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Roles of Environmental Cues for Embryonic Incubation and Hatching in Mudskippers

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Synopsis Reproduction on mudflats requires that eggs are protected from different environmental challenges during development and hatch when environmental conditions are favorable for survival of juveniles. Mudskippers are air-breathing, amphibious gobies of the subfamily Oxudercinae, and one of a few vertebrates that reside on mudflats. They excavate burrows in mudflats and deposit eggs in them. However, these burrows are filled with extremely hypoxic water, in which eggs could not survive. To secure embryonic development within their burrows, the burrow-guarding parental fish (a male or mating pair) store fresh air in an egg chamber, located near the bottom or at mid-depth in a burrow, by transporting mouthfuls of air during each low tide. The Japanese mudskipper, *Periophthalmus modestus*, is the best-studied species regarding reproductive strategies. The air-supplying behavior appears to be predominantly governed by the oxygen levels within egg chambers, but also by some other factor that is possibly related to the tidal cycle. When embryonic development is complete, the burrow-guarding male *P. modestus* removes the air from the egg chamber and releases the air outside the burrow on a nocturnal rising tide. Consequently, the tide floods the egg chamber and induces hatching. Because *P. modestus* eggs only have a 5–6 day window for hatching competence, the male's initial selection of the position for the burrow in the intertidal zone and the timing of spawning relative to the tidal cycle are both important factors in hatching success. This is particularly crucial for those burrows in higher intertidal zones, which may be reached

only by high spring tides. Not much is known for other mudskippers, but it is likely that they also employ similar reproductive strategies. The objective of this review is to summarize available information on reproductive strategies of mudskippers, and to discuss future directions to better elucidate mechanisms and adaptive significance for the reproduction of mudskippers. Further comparative studies with both mudskippers and other oxudercine gobies dwelling mudflats could shed new light on how vertebrates solved problems of reproduction when they expanded habitats to environments in an air-water interface.

Introduction

Intertidal spawning is known from nine orders of teleosts comprising 25 families, of which 14 families include resident species; i.e., fishes that live exclusively or during a part of their life in the intertidal zone after settlement (DeMartini 1999). Most resident intertidal fish deposit demersal eggs on surfaces that may become exposed to air during low tide. Parental care is seen in many of them; however, the type of shore where eggs are deposited is a decisive determinant of the type and extent of this behavior. Fishes that spawn on sandy shores lack parental care because there is no place for the adult to safely remain near the brood (e.g., the grunion *Leuresthes tenuis* and the capelin *Mallotus villosus*, Martin et al., 2004). In contrast, rocky

shores offer microhabitats that are humid and that shelter (i.e., under boulders, rock crevices, or rock surface covered by draping macroalgae) the egg-tending fish during periods of emersion. Accordingly, blennies, stichaeids and other species in these habitats often show parental care of eggs and some endure periods of exposure to air during low tide by breathing air (Coleman 1999; Martin and Bridges, 1999). The dynamic nature of muddy shores precludes the option of laying eggs on the surface: Eggs deposited on the surface of a mudflat risk being buried by sediment or carried out to sea by a receding tide.

Egg incubation in the intertidal zone is thought to offer both benefits and costs (DeMartini 1999). Benefits include protection from predators of both adult spawners and the offspring, and possibly a higher rate of development due to increased availability of oxygen and favorable temperatures. The costs include the time and energy expended in parental care and the physiological stress of desiccation, deterioration of water quality and overheating of egg-tending parents and embryos in the harsh intertidal environment. When intertidal incubation of eggs takes place in isolated pools or burrows, there is less contact between the eggs and the flow of tidal water. Intertidal egg broods are also subject to predation, both by egg-guarding adults (filial cannibalism) and by terrestrial predators.

Gobies occur mainly in marine habitats, but some live in fresh and brackish environments. They are largely bottom dwellers, lay demersal eggs, and show parental care of the eggs

(Patzner et al., 2011). Of the 1875 marine gobies, 54 species occur in rocky intertidal habitats (Chotknowski et al 1999). The total number of gobies inhabiting in mudflats is not known: The subfamily Oxudercinae, which includes mudskippers, has about 40 species (Murdy 2011a). There are also several mudflat-dwelling species in Amblyopinae (Murdy 2011b). Gobies usually deposit a single layer of demersal eggs on the undersurface of a solid object such as a stone or dead shell, or in a burrow. Deposition of eggs in burrows is known for species in Gobiinae, Gobionellinae and Oxudercinae (Breder and Rosen 1966; Gaisner 2005; Takegaki and Nakazono 1999a; Takegaki 2000), and likely occurs in other subfamilies. Parental care by males is the most common among gobies, but care by the female alone and biparental care both also occur (Blumer 1982). The forms of parental care by gobies include guarding eggs, nest/burrow building and/or cleaning of the substrate, fanning, internal gestation, and removal of dead or diseased eggs. The majority of gobies are iteroparous (i.e., reproducing more than once during a life span, Breder and Rosen 1966; Miller, 1984). Hatched larvae usually lead a planktonic life-stage for variable periods of time ranging from two weeks to over 200 days (Borges et al., 2011).

Mudskippers are specialized amphibious gobies in the four genera of Oxudercinae (*Boleophthalmus*, *Periophthalmodon*, *Periophthalmus* and *Scartelaos*, Clayton 1993; Graham 1997; Graham and Wegner, 2010; Ishimatsu and Gonzales, 2011; Murdy 2011a), although other

oxudercine gobies also show some degree of amphibious nature (Murdy 1989). Despite that

ample data are available on various aspects of mudskipper's behaviors on the exposed surface of

mudflats (Clayton, 1993), far less is known about their life in the mud. The present review

attempts to summarize what is known about the reproduction of mudskippers within their

burrows, and discuss selected important topics for future research.

Reproduction of mudskippers

Most notable of the reproduction in mudskippers are the storage and maintenance of air

within egg chambers by egg-guarding parental fish, embryonic development therein, and the

mechanism for the induction of embryonic hatching by actively flooding the chambers by the

parental fish, even though these are described unequivocally only for a single species of

mudskippers (Ishimatsu et al. 2007). Table 1 summarizes basic aspects of reproductive biology

known for five species of mudskippers. Difficulties of collecting accurate data in soft muddy

habitats apparently are responsible for uncertainties and often conflicting descriptions on the

reproduction of mudskippers (Clayton 1993). For example, *Boleophthalmus dussumieri* and *B.*

pectinirostris both have been reported to spawn once in a year in some studies, but several times

in some other studies (Clayton 1993; Washio et al. 1993).

All mudskippers studied so far excavate burrows in their habitats in mudflats and use them for refuge from predators, for protection from desiccation and extreme temperatures, and for incubation of eggs (Clayton 1993). Burrows of mudskippers vary in shape and size between species or even within a species (see Brillet 1976), but those built during a reproduction season often have a specialized chamber that contains eggs on the ceiling (Fig. 1). Even though reproduction is not the exclusive function of their burrows, laying and incubating eggs in burrows are critically important elements affecting both the benefits and costs of parental care. In some species, the burrows are tended by a male fish (*Periophthalmus magnuspinnatus*, *P. modestus* and *Boleophthalmus pectinirostri*s), but the male and female both appear to be involved in others (*Periophthalmodon schlosseri* and *Scartelaos histophorus*, Table 1). Depending upon the position of the burrow relative to tidal amplitude, openings of burrows may be exposed to air as much as twice each day or remain emergent for several tidal cycles. At the time of spawning, these burrow openings may be plugged by mud from inside (Hong et al. 2007), thus isolating a spawning pair and their eggs on the chamber wall from predators.

The cost of spawning in burrows is the energetic expenditure for digging and maintaining it, and for protecting the embryos from hypoxic stress (Gordon, 1995). The water in mudskippers' burrows is known to be extremely hypoxic (Gordon et al., 1978; El-Ziady et al., 1979; Ishimatsu et al., 1998, 2000, 2007). Burrows are excavated by mouth and then spitting mud pellets onto

the mudflat's surface during either emersion (*Periophthalmus*) or submersion (*Boleophthalmus*, Ishimatsu et al., personal observation) of the mudflat. Mudskipper burrows can be deep, some exceeding the vertical dimension of 1.3 m (*Periophthalmodon schlosseri*, Ishimatsu et al. 1998) or deeper (*Boleophthalmus boddarti*, Clayton and Vaughan 1986; see also Atkinson and Taylor 1991 for a review of fish burrows and burrowing), which requires a substantial expenditure of energy. If unattended, burrows in mudflats will become filled with sediment carried by the tidal flow. Therefore, these burrows need continued maintenance by a burrow-guarding fish, which has implications for bioturbation (Atkinson and Taylor 1991).

The problem of hypoxic water is solved by deposition of the eggs in an egg chamber that the male fish fills with air (Ishimatsu et al., 1998, 2007, 2009). After spawning, the male, in addition to guarding the eggs, must also ensure that the supply of air surrounding the eggs is sufficient to meet their O_2 requirements (Ishimatsu et al., 2007). Hatching of eggs in air requires an additional behavioral innovation since mudskipper eggs, like the eggs of other intertidally spawning fishes, need to be submerged in water for hatching (Martin, 1999). To achieve this, mudskippers developed the behavior of expelling air in the egg chambers by repeating gulps of the air during high tide as the last step of their egg care.

Evolution of the storage of air in burrows

We suspect that the habit of storing air in burrows initially evolved as an adaptation to the dual

requirements for securing an oxygen source for adult fish during confinement in a burrow at

high tide, and ensuring embryonic development in burrows. With higher specialization of

physiology and behavior, some mudskippers such as *Periophthalmus* spp. tend to remain

emergent even during inundation of the mudflat (Ikebe and Oishi 1996; Baeck et al. 2008), but

they retain the strategy of storing air in the burrow, a behavior that is essential for reproduction.

This argument is based upon the maintenance of air in the burrow reported for *Scartelaos*

histophorus. Lee et al. (2005) demonstrated that both male and female *S. histophorus* deposited

air in a laboratory burrow chamber under simulated low-tides. When the PO_2 of water in the

burrow declined to 4.8 kPa during confinement in the burrow under simulated high-tides, the

fish switched from aquatic to aerial breathing, utilizing air deposited in the chamber as an

oxygen reservoir. A booklet on the conservation of endangered species on Okinawa Island,

Japan, states that eggs of *S. histophorus* are attached to the ceiling of the egg chamber. Whether

air is present in the egg chambers was not stated, but is implied from the behavior of gulping air

at the surface and carrying it into the burrow (Okinawa Prefecture, 2000). Similarly, Hong et al.

(2007) presumed that males and females of *B. pectinirostris* both store air in their burrows both

during breeding and non-breeding seasons. Male and female *B. pectinirostris* both maintain

burrows throughout the year, and the fish use them as a retreat during high tide, as in all other

Boleophthalmus species (Polgar and Crosa 2009). During the breeding season, an egg chamber

is built in the burrows of male fish; eggs are spawned in a monolayer on the ceiling of the

chamber (Hong et al. 2007). Hong et al. (2007) demonstrated that a dye infused into burrows of

male *B. pectinirostris* would not stain eggs laid on the upper wall of chambers, and suspected

that the chambers were filled with air.

In a more terrestrially adapted mudskipper, *Periophthalmus modestus*, males but not

females, excavate burrows during the breeding season (Ishimatsu et al. 2007). These burrows

have the shape of a'J', and the upturned terminus is used as an egg chamber (Fig. 1).   Burrows

of the males showing courtship displays contained air, indicating that deposition of air occurs

before spawning, possibly to oxidize the chamber wall and thereby reduce oxygen loss into the

surrounding mud (Ishimatsu et al. 2007). *Periophthalmus modestus* also digs burrows for

overwintering, but probably does not excavate them during non-breeding seasons of warmer

months (Baek et al. 2008).

Periophthalmus magnuspinnatus might represent a transitional stage between the one seen

in *Boleophthalmus* and *Scartelaos* (oxygen reservoir during confinement of fish in the burrow

and while embryos are developing) to the one in *P. modestus* (embryonic development only). In

comparison, male and female *P. magnuspinnatus* both likely possess burrows throughout the

year, but it is only male's burrows that contain eggs (Baek et al. 2008). According to the description by Baek et al. (2008), some *P. magnuspinnatus* remain emergent but others retreat into burrows during high tide. The hypothesis needs to be tested by further field observations on *P. magnuspinnatus*. The quantity and quality of the field-observation data are not always satisfactory about mudskipper's natural history because of the difficulties that one often encounters when attempting to observe these quick and timid fishes in their very soft muddy habitats.

The evidence for the deposition of air in mudskipper burrows is strong, but still there was some degree of uncertainty because the destructive methods used might have caused artifactual trapping of air (Ishimatsu et al. 2007), the evidence was indirect (Hong et al. 2007), or the experiment was conducted under artificial conditions in the laboratory (Lee et al. 2005). However, these uncertainties have been dispelled by the direct endoscopic observations of air-filled egg chambers in a tropical, giant mudskipper, *Periophthalmodon schlosseri* (Ishimatsu et al. 2009). Air was present in burrows both with and without spawned eggs. The presence of air in egg-less burrows supports the idea that deposition of air takes place prior to oviposition, because eggs of *P. schlosseri* need submersion for hatching (Tsuhako et al., 2003), as has been observed for *P. modestus* (Ishimatsu et al., 2007). Although evidence for storage of air is not unequivocal for the other species listed in Table 1 and needs more rigorous examination, it is

probably prevalent in burrows of all four genera of mudskippers.

Role of oxygen in parental care by mudskippers

The role of oxygen in parental care is best known for *P. modestus* (Ishimatsu et al. 2007). In that study, continuous determinations were made for the oxygen concentration of the gas stored within egg chambers. Prior to observations, an upper portion of a burrow were half-destroyed, and the roof of the egg chamber was instrumented with an oxygen electrode for determination of the oxygen concentration of the gas in the chamber, an endoscope for observation of the burrow-guarding male, and a thin tube for manipulation of the gas in the chamber. In addition, a pair of impedance electrodes were inserted into the burrow section below the chambers to monitor the movements of the male through the vertical shaft. After reconstruction of the once-destroyed portion of the burrows, data were continually obtained until the eggs hatched. The results demonstrated that the gas in the egg chambers of *P. modestus* burrows showed regular fluctuations in oxygen concentration synchronized with the tidal cycles of emersion and submersion of the mudflat (Fig. 2). Thus, oxygen concentration increased during emersion of the burrow at low tide, and decreased during submersion at high tide. The correlation between male activity (impedance data) and the increase in PO_2 of the egg chambers (Fig. 2) most likely

reflects transport of mouthfuls of fresh air into the chamber by the male. That the decline of PO_2 in egg-chamber gas reflects the respiration of the eggs is suggested by the product of the measured rate of oxygen consumption of a developing mudskipper egg [76.5 ± 12.0 nl h^{-1}/hatch-competent egg (Etou et al. 2007)] and the estimated number of eggs in the chamber (5200). Calculations demonstrated that oxygen consumption of the eggs accounts for 75–100% of the observed rate of oxygen depletion of the egg chamber (Ishimatsu et al. 2007). Impedance signals recorded as an index of males' burrow guarding behavior occurred only during high low tide. Endoscopic observations verified that eggs remained in air, and the males occasionally entered the chambers. Even though these lines of evidence are available only for a single species of mudskipper, they strongly indicate that the males guarding burrows gulp air and release it into the egg chamber, thereby replenishing the oxygen store that was depleted, mainly by respiring embryos, during the preceding high-tide. The facts that water in the burrow is always severely hypoxic, and that embryos of *P. modestus* perished when incubated in simulated burrow water (Etou et al. 2007) suggest that deposition of air in an egg chamber is a general reproductive strategy for other mudskipper species (Table 1), and possibly also for those non-mudskipper fishes that supposedly lay eggs in muddy burrows (e.g. *Odontamblyopus lacepedii* and other eel gobies, see Gonzales et al., 2006, 2008).

Oxygen in egg chambers promptly recovered when it was experimentally reduced by

injections of a volume of hypoxic gas through a thin tube fixed to an egg chamber (Fig. 3). The injections were made ca. 1 h before the burrow was covered by water of the rising tide and while the burrow-guarding male was on the mudflat surface. Fig. 3A,C show the gradual rise in egg-chamber PO_2 that occurred in two burrows from the beginning of the low-tide period through about 7 h and up to the time of hypoxic-gas introduction (arrowheads). In response to the sudden drop of egg-chamber PO_2, the male fish was able to rapidly restore the PO_2 within 1 h. When the frequency of air-supplying behavior (fa) is plotted against egg-chamber PO_2 for the periods of the rapid recovery following the injection and the undisturbed periods prior to the injections, the fa obtained following the injections lies far above the calculated fa–PO_2 relationships for the undisturbed periods prior to the injections (Fig. 3B,D). These results imply that air-supplying behaviour was not solely modulated by air PO_2 of egg chambers but also by some other factor that is possibly related to the tidal cycle.

Oxygen sensing and air-supplying behavior

The supply of oxygen to developing embryos through fanning is one of the most important functions of parental care among fishes (Keenleyside 1979; Blumer 1982). Brood-guarding aquatic fish ventilate their brood by increasing the frequency and/or time allocated to fanning (Takegaki and Nakazono 1999b; Jones and Reynolds 1999; Lissåker and Kvarnemo 2006).

Even though ample data are available on the role of oxygen as a controlling factor for

ventilation of the gills (Perry et al. 2009), the egg-fanning responses must require something

more than the classical autonomic control of ventilation. Regulation of the air-supplying

behavior shown by mudskippers is even more complex than the fanning responses of aquatic

fishes, and must include sensing the oxygen levels of the egg chamber, and modulating

locomotive transport of oxygen from above the water's surface down into the chamber to the

extent that the level of oxygen is sufficiently high that embryos do not suffer hypoxic stress

during subsequent submersion of the burrow. Oxygen sensors are believed to reside in the gills,

and evidence indicates a role for neuroepithelial cells in the filaments for zebrafish and catfish

(Perry et al. 2009; Nikinmaa 2010). No data are currently available on oxygen sensors in

mudskippers.

CO$_2$ can also be an additional candidate for the regulation of the air-supplying behavior.

The PCO$_2$ of the egg-chamber gas ranges from 0.4 to 2.5 kPa for *P. modestus* (Ishimatsu et al.

2007), and from 0.4 to 12 kPa (but this extremely high PCO$_2$ was obtained when gas PO$_2$ was

nearly zero) for *Pn. schlosseri* (Ishimatsu et al. 1998). Even though data are limited, an

insignificant ventilatory response to 2 kPa of PCO$_2$ demonstrated for *Pn. schlosseri* (Aguilar et

al. 2000) is indicative of a minor, if any, role of CO$_2$ in air-supplying behavior for mudskippers.

Role of tides in parental care by mudskippers

Spawning by mudskippers is seasonal, and therefore temperature and day length are expected to affect gonadal development, particularly in temperate latitudes, as in other fishes (Clayton 1993). In addition, tidal or lunar cycles would play a decisive role for spawning by intertidal fishes. Many of the well-known examples are tidal migrants, which spawn at high tide when spawning grounds are available (DeMartini 1999). Mudskippers are fundamentally different from these fishes in that courtship and subsequent spawning occur at low tide when mudflats are emergent (Clayton 1993; Matoba and Dotsu1977).

Mudskippers may excavate burrows in the higher intertidal zones that are submerged only by spring high tides. Ishimatsu et al. (2007) revealed that some of the *P. modestus* burrows in these zones remained emergent until the day of hatching (Fig. 2). For burrow-guarding males of these burrows, it is crucially important to ensure that burrow openings will be covered by the rising tide at the time of hatching. This requires (1) recognition of the vertical location of burrows in the intertidal zones in relation to tide and (2) timely spawning. How a male mudskipper determines the site of excavation for a breeding burrow in intertidal zones is yet to be studied. Unless openings of the burrow are inundated, the larvae cannot be dispersed to open water, even if eggs are induced to hatch. If the larvae were to remain in a burrow for an extended period of time, they would not survive in a small volume of severely oxygen–depleted

water filling the burrow (Etou et al. 2007). In addition, the hatched larvae would be subjected to extremely risky conditions of the high intertidal zone (high temperature and intense solar irradiation) and to higher risk of predation by terrestrial carnivores. It is also necessary that the timing of spawning be adjusted such that future hatching will coincide with submersion of the burrow. Ishimatsu et al. (2007) showed that eggs of *P. modestus* have a 5-6 day window, during which eggs can hatch upon submersion. This would afford male *P. modestus* a correspondingly narrow period of courtship. The windows for hatching in mudskippers are thus expected to be longer for the species occupying the higher intertidal zone as compared with more fully aquatic species.

Cues for the hatching of eggs

Mudskipper eggs cannot hatch in air and must be submerged for hatching to occur (Brillet 1976; Tsuhako et al. 2003; Ishimatsu et al. 2007). Reasons for the necessity of immersion for hatching are not currently clear, but there is evidence that shortage of oxygen upon submersion is responsible for the hatching of embryos that have developed to hatching readiness in air (Yamagami 1988; Martin 1999). Embryos of *P. modestus* develop in air stored in an egg chamber, and thus are exposed to a range in PO_2 of 13-17 kPa (Fig. 2), but then suddenly are

submerged to water having a PO_2 of only 1.5 kPa when the male parent floods the egg chamber,

assuming that the PO_2 of the water at the time the male induces hatching is identical to that of

standing water sampled from the depth of > 5cm in the vertical shaft (Ishimatsu et al. 2007).

This sudden drop in environmental oxygen is supposedly a strong stimulus triggering hatching

of *P. modestus* embryos. Taking into account the reduction in oxygen uptake by *P. modestus*

embryos ready to hatch (Etou et al. 2007) and assuming that the entire volume [0.193 µL,

calculated from the longer axis length of 0.90 mm, the shorter axis length of 0.64 mm

(Kobayashi et al. 1972), and assuming the eggs as spheroids] of an egg is in equilibrium with

the air of the egg-chamber, the embryo would consume all of the oxygen reserve in the egg

capsule within several seconds. This calculation is admittedly too simplistic, but nevertheless

indicates that embryos are rapidly subjected to hypoxic conditions once submerged.

Since induction of hatching in mudskippers is a phenomenon initiated and completed by a

male guarding the burrow, identifying the possible cues for hatching is equivalent to asking how

and what cues are sensed by the male. How does the male sense that his eggs are mature enough

to be released from his burrow? Are there some chemical or physical cues emitted from

thousands of embryos awaiting hatching? Visual cues from the embryos are unlikely since the

inside of egg chambers must be nearly completely dark. The Y-shape configuration of the upper

portion of the burrow (e.g. most *Periophthalmus* species), high turbidity of water in the burrow,

or upturned/acclivitous disposition of an egg chamber derived from the almost vertical main shaft of the burrow would preclude penetration of light into the chamber.

Selected questions about mudskippers' reproductive behavior

The above discussion summarizes the current knowledge about reproduction of a few mudskipper species for which data are available. Apparently, data are far from sufficient to gain insight into how reproductive behaviors in mudskippers have developed from aquatic ancestral gobies. The following lists some of the important questions to be solved by future studies.

1. Can males recognize vertical location of their burrows when excavating for breeding?

If burrows are too high in intertidal zones, reproduction is not possible since hatched juveniles cannot be dispersed. This question should be addressed first by determining vertical locations of mudskipper burrows in relation to periods of tidal inundation at respective sites. The embryos developing in those burrows may be subjected to higher degrees of stress from lower humidity and excessive heat. There might be "failed" burrows that are too high to be covered by high spring tide.

2. Do spawning and fertilization of eggs occur in air?

As stated earlier, air occurs in the burrows of courting male *P. modestus*, which suggests that

spawning and fertilization both occur in air (Ishimatsu *et al.*, 2007). The presence of air in egg

chambers of *Pn. schlosseri* burrows where no eggs were found (Ishimatsu *et al.*, 2009) may also

represent the deposition of air prior to laying of the eggs. The findings that spawning and

fertilization of eggs both occur in air-exposed rock crevices by the amphibious blenny *Andamia*

tetradactyla pointed out that it is possible for a fish to lay eggs and fertilize them in air (Shimizu

et al. 2006). Direct video recording of burrow spawning must answer this question.

3. Are the eggs of mudskippers specialized for developing in air?

There is no detailed study on morphology of mudskipper eggs. A priori, there seems to be no

specialization required for the embryonic development in air, as long as desiccation is avoided.

The air filling egg chambers of mudskippers is thought to be saturated with water vapor, since

the mud particles of the mudskipper habitats are very fine, e.g., ca. 7 µm for the habitat of *P.*

modestus in Ariake, Japan (Ishimatsu et al. 2007), the burrows are submerged by tide twice a

day (except those build in higher intertidal zones), and those soft substrata usually contain a

large amount of water (Little 2000). Embryos of a purely aquatic fish, *Oryzias latipes*, do

develop normally in moist air and hatch when immersed in water (Yamagami 1988).

4. Where do mudskippers have oxygen sensors for maintaining egg-chamber O$_2$?

Stronger ventilatory responses were provoked by aerial hypoxia rather than aquatic hypoxia in

Periophthalmodon schlosseri (Aguilar et al., 2000), which implies that a role of oxygen sensors

on the epithelial surface for air breathing is predominant compared with any oxygen sensors on the gills, or that oxygen levels in blood is crucial for the regulation of aerial ventilation. Histochemical investigation for detection of neuroepithelial cells (Coolidge et al. 2008) should prove whether there are putative oxygen sensors or not on the aerial gas exchange surfaces (the bucco-opercular cavities or possibly skin) and the gills of mudskippers.

5. What triggers the induction of hatching?

This includes two aspects. One relates to potential signals given by embryos to an egg-tending male such that he "realizes" that the embryos are ready to hatch. Vibrational cues might be sensed by the male since mudskippers' embryos show increasingly vigorous turning inside the egg shell as development proceeds (Kobayashi et al. 1972). Also, the male might sense some kind of gaseous substances released from embryos. Oxygen is unlikely to be involved since there are no obvious changes of oxygen concentration in an egg chamber toward the end of incubation (Ishimatsu et al. 2007). Killing embryos by injecting carbon monoxide shortly before expected time of hatching might make clear if vibrational cues are involved. The other aspect relates to on what cue the male relies to initiate the behavioral induction of hatching at right time. Ambient light is likely involved in this decision since hatching always occurred during rising tides at dusk or at night, and never happened during diurnal high tides (Ishimatsu et al. 2007).

6. How are hatched larvae dispersed from within a burrow?

Rapid escape after hatching is crucial for larvae, because Etou et al. (2007) showed that survival of newly hatched larvae rapidly decreased with time in simulated hypoxic burrow water. Whether or not the guarding male is involved in releasing larvae from its burrow remains to be determined. Artificial induction of larval hatching in burrows from which a guarding male was removed suggested that relatively few of the hatched larvae were able to find their way out of the burrow (Ishimatsu et al. 2007). Direct endoscopic observation is difficult due to the high turbidity of water in the burrows, but should be the most direct way to answer this question.

References

Aguilar NM, Ishimatsu A, Ogawa K, Khoo KH. 2000. Aerial ventilatory responses of the mudskipper, *Periophthalmodon schlosseri*, to altered aerial and aquatic respiratory gas concentrations. Comp Biochem Physiol 127A:285-292.

Atkinson RJA, Taylor AC. 1991. Burrows and burrowing behaviour of fish. In: Meadows PS, Meadows A, editors. The environmental impact of burrowing animals and animal burrows. Symp Zool Soc Lond 63:133-155.

Baek GW, Takita T, Yoon YH. 2008. Lifestyle of Korean mudskipper *Periophthalmus magnuspinnatus* with reference to a congeneric species *Periophthalmus modestus*. Ichthyol Res 55:43-52.

Blumer LS. 1982. A bibliography and categorization of bony fishes exhibiting parental care. Zool J Linn Soc 76:1-22.

Borges R, Faria C, Gil F, Gonçalves EJ. 2011. Early development of gobies. In: Patzner R, Van Tassell JL, Kovačić M, Kapoor BG, editors. The biology of gobies. Enfield: Science Publishers. p. 403-462.

Breder CM, Rosen DN. 1966. Modes of reproduction in fishes. New York: Natural History Press. 941 p.

Brillet C. 1976. Structure du terrier, reproduction et comportement des jeunes chez le poisson

amphibie *Periophthalmus sobrinus* Eggert. Rev Écol Terre Vie 30:465-483.

Chotknowski, MA, Buth DG, Prochazka K. 1999. Systematics of intertidal fishes. In: Horn MH, Martin KLM, Chotknowski MA, editors. Interdial fishes: Life in two worlds. San Diego: Academic Press. p. 297-331.

Clayton DA. 1993. Mudskippers. Oceanogr Mar Biol Annu Rev 31:507-577.

Clayton DA, Vaughan TC. 1986. Territorial acquisition in the mudskipper *Boleophthalmus boddarti* (Teleostei, Gobiidae) on the mudflats of Kuwait. J Zool Lond 209A:501-519.

Coleman RM. 1999. Parental care in intertidal fishes. In: Horn MH, Martin KLM, Chotknowski MA, editors. Interdial fishes: Life in two worlds. San Diego: Academic Press. p. 165-180.

Coolidge EH, Ciuhandu CS, Milsom WK. 2008. A comparative analysis of putative oxygen-sensing cells in the fish gill. J Exp Biol 211:1231-1242.

DeMartini EE. 1999. Intertidal spawning. In: Horn MH, Martin KLM, Chotknowski MA, editors. Interdial fishes: Life in two worlds. San Diego: Academic Press. p. 143-164.

El-Ziady S, Eissa SM, Al-Naqi Z. 1979. Ecological studies on the mud-skipper fish, *Periophthalmus chrysospilos* (Bleeker) in Kuwait. Bull Fac Sci Cairo Univ 48:113-133.

Etou A, Takeda T, Yoshida Y, Ishimatsu, A. 2007. Oxygen consumption during embryonic development of the mudskipper (*Periophthalmus modestus*): Implication for the aerial development in burrows. In: Fernandes MN, Rantin FT, Glass ML, Kapoor BG, editors.

Fish respiration and environment. Enfield: Science Publishers. p. 83-91.

Gaisner A. 2005. Parental care and reproductive behavior of the clown goby, *Microgobius gulosus*, with observations on predator interactions. Env Biol Fish 73:341-356.

Gonzales TT, Katoh M, Ishimatsu A. 2006. Air breathing of aquatic burrow-dwelling eel goby, *Odontamblyopus lacepedii* (Gobiidae: Amblyopinae). J Exp Biol 209:1085-1092.

Gonzales TT, Katoh M, Ishimatsu A. 2008. Intertidal burrows of the air-breathing eel goby, *Odontamblyopus lacepedii* (Gobiidae: Amblyopinae). Ichthyol Res 55:303-306.

Gordon MS. 1995. Functional evidence from living vertebrates. In: Gordon MS, Olson EC, editors. Invasions of the land: The transitions of organisms from aquatic to terrestrial life. New York: Columbia University Press. p. 216-250.

Gordon MS, Ng WW-s, Yip AY-w. 1978. Aspects of the physiology of terrestrial life in amphibious fishes. III. The Chinese mudskipper *Periophthalmus cantonensis*. J Exp Biol 72:57-75.

Graham JB. 1997. Air-breathing fishes: Evolution, diversity, and adaptation. San Diego: Academic Press. 299 p.

Graham JB, Wegner NC. 2010. Breathing air in water and in air: The air-breathing fishes. In: Nilsson GE, editor. Respiratory physiology of vertebrates: Life with and without oxygen. Cambridge: Cambridge University Press. p. 174-221.

Hong W, Chen S, Zhang Q, Wang Q. 2007. Reproductive ecology of the mudskipper

   Boleophthalmus pectinirostris. Acta Oceanol Sinica 26:72-81.

Ikebe Y, Oishi T. 1996. Correlation between environmental parameters and behaviour during

   high tides in *Periophthalmus modestus*. J Fish Biol 49:139-147.

Ishimatsu A, Gonzales TT. 2011. Mudskippers: Front runners in the modern invasion of land.

   In: Patzner R, Van Tassell JL, Kovačić M, Kapoor BG, editors. The biology of gobies.

   Enfield: Science Publishers. p. 603-632.

Ishimatsu A, Hishida Y, Takita T, Kanda T, Oikawa S, Takeda T, Khoo KH. 1998.

   Mudskippers store air in their burrows. Nature 391:237-238.

Ishimatsu A, Takeda T, Kanda T, Oikawa S, Khoo KH. 2000. Burrow environment of

   mudskippers in Malaysia. J Biosci 11:17-28.

Ishimatsu A, Yoshida Y, Itoki N, Takeda T, Lee HJ, Graham JB. 2007. Mudskippers brood their

   eggs in air but submerge them for hatching. J Exp Biol 210:3946-3954.

Ishimatsu A, Takeda T, Tsuhako Y, Gonzales TT, Khoo KH. 2009. Direct evidence for aerial

   egg deposition in the burrows of the Malaysian mudskipper, *Periophthalmodon schlosseri*.

   Ichthyol Res 56:417-420.

Jones JC, Reynolds JD. 1999. Costs of egg ventilation for male common gobies breeding in

   conditions of low dissolved oxygen. Anim Behav 57:181-188.

Keenleyside MHA. 1979. Diversity and adaptation in fish behaviour. Berlin: Springer-Verlag.

   208 p.

Kobayashi T, Dotsu Y, Takita T. 1971. Nest and nesting behavior of the mud skipper,

   Periophthalmus cantonensis in Ariake Sound. Bull Fac Fish Nagasaki Univ 32:27-40 (in

   Japanese).

Kobayashi T, Dotsu Y, Miura N. 1972. Egg development and rearing experiments of the larvae

   of the mud skipper, *Periophthalmus cantonensis*. Bull Fac Fish Nagasaki Univ 33:49-62 (in

   Japanese).

Lee HJ, Martinez CA, Hertzberg KJ, Hamilton AL, Graham JB. 2005. Burrow air phase

   maintenance and respiration by the mudskipper *Scartelaos histophorus* (Gobiidae:

   Oxudercinae). J Exp Biol 208:169-177.

Lissåker M, Kvarnemo C. 2006. Ventilation or nest defense   parental care trade-offs in a fish

   with male care. Behav Ecol Sociobiol 60:864-873.

Little C. 2000. The biology of soft shores and estuaries. Oxford: Oxford University Press. 252

   p.

Martin KLM. 1999. Ready and waiting: Delayed hatching and extended incubation of

   anamniotic vertebrate terrestrial eggs. Amer Zool 39:279-288.

Martin KLM, Bridges CR. 1999. Respiration in water and air. In: Horn MH, Martin KLM,

Chotknowski MA, editors. Interdial fishes: Life in two worlds. San Diego: Academic Press. p. 54-78.

Martin KLM, Van Winkle RC, Drais JE, Lakisic H. 2004. Beach-spawning fishes, terrestrial eggs and air breathing. Physiol Biochem Zool 77:750-759.

Matoba M, Dotsu Y. 1977. Prespawning behavior of the mud skipper *Periophthalmus cantonensis* in Ariake Sound. Bull Fac Fish Nagasaki Univ 43:23-33 (in Japanese).

Miller PJ.1984. The tokology of gobioid fishes. In: Potts GW, Wootton RJ, editors. Fish reproduction: Strategies and tactics. London: Academic Press. pp. 119-153.

Murdy E. 1989. A taxonomic revision and cladistic analysis of the oxudercine gobies (Gobiidae: Oxudercinae). Rec Aust Mus Suppl 11:1-93.

Murdy E. 2011a. Systematics of Oxudercinae. In: Patzner R, Van Tassell JL, Kovačić M, Kapoor BG, editors. The biology of gobies. Enfield: Science Publishers. p. 99-106.

Murdy E. 2011b. Systematics of Amblyopinae. In: Patzner R, Van Tassell JL, Kovačić M, Kapoor BG, editors. The biology of gobies. Enfield: Science Publishers. p. 107-118.

Nikinmaa M. 2010. Sensing oxygen. In: Nilsson GE, editor. Respiratory physiology of vertebrates: Life with and without oxygen. Cambridge: Cambridge University Press. p. 14-48.

Okinawa Prefecture. 2000. Tokage-haze no hanashi (An account of *Scartelaos histophorus*).

Okinawa Sekkei Service, Okinawa (in Japanese). 12 p.

Patzner R, Van Tassell JL, Kovačić M, Kapoor BG. 2011. The biology of gobies. Enfield: Science Publishers.

Perry SF, Esbaugh A, Braun M, Gilmour KM. 2009. Gas transport and gill function in water-breathing fish. In: Glass ML, Wood SC, editors. Cardio-respiratory control in vertebrates: Comparative and evolutionary aspects. Berlin: Springer-Verlag. p. 5-42.

Polgar G, Crosa G. 2009. Multivariate characterisation of the habitats of seven species of Malayan mudskippers (Gobiidae: Oxudercinae). Mar Biol 156:1475-1486.

Shimizu N, Sakai Y, Hashimoto H, Gushima K. 2006. Terrestrial reproduction by the air-breathing fish *Andamia tetradactyla* (Pisces; Blenniidae) on supralittoral reefs. J Zool Lond 269:357-364.

Takegaki T. 2000. Monogamous mating system and spawning cycle in the gobiid fish, *Amblygobius phalaena* (Gobiidae). Env Biol Fish 59:61-67.

Takegaki T, Nakazono A. 1999a. Reproductive behavior and mate fidelity in the monogamous goby, *Valenciennea longipinnis*. Ichthyol Res 46:115-123.

Takegaki T, Nakazono A. 1999b. Responses of the egg-tending gobiid fish *Valenciennea longipinnis* to the fluctuation of dissolved oxygen in the burrow. Bull Mar Sci 65:815-823.

Townsend KA, Tibbetts IR. 2005. Behaviour and sexual dimorphism of the blue mudskipper,

Scartelaos histophorus (Pisces: Gobiidae). Proc Roy Soc Queensland 112:53-62.

Tsuhako Y, Ishimatsu A, Takeda T, Khoo KH, Tachihara K. 2003. The eggs and larvae of the

giant mudskipper, *Periophthalmodon schlosseri*, collected from a mudflat in Penang,

Malaysia. Ichthyol Res 50:178-181.

Washio M, Komiya S, Takita T. 1993. Maturation of the mudskipper *Boleophthalmus*

pectinirostris distributed in the mud flats of the Midori River, Kumamoto Prefecture. Nippon

Suisan Gakkaishi 59:575-580.

Yamagami K. 1988. Mechanisms of hatching in fish. In: Hoar WS, Randall DJ, editors. Fish

physiology Vol. XI The physiology of developing fish, Pt A Eggs and larvae. San Diego:

Academic Press. p. 447- 499.

Zhang QY, Hong WS, Dai QN, Zhang J, Cai YY, Huang JL. 1989. Studies on induced

ovulation, embryonic development and larval rearing of the mudskipper (*Boleophthalmus*

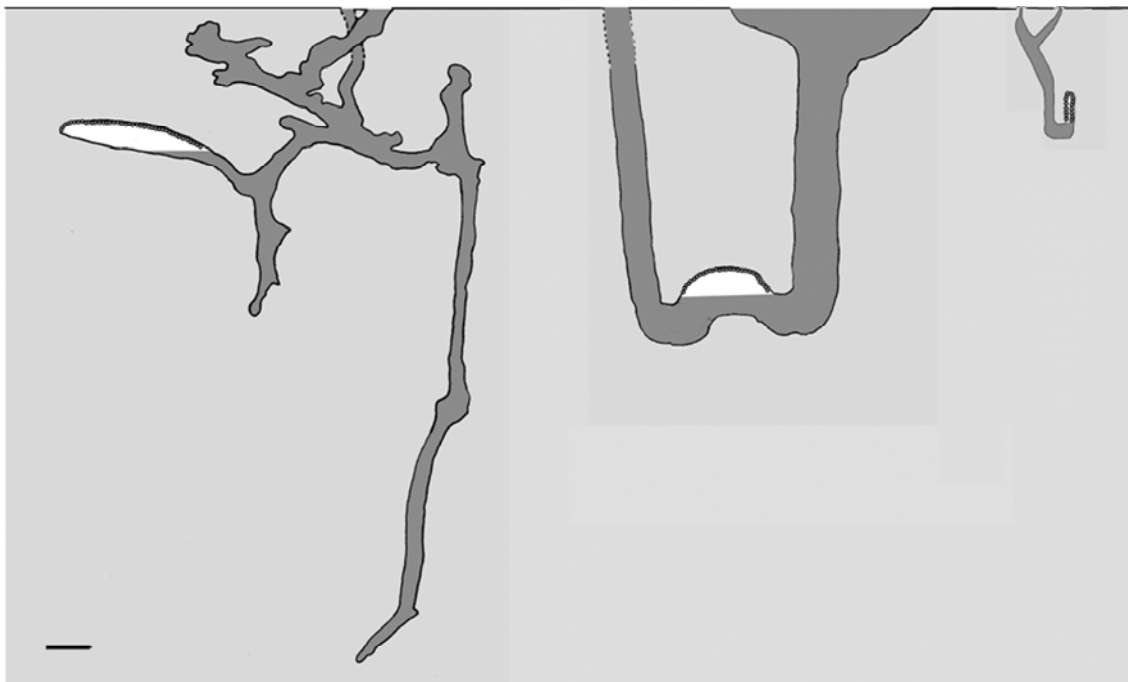*pectinirostris*). Aquaculture 83:375-385.

Figure legends

Fig. 1 Mudskipper burrows (left, *Boleophthalmus pectinirostris*; middle, *Periophthalmodon schlosseri*; right, *Periophthalmus modestus*). Dark gray areas represent water within the burrow. Egg chambers are filled with air by a male (or mating pair) that guards the burrow during emersion. Eggs are deposited on the ceiling of the egg chamber. The scale represents 10 cm, and apply to all diagrams.. Left: Based on plastic casts prepared by Toba and Ishimatsu (unpublished). Middle: Modified from Ishimatsu et al. (1998) and Ishimatsu et al. (2000). Right: Modified from Matoba and Dotsu (1977). Note that the structural details of mudskipper burrows can be highly variable even within a species (see Brillet 1976).
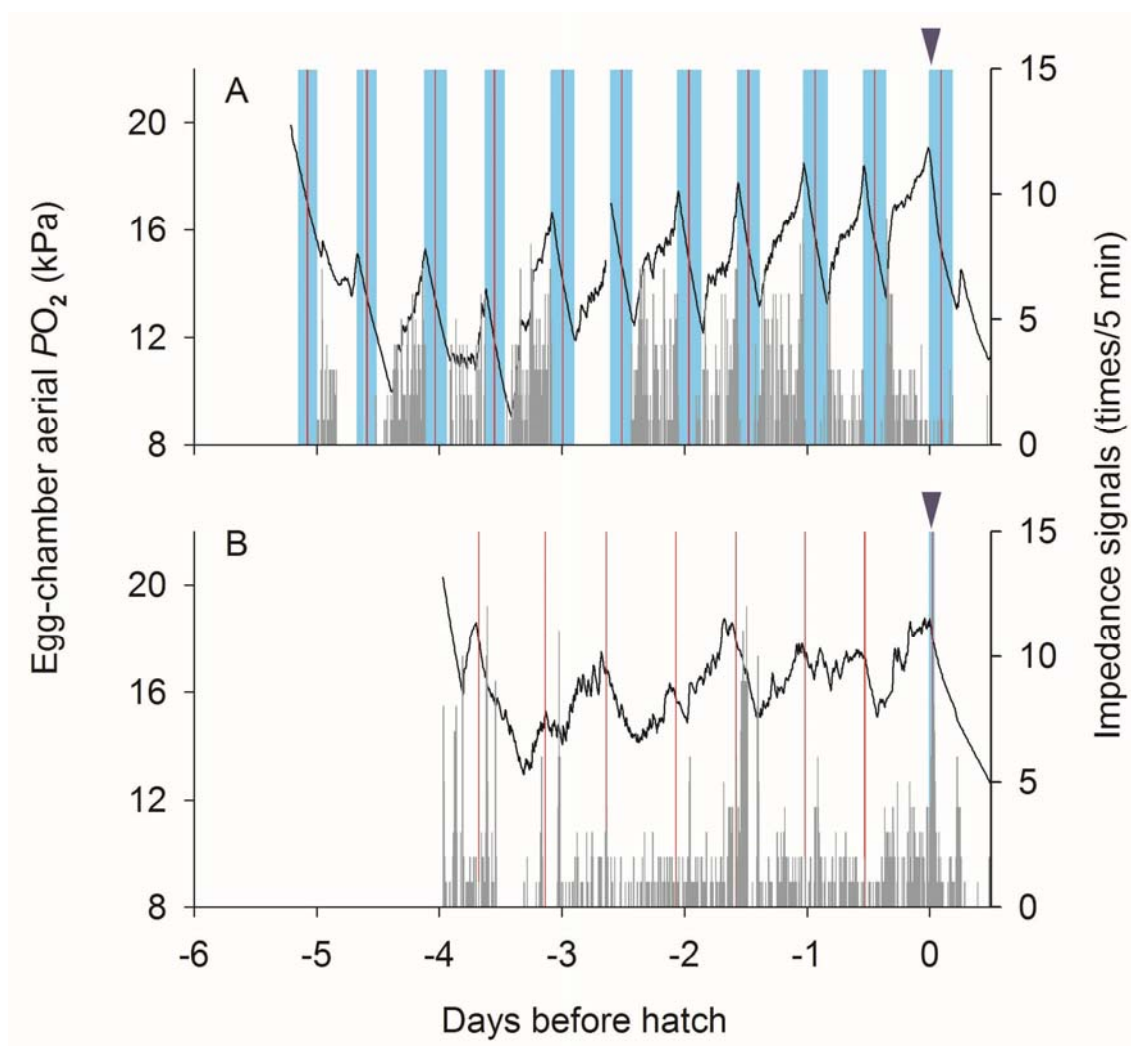
Fig. 2 Continuous records of the PO₂ of the air in the egg chambers of *Periophthalmus modestus* (black line) and the timing of the male's visits to egg-chambers (indicated by impedance signals, grey spikes) in relation to the tidal cycle. Acquisition of data began with the day of instrumentation and continued until hatching was initiated by the male on a rising tide (blue triangle). The beginning of day zero was set to the time when a burrow was covered by an incoming tide, following which hatching occurred. The initial high PO₂ reflects the opening of the egg chamber to air during instrumentation. (**A**) Record for a burrow regularly covered (light blue bars) and uncovered by the tidal oscillation (red vertical lines show the time of the highest tide). (Note: There are no data for impedance for day −3 due to a technical problem.) (**B**) Record for a burrow located sufficiently high on the mudflat to be continually exposed to air until day zero when the rising tide covered the burrow (blue bar) and hatching occurred (blue triangle). From Ishimatsu et al. (2007) with permission.
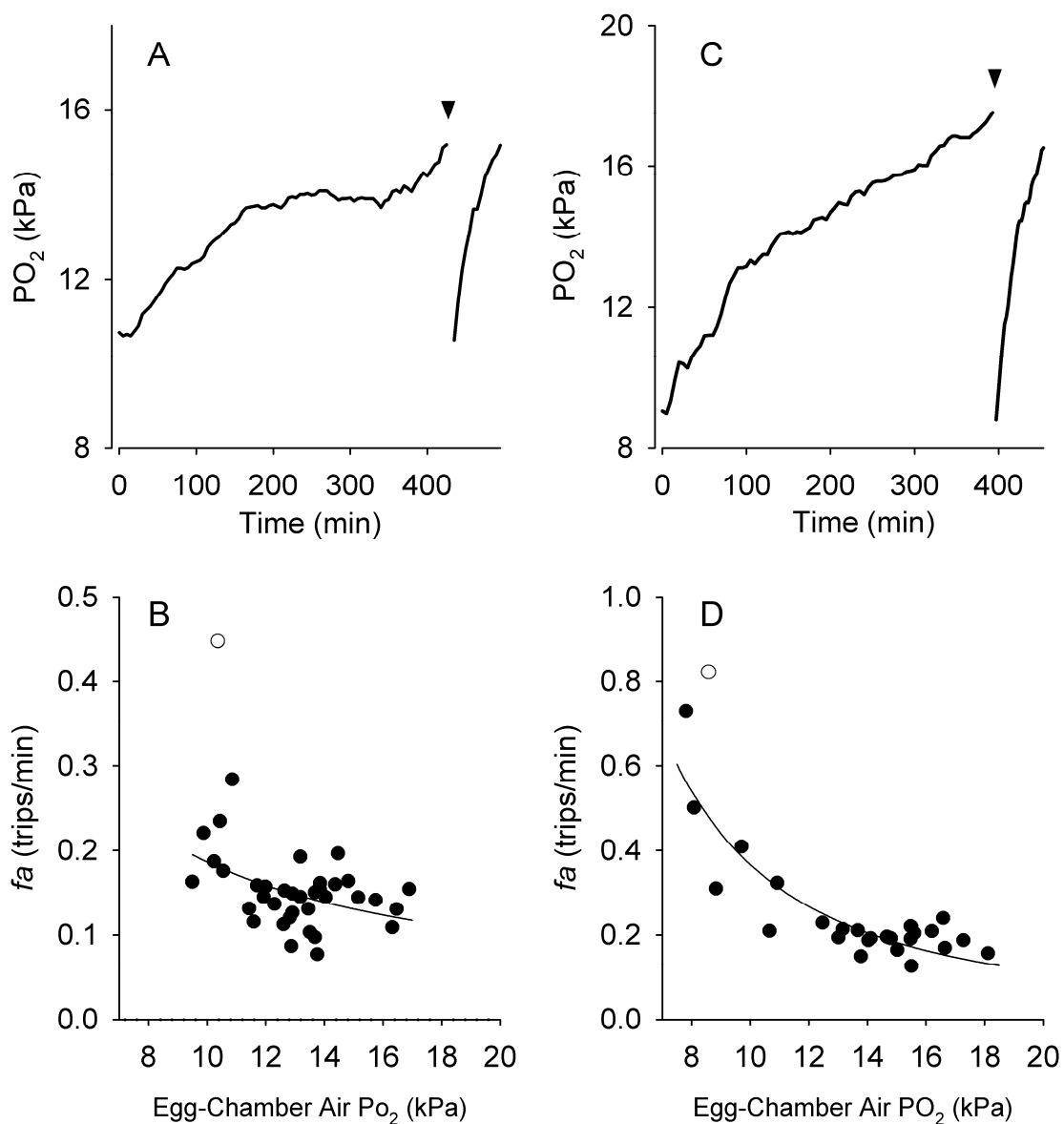
Fig. 3 Effect of injection of hypoxic gas into an egg-chamber (**A,C**) and estimated frequency of air-supplying behavior (**B,D**) by two male *Periophthalmus modestus.* **A** and **C** show the significantly higher rate of PO_2 increase observed following the injection of hypoxic gas than at the beginning of the low-tide period with nearly identical initial PO_2.   **B** and **D** demonstrate an inverse relationship between egg-chamber PO_2 and frequency of air-supplying behavior (*f*a) as determined for five (**B**) or four (**D**) complete low-tide periods preceding hypoxic-gas injection (solid symbols) and *f*a after injection (open symbol). From Ishimatsu et al. (2007) with permission.

Table 1 Basic data on the reproduction of mudskippers

Species	Spawning in burrows	Egg care	Storage of air in burrows	Hours to hatch	Larval period (days)	Transition to amphibious life after hatching (days)
Periophthalmodon schlosseri	Yes (1)	Biparental? (2)	Yes (1,2)	Unknown	Unknown	Unknown
Periophthalmus magnuspinnatus	Yes (4)	Male (4)	Yes? (4)	Unknown	Unknown	Unknown
Periophthalmus modestus	Yes (5,6)	Male (5)	Yes (5)	170 -175 (7)*	45 (7)**	50 (7)**
Boleophthalmus pectinirostris	Yes (8)	Male (8)	Yes? (8)	87 (9) †	28 (9) ††	Unknown
Scartelaos histophorus	Yes (10)	Biparental? (11)	Yes (10,12)	Unknown	30 (10)	Unknown

*19-20°C, **23-28°C, †27-29°C, ††25-29°C, (1) Ishimatsu et al. (2009), (2) Ishimatsu et al. (1998), (3) Tsuhako et al. (2003), (4) Baek et al. (2008), (5) Ishimatsu

et al. (2007), (6) Kobayashi et al. (1971), (7) Kobayashi et al. (1972), (8) Hong et al. (2007), (9) Zhang et al. (1989), (10) Okinawa Prefecture (2000), (11)

Townsend and Tibbetts (2005), (12) Lee et al. (2005)