Mudskippers brood their eggs in air but submerge them for hatching

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Summary

Intertidal mudflats are highly productive ecosystems that impose severe environmental challenges on their occupants due to tidal oscillations and extreme shifts in habitat conditions. Reproduction on mudflats requires protection of developing eggs from thermal and salinity extremes, O_2 shortage, dislodgement by currents, siltation and predation. Mudskippers are air-breathing, amphibious fishes, and one of few vertebrates that reside on mudflats. They lay their eggs in mud burrows containing extremely hypoxic water, raising the question of how the eggs survive. We found that the Japanese mudskipper *Periophthalmus modestus* deposits its eggs on the walls of an air-filled chamber within its burrow. To ensure adequate O_2 for egg development, the burrow-guarding male mudskipper deposits mouthfuls of fresh air into the egg chamber during each low tide, a behaviour that can be upregulated by egg-chamber hypoxia. When egg development is complete the male, on a nocturnal rising tide, removes the egg-chamber air and releases it outside the burrow. This floods the egg chamber and induces egg hatching. Thus, *P. modestus* has developed a reproductive strategy that allows it to nurture eggs in this severe habitat rather than migrating away from the mudflat. This requires that mudskipper eggs be specialized to develop in air and that the air-breathing capacity of the egg-guarding male be integrated in a complex behavioural repertoire that includes egg guarding, ferrying air to and from the egg chamber, and sensing O_2 levels therein, all in concert with the tidal cycle.

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Introduction

Mudskippers (family Gobiidae, subfamily Oxudercinae) are specialized teleosts adapted for amphibious life on mudflats. During low tide, mudskippers volitionally emerge from water and feed, defend territories, and engage in courtship on the mud surface (Clayton, 1993). Mudskipper terrestrial activity is supported by a capacity for aerial respiration, to which epithelia in the gills, the buccal-branchial cavity, and the skin all contribute in varying proportions, depending on species (Graham, 1997). The large buccal-branchial cavity of mudskippers is important for holding a substantial air volume for respiration, as well as for territorial displays and burrowing (Graham et al., 2007). For example, the Malaysian mudskipper *Periophthalmodon schlosseri* (Pallas) has a buccal-branchial cavity volume that is 16% of its body volume (Aguilar et al., 2000), which is much larger than the cavity volume of 2–6% reported in gobies not specialized for amphibious air breathing (Gee and Gee, 1991; Gonzales et al., 2006). The anatomical and physiological specializations of mudskippers enable them to maintain metabolic rates in air that are generally equal to or higher than in water (Graham, 1997; Kok et al., 1998; Takeda et al., 1999).

In contrast to activities on the mudflat surface, little is known about mudskipper behaviour in burrows during high tide. Most mudskippers excavate burrows and use them as a refuge from predators, for protection from desiccation and temperature extremes, and for nesting. Although spawning in burrows affords egg protection, the severe hypoxia of burrow water (Ishimatsu et al., 2000) raises the question of what mechanisms are used to ensure adequate O_2 for developing mudskipper eggs (Gordon, 1995). Mudskipper burrows often have one or more horizontal or upturned chambers near their terminus where monolayers of fertilized eggs develop (Brillet, 1976; Clayton and Vaughan, 1986; Kobayashi et al., 1971). Recent studies document an air-deposition behaviour by burrow-guarding mudskippers in the field (Ishimatsu et al., 1998; Ishimatsu et al., 2000) and in artificial laboratory burrows (Lee et al., 2005), prompting the hypothesis that eggs are spawned and develop within a burrow's air-containing space. We tested this and related aspects of burrow incubation of eggs by conducting field and laboratory studies of the Japanese mudskipper *Periophthalmus modestus* Cantor. In the spawning season (mid-May to late August in our study area) male *P. modestus* excavate

'J' shaped burrows (Fig. 1) having two or three openings on the mudflat surface (Kobayashi et al., 1971). Using nuptial colouration and courtship displays the male attracts a female into its burrow, where spawning occurs, and the fertilized eggs are deposited in the terminus (Matoba and Dotsu, 1977). Thereafter, the male tends its burrow for about 1 week until the eggs hatch.

Field and laboratory studies were carried out to determine the role of the male in securing egg development and hatching in burrows. The questions we studied were as follows. (1) Does burrow air occur in the egg chamber? (2) Does a burrow-guarding male deposit air before or after egg spawning? (3) How are the oxygen levels of the egg chamber maintained during egg-incubation periods? (4) Does egg chamber hypoxia stimulate male's behaviour to restore egg-chamber P_{O_2}? (5) Do mudskipper eggs really develop in air, and what is the trigger for hatching? Integration of the data obtained provides strong evidence that during each low tide the burrow-guarding male adds air into an air-filled egg chamber, thereby refreshing egg-chamber O_2. The air-adding behaviour is at least partially governed by egg-chamber P_{O_2}. Once egg development is complete, the male removes egg-chamber air to induce hatching.

Materials and methods

Field studies on *Periophthalmus modestus* Cantor burrows occurred from 1998 through 2006 on mudflats in Ashikari, Saga Prefecture, Japan. The burrows of *P. modestus* could be easily differentiated from those of two other sympatric intertidal gobies [*Boleophthalmus pectinirostris* (Linnaeus), also an oxudercine, and *Odontamblyopus lacepedii* (Temminck & Schlegel), subfamily Amblyopinae] by the presence of mud pellets (i.e. mouthfuls of mud ejected during burrow excavation and maintenance) around the openings.

Burrow respiratory environment

Samples for burrow-water P_{O_2} determinations were taken in glass tuberculin syringes (1 ml volume), the tips of which were inserted to a depth of at least 5 cm into the water standing in burrow shafts at low tide. The filled syringes were immediately stored in ice water and transported to the Saga Prefectural Ariake Fisheries and Development Center where, following anaerobic centrifugation to separate the suspended mud particles, the P_{O_2} of the supernatant in each was measured using an O_2 electrode (E101, Cameron Instrument Co., TX, USA) and a meter (OM200, Cameron Instrument Co.). The total gas volume and its P_{O_2} and P_{CO_2} levels were compared for 20 guarded egg chambers (i.e. eggs present) and 12 pre-spawning egg chambers (i.e. male courting, no eggs in the chamber). Egg-chamber gas was obtained by forming a shallow depression over the burrow, lining it with plastic sheeting and placing a thin layer of water therein, and then compressing the underlying burrow to force air up under the plastic where it could be withdrawn into a glass syringe for determination of volume and gas content. Gas P_{O_2} and P_{CO_2} were measured by chromatography (Shimadzu GC-14B, Kyoto, Japan). Determinations were also made for both the average particle size of the mud comprising the layer between the mudflat surface and the egg chamber (Shimadzu SALD-3100 Laser Diffraction Particle Size Analyzer) and the redox potentials (ORP electrode, 9678BN and 230Aplus meter, ThermoOrion, MA, USA) of mud from the egg-chamber surface, the shaft walls, and at sites remote from the burrow. A combination pH electrode (ThermoOrion 8163BN) was used as a reference electrode to improve stability of the redox measurements.

Egg-chamber observations

Burrow structure and the instrumentation procedures are detailed in Fig. 1. For each burrow selected for instrumentation the first step was to remove standing water in the shaft to prevent it from accidentally inundating the eggs when the egg chamber was opened. The mud over the chamber and surrounding the burrow shaft was then excavated down to the level of the chamber's roof. When the chamber was opened, the presence of eggs was verified and their state of development assessed by immersing a small number of eggs in water. If immersed eggs did not hatch within 5 min, the chamber was considered to contain relatively young eggs and instrumentation proceeded. The roof of the egg-chamber was fitted with the upper-half of a plastic bottle into which both an endoscope (HSCI-S10M, HOGA, Kyoto, Japan) and an O_2 electrode (ThermoOrion 083025A) had been mounted. For two burrows a small Teflon™ tube (1 mm i.d.) was also inserted into the chamber roof so that egg-chamber O_2 levels could be experimentally modulated (see below). Stainless steel impedance electrodes were inserted in the burrow section below the egg chamber (Fig. 1). Mud was then replaced to cover and secure the instruments and the section of burrow shaft destroyed
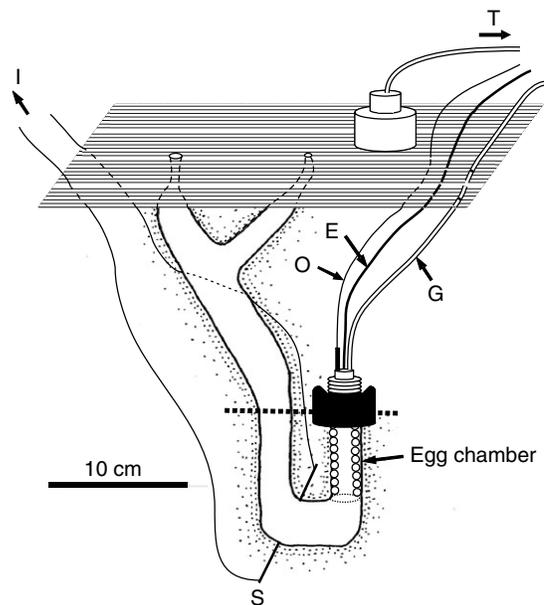

Fig. 1. Three-dimensional drawing of a *Periophthalmus modestus* burrow showing monitoring device positions: O_2 electrode (O), endoscope camera (E), tube for gas injection (G), stainless-steel electrodes (S) for impedance measurement (I), and pressure transducer tube connection (T). Thick dotted line above egg chamber shows the level of burrow excavation required for instrumentation. Thin broken line under the egg chamber shows approximate position of the air–water interface.

by digging was reconstructed. Unless egg-chamber excavation and instrumentation caused the burrow-guarding male to abandon the nest completely (in such cases the eggs did not hatch), it would re-enter the burrow and resume its activities. However, the male usually did not use the hand-constructed opening, but built new connections to the vertical shaft. To monitor the tidal cycle, a Tygon™ tube connected to a pressure transducer (Keyence AP-81A/AP-12, Osaka, Japan) was deployed on the mud surface at nearly the same height as the reconstructed burrow opening.

Cables connecting the measuring devices to their monitors (ca. 10 m long) extended to a tent pitched on a nearby pier (ca. 3 m above the mudflat surface). Signals from the O_2 electrode were fed to and saved every 5 min in an O_2 meter (ThermoOrion 835A). The O_2 electrode (a nominal drift of 0.1% per day) was calibrated with humidified N_2 gas and air immediately before egg-chamber instrumentation and after termination of the observations. Egg-chamber P_{O_2} data were corrected for hydrostatic pressure using measured water depth and specific gravity of 1.02 for seawater, and drift corrections assumed a linear drift over the observation period. Endoscope video recording was kept minimal to lessen the effects of light on the guarding male's behaviour. Signals from the tide-monitoring pressure transducer were digitized and stored every 5 min in a data acquisition system (Keyence NR-1000).

Preliminary laboratory experiments indicated that the passage of dead *P. modestus* through a pipe generated impedance signals of more than ±500 mV, and it was expected that this criterion could be used to indicate the number of trips made by the male into the burrow chamber. Burrow impedance signals were amplified with an impedance converter (model 2991, UFI, CA, USA), digitized by another data acquisition system (Keyence NR-2000), and stored in memory cards (32 MB) at a sampling rate of 2 Hz. The digitized data were analysed with software (Wave Shot v.1.05 and Flex Logger v.1.07, Keyence).

The two burrows in which the egg chamber had been fitted with a small Teflon™ tube (Fig. 1) were used to test the effects of a reduction in egg-chamber O_2 level on fish passage through the burrow section connecting to the chamber (indicated by impedance spikes) as a possible indicator of air-adding behaviour. For these tests humidified hypoxic-gas mix, in which the P_{CO_2} was adjusted to minimize changes in egg-chamber P_{CO_2}, was introduced into the egg chamber to reduce its P_{O_2} to within 0.25 kPa of the levels observed at the beginning of the low tide period. Hypoxic gas injections were made approximately 1 h before the burrow was covered by water of the rising tide and while the burrow-guarding male was on the mudflat surface. After egg incubation and hatching were completed, egg-chamber air was withdrawn through the Teflon™ tube to determine the volume for the computation of air-adding frequency (see Appendix).

Laboratory studies of aerial egg development and hatching

Five intact egg chambers were transported to the laboratory for studies of egg-hatching success in relation to time and for comparisons of the hatching success of eggs in air and in water. After clearing mud from around the chamber, the column of mud surrounding the intact egg chamber (i.e. a complete, mud-walled, air-filled chamber having a ventral opening into the

burrow shaft) was extracted by sliding a bottomless plastic bottle over it, placing a plastic plate under the open end of the bottle and lifting it out. Only egg chambers with relatively young eggs (as defined above) were extracted and, once in the laboratory, the chambers were incubated in high humidity at 23°C. About 50 eggs were removed from each chamber and used in daily 4 h tests of hatching competence (done by immersing eggs in freshwater, 50% seawater and full-strength seawater). Also tested was the viability of eggs kept submerged in hypoxic water. The total number of eggs in each egg chamber was estimated from the density of eggs and total surface area of the chamber.

Results
Burrow respiratory environment

The standing water in the burrow shafts was extremely hypoxic [P_{O_2}=1.47±1.14 kPa (mean ± s.d., N=13); the P_{O_2} of air-equilibrated water is 20.6 kPa]. The average gas volume of the egg chambers was 47±19 ml (N=17) with a P_{O_2} of 13.8±3.0 kPa and a P_{CO_2} of 1.11±0.57 kPa (N=20) and, as in other mudskipper burrows (Ishimatsu et al., 1998; Ishimatsu et al., 2000), there was a significant inverse relationship between P_{O_2} and P_{CO_2} (P_{CO_2}=2.65–0.11P_{O_2}, r=–0.577, N=20, P=0.008, Fig. 2). The egg-chamber volumes of 'still courting' (i.e. no eggs present) and 'egg-guarding' males did not differ (47±19 *vs* 43±15 ml, N=12) and, while the P_{O_2}–P_{CO_2} regression slopes of the two were also not different (ANCOVA, P=0.141), the mean P_{O_2} of the 'no-egg' chambers was significantly less (6.15±4.66 kPa *vs* 13.8±3.0 kPa, P=0.04, *t*-test, d.f.=30).

The method of egg-chamber excavation did not allow visual verification that the egg chambers contained air (i.e. we could not rule out the possibility that water had drained out of the chamber during excavation). However, our supposition that the egg chambers normally contain air was supported on the basis of the colour and the redox potentials measured for the mud lining the chamber wall. Mud on the wall of the egg chamber
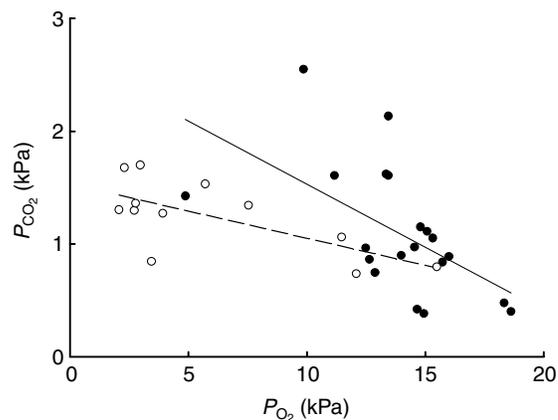

Fig. 2. Relationship between P_{O_2} and P_{CO_2} of *Periophthalmus modestus* egg-chamber air determined for burrows with egg-guarding males (solid circles) and courting males (open circles). Regression equations are P_{CO_2}=2.62–0.11P_{O_2} (r=–0.577, N=20, P=0.008) for burrows with guarding males, and P_{CO_2}=1.53–0.05P_{O_2} (r=–0.678, N=12, P=0.015) for burrows with courting males. Slopes are not significantly different from each other (ANCOVA, P=0.141).

had a uniform brownish-gold colour rather than the dark grey colour of mud on the surface of other burrow regions. Also, the positive redox potential measured for egg-chamber mud (82.9±23.4 mV, reflecting a greater oxidation state) was significantly higher than those of mud lining the burrow vertical shaft below the water line (–29.3±60.2) and mud positioned at the same depth (20 cm under the surface) but not near the burrow (–117.1±22.4, $N=8$; $P<0.01$ Sheffé test for pairwise comparisons). The average particle size of mud comprising the layer between the mudflat surface and the egg chamber was 7.3±0.5 μm ($N=5$). The estimated average number of eggs in a *P. modestus* burrow was 5200±765 ($N=11$).

Maintenance of egg-chamber O_2 by male's air-adding behaviour

Of 44 attempts to instrument burrows, only 6 records were obtained in which egg-chamber P_{O_2}, endoscope video recordings, and impedance data spanned 4 days or longer.

The P_{O_2} records documented tidal-cycle related changes; O_2 increased during low tide but dropped during high tide (Fig. 3A). The corresponding endoscope records verified that the eggs remained in air, that the male occasionally entered the egg chamber and that it was not adversely affected by the presence of air therein. The quality of the simultaneous burrow impedance records did not enable full quantification of male movement pattern. However, a semi-quantitative pattern of fish movement was discernible and, in the majority of cases, this showed that the male's passage through the burrow section under the egg chamber (and entry into the egg chamber) occurred mainly during low tide (Fig. 3A). The increase in egg-chamber P_{O_2} during low tide was a common finding for all of the instrumented burrows. The average egg-chamber P_{O_2} was 12.8±0.99 kPa ($N=4$) at the beginning of low tide exposure (i.e. burrow opening exposed to air) and was 16.8±0.32 kPa at the end of the low tide. The correspondence between the increase in P_{O_2} and the impedance records suggests that during low tide the male adds gulps of air to the egg chamber and this raises P_{O_2}.

More irregularity in the impedance records was found for burrows that were sufficiently high on the mudflat to remain uncovered by the highest neap tides; these showed impedance signals during both low and high tide. While also reflecting the tidal cycle, the P_{O_2} in these higher-positioned egg chambers showed small, intermittent step increases during 'high tide' that also correlated with impedance-indicated movement. In one extreme case, the monitored burrow remained uncovered for 4 days after instrumentation. The subsequent increase in tidal amplitude and the covering of the burrow by rising water coincided with hatching (Fig. 3B).

Egg-chamber P_{O_2} as a possible modulator of air-adding behaviour

To examine possible role of egg-chamber hypoxia as a modulator of air-adding behaviour, a volume of hypoxic gas was injected into the egg chamber to reduce P_{O_2} to within 0.25 kPa of the level observed at the beginning of the low tide period. Fig. 4A,C show the gradual rise in egg-chamber P_{O_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
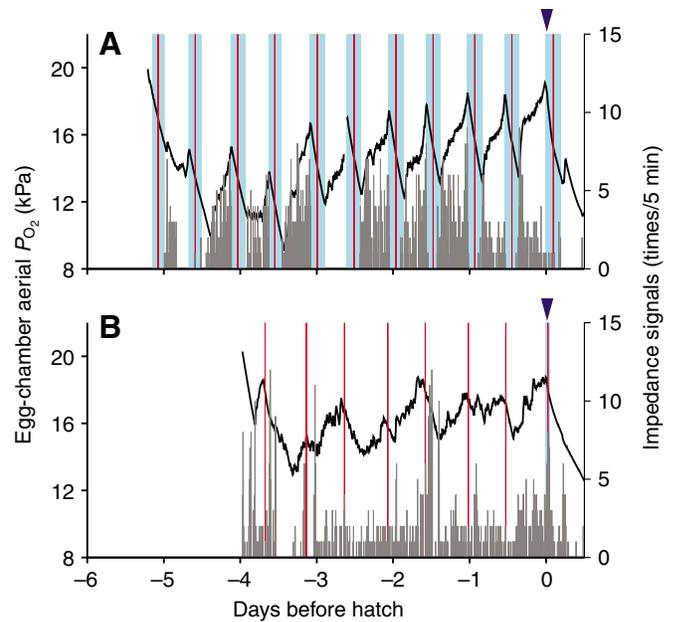

Fig. 3. Continuous records of *Periophthalmus modestus* egg-chamber air P_{O_2} (black line) and the timing of the male's egg-chamber visits (indicated by impedance signals, grey spikes) in relation to the tidal cycle. Data acquisition began with the day of instrumentation and continued until egg hatching, initiated by the male on a rising tide (blue triangle). The beginning of day zero is set to the time when a burrow was covered by an incoming tide, following which egg hatching occurred. The initial high P_{O_2} reflects the opening of the egg chamber to air during instrumentation. (A) Record for a burrow regularly covered (blue bars) and uncovered by the tidal oscillation (red lines show the highest tide). (Note: No impedance data 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).

introduction (arrowheads). Although hypoxic gas injection reduced egg-chamber P_{O_2} to near the level observed at the beginning of each low tide, the male fish was able to rapidly restore the P_{O_2} within 1 h. For both of these burrows ANCOVA comparisons show a significantly greater rate of P_{O_2} increase for the 60 min after hypoxia injection than for the first 60 min of low-tide exposure, which implies that air-adding behaviour was not solely modulated by egg-chamber air P_{O_2}. This possibility is further supported by the fact that the air-adding frequency (f_a) following hypoxic gas injection lies far above the calculated f_a–P_{O_2} relationships (Fig. 4B,D).

Laboratory studies of mudskipper eggs

Eggs from the incubated chambers developed normally in air and had embryos ready for hatching (indicated by frequent turning) within 6–7 days of their estimated fertilization time (Kobayashi et al., 1972). This contrasts to eggs from these same burrows which, when placed in hypoxic water ($P_{O_2}\approx2$ kPa, simulating typical burrow-water conditions), succumbed within 2 days.

Although the laboratory-incubated eggs developed in air, they did not hatch and eventually died. It was found that water

immersion (either in freshwater or seawater) was necessary to trigger egg hatching. Hatching competence tests on developing eggs over a 12-day period showed a 5–6 day window within which immersion must take place to ensure a mean successful hatch rate of 80% (Fig. 5). Eggs remaining in air beyond this time had a reduced hatching competence. Although hatching occurred in water of different salinities (i.e. freshwater, 50% seawater and full-strength seawater), larvae induced to hatch in freshwater did not survive unless transferred to higher salinities.
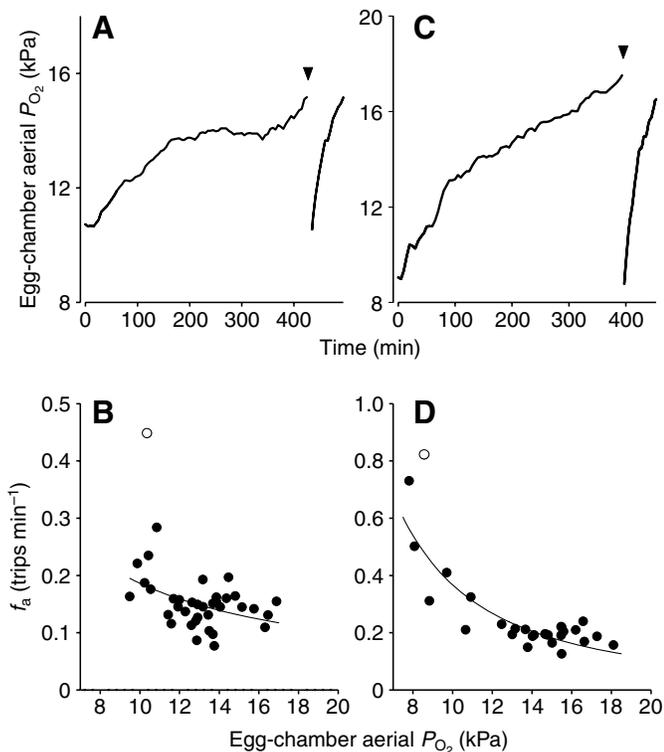

Fig. 4. Effect of hypoxic gas injection on egg-chamber P_{O_2} (A,C) and estimated air-adding frequency (B,D) of two male *Periophthalmus modestus*. (A,B) Burrow instrumented on June 30, 2004. (A) Real-time records of the low-tide increase in egg-chamber P_{O_2} contrasted with the significantly higher (3.2×, $P<0.0001$, ANCOVA) P_{O_2} increase rate observed over 60 min following hypoxia injection (arrowhead) than at the beginning of the low-tide period with nearly identical initial P_{O_2}. (B) An inverse relationship between egg-chamber P_{O_2} and air-adding frequency (f_a) as determined for five complete low-tide periods preceding hypoxic-gas injection (solid symbols) and f_a after injection (open symbol). Egg-chamber P_{O_2} at the beginning of each 60 min segment is plotted against f_a (see Appendix). Regression line equation; $f_a=1.39P_{O_2}^{-0.872}$ ($P=0.006$, $r=0.449$, the post-hypoxia value is not included in the regression). (C,D) Burrow instrumented on July 29, 2004. (C) Real-time records of egg-chamber P_{O_2}. Hypoxia injection (arrowhead) caused 3.2-fold higher P_{O_2} increase than at the beginning of the low-tide period ($P<0.0001$, ANCOVA). (D) An inverse relationship determined for four low-tide periods and higher f_a immediately following hypoxia injection, as in A. Regression line equation; $f_a=19.68P_{O_2}^{-1.729}$ ($P<0.0001$, $r=0.881$). Solid and open symbols represent data points obtained before and after hypoxic-gas injection, respectively, as in B.

Hatch induction caused by air removal and egg-chamber flooding

Endoscope video records (see Movie 1 in supplementary material) documented the male's role in hatch induction (Fig. 6, Table 1). Approximately 80 min after the burrow openings were inundated by the rising tide, the egg-guarding male triggered egg hatching by expelling air from the egg chamber, which immersed the eggs. Air removal was done by transporting air gulps into the burrow shaft leading to the openings (Fig. 1) and releasing them. As this was occurring bubbles were observed exiting from the burrow openings. Video records showed that flooding the chamber required the removal of an average of 103±42 air gulps, which gave an almost exact volume of total egg-chamber air (47 ml) based on the buccal-branchial capacity of *P. modestus* (0.46 ml in a 2.6 g fish, Table 1). Hatching occurred during evening or nocturnal rising tides (Fig. 7) but was not correlated with the lunar cycle.

Discussion
Mudskipper reproduction as an adaptation to the mudflat environment

Several littoral and sublittoral fish species deposit demersal eggs on surfaces in the intertidal zone that will emerge from the water as the tide recedes after spawning. This affords the eggs protection from aquatic predators and development in a habitat that is warmer and has greater O_2 availability. After completing development, the embryos hatch typically by inundation in high tide to be scattered as pelagic larvae (DeMartini, 1999; Helfman et al., 1997; Martin et al., 2004). Mudskippers differ from these fishes that spawn at the water's edge since a lack of cover and the extreme conditions on the mudflat preclude placement of eggs on the open surface and make it impossible for an adult mudskipper to remain with the eggs and care for them. Sequestration in burrows protects mudskipper eggs from predation and tidal extremes in current flow, temperature and salinity. However, the burrow water is extremely hypoxic (Gordon, 1995; Gonzales et al., 2006) (data in the present study), so this requires a solution for the O_2 supply problem
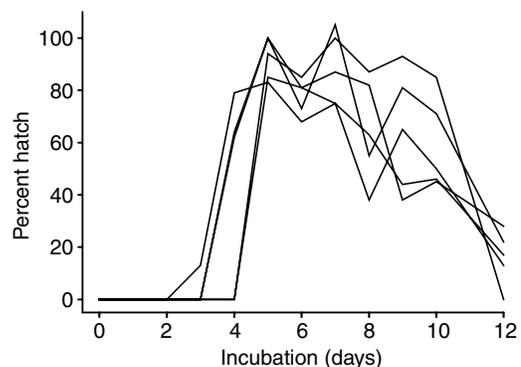

Fig. 5. Daily determinations of the hatching percentage of *Periophthalmus modestus* eggs taken from five intact, air-filled egg chambers (separate lines) that were incubated in the laboratory. Hatching was induced by submerging about 50 eggs removed from the chambers in 50% seawater.

Table 1. *Parameters related to the hatch-induction behaviour of Periophthalmus modestus*

Mass of the burrow-guarding male (g)	2.6±0.5 (17)
Number of gulps taken	103±42 (8)
Buccal-branchial cavity gas volume (ml)	0.46*
Estimated gas volume removed by the male (ml)	47.4[†]
Measured egg-chamber gas volume (ml)	47±19 (17)
Time of hatch-induction behaviour (min)	16±7 (8)
Time between burrow-opening inundation and beginning of hatch induction (min)	83±21 (5)
Time between the end of hatch induction and the highest tide (min)	31±18 (4)
Time between the end of hatch induction and burrow-opening emersion (min)	161±28 (4)

Values are means ± s.d. (*N*).
*Calculated from: volume (ml)=0.076+0.148×body mass (g) [*r*=0.948, *N*=11, *P*=8.7×10^{-6} (Graham et al., 2007)].
[†]The product of buccal-branchial cavity gas volume and the number of gulps (see text).

which, as demonstrated here for *P. modestus*, is done by egg development in air and refinements in parental care.

This study documents the critical importance of the male mudskipper in excavating a burrow, complete with an air chamber, at a position in the intertidal zone where it will have access to atmospheric air during low tide. Even though direct evidence for the presence of air in egg chambers could not be obtained, the differences in mud colour and the positive redox potential of chamber mud, the successful aerial egg development in the laboratory, and the burrow-guarding male's air-adding behaviour all attest to air deposition in egg chambers of intact *P. modestus* burrows. It should be noted that for another mudskipper, *Periopthalmodon schlosseri*, which is much larger than *P. modestus*, it has been possible to confirm the presence of air in egg chambers of intact burrows by directly inserting an endoscope through a much larger vertical shaft (A. Ishimatsu, T. Takeda, Y. Tsuhako and K. H. Khoo, manuscript in preparation).

Just as air access at low tide is required for egg-chamber O_2 replenishment during incubation (Fig. 3), submersion of the burrow opening by a rising tide is necessary for the male to induce hatching and larval escape by flooding the egg chamber (Fig. 6). In addition to being in synchrony with the tidal cycle, the male's egg-chamber management behaviour is also modulated by its P_{O_2} (Fig. 4). The aerial-egg development and water-induced hatching demonstrated for *P. modestus* in this study agrees with observations of Brillet (Brillet, 1976) who, while not detailing methodology, reported that the eggs of *P. sobrinus* [=*P. argentilineatus* Valenciennes (Murdy, 1989)] required both air incubation and subsequent submersion for hatching and did not survive if incubated in water or kept in air but not immersed after development.

Air-adding behaviour

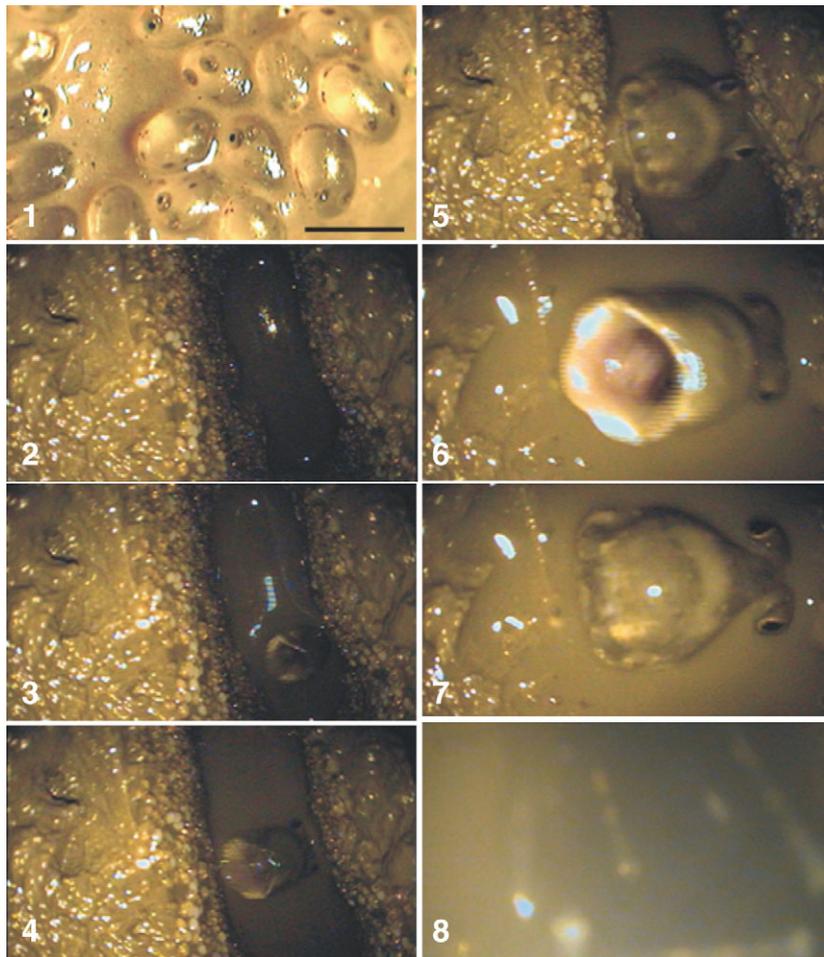On the basis of the presence of air in the egg chambers of males engaged in courtship (i.e. no

Fig. 6. Interior view of *Periophthalmus modestus* egg chamber. (1) Eggs on the mud wall of a laboratory-incubated chamber (scale bar 1 mm). (2–7) Endoscope video frames documenting hatch-induction behaviour (June 19, 2002). Vertical view is down, through the egg chamber (note glistening monolayer of eggs on the mud wall), to the burrow-water surface. The male appears at the surface with its mouth open, and becomes progressively larger as it moves closer to the camera. Removing mouthfuls of air (3,4,6,7) raises the water level, which immerses the eggs, causing them to hatch. (8) Hatched larvae [average total length 2.84 mm (Kobayashi et al., 1972)] swimming in the flooded egg chamber 48 min after frame 7 (see also Movie 1 in supplementary material).

eggs present), we speculate that both spawning and fertilization occur in air, although this has not been observed. Details about fish aerial spawning behaviour were recently described for the rockhopper blenny *Andamia tetradactyla* (Bleeker) (Shimizu et al., 2006). The female deposits a monolayer of eggs in rock crevices along the splash zone, which the male fertilizes by rubbing its genital papillae over them. *Periophthalmus modestus* and other mudskippers may have a similar spawning behaviour and, while this is not documented, egg-chamber videos (Movie 1 in supplementary material) show that males can emerge into the air phase and both turn and crawl on the mud and egg surfaces.

The comparable volumes of the 'no-egg' and 'egg-containing' chambers imply that the egg chamber is maximally filled with air at construction and that air additions during egg incubation displace excess air into the burrow shaft from where it ultimately exits the burrow. The pre-spawning deposition of air inside an egg chamber may be beneficial for maintaining egg-chamber P_{O_2} during the incubation period because it would oxidize the highly reducing surrounding mud and thus lessen the total O_2 need once eggs are added.

The correlation between male activity (impedance data) near the egg chamber and its increase in P_{O_2} (Fig. 3) most likely reflects transport of mouthfuls of fresh air into the chamber. Because burrow shafts are water-filled during low tide, it is not possible that air in the burrow was trapped during low tide as suggested for *P. sobrinus* (Brillet, 1976). Also, the small particle size of the overlying dense mud, its low redox potential, and the egg-chamber's position 20 cm under the mud surface (Fig. 1), exclude the possibility that the high egg-chamber P_{O_2} results from the diffusion of atmospheric air. That the decline in egg-chamber P_{O_2} during high tide reflects egg-mass respiration is suggested by the product of the measured rate of O_2 consumption of a developing mudskipper egg [76.5±12.0 nl h^{-1}/ hatch-competent egg (Etou et al., 2007)] and our estimate of the

number of eggs in the chamber (5200). Assuming an egg-chamber air volume of 47 ml and a temperature of 24°C, the respiring egg mass would consume 1.1–2.0 ml of O_2 during a daytime high-tide period on the day of hatching (duration ranging 170–310 min, $N=4$; note that hatching occurred at the nocturnal high tide of the same day), and this accounts for 75–100% of the observed egg chamber aerial O_2 depletion of the high-tide periods (1.2–2.3 ml O_2) calculated from the slope of egg-chamber air P_{O_2} over time (–0.013 to –0.018 kPa min^{-1}).

The lowest egg-chamber P_{O_2} levels recorded at the end of high tide ranged from 8.2 to 14.5 kPa ($N=8$). These are above the critical P_{O_2} (i.e. the O_2 partial pressure below which aerobic metabolism declines) of a *P. modestus* egg [4.3 kPa (Etou et al., 2007)]. Although the high-tide decline in P_{O_2} did not reach the eggs' critical P_{O_2}, this level would have eventually been reached if the male had not added air during the subsequent low tide. Also, variables such as chamber volume, egg mass, the burrow's position in the intertidal zone, tidal amplitude, and whether or not the male also requires egg-chamber O_2 for respiration could all combine to cause greater reductions in egg-chamber O_2 during high tide.

Most nest-guarding fishes respond to hypoxia with fanning or other ventilation behaviours to augment O_2 delivery to the eggs (Jones and Reynolds, 1999; Takegaki and Nakazono, 1999). For species guarding eggs in the intertidal zone, fanning may be needed during low tide when pools are isolated and convective O_2 delivery is reduced. In this respect the air-adding behaviour of *P. modestus* parallels fanning in being hypoxia-induced and linked to the tidal cycle. A principal difference between the two is that, while requirements for fanning can vary depending on variations in the physical and biotic factors around an underwater nest, the small, mud-enclosed burrow egg chamber will always require the addition of air, which can only be done during low tide. The male must therefore ensure that there is sufficient O_2 to meet the requirement of the eggs during the subsequent high tide.

The mechanism enabling *P. modestus* to monitor O_2 levels in its egg chamber may not differ from the O_2 sensing mechanisms of other fish species. Most fishes have O_2-sensitive chemoreceptors that monitor the external environment and these are often positioned in the buccal-branchial cavity, associated with the gills or other structures (Milsom and Burleson, 2007). These external receptors have an important role in rapid responses to hypoxia, which appears important for air-breathing fishes (Graham, 1997). For *Pn. schlosseri* aerial hypoxia is a more important driver of air ventilation than aquatic hypoxia (Aguilar et al., 2000). While the air-adding behaviour of *P. modestus* is likely a ventilatory response driven by external O_2 receptors, it requires a more sophisticated behavioural combination of air gulping at the water surface, transporting mouthfuls of air down through the burrow shaft, releasing these into the egg chamber and repeating this action until egg-chamber P_{O_2} reaches a level sufficient to sustain the O_2 consumption of the eggs during the following high-tide period. The injection of hypoxic gas into the egg chamber late in the low-tide period increased the male's air-adding frequency and egg-chamber O_2 was restored to its pre-hypoxia injection level within 1 h (Fig. 4). The far higher rate of air addition triggered by this treatment implies that the
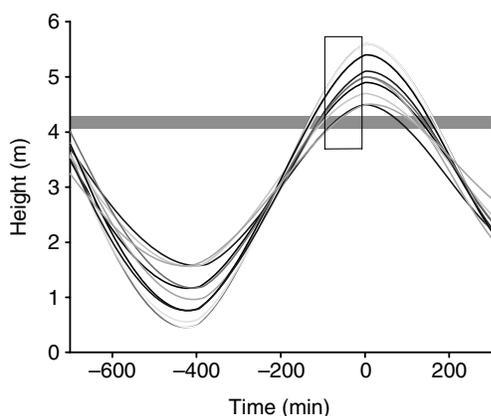

Fig. 7. Relationships between tidal height and the timing of hatch induction of *Periophthalmus modestus*. The horizontal grey bar represents heights of mudflat surface in which openings of *P. modestus* burrows were located. Different curves show changes in tidal height during the last tidal cycles of egg incubation periods determined for 11 burrows. Curves are adjusted to the peaks of flood tide (time zero). The rectangle encloses the curves for an approximate range in which hatch induction occurred.

male somehow modulates this behaviour by integrating information on egg-chamber P_{O_2} that must be attained by the end of low tide and the time available before the next submersion. This could be tested through hypoxic gas injections at different times during low tide.

A male *P. modestus* can readily add air during low tide, but numerous obstacles prevent this at high tide. When the burrow is submerged (by as much as 2 m at high tide in our study site), the male would need to swim to the surface, gulp air, and return it to the burrow. Direct observations of male activity during high tide are difficult because the water covering the mudflat at high tide is extremely turbid. Nevertheless, mudskippers were never observed surfacing to gulp air during high tide. If the male's primary duty is to guard the nest, it may spend high tide near the burrow openings where, while intercepting potential predators, it could respire aquatically (Tamura et al., 1976). Both endoscope and impedance data suggest that the male does not spend much time in the egg chamber during high tide. However, this conclusion is tentative because endoscope recording times were kept minimal to lessen disturbance from its light and the lower number of impedance spikes at high tide does not indicate that the fish is not in the egg-chamber.

Induction of hatching by air removal

Inundation is the most common hatch mechanism for the aerially spawned eggs of fishes (Yamagami, 1988) and amphibians (Warkentin, 2002). Egg submergence may be by rain-caused flooding or by cyclic change in water height (spring tides). In cases where immersion occurrence is less predictable, extra yolk supply within the egg extends hatch competency (Moffatt and Thomson, 1978). Egg hatching for *P. modestus* differs from these general examples in that, once development is complete, the guarding male triggers hatching by inundating the egg chamber. This behaviour, which usually occurs during a nocturnal rising tide (Fig. 7), may be initiated through the male's capacity to sense that egg development is complete (e.g. increased vibrations from turning larva) or through an endogenous factor (e.g. a tidal rhythm) regulating its behaviour. However, because *P. modestus* eggs only have a 5–6 day window for hatching competence, the male's initial selection of burrow position within the intertidal zone and the timing of spawning in its burrow relative to the tidal cycle are important factors in hatching success.

It is not known how hatched larvae make their way from the egg chamber to the open water where they will live in the plankton for about 50 days (Kobayashi et al., 1972). Preliminary field experiments suggest that relatively few of the hatched larvae are able to find their way out of the burrow. However, rapid escape is important as survival of newly hatched larvae kept in hypoxic water (P_{O_2}=2.0 kPa) quickly decreased with time (35% in 3 h and <10% in 8 h) (Etou et al., 2007). Whether or not the guarding male is involved in transporting larvae out of the burrow remains to be determined.

The origin of subterranean aerial brooding in mudflat gobies

Fishes in the family Gobiidae are typically substrate brooders and attach adhesive eggs to rocks, in depressions, or to the inside of enclosed spaces including burrows (Breder and Rosen, 1966; Thresher, 1984). In addition to egg guarding, male gobies carry

out other parental-care behaviours such as preening and fanning that have been described for other species (Blumer, 1982; Breder and Rosen, 1966). As common among gobies, these traits appear to have developed before mudskipper's invasion of the land and have set a stage for the development of their novel reproductive strategy. Acquisition of air-breathing capability is a vital prerequisite for brooding eggs in a burrow filled with hypoxic water, which demands the storage of air within the burrow and aerial egg development. Several of the other species classified with mudskippers in the subfamily Oxudercinae also live in burrows and have the capacity to gulp air at low tide, suggesting that they are also air breathers (Graham et al., 2007). Another mudflat dweller, the eel goby *Odontamblyopus lacepedii*, which is sympatric with *P. modestus*, has recently been shown to be a facultative air breather (Gonzales et al., 2006). *Odontamblyopus*, however, is not amphibious and is restricted to its extensive mudflat burrow where, during low tide, it breathes air to sustain metabolism. The eel goby's reproductive behaviour is not known, however, fish that spawned in captivity attached their eggs along the upper, inner side of the submerged clay pipes in their holding tank (Dotsu and Takita, 1967), implying that burrow spawning may occur. If *O. lacepedii* and some of the other oxudercines are burrow-brooders then, because of severely hypoxic burrow water (Gonzales et al., 2006; Ishimatsu et al., 2000), both air storage and air-adding behaviour during low tide are likely requirements for reproductive success. Investigations into the reproductive strategies of other gobies of Oxudercinae and related subfamilies would shed light onto the evolution of subterranean aerial brooding of mudskippers.

Conclusion

In summary, mudskippers maximize their potential for reproductive success in the mudflat habitat with eggs that are capable of developing within the air placed in the burrow egg chambers by the male. In addition to guarding the nest, the male carries out elaborate egg-care behaviours that include gulping air and transporting it to the egg chamber at low tide to ensure adequate oxygen supply for the subsequent high tide (when air cannot be obtained), the orchestration of submergence hatch induction by removing air and thus flooding the egg chamber during a rising tide, and possibly the facilitation of burrow escape by the newly hatched larvae.

Appendix

The frequency of air addition to an egg chamber was estimated from the egg-chamber volume, the male's buccal-branchial chamber capacity (Table 1), and the observed changes in O_2 concentration of the egg-chamber air during successive low-tide periods including one in which hypoxic gas injection was made. Each low-tide period was divided into approximately 60 min segments, and assuming that the rate of O_2 decline recorded during a preceding high tide (Fig. 3A) remained constant throughout the subsequent low-tide period, air-adding frequency (f_a) could be calculated for each segment by:

$$f_a = \frac{[F_{O_2,f} - F_{O_2,i} - ST]V_e}{F_{O_2,e}V_mT}, \tag{A1}$$

where $F_{O_2,i}$ and $F_{O_2,f}$ are the initial and final fractional concentrations of egg-chamber O_2 (expressed as %) for each segment, S is the rate of F_{O_2} decline during a preceding high-tide period (% min^{-1}, r^2 ranging from 0.946–0.997), T is segment duration (approximately 60 min), V_e is egg-chamber volume (ml) measured after egg incubation and hatching were complete (see Materials and methods), V_m is the volume of air (0.46 ml; estimated buccal-branchial cavity gas volume of a burrow guarding male, see Table 1) added to the chamber on each trip (ml trip^{-1}; Table 1), and $F_{O_2,e}$ is the fractional O_2 concentration of ambient air. Further assumptions underlying f_a estimation are: (1) the male added its maximum possible air volume on each trip to the egg chamber (i.e. there was no residual gas volume in the buccal-branchial cavity after the male expired air into the egg chamber), (2) the added air volume was immediately mixed with the air in the chamber, and (3) the volume of air in the egg chamber remained constant (i.e. the addition of new air overflowed the equal volume of the mixed air).

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