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Reproduction, development and parental care in two direct-developing flatworms (Platyhelminthes: Polycladida: Acotylea)

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Variations in reproductive modes, egg production, and the effects of parental care on hatching success are compared between *Pleioplana atomata* and *Imogine zebra*. *Pleioplana atomata* transfers sperm via true copulation, whereas *I. zebra* dermally impregnates spermatophores onto the dorsal surface of partners. *Pleioplana atomata* lays up to 750 large eggs over a 6-week period, and *I. zebra* individuals lay up to 1346 small eggs in 12 days. Female fecundity is positively correlated with body size in both species. Developmental time lines are temperature-dependent, and juvenile worms hatch after 3 and 6 weeks for *I. zebra* and *P. atomata*, respectively. Covering of egg masses by the adult is observed for both species and although this parental care is not necessary for egg development or hatching, it plays a significant role in the hatching success of *P. atomata*. In *I. zebra*, parental covering of egg masses may play a role in eggshell development.

Keywords: reproduction; direct development; parental care; marine flatworms

Introduction

Polyclad reproduction and development have been the focus of numerous studies dating to the earliest investigations of polar body formation, fertilization and cleavage patterns (Hallez 1879; Selenka 1881; Lang 1884; Götte 1878, 1882; Surface 1907). Since then, different aspects of reproductive behaviour, embryogenesis and hatching have been recorded for a number of polyclad species (Kato 1940; Christensen 1971, Lytwyn and McDermott 1976; Anderson 1977; Ballarin and Galleni 1984). Today, polyclads are models for understanding spiralian development (Boyer and Henry 1995; Henry et al. 1995), and are used in studies of cell lineage tracing using fluorescent and immunohistochemical approaches (Boyer et al. 1996, 1998; Younossi-Hartenstein and Hartenstein 2000).

As is true for other flatworms, polyclads are simultaneous hermaphrodites, with concurrently functional male and female reproductive structures. Therefore, insemination can potentially be reciprocal with each partner contributing and receiving sperm via direct sperm transfer. Alternatively, insemination may be unilateral where one animal performs as the male and the partner animal acts as the female. Unilateral insemination is achieved by indirect sperm transfer, which involves either hypodermal insemination or dermal impregnation. During hypodermal

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insemination, one individual injects sperm anywhere through the epidermis of the partner animal, using an armed penis (Hyman 1951). In dermal impregnation, spermatophores are deposited by the male copulatory organ of one individual onto the dorsal surface of the partner. Deposited sperm are absorbed through the epidermis and then move through the parenchyma to the eggs (Hyman 1951; Galleni and Gremigni 1989). The female gonopore is used only during the process of egg laying.

In many simultaneous hermaphrodites reciprocity of sperm exchange is the rule. Precopulatory behaviour of reciprocally inseminating individuals has been studied extensively in triclad flatworms (Vreys and Michiels 1995, 1997; Vreys et al. 1997) and in the microturbellarian *Macrostomum* sp. (Schärer et al. 2004). Hyman (1951) contends that copulatory behaviour among all free-living, reciprocally inseminating flatworms is similar. Generally, copulation involves pressing the genital regions together, commonly elevating them in the process and a mutual insertion of the copulatory structures into the respective female gonopores. The duration of copulation is highly variable within the group, ranging from minutes to hours (Hyman 1951; Kato 1940). In polyclads with true copulation, sperm is deposited through the female gonopore and probably stored in Lang's vesicle. From there, sperm moves either into the oviducts or the uteri to fertilize the eggs. True copulation has been linked to the presence of a Lang's vesicle in the female reproductive system (Galleni and Gremigni, 1989).

Parental care in which adults cover laid egg masses with their bodies, has been shown in *Echinoplana celerrima* Haswell, 1907 (Lee 2006), and in many stylochids (Pearse and Wharton 1938; Rzhepishevskjj 1979; Galleni et al. 1980; Murina et al. 1995; Merory and Newman 2005), and may represent a form of guarding against potential predators. However, hatching success was not dependent on time spent guarding the eggs even in the presence of putative flatworm predators (Lee 2006). Furthermore, at least two planocerid polyclads are known to secrete tetrodotoxin into their eggs (Miyazawa et al. 1986; Tanu et al. 2004), affording potential protection against predation. Therefore, the function of covering laid egg masses by adults may be multifaceted and may differ among species.

The major goals of this study were threefold. First, we describe the reproductive behaviour, egg laying and developmental time line of the acotyleans *Pleioplana atomata* (Müller OF, 1776), and *Imogine zebra* (Verrill, 1882). Second, we also examine potential correlations between the size of the parent animal and the number of eggs and egg batches laid. Finally, we assess the effect of parental care on egg hatching rate in *P. atomata* and *I. zebra* and discuss possible functions of this behaviour.

Materials and methods

Specimen collection

Pleioplana atomata was collected from under rocks in the intertidal and shallow subtidal zones in March 2006 and 2007 at Odiorne Point, New Hampshire, USA (43°02.395′N 70°42.899′W). Many specimens were found hiding under clusters of the green sea urchin *Strongylocentrotus droebachiensis* (Müller OF, 1776). Mature specimens were identified by the presence of sperm in the sperm ducts visible by eye. *Imogine zebra* was obtained from the Marine Biological Laboratory (Wood's Hole, MA). The specimens had been collected from gastropod shells that were inhabited by

the hermit crab, *Pagurus pollicaris* Say, 1817. Measurements of adult body length (in mm) were taken from live animals when they were in a relaxed and quiescent state.

Reproductive behaviour

Pleioplana atomata and *I. zebra* adults were kept at 15°C in constant darkness; they were not fed, and the seawater was changed every 5 days. After an acclimation time of 2 days, similar-sized, mature specimens of *P. atomata* were placed in Petri dishes and reproductive behaviour was recorded. *Imogine zebra* were not acclimated, instead animals were placed together immediately. Precopulatory and copulatory behaviours were recorded digitally as iMovies, using a Canon XL1s. Mode of insemination was noted, as well as length of time of copulations (in min).

Reproductive effort and embryonic development

After recording reproductive behaviour, individual specimens of both species were isolated into separate plastic bags. Over the course of 6 weeks, estimators of reproductive effort based on the number of batches of eggs and the number of eggs per batch were obtained for each individual flatworm.

The animals deposited eggs on the sides of the plastic bags, which could then be examined and counted under a Nikon SMZ-U stereomicroscope. To document embryonic development, photographs were taken every 24 hours using a Nikon CoolPix 990 digital camera attached to the stereomicroscope. Embryos of *P. atomata* were kept in plastic bags at 15°C during their development, whereas embryos of *I. zebra* were kept in Petri dishes at 22°C in the laboratory under natural light cycles, and water was changed three times a week.

Parental care

The influence of parental care on hatching success was determined by calculating the percentage of eggs in each batch that developed to hatching stage in the presence or absence of a parent. Two approaches were used to assess the effects of parental care on hatching success. In the first approach (Treatment 1), 14 adult individuals of *P. atomata* and six adult individuals of *I. zebra* were each divided into two equal groups. In one group, adults remained with their egg batches over the developmental period but in the second group, every egg batch laid was removed and reared in the absence of adults. The second approach was designed to exclude the effects of individual variability of fecundity (Treatment 2). Eight adult individuals of *P. atomata* and three adult specimens of *I. zebra* were used. In this case, the first three egg batches laid were removed and reared in the absence of the parent. The next three egg batches laid by the individual were kept in the plastic bag with the adult and used in the parental care treatment.

A possible variable that could confound the results of Treatment 2 is whether egg viability decreases over time with every batch laid. Data from Treatment 1 provided a time series of egg batches from many individuals, allowing us to test egg viability over time. The first six egg batches were divided into early (batches 1–3) and late (batches 4–6) groups. Twelve individuals of *P. atomata* and eight individuals of *I. zebra* laid over six batches each and their hatching rate was analysed.

Data analysis

For both species, the number of eggs and batches laid was plotted against adult body length and the relationship and significance between them was evaluated using pairwise Spearman rank correlations (r_s). Analysis of variance (ANOVA) was used to test hypotheses about the effects of parental care on the percentage-hatching rate of egg batches. All data were arcsine transformed. Data of Treatment 1 were analysed using a one-way ANOVA with 'parental care' as a fixed factor. Because the number of batches per individual varied, the variation in hatching success was analysed for a subset of batches: four per *P. atomata* adult and six per *I. zebra* adult. Treatment 2 data were analysed on a two-way nested ANOVA (fixed factor=parental care, random factor=individual). A one-way ANOVA was used to assess variation in egg viability (hatching rate) between early-laid and later-laid batches. All tests were carried out using MINITAB 13.30. Homogeneity of variance was tested using Levene's test.

Results and discussion

Reproductive behaviour

Before copulation, *P. atomata* individuals glided over and around each other (Figure 1A). After raising their caudal ends, both individuals prodded the ventral surface of partners with an everted penis (Figure 1B,C). This may represent attempts at finding the female gonopore. The animals copulated (Figure 1D), and transferred sperm directly into the female gonopore. Sperm transfer was assumed to be reciprocal. Copulation lasted for 18.2 ± 5.3 minutes (mean $\pm$ SD) (maximum 49 minutes; $n=10$, eight pairs).

Sexually interacting individuals of *I. zebra* glided over and around each other (Figure 2A), raised their caudal ends repeatedly (tail twitching), everted their penes, and prodded the dorsal surface of their partners (Figure 2B). Usually just one individual undertook this behaviour but occasionally, prodding occurred reciprocally. Alternatively, many individuals became involved, in which case one individual received sperm and at the same time, donated sperm to a third worm (Figure 2D). Mode of insemination was by dermal impregnation (Figure 2C) and pairs of worms took turns to donate and receive sperm. Spermatophores up to 500 µm in size were deposited onto the epidermis (Figure 2E). Mean length of time leading to sperm transfer was 8.2 ± 5.6 minutes (mean $\pm$ SD) ($n=10$, eight pairs).

Hypodermic insemination and dermal impregnation allow hermaphrodites to skew sexual interactions in favour of sperm donation instead of the presumably more costly sperm receipt. The role of each worm may be determined by precopulatory behaviour (Michiels and Newman 1998). Precopulatory behaviour involving violent bouts of penis fencing that usually leads to unilateral insemination has been reported for pseudocerotid polyclads (Michiels and Newman 1998). Among individuals of *I. zebra*, dermal impregnation was less violent and consequently resulted in less physical damage. Sperm donation, although not simultaneous, was generally reciprocal. Worms mated with many individuals and there appeared to be little discrimination in mate choice.

Direct sperm transfer is thought to be the rule among polyclads that possess a well-developed female tract with a Lang's vesicle (Galleni and Gremigni 1989; Table 1). The function of Lang's vesicle is unknown. Bock (1913) considered it a

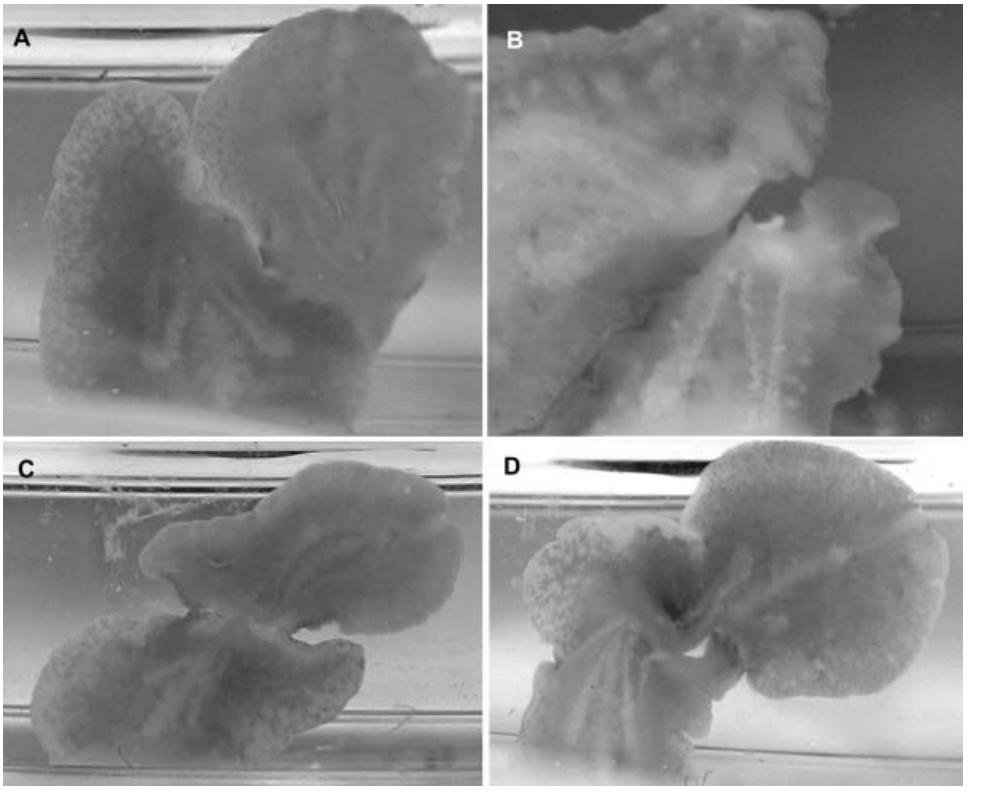

Figure 1. *Pleioplana atomata* reproductive behaviour: (A) initial encounters, (B) and (C) reciprocal prodding of ventral surface, and (D) copulation. Images are video stills.

secretory organ involved in the digestion of excess sperm and prostatic secretions. This function is supported by the fact that in some polyclads, the female reproductive tract is connected to the intestine (Prudhoe 1985). Kato (1940) on the other hand, proposed that Lang's vesicle is a sperm storage organ from which eggs will be fertilized later. The two proposed functions are not mutually exclusive. In fact, it is possible that Lang's vesicle initially functions as a storage organ for sperm received during copulation, followed by a digestive role in the removal of excess and aging sperm. Hence, species possessing a Lang's vesicle will assure sperm entry into the vesicle via true copulation (Galleni and Gremigini 1989). Species lacking a well-developed Lang's vesicle, on the other hand, will use indirect sperm transfer.

Although our observations on *P. atomata* and *I. zebra* support these hypotheses, the connection between mode of insemination and the presence/absence of a Lang's vesicle is not as clear-cut for other species (Table 1). Kato (1940) for example, recorded true copulation in species lacking Lang's vesicle (Table 1). On the other hand, it does seem unlikely that species using indirect sperm transfer would possess a Lang's vesicle, if it functions in sperm storage and digestion. More detailed observations of copulatory behaviour in diverse species are needed to draw conclusions between reproductive system form and function.

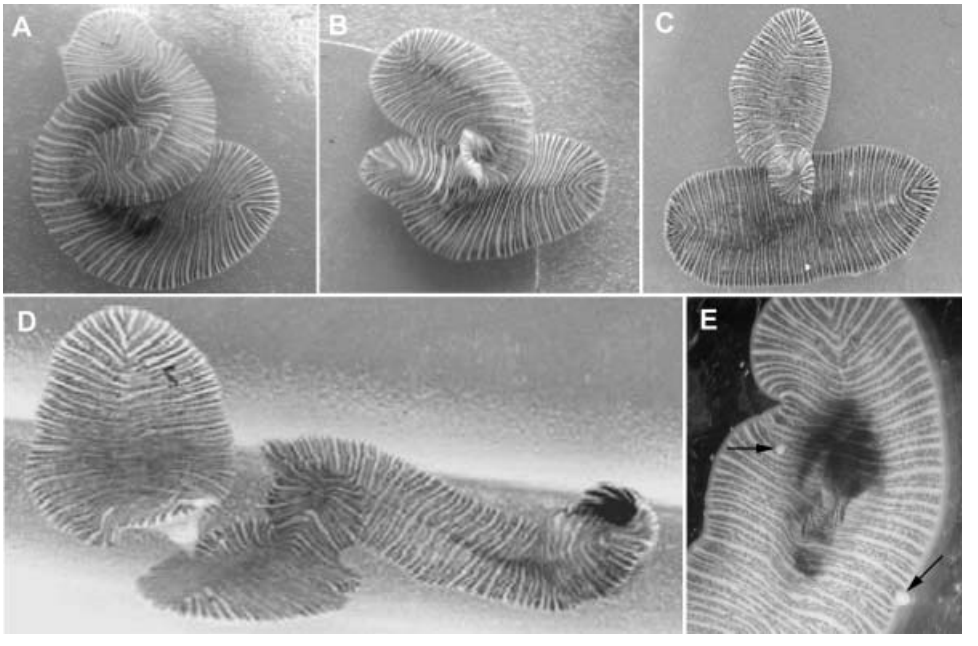

Figure 2. *Imogine zebra* reproductive behaviour: (A) initial encounter and gliding over each other, (B) prodding of dorsal surface, (C) unilateral sperm transfer, (D) sperm transfer among multiple animals, (E) spermatophores on dorsal surface. Images are video stills.

Adult size and reproductive effort

Egg masses of *P. atomata* and *I. zebra* were irregular in shape (Figure 3A,B). The eggs of *P. atomata* were less regularly spaced, occasionally positioned on top of each other, and covered with a thick, gelatinous coating. Egg plates of *I. zebra* consisted of a single layer of closely packed and regularly spaced egg capsules. In both species, egg capsules consisted of transparent membranes that enclose round, cream-coloured, entolecithal eggs. Generally, there was one egg per capsule, although occasionally two eggs per capsule were found in *P. atomata* (Figure 3C). Most of these developed into two conjoined juveniles that adhered to each other even after hatching (Figure 3D).

Differences in the shape of egg batches may be the result of different habitat conditions of the respective species. Egg masses of *P. atomata* are attached firmly to the substrate with a thick adhesive, possibly because the species inhabits the wave-exposed temperate littoral and sub-littoral zones. The more delicate, thinly coated egg plate of *I. zebra* on the other hand, is deposited within the body whorl of gastropod shells (Lytwyn and McDermott 1976) and therefore, may require less protection from the mechanical disturbances of the environment.

Mature *P. atomata* individuals ranged in length from 8 to 20 mm (mean $\pm$ SD 13.7 $\pm$ 3.46 mm; n=43). Egg diameter measured from 290 to 480 µm (385 $\pm$ 55.9 µm; n=10). Eggs within a batch numbered from 6 to 144, and four to 15 batches were produced over a 6-week period. An animal of 18 mm laid the highest number of eggs with 750 eggs over nine batches. The number of eggs produced showed a significant positive relationship with animal length (r_s=0.52, P=0.05, n=15) (Figure 4A), as did

Table 1. Comparison of modes of insemination and types of development with the presence or absence of a Lang's vesicle among various acotylean polyclads.

Taxon	Lang's vesicle	Mode of insemination	Development	Reference
Family: Callioplanidae				
Kaburakia excelsa Bock, 1925	Absent	Unknown	Direct	Shinn (1987)
Koinostylochus elongatus (Kato, 1937) (=*Pseudostylochus elongatus* Kato, 1937)	Present; well-developed	Unknown	Direct	Kato (1940)
Koinostylochus ostreophagus (Hyman, 1955) (=*Pseudostylochus ostreophagus* Hyman, 1955)	Present; small	Unknown	Direct	Woelke (1956)
Family: Cryptocelidae				
Cryptocelis alba Lang, 1884	Absent	Unknown	Direct	Lang (1884)
Family: Discocelidae				
Discocelis tigrina (Blanchard, 1847)	Horseshoe-shaped	Unknown	Direct	Lang (1884)
Family: Euplanidae				
Euplana gracilis (Girard, 1850)	Absent	Unknown	Direct	Christensen (1971)
Family: Gnesioceridae				
Echinoplana celerrima Haswell, 1907	Small; rudimentary	Unknown	Direct	Ballarin and Galleni (1984)
Family: Leptoplanidae				
Hoploplana villosa (Lang, 1884)	Absent	Unknown	Direct	Kato (1940)
Hoploplana inquilina (Wheeler, 1894) (=*Planocera inquilina* Wheeler, 1894)	Absent	Dermal impregnation	Müller's larva	Wheeler (1894); Surface (1907)
Leptoplana tremellaris (Müller, 1774)	Small; rudimentary	Unknown	Direct	Selenka (1881)
Family: Notoplanidae				
Notocomplana litoricola (Heath and McGregor, 1912) (=*Freemania litoricola* (Heath and McGregor, 1912)	Present	Unknown	Direct	Shinn (1987)
Notoplana alcinoi (Schmidt, 1861)	Present	Unknown	Direct	Selenka (1881)
Notoplana australis (Schmarda, 1859)	Present	Unknown	Götte's larva	Anderson (1977)
Notoplana delicata (Yeri and Kaburaki, 1918)	Present	Unknown	Direct	Kato (1940)
Notoplana humilis (Stimpson, 1857)	Present	Copulation	Direct	Kato (1940)

Table 1. (*Continued*)

Taxon	Lang's vesicle	Mode of insemination	Development	Reference
Family: Planoceridae				
Planocera multitentaculata Kato, 1944	Highly reduced	Unknown	Müller's larva	Kato (1940)
Planocera reticulata (Stimpson, 1855)	Highly reduced	Unknown	Intra-capsular Müller's larva	Kato (1940)
Family: Pleioplanidae				
Pleioplana atomata (Müller OF, 1776) (=*Notoplana atomata* (Müller OF, 1776))	Present, well-developed	Copulation	Direct	Remane (1929); this study
Family: Pseudostylochidae				
Pseudostylochus intermedius Kato, 1939	Present; well-developed	Unknown	Direct	Teshirogi et al. (1981) Tajika and Ishida (1999)
Pseudostylochus obscurus (Stimpson, 1857)	Present; well-developed	Copulation	Direct	Kato (1940)
Family: Stylochidae				
Distylochus pusillus (Bock, 1913)	Absent	Unknown	Direct	Teshirogi et al. (1981)
Imogine aomori Kato, 1937 (=*Stylochus aomori* Kato, 1937)	Absent	Unknown	Götte's larva	Kato (1940)
Imogine ijimai Yeri and Kaburaki, 1918 (=*Stylochus ijimai* Yeri and Kaburaki, 1918)	Absent	Unknown	Götte's larva	Rho (1976)
Imogine lateotentare Lee, Beal and Johnston, 2006	Absent	Unknown	Götte's larva	Lee et al. (2006)
Imogine mcgrathi Jennings and Newman, 1996	Absent	Unknown	Götte's larva	Jennings and Newman (1996)
Imogine mediterraneus Galleni, 1976 (=*Stylochus mediterraneus* Galleni, 1976)	Absent	Unknown	Götte's larva	Galleni (1976)
Imogine orientalis Bock, 1913 (=*Stylochus orientalis* Bock, 1913)	Absent	Unknown	Direct	Chen et al. (1990)

Table 1. (*Continued*)

Taxon	Lang's vesicle	Mode of insemination	Development	Reference
Imogine uniporus Kato, 1944 (=*Stylochus uniporus* Kato, 1944)	Absent	Copulation	Götte's larva	Kato (1940)
Imogine zebra (Verrill, 1882) (=*Stylochus zebra* (Verrill, 1882))	Absent	Dermal impregnation	Direct	Lytwyn and McDermott (1976); this study
Stylochus ellipticus (Girard, 1850)	Absent	Unknown	Müller's larva	Provenzano (1959)
Stylochus flevensis Hofker, 1930	Absent	Unknown	Götte's larva	Hofker (1930)
Stylochus frontalis Verrill, 1893 (=*S. inimicus* Palombi, 1931)	Absent	Copulation	Direct	Pearse and Wharton (1938)
Stylochus neapolitanus (Delle Chiaje, 1841)	Absent	Unknown	Direct	Lang (1884)
Stylochus pilidium (Götte, 1881)	Absent	Unknown	Götte's larva	Lang (1884)
Stylochus pygmaeus Merory and Newman, 2005	Absent	Unknown	Götte's larva	Merory and Newman (2005)
Stylochus tauricus Jacubowa, 1909	Absent	Unknown	Götte's larva	Murina et al. (1995)
Family: Stylochoplanidae				
Stylochoplana maculata (Quatrefage, 1845)	Present; round	Copulation	Direct	Remane (1929)
Stylochoplana parasitica Kato, 1935	Present; round	Unknown	Direct	Kato (1940)
Family: Theamidae				
Theama mediterranea Curini-Galletti *et al.* 2008	Absent	Unknown	Direct	Curini-Galletti et al. (2008)

Nomenclature *sensu* Faubel (1983); names in parenthesis correspond to names used in listed references.

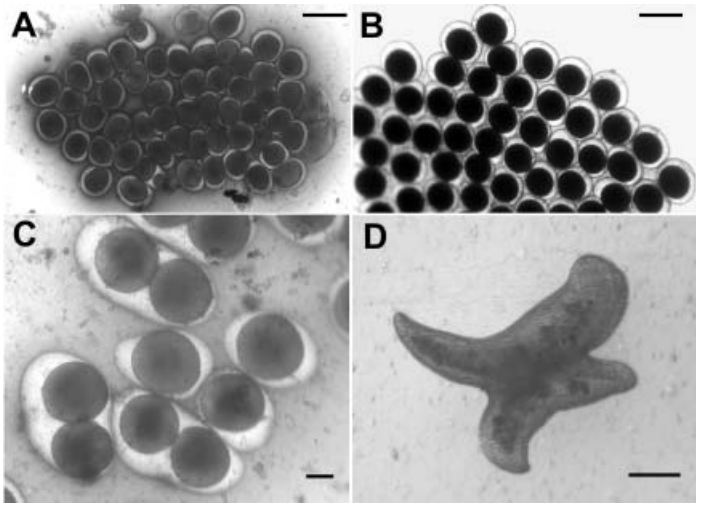

Figure 3. Egg masses: (A) *Pleioplana atomata*, (B) *Imogine zebra*. Scale bars, 500 µm. *Pleioplana atomata*: (C) two eggs per egg capsule, scale bar 150 µm, (D) conjoined hatchlings, scale bar 100 µm.

the number of egg batches (r_s=0.54, P<0.05, n=15) (Figure 4B). Furthermore, there was a positive correlation between the number of eggs and number of egg batches (r=0.54, P<0.04, n=15).

Mature *I. zebra* individuals ranged in length from 12 to 28 mm (mean ± SD 17.6 ± 4.3 mm; n=9). Egg diameter measured 175–225 µm (203 ± 14.8 µm; n=14). Eggs within a batch numbered from 10 to 490, and six to 17 batches were produced in 12 days. An animal of 28 mm laid the highest number of eggs with 1346 eggs over 14 batches. The number of eggs produced showed a significant positive relationship with animal length (r_s=0.96, P<0.001, n=7) (Figure 4A). However, there was no correlation between length and number of egg batches (Figure 4B) and between number of batches and number of eggs produced. *Imogine zebra* is known to have large eggs and relatively low fecundity compared to other stylochids (Chintala and Kennedy 1993).

Adult size is a positive predictor of female fecundity, with larger individuals producing more eggs. In *P. atomata*, the number of batches was also an indicator of reproductive success, but this was not the case for *I. zebra*. Another polyclad, *Stylochus ellipticus* (Girard, 1850) shows no size-related female fecundity (Chintala and Kennedy 1993). However, the freshwater triclad *Dugesia gonocephala* (Duges, 1830) shows significantly increased reproductive success with increased size and actively selects mates based on size (Vreys and Michiels 1995). In hermaphrodites where copulations are reciprocal and size is a positive indicator of female fecundity, larger partners are favoured. It is therefore possible that size assortative precopulatory behaviour is also occurring in polyclads.

Developmental time line and description of embryonic stages

Time from oviposition to hatching for *P. atomata* and *I. zebra* was 6 and 3 weeks, respectively (Table 2). Both are temperate species but the distribution of *P. atomata*

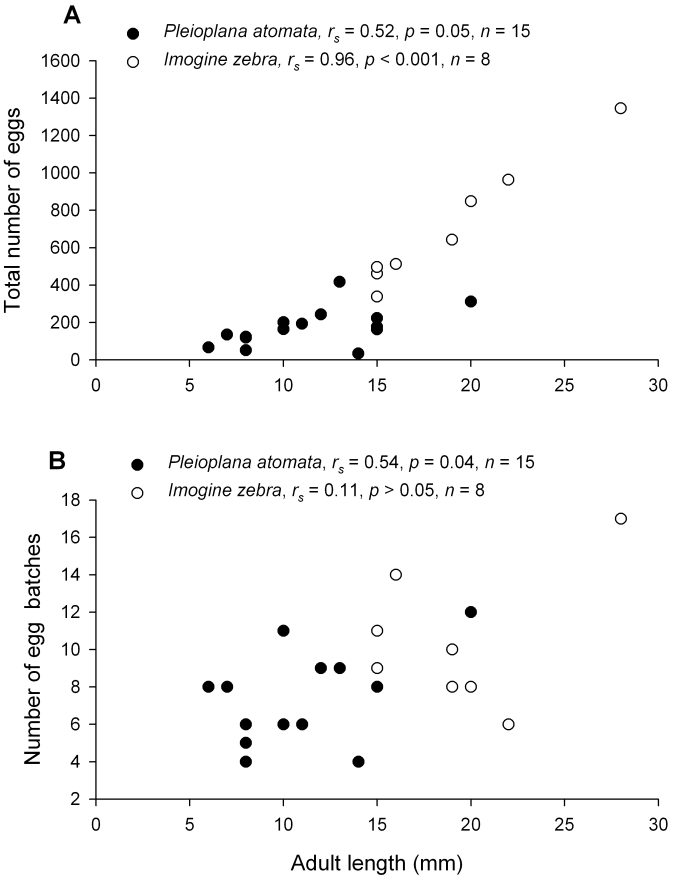

Figure 4. Relationship between adult length (in mm) and (A) egg production and (B) the number of egg batches in *Pleioplana atomata* and *Imogine zebra*.

in the western Atlantic extends to higher latitudes (north of Cape Cod to Newfoundland), whereas *I. zebra* is found south of Cape Cod (Pollock 1998). Consequently, we reared the embryos of *P. atomata* at a lower temperature (15°C) than that used for *I. zebra* embryos (22°C). Developmental times of many invertebrates have been shown to correlate strongly with temperature, with lower temperatures generally resulting in slowed development (Hoegh-Guldberg and Pearse 1995). In fact, preliminary observations reveal rapid developmental time lines for tropical and subtropical polyclads (Bolaños, personal observation). Therefore, it is highly likely that the extended developmental times observed for *P. atomata* are a result of rearing temperatures. In the following, we discuss the developmental stages of *I. zebra* in detail. These observations were made on eggs reared at 22°C in the absence of adult animals. *Pleioplana atomata* underwent comparable stages, although at a delayed pace (Table 2).

After oviposition and before the formation of polar bodies, numerous short pseudopodia appeared on the surface of the egg (i.e. blebbing; Figure 5A). These are extensions of the egg membrane and indicate the completion of meiosis of the egg cell. Egg blebbing has been described previously for many other polyclad species (Wheeler 1894; Kato 1940; Younossi-Hartenstein and Hartenstein 2000). The

Table 2. Time line of embryonic development of *Imogine zebra* and *Pleioplana atomata* (rearing temperature 22°C and 15°C, respectively).

Day	Hour	*Imogine zebra*	*Pleioplana atomata*
01	0	Oviposition	Oviposition
	3	Pseudopodia, blebbing	
	12	2 cells	
	16	4 cells	
	19.5	8 cells	
	22	16 cells	
02	13	32 cells	
02	22	64 cells	
03	0		2 cells
03	14		4 cells
04	0		8 cells
05		Gastrulation	
06		Cilia appear, slow rotation	
10		Two eyes	
11		Muscular contractions	Gastrulation
14		Four eyes, formation of mouth	Rotation by ciliary action
18		Formation of pharynx and intestinal branches	
21			Muscular contractions
22		Elongate, worm shape, hatching	
29			Two eyes
30			Contraction and rotation
32			Strong muscular contractions, bending of worm
37			Four pairs of eyes
39			Five pairs of eyes
42			Six pairs of eyes - hatching starts

pseudopodia disappear once polar bodies have been formed and the zygote assumes a smooth, round shape (Kato 1940). First cleavage occurred 2 hours after oviposition and resulted in two equal-sized blastomeres (Figure 5B). Cleavage progressed in a typical quartet spiral pattern (Figure 5B–D); divisions were complete but unequal after the first two, as described for numerous other polyclad species (Surface 1907; Kato 1940; Anderson 1977; Boyer 1986). A solid morula formed on day 3 (Figure 5E). Gastrulation via epiboly occurred on day 5 and the blastopore was clearly visible (Figure 5F). On day 6, the developing embryos started to rotate slowly within the eggshells (88 seconds/complete turn), presumably as the result of ciliary action. At that time, the ectoderm was distinct, as was a concentration of large yolk globules in the interior of the embryo. During the next 4 days, the embryos began to rotate rapidly and on day 10, the first pair of eyes appeared (Figure 5G). Also, the colour of the yolk gradually changed to brown, and eventually the granules were being absorbed rapidly. At this stage, the embryos occupied the entire space within the eggshells; strong muscular contractions became evident on day 11.

On day 14, a second pair of eyes appeared and a large, disc-shaped mouth was fully defined. Over the next 4 days, the embryo started to elongate, the pharynx and

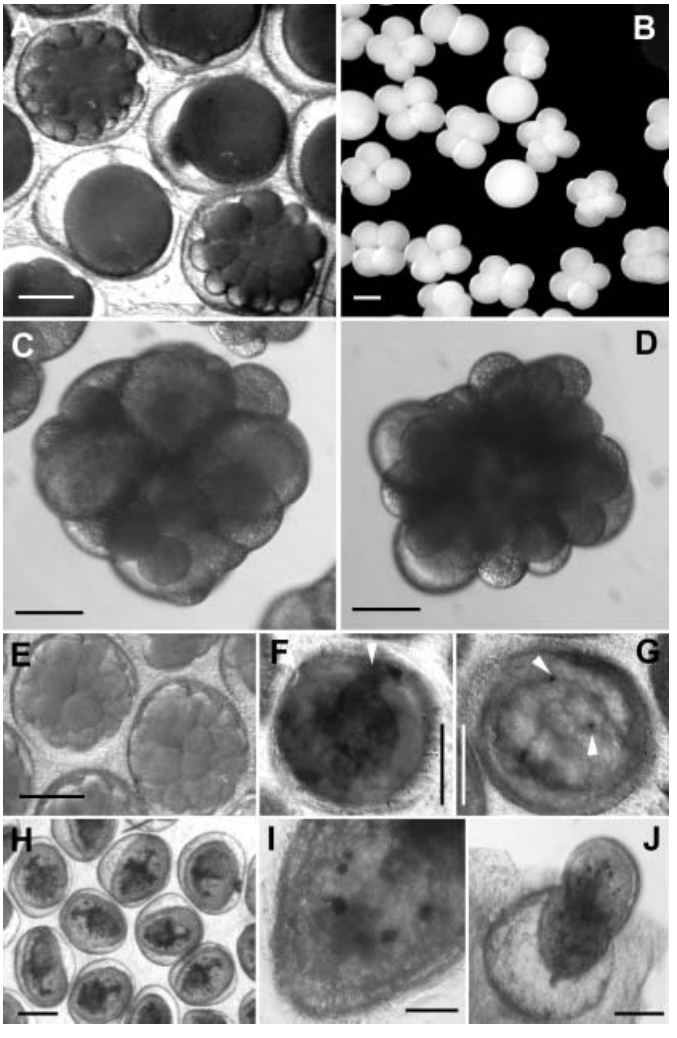

Figure 5. *Imogine zebra*, developmental stages. (A) egg blebbing, (B) two-cell to eight-cell stage, (C) 16-cell stage, (D) 32-cell stage, (E) solid morulae, (F) gastrulation by epiboly, arrowhead indicates blastopore, (G) embryo with one pair of eyes (arrowheads), (H) embryos with noticeable pharynges, (I) embryo with two pairs of eyes, (J) juvenile worm hatching out of eggshell. Scale bars (C, D, I) 50 µm, all others 100 µm.

intestinal branches became noticeable (Figure 5H), a third pair of eyes developed, and the entire embryo was covered by cilia (Figure 5I). By day 22, the embryos had taken on a characteristic, elongate worm-like shape with well-defined anterior and posterior ends. At 3 weeks, the juvenile *I. zebra* hatched (Figure 5J). In *P. atomata* hatching occurred at 6 weeks. A 3-week time-frame from oviposition to hatching is consistent with reports for other direct-developing polyclads. Kato (1940) noted the same time span for *Notoplana delicata* (Yeri and Kaburaki, 1918) and *Notoplana humilis* (Stimpson, 1857), as well as for *Pseudostylochus obscurus* (Stimpson, 1857) and *Koinostylochus elongatus* (Kato, 1937) (reported under the name *Pseudostylochus*

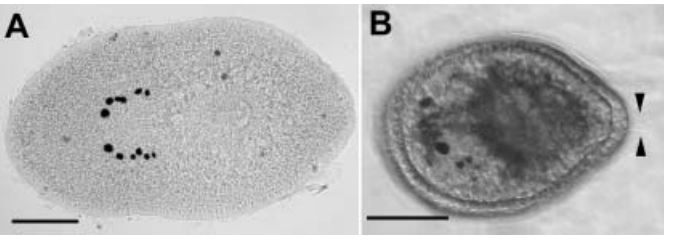

Figure 6. Hatchlings (A) *Pleioplana atomata* and (B) *Imogine zebra*, arrowheads indicate caudal cilia. Scale bars, 100 µm.

elongatus). Indirectly developing acotyleans however, tend to have shorter developmental times, e. g. 7–8 days for *Imogine aomori* Kato, 1937, *I. uniporus* Kato, 1944 (Kato 1940), and *I. mcgrathi* Jennings and Newman, 1996 (Younossi-Hartenstein and Hartenstein 2000).

Newly hatched juveniles of *P. atomata* were about 490 µm long, tapered at both ends and dorsoventrally flattened (Figure 6A). Six pairs of eyes were arranged around the cerebral ganglion and anterior end of the pharynx. Sensory cilia were evenly positioned around the peripheral margin. *Imogine zebra* hatchlings were smaller (300 µm) in comparison, elongate and cylindrical (Figure 6B). Three pairs of eyes were arranged anteriorly, cilia covered the external surface and two longer caudal cilia were present at the posterior end (Figure 6B).

The Polycladida is the only order among free-living flatworms to encompass species that develop indirectly through larvae as well as species that develop directly (Table 1). Larvae either have eight (Müller's larvae) or four (Götte's larvae) ciliated lobes, which they use for swimming (Hyman 1951). Generally, Müller's larvae are found among members of the suborder Cotylea (polyclads with a ventral sucker present; Lang 1884), whereas Acotylea (ventral sucker absent; Lang 1884) may or may not pass through a larval stage. There is no phylogenetic correlation with family or even genus for the presence or absence of a larva among the Acotylea (Kato 1940; Ballarin and Galleni 1984; Table 1).

Notoplanidae has long been recognized as a heterogeneous assemblage of acotylean species, and taxonomic revisions of the family had been attempted by Bock (1913) and by Marcus and Marcus (1968). Based on the characteristically subdivided lining of the prostatic vesicle of *Notoplana atomata* (Müller OF, 1776), Faubel (1983) established the family Pleioplanidae and designated *N. atomata* as its type (*Pleioplana atomata*). Despite the reclassifications by Faubel (1983), the Notoplanidae remains in need of revision. Therefore, we include comparisons on the development of genera closely related to *Pleioplana*. Development has been described for *Notoplana alcinoi* (Schmidt, 1861), *N. delicata* (reclassified as *Pleioplana delicata* by Faubel, 1983) and *N. humilis* (reclassified as *Notocomplana humilis* by Faubel, 1983). All three species hatch as juvenile worms (Selenka 1881; Kato 1940). However, one species, *N. australis* (Schmarda, 1859) develops into a Götte's larva (Anderson 1977).

Similarly, the development of 16 species belonging to the family Stylochidae has been described (Table 1). Five of these are direct developers (Lang 1884; Pearse and Wharton 1938; Lytwyn and McDermott 1976; Teshirogi et al. 1981; Chen et al. 1990), while the remaining species develop via a Götte's larva (Lang 1884; Hofker

1930; Kato 1940; Galleni 1976; Rho 1976; Murina et al. 1995; Jennings and Newman 1996; Merory and Newman 2005; Lee et al. 2006) except for *Stylochus ellipticus*, which develops via a Müller's larva (Provenzano 1959). Among Leptoplanidae, a Müller's larva is known for *Hoploplana inquilina* (Wheeler, 1894) (Surface 1907) but direct development has been described for its congener, *H. villosa* (Lang, 1884) (Kato 1940). Among acotyleans, mode of development is not taxonomically significant (Table 1). On the other hand, all cotylean species studied to date possess a Müller's larva; no Götte's larvae have been recorded for the group (Ruppert 1978).

Effects of parental care on hatching success

Parental care in the form of adult worms covering egg masses with their bodies (Figure 7A) was observed for both species. In one instance, we observed guarding behaviour of an *I. zebra* parent animal against a gastropod approaching the egg masses of the flatworm (Figure 7B). For *P. atomata*, analysis of variance of Treatment 1 revealed a highly significant difference in hatching success between embryos with and without parental care (one-way ANOVA: $d.f._{1,54}$, $F=19.44$, $P<0.001$). The mean hatching success for embryos with parental care was 65.1% and

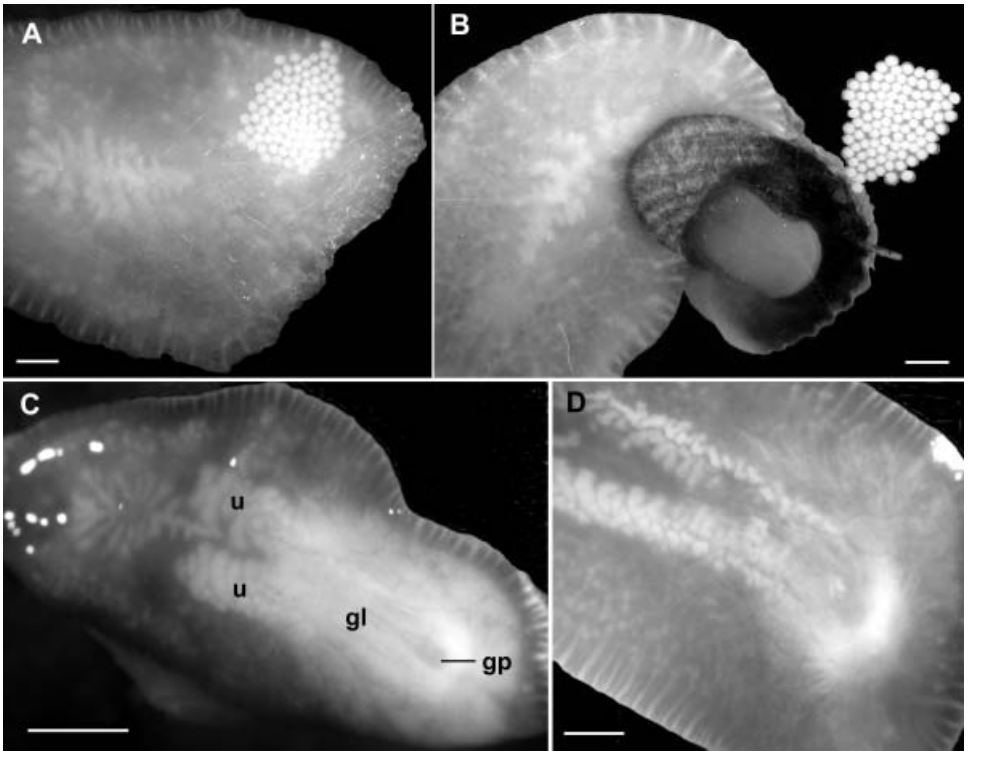

Figure 7. *Imogine zebra* parental care: (A) covering newly laid eggs, (B) attacking a gastropod near a recently laid egg plate, scale bars, 1 mm. *Imogine zebra*, ventral views: (C) before oviposition, showing egg-filled uteri (u), filled ventral glands (gl), and gonopore (gp), scale bar 5 mm; (D) immediately after oviposition, showing marked decrease in gland contents, scale bar 2 mm.

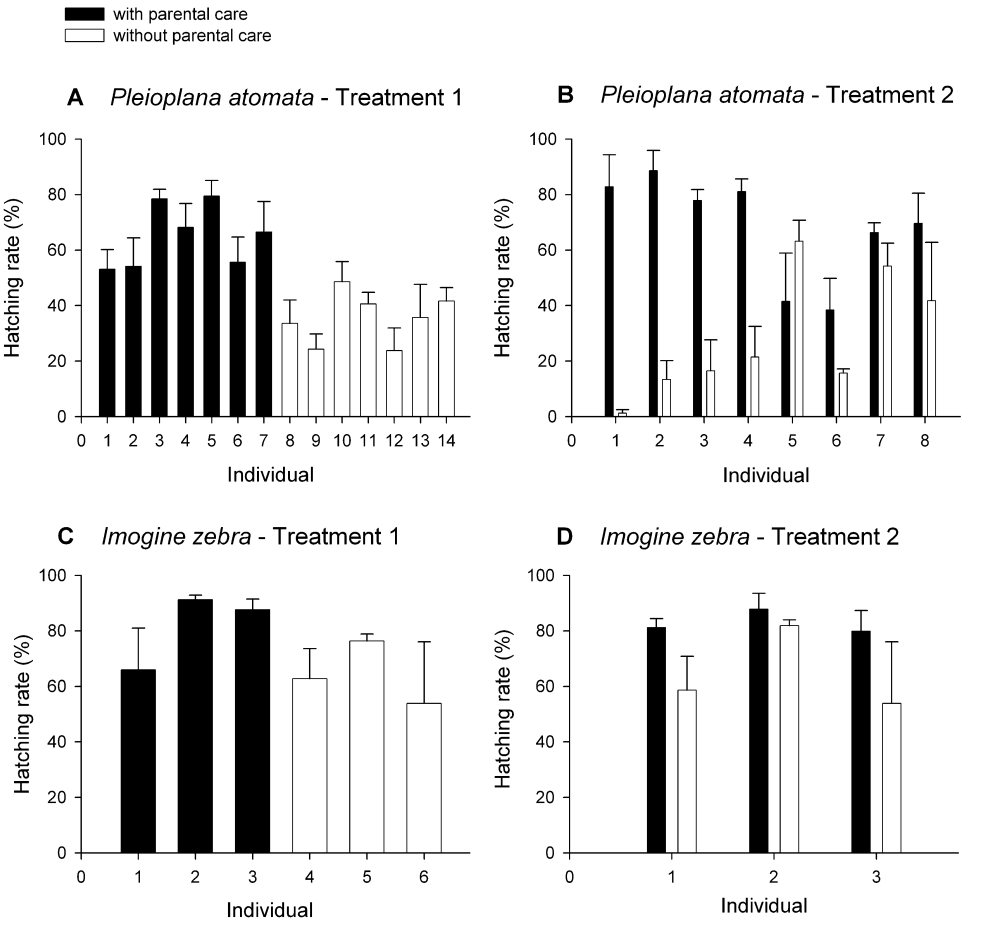

Figure 8. Variation in mean hatching rate (±1 SE) per batch of eggs with and without parental care in *Pleioplana atomata* and *Imogine zebra*. (A, C) Treatment 1 – egg hatching rate for all batches per individual; black bars – with parental care, white bars – without parental care, (B, D) Treatment 2 – three egg batches with parental care and three batches without parental care per individual.

without parental care was 35.6% (Figure 8A). The variation in hatching rate between eggs in the first three and second three batches of each individual was not significant (one-way ANOVA: d.f.$_{1,70}$, F=1.82, P>0.05), therefore variation in viability between early-laid and late-laid eggs should not confound the results in Treatment 2. Taking variation of individual fecundity into account (Treatment 2), the two-way nested ANOVA showed significant care and individual effects; however, parental care was the most important factor (Figure 8B, Table 3).

Imogine zebra adults covered the egg plates continually for up to 15 minutes after oviposition (Figure 7A). Hatching success was not influenced by the presence or absence of the parent (Treatment 1, one-way ANOVA: d.f.$_{1,34}$, F=0.01, P>0.05, Figure 8C; Treatment 2, Figure 8D and Table 3). However, the viability in the eggs of later-laid batches was significantly less than those laid earlier in the experiment (one-way ANOVA: d.f.$_{1,46}$, F=6.09, P<0.02). As the effect of laying-sequence on egg

Table 3. Summary of two-way nested ANOVA of hatching rate of *Pleioplana atomata* and *Imogine zebra* eggs with and without parental care (Treatment 2).

	Source	d.f	*MS*	*F*	*P*
Pleioplana atomata	Parental care	1	0.016	14.59	0.002
	Animal (Parental care)	14	0.015	3.16	0.003
	Error	32	0.011		
	Total	47			
Imogine zebra	Parental care	1	0.0026	1.92	0.238
	Animal (Parental care)	4	0.0013	2.32	0.117
	Error	12	0.0006		
	Total	17			

viability is variable between species and can be significant, future studies should minimize this effect by using every other batch laid by the individual for the two conditions tested.

One batch of eggs was removed from the adult immediately after oviposition and reared in filtered seawater. No eggshells formed and eggs were not arranged into a plate but cleavage proceeded normally. The function and the factors determining the evolution of covering behaviour in polyclad parental care are not clear. Lee (2006) suggested that it is a 'brooding' behaviour affording protection to the developing embryos. However, it does not appear to be necessary for either development or hatching under laboratory conditions (Lee 2006; this study). In the four species where the contribution of 'brooding' to hatching success has been quantified, only *P. atomata* showed a significantly increased hatching rate in the presence of a parent. In *I. zebra* (this study), *Echinoplana celerrima* and *Stylochus pygmaeus* Merory and Newman, 2005 (Lee 2006) hatching rates were not influenced by the presence or absence of parents. Because egg-covering behaviour still occurred under controlled laboratory conditions where potential predators were absent, we infer that this behaviour is probably not related to the protection of developing embryos.

In *I. zebra*, the role of covering behaviour immediately after oviposition may be linked to eggshell formation and possibly the adhesion of egg masses to the substrate. Eggshell formation involves shell-forming granules in the egg, as well as secretions of shell glands discharged into the female atrium as eggs are deposited (Ishida and Teshirogi 1986). Soon after oviposition, the shell-forming granules swell through water absorption and separate from the egg to form the eggshell. Eggs of *I. zebra* collected within a minute of oviposition and developing in the absence of an adult, showed no eggshell formation. Still these eggs developed normally.

An examination of the ventral surface of *I. zebra* immediately before egg laying revealed not only uteri loaded with eggs but also glands prominently filled with materials ready for release (Figure 7C). Immediately after oviposition, these glands showed a marked decrease in contents (Figure 7D). Hence, it is possible that the covering behaviour of the adult worm plays a role in the formation and tanning of eggshells. Additionally, covering behaviour may result in the adhesion of the egg masses to the substrate. Kato (1940) too, describes adult worms covering cemented egg masses with gelatinous secretions from ventral glands. Further work on covering behaviour and its role in shell formation is clearly required.

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