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Ecology of the Australian mudskipper *Periophthalmus minutus*, an amphibious fish inhabiting a mudflat in the highest intertidal zone

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Running headline: Ecology of *Periophthalmus minutus*

Abstract. A population of *Periophthalmus minutus* inhabiting a mudflat in the highest intertidal zone in Darwin was investigated for surface activity, feeding and reproduction in relation to environmental conditions in dry (August) and wet seasons (February). On days with tidal inundation, the fish were diurnally active on exposed mudflat surface at low tide, but retreated into burrows during daytime inundation and at night. Temperature above 40°C and heavy precipitation suppressed the daytime surface activity of the fish. During neap tides, the mudflat remained uncovered by the tide for nine days in both seasons. The fish confined themselves in burrows without ingested food throughout the nine-day period in August, but they remained active on mudflat surface and kept foraging in February. The salinity of burrow water during the nine-day emersion was extremely high (72 ± 6 psu, mean ± SD) in August, but lower (46 ± 9), though still higher than the open seawater value (34), in February. The burrows had a shape of "J" in February, but were straight with no upturn in August. Fertilized eggs were collected from the upturned portion, and hatched upon submersion. Juveniles occurred in water pools on the mudflat surface in March.

Keywords ecology, environmental stress, natural history, *Periophthalmus minutus*, reproductive strategy

Introduction

Mudskippers are amphibious euryhaline gobies inhabiting intertidal mudflats of east Asia, the South Pacific islands and northern Australia, westward across South-East Asia and the Arabian Peninsula to both the east and west coasts of Africa (Murdy 1989). Being residents of mudflats, they are regularly exposed to environmental fluctuations such as cyclic submersion/emersion of the habitat, daily and seasonal changes in temperature, and variable salinities caused by tide, evaporation, and freshwater runoff after heavy precipitation (Ishimatsu and Gonzales 2011). Among the four genera of mudskippers (*Boleophthalmus*, *Periophthalmodon*, *Periophthalmus*, and *Scartelaos*), species of *Periophthalmus* are usually regarded as the most terrestrial (Clayton 1993), actively foraging, courting, and defending territories on exposed mudflat surface during low tide. Still, they usually stay near the water's edge probably to satisfy the needs for water and ion balance (Dall and Milward 1969), cutaneous respiration (Graham 1997), and excretion of nitrogenous wastes (Ip *et al*. 2004). *Periophthalmus minutus* Eggert is distributed in the coasts of Southeast Asian countries and northern Australia (Murdy 1989). Nursall (1981) reported that the fish inhabited uppermost intertidal zones, including mudflats near and inside *Ceriops* thickets, in landward fringes of *Avicennia* forests, and in halophyte-bearing flats between and beyond these mangrove trees (note this species was referred to as "red-fin" in Nursall 1981, see Murdy 1989). More recently, Takita *et al.* (2011) confirmed the occurrence of *P. minutus* in the highest intertidal zones in northern Australia, and gave some accounts of the fish's natural history. Thus, *P. minutus* is thought to be one of the most terrestrially adapted species even among *Periophthalmus* mudskippers, and could provide insights into how ecology, physiology and behaviour of an originally aquatic vertebrate can be altered in transition from an aquatic to a terrestrial mode of life. Nevertheless, to our knowledge, only anecdotal information is available on any aspect of the biology of *P. minutus*. During the course of our field surveys, we encountered a population of *P. minutus* inhabiting a mudflat near Darwin, which remained uncovered by the tide for nine days or even longer (Itoki *et al*. unpublished data). Thus, this *P. minutus* population may represent an extreme case of mudskipper's adaptation to arid semi-terrestrial habitats. Our main interest was to examine how environmental conditions affect their most vital life-history traits, i.e., emergence from or retreat into their burrows, feeding and reproduction. Field surveys were conducted in November in 2000, March, July, and August in 2001, January and August in 2002, and January and February in 2003. Almost all rainfall occurs during the months from November to April (wet season), and the precipitation is near zero in June through August (dry season) in Darwin (May *et al*. 2002). In this paper, we report on the results obtained mainly in August 2002 and February 2003 when the most intensive investigations were conducted, but also include data for the other years to complement data on reproductive activity.

Materials and methods

Study site

Field observations of the mudskipper, *P. minutus*, were carried out at a highest intertidal mudflat surrounded by mangrove trees (*Lumnitzera racemosa*) near Darwin, Northern Territory, Australia (12°34’03” S, 130°53’09” E). The mudflat (ca. 250 × 100 –150 m) was located near a road in the Middle Arm of Port Darwin leading to Channel Island, and connected to the shore through three narrow channels (ca. 50 cm wide). There was no noticeable freshwater inflow to the flat. The mudflat's seaward fringe was approximately 250-300 m away from the shore (Fig. 1).

Environmental measurements

The height of the study site (ca. 6.9 m above the chart datum) was estimated by comparison of measured water depth in the study site and tidal prediction values for Darwin (© National Tidal Facility, The Flinders University of South Australia). Maximum daily water depths were then estimated from the differences between the reported daily maximum tidal heights and the height of the mudflat. The water table in *P. minutus* burrows was determined by inserting a graduated rubber tube (4 × 6 mm) into the burrows. Burrow water was sampled as much as possible with a syringe connected to a rubber tube and immediately analysed for volume and dissolved oxygen concentration. Temperature inside an artificial burrow (diameter 15 cm, depth 60 cm, Fig. 1) was determined with a thermo recorder (TR-50A, T&D Corporation, Japan) at 10 cm below the mudflat surface. Surface temperature of the mudflat was recorded with another thermo recorder buried at the depth of 0.2-0.5 cm beside the artificial burrow. Dissolved oxygen concentration was measured with an oxygen meter (YSI model 55) and an electrode, which was mounted in a custom-made cuvette containing a magnet bar to anaerobically stir sample water during measurements. The cuvette was placed in a container filled with seawater from each sampling site to minimize temperature change during the measurement. Air-equilibrated seawater was used for calibrating the electrode. The remaining water samples were kept in syringes, stored in ice water, and brought back to the laboratory. After filtration, the samples were analysed for salinity with a refractometer (S/Mill-E; Atago, Japan), and pH with a portable pH meter (MP-125, Mettler Toledo). In addition, surface mud was sampled at the time of fish counting in the morning at six points near the transects (see *Counting of emergent fish*), and analysed for water content by drying at room temperature (August 2002) or with a hot plate (February 2003). Salinity of pool water on the mudflat was also determined in both years. Precipitation records for the study period were obtained from the web site of the Bureau of Meteorology, Australian Government (Site Name, Channel Island, Site Number 14009; August 2002, http://www.bom.gov.au/jsp/ncc/cdio/weatherData/av?p_display_type=dailyDataFile&p_stn_num=14009&p_startYear=2002&statType=Rainfall+of+....&p_nccObsCode=136; February 2003, http://www.bom.gov.au/jsp/ncc/cdio/weatherData/av?p_display_type=dailyDataFile&p_stn_num=14009&p_startYear=2003&statType=Rainfall+of+....&p_nccObsCode=136). Note that precipitation for February 4^{th} to 6^{th} was summed in this report and reported as of 6^{th}. We confirmed that there was no rain on February 4^{th} or 5^{th}, 2003.

Counting of emergent fish

Three transects (each 50 m long and 1 m wide) were laid on the mudflat where *P. minutus* was abundant (Fig. 1). We counted the number of emergent mudskippers within 50 cm each from the midline of the transects before, during and after the mudflat emersion in 2002 and 2003. Counting was carried out between 10:00 and 12:00 except the days of diel observation (August 12^{th}, 15^{th}, 21^{st} and 24^{th} in 2002, and February 5^{th}, 8^{th}, 13^{th} and 17^{th} in 2003) when counting lasted from 1.0-1.5 h before sunrise to 1.0-1.5 h after sunset.

Analysis of gut content

Six individuals of *P. minutus* were sampled between 9:00 and 11:00 on August 11th, 13th, 16th, 19th and 22nd in 2002 and on February 4th, 6th, 9th, 12th, 15th and 18th in 2003. Fish were caught with nylon hand nets when they were on the mudflat surface or by excavating the burrows when they were not seen on the surface. The captured fish were killed with a sharp blow on the head, and at the site of capture the body weight was measured to the nearest 10 mg and total length to the nearest 0.1 mm, and preserved in 10% formalin after transecting the medulla and opening the abdominal cavity. In the laboratory, the entire alimentary tract was isolated to identify and enumerate individual food items.

Burrow casting

Burrows of *P. minutus* were cast following the method described in Ishimatsu *et al.* (2000). Briefly, a methyl methacrylate resin was mixed with a polymeriser immediately before casting, and poured into the burrows. The casts were excavated after three days to ensure complete resin setting. The numbers of burrow cast made were 10 in August 2001 (a preliminary survey) and 21 in February 2003. After removing any mud remaining adhered to them, the burrow casts were photographed, and the vertical dimensions were determined.

Reproduction

Fish were sampled in November 2000, March, July, August 2001, January and August 2002, and January and February 2003 to examine annual changes in the gonad-somatic index (GSI). Fish were fixed in a 10% formalin solution immediately after collection. Gonads were excised from the preserved specimens to calculate GSI as (gonad weight/body weight) × 100, and to determine gonad developmental stages with the conventional histological technique. Briefly, the preserved gonads were dehydrated in an ethanol series, cleared in 100% butanol, and embedded in paraffin. Cross-sections were cut to 5-µm thickness from the middle portion of the gonad in each individual, and stained with Mayer's hematoxylin and eosin. Each section was mounted on a microscope slide, and gonadal development was evaluated by using a light microscope. Possible relationship between spawning and lunar cycle was studied by examining the gonads from the fish used for the analysis of gut content in 2003.

Two intact eggs chambers with surrounding mud were excised and transported to the laboratory in 2003 to study egg-hatching success as reported by Ishimatsu *et al*. (2007). The egg chambers were incubated in air with high humidity at room temperature. Some 10-40 eggs were removed each day from the egg-chamber wall with a spatula, immersed in seawater, and the number of hatched larvae was counted 15 and 20 min after immersion.

Statistics

One-way analysis of variance was used to analyse daily changes in water table in the burrows, burrow water chemistry (salinity, pH and DO), and mud water content. Where a significant difference was detected, test of Dunnett was applied for post hoc comparison (SPSS 16.0). Data are expressed as mean ± standard deviation wherever possible.

Results

Environmental conditions

The mudflat remained exposed for nine days during neap tide both in the dry season (from 15th through 23rd, August 2002, Fig. 2A) and in the wet season (from 7th through 15th, February 2003, Fig. 3A). Daily inundation of the mudflat lasted for only 1 to 2 h in the morning (2002) or in the evening (2003), during the few days preceding the nine-day emersion. Water depth reached a depth of 1.1 m at the highest spring tide (data not shown). The mudflat substratum consisted of silty estuarine clay.

In August 2002, no rainfall was recorded throughout the month. Water content of the surface mud was 38.9 ± 0.5% (SD, N = 6) on 14th, decreased to 20% on 17th, and remained at this level thereafter (F(6,33) = 78.56, P < 0.001, Fig. 2A). The water table in the mudskipper burrows significantly lowered as the emersion period prolonged (F(4,25) = 384.51, P < 0.001, Fig. 2B). Of the 50 burrows we attempted water sampling on the last day of emersion (Aug. 23rd), water could be sampled from only 35 burrows, which contained 17.7 ± 11.6 ml. No water pool was observed on the mudflat surface from 16th through 23th.

The salinity of burrow water averaged 72 ± 6 psu (N = 30) for the entire 2002 study period, and did not change significantly with time (F(4,23) = 0.417, P = 0.795, Fig. 2C). Surprisingly, the pool water was similarly high in salinity, with the highest value (85) determined on the first day of re-inundation (Aug. 24th). The burrow water pH decreased transiently during the emersion, but recovered with time (F(4,23) = 4.331, P = 0.009, Fig. 2D). The oxygen saturation of burrow water varied largely between burrows, particularly towards the end of the emersion period, but it did not significantly change with time (F(2,15) = 3.243, P = 0.068, Fig. 2D).

In contrast to 2002, it rained nearly every day during the 2003 emersion period in February, with daily precipitation ranging from 0 to ca. 70 mm (Fig. 3E). The water content of surface mud significantly fluctuated with time (F(14,25) = 47.118, P < 0.001), but remained > 30% throughout the study period (Fig. 3A). The water table in mudskipper burrows dropped on 9th, after two clear days, but otherwise remained at the mudflat surface level (F(5,30) = 178.216, P < 0.001, Fig. 3B). Water pools remained on the mudflat surface throughout the emersion period except on 9th.

The average salinity of burrow water was 46 ± 10 psu (N = 36) for the entire 2003 study period, in spite of the precipitation (F(9,50) = 4.655, P < 0.001, Fig. 3C). In contrast, the mean salinity of surface pools fluctuated largely with rain (7 to 54 psu). The burrow water pH was stable throughout the study period, averaging 7.13 ± 0.34 (F (5,30) = 2.403, P = 0.06, Fig. 3D). As in 2002, the oxygen saturation of burrow water varied largely between burrows. It transiently decreased on Feb. 10th, and then recovered (Fig. 3E).

Number of emergent fish

P. minutus was abundant where the halophyte (*Suaeda arbusculoides*) or low mangrove thickets (*Avicennia alba* and *Lumnitzera racemosa*) covered the mudflat, but was rarely observed in the central open area of the mudflat (Fig. 1). In August 2002, the numbers of fish observed on the exposed surface at 10:00-12:00 ranged from 15 ± 9 to 19 ± 6 /100m^2 on 11th through 14th (Fig. 4A). The number of emergent fish markedly declined to only 1 /100m^2 in A and C and 0 in B on 15th and none was confirmed from 16th to the end of the emersion period in any transects. Fish occurred inside in 31 out of 36 burrows studied on 19th, mostly remaining still in the bulbous chamber (see *Burrow morphology*). On the 1st day of re-inundation, the number of emergent fish increased to the same levels as in the pre-emersion period. In contrast to the observation in 2002, the number of emergent fish during the nine-day emersion period in February 2003 was nearly the same or even higher, as compared with the data in the pre- and post-emersion periods, except on 9th (Fig. 4B, note that the rain on 9th shown in Fig. 3E started after fish counting). A similarly low number was recorded on 16th when fish counting was done in heavy rain.

Diel counting demonstrated that *P. minutus* was diurnally active on days with tidal inundation (e.g., Aug. 12th and Feb. 17th, Fig. 5). *P. minutus* retreated into its burrows with the flood tide and remained inside during habitat inundation (Takita *et al*. 2011). On days without inundation, fish did not appear on the mudflat surface in the dry season (Aug. 21st, Fig. 5) but they were active on the surface in the wet season (Feb. 8th, Fig. 5). Air temperature of above 40°C at midday (Feb. 8th) or heavy rain (Feb. 17th) transiently reduced the number of emergent fish. On Feb. 17th, it squalled intermittently from before sunrise till around 13:00, which submerged the transects by the 5th counting (12:40-12:47). Daily temperature fluctuation was smaller at 10 cm below the mudflat surface (as determined inside the artificial burrow) than at the mudflat surface, particularly during the continued emersion period in August (Fig. 5).

Gut content

Before the nine-day emersion in August 2002, most fish ingested animal food. The animal food items identified in 2002 consisted of crabs (in 8 out of 12 fish examined on August 11th and 13th), insects other than ants (6), ants (3), bivalves (3) and a gastropod (1). However, the gut of 17 fish among 18 examined during the emersion (16th, 19th and 22nd) was filled only with mud. In February 2003, most fish fed animal food in all sampling days including during the nine-day emersion period, except 9th when the gut content was solely composed of mud. The food items consisted of amphipods (in 8 out of 12 fish examined on February 4th and 6th), insects other than ants (7), copepods (5), crabs (2) and unidentified animal tissues (5) prior to the emersion, but they shifted to small gastropods (7 out of 12 fish examined on February 12th and 15th) and insects other than ants (5) during the emersion. After re-inundation, crabs became the dominant food item again (all fish examined on February 18th).

Burrow morphology

Burrows of *P. modestus* had two or three surface openings with turrets of 1 to 3 cm high (see also Takita *et al*. 2011) in both dry and wet seasons. A shaft extends diagonally from each turret to meet at 3 to 6 cm below the openings where the lumen is moderately distended and forms a bulbous chamber (Fig. 6). A single vertical shaft (1.5 to 2.5 cm in diameter) extends further downward from the chamber. The vertical shaft was nearly straight in the dry season (Fig. 6A), but had one or occasionally two upturns at the bottom in the wet season (Fig. 6B) with a few exceptions (of 21 casts, two lacked an upturn). Such an upturn was never observed in the dry-season casts. The mean lengths of the vertical shafts were 33.4 ± 6.8 cm in dry season ($N = 10$), and 35.4 ± 5.8 in wet season ($N = 21$), which was not significantly different from each other ($P = 0.395$, $df = 29$, t-test).

Reproduction

Monthly changes in the gonado-somatic index (GSI) showed a clear peak in January to February in both sexes (Fig. 7). GSI was also high in November in males (Fig. 7B), while such a trend was less obvious in females (Fig. 7A). Ovaries in late vitellogenic stages were confirmed only in January and

February. Testes in the functional maturation stage were predominant in November and January, while a small percentage of male fish was also in this maturation stage in all other months. The daily sampling in February 2003 demonstrated that GSI of female fish increased from the values of 5-6% (N = 6) to 12% (N = 1) on the day of half moon (Feb. 9th), notably decreased to 1 on Feb. 12th, and remained low thereafter (Fig. 7C).

Fertilized eggs were collected from two burrows on Feb. 13th, 2003. The eggs were laid in a monolayer on the wall of upturned egg chambers. The two batches of eggs developed normally in humidified air in the laboratory, as has been observed for *P. modestus* (Ishimatsu *et al*. 2007). Sample eggs from one batch hatched upon immersion with hatching rates of 30% on Feb. 17th, 62% on Feb. 18th, and ca. 100% on the following several days. Eggs of the other batch did not hatch during the study period, although embryos developed to the stage that they actively wriggled within the egg capsules with the heart beating. Two adult fish were found to occupy each of two turrets of a single burrow on Feb. 8th (the egg batch from this burrow hatched) and 9th (Fig. 8). Though we were unable to collect them, we suspected that they were a mating pair on the basis of the different colour patterns of the two fish and the courtship-like behaviour shown by them. Juveniles were collected in March, 2003 from water pools remaining after the tide ebbed from the mudflat.

Discussion

Mudskippers may provide insights into how aquatic vertebrates alter their ecology, physiology and behaviour when they colonize a habitat that is increasingly remote from subtidal environments. Life on land obviously requires different bodily structures, functions and lifestyles than has evolved among obligate aquatic animals, due mainly to the different physical and chemical properties of air and of water (Willmer *et al*. 2005). Intertidal zones occur in between aquatic and terrestrial biospheres, and are characterised by cyclical variations and spatial gradients in environmental properties. Mudflats are one of the most productive ecosystems of the earth (Willmer *et al.* 2005), but at the same time pose severe challenges to inhabitants due to extreme environmental fluctuations driven by tidal cycles (Little 2000). *P. minutus* has evolved behavioural, physiological, and perhaps molecular mechanisms to withstand these arid conditions, and the fish thrives there. We obtained evidence that the fish even reproduced there in the wet season when environmental conditions were more benign.

Environmental effects on the surface activity of P. minutus

Surface activity of *P. minutus* was confirmed only during daytime habitat emersion, but not during nighttime emersion or habitat submersion. Our preliminary observations demonstrated no or only few emergent fish at night even on days of full moon, which agrees with the data obtained on February 17th, 2003 (lunar age 16.0, Fig. 5). The decisive role of light condition for emergence is apparent from the diel observations in which nearly no fish was counted on the surface at the earliest counting time of the day and shortly after sunset, irrespective of the presence or absence of daily inundation and therefore the degree of water availability on the mudflat surface (Fig. 5). Temperature remained relatively stable around both sunrise and sunset, and cannot be responsible for the rapid changes in the number of emergent fish at those times.

The importance of water availability for surface activity is supported by the total absence of emergent fish during the nine-day habitat emersion in August 2002 when there was no rain (Fig. 4A), and by no apparent change or even a higher number of emergent fish observed in February 2003 when the effects of rain kept the habitat damp (Fig. 4B). The sharp difference between the two years verifies that surface activity of *P. minutus* was not governed by the tidal phase. In addition, the sharp decline of emergent fish recorded on February 9th (Fig. 4B) is most likely due to habitat desiccation caused by little rainfall in the preceding two days (Fig. 3E). The rain started to fall on 9th after daily counting of the emergent fish in the morning. Salinity of surface pool water sharply increased from 11.2 ± 0.8 on 6th to 54.7 ± 20.0 on 8th (Fig. 3C), also causing strong dehydration of the mudflat surface during these two days. In fact, we observed drying up of surface water pools and sun-cracking of surface mud on February 9th.

It should be noted, however, that heavy precipitation and high temperature extremes (> 40°C) could override the stimulating effect of water availability on fish emergence. The low number of emergent fish recorded on February 16th was presumably due to the rapid flooding of the habitat by heavy precipitation, which entirely covered the mudflat to the depth of 2 cm (Fig. 4B). Similarly, the midday drop of emergent fish number on February 17th was likely due to torrential downpour of the day in the morning hours (Fig. 5). The effect of temperature extremes on fish emergence during day time could be best attested by the transient midday reductions observed on February 8th (Fig. 5). A similar midday drop occurred also on February 5th (data not shown). No rain was recorded on either day (Fig. 3E). Thus, we tentatively conclude that light conditions set the basic rhythm for the surface activity of *P. minutus* in our study site, and water availability during daytime acts as a primary determinant of surface activities, which is further modulated by precipitation and extreme high temperatures. Obviously, the latter three conditions are interrelated. There is some uncertainty about the daily activity pattern of other mudskippers, and it seems possible that the pattern differs between species and even between populations of the same species from different localities (Stebbins and Kalk 1961; Gordon *et al*. 1968; Colombini *et al*. 1995). Retreating into burrows due to heavy rain was reported for *P. modestus* by Ikebe and Oishi (1997).

Feeding activity of P. minutus

The fish appear to have not fed during the continual emersion of the habitat at neap tide in the dry season. This probably relates to behavioural avoidance of too much dehydration and thermal stress by *P. minutus* by retreating into burrows. At the same time, availability of feed animals on the mudflat surface was apparently extremely low, as arid conditions prevailed. In contrast, the fish kept feeding during the nine-day emersion period in the wet season. Feed organisms were more variable in the wet season, including a large number of copepods only in the wet season. The presence of ants in the gut suggests opportunistic feeding habits of *P. minutus*, ingesting animals not only resident in the mudflat but also migrating from land. Colombini *et al*. (1996) analysed feeding behaviour of *P. sobrinus*, and suspected that a low number of prey ingested but with a greater diversity was due to “a drying-up of mud surface which probably causes a quantitative and qualitative change in prey availability”. Clayton and Snowden (2000), studying the relationship between surface activity of *P. waltoni* and prey (crab) activity and environmental conditions, reported that little activity was observed on dry mud, and implied that it is due to the absence of crabs on the dry surface.

On August 17th, 20th and 23rd of 2002 and on February 9th of 2003, only mud was found to have been ingested in all the fish examined. These are the days preceded by two days (2003) or more (2002) clear days, when water table inside burrows was more than 20 cm below the mudflat surface (Figs. 2 and 3). Thus, water was poorly available for the fish, which confined themselves in burrows on those days (Fig. 4). Thus, the fish might have drunk muddy burrow water to obtain whatever water available for them, even though it would impose a high salt load which should be eliminated. Otherwise, the mud may have been ingested for microscopic organisms, detritus or other interstitial food.

Reproduction in the habitat

P. minutus doubtlessly reproduces in our study site. The fertilized eggs were found in the upturned egg chambers, which only occurred in February (Fig. 6B), but not in August (Fig. 6A). The J-shaped morphology and the location of egg deposition in *P. minutus* burrows are identical with the findings for *P. modestus* (Ishimatsu *et al*. 2007). Ishimatsu *et al*. (2007) reported that *P. modestus* stores air in egg chambers and replenishes it during low tide to ensure an adequate O_2 supply for developing embryos. When embryos are ready to hatch, the male *P. modestus* removes the air in the egg chamber, and releases it outside the burrow on a nocturnal rising tide. This behaviour floods the egg chamber and induces the eggs to hatch. The embryonic development in air-filled egg chambers was recently reported by direct endoscopic observations of the burrows of a Malaysian mudskipper, *Periophthalmodon schlosseri* (Ishimatsu *et al*. 2009). We were unable to confirm whether air occurs in *P. minutus* burrows, since the hard substratum did not allow us to apply the gas-collection method used for *P. modestus* or *Pn. schlosseri* (Ishimatsu *et al*. 1998). However, the fact that *P. minutus* burrows had a J-shaped terminus only when eggs were found strongly suggests that the fish uses the same reproductive strategy as has been found for *P. modestus*. The occurrence of fertilized eggs from a burrow tended by two adult fish suggests biparental care of the eggs in *P. minutus*. Parental care by males is the most common, but biparental care is also known for gobies (Ishimatsu and Graham 2011; Takita *et al*. 2011).

Physiological basis for the environmental adaptation of P. minutus

The observed burrow confinement during high tide indicates that *P. minutus* is able to maintain its metabolism at least for several hours by respiring aquatically. The dissolved oxygen level of burrow water during mudflat inundation is likely higher than 30% of air saturation, deducing from the data we obtained toward the end and after the onset of daily inundation (Fig. 3D). The fish would thus be able to satisfy its oxygen demand in the moderately hypoxic conditions of the burrows or by respiring in free water covering the mudflat surface during high tide, which presumably has a higher oxygen concentration. Storage of air in burrows and its use by *P. minutus* during burrow confinement at high tide, as suggested by Ishimatsu *et al*. (1998) for *Periophthalmodon schlosseri*, should be unlikely at least for the non-breeding season on the basis of the straight shape of *P. minutus* burrows as shown by casts made in August (Fig. 6A).

The observed effects of reduced water availability on fish emergence may not be related to salinity tolerance of *P. minutus*. Mudskippers are in general euryhaline, equipped with an ability to adapt to a wide range of environmental salinity (Clayton 1993; Sakamoto *et al*. 2000; Sakamoto and Ando 2002; Wilson *et al*. 1999, 2000). We have recently demonstrated that *P. minutus* is highly euryhaline too; no mortality occurred during two-week exposure to 200% seawater, with free access to land (Itoki *et al*. 2012). Moreover, *P. minutus* has survived for more than 3 years in 200% seawater in the laboratory at

Kyushu University, where free choice of water and land was allowed to the fish (Takeda, unpublished data). *P. minutus* has ion-transporting mitochondria-rich cells in the inner and outer opercular epithelia and in the skin adjacent to the inner base of the pectoral fin, with densities one order higher than those found for *P. novaeguineaensis* (Itoki *et al.* 2012), which also occupied highest intertidal zones but supposedly with higher water availability (Takita *et al*. 2011). The highly developed population of these ion-transporting cells would help maintain ionic homeostasis against potential excessive ionic loads gained by ingesting burrow water during confinement at neap tide. On the other hand, the suppressive effects of heavy rain fall might relate to the fish's limited tolerance to low salinities. Exposure of *P. minutus* to freshwater resulted in a 50% mortality and a significant drop of plasma sodium concentration after four days (Itoki *et al.* 2012). A laboratory experiment on microhabitat selection by *P. cantonensis* (= *P. modestus*, see Murdy 1989) demonstrated that the fish avoided freshwater irrespective of season and time of day (Gordon *et al*. 1985). In contrast, such avoidance of freshwater was not observed for early juvenile *Boleophthalmus pectinirostris* (Chen *et al*. 2008). Questions remain as to how *P. minutus* conserve energy, endure probable buildup of metabolic end products, and prevent excessive loss of body water inside their burrows during the prolonged habitat emersion in the dry season.

Conclusion

The population of *Periophthalmus minutus* investigated in the present survey could exemplify an extreme case in the invasion of land by fishes. Yet, the fish appeared dormant during the harshest periods of prolonged emersion, withstanding the stressful environmental conditions by retreating into burrows without foraging for food. The fish presumably retains the same reproductive strategy as known for other mudskipper species from littoral mudflats, but restricts its reproductive window to the wet season when environmental conditions are less hostile. We found that another population of *P. minutus* inhabits an open intertidal mudflat that is presumably regularly inundated by the tide (at the Hope Inlet in Shoal Bay, northwest of Port Darwin, see also Takita *et al*. 2011). There, *P. minutus* occupied a higher zone of the flat whereas a lower zone was occupied by *P. novaeguineaensis*. Comparison of behavioural, physiological and biochemical traits between these three populations might provide useful insights into how fish can adapt to environments increasingly distant from the water's edge.

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Figure legends

Fig. 1. A map of the study site. The upper panel shows the location of the study site (*), which is enlarged in the lower panel. Three transects (A, B and C) were set: The transect A was in an open area between two meadows of the halophyte *Suaeda arbusculoides* (light gray zones), the transect B inside one of the meadows, and the transect C in a mixed thicket of low mangrove trees (*Avicennia alba* and *Lumnitzera racemosa*, both 0.2-1.0 m high, black triangles), nearly perpendicular to the transects A and B. The mudflat was surrounded by *L. racemos*a forests of over 2 m high (dark gray zones). The mudflat was connected to the beach through three narrow channels but had no noticeable freshwater inflow from landward. An open circle to the right of the transect B indicates the location of the artificial burrow (see text). Two solid circles to the southern end of the mudflat indicate the location of a power pylon. RTC: Road to Channel Island.

Fig. 2. Environmental conditions of the mudflat and within burrows preceding, during and following a 9-day continued emersion of the habitat of *Periophthalmus minutus* recorded in August 2002 (dry season). (A) Changes in maximum daily water depth (bars) and water content of emerged surface mud (solid circles). The water content of bottom-mud samples from water pools is also given (open circles). (B) Water table of burrows in relation to burrow vertical dimension (mean ± SD, hatched area). Burrow vertical dimension was determined from measurements of 10 casts made (see text). (C) Daily changes in the salinity of burrow water (solid circles) and of surface pool water on the mudflat (open circles). (D) The pH (open circles) and dissolved oxygen (DO) concentration (solid circles) of burrow water. DO is shown as percentage air saturation. Values are given as mean ± SD wherever possible.

Asterisks indicate statistically significant difference from the respective initial values ($P < 0.05$, Dunnett test). N = 6 for all the data except the salinity and pH data on Aug. 19 where N = 4, and all data for water pool salinity where N = 1.

Fig. 3. Environmental conditions of the mudflat and within burrows preceding, during and following a 9-day continued emersion of the habitat of *Periophthalmus minutus* recorded in February 2003 (wet season). (A) Changes in maximum daily water depth of the mudflat (bars), and water content of the surface mud (solid circles). (B) Water table of burrows (solid circles) in relation to burrow vertical dimension (mean ± SD, hatched area). Burrow vertical dimension was determined from measurements of 21 casts made (see text). (C) Daily changes in the salinity of burrow water (solid circles) and of surface pool water (open circles). (D) The pH (open circles) and dissolved oxygen (DO) concentration (solid circles) of burrow water. DO is shown as percentage air saturation. (E) Daily precipitation (data are obtained from the web site of the Bureau of Meteorology, Australian Government, see text). Values are given as mean ± SD wherever possible. Asterisks indicate statistically significant difference from the respective initial values ($P < 0.05$, Dunnett test). N = 6 for all the data except the data for surface pool water salinity where N varied from 1 to 7. No statistical analysis was applied to the data of surface pool water. Note that the mud samples for water content measurement were collected from either submerged (Feb. 6^{th} and 14^{th} through 17^{th}) or emerged surface (the other days).

Fig. 4. Changes in the number of emergent *Periophthalmus minutus* observed along the three transects. (A) Data obtained in August 2002 (dry season). (B) Data obtained in February 2003 (wet season).

Mudskippers within 50 cm on both sides of the three 50-m long transects (transect A, solid circles; transect B, open circles; transect C, solid triangles) were counted at 10:00 to 12:00. Hatched areas represent days with tidal inundation of the study site. Note nearly total absence of emergent fish during the 9-day habitat emersion in 2002, but not in 2003. Hatched rectangles indicate the period of mudflat inundation.

Fig. 5. Diel changes in the number of emergent *Periophthalmus minutu*s recorded on two days each in August 2002 (dry season) and February 2003 (wet season). Mudskippers within 50 cm on both sides of the three 50-m long transects (transect A, solid circles; transect B, open circles; transect C, solid triangles) were counted from 1.0-1.5 h before sunrise until 1.0-1.5 h after sunset. Also given are temperatures of the surface mud (solid lines) and at the depth of 10 cm into an artificial burrow (dotted lines). Time of sunrise and sunset is given as vertical lines. The period of mudflat inundation is indicated by hatched rectangles. The double-headed arrow in the bottom panel indicates the approximate period of intermittent squalls with heavy rain on Dec. 17th.

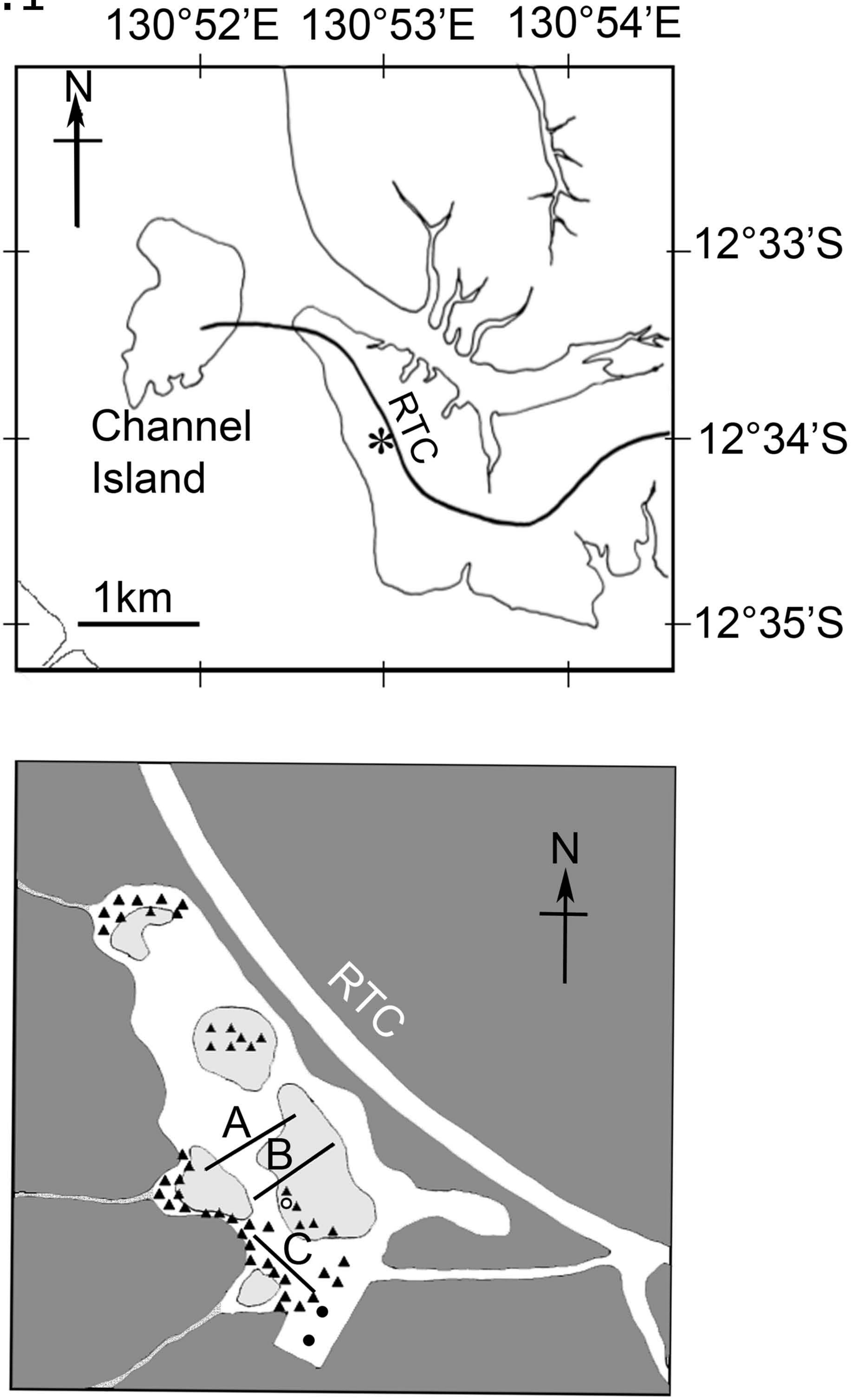
Fig. 6. Casts of *Periophthalmus minutus* burrows. (A) A cast made in August 2002 (dry season). Note that the vertical shaft is straight and lacks an upturn at the bottom. (B) A cast made in February 2003 (wet season). Note the presence of an upturn at the bottom of the vertical shaft, presumed to be the lower portion of an egg chamber. The vertical lengths of A and B are 37 and 36 cm, respectively.

Fig. 7. Seasonal changes in gonad somatic index (GSI) of female (A) and male (B) *Periophthalmus*

minutus obtained for 2000 (triangles), 2001 (diamonds), 2002 (squares) and 2003 (circles). (C) Daily changes in GSI of female *P. minutus* obtained in February 2003. The circles above the panel represent days of half (black and white) and full (white) moon on Feb. 9th and 17th, respectively. Mean ± SD. The numbers of samples are given in parentheses.

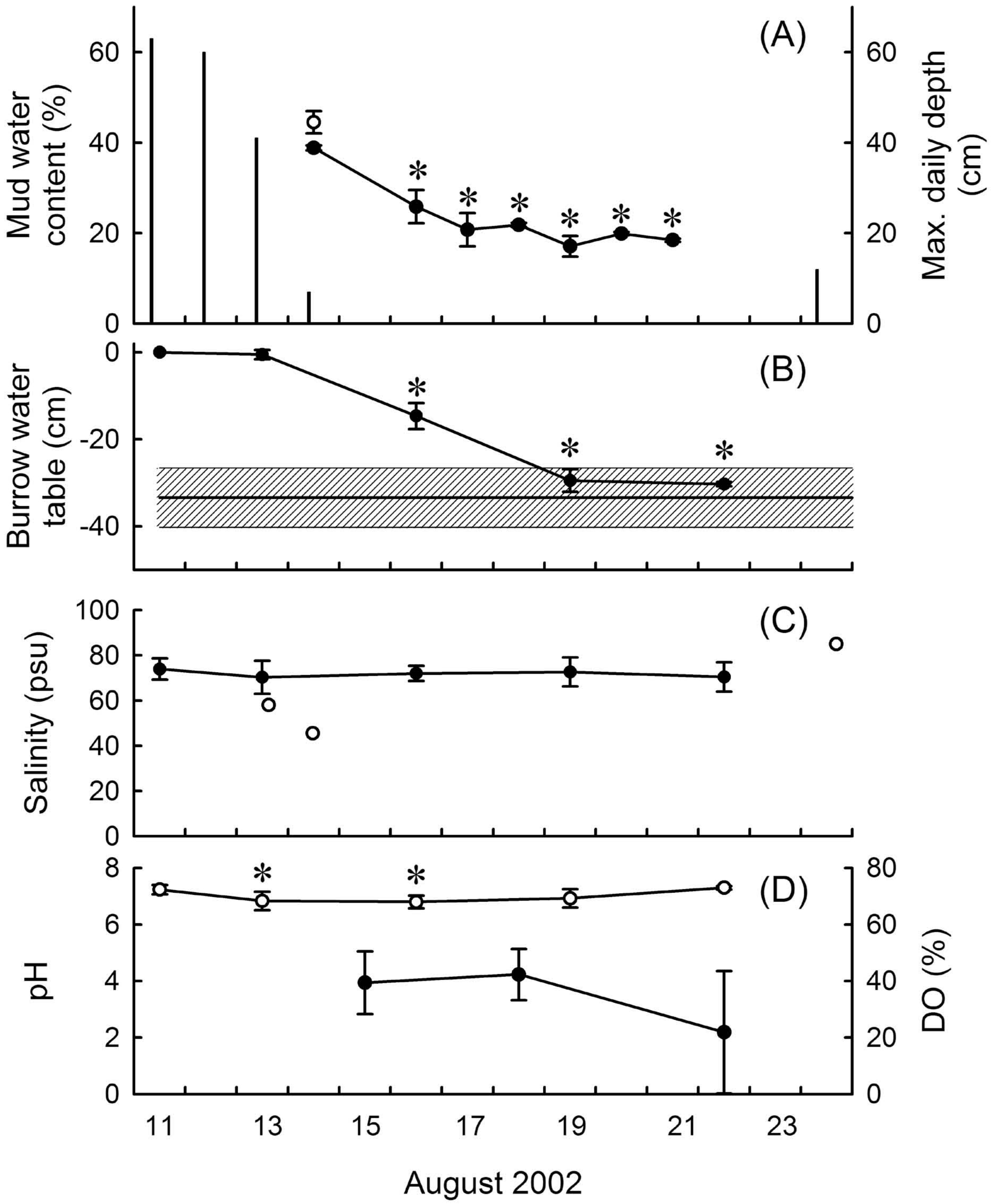
Fig. 8. Two individuals of *Periophthalmus minutus* resting in two openings of a single burrow. The photograph was taken on February 9th 2003. Fertilized eggs were collected from the egg chamber of this burrow.

Fig.1



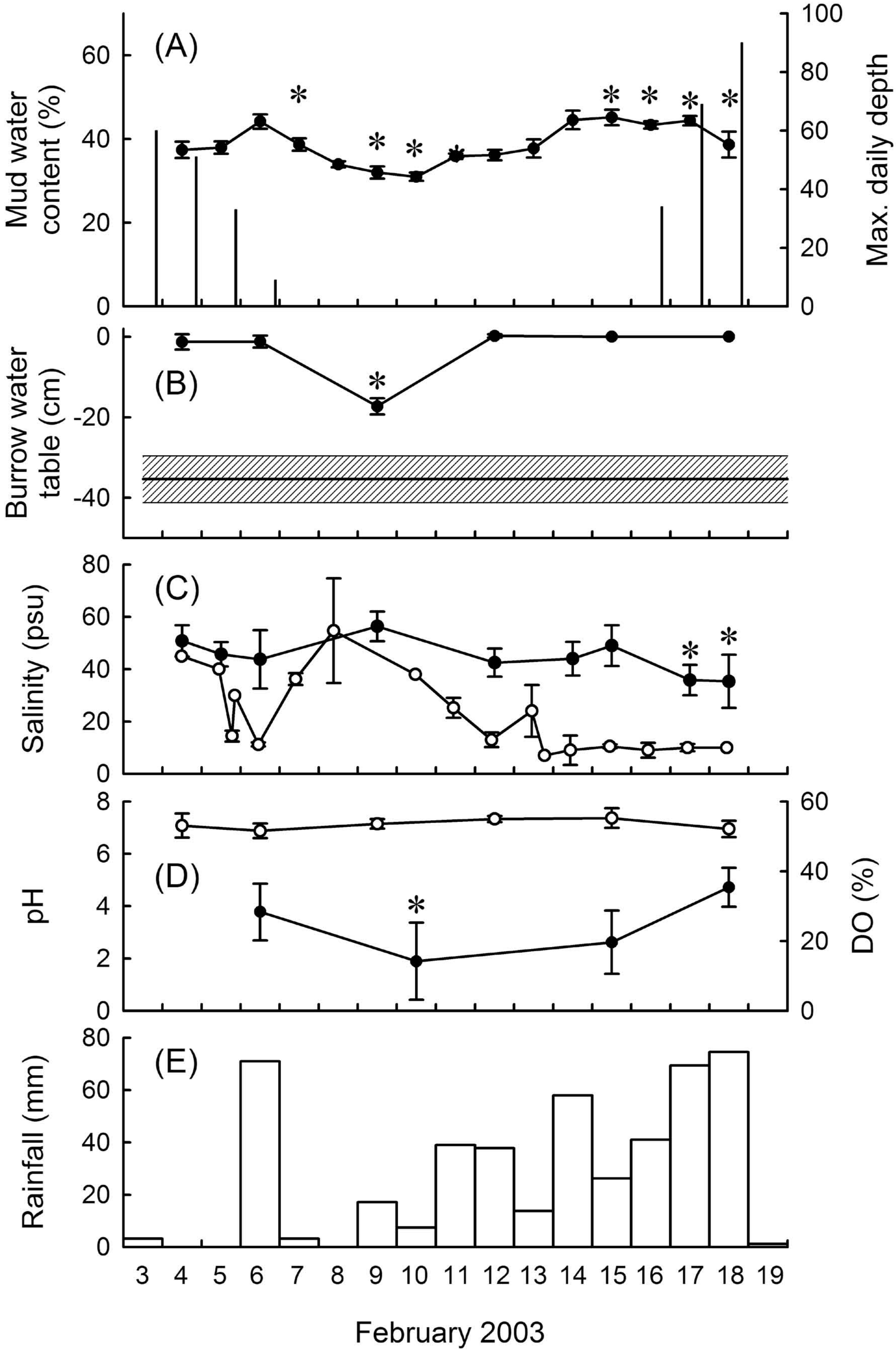
130°52'E
130°53'E
130°54'E
N
12°33'S
12°34'S
12°35'S
RTC
Channel
Island
1km
RTC
N
A
B
C

Fig.2



(A)
Mud water content (%)
Max. daily depth (cm)
60
40
20
0
(B)
Burrow water table (cm)
0
-20
-40
(C)
Salinity (psu)
100
80
60
40
20
0
(D)
pH
DO (%)
8
6
4
2
0
80
60
40
20
0
11
13
15
17
19
21
23
August 2002

Fig.3



(A)
Mud water content (%)
Max. daily depth (cm)
0
20
40
60
80
100
(B)
Burrow water table (cm)
0
-20
-40
(C)
Salinity (psu)
(D)
pH
DO (%)
(E)
Rainfall (mm)
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
February 2003

Fig.4

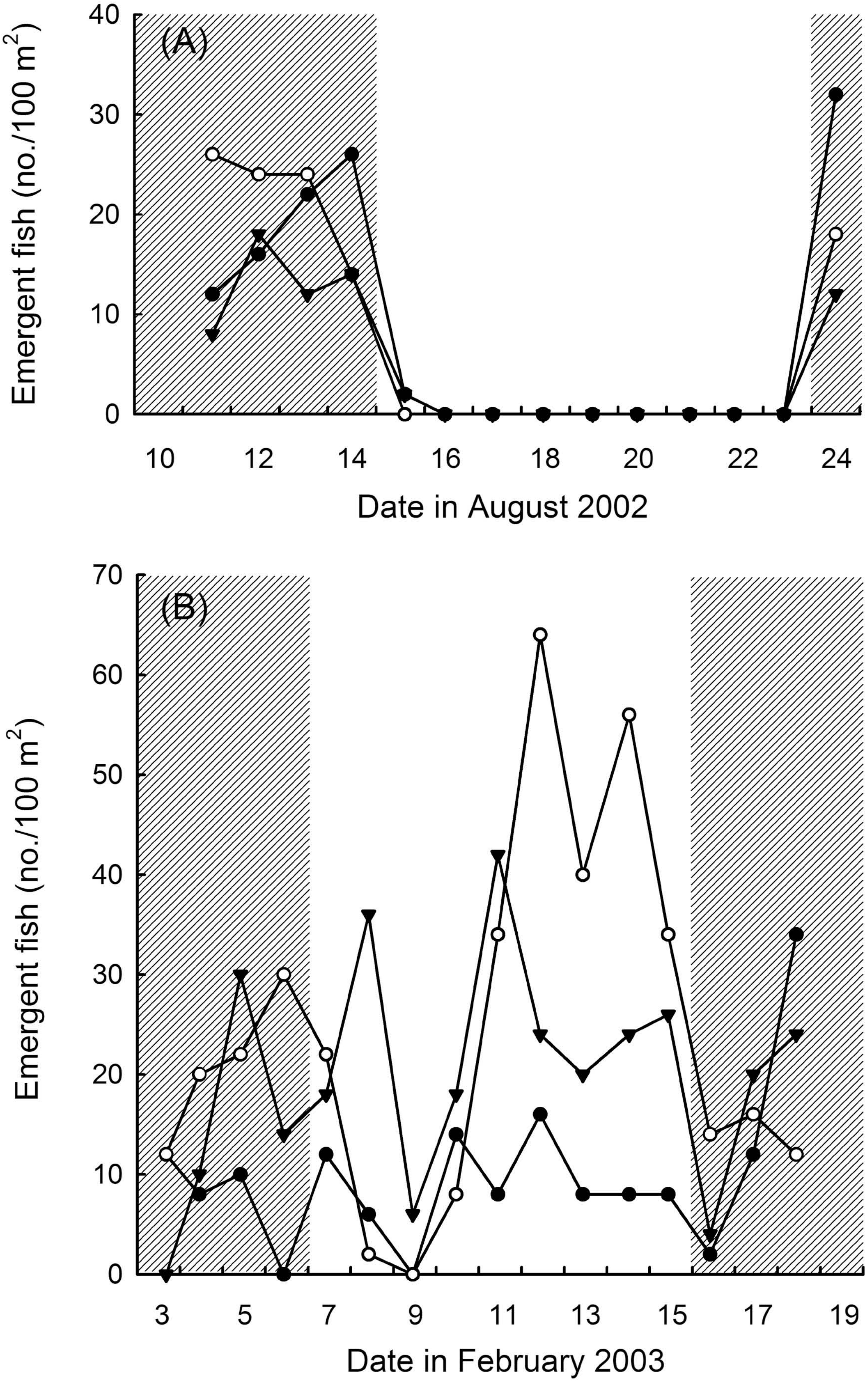
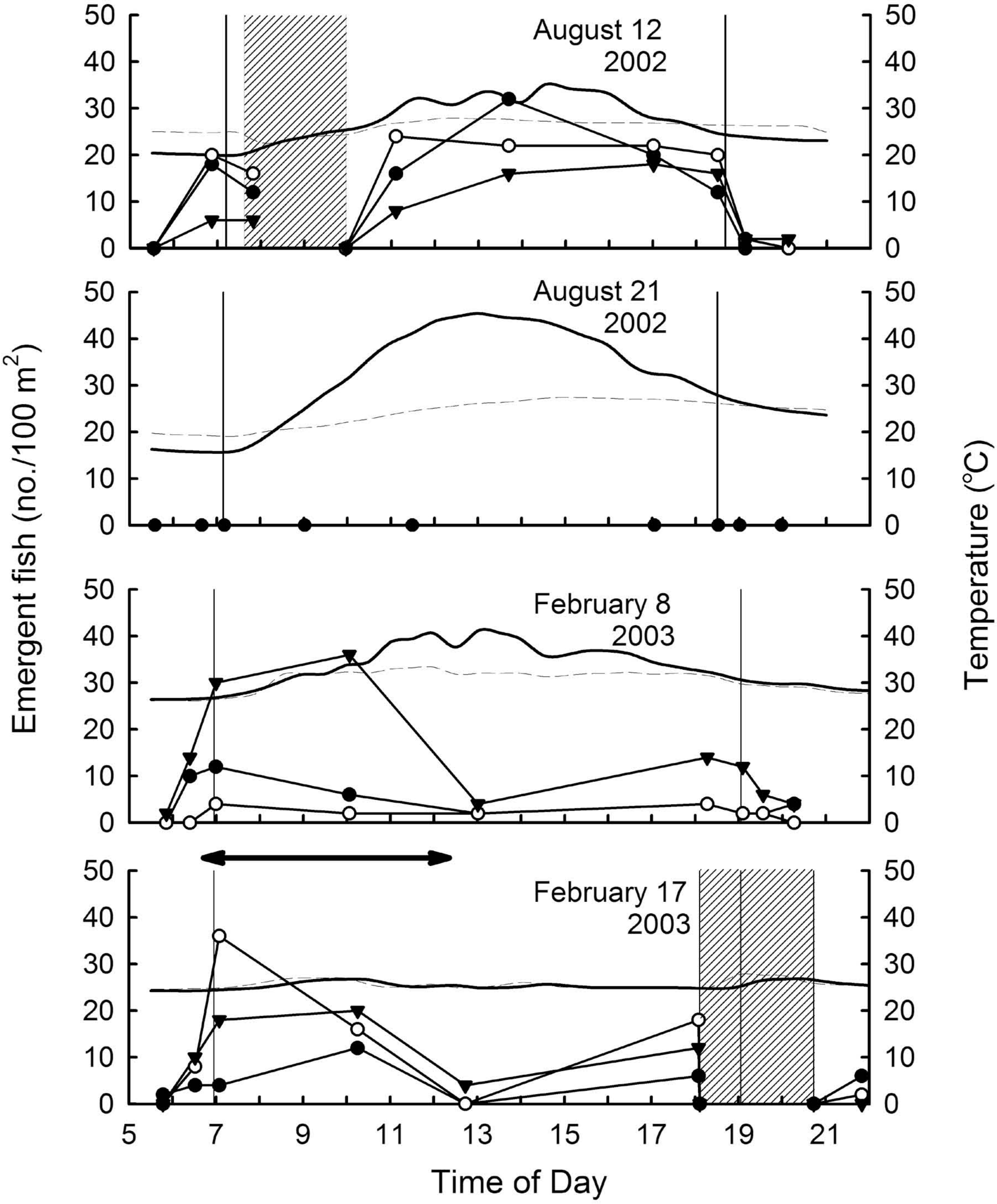

(A)
Emergent fish (no./100 m2)
0
10
20
30
40
10
12
14
16
18
20
22
24
Date in August 2002
(B)
Emergent fish (no./100 m2)
0
10
20
30
40
50
60
70
3
5
7
9
11
13
15
17
19
Date in February 2003

Fig.5



August 12
2002
August 21
2002
February 8
2003
February 17
2003
Emergent fish (no./100 m^2)
Temperature (°C)
Time of Day
0
10
20
30
40
50
5
7
9
11
13
15
17
19
21

Fig.6

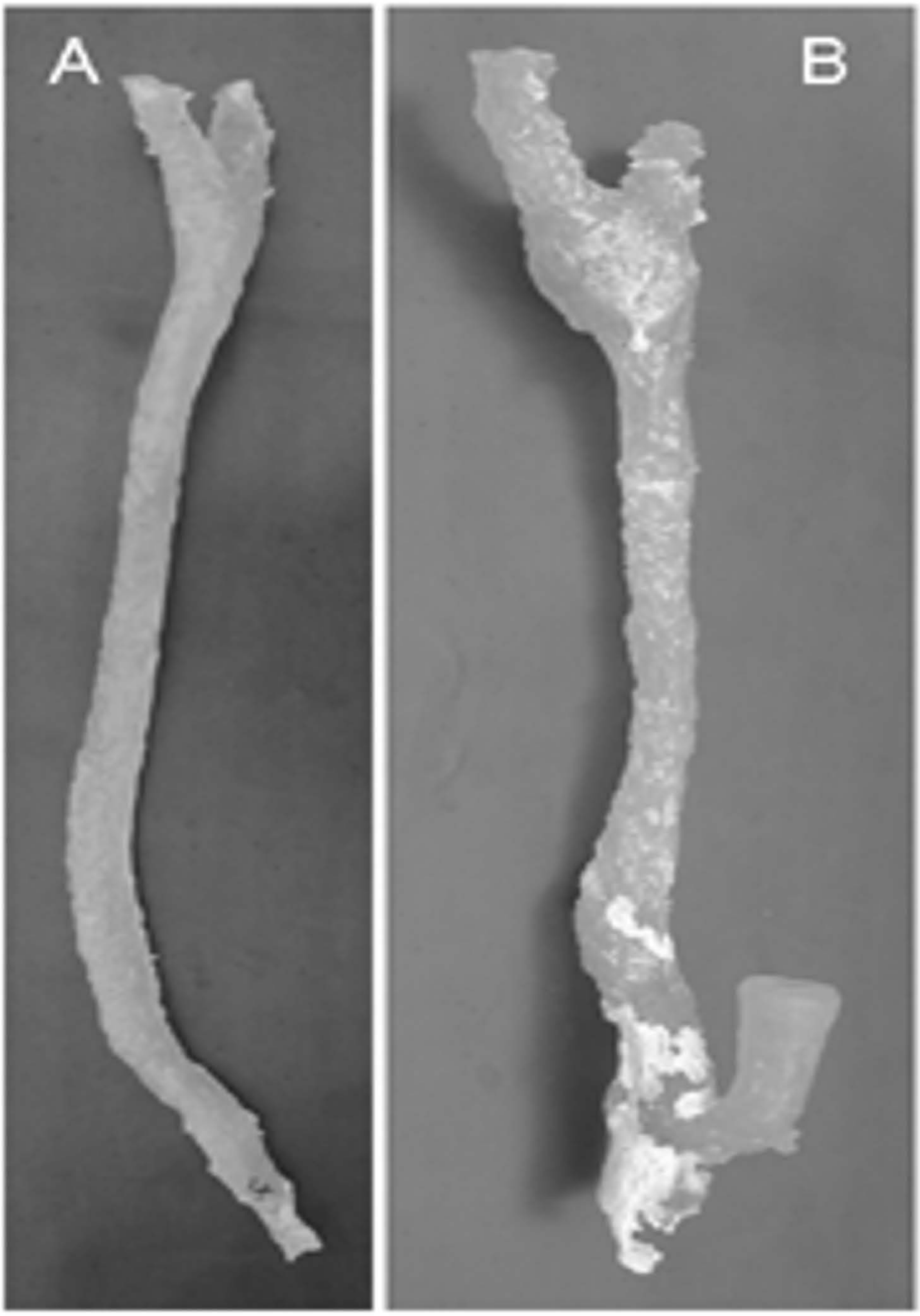


Fig.7

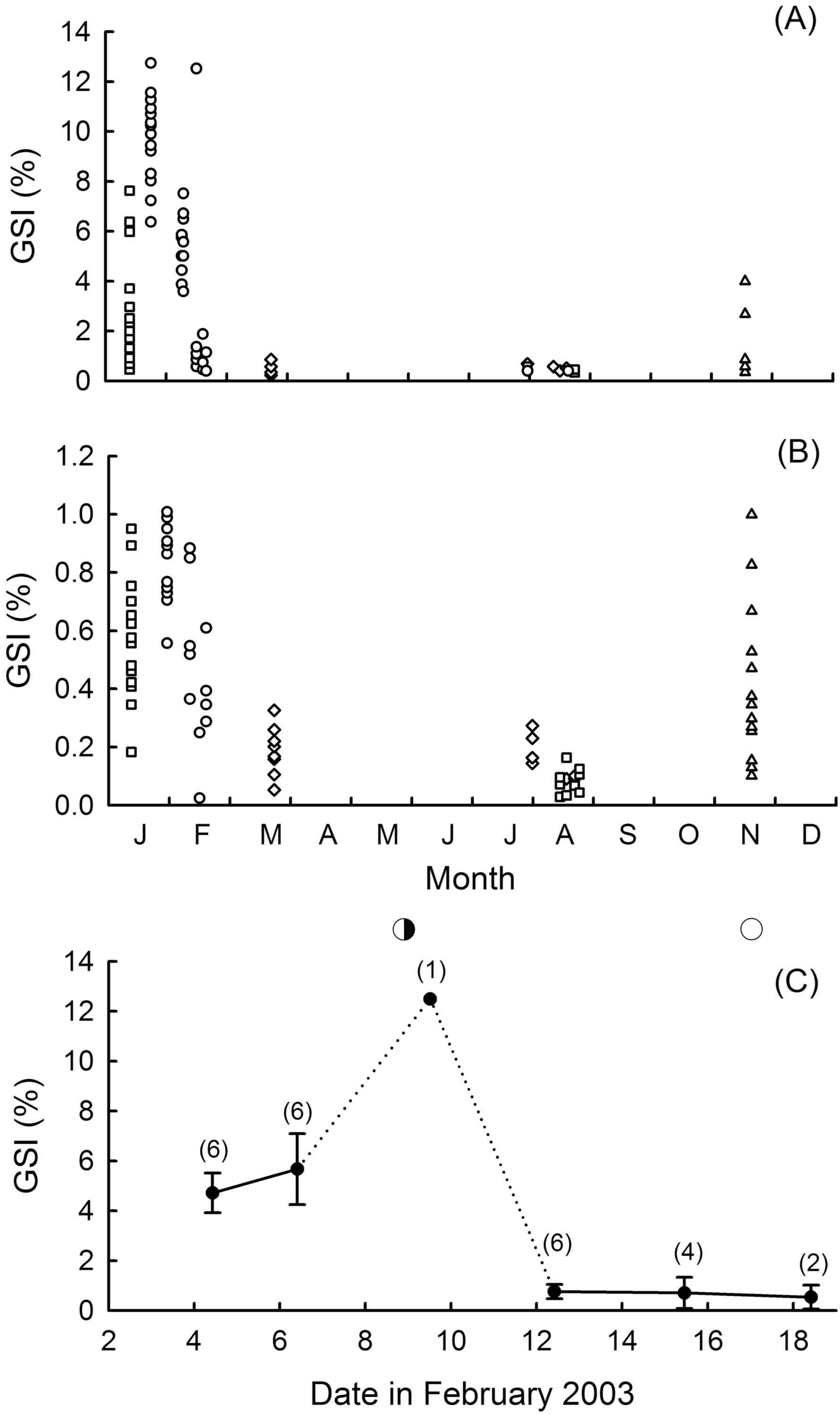

(A)
GSI (%)
14
12
10
8
6
4
2
0
(B)
GSI (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
J F M A M J J A S O N D
Month
(C)
GSI (%)
(6)
(6)
(1)
(6)
(4)
(2)
2 4 6 8 10 12 14 16 18
Date in February 2003

Fig.8