Molluscan Research

Reproductive variance in planar spawning Chromodoris species (Mollusca: Nudibranchia)

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Reproductive variance in planar spawning *Chromodoris* species (Mollusca: Nudibranchia)

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Although variance in life history parameters is well known from comparisons among broad phylogenetic groups of marine invertebrates, there is still an outstanding need to increase empirical studies that compare closely related species. If the species under study share a recent common ancestor and developmental strategy, there is an opportunity to contrast maternal investment against interspecific variation while controlling for evolutionary distance. Furthermore, when these species co-occur, it allows for exploration of potential character displacement. We examined egg size and other factors related to reproduction in four closely related species, co-occurring nudibranchs belonging to the monophyletic *Chromodoris* planar spawning clade. The duration of oviposition appeared to be conserved and may be phylogenetically constrained in these four co-occurring species. In contrast, egg size differed significantly among species, but was not influenced by parental body length or position within the egg mass for any of the species. The number of egg mass whorls also varied, but did not correspond to the interspecific differences in parental body length. These results suggest that some significant differences exist among these sympatric *Chromodoris* species that may be candidate traits for character displacement. These characters would need to be re-measured in geographic areas where the studied *Chromodoris* species do not co-occur.

Keywords: nudibranchs; egg size; character displacement; phylogenetic constraints

Introduction

The type of development shown by an organism strongly impacts its ability to disperse within its environment and underlies many important ecological and evolutionary aspects of its biology. Factors leading to the ecological success of a larval cohort are influenced not only by the set of genes they have inherited and the environment they experience, but also by the environment and phenotype of their mother. These latter "maternal effects" have received much recent attention and have demonstrated themselves to be a non-genetic influence on variation in larval fitness and phenotype (Marshall *et al.* 2008a, 2008b), and hence is one of the most important influences on offspring performance (Wade 1998). In contrast, the phylogenetic constraints that are encoded in genomic DNA are much less well understood (but see Eckelbarger and Watling 1995; Levitan 2000; Collin 2004), and reviews on the subject have consistently highlighted the need for more empirical studies (Bernado 1996; Marshall and Keough 2007; Moran and McAlister 2009). When closely related sympatric species fill the same ecological niche, interspecific competition should be reduced by the process of character displacement (Brown and Wilson 1956). That is, selective forces should increase differences in traits considered ecologically important in order to reduce competition,

reinforce reproductive barriers, and maximize individual fitness in areas where similar species occur together. Given that reproductive barriers can directly impact speciation processes, variance in reproductive parameters should be especially important to examine. However, the paucity of the data on observed reproductive differences among sympatric, closely related species limits the exploration of this hypothesis.

The influence of phylogeny on life history traits has been examined in some marine invertebrate groups (Spight 1979; Lessios 1990; Collin 2004), but in nudibranchs it remains essentially untested. Nudibranch egg size can range from 90 to 380 μm within a single genus, and this level of variation is common (Hadfield and Switzer-Dunlap 1984). In fact, variance is very high for most measured life history parameters across "opistho-branchs" (now recognized as a paraphyletic group nested inside Heterobranchia, see Schrödl *et al.* 2011), such as size at first reproduction, number of eggs laid, and egg diameter (reviewed in Hadfield and Switzer-Dunlap 1984). Egg size variation within marine invertebrate species has been attributed to parental food supply and quality (Qian and Chia 1991; Marshall and Keough 2007), differential yolk distribution during gametogenesis (Lonsdale and Levinton 1985), fertilization efficiency

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(Levitan 1993), temperature (Sheader 1996; Simonini and Prevedelli 2003; Collin and Salazar 2010), salinity (Gimenez and Anger 2001), and adult environment (George 1994; Rossiter 1996; Pond *et al.* 1996; Marshall *et al.* 2008b). Within heterobranchs in particular (which includes nudibranchs), intraspecific egg size variation has been linked to spawning history (Jones *et al.* 1996; Ito 1997), hatching success (Sprenger *et al.* 2010), genetic drift (Jones *et al.* 1996; Lambert *et al.* 2000), and maternal body size (Chester 1996; Lambert *et al.* 2000; Allen *et al.* 2009). In contrast, interspecific egg size variation appears mostly attributed to differing larval developmental strategies (Hadfield and Switzer-Dunlap 1984; Vendetti *et al.* 2012).

One strongly supported clade of *Chromodoris* (Alder & Hancock, 1855) species (Wilson and Lee 2005; Turner and Wilson 2008) is now recognized as *Chromodoris* sensu stricto (Johnson and Gosliner 2012). All of these species lay planar shaped egg masses (Wilson 2002), unlike any other species that may have been previously associated with the genus. These published phylogenetic topologies clearly demonstrate that this particular clade not only has a long branch length leading to it, but also that the species within the clade show very short branch lengths compared with all other species in the entire family, often form a polytomy, and are likely to comprise a very recent radiation. Furthermore, these planar spawning *Chromodoris* are also linked ecologically — all prey upon sponges containing the secondary metabolite Latrunculin A (Rudman and Bergquist 2007), which no other *Chromodoris* species feed on. Many of these closely related planar spawning *Chromodoris* species are found co-occurring throughout the "Coral Triangle" region, and when utilizing the same larval developmental strategy, provide an excellent opportunity to examine whether reproductive traits among these species remain phylogenetically conserved.

Materials and methods

Four species of planar spawning *Chromodoris* were examined in this study: *Chromodoris annae* Bergh, 1877, *Chromodoris lochi* Rudman, 1982, *Chromodoris magnifica* (Quoy & Gaimard, 1832), and *Chromodoris willani* Rudman, 1982. During the austral winter 2002, 14–22 specimens of each species were collected using SCUBA from the Wakatobi Marine National Park in the Tukang Besi Archipelago, southeast Sulawesi (5°45′S, 123°45′E). The extended crawling length of each specimen was measured and recorded after collection, as it is known to correlate strongly with body weight within a population (Wilson, N.G., unpublished observations). Specimens were held and maintained individually in small plastic aquaria that were cleaned daily with a complete water change. Ambient air temperatures reached a daily average of 35°C.

Most mature nudibranchs spawn readily in captivity, presumably due to capture and handling stress (Hadfield and Switzer-Dunlap 1984). The collected specimens were under constant observation during daylight hours (∼12 h) and the time taken to spawn after collection was measured to the nearest day. Observations were taken only from the first egg mass laid by each individual. The duration of oviposition was timed to the nearest 15 min, and egg mass samples were taken as soon as spawning concluded. Samples from both ends of the coil were fixed in 4% neutrally buffered formalin. The colour and number of whorls in the coil were noted, and the remainder of the egg mass was removed from the aquaria and placed in a Petri dish containing seawater. These dishes were also cleaned daily and the seawater changed. The time taken for the embryos to hatch into veligers was measured to the nearest day. The larvae were observed under a compound microscope in order to note the morphology of the larval retractor muscle and remaining yolk and thus ascertain the larval type according to Thompson (1967). Any successive egg masses were recorded and sampled in the manner described above, but were not used in the same analyses as the first-laid masses.

The preserved egg samples were examined at the University of Glamorgan, Wales. The diameters of 20 uncleaved ova (herein termed eggs) were collected from both ends of each egg mass, and were measured under a compound microscope connected to a computer. Digital images were then taken with Motic 2002 Images software. The average measurement of these 20 eggs represents a single observation in the data set, so the total data set (58 sampled egg masses) is represented by 1160 egg measurements. Any sample in which the eggs had already started cleaving was discarded, as the remaining uncleaved ova being measured may have been abnormal. Data analysis was carried out with Microsoft Excel and IBM SPSS Statistics v11 for Windows, with statistical significance set at $p < 0.05$. Data were accepted as having a normal distribution if skew and kurtosis values fell into a range of ± 2 after removing outliers. Although normally distributed data may actually be quite rare (Micceri 1989), we acknowledge that any deviations, combined with unequal sample sizes, may render parametric *t*-tests less robust. We have highlighted any normality deviation that cannot be attributed to zero variation in the data set or a small sample size ($n < 3$ for skew calculations, $n < 4$ for kurtosis calculations) in Table 1.

Results

Egg mass production, colour, and size

Approximately half of the animals collected laid an egg mass after collection. This ranged from 46% in *C. magnifica* to 89% in *C. lochi* (see Table 1). *C. willani* was the fastest to spawn post-collection and *C. magnifica* the

Table 1.   Mean values of reproductive traits for sampled *Chromodoris* species.

	Chromodoris lochi	*Chromodoris annae*	*Chromodoris willani*	*Chromodoris magnifica*
Inner egg size (μm $\pm$ SE)	113.860 $\pm$ 0.84 ($n = 15$)	112.642 $\pm$ 1.46 ($n = 5$)	119.003 $\pm$ 2.47 ($n = 6$)	120.482 $\pm$ 1.07 ($n = 2$)
Outer egg size (μm $\pm$ SE)	111.533 $\pm$ 1.47 ($n = 14$)	**113.143 $\pm$ 1.93** (***n* = 5**)	116.438 $\pm$ 3.34 ($n = 6$)	118.805 $\pm$ 4.72 ($n = 5$)
Mean egg size (μm $\pm$ SE)	112.732 $\pm$ 0.84 ($n = 29$)	112.892 $\pm$ 1.14 ($n = 10$)	117.721 $\pm$ 2.02 ($n = 12$)	119.284 $\pm$ 2.33 ($n = 7$)
Body length (mm $\pm$ SE)	34.26 $\pm$ 1.30 ($n = 19$)	36.50 $\pm$ 2.66 ($n = 10$)	38.78 $\pm$ 3.44 ($n = 9$)	47.33 $\pm$ 1.72 ($n = 12$)
Time to spawn (days $\pm$ SE)	1.5 $\pm$ 0.3 ($n = 16$)	**1.3 $\pm$ 0.2** (***n* = 9**)	$\pm$0 ($n = 9$)	**3.2 $\pm$ 1.2** (***n* = 6**)
Oviposition duration (h $\pm$ SE)	3.07 $\pm$ 0.30 ($n = 11$)	2.33 $\pm$ 0.39 ($n = 8$)	**2.15 $\pm$ 0.42** (***n* = 4**)	4.25 $\pm$ 0.98 ($n = 3$)
Number of whorls ($\pm$ SE)	3.4 $\pm$ 0.2 ($n = 17$)	**3.4 $\pm$ 0.2** (***n* = 9**)	3.7 $\pm$ 0.3 ($n = 3$)	4.8 $\pm$ 0.6 ($n = 5$)
Percent to spawn	89%	59%	60%	46%

Standard error values and sample sizes are included. Bold values are those that fall outside the accepted normality range, and which cannot be attributed to an inadequate sample size or zero variation within the data set.

slowest, and this was the only significant comparison between any of the species for the time taken to spawn after collection from the field (Student's *t*-test, $p = 0.049$).

The planar egg masses produced by all four species were relatively translucent, as is typical for this *Chromodoris* clade, and were laid from the centre spiralling outwards. The colour of the ova differed slightly, giving an overall hue to the egg masses. *C. annae* laid a cream-coloured egg mass with an average of 3–4 whorls, and *C. lochi* produced white egg ribbons with an average of 3–4 whorls. *C. magnifica* laid a pale orange egg mass with an average of 5 whorls, and *C. willani* laid a cream-coloured egg mass similar to that of *C. annae*, with an average of 3–4 whorls (Table 1).

The mean number of whorls deposited varied significantly among the four species (one-way analysis of variance (ANOVA), $p = 0.010$), and Tukey's *post hoc* tests ($p < 0.05$) revealed that the egg masses of *C. magnifica* contained significantly more whorls than those of *C. annae* and *C. lochi*, but not *C. willani* (Figure 1). Due to this significant variation, we did not pool species data for examining the relationships between the number of whorls laid against other parameters, namely body length or duration of oviposition.

Oviposition and the influence of body size on reproduction

Although the number of whorls deposited varied significantly between the species, the duration of oviposition did not (Table 1). On average, *C. magnifica* took the longest to deposit an egg mass, followed by *C. lochi*, *C. annae*, and *C. willani*, although these differences among species were not statistically significant (one-way ANOVA, $p = 0.058$). The duration of oviposition was found to positively
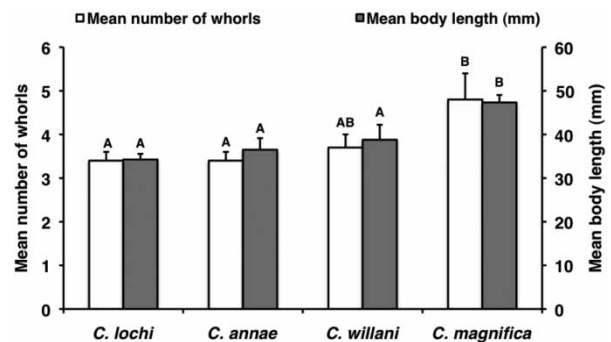

Figure 1.   Mean number of whorls and mean body lengths plotted as a function of species. Standard error bars are included. Different letters indicate significantly different means (one-way ANOVA followed by Tukey's tests for multiple comparisons, $p < 0.05$), while means with the same letter indicate no significant difference.

correlate with the number of whorls being deposited, but this was significant only for *C. lochi*, which notably had the largest sample size for these data ($n = 11$; linear regression, $r = 0.859$, $p = 0.001$).

There was significant variation among the mean parental body lengths of the species (one-way ANOVA, $p = 0.000$). *C. magnifica* had the greatest mean body length overall, and Tukey's *post hoc* tests ($p < 0.05$) revealed that it was significantly larger than *C. annae*, *C. lochi*, and *C. willani* (Figure 1). Because of these differences, we did not pool across species for investigating the effect of parental body length on egg size, duration of oviposition, or number of whorls laid.

Within a species, body length did not correlate with the duration of oviposition (linear regression: *C. annae* $p = 0.322$; *C. lochi* $p = 0.400$; *C. magnifica* $p = 0.682$; *C. willani* $p = 0.485$). Linear regression analysis also showed that body length did not have an effect on the
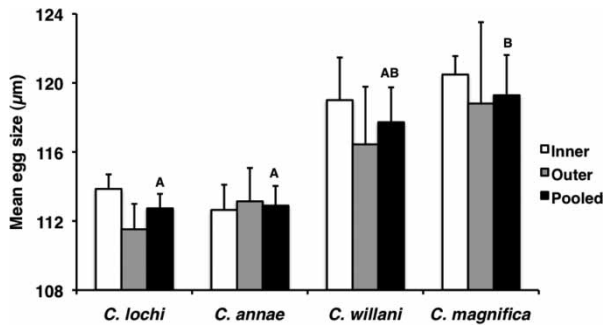
Figure 2.   Mean values for egg size in the inner part of the egg coil, outer part of the egg coil, and pooled (inner and outer measurements combined) plotted as a function of species. Standard error bars are included. Different letters indicate significantly different means (one-way ANOVA followed by Tukey's tests for multiple comparisons, $p < 0.05$), while means with the same letter indicate no significant difference.

number of whorls laid for *C. annae* ($p = 0.392$), *C. lochi* ($p = 0.990$), or *C. magnifica* ($p = 0.436$), but the sample size was too small to test *C. willani*.

Egg size and development time

Although there was a trend for the uncleaved ova diameter (= egg size) to be larger in the innermost (and thus earliest laid) part of the egg mass compared with the outermost part of the coil, this size difference was not significant for any tested species (paired two-sample *t*-tests: *C. annae* $p = 0.690$; *C. lochi* $p = 0.098$; *C. willani* $p = 0.992$; *C. magnifica* had too few data). The mean egg size for each species was thus calculated by pooling both inner and outer measurements when available, and this mean was subsequently used to test comparisons with parental body length.

The mean egg sizes among the four species were significantly different (one-way ANOVA, $p = 0.004$). Figure 2 shows that the mean egg sizes for *C. annae* and *C. lochi*

were very similar, as were those of *C. magnifica* and *C. willani*. Tukey's *post hoc* tests ($p < 0.05$) revealed that the mean egg size of *C. magnifica* was significantly larger than that of both *C. annae* and *C. lochi*, although mean egg size in *C. willani* was not significantly different from that of any other species. Egg size did not correlate with parental body length within any of the species (Figure 3) (linear regression: *C. annae* $p = 0.344$, $R^2 = 0.43$; *C. lochi* $p = 0.632$, $R^2 = 0.024$; *C. magnifica* $p = 0.366$, $R^2 = 0.27$; *C. willani* $p = 0.736$, $R^2 = 0.044$).

All species studied here produced planktotrophic veliger larvae, which is a developmental strategy involving small ova and a short embryonic period (Thorson 1950). The length of time taken by the embryos to hatch into veligers was measured to the nearest day, and all species took 5–7 days to hatch.

Discussion

Egg size can predict many downstream factors of ecological success, as it influences factors such as hatching size (Barnes and Barnes 1965; Spight 1976; Perron 1981), hatching success (Marshall *et al.* 2002; Sprenger *et al.* 2010), settlement success (Marshall *et al.* 2003; Levitan 2006), and dispersal distance (Thorson 1950; Levin 2006). We show here that egg size still varied significantly among our studied *Chromodoris* species, despite a recent shared evolutionary history and a common larval development strategy. Furthermore, variation in egg size and number of whorls in the egg mass was not found to be functions of parental body length for any species, even though significant interspecific variation existed among parental body lengths. Although several studies have demonstrated that egg size in marine molluscs can be positively correlated with maternal size (nudibranch — Lambert *et al.* 2000; sacoglossan — Gianguzza *et al.* 2005; sacoglossan — Allen *et al.* 2009), others have found no statistical link between these two parameters (bivalve
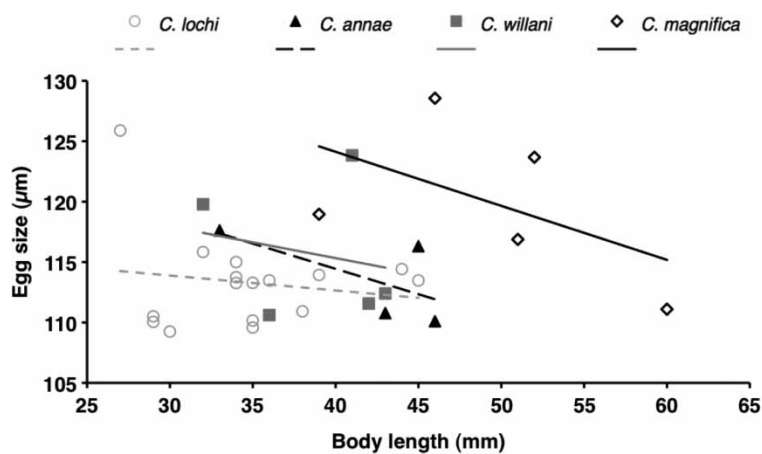

Figure 3.   Linear regression plot of parental body length plotted against egg size for the sampled species.

— Phillips 2007; caenogastropod — Collin and Salazar 2010).

There are a number of examples of variance in reproductive traits between sympatric sister species of marine invertebrates that can be explained by differing reproductive strategies (Pickford 1949; Byrne *et al.* 1999; Ellingson and Krug 2006). This also applies to cases of poecilogony (Levin 1984; Vendetti *et al.* 2012) in which differences in reproductive investment drive large differences between two egg size classes. However, examples that demonstrate significant variance in egg size among co-occurring closely related species that share the same reproductive strategy remain particularly rare. For example, Levitan's (1993) well-known study showed significant differences in the egg sizes among three co-occurring species of *Stronglyocentrus* sea urchins, but subsequent phylogenetic analyses showed that these taxa were not sister species (Biermann *et al.* 2003). In Byrne's (2006) phylogeny of the Asterinidae (28 species), only one well-supported species pair showed large differences in egg size and a shared developmental strategy (in *Parvulastra*).

In general, larger individual heterobranchs are known to lay larger egg masses (Thompson 1967; Kandel and Capo 1979; Lambert *et al.* 2000). Because it is assumed that larger mothers produce larger offspring (Sakai and Harada 2001), a link between parental body length and egg size might also have been expected in the present study. Although the *Chromodoris* species studied here exhibited considerable variation among parental body length and egg size, we found no statistically significant correlations between these two traits within any of the species. Hence, parental body size cannot account for the observed interspecific differences in egg size. In their study of the dorid nudibranch *Adalaria proxima*, Jones *et al.* (1996) likewise found the mean egg size of the first spawn mass to be unrelated to adult body size or developmental time, and instead attributed the intraspecific variation in larval traits to genetic drift. Gianguzza *et al.* (2005) similarly found no link between egg size and parental size. The results of the current study also indicate that the dominant factor in determining egg size in heterobranchs appears to be genetic rather than influenced by maternal provisioning.

The only attribute we found to be conserved across all four species was the duration of oviposition, despite there being significant differences in the number of whorls per egg mass between species. However, this requires further examination because a direct relationship between the duration of oviposition and number of whorls did exist within *C. lochi*, in that the longer a specimen took to deposit an egg mass, the more whorls it contained. Although this trend was only found to be statistically significant within *C. lochi*, it is possible that this pattern could have been demonstrated in the other species had their data sets been larger.

Nudibranchs are semelparous spawners and typically produce several egg masses over a reproductive period (Todd *et al.* 2001). Previous studies (Chester 1996; Jones *et al.* 1996) have shown that egg size decreases over consecutive spawns due to energy expenditure. A prediction could thus be made that eggs in the inner part of the coil may be larger than those in the outer part because they are deposited first and thus could have been subject to higher energy investment. However, the findings of the present study do not support this hypothesis, as egg size did not significantly vary within a spiral in any of the sampled species. Furthermore, only six specimens deposited a second egg mass (not included in any other tests), which were too few for examining changes in egg size over successive spawns. Qualitative observations of the second egg masses revealed fewer whorls, the gelatinous matrix to typically be thinner or less robust, and the mass was more likely to break up during examination.

As significant discrepancies in reproductive parameters can exist even among closely related species of nudibranchs, a great deal of work remains to be done to explain the mechanisms underlying such variation. Although maternal effects are undoubtedly an important contribution to larval traits, we must be careful to also consider the input of evolutionary forces when evaluating phenotypic variation among offspring. To further elucidate the contributions of genetic drift versus character displacement and reproductive isolation, it is important to examine these same parameters in additional populations that do not co-occur with other planar spawning *Chromodoris* species.

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