

Brooding behaviour and reproductive success in two species of free-living simultaneous hermaphrodites

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Abstract Although polyclads are amongst the most structurally simple of the triploblastic metazoans, they adopt a wide range of reproductive strategies. Parental care behaviour in this group is yet to be quantified for any species. We assessed the significance of brooding behaviour to the reproductive success of two free-living marine flatworms. *Echinoplana celerrima* and *Stylochus pygmaeus* were collected from the field and placed in pairs in containers of filtered seawater where they laid batches of eggs. Both parents were then removed from half of the containers and the brooding behaviour and hatching success of eggs were quantified. There were interspecific differences in brooding behaviour. Egg masses were covered by one *E. celerrima* parent for $12 \pm 2\%$ of time, whereas egg masses of *S. pygmaeus* were covered by one or both parents simultaneously for $85 \pm 8\%$ of time. Egg batches were abandoned by both species immediately prior to the onset of hatching (10–12 days). Hatching success was generally high ($\sim 90\%$) and brooding did not enhance the hatching success of eggs. We assessed the significance of parental care to hatching success of *E. celerrima* egg masses in the presence of three potential egg predators; in the presence of other organisms. *E. celerrima* devoted less time to brooding; however, hatching success

was not affected. The amount of time spent brooding eggs differed greatly between the two polyclad species but was not essential to their reproductive success under benign conditions. Parental care may be of adaptive value under more stressful environmental conditions commonly experienced in estuarine environments such as lowered salinity, increased hypoxia or turbidity. Covering egg batches may play an additional role of advertising sexual status and a willingness to care for eggs.

Introduction

Animals display a wide range of parental care behaviour to ensure the survival, growth and breeding success of their offspring (Clutton-Brock 1991). ‘Parental care’ is a descriptive term referring to any form of parental behaviour that has the potential to increase the fitness of a parent’s offspring and in some species it is required for egg hatching and egg development (Barnard 2004). The brooding of eggs is a common parental care behaviour adopted by small invertebrates (Barnard 2004). Brooding is more commonly associated with small adult size, possibly because larger adults have a relatively greater capacity to produce large numbers of offspring than their capacity to brood them (Strathmann and Strathmann 1982; Strathmann 1985).

The contribution that the parental care makes to the viability of offspring has been studied in a wide range of taxonomic groups (Charrassin et al. 1998; Eggert et al. 1998). Brooding behaviour has been widely observed amongst marine invertebrates, such as amphipods (Dick et al. 2002; Thiel 2003), crabs (Fernandez et al. 2000), oysters (Foighil and Taylor 2000) sea stars (Strathmann et al. 1984; Bosch and Slattery 1999; Byrne 2005) and polychaetes (Sella

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1991; Sella and Lorenzini 2000). In general, amongst marine invertebrates, brooding behaviour has been associated with adverse environmental conditions, such as low oxygen availability within the embryo mass (Baeza and Fernandez 2002; Lardies and Fernandez 2002; Brante et al. 2003) although protection of offspring from predation is arguably one of the strongest drivers for the evolution of parental care (Aoki and Kikuchi 1991; Kutschera and Wirtz 2001; Thiel 2003; Taborsky and Foerster 2004).

Marine flatworms (polyclads) are simultaneous hermaphrodites and much attention has been paid to their mating behaviour including observations of hypodermic insemination and tests of sex allocation theory (Michiels 1998; Michiels and Newman 1998; Schärer et al. 2004; Schärer et al. 2005). There exist, however, only anecdotal reports of parental care behaviour for this group (Pearse and Wharton 1938; Prudhoe 1985; Murina et al. 1995; Merory and Newman 2005).

Polyclads are common mobile predators found on hard substrata and are closely associated with sessile invertebrate assemblages (Lee et al. 2006). Adults lay eggs in chains or plate-like masses, which are usually covered with a sticky gelatinous substance secreted from the glands lying in the ventral parenchyma and from the glands opening into the vagina. In this way, eggs are fastened firmly to the substratum but the egg casing may also provide protection in the form of toxic exudates (Prudhoe 1985). Flatworm parents may provide indirect protection to their offspring using empty barnacle shells as a cradle (Murina et al. 1995). Moreover, it has been suggested that flatworms brood offspring by covering the egg masses with their body for several days (Pearse and Wharton 1938; Merory and Newman 2005). Adult flatworms are thought unlikely to be the subject to heavy predation because of the highly toxic or distasteful chemical compounds, such as tetrodotoxin and staurosporine derivatives in their epidermis (Newman and Cannon 2003). Eggs, larvae and juvenile stages, however, may be susceptible to predation due to the weaker potency of epidermal secretions (Prudhoe 1985). Nevertheless, studies are yet to identify putative predators or examine the significance of parental care to the reproductive success of flatworms.

We predicted that the parental care would increase the reproductive success of flatworms and that brooding behaviour would intensify in the presence of potential egg predators. We investigated the contribution of brooding behaviour by *Echinoplana celerrima* and *Stylochus pygmaeus* to the hatching success of their eggs. We also determined whether reproductive behaviour of *E. celerrima* was altered by the presence of three potential flatworm egg predators: the whelks *Morula marginalba* (Blainville) and *Lepsiella hanleyi* (Angas), and the co-occurring predatory flatworm *S. pygmaeus*.

Materials and methods

Adults of two flatworm species, *E. celerrima* ($N = 92$) and *S. pygmaeus* ($N = 12$) were collected from artificial settlement panels deployed at depth of 3 m from Kurnell Pier, on the southern margin of Botany Bay, New South Wales, Australia (33°59.92'S, 151°2.62'E). Collection of flatworms was made difficult by the mobility and extreme delicacy of the worms. Worms were collected from sessile invertebrate assemblages using fine paint brushes during the 2004–2005 reproductive season (December–March). Flatworms were monitored for 2 days prior to experimental trials to allow them to recover from collection and transport stress. Flatworms that exhibited signs of collection injury were not used in experimental trials.

Parental care: brooding behaviour and hatching success of eggs

Ten pairs of similar sized *E. celerrima* and six pairs of similar sized *S. pygmaeus* were placed in separate plastic containers with 1 L of filtered seawater and allowed to mate. All experiments were conducted in a constant-temperature room ($23 \pm 0.5^\circ\text{C}$) subjected to 11:13 light:dark cycle. Salinity (33.7–34.5 ppt), pH (7.93–7.98), temperature ($23\text{--}23.5^\circ\text{C}$) and dissolved oxygen ($5.56\text{--}5.74\text{ mg L}^{-1}$) were monitored daily using an YSI 556 MPS (Yellow Springs, OH, USA) water quality metre and remained constant throughout the experiments. Filtered seawater was changed daily. Polyclads tend to hide from light so a loose black perspex panel ($6 \times 6 \times 0.5\text{ cm}$) was included in each container to provide a sheltering place for the adult flatworms. The first batch of eggs laid was treated as the focal egg batch with subsequent egg batches being immediately and carefully removed with a spatula. The containers were randomly assigned to two treatments: containers holding eggs and a pair of flatworm parents (parental care) and containers from which the flatworms were removed immediately after the focal egg batch had been laid (no care). The number of replicates in the treatment and control was constant for each flatworm species (*E. celerrima*, $n = 5$ and *S. pygmaeus*, $n = 3$). The number of replicates was restricted by the availability of mature adult worms of a similar size; however, the low variability in results indicates that replication was sufficient for our tests. Marine flatworms can survive unfed for many weeks and will continue to lay eggs (Chintala and Kennedy 1993). Flatworms were not fed for the duration of the experiments.

The number of eggs in the focal egg batch was censused by examination of a high resolution digital image. Digital photographs of the focal egg batch were taken daily using Olympus compound microscope (SZX-ILLK200) connected to Pixelink (PL-A 642) with a magnification of $40\times$. The number of eggs was counted from the photographs

using Image-Pro Express 4.0.1 software. Hatched flatworm larvae are planktonic immediately post-hatching and were collected daily by pouring the contents of the experimental container into a sample jar and preserving the solution in 7% formalin. Freshly filtered seawater (1 L) was then replaced in each treatment container. This was done for 10 days after the focal batch had begun to hatch. Multiple solutions of preserved larvae from the same replicate container were then pooled by pouring all through a 10 μm sieve. The larvae were then resuspended in a single 500 mL sample solution. Triplicate estimates of flatworm larval numbers in each sample were made by counting the number of larvae in three 20 mL aliquots. Hatching success of flatworm eggs was calculated as the percentage of number of larvae out of the number of eggs. Flatworm activity was recorded every 4–6 h from when the first egg batch was observed until 4 days after larvae were first observed hatching from the eggs. This included night-time observations conducted with the aid of a flashlight. Flatworms were considered to be actively “brooding” if their body covered any part of an egg batch.

Potential egg predators

Parental care experiments as described above were again conducted with *E. celerrima* except for the addition of a third factor of potential predator presence or absence. Replicate containers of paired *E. celerrima* were randomly assigned to one of the four treatments: (a) “parents and predator”—parents retained and predator added, (b) “parents only”—parents retained but predator-free, (c) “predator-only”—parents removed and predator added, (d) “eggs only”—parents removed and predator-free. One individual “predator” of one species (whelks, *Morula marginalba*, *Lepsiella Hanleyi* or flatworm *Stylochus pygmaeus*) was added to the experimental container after the focal egg batch was laid. There were five replicates of each treatment for the trial involving *M. marginalba* and three replicates of each treatment for the trials involving *L. hanleyi* and *S. pygmaeus*. *M. marginalba* was collected from the intertidal rock platform at Clovelly headland New South Wales, Australia (33°5'S, 151°16'E). The potential egg predators *L. hanleyi* and *S. pygmaeus* were collected from empty barnacle shells on artificial settlement panels deployed at depth of 3 m from Kurnell Pier. *S. pygmaeus* were collected with a paintbrush whilst *M. marginalba* (Blainville) and *L. hanleyi* (Angas) were collected by handpicking. Each individual was kept separately in well-aerated filtered seawater for 2 days prior to experimental trials to allow them to recover from collection and transport stress. They were not fed during the recovery period.

Flatworm brooding behaviour, egg laying and hatching success were quantified as above.

Results

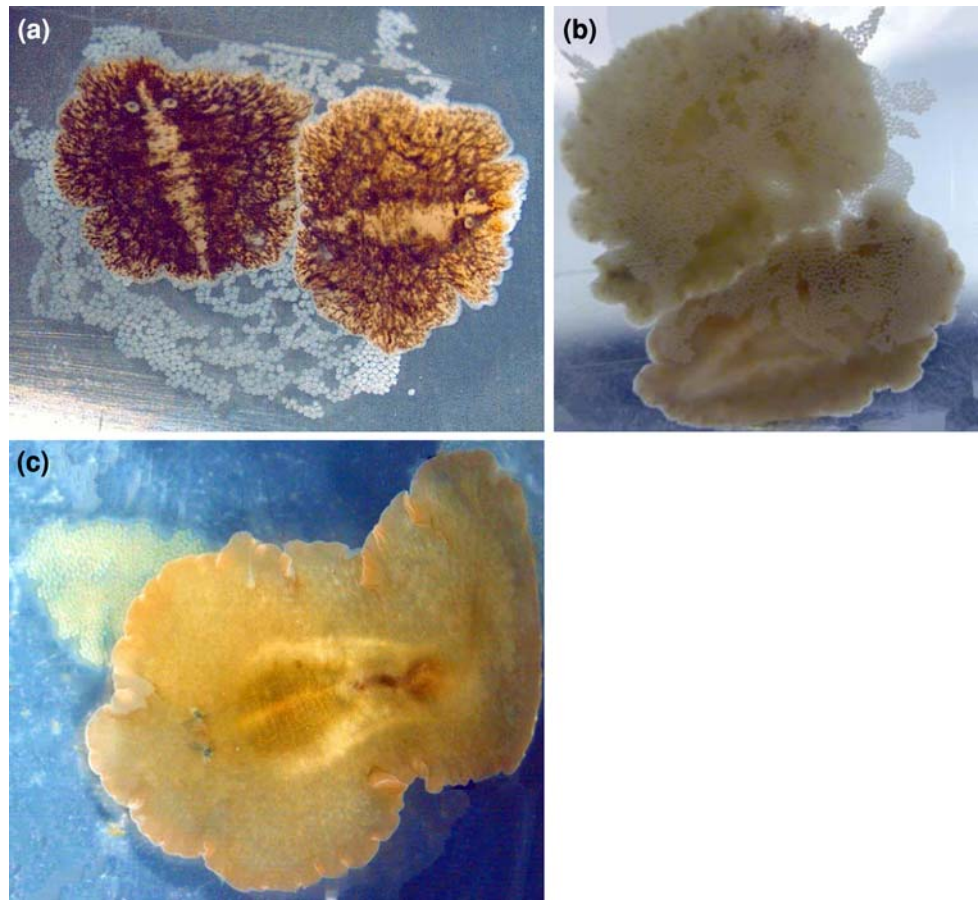
General mating and parental care behaviour

Both species were observed engaging in mutual cross fertilisation. Before mating, the two individuals in the container were observed moving towards each other, touching and then winding themselves around each other. Both species chose to lay egg batches on the sides of the container walls such that in order to brood they must leave their dark perspex shelter. Worms laid eggs in plate-like masses which were fastened firmly to the substratum with a sticky gelatinous substance. *Echinoplana celerrima* eggs were individually encased whilst *S. pygmaeus* egg capsules each contained 12 embryos arranged in a sphere. Flatworm parents brooded offspring by covering the egg masses with their body (Fig. 1) and they were observed undulating their bodies above the eggs as if to increase water exchange.

Interspecific differences in brooding behaviour

There were interspecific differences in brooding behaviour between *E. celerrima* and *S. pygmaeus*. Both *S. pygmaeus* parents could be involved in brooding concurrently on the one egg batch. In contrast, only one *E. celerrima* parent was involved in brooding at any one time. It was not possible to determine if the brooding individual had also laid the egg batch or if both worms had contributed to the one egg batch. The focal egg batch of *E. celerrima* was covered by one of the parents for approximately 12% of time, whereas, the focal egg batch of *S. pygmaeus* was covered by one or both parents simultaneously for approximately 85% of the time (Fig. 2a, ANOVA: $F_{1,6} = 123.2$, $P = 0.00$). Both species brooded the focal egg batch for up to 10 days until they began to hatch; whereupon brooding activity ceased entirely (Fig. 3). Egg batches varied greatly between parents and also between species. *S. pygmaeus* egg batches could contain more than double the number of eggs of *E. celerrima* batches but they were highly variable (*S. pygmaeus*: $2,210 \pm 1,030$, *E. celerrima*: 840 ± 85 , ANOVA: $F_{1,14} = 3.779$ $P = 0.072$). In the first trial, *E. celerrima* laid only 1.8 ± 0.2 egg batches over 10 days whereas *S. pygmaeus* laid 3.0 ± 1.0 egg batches. In the later trial *E. celerrima* laid 6.7 ± 1.5 egg batches in the ‘parents’ only treatment. All egg batches laid after the first “focal egg batch” were immediately removed from the containers (“Materials and methods”). It would appear that the frequency of egg laying may be highly seasonal and a comparison between species

Fig. 1 Photographs of flatworms brooding eggs: **a** *Stylochus pygmaeus* pair brooding eggs. **b** Image taken from underneath a pair of *Stylochus pygmaeus* worms brooding eggs. **c** *Echinoplana celerrima* brooding eggs



would require closer examination throughout the breeding season (November–April in the southern hemisphere).

Effects of brooding on hatching success

Hatching success of *E. celerrima* and *S. pygmaeus* eggs was generally high and was not influenced by the presence or absence of parents (Fig. 2b, ANOVA: *E. celerrima*: $F_{1,8} = 0.85$, $P = 0.38$; *S. pygmaeus*: $F_{1,4} = 0.07$, $P = 0.81$). *E. celerrima* eggs took longer to begin hatching (12 ± 0.4 days) than *S. pygmaeus* eggs (10 ± 1 days) but this was not influenced by the presence or absence of parents (ANOVA: *E. celerrima* $F_{1,8} = 0.2$, $P = 0.64$; *S. pygmaeus*: $F_{1,4} = 0.1$, $P = 0.81$). Daily photographs confirmed that no additional eggs were added to the egg batch after it had been laid.

Behaviour and hatching success in the presence of potential predators

Echinoplana celerrima reduced the proportion of time spent brooding in the presence of other organisms (Fig. 4a, ANOVA: *Morula marginalba*: $F_{1,8} = 6.756$, $P = 0.032$; ANOVA planned comparisons, *Lepsiella hanleyi*: $F_{1,4} = 24.836$, $P = 0.002$, *S. pygmaeus* $F_{1,4} = 27.596$,

$P = 0.002$). *E. celerrima* displayed different behaviour when confronted by different potential egg predators. *E. celerrima* avoided contact with the whelk *L. hanleyi* or the flatworm *S. pygmaeus* whilst staying near, but not on top of, the focal egg batch. In contrast, *E. celerrima* spent $21 \pm 5\%$ of its time closely associated with the whelk *M. marginalba*, with both parents simultaneously surrounding the whelk's shell apparently rendering it unable to move. Although *E. celerrima* parents were sometimes actively associated with the potential predator, hatching success of *E. celerrima* eggs was not influenced by the presence of parents or potential flatworm egg predators (Fig. 4b; Table 1). Moreover, the time taken for eggs to begin hatching (~ 12 days) did not differ between treatments (Table 1). The presence of predators did not affect the number of egg batches laid by *E. celerrima* ($P > 0.05$).

Discussion

Protection of offspring is one of the most important functions of parental care behaviour (Clutton-Brock 1991) and in this study the protection of offspring was quantified for the first time in free-living flatworms. *S. pygmaeus* spent

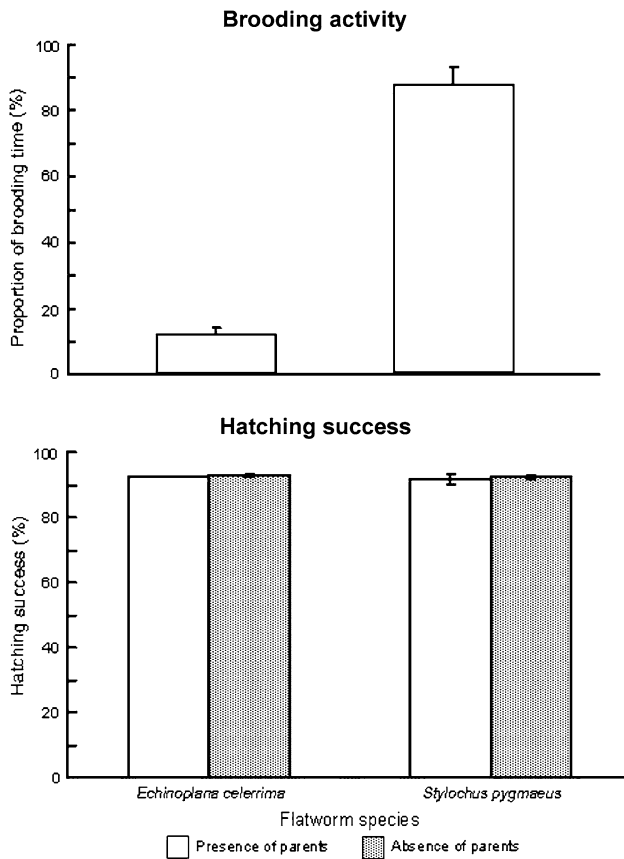


Fig. 2 a Proportion of time spent brooding by *Echinoplana celerrima* and *Stylochus pygmaeus* and b mean hatching success of flatworm eggs in the presence and absence of parents. Error bars represent mean (± 1 SE)

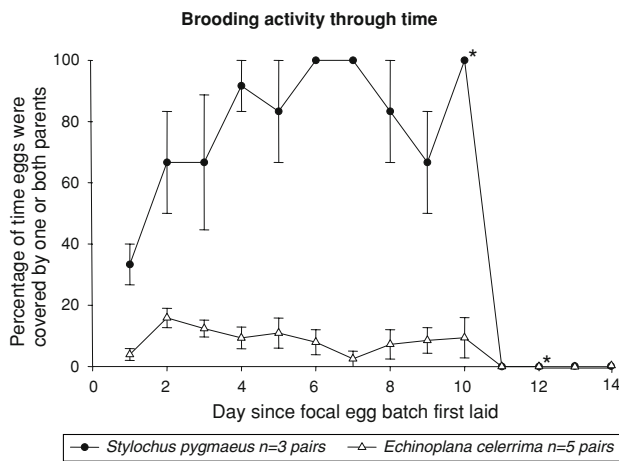


Fig. 3 Proportion of time spent brooding by *Echinoplana celerrima* and *Stylochus pygmaeus*. Daily values represent the average amount of time spent brooding from observations made every 4–6 h for a period of 14 days. Day 1 represents the first day that an egg batch was observed. The star represents the average day on which flatworm eggs began hatching

vastly more time covering egg masses than *E. celerrima* but both species abandoned egg batches immediately prior to the commencement of hatching. This strengthens our

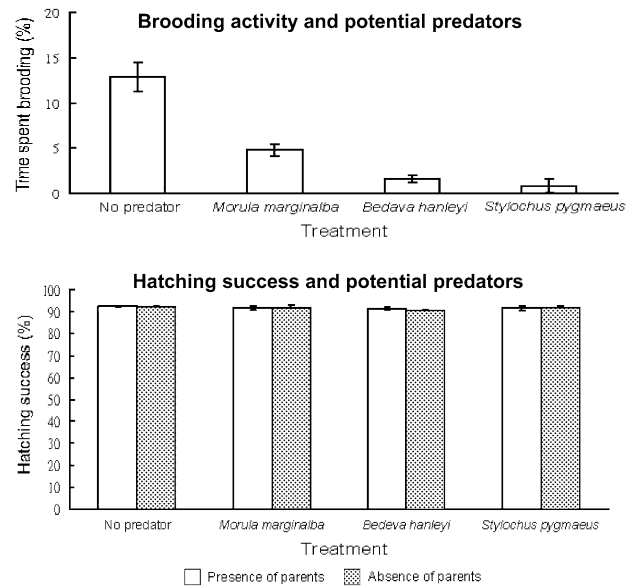


Fig. 4 a Time spent brooding in the presence and absence of potential predators and b mean hatching success of *E. celerrima* eggs in the presence and absence of *E. celerrima* parents and potential predators *Morula marginalba*, *Lepsiella hanleyi*, *Stylochus pygmaeus*. Error bars represent mean (± 1 SE). To avoid repetition the ‘no-predator’ bars are the result of pooling the control treatment values from each of the separate predator trials

hypothesis that the act of covering egg masses was indeed brooding activity. However, we also recorded a consistently high hatching success rate regardless of the presence or absence of parents, or the level of parental care provided.

Predation on eggs and juveniles has been posited as the major selective pressure driving the evolution of parental care in some invertebrates (Kutschera and Wirtz 2001). *Morula marginalba*, *Lepsiella hanleyi* and *S. pygmaeus* are common organisms co-occurring with the flatworm *E. celerrima* and they are voracious predators of a range of marine invertebrate phyla. Changes in the brooding behaviour of *E. celerrima* were observed in the presence of these organisms. Contrary to expectations, *E. celerrima* spent more time moving around the containers or staying beside the egg batch instead of covering it when any of the three other species were present. Guarding behaviour is an important function of parental attendance and is believed to be useful in protecting the egg masses from predators (Blumer 1986; Markman et al. 1995; Asoh and Yoshikawa 2001; Li and Jackson 2003). However, neither the whelks nor the predatory flatworm were observed actively preying on eggs and the presence of these organisms did not affect the hatching success of eggs. All three potential predators have been observed preying on barnacles, and have been observed co-existing with *E. cellerimma* in the field (personal observations). Empty barnacle shells are a popular place for flatworms to lay egg batches, so it is very likely that the potential predators encounter egg masses. However it is not

Table 1 Summary of two-factor ANOVA of the hatching success and the time taken for the *Echinoplana celerrima* eggs to finish hatching in the presence and absence of parental care and the potential predators *Morula marginalba*, *Lepsiella hanleyi* and *Stylochus pygmaeus*

Source	df	MS	F	P
Hatching success of the eggs				
Presence of <i>Morula marginalba</i>	1	1.973	0.762	0.397
Presence of parents	1	0.221	0.085	0.775
Interaction (<i>M. marginalba</i> × parents)	1	0.005	0.002	0.965
Residual	14	2.589		
Presence of predator (<i>L.hanleyi</i> or <i>S. pygmaeus</i>)	2	1.508	1.155	0.348
Presence of parents	1	1.850	1.416	0.257
Interaction (predator × parents)	2	0.933	0.714	0.509
Residual	12	1.306		
Time taken for the eggs to begin hatching				
Presence of <i>Morula marginalba</i>	1	0.155	0.453	0.511
Presence of parents	1	0.251	0.734	0.405
Interaction (<i>M. marginalba</i> × parents)	1	0.155	0.453	0.511
Residual	14	0.342		
Presence of predator (<i>L.hanleyi</i> or <i>S. pygmaeus</i>)	2	3.389	2.179	0.156
Presence of parents	1	1.389	0.893	0.363
Interaction (predator × parents)	2	0.389	0.250	0.783
Residual	12	1.556		

certain that they actually attack or prey on them. Hence, ‘guarding’ may be a response to the presence of another organism, rather than specifically an attempt to protect eggs.

The clear absence of any effect of brooding on the hatching success of flatworm eggs may stem from the benign nature of the physical and chemical environment maintained during experiments. Flatworms were held in well-controlled laboratory conditions with daily refreshment of filtered field seawater and a constant room temperature. Flatworm eggs may not require parental care in order to hatch under these conditions. In studies of the significance of brooding in polychaete worms of the genus *Ophryotrocha*, eggs developed equally well under the laboratory conditions if they were cared for by both parents, one parent, an unrelated individual, or even without any parental care (Sella 1991). Our flatworms were observed undulating their bodies whilst brooding the egg masses, which may be interpreted as an attempt to increase water exchange. If water exchange is an important benefit of brooding this might explain the large interspecific differences in time spent on parental care. *Stylochus pygmaeus* eggs were packed more compactly (12 eggs in each egg casing) than *E. celerrima* eggs and may therefore require greater water exchange to maintain adequate oxygen levels. If water exchange is important then one would predict that brooding will increase the survival of offspring under poor water quality conditions (e.g. hypoxia). However, our attempts to imitate adverse environmental conditions, including lowering dissolved oxygen or increasing turbidity, caused immediate mortality of adult flatworms (unpublished data). Adult flatworms possess a large permeable surface area and are

known for their low tolerance to physiological stresses (Sagasti et al. 2000; Lee et al. 2006). It is possible that adult flatworms are more susceptible to poor water quality conditions than their eggs. This would have major implications for the cost-benefit analysis of parental care and further work is required to assess the relative tolerance of flatworm eggs and their parents to common variations in water quality.

Brooding egg batches might also signal that sexually mature receptive flatworms are nearby (King et al. 2003) and/or brooding may increase the likelihood that an individual is chosen as a mate (Hale and St Mary 2007). Hale and St Mary (2007) show that under certain environmental conditions, nest-tending by male flagfish increases their chance of spawning, whilst King et al. (2003) observed that squid egg mops provide sensory cues that increase the agonistic behaviour of males in competition for female mates. The large interspecific differences in polyclad parental care that we observed might then be related to the population frequency of mature hermaphrodites. If the frequency of mature ovigerous hermaphrodites is low then both parents tend to stay together as a way to ensure a partner for the next mating (Charnov 1982; Ghiselin 1987). *Echinoplana celerrima* is the most common flatworm in NSW and its relative density is generally higher in natural populations than the density of *S. pygmaeus* (unpublished observations). Hence, remaining tightly associated with an egg mass may not greatly increase the probability of *E. celerrima* encountering and/or being chosen as a mate, whereas, the benefit to a less common worm such as *S. pygmaeus* may be far greater.

The fact that both flatworm species abandoned eggs just prior to hatching suggests that brooding is indeed an active

behavioural choice of these simple animals. This represents a preliminary study and further research is required to explore the ecological and evolutionary significance of this behaviour under a range of environmental conditions.

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