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Penis fencing, spawning, parental care and embryonic development in the cotylean flatworm *Pseudoceros indicus* (Platyhelminthes: Polycladida: Pseudocerotidae) from Singapore

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Abstract. The reproductive behaviour of a pair of *Pseudoceros indicus* from Singapore was observed in the laboratory. Pre-copulatory behaviour included penis fencing, i.e., using their extensively everted penis to stab each other on any part of the body while avoiding being inseminated. However, successful injection resulted in the transfer of sperm in the form of a white spermatophore on the dorsal and/or ventral side of the flatworm. Most of the time, both flatworms received sperm after each bout of penis fencing. The sperm bundles spread across the body as white streaks and were absorbed within a day. After 18 days following at least six insemination occasions, oviposition occurred. The resulting irregularly shaped egg plate consisted of a single layer of eggs tightly packed together. One of the flatworms laid seven batches of eggs over 50 days while another individual laid four batches over 52 days. For both individuals, new egg batches were always added next to old ones, except for their respective last batches, which were deposited on top of the old ones. Reproductive outputs of the two flatworms were 1,307 eggs and 3,073 eggs, respectively. The adults exhibited parental care by covering the egg plates with their bodies until hatching, with decreasing levels of intensity and frequency over time. Eight-lobed Müller's larvae hatched 27 days after oviposition. Each larva measured 282 × 197 µm in size and possessed three eye spots.

Key words. Invertebrate, polyclad, reproduction, oviposition, egg mass, larvae

INTRODUCTION

Members of the order Polycladida are free-living, almost exclusively marine, flatworms. Many polyclads are colourful animals and different species can often be easily distinguished from each other by their distinct surface coloration. There are, however, also a large number of species that appear similar and can only be differentiated by examination of the reproductive organs (Newman & Cannon, 1995). While all polyclads are hermaphrodites and have internal fertilisation (Prudhoe, 1985), they employ diverse strategies at the different stages of reproduction. Precopulatory behaviour, mode of insemination, oviposition, parental care and embryonic development are often species-specific. Such information can be used to complement taxonomic characters in the identification of polyclads, especially when specimens cannot be sacrificed for histological analyses. There are approximately 800 described species of polyclads but data on reproductive biology are available for less than 8% of these polyclads (Rawlinson, 2014). Furthermore, almost all of these studies are based on temperate species, thus resulting in a large gap of knowledge regarding tropical species (Rawlinson, 2014).

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Polyclads employ three methods of insemination, i.e., direct copulation, dermal impregnation or hypodermic insemination. Direct copulation occurs when one animal uses its male reproductive organ to transfer sperm directly to another individual via the female reproductive organ (Rawlinson et al., 2008). In dermal impregnation, one animal deposits its spermatophores onto the surface of another individual, and the sperm is then absorbed through the epidermis to reach the eggs (Poulter, 1975). In hypodermic insemination, one animal injects sperm through any part of the epidermis of another individual using an armed penis (Newman et al., 2000; Newman & Cannon, 2003). This mode of sperm transfer is often associated with the precopulatory behaviour 'penis fencing'.

Penis fencing entails a fight between two flatworms using their armed penis to attempt to stab each other while avoiding impregnation (Michiels & Newman, 1998). This behaviour allows hermaphrodites to skew sexual interaction in favour of sperm donation over the cost of sperm receipt. Hypodermic insemination follows when one flatworm successfully uses its penis to inject male reproductive material anywhere through the epidermis of its partner (Newman et al., 2000; Newman & Cannon, 2003).

Polyclads lay benthic egg masses and each egg capsule may contain single or multiple embryos (Rawlinson et al., 2008). Juveniles of most acotyleans undergo direct development and hatch without undergoing metamorphosis (Rawlinson et al., 2008; see however Boyer, 1986, 1987, 1989). In contrast, indirect development is more prevalent among cotyleans (Ching, 1977; Drobysheva, 2003; Johnson & Forward Jr., 2003; Semmler & Wanninger, 2010; Gammoudi et al., 2012b), and the free-swimming larvae of these species usually hatch as Müller's, Götte's or Kato's larvae that will then undergo metamorphosis (Rawlinson et al., 2008).

The polyclad flatworm *Pseudoceros indicus* Newman & Schupp, 2002 is a common species widely distributed in the Indo-Pacific (Newman & Schupp, 2002; Newman et al., 2003; Apte & Pitale, 2011; Sreeraj & Raghunathan, 2013). However, studies on this species have focused mainly on its chemical compounds (Van Alstyne et al., 2006; Schupp et al., 2009). The only ecological information available concerns its choice of microhabitats (i.e., in between mangrove roots and under rocks from mudflats) and diet preference of the ascidians *Eudistoma toealensis* and *E. viridis* (Schupp et al., 1999; Newman & Schupp, 2002). In this study, we document for the first time the reproductive aspects of this cotylean polyclad.

MATERIAL AND METHODS

Specimen collection. A pair of *Pseudoceros indicus* were hand-collected with a soft paint-brush from the underside of a rock at the mouth of Changi Creek in the East Johor Strait, Singapore (1°23.550'N, 103°59.120'E) on 25 July 2013.

Study species. Based on the observations of living animals, they closely resembled *Pseudoceros indicus* Newman & Schupp, 2002, in having an elongated, oval-shaped and cream coloured body with blue spots along its margin. These marginal spots are uneven in size and continue over the pseudotentacles. However, these blue spots were not as distinctively separated from each other as described by Newman & Schupp (2002) and in some areas appeared to merge into each other to form a continuous line. The horseshoe-shaped cluster of cerebral eyespots was also present, as described for this species.

Laboratory observations. Adult flatworms, eggs and larvae were maintained under a 12:12 h light-dark cycle at an average temperature of 29.5°C. Seawater with a salinity of about 30 ppt was changed every 1–2 days. Both flatworms were initially kept in the same plastic container (13 × 9 × 3 cm) with a pebble (2 × 1.5 cm) added into the container to mimic the natural environment. After insemination, the flatworms were isolated into separate plastic containers labelled with "Individual 1" and "Individual 2" and a pebble was added into each container. Occurrences of penis fencing, insemination, absorption of sperm bundles, egg deposition and parental care were observed, photographed and recorded at a time interval of 1–8 h while the development of eggs were observed and photographed every 1–3 days. Hatched larvae were collected and maintained in small beakers labelled with their respective hatching dates. These larvae were then observed and measured using a stereomicroscope (Olympus SZH10). Both adult flatworms were fed only once or twice, with a small colony of the ascidian *Eudistoma* sp. to "Individual 1" on 10 October 2013 and to "Individual 2" on 9 and 11 October 2013. Only "Individual 2" consumed the ascidian. To minimise the accumulation of mucus produced by the flatworm parents on the egg plates, excess mucus was gently removed using a soft paintbrush so that development of the eggs can be clearly observed. In addition, empty egg capsules were removed when there was an accumulation of organic material that hindered our observations on the development of the other eggs. "Individual 1" and "Individual 2" were maintained in the laboratory until death, for 110 days and 113 days, respectively.

RESULTS

Penis fencing and insemination. Precopulatory and copulatory behaviours were observed during both day and night. Penis fencing between the pair of flatworms was first observed about two hours after field collection. A total of six penis-fencing sessions were observed before the first oviposition event (Table 1). Penis fencing began when both flatworms had their penis everted, which can be seen clearly from the antero-ventral view (Fig. 1A). During each bout, both individuals tried to strike each other while avoiding being stabbed. Penis fencing (Fig. 1B) and insemination (Fig. 1C) occurred simultaneously and the whole process lasted from 10 minutes to more than an hour. Hypodermic inseminations occurred on both dorsal (Fig. 1D) and ventral (Fig. 1E) surfaces of the body. Sperm was transferred in single or multiple ball-shaped spermatophores (Fig. 1F). Both flatworms received sperm after each bout of penis fencing. Sperm bundles spread across the body as white streaks, and were absorbed within a day (Fig. 2A–F).

Oviposition. During egg-laying, the adult flatworm held tightly to the pebble even when disturbed during the process of changing seawater or when touched with a soft paint brush during photography. All eggs were deposited on the pebble in the respective containers of the flatworms. The eggs were tightly packed in a single layer. Each batch of eggs was laid adjacent to previous batches with the perimeters often touching. The outline of the egg plate was somewhat irregular. "Individual 1" laid at least seven egg batches at intervals of 1–15 days over a 50-day period between 20 August–8 October 2013 (Fig. 3A–L). Oviposition occurred in four separate sessions at intervals of 3–28 days for "Individual 2" within 52 days between 31 August–21 October 2013 (Fig. 4A–G). Reproductive output of "Individual 1" was at least 3,073 eggs and that of "Individual 2" was 1,307 eggs (Table 1). For both individuals, their first batch contained the highest number of eggs; "Individual 1" laid 1,920 eggs and "Individual 2" laid 513 eggs. Freshly deposited eggs appeared yellow, while eggs in advanced stages of development appeared red. A mucus layer became apparent during the advanced stages of egg development, possibly due to biofilm formation and attachment of debris. After hatching, the empty egg capsules appeared translucent. The last batches of eggs for both flatworms were deposited on top of previous batches, regardless of whether the latter batches of eggs have hatched (Fig. 3J) or were still developing (Fig. 4E).

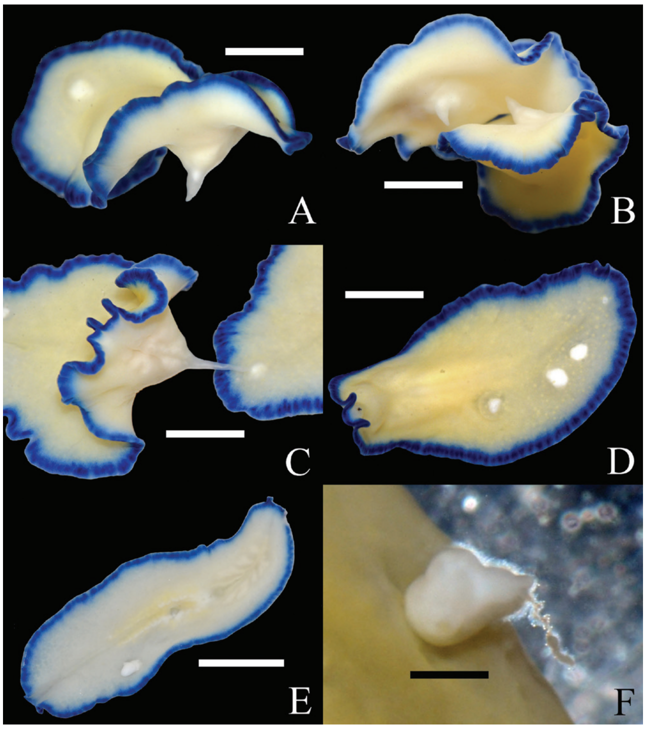

Fig 1. *Pseudoceros indicus*. A, Individual with everted penis; B, Penis fencing in a mating pair; C, Insemination at the posterior region of another flatworm by hypodermic injection; D, Sperm bundles on the dorsal surface; E, Sperm bundle on the posterior ventral surface; F, Detail of sperm bundle. Scale bars = 5 mm (A–E); 1.5 mm (F).

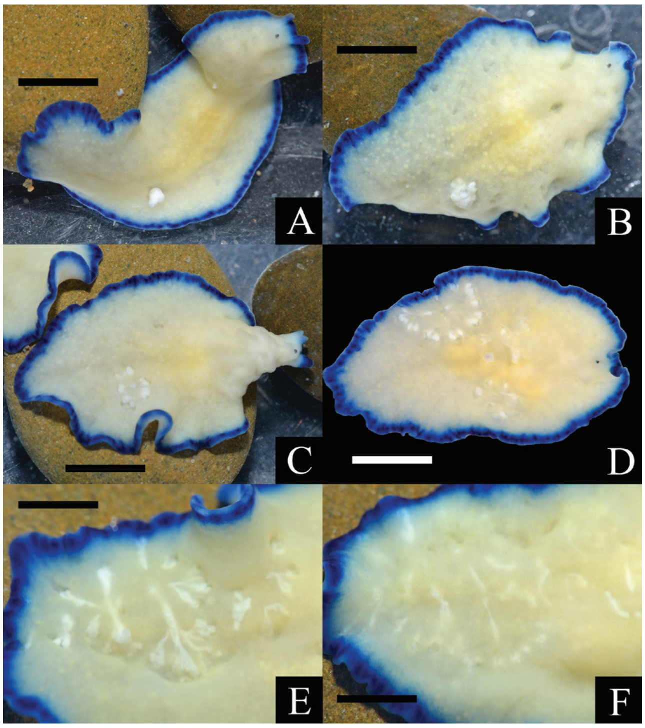

Fig 2. *Pseudoceros indicus*. A, Sperm bundle deposited on the dorsal surface after hypodermic insemination, and its spreading across the body; B, after 107 min; C, after 128 min. D, At least two sperm bundles spreading across the body in another insemination session; Streaks of sperm spreading across the epidermis; E, 7h; F, 13h after insemination. Scale bars = 5 mm (A–D); 2 mm (E, F).

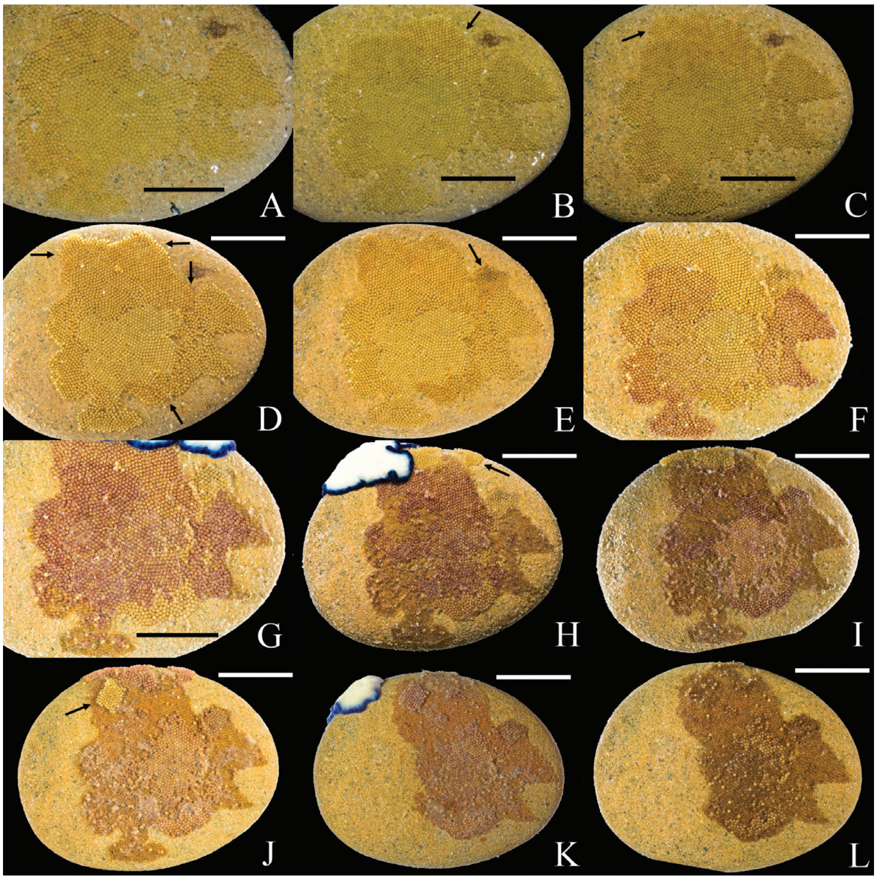

Fig. 3. Eggs of "Individual 1" of a mating pair of *Pseudoceros indicus* deposited on an elliptical surface of a pebble: A, 1st; B, 5th; C, 6th; D, 13th; E, 16th; F, 23rd; G, 28th; H, 30th; I, 35th; J, 50th; K, 67th and L, 72nd day after oviposition. K, L, Empty egg capsules were removed. Arrows indicate subsequent batches of eggs deposited since the first day, as shown in A. Scale bars = 5 mm.

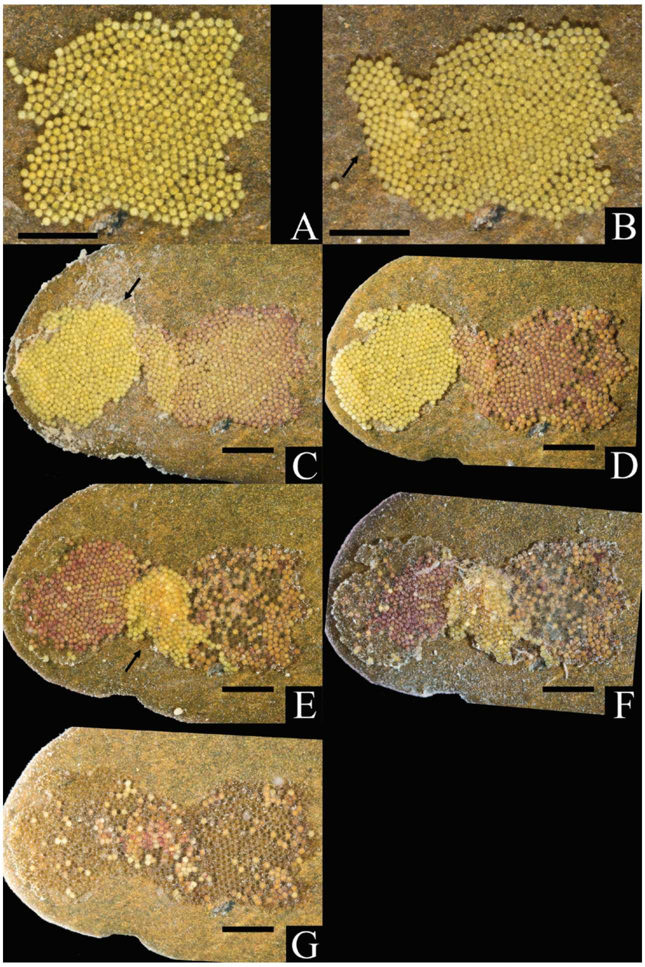

Fig. 4. Eggs of "Individual 2" of a mating pair of *Pseudoceros indicus* on the: A, 1st; B, 4th; C, 24th; D, 40th; E, 52nd; F, 58th; G, 80th day after oviposition. Arrows indicate subsequent batches of eggs deposited since the first day, as shown in A. Scale bars = 2.5 mm.

Parental care. Both flatworms covered eggs with their bodies until hatching, with decreasing intensity and frequency. Freshly laid eggs were covered for almost the entire time on the first day. On the second day, the eggs were abandoned occasionally for 1–2 hours. Subsequently, the parent spent more time away from each batch of eggs, for up to 8 hours at a time. Adults were rarely seen to be covering eggs that have turned red.

Embryonic development. There was only one embryo per egg capsule (capsule diameter approximately 280 µm). Freshly deposited eggs each possessed a dark pigment spot (Fig. 5A). The eggs developed a fine granular surface (Fig. 5B), before the zygote divided into two equally sized blastomeres (Fig. 5C), followed by four equally sized blastomeres (Fig. 5D) within a day after the eggs were deposited. The 8-cell stage was attained on the third day, showing the typical quartet spiral pattern of cleavage with larger macromeres and smaller micromeres (Fig. 5E). Further cell divisions (Figs. 5F, G) were evident from the fourth day onwards. The mesentoblast stage was reached on the sixth day. By day 15, each embryo had developed one or two eyespots, and the embryos could be observed rotating slowly inside the egg capsule (Fig. 5H, I). A full set of three eyes (Fig. 5J) was present by day 16, during which red spots began to appear on the embryos that were morphologically characteristic of the Müller's larva. Over the next few days, more red spots appeared on the larvae. By day 18, the larvae were almost completely covered with red spots and were rotating more actively inside the egg capsules. Hatching of the first larvae occurred on day 27, through a preformed circular operculum at the dorsal side of the capsule (Fig. 5L). The larvae swam immediately upon hatching, and exhibited positive phototaxis. Hatching generally occurred at night.

Larvae. The larvae hatched with eight lobes, with two lobes located apically and six lobes latero-ventrally (Fig. 5K, M–O). Linear dimensions of this Müller's larvae were 282 × 197 µm.

DISCUSSION

In this study, the reproductive biology of *Pseudoceros indicus* was documented for the first time. According to Gammoudi et al. (2012b), the possession of an armed penis does not necessarily imply hypodermic insemination as a mode of sperm transfer. However, this study observed that the penis of *P. indicus* is armed with a long and sharp stylet as described by Newman & Schupp (2002), and the specimens in our laboratory used it to transfer sperm bundles via hypodermal injection after bouts of penis fencing. The strike and avoidance behaviours observed during penis fencing are similar to those documented in *Pseudoceros bifurcus* (see Michiels & Newman, 1998), except that there was no obvious tissue damage caused by hypodermic insemination. Other polyclads that perform hypodermic insemination include the cotylean *Maritigrella crozieri* (see Newman et al., 2000) and the acotylean *Hoploplana inquilina* (see *Planocero inquilina* in Wheeler, 1893). The precopulatory behaviour of *M. crozieri* resulted in bilateral and simultaneous insemination (Newman et al., 2000). However, the deliberate striking and avoiding behaviours observed in *P. indicus* and *P. bifurcus* strongly suggest that these two species intend to transfer sperm unilaterally even though all sparring individuals became recipients eventually.

In *P. indicus*, *P. bifurcus* and *M. crozieri*, white globules of sperm bundles appear on the epidermis after insemination. Spermatophores are also deposited on the body surface in species that employ dermal impregnation, such as the cotyleans *Pseudoceros canadensis* and *Thysanozoon brocchii* and the acotyleans *Echinoplana celerrima* and *Imogine zebra* (Rawlinson et al., 2008; Semmler & Wanninger, 2010; Gammoudi et al., 2012b). As observed in *P. bifurcus* and *M. crozieri*, sperm spread across the body of *P. indicus* after insemination and was absorbed after about one day.

Polyclads are known to lay eggs on glass and plastic surfaces of the containers in which they are kept (Lytwyn & McDermott, 1976; Newman & Cannon, 1994; Johnston & Lee, 2008; Gammoudi et al., 2012a). However, the two individuals observed in this study deposited all their eggs on the small pebble provided, even though there was plenty of space available on the plastic containers. Furthermore, both

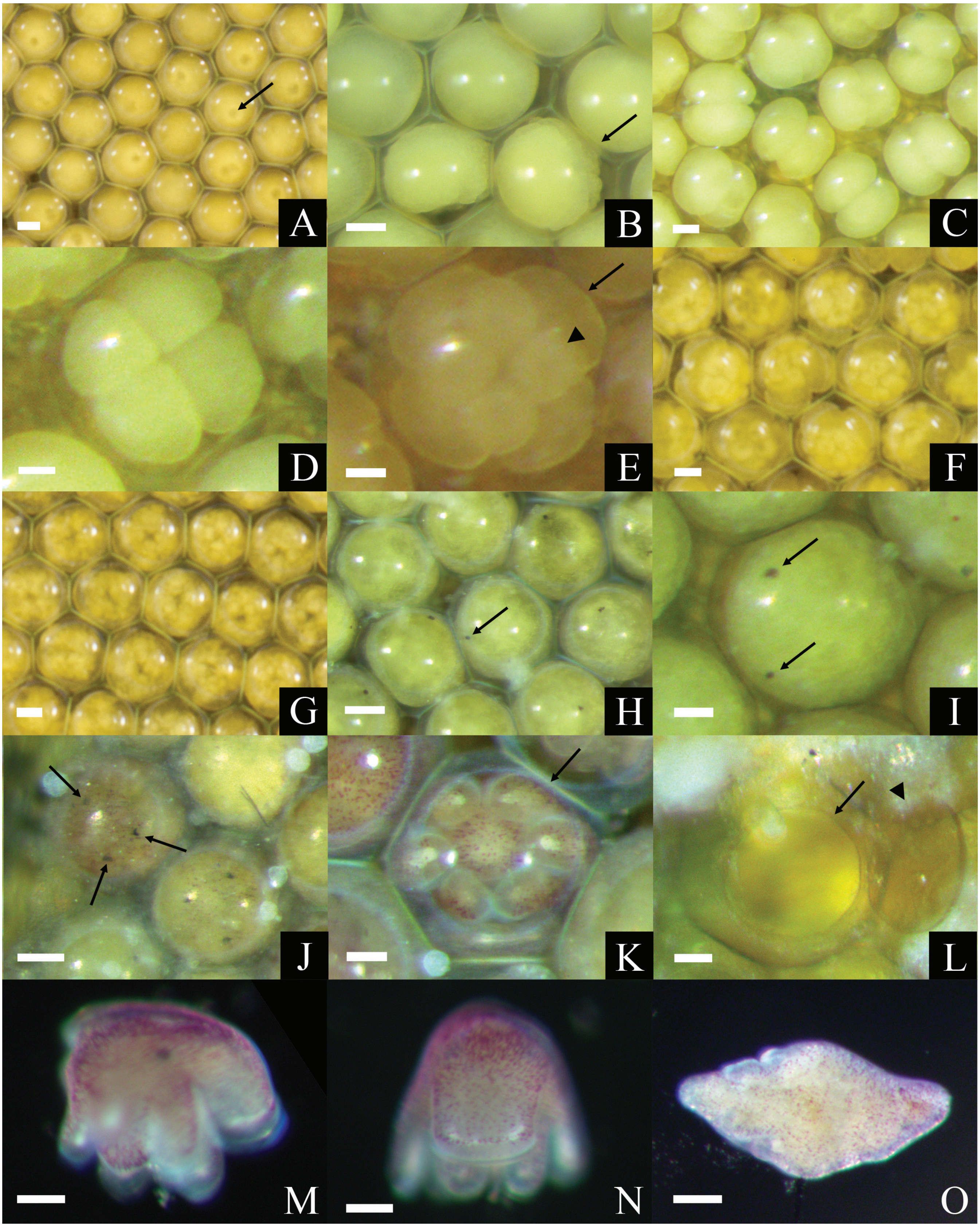

Fig. 5. Embryonic development of *Pseudoceros indicus*. A, 1-cell stage showing a dark pigmented spot (arrow); B, Granularities (arrow) on the cell surface; C, 2-cell stage; D, 4-cell stage; E, 8-cell stage viewed from the animal pole showing the typical spiral pattern with the larger macromeres (arrow) and the smaller micromeres (arrowhead); F, "multi-cell" stage; G, "multi-cell" stage; H, Embryo with one eye (arrow); I, Embryo with two eyes (arrows); J, Embryo with three eyes (arrows); K, Ventral view of unhatched larvae, showing the six lobes (arrow); L, Empty egg capsule (arrow) with the operculum (arrowhead) opened after hatching; M, Hatched Müller's larva, lateral view; N, Front view of larva with dorsal side facing up; O, Dorsal of larva. Scale bars = 100 µm (A–C, F–H, J); 50 µm (D, E, I, K–O).

Table 1. Timeline of reproductive activities in a mating pair of *Pseudoceros indicus* from Singapore.

Date	Day	Observation	
		Individual 1	**Individual 2**
25 July	01	Flatworms collected and kept in same container Penis fencing and insemination	
26 July	02	Sperm bundles spread across body and absorbed	
28 July	04	Penis fencing and insemination	
29 July	05	Sperm bundles spread across body and absorbed	
30 July	06	Penis fencing and insemination	
31 July	07	Penis fencing and insemination	
3 August	10	Penis fencing and insemination Flatworms kept in separate containers	
4 August	11	Sperm bundles spread across body and absorbed	
14 August	21	Flatworms kept in same container Penis fencing and insemination for an unusually long period Sperm bundles spread across body and absorbed	
15 August	22	Presence of sperm bundles Sperm bundles spread across body and absorbed	
20 August	27	Oviposition (1,920 eggs)	
21 August	28	Flatworms kept in separate containers	
24 August	31	Oviposition (339 eggs)	
25 August	32	Oviposition (143 eggs)	
31 August	38		Oviposition (513 eggs)
1 September	39	Oviposition (284 eggs)	
3 September	41		Oviposition (108 eggs)
4 September	42	Oviposition (78 eggs)	
18 September	56	Oviposition (> 223 eggs)	
23 September	61		Oviposition (480 eggs)
8 October	72	Oviposition (86 eggs)	
21 October	85		Oviposition (206 eggs)
15 November	110	Died	
18 November	113		Died

P. indicus individuals chose to lay their last egg batches on top of older, still developing, egg batches when space on the pebble became limited. We do not know if *P. indicus* selects or prefers a specific type of substratum for oviposition.

Trade-offs between egg/offspring number and size are well documented in many organisms (Messina & Fox, 2001), including caridean shrimps (Clarke, 1993) and copepods (Guisande et al., 1996). Indeed, there is an inverse relationship between egg number and size in polyclads. The reproductive output of *Pleioplana atomata*, *Pseudoceros indicus*, *Imogine zebra* and *Stylochus ellipticus* are 750 eggs (Rawlinson et al., 2008), 1,307–3,073 eggs (this study), 1,346–9,549 eggs (Lytwyn & McDermott, 1976; Rawlinson et al., 2008) and 156,000 eggs (Chintala & Kennedy, 1993), respectively. The egg diameters of *Pleioplana atomata*, *Pseudoceros indicus*, *Imogine zebra* and *Stylochus ellipticus* are 385 µm (Rawlinson et al., 2008), 282 µm (this study), 200–220 µm (Lytwyn & McDermott, 1976; Rawlinson et al., 2008) and 69 µm (Chintala & Kennedy, 1993), respectively.

The shape of polyclad egg plates is diverse and appears to be species specific. The egg plates of *Echinoplana celerrima* and *Leptoplana mediterranea* are ribbon-shaped (Gammoudi et al., 2012a, b) whilst those of *Hoploplana inquilina* and *Thysanozoon brocchii* have a spiral-shaped egg plate (Wheeler, 1893; Gammoudi et al., 2012b). Irregularly shaped egg plates are more common and have been observed in the acotyleans *Imogine zebra*, *Pleioplana atomata* and *Stylochus pygmaeus* (Lytwyn & McDermott, 1976; Johnston & Lee, 2008; Rawlinson et al., 2008) and the cotyleans *Prosthiostomum siphunculus* and *P. indicus* (Gammoudi et al., 2012b; this study).

Pseudoceros indicus laid six batches of eggs over seven weeks. Spawning multiple times over a prolonged period of time is common among polyclads (Chintala & Kennedy, 1993; Johnston & Lee, 2008; Rawlinson et al., 2008, 2011), with some species depositing up to 17 egg batches. This reproductive strategy resembles the intermittent spawning documented in molluscs, and may possibly be an adaptation to unstable habitats and environmental stresses, such as fluctuations in temperature, salinity, or dessication (see Rocha et al., 2001; Hodgson, 2010). Hatching in batches may increase the survival success rates of the larvae.

The adults of *Pseudoceros indicus* exhibited parental care, or brooding, by covering the laid eggs with their bodies until hatching. Brooding has also been observed in the direct-developing acotylean polyclads *Echinoplana celerrima*, *Imogine zebra*, and *Pleioplana atomata* (Johnston & Lee, 2008; Rawlinson et al., 2008) but not in *Leptoplana mediterranea* (Gammoudi et al., 2012a). Interestingly, the acotylean *Stylochus pygmaeus* produces planktotrophic Götte larvae (Merory & Newman, 2005) but was also observed to brood their eggs. Although Rawlinson et al. (2008) showed that the egg-covering behaviour in *Pleioplana automata* significantly increased the hatching rate of embryos, this behaviour did not appear to enhance hatching success in our study. We feel it is more likely to play a role in the formation and tanning of egg capsules as well as in the adhesion of the egg plates to the substratum (Merory & Newman, 2005; Johnston & Lee, 2008; Rawlinson et al., 2008), but this behaviour may also have evolved in parallel with having larger embryos and larvae (see below).

In comparison with the few cotylean flatworms whose larval developmental period is known, the time taken (i.e., 27 days at 29.5°C) for Müller's larvae of *P. indicus* to develop and emerge from the egg capsules appears to be longer than other species. For instance, the time taken for embryos of the temperate flatworm *Pseudoceros canadensis* to develop into swimming larvae was 12 days at 12–13°C (Semmler & Wanninger, 2010). Similarly, the embryonic development time of another pseudocerotid flatworm *Thysanozoon brocchii* was 14 days at 17°C (Gammoudi et al., 2012b). However, the development time of *T. brocchii* increased to 25 days with a decrease of only 2°C (Gammoudi et al., 2012b). The prosthiostomid flatworm *Prosthiostomum siphunculus* also experienced a two-fold increase in development time, from 13 days to 23 days, when the temperature was decreased from 17°C to 15°C (Gammoudi et al., 2012b). The euryleptid flatworms *Maritigrella crozieri* and *Stylostomum sanjuania* have the same development time (i.e., 7–8 days) but at very different temperatures, of 24–27°C (Johnson & Forward Jr., 2003) and 12–13°C (Semmler & Wanninger, 2010), respectively. It is also interesting to note that the embryos and larvae of *P. indicus* were substantially larger in size than those of other cotylean flatworms whose development is known (282 µm in *P. indicus*, compared to 90–150 µm in the abovementioned species). The evolution of parental care in flatworms may have taken place in tandem with an increase in the size of the embryos and larvae, possibly associated with the lengthening of the incubation period.

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