that only male *L. trisignatus* could care for their eggs; however, Pietsch and Arnold (2020) later observed that *Lophiocharon trisignatus* females cared for their eggs. This confusion is likely due to the difficulty in determining the sex of frogfishes based on their external morphologies, although the females are usually are bigger than the males (Pietsch et al. 2013). Therefore, detailed studies of reproduction are needed to fully understand the early-life history of frogfishes exhibiting various reproductive strategies.

Here, we report the sequences of spawning and egg care behavior of marble-mouth frogfish (*L. lithinostomus*). This species inhabits coastal reefs of Indonesia and the Philippines and resembles algae-covered rocks (Arnold and Pietsch 2012; Arnold et al. 2014). There have been no reports on the species' reproductive behavior. In addition to the analysis of the reproductive behavior, we report the unique egg structure adapting to the egg care behavior by female parents and the morphology of newly hatched juveniles.

Moreover, we review the evolution of parental care behaviors and egg types, pelagic or demersal eggs, among frogfish families using limited available reports.

Materials and methods

Studied Fish

Ten adult *L. lithinostomus*, including four males with a standard length (SL) of 105.7 ± 15.6 mm and six females with an SL of 120.0 ± 10.1 mm, were obtained from the ornamental fish company (Kamihata Fish Industries LTD, Hyogo, Japan) and identified according to Pietsch (2004). The sexes of these individuals were determined by dissection when they died. Moreover, we used one female with an SL of 89.5mm bred by the Marine Science Museum, Fukushima. Thus, a total of 11 *L. lithinostomus* individuals were used in this study. They were individually identified by observers using their unique coloration and body morphology. The individuals used here have been registered as voucher specimens in the Marine Science Museum, Fukushima (registration number: AMF0053 and 0115).

Fishkeeping and reproduction

We used two water tanks in this study. Four fish (two males and two females) were held in a 1-m^3 water tank (1.0 × 1.0 × 1.0 m) at 26.0°C from 2016 to 2019, and six fish (two males and five females) were held in a 0.45-m^3 water tank (0.7 × 1.0 × 0.6 m) at 25.0 °C from 2018 to 2019. The fish were fed silver-stripe round herrings (*Spratelloides gracilis*) once daily. Between 2016 and 2019, 28 spawning events were observed.

Behavioral observation

When tracking behavior by the males toward the females was observed, we videotaped their reproductive behaviors and continued throughout the day until spawning was observed. Then, we recorded individuals that cared for eggs and the lateral surface of the body to which the eggs were attached.

We quantified the investment in offspring by parents by conducting detailed behavioral observation of four reproductive events (n = four females). Female parents attached spawned eggs to the left or right flank and exhibited fanning behavior toward the eggs using their dorsal fin. We count the fanning behavior for 10 min per day. This behavioral observation was conducted between 8:00 and 17:00. Lastly, statistical analysis was performed using R (R development core team 2020).

Morphology of Eggs and newly hatched juvenile

Because one female (137.8 mm SL) renounced an egg mass twice during observation, we used these the egg masses for measurements of egg morphology. The number of eggs per clutch was estimated by comparing the weight of fifty eggs and the weight of egg mass. Egg size was measured using a stereomicroscope (ZEISS SteREO Discovery.V12, ZEISS Research Microscopy Solution, Ltd., Germany). The egg mass was not consumed by other fish because we sampled them right after it was renounced.

We observed the morphology of newly hatched juveniles by fixing them in 10% formalin (n = 10). Their standard lengths were measured to the nearest 0.1 mm under a microscope, according to Hubbs and Lagler (1958), after they were overdosed with an anesthetic (MS-222, Wako Pure Chemical Industries). The number of fin ray of all fins of the newly hatched juveniles were counted and compared with those of adult fish. The observed individuals were deposited as voucher specimens (registration number: AMF0127). We classified the newly hatched fish as "juveniles," according to the developmental classification by Kendall et al. (1984) because their fin rays already attained the full complement right after hatching.

Review of the evolution of egg care in the frogfish family

To understand the functional role of egg care behaviors and morphology of eggs in *L. lithinostomus*, we performed a phylogenic analysis to examine the evolution of egg care behaviors in the frogfish family. The data on the reproductive characteristics, such as the size and number of eggs, egg types such as pelagic or demersal, and egg care behaviors, were collected from the primary literature. Eventually, we found a total of 14 reports on the reproduction of 14 frogfish species. The egg care behaviors were classified into four types according to Arnold et al. (2014): 1) Attaching to the body of the parent (A-type); 2) carrying within a pocket of parent (P-type); 3) guarding in the nest (N-type); 4) no egg care. In the species with A-type behavior, an egg mass is attached to the lateral side of the parent’s body and nurtured (Pietsch and Grobecker 1980; Pietsch and Arnold 2020). In the species with P-type behavior, the parents embrace their egg masses by curling their dorsal and anal fins (Pietsch and Grobecker 1987; Pietsch et al. 2009; Arnold and Pietsch 2018; Pietsch and Arnold 2020). Finally, in species with N-type behavior, the parents spawn specific substrates and care for their egg mass (Kuiter 1993; Liem 1998; Arnold et al. 2014; Bray and Gomon 2021). We also recorded the flank that the A-type and P-type parents carried their egg mass because we found a left-bias in egg caring on the flank in *L. lithinostomus*.

In addition, the presence or absence of egg care and egg types (pelagic or demersal) in major species relative to frogfishes were recorded. Reports on the presence or absence of egg care and egg types for coffinfish *Chaunax abei* (Mimori 2015), anglerfish *Lophius litulon* (Ishikawa et al. 2022), and batfish *Ogcocephalus nasutus* (Christie 2016) were obtained. Although footballfishes (e.g., *Himantolophus appelii*) are also a major species relative to frogfishes (Rabosky et al. 2018), we could not find reports on their reproduction because they inhabit the deep sea.

For the phylogenetic analyses, the phylogenetic tree of frogfishes and related species was obtained or modified from a previously published tree (Rabosky et al. 2018). This tree was estimated using RAxML and dated using treePL, and non-target species were excluded using the “droptip” function in R ver. 4.0.3 (R Core Team. 2014), package “ape” (Paradis et al. 2004). Ancestral states of the egg were assessed by using Mesquite Version 3.61 (Maddison and Maddison 2019) with the likelihood method in a Markov, k-status, 1-parameter model, using the modified tree. The reconstruction of ancestral states was conducted using 11 frogfish species and their related species because of their clear phylogenetic relationships (Rabosky et al. 2018).

Results

Spawning behavior

During observation, we successfully videotaped three spawning behaviors (Supplementary S1). Adult males and females were usually solitary in the water tank; however, multiple males began to follow females approximately seven days before spawning. When spawning began, a male ejaculated when a female released an egg mass from its gonopore. Before the egg mass was attached to the side of the female, the male left the female (Fig. 1a). A female attached the spawned egg mass to its side using its caudal fin to wrap it around the lateral surface of its body. The egg mass was not released into the water but transferred from the gonopore to the lateral surface of the body (Fig. 1b). A female protected an egg mass by covering it with her dorsal, anal, and caudal fins and occasionally fanned it with her dorsal fin (Supplementary S1).

Egg care and laterality of female parents

All egg care behaviors (n = 28) were performed only by the female parents, and no males cared for the eggs, based on the spawning events by seven females. While one of the females cared for its eggs on its right flank three times, 89.2 % of the egg masses (25 out of 28 spawning events) were cared for on the left flank of the parent, suggesting a significant bias towards egg care on the left flank (Pearson's chi-squared test χ^2_1 =37.605; $p < .001$). The number of fanning behaviors towards attached eggs varied considerably, ranging from 0 to 537 per 10 min with a mean of 201.9 ± 148.6; it increased from the day after spawning until fifteen days after spawning. However, after that, the number of fanning behaviors remained unchanged (Fig. 2). Hatching began 22 to 23 days after spawning; almost all juveniles hatched by days 27 to 28. The juveniles were scattered throughout the tank after hatching.

Morphological characteristics of eggs and newly hatched juveniles

The egg mass had an elliptic shape with a mean major axis of 91.2 mm and a mean minor axis of 62.5 mm. The eggs were round; their average length was 2.9 ± 0.3 mm, ranging from 2.4 to 4.1 mm (*n* = 50; from two egg masses from one female). The coloration of eggs was semi-transparent white just after spawning. Eye pigmentation was confirmed 11 days after spawning. Interestingly, demersal eggs had a unique structure, an s-shaped hook (Fig. 3). Although juveniles (*n* = 10) had yolk sacs just after hatching, all their fin rays already attained the full complement compared with the adults (Fig. 4). The standard length (SL) of the newly hatched juveniles (*n* = 10) was 5.70 ± 0.20 mm, ranging from 5.40 to 6.09 mm. They already had esca, which was the distinctive protuberance of this group used for predation as a lure; in addition, they could swim freely and often they could attach to substrates in the water tank.

Evolution of egg care among frogfish family

We obtained 14 anecdotal or qualitative reports in the field associated with egg care behaviors of frogfishes (Table 1). The species in Antennariinae do not display egg care behaviors and have pelagic eggs. In contrast, the species in Histiophryninae have three types of egg care behaviors, i.e., attaching to the body of the parent (A-type), carrying within a pocket (P-type), and guarding at the nest (N-type). The species in Histiophryninae have demersal eggs. Any form of egg care behavior has not been reported from the species related to frogfishes. Ancestral state reconstruction revealed that P-type and N-type egg care behaviors had been evolved independently from the no-care species in Histiophryninae (Fig. 5). In addition, A-type care had been evolved from a group within the P-type egg care (Fig. 5).

Discussion

In this study, we investigated the reproductive behavior of marble-mouth frogfish (*Lophiocharon lithinostomus*). We found that multiple males stalked breeding females before spawning, although only one male could participate in the spawning event. Similar behavior by adult males before spawning has been observed in several fishes and functions as mate guarding (Yokoi et al. 2016). Moreover, female filefish (*Rudarius ercodes*) move around and choose any male following them as their reproductive partners (Akagawa et al. 1998). Thus, the behavior of *L. lithinostomus* will be regarded as a process of mate choice or mate guarding. For *L. lithinostomus*, fertilization occurred immediately after the onset of spawning, and the male left the female before the entire egg mass was laid. The time difference between fertilization and spawning did not affect the fertilization rate, and most of the eggs were fertilized (Mori personal observation). Fertilized eggs were covered with a gelatinous curtain. Since the activity time of the sperms in the ovarian cavity fluid of females plays an important role in fertilization, the gelatinous membrane covering the eggs may play a role similar to the ovarian cavity fluid (Hayakawa and Munehara 2001).

We found that only females attached egg masses to their flanks and began to fan the egg masses after spawning. Also, regarding *L. trisignatus*, a related species to *L. lithinostomus*, only female parents care for egg masses (Pietsch and Arnold 2020). In many species in the frogfish family (Antennariidae), the females are larger and have larger abdomens than the males (Pietsch et al. 2013). Moreover, according to the reports on the sexual dimorphism in frogfishes, there are sexual differences in the number of dermal spinules in striated frogfish (*Antennarius scaber*) (Breder and Rosen 1966). However, no definitive sex differences have been found among frogfishes. Therefore, the study of the sex of the frogfishes caring for egg mass should be reconsidered.

More generally, the morphologies of newly hatched and developing teleost fishes are incomplete compared with adults (Mosher 1954; Ehrlich et al. 1976; Martin and Drewry 1978; Kendall et al. 1984; Satoh et al. 2017). In some species, the mouth and anus of the newly hatched larvae do not open, making feeding impossible (Satoh et al. 2017). Even with an open mouth, it would be difficult for the larvae to catch small prey with fin folds due to their low swimming ability (Satoh et al. 2017). Therefore, morphological ontogeny can highly influence early-life history, especially foraging ecology (Satoh et al. 2017). For example, the newly hatched larvae of frogfishes in the Antennariinae with pelagic eggs, such as *Histrio histrio*, do not have advanced organogenesis (Mosher 1954). In contrast, the newly hatched juveniles of *L. lithinostomus* already have open mouths, and all their fin rays attain the full complement, suggesting that they already have had robust foraging abilities immediately after hatching.

The dispersal of many marine fishes is commonly concentrated in egg or early larval stages (Barlow 1981; James et al. 2002). However, the dispersal ability of *L. lithinostomus* would be even lower than the pelagic egg group, such as *Histrio histro* and *Antennarius striatus,* because *L. lithinostomus* eggs do not float and are cared for by the female parents. Indeed, frogfishes in Histiophryninae have a smaller distribution area than those in Antennariinae and highly depend on the specific local area (Arnold and Pietsch 2012). In general, species with a low dispersal ability are more susceptible to local environmental changes (Baguette et al. 2012; Gonçalves et al. 2016). Recently, the deterioration of the coastal environment, including coral reefs, has been reported (Kennish 2002). Trends in species such as *L. lithinostomus*, which are presumed to be less tolerant of environmental changes, should be all eyes.

According to the ancestral state reconstruction, three types of egg care had evolved only among the species of Histiophryninae, which have demersal eggs, from non-egg care species. These findings suggested that egg care behaviors had co-evolved with demersal eggs. Demersal eggs are generally larger than pelagic eggs, and the fry can hatch with advanced organogenesis (Sargent et al. 1987; Araujo‐Lima 1994). Indeed, we found newly hatched juveniles of *L. lithinostomus* had highly advanced organogenesis. However, demersal eggs stick to substrates and thus, cannot change location. Therefore, demersal eggs will be exposed to high predation pressure unless they have a chemical defense or camouflage coloration (Eisner et al. 2000; Ruxton et al. 2004; Skelhorn

2015). The frogfishes of Histiophryninae may have evolved egg care as a counter-strategy to avoid the high predation pressure applied on demersal eggs.

The N-type egg care is thought to have evolved independently of these lineages. Also, we found that A-type egg care may have evolved from P-type egg care. It would be very unusual for diverse modes of egg care to evolve among closely related species. In species with P-type egg care, female parents embrace their egg masses by curling dorsal and anal fins into a pocket-like structure (Arnold et al. 2014). In contrast, egg masses are adhered to the flank for fishes with A-type egg care, such as *L. lithinostomus*. We found an s-shaped hook structure in the egg masses of *L. lithinostomus.* This structure may function as adhesives to attach the egg masses to the flank of female parents so that the eggs are tightly glued to the parental body. Indeed, when a female parent abandoned the glued eggs, she shook her body vigorously to remove the eggs. (Mori personal observation).

The number of eggs varies between egg care types. For example, speceis with N-type has the highest number of eggs at approximately 5000 eggs (n = 1 species), followed by A-type with 320 to 1311 eggs (n = 2 species) and P-type with 105 to 220 eggs (n = 3 species) (Table 1). The number of eggs by N-type fish will not be limited because the fish spawn egg masses on a substrate (Arnold et al. 2014). However, in A-type and P-type, the size of egg masses will be restricted by the flank of the parents because the fish attach or entrap their eggs to the body (Arnold et al. 2014). Although they are constrained by the number of eggs they can spawn in one reproductive event, the parents with A- and P-type egg care can migrate while raising their eggs. Additionally, the species with A-type egg care have more eggs than those with P-type egg care (Table 1). In general, oxygen demand, hence the frequency of fanning, increases with embryonic development (Abe and Munehara 2005). Unlike a P-type species, an A-type species can use its dorsal fins for fanning behavior, likely allowing it to hold more eggs, even though the number of eggs is limited by body size (Bagenal and Braum 1978; Warner 1984). Although these comparisons are constrained by the simplicity of the evolution of egg care modes among frogfishes, they suggest that the number of eggs will vary depending on the egg care mode.

Finally, we found that *L. lithinostomus* females preferred to use the left side of their bodies for egg care. Interestingly, they also appeared able to use their right flank for egg care because one female used the right flank. Lateralized (left-right asymmetry) morphology and behavior among fishes have been reported in many species (Takeuchi et al. 2016; Hori et al. 2017). Because left-biased egg care was not detected in species with A-type egg care (L: R = 3:2 from 5 reports for 3 species, Table 1) but *L. trisignatus* also displayed left-biased egg care similar to *L. lithinostomus* (Table 1), this tendency may have co-evolved with A-type egg care. However, we could not determine why *L. lithinostomus* females used their left side for egg care; this interesting phenomenon requires further morphological or evolutionary validation.

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Table 1. Comparison of reproduction modes for frogfish family Antennaridae.

Species	Egg size (mm)	The number of eggs	Type of egg	Parental care mode	Laterality of body side used for egg care	Reference(s)
Antennariinae						
Antennarius striatus	0.6	89635	pelagic	No care	–	Fujita and Uchida 1959
Antennarius nummifer	0.7	73000-288000	pelagic	No care	–	Piestch and Grobecker 1987
Histrio histro	0.7	48800	pelagic	No care	–	Ray 1961
Histiophryninae						
Histiophryne cryptacanthus	3.6–4.2	115	demersal	P-type	L:R = 1:0 (*n* = 1 report)	Piestch and Grobecker 1987
Histiophryne bouganivilli	3.2–3.9	105	demersal	P-type	L:R = 1:1 (*n* = 2 reports)	Piestch and Arnold 2020
Histiophryne psychedelica	3.0–4.0	220	demersal	P-type	L:R = 1:1 (*n* = 2 reports)	Piestch et al. 2009; Piestch and Arnold 2020
Histiophryne narungga	No data	No data	demersal	P-type	No data	Arnold and Piestch 2018
Lophiocharon trisignatus	3.2–3.6	650	demersal	A-type	L:R = 4:0 (*n* = 4 reports)	Piestch and Grobecker 1980; Piestch and Arnold 2020
Lophiocharon lithinostomus	2.1–4.1	320-1311	demersal	A-type	L:R = 26:3 (*n* = 3 reports)	I.O.P Diving News 2001; Piestch and Arnold 2020; Present study
Echinophryne crassispina	No data	150	demersal	N-type	–	Liem 1998; Bray and Gomon 2021
Echioophryne reynoldsi	No data	No data	demersal	N-type	–	Bray and Gomon 2021
Rhycherus filamentosus	5.0	5000	demersal	N-type	–	Kuiter 1993; Bray and Gomon 2021
Porophryne erythrodactylus	No data	No data	demersal	N-type	–	Arnold et al. 2014
Phyllophryne scortea	No data	No data	demersal	N-type	–	Bray and Gomon 2021

A-type: attaching to the body of the parent, P-type: carrying within a pocket of parent, N-type: guarding in the nest.

Figure and legends

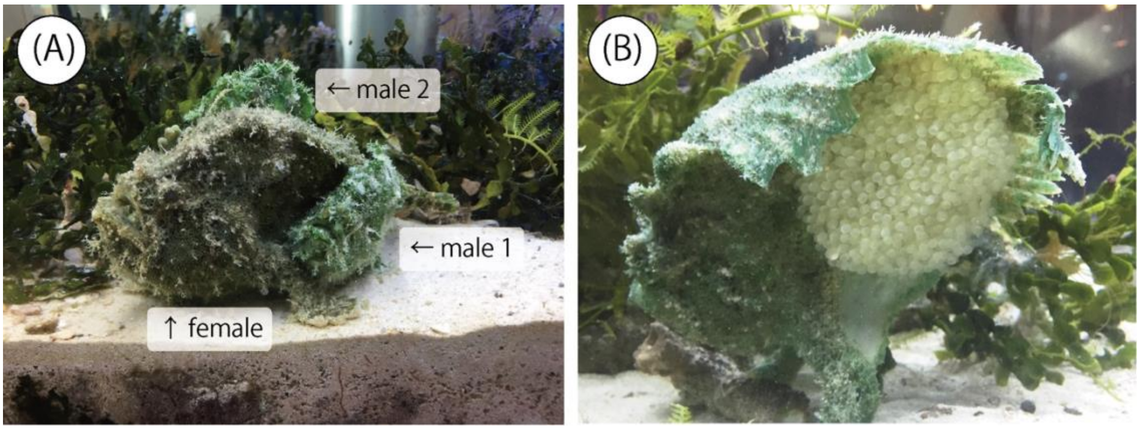

Figure 1. A Photograph of a gravid female (dark brown color) marble-mouth frogfish *Lophiocharon lithinostomus*) surrounded by two courting males (green color). **B** Female marble-mouth frogfish with egg mass attached on the left side of bodies.

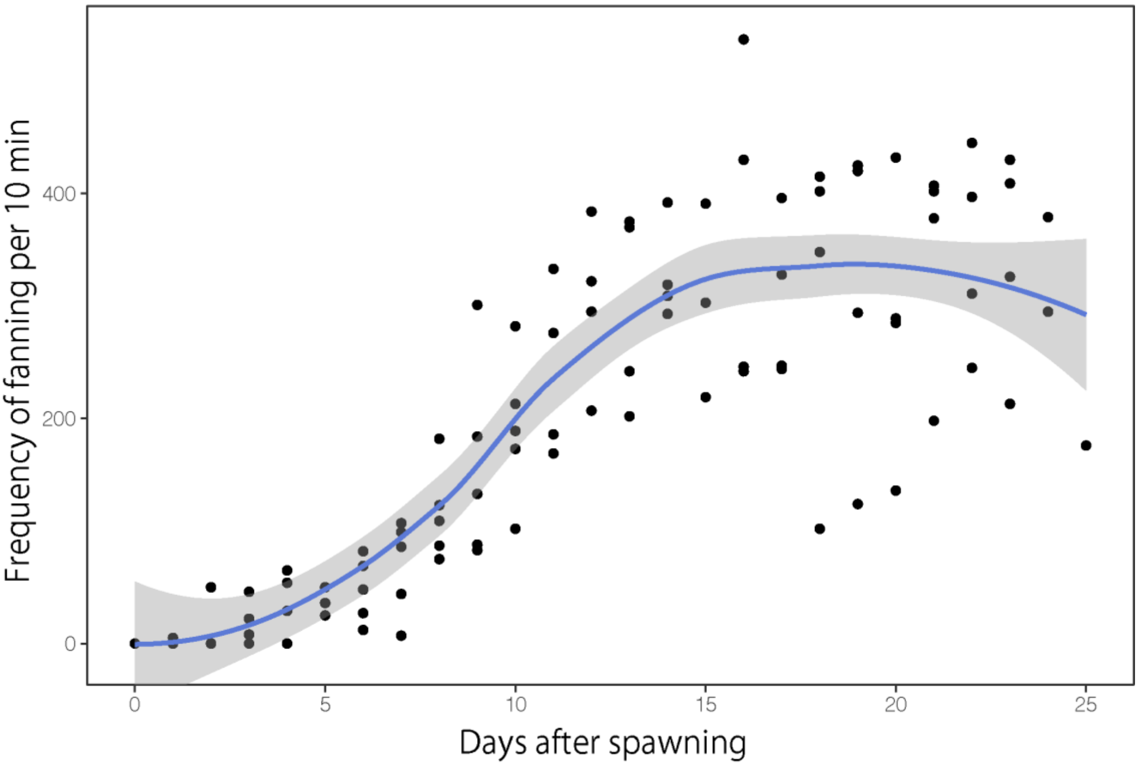

Figure 2. Relationship between days after spawning and the frequency of fanning behavior by female marble-mouth frogfish *Lophiocharon lithinostomus.* A solid line and a grey area indicate the loess regression and 95% Confidence interval, respectively.

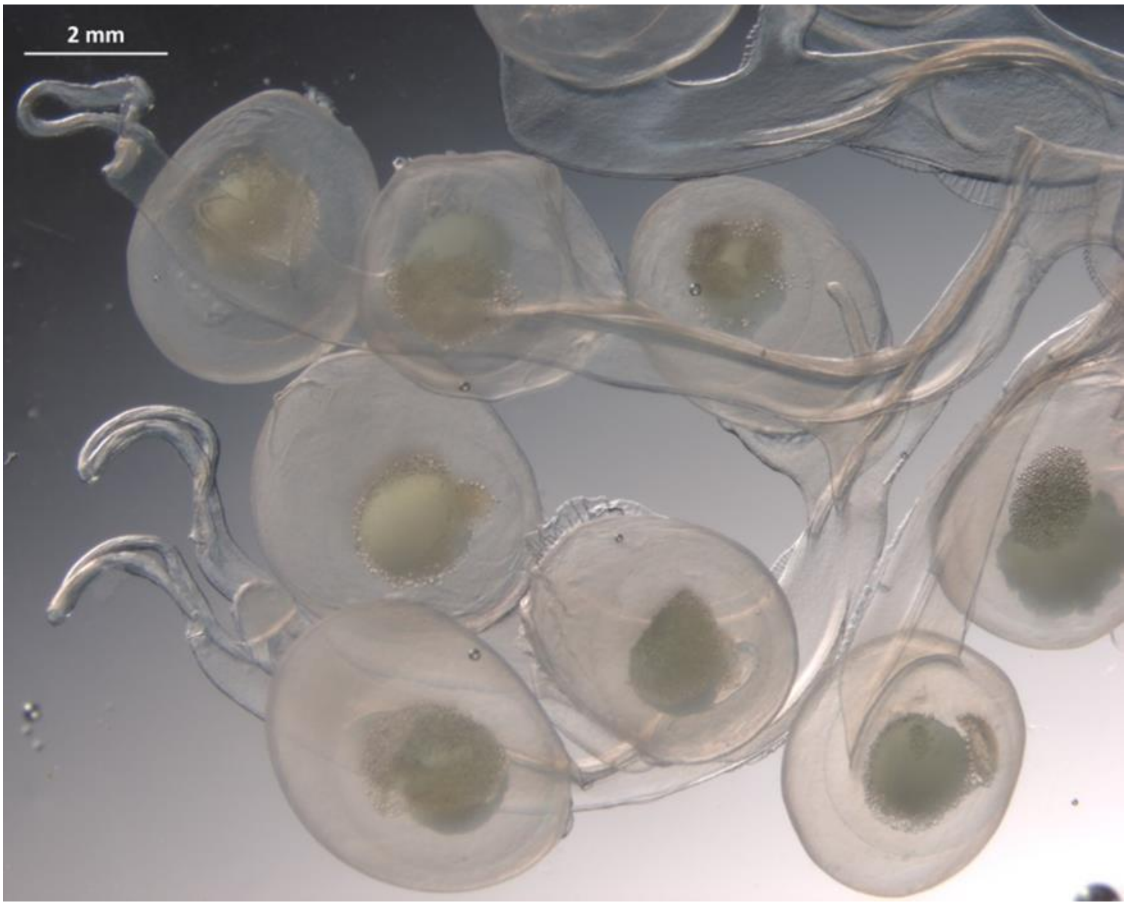

Figure 3. Photograph of fertilized eggs and gelatinous curtains with S-shaped hooks surrounding the eggs. White scale bar: 2 mm.

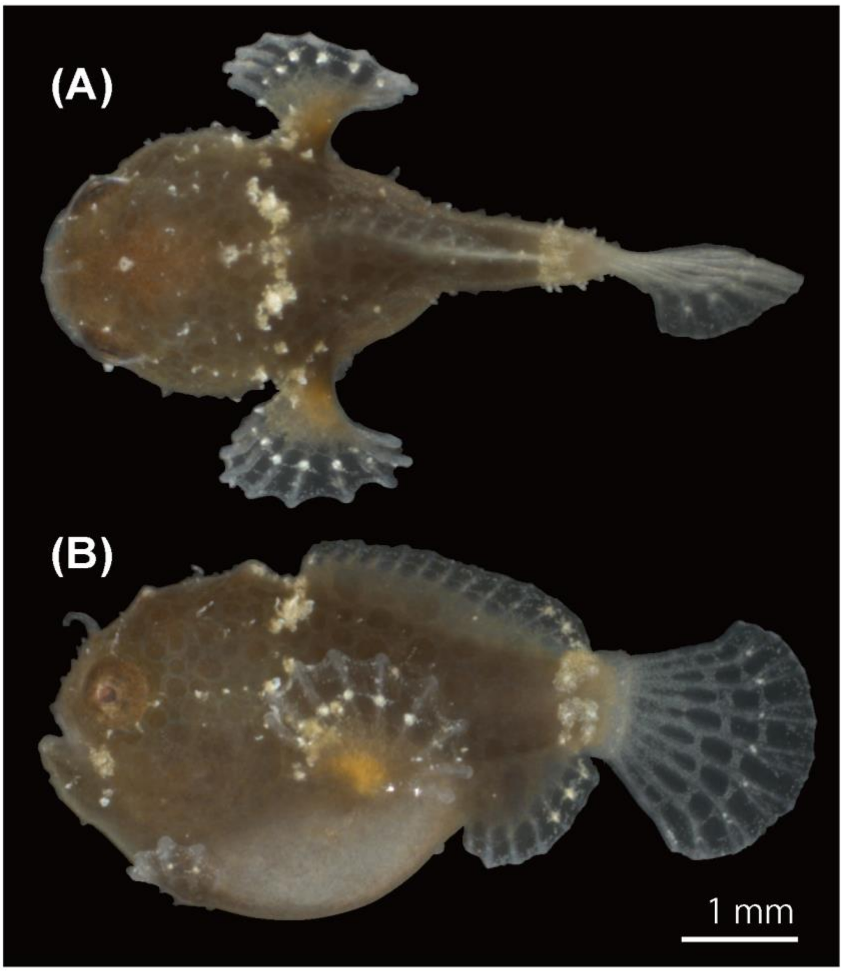

Figure 4. Photographs of a marble-mouth frogfish *Lophiocharon lithinostomus* juvenile just after hatching. **A** Overhead view. **B** Lateral view. White scale bar: 1 mm.

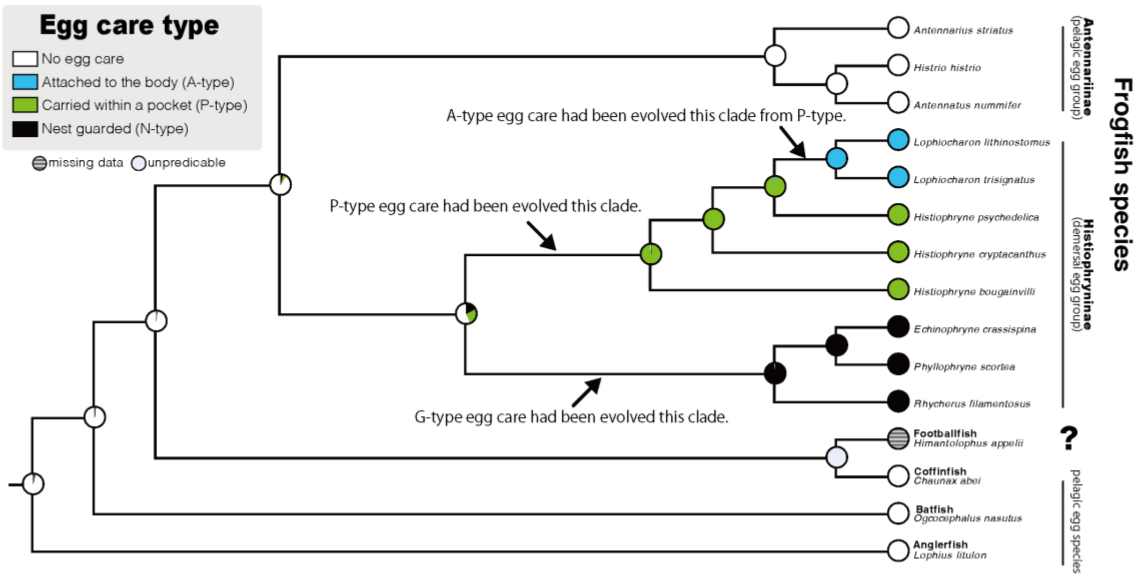

Figure 5. Evolution and phylogenetic distribution of the forms egg care in Antennariidae and related species. The ultrametric tree was modified from Rabosky et al. (2018). Egg care behaviors were classified into four types: 1) Attaching to the body of the parent (blue); 2) carrying within a pocket of a parent (green); 3) guarding in the nest (black); 4) no egg care (white).

ESM S1

Spawning and fanning behavior of marble-mouth frogfish *Lophiocharon lithinostomus.*