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 pectinirostris), but the male and female both appear to be involved in others (Periophthalmus 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 ($f_a$) 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 $f_a$ obtained following the injections lies far above the calculated $f_a$–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.

CO₂ can also be an additional candidate for the regulation of the air-supplying behavior. The PCO₂ 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 PCO₂ was obtained when gas PO₂ was nearly zero) for *Pn. schlosseri* (Ishimatsu et al. 1998). Even though data are limited, an insignificant ventilatory response to 2 kPa of PCO₂ demonstrated for *Pn. schlosseri* (Aguilar et al. 2000) is indicative of a minor, if any, role of CO₂ 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 Dotsu 1977).

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; Tshako 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 PO₂ of 13-17 kPa (Fig. 2), but then suddenly are
submerged to water having a PO2 of only 1.5 kPa when the male parent floods the egg chamber, assuming that the PO2 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 O₂?**

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.

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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 PO$_2$ 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 PO$_2$ 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

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