

Original Article

Burrow residence of males and females of the mudskipper *Periophthalmus modestus* during reproduction

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Abstract: Pre-spawning reproductive behavior on the mudflat surface was reported previously for the mudskipper, *Periophthalmus modestus*, but it remains unknown how a pair spawns and fertilizes eggs within the spawning chamber of a burrow. In this study, we recorded reproductive behaviors from burrow entry of a pair to the departure of a female. In those cases, where spawning was suspected from a drastic reduction in the distension of the female's belly (N = 4), the female remained inside a burrow for 240-350 min without coming out onto the mudflat surface. In contrast, the male often but irregularly emerged from the burrow. In one case, a second male entered a burrow 169 min after the burrow entry of a female, resulting in her desertion from the burrow without spawning. During burrow cohabitation of the pair, burrow openings were often clogged by mud from inside. After the female left the burrow, the male remained inside the burrow for variable periods. Then, the male guarded the burrow for about one week. Upon termination of the burrow guarding, the male began excavating a new burrow near the previous one or repaired the burrow that he had guarded. A hypothesized scenario is proposed for the reproduction of this species for further investigation.

Article history:

Received 28 June 2020

Accepted 21 October 2020

Available online 25 December 2020

Keywords:

Courtship

Mudskipper

Periophthalmus modestus

reproduction

Introduction

Reproduction at the air-water interface is a formidable task for animals that are in the process of habitat expansion from water to land. Although amphibians show an impressive variety of reproductive strategies from laying eggs in subterranean, terrestrial or arboreal nests to oviposition to female's back or eggs retained in oviduct (Wells, 2007), reproduction at the air-water interface is known in only 104 among over 35,000 species of extant fishes (Ishimatsu et al., 2018). It requires the preparation of a nest or a burrow, or exploitation of a spawning site that protect small anamniotic eggs from desiccation while securing a sufficient supply of oxygen.

Mudskippers (Oxudercinae, Gobiidae) are among those rare examples. They store a volume of air in the spawning chamber of the burrow that they excavate in muddy substratum so that embryos develop in air, not in water. This is presumably to protect embryos from nearly anoxic environments prevailing in the

subsurface sediments of muddy shores (Little, 2000). The air storage in a spawning chamber has been confirmed for only four species of mudskippers, *Boleophthalmus pectinirostris* (Toba and Ishimatsu, 2014), *Periophthalmodon schlosseri* (Ishimatsu et al., 1998, 2009), *Pn. septemradiatus* (Mai et al., 2019), and *Periophthalmus modestus* (Ishimatsu et al. 2007), but it is probably prevalent among other mudskippers too (Ishimatsu and Graham, 2011; Martin and Ishimatsu, 2017). Apart from the air-filled spawning chamber, mudskipper burrows are filled with extremely hypoxic water (Gordon, 1995; Ishimatsu et al., 1998, 2007).

Periophthalmus modestus is distributed along the Pacific coast from the Tokyo Bay to Okinawa in Japan (Takita and Ishimatsu, 2015). In mudflats in the Ariake Sea of the Kyushu Island, the breeding season of *Ps. modestus* lasts from May to August (Ishimatsu et al., 2007), during which the fish breeds several times (Soyano et al., 2008a). Males dig J shaped

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burrows with two or three openings on the mudflat surface (Kobayashi et al., 1971). To attract females, male fish change body color from grey to pinkish, raise and wave the tails, and make jumps with fins stretched. When a gravid female approaches a male, he leads her to his burrow by slow, wiggling movements (Matoba and Dotsu, 1977; Baeck et al., 2008). The spawning chamber is located at the terminus of a burrow and it is the site of oviposition (Kobayashi et al., 1971; Matoba and Dotsu, 1977; Ishimatsu et al., 2007). After spawning, the female leaves the burrow, but the male guards it for about a week. During low tide when burrow openings are uncovered by water, the burrow-guarding male gulps fresh air and adds it into the spawning chamber. During high tide when the burrow is inundated, the male remains inside the burrow. When the eggs are competent to hatch, the male expels the air from within the spawning chamber at nighttime high tide and submerges the eggs for hatching (Ishimatsu et al., 2007).

One of the major remaining questions regarding mudskipper reproduction is whether the spawning chamber is filled with air or water at the time of spawning and fertilization. Presently, there is conflicting circumstantial evidence in this regard. The facts that support aerial spawning and fertilization include: (1) Air was collected from the burrows of courting male *Ps. modestus* (Ishimatsu et al., 2007). Because there is only one clutch in a spawning chamber (Kobayashi et al., 1971), these burrows are inferred to contain no eggs, (2) the presence of air was confirmed by direct video-recording in the spawning chambers of *Pn. schlosseri* burrows where no eggs were found (Ishimatsu et al., 2009), and (3) the amphibious blenny *Andamia tetradactyla* spawns and fertilizes eggs in air-exposed rock crevices (Shimizu et al., 2006), the only known example for subaerial piscine reproduction. On the contrary, the following observations are in favor of oviposition in water: (1) A preliminary observation showed that a male *Ps. variabilis* probably added air only after spawning and fertilization in an aquarium (Rupp, 2018); and (2) *Pn. schlosseri* can weigh more than 200 g (Ishimatsu

et al. 1999), which makes it unlikely for those individuals to cling onto the ceiling of a spawning chamber filled with air for spawning and fertilization.

This study aimed to determine the burrow residence duration of the male and the female of *Ps. modestus* during spawning and its relationship with tide, with the hope that the data will benefit future investigations to answer the question. The obtained knowledge would also help us understand the possibilities and limitations of fish reproductive strategies at the air-water interface, and may also shed light on the earliest steps of evolution of vertebrate reproduction from water to land.

Materials and Methods

This study was conducted in the mudflat along the Fukushoe River, Ogi City, Saga Prefecture, Japan (33°12'22.24"N, 130°13'41.97"E) in May and June, 2016. Reproductive behavior before, during and after the cohabitation in a burrow was recorded and analyzed for five pairs of *Ps. modestus* (4 complete cycles and 1 incomplete cycle, see below). Even though the total number of observations was more than 50, the rest had to be canceled because female fish did not enter a burrow or stay inside for only a very short period. During daytime low tide, a video camera (Sony FDR-AX100E 4K, Japan) was set on a concrete pier to record the timing of male and female entry into a burrow and their residence time within the burrow, and the frequency of male emerging from the burrow. Video recording started at 1015 to 1330 and lasted for 380 to 410 min, except one case (Pair 2) in which recording lasted only 250 min. A digital camera (Nikon D800, Japan) with a telephoto lens (Nikon AF-S VR 70-300mm, Japan) was used for capturing selected events or changes in burrow openings during burrow residence.

The spawning was assumed to have occurred if the following two conditions were satisfied; (1) A male continued guarding his burrow after a female had left; and (2) the distension of the female's abdomen obviously decreased between entry into and exit from a burrow (Fig. 1). Larval hatching was assumed to have occurred when a male fish stopped guarding his

Table 1. Burrow residence of male and female *Periophthalmus modestus* in relation to tide.

Pair	Date	Lunar age	Emersion of the burrow opening (A)	Male entry	Female entry	Female departure (B)	Inundation of the burrow opening (C)	(A) to (C) (min)	(B) to (C) (min)	Spawning
1	25/5	18.7	13:14	13:16	13:16	18:37	22:18	544	221	Yes
2	29/5	19.7	15:21	15:19	15:19	18:09	2:32 (30/5)	671	503	No
3	7/6	2.4	12:34	13:22	13:22	18:07	20:33	479	146	Yes
4	8/6	3.4	13:10	13:44	13:45	19:30	19:32	382	2	Yes
5	15/6	10.4	6:19	10:16	10:17	14:17	16:48	629	151	Yes

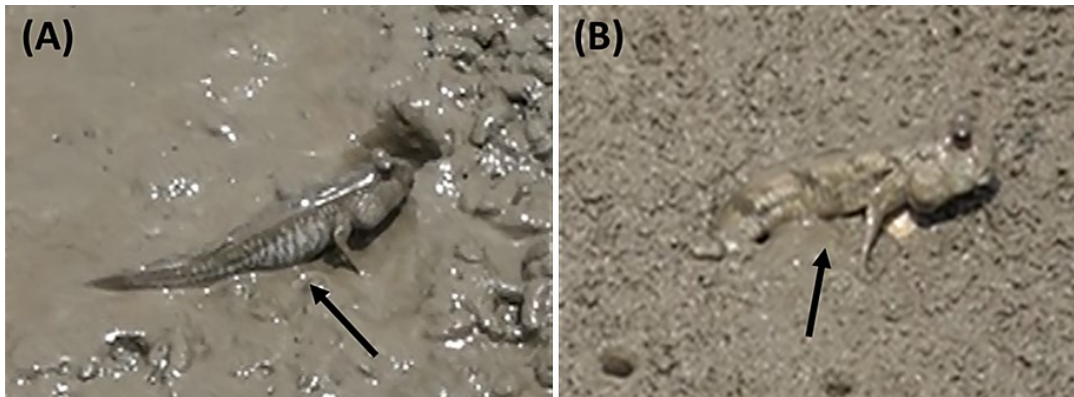


Figure 1. Comparison of the belly distension (arrows) before and after burrow residence of a female *Periophthalmus modestus*, suggesting that spawning occurred. (A) The image was taken prior to burrow entry of the female. (B) The image was taken at the exit of the female after spending 285 min within the burrow. The two still images were cut from the video footage recorded on June 7, 2016.

burrow and started to dig a new burrow or repair the burrow that he had guarded.

When a gravid female approached a male showing courtship display near his burrow, the male fish escorted the female to an opening of the burrow. Then, the male usually entered the burrow first and the female followed within 2 min, except in one pair in which the female entered a burrow first (Fig 2. and Table 1). On the way to the burrow opening, the female often stopped and stared at the male's courtship display. In some other cases, the male bit the female's operculum, which caused the female to follow the male again. In still some other cases, the male fish came out from the burrow which he had entered, and escorted the female again while she hesitated to enter. In many cases, a female followed a male at some distance from him and ran away for food or another male.

Results

Four pairs were successfully recorded from the entry of the pairs until the female's departure (Pairs 1 and 3-5, Fig. 3). Spawning was presumed to have occurred

in these four cases. In these cases, females stayed continuously inside the burrow for 245 ± 38 min (SD) (Fig. 3). In contrast, the male came out onto the mudflat surface and returned into the burrow after usually less than a minute but as long as 10 min in some cases (Fig. 3). When a male was outside the burrow, he remained around the openings and protected his burrow from conspecifics and crabs. The male occasionally fed. The frequency of male's emersion largely varied between individuals (Table 2). The males of Pairs 3 and 4 showed prolonged sojourn in the burrows toward the later phase of the female's burrow residence, while no such pattern was observed for the males of Pairs 1 and 5. Fish clogged one or more burrow openings during the female fish confinement (Fig. 4). Recording of one pair (Pair 2, Fig. 3) was terminated when another male entered the burrow 169 min after the female entered the burrow. The female deserted the burrow in one min (i.e., 170 min after her entry).

After presumed spawning in Pairs 1 and 3-5, the males of Pairs 1, 4 and 5 mostly remained inside the burrow until the observation was terminated due to

Table 2. Burrow confinement and emersion of male and female *Periophthalmus modestus*.

Pair	Male		Female	
	Period of burrow*	Number of emersions*	Period of burrow	Number of emersions
1	178	52	309	0
2	119	13	170	0
3	239	10	285	0
4	265	35	345	0
5	178	15	240	0

*During the female's residence in a burrow.

Table 3. Period of egg incubation in *Periophthalmus modestus*.

Pair	Spawning date	Hatching date	Incubation days
1	May 25, 2016	June 3, 2016	9
3	June 7, 2016	June 15, 2016	8
4	June 8, 2016	June 15, 2016	7
5	June 15, 2016	June 22, 2016	7



sunset (Male 1 and 4) or rising tide (Male 5), but the burrow until sunset despite that spawning did not

Figure 2. Composite photographs of the on-surface behavior of a male and a female *Periophthalmus modestus* during presumed spawning inside a burrow. (A) After completing excavation of a burrow, the male protrudes the head, searching for a female; (B) the male turning to a nuptial color (front) is trying to attract the female (back) with distended abdomen; (C) the male is entering the burrow (the female about to enter the burrow); (D) the female is entering the burrow (after male). Note that the size of the female's belly is nearly as large as or larger than the size of the burrow opening; (E) The female came out from the burrow and is leaving it; (F) The male is restoring the burrow for the next spawning round (pair 3).

male of Pair 3 repeatedly emerged (Fig. 5). Thereafter, all the males guarded the burrow for 7-9 days (Table 3). Upon termination of the burrow guarding, the males either dug a new burrow or repaired the burrow that he had guarded. Male 2 remained inside the

happen.

Discussions

The most noticeable difference in burrow residence between males and females is that females remained

Table 4. Duration of nest residence and spawning in gobies observed under captivity.

Species	Nest residence before spawning	Spawning (h)	Nest type	Number of eggs per extrusion	Fecundity	Ref
Gobiidae						
<i>Bathygobius soporator</i>	>0.5 h	3-9	On surface	10-30	15000, 17500, 18000	(1)
<i>Glossogobius olivaceus</i>	10 h to 6 d	3.5-9.5	On surface	No data	60800	(2)
<i>Leucopsarion petesii</i>	14-22 d	1-2.5	Burrow	3-6	No data	(3)
<i>Pterogobius elapoides</i>	1-2 d	1	On surface	No data	1800	(4)
Eleotridae						
<i>Eleotris oxycephala</i>	No data	2-3.75	On surface	3-10	37800, 178000	(5)
Odontobutidae						
<i>Odontobutis obscura</i>	12 h	6-12	On surface	5-10	960	(6)

(1) Tavolga 1954; (2) Senta and Wada 1970; (3) Akiyama and Ogasawara 1994; (4) Dôtu and Tsutsumi 1959; (5) Matsuo and Takahama 2001; (6) Mashiko 1976.

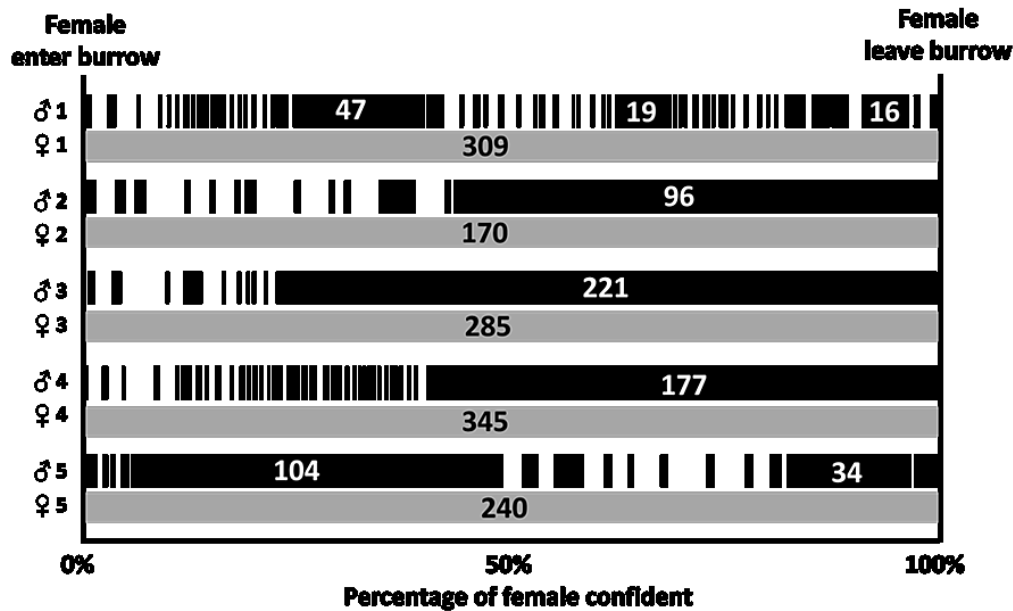


Figure 3. Burrow residence of male and female *Periophthalmus modestus* from female's entry into the burrow (on the left) to the female's departure from the burrow (on the right). Five pairs were recorded. The colored bars represent time when a male and a female remained inside a burrow. The numbers in the bars indicate the duration of long confinement (min). No spawning occurred in the pair 2.



Figure 4. *Periophthalmus modestus* clogged a burrow opening from inside (red arrows). (A) fish started to clog the opening; (B) the burrow opening was partly clogged; (C) the burrow opening was fully clogged.

inside a burrow once they entered it, whereas males repeatedly came out and stayed on the mudflat surface for a variable, but usually short, period of time before returning into the burrow. In two cases (Pairs 3 and 4),

the male's burrow residence became prolonged and continuous in the later phase of female's residence (Fig. 3), and extended beyond the time of female's deserting the burrow (Fig. 5). The pattern is somewhat

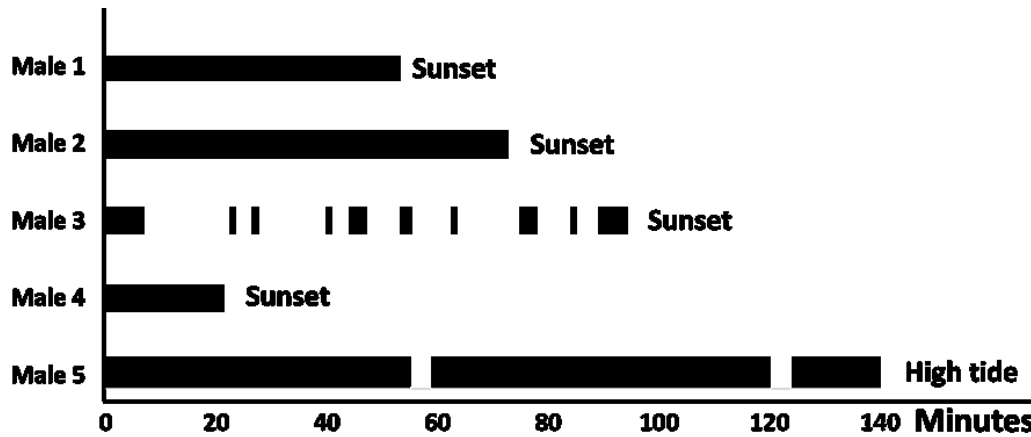


Figure 5. Burrow residence of male *Periophthalmus modestus* after a female left his burrow (min). The observations were terminated at the sunset (pair 1 to pair 4) or at mudflat inundation (pair 5).

different in Pair 1, in which the male repeated short sojourn inside his burrow interspersed with three occasions of longer (16 to 47 min) residence. The male of Pair 5 showed a long (ca. 100 min) burrow residence in the early phase, followed by irregular pattern of emersion and confinement. This male remained confined more than 50 min after the female's departure. Since direct evidence is currently lacking about the reproductive behavior of any mudskipper within a burrow, we propose a hypothesized scenario for reproduction of *Ps. modestus* at the end of this section for the future study, on the basis of available information on goby reproduction and fish reproduction in general.

Gobies typically attach adhesive eggs on the underside of rocks, depressions, or on the ceiling of enclosed spaces, including burrows (Miller 1984). These spawning sites are usually prepared by a male prior to his nuptial display. Following male-female interactions, a couple will enter the nest or the burrow and remain inside for variable periods of time before spawning (Table 4). It is not always clear whether either of the couple would make short excursions to outside after their entry into the nest/burrow. The actual time of spawning in the nest/burrow lasted for 1 to 9.5 h, during which the female extruded 3-30 eggs from the urogenital papilla at each spawning bout. The tip of the urogenital papilla presumably has a tactile sensory function, because it has been repeatedly observed that the female avoided spawning over the already laid eggs and oviposited only on the bare

surface (Tavolga, 1954; Matsuo and Takahama, 2001). When the spawning was completed, the female left the nest/burrow while the male continued to stay in it for parental care.

The testis of gobies is accompanied with a unique accessory gland called sperm duct gland or seminal vesicles (Chowdhury and Joy 2007; Mazzoldi et al., 2011). The sperm duct gland has been reported for 8 species of mudskippers by Fishelson (1991) and is also confirmed for *Ps. modestus* (Tran Xuan Loi, Graduate School of Fisheries and Environmental Sciences, Nagasaki University, personal communication). The physiological roles of the sperm duct gland have not been fully elucidated (Chowdhury and Joy, 2007), but one probable function is to prolong sperm availability for egg fertilization by slowly releasing sperm from within a viscous secretion from the gland, which is glued onto the nest/burrow surface. Male gobies were observed to rub the urogenital papilla before, during and after oviposition by a female fish (Tavolga, 1954; Marconato et al. 1996; Ota et al., 1996). Hence, the male goby does not need to accompany the female throughout oviposition that may last for several hours (Table 4), but can guard the nest or burrow from intruders.

Prior to spawning, oocytes must go through the process of final oocyte maturation, accumulating yolk materials and increasing the size by water uptake, and are shed from the ovarian follicles (ovulation) (Kagawa, 2013). Gonadal development in fish is regulated by environmental factors such as

temperature, light, lunar cycle and social interaction, which generate endocrine changes that drive the gonad maturation (Pankhurst and Porter, 2003). The process of final oocyte maturation to ovulation takes 1.5 to 2 days in marine fishes (Alvariño et al., 1992; Shein et al., 2004; Shiraishi et al., 2008). In addition, the process of ovulation itself takes several hours up to 24 h (Alvariño et al., 1992; Goetz and Garczynski, 1997; Soyano et al., 2008b). Ovulated eggs are retained within the female's body but must be released and fertilized within a certain period of time. Otherwise, they degenerate and are progressively resorbed (Samarin et al., 2015). The duration of egg fertility inside female's body varies largely among species and temperature, ranging from 30 min to 12 h for most tropical and temperate species but it can be as long as 30 to 40 days in cold water species (Samarin et al., 2015). Larval hatching was almost completely inhibited when ovulated eggs were fertilized 12 h after ovulation in the sevenband grouper (Soyano et al., 2008b).

We hypothesize the following scenario for reproduction of *Ps. modestus* within its burrow. (1) Gravid females have nearly or completely finished ovulation before burrow entry. We presume this because the total duration needed for final oocyte maturation and ovulation is much longer than the observed period of female's residence in a burrow (240 to 350 min). Therefore, final oocyte maturation must have been terminated and ovulation may have been partly or fully completed before female's entry into a burrow. (2) We assume that the spawning chamber is filled with air prior to spawning for the following three reasons. First, a female presumably cannot maintain metabolism during spawning that may last for several hours if the burrow including the spawning chamber is filled with hypoxic water (water P_{O_2} of *P. modestus* burrow is 1.5 kPa, only 7% of air saturation, Ishimatsu et al., 2007). Yet, we cannot preclude the possibility that the female will swim up to the air-water interface of the burrow and gulp air. Second, fish embryos are generally sensitive to hypoxic conditions (Hassel et al., 2008; Elshout et al., 2013; DePasquale et al., 2015). Rombough (1988)

summarized early data on metabolic intensity during early developmental stages and showed that mass specific oxygen consumption can be high during cleavage, suggesting high sensitivity to hypoxia. On the other hand, how hypoxia affects sperm motility and fertilization success is scarcely known for fishes (Dzyuba and Cosson, 2014). Third, burrow water is thought to contain high concentrations of ammonia (Ip et al., 2004), which disrupts embryogenesis in fishes (Bardon-Albaret and Saillant, 2016). (3) The time between female's entry to spawning is estimated to be at least 170 min. This is based on the observation of the Pair 2, in which the female left the burrow without spawning due to the entry of an intruder male. This would allow the time for spawning ranging from 70 to 180 min, i.e. the difference between female's burrow residence time except the Pair 2 (240 to 350 min, Table 2) and the presumed time before spawning (170 min). The fecundity of *Ps. modestus* was reported as 5200 ± 765 eggs/burrow (Ishimatsu et al. 2007). This means 29 to 74 eggs spawned per min. (4) A male fertilizes the eggs during, after spawning, and even before spawning as in other gobies (Ota et al., 1996). A male *Andamia tetradactyla*, the only fish for which spawning and fertilization in air is known, rubs the urogenital papilla on the eggs after a female finishes spawning (Shimizu et al., 2006). The sperm duct gland was found in some blennies (Chowdhury and Joy, 2007), yet it remains unknown if *A. tetradactyla* has it or not. The unpublished observation that *Ps. variabilis* added air only after spawning in an aquarium needs confirmation.

Clogging of burrow openings after female's entry for reproduction was reported for gobies, for example, *Leucopsarion petersii* (Akiyama and Ogasawara 1994) and *Valenciennesa longipinnis* (Takegaki and Nakazono 1999). Hong et al. (2007) stated that those burrows of the mudskipper, *Boleophthalmus pectinirostris*, always contained a spawning chamber and the eggs therein when the openings were clogged by mud.

Acknowledgements

We are grateful to S. Noma and M. Murata of the

Institute for East China Sea Research, Nagasaki University for their help during the field study in Ogi City, Saga.

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